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Surface functionalization of cellulosic substrates by using chemical and biotechnological methods

Doctoral Thesis

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Als meus pares i germana

A la Joana

Agraïments

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Nomenclature

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AFM	Atomic force microscopy
AS	Acetosyringone
ATR	Attenuated total reflectance
α-TP	α-tocopherol
AV	Acetovanillone
β-CD	Beta-Cyclodextrin
BG	Butyl gallate
BS	Backscattering
β-S	β-sitosterol
BUFF	Buffer
CA	Caffeic acid
CD	Cyclodextrin
CE	Catalytic efficiency
CFA	Coniferyl aldehyde
CHIT	Chitosan
CMC	Critical micelle concentration
CNF	Cellulose nanofibrils
COOH	Carboxyl groups
CSt	Cationic starch
CV	Cyclic voltammetry
D	Dissipation
digCHX	Chlorhexidine digluconate
DLS	Dynamic light scattering

ECF	Elementary chlorine free
EG	Ethyl gallate
ESEM	Environmental scanning electron microscopy
F	Freqüency
FA	Ferulic acid
FCS	Formed cellulosic substrates
FS	Functionalization solution
FSU	Functionalization solution uptake
FTIR	Fourier transform infrared spectroscopy
GA	Gallic acid
IS	Isoamyl salicylate
KFS	Control functionalization solutions
Lacc	Laccase
LG	Lauryl gallate
LMS	Laccase-mediator system
MG	Methyl gallate
MGCh	Methyl-glycol chitosan
MIC	Minimum inhibitory concentration
MS	Methyl syringate
MW	Molecular weight
NFC	Nanofibrillar cellulose
OG	Octyl gallate
OH	Hydroxyl functional group
PAAE	Polyamidoamine-epichlorohydrin
pCA	Para-coumaric acid
PEI	Polyethylene-imine

PG	Propyl gallate
pHB	Para-hidroxibenzoic acid
PhC	Phenolic compound
PS	Paper sheets
QCM	Quartz-crystal microbanalce
ROP	Ring opening polymerization
RV	Resveratrol
SA	Syringaldehyde
SAD	Sinaplyl aldehyde
°SR	Schopper-Riegler degree
SEM	Scanning Electron Microscopy
SFE	Surface free energy
SG	Stearyl gallate
SL	Lignosulfonate
SPA	Sinapic acid
StD	Standard deviation
TA	Tannic Acid
TEAC	Trolox equivalent antioxidant activity
TPH	4-[4-(trifluoromethyl)phenoxy]phenol
TR	Transmission
TRP	2,4,6-tris(1-phenylethyl)phenol
UV-Vis	Ultraviolet–visible spectroscopy
V	Vanillin
VA	Violuric acid
WCA	Water contact angle
WDT	Water drop test

WG	Weight gain
WVTI	Water Vapor Transmission Index

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Abstract

This thesis deals with the surface modification of cellulosic substrates by use of chemo–enzymatic methods. Functionalizing cellulose is intended to adjust its properties for various purposes, but particularly for obtaining a chemical feedstock for the production of cellulose derivatives with a variety of uses. At present, cellulosic substrates are chemically or enzymatically modified by treating fibres in aqueous suspensions prior to formation substrates. In this doctoral work, treatments were applied to previously formed cellulosic substrates (FCS), providing advantages in terms of sheet formation, chemical consumption and manufacturing speed. A non-enzymatic and an enzymatic approach to substrate functionalization were examined, with emphasis on the latter.

This work is part of the research conducted by the CELBIOTECH Group (UPC-BarcelonaTech) within the framework of Spain's MICINN Projects FUNCICEL (CTQ2009-12904), BIOSURFACEL (CTQ2012-34109) and BIOFIBRECELL (CTQ2010-20238-CO3-01), and the BIORENEW integrated project of the Sixth Framework Program (NMP2-CT-2006-026456).

β-cyclodextrins (β-CD) were used to facilitate the chemical modification of three different commercial FCS. Grafting of the substrates was assessed by scanning electron microscopy and FTIR analysis. Drug delivery kinetics were analyzed by UV spectroscopy after loading of the β-CD-grafted substrates with chlorhexidine digluconate (digCHX). Several grafted substrates were found to absorb substantial amounts of active molecules and to release them over a long period (about 20 days).

The use of enzyme systems for surface treatments of FCS is still in its infancy. This doctoral work was intended to fill the gap as far as possible. In preliminary tests, the surface hydrophobization was accomplished onto commercial FCS by using laccase in combination with hydrophobic compounds. The efficiency of the method was increased by using lignosulfonates, which improved the surface distribution of hydrophobic compounds, increased hydrophobicity and helped preserve enzyme activity. The influence of processing conditions including LG dose and treatment time was examined, with a view to their optimization for further increased hydrophobicity.

Surface enzyme treatments evolved into the preparation of a functionalization solution (FS), consisting of an enzymatic product that was subsequently applied to the surface of FCS. This method was also effective and provided major advantages for industrial

implementation; in fact, it is the subject of an international patent application. Surface application of the functionalization solution to FCS was found to confer them hydrophobic and antioxidant properties by effect of physico–chemical interactions between FS and the substrate. Cellulose model surfaces were used to assess FS adsorption, and the WDT, WCA, SEM, AFM and SFE techniques to characterize the treated FCS. Efficient fibre bonding and chemical functionalization were confirmed by thorough washing and Soxhlet extraction of the substrates. As shown here for the first time, surface enzyme treatments have the potential to confer advanced properties to FCS. The performance of the proposed cellulose functionalization technique was assessed by using laccases from various microorganisms.

A better understanding of the reaction mechanisms and physico–chemical interactions behind the laccase treatments, and also of FS–substrate interactions, was acquired by using various analytical techniques including DLS, QCM, FTIR, UV-VIS spectroscopy, Z-potential, Turbiscan® and cyclic voltammetry. The chemical structure of the resulting enzyme-oxidized molecules, the sequence for the oxidation reaction, and the potential grafting mechanisms of enzyme-modified compounds to cellulose, were proposed. The effects of the alkyl chain length of the gallates studied and curing treatments used were analysed in terms of the development of hydrophobic and antioxidant properties.

Resum

La present tesi tracta sobre la modificació superficial de substrats cel·lulòsics utilitzant mètodes químics i enzimàtics. La funcionalització de la cel·lulosa consisteix en ajustar-ne les seves propietats, per a poder-la utilitzar en gran varietat d'aplicacions. Habitualment aquesta modificació s'aconsegueix tractant les fibres en suspensió aquosa i abans de la formació del substrat. No obstant, en la present tesi els tractaments s'apliquen utilitzant substrats cel·lulòsics ja formats (FCS), obtenint una sèrie d'avantatges en termes de formació, consum de químics, i velocitat de fabricació. S'han estudiat dos enfocaments diferents per a la funcionalització: tècniques no-enzimàtiques i enzimàtiques. Els esforços s'han centrat en l'enfoc enzimàtic.

Aquest treball es va dur a terme en el grup de recerca CELBIOTECH (UPC-BarcelonTech), en el marc dels projectes FUNCICEL (CTQ2009-12904) , BIOSURFACEL (CTQ2012-34109) , BIOFIBRECELL (CTQ2010-20238-CO3-01), del MICINN Espanyol i el Projecte integrat BIORENEW (NMP2-CT-2006-026456) del sisè Programa Marc.

Pel que fa a la funcionalització no-enzimàtica, s'han utilitzat β-ciclodextrines (β-CD) per a la modificació superficial de tres FCS comercials. L'empelt s'ha avaluat mitjançant microscòpia electrònica de rastreig i anàlisi FTIR. Els substrats empeltats amb β-CDs s'han carregat amb digluconat de clorhexidina (digCHX), i s'ha analitzat l'alliberament d'aquest principi actiu utilitzant espectroscòpia UV. Diversos substrats han estat capaços de retenir quantitats significatives de digCHX, i de mantenir-ne l'alliberament durant períodes de temps de fins a 20 dies.

La funcionalització enzimàtica per al desenvolupament de derivats de la cel·lulosa ha guanyat interès en la comunitat científica i industrial darrerament. No obstant, l'ús de sistemes enzimàtics superficials encara es troba en una fase molt prematura; la present tesi fa èmfasi en aquest aspecte. En un primer estudi s'analitza la hidrofobització superficial de FCS comercials, mitjançant una lacasa en combinació amb compostos hidròfobs. L'eficiència s'ha vist augmentada amb l'ús de lignosulfonats (SL), els quals milloren la distribució superficial, augmenten els nivells d'hidrofobicitat, i ajuden a preservar l'activitat enzimàtica. La dosi de LG i el temps de tractament s'han optimitzat resultant en una major hidrofobicitat.

Seguidament, els tractaments han evolucionat cap a un nou mètode basat en l'obtenció d'una solució funcionalitzadora (FS), consistent en un producte derivat d'una reacció

enzimàtica, el qual és aplicat posteriorment a la superfície dels FCS. Aquest mètode també s'ha mostrat efectiu, i proporciona avantatges importants respecte a la possible aplicació industrial; de fet, el nou mètode ha permès realitzar una sol·licitud internacional de patent. L'aplicació de la FS a la superfície de FCS els confereix propietats hidrofòbica i antioxidant gràcies a les interaccions fisicoquímiques que es produeixen. S'han utilitzat superfícies model de cel·lulosa per tal d'avaluar l'adsorció de la FS, i s'han emprat les tècniques de WDT, WCA, SEM, AFM i SFE per caracteritzar-les. La força de l'enllaç s'avalua mitjançant rentats agressius i extraccions Soxhlet. Es demostra per primer cop el potencial dels tractaments enzimàtics superficials per a conferir propietats avançades als substrats cel·lulòsics. La tècnica també s'ha assajat emprant diverses lacases.

S'han utilitzat diverses tècniques d'anàlisi per estudiar els mecanismes de reacció, i les interaccions FS-substrat. Les tècniques han estat: DLS, QCM, FTIR, espectroscopia UV-VIS, potencial-Z, Turbiscan® i Voltametria Cíclica. S'ha proposat l'estructura química de les molècules resultants de l'oxidació del LG, els possibles mecanismes d'oxidació, i possibles mecanismes d'empelt entre els compostos modificats i la cel·lulosa. També s'ha analitzat l'efecte de la longitud de la cadena alquílica i dels tractaments tèrmics en les propietats antioxidant i hidrofòbica.

Chapter 1

Introduction and Objectives

Summary

This chapter introduces the reader to the topics dealt with in this thesis and provides an overview of the state of the art in currently available techniques for functionalizing cellulosic materials to obtain specialty papers. The chapter describes lignocellulosic materials in terms of origin, structure and processing methods, and then approaches paper-based materials with emphasis on specialty papers, their marketing forms and physical characteristics, and the increasing interest in their production by using renewable sources and environmentally friendly methods. Two major approaches to functionalization are considered: non-enzymatic and enzymatic. The raw materials and enzyme systems used, and their properties, are also examined.

1.1 Paper materials

Paper can be defined in board terms as a laminar material consisting of interlacing cellulose fibres of natural origin formed by deposition onto a mesh from an aqueous suspension. However, the term "paper" is currently applied to a broader range of products including many types of laminar materials, whether fibrous, cellulose-based or otherwise. Paper has traditionally been obtained from cellulosic plant fibres; however, it can also be produced from animal fibres (e.g. wool, fur, hair, silk), mineral fibres (e.g. asbestos), synthetic fibres (e.g. rayon, nylon) or even ceramics, metals, glass and various other materials. Today, paper is used in printing and writing media, packaging and shipping containers, absorbent tissues and towels, moulded products and a variety of specialty papers.

The manifold properties of paper can be obtained by appropriate selection of the type of fibre used, its treatment, the additives employed, and the way the paper machine is operated to form, consolidate, and dry sheets (Emerton, 1980). In addition, paper can be modified by using a number of conversion processes such as functionalization, which was the main subject of this doctoral work.

1.2 Fribrous raw materials for papermaking

The main sources of fibres for papermaking are living plants, and among them those derived from the trees for being the ones that yield the highest proportion of their weight as fibres suitable for papermaking. Papermaking fibres can be classified into two broad categories according to origin, namely:

- **Non-plant fibers**, which include animal fibres (wool, hair, silk), mineral or ceramic fibres (asbestos, glass, metals, carbon) and synthetic fibres (polyethylene, polypropylene, polystyrene or polyvinylchloride obtained by extrusion or spinning/bonding).
- **Plant fibers**, which can come from woody or non-woody plants. A woody plant is a plant that produces wood as its structural tissue. Woody plants are usually trees, shrubs, or climbing woody vines ("lianas"). Wood is a structural cellular adaptation that allows woody plants to grow from above ground stems year after year. The main sources of non-woody raw materials are monocotyledons, including cereal straw and bagasse, or plants grown specifically for their fiber, such as bamboo, reeds, and some other grass plants such as flax, hemp, kenaf, jute, sisal, or abaca.

Plant life can be classified as depicted in Fig. 1-1. Among all the existing species, woody gymnosperms and angiosperms are the most widely used for papermaking. Gymnosperms are commonly referred to as "conifers". The conifers used by the forest products industry are all "trees" and they are sources of "long fibered" wood pulp; they are also commonly referred to as *softwoods*. Woody angiosperms include trees that are used as sources of "short-fibered" wood pulp and commonly referred to as *hardwoods*.

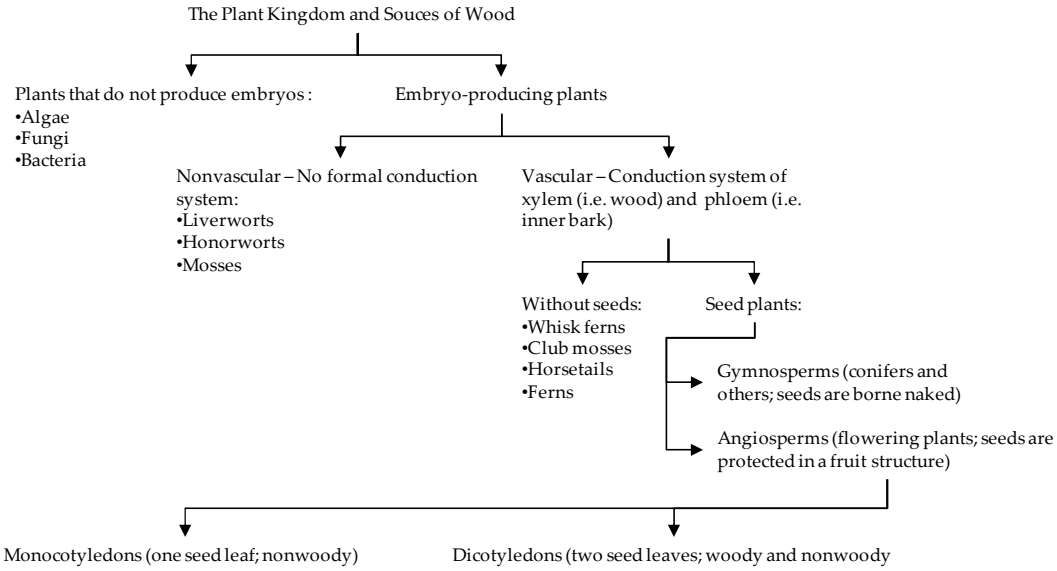

Figure 1-1 Plant life classification (Parham, 1983).

Plant fibres or "tracheids" consist of three structural polymers (the polysaccharides cellulose and hemicelluloses, and the aromatic polymer lignin) in addition to various minor non-structural components including proteins, extractives and minerals. Of these three major constituents of wood, cellulose is essential for papermaking and hemicelluloses are mainly beneficial; on the other hand, lignin is detrimental and its removal from between and within tracheid walls is the subject of chemical pulping and, to a large extent, bleaching.

Wood is extremely anisotropic by effect of 90–95% of its cells being elongated and vertical (i.e. aligned parallel with the tree trunk) (Fig. 1-2a). The other cells (5–10%) are arranged in radial directions but none tangentially. The middle lamella, which is the region between contiguous fibres, contains abundant lignin and ensures adhesion between fibres. The wall of a typical tracheid consists of several layers, namely: the primary wall

(P), the external secondary wall (S1), the middle secondary wall (S2) and the internal secondary wall (S3) (Fig. 1-2b). Each layer has its cellulose microfibrils arranged in a specific manner which dictates the mechanical and physical properties of the wood in that cell. Microfibrils may be aligned irregularly (as in the primary cell wall, P) or at a specific angle to the cell axis (as in S1, S2, and S3). The inner part of each tracheid is called the "lumen" and is empty.

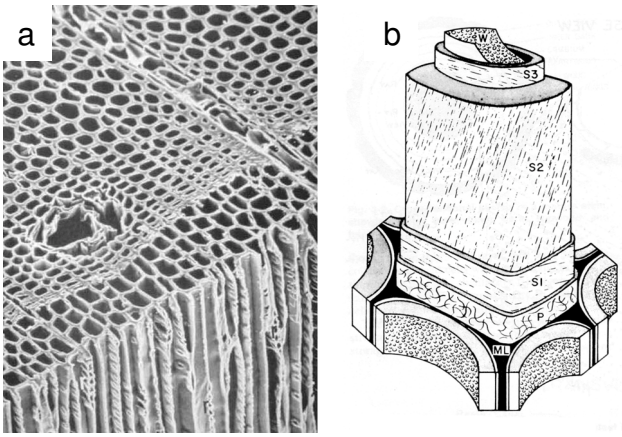

Figure 1-2 Cross-section of woody tissue, showing xylem tracheids (a) and diagram of cell wall organization (b) (Parham, 1983).

1.2.1 Cellulose

At present, all paper is made of cellulose in one form or another and in different degrees of purity. Cellulose is a carbohydrate of empirical formula $(C_6\text{-}H_{10}\text{-}O_5)_n$, where "n" is the number of repeating sugar units or "degree of polymerization" (DP), from which its composition may be calculated to be 44.4% carbon, 6.2% hydrogen and 49.4% oxygen. Cellulose was discovered in 1838 by Payen, and is the dominant component of all types of plant tissues and fibres. Cellulose is the most abundant organic polymer on the face of the earth. It is a glucan polymer of β-D-glucopyranose units linked together by β-(1,4) glycosidic bonds (Fig. 1-3). In fact, the building block for cellulose is cellobiose because the repeating unit in cellulose is a two-sugar unit. The number of glucose units in a cellulose molecule is referred to as the "degree of polymerization" (DP), and the average DP for plant cellulose ranges from a low of about 50 for sulfite pulp to approximately 600, depending on the particular determination method used (Stamm, 1964). Therefore, the molecular weight of cellulose can be estimated to range from 10 000 to 150 000.

Cellulose is insoluble in water and generally relatively inert, but susceptible to oxidation of its primary alcohol groups.

The β-(1,4) glycosidic bonds in cellulose provide a potentially straight molecule. The linearity of cellulose molecules has far-reaching implications on papermaking, because formed paper will consist in cell units possessing the mechanical properties of plant fibres.

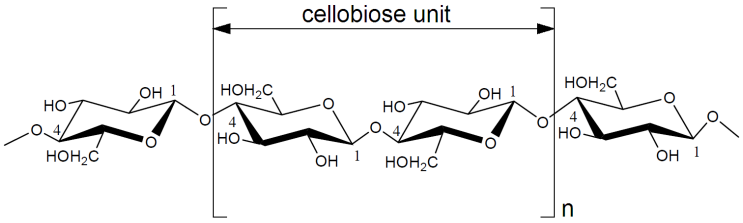

Figure 1-3 Partial structure of cellulose.

Cellulose molecules are randomly oriented and tend to form intracrystalline regions. Also, cellulose has a strong tendency to form intra- and inter-molecular hydrogen bonds via hydroxyl groups on its linear chains that stiffen the straight backbone and promote aggregation into a crystalline structure, thereby allowing cellulose to adopt a large variety of partially crystalline fibre structures and morphologies. Most plant-derived cellulose is highly crystalline and may contain as much as 80% crystalline regions. Also, most plants consist of approximately 45–50% cellulose on a dry weight basis, with figures as high as nearly 90% for cotton or as low as about 30% for stalk fibres.

Fig. 1-4 illustrates the hierarchical structure of plant cell walls. Macrofibrils (roughly 10–25 nm in diameter), which form the primary wall, consist of bundles of microfibrils about 3–4 nm in diameter which are bound together by hemicellulose and either pectin or lignin. Microfibrils in turn are bundles of cellulose chains. Approximately 36 individual cellulose molecule chains connected to each other by hydrogen bonds form larger units known as “elementary fibrils”, which are packed into larger microfibrils. Cellulose microfibrils exhibit crystalline regions separated by shorter, non-crystalline regions.

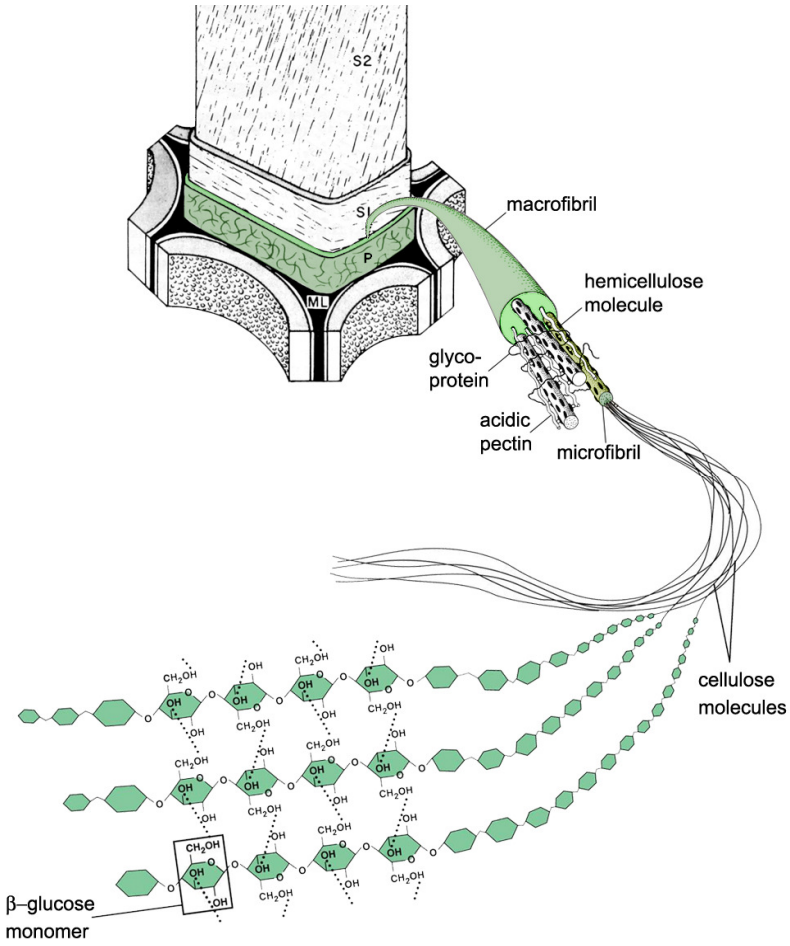

Figure 1-4 Hierarchical structure of plant cell walls.

1.2.2 Hemicellulose

Unlike cellulose, hemicelluloses have low DP values (only 50–300); also, they bear side groups on their chains and are essentially amorphous. The hemicellulose fraction of plants consists of a collection of polysaccharide polymers mainly containing the sugars D-xylopyranose, D-glucopyranose, D-gallactopyranose, L-arabinofuranose, D-mannopyranose and D-glucopyranosyluronic acid in addition to minor amounts of other sugars. Hemicelluloses usually consist of more than one type of sugar unit and are sometimes named after the sugars they contain. While cellulose is crystalline, strong and resistant to hydrolysis, hemicellulose has a random, amorphous structure of little strength; also, it is easily hydrolysed by dilute acid or base, as well as by a myriad hemicellulase enzymes.

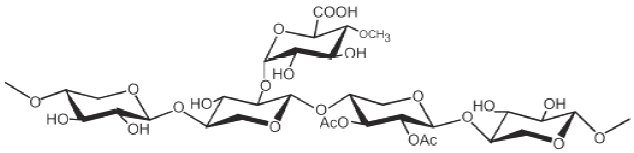

Figure 1-5 Partial structure of glucuronoxylan, a hardwood hemicellulose.

Softwood hemicelluloses are largely gallactoglucomannans, whereas hardwood hemicelluloses are mostly xylan (Fig. 1-5). Both provide a supportive matrix for cellulose microfibrils and appear to distribute relatively uniformly across fibre walls.

1.2.3 Lignin

Lignin is an amorphous, highly branched, three-dimensional phenolic polymer consisting of phenylpropane units (Fig. 1-6) that is present as an encrustant in all woody tissues; indeed, lignin is the second most common class of organic molecule in living organisms, being about half as abundant as cellulose. Lignin can be classified in various ways, but most often according to the structural elements it contains (Sjöström, 1981). Lignin in all plants consists mainly of three basic building blocks (guaiacyl, syringyl and p–hydroxyphenyl moieties) but can also contain other aromatic units in many plants (Fig. 1-7).

Lignin structure varies markedly between plant species. Thus, its phenylpropane unit can be substituted at the α, β, and γ positions to form various combinations linked together by ether and carbon-to-carbon linkages (see Fig. 1-7). Lignin in wood distributes throughout the secondary cell wall but concentrates in the middle lamella. By effect of the difference in volume between the middle lamella and secondary cell wall, about 70% of all lignin is located in the secondary wall. Lignin in plants acts primarily as an encrusting agent for the cellulose/hemicellulose matrix; unsurprisingly, lignin is often referred to as the "plant cell wall adhesive". In fact, lignin forms a cementing matrix that holds together cellulose fibrils in each tracheid, and tracheids to one to another; this confers plants substantial mechanical strength and allows some to grow very tall (Emerton, 1980). Lignin is much less hydrophilic than both cellulose and hemicelluloses —almost to the point of being hydrophobic—; as a result, it confers the walls of dead cells some hydrostability that may be essential for bulk water conduction in tissues. Both lignin and extractives present in plants reduce the digestibility of grasses to animals (Jung and Deetz, 1993). Lignin can associate to hemicelluloses and form lignin–carbohydrate complexes that are resistant to hydrolysis even under pulping conditions.

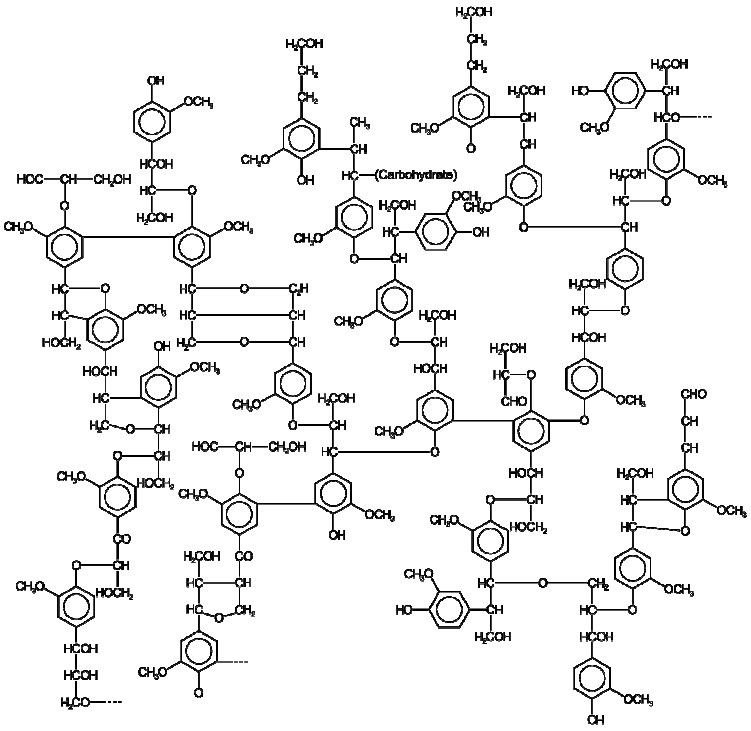

Figure 1-6 Partial structure of a hypothetical lignin molecule (Glazer and Nikaido, 1995).

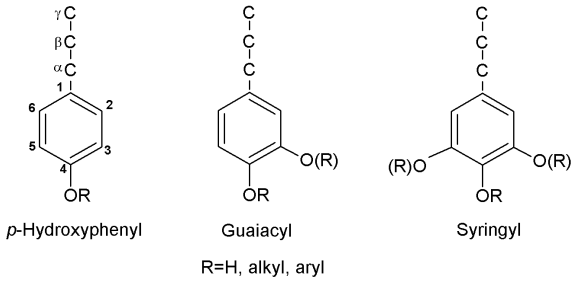

Figure 1-7 Building blocks of lignin.

Wood contains a highly substantial amount of lignin (18–37%, but typically 23–30% for most species). Obtaining quality papermaking fibres entails removing most lignin, which is one of the chief functions of a pulp mill. Most fortunately for papermaking, as well as textile work, cellulose is quite resistant to the action of alkaline materials such as those used to wash textile goods and remove impurities from fibrous raw materials — otherwise, papermaking from chemically prepared wood fibres would be impossible.

Acids have a more drastic action on cellulose: they weaken fibres. However, the sulfite process, which uses carefully controlled acid conditions, has enabled the production of fibres from many kinds of wood.

Pulping processes produce large amounts of lignin derivatives in waste liquors. The chemistry of lignin has aroused increasing interest in the hope that a fuller knowledge of its nature and structure might lead to the development of a major commercial outlet for this plant component. For example, lignosulfonates, which are by-products from the sulfite pulping process, have found use as binders, surfactants and concrete plasticizers. During the sulfite process, native lignin is released from pulp fibres through sulfonation and washed away. As a consequence, the lignosulfonate molecules contain both hydrophobic and hydrophilic moieties, and form a polyelectrolyte which has found many technical applications as a dispersing or binding agent (Vishtal and Kraslawski, 2011).

1.3 Paper production

Paper consists of a flat, felted structure made of cellulose fibres (Fig. 1-8) from natural sources (annual plants and trees) and/or recycled fibres. These fibres are intertwined and held together both mechanically and by hydrogen bonding, forming a thin web-like structure. The average size of an individual fibre ranges from 0.5 to 4 mm in length and 12 to 50 microns in width (Garcia-Hortal, 2007). However, these values differ markedly from species to species. For papermaking, the fibers are arranged in aqueous suspension. Within the aqueous medium the fibers are individualized, isolated, and suspended in the medium by mechanical agitation. Agitation facilitates homogeneous distribution of fibres in suspension and, once stopped, suspended fibres tend to settle and aggregate.

The papermaking process begins with reception of wood logs in the wood yard. The logs are debarked and cut into small chips (2 to 3 cm). The next step involves individualizing cellulose fibres by using mechanical or chemical methods, or a combination of both, to disperse or suspend the fibres in water and obtain so called paper “pulp”. The most commonly used process for individualizing cellulose fibres is the “kraft” chemical process. In the kraft process, wood chips are cooked in a digester containing an alkaline medium to dissolve lignin and individualize cellulose fibres. Then, the fibres are subjected to a bleaching sequence involving treatment with hydrogen peroxide, oxygen, chlorine dioxide or even enzymes. Once individualized and bleached, the fibres usually undergo further conversion before papermaking. Thus, a refining stage is frequently used to modify the fibres physically and increase their ability to form hydrogen bonds in order

to develop paper strength. Some pulp preparation processes additionally use chemical treatments. Finally, the pulp enters the paper machine at a specific consistency and the machine forms, presses, dries, calenders and winds sheets. Thus, one can realize that depending on the stage of the process, the equipment and operations work with different type of matter (wood, fiber suspension, or paper).

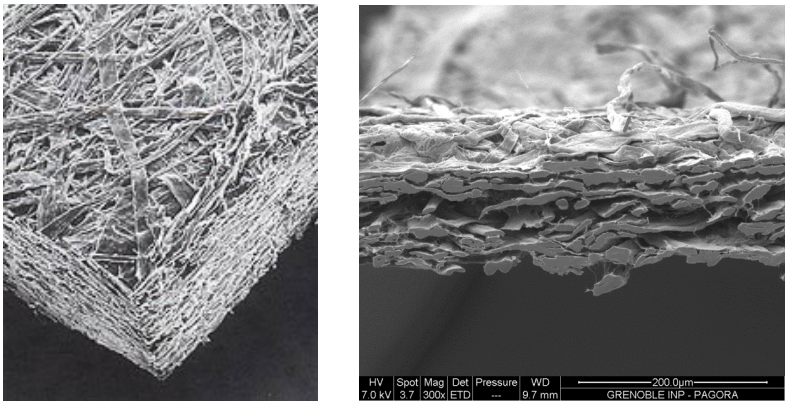

Figure 1-8 SEM images of cross-sections of two different types of papers. Individual fibres can be easily identified.

1.3.1 Pulping

There are several methods of manufacturing cellulose pulp from raw materials. The two main approaches use chemical or mechanical means, or combinations of both. Pulp for paper and cardboard production is typically obtained by using hybrid chemical and mechanical methods that provide intermediate yields. Generally, chips are partly softened or digested with chemicals and then mechanically converted to fibre. Chemi–mechanical pulping, which is typically used with hardwood, softens chips prior to the usual mechanical action and provides yields in the 80–90% range. Semi-chemical pulping requires heavier cooking and delignification prior to mechanical refining.

1.3.1.1 Mechanical pulping

The most common mechanical pulping method is the groundwood process, where a roundwood bolt is pressed lengthwise against a rough, revolving grinding stone. Wood fibres are torn out of the wood, abraded and removed from the stone surface with water. Mechanical pulping converts up to 95% of the dry weight of the wood input into pulp, but the mechanical action requires a large energy input. The pulp forms highly opaque

paper with good printing properties, but the paper is relatively weak and turns easily to yellow with exposure to light. Newsprint is a major product of mechanical pulp, which is generally produced from long-fibred softwood (conifer) species.

1.3.1.2 Chemical pulping

In chemical pulping, wood fibres are separated by dissolving away the lignin component; this leaves behind a fibre that retains most of its cellulose and some hemicellulose. The yields of chemical processes typically fall in the region of 40–50% dry weight of the original wood input.

One of the most commonly used chemical processes is the kraft (sulfate) process, which dominates the papermaking industry by virtue of its excellent chemical recovery and pulp strength. In fact, the kraft process accounts for 91% of chemical pulp and 75% of all pulp produced worldwide each year. The high efficiency of the process relies on the use of effective chemical (alkali and sulfidity) conditions (Smook, 1982). One other commonly used chemical process is the sulfite process, which uses various chemicals to attack and remove lignin. Sulfite pulp is brighter and more easily bleached, but weaker, than kraft pulp; it is produced in several grades, but mostly in bleached grades. Yields are generally in the range 40–50%, but tend to the lower end in bleached grades. The sulfite process is more sensitive to species characteristics than is the kraft process; thus, the former is usually intolerant of resinous softwood, tannin-containing hardwood and any bark-containing furnish.

1.3.1.3 Semi-chemical pulping

Semi-chemical pulping uses a combination of chemical and mechanical methods. Wood chips are partially cooked with chemicals and the remainder of the pulping is accomplished mechanically.

1.3.2 The paper machine

The paper machine (where the sheet is formed) is basically divided into four sections: the forming, the pressing, the drying, and the calendaring sections. Figure 1-9 shows a simplified layout of a paper machine.

The paper web formation area, also called the "wet end", includes the headbox, which holds the fibre suspension, the wire and all elements needed to remove water in large amounts from the fibre suspension (basically, foils and suction boxes). The press area or "wet press section" consists of hard rolls which press the paper sheet to force water out of

the paper structure by effect of pressure differences and to consolidate the sheet. The drying section comprises a series of hot-press rollers that facilitate evaporation of water from the sheet structure by heating. Finally, the calendering section consists of a series of metallic cylinders intended to improve the surface characteristics of the sheet. Besides these basic processes, the paper is subjected to other operations to improve its characteristics (e.g. surface spraying with starch, size press-coating, creping). These operations are integrated into sections or between sections of the paper machine.

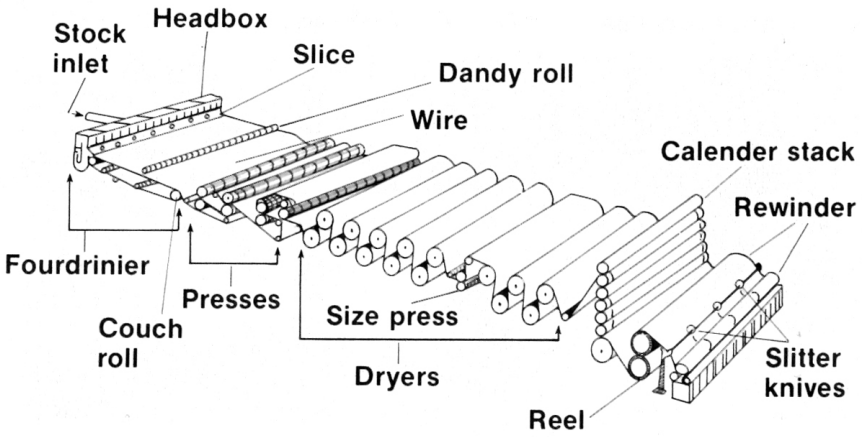

Figure 1-9 Basic parts of a typical *Fourdrinier* paper machine (Kline, 1982).

When the cellulose fibres, in the form of a fibre suspension or "stock", are ready for papermaking, they enter the paper machine via the headbox. The headbox and slice are used to deliver a ribbon of stock to the wire at uniform dilution, thickness and speed (Kline, 1982). The slice is a narrow opening in the headbox through which the stock flows. The headbox and the slice work together to control the volume or weight of fibers as well as the flow rate (viz. the velocity of the flow relative to the speed of the wire).

In order to form a paper sheet, the fibre suspension is deposited onto a "wire" that is usually made of plastic or metal (Fig. 1-10). The wire, which in morphological terms is tipically a continuous belt of woven material, has a certain "mesh opening" (Fig. 1-10a). During the sheet formation process (i.e. when the pulp is deposited onto the wire), water seeps through the mesh opening and fibres are trapped and deposited onto the wire surface (Fig. 1-10b).

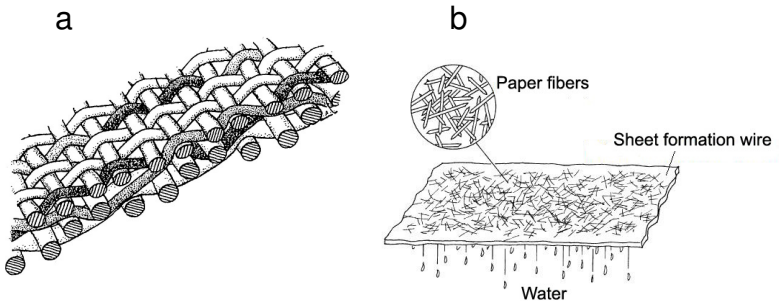

Figure 1-10 Schematic depiction of a formation wire (a), and de-watering of the pulp on the wire surface (b).

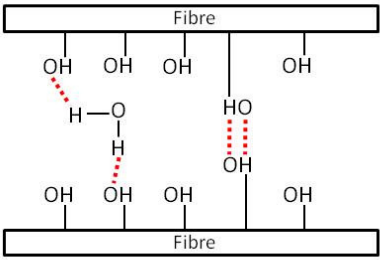

Figure 1-11 Hydrogen bonding between two adjacent fibers.

The fibres are then bonded together to form the paper structure. The wire travels over a series of rolls and/or devices called hydrafoils to have water removed. As the stock and wire pass through the wet end, water is removed first by gravity, next by low pressure generated on the back side of the rolls and foils, and finally by suction devices located under the wire. Much of the water constituting the aqueous suspension is drained through the forming wire, but a small portion remains trapped by capillary action between fibres, thus preventing intimate contact. This phenomenon hinders formation of a greater number of hydrogen bonds between fibres.

The wet web is subsequently passed through a series of hard rollers (the press section) while it is entrapped with a felt. The structure of the felt and the pressure between the rollers allows residual water between fibres to be forced out by squeezing. The felt is a smooth non-woven cloth which absorbs water from the paper; therefore, it must be porous enough to absorb water when compressed. After the paper web leaves the press

section, it is passed around a series of steam-filled drums (dryer cans) where any residual water is removed by evaporation.

The dryer section usually includes a “size press” (viz. a coating device that is used to apply chemicals or pigmented solutions to the surface of the web in order to improve its surface characteristics). The most commonly used pigments include clay, calcium carbonate and titanium dioxide; they are typically applied in water, using an adhesive to hold them onto the surface of the web after it dries. Since the pigment and the chemical solutions in the size press are used in water, further drying is required. Finally, the paper web can undergo operations such as “calendering”, which makes the paper smoother and helps control its thickness, and “reeling”, by which the web is wound into a roll.

Initially, paper fibres are bound by hydrogen bonds with water molecules; because the water is subsequently removed from the sheet structure, the fibres are held together by hydrogen bonds involving hydroxyl groups present in cellulose chains (Fig. 1-11).

1.3.3 Paper grades

The term “paper” encompasses a wide range of cellulose-based products the characteristics of which depend on the intended use and specifications of the end-product. Paper can be classified in a number of ways. One is according to the nature of the pulping process used (viz. chemical, mechanical, semi-chemical or mixed). Another classification is based on the type of raw material used: virgin or recycled fibres and long or short fibres, which vary with the particular species used as fibre source (pine, fir, eucalyptus, birch, poplar, hemp, bamboo, linen). The most widely used classification of paper products is according to use (newsprint, printing and writing paper, sanitary paper for packaging, specialty paper).

Many types of paper classed as “special” are in fact used to manufacture packaging, either as single components or as parts of complex materials. One possible definition of special paper is "paper that is suitable for special purposes as it has unique properties and characteristics which are considered to be out of the standards of the paper industry". Examples of specialty papers include tea bag paper, label paper, fireproof paper, carbonless paper, security paper, thermal paper, filter paper, antibacterial paper and anti-slip paper, which are obtained by using a variety of processes differing in environmental impact. The specialty paper market tends to be a niche with the potential to generate high profit margins relative to “commodity-considered” products since prices are fairly stable over time. Specialty paper is produced in small amounts by using complex processes that warrant a higher price. About 2900 types of specialty papers are

currently produced in the world that can be classified into eight different segments according to use and application sector (Fig. 1-12). Also, more than 19 million metric tons of specialty paper is produced and used each year, and the figure is likely to increase in the future (Lehmann, 2008).

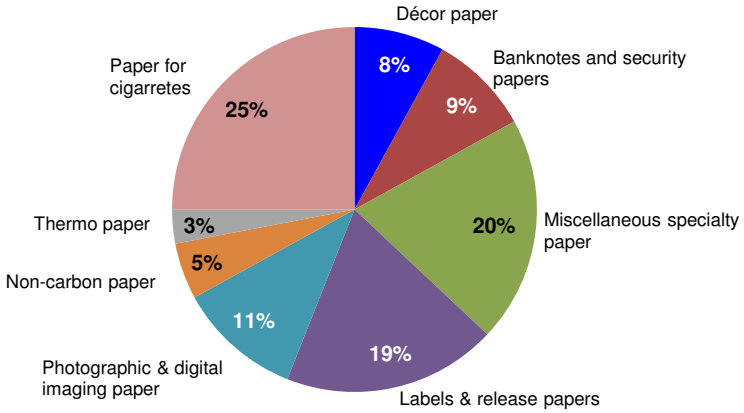

Figure 1-12 Distribution of specialty papers according to number of paper machines worldwide (total, around 600). Source: Voith Paper, (2008).

1.3.4 Functionalization

Functionalization is one of the most commonly used methods for obtaining specialty papers. By definition, the functionalization of a given material involves conferring it a specific function. In paper-based materials, functionalization introduces new properties not present in common paper. For example, "grafting" anchors functional chemical groups onto a surface and confers the surface new or modified properties as a result. In paper terms, functionalization occurs at the fibre surface or paper sheet surface level.

Chemical functionalization of cellulose involves reactions of hydroxyl groups such as esterification, etherification, intermolecular cross-linking and macrocellulosic free radical reactions. Chemical functionalization continues to play a dominant role in improving the overall usefulness of cellulosic polymers. The accessibility to hydroxyl groups and their reactivity has opened up new prospects for preparing specific molecular structures for future application (Varshney and Naithani, 2011). Several studies have demonstrated the functionalization of cellulose-based materials with a variety of techniques with a view to introducing novel properties. For example, Ly *et al.*, (2010) developed a method for functionalizing cellulose fibres and filter paper by using oligoethers to obtain variably

hydrophilic or hydrophobic modified fibres; Fragouli *et al.*, (2012) reported one for rendering magnetic properties to any kind of commercially available cellulose material; Waly *et al.*, (2012) succeeded in preparing flame-retardant cellulosic cotton fabrics; and Mbakidi *et al.*, (2013) obtained paper with photoantibacterial activity for biomedical use. In fact, cellulose- and paper-based materials can be conferred a wide range of properties such as conductivity (Jabbour *et al.*, 2012), antioxidant capacity (Silva *et al.*, 2011), water pollutant adsorption (Wojnárovits, Földváry and Takács, 2010) or odour and pH control (Dutkiewicz, 2006), among others.

Recent studies have shown that cellulose and paper can also be functionalized by using enzymes with the added advantage of environmental friendliness. Surface-modifying fibres to obtain hydrophobic cellulose (Garcia-Ubasart *et al.*, 2012; Cusola *et al.*, 2013), improve mechanical resistance (Aracri and Vidal, 2012), or obtain antimicrobial properties (Fillat *et al.*, 2012) are but a few successful applications of enzyme-catalysed cellulose functionalization.

1.4 Biotechnology in the pulp and paper industry

The increasing social awareness of climate change, consumers' perception, global competition, the need to comply with stringent environmental legislation and competition have aroused increasing interest in biotechnology in the pulp and paper industry (Viikari, 2002). Biotechnological methods have the potential to provide substantial improvements in traditional pulp and paper manufacturing processes thanks to their specificity and potential environmental advantages. A high global R&D interest in the development and use of enzymes by the pulp and paper industry currently exists as reflected in the large number of scientific papers published and patents filed on this topic, which has grown steadily since 1995 (Demuner, B., Pereira Junior, N., Antunes, A., 2011).

The use of enzymes for pulp and paper manufacturing has grown, and a number of enzyme-based processes have found industrial implementation, in recent years. Such processes include enzyme-aided bleaching, direct delignification with oxidative enzymes, energy-saving refining with cellulases, pitch removal with lipases, freeness enhancement with cellulases and hemicellulases, enzymatic de-inking, modification of fibre surfaces, and the combined use of laccases and mediators for lignin removal (Jeffries, 2008). Enzymes provide substantial, increasingly important advantages over chemical catalysts in that they are biodegradable, derived from renewable resources, usable under relatively

mild conditions of temperature and pH, and usually exquisitely selective as regards both reactant and product stereochemistry (Cherry and Fidantsef, 2003).

Biotechnology has also been recently used for the targeted modification of lignocellulosic fibres with the aim of conferring new properties or improving existing ones, as discussed in the previous paragraphs.

1.4.1 Laccases

Enzymes are proteins widely distributed in nature and an essential part of life. Chemically, enzymes contain one or more chains of hundreds of amino acids in a highly complex three-dimensional structure, which is extremely important for their action. Thus, enzymes act as biological catalysts for biochemical reactions in all living organisms, but are also reliable, convenient processing aids for many technical industries (Call and Mucke, 1997).

Laccase (EC 1.10.3.2) is a multicopper blue oxidoreductase of average molecular weight 60–70 kDa that was discovered by Yoshida in 1883. Laccases are expressed by white-rot fungi and other organisms that play a crucial role in the terrestrial carbon cycle by helping to degrade lignocellulosic materials such as wood. One of the current hottest uses of enzymes to bleach wood pulp is in combination with a redox mediator. Laccase acts on phenolic substrates and aliphatic or aromatic amines by catalysing the oxidation of their phenolic hydroxyl groups to phenoxy radicals while dioxygen (O_2) is reduced to water (Fig. 1-13) (Widsten and Kandelbauer, 2008).

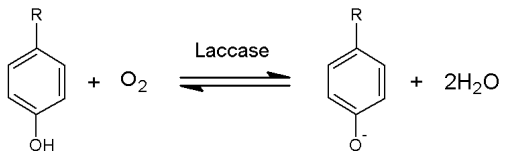

Figure 1-13. Reaction of laccase with a phenolic compound.

Oxidation of substrates by laccases is made difficult by their rather low redox potential (~0.5–0.8 V); this precludes oxidizing non-phenolic (C4-etherified) lignin units, which have a high redox potential (>1.5 V). Also, laccases are bulky molecules, which prevents them from penetrating deep into wood or fibres. Because of these limitations, laccases by themselves can only oxidize phenolic lignin units —which account for less than 20% of all lignin in native wood— at the substrate surface. However, laccases can be used in

combination with an oxidation mediator: a small molecule capable of extending the enzyme action to non-phenolic lignin units and overcoming the reduced accessibility problem (Barreca *et al.*, 2003). In so-called "laccase–mediator systems" (LMS), the mediator is first oxidized by laccase and then diffuses into cell walls to oxidize lignin otherwise inaccessible to laccase (Fig. 1-14).

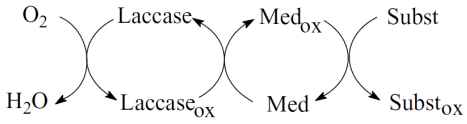

Figure 1-14. Oxidation of a generic non-phenolic substrate (Subst) using the laccase-mediator system.

Researchers worldwide are competing to develop effective, industrially applicable bleaching systems using a LMS. For a long time, laccase aroused little interest for bleaching wood pulp because it was known to have too low a redox potential to attack non-phenolic lignin structures —which accounted for 90% of the composition of the target structures. This perception changed when laccase proved able to oxidize non-phenolic structures in the presence of simple, low-molecular weight compounds acting as redox mediators. Interest in laccases was further increased when researchers later demonstrated that a laccase–redox mediator system can also efficiently delignify and demethoxylate lignin in kraft pulp.

Laccases and peroxidases have been thoroughly investigated for biopulping, biobleaching, de-inking and effluent processing by the pulp and paper industry. However, the use of laccases and LMS to develop or improve paper properties has gained increasing attention and recent research has shifted to the functionalization of pulp in order to produce novel paper products. The enzyme-assisted grafting of functional compounds to pulp has provided novel opportunities to alter the physico–chemical properties of the resulting paper products (Nyanhongo *et al.*, 2011). Several studies have examined the improvement in physical properties of various types of fibres (Wong, Richardson and Mansfield, 2000; Grönqvist *et al.*, 2003), and the grafting of aromatic compounds onto fibres as a means of conferring new or enhancing existing fibre properties (Cusola *et al.*, 2013; Fillat *et al.*, 2012; Elegir *et al.*, 2008; Garcia-Ubasart *et al.*, 2011; Aracri *et al.*, 2010). The laccase-mediated direct oxidation of phenolic substrates produces highly unstable and reactive phenoxy radicals. These radicals can undergo further enzymatic oxidation to quinones or spontaneous non-enzymatic coupling reactions (e.g. hydration, polymerization, oxidation, reduction) to give polymers (dimers,

mainly) or be grafted onto cellulosic substrates. Laccase can also couple a typical laccase substrate with substances that are not substrates for the enzyme to give new heteromolecular hybrid molecules (Polak and Jarosz-Wilkolazka, 2012).

1.5 Objectives and structure of the thesis

This thesis deals with the development of new techniques for obtaining value-added materials by chemical or enzymatic surface functionalization of cellulosic substrates. This is one of the goals of the research line "Application of Biotechnology for Pulp Fibre Modification" currently under way at the Textile and Paper Engineering Department of the Universitat Politècnica de Catalunya – BarcelonaTech.

The pulp and paper industry worldwide is increasingly seeking methods to replace traditional manufacturing processes with environmentally friendlier alternatives. Naturally occurring enzymes, which are known to act as catalysts for a variety of chemical processes in living organisms, may allow a number of synthetic chemicals used in industrial processes to be effectively superseded with the additional advantage of a reduced environmental impact.

The oxidation of phenolic compounds by laccases has been extensively studied. For example, laccase has been shown to play a central role in the delignification of wood and pulp. Also, recent studies have shown laccase systems to catalyse the grafting of low-molecular weight compounds onto cellulosic fibres. In this doctoral work, cellulosic substrates were efficiently surface-modified by using chemical and enzymatic methods to obtain value-added products. Efforts, however, focused on the enzymatic approach.

The paper manufacturing industry applies enzyme treatments to fibre suspensions in the blending chests (i.e. before sheet formation). Industrially, introducing chemicals or enzymes before paper formation can alter the natural interaction between fibres and lead to paper with impaired strength-related properties and less easily recycled effluents as a result. For this reason, one of the central goals of this doctoral work was to develop effective enzymatic treatments for surface application after formation of the paper sheets.

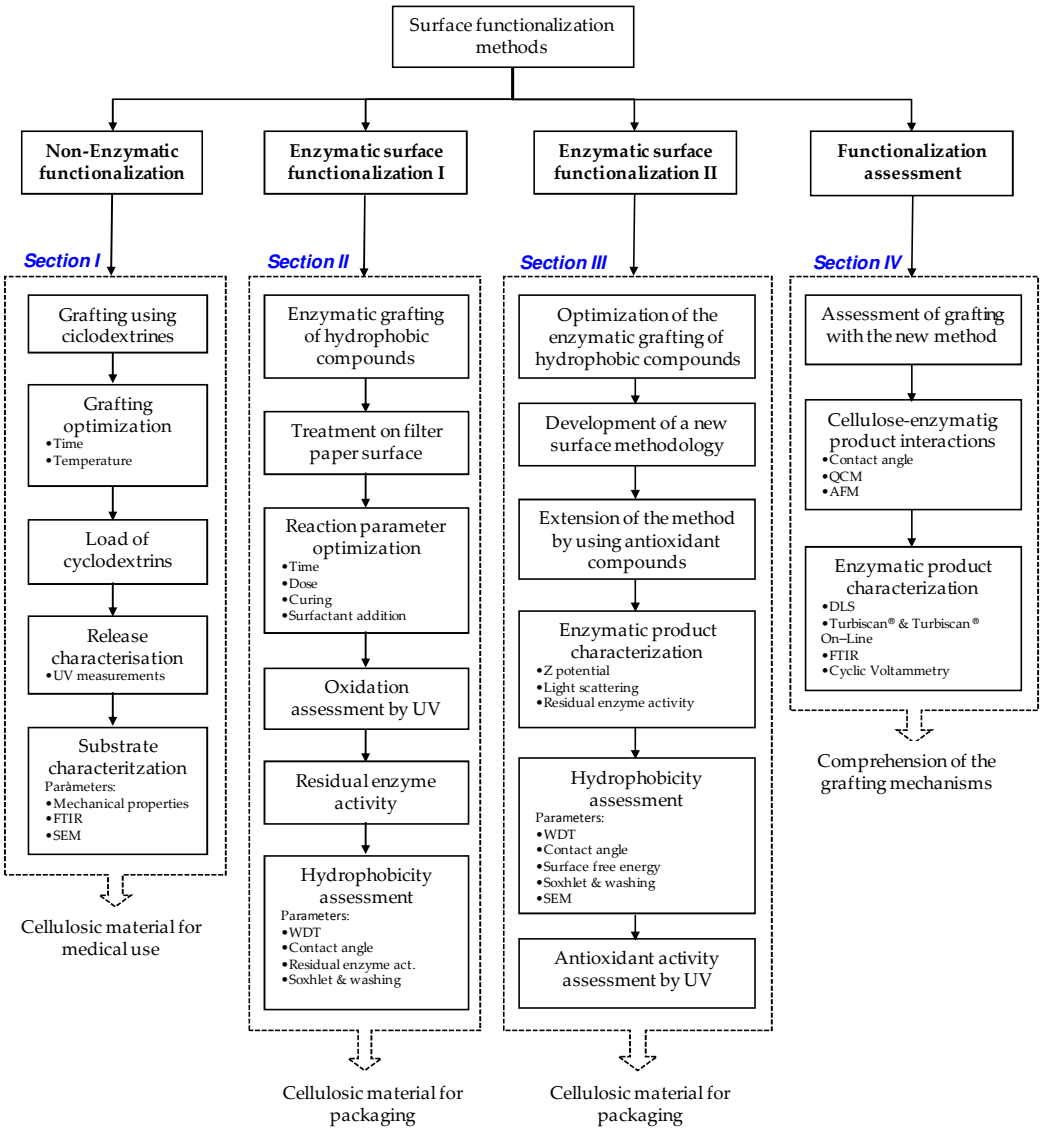

Figure 1-15. General overview of the work plan of the thesis.

The main objective of this work was thus:

To develop new surface treatments preferably using enzymatic methods (specifically, a laccase–mediator system) for the surface functionalization of cellulosic paper products with a view to obtaining high added-value materials.

The research work to be conducted to this end encompassed four main parts (Fig. 1-15). The first part involved obtaining advanced antiseptic-releasing cellulosic materials by functionalization with a non-enzymatic method. The second and third were concerned with the use of surface enzymatic systems to obtain cellulosic materials with a high hydrophobicity and other advanced properties, respectively. Finally, the fourth part involved using various analytical methods to elucidate the nature of the underlying grafting mechanisms.

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Chapter 2

Materials and Methods

2.1. Raw material

The raw materials used in the present thesis were mainly paper-based products, which were commercial grade or laboratory-made. A special interest in treating already formed paper structures arose, because of the lack of studies aiming to functionalize materials like paper, presenting a 3D heterogeneous fibrous structure. In addition, functionalization of paper was commonly achieved by treating the pulp stock (fibers in aqueous suspension) prior to the manufacture of the paper sheet. Here, the main goal is to achieve the functionalization of already formed cellulosic substrates, by means of surface treatments.

Three raw materials were used in the non-enzymatic functionalization studies using β-Cyclodextrins, namely: a noncoated paper (70 g/m^2), a 100% cotton-based medical bandage (70 g/m^2), and a medical crepe paper (60 g/m^2, from Ahlstrom, France) used for sterilization purposes. The main raw materials used in the enzymatic functionalization studies using Laccase were Eucalyptus ECF pulp supplied by ENCE® (Spain), and filter paper sheets (73 g/m^2, 170 μm thickness, and 0.260 KN/m wet strength) purchased from

FILTERLAB®, Table 2-1 (Sant Pere de Riudebitlles, Barcelona–Spain). Unbleached flax and sisal pulps from CELESA® (Tortosa, Spain) were also used in a lower extent. Refined and non-refined samples from the eucalyptus, flax, and sisal pulps were used to prepare handsheets in a Rapid-Köten lab former according to ISO 5269-2:2004, while filter paper sheets were used as received.

Table 2-1 Properties of the paper filter sheets

Property	Mean	StD
Bursting Strength Index [kN/g]	2.23	0.23
Tear Strength Index [mNm²/g]	12.36	1.44
Folding Endurance [log n]	3	0.3
Tensile Strength Index [Nm/g]	38.38	5.43
Wet Tensile Strength Index [Nm/g]	6.72	0.73
Zero-Span [N/cm]	73	9

Cellulose model surfaces were considered for the analysis of physicochemical interactions between cellulose and the enzymatic systems (chapter 6). Models consisted on bare silica wafers, QCM-D silica sensors obtained from Q-Sense (Västra Frölunda, Sweden), and nanofibrillar cellulose (CNF) obtained from bleached hardwood pulp (birch). The nanofibrils were produced following the method described by Ahola *et al.*, (2008).

CNF was obtained by mechanical treatment (five times with Masuko grinder) and then further disintegrated by microfluidization with 20 passes. The individual cellulose nanofibrils were then produced using mechanical stirring and tip ultrasonication (10 min, 25% amplitude). The resulting CNF suspension was centrifuged at 10,400 rpm for 45 min and nanofibrils were then collected from the supernatant by pipetting.

2.2. Functionalization with β-cyclodextrins

2.2.1. β-Cyclodextrin and complexation

β-Cyclodextrins (β-CD) are cyclic oligosaccharides consisting of seven D-glucose units linked by α-1,4-glycosidic bonds as shown in Fig. 2-1. This is similar to the starch components amylose and amylopectin (Dodziuk, 2008; Szejtli and Osa, 1996; Bender and Komiyama, 1978). In all cyclodextrins, the glucose units are arranged in such a way that

the hydroxymethylene groups are pointing downwards, whereas the hydroxyl groups are pointing upwards. As a result of this, a hydrophilic outer space and a hydrophobic inner space are formed leading to the particular ability of the cyclodextrins to form host–guest complexes with organic components having appropriate diameters and physical interactions (Bender and Komiyama, 1978; Szejtli, 1988). The formation of the mentioned inclusion complexes are successfully exploited in different fields such as food manufacturing, cosmetics, pharmaceuticals, analytical and organic chemistry.

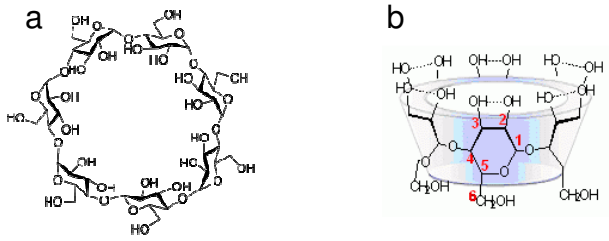

Figure 2-1 General structure of the β-cyclodextrins (a) and representation of their cup-shaped structure (b).

In the present thesis β-CDs were used to obtain new cellulosic surface-modified materials able to release antiseptic molecules. The antiseptic property was achieved by encapsulation of chlorhexidine digluconate (digCHX) into the β-CDs cavities. The size, and the physical properties of the digCHX molecule was optimal for the complex formation. β-CDs, and chlorhexidine digluconate were commercial chemical grade products, supplied from Sigma-Aldrich®.

2.2.2. Cyclodextrin grafting

The grafting of paper by β-CD was based on the pad-dry-cure textile finishing method, previously reported (Martel *et al.*, 2003). All the samples (4 cm x 4 cm) were oven dried at 105℃ for 20 min before soaking and immediately weighted. After, each single paper sheet was soaked into 100 mL aqueous solution containing 10 g of β-CD, 3 g of catalyst (sodium dihydrogen hypophosphite), and 10 g of crosslinking agent (citric acid). Then, the samples were dried at 105℃ during 10 min. The grafting reaction occurs via a polyesterification reaction which is described in chapter 3; the structures of the cyclodextrin/cellulose/citric acid adducts were described in detail in previous publications (Martel *et al.*, 2002). The grafting reaction occurred in a ventilated stove at different temperatures (between 130℃ and 160℃) and times (between 5 and 50 min). After the reaction, the ensuing specimens were abundantly washed with hot (80℃)

distilled water to remove the nonlinked CDs, oven dried (at 104ºC for 30 min) and weighted. The weight gain (WG) (%) representing the yield of the grafting reaction was calculated using the expression:

$$WG(\%) = (M_f - M_i)/M_i \times 100 \quad [2\text{-}1]$$

where M_i and M_f are the sample weights, before and after the grafting treatment, respectively. The data reported in this work are the average values of three experiments.

2.2.3. Adsorption of digCHX

β-CD-modified and unmodified sheets were dried at 105ºC for 20 min before being weighted. Then, the samples were treated with the antibacterial agent by dipping at room temperature into 20% (w/v) chlorhexidine digluconate (digCHX) solution, for 1 h. Afterward, the sheets were dried with blotting paper to remove the excess of solution and oven dried at 40ºC in a ventilated oven during 24 h. The resulting sheets were finally considered totally dried (without any residual humidity) and immediately weighted. The weight gain (ΔM_{digCHX}) represents the amount of antiseptic agent loaded onto sample sheets, as calculated from the following equation:

$$\Delta M_{digCHX} = M_{f2} - M_f \quad [2\text{-}2]$$

where M_f is sample weight after the grafting treatment and M_{f2} is the sample weight after loading with the antiseptic agent. The amount of CHX adsorbed has been reported in grams per gram of the substrate, as follows:

$$g_{digCHX}/g_{impregnated paper} = \Delta M_{digCHX}/M_{f2} \quad [2\text{-}3]$$

2.3. Functionalization using enzymatic systems

2.3.1. Laccases

The most used enzyme in the present thesis was a commercial grade laccase (Lacc) from *Trametes Villosa* (Tv) with an activity of 588 U/mL supplied by Novozymes® (Bagsvaerd, Denmark). Additionally, other commercial grade laccases from *Cerrena Unicolor* supplied by Fungal Bioproducts® (Fb) with an activity of 1660 U/mL, and a laccase from *Myceliphthora Thermophila* (Mt) with an activity of 532 U/mL supplied by Novozymes®, were also tested. Laccase activity was followed by measuring the oxidation of 2,2'-azino-bis(3-ethylbenzylthiazoline-6-sulphonate) (ABTS) in 0.1 M sodium acetate buffer (pH 5) at 436 nm (ε_{436} = 29300 M^{-1} cm^{-1}). One activity unit is defined as the amount of laccase that transforms 1 μmol/min of ABTS to its cation radical at 25ºC.

2.3.2. Phenolic compounds (PhC)

To achieve the functionalization of paper sheets by means of enzymatic treatments, the enzyme (laccase) was used in combination with phenolic compounds (PhC) which were substrates (or mediators) for the enzyme. The compound would be grafted onto the surface of cellulosic material upon its chemical modification by the enzyme. The main phenolic compound used in this work was lauryl gallate (LG) supplied by Sigma-Aldrich®. Soluble sulfonated kraft lignin (SL) or lignosulfonate, was obtained from Borreegard® (Sarpsborg, Norway), and used as received. SL was used in several treatments to enhance the dispersion of non-soluble phenolic compounds (such as LG). Lignosulfonates are water-soluble anionic polyelectrolyte biopolymers; they are salts of lignosulfonic acid that has been formed when pulp is manufactured by the sulphite method. The lignosulfonates are of varied composition because the woods are different, the extent of the lignin degradation can be different and a different number of sulfonic groups can have been added. The distribution of non-polar and polar groups, including the hydroxyl and sulfonic acid groups formed during the degradation of lignin, decides the properties of the particular lignosulfonate.

Other phenolic compounds used to confer hydrophobic or antioxidant properties to cellulose-based materials were: caffeic acid (CA), para-hydroxybenzoic acid (pHB), acetosyringone (AS), syringaldehyde (SA), p-coumaric acid (pCA), vanillin (V), acetovanillone (AV), ferulic acid (FA), coniferyl aldehyde (CFA), sinapic acid (SPA), sinapyl aldehyde (SAD), violuric acid (VA), methyl syringate (MS), gallic acid (GA), methyl gallate (MG), ethyl gallate (EG), propyl gallate (PG), butyl gallate (BG), octyl gallate (OG), stearyl gallate (SG), β-sitosterol (β-S), α-tocopherol (α-TP), 4-[4-(trifluoromethyl)phenoxy]phenol (TPH), isoamyl salicylate (IS), 2,4,6-tris(1-phenylethyl)phenol (TRP), tannic acid (TA) and resveratrol (RV), all supplied by Sigma-Aldrich®.

2.3.3. Pre-treatment of paper sheets with cationic compounds

In the cases in which paper sheets with cationic character were required, they were obtained either by treating the fibers (pulp suspension) or the surface of already-formed paper sheets (coating sauce) using several cationic compounds. Two commonly used compounds related to wet strength paper additives (polyamidoamine-epichlorohydrin resin, and cationic starch, Figs. 2-2a and 2-2b), and a non-papermaking additive (Methyl-glycol chitosan, Fig. 2-2c) were used as sources of cationic charge-conferring compounds.

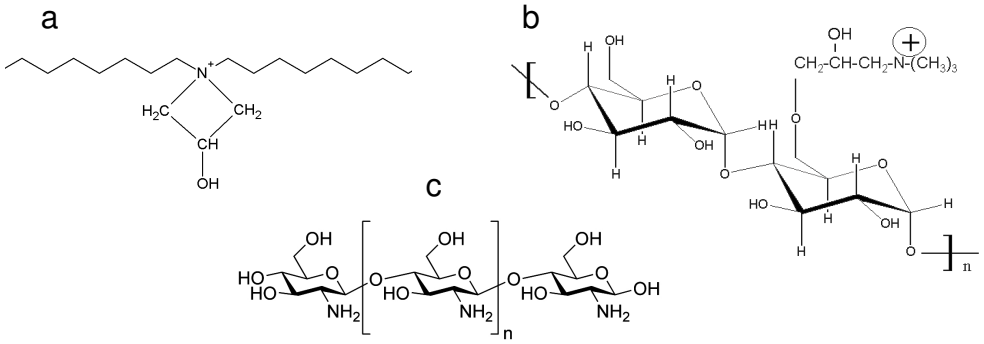

Figure 2-2 Structure of an azetidinium group, a source of cationic charge and reactivity on PAAE wet-strength resins (a), chemical structure of part of a cationic starch molecule (b), and part of a common chitosan molecule (c).

Eka WS 505 polyamidoamine-epichlorohydrin (PAAE) resin (20 ± 0.5% (w/w) of solid content, 1060 ± 20 Kg/m^3 (at 20℃) density, and 70-130 mPa·s Brookfield viscosity) was kindly supplied by Azko Nobel Chemicals® (El Prat de Llobregat, Spain). PAAE resin was applied at 0.7% of solids onto fibers, pH 7, and diluted in water at 10% (w/w). The paper pulp was stirred vigorously and the PAAE resin was added using a disposable pipette; the whole was left under agitation for 5 minutes before obtaining the laboratory handsheets.

The cationic starch (CSt) was kindly supplied by Roquette® (Benifaió, Valencia, Spain), and it was cooked at 5% consistency at 90℃ during 1 hour. Afterwards, the pulp stock was treated with 1% (w/w) of CSt, diluted in water at 0.5% (w/w), before obtaining the handsheets.

Methyl-glycol chitosan (MGCh) was supplied by Sigma-Aldrich®. The chitosan coating sauce was prepared by mixing 0.1 g of finely grinded MGCh powder with 5mL of acetic acid at 1% (w/w), and stirred at room temperature for 30 minutes. Afterwards, the coating colour was applied on the surface of the laboratory-made eucalyptus sheets using a mayer bar, and sheets were allowed to dry under normalized atmosphere (23℃, 50% RH).

2.3.4. Enzymatic treatments in the presence of paper sheets

The enzymatic treatments using finished paper sheets (PS) were performed by cutting paper into 4cm diameter circular sheets. Treatments were performed in an agitated bath, using 250 mL erlenmeyers. The treatment conditions were based on those reported on previous studies by Garcia-Ubasart *et al.*, (2011) by means of treating the fibers

suspension. Laccase-lauryl gallate (LG+Lacc) treatments were performed by using an amount of 1.5 g of paper sheets at 3 % consistency in 50mM sodium tartrate buffered at pH 4, and 40U/g odp (oven dried pulp) laccase. LG was applied at 4 and 8% (w/w). The PS were soaked in the sodium tartrate solution placed into the erlenmeyers one by one. Afterwards, the LG was added into the erlenmeyers and the set was put under agitation for 10 minutes at 50°C, to homogenize and let the set reach the treatment temperature. Finally, the enzyme was added and the erlenmeyers were put under agitation at 50°C during the established treatment time (1 to 4 h). The reaction was stopped by cooling the erlenmeyers with cold water, removing the paper sheets one by one, and extending them onto blotting paper. In the cases in which a surfactant was applied to improve the LG dispersion, SL (soluble sulfonated lignin) was added at 4%(w/w) into the reaction vessel at the same moment as LG.

2.3.5. Sonication

Several compounds used in the present thesis to achieve the specific properties in cellulosic substrates presented a very low surface free energy and were thus highly hydrophobic. As a result, those compounds were insoluble in the treatment solution and poorly dispersed on the paper surface after treatments. In fact, adducts easily formed aggregates that were still present after the enzymatic reaction. For this reason, the introduction of an ultrasound step prior to the enzymatic reaction allowed the aggregates to be efficiently disintegrated and improved dispersion of the compounds and interaction with the enzyme. Aqueous solutions of the non-soluble PhC in 50 mM sodium tartrate buffer at pH 4 were sonicated on a Hielscher® Ultrasonic Processor UP100H at 100% amplitude for 30 min. The resulting consisted in a homogeneous and well-dispersed mixture of the compound in the aqueous medium. However, the obtained suspension needed to be stirred constantly, otherwise it precipitated rapidly.

2.3.6. Functionalization solution (FS) preparation and application

The treatment conditions were based on those reported in a previous study (Cusola *et al.*, 2013) by means of treating paper sheets. However, in the present treatments the main difference was that the enzymatic reaction was performed with no presence of paper sheets into the reaction vessels, and the resulting post-enzymatic solution, namely: functionalization solution (FS), was used to impregnate the paper sheets by dipping them into this solution.

The enzymatic treatments were performed in an Ahiba Spectradye dyeing apparatus from Datacolor® equipped with closed vessels 250 mL in volume with final

concentrations equivalent to 50mM sodium tartrate buffer (pH 4), PhC (1.2 g/L), lignosulfonate (1.2 g/L) and 1.2 U/mL laccase. SL was introduced in the reaction to improve dispersion of the PhC. If the PhC was hydrophobic and no-solubilisation in water was produced, then it was applied as a colloidal suspension prepared upon ultrasonication, achieving a reduction in the PhC effective size and ensuring homogeneous dispersion. The PhC and sulfonated lignin (or lignosulfonate, SL) were added into the beakers and kept under agitation for 10 minutes at 50℃. Following, the enzyme was added and the beakers were kept stirred during 4 h reaction. The reaction was stopped by quenching the beakers with cold water. Several control treatments to obtain control functionalization solutions (KFS), were also performed to elucidate the role of each of the compounds present in the FS (Table 2-2).

The obtained solution (functionalization solution, FS or KFS) was applied at room temperature to the paper sheets by impregnation/immersion. With this purpose, the paper sheets were cut into circular pieces 4 cm in diameter and soaked in the FS or KFS by keeping the sheet submerged for 3 seconds. Then the paper sheet was removed, spread onto blotting paper, and allowed to dry in a normalized atmosphere (23°C, 50% RH) or using forced-drying devices (by the drying section of the Rapid-Köten lab former, or by oven-drying).

Table 2-2 Conditions used to obtain the aqueous functionalization (FS) and control systems (KFS) for the paper surface treatments. The control treatments involved various combinations of the compounds present in the FS (PhC, SL and Lacc).

	System	Phenolic compound dose (g/L)	Sulfonated lignin dose (g/L)	Laccase dose (U/mL)
FS	PhC+SL+Lacc	1.2	1.2	1.2
KFS	PhC	1.2	—	—
	SL	—	1.2	—
	Lacc	—	—	1.2
	PhC+Lacc	1.2	—	1.2
	SL+Lacc	—	1.2	1.2
	PhC+SL	1.2	1.2	—

2.3.7. Cellulose model surfaces

Model surfaces for adsorption tests using the FS and KFS, consisted of bare silica wafers (Fig. 2-3a) and QCM-D silica sensors (Fig. 2-4a) obtained from Q-Sense (Västra Frölunda, Sweden). For adsorption experiments using CNF, silica sensors were first cleaned by

immersion for 20s in 10% NaOH-solution followed by rinsing with Millipore water and drying under nitrogen. Afterwards the sensors were pre-treated with polyethylene-imine (PEI) polymer and dried followed by spin coating with the previously mentioned dispersion of nanofibrillar cellulose (CNF) at 3000 rpm for 1 min. Finally, the CNF-coated sensors were rinsed with Millipore water, and dried in an oven at 80°C for 15 min and stored until use.

Silica was used as model for cellulose since it contains free silanols and geminal silanol groups on its surface (Fig. 2-3b), which simulate the free hydroxyl groups of cellulose. By using silica it is also possible to avoid the contributions from the otherwise soft nature of cellulose substrates: silica, unlike cellulosic, presents a flat and smooth surface with no porosity and reduced roughness (no swelling, hydration, diffusion, etc.).

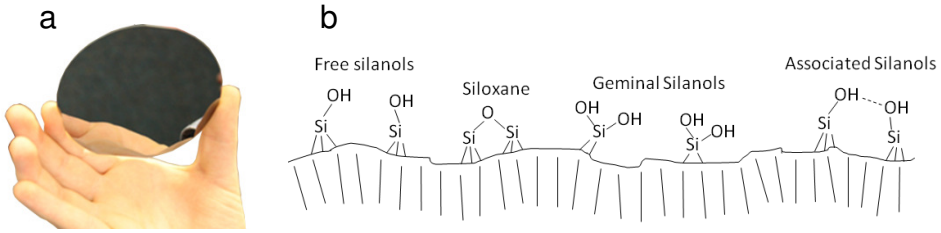

Figure 2-3 Silica wafer used in adsorption tests (a), and schematic depiction of the different types of hydroxyl groups on a silica surface (b).

2.4. Assessment of grafting

In the present thesis several techniques were used to achieve the surface functionalization of cellulosic materials. Although the main interest was focused on the functionalization or grafting using enzymatic processes, a non-enzymatic process was also considered. After grafting of cellulosic materials to achieve a specific property, the modified materials needed to be characterized in terms of finding evidences that such grafting had took place. For this reason, several tests and analytical techniques were used, depending on which kind of functionalization was produced.

2.4.1. FTIR analysis

In the functionalization by using β-CDs, and in that obtained upon enzymatic treatments, Fourier transform infrared spectroscopy (FTIR) was conducted in order to assess the grafting, by comparing non-treated and treated paper sheets using a Perkin Elmer PARAGON 1000 FTIR spectrometer equipped with spectrum software. The spectra were obtained by analyzing directly the surface by using ATR system (GoldenGate, JASCO).

The FTIR spectra were collected with a resolution of 4 cm^{-1} in the range of 4000–400 cm^{-1}. Thus, 16 scans were taken within this interval.

For the FTIR analysis of the enzyme-modified PhC, 200 mL of the FS and KFS (see table 2-2) were prepared using the doses and reaction conditions explained previously. Then, the solutions were centrifuged at 6000rpm during 90 min, and the supernatant eliminated from the sample. Afterwards, the sample was allowed to dry at room temperature until a dry solid residue was obtained, and the solid was placed into a desiccator overnight. Finally, it was finely grinded and mixed with potassium bromide for KBr pellets preparation. Samples were measured using a Nicolet 6700 FT-IR Spectrometer equipped with spectrum software in the range of 4000–400 cm^{-1}.

In the ATR-FTIR experiments described in chapter 8, the solid residue was directly analyzed on a Perkin Elmer Spectrum 100 FT-IR spectrometer equipped with universal ATR sampling accessory by performing 32 scans at 1 cm^{-1} intervals over the wavenumber range 4000–550 cm^{-1}. The solid residues were placed directly on the ATR accessory, and the measuring cell was washed with de-ionized water and ethanol between measurements.

2.4.2. Scanning Electron Microscopy Imaging

The analysis of the surface and cross-section of the treated and non-treated substrates with the β-CDs technique was performed using a scanning electron microscope (SEM) (Quanta 200, FEI Company, Hillsboro, Oregon) under moderate vacuum at an operating voltage of 7 kV. Dried cellulose-based fibre mats were gold-coated by sputtering for 15 s.

SEM microscopy was also used to analyze the surface of paper sheets which were treated using the enzymatically-obtained functionalization solution (FS). Analyses were performed with a JEOL JSM-6400 microscope under moderate vacuum at an operating voltage between 5 and 10 kV. The samples were previously cut in small pieces and coated in a sputter coater SCD005 with a very thin layer (12 nm thick) of gold-palladium, in order to obtain a conductive surface.

2.4.3. Washing and Soxhlet extractions

The grafted papers using β-CDs and those treated with enzymatic treatments were abundantly washed with hot (80℃) deionized water during 30 min after the respective reaction processes to ensure the removal of the fraction of compounds failing to bind covalently to fibres. After washing, sheets were removed one by one and extended onto blotting paper, and left drying under normalized atmosphere (25°C, 50% RH).

Soxhlet extractions were also performed onto paper sheets using acetone. To this end, 2g of small paper pieces were added to a cellulose extraction thimble and placed in the extractor; 50mL of acetone was then added to a flask which was also fitted to the extractor. Extraction was carried out in three steps, a first 45 minutes boiling step (140℃) in which sheets were completely soaked into acetone, a second rinsing step in which sheets were rinsed with acetone during 1.5 hours, and a third recovering step in which acetone is evaporated from the sample for 15 minutes. After the extraction, samples were placed onto blotting paper and left for complete drying under normalized atmosphere (25℃, 50% RH).

2.4.4. Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

Many important physical and chemical processes can be followed by observing the associated mass changes. In this sense, a quartz crystal microbalance (QCM) consists of a thin quartz disk with electrodes plated on it as can be seen in Fig. 2-4a. The application of an external electrical potential to a piezoelectric material produces internal mechanical stress. As the QCM is piezoelectric, an oscillating electric field applied across the device induces a mechanical oscillation of characteristic frequency, f, to the crystal. Then, the deposition of a thin film of any material on the crystal surface decreases the frequency in proportion to the mass of the film, providing information on the amount and viscoelastic properties of bound mass. A typical experimental apparatus set up is shown in Fig. 2-4b.

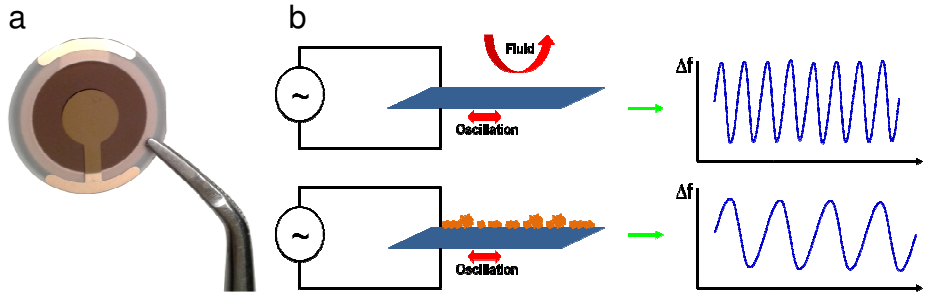

Figure 2-4 Image of a QCM-D silica sensor (a), and schematic representation of the QCM-D device operation and the effect of mass deposition on the surface of the electrode (b).

In situ QCM-D experiments were performed using a Q-Sense E4 instrument (Västra Frölunda, Sweden) operated in continuous mode. Any molecular interaction between the functionalization solution (FS) (and control systems) with the substrate will produce a shift in the resonance frequency, f, which can be monitored and used to quantify any gain

in mass (for example, via adsorption). A linear relationship between the shift of resonance frequency (Δf) and the change in mass (including the contribution of coupled water) due to adsorption on the surface of the sensor can be determined via the Sauerbrey model, which assumes that the adsorbed layer is thin, homogeneous and rigid (i.e. the layer has mechanical properties close to those of quartz) (Sauerbrey, 1959):

$$\Delta m = -C\Delta f / n \qquad [2\text{-}4]$$

where Δm, Δf, C and n, are the variation of mass [ng·cm^{-2}], the resonance frequency [Hz], a constant for the crystal [ng·Hz^{-1}·cm^{-2}] and the overtone number (n=1 for the fundamental frequency), respectively. For the present setup, C equals 17.7 ng/cm^2·s^{-1} at f = 5 MHz. The third overtone (n=3) was used in interpreting the QCM data in this study.

In addition to resonance frequencies, QCM-D measures energy dissipation of the system. The changes in dissipation, D, was thus monitored to determine the frictional (viscous) losses in the adlayer. D is related to the viscoelastic properties of the layer(s) deposited on the sensing electrode and can give indication of its stiffness. For example, the presence of a soft layer on the electrode enhances the damping of the vibration and result in a higher dissipation. The energy dissipation D is related to the frequency Δf and the decay time τ as follows:

$$D = \pi \Delta f \tau \qquad [2\text{-}5]$$

where τ values are obtained by periodically disconnecting the oscillating crystals from the main circuit via a computer-controlled relay.

Time-resolved adsorption was followed on bare silica sensors and on silica sensors covered with a CNF film. The QCM-D measurements were performed under continuous flow rate of 100 μL/min at 25°C.

2.4.5. Atomic force microscopy (AFM)

Atomic force microscopy or AFM is a method of measuring surface topography in the nanometer scale. The AFM determine the local height of a surface by "touching" it by means of a sharp tip with a radius of 20 nm. Variations in tip height are recorded while the tip is scanned repeatedly across the sample, producing a topographic image of the surface. The tip is attached to a flexible microcantilever which bends under the influence of force. The bending produces a change of angle of inclination, measured by bouncing a laser beam off of the cantilever and into a position-sensitive detector (split photodiode), the output of which gauges small movements of the laser spot. The vertical tip movement in turn is quantified from the cantilever bending.

A Nanoscope IIIa Multimode scanning probe microscope (Digital Instruments, Inc., Santa Barbara, CA) was used to characterize the surface topography of the QCM-D silica sensors before and after surface treatment. The images were scanned in tapping mode using a J-scanner and silicon cantilevers (NSC15/AIBS from Micromasch, Tallinn, Estonia). The radius of curvature of the AFM tip according the manufacturer was less than 10 nm, and typical resonance frequency of the cantilever was 325 kHz. At least two different areas were analyzed on each sample; scan sizes included 10x10, 5x5, and 1x1 μm^2. AFM images where flattened following first order conversion. Image analysis was performed using Nanoscope software (ver. V6.13 R1, Digital Instruments, Inc.) from which rms roughness and Z-sections in line profiles were determined.

2.5. Enzymatic products characterization

Enzymatic products refer to the aqueous solutions obtained upon enzymatic reactions of phenolic compounds (PhC) with the Laccase (and the corresponding controls). In the case of a complete treatment (not a control treatment), the solution will contain: the enzyme (laccase), the phenolic compound oxidized by the enzyme (mediator, or functionalizing compound), and/or an enhancer (compound which is added to improve the effectiveness of the enzyme, a lignosulfonate in the present thesis).

In the case of the enzymatic treatments in the presence of the paper sheets, the enzymatic product refers to the effluent resulting from the treatment of the sheets.

2.5.1. UV-VIS spectra and residual laccase activity

The oxidation of the compounds caused by the laccase was analyzed by UV-visible spectroscopy, using a Thermo Scientific Evolution 600 spectrophotometer. The tests consisted in obtaining the UV-visible absorbance spectrum of the effluents between 190 and 900 nm. For this purpose, the reference quartz cubette was filled with the solutions to be characterized (buffer+enzyme). The effluents resulting from treatments were filtered using 0.2 μm filters, and placed in the quartz cubette destined to measure.

For the non-enzymatic functionalization studies using cyclodextrins, UV-Vis spectroscopy was used to determine the amounts of digCHX released from the functionalized paper under aqueous environment. The absorbance values of the aliquots were measured at 254 nm using a UNIcam UV500 Thermospectronic spectrophotometer.

Residual laccase activity was measured in control and enzymatic treatments considering the absence/presence of paper sheets, by measuring the oxidation of 2,2'-azino-bis(3-ethylbenzylthiazoline-6-sulphonate) (ABTS) in 100 mM sodium acetate buffer, pH 5 at a

wavelength of 436 nm, and using 29300 $M^{-1}cm^{-1}$ as the molar extinction coefficient at 25°C. Residual laccase activity values were corrected by the dilution factor, and expressed as activity units per g or mL.

2.5.2. Particle size analysis (DLS)

Dynamic Light Scattering (DLS) is also known as Photon Correlation Spectroscopy. This technique is used to determine the size of particles typically in the sub micro region. Shining a monochromatic light beam, such as a laser, onto a solution with spherical particles in Brownian motion causes a Doppler Shift when the light hits the moving particle, changing the wavelength of the incoming light. This change is related to the size of the particle. It is possible to compute the sphere size distribution and give a description of the particle's motion in the medium by measuring the diffusion coefficient of the particle, and using the autocorrelation function.

The time-dependent fluctuations in the intensity of scattered light are analyzed using an autocorrelator which determines the autocorrelation function of the signal. The correlation function of the signal G, decays at an exponential rate which is dependent on the diffusion of the particles being measured (Kaszuba *et al.*, 2008) (ISO13321:1996).

$$G = \int I(t)I(t+\pi)dt = B + Ae^{-2qD\tau} \quad [2\text{-}6]$$

where B is the baseline, A the amplitude, D the translational diffusion coefficient, and q is the scattering vector. The scattering vector "q" is defined as follows:

$$q = \frac{4\pi\tilde{n}}{\lambda_0}\sin\left(\frac{\theta}{2}\right) \quad [2\text{-}7]$$

where ñ represents the solvent refractive index, λ_0 the vacuum wavelength of the laser, and θ the scattering angle. The DLS technique measures the diffusion coefficients of molecules or particles undergoning Brownian motion, and the size of a particle is calculated from the translational diffusion coefficient by using the Stokes-Einstein equation:

$$d(H) = \frac{kT}{3\pi\eta D} \quad [2\text{-}8]$$

where d(H) is the hydrodynamic diameter, D the translational diffusion coefficient, T the temperature, K the Boltzmann's constant and η the viscosity. The diameter obtained by this technique is the diameter of an ideal sphere that has the same translational diffusion coefficient as the particle.

In the present thesis, the FS and all the control solutions (KFS) of the treatment using laccase, LG, and SL were measured using DLS in order to determine the particle size. Size

was obtained from the correlation function. It is difficult with DLS to measure a mixture of large (e.g. 1000 nm) and small (e.g. 10 nm) particles, because the contribution to the total light scattered by the little particles will be extremely small; there is a danger that the light from the larger particles swamp the scattered light from the smaller ones (Malvern technical note MRK656-01).

Hydrodynamic diameter and size distributions of the colloidal particles present in the FS as well as control treatments were determined using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.) light scattering (DLS) device.

2.5.3. Z-Potential tests

The stability of the enzymatic products was characterized by measuring the electrophoretic mobility of the solutions using a Malvern Zetamaster device equipped with a laser Doppler velocimeter.

Electrophoresis is an electrokinetic phenomenon by which charged surfaces (colloids) move with respect to a stationary liquid under the action of an applied electric field (Shaw, 1992). Charged particles suspended in the electrolyte are attracted towards the electrode of opposite charge. Viscous forces acting on the particles tend to oppose this movement. When equilibrium is reached between these two opposing forces, the particles move with constant speed. The velocity of the particle is dependent on several factors, such as the strength of the electric field, the voltage gradient, the dielectric constant of the medium, or its viscosity. The velocity of a particle in an electric field is commonly referred to as its electrophoretic mobility. The zeta potential (Z-potential) is a value related to the stability of colloidal dispersions, and can be determined by measuring the electrophoretic mobility.

The zeta potential of the particle can be obtained by applying the Henry equation:

$$U_E = 2\varepsilon z f(ka)/3\eta \qquad [2\text{-}9]$$

Where z is the Zeta potential, U_E the electrophoretic mobility, ε the dielectric constant, η the viscosity of the medium, and f(ka) the Henry's function. To determine f(ka), two values are generally used, either 1.5 or 1.0. When measuring zeta potential in aqueous solutions of moderate electrolyte concentration (electrolytes containing more than 10-3M molar salt), and particles larger than 0.2 microns, a value of 1.5 is used and this is referred to as the Smoluchowski approximation. For smaller particles in low dielectric constant media f(ka) becomes 1.0, and allows an equally simple calculation. This is referred to as the Huckel approximation. Non-aqueous measurements generally use the Huckel approximation.

2.5.4. *Turbiscan® and Turbiscan®-OnLine tests*

The stability of the FS and control products was evaluated using a Turbiscan® MA 2000 (for space-resolved measurements), and a Turbiscan®-On-Line (for time-resolved measurements) analyzers (Formulaction, France). The aqueous products after the enzyme treatments were placed in a 80 mm long cylindrical glass cell. The detection head consisted of a pulsed near-infrared light source (λ=850 nm) and two synchronous transmission and backscattering detectors. As a result it gets objective information on time and space dependence of both signals. Turbiscan® is able to detect migration of particles (sedimentation or creaming) and particle size changes (originated in flocculation, agglomeration or coalescence). The optical sensors collected the transmission and backscattering signals through a vertical scanning of the sample with a step of 40 μm at given time intervals (Fig. 2-5).

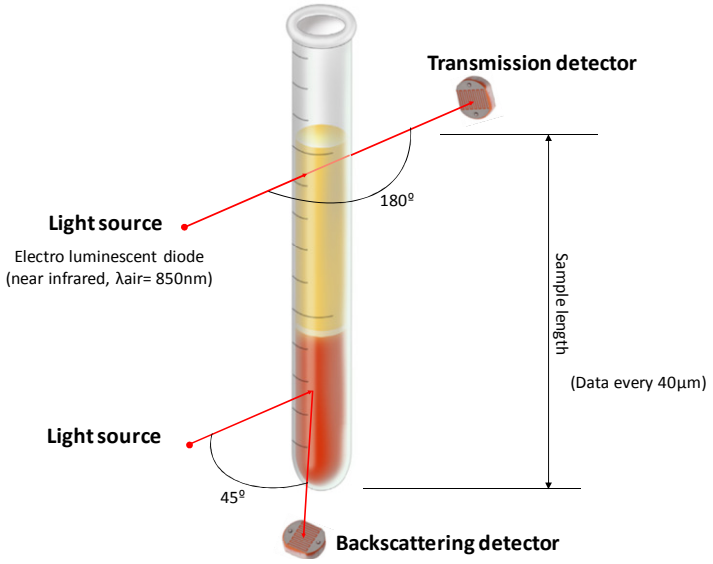

Figure 2-5 Turbiscan® cylindrical measuring cell with the transmission and backscattering measurement geometries.

In a first set of experiments, scans were performed every min, during 30 minutes, after, the time interval between data points was increased in order to evaluate the long-time stability. The experimental results are represented as a set of curves indicating the percentage of transmitted light as a function of the sample tube height.

The optical analyzers in Turbiscan® detect the light flux backscattered at 135° by a dispersed and low-transparency medium in a cylindrical cell. The backscattered flux (BS) can be linked to λ^* (as first approximation, BS is inversely proportional to λ^* square root):

$$BS = 1/\sqrt{\lambda^*} \quad [2\text{-}10]$$

$$\lambda^* = 2d/3\phi(1-g)Qs \quad [2\text{-}11]$$

where λ^* (photon transport mean free path) represents the mean distance travelled by photons into the dispersion medium, Ø is the volume fraction of the particles, and d is the mean diameter. Qs and g are parameters given by Mie theory (Mie, 1908).

Transmission measurements were performed by the Turbiscan® by sending a light beam through the cell and detecting the photons that cross the dispersion, without being diffused. The Beer-Lambert law gives an analytical expression of the transmitted flux (T) measured as a function of λ:

$$T = T_0 \cdot e^{(-2r_i/\lambda)} \quad [2\text{-}12]$$

$$\lambda = 2d/3\phi Qs \quad [2\text{-}13]$$

where λ is the photon mean free path, r_i is the internal radius of the measurement cell, and T_0 denotes the transmittance of the continuous phase. The stability of FS and control treatments were evaluated by analyzing the variation of the backscattered flux (BS) and transmitted flux (T); both parameters are dependent on the particle volume fraction Ø and the particle mean diameter.

2.5.5. Cyclic voltammetry

Cyclic voltammetry is the most widely used technique for acquiring qualitative information about electrochemical reactions. It offers a rapid location of redox potentials of the electroactive species; it can provide qualitative information about the number of oxidation states and their stability, as well as the rate of heterogeneous electron transfer reactions. It can also be used in combination with simulation software to calculate rates of homogeneous and heterogeneous reactions.

In cyclic voltammetry, the potential of a small, stationary working electrode is changed linearly with time starting from a potential in where no electrode reaction occurs and moving to potentials where reduction or oxidation of a solute (material under sudy) occurs (Fig. 2-6). After traversing the potential region in which one or more electrode reactions take place, the direction of the linear sweep is reversed and the electrode

reactions of intermediates and products, formed during the forward scan, often can be detected (Evans *et al.*, 1983).

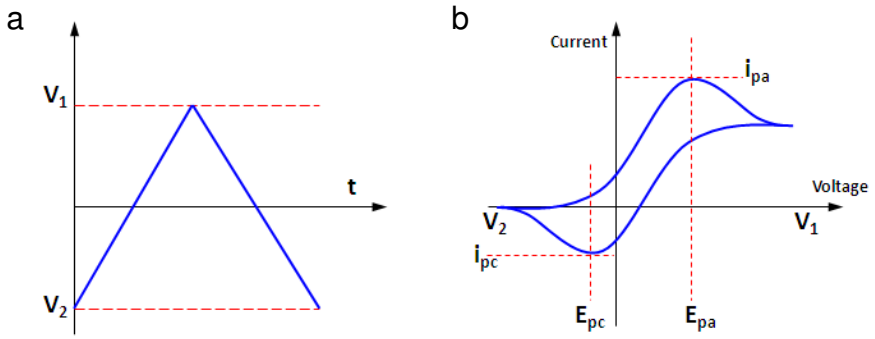

Figure 2-6 Voltage swept between two values at a fixed rate (a), and typical cyclic voltammogram recorded for a reversible single electrode transfer reaction, in which the solution contains only a single electrochemical reactant (b).

A typical electrochemical cell for cyclic voltammetry measurements consists of three electrodes and an electrolyte (Fig. 2-7). The electrolyte is needed in order to provide electrical conductivity between the electrodes. The first of the three electrodes is known as the test or working electrode. This is the electrode at which the electrochemical phenomena (reduction or oxidation) being investigated are taking place. The second functional electrode is the reference electrode. This is the electrode whose potential is constant enough to be taken as the reference standard, against which the potentials of the other electrodes present in the cell can be measured. Commonly used reference electrodes are the silver-silver chloride electrode (Ag/AgCl/4M KCl, e=0.222 V) or the Kalomel electrode (Hg/HgCl/KCl). The final functional electrode is the counter or auxiliary electrode, which serves as a source or sink for electrons, so that current can be passed from the external circuit through the cell. In general, neither its true potential nor current is ever measured or known.

Cyclic Voltrammetry has been used to study the chemistry of gallates and the effect of lignosulfonates (SL) in the redox processes. Voltammetric studies were performed using a μAutolab Type III (EcoChemie, The Netherlands) potentiostat/galvanostat controlled by Autolab GPES software version 4.9. All experiments were carried out in a thermostatic, 20 mL, three-electrode configuration cell (Metrohm). The working electrode was a glassy carbon electrode with a surface diameter of 3 mm (Metrohm, The Netherlands). A platinum electrode and a silver–silver chloride (Ag/AgCl) electrode

(Metrohm, The Netherlands) were used as counter and reference electrode, respectively. The glassy carbon surface was renewed by polishing with 1.0 and 0.3 μm α-alumina (Micropolish, Buehler, Germany) on a microcloth polishing pad (Buehler, Germany), followed by washing in an ultrasonic bath (Selecta, Spain) for 2 min.

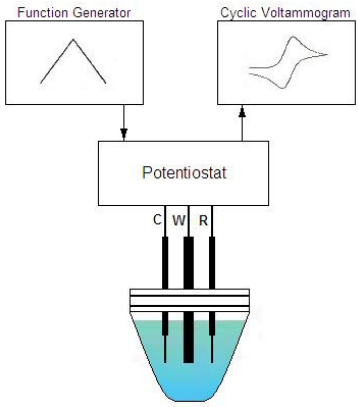

Figure 2-7 Schematic representation of a cyclic voltammetry cell and electrodes.

Voltammetric responses were recorded in 50 mL of 80/20 (v/v, %) aqueous-ethanol solution, containing 0.1 M tartrate buffer pH 4, and 0.5 mM of the compound to be studied (gallate compound) in the presence and absence of lignosulfonate (0.17 mg/mL). Potential was scanned from 0 to 800 mV vs. Ag/AgCl at a scan rate of either 5 mV/s or 200 mV/s.

In the cases where a specific compound was used as oxidation enhancer, the catalytic efficiency (CE) was determined. The CE is the increase in anodic peak current of the compound acting as catalyst (or enhancer) in the presence of the compounds to be oxidized. The CE was calculated as $\Delta I/IE$, ΔI being the increase in anodic current at 700 mV of the enhancer-compound mixture, and IE the oxidation current of the enhancer alone at the same potential.

2.6. Cellulosic material characterization

2.6.1. Hydrophobic behavior assessment

The hydrophobicity of the treated cellulosic materials was assessed either by using a Tappi standard method entitled Water Drop Test (WDT) (measured by dropping a drop of water on the surface of a specimen and determining the time in seconds for the drop to be completely absorbed), or by means of a contact angle goniophotometer (device using

an optical subsystem to capture the profile of a pure liquid on a solid substrate). Both methods give complementary information on the interaction between the treated cellulosic materials and water. Water Drop Test provides information about the absorption and capillary interactions of water in the structure of paper, while contact angle measurements are more related to the surface energy of the material. At least ten measurements of each test were conducted.

2.6.1.1. Water drop test (WDT)

The water drop test (WDT) was performed according to Tappi standard T835 om-08. Previously, the paper was conditioned according to ISO 187. The WDT consists in placing a drop of deionized water on the surface of paper and recording the time needed for complete absorption, which is signaled by the vanishing of the specular gloss of the drop. Fifteen measurements per treated paper sample were made. The geometry and characteristics of the setup are shown in Fig. 2-8.

The water drop test is used as a measure of the acceptability of tissue, towelling and blotter papers in sorptive tasks. Other test liquids such as ink, oil and milk can be used to assess the comparative performance of papers for specific applications.

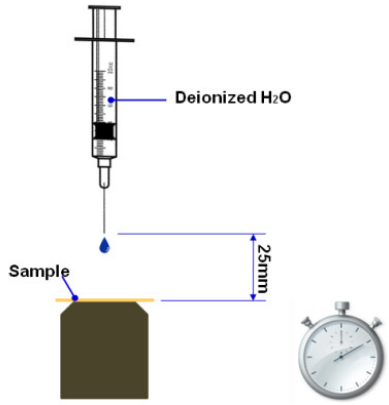

Figure 2-8 Geometry and characteristics of the setup for WDT measurement.

2.6.1.2. Water contact angle measurement (WCA)

The water contact angle (WCA) technique provides information about the affinity of a liquid to a solid surface. If water is used to measure the contact angle one can deduce the hydrophobic (great angle) or hydrophilic (small angle) character of the surface. It is generally assumed that, if a surface is hydrophobic, then its WCA will be greater than

90°; otherwise, the surface is assumed to be hydrophilic (Adamson, 1990). The contact angle is determined by using the sessile drop method. This method consists in dispensing a water drop of about few microliters from the needle of a precision syringe, and slowly approaching the solid material (from which the surface free energy value is required) until the contact between the drop and the surface is produced. The contact angle is the angle formed between the tangent to the drop's profile and the tangent to the surface at the intersection of the vapor, the liquid and the solid (Fig. 2-9a).

If several reference liquids are used, the surface energy of the solid can be calculated, discriminating between polar and dispersive components. The most common models are Good & Van Oss model or Owens & Wendt model (Owens and Wendt, 1969).

The water contact angle of the paper was measured by using a Dataphysics OCA15EC contact angle goniophotometer (Fig. 2-9b), using an image capture ratio of 25 frames/s. A 4 μL water drop was delivered to the sample surface. At least 10 measurements were made for each liquid probe. Also, changes in contact angle were monitored until complete absorption of each water drop.

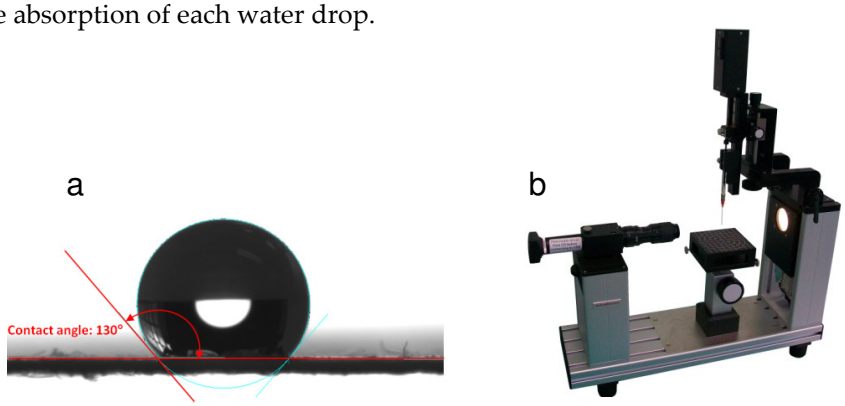

Figure 2-9 Contact angle determined for a water drop delivered onto the surface of a paper sheet (a), and contact angle goniophotometer Dataphysics® OCA15EC (b).

2.6.2. Antioxidant activity

2.6.2.1. Antioxidant activity of pure compounds, enzymatic systems, and (FS)

The antioxidant activity of the enzymatic systems was assessed by a procedure consisting in the quantification of the $ABTS^{\bullet+}$ radical decoloration described by several authors (Re *et al.*, 1999; Kubo *et al.*, 2000). The decoloration is due to the interaction of this radical with hydrogen or electron donor species. The method consists in the addition of the antioxidant compound to a pre-formed $ABTS^{\bullet+}$ radical solution and quantifying the

remaining $ABTS^{\bullet+}$ after a fixed time period, by means of UV spectrophotometry. The reduction in $ABTS^{\bullet+}$ concentration, induced by a certain concentration of antioxidant, is related to that of trolox and gives the TEAC (trolox equivalent antioxidant activity) value of that antioxidant (Arts *et al.*, 2004). Experiments were performed on a Thermo Scientific Evolution 600 spectrophotometer. ABTS was dissolved in water to a 7 mM concentration, and ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting the ABTS stock solution with 2.45 mM potassium persulfate (final concentration). The mixture was allowed to stand in the dark at room temperature for 12–16 h before use. Then, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (± 0.1) at 752 nm and equilibrated at 30°C.

Solutions of antioxidants and trolox with concentrations ranging from 0 to 2.5 mM were prepared in ethanol and conditioned at 30°C. After, 1 mL of diluted $ABTS^{\bullet+}$ solution (A = 0.70 ±0.1 at 752 nm) was added to 10 μL aliquots of each solution to obtain a final concentration ranging from 0 to 25 μM. The absorbance value of each solution at 752 nm was monitored every minute up to 6 minutes, or until stabilization. The inhibition % can be calculated as follows:

$$inhibition\,\% = (A_i - A_f)/A_i \times 100 \qquad [2\text{-}14]$$

where Ai is the initial $ABTS^{\bullet+}$ absorbance value, and Af is the $ABTS^{\bullet+}$ absorbance value after contact with the antioxidant compound aliquot.

The inhibition % was plotted against the concentration, and the slope of the curve was compared to that of trolox to calculate the TEAC. The TEAC was obtained from the ratio between the slope of the curve of the studied compound and the slope of the curve obtained for trolox.

2.6.2.2. Antioxidant activity of cellulosic substrates

The method to evaluate the antioxidant capacity of the treated papers was also based on the procedure which consists in the quantification of the $ABTS^{\bullet+}$ radical inhibition, but in this case, the method was also based on the method developed by Serpen *et al.*, (2007) for the measurement of the antioxidant activity of insoluble food components. Experiments were also performed on a Thermo Scientific Evolution 600 spectrophotometer. The ABTS radical cation ($ABTS^{\bullet+}$) was produced in the same way as explained in the previous paragraph for the measurement of the antioxidant activity of enzymatic products.

For the study of the antioxidant capacity of the treated papers, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.1) at 752 nm and equilibrated at 30°C. The 1 mL reference quartz cubette was filled with $ABTS^{\bullet+}$, recording its initial absorbance value at 752 nm. Samples of 10 mg of the treated paper sheets were cut in small pieces,

weighed and introduced into microcentrifuge tubes. A volume of 1.7 mL of the $ABTS^{\bullet+}$ solution was added to each one of the microcentrifuge tubes. Next, the tubes were agitated using a vortex mixer for 2 minutes to facilitate the surface reaction, centrifuged for 4 minutes at 6000 rpm, and allowed to stand for 30 minutes before conducting the absorbance tests. The absorbance measures at 752 nm of the centrifuged tubes were performed by taking 2-14 mL of the supernatant part of the tubes and placing it into a methacrylate cubette. The absorbance value of each sample at 752 nm was monitored every minute up to 6 minutes, or until stabilization. The inhibition % is calculated as indicated in equation 2-14, however in this case (paper samples) the Ai is the initial $ABTS^{\bullet+}$ absorbance value, and Af is the $ABTS^{\bullet+}$ absorbance value after contact with the paper samples.

2.6.3. Paper physical properties

The physicomechanical properties of the paper samples after treatment with FS were assessed by using commercial grade laboratory filter paper in accordance with ISO standards. The target properties were Bendtsen permeability (ISO 5636), burst strength (ISO 2758), tear strength (ISO 1974), folding endurance (ISO 5626), tensile strength (ISO 1924), wet tensile strength (ISO 3781), $Cobb_{60}$ (ISO 535) and zero-span tensile strength (ISO 15361).

At least 10 specimens per analysis were used to obtain a mean value and its standard deviation. The target properties were assessed in untreated, sodium tartrate (buffer)-treated, and FS-treated paper sheets.

2.6.3.1. Air permeability

The measurement of permeability describes the potential air flow through a sheet in z-direction. Permeability is assessed by measuring the time needed for a certain volume of air to flow through the structure of the sheet. The measurement is done with a defined air pressure (1.47 kPa) between the two surfaces of the sheet and with a defined measurement area (10 cm^2).

2.6.3.2. Burst strength

In the bursting strength method the sample is held between two annular clamps and hydrostatic pressure is applied through a rubber diaphragm of 30.5 mm diameter. Bursting Strength is measured as the maximum hydrostatic pressure required to rupture the sample by constantly increasing the pressure.

2.6.3.3. Tear strength

Tearing resistance is a measure of the force perpendicular to the plane of the paper necessary to tear a single (or multiple) sheet/s through a specified distance after the tear has already been started. In the Elmendorf-type tearing tester, the work necessary in tearing is measured by the loss in potential energy of the pendulum.

2.6.3.4. Folding endurance

The folding-endurance test is used to measure the ability of a paper to maintain its strength after repeated folding. In this method a strip of 15 mm wide and 100 mm length is held under tension and subjected to repeated folding; the number of folds necessary to cause failure is taken as a measure of folding endurance.

2.6.3.5. Tensile strength & wet tensile strength

Stress-strain curves provide a fundamental engineering description of the mechanical behavior of paper when subjected to tensile stress. The tensile strength test measures the force required to produce a rupture in a strip of paper, measured in machine or cross directions, expressed in kN/m. Tensile strength is indicative of fiber strength, fiber bonding and fiber length. Tensile strength can be used as a potential indicator of resistance to web breaking during printing or converting. In the tests, the specimen is subjected to uniaxial tension until failure. Tests were conducted on 15 mm wide specimen strips, using a computer interfaced to a J.J. Lloyd universal testing machine (model T5K).

Wet strength is the ability of the paper to retain its tensile strength when saturated with water. Wet tensile strength was conducted as the dry tensile strength method, but using strips previously soaked in de-ionized water for 5 s.

2.6.3.6. $Cobb_{60}$

Water absorbency is a measure of the amount of water absorbed by the wetted surface of paper and board materials. The Cobb test determines the amount of water absorbed into the surface by a sized (non-bibulous) paper, paperboard, and corrugated fibreboard paper or paperboard sample in a set period of time. $Cobb_{60}$ value represents the grams of water absorbed by 1 m^2 paper surface during flooded contact with 1 cm water column for 60 seconds. Water absorbency is expressed in g/m^2.

2.6.3.7. Zero-span tensile strength

The zero span test measures the tensile load bearing capability of fibres in a test specimen clamped between two jaws in contact with each other at the time of tensile failure. The zero span tensile strength value is used to assess the average axial tensile strength of individual fibres and is independent of the relative bonded area in the measured sheets.

2.6.4. Surface free energy (SFE)

The surface free energy (SFE) of treated and untreated sheets was measured with a Dataphysics® OCA15 contact angle goniometer by applying 4 μL droplets of deionized water, ethylene glycol, and 99% diiodomethane to the surface of paper sheets and determining the corresponding contact angles. At least five droplets of each liquid were dispensed in different regions of the sheets and the average value obtained was used to calculate the surface free energy. The SFE results were evaluated by using the goniometer in the OWRK mode, which is based on Owens's three-liquid method.

The basis of the Owens-Wendt method for the calculation of the surface free energy of a solid material relies on the Young's equation (Young, 1805). Its modified form is as follows:

$$\gamma_S = \gamma_{SL} + \gamma_L \cos\theta \qquad [2\text{-}15]$$

where γ_S is the SFE of the solid, γ_{SL} the SFE corresponding to the solid-liquid interface, γ_L the SFE of the measuring liquid, and θ the contact angle between the solid and the measuring liquid.

Owens and Wendt, (1969) assumed that all the possible interactions between a liquid and a solid material i.e.: polar, hydrogen (related to hydrogen bonds), induction, and acid-base components, could be associated with the polar interaction (γ_S^p). Consequently, the following equation was obtained:

$$\gamma_{SL} = \gamma_S + \gamma_L - 2\left(\gamma_S^d \gamma_L^d\right)^{0.5} - 2\left(\gamma_S^p \gamma_L^p\right)^{0.5} \qquad [2\text{-}16]$$

The combination of Eq. [2-15] with Eq. [2-16] leads to the following relationship:

$$\gamma_L\left(1+\cos\theta\right) = 2\cdot\left(\gamma_S^d \gamma_L^d\right)^{0.5} + \left(\gamma_S^p \gamma_L^p\right)^{0.5} \qquad [2\text{-}17]$$

Because two unknowns, γ_S^d and γ_S^p, appear in Eq. [2-17], this formula is insufficient to determine the SFE of a given solid. Thus, the contact angle has to be measured using a minimum of two measuring liquids, which would yield two equations in the form of Eq. [2-17]. As a result, a system of two linear equations is obtained, and the γ_S^d and γ_S^p determined.

If we divide all the previous expression in Eq. [2-17] by $(\gamma_L^d)^{0.5}$ we obtain the equation of a line (Eq. [2-18]), from which the slope and y-intercept (corresponding to the parameters of the solid $(\gamma_S^p)^{0.5}$ and $(\gamma_S^d)^{0.5}$ respectively) can be also determined by using several liquids with different polar and dispersive components:

$$\frac{\gamma_L(1+\cos\theta)}{2\cdot(\gamma_L^d)^{0.5}} = (\gamma_S^d)^{0.5} + (\gamma_S^p)^{0.5}\frac{(\gamma_L^p)^{0.5}}{(\gamma_L^d)^{0.5}} \quad [2\text{-}18]$$

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Chapter 3

Cyclodextrin Functionalization of Several Cellulosic Substrates for Prolonged Release of Antibacterial Agents

Abstract

Several cellulosic substrates have been chemically-functionalized with cyclomaltoheptaose (β-cyclodextrin, β-CD) using citric acid as a crosslinker agent to obtain new surface-modified materials able to release antiseptic molecules over a prolonged period, in view of their use in medical domain. Three different commercial cellulosic substrates were used, namely: (i) an uncoated paper, (ii) a crepe paper, and (iii) a medical bandage. They were successfully grafted by a crosslinked polymer consisting on β-CD molecules as assessed by scanning electron microscopy and Fourier transform infrared spectroscopy analysis. Several time–temperature kinetic cycles were performed to reach the optimum curing parameters. The grafted and nongrafted samples were loaded with chlorhexidine digluconate (digCHX), a widely used antiseptic agent. The drug-delivery kinetics of the encapsulated digCHX was carried out by immersing the sample under investigation into an aqueous medium, and the quantity of the released digCHX was measured, as a function of time, by UV spectroscopy. The optimal grafting conditions were established on the basis of the highest weight gain. These samples did not give the best release performance. Nevertheless, several grafted substrates were able to uptake an appreciable amount of active molecules and release them over a prolonged time of about 20 days.

3.1. Introduction

Current investigations tend to search new functionalities for papers and cellulose-based substrates. Deactivation of pathogen substances (Daoud, Xin and Zhang, 2005), liberation of flavoring agents, or liberation of fragrances (Hu *et al.*, 2006), or cellulosic fabrics for insecticide delivery (Romi *et al.*, 2005) are examples of new functionalities for papers. Papers and paper-based cellulosic substrates with enhanced functionalities apart from conventional ones (printing or packaging) are called specialty or active papers. The main sectors where active papers are reaching high interest are packaging and medical. The objective of surface functionalization is to provide new properties for cellulose based materials. From this point of view, the use of cyclodextrins seems to be a good method to functionalize cellulosic substrates (Bergamasco, Zanin and Moraes, 2007; Lo Nostro, Fratoni and Baglioni, 2003).

β-Cyclodextrin (β-CD) produced by enzymatic degradation of starch is a cyclic oligosaccharide consisting of seven D-glucose units linked by α-(1→4) glycosidic bonds (Dodziuk, 2008; Szejtli and Osa, 1996; Duchene, 1991; Szejtli, 1988; Szejtli, 1982; Bender and Komiyama, 1978). More generally, β-CDs contain a lipophilic central cavity and a hydrophilic outer surface. Due to the chair conformation of the glucopyranose units, the cyclodextrins are shaped like a truncated cone rather than perfect cylinders. β-CDs are able to form host–guest complexes with hydrophobic molecules given the unique nature imparted by their structure (Dodziuk, 2008; Szejtli, 1988; Bender and Komiyama, 1978). As a result, these molecules have found a number of applications in a wide range of fields thanks to their encapsulation ability (Krasowska and Bojarski, 1991; Grigoriu, Luca and Grigoriu, 2008; Uekama, 1983). In addition to the above-mentioned pharmaceutical applications for drug release, β-CDs can be used in environmental protection; they can effectively immobilize toxic compounds like trichloroethane or heavy metals inside their rings, or they can form complexes with stable substances like trichlorfon (an organophosphorus insecticide), or sewage sludge, enhancing their decomposition (Gruiz *et al.*, 1996; Yoshii *et al.*, 2001). Other interesting applications arise from their ability to form inclusion complexes with fragrance/flavor materials like menthol to obtain, for example, polymeric fibers with odor properties (Uyar, Hacaloglu and Besenbacher, 2009). This study applies a technique consisting in grafting a β-CD crosslinked polymer on the surface of cellulosic substrates with different morphologies, to confer them bactericidal properties through the encapsulation of active agent molecules.

To date, studies establishing the most appropriate pathway of grafting cyclodextrins onto textiles (Martel *et al.*, 2002a; Martel *et al.*, 2006; Voncina, Vivod and Chen, 2009) and nonwovens (Martel *et al.*, 2002b; Le Thuaut *et al.*, 2000; Martel *et al.*, 2000) were

performed. Moreover, studies for grafting cyclodextrins onto cellulosic fabrics loaded by miconazole nitrate (Wang and Cai, 2008) or silver ions (Bajpai, Gupta and Bajpai, 2010) were also carried out, to prepare new fabrics with antimicrobial properties. In the medical domain, cyclodextrins were used to obtain polyvinylidene fluoride regenerative membranes for periodontal applications (Tabary *et al.*, 2007), to produce bone implants with drug release properties (Hoang Thi *et al.*, 2010; Tang *et al.*, 2012), polyvinyl alcohol hydrogels for sustained release of ocular therapeutics (Xu *et al.*, 2010), or to fabricate functionalized polyester vascular prosthesis with a postoperation release of antibiotics to prevent infections (Blanchemain *et al.*, 2011). Nowadays, these studies are extended for commercial cellulosic materials, to provide them new or added bactericidal properties, thus yielding a final product with the highest added value possible; this is one of the reasons why medical application is targeted. In the present chapter, we compare for the first time different kinds of paper substrate, and an optimum of grafting is proposed. An additional challenge consists on maintaining the release for within a prolonged period of time.

3.2. Materials and methods

3.2.1. Cellulosic substrates

The three different cellulosic substrates used in this work were a noncoated paper (70 g/m^2), a 100% cotton-based medical bandage (70 g/m^2), and a medical crepe paper (60 g/m^2, from Ahlstrom, France) used for sterilization purposes. Citric acid, sodium dihydrogen hypophosphite ($NaH_2PO_4 \cdot H_2O$), β-CDs, and chlorhexidine digluconate were commercial chemical grade products, supplied from Aldrich Chemicals (Saint Quentin Fallavier, France). They were used as received.

3.2.2. Cyclodextrin grafting process

β-CDs were grafted onto cellulosic substrates using a pad-dry-cure textile finishing method. All the samples were soaked into 100 mL aqueous solution containing β-CD, catalyst (sodium dihydrogen hypophosphite), and crosslinking agent (citric acid). Then, the samples were dried. The grafting reaction occurs via a polyesterification reaction; it occurred in a ventilated stove at different temperatures (between 130℃ and 160℃) and times (between 5 and 50 min). After the reaction, the ensuing specimens were abundantly washed, oven dried (at 104℃ for 30 min) and weighted. The weight gain (WG) (%) representing the yield of the grafting reaction was calculated using the expression [2-1] as explained in the "Materials and Methods" chapter.

3.2.3. Drug adsorption

The previously grafted sheets using β-CDs were dried at 105℃ for 20 min before being weighted. Then, the samples were treated with antibacterial agent as explained in the "Materials and Methods" chapter by dipping into 20% (w/v) chlorhexidine digluconate (digCHX) solution. The weight gain (ΔM_{digCHX}) represents the amount of antiseptic agent loaded onto sample sheets, as calculated from equation [2-2]. The amount of CHX adsorbed has been also reported in grams per gram of the substrate (equation [2-3]).

3.2.4. UV Spectroscopy and release analyses

An UV spectrophotometer (UNIcam UV500 Thermospectronic) was used to characterize the prolonged liberation behavior by measuring concentration of the digCHX. Each experiment was conducted in triplicate. Samples were dipped in distilled water into vials filled with 30 mL, waiting for release under constant stirring. At pre-established intervals, the water was completely renewed and the drug content in the withdrawn fluid was determined by UV spectrophotometry. The first measure was taken at few hours after the beginning of the release kinetic, while the following ones were taken at regular intervals of 24 h. Measures taken every 24 h give the number of cycles with renewed distilled water the functionalized support can carry on releasing the active agent, that is, the prolonged liberation behavior.

3.2.5. Scanning electron microscopy imaging

An analysis of the surface and cross-section of the different substrates was performed using a scanning electron microscope (SEM) (Quanta 200, FEI Company, Hillsboro, Oregon) under moderate vacuum at an operating voltage of 7 kV. Dried cellulose-based fibre mats were gold-coated by sputtering for 15 s.

3.2.6. Fourier transform infrared spectroscopy

A Perkin Elmer PARAGON 1000 FTIR spectrometer equipped with spectrum software was used to perform the Fourier transform infrared spectroscopy (FTIR) analyses. The spectra were obtained by analyzing directly the surface by using ATR system (GoldenGate, JASCO). The FTIR spectra were collected with a resolution of 4 cm^{-1} in the range of 4000–400 cm^{-1}. Thus, 16 scans were taken within this interval.

3.2.7. Cobb absorption, bendtsen roughness, and bendtsen permeability

Properties that would have an influence on the functionalization and liberation behavior of substrate were tested, namely: Cobb absorption, Bendtsen roughness, and Bendtsen

permeability. All these parameters may play an important role on the way the β-CD solution and the digCHX are captured by the sample surface and internal diffusion on the sheet structure. All these tests were carried out according to commonly used international standards (Cobb absorption: EN 20535-1994; Bendtsen roughness: NF Q03-049-1972; Bendtsen permeability: NF Q03-076-1986).

3.3. Results and discussion

3.3.1. Cyclodextrin grafting

The WG of the sheets results from the grafting of the cellulose fibers by a crosslinked polymer formed between β-CD and the citric acid. This occurs via a polyesterification reaction, as previously reported for material treated with other CDs derivatives. Under the influence of heat and catalyst, the polycarboxylic acid (citric acid) is dehydrated. A first esterification reaction occurs between the formed anhydride and a hydroxyl function of the cyclodextrin. In a second step, an anhydride is formed between the other two remaining acid functions; a second esterification reaction can then take place either with the cellulose which is going to be functionalized (—OH functions of cellulose, for example) or with an hydroxyl of another cyclodextrin to form a crosslinked polymer (Martel *et al.*, 2002a; Blanchemain *et al.*, 2011; Martel *et al.*, 2003). The reaction between citric acid and cyclodextrins results in a three-dimensional polymer network whose structure is based on cyclodextrin moieties linked to each other via esterified citric acid residues carrying free carboxylic groups. Depending on the pH, those free carboxylic groups will appear under carboxylate form and experience an ionic interaction with the amino groups present in digCHX molecules. Therefore, the loading of the digCHX onto the functionalized cellulosic substrates can be expected to occur either entrapped into the β-CD cavity by hydrophobic complexation, or adsorbed onto the polymer structure via acid–base interactions (Blanchemain *et al.*, 2012).

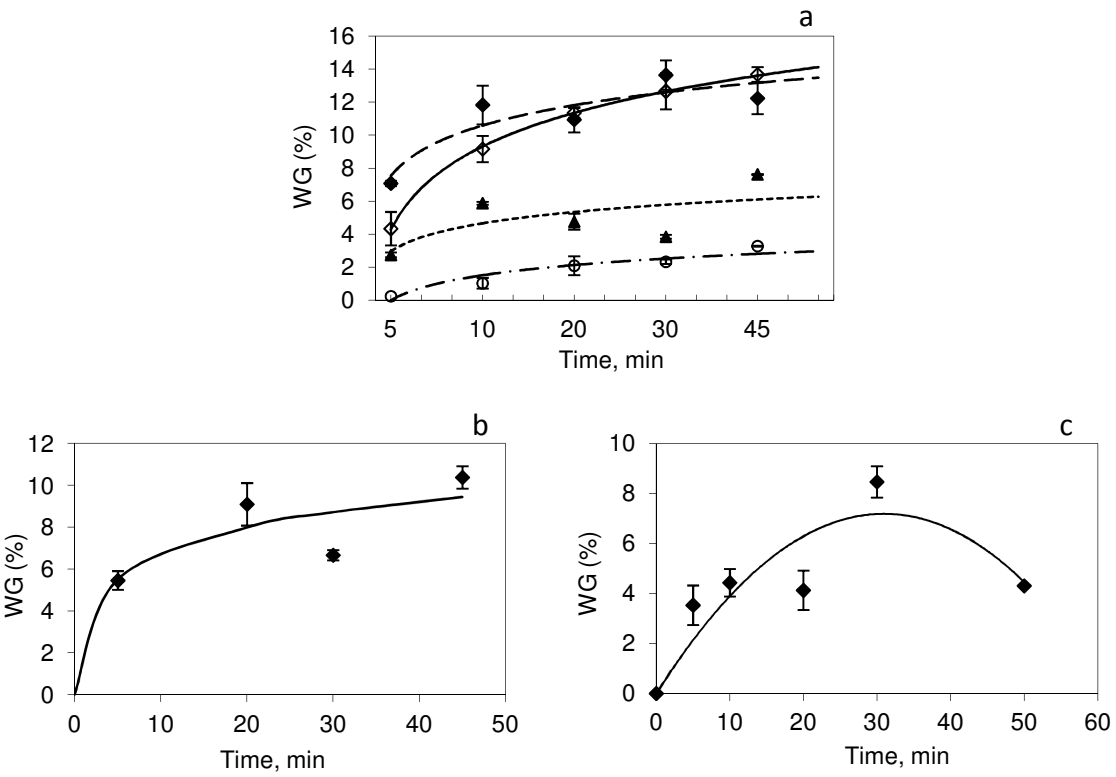

Figure 3-1 Time–temperature kinetics for noncoated paper (a). Key: 130℃ (dash-point curve, ○); 140℃ (point-curve, ▲); 150℃ (continuous curve, ◊); 160℃ (dashed curve, ♦). (b) and (c) show the weight increase kinetics for medical bandage and crepe paper, respectively, at fixed conditions of 150℃ for 30 min. Grafting yield is expressed as the WG (%) of the sheets upon reaction.

Fig. 3-1a reports the WG values of the treated noncoated paper for up to 45 min at temperatures between 130℃ and 160℃. Regression lines were used to indicate the observed trend. The highest WG was observed after 30 min curing at 150℃ and 160℃, as shown in Fig. 3-1a. In fact, a maximum WG of 13 ± 1% was achieved within 30 min at 160℃ and for 45 min at 150℃. At lower temperatures, the WG increased more smoothly and reached around 3.3% and 7.6%, after 45 min of curing, for 130℃ and 140℃, respectively. Thus, working at 150℃ for 30 min was fixed as the optimal condition of curing. The WG under these conditions for the noncoated paper was around 13%.

The same temperature was used in the case of the medical bandage and crepe paper. For those substrates, only the curing time was studied, as reported in Figs. 3-1b and 3-1c. The

best curing time for both materials was also set at 30 min. In the case of the medical bandage, the curve reached a plateau after 30 min; for crepe paper, functionalization tends to decrease after such a delay, possibly due to loss of fibers in fabric after washing, caused by the degradation and embrittlement of cellulose with temperature. The WG was about 9% and 8%, for the medical bandage and crepe paper, respectively.

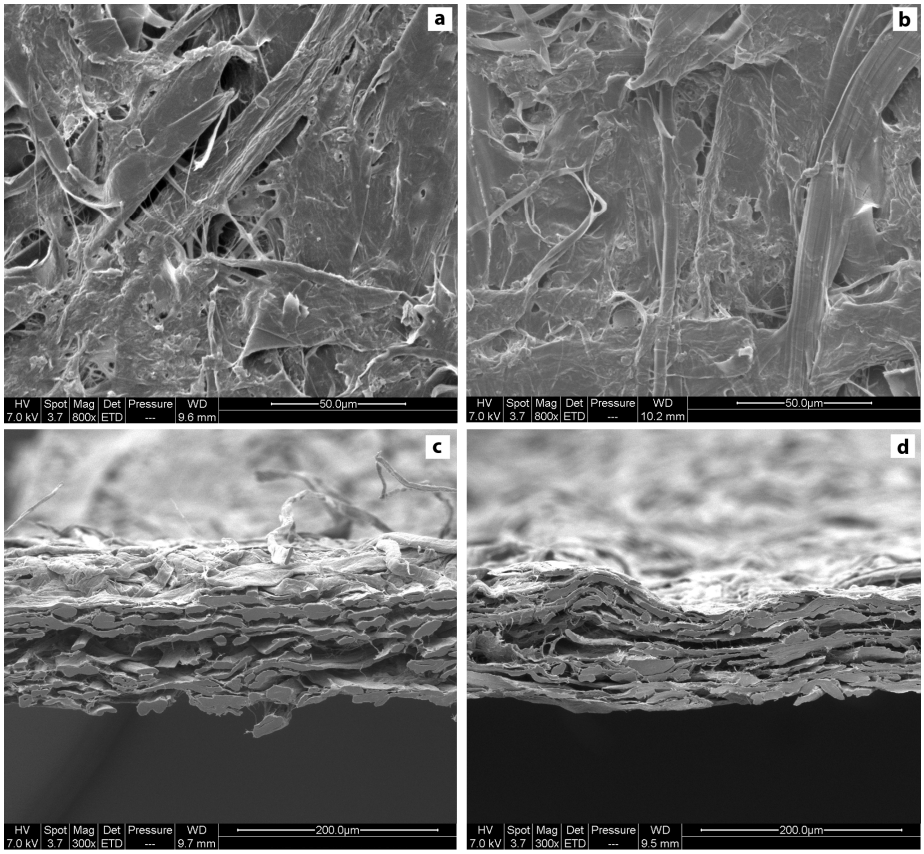

Figure 3-2 SEM images of the noncoated paper surface (a) initial, and (b) grafted with WG = 13 ± 1%; images (c) and (d) correspond to the crepe paper cross-section; (c) initial, and (d) grafted with WG = 8 ± 1%.

The surface morphology of the reference and that of the CD-grafted samples were analyzed by SEM, as displayed in Fig. 3-2. Very few differences were observed after grafting, and material with similar features was achieved. Nevertheless, the compactness of the treated samples seems to be higher. Moreover, porous structure is maintained in both cases, and there is no formation of continuous film at the surface of the grafted materials. This may be due (at least partially) to the grafting but most probably to the role of citric acid and/or drying process at high temperature and under released constraints. The latter two phenomena are well known in papermaking and arise from the partial collapsing (physical for drying and chemical for crosslinking with citric acid) of the fiber cell walls (Caulfield, 1994).

Table 3-1 shows the mean results of paper physical properties obtained with the Bendtsen and Cobb tests for 10 specimens of each substrate. Bendtsen permeability and roughness were not possible to measure on the medical bandage, because it had a very open structure. As mentioned before, the method used to graft the β-CD onto the cellulosic sheets was a bulk-type approach, that is, their soaking into an aqueous citric acid and β-CD solution. This is the reason why the parameters which most probably correlate properly the effect of such a modification are the WG and the Cobb absorption. Indeed, the standardized Cobb absorption test (in g/m^2) gives the amount of water which is retained by unit area after a certain period of time.

Table 3-1 Bendtsen and Cobb values for the three substrates.

Substrate	Bendtsen Permeability [$cm^3/cm^2 \cdot Pa \cdot s$]		Bendtsen Roughness [mL/min]		Cobb absorption [g/m^2]	
	Mean	StD	Mean	StD	Mean	StD
Non-coated	0.39	0.03	304	12	56	1
Crepe paper	3.15	0.05	2534	45	15	1
Medical bandage	—	—	—	—	36	3

As the citric acid and β-CD solution behave similarly to water, higher is the impregnation level, bigger is the absorbed amount of citric acid and β-CD. It is therefore easy to understand that the amount of grafted β-CD onto the fibers is higher. Figure 3-3 shows the relationship between the amounts of citric acid and β-CD on the different substrates after curing (represented by WG) and the Cobb absorption. As expected, the noncoated paper was better impregnated by the aqueous solution, thus leading to the highest

grafting level, whereas samples based on crepe papers which has the lower Cobb values consequently underwent much less to the grafting operation and gave the lower WG.

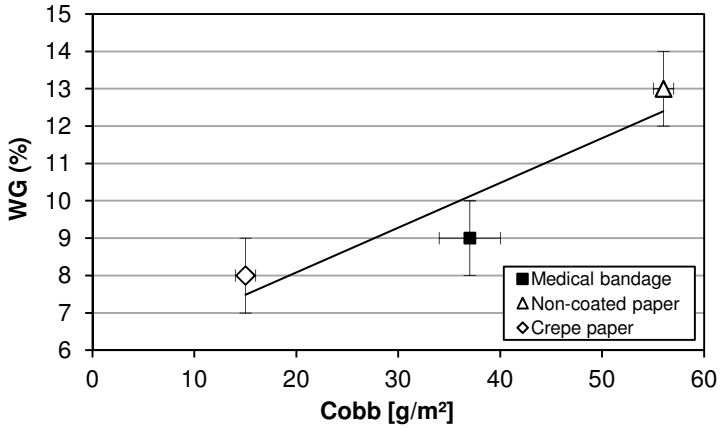

Figure 3-3 Relationship between the Cobb absorption, expressed in g/m², and the amount of β-CD grafted on the substrate expressed as WG increase (%).

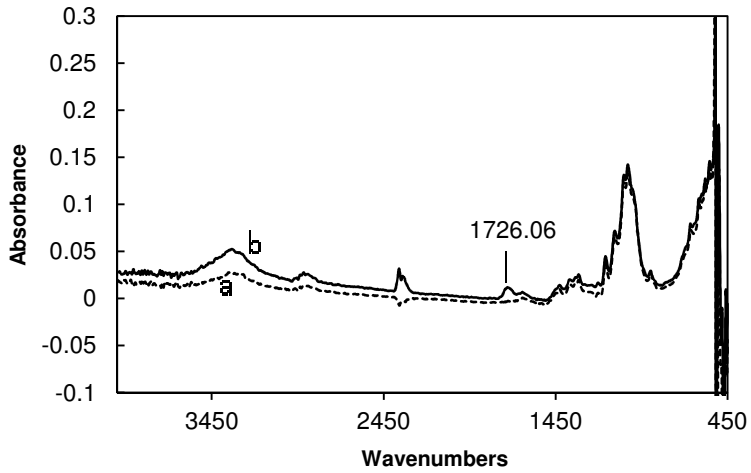

Figure 3-4 ATR-FTIR spectra of (a) crepe paper and (b) crepe paper treated with β-CD after washing.

FTIR spectra of the reference samples and those corresponding to the grafted sheets were compared. The esterification reaction that occurs with the —OH functions of cellulose macromolecules (or those of cyclodextrins) produces chemical functions (ester groups)

which are not present in the cellulose. The detection of these groups in the modified samples evidence that the grafting reaction had took place (Yang, 1999; Yang, Xu and Wang, 1996). The retained material could be either adsorbed and crosslinked at the surface of the samples or chemically bonded to it. Of course both mechanisms could occur simultaneously. The elimination of non-crosslinked and/or nongrafted molecule is assessed considering the washing procedure. Figure 3-4 shows a comparison of the FTIR curves for initial crepe paper before and after modification. The spectrum of the surface of the grafted sheets shows a peak of C=O stretching vibration observed at 1726 cm^{-1}. This signal was not detected in the spectrum associated with the initial substrate. Similar results were obtained for the noncoated paper and for the medical bandage.

3.3.2. Release studies of noncoated paper

UV spectrophotometry was used to characterize the release of the digCHX retained by the β-CD. The use of the digCHX was suitable in terms of molecular size to enter the β-CD cavities, since it was reported in previous works (Tabary *et al.*, 2007). The controlled release effect of digCHX from CD-finished cellulosic substrates is explained by the inclusion of this molecule into the internal cavity of CD (Boschin *et al.*, 2006), and also due to ionic interactions of digCHX with the free carboxylic groups on the crosslinked polymer, as explained in previous sections. More precisely, ionic exchange probably exists between the gluconate counter ions of the positively charged digCHX and the carboxylate groups present on the grafted substrates. The digCHX molecule has two absorbance peaks between 200 and 300 nm. The peak corresponding to a wavelength of 254 nm (λ_{max}) was used to determine the digCHX concentration using the Beer Lambert law (Barao de Aguiar, M.M.G. *et al.*, 2010; Wu *et al.*, 2005; Yao *et al.*, 2009; Zhan and Pan, 2006). Despite knowing the optimal grafting time and temperature for each substrate, other grafting conditions were tested in terms of release. The idea was to confirm the hypothesis which considers that the maximum number of grafted cyclodextrins corresponds to the highest release level of active agent. The first set of experiments was carried out on each substrate separately. It is important to notice that, the released amounts of digCHX in the following results may appear to be weak, but they are interesting from the point of view of bacterial inhibition, as it will be discussed in next paragraphs.

Table 3-2 Characteristics of the samples tested in terms of liberation behavior, and the amount of the loaded digCHX.

Non-coated paper	**Number of samples**	**Loaded digCHX in g**	**g digCHX/g of loaded paper**
5min. 150°C	3	0.042±0.002	0.271±0.011
30min. 150°C	3	0.028±0.002	0.19±0.01
Non-grafted and oven dried at 150°C for 30 min	3	0.033±0.002	0.24±0.01
Non-grafted and not oven dried	3	0.035±0.003	0.24±0.02
Crepe paper	**Number of samples**	**Loaded digCHX in g**	**g digCHX/g of loaded paper**
5min. 150°C	3	0.045±0.001	0.219±0.003
Non-grafted and oven dried at 150°C for 30 min	3	0.032±0.005	0.181±0.010
Medical bandage	**Number of samples**	**Loaded digCHX in g**	**g digCHX/g of loaded paper**
5min. 150°C	3	0.045±0.007	0.178±0.025
Non-grafted and oven dried at 150°C for 30 min	3	0.035±0.001	0.145±0.006

For noncoated paper, two grafting levels (5 and 30 min at 150℃) were tested and compared with the references. Table 3-2 summarizes the characteristics of samples and the amount of the loaded digCHX into the substrate before setting up the kinetics of the release. Concerning noncoated paper, all samples tend to retain similar amounts of digCHX, except those which were grafted at the optimum time and temperature, as described previously. This fact can be explained from the point of view of the sheet structure. After grafting, the structure of sheets becomes more closed (Fig. 3-2), and the cavities are filled by β-CDs. Moreover, the citric acid causes the intrafibers and interfibers crosslinking, yielding substrates with lower capacities of absorbing active agent (i.e., lower porosity and affinity to waterbased baths). It is important to notice that this fact is not worrisome as the amount of digCHX which is filled into the surface hollows will not necessarily charge the cavities of β-CDs and will be quickly released. What we want to characterize is the lasting release ability of the grafted β-CDs.

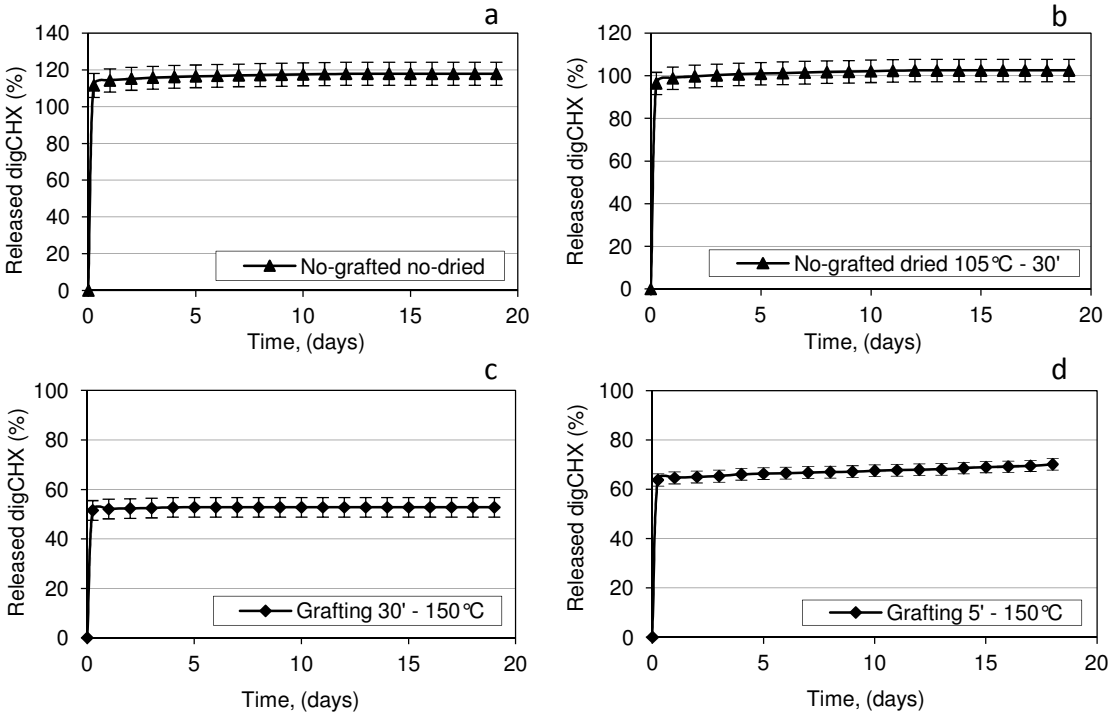

Figure 3-5 Accumulated release kinetics for noncoated paper samples compared to their theoretical loaded amounts (expressed as % of the initially loaded digCHX). (a and b) show the nongrafted samples while (c and d) show the grafted ones.

Fig. 3-5 displays the kinetic of digCHX release for the nongrafted and grafted samples and shows that the major part of digCHX is released already after 12 h. A plateau is classically observed for each sample, and a slight but lasting release is observed for the grafted materials. This gives an indication about the prolonged release of active material for the grafted paper. Figures 3-5a, and 3-5b provide evidence that for nongrafted samples the released amount of digCHX corresponds to that initially loaded (100%). Figures 3-5c, and 3-5d show how the initially loaded amount is not achieved after 19 days, indicating that a significant amount of digCHX still remains inside the CDs cavities.

Fig. 3-6 shows the prolonged digCHX release after successive washing for noncoated paper samples that were grafted at different degrees. The release is represented as a percentage of the initially digCHX loaded amount. It is obvious to expect that the maximum amount that can be released for one sample is theoretically 100%. However, the initially loaded amount of digCHX (which corresponds to 100%) is calculated by a

mass difference method as explained in the experimental section. Only the initially loaded digCHX amount is estimated by mass difference; the reason is because there are samples that never release the entire active agent after the period of the kinetic study, so there is no other way to know the loaded digCHX at the beginning. It is important to notice that the difference between the UV measurements and the mass difference method used to calculate the amounts of digCHX may lead to a slight discrepancy, as already shown in Fig. 3-5a where the released amount is slightly above 100%. However, Fig. 3-5b confirms that such difference could be neglected.

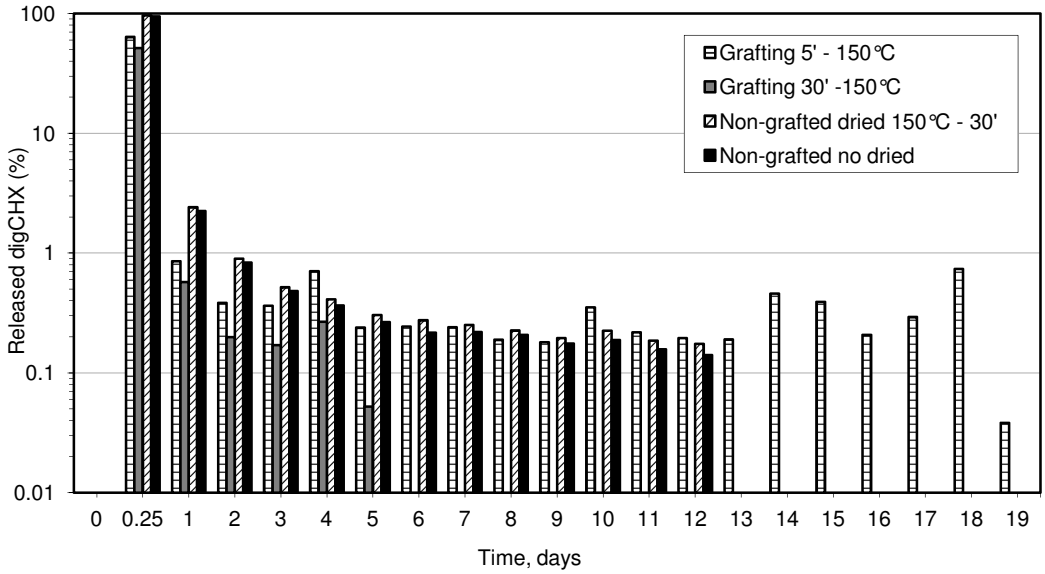

Figure 3-6 Prolonged digCHX release for noncoated paper samples as (%) of the initially loaded digCHX in the sheets.

The release values after 12 h evidence that the main part of the loaded digCHX is released in the first hours. Nongrafted samples released practically the total amount of the loaded active agent, while grafted specimens tend to release only about 50% after a first cycle of measurement (curing conditions 30 min-150℃), and 60% (curing conditions 5 min-150℃). Several conclusions can be drawn from Fig. 3-6 comparing the tested samples. The grafted samples using optimal conditions (30 min-150℃) were found to stop the release of the active digCHX molecules after 5 days. The release kinetic is considered accomplished when the concentration of digCHX is so weak that its dosage became impossible to detect from the absorbance peak. Nongrafted samples (dried and nondried) have found to release the total amount of digCHX molecules within the first 12 days,

even if it is worth to mention that the major part of the active agent is released within the first measurement. Samples grafted for 5 min at 150℃ were found to carry on the releasing after the highest number of washings. The effect of β-CD grafting is clearly observed confirming that such a grafting procedure strongly prolonged the liberation behavior of the active molecules.

However, samples grafted using the optimum curing time and temperature (the highest WG) did not provide the highest release performance. The reason might be the strong crosslinking density with citric acid. At the optimal conditions, a high amount of β-CDs are grafted on the substrate, but the strong cross-linking of the β-CDs layer, the citric acid, and the fibers with themselves produce a closed mesh, which makes cyclodextrin's cavities less accessible to the active molecules. From this point of view a lower grafting level lead to a higher prolonged release behavior, suggesting the necessity of finding a compromise.

The released amounts in the range of 0.2–0.5% correspond to digCHX concentrations around 4–10 mg/L. They may appear to be weak, but they are interesting from the point of view of bacterial inhibition (Woodcock, 1988). At relatively low concentrations, the action of chlorhexidine is bacteriostatic, and at higher concentrations, it becomes rapidly bactericidal, with the actual levels varying somewhat from species to another. Chlorhexidine minimum inhibitory concentration (MIC) varies from 0.25 to 128 mg/L for a wide range of gram-positive and gram-negative bacteria (Graham W. Denton, 1991). Chlorhexidine's MIC against microorganisms found in endodontic infections was found to be in a range from 2.67 to 80.00 μg/mL (do Amorim Crystiane, Aun and Mayer Marcia, 2004). Chlorhexidine's MICs were ranged from 0.625 to 50.00 μg/mL, in the case of pathogenesis related with skin diseases (Odore, Colombatti and Re, 2000).

The digCHX loaded in the nongrafted samples is completely released, while the accumulated release amounts for the grafted samples never reaches the 100% after 20 days of release kinetic. This means that there's a certain amount (probably negligible) of digCHX which remains strongly entrapped into the CDs cavities. The experience showed that when a longer period of time between two UV measurements is used (longer than 24 h), the amounts of released digCHX increase. It is therefore possible that 24 h is not enough to allow the entrapped digCHX to be liberated from the CDs, after a few days of starting the experiment. In spite of the impossibility to perform UV measurements (due to the small amounts released), a significant amount still remains onto the grafted sheets. The criterion used was to consider that experiments were finished when it was not possible to perform daily measurements. Such a rule was applied for all the investigated substrates.

3.3.3. Comparison between the cellulosic substrates

Taking into account the previous results, grafted samples for 5 min at 150°C were tested for the liberation behavior on the other two cellulosic substrates. Table 3-2 summarizes the properties of the samples and the amount of the loaded digCHX on each substrate, before starting the investigation of the release kinetics. Similar observations on the digCHX release kinetics were obtained for crepe paper and medical bandage. The results show that in all cases the main part of the loaded digCHX is released in the first few hours.

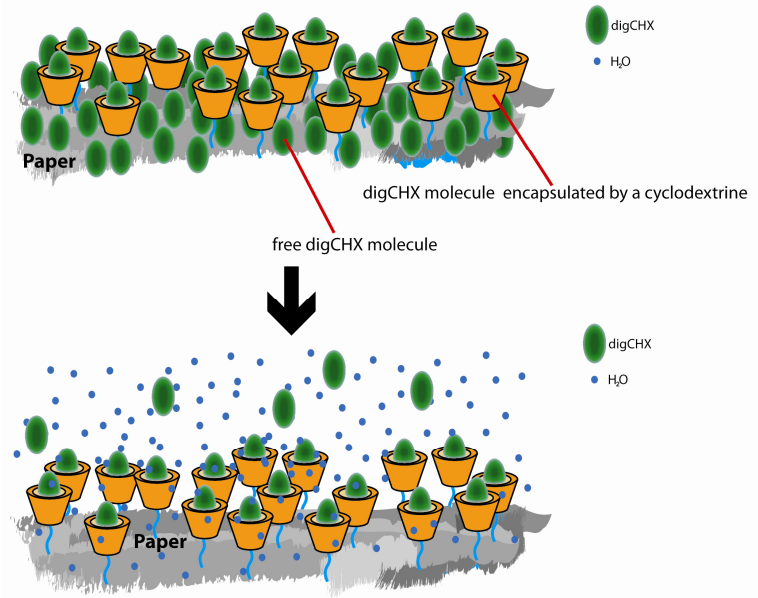

Figure 3-7 Schematic representation of massive release.

For crepe paper, grafted samples retained around 30% of the digCHX after 12 h while, for the same delay, the nongrafted samples retained less than 4%. The release of digCHX was found to stop after 4 days for the nongrafted samples, whereas for the grafted samples (5 min-150°C) 9 days were necessary to reach the decay of the digCHX liberation. As previously reported, the sum of the released amounts for the grafted samples does never reach the 100% after 9 days.

For the medical bandage, the retention of the digCHX, after the first measurement (12 h), was around 30% and 10% for the grafted and nongrafted samples, respectively. After 2 days, nongrafted samples did not release the digCHX anymore, whereas for grafted samples (5 min-150°C), this delay was extended to 4 days. Concerning the theoretical

loaded amount of digCHX, it is completely released after 3 days for nongrafted samples, while for the grafted samples it continued releasing, even at least, after 4 days.

It is therefore possible to conclude that grafted samples are capable to retain the active molecules and to release them for much longer periods. The nongrafted samples also retained digCHX molecules but their release was so fast that after few days they stopped to liberate such active molecules.

In a general way, all functionalized (grafted) substrates are unable to achieve the theoretical loaded digCHX levels within the period of release measurements, indicating that the active ingredient remains strongly retained by cyclodextrins and its release is much more slowed. Initially loaded digCHX consists of linked and nonlinked molecules on the grafted sheet's surface. When the study of liberation kinetic is started, there is a first fast step in which a massive release is produced (Fig. 3-7). During this period, the water immersion produces an easy release of all the free digCHX molecules which are not strongly linked to CDs. Then, a very weak but steady step of releasing starts for the grafted samples. Nongrafted samples have not digCHX molecule anymore and consequently, the release is stopped. The ability of CDs to retain the active agent is clearly the reason for the prolonged release of the grafted samples.

3.3.4. Relationship between loaded digCHX and Cobb absorbance

Cobb absorbance has an influence on the ability of samples to be loaded by digCHX, as shown in Fig. 3-8. Taking into account the paper substrates, we can observe the higher is the Cobb absorption the greater is the loaded digCHX into the sheets. As previously explained, the load of digCHX is performed by soaking the sheets into an aqueous environment containing the active agent. The Cobb absorption has proven to be a good indicator to determine that the grafting and the loading of the grafted cellulose-based increase with increasing the Cobb values. Figure 3-8 also shows that grafted samples increase the ability of the sheets to be loaded with digCHX, for all the substrates.

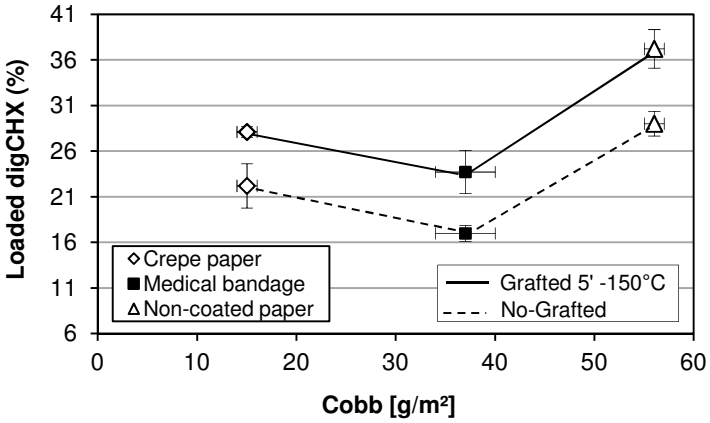

Figure 3-8 WG due to the loaded digCHX vs. Cobb absorption (in g/m²) aiming at comparing the capacity of uptaking digCHX of the grafted and the nongrafted samples.

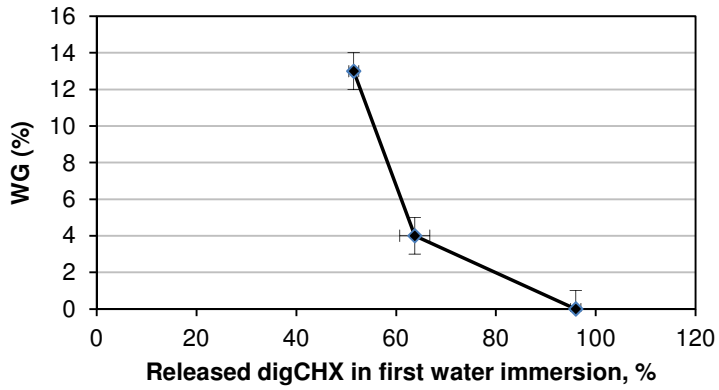

Figure 3-9 Relationship between the WG (%) and the released digCHX for the noncoated paper samples after the first water immersion of the liberation kinetic. The released digCHX is expressed as % of the initially loaded.

3.3.5. Relationship between grafting level and digCHX retention

The ability to retain active agent when sheets are soaked into an aqueous environment under stirring is strongly related to the amount of grafted cyclodextrin within the sheets, as illustrated in Fig. 3-9. In fact, this figure shows the relationship between the grafting level and the released digCHX, after the first cycle of water immersion for the noncoated paper samples. Higher is the grafting level, higher is the substrate ability to retain active molecules in the aqueous environment.

3.3.6. Comparison of substrates in terms of liberation behavior

The three tested substrates present different release behavior. Thus, the released amount of digCHX of the grafted samples (grafted 5 min-150℃), as a function of time, is shown in Fig. 3-10a. The noncoated substrate was found to retain the highest amounts of digCHX (release only about 65% in the first measurement). Moreover, it releases active agent for the longest time (19 days). Retention levels for crepe paper and the medical bandage are similar, but the release time lasts 9 days for crepe paper, and only 3 days for medical bandage. Grafted samples never reached the theoretical loaded amounts of digCHX when the kinetics were stopped.

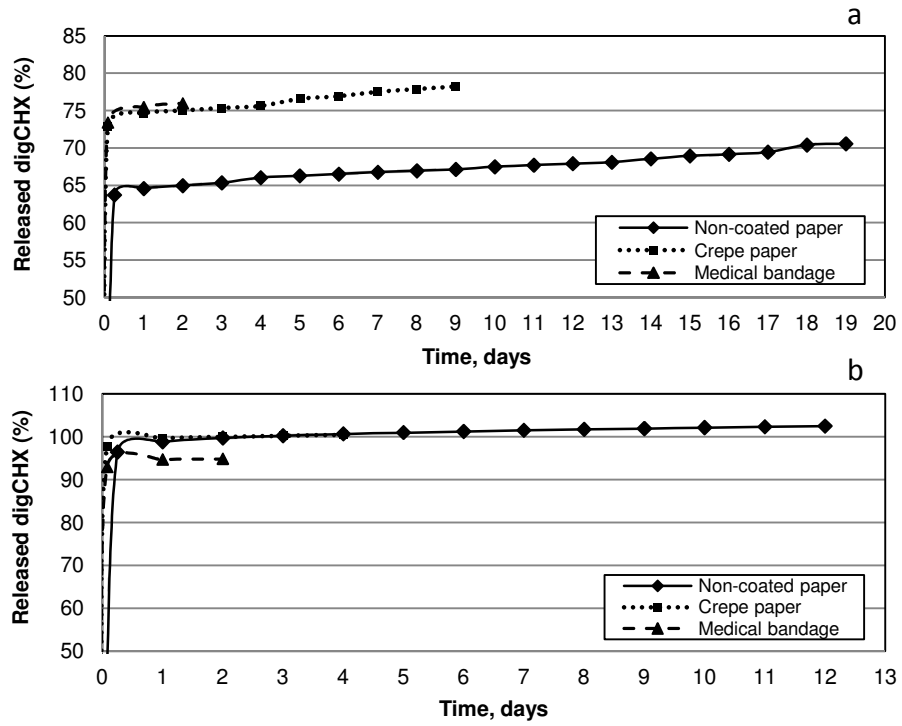

Figure 3-10 Release kinetics of (a) grafted and (b) nongrafted samples. The grafting conditions are 5 min at 150℃. The released digCHX is expressed as % of the initially loaded.

Considering the nongrafted samples (dried at 105℃ for 30 min) (Fig. 3-10b), the noncoated paper is also the best substrate in terms of the release, since it lasted 12 days. The crepe paper and the medical bandage have released digCHX for periods of 4 and 2 days, respectively. In the nongrafted samples, the active agent released amounts were close to 100% and the release kinetics stopped earlier.

3.4. Conclusions

This study opens a new way of providing bactericidal properties to cellulose-based materials and more specifically to finished commercial paper substrates with different morphologies. Different kinds of paper substrates are compared for the first time, and an optimum of cellulose grafting is proposed. All the studied substrates can be successfully functionalized using the CDs technique, obtaining weight gain between 7% and 15%, depending on the initial substrate properties. It has been found that in all substrates the functionalized samples increased the substrate ability to maintain the release for longer time periods, reaching periods up to 20 days. The optimum grafting conditions, (based on the experimental conditions giving the highest WG) does not give the best material in terms of digCHX release performance. Thus, the best performing substrates in terms of digCHX delivery were those in which the WG was situated between 3% and 7%, that is, 5 min at 150ºC. Cobb absorption test was found to be a good indicator to select the substrates to be grafted, as well as their ability to be loaded with active agent. The obtained results allow building the foundation of a new area dealing with ''biologically active cellulose paper substrates.'' Since the functionalization treatment is applied superficially, the paper manufacturing process is not affected, and the technique becomes very versatile to be potentially applied to a wide range of paper grades. However, this work is a first approach on the use of this functionalization technique applied to this kind of materials, and antimicrobial analysis will be carried out to confirm and characterize the antimicrobial effect.

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Chapter 4

Application of Surface Enzyme Treatments using Laccase and a Hydrophobic Compound to Paper-based Media

Abstract

An innovative method for the surface hydrophobization of cellulosic substrates based on an enzyme treatment is proposed. Commercial finished paper was hydrophobized by using laccase from *Trametes Villosa* in combination with lauryl gallate (LG) as hydrophobic compound. The efficiency of the method was increased by the use of a lignosulfonate as a natural dispersant to improve the surface distribution of LG on the paper, raise its hydrophobicity and help preserve the enzyme activity. No similar threefold effect from a single enzyme treatment was previously reported. Paper hydrophobicity was measured with the water drop test (WDT) and temporal changes in contact angle with a goniometer. The influence of processing conditions including the LG dose, treatment time and temperature was also examined. The hydrophobicity levels obtained with the proposed method were similar to those of commercial paper. Using a high temperature resulted in further increased hydrophobicity. Efficient fiber bonding and chemical functionalization were confirmed by thorough washing and Soxhlet extraction of the paper. Enzyme treatments have the potential to improve the surface properties of paper-based media.

4.1. Introduction

The use of environmentally friendly biotechnological systems in industrial processes has become a field of increasing interest and study in recent years, especially in a global scenario of growing environmental awareness in companies and institutions. In this context, enzymes have emerged as effective alternatives to the chemicals traditionally used by the paper industry; in fact, enzymes afford milder treatment conditions, cause less damage to fibers, can be obtained from renewable resources and are biodegradable (Ma and Jiang, 2002). Enzymes are proteins widely occurring in nature that act as biological catalysts for biochemical reactions in all living organisms; also, they have proved reliable, convenient processing aids for a number of technological industries. Like all catalysts, an enzyme is a reaction partner that increases the rate or velocity of a chemical reaction without itself being changed in the overall process (Nair *et al.*, 2010).

A wide range of enzymes have found use in a variety of industrial processes including the production of laundry detergents, bread making, textiles, and pulp and paper manufacturing (Cherry and Fidantsef, 2003). Enzyme-based applications in the pulp and paper industry are currently not only technically feasible but also cost-effective. Laccases are enzymes belonging to the so-called "blue-cooper family" of oxidases. Research has shown laccases in combination with a mediator (viz. a laccase–mediator system, LMS) to be useful for delignification (Fillat and Roncero, 2009) and bleaching purposes (Aracri, Colom and Vidal, 2009; Valls *et al.*, 2010a; Fillat, Roncero and Vidal, 2011), and also for functionalizing fibers (Cadena *et al.*, 2011; Aracri *et al.*, 2010; Andreu and Vidal, 2011; Fillat *et al.*, 2012; Gaffar Hossain *et al.*, 2010) and altering chemical or refining properties of pulp (Cadena, Vidal and Torres, 2010; Mansfield, 2002). Functionalizing treatments improve some paper properties including hydrophobicity, internal sizing, antioxidant and antimicrobial resistance, and wet strength. Recent research has shown that cellulose can be efficiently hydrophobized by applying an enzyme treatment to a fiber suspension (Garcia-Ubasart *et al.*, 2012).

Cellulose fibers for papermaking are placed in an aqueous suspension prior to forming paper sheets. The suspension is usually supplied with various additives to confer additional properties to paper. Thus, fillers, dyes, sizing agents and other chemicals are typically mixed with cellulose fibers to confer smoothness, color, fibered appearance or other desirable attributes. The absorption of liquids into the structure of paper is a key factor for the end-use performance of paper products (e.g. cups, bags, packaging boxes, liquid containers) and for the runnability of the papermaking process (e.g. in size press or printing paper) (Neimo, 1999; Hubbe, 2007). Papermakers reduce the rate of liquid absorption into the paper structure by treating the stock with hydrophobic substances

(Hossain *et al.*, 2010) and, as noted earlier, this can also be accomplished with biotechnological methods (Garcia-Ubasart *et al.*, 2012). However, introducing chemicals or enzymes before paper formation hinders recycling of effluents; also, it can affect the natural interaction between fibers and adversely alter some properties such as strength, which often forces manufacturers to make some compromise in this respect. In any case, enzyme treatments on finished paper provide an effective alternative to stock treatments (cellulose fibers in an aqueous suspension) and confer new properties to paper without affecting the production process.

In this work, an enzyme treatment —which is traditionally used on fiber suspensions— was for the first time applied to finished paper in order to improve its hydrophobicity. As shown here with the laccase–lauryl gallate system, an enzyme treatment can be directly applied to paper-based media in order to avoid the shortcomings of using fiber suspensions.

4.2. Materials and methods

4.2.1. Paper, enzyme and chemicals

Filter paper sheets from FILTERLAB® (Sant Pere de Riudebitlles, Barcelona, Spain) were used for functionalization. The enzyme used was laccase from *Trametes villosa* supplied by Novozymes (Denmark). Lauryl gallate (LG) was purchased from Sigma–Aldrich. Various soluble sulfonated lignins from softwoods and hardwoods (SL1 to SL4), with molecular weights ranging from 5.9 to 28.4 kDa, and total sulfur content ranging from 3.1 to 6.9% were obtained from Borreegard (Sarpsborg, Norway) and used as received.

4.2.2. Laccase treatments

Paper filter sheets (PS) were cut into circular pieces 4 cm in diameter and subjected to the enzyme treatments in 250 mL Erlenmeyer flasks. The treatment procedure and conditions were explained in detail in the "Materials and Methods" chapter. In the present study, LG was used at two different concentrations (4 and 8% (w/w)) in combination with the laccase (LG+Lacc). When the enzymatic reaction was finished, the paper sheets were removed one by one, spread onto blotting paper, and left allowed to dry in standard atmosphere (23°C, 50% RH). Sulfonated lignin (SL) was added at a 4% (w/w) concentration to the reaction vessel simultaneously with LG. This treatment was designated LG+SL+Lacc. Several control treatments were performed under the same conditions as the standard enzyme treatments. Such treatments involved the following substances: lauryl gallate (LG control), sulfonated lignin (SL control), enzyme (Lacc

control) and enzyme+SL (SL+Lacc control). The results of the control treatments were compared to the properties of the initial paper sheets.

4.2.3. UV–Vis spectra and residual laccase activity

The oxidation of lauryl gallate (LG) and sulfonated lignin (SL) by laccase (Lacc) was examined by UV–Vis spectrophotometry, using a Thermo Scientific Evolution 600 instrument. Residual laccase activity after the control and enzyme treatments was measured in the presence and absence of paper. One activity unit was defined as the amount of laccase transforming 1 μmol/min ABTS to its cation radical (ε_{436} nm = 29,300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer at pH 5 at 25℃.

4.2.4. Contact angle measurements and water drop tests

The water contact angle (WCA) of the paper was measured by using a Dataphysics OCA15EC contact angle goniophotometer, using an image capture ratio of 25 frames/s. 4 μL water drops were delivered to the samples surfaces. At least 10 measurements were made for each liquid probe. Also, changes in contact angle were monitored until complete absorption of each water drop. The water drop test (WDT) was performed according to Tappi standard T835 om-08. Fifteen measurements per treated paper sample were made.

4.2.5. Heat treatments

Heat treatments were performed by using the drying section of a Rapid Köten lab former. A constant temperature of 90℃ and a drying time of 20 min were used.

4.2.6. Washing and Soxhlet extractions

As explained in the "Materials and Methods" chapter, the bonding strength between fibers in the paper and the ingredients of the enzyme reaction was evaluated by soaking the paper in beakers holding hot water (80℃) for 30 min under constant stirring. After washing, the paper was spread onto blotting paper and allowed to dry under a normalized atmosphere (25℃, 50% RH). The paper was also subjected to Soxhlet extraction with acetone. After extraction, samples were placed onto blotting paper and allow drying under a normalized atmosphere (25℃, 50% RH).

4.3. Results and discussion

4.3.1. Laccase–lauryl gallate treatments

The primary purpose of this work was to assess the ability to increase the hydrophobicity of paper-based media by direct treatment with laccase in combination with a hydrophobic compound. The laccase used was from *Trametes Villosa* and the hydrophobic compound lauryl gallate (LG), used at a dose of 4% (w/w) for a variable time from 1 to 4 h. The laminar cellulosic product used was commercial filter paper. Paper sheets were treated with LG+Lacc and assessed for hydrophobicity, using parallel control treatments for comparison. The paper used in this study presented in its formulation wet strength resins, allowing the soaking the sheets in buffer medium for the enzymatic treatment without experiencing disintegration. Several ISO standard methods to test the strength before and after soaking in buffer showed no alteration on the properties of the paper (Table 4-1).

Table 4-1 Properties of the filter paper sheets before and after impregnation with the buffered aqueous solution.

	Before impregnation		After impregnation	
Property	**Mean**	**StD**	**Mean**	**StD**
Bursting Strength Index [kN/g]	2.23	0.23	2.36	0.31
Tear Strength Index [mNm²/g]	12.36	1.44	11.58	0.5
Folding Endurance [log n]	3	0.3	3	0.1
Tensile Strength Index [Nm/g]	38.38	5.43	46.03	3.23
Wet Tensile Strength Index [Nm/g]	6.72	0.73	6.56	0.83
Zero-Span [N/cm]	73	9	85	6

The most immediate inference from the results is that applying a laccase treatment to finished paper increases its hydrophobicity as measured with the water drop test (WDT) and in terms of contact angle (WCA). However, unlike treatments on fiber suspensions, LG distributed rather unevenly on the paper surface and led to an inconsistent hydrophobic response. As shown in Fig. 4-1a, dark spots of oxidized LG appeared on the paper surface as a result. The LG compound is highly hydrophobic and was insoluble in the treatment medium; therefore it was oxidized by the laccase, but provided the mentioned uneven distribution along the surface after the treatment.

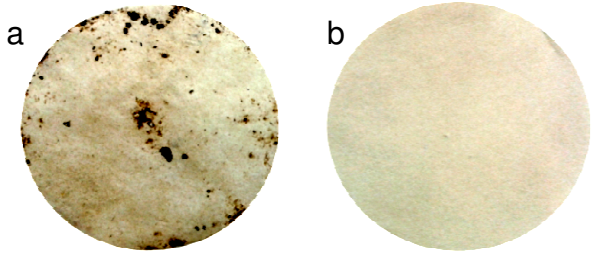

Figure 4-1 Surface images of filter paper sheets after enzyme treatment with LG+Lacc (a) and LG+SL+Lacc (b).

4.3.2. Improving LG distribution: natural surfactants

After having seen how the bad solubility of the LG compound affected its distribution on the surface of the paper sheet, an effective dispersant for LG not altering its properties was therefore required. The main problems with many synthetic surfactants such as linear alkylbenzene sulfonates (LAS) and alkylphenol ethoxylates (APE) are their high production costs, and their potentially deleterious effects on health and the environment. Lignosulfonates are generated in large amounts by the cellulose sulfite pulping industry, which has conducted vast research to find potential uses for this lignin byproduct. Lignosulfonates have found widespread use as an available renewable bioresource for producing different commercially attractive end-products including dispersants, detergents and surfactants. These compounds are known to reduce water surface tension by effect of the diphilic structure of their molecules, which contains a hydrophobic framework and a hydrophilic macromolecular component: ionogenic functional groups (Trufanova, Parfenova and Yarygina, 2010). The potential environmental effects of lignosulfonates have been the subject of extensive research which has shown them to be harmless to plants, animals and aquatic life if properly manufactured and used (Lauten, Myrvold and Gundersen, 2010; Shulga *et al.*, 2011). A lignosulfonate was introduced to the enzyme treatment aiming to be adsorbed at the interface between the two immiscible phases LG and water. The interfacial tension at the water-LG interface, which arises because of a similar imbalance of attractive forces as at the water surface, will be reduced by this adsorption.

The critical micelle concentration (CMC) of a surfactant is the concentration at which the surface active ions or molecules in solution associate to form larger units. These associated units are called micelles (self-assembled structures), and the first formed aggregates are generally approximately spherical in shape. Each surfactant molecules have a characteristic CMC value at a given temperature and electrolyte concentration.

The surface tension of liquids when gradually increasing the surfactant concentration strongly decreases until reaching the CMC; after reaching the CMC, no significant decrease on the surface tension is observed, remainimg relatively constant or changing with a lower slope. The CMC is an important characteristic of a surfactant, providing information about the optimal dosing in a given system. The CMC of the SL was determined by measuring the surface tension of water solutions with increasing SL concentration, in order to determine the optimal SL dose for its introduction in the enzymatic treatment. Surface tension was determined using the pendant drop method, in which the surface tension is determined by fitting the shape of the drop (in a captured video image) to the Young-Laplace equation which relates interfacial tension to drop shape. The software of the OCA15 (Dataphysics®) device does this automatically.

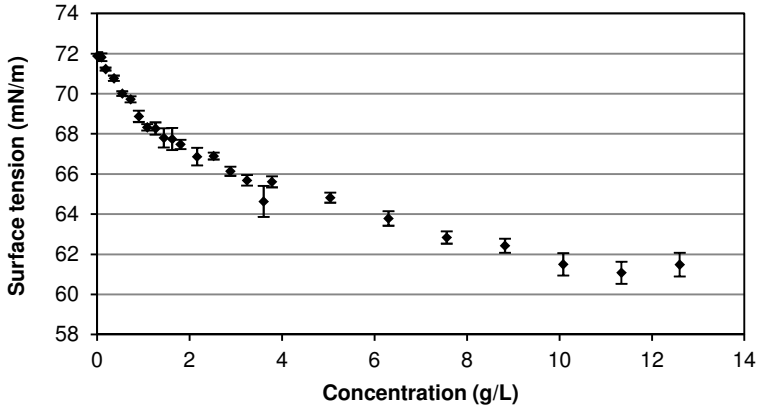

Figure 4-2 Evolution of the surface tension of water-SL mixtures with increasing SL concentration. CMC located at 10g/L.

As shown in Fig. 4-2, the water surface tension (72.8 mN/m) is clearly decreased by the gradual addition of SL, confirming that the SL was acting as a surfactant in aqueous mediums. The CMC is located around a SL concentration of 10 g/L, decreasing the water surface tension to a maximum of 61 mN/m. Despite the SL concentration to obtain the highest reduction of the water surface tension was found, the concentration was relatively high; since interesting reductions on the surface tension were also obtained for lower concentrations, it was decided to use the same dosage as the used for the LG compound.

Based on the foregoing, sulfonated lignins (SL) were added at the same dose as LG as natural surfactants to the LG+Lacc treatment. The paper samples subjected to the LG+SL+Lacc treatment exhibited an evenly hydrophobic surface (see Fig. 4-1b). This

novel result is very promising as it solves the distribution issues of using a sulfonated lignin as a natural surfactant —a problem never addressed before in enzyme treatments involving a hydrophobic compound. In addition, the ability to apply an enzymatic treatment directly to paper-based media circumvents the shortcomings of suspension-based enzyme treatments as regards effluent recycling, interactions between fibers and losses in some strength-related properties.

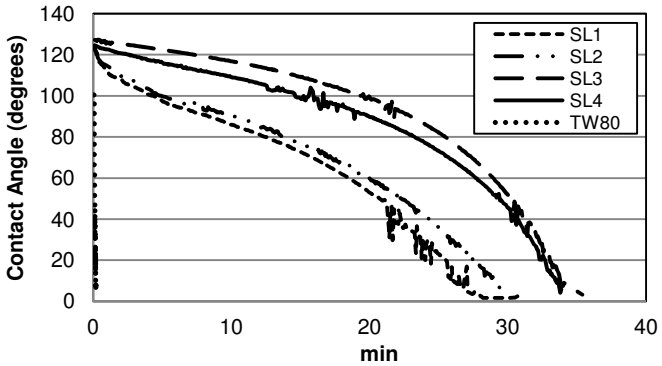

Figure 4-3 Temporal variation of the water contact angle in paper sheets treated with the Lacc–LG system in the presence of various lignosulfonates and TWEEN80®.

Four types of sulfonated lignins (SL1–SL4) were used to identify the best candidate in terms of hydrophobicity improvement and compared with TWEEN80® (TW80), a nonionic surfactant widely used in laccase treatments, in this respect. Five enzyme control treatments were also performed in parallel with each of the previous surfactants under the above-described conditions. The hydrophobic character of the resulting paper sheets was assessed via contact angle measurements of a water drop at variable times.

Fig. 4-3 shows the differences in absorption behavior of the paper in terms of contact angle. As can be seen, SL3 and SL4 were the lignosulfonates affording the strongest hydrophobization (viz. the longest absorption times and highest contact angles). The two were similarly efficient, but SL3 provided slightly higher contact angles, so it was adopted for further testing. As can also be seen from the figure, the contact angle of the sheets treated with TWEEN80® dropped to 0° within 2 s after deposition of the water drop; this surfactant was thus less efficient than the lignosulfonates in improving LG distribution on the paper surface. The results therefore reflected the favorable effect of lignosulfonates on hydrophobicity and LG distribution.

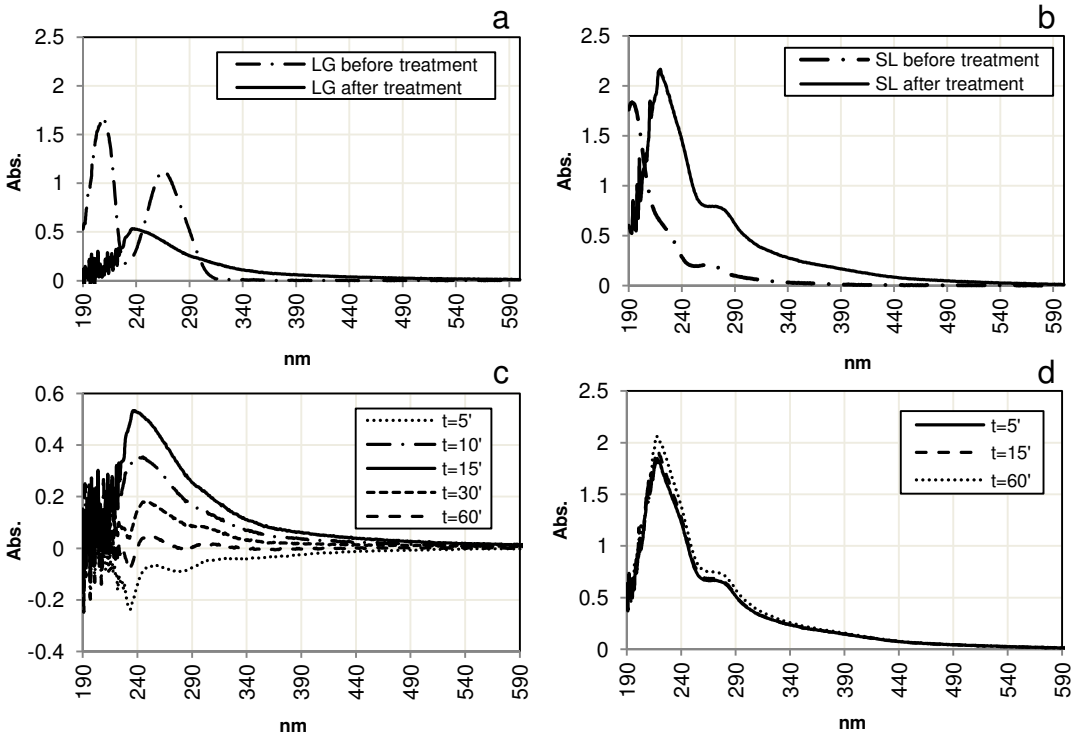

Figure 4-4 UV–Vis spectra for lauryl gallate (a) and sulfonated lignin (b) before and after enzymatic oxidation. Temporal variation of the absorbance spectra by effect of the LG+Lacc (c) and SL+Lacc treatment (d).

4.3.3. Lauryl gallate (LG) and sulfonated lignin (SL) oxidation

A change in the appearance of the effluents reflecting alteration of the reactants by the enzyme was observed after the LG+SL+Lacc treatments. This led us to monitor the oxidation of LG and SL catalyzed by laccase via absorbance changes in the spectrum at different times (5, 10, 15, 30, 45, 60 and 75 min). Figure 4-4a and 4-4b show the UV–Vis spectra for LG and SL before and after 15 min of enzyme treatment. As can be seen, LG exhibited two absorbance peaks at 220 and 275 nm (Fig. 4-4a), and SL another two at 210 and 280 nm (Fig. 4-4b), before the enzyme treatment. After treatment, the spectrum for SL retained the original peaks and shape for pure lignin, but was shifted to the right (Fig. 4-4b). On the other hand, the spectrum for LG (Fig. 4-4a) was markedly altered by the enzyme treatments and only one peak (that at 240 nm) was observed after the reaction. These results suggest that the enzyme oxidizes LG strongly and the sulfonated lignin slightly.

Figs. 4-4c and 4-4d show the variation of the absorbance spectra for LG and SL by effect of the LG+Lacc and SL+Lacc enzyme treatments. As can be seen from Fig. 4-4c, the peak at 240 nm increased during the first 15 min of LG+Lacc treatment, after which it decreased gradually, which is suggestive of enzyme inhibition or cyclic enzymatic oxidation–reduction of LG after the first 15 min. On the other hand, the spectrum for the SL+Lacc treatment (Fig. 4-4d) exhibited little change with time, probably because SL was rapidly oxidized by the enzyme and underwent no further reaction or change as the treatment progressed. Oxidation measurements in the presence and absence of paper sheets were used to assess the influence of PS on the oxidation of LG and SL. The presence of PS in the enzymatic reaction seemingly helped to protect SL from oxidation and altered the shape of the UV–Vis spectra for LG.

4.3.4. Effect on enzyme activity

The variation of the enzyme activity after oxidizing LG and SL was monitored by measuring its residual activity in the effluents from treatments at each reaction time (5, 10, 15, 30, 45, 60 and 75 min). Measurements were made in the presence and absence of paper sheets (PS) in the medium. The thermostability of the laccase from *Trametes Villosa* in the buffer was studied by Ibarra *et al.*, (2006), reporting that the activity experienced a decrease of the 50% after incubation during 4h at 50ºC. Therefore, in order to ensure that significant enzyme activity will remain after the treatments, and to enhance the differences given by the presence/absence of the SL and LG compounds, the maximum reaction time was set at 75minutes for the residual activity measurements.

As can be seen from Fig. 4-5a, the enzyme activity decreased gradually to 0 after 75 min of treatment with LG+Lacc; also, the presence of PS allowed the activity to be preserved for 15 min, after which it fell considerably as reflected in the absorbance decrease observed in Fig. 4-4c. By contrast, enzyme activity in the SL+Lacc treatments was reduced by only one half after 75 min (Fig. 4-5b). The residual activity of the effluents from the LG+SL+Lacc treatments exhibited an interesting feature. Thus, as can be seen from Fig. 4-5c, the enzyme activity was also reduced by one half after 75 min compared to 100% with the LG+Lacc treatment. This suggests that sulfonated lignin acts not only as a natural dispersant for LG, but also as a preservative for enzyme activity. The inactivation of laccase by synthetic mediators has previously been reported (Aracri, Colom and Vidal, 2009; Valls *et al.*, 2010b; Fillat, Colom and Vidal, 2010). In these works the enzymatic treatment was applied in a suspension of fibres instead of paper sheet, and it was obtained that the presence of pulp lignin and natural mediators also preserved the enzymatic activity and it is stated that lignin acts as a reductive substrate for the oxidized

mediator. The addition of SL to the LG+Lacc treatments also increased the hydrophobicity of the paper sheets. Therefore, sulfonated lignin has three favorable effects on the enzyme treatment.

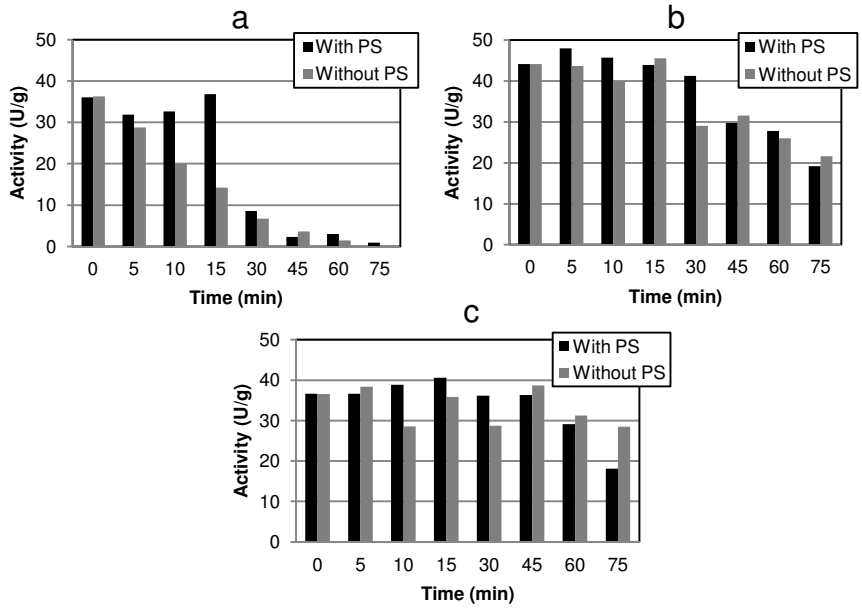

Figure 4-5 Residual enzyme activity at different times of treatment with (a) LG+Lacc, (b) SL+Lacc and (c) LG+SL+Lacc in both the presence and absence of paper sheets (PS). (Error: ±3 U/mL).

4.3.5. Hydrophobicity and influence of the reaction time

As revealed by the previous tests, some residual enzyme activity remained after 75 min of treatment with LG+SL+Lacc. This led us to examine the variation of hydrophobicity with the reaction time, using the Tappi normalized Water Drop Test (WDT). This test is a simple method involving placing a drop of deionized water on the surface of paper and recording the time needed for complete absorption, which is signaled by vanishing of the specular gloss of the drop. The initial absorption time for untreated paper sheets was 4 ± 0.85 s.

The treatment time was gradually increased until the hydrophobicity of the paper leveled off. As can be seen from Fig. 4-6a, a slight increase in hydrophobicity was observed after 1 h of treatment. After 2 h, complete absorption of the water drop took 7–6 min. A sharp increase in hydrophobicity was observed between the third and fourth hours of treatment. Also, after 4 hours of treatment, the drop needed around 50 min to be

completely absorbed and the absorption time leveled off above 2300 s (about 40 min). The paper sheets subjected to the LG, SL and SL+Lacc control treatments exhibited no increase in hydrophobicity with time. On the other hand, those treated with LG+Lacc exhibited a slight increase but never approached the hydrophobicity levels of the LG+SL+Lacc treatment. Figures 4-6b and 4-6c show the absorption differences between an untreated paper sheet and one treated for the optimum time established in the hydrophobicity tests (4 h).

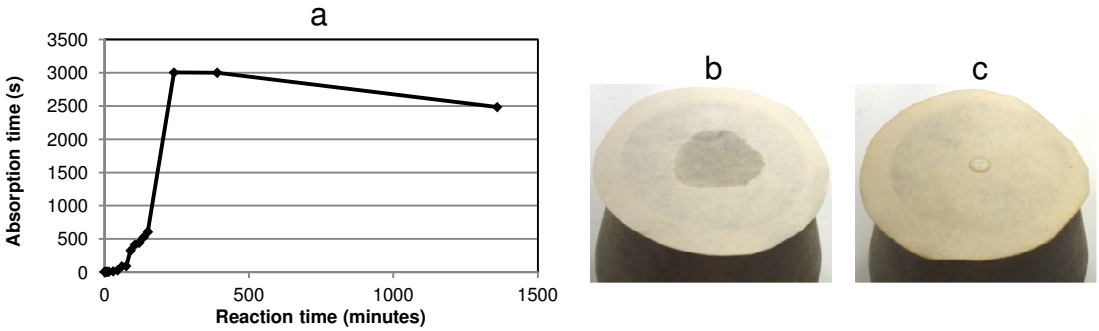

Figure 4-6 Temporal variation of the hydrophobicity of the treated paper sheets (a), and differences in absorption of the water drop after 5 s of deposition on untreated (b), and treated paper (c).

The hydrophobicity of the treated sheets was also assessed via water contact angle (WCA) measurements made with a goniophotometer. This instrument comprises a high-resolution video camera, an automatic syringe and a needle of calibrated diameter. The dosing volume and image capture parameters are controlled by analysis software. The procedure involves placing a known water volume onto the solid surface to be characterized for hydrophobicity and measuring the angle between the tangent of the water drop profile and the sample surface. If the contact angle is greater than 90°, then the surface is hydrophobic; otherwise, it is assumed to be hydrophilic. Placing a 4 μL water drop onto untreated paper provided a contact angle of 16 ± 3° (Fig. 4-7a); therefore, the untreated paper surface was clearly hydrophilic. On the other hand, placing a drop onto treated sheets led to a contact angle of 121 ± 2°, consistent with their hydrophobic character (Fig. 4-7b).

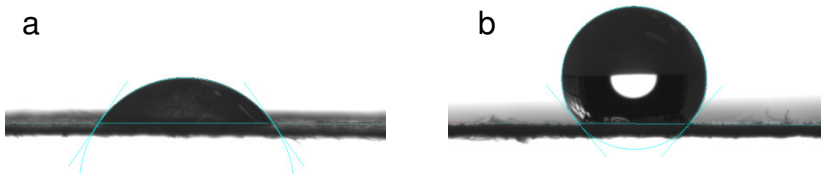

Figure 4-7 Hydrophobicity of filter paper sheets as measured with a contact angle goniometer in untreated paper (a) and enzyme-treated paper (b).

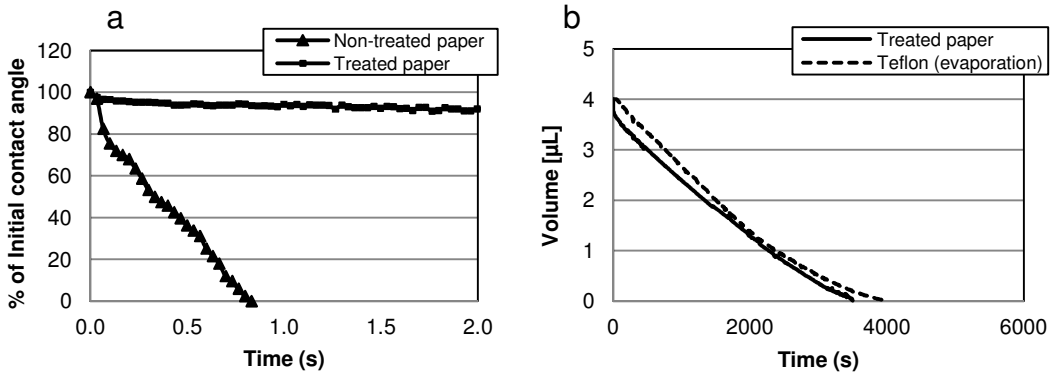

Figure 4-8 Temporal variation of the contact angle in untreated and treated paper (a), and comparison of water absorption in treated paper and Teflon® (b).

Water drops were found to remain onto the surface of the treated sheets for a long time before being completely absorbed. This led us to examine the variation of the contact angle with time. Changes in contact angle after deposition of the drop were due to a decrease in volume and promoted mainly by absorption in the sheet structure and evaporation —the latter, however, was only influential after a long time. The contact angle was measured at a constant rate of 25 measures/min for 2 h after deposition of the water drop. The angle fell very quickly (less than a second) to 0° in untreated paper but took about one hour to reach the same level in treated paper, thereby reflecting the long-lasting hydrophobicity of the treated sheets (Fig. 4-8a). This result was compared with that obtained with Teflon®, a material with a water contact angle similar to that of our treated sheets; because Teflon® is completely non-absorbent, a water drop deposited onto its surface can only disappear by evaporation. A 4 µL drop was placed on the surface of treated sheets and Teflon® and the resulting volume decrease measured until complete disappearance of the drop. As can be seen from Fig. 4-8b, the curves for the two materials were very similar. Therefore, the treated sheets were nearly as hydrophobic as a perfect nonabsorbent surface. Although the curve for the paper treated with LG+SL+Lacc fell

slightly below that for Teflon® owing to very slight absorption in the former, the volume decrease in the drops deposited onto the treated sheets was clearly due to evaporation. These results confirm that one can obtain highly hydrophobic paper surfaces by applying an appropriate enzyme treatment directly to finished paper.

4.3.6. Influence of heating and the LG dose on hydrophobicity

The initial conditions were modified to examine their influence on the results of the water drop test. Heating and the LG dose were found to be the individual factors most markedly influencing the absorption behavior of the paper sheets. The paper was subjected to enzyme treatments with laccase, sulfonated lignin and lauryl gallate (LG+SL+Lacc) where the proportion of LG ranged from 4% (w/w) (LG_4) to 8% (w/w) (LG_8). Heat treatments (LG_4+Heat and LG_8+Heat) were performed in a Rapid Köten lab former as described above. The pH, time and temperature conditions used were the same as in the previous tests. As can be seen from Fig. 4-9, doubling the proportion of LG increased hydrophobicity as measured with the WDT from 90 to 350 s. Also, heating dramatically increased the drop absorption time (to WDT values around 1200 s). Heating and a high LG dose had a synergistic effect on hydrophobicity, which was raised to levels in the region of 1700 s.

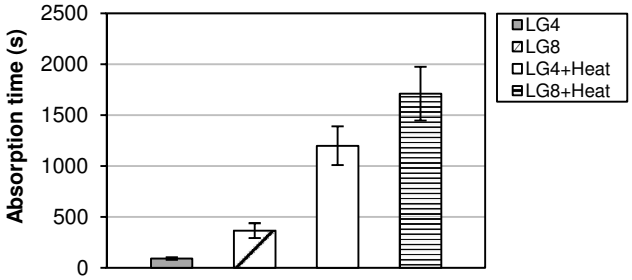

Figure 4-9 Influence of the process conditions on the hydrophobicity of paper sheets as measured via WDT. LG_4 and LG_8 denote treatment with an LG dose of 4 and 8%, respectively; and LG4+Heat and LG8+Heat heating in addition to the previous treatments.

4.3.7. Evaluation of bonding strength

As stated before, the sheets obtained with the LG+SL+Lacc treatment exhibited high hydrophobicity levels. The results were suggestive of a chemical or physicochemical interaction between enzyme-modified LG and paper fibers. Strong washings with hot water and Soxhlet extractions with acetone were used to assess the paper bonding strength of the treated sheets.

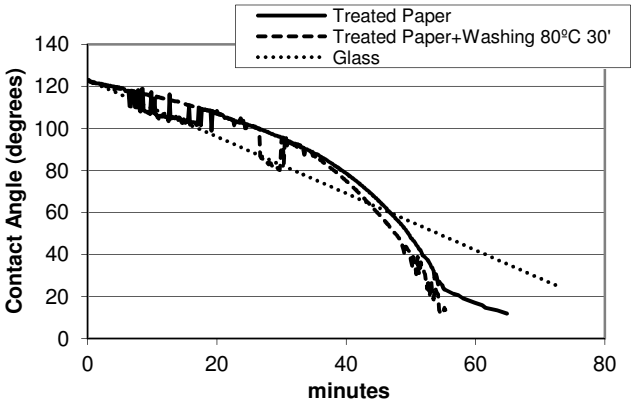

Figure 4-10 Variation of the water contact angle in LG+SL+Lacc treated sheets as such and after washing with hot water (80℃) for 30 min.

Fig. 4-10 shows the variation of the water contact angle for an LG+SL+Lacc treated paper sheet as such and after washing with water at 80℃ for 30 min. The results were very similar, which indicates that the strong washing conditions used failed to break the bonds between LG and paper fibers and hence that the bonds were very strong. Recently, a covalent binding of phenolic compounds with flax and sisal fibres has been evidenced when the laccase system was applied in fibre suspension (Aracri *et al.*, 2010), but no data existed for the case of paper sheets until now; this is the first time that the laccase system is applied in commercial finished paper. The figure also shows the variation of the contact angle of a drop deposited onto a glass surface as a model of a completely nonabsorbent material; as can be seen, the curve differed markedly from those for the paper sheets as a result of the presence of absorption phenomena in the sheets.

Soxhlet extractions were carried out on untreated sheets and sheets subjected to the LG+SL+Lacc treatment, using acetone for 2.5 h. The initial contact angle of the sheets was measured after complete drying. The contact angle for the untreated sheets after Soxhlet extraction was 30° ± 3, and that for the treated sheets 123° ± 3. These results suggest that LG remained strongly attached to the paper fibers, even after acetone extraction.

4.4. Conclusions

Applying an enzyme treatment to finished paper increases its hydrophobicity by virtue of the threefold favorable effect of sulfonated lignin (SL) as a dispersing agent, hydrophobicity enhancer and enzyme activity preserver. The hydrophobic compound used, lauryl gallate (LG), is oxidized by the enzyme, and so is SL, albeit only slightly. Increasing the treatment time increases the hydrophobicity of the paper, and so do

heating and raising the LG dose. Also, as inferred from contact angle measurements, treated paper acquires long-lasting hydrophobicity similarly high to that of a perfect nonabsorbent surface and resisting severe washing with hot water and Soxhlet extraction by virtue of the high bonding strength between enzyme-modified LG and paper fibers.

4.5. References

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Chapter 5

Coating of Cellulose-based Materials by an Innovative Functionalization Bioproduct

Abstract

The present chapter describes an innovative method for the hydrophobization of cellulosic material by impregnation with an enzymatically obtained functionalization solution (FS). Application of FS to the surface of previously formed cellulose sheets was found to confer them hydrophobic properties. The absorbance of functionalized sheets was assessed with the water-drop test (WDT), and their hydrophobicity from their contact angle (WCA) and the surface free energy (SFE) as determined with a goniophotometer. The proposed method is an effective choice for the hydrophobization of paper sheets, with absorption times of up to 4000 s and WCA values around 130°. Treating eucalyptus paper sheets dramatically decreased SFE (from 55 to 10 mJ/m^2), and evidences on the grafting of the FS onto the cellulosic sheets were assessed by using ATR-FTIR. The stability of FS and the control solutions was characterized in terms of Z potential and light scattering measurements. The proposed enzymatic method is simple, expeditious and suitable for industrial implementation with no additional investments.

5.1. Introduction

Paper and board manufacturing processes include operations intended to improve quality in terms of mechanical resistance, optical rendering or barrier properties. The ability of paper to resist wetting and penetration by liquids such as water, hot and cold drinks, juices, inks, oils or organic solvents is important with a view to manufacturing some paper-based products such as paper cups, packaging and printing media (Bordenave, Grelier and Coma, 2010; Hubbe, 2007). However, wetting strength in paper can only be accomplished by making it hydrophobic.

Surface functionalization is the process by which functional groups are added to a surface in order to modify its properties. One way of hydrophobizing paper substrates is by grafting hydrophobic compounds onto lignocellulosic fibers. This, however, entails chemically altering the paper surface or using a product or reagent to bind functional groups to paper fibers. Enzymes have been shown to catalyze coupling reactions with potential use in various areas, including the field of lignocellulosic materials (Fackler *et al.*, 2008; Grönqvist *et al.*, 2006). This has aroused increasing industrial interest in them in recent years. Enzymes provide substantial advantages over chemical catalysts by virtue of their being derived from renewable resources, usable under relatively mild conditions of temperature and pH, and exquisitely selective in both reactant and product stereochemistry. Societal interest in green chemistry and advances in biotechnology have brought to the forefront the use of enzymes to address many of the challenges of modern synthetic organic chemistry (Cherry and Fidantsef, 2003; Ma and Jiang, 2002). Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.3) is a blue multi-copper oxidase capable of catalyzing the oxidation of various low-molecular weight compounds while concomitantly reducing molecular oxygen to water. Recently, this enzyme has garnered increasing attention as an oxidative catalyst for fine chemical synthesis thanks in part to its high stability, selectivity for phenolic substructures and ability to act under mild reaction conditions (Witayakran and Ragauskas, 2009).

Laccases have been successfully used in biobleaching (Fillat and Roncero, 2009; Valls and Roncero, 2012), and also to functionalize cellulose fibers for purposes such as internal sizing of paper (Garcia-Ubasart *et al.*, 2011), or obtaining paper sheets with antimicrobial properties (Fillat *et al.*, 2012), or improved wet strength properties (Aracri, Roncero and Vidal, 2011). Also, Garcia-Ubasart *et al.*, (2012) and Sipponen *et al.*, (2010) obtained hydrophobic fibers by applying laccase in combination with hydrophobic phenolic compounds to a fiber suspension. However, all previous enzyme treatments were conducted in aqueous suspensions consisting of cellulose fibers, an enzyme and the functional compound (i.e. before the paper or paper substrate was formed).

Industrially, introducing chemicals or enzymes before paper formation can alter the natural interaction between fibers and lead to paper with impaired strength-related properties and less easily recycled effluents as a result. In previous work (Cusola *et al.*, 2013, chapter 4), this problem was addressed by applying a laccase treatment for the first time to finished paper in order to improve its hydrophobicity and avoid the shortcomings of the procedure traditionally applied to fiber suspensions. Although an effective method for hydrophobizing finished paper via enzymatic coupling was developed, the processing time was rather long. In fact, paper sheets required soaking in the enzyme solution for 4 h, which is a strong hindrance for industrial application since paper machines can currently operate at rates in the region of 1500 m/min. Therefore, the previously reported method by Cusola *et al.*, (2013) (chapter 4), was not feasible to be implemented industrially in the papermaking process due to the long reaction time required.

In this work, we developed a new, fast method for the surface treatment of paper sheets that is compatible with these paper processing rates. The surface treatment has the advantages that it does not affect sheet formation, uses small amounts of chemicals per functionalized area unit, is compatible with the high throughput of paper machines, can be implemented at various points in the industrial process and can be applied to sheet surfaces with existing devices. The proposed method provides an innovative tool for functionalizing paper surfaces by using the enzyme laccase to make them hydrophobic. The functionalization solution is compatible with most papermaking technologies (size pressing, speed sizing, spraying). The method is the basis for a patent filed by Cusola *et al.*, (2012).

5.2. Materials and methods

5.2.1. Paper, enzyme and chemicals

Filter paper sheets (PS) were purchased from FILTERLAB. Eucalyptus ECF pulp was supplied by ENCE® (Spain), and unbleached flax and sisal pulps were kindly supplied by CELESA®. The enzyme used was laccase (Lacc) from *Trametes Villosa* supplied by Novozymes® (Bagsvaerd, Denmark). Lauryl gallate (LG) and methyl-glycol chitosan (MGCh) were purchased from Sigma–Aldrich. Soluble sulfonated kraft lignin (SL) was obtained from Borreegard and used as received. Eka WS 505 PAAE resin was supplied by Azko Nobel Chemicals®. Cationic starch (CSt) was complimentarily supplied by Roquette®. The chitosan coating sauce was prepared by mixing 0.1 g of finely ground MGCh powder with 5 mL of 1% (w/w) acetic acid and stirred at room temperature for 30

min, after which coating color was applied onto the surface of laboratory-made eucalyptus sheets with a Mayer bar. The eucalyptus, flax and sisal pulp samples were refined to a variable degree (°SR) according to ISO 5264-1:1979 on a Valley mill. More details on the compounds are given in chapter 2.

5.2.2. Preparation of laboratory handsheets

Refined and unrefined samples from eucalyptus, flax, and sisal pulp were used to prepare handsheets in a Rapid-Köten lab former according to ISO 5269-2:2004. Filter paper sheets were used as received. All sheets were cut into circles 4 cm in diameter for surface treatment.

5.2.3. Sonication

An aqueous solution of lauryl gallate (LG) in 50 mM sodium tartrate buffer at pH 4 was sonicated on a Hielscher® Ultrasonic Processor UP100H at 100% amplitude for 30 min.

5.2.4. Laccase treatments to obtain the functionalization solution (FS) and surface application to paper sheets

In the work described in the present chapter, the enzymatic reaction was performed in the absence of paper sheets (PS) and the resulting functionalization solution (FS), was used to impregnate paper sheets by dipping. Enzymatic treatments were performed using 250 mL beakers containing final concentrations equivalent to 50 mM sodium tartrate buffer (pH 4), 1.2 g/L LG, 1.2 g/L lignosulfonate and 1.2 U/mL laccase. LG was used as a colloid suspension obtained by sonication. The resulting, functionalization solution (FS) was then applied to the surface of paper sheets by dipping, and the sheets were allowed to dry in a normalized athmosphere (23℃; 50% RH). Various control treatments were also performed by using control functionalization solutions (KFS) and the results compared with those for the paper sheets before any surface treatment (see Table 2-2, where PhC corresponds to LG in the present case). More details on the laccase treatments are shown in chapter 2.

5.2.5. Hydrophobicity assessment

Contact angles were measured with a Dataphysics® OCA15 contact angle goniometer, using a 4 μL water drop for delivery to the sample surface in each measurement. Surface free energy (SFE) was determined by applying 4 μL droplets of deionized water, ethylene glycol and 99% diiodomethane to the surface of paper sheets and determining the corresponding contact angles. Water drop tests (WDT) were performed on each treated

paper specimen according to Tappi standard T835 om-08. Previously, the paper sheets were conditioned according to ISO 187.

5.2.6. ATR-FTIR analysis

ATR-FTIR spectra of paper sheets were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer equipped with universal ATR sampling accessory. The spectra wavenumber range was 4000-550 cm^{-1} and the number of scans was 32, with a wavenumber resolution of 1 cm^{-1}. Paper samples were placed directly on the ATR accessory and the measuring cell was washed using deionized water and ethanol between measurements.

5.2.7. Z potential tests and paper properties

The stability of enzymatic products was assessed by measuring the electrophoretic mobility of the solutions with a Malvern Zetamaster device equipped with a laser Doppler velocimeter. The physicomechanical properties of the paper samples after treatment with FS were assessed by using commercial grade laboratory filter paper in accordance with ISO standards. The target properties were Bendtsen permeability (ISO 5636), burst strength (ISO 2758), tear strength (ISO 1974), folding endurance (ISO 5626), tensile strength (ISO 1924), wet tensile strength (ISO 3781), $Cobb_{60}$ (ISO 535) and zero-span tensile strength (ISO 15361).

5.2.1. Soxhlet extraction and washing tests

Bonding strength between fibers and hydrophobic compounds in FS was assessed by subjecting the sheets to severe washing and extraction with solvents prior to determining their hydrophobicity. Washings were carried out by soaking the paper in beakers containing hot water at 80℃ for 30 min under constant stirring. Paper samples were also subjected to Soxhlet extraction with acetone.

5.3. Results and discussion

Lauryl gallate (LG) was the compound used to confer the hydrophobic behavior to the paper sheets. The main difference with respect the previously reported method (Cusola *et al.*, 2013, chapter 4), is that in this case LG is enzymatically modified alone (without the presence of any cellulosic material) before coating the surface of paper media using the resulting product.

LG has a very low surface free energy and is thus highly hydrophobic. As a result, it was insoluble in the treatment solution and poorly dispersed on the paper surface after reaction. In fact, LG easily formed aggregates that were still present after the enzymatic reaction. This led us to use sulfonated lignin (SL) in order to improve LG dispersion as in previous work (Cusola *et al.*, 2013). Nowadays, the introduction of an ultrasound step prior to the enzymatic reaction allowed LG aggregates to be efficiently disintegrated and improved dispersion of the compound and interaction with the enzyme. LG was added to an aqueous treatment solution buffered with 50 mM sodium tartrate at pH 4 and sonicated at 100% amplitude for 30 min. This was followed by addition of SL and filter paper sheets, after which the procedure continued identically as described elsewhere (Cusola *et al.*, 2013). The "a" and "b" bars in Fig. 5-1 illustrate the differences in WDT measurements between paper sheets treated with sonicated and unsonicated LG using the previously reported method (chapter 4). Sonication seemingly increased hydrophobicity in the sheets by effect of the disintegration of LG aggregates leading to a better distribution of LG during the enzymatic treatment and providing an increased amount of LG available for oxidation by laccase. Based on these results, all LG solutions subsequently used were previously sonicated.

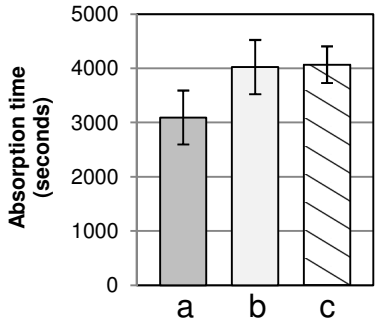

Figure 5-1 Hydrophobicity as measured with the WDT of filter paper sheets treated with the previously reported method (Cusola *et al.*, 2013) (a), treated with the previously reported method with sonication (b) and treated with the new method (c).

5.3.1. Hydrophobization of paper sheet surfaces

The above-described method for functionalizing paper surfaces via an enzymatic reaction involving a hydrophobic compound (LG) (Cusola *et al.*, 2013, chapter 4), has a serious shortcoming: too long a reaction time. These long reaction periods appear as limiting factors for industrial application. Thus, the process involves immersing paper sheets in an enzymatic reaction medium at 50℃ for 4 h, which represents a clear hindrance for implementation in the manufacture processes of cellulosic substrates.

Surface functionalization is the process by which functional groups are added to a surface in order to modify its properties. Grafting of suspended fibers with laccase-mediator systems previously revealed that the phenolic compound used as mediator was covalently bound to pulp fibers (Fillat, Roncero and Vidal, 2012), especially if the pulp contained hexenuronic acid (HexA) groups (Cadena *et al.*, 2011; Aracri *et al.*, 2010). Seemingly, the mediator was first oxidized by laccase and then bonded to lignocellulosic fibers. Therefore, the phenolic compound might be the agent of the fiber changes conferring new properties to pulp.

Based on the foregoing, we addressed the problem posed by the long reaction time by having the enzyme react with lauryl gallate as phenolic compound, albeit in the absence of paper sheets, in order to obtain a post-enzymatic solution containing an enzyme-modified phenol. The resulting, functionalization solution (FS) was applied to paper sheets by impregnation/immersion, after which the sheets were allowed to dry in a normalized atmosphere (23°C, 50% RH). The ensuing method involves the following steps: (1) initial sonication of LG in the reaction buffer (50 mM sodium tartrate); (2) enzymatic reaction under conditions described elsewhere, but in the absence of paper sheets to obtain the FS; (3) impregnation of paper sheets with FS; and (4) drying of impregnated sheets under a normalized atmosphere or with forced drying.

The resulting sheets were subjected to the Water Drop Test (WDT) and the results compared to those for sheets that were functionalized by using the previously reported procedure (Cusola *et al.*, 2013, chapter 4). As can be seen from bar "c" in Fig. 5-1, the WDT values for the sheets treated with the new method were higher to those provided by the previous method. Control functionalization solutions (KFS) were obtained by using various modifications of the original standard procedure to assess the influence of each individual agent, namely: laccase (Lacc), lauryl gallate (LG) and lignosulfonate (SL). The solutions were prepared in the absence of paper and then used to impregnate filter paper sheets by immersion as described above. Then, the sheets were allowed to dry under a normalized atmosphere (23°C, 50% RH) and subjected to the WDT. The LG, SL and laccase combinations used to prepare the control solutions are listed in Table 2-2. Figure 5-2 shows the WDT values of the filter paper sheets impregnated in the different control solutions (LG, LG+SL, SL, Lacc, LG+Lacc and SL+Lacc) and the functionalization solution (LG+SL+Lacc). As can be seen, none of the compounds improved hydrophobicity by itself, and only the LG+Laccase control solution increased it significantly. In fact, only the tree compounds in combination (LG+SL+Lacc) resulted in a dramatic increase in paper hydrophobicity. Also, the combined contribution of the LG+Lacc and SL+Lacc solutions to hydrophobicity as measured with the WDT was lower

than that of the LG+SL+Lacc solution. This confirms the presence of a synergistic effect between LG and SL, and is consistent with previous results (Cusola *et al.*, 2013) based on which SL improves LG dispersion. Therefore, the LG+SL+Lacc combination provided a functionalization solution (FS) conferring high hydrophobicity to paper sheets after impregnation for a short time.

The results were suggestive of chemical or physicochemical interaction between enzyme-modified LG and paper sheets. The treated sheets were subjected to strong washing with hot water and Soxhlet extraction with acetone to assess the strength of paper–chemical linkages. The measured contact angles (WCA) for the treated sheets were insignificantly decreased by hot washing and Soxhlet extraction (about 4 and 5% respectively); this suggests that LG was strongly bound to the paper sheets even after washing and acetone extraction.

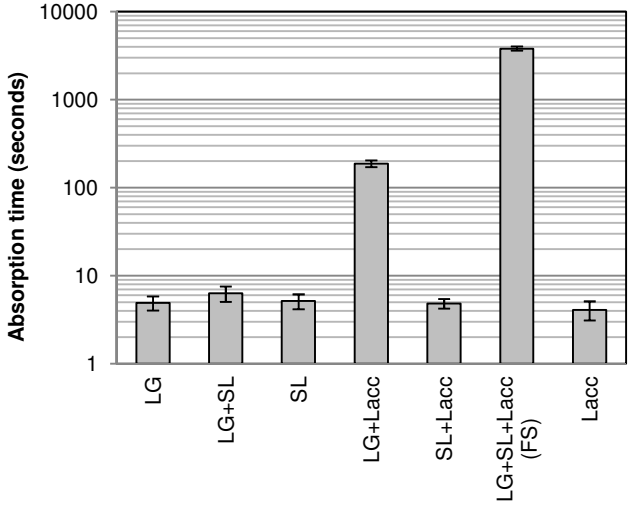

Figure 5-2 WDT of filter paper sheets after impregnation with the control solutions (LG, LG+SL, SL, LG+Lacc, SL+Lacc, Lacc) and the complete functionalization solution (LG+SL+Lacc).

Various other physical properties of the treated paper sheets were also evaluated, using ISO standard methods, with a view to determining whether the hydrophobization treatments altered the morphology of the sheets and detracted from their initial strength. Based on the results (shown in Table 5-1), Bendtsen permeability, burst strength, folding endurance, tensile strength and zero-span tensile strength were practically unaltered by FS treatment. By contrast, wet tensile strength was improved by 35% relative to control,

and the $Cobb_{60}$ of treated sheets reached a value about the 22 g/m² while it could not be measured on control samples (untreated samples) due to complete absorption of water. Therefore, hydrophobization with FS had no deleterious effect on strength-related properties of the paper sheets and even increased their wet tensile strength.

Table 5-1 Mechanical properties of the filter paper sheets before and after impregnation with the FS.

	Before impregnation		After impregnation with the FS	
Property	**Mean**	**StD**	**Mean**	**StD**
Bursting Strength Index [kN/g]	2.23	0.23	2.41	0.25
Tear Strength Index [mNm²/g]	12.36	1.44	11.95	0.92
Folding Endurance [log n]	3	0.3	3	0.1
Tensile Strength Index [Nm/g]	38.38	5.43	36.77	4.07
Wet Tensile Strength Index [Nm/g]	6.72	0.73	9.10	2.55
Zero-Span [N/cm]	73	9	79	6
$Cobb_{60}$ [g/m²]	-	-	22	2.07

The proposed enzymatic method is greatly important and innovative because it avoids the need to treat sheets during the enzymatic reaction and substantially expedites the manufacture process as a result. It thus represents a step forward in the fast hydrophobization of paper surfaces by using an enzymatic treatment involving a natural hydrophobic compound such as lauryl gallate. In addition, the method is suitable for use by the papermaking industry without the need for additional investments. Thus, it can be implemented by using common papermaking technologies such as size pressing, speed sizing or spraying at the end of the drying section. Because the enzymatic functionalization solution (FS) can be produced batchwise off the paper manufacturing line, the reaction time should pose no problem.

Table 5-2 WDT values for refined and unrefined handsheets before and after impregnation with FS.

Handsheets	**Initial [seconds]**		**Initial + FS [seconds]**		**Refined [seconds]**		**Refined + FS [seconds]**	
	Mean	**StD**	**Mean**	**StD**	**Mean**	**StD**	**Mean**	**StD**
Eucalyptus ECF	1	-	77	13	26	3	422	85
Unbleached Sisal	0.5	-	62	13	4	1	215	44
Unbleached Flax	1	-	705	123	4	1	663	81

5.3.2. Application of the new method to paper substrates

In order to extend the use of the functionalization solution (FS) to other types of paper-based substrates and assess the influence of pulp type, laboratory sheets were obtained from various raw materials and subjected to the WDT after impregnation with FS. Eucalyptus ECF, unbleached sisal and flax pulp samples were used to obtain laboratory handsheets. Sheets were impregnated with FS and allowed to dry under the conditions described in the previous section. Table 5-2 shows the WDT results for the laboratory sheets before (initial) and after impregnation with FS. An obvious increase in water repellency was observed with respect to the initial, untreated sheets after impregnation. However, the measured WDT values were far from those obtained for the filter paper in the previous tests, which easily reached absorption times in the region of 4000 s (Fig. 5-2). The main differences between the commercial filter paper sheets and our laboratory sheets were as follows: filter paper sheets were manufactured from refined pulp, which strengthens fiber linkages via hydrogen bonds; also, they may contain various additives including water resistance agents. We therefore aimed to identify the specific factor of filter paper sheets leading to optimal retention of the hydrophobic compounds present in FS in order to extend the use of the proposed method to other paper types.

5.3.3. Effect of refining

Refining pulp before paper manufacturing is a common practice intended to optimize linkages between paper fibers. Among other effects, refining causes the final structure of paper to appear more closed by effect of its reducing paper porosity; also, it increases the water retention value (WRV) of fibers, which makes paper more absorbent and increases its ability to retain liquids.

New laboratory handsheets were obtained by refining the pulp samples in a PFI mill at 4500 or 6000 revolutions in order to check whether refining was the specific factor behind the retention of hydrophobic compounds present in FS on the surface of paper sheets. As can be seen from Table 5-2, untreated refined sheets exhibited an increased water retention capacity, thereby confirming the previous hypothesis about the refining process. The increase was very slight for sisal and flax sheets relative to eucalyptus sheets. Impregnation with FS substantially increased the WDT values for the refined eucalyptus and sisal sheets, but maintained those for the refined flax sheets. Refining seemingly increased the ability of paper to retain FS, but depending on the particular type of pulp. Because the WDT values for FS-impregnated eucalyptus and sisal sheets were similar to each other but rather different from these for flax sheets, the type of pulp used obviously influences FS retention. In any case, the WDT values obtained were still

far from those for filter paper sheets. Therefore, refining cannot be the key factor causing retention of hydrophobic compounds present in FS.

5.3.4. Effect of additives

Once refining was confirmed not to be the main factor leading to optimal retention of FS on the paper sheets, we focused on additives. The wet strength of filter paper is to some extent dictated by its uses. Paper loses most of its strength when exposed to high humidity or soaked in water. In fact, paper exposed to high humidity absorbs water and swells (Hubbe, 2007); as a result, hydrogen bonds are destroyed (Hossain *et al.*, 2010) and the paper loses most of its original dry strength. There are several methods for conferring wet strength to paper; most use resins such as urea-formaldehyde (UF), melamine-formaldehyde (MF), polyamidoamine-epichlorohydrin (PAAE) and various other polymers including polyethyleneimine (PEI), glyoxalated polyacrylamide (G-PAM) and dialdehyde starch (DAS).

The effect of additives in the FS retention was assessed by producing laboratory handsheets from eucalyptus ECF pulp, which were treated with a wet strength resin consisting of polyamidoamine-epichlorohydrin (PAAE). After impregnation with FS, sheets were allowed to dry under a normalized atmosphere (23℃, 50% RH) and assessed with the WDT. Before sheet formation, the pulp stock was processed as explained under Materials and Methods. Therefore, the PAAE pre-treated sheets were surface-treated with FS and tested. The WDT value obtained after complete drying, 4807 ± 231 s (Table 5-3), was slightly higher than that for filter paper and confirmed that the wet strength resin was responsible for the optimal retention of FS on the paper sheets.

Table 5-3 WDT of eucalyptus handsheets treated with the wet strength agents PAAE and cationic starch (CSt), and also with methyl glycol chitosan (MGCh), before and after treatment with the functionalization solution (FS).

	PAAE [seconds]	PAAE + FS [seconds]	CSt [seconds]	CSt + FS [seconds]	MGCh [seconds]	MGCh + FS [seconds]
Mean	1	4807	1.77	4482	4.7	5088
StD	-	231	0.6	209	0.5	320

In order to determine why the PAAE resin resulted in optimal retention, another widely used wet-strength compound consisting of cationic starch (CSt) was tested. Eucalyptus pulp was pretreated with CSt as described under Materials and Methods, and used to prepare laboratory handsheets for water drop testing. The WDT value obtained, 4482 ±

209 s (Table 5-3), confirms that not only the PAAE resin was effective in improving retention of FS. The most obvious connection between PAAE and cationic starch is their cationic character. In the resin, the cationic character is provided by azetidinium groups present in the chain of the reticulated PAAE (Fig. 5-3a); in cationic starch, it is provided by tertiary or quaternary amino groups added to the starch molecule as cationic substituents (Fig. 5-3b).

The results suggested a physicochemical effect: electrical attraction between FS and cationic groups in the wet strength additives. PAAE is a widely used additive in papermaking but produces somewhat toxic effluents. In order to confirm that cationicity was the main property improving FS retention and develop an environmentally friendly process, we used a new treatment based not on a wet-strength additive, but rather on a natural cationic product.

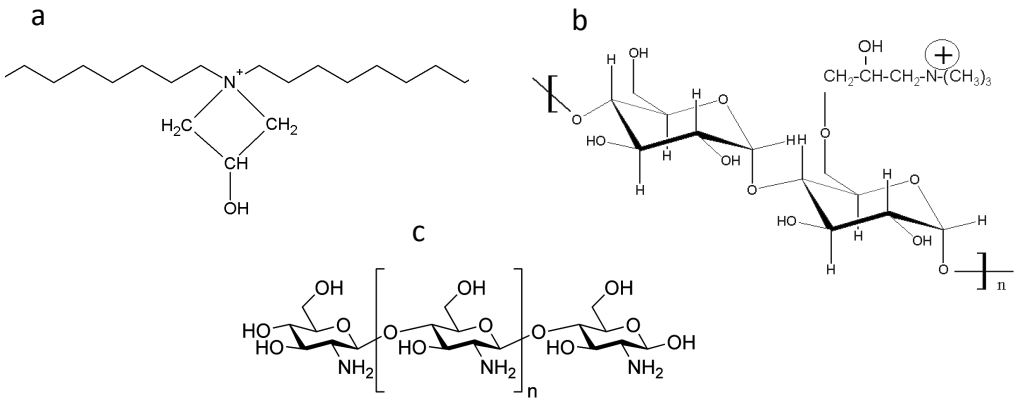

Figure 5-3 Structure of an azetidinium group, a source of cationic charge and reactivity in PAAE wet-strength resins (a), chemical structure of a portion of a cationic starch molecule (b) and part of a common chitosan molecule (c).

The treatment involved coating eucalyptus handsheets with a thin layer of chitosan. We selected this substance because it is a natural product with cationic character given by its amino groups (Fig. 5-3c), it was previously found to exhibit affinity for and effective adsorption onto cellulosic materials (Orelma *et al.*, 2011), and because previous works already reported the enzymatic grafting of phenolic compounds onto chitosan with the aim of obtaining hydrophobic surfaces (Chen *et al.*, 2000). A chitosan coating was obtained from methyl glycol chitosan (MGCh) by using a procedure based on the method of Rugmini *et al.*, 2010 and described in the "Materials and Methods" chapter. The MGCh

sauce was applied onto paper sheets which were then allowed to dry and coated with FS. The WDT value for the MGCh-coated sheets was 5088 ± 320 s (Table 5-3) and confirms that cationicity was the main factor causing adsorption of FS on the surface of the sheets. It is therefore possible to obtain functionalized sheets by using natural, eco-friendly products and processes. As can be seen from Table 5-3, the eucalyptus sheets exhibited no change in WDT upon treatment with PAAE or cationic starch, and only a small increase when coated with chitosan —by effect of the thin film slightly clogging sheet pores.

Water can penetrate paper through pores (inter-fiber penetration) and diffuse through fibers (intra-fiber penetration) (Lindström, 2009). Both penetration paths were dramatically hindered by application of the functionalization solution to sheets previously treated with a cationic additive. The need of adding cationic compounds to paper to enhance the functionalization using the FS does not represent an extra step in the manufacture of paper products, since such additives are used in the common papermaking processes.

5.3.5. Hydrophobic characterization of treated paper sheets

Up to this point, the hydrophobicity of treated sheets was measured with the water drop test (WDT), which however, assesses absorptive behavior rather than hydrophobic character proper. Other techniques such as that based on contact angle (WCA) measurements afford more accurate characterization of hydrophobic behavior. The contact angle technique involves placing a small water droplet on the surface of a sheet and measuring the angle at which the liquid interface meets the sheet surface at the precise moment of the contact. It is generally assumed that, if a surface is hydrophobic, then its WCA will be greater than 90°; otherwise, the surface is assumed to be hydrophilic (Adamson, 1990). Figure 5-4 shows WCA for eucalyptus sheets treated with wet strength additives and then with FS as described in the previous section for measurement and comparison.

As can be seen, the contact angle of the eucalyptus sheets treated with PAAE resin, 50°, was very similar to that for untreated (Initial) sheets. On the other hand, WCA for the sheets treated with the cationic starch (CSt), was lower than the value for the initial sheets; this can be ascribed to the hydrophilicity of starch present in the sheets. The sheets coated with a layer of chitosan (MGCh) exhibited a substantial increase in WCA with respect to the initial sheets by effect of chitosan forming a very thin film that clogged paper pores and hindered penetration of the water drop at the measurement time.

However, the most dramatic increase in WCA was caused by application of FS, which raised it to about 130° irrespective of the particular additive used (PAAE, MGCh and CSt). Interestingly, WCA after impregnation with FS was very similar in all sheets despite the large differences prior to application of the functionalization solution; this was especially apparent in comparing the starch modified sheets (CSt) and the chitosan coated sheets (MGCh), which had a rather different initial contact angle (30° and 100°, respectively) but an identical value (130°) after application of FS. Figure 5-4 also shows WCA for untreated filter paper sheets prior to (15°) and after application of FS (120°).

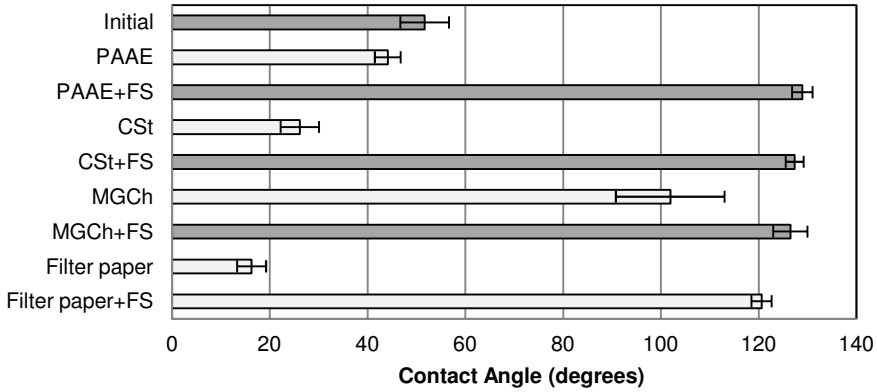

Figure 5-4 Contact angle of the eucalyptus sheets treated with the wet strength agents PAAE and cationic starch (CSt), and with methyl glycol chitosan (MGCh), and also of filter paper, before and after application of the functionalization solution (FS). "Initial" refers to the initial, untreated eucalyptus pulp.

The above-described results reveal that the cationic character of paper sheets is the key to achieving highly efficient hydrophobization with the proposed functionalization method as it facilitates electrical attraction between negatively charged hydrophobic compounds present in FS and positively charged sheet surfaces.

5.3.6. Evaluation of surface free energy

The hydrophobic behavior of a surface is dependent on its surface free energy (SFE) (Bartell and Zuidema, 1936), which derives from the unsatisfied bonding potential of molecules at the surface of a solid material. In other words, SFE is the energy resulting from the disruption of intermolecular bonds that occurs when a surface is created (Baidakov, 2012). In contrast, molecules in the bulk of a material have less energy because they are subject to interactions with other molecules in all directions. Molecules at the

surface try to reduce this free energy by interacting with other molecules in an adjacent phase, whether solid, liquid or gaseous.

The free energy of a surface is a measure of its readiness to interact with other surfaces or liquids. In this work, SFE was used to assess the tendency of treated sheets to interact with water. A high SFE value is indicative of easy wetting by water and leads to a low contact angle.

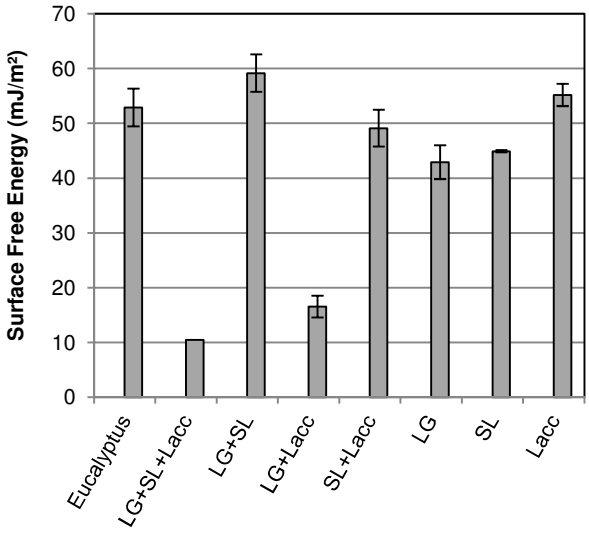

Figure 5-5 Surface Free Energy (SFE) of PAAE-pretreated eucalyptus sheets before (ref. Eucalyptus) and after impregnation with FS (LG+SL+Lacc) and control solutions (LG+SL, LG+Lacc, SL+Lacc, LG, SL and Lacc).

Fig. 5-5 shows the SFE values of PAAE-treated eucalyptus sheets before and after impregnation with FS and the control solution (KFS). As can be seen, SFE for PAAE-treated sheets to which no FS or KFS was applied ranged from 50 to 60 mJ/m^2, which is consistent with previously reported values for this material (Dourado *et al.*, 1998; Persin *et al.*, 2004). Only the LG+Lacc control treatment and the FS treatment reduced SFE to a significant extent. Application of FS was especially effective in this respect; thus, it reduced SFE from an initial value around 55 mJ/m^2 to one in the region of 10 mJ/m^2. The reduction in SFE testifies to the hydrophobizing ability of FS and is consistent with the WDT results. By contrast, none of the control treatments (LG+SL, SL+Lacc, LG, SL and Lacc) was efficient in reducing SFE, which remained at levels near 50 mJ/m^2 after treatment.

5.3.7. ATR-FTIR analysis of treated PS

ATR-FTIR analysis was used to find evidences about the grafting of the FS onto the paper sheets. Figure 5-6 shows the IR absorbance spectra for the non-treated eucalyptus sheets (a), PAAE-treated eucalyptus sheets (b), and PAAE-treated eucalyptus sheets which were additionally treated with each one of the KFS and the whole FS product (c, d, e, f, g, h and i); figure also shows the IR spectrum of the pure LG compound (j).

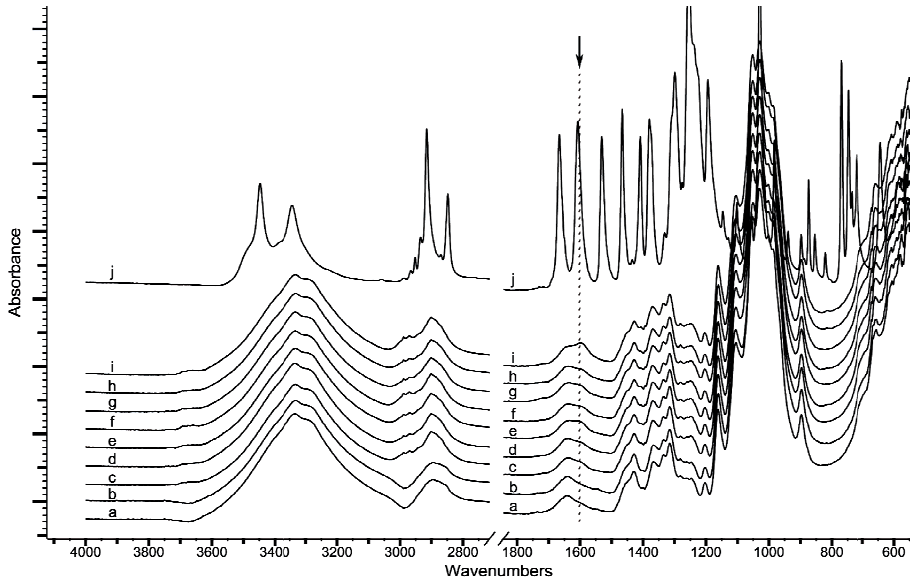

Figure 5-6 FTIR spectra of the bare eucalyptus sheets and PAAE-treated eucalyptus sheets before and after treatment using the FS, and KFS. Key: a) Eucalyptus; b) Eucalyptus+PAAE; c) Lacc; d) SL; e) LG; f) LG+SL; g) LG+Lacc; h) SL+Lacc; i) FS; j) pure LG compound.

The global aspect of the IR curves is almost identical, only noticeable changes are observed at the wavelength of 1600 cm^{-1}. A new peak at 1600 cm^{-1} appeared in the IR spectrum of the FS-treated and KFS-treated eucalyptus sheets with respect the non-treated ones. It can be observed how the intensity of this absorbance signal is similar for the Lacc (c), SL (d), LG (e), LG+SL (f), LG+Lacc (g) and SL+Lacc (i) controls. This peak may correspond to the conjugated C=C stretching frequency of the aromatic rings in the LG and SL molecules. Considering the IR curve of the FS-treated sheets (i), the intensity of the peak appearing at the 1600 cm^{-1} is stronger than that observed in the previous controls; since the FS contains both compounds (LG and SL), a possible explanation could be that the presence of higher amounts of phenols on the paper surface would had

enhanced the signal intensity of the C=C stretching. However, if taking into account these observations, the intensity of the peak corresponding to the LG+SL control (f) should have also been stronger, but in this control the LG and SL products were not modified by the enzyme, so lower amounts of molecules would had been retained onto the surface. The interaction between the enzyme-modified products in the FS (LG and SL), and the cationic substrate (PAA-treated sheets), produce the retention of higher amounts of moieties onto the surface; the occurrence of the peak at 1600 cm^{-1} upon the treatment of the sheets using the FS can only be ascribed to the presence of LG molecules in the FS-treated papers. The peak at 1600 cm^{-1} is clearly defined when considering the IR curve of the pure LG in Fig. 5-6 (j). The absorbance of this peak in the IR curves of papers was weaker due to the low concentration of the LG compound, compared to the IR of the pure LG. However, the occurrence of the peak was easily detected in the paper samples.

5.3.8. Characterization of the functionalization solution

The enzymatic reaction taking place in the presence of lauryl gallate (LG), lignosulfonate (SL) and laccase (Lacc) caused apparent changes in LG. As stated before, LG has a very low solubility in water. This was the main reason for introducing SL in the enzymatic reaction (Cusola *et al.*, 2013), and that reason for sonicating LG before it. As can be seen in Fig. 5-7, all the control solutions (LG, SL, LG+SL, LG+Lacc and SL+Lacc) and the complete functionalization solution (LG+SL+Lacc) exhibited a marked, composition-dependent color change after the enzymatic reaction. The fact that no color change was observed in the individual LG and SL solutions suggests that the reaction conditions (temperature, pH, reaction time) somehow did not alter the structure or chemical nature of the two compounds. The initial LG control, which consisted of sonicated LG suspended in the reaction buffer, became a colloid suspension upon sonication. The suspension, however, was highly unstable and LG particles rapidly aggregated and precipitated. This is why the LG control in Fig. 5-7 was a clear solution containing an LG precipitate in the bottom of the beaker. Adding SL to the LG solution increased its turbidity by effect of SL, increasing LG dispersion in the aqueous medium and preventing complete precipitation as in the LG control solution. Adding laccase caused a marked color change in the SL solution, possibly as a result of the compound being oxidized by the enzyme (Cusola *et al.*, 2013). Thus, a solution containing LG+Lacc but no SL differed markedly in color from one of LG alone; interestingly, however, no oxidized LG precipitate formed from the turbid solution. This can be ascribed to laccase causing molecular changes in LG that increased its stability in the aqueous medium.

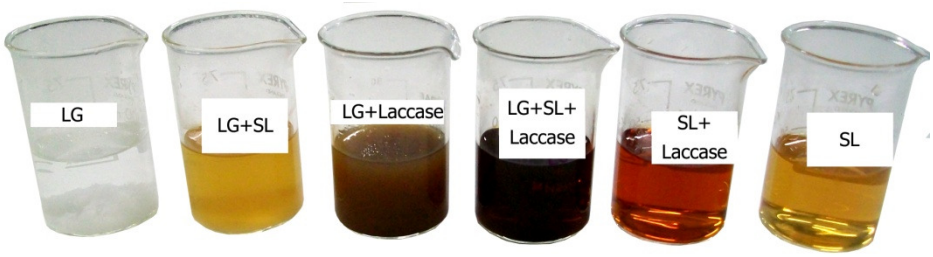

Figure 5-7 Color changes in the control solutions KFS (LG, LG+SL, LG+Lacc, SL+Lacc and SL) and complete functionalization solution, FS (LG+SL+Lacc).

In any case, the most interesting results were obtained with a combination of the three compounds: LG, SL and laccase (FS). The marked color changes observed were due to oxidation of SL and LG, and resulted in dramatically reduced turbidity with respect to the LG+Lacc solution. This suggests that the presence of the SL induces further changes in the molecular structure of LG which possibly reduce the size of LG particles or facilitate its complete dissolution in the aqueous medium. In fact, no large LG particles were found in FS previously used to hydrophobize paper sheets; also, the solution was stable and contained no precipitated material.

The stability of the control solutions was assessed and compared via Z potential, which is illustrative of the behavior of colloid suspensions. Z potential is the potential difference at the junction between the Stern layer of a charged particle and the dispersing medium containing the particle (Cadena *et al.*, 2009). The greater Z potential is, whether positive or negative, the higher are the repulsion forces between particles that help discrete particles remain in suspension and prevent the formation of larger, faster settling agglomerates.

Fig. 5-8 shows the Z potential values of the control solutions (LG, SL, LG+SL, SL+Lacc and LG+Lacc) and the complete functionalization solution (LG+SL+Lacc) as well as that of water for reference. It should be noted that distilled water contains no suspended particles, so its Z potential is near-zero. The sonicated LG solution (Fig. 5-8) had Z potential ≈ –30 mV and was thus scarcely stable. The SL solution had Z potential ≈ –50 mV and was thus highly stable —in fact, SL never precipitated in the aqueous medium. An interesting result that confirmed the hypothesis previously made from the appearance of the solutions (Fig. 5-7) was obtained with the LG+SL combination; thus, its Z potential was about –45 mV, which indicates that adding SL to the LG solution substantially increased its stability.

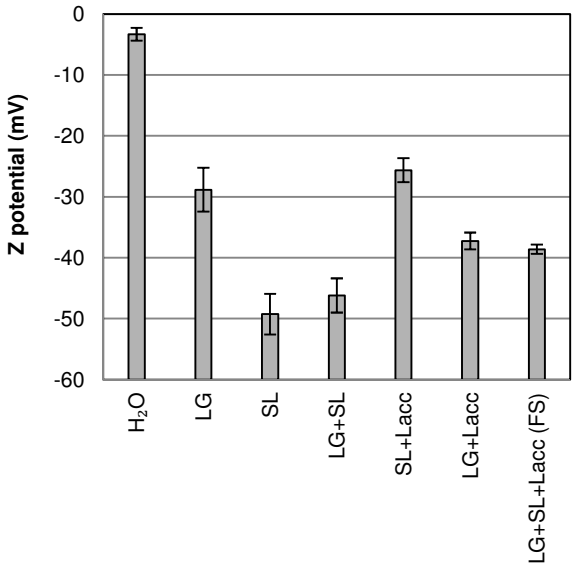

Figure 5-8 Z potential values of the control solutions KFS (LG, SL, LG+SL, SL+Lacc and LG+Lacc) and the complete functionalization solution, FS (LG+SL+Lacc).

On the other hand, the combination of SL with laccase exhibited a significantly reduced Z potential (ca. –35 mV) compared with to SL alone; however, the solutions (Fig. 5-7) exhibited no visual signs of agglomeration, turbidity or large particles. One possible explanation for this result is that the reaction with laccase may have partially dissolved SL and reduced Z potential to a near-zero level closer to the electrokinetic properties of water. Combining LG with laccase increased its Z potential to about –40 mV and improved its LG stability even though the solution still looked turbid to the naked eye (Fig. 5-7). The most interesting result was derived from the measurements of the solutions containing LG, SL and laccase (FS), which exhibited all above-described effects simultaneously. One would have expected the synergistic effect of SL in combination with laccase to result in a dramatic increase in Z potential with respect to LG alone; however, Z potential remained at ca. –38 mV and, again, the solutions looked clear and contained no suspended particles (Fig. 5-7). As before, the enzyme may have facilitated dissolution of SL and LG, thereby reducing the Z potential of the solution to the level for water.

In a subsequent test, solutions were viewed from the top of the beakers while coherent light from a laser beam was passed through the solution. Figure 5-9a shows the viewing geometry. The top image illustrates the response of laser light in the absence of solution turbidity and the bottom image its scattering when the solution was turbid. As can be

seen in Fig. 5-9b, the LG solution exhibited marked light scattering. This solution contained visible suspended LG particles and was scarcely stable, so it precipitated very rapidly in the absence of agitation. By contrast, the SL solution caused no light scattering and the laser beam crossed the whole beaker section freely. Therefore, SL was virtually completely dissolved in the aqueous medium, where it was highly stable as confirmed by its Z potential. The LG+SL solution diffused the laser beam; unlike the LG solution, however, diffusion was much more uniform and no suspended particles were visible to the naked eye, which further confirms that SL improved LG dispersion and stability in the aqueous medium.

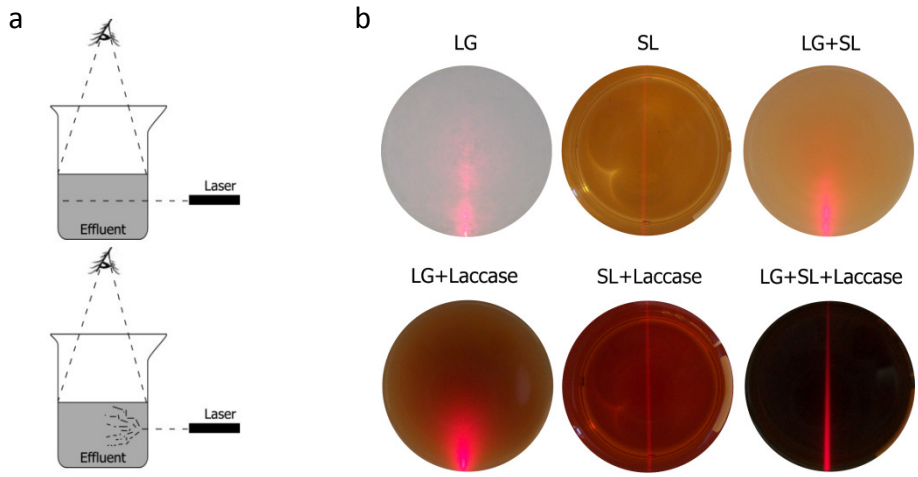

Figure 5-9 Viewing geometry of a laser beam passing through solutions held in glass beakers (a), and through FS and the control solutions (KFS) (b).

The laser beam crossed the SL+Laccase containing beaker without refraction or diffusion; however, the beam intensity was seemingly increased relative to the SL solution —which suggests increased solubilization of SL by effect of the enzyme and is consistent with the Z potential of the solution. The laser beam was thoroughly diffused by the LG+Laccase solution. Although this is suggestive of increased turbidity, diffusion was highly uniform and no LG particles were visible. This behavior departs from that of the LG and LG+SL solutions; thus, the former contained suspended and precipitated LG, and the latter some precipitated LG. Finally, the laser beam crossed the whole section of the beaker containing the LG+SL+Laccase solution (FS) and the measured laser intensity was higher than with the other solutions. Laccase thus seemingly facilitates dissolution of LG and SL in the aqueous medium, and prevents the formation of suspended particles of either.

The absence of large particles in FS is of paramount importance for the potential industrial application of the proposed method as it can facilitate the surface application of aqueous FS by using existing industrial devices such as size presses, metering bars or sprayers. Also, FS sprayed onto sheet surfaces distributes uniformly over the product and confers it with hydrophobic properties throughout.

5.4. Conclusions

The work described in the present chapter reports an innovative green methodology for preparing a functionalization solution (FS) via an enzymatic reaction between laccase and lauryl gallate. Application of FS to the surface of finished paper substrate makes it hydrophobic. Sonicating the solution has a favorable effect on lauryl gallate and adding sulfonated lignin (SL) as dispersing agent boosts hydrophobicity. Treated sheets were assessed for this property from WDT and WCA measurements, which provided values around 4000 s and 130°, respectively. One important finding was that the cationic character of the sheets was the key to achieving a high level of functionalization by effect of electrical attraction between the negatively charged hydrophobic compounds present in FS and the positively charged sheets. The ATR-FTIR analysis revealed the appearance of a peak at 1600 cm^{-1} for the FS-treated eucalyptus sheets, providing evidences on the grafting of the FS onto the surface. As confirmed by Z potential measurements, the functionalization solution (FS) is highly stable; also, it contains no large particles in suspension, which enables its application with existing industrial surface-treatment devices.

This simple, expeditious method improves on the existing enzymatic alternative (reported in chapter 4) and is amenable to industrial application in the future.

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Chapter 6

A Facile, Green Method for Hydrophobizing Silica and Films of Cellulose Nanofibrils via Laccase-Mediated Coupling of Non-polar Particles

Abstract

Hydrophobization of silica and cellulose nanofibrils (CNF) was accomplished by enzymatic modification with the enzyme laccase (Lacc). Hydrophobic chains of the insoluble surfactant dodecyl-3,4,5-trihydroxybenzoate (LG) were coupled onto CNF and silica surfaces by treatment with a multicomponent functionalization system (FS) consisting of products of the reaction between LG, Lacc and sulphonated lignin (SL). Dynamic light scattering (DLS) and space/time-resolved light scattering (back and transmitted scattering) were used to determine the status of FS dispersion. Also, quartz crystal microgravimetry (QCM-D) was used to monitor the hydrophobization process in situ in real time. Relevant characteristics of the resulting adsorbed layers were determined from atomic force microscopy (AFM) and water contact angle (WCA) measurements. The laccase treatment reduced the size (DLS) of associated LG structures from polydisperse particles (average size ~4 μm) to about 200 nm; also, addition of SL further reduced LG particle size down to 81 nm and boosted surface enzymatic reactions. Efficient adsorption of FS onto CNF and silica surfaces increased their WCA by 88° and 78°, respectively. Finally, conferring silica and CNF cationic character with chitosan was found to boost adsorption of FS.

6.1. Introduction

The use of renewable and biodegradable materials has grown enormously in recent years. Their global market is expected to expand in the near future following the rising of societal awareness in response to climate change and consumer perception (Johansson *et al.*, 2012). Cellulose, which is the most abundant natural polymer in the biosphere, possesses interesting properties such as hydrophilicity, chirality, biodegradability, biocompatibility and recyclability. Also, it can be modified in a wide variety of manners. Nanosized cellulose (cellulose nanofibrils, CNF) has attracted attention as a renewable resource for conversion into high added value products and advanced functional materials by the packaging, biomedical, adhesive, composite, electronic and automotive industries (Faruk *et al.*, 2012; Hamad, 2006; Kolakovic *et al.*, 2012; Siqueira, Bras and Dufresne, 2010; Zhang *et al.*, 2013). CNF differs significantly from common cellulose fibres; thus, it has a higher aspect ratio and specific surface area, in addition to a greater intrinsic strength and flexibility (Saito *et al.*, 2013; Ferrer *et al.*, 2012).

However, expanding the uses of CNF requires its hydrophobization. Waterproof coatings and films, packaging materials and hydrophobic composites are but a few examples of hydrophobized CNF. Hydrophobization of CNF facilitates industrial processing operations such as size pressing, printing or conversion (Li, Zhang and Wang, 2008; Ogihara *et al.*, 2012). To be useful for packaging purposes, CNF must be treated with environmentally friendly hydrophobizing agents to facilitate recycling, which is not the case with the currently used conventional waxes and fluoropolymers (Stanssens *et al.*, 2011).

The wettability of a surface is a function of the combined contribution of its topography and low surface energy. Most methods for obtaining a high hydrophobicity minimize contact between water droplets and the surface by producing hierarchical micro and nanostructures. The hydrophobization method of choice depends on the particular substrate in each case. For example, “wet chemical reaction” and “electrochemical deposition” methods are commonly applied to metals, whereas “self-assembly” and “layer-by-layer” deposition methods are preferred for glass substrates, and textiles and other substrates are often modified via sol–gel and polymerization reactions (Guo, Liu and Su, 2011). These and other cellulose hydrophobization methods are the subject of a comprehensive review by Song and Rojas, (2013).

Green chemistry approaches based on enzyme reactions have aroused increasing interest in the scientific and industrial communities with a view to developing sustainable CNF-based products. Enzymatic functionalization of cellulose fibres by using phenolic

moieties to introduce antimicrobial or antioxidant effects, among others, was recently accomplished (Fillat *et al.*, 2012; Aracri *et al.*, 2010; Cadena *et al.*, 2011).

The use of enzyme systems for surface hydrophobization is still in its infancy, however. A method using laccase enzymes in combination with hydrophobic phenolic compounds has proved effective to confer suspended cellulose fibres and CNF hydrophobic properties (Garcia-Ubasart *et al.*, 2012; Saastamoinen *et al.*, 2012). Interestingly, the treatment can be applied to aqueous suspensions.

In this work, we devised a novel route to further exploit laccase enzymes with a view to hydrophobizing films or mats of cellulose and CNF (e.g. paper, non-wovens) by direct surface application of the enzyme together with a co-adjuvant to strengthen physico–chemical interactions in aqueous systems. Specifically, we examined physico–chemical interactions of silica and CNF with a multi component system (FS) obtained by enzymatic reaction of laccase with a short-chain organic molecule, dodecyl-3,4,5-trihydroxybenzoate (commonly known as lauryl gallate, LG), and sulphonated lignin (SL). FS proved effective in hydrophobizing CNF by simple immersion, spraying or size-pressing. The proposed methodology meets the requirements for facile application and renewability, and uses a green chemistry approach to facilitate recycling.

6.2. Materials and methods

6.2.1. Enzyme and chemicals

The enzyme used in this work was laccase from *Trametes Villosa* supplied with an activity of 588 U/mL by Novozymes®. Dodecyl 3,4,5-trihydroxybenzoate (LG) and chitosan (CHIT, 50–190 kDa molecular weight, 75–85% de-acetylated) were purchased from Sigma Aldrich®. The C_{12} chain in LG provided the non-polar moiety needed to confer cellulose water repellency. Soluble sulphonated kraft lignin of 5.9 kDa MW and 5% total sulphur content was obtained from Borreegard® (Sarpsborg, Norway) and used as received. Nanofibrillar cellulose (CNF) was obtained from bleached hardwood (birch) pulp by mechanical treatment as described in the "Materials and Methods" (chapter 2).

6.2.2. Substrate preparation

Model surfaces for adsorption tests consisted of bare silica wafers and QCM-D silica sensors purchased from Q-Sense (Västra Frölunda, Sweden). For adsorption experiments using cellulose the silica sensors were pretreated with polyethyleneimine (PEI) polymer,

followed by spin coating with a dispersion of nanofibrillar cellulose (CNF) at 3000 rpm for 1 min.

6.2.3. Functionalization Solution (FS) and surface treatment

The functionalization solution (FS) was the product of the enzymatic reaction between *Trametes Villosa* laccase (Lacc) and the low-surface energy phenolic species dodecyl-3,4,5-trihydroxybenzoate (LG) in the presence of lignosulfonate (SL).

The enzymatic treatments involved reaction of the enzyme with the hydrophobizing agent, LG, in FS. FS was prepared in a stirred bath, using 250 mL beakers. The preparation conditions were based on previous reports (Cusola *et al.*, 2013; chapter 5). Treatments were performed by using final concentrations equivalent to 0.1 M sodium acetate buffer (pH 4), 1.2 g/L LG, 1.2 g/L SL and 1.2 U/mL laccase. Because LG is hydrophobic and insoluble in water, it was used as a colloid suspension prepared by sonication on a Hielscher® Ultrasonic Processor UP100H apparatus at 100% amplitude for 30 min. This reduced the effective size of LG and ensured homogeneous dispersion. In any case, LG remained quite stable after treatment with the enzyme. LG and SL were placed in beakers, stirred at 50°C for 10 min, supplied with the enzyme and kept under stirring for 4 h. The reaction was stopped by quenching with cold water. The role of each FS component was elucidated by using various control treatments (Table 2-2; in the present chapter PhC refers to LG). Potential interferences from residual large LG particles with QCM measurements were avoided by passing FS and the controls through syringe filters of 0.45 μm pore size. In the cases where chitosan was used for QCM-D analysis, it was diluted to 0.5 g/L in sodium acetate buffer before use in the QCM-D device.

6.2.4. Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

In situ QCM-D tests were performed on a Q-Sense E4 instrument (Västra Frölunda, Sweden) operated in the continuous mode. Molecular interactions between FS (or the controls) with the substrate caused a shift in resonance frequency (f) which was monitored in order to quantify any gain in mass (e.g. by adsorption). The third overtone (n = 3) was used to interpret QCM data. Changes in dissipation (D) were also monitored in order to determine frictional (viscous) losses in the adlayer. D is related to the viscoelastic properties of the layer(s) depositing onto the sensing electrode and hence a measure of stiffness. Time-resolved adsorption was monitored with silica sensors, both bare and coated with a CNF film. QCM-D measurements were made at a continuous flow rate of 100 μL/min at 25°C.

6.2.5. Atomic force microscopy

A Nanoscope IIIa Multimode scanning probe microscope from Digital Instruments, Inc. (Santa Barbara, CA, USA) was used to characterize the surface topography of the QCM-D silica sensors and CNF films before and after surface treatment. At least two different areas in each sample were examined, using a scan size of 10x10, 5x5 or 1x1 μm^2. AFM images where flattened following first-order conversion.

6.2.6. Particle size analysis

The hydrodynamic diameter and size distribution of the colloid particles present in FS and the controls were determined by using a Zetasizer Nano ZS dynamic light scattering (DLS) instrument from Malvern Instruments (Malvern, UK).

6.2.7. Water Contact Angle measurement

Water contact angles (WCA) were measured with a CAM-200 contact angle goniometer from KSV Instruments, Ltd. (Helsinki, Finland) by depositing a 4 μL water drop onto the substrate from above and using a high-resolution digital camera to capture the drop profile. Measurements were made upon contact, once the water drop had stabilized —the time required for the initial transient fluctuations to cease was 2 s.

6.2.8. Turbiscan® and Turbiscan On-Line® tests

The stability of FS and the controls was assessed by using Turbiscan® MA 2000 and Turbiscan On-Line® analysers from Formulaction (L'Union, France). The detection head consisted of a pulsed near-infrared light source (λ = 850 nm) and two synchronous transmission and backscattering detectors. More details on the measuring technique are given in the "Materials and Methods" (chapter 2).

6.3. Results and discussion

6.3.1. Particle size analysis with DLS

Some authors have successfully hydrophobized various types of surfaces by using a combination of surface topography and low surface energy (Ogihara *et al.*, 2012; Roach, Shirtcliffe and Newton, 2008), most often by creating hierarchical micro and nanostructures on the surfaces (Mammen *et al.*, 2012). Because all treatments in this work were applied after passage through syringe filters of 0.45 μm pore size, bare silica and nanofibrillar cellulose surfaces were treated by adsorption of nanoparticles present in FS

and the controls. This was an indication that adsorption was building a nanostructure on the surfaces, and also that particle size would play a central role in defining their hydrophobicity. The particle size of FS and the controls before and after filtration was determined by dynamic light scattering (DLS); the results are shown in Fig. 6-1. The LG control, which consisted of sonicated LG particles (as shown in table 2-2) exhibited a highly polydisperse size distribution with a major concentration of particles about 5 μm in size. After filtration, no particles were detected by DLS, indicating that virtually all the LG was retained by the filter and there were no particles under 450 nm. However, the control LG+Lac presented a monodisperse particle size around 300 nm before filtration and the particle size was reduced to 200 nm after filtration; this result is very interesting since it shows the clear effect of the LG after reaction with Lac enzyme. The particle size of the control treatment LG+Lac after filtration was due to the presence of LG particles having a diameter under the pore size (450 nm), because the control with the enzyme alone (Laccase) presented a smaller particle size around 40 nm after filtration. Additionally, the size of the Laccase as far as DLS measurements had to be due to enzyme aggregates, because the typical Laccase individual molecule dimensions are around 5 nm as reported elsewhere (Klis *et al.*, 2007; Molitoris *et al.*, 1972; Bogdanovskaya *et al.*, 2002). The control SL presented a particle size around 400 nm before filtration and 60 nm after filtration; this might be due to the shear forces generated during the filtration, which might destroy the SL aggregates and, in fact, this was a general observation that can be extended to the other treatments; for example, in the control with the laccase alone, in which the particle size passed from a particle size of 500 nm to 40 nm after filtration. The particle size of the SL was also reduced by means of the reaction with the enzyme as can be seen in Fig. 6-1 comparing the SL and SL+Lacc non-filtered controls which presented a particle size about 400 and 200 nm respectively. The SL control reduced the particle size dramatically after filtration to a size around 55 nm due to aggregate destruction, while the SL+Lac treatment maintained the particle size at the 200 nm after filtration. In the control consisting in the mixture LG+SL the particle size was around the 5 μm before filtration (the same size as the LG alone before filtration), and after the filtration the particle size was reduced to around 50 nm, which was the particle size of the SL alone after filtration, indicating the retention of the LG particles and the no-retention of the SL particles that could pass through the pore filters.

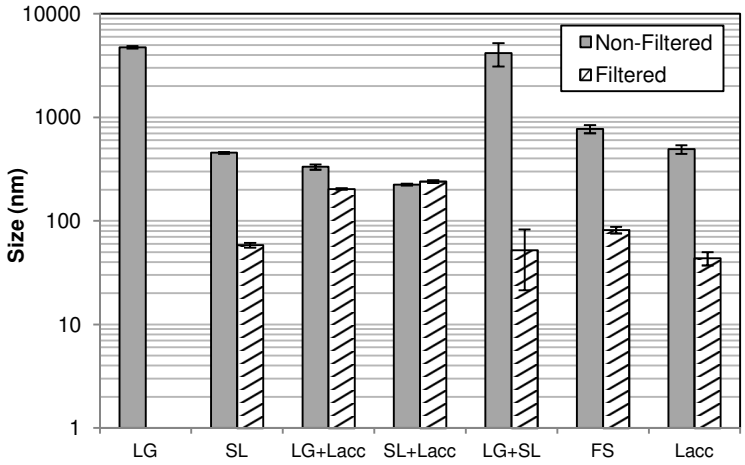

Figure 6-1 Particle size of FS and component systems as measured by DLS before and after passage through syringe filters of 0.45 μm pore size.

The particle size of FS after reaction with the enzyme was about 800 nm. Although there was a marked reduction in size of LG particles, FS remained highly polydisperse. The particle size of unfiltered FS was greater than that of the unfiltered LG+Lac control, possibly because of the presence of residual large unoxidized LG particles. However, the fact that filtered FS particles were approximately 80 nm in size and non-polydisperse suggests that the LG particles passing through the filter were smaller than those of the LG+Lac control. Therefore, FS was the solution containing the smallest LG particles after filtration.

DLS measurements exposed the effect of the enzyme in reducing LG particle size. Passage through 0.45 μm cut-off pore size syringe filters afforded better understanding of the effect of each treatment and control, especially with highly polydisperse samples. The whole suspension containing the three main components (FS) was that leading to the smallest LG sizes after filtration as a result of the favourable effect of SL —the LG+Lac control contained larger particle sizes after filtration.

6.3.2. Turbidity analysis

FS and the controls consisted of aqueous suspensions of variable turbidity and stability. The LG control, which was an aqueous suspension of sonicated LG at the concentration used in the enzymatic reaction, was highly unstable: particles precipitated within a few minutes and required continuous stirring to maintain a homogeneous distribution in the aqueous medium. However, the enzymatic reaction and its combination with SL increased its stability by effect of the above-described reduction in particle size and the

surfactant action of SL. As it is discussed in the next section, the adsorption curve for FS on the QCM-D sensors obtained from frequency changes never reached a plateau — which indicates steady deposition of particles onto the surface. The 5 h deposition of FS on silica in the QCM-D tests evidenced that the curve followed the same trend, with no sign of change or stabilization; additionally, deposition resulted in strong adsorption — no significant changes in Δf were observed after rinsing. The instability of FS particles in the aqueous medium may be behind this phenomenon.

The turbidity and stability of FS and the controls was assessed from light transmission (TR) and backscattering (BS) measurements made with a Turbiscan® analyser. Transmission and backscattering of light through the length of a glass test tube containing a liquid provides information about the specific phenomenon involved: sedimentation, coalescence, flocculation, creaming. The Turbiscan® analyser scans the entire length of the tube at preset intervals while the sample remains stationary. Figure 6-2 shows the light transmission results for FS and the controls as obtained from scans performed at 5 min intervals for 1 h. Figure 6-2a shows the TR measurements for the LG control; as can be seen, the measured percent transmission increased steadily, which indicates clarification of the fluid by effect of sedimentation and aggregation of LG particles, and confirms the high instability of this control. As can be seen, the upper portion of the test tube exhibited higher transmission values indicating that this zone clarified as particles settled and aggregated. Figure 6-2b shows the transmission values for the SL control. SL gives a highly stable, transparent, non-precipitating dissolution in aqueous media as confirmed by superimposing the transmission curves obtained in the different scans. This was also the case with the SL+Lac control (see Fig. 6-2d).

Although the transmission values for the LG+Lac control (Fig. 6-2c) are suggestive of a high stability, the liquid was very dark; this resulted in strong absorption of light by the aqueous medium and led to very low transmission measurements (about 3%). However, the effect of Laccase in reducing LG particle size —and increasing the stability of the suspension as a result— was quite clear and the results were consistent with those of the DLS and AFM analyses. Figure 6-2e shows the variation of the percent transmission for FS. Although the FS suspension was slightly unstable, the medium was more transparent, with transmission values from 50% to 60% throughout the length of the test tube. Note the difference in this respect from the LG+Lac control (Fig. 6-2c), which exhibited very low transmission as a result of the large particles present in it preventing the passage of light through the test tube, and the smaller particles of FS —closer to molecular size— allowing it. Figure 6-2f, which corresponds to the Lac control, testifies to the high stability of laccase in the aqueous medium.

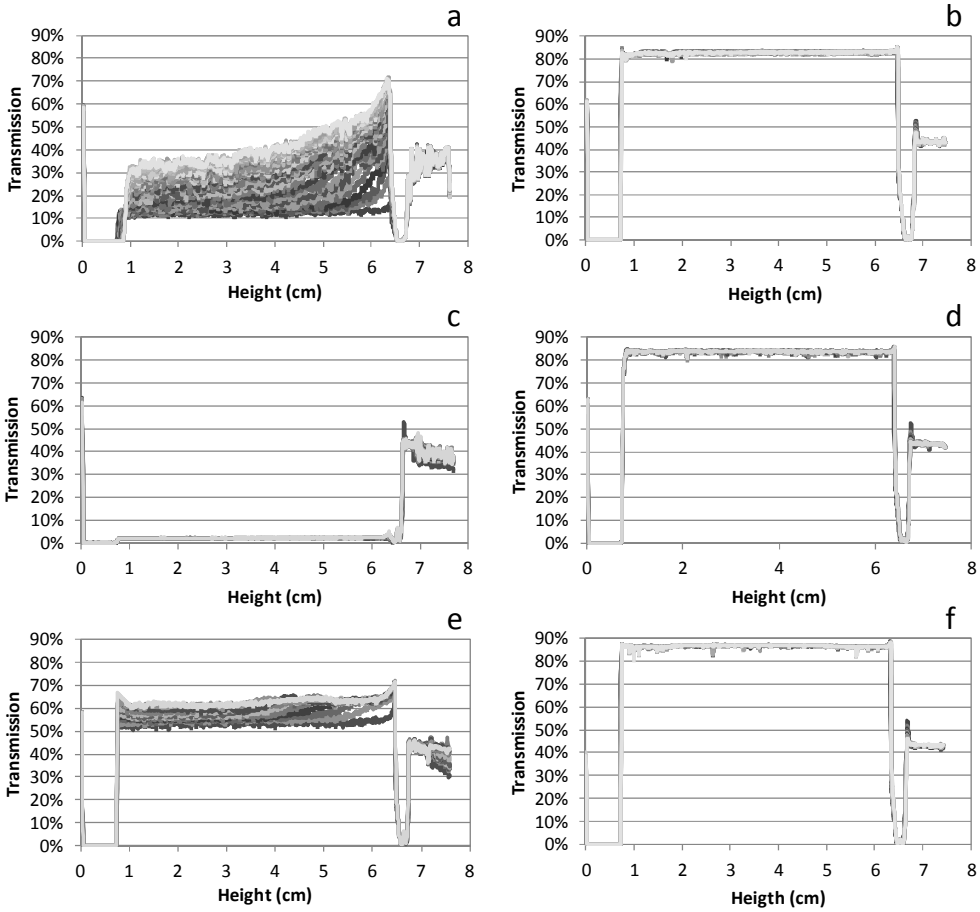

Figure 6-2 Light transmission values (%) for the FS suspension and related systems. The different profiles are represented in such a way that the intensity of grey decrease with time. Key: Transmission of LG (a), SL (b), LG+Lacc (c), SL+Lacc (d), FS (e) and Lacc (f) systems.

The enzyme was found to have several effects on LG, namely: reducing its particle size, stabilizing its suspension and increasing its darkness through oxidation of LG. Also, the presence of SL seemingly enhanced the size reduction of LG and increased the transparency of the aqueous medium. In order to better understand the action of the enzyme and SL on LG, and also to examine changes in particle size, we made on-line transmission and backscattering measurements of LG+Laccase, SL+Laccase and FS during the enzymatic reaction. Measurements were performed on a Turbiscan On-Line® analyser, which affords real-time characterization of liquid dispersions. The experimental set-up consisted of an Erlenmeyer flask holding the reactants and a thermostating system

(Fig. 6-3). The reacting fluid in the Erlenmeyer flask was connected to the inlet and outlet of a peristaltic pump. The pump was used to transfer the liquid reaction mixture to a measuring cell at 10 s intervals for measurement of TR and BS, and then to recirculate the sample. Fig. 6-4 shows the TR and BS values measured during 27 h of reaction. The transmission and backscattering values for the SL+Lac control remained unchanged during the enzymatic reaction. In fact, both parameters changed very slightly at the time the enzyme was incorporated, but oxidation of SL by the enzyme was virtually instantaneous and no further changes were observed over the next 30 h. The transmission values for LG+Lacc and FS fell to 0% within 4–5 h after the enzyme was introduced. Then, transmission rose to about 50% for FS but only slightly (10%) for LG+Lacc. This increase in transparency was necessarily due to the presence of SL in the reaction medium enhancing the action of the enzyme as noted in relation to the AFM and Turbiscan® measurements in the previous sections. Note that the 50% transmission obtained after the FS treatment exceeded the value at the start of the reaction (before the enzyme was added): 30%.

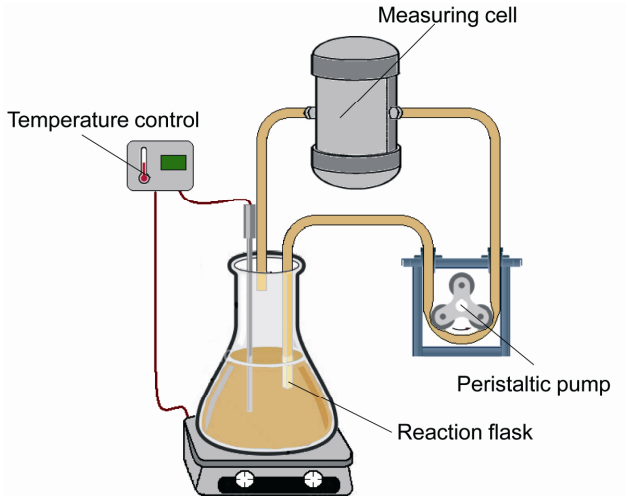

Figure 6-3 Schematic depiction of the Turbiscan On-Line® experimental set-up for the continuous measurement of light transmission and backscattering.

The initial decrease in transmission may have resulted from the enzyme oxidizing LG particles —which were initially several microns in size as estimated from DLS measurements— only at the surface, and oxidized LG having a very dark colour caused increased absorption of light by the aqueous medium. After reaction, particle size was reduced, so more light was allowed to pass through the test tube and increased TR as a result. Another possibility would be that longer enzymatic treatment caused further

changes in the chemical structure of LG, yielding non-colored molecules. Backscattering evolved similarly to TR. Thus, an initial dramatic decrease in BS was observed by effect of the oxidation of LG with the LG+Lacc and FS treatments, and most incident light was absorbed by the medium. BS for the FS suspension increased to levels above 20% after 4–5 h through clarification and colour change in the medium, but remained virtually constant at about 5% with the LG+Lacc treatment.

The Turbiscan On-Line® analyser was also used to compute the mean diameter d of the particles by using the Mie theory and volume fraction Ø of the medium. The particle size of the LG+Lac control, which was about 17 μm before the enzymatic reaction, was reduced to about 177 nm after 27 h; likewise, that for the FS suspension was reduced from about 18 μm to 81 nm after an identical reaction time. These results confirm the conclusions drawn from the TR and BS data, and hence that SL acted as an enhancer of the enzyme activity.

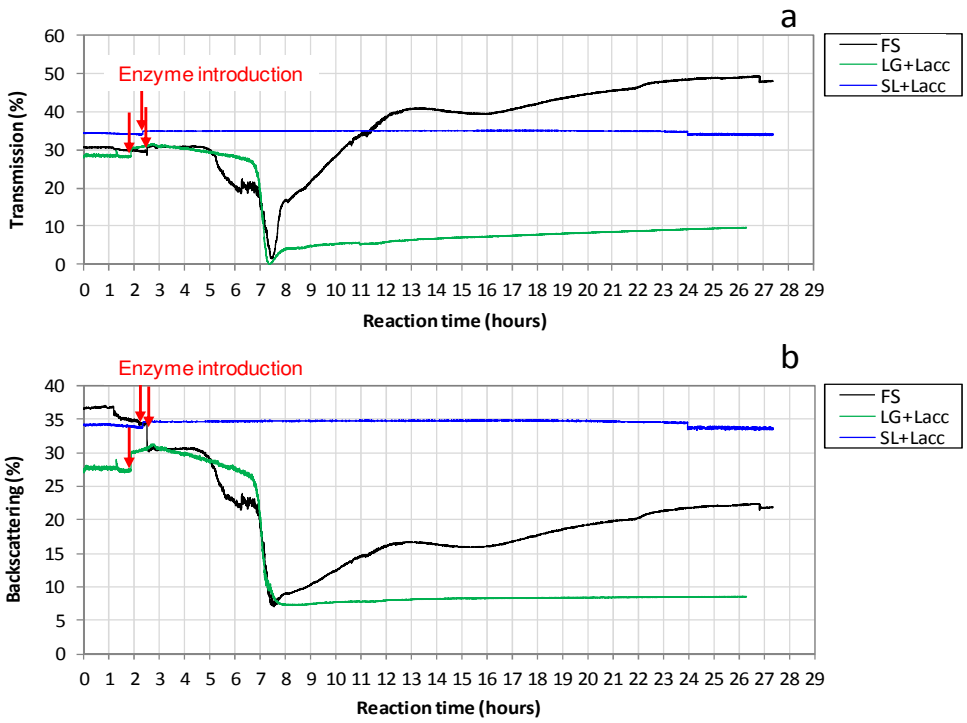

Figure 6-4 Percent transmission (a) and backscattering (b) of FS, LG+Lac and SL+Lac as measured within 24 h of treatment.

6.3.3. Adsorption of FS and FS components on silica

Silica was used as a model for cellulose by virtue of its containing free silanols and geminal silanol groups —which mimic hydroxyl groups in cellulose— on its surface and also of its being subject to none of the limitations of soft materials such as cellulose substrates —unlike cellulose, silica has a flat, smooth, non-porous surface that prevents swelling, hydration and diffusion.

Fig. 6-5a shows the QCM isotherms obtained in the form of the variation of the negative value of the frequency shift in terms of mass uptake by the silica surface as a function of time. After the buffer solution (0.1 M sodium acetate) was delivered for 3 min or until no change in Δf was observed, introducing filtered FS dramatically reduced the frequency by effect of adsorption or deposition of FS onto the silica surface. In order to determine whether the frequency decrease was due to aggregate deposition or molecular adsorption, the sensor was rinsed with buffer after 70 min. Although rinsing FS off the silicate surface caused a slight increase in Δf, the initial frequency value was never restored, which was taken as evidence of net adsorption. The difference between the frequency at the beginning (initial signal in the buffer solution) and that after the final rinsing can be assigned to a firmly adsorbed layer of FS on the silica surface, equivalent to $\Delta f \approx -28$ Hz after 70 min adsorption. This result indicates physico–chemical affinity between silica and FS. Note that the adsorption curve based on frequency changes in FS never reached a plateau; rather, Δf decreased linearly with time throughout.

Fig. 6-5b shows the results of similar tests involving additional rinsing with an electrolyte solution applied before the final rinsing with buffer. This was intended to increase the conductivity of the medium in order to assess the contribution of electrostatic affinity between silica and FS. No desorption, but rather substantially reduced dissipation, was observed after rinsing with electrolyte (0.1 M NaCl), possibly as a result of compaction of the highly hydrated adsorbed layer.

Fig. 6-5c shows the isotherms obtained by coating the silica surface with a thin layer of chitosan prior to application of FS. As can be seen, the chitosan coating led to stronger, faster adsorption of FS relative to uncoated silica (Figs. 6-5a and b) and adsorption reached a plateau at an Δf value of ca. –160 Hz indicating much higher adsorption of FS components onto the silica surface. Chapter 5 discusses the electrostatic affinity between FS and cationically charged surfaces in greater detail.

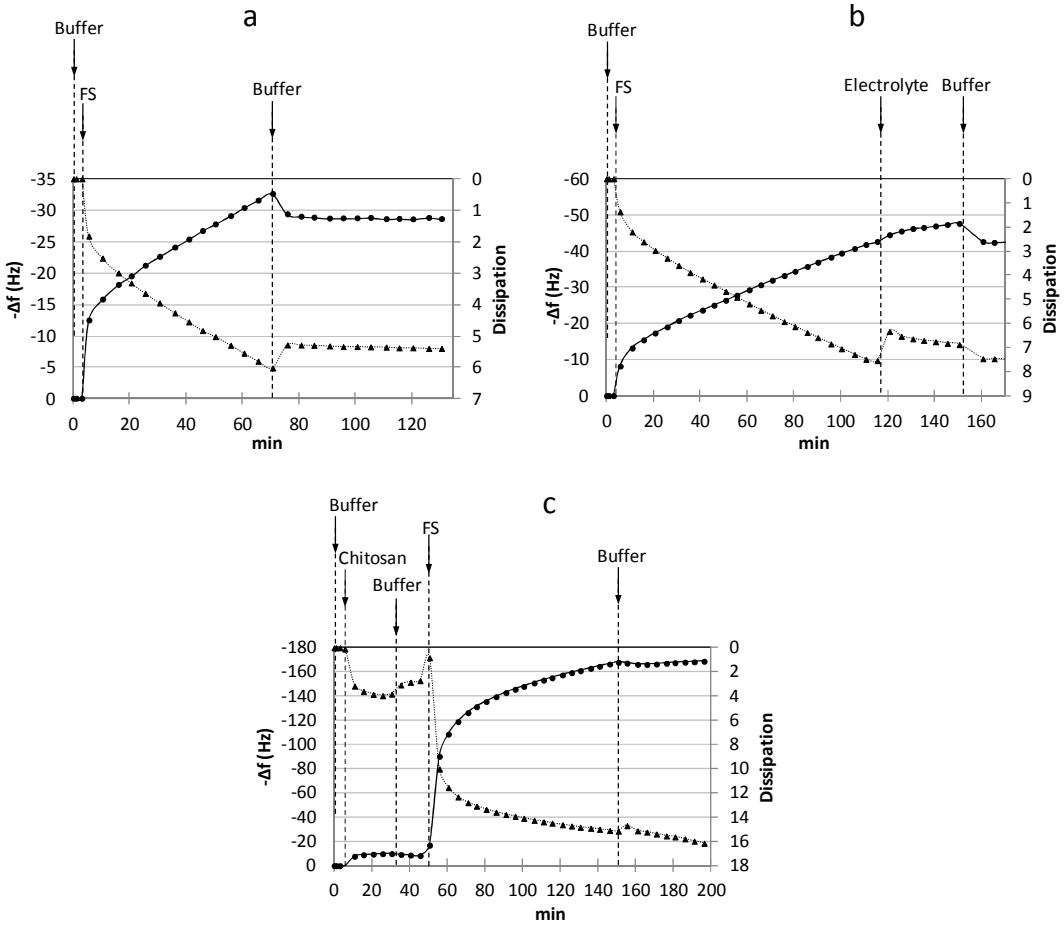

Figure 6-5 QCM Δf and ΔD isotherms obtained by injection of FS onto silica surface and subsequent rinsing with background buffer (a) or electrolyte (0.1 mM NaCl solution) followed by rinsing buffer solution (b). (c) Results for similar tests on chitosan-coated silica surfaces. Key: $-\Delta f$ and dissipation D signals are indicated by "●" and"▲" symbols, respectively.

Control treatments (Table 2-2) were also used to elucidate the role of each component of FS in its adsorption onto silica. The SL control was efficiently adsorbed onto the silica surface; as a result, Δf was about –14 Hz, which indicates the presence of a thin, though firmly attached SL layer (Fig. 6-6a). As can be seen in Fig. 6-6b, the LG control exhibited a very weak adsorption signal (less than –2 Hz after 60 min) suggestive of negligible adsorption. This is interesting since applying LG after reaction with the enzyme system led to completely different results (Figs. 6-5a-c). As stated before, the LG control was expected to contain large particles of low stability even after sonication; also, the weak adsorption signal obtained was ascribed to filtration (passage through syringe filters of

0.45 μm pore size) removing LG particles. However, the high adsorption of the LG+Lacc control observed in Fig. 6-6d ($\Delta f \approx -389$ Hz after rinsing) suggests extensive adsorption and the formation of a thick adsorbed layer.

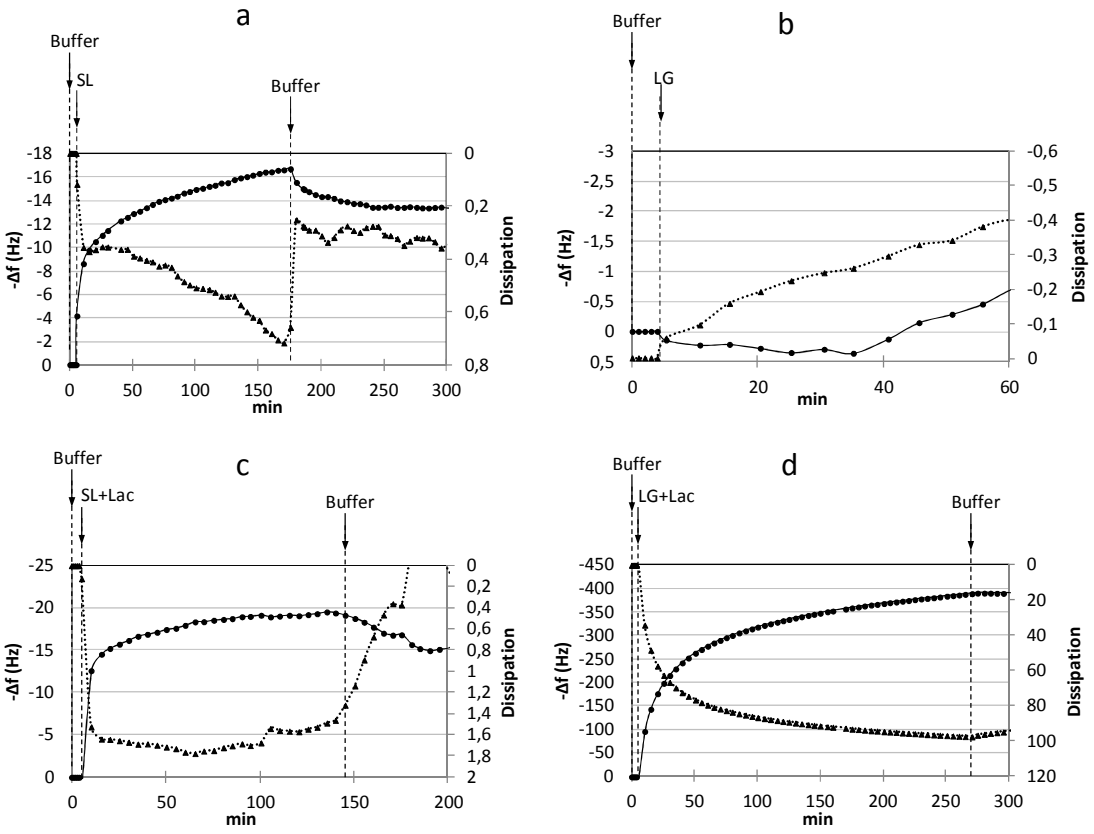

Figure 6-6 QCM's Δf and ΔD profiles upon adsorption on silica surfaces of SL (a), LG (b), SL+Lacc (c), LG+Lacc (d). Key: $-\Delta f$ and dissipation D signals are indicated by "●" and"▲" symbols, respectively.

Interestingly, the enzyme treatment reduced the particle size of LG in the LG+Lacc control and allowed LG particles to pass through the filter; this confirms the presence of small LG particles (less than 450 nm) available for adsorption onto the silica surface. Figure 6-6c shows the adsorption data for the SL+Lacc treatment; based on it, SL was also adsorbed onto the silica surface upon reaction with laccase —a plateau similar to that for SL alone was reached at $\Delta f \approx -14$ Hz. The particle size of LG was expected to play a prominent role in the surface modification by FS by introducing variable hydrophobicity

depending on the particular conditions. Application of enzyme solutions (Lacc control) led to Δf values about –38 Hz after 60 min. Overall, there is evidence that the three components of FS (LG, SL and Lacc) can be adsorbed to a different extent onto a silica surface.

6.3.4. Hydrophobization of CNF films

Similarly to silica surfaces, adsorption of FS and the control systems onto nanofibrillar cellulose (CNF) substrates was investigated via QCM-D tests. Figure 6-7a illustrates the adsorption of FS onto CNF-coated sensors. As can be seen, Δf was about –40 Hz after 85 min of adsorption and rinsing with background buffer; this is suggestive of a high affinity between FS and CNF. A comparison with the results for silica reveals that FS was adsorbed to a similar extent on both types of surface. Figure 6-7b shows the results of similar tests where CNF was coated with a layer of chitosan prior to contact with FS. Under these conditions, Δf was about –110 Hz; therefore, the chitosan effectively increased the adsorption of FS onto CNF, as it did with silica. Figures 6-7c and 6-7d illustrate the adsorption of the SL+Lacc and LG+Lacc controls onto CNF; Δf was about –14 Hz and –564 Hz, respectively. The SL+Lacc control was adsorbed in very similar amounts onto CNF and silica, whereas the LG+Lacc control was adsorbed to a much greater extent onto CNF.

Adsorption onto silica, and onto chitosan-coated and uncoated CNF, upon treatment with FS (Figs. 6-5 and 6-7) led to no plateau in the frequency plots. This can be taken as evidence of slow, extensive accumulation of FS components onto the substrate surface, possibly in the form of multilayers. Δf after 90 min contact of FS with anionic surfaces not coated with chitosan was –42 Hz for silica and –40 Hz for CNF; therefore, both substrates were modified to a similar extent by FS. However, the presence of a chitosan coating caused a greater frequency change upon the FS treatment; thus, Δf after 60 min contact was –145 Hz for silica and –100 Hz for CNF. This result reflects much more marked adsorption onto cationic substrates.

All FS components (Laccase, LG and SL) also accumulated on the surface of silica and CNF to a different extent with the SL, Lacc, SL+Lacc and LG+Lacc treatments. Based on the results, LG+Lacc was adsorbed to a greater extent than the other controls on both silica and CNF.

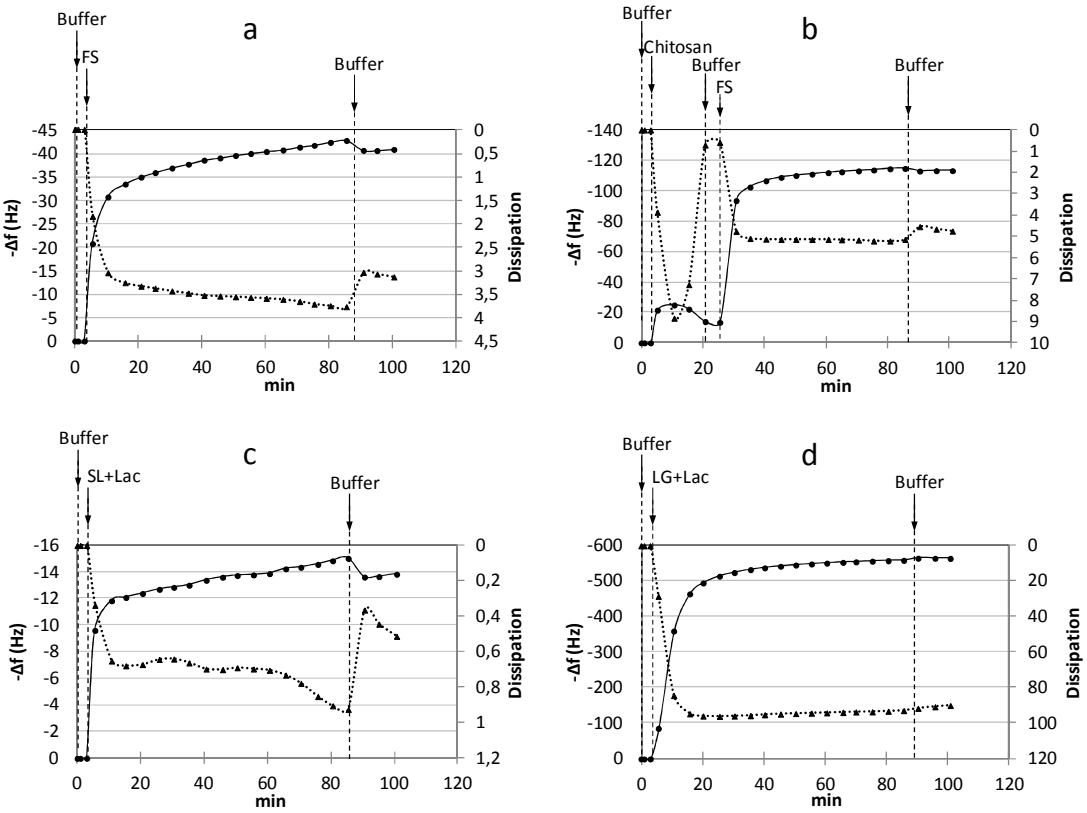

Figure 6-7 QCM Δf and ΔD profiles obtained upon adsorption of FS onto bare CNF (a) and chitosan-coated CNF (b). The profiles obtained upon treatment of CNF substrates with SL+Lacc (c), and with LG+Lacc (d) are also shown. Key: $-\Delta f$ and dissipation D signals are indicated by "●" and"▲" symbols, respectively.

6.3.5. Attachment of enzyme-modified LG moieties onto cellulose

The mechanism behind the oxidation of gallic acid proposed by Tulyathan *et al.*, (1989) was used here as the starting point to understand the reaction between Lacc and LG. Laccases exhibit specificity for phenols, *o*- and *p*-diphenols, methoxyphenols, aminophenols, benzenethiols and hydroxyindoles. Also, the formation of quinones by the oxidation of phenolic compounds using laccases has been widely reported (Navarra *et al.*, 2010; Witayakran, Zettili and Ragauskas, 2007). The underlying mechanism involves the oxidation of –OH groups in LG to form phenoxy radicals which are further oxidized to quinones and open-ring acid products (Gess and Dence, 1971). Formation of dimers by regeneration of the hydroquinones is expected to be difficult owing the steric hindrance posed by the long hydrocarbon chain of LG. The reaction yields a molecule bearing

carboxyl groups anchored to the alkyl chain through the original ester. The formation of quinones can be identified by the dark colour of the enzymatic products of LG.

We hypothesize that attachment of LG moieties to the surfaces occurs via a Fischer esterification reaction between hydroxyl functions on the surface of silica or CNF and carboxyl groups formed in LG upon enzymatic reaction (Gess and Dence, 1971; Gess, 1995; Sobkowicz, Braun and Dorgan, 2009) (Fig. 6-8). The sodium tartrate buffer used in the enzymatic reaction, which derived from tartaric acid, may have acted as a strong acid catalyst for the reaction by promoting protonation of carboxyl groups. The reaction must be reversible and the composition of the reaction mixture or equilibrium position is thermodynamically controlled. Ensuring good ester yields requires efficient removal of water by drying. Therefore, the presence or addition of water is of some concern here since it can shift the composition of the reaction mixture away from formation of the ester and towards the carboxylic acid.

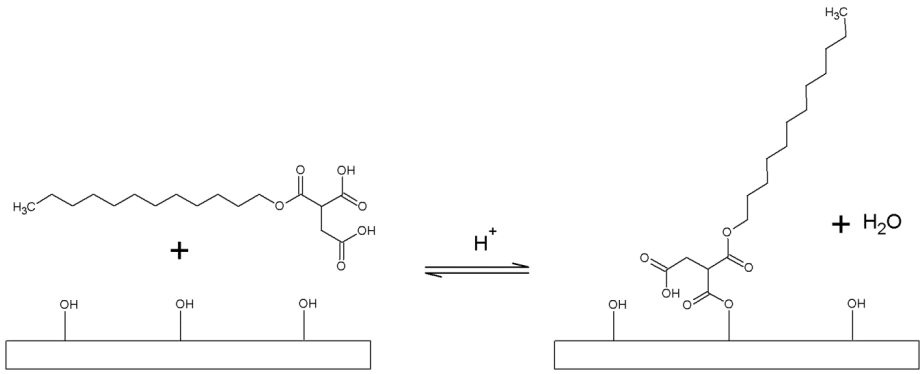

Figure 6-8 Attachment of enzyme-modified LG moieties onto the surface of silica or CNF via Fischer esterification reaction and drying.

The results of the QCM-D tests were suggestive of attachment of increased amounts of LG moieties onto silica and CNF substrates previously coated with chitosan. This effect can be ascribed to physico–chemical attraction between anions in FS (in the form of carboxyl groups) and cationic chitosan. Electrochemical attraction can boost retention/attraction of moieties on a surface for subsequent esterification as described above.

In previous results (Cusola *et al.*, 2013; chapters 4 and 5), the bonding strength of LG molecules attached onto cellulose-based materials was found to be very high. In the previous works, we used the same system (FS) to easily hydrophobize cellulose paper sheets and assessed the bonding strength by subjecting treated sheets to heavy washing

(80°C for 30 min) and Soxhlet extraction with acetone. No alteration of the hydrophobic behaviour of the sheets was observed, however, which further confirms the strong attachment of LG molecules.

6.3.6. Water contact angle and surface hydrophobicity

Water contact angle (WCA) measurements of silica and CNF surfaces treated with FS and the controls were used to relate adsorption to hydrophobization of silica and CNF. To this end, bare silica and CNF were subjected to the experimental sequences described in previous sections (see also Figs. 6-5, 6-6 and 6-7). As shown in Fig. 6-9a, WCA for silica was slightly increased (from 15° to 20°) by exposure to the buffer solution (BUFF). Also, treating bare and chitosan-coated silica with FS increased WCA to about 35° and 50°, respectively; therefore, as expected from the adsorption curves, the presence of a chitosan coating resulted in further hydrophobization.

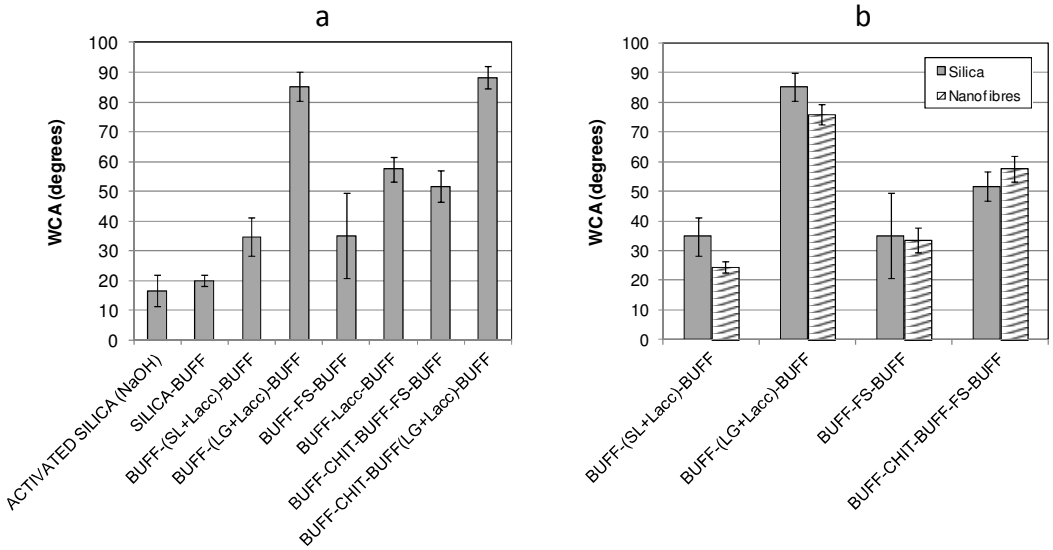

Figure 6-9 Water contact angle for silica surfaces treated with FS and respective components and rinsing sequences (a). Comparison of WCA results after treatment of bare silica, and CNF after treatment with FS and respective component solutions (b) (BUFF= buffer rinsing).

The increased hydrophobicity observed was seemingly directly related to the amounts of material being adsorbed onto the surfaces. Thus, treatment with the SL+Lacc and Lacc controls increased the contact angle to about 35° and 55°, respectively. The most effective treatment as regards increasing WCA or surface hydrophobicity was the LG+Lacc

control, which led to a contact angle of 85 and 88° for bare and chitosan-coated silica, respectively. Figure 6-9b compares WCA upon treatment of silica and CNF with FS (with and without pre-coating with chitosan) and also with the LG+Lacc and SL+Lacc controls. As can be seen, silica and CNF exhibited similar WCA values: the LG+Lacc control was the treatment leading to the highest WCA; also, an identical increasing trend in WCA was observed by effect of chitosan coating prior to treatment of CNF with FS.

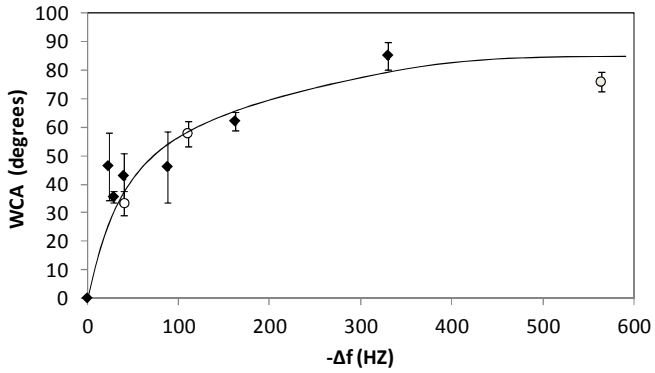

Figure 6-10 Relationship between the WCA of the substrate and the adsorbed mass (from the shift in QCM frequency, $-\Delta f$) after treatment with FS and LG+Lacc solutions. Key: Silica and nanofibre substrates are indicated by "♦" and"○" symbols, respectively.

A relationship between the amount of LG adsorbed and the hydrophobicity introduced by the treatments clearly exists. Figure 6-10 summarizes the QCM and WCA results of LG adsorption onto silica and CNF upon treatment with FS or the LG+Lacc control. WCA was measured with sensors previously rinsed with Milli-Q water and dried with nitrogen after the QCM adsorption tests described in the previous sections. The greater adsorption was, the higher was WCA; however, WCA values tended to a plateau and levelled off above a Δf value of ca. –300 Hz.

6.3.7. Analysis of silica surfaces by AFM

The surfaces of the QCM-D silica sensors treated with FS and the controls were imaged by AFM (Fig. 6-11). Figure 6-11a shows the surface topography of the bare silica surface, which is featureless and smooth (rms roughness less than 1 nm). Figure 6-11b corresponds to the silica sensor after treatment with laccase (Lacc); as can be seen, the enzyme was efficiently adsorbed onto the silica sensor and surface roughness was in the region of a few nanometres. However, larger particles —possibly enzyme aggregates— unevenly distributed on the surface were observed; also, the laccase was non-purified

commercial-grade enzyme so, possibly, the enzyme cocktail contained several other additives which were also adsorbed onto the silica surface.

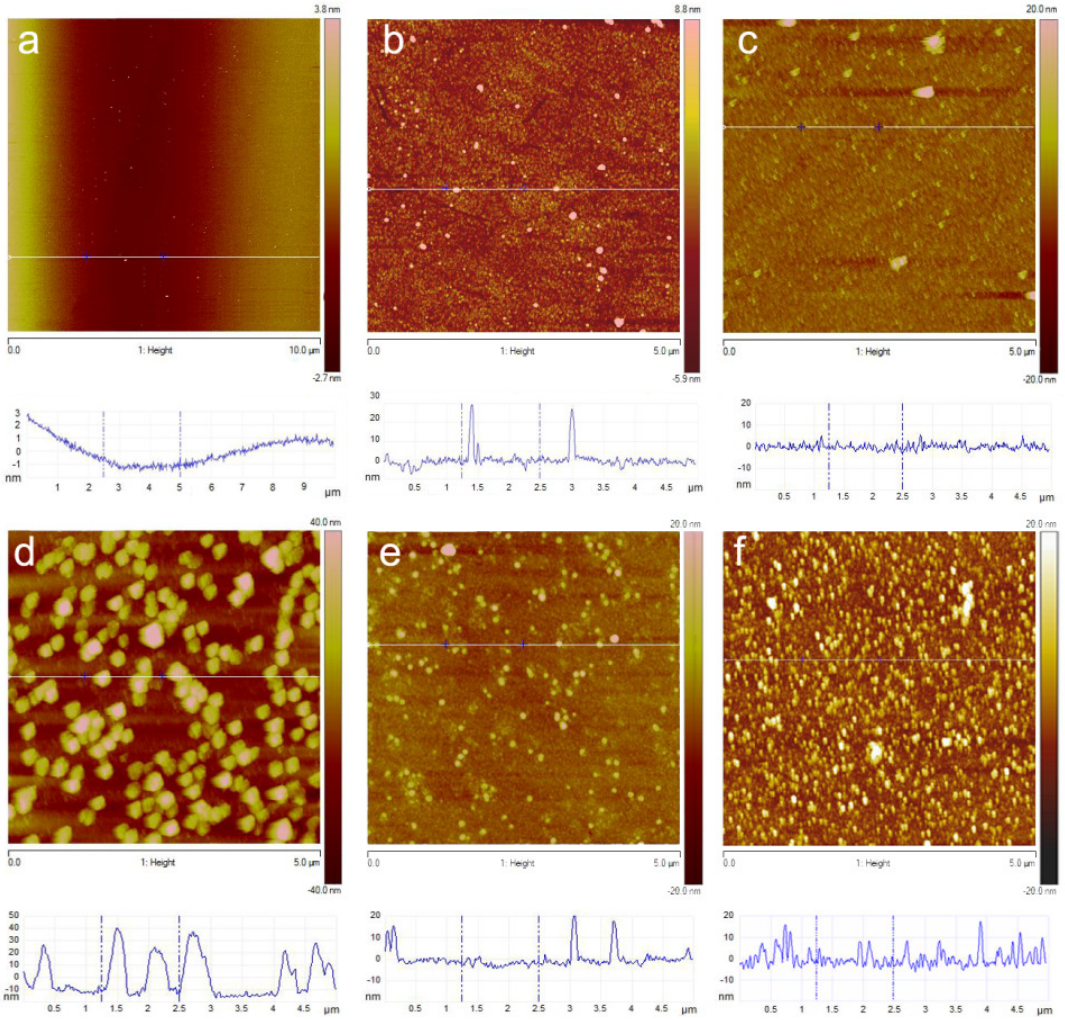

Figure 6-11 AFM images of silica surfaces after adsorption tests using FS, its individual components and binary combinations thereof. Key: Untreated bare silica surface (a) and silica surface after adsorption of Lacc (b); SL+Lacc (c), LG+Lacc (d) and FS (e). An image of FS adsorbed onto chitosan-coated silica is also included (f). Scanned area: (a) 5×5 µm², (b–e) 10×10 µm².

Fig. 6-11c shows the surface of the silica sensor treated with the SL+Lacc control; it had a typical rms surface roughness of only few nanometres which exhibited few unevenly

distributed aggregates of SL or Laccase. The aggregates may be responsible for the relatively large particle sizes found with the Lacc and SL+Lacc controls (see the "Particle size analysis" section). Figure 6-11d shows the surface topography of the silica sensor that was treated with the LG+Lacc control, which exhibits uniformly distributed large adsorbed particles about 200–300 nm in diameter and 20–40 nm in height. Such particles can only be enzyme-treated LG, which was not observed with the previous controls. The particle diameters of Fig. 6-11d are similar to those calculated from the DLS measurements with the LG+Lacc control (see "Particle size analysis" section). The silica surface treated with FS (Fig. 6-11e) contained uniformly distributed LG particles about 100 nm in diameter and 10–20 nm in height. The particle size of adsorbed FS as determined by AFM was also similar to that obtained from DLS measurements. Note that SL in FS boosted the action of the enzyme by reducing the size of LG particles relative to the LG+Lacc control. The effect of the chitosan coating on the silica surface was also apparent in the AFM images. Thus, as can be seen from Fig. 6-11f, the chitosan cationic layer significantly increased the amount of LG particles from FS that was adsorbed onto the surface. This effect was also observed in the previous QCM-D analysis.

A strong relationship between WCA for the treated silica surfaces and their topography was observed. The LG+Lacc control was the individual treatment resulting in the highest contact angle, and also that which conferred the silica surface its highest roughness, followed by FS —with and without the prior cationization of the silica surface—, which was also efficient in increasing the hydrophobicity of the silica. The enzyme (Lacc) control was also capable of significantly raising the hydrophobicity of the silica surface, possibly as a result of the presence of large aggregates and other particles as discussed before. The highest contact angles for silica and CNF were obtained by treating their surfaces with the LG+Lacc control. However, the angles never exceeded 90° and, as can be seen from Fig. 6-10, WCA tended to level off. This was possibly the result of treating a very smooth, clean silica or CNF surface with the LG+Lacc products causing the accumulation of a nanosized structure when surface hydrophobicity is known to be maximal for hierarchical structures combining micro- and nanoroughness. Our silica and CNF substrates lacked microroughness, so WCA cannot have been increased by its effect. This may also be the reason for the increased contact angles observed in paper-based surfaces treated with FS (higher than those observed for silica and CNF, see chapter 5); thus, the paper possessed microroughness by itself, so treating it with FS facilitated the formation of the above-mentioned hierarchical structure and led to increased contact angles (see WCA values in chapter 5) (see Fig. 6-12).

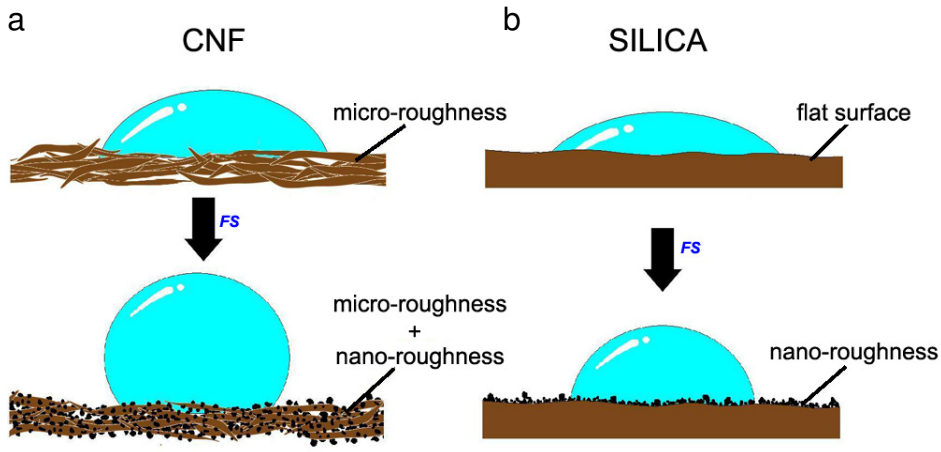

Figure 6-12 Hierarchical structure formed on paper (a) and silica (b) upon treatment with FS.

6.4. Conclusions

Adsorption of FS components (Lacc, SL and LG) onto silica and CNF surfaces was boosted by previously coating the substrates with a layer of chitosan. Treatment with FS increased WCA for CNF from 20° to about 90°. A direct relationship between the amount of LG adsorbed onto silica or nanofibres and WCA was observed, and also one between the roughness of the treated silica and CNF surfaces as determined by AFM and their WCA values.

DLS measurements revealed an effect of the enzyme on LG: reducing particle size from several microns down to 300 nm. The Lacc treatment in the presence of SL reduced LG particle size to 80 nm through a dispersive effect of SL. Light transmission and backscattering data confirmed the effects of laccase (Lacc) on LG: reducing particle size and increasing dispersion stability.

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Chapter 7

Conferring Antioxidant Capacity to Cellulosic Substrates by Using Enzymatic Products

Abstract

A new, industrially feasible method for conferring cellulosic substrates antioxidant properties by using enzymatic products was developed. The method allows cellulose surfaces such as those of paper sheets (PS) to be conferred antioxidant capacity by using a functionalization solution (FS) obtained from an enzymatic reaction. Various laccases and different phenolic compounds potentially possessing antioxidant action were used to prepare the functionalization solutions. Antioxidant capacity was assessed by using UV spectrophotometry to monitor the inhibition of ABTS radical cation (ABTS•+) in the presence of antioxidants. Based on the results, enzymatic modification of the phenolic compound in the functionalization solution increases the bonding strength of its components to cellulosic materials. The level of antioxidant capacity achieved depends on the enzyme type, the chemical structure of the compounds in FS, and the presence of lignosulfonates (SL) in it. The potential of the proposed method for conferring antioxidant properties to paper-based media by surface application of a product obtained from an enzymatic reaction is demonstrated here for the first time.

7.1. Introduction

The search for renewable, sustainable alternatives to petroleum-derived materials has gained interest in recent years. Cellulose can be an effective choice here by virtue of its natural abundance, renewable nature and unique properties. Therefore, special attention has been focused on the search for new and more valuable applications for cellulose. The development of innovative materials with new or enhanced properties for use in the packaging industry has also aroused much interest lately, particularly as regards active packaging materials. Active packaging is an innovative concept that can be defined as a type of packaging that extends the shelf-life of foods while maintaining their safety and quality (Odzemir and Floros, 2004; Suppakul *et al.*, 2003), which is currently a central concern for the food industry. Antioxidant capacity is one of the most desirable properties for a material used to manufacture food-containing packaging. In fact, the quality and shelf-life of packed food are known to be diminished by the action of oxygen causing colour and flavour alterations, lipid and fat oxidation and microbiological spoilage. Accordingly, the most common approaches to preventing food oxidation involve creating oxygen barriers by using glass, metal or plastics in combination with oxygen scavengers in packaging materials (Raheem, 2013; López-de-Dicastillo *et al.*, 2012). However, the reagents used in oxygen scavenger systems can migrate off the packaging or be channeled to the environment after use.

An antioxidant compound can be defined as a substance whose action can inhibit oxidation rate of a free radical. Oxidation reactions can in fact produce free radicals and the radicals start chain reactions that are deleterious to cells. Antioxidants terminate such reactions by removing free radical intermediates and inhibit other oxidation reactions by oxidizing themselves. Traditionally, food producers have prevented oxidation reactions by using synthetic antioxidants; however, their direct addition to foods limits their activity owing to their lack of selectivity to target the food surface —where most oxidative reactions occur. This has aroused considerable interest, promoted by consumer's demands, in using non-detrimental antioxidants and reducing their presence in food.

The most common method for obtaining packaging materials with antioxidant properties involves incorporating appropriate natural antioxidants into plastic materials and assessing the risk of their migrating off the polymer on contact with food. In this context, López-de-Dicastillo *et al.*, (2012) studied antioxidant packaging films consisting of ethylene vinyl alcohol (EVOH) copolymers, Barbosa-Pereira *et al.*, (2013) assessed tocopherol-containing active packaging films made of low density polyethylene (LDPE) and Kanatt *et al.*, (2012) investigated active chitosan–polyvinyl alcohol films with natural

mint and pomegranate peel extracts. Cellulose derivatives such as microcrystalline, carboxymethyl and ethyl cellulose have previously been used as carrier materials to prepare advanced materials with antioxidant properties (Jansen *et al.*, 2011; Serrano-Cruz *et al.*, 2013; Hsu and Kilmartin, 2012); by contrast, paper has seemingly never to date been used as carrier material for this purpose.

The use of enzymes in technological processes has provided an environmentally friendly, cost-effective alternative to the traditional chemicals used in industrial processes. Specifically, laccase–mediator systems have brought about especially interesting advances in the pulp and paper industry, and allowed lignocellulosic materials with improved properties to be developed (Valls and Roncero, 2012; Shogren and Biswas, 2013; Aracri, Roncero and Vidal, 2011; Gao and Cranston, 2008; Saastamoinen *et al.*, 2012). One interesting application of laccase systems exploits their ability to graft low-molecular weight compounds onto lignocellulosic materials (Aracri *et al.*, 2010; Fillat *et al.*, 2012).

In this work, we developed a new methodology for applying a functionalization solution (FS) obtained by enzymatic reaction to the surface of previously formed paper media. The main objective of the treatment was to obtain a paper-based material with antioxidant properties useful for packaging applications.

7.2. Materials and methods

7.2.1. Paper, enzyme and chemicals

Handsheets made from Eucalyptus ECF fibers (supplied by ENCE, Spain), and Filter paper sheets from FILTERLAB® (Sant Pere de Riudebitlles, Barcelona, Spain) were used for functionalization. The enzymes used were a laccase from *Trametes villosa* (Tv) with an activity of 588U/mL, a laccase from *Myceliphthora Thermophila* (Mt) with an activity of 532 U/mL both supplied by Novozymes® (Bagsvaerd, Denmark), and a laccase from *Cerrena Unicolor* supplied by Fungal Bioproducts® (Fb) with an activity of 1660 U/mL. Potassium persulfate (di-potassium peroxdisulfate), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and ethanol were obtained from Sigma-Aldrich. Twenty-five synthetic and natural phenolic compounds (PhC) potentially possessing antioxidant capacity were used in the laccase treatments, namely: caffeic acid (CA), para-hydroxybenzoic acid (pHB), gallic acid (GA), acetosyringone (AS), syringaldehyde (SA), p-coumaric acid (pCA), vanillin (V), acetovanillone (AV), ferulic acid (FA), coniferyl aldehyde (CFA), sinapic acid (SPA), sinapyl aldehyde (SAD), violuric acid (VA), methyl syringate (MS), ethyl gallate (EG), propyl gallate (PG), octyl gallate (OG), lauryl gallate (LG), β-sitosterol (β-S), α-tocopherol

(α-TP), 4-[4-(trifluoromethyl)phenoxy]phenol (TPH), isoamyl salicylate (IS), 2,4,6-tris(1-phenylethyl)phenol (TRP), tannic acid (TA) and resveratrol (RV), all supplied by Sigma–Aldrich. Soluble sulfonated kraft lignin (SL) was obtained from Borreegard (Sarpsborg, Norway), and used as received.

7.2.2. Obtention of the functionalization solutions (FS)

The enzymatic treatments were performed in an Ahiba Spectradye dyeing apparatus equipped with closed vessels. The treatment conditions were based on those described in the "Materials and Methods" chapter. Laccase-phenolic compound (Laccase-PhC) treatments were performed in 250 mL beakers containing final concentrations equivalent to 50 mM sodium tartrate buffer (pH 4), 1.2 g/L PhC, and 1.2 U/mL laccase. In the case of using SL, it was applied at 1.2 g/L. A single treatment was carried out with each phenolic compound (PhC). The resulting reaction products constituted a functionalization solution (FS) that was used to treat the surface of the paper sheets (PS). LG compound was used as a colloid suspension obtained by sonication.

7.2.3. Paper sheets (PS) treatment with the FS

Eucalyptus fibres above described were used to prepare handsheets according to ISO 5269-2:2004 on a Rapid-Köten lab former. Filter paper was used as received. Samples (4 cm^2) of both paper substrates (handsheets and filter paper sheets) were cut off and soaked in each one of the functionalization solutions derived from the Laccase-PhC enzymatic reactions, spread onto blotting paper, and allowed to dry under a standard atmosphere (23°C, 50% Rh).

7.2.4. Antioxidant capacity of the functionalization solutions (FS), and the pure phenolic compounds

The antioxidant capacity of the FSs and the pure phenolic compounds (PhC) was assessed by monitoring ABTS•+ radical decolorization as described in the "Materials and Methods" chapter. To this end, the FS or PhC concerned was added to a pre-formed ABTS•+ radical solution, and the amount of radical cation remaining after a preset time quantified by UV spectrophotometry. The reduction in ABTS•+ concentration, induced by a given concentration of FS or PhC, was related to that of trolox to calculate the antioxidant activity as TEAC (trolox equivalent antioxidant activity). Tests were performed on a Thermo Scientific Evolution 600 spectrophotometer.

7.2.5. Antioxidant capacity of treated papers

The ABTS radical cation (ABTS•+) is a chromophore obtained by oxidizing ABTS with potassium persulfate that absorbs UV light at 415 nm, 654 nm, and 754 nm. The antioxidant capacity of the paper sheets (PS) treated with the 25 functionalization solutions (FS) was assessed in terms of ABTS•+ decolorization by quantifying the reduction of the radical cation as the percent inhibition of the absorbance at 752 nm. Experiments were performed on a Thermo Scientific Evolution 600 spectrophotometer. ABTS radical cation (ABTS•+) was produced in the same way as explained in the previous paragraph for the measurement of the antioxidant capacity of FS and PhC. After the initial absorbance at 752 nm of the ABTS•+ solution was measured, 10 mg samples of treated PS were cut into small pieces, weighed and placed in microcentrifuge tubes. A volume of 1.7 mL of the ABTS•+ solution was then added to each of the microcentrifuge tube and the tube agitated on a vortex mixer for 2 minutes to facilitate surface reaction, centrifuged at 6000 rpm for 4 min and allowed to stand for 30 minutes pior to measuring the absorbance at 752 nm by taking 1 mL aliquots of the supernatant and placing it into methacrylate cuvettes. Each sample was monitored for its absorbance at 752 nm at 1 min intervals for 6 minutes or until stabilization, whichever occurred first. The resulting inhibition was calculated as a percentage by using equation 2-14. The procedure is described in greater detail in the "Materials and Methods" chapter.

7.3. Results and discussion

7.3.1. Screening of antioxidant capacity in PS upon treatment with the functionalization solution (FS).

Preliminary control tests were used to measure the antioxidant capacity of the eucalyptus handsheets (untreated paper) and the enzyme (PS impregnated with a laccase solution). These substrates exhibited 5% and 7% inhibition, respectively, so the controls possessed little antioxidant capacity. Figure 7-1 shows the antioxidant capacity of paper sheets previously treated with the functionalization solutions obtained by using the 25 phenol compounds. If one considers the low antioxidant capacity of the PS and Lacc controls, all enzymatic solutions increased that of the treated PS (Fig. 7-1). This was particularly so with the functionalization solutions prepared from ferulic acid (FA), octyl gallate (OG), lauryl gallate (LG), 2,4,6-tris(1-phenylethyl)phenol (TRP) and resveratrol (RV).

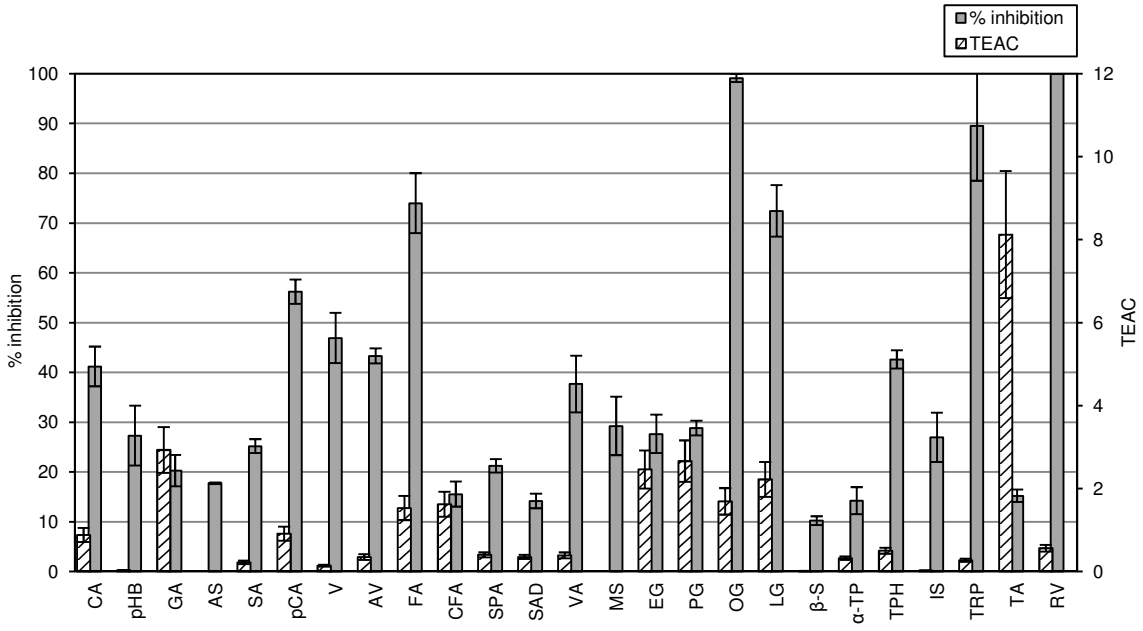

Figure 7-1 Antioxidant capacity of PS after surface treatment with each of the 25 functionalization solutions obtained by reaction with Laccase, and TEAC values for the pure compounds. Antioxidant capacity is expressed as percent inhibition.

However, it should be borne in mind that the PS samples were treated with products of the reaction of the above-mentioned compounds with laccase; as it will be discussed in greater detail in chapter 9, the enzyme altered the chemical structure of the phenolic compounds. Therefore, antioxidant capacity may have been conferred by the enzymatically modified compounds, but the effect of the unmodified (pure) compounds must also be characterized to justify the need for an enzymatic treatment. As can be seen from the TEAC values for the pure compounds, also shown in Fig. 7-1, some compounds conferred no antioxidant capacity by themselves, but only after enzymatic reaction and application onto the surface of PS; however, the opposite effect was observed for the TA compound. One can therefore use a suitable phenolic compound to prepare an effective post-enzymatic solution for efficiently functionalizing the surface of cellulosic substrates. In any case, we judged it essential to examine the influence of the enzyme type, strength of grafting and paper type in order to more accurately assess antioxidant capacity.

In the work described in chapters 4 and 5, we used a surfactant (lignosulfonate, SL) to prepare the functionalization solution (FS) for hydrophobizing cellulosic substrates; we therefore thought it necessary to also elucidate the potential role of this compound in antioxidant capacity development. The following sections describe the experiments used

to these ends. The compounds, enzymes and paper substrates used in each study were chosen in accordance with the particular target parameter.

7.3.2. Effect of the enzyme on antioxidant capacity and bonding strength of FS

The effect of the enzyme on the development of antioxidant capacity with FS was assessed by using two of the compounds previously found to result in high, similar inhibition, namely: ferulic acid (FA) and lauryl gallate (LG). Both also had a similar TEAC value (Fig. 7-1). Tests were conducted on eucalyptus PS, using laccase from *Trametes villosa* (Tv). In order to evaluate the bonding strength of FS to the cellulosic material, antioxidant capacity was also measured in treated PS previously subjected to heavy washing with hot water at 80ºC for 30 min.

Fig. 7-2 illustrates the antioxidant capacity, as percent inhibition, of paper sheets treated with functionalization solutions containing FA or LG in pure or enzymatically modified form. The control solutions (KFS) of pure FA and LG were obtained identically with FS but using no enzyme (see Section 2.3.6). As can be seen, the enzymatically-modified ferulic acid and lauryl gallate (LacTv+FA and LacTv+LG), increased the antioxidant capacity of PS to some extent. This result justified using the enzyme to develop antioxidant capacity.

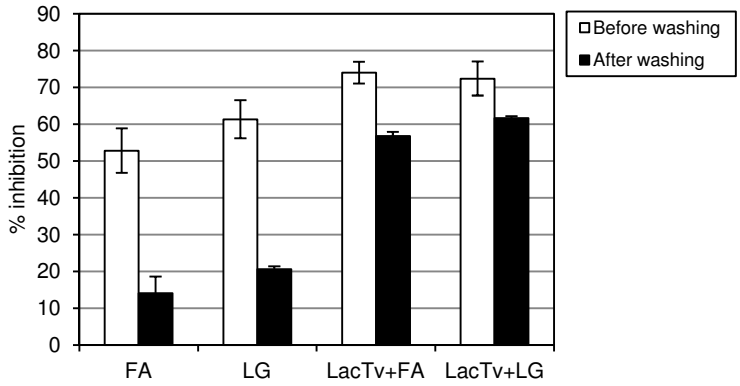

Figure 7-2 Antioxidant capacity of the PS after surface treatment with enzyme-modified and unmodified FA and LG, before and after heavy washing.

As can be seen from Fig. 7-2, the antioxidant capacity of pure (enzymatically unmodified) FA and LG was dramatically reduced by the washing treatment in relation to their enzymatically modified counterparts. Thus, antioxidant capacity remained high upon

washing of the substrates treated with the enzymatically modified compounds. This provides an indication of the extent to which the antioxidant chemicals remain attached to the cellulosic material after washing and is very important in relation to the potential migration of chemicals from food packaging. Food packaging can in fact be a source of chemical food contaminants and increasing concern with risk of chemicals migrating into packaged foods has raised the demand for low-migration materials in Europe.

These results show that the chemical modification introduced by the enzyme not only boosts antioxidant capacity, but also increases the bonding strength of the antioxidants to cellulosic materials. Therefore, using enzymes to develop antioxidant capacity in PS with the proposed methodology is well justified.

7.3.3. Effect of enzyme type

The influence of enzyme type on the development of antioxidant capacity in PS was assessed by treating the LG compound using three different laccases, namely: a laccase from *Trametes villosa* (Tv), a laccase from *Cerrena Unicolor* (Fb) and a laccase from *Myceliphthora Thermophila* (Mt). The enzymes were used to obtain three different FS that were subsequently applied to filter PS prior to measuring antioxidant capacity.

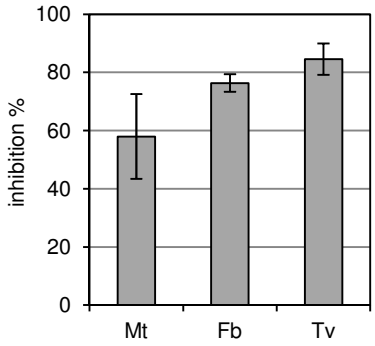

Figure 7-3 Antioxidant capacity of the filter PS after surface treatment with the enzyme-modified LG compound solution obtained by using three different enzymes, namely: Mt, Fb and Tv enzymes.

As can be seen from Fig. 7-3, antioxidant capacity was markedly dependent on the particular enzyme used. Based on the results, the best enzyme candidate was Tv, followed by Fb and Mt. Results demonstrated that the antioxidant capacity can be increased by up to 30% (Tv with respect to Mt) depending on the nature of the enzyme. The results also suggest that the different enzymes may have caused different chemical

changes in LG, thereby producing LG-modified molecules differing in antioxidant properties. In this section we only examined the effect on LG, so other compounds may behave differently.

7.3.4. Effect of the addition of SL on antioxidant capacity

As stated in chapters 4 and 5 we introduced a lignosulfonate (SL) to prepare the functionalization solution (FS). The study described in the present section was aimed at clarifying the effect of this additive on antioxidant activity in PS treated with the proposed methodology. For this purpose, we selected the six compounds exhibiting the highest antioxidant capacity as shown in Section 7.3.1 (viz. FA, LG, OG, TRP, RV and pCA) and examined the effect of adding SL. The study was conducted with filter PS and the enzyme Fb. Figure 7-4 illustrates the antioxidant capacity of PS treated with solutions of the pure compounds, and the effect of including SL and the enzyme in the formulation of FS; also, it shows the antioxidant capacity of each treated PS sample after heavy washing.

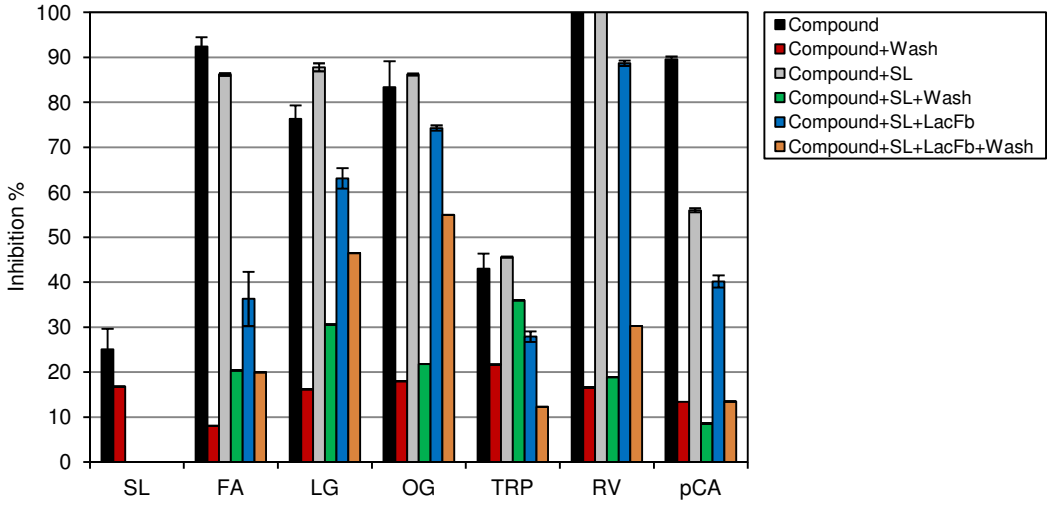

Figure 7-4 Antioxidant capacity of filter PS after surface treatment with solutions of the pure compounds alone and in combination with SL or SL+Lacc before and after heavy washing with hot water (80°C) for 30 min.

The control tests used to assess antioxidant capacity in filter PS samples, both untreated and impregnated with a LacFb solution, provided 6 ± 1% and 9 ± 1% inhibition, respectively; therefore, neither the paper nor the enzyme by itself was able to develop antioxidant capacity. A comparison of the black and grey bars in Fig. 7-4 exposes the

effect of adding SL to the formulation, which differed with the particular phenolic compound. Thus, with FA and pCA, adding SL decreased antioxidant capacity; with the other phenols (LG, OG, TRP and RV), however, the presence of SL increased antioxidant capacity. Overall, SL increased the antioxidant capacity of PS treated with the pure compounds. As regards washing, the dramatic decrease in antioxidant capacity apparent from the black and red bars in Fig. 7-4 indicates that the phenolic compounds were weakly adsorbed onto the PS surface and thus easily removed from it by washing. As can be inferred from the green bars in Fig. 7-4, the antioxidant capacity of washed PS was retained to a greater extent when SL was added to all pure compounds, except for pCA. This result indicates that the presence of SL may have increased retention of the phenolic compounds onto PS surfaces since the increased antioxidant capacity values observed cannot be ascribed to SL alone —the antioxidant capacity of which is also shown in Fig. 7-4.

The antioxidant capacity of PS treated with functionalization solutions containing the phenolic compound, SL, and the enzyme (LacFb) (blue bars in Fig. 7-4) was lower than that obtained with the pure compounds (black bars in Fig. 7-4) and the phenolic compounds plus SL (grey bars in Fig. 7-4). Washing, however, reduced the antioxidant capacity of PS treated with the compound+SL+LacFb solution (orange bars in Fig. 7-4) to levels similar to those obtained with the compound+SL solutions (green bars in Fig. 7-4); by exception, with LG, OG and RV antioxidant capacity remained high after washing — which suggests stronger binding of the phenolic compounds to the paper substrate by effect of the action of the enzyme on the compounds. These results are consistent with those described in Section 7.3.2 and suggest a favourable effect of the enzyme in the form of chemical changes in the phenolic compounds facilitating their binding to the cellulosic material. Using SL had a favourable effect on the development of antioxidant capacity in PS treated with the proposed method; the effect, however, was dependent on the particular phenolic compound. In fact, the antioxidant capacity of a mixture of phenolic compounds was previously found to be smaller than the sum of the antioxidant capacity of each individual compound to an extent depending on the particular PhC and its microenvironment. Interactions between compounds can have inhibitory or synergistic effects (Kuskoski *et al.*, 2005; Arts *et al.*, 2001; Blauz *et al.*, 2008). The presence of Lacc in the treatment solution decreased the initial antioxidant capacity of the phenolic compounds, but had a favourable overall effect since it seemingly led to stronger binding of the compounds to cellulosic substrates as shown by the results for paper samples heavily washed with hot water.

7.3.5. Antioxidant capacity of the Lacc-SL-LG (FS) system

Previous studies showed LG to be effective in hydrophobizing cellulosic substrates by effect of a surface enzymatic treatment, and the effect to be enhanced by the action of the natural surfactant sulfonated lignin improving distribution, hydrophobicity and enzyme activity stability (Cusola *et al.*, 2013; chapters 4, 5 and 6). Also, as stated above, impregnating PS with the enzymatic product of the reaction of laccase with LG conferred them antioxidant capacity. The study described in this section was aimed at characterizing antioxidant capacity in the functionalization solution (FS) and elucidating the role of its individual components in the development of this property.

The antioxidant capacity of the LG, SL, LG+SL, SL+Lacc, LG+Lacc controls, and that of the whole treatment (FS), was assessed using by a methodology based on previously reported procedures (see section 2.6.2.1). Solutions of the mentioned controls and FS at concentrations ranging from 0 to 2.5 mM were used to measure antioxidant capacity as a function of concentration (see Fig. 7-5), the slopes of the resulting curves being compared with that for trolox to calculate TEAC (viz. the trolox equivalent antioxidant capacity; see section 2.6.2.1) (table 7-1). Trolox equivalency is widely used as a benchmark for antioxidant capacity; a TEAC value higher than unity is indicative of a high antioxidant capacity.

As can be seen from the results, the LG and LG+SL controls were those exhibiting the highest TEAC values (1.96 and 1.38, respectively) and hence the highest antioxidant capacity. However, these controls consisted of unmodified LG and SL, so TEAC was considerably decreased upon treatment with the enzyme: to 0.39 with the LG+Lacc control and 0.19 with the functionalization solution (LG+SL+Lacc). This result is suggestive of changes in the phenolic compound (LG) upon enzymatic reaction altering its chemical structure and hence its antioxidant capacity. The TEAC value for the SL and SL+Lacc controls (0.4 and 0.03, respectively) are suggestive of poor antioxidant capacity in SL —and even poorer after the enzymatic reaction.

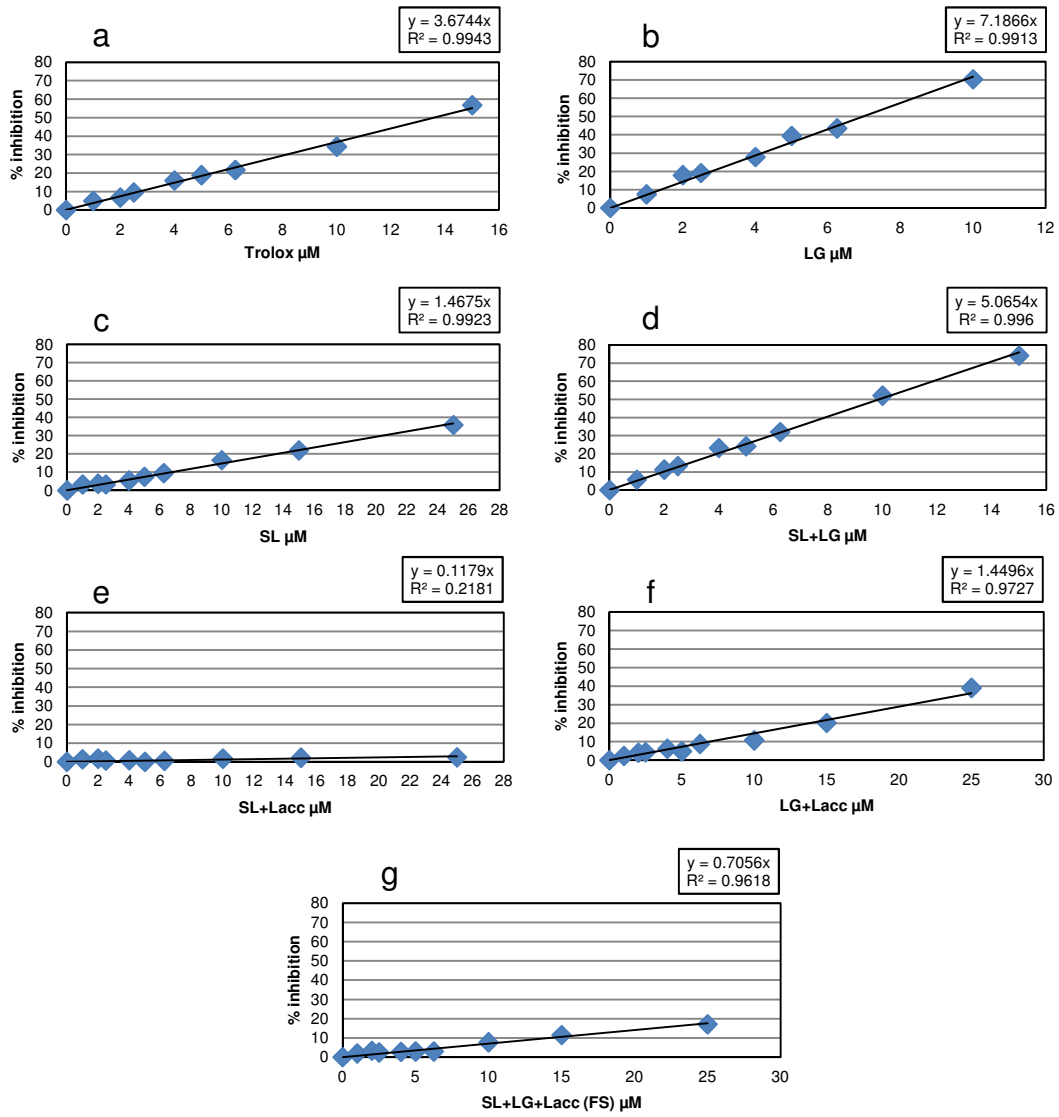

Figure 7-5 Variation of inhibition with the concentration of Trolox (reference) (a), LG (b), SL (c), LG+SL (d), SL+Lacc (e), LG+Lacc (f) and FS (g).

Table 7-1 Slopes of the curves obtained from the representation of the percent inhibition versus concentration (Fig. 7-5), and TEAC values of Trolox, FS, and controls.

System	Slope	TEAC
Trolox	3.67	-
LG	7.19	1.96
SL	1.47	0.4
LG+SL	5.07	1.38
LG+Lacc	1.45	0.39
SL+Lacc	0.11	0.03
FS (LG+SL+Lacc)	0.71	0.19

Based on the results, the LG+Lacc and LG+SL+Lacc (FS) enzymatic products, which consisted in aqueous solutions obtained by enzymatic reaction with laccase, possessed no antioxidant capacity. These results contradict those previously obtained for PS treated with these solutions. In fact, as shown in Section 7.3.1, the PS samples subjected to the LG+Lacc treatment exhibited marked antioxidant capacity. There are at least two plausible explanations for this phenomenon, namely:

a) Although the LG+Lacc system has no antioxidant capacity, interacting with cellulose in PS may cause chemical rearrangement of the LG molecule by effect of its grafting onto cellulose and lead to the formation of new structures with antioxidant properties.

b) Although the slope of the inhibition curves for LG+Lacc and FS were very low, the concentrations actually deposited onto the treated PS samples were unknown. In fact, the antioxidant capacity of PS was measured by using a certain amount of paper (10 mg), but the exact amounts of LG+Lacc and FS attached to the PS surface could not be determined.

The likelihood of the former hypothesis can be judged partly from the discussion of potential reaction products and grafting adducts (this issue is discussed in greater detail in chapter 9). That of the latter requires at least a rough estimate of the amounts of FS products —and hence of modified LG— deposited onto the paper sheets by the treatment. As can be seen in Fig. 7-1, the samples treated with the LG+Lacc solution exhibited an antioxidant value of 73 ± 5% inhibition, whereas the antioxidant capacity of PS treated with FS was 74 ± 5% inhibition.

Based on the slopes of the curves for LG+Lacc and FS in Fig. 7-5, obtaining 73% inhibition required the paper to bear or release amounts of LG+Lacc or FS products in the region of 50 μM and 103 μM, respectively.

The amounts of FS the paper was able to absorb by impregnation were evaluated by using the mass-difference method described in annex A to determine FS uptake by the paper substrate (commercial filter paper of 62 g/m² grammage). The FS uptake (FSU) for the paper was about 68 g/m². Since the concentration of LG in FS was 3.55 mmol/L and the density of FS 1 g/mL, the amount of FS borne by 1 g of treated paper was calculated from the following equation:

$$FSU \times G \times \delta_{FS} \qquad [7\text{-}1]$$

where FSU is the FS uptake, G the paper grammage and δ_{FS} the FS density. The amount of FS present in 1g of paper was thus calculated to be:

$$\frac{68 g_{FS}}{1 m^2} \times \frac{1 m^2}{62 g_{paper}} \times \frac{1 mL_{FS}}{1 g_{FS}} = 1.1 \, mL_{FS} \Big/ g_{paper} \qquad [7\text{-}2]$$

Since we used 10 mg of paper to determine the antioxidant capacity of the paper sheets, the concentration of LG in FS allowed us to easily estimate the amount of LG present in each paper sample as:

$$\frac{1.1 mL_{FS}}{g_{paper}} \times 0.01 g_{paper} \times \frac{3.5 \times 10^{-3} mmol}{1 mL_{FS}} = 3.9 \times 10^{-3} mmol = 0.039 \mu mol \qquad [7\text{-}3]$$

Finally, if complete release of LG present in the paper into the volume of ABTS solution used (1.7 mL, see Section 2.6.2.2) is assumed, then the concentration of LG leading to the previously measured inhibition value in treated PS (73%) was:

$$\frac{0.039 \mu mol}{1.7 mL_{ABTS}} \times \frac{1000 mL}{1 L} = 23 \mu M \qquad [7\text{-}4]$$

Based on the curve of Fig. 7-5g for FS inhibition, an amount of 23 μM of FS could only have caused about 15% inhibition. Therefore, the amount of LG present in FS —which essentially consisted of enzyme-modified LG molecules— leading 73% inhibition in treated PS were not able to develop such inhibition percentage when considered in solution without the presence of paper. This result suggests that hypothesis (b) above is not valid and strengthens the likelihood of hypothesis (a).

The previous estimates were obtained on the assumption that FS distributed evenly across PS upon impregnation —which was supported by visual inspection of the PS specimens after impregnation.

7.3.6. Comparison of TEAC for the pure compounds and the antioxidant capacity of PS conferred by the enzymatic functionalization solution (FS)

As shown in the previous section, the antioxidant capacity of PS treated with the enzymatic solutions can be ascribed to chemical rearrangement in chemically modified LG molecules upon interaction with the cellulosic material. The LG control treatment involved impregnating PS with an LG solution at the same concentration used in the reaction. In the study described in this section, we examined the potential relationship between TEAC for the pure compounds and the final antioxidant capacity conferred to PS by the functionalization solution (FS). The phenolic compounds studied were selected from among those providing the highest antioxidant capacity upon treatment of the paper substrate (see Section 7.3.1), namely: FA, OG, LG, TRP, RV and pCA. Antioxidant capacity, expressed as percent inhibition, was measured after washing the treated PS samples. Figure 7-6 shows the selected compounds in increasing sequence of TEAC; as can be seen, LG exhibited the highest value and TRP the lowest.

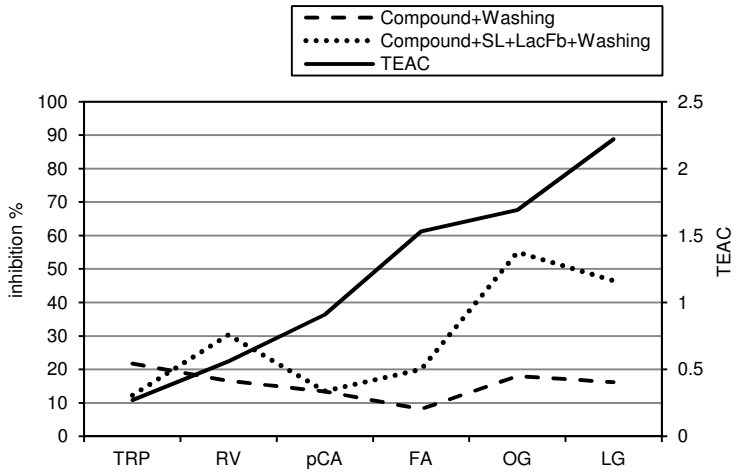

Figure 7-6 TEAC values for the pure compounds (solid lines), and percent inhibition of PS impregnated with the pure compounds (dashed lines) and with laccase-treated compounds (compound+SL+LaccFb, dotted lines). The studied compounds were FA, OG, LG, TRP, RV and pCA.

There was no clear-cut relationship between TEAC for a given compound and its ability to develop antioxidant capacity in PS upon enzymatic modification and application under the form of a functionalization solution to PS. However, a slight tendency of antioxidant capacity to increase with increasing TEAC was observed. The PS samples

treated with solutions of the pure, enzymatically untreated compounds exhibited no such tendency, so their low inhibition values can be ascribed to the loss of phenolic product upon washing of the substrate. No such loss was observed —inhibition remained high— from PS treated with enzymatically modified compounds. These results testify to the favourable effect of the enzyme, which alters the chemical structure of the phenolic compounds and facilitates their anchoring to cellulose fibres as a result.

The chemical structure of the phenolic compounds may play a prominent role in the intended correlation. Since in the present section only six compounds were analyzed, the study could be extended to additional compounds exhibiting also high correlation. Then, it would be possible to establish curve given by several compounds with increasing antioxidant capacity degrees (assessed by the TEAC) that would be useful to predict the antioxidant capacity of the FS-treated PS by only measuring the TEAC value of the compound, without the need to launch the enzymatic reaction and PS treatment. Such a curve might also be useful to ascertain whether antioxidant capacity depends on the chemical structure of the particular phenolic compound.

7.4. Conclusions

A surface treatment of cellulose substrates with the aqueous products derived from the reaction of the enzyme laccase with specific phenolic compounds provides an effective method for developing antioxidant properties in cellulosic materials. Using the enzyme to introduce chemical changes in the compounds is well justified since, as confirmed by washing tests, it strengthens binding of the modified products to cellulosic materials. The enzyme type used strongly influences the ability of the functionalization solution (FS) to confer antioxidant capacity to paper sheets (PS). Our results suggest that different enzymes cause also different chemical changes in the phenolic compounds, thus leading to differences in antioxidant behaviour. The addition of SL to the functionalization solution has a favourable effect on antioxidant capacity development with most phenolic compounds —and an only slightly adverse impact on that of the others. This is quite interesting because the presence of SL in FS is desirable; in fact, SL provides a number of benefits by acting as an oxidation enhancer, dispersing agent and hydrophobicity developer, among other roles. The LG+Lacc treatment and FS (LG+Lacc+SL) were found to efficiently confer antioxidant capacity to PS; however, they exhibit no antioxidant capacity by themselves. Thus, the enzyme causes chemical changes in the LG molecule that reduce its antioxidant capacity, but interaction of enzyme-modified LG with a cellulosic material causes further changes in LG that seemingly restore its antioxidant capacity. The TEAC value for each phenolic compound and its ability to develop

antioxidant capacity in PS were found to be slightly correlated. Correlation might be stronger if a greater number of phenolic compounds were examined. Also, correlation curves thus obtained might be useful with a view to predicting antioxidant capacity in FS-treated paper sheets simply by measuring TEAC for each phenolic compound.

7.5. References

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Chapter 8

Effect of the Chain Length of Alkyl Gallates on Hydrophobicity and Antioxidant Properties

Abstract

This chapter examines the influence of the alkyl chain length in gallates on the development of hydrophobic and antioxidant properties in paper-based materials by use of an enzymatic treatment. To this end, various members of the gallate homologous series were used to treat cellulosic substrates that were then assessed for hydrophobicity and antioxidant properties. Hydrophobicity was evaluated from water drop test (WDT) and contact angle (WCA) measurements, and antioxidant capacity by using UV spectrophotometry to monitor the inhibition of ABTS radical cation (ABTS•+) in the presence of paper substrates. The results of this study expose the influence of the alkyl chain length on such properties. Paper sheets (PS) were additionally subjected to curing treatments with the aim of understanding the influence of heat on the development of hydrophobic and antioxidant properties in the substrates. Finally, chemical changes in gallates of variable chain length were studied by FTIR-ATR analysis, which revealed major alterations in the phenolic moieties of the gallates but none in their hydrocarbon chains. The study was conducted by using three different enzymes.

8.1. Introduction

The effectiveness of an enzymatic system consisting of a functionalization solution (FS), containing the enzyme laccase (Lacc), lauryl gallate (LG) and lignosulfonate (SL), in conferring cellulosic materials hydrophobic and antioxidant properties is demonstrated in chapters 4 to 7. Also, the favourable effect of a curing treatment on the hydrophobicity of paper sheets previously subjected to an FS treatment is discussed in section 4.3.6.

Lauryl gallate (LG) is the n-docecyl ester of gallic acid; however, the homologous series of gallates includes a number of similar compounds of variable chain length that may be useful for the enzymatic surface modification of cellulosic materials. Alkyl gallates (viz. 3,4,5-trihydroxybenzoic acid alkyl esters) are widely used as food antioxidants (Aruoma *et al.*, 1993) even though they are not natural compounds. Also, some alkyl gallates have proved useful as multifunctional food and pharmaceutical additives by virtue of their antibrowning (Kubo *et al.*, 2000), antifungal (Hsu *et al.*, 2009), and antibacterial effects (Silva *et al.*, 2013; Gunckel *et al.*, 1998). In addition, they have been shown to possess amphiphilic and anticancer properties (Gonzenbach *et al.*, 2006; Locatelli, Filippin-Monteiro and Creczynski-Pasa, 2013; Ortega *et al.*, 2003).

Several studies have examined the use of gallates of variable alkyl chain length to modify the properties of a wide variety of materials such as cellulose (Garcia-Ubasart *et al.*, 2012), wool (Gaffar Hossain *et al.*, 2010), chitosan (Vartiainen *et al.*, 2008), titanium diodixe (TiO_2) (Dzunuzovic *et al.*, 2013), and ceramics (Studart *et al.*, 2006), which are conferred advanced properties such as improved thermal and thermo-oxidative stability (polymers, foams) or dispersibility by gallates. However, little is known about the ability of gallates to confer advanced properties to paper-based materials, and even less about the effect of the gallate chain length on enzymatic oxidation reactions. Given the multiple inherent properties of alkyl gallates, one can hypothesize that, if they can bind chemically to the surface of virtually all types of materials, they may be useful to confer them advanced properties. The innovative aspect of this chapter, and indeed of the whole thesis, is that gallates can be chemically bonded by using biotechnological techniques.

In this work, we examined the effects of eight enzymatically modified alkyl gallates on the hydrophobicity and antioxidant activity of cellulose-based materials with a view to elucidating the role of the hydrophobic moiety (alkyl chain) and gallic acid moiety on these two properties. Chapter 4 discusses the effect of heat treatments on paper hydrophobicity; this, deals with the effect of curing. We additionally studied the chemical structure of pure and enzymatically modified gallates by IR analysis to elucidate the influence of their chain length on the enzymatic oxidation.

8.2. Materials and methods

8.2.1. Enzyme and chemicals

Filter paper sheets (PS) from FILTERLAB® (Sant Pere de Riudebitlles, Barcelona, Spain) were used for functionalization. The enzymes used in this work were a laccase from *Trametes villosa* (Tv) with an activity of 588U/mL, supplied by Novozymes® (Bagsvaerd, Denmark), a laccase from *Cerrena Unicolor* supplied by Fungal Bioproducts® (Fb) with an activity of 1660 U/mL and a laccase from *Myceliphthora Thermophila* (Mt) with an activity of 532 U/mL also supplied by Novozymes®. Eight compounds from the gallate homologous series were used in the laccase treatments, namely: gallic acid (GA), methyl gallate (MG), ethyl gallate (EG), propyl gallate (PG), butyl gallate (BG), octyl gallate (OG), lauryl gallate (LG) and stearyl gallate (SG). Tannic acid (TA) was additionally used to study the antioxidant properties. All compounds were supplied by Sigma–Aldrich.

8.2.2. Enzymatic products obtention

The enzymatic reaction was used to obtain several functionalization solutions (FS), which were then used to impregnate paper sheets (PS) by dipping. Enzymatic treatments were performed in 250 mL beakers containing final concentrations equivalent to 50 mM sodium tartrate buffer (pH 4), 1.2 g/L LG (or other phenolic compound, PhC) and 1.2 U/mL laccase. LG and SG were used as colloid suspensions obtained by sonication. The resulting FSs were used to treat the surface of paper sheets (PS) or concentrated to obtain powder for FTIR-ATR analysis.

8.2.3. Treatment of paper sheets (PS) with the enzymatic products

Samples of 4 cm^2 were cut from the filter PS and soaked in each of the laccase–PhC products derived from the enzymatic reaction, spread onto blotting paper and allowed to dry under a standard atmosphere at 23°C at 50% relative humidity.

8.2.4. Assessment of hydrophobicity

Contact angles were measured with a Dataphysics® OCA15 contact angle goniometer, using a 4 μL water drop for delivery to the sample surface in each measurement. The water drop test (WDT) was applied on each treated paper specimen according to Tappi standard T835 om-08. Previously, the paper sheets were conditioned according to ISO 187.

8.2.5. Antioxidant activity of paper sheets

Tests for antioxidant activity were performed on a Thermo Scientific Evolution 600 spectrophotometer. ABTS radical cation (ABTS•+) was produced as explained in the "Materials and Methods" section, and the initial absorbance of the ABTS•+ solution was recorded at 752 nm. Then, samples of 10 mg of treated PS were cut into small pieces, weighed and placed in microcentrifuge tubes that were supplied with a volume of 1.7 mL of ABTS•+ solution. Next, the tubes were agitated on a vortex mixer for 2 min to facilitate surface reaction, centrifuged at 6000 rpm for 4 min and allowed to stand for 30 min before conducting the absorbance tests. Absorbance measurements at 752 nm of the centrifuged tube contents were made by using 1 mL of supernatant in a methacrylate cuvette. The absorbance at 752 nm of each sample was monitored at 1 min intervals for 6 min or until stabilization, whichever occurred first.

8.2.6. ATR-FTIR analysis

For the FTIR analysis of the enzyme-modified gallates, 200 mL of the aqueous enzymatic product resulting from the reaction of the laccase with each gallate (or PhC) was prepared by using the doses and reaction conditions described above. The enzymatic products thus obtained, which exhibited liquid properties, were concentrated on a centrifuge or rotavapor and dried. The resulting solid residue was finely ground to obtain a fine powder which was then analyzed on a Perkin Elmer Spectrum 100 FT-IR spectrometer equipped with universal ATR sampling accessory. Pure gallates (i.e. gallates subject to no enzymatic modification) were also analysed in parallel. Each spectrum was the average of 32 scans performed at 1 cm^{-1} intervals over the wavenumber range 4000–550 cm^{-1}. Samples were placed directly on the ATR accessory and the measuring cell was washed with de-ionized water and ethanol between measurements.

8.3. Results and discussion

All tests discussed below were conducted in triplicate, using each of the three laccases described in the Materials and Methods section. However, only the results obtained with laccase from *Trametes villosa* are reported because those provided by the other two enzymes were very similar —and also because most of the tests performed in this doctoral work were done with *Trametes villosa* laccase.

8.3.1. Influence of the chain length of alkyl gallates and curing on hydrophobicity

The hydrophobic nature of LG must be due to its alkyl chain. Several studies have examined the influence of alkyl chains of variable length on the development of surface hydrophobicity in various materials (Darmanin and Guittard, 2012; Vanzo, Bratko and Luzar, 2012; Jin *et al.*, 2012); none, however, has focused on the effect of a long gallate alkyl chain on the development of hydrophobicity in previously formed cellulose sheets. The primary goal of this study was thus to improve our understanding of the relationship between hydrophobicity and alkyl chain length in the homologous series of gallic acid and its alkyl esters (Fig. 8-1).

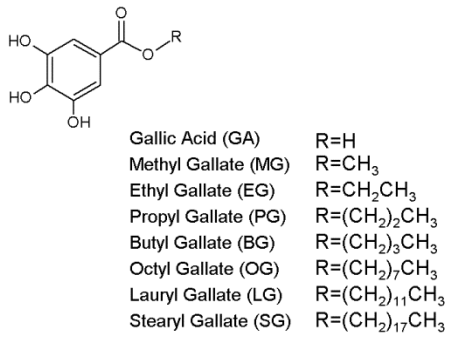

Figure 8-1 Chemical structure of gallic acid and its alkyl esters.

Each gallate was oxidized by the action of laccase under the reaction conditions described in chapter 5, albeit in the absence of SL from the medium. The resulting products, which exhibited liquid properties, were used to impregnate filter paper sheets (PS) that were then allowed to dry and assessed for hydrophobicity from water drop test (WDT) and contact angle (WCA) measurements as described under "Materials and Methods". A control treatment involving unmodified filter PS was also used for comparison. In

addition, the treated sheets were subjected to a curing treatment in order to better understand its effect on the development of hydrophobicity.

Fig. 8-2 shows the variation of hydrophobicity as assessed from WDT values with the chain length of the gallate (on the x-axis). Based on the curve for uncured PS, hydrophobicity as measured from either WDT or WCA values increased with increasing length of the hydrocarbon chain of the gallate. In treated uncured PS, however, hydrophobicity peaked between OG and LG (i.e. a chain length of 8–12 carbon atoms) and then decreased markedly with increasing length. Therefore, our selection of alkyl chain lengths, based on past experience with LG compounds, afforded optimal hydrophobicity in PS. Hydrophobicity increased steadily from 0 to 5 carbon atoms; however, the effect was maximal for chains 5–15 atoms long.

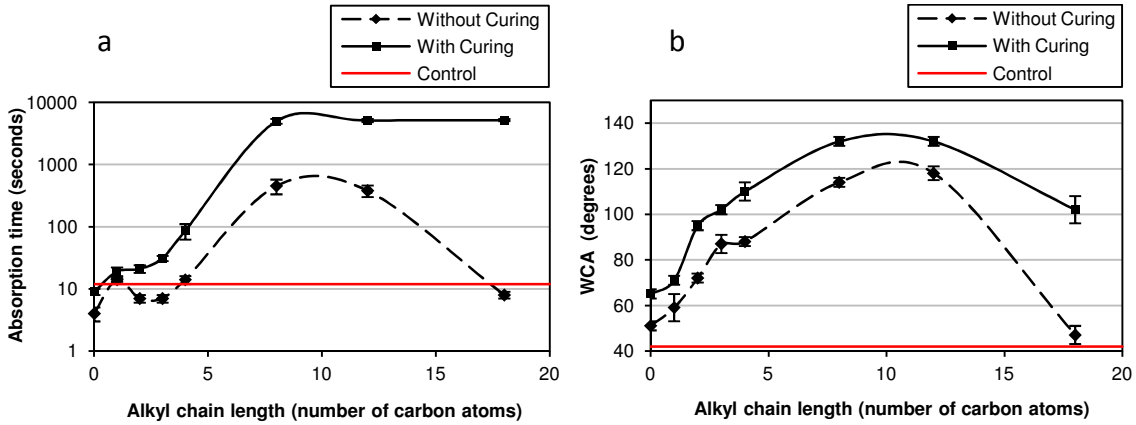

Figure 8-2 Hydrophobicity as determined from WDT (a) and contact angle measurements (b) of filter paper sheets (PS) treated with functionalization solutions (FS) containing gallates of variable alkyl chain length.

We had previously found curing treatments to have a favourable effect on the development of hydrophobicity in PS (see Section 4.3.6). This led us to subject PS samples treated with FS containing gallates of variable alkyl chain length to a heat-curing treatment involving placing treated samples in an oven at 150℃ for 30 min. Figure 8-2 shows the variation of the WDT and WCA values for the cured samples; as can be seen, all treated samples exhibited significantly increased hydrophobicity —in some cases by as much as one order of magnitude— irrespective of the length of the gallate alkyl chain. Unlike the uncured samples, the hydrophobicity of which was considerably decreased by the long-chain gallates, this property levelled off at high levels in terms of WDT (Fig. 8-

2a). Long-chain gallates also reduced WCA in cured sheets, albeit to a lesser extent than in uncured samples.

The functionalization solutions obtained by reacting the enzyme with each gallate exhibited a marked colour change from the initial product (before reaction) except in SG. In fact, the enzymatic reaction of SG provided a functionalization solution with little signs of oxidation (viz. little colour change). Also, SG was scarcely dispersed in the reaction medium, even after sonication. Possibly, the long alkyl chain of SG introduced considerable steric hindrance that may have affected the action of the enzyme by preventing the phenol moiety from accessing its catalytic sites. In addition, the low solubility and dispersibility of the compound may have led to the formation of aggregates further hindering the action of the enzyme. Since as stated in chapters 4 and 5, enzymatic oxidation of the gallate is essential for effective hydrophobization, partial or little oxidation of SG must have been the main reason for the low hydrophobicity of PS treated with an SG-containing functionalization solution.

The increased, stable hydrophobicity obtained with the long-chain gallates upon curing can be ascribed to chemical reactions between FS and PS taking place at high temperatures, and also to the spatial distribution of the gallate over the substrate. Thus, functionalizing PS at room temperature with FS obtained by reacting the enzyme with long-chain gallates caused chemical changes that facilitate physico–chemical adsorption by positively charged paper as shown in chapter 5. This led to a cellulosic substrate containing gallate molecules randomly adsorbed onto its surface that introduced substantial hydrophobicity (Fig. 8-3a). However, the curing treatment may have triggered new coupling reactions only occurring at increased temperatures (e.g. esterification) and causing the hydrophobic tail of the gallates to face outwards from the substrate (Buriak, 2002; Sieval *et al.*, 2001), thereby further increasing its hydrophobicity (Fig. 8-3b). The chemical nature of enzyme-modified LG and its interaction with cellulosic materials will be discussed in chapter 9. In addition, small particles of gallate aggregates may have melted onto the surface by effect of the high temperatures, thereby increasing the nanoscale roughness of the cellulosic substrate and increasing its hydrophobicity as a result (see chapter 6).

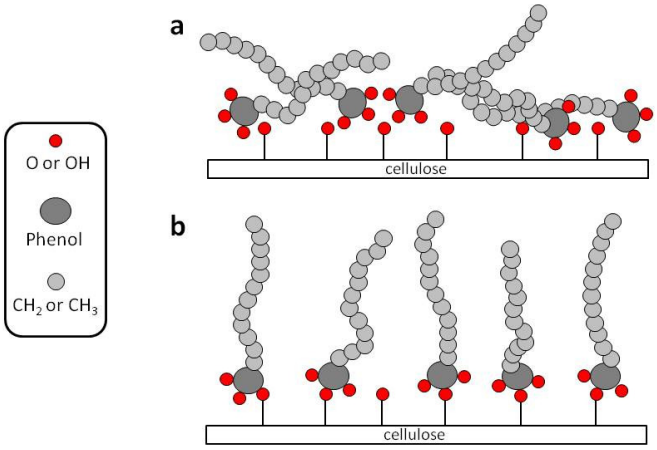

Figure 8-3 Schematic depiction of gallate molecules randomly adsorbed onto the cellulosic substrate upon treatment with FS at room temperature (a) and after curing at 150°C for 30 min, which caused the alkyl chain to face outwards from the substrate (b).

8.3.2. Effect of the alkyl chain length of the gallates on the development of antioxidant activity

According to some authors, hydroxyl groups on the aromatic ring of phenolic compounds are responsible for their antioxidant properties (Arts *et al.*, 2004). Therefore, the differences in antioxidant properties between unmodified LG and laccase-modified LG described in chapter 7 must have arisen from chemical changes in LG introduced by the enzyme. In fact, laccase causes chemical alterations in the phenol moiety of LG.

However, as also explained in chapter 7, when enzyme-modified LG interacts with cellulosic materials, chemical re-arrangement of the molecule restores the antioxidant capacity of LG. Thus, the complex formed between enzyme-modified LG and paper possesses antioxidant properties. The present study was undertaken to elucidate the influence of the alkyl chain length on the development of such properties. For this purpose, we selected several compounds from the gallate family because they possess an identical core structure (the phenol) and differ only in the length of the hydrocarbon chain. Each gallate was oxidized by the action of laccase and the resulting products, which exhibited liquid properties, were used to impregnate filter PS. Once the treated PS were dry, their antioxidant capacity was assessed as explained under "Materials and Methods". A control consisting of untreated PS samples, one containing the enzyme (viz. paper impregnated with enzyme solution) and one consisting of tannic acid (TA) were also examined for comparison. As can be seen from Fig. 8-4, the paper itself (without

modification) and the laccase control (PS impregnated with the enzyme solution) exhibited very weak inhibition and were thus deemed inactive in terms of antioxidant capacity.

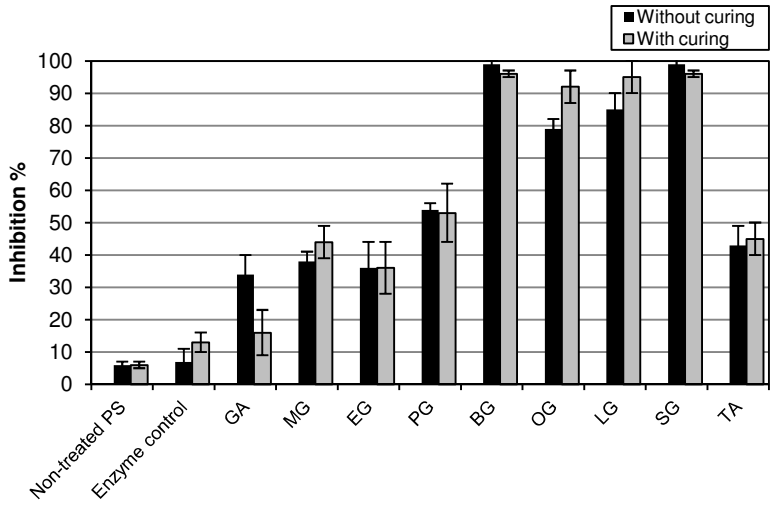

Figure 8-4 Antioxidant activity of PS upon surface treatment with each product of the gallate family as prepared by reaction with laccase. The results are expressed as percent inhibition.

The figure also shows the variation of antioxidant activity with the gallate chain length. Based on the results, the antioxidant capacity of treated PS increased gradually with increasing chain of the gallate but levelled off beyond OG. Since the action of the enzyme focused on the phenol, an identical chemical modification can be expected to have been undergone in all the gallates, and hence no chemical modification in the hydrocarbon chains —as also supported by the FTIR results discussed in detail in chapter 9.

Tannic acid (TA) was also included in the analysis because it contains a large number of phenols and other groups potentially influencing the development of antioxidant properties. As can be seen from Fig. 8-4, TA inhibited oxidation by about 40%. Based on the results, successfully introducing antioxidant capacity with this technique relies more heavily on the presence of a long aliphatic chain in the enzyme-modified compound than on a large number of aromatic rings in its structure.

As shown in previous chapters, heat treatments have a favourable effect on hydrophobicity in treated PS. This led us to also examine the effect of a curing treatment on antioxidant capacity development in treated PS. To this end, PS samples treated with

enzyme-modified gallates were oven-cured at 150°C for 30 min. Figure 8-4 illustrates the effect of the curing treatment; as can be seen, the treatment had no appreciable effect on the antioxidant capacity of PS.

8.3.3. FTIR-ATR analysis of the increasing alkyl chain length gallates and effect of the enzymatic modification

The chemical modifications potentially undergone by LG will be discussed in chapter 9. In the present work, we performed an IR study using other compounds of the gallate family in order to strengthen our understanding of the relationship between the structure of the gallates and their chemical changes by effect of enzymatic modification. Each gallate studied was oxidized with laccase under the reaction conditions described in chapter 5, albeit in the absence of SL. The products thus obtained, which exhibited liquid properties, were concentrated on a centrifuge or rotavapor and dried. The resulting powders, which consisted of enzimatically modified gallates, and the pure gallates, were measured by FTIR-ATR. Figure 8-5 shows the IR spectra for the pure (Fig. 8-5a) and enzyme-modified gallates (Fig. 8-5b) over the wavenumber range 3600–2600 cm^{-1}, and Fig. 8-6 those for the same compounds in the spectral region from 1750 to 600 cm^{-1}.

Based on Fig. 8-5, the hydrocarbon chain remained intact after reaction with the enzyme; in fact, the peaks for methyl ($–CH_3$, 2850 cm^{-1}) and methylene groups ($–CH_2$, 2916 cm^{-1}) remained unchanged. This was particularly so for the long-chain gallates and, especially, for PG and longer gallates. The enzyme caused no chemical changes in the alkyl chain. The peaks for $–CH_2$ and $–CH_3$ groups in the hydrocarbon chain increased with increasing chain length of the gallate; this was especially so for methylene groups by effect of their number increasing with increasing chain length. This latter finding was observed in both the pure gallates and the enzyme-modified compounds.

The broad band from 3570 to 3200 cm^{-1} for the pure gallates (Fig. 8-5a) corresponds to hydroxyl groups in their phenol moiety. The large width of the band was due to intra- and inter-molecular hydrogen bonding interactions typical of such groups (Coates, 2000). The band was very similar in shape and strength for all pure gallates by effect of their containing an identical number of hydroxyl groups. However, reaction with the enzyme decreased the band by effect of the loss of hydroxyl groups and made it even broader by effect of an increased number of hydrogen bonding interactions in the remaining groups. This latter finding was observed in all gallates. Modification by the enzyme may have caused the formation of carboxyl groups (see chapter 9); hydroxyl functions in carboxyl groups typically establish hydrogen bonds and form stable dimers as a result —which reflected in a shift in the band to lower frequencies.

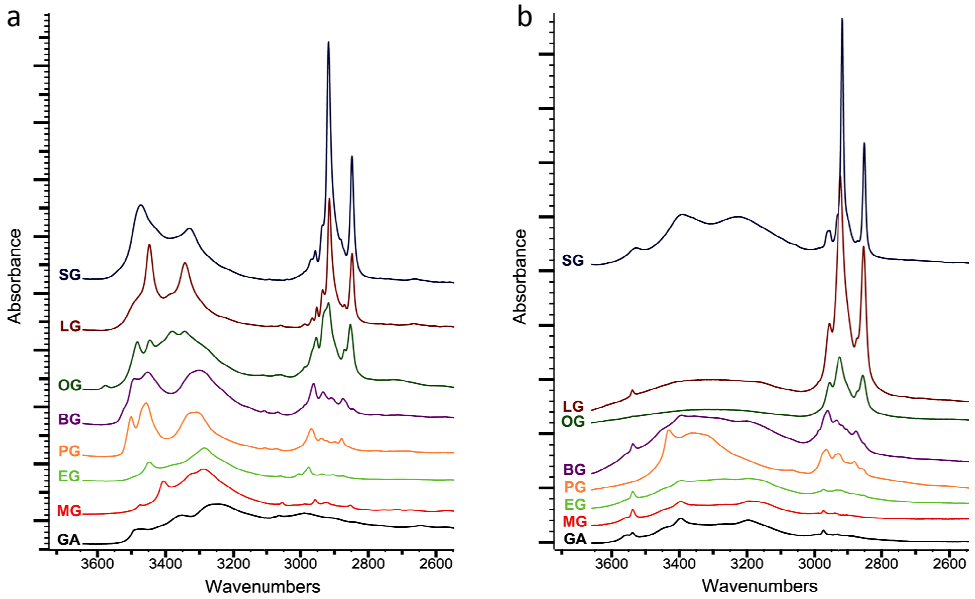

Figure 8-5 IR spectra for pure (a) and enzyme-modified gallates (b) in the range 3600–2600 cm^{-1}.

The carbonyl group in each pure gallate (Fig. 8-6a) was found to absorb at a different frequency (see table 8-1). In the pure GA, the carbonyl peak should have appeared at 1725–1700 cm^{-1} (C=O in the ester group); however, the peak appeared at a lower frequency, probably because the carbonyl group was conjugated with the aromatic ring, and also because of the tendency of the carbonyl group in the ester to experience hydrogen bonding interactions. Hydrogen bonding must have reduced the vibration frequency of the C=O, and also led to the appearance of the broad absorption signal in the range 3300–2500 cm^{-1}. In MG, the peak for C=O in the ester appeared at 1669 cm^{-1} rather than at 1750–1725 cm^{-1} as one would have expected. The decreased frequency of this peak can also be ascribed to hydrogen-bonding interactions. Overall, the carbonyl peak for all pure gallates was shifted to lower frequencies. One interesting finding was that the peak appeared at a gradually decreasing wavelength in PG and longer gallates. This suggests that an increased chain length decreases the vibrational frequency of the C=O group, possibly because a long hydrocarbon chain can "fold" above the phenol moiety and weaken the vibration of the C=O bond. The phenomenon is illustrated in Fig. 8-7.

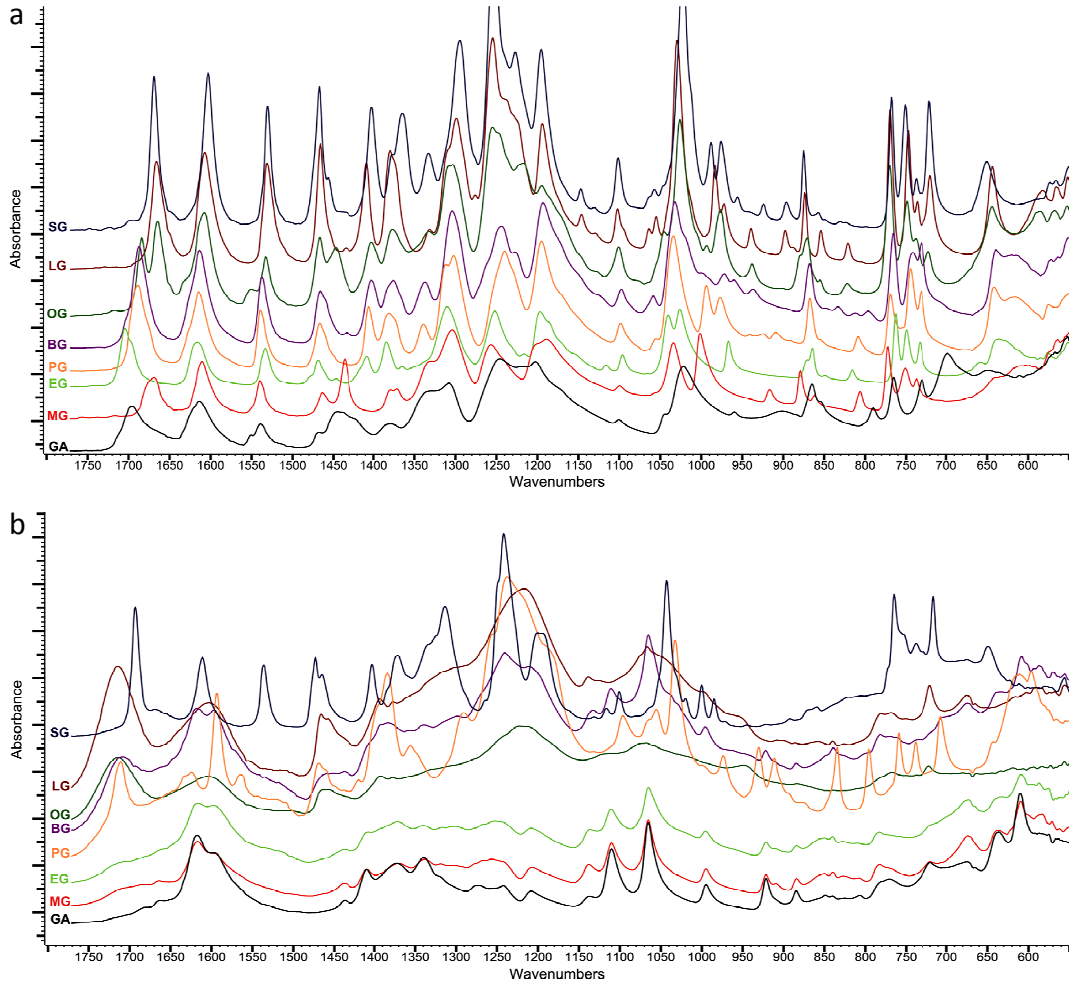

Figure 8-6 IR spectrum of the pure (a), and enzyme-modified (b) gallates in the zone ranging from the 1750 to the 600 cm^{-1}.

Table 8-1 IR absorption frequencies for the carbonyl group (C=O) in the pure gallates.

Gallate	Carbonyl frequency
GA	1697 cm^{-1}
MG	1669 cm^{-1}
EG	1704 cm^{-1}
PG	1689 cm^{-1}
BG	1687 cm^{-1}
OG	1684 cm^{-1} (double)
LG	1666 cm^{-1}
SG	1668 cm^{-1}

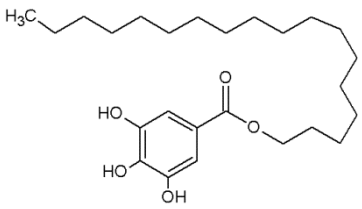

Figure 8-7 Schematic depiction of folding of the alkyl chain in SG above the phenol moiety.

The spectrum for SG was unchanged by the action of laccase: its peaks were identical in shape and strength before and after reaction. This suggests that SG is only slightly oxidized or modified by the enzyme. In fact, the reaction product exhibited no colour, so no oxidation to a yellowish o-quinone occurred. The resulting o-quinones usually undergo side reactions, either between one another or with other substances in their immediate vicinity, to form a variety of colored compounds (Friedman, 1996; Bittner, 2006; Parveen *et al.*, 2010). One possible explanation here is that the long hydrocarbon chain of the SG molecule may have prevented the phenol moiety from accessing the catalytic site of the enzyme, thereby inhibiting its oxidation through steric hindrance — possibly by effect of the above-discussed folding phenomenon. In fact, some authors have suggested that the rate of oxidation of gallates by the enzyme tyrosinase decreases with increasing alkyl chain length of the substrate (Kubo *et al.*, 2000). We observed similar effects with laccase here.

Enzymatic modification of GA, MG and EG caused their carbonyl peak to disappear from the spectrum. Longer gallates (PG, BG, OG and LG), exhibited the peak at the same frequency (1714 cm^{-1}), except in SG which has not been enzymatically-modified as discussed in the previous paragraph. Therefore, this enzyme may have induced similar chemical changes in PG, BG, OG and LG.

The peak at about 1300 cm^{-1} for the unmodified compounds may be due to vibration of the C–O bond in the ester. This peak was observed in the spectra for all unmodified gallates (Fig. 8-6a) but was absent from those for enzymatically treated GA, MG and EG (Fig. 8-6b). The peak may have been concealed by the broad signal in the spectra for PG, BG, LG and OG. With GA, MG and EG, this finding is consistent with the above-discussed disappearance of the carbonyl peak.

All pure gallates exhibited three well-defined peaks at the same vibrational frequencies in the range 1615–1450 cm^{-1} and corresponding to vibrations of C=C-C links in the aromatic ring (Fig. 8-6a). The central peak (1540–1520 cm^{-1}) disappeared upon reaction with the

enzyme (Fig. 8-6b) and thus reflected the changes undergone by the aromatic ring. SG retained the peak, but, as stated before, it underwent no enzymatic modification.

The peak at 720 cm^{-1} for pure OG, LG and SG (Fig. 8-6a) corresponds to C–C skeletal vibrations in the hydrocarbon chain. This peak was only detected in OG and longer gallates, and its strength increased with increasing chain length. Also, it remained unchanged and exhibited an identical trend upon enzymatic reaction of the gallates, which provides further evidence that the alkyl chain was not modified by the enzyme.

The many peaks observed for the pure compounds in the range 1225–950 cm^{-1} might correspond to C–H in-plane vibrations of the aromatic ring and those at 900–670 cm^{-1} to out-of-plane vibrations of the same bonds. These peaks were significantly altered by the enzymatic reaction —some even disappeared completely—, which suggests modification of the aromatic ring (Figs. 8-6a and 8-6b).

The peak at 1200 cm^{-1} for the unmodified (pure) gallates (Fig. 8-6a) might correspond to vibration of phenolic C–OH bonds since all compounds exhibited one. Enzymatic modification caused the peak to disappear from the spectra for some (GA, MG, EG) and may be masked by the broad band at higher frequencies (about 1225 cm^{-1}) for others (PG, BG, OG and LG) (Fig. 8-6b).

8.4. Conclusions

Based on the results of the water drop test (WDT) and water contact angles (WCA), the hydrophobicity of gallate-treated paper sheets (PS) is related to the length of the gallate alkyl chain. Thus, hydrophobicity increased with increasing chain length, peaked between octyl gallate (OG) and lauryl gallate (LG), and then decreased as chain length increased in longer gallates. A curing treatment substantially increased hydrophobicity irrespective of chain length. Also, no decrease was observed after hydrophobicity peaked with the gallates from OG to LG. The antioxidant capacity of treated PS increased gradually with increasing chain length of the gallate. Therefore, the presence of a long alkyl chain seems to be more important than possessing a large number of aromatic rings here. Antioxidant capacity levelled off in octyl gallate (OG) and was unaffected by a curing treatment. FTIR-ATR analysis of the functionalization solutions revealed that the alkyl chain of the gallates remained unaltered upon enzymatic modification. However, the enzyme caused major changes in the peaks for their phenolic moiety, which suggests opening of the ring and formation of carboxyl groups. Stretching vibrations in the carbonyl group and hydroxyl groups were considerably altered by effect of hydrogen-

bonding interactions in both pure and modified gallates. The three enzymes used provided similar results in all the experiments.

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Chapter 9

Elucidating the Chemical Nature of the Functionalization Solution

Abstract

The chemical processes involved in the production of the functionalization solution (FS) by reacting the enzyme laccase (Lacc) with the hydrophobic compound lauryl gallate (LG) in the presence of lignosulfonate (SL) were investigated. Cyclic voltrammetry (CV) was used to study the chemistry of several gallates and the effect of the lignosulfonates (SL) on the redox reactions involved. The intensity of the anodic peak in the voltammograms was found to decrease with increasing chain length of the gallate; also the electrochemical behaviour of LG differed from that of other gallates. The fact that the voltammetric curves for SL and LG intersected at a potential of 478 mV indicates an enhancing effect of SL on LG oxidation at high potentials (above 478 mV). The chemical structure of the resulting oxidized form of LG was elucidated by using Fourier transform infrared (FTIR) spectroscopy and a plausible oxidation mechanism developed from the results. Based on them, the hydrocarbon chain of LG remains unaltered after oxidation with the enzyme, the aromatic ring is broken to form acid products and the resulting products are strongly hydrogen-bonded. Grafting of enzyme-modified LG to cellulose is explained in the light of various mechanisms. Residual enzyme activity measurements confirmed the favourable role of SL in the treatments, and SEM images of paper samples treated with FS and control solutions (KFS) revealed the presence of LG particles attached to the surface of paper fibres.

9.1. Introduction

The use of chemical additives such as antioxidants, antimicrobials or antifungals to manufacture food packaging and medical materials has become increasingly important in recent times (Suppakul *et al.*, 2003; Elegir *et al.*, 2008; Gao and Cranston, 2008; Cusola *et al.*, 2013a; Raheem, 2013). Currently, a number of foods produced in one area are shipped to another for processing or distribution. Several months or years may elapse from the time the food is processed until it is consumed. In the medical domain, healthcare facilities are under increasing pressure to reduce instances of nosocomial infections; since most cellulosics are fluid-absorbent and in close contact with skin, conditions for bacterial growth are nearly ideal, so antimicrobial cellulosics can help reduce available transfer vectors for pathogens (Twomey, Beitz and Johnson, 2009). Gallic acid and its alkyl esters constitute an important group of biogenic molecules possessing a wide spectrum of interesting properties such as antibacterial (Silva *et al.*, 2011), antioxidant (Kubo *et al.*, 2010), antifungal (Hsu *et al.*, 2009) and antitumoral action (Serrano *et al.*, 1998; Ortega *et al.*, 2003). Since most of these properties are usually required specifications in the manufacturing of materials for packaging or medical uses, using alkyl gallates in their production is indeed warranted.

The interest in using biotechnological methods to obtain materials with improved properties has also grown substantially in recent years (Gübitz and Paulo, 2003; Aljawish *et al.*, 2012; Liu, Qin and Li, 2013). Using enzymes to produce advanced materials has some advantages over chemical methods including the ability to operate under milder conditions, the high specificity and efficiency of enzymes, and their easy control and environmental friendliness (Skals *et al.*, 2008; Kirk, Borchert and Fuglsang, 2002). Laccases (EC 1.10.3.2) are blue copper oxidases capable of oxidizing a variety of aromatic compounds by using oxygen as electron acceptor and giving water as a by-product. Laccases provide an eco-friendly approach to modifying cellulose and enzyme grafting improves the physical properties of cellulosic materials. The ability of laccases to graft low molecular weight phenolic compounds onto cellulose has been demonstrated by several authors in recent years (Aracri, Roncero and Vidal, 2011; Fillat *et al.*, 2012; Cadena *et al.*, 2011; Aracri *et al.*, 2010).

Alkyl gallates have gained interest lately for the laccase-catalysed grafting of such compounds onto cellulose surfaces with a view to developing new substrates with enhanced properties. For example, Gaffar *et al.*, (2010) studied the laccase-catalysed grafting of various gallates on wool to obtain multifunctional textile materials with antioxidant, antibacterial and water-repellent properties. Also, lauryl gallate (LG) was recently used to hydrophobize cellulose fibres (Garcia-Ubasart *et al.*, 2012), nanofibrillar

cellulose (Saastamoinen *et al.*, 2012) and the surface of paper sheets (Cusola *et al.*, 2013b, chapter 4).

Several studies have shown that phenolics attach to cellulose trough cellulose-bound lignin (Grönqvist *et al.*, 2006; Kudanga *et al.*, 2008; Aracri, Colom and Vidal, 2009; Suurnäkki *et al.*, 2010). This requires laccase-assisted activation of fibre surfaces followed by radical coupling. However, recent research (chapter 5) has exposed alternative routes for attaching phenolic compounds to cellulose-based materials; an enzyme-modified phenol is obtained that can be subsequently grafted without the need for enzymatic activation of the cellulosic material. There is, however, little knowledge about the nature of the chemical changes in alkyl gallates caused by laccase or the mechanisms by which the enzyme-modified molecules bond to cellulose surfaces.

In previous work (chapter 5), we successfully functionalized cellulosic materials by using enzyme-modified gallates in the presence of lignosulfonates (SL). In this work, we investigated the chemical changes undergone by the gallates on laccase oxidation and the mechanisms by which they are grafted onto cellulose. The study began by using cyclic voltammetry (CV) to examine the oxidation of alkyl gallates, lignosulfonates and their combinations by reaction with laccase. The choice of CV was dictated by the facts that laccases are enzymes undergoing oxidation processes and their effect can be simulated by using a working electrode to record, in a controlled manner, changes in the applied potential. Subsequent tests focused on lauryl gallate (LG) and involved using FTIR spectroscopy to investigate the chemical changes undergone by this compound upon enzymatic reaction. The results were used to establish various potential mechanisms for the grafting of enzyme-modified LG to cellulose. The role of lignosulfonate (SL) in the process was also identified by measuring the residual enzyme activity after several hours of enzymatic reaction. Finally, eucalyptus paper sheets were treated with enzyme-modified LG and controls for analysis by SEM.

9.2. Materials and methods

9.2.1. Paper, enzyme and chemicals

The enzyme used was laccase (Lacc) from *Trametes villosa* supplied by Novozymes® (Bagsvaerd, Denmark). Gallic acid (GA), ethyl gallate (EG), propyl gallate (PG), octyl gallate (OG) and lauryl gallate (LG) were purchased from Sigma–Aldrich®. Soluble sulphonated kraft lignin (SL) was obtained from Borreegard® and used as received. Eka WS 505 polyamidoamine–epichlorohydrin (PAAE) resin was supplied by Azko Nobel Chemicals®. Further details about these compounds are given in chapter 2. Eucalyptus

ECF pulp was supplied by ENCE® (Spain) and used to prepare handsheets on a Rapid-Köten lab former according to ISO 5269-2:2004. Prior to sheet formation, the pulp was treated with wet-strength PAAE resin at a dose of 0.7% of solids onto fibres (pH 7), and diluted to 10% (w/w) with water prior to application to the fiber suspension.

9.2.2. Functionalization solution (FS) preparation and application

The enzymatic reaction was performed in the absence of paper sheets and the resulting functionalization solution (FS) subsequently used to impregnate paper sheets by dipping. Enzymatic treatments were performed by using 250 mL beakers containing final concentrations equivalent to 50 mM sodium tartrate buffer (pH 4), 1.2 g/L LG, 1.2 g/L lignosulfonate (SL) and 1.2 U/mL laccase. In the enzymatic reaction, the LG was used as a colloid suspension obtained by sonication. The resulting, functionalization solution (FS) was applied to the surface of paper sheets by dipping and the sheets were allowed to dry in a normalized atmosphere prior to SEM analysis. Aliquots of FS were centrifuged at 6000 rpm for 90 min and the solid precipitate formed recovered for the FTIR analysis. Various control treatments involving a control functionalization solution (KFS) were also performed and their results compared with those for paper sheets subjected to no surface treatment (see Table 2-2). The enzymatic treatments are described in greater detail in chapter 2.

9.2.3. Cyclic voltammetry

Voltammetric tests were performed on a μAutolab Type III (EcoChemie®, The Netherlands) potentiostat/galvanostat controlled by Autolab GPES software version 4.9. Voltammetric responses were recorded in 50 mL of a 80/20 (v/v, %) water–ethanol mixture containing 0.1 M tartrate buffer at pH 4 and a 0.5 mM concentration of the target compound (gallate) in the presence or absence of lignosulfonate (0.17 mg/mL). The potential was scanned from 0 to 800 mV vs Ag/AgCl at a rate of either 5 or 200 mV/s.

9.2.4. FTIR analysis

For FTIR analysis of enzyme-modified LG, 200 mL of KFS consisting of LG+Lacc enzymatic product (see table 2-2) was prepared by using the above-described doses and reaction conditions. The resulting solid residue was finely ground and mixed with potassium bromide to obtain KBr pellets. Samples were measured over the wavenumber range 4000–400 cm^{-1} on a Nicolet 6700 FT-IR spectrometer governed by spectral software. The procedure is described in greater detail in chapter 2.

9.2.5. Residual enzyme activity

Residual laccase activity was determined by monitoring the oxidation of 2,2′-azino-bis(3-ethylbenzylthiazoline-6-sulfonate) (ABTS) in 100 mM sodium acetate buffer (pH 5) via spectral measurements at 436 nm. A molar extinction coefficient of 29,300 M^{-1} cm^{-1} at 25°C was used. Residual laccase activity values were corrected by the dilution factor and expressed as activity units per gram or millilitre. Measurements were made on a Thermo Scientific Evolution 600 spectrophotometer.

9.2.6. SEM analysis

Scanning electron microscopy (SEM) was used to examine the surface of paper sheets treated with the functionalization solution (FS) and control solutions (KFS). Micrographs were obtained with a JEOL JSM-6400 microscope operating at 5–10 kV under moderate vacuum. Specimens were previously cut into small pieces and coated with a very thin (12 nm) Au–Pd layer on an SCD005 sputter coater to obtain a conductive surface.

9.3. Results and discussion

9.3.1. Electrochemical interaction between lignosulfonate and gallates

The chemistry of gallates and the effect of lignosulfonates (SL) on the redox processes involved were studied by using cyclic voltammetry (CV). Although the oxidation of alkyl gallates by laccase had previously been in this manner (Gaffar Hossain *et al.*, 2010), no specific information about the effect of SL on the oxidation of gallates of increasing chain length with this technique had been reported. In this work, various gallates of increasing chain length including gallic acid (GA), ethyl gallate (EG), propyl gallate (PG), octyl gallate (OG) and lauryl gallate (LG) were combined with SL and examined by cyclic voltammetry.

Fig. 9-1 shows the cyclic voltammograms for the pure gallates as obtained at a scan rate of 5 mV/s. As can be seen from the figure, all gallates underwent irreversible oxidation; they exhibited a strong anodic peak and no cathodic peaks —which would have indicated reduction of oxidized compounds. Therefore, oxidized intermediates (phenoxy radicals) were rapidly removed by chemical reactions such as phenoxy radical coupling or the formation of quinones or open-ring acid products. As can be seen, the intensity of the anodic peak, which was related to the current intensity supplied by the electron flow in the oxidation process, decreased steadily with increasing chain length of the gallate. The reduction may have resulted from the high hydrophobicity and low solubility of

long-chain gallates and their tendency to aggregate hindering oxidation. One other reason might be steric hindrance in the longer gallates (OG and LG) preventing them from approaching the surface of the electrode and increasing the resistivity of the medium as a result.

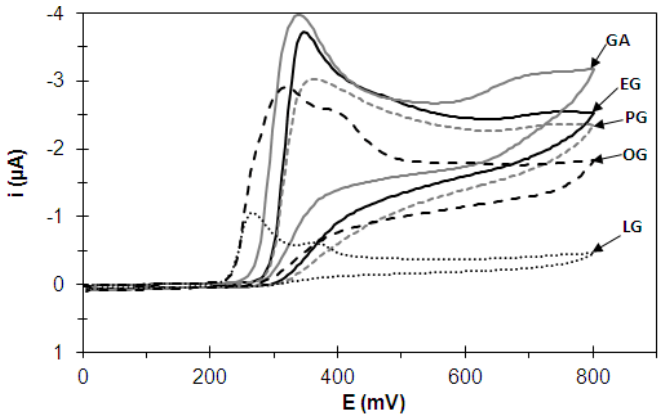

Figure 9-1 Cyclic voltammograms for 0.5 mM gallate solutions in a 80/20 (v/v) mixture of 0.1 M tartrate buffer and EtOH at pH 4 as obtained at a scan rate of 5 mV/s.

All studied gallates exhibited an anodic peak at 250–400 mV; however, after the decay of the initially oxidized species, all underwent further oxidation at higher potentials (above 600 mV). GA, EG and PG exhibited a sharp, well-defined anodic peak at 250–400 mV; however, formation of a second oxidized species at higher potentials was only observed in OG and LG. With these two gallates, the initial species were oxidized at 313 and 268 mV, respectively, and the second species at 396 and 361 mV. As can also be seen from Fig. 9-1, the first anodic peak was shifted to lower potentials as the length of the gallate chain was increased; this indicates that long-chain gallates are oxidized at lower potentials (but less strongly as suggested by their lower peaks). Although the cyclic voltammograms for the gallates in the presence of SL afforded no clear-cut conclusion about the effect of the gallate chain length on the anodic peak (results not shown), the intensity of the oxidation peak at higher potentials (750 mV) was found to follow the previous trend.

Additional voltammetric measurements of the studied gallates at higher scan rates (200 mV/s) were also made in order to ascertain whether more reversible processes could take place under such conditions and assess the stability of the resulting radicals. Based on the results, all studied gallates underwent irreversible oxidation, even at increased scan rates; this is indicative of poor stability in the oxidized species. However, OG and LG, which

exhibited peaks for two different species at 5 mV/s, gave a single anodic peak (indicating formation of a single species) at higher rates (results not shown).

Fig. 9-2 shows the cyclic voltammograms at slow scan rate (5 mV/s) for the target compounds in the absence and presence of SL. As can be seen, SL increased the anodic peaks for PG and LG. The oxidation current for SL was very weak; therefore, the lignosulphate was assumed to be electroinactive in the potential range of appearance of the anodic peaks for the gallates. For this reason, the increased anodic peak current for PG, OG and LG observed in the presence of SL was ascribed to regeneration of the gallate at the electrode surface and oxidation of SL. As noted earlier, the phenoxy radicals produced by oxidation of the gallates exhibited very low stability; however, the radicals were long-living enough to oxidize SL. This effect of SL on the anodic current falls into the homogeneous redox catalysis class of electrochemical reactions (Andrieux and Savéant, 1986) and was previously observed in studies on the effect of various types of lignin products in combination with commonly used laccase mediators (Aracri, Tzanov and Vidal, 2013; González Arzola, Arévalo and Falcón, 2009). The cyclic voltammogram for OG in the presence of SL exhibited a very slight increase in anodic current; by contrast, those for GA and EG in the presence of SL exhibited no increase, but rather a decrease. A decreased anodic current of GA in the presence of a ligninolytic compound was previously observed by Díaz-González, Vidal and Tzanov (2011).

The present voltammetric study was conducted on several representative gallate compounds with variably long chains in order to gain insight into the electrochemical behaviour of these compounds in the presence of lignosulfonates. However, the focus was placed on LG and its interaction with SL because previous studies had exposed a favourable effect of lignosulfonates on enzymatic treatments involving laccase and LG (Cusola *et al.*, 2013b, chapters 4 and 5). In such studies, the product of the oxidation of LG by laccase was anchored to the surface of cellulosic materials and the grafting reaction assumed to occur upon oxidation of LG; therefore, oxidation of the compound was essential in order to ensure grafting. Since introducing SL improved grafting, stability and particle size reduction, and also helped preserve the enzyme activity, we suspected that SL was acting as an enhancer for the oxidation of LG. As can be seen from the voltammetric curves of Figs. 9-1 and 9-2, the electrochemical behaviour of LG differed markedly from those of the other gallates; thus, the former gave lower currents —by effect, as noted earlier, of its low solubility. The electrochemical behaviour of LG in the presence of SL therefore warrants special attention. As can be seen from Fig. 9-2, the anodic currents for GA, EG, PG and OG, and that for SL, differed in magnitude; therefore, SL was assumed to be electroinactive for these gallates. However, based on the

curves for LG and SL in Fig. 9-2, the anodic currents were of a similar magnitude. The key observation to understand the effect of SL on LG is the fact that the curves for the two compounds intersected at a potential of 478 mV. Below 478 mV, the anodic current of LG fell above that of SL, but the opposite was observed at higher potentials. This finding allows one to understand the chemical redox interactions between LG and SL when combined (dashed curve). Thus, at potentials from 0 to 475 mV, SL is much less electroactive than LG and the main oxidation species at the electrode surface is provided by LG. The slightly increased anodic peaks for the LG+SL system relative to LG alone was a result of the above-mentioned regeneration of LG —to a small extent— at the electrode surface through oxidation of SL. Above 478 mV, the anodic current of SL surpassed that of LG; therefore, when combined (LG+SL), the increase in anodic current is due to the regeneration of SL and oxidation of LG. An increase in anodic charge at high potentials in the presence of lignin was previously observed by Aracri et. al., (2013) and suggested oxidation of non-phenolic lignin components. In this work, oxidation of such components would have induced oxidation of LG at high redox potentials. The overall effect on LG in the LG+LS system was as follows: at low potentials (0–478 mV), LG was oxidized mainly by the electrode —with slight regeneration caused by lignin—; at higher potentials (above 478 mV), the electrode was unable to oxidize the previously formed LG species, so its role was assumed by SL. The overall effect was that SL enhanced the oxidation of LG by acting specifically in those potential ranges were the electrode was less active and could not carry on with the oxidation of the LG (Fig. 9-3).

If the previous findings are extrapolated to laccase–LG interactions in the presence of SL, the role of the electrode in the voltammetric process is assumed by laccase. Unlike with the voltammetric electrode, the redox potential of the enzyme did not initially increase and then decreased steadily; rather, it remained constant at a given potential. Some authors have reported the redox potentials for laccases to be about 700 mV (Shleev *et al.*, 2004; Morozova *et al.*, 2007). Therefore, based on the voltammograms for LG in Fig. 9-2, the anodic current of SL at the working potential for laccases exceeded that of LG, so the oxidation mechanism of LG by laccase in the presence of SL must be similar to that described above involving enzyme-oxidized LG, whose action is enhanced by the presence of SL.

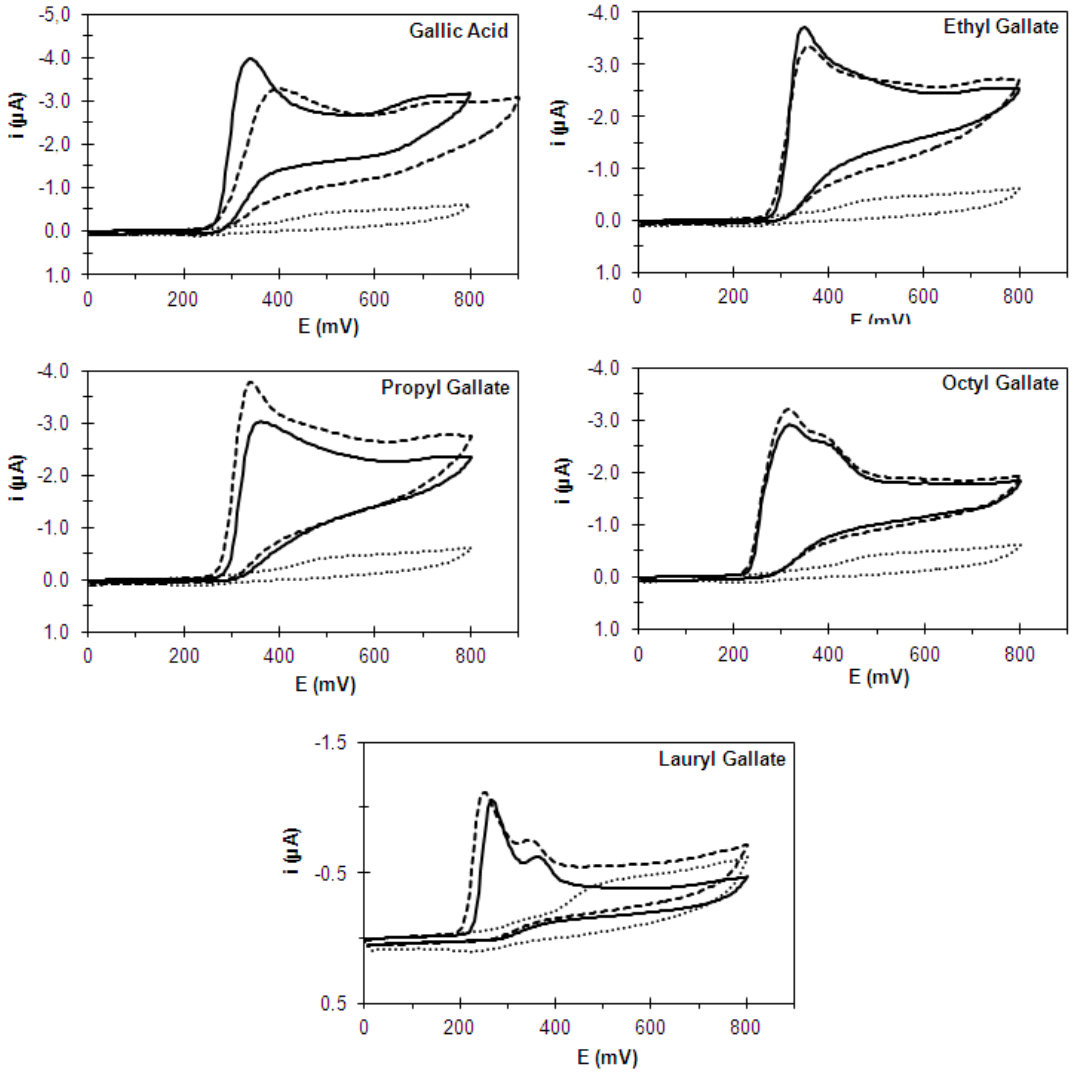

Figure 9-2 Electrode responses of 0.5 mM gallate solutions (solid lines —), a 0.169 mg/mL solution of SL (dotted lines •••), and gallates plus SL (dashed lines – –), all in tartrate buffer at pH 4 at room temperature, as obtained at a scan rate of 5 mV/s.

The electrochemical behaviour of the studied gallates, both alone and in combination with SL, was examined at a scan rate of 5 mV/s in two consecutive scans. All gallates and their combinations with SL exhibited an identical tendency in the first and second scans. Their behaviour is summarized by the example of Fig. 9-4a, which corresponds to LG+SL. The second scan revealed a marked decrease in oxidation current probably due to polymerization at the electrode surface of the species previously formed via phenoxy

radical coupling. Also, the anodic peaks in the second scan were shifted to the right, thus indicating that higher potentials were needed to oxidize LG owing to the presence of the previously generated polymer layer. Figure 9-4b compares the second scan curves for LG alone and in combination with the SL. As can be seen, the anodic peak for LG+SL was lower, which suggests that the presence of SL boosted radical coupling of LG oxidized species and favoured the formation of a thicker polymerized layer on the electrode surface as a result.

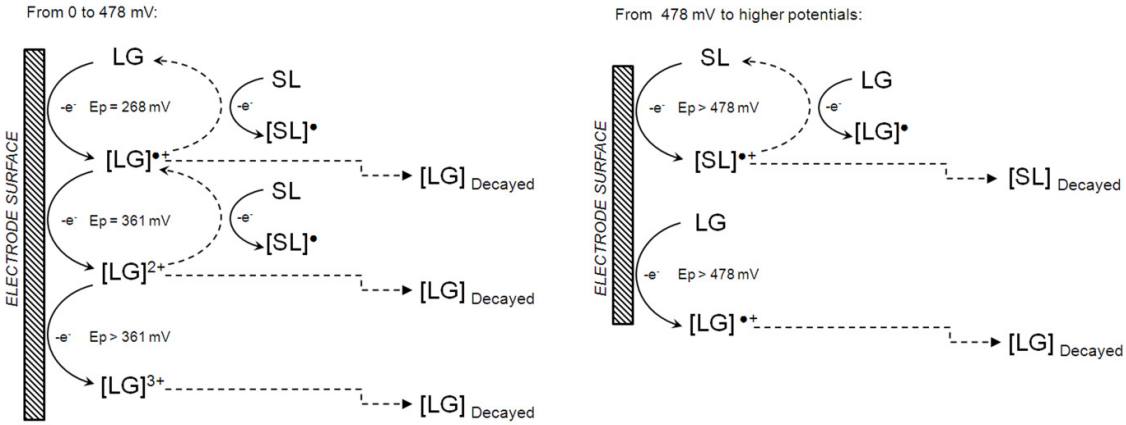

Figure 9-3 Schematic representation of redox catalysis for LG and SL.

Table 9-1 illustrates the effect of the gallate chain length on the redox potential in the presence of SL. The oxidation potentials for GA, EG and PG increased slightly with increasing chain length; by contrast, that for OG exhibited a substantial decrease that was even more pronounced in LG —about one order of magnitude. Previous studies examined catalytic efficiency (CE) as the increase in anodic peak current of the compound acting as catalyst in the presence of lignin or lignin model compounds. In such studies, lignin was assumed to be electroinactive and its oxidation to involve regeneration at the electrode surface of the catalyst (Aracri, Tzanov and Vidal, 2013; Bourbonnais, Leech and Paice, 1998). Table 9-1 shows CE for the studied gallates. Based on the cyclic voltammograms for GA, EG, PG and OG in the presence of SL (Fig. 9-2), SL was less electroactive than the gallates; this suggests that the gallate was the species acting as enhancer and SL the oxidized species (CE at 700 mV). By contrast, the main electroactive species in the LG+SL system at 700 mV was SL, which suggests that LG was oxidized through SL reduction at the electrode surface. The increase in anodic current upon

reduction of the enhancer at the electrode surface was calculated as ΔI/IE (table 9-1), ΔI being the increase in anodic current at 700 mV and IE the oxidation current of the enhancer at the same potential.

Table 9-1 Redox potential of the studied gallates and catalytic efficiency of GA, EG, PG and OG against SL, and of SL against LG. (Substrate-to-enhancer weight ratio 1:1).

Compound	Ep,a (mV)	CE (at 700 mV)
GA	337	-0.05
EG	342	0.06
PG	361	0.18
OG	317	0.04
LG	259	0.15*

* CE for LG was calculated by assuming SL to be the enhancer and LG the oxidized substrate.

As can be inferred from Table 9-1, GA was ineffective, EG and OG had a very slight positive effect, and PG exhibited a substantial positive effect on the oxidation of SL components at 700 mV. The effect of SL as enhancer of the oxidation of LG was significant (CE about 15%).

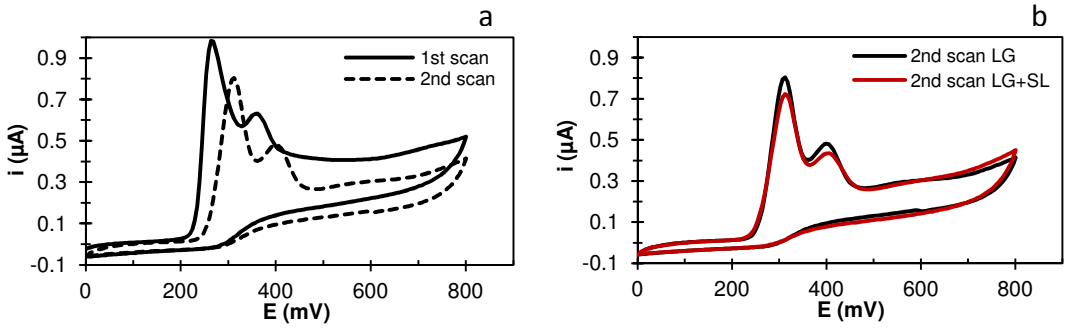

Figure 9-4 Two-scan cyclic voltammogram for 0.5 mM LG+SL in 80/20 (v/v) 0.1 M tartrate buffer–EtOH mixture at pH 4 as obtained at a scan rate of 5mV/s (a); and comparison of the second scans for LG alone and LG+SL at the same scan rate (b).

9.3.2. Enzyme-modified LG analysis by FTIR

Fig. 9-5 shows the IR absorbance spectra for LG before (LG) and after oxidation by the enzyme (LG+Lacc). The overall shape of the spectrum for LG changed upon enzymatic modification; thus, the initial spectrum contained a number of strong, well-defined peaks, whereas that for enzyme-treated LG was very smooth.

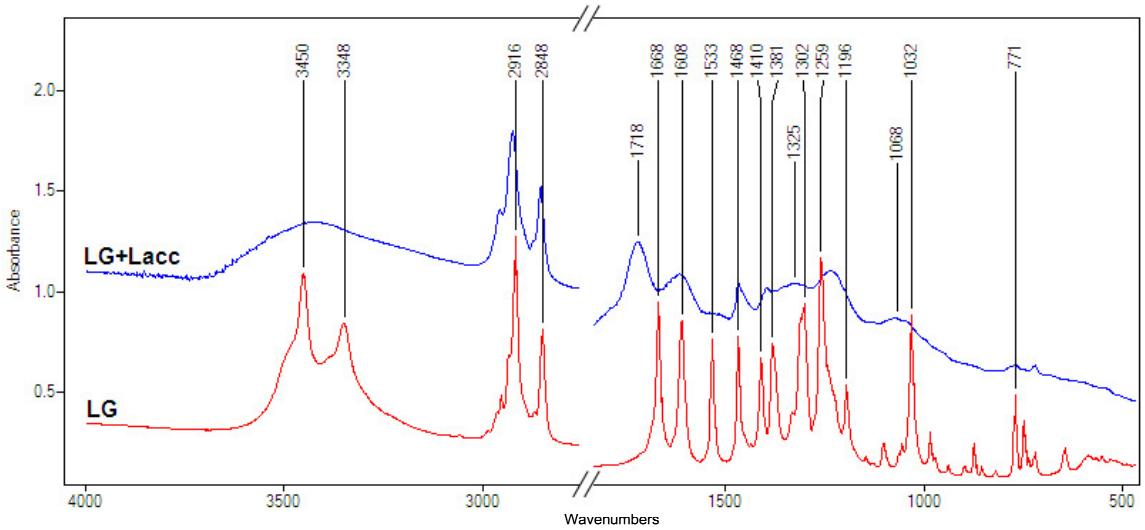

Figure 9-5 FTIR spectra for the LG before and after modification by the enzyme.

The peaks at 2848 and 2916 nm corresponded to methylene and methyl groups in the LG hydrocarbon chain. As can be seen, this zone in the IR spectrum remained unchanged upon enzymatic modification of LG, thus indicating that the hydrocarbon chain was probably not altered by the enzyme. The adsorption band at 1668 nm for unmodified LG must correspond to C=O stretching in the ester —which usually appears at higher frequencies in esters. The reason for the decreased frequency of this band may be due to the connection of the ester with the aromatic ring (an aryl ester) (Coates, 2000), and also hydrogen bonding interactions of the ester group with hydroxyl groups in the same molecule (intra-molecular interactions) or in others (inter-molecular interactions) (Coleman *et al.*, 1988) as illustrated in Fig. 9-6. Intra-molecular interactions should be independent of the gallate chain length; in fact, the C=O stretching band for the ester group in other gallates appeared at the expected frequencies. A longer chain may thus prevent inter-molecular interactions and promote intra-molecular interactions.

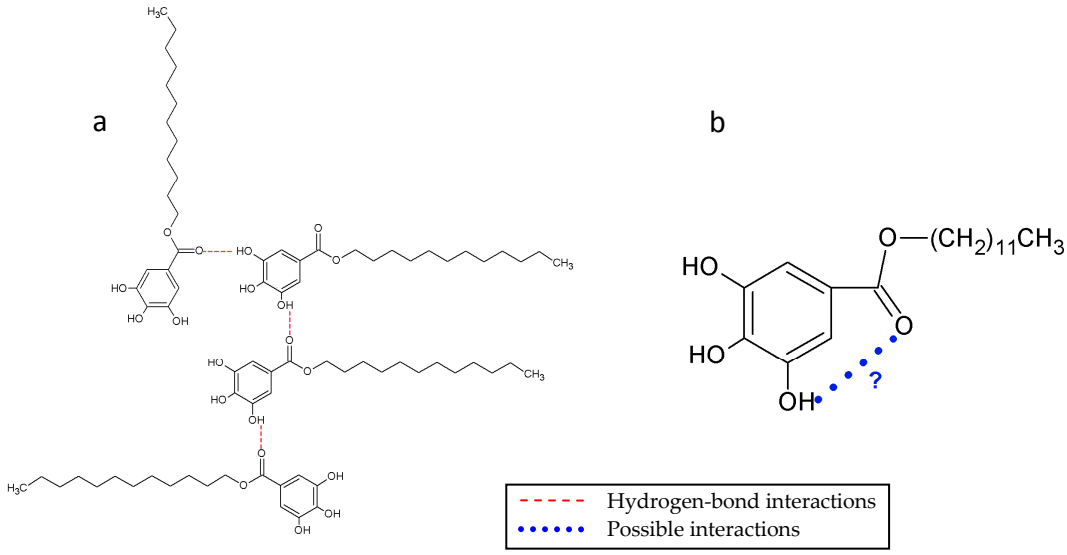

Figure 9-6 Hydrogen bonding of C=O in the ester group of various LG molecules (inter-molecular interactions) (a) and interactions within the same molecule (intra-molecular interactions) (b).

The carbonyl peak appeared at higher frequencies in the spectrum for enzyme-modified LG (1718 nm). Since the action of the enzyme focuses on the phenol (Widsten and Kandelbauer, 2008), the shift may be a result of some alteration of the aromatic ring such as cleavage and the formation of a carboxylic acid (Glasser, 1980), whose peaks usually appear in a very specific frequency range (1725–1700 nm). The ester peak did not disappear and, probably, the ester and carboxylic acid peaks merged at 1718 nm. Further evidence of opening/degradation of the phenolic ring was obtained from the LG peaks observed in the 1600-1450 nm range, which correspond to C=C–C aromatic ring bonds and were considerably different in the spectrum for enzyme-modified LG (specifically, the peak at 1533 nm disappeared altogether).

Stretching vibrations of the hydroxyl group in the initial LG molecule [step (1) in Fig. 9-7] reflected in a broad band in the range 3600–3300 nm containing peaks at 3450–3348 nm. There were no strong peaks for unbonded hydroxyl groups in the range 3645–3600 nm; rather, the broad band at 3600–3300 nm is suggestive of H-bonding of hydroxyl groups in the same (intra-molecular hydrogen bonding) or neighboring molecules (inter-molecular bonding). The reduction and further broadening of this band observed in enzyme-modified LG was also an indication of the disappearance of hydroxyl groups and formation of carboxyl groups; a highly characteristic large shift to lower frequencies by

effect of carboxyl groups establishing extremely strong hydrogen bonds (Fig. 9-8) to form stable dimers was observed.

The reaction mechanism for the oxidation of gallic acid proposed by Tulyathan et. al., (1989), in combination with our FTIR results, can be used to understand the nature of the interaction between Lacc and LG. Laccases exhibit specificity for phenols, diphenols, aryldiamines, aminophenols, benzenethiols and hydroxyindoles; this was confirmed by the FTIR results, which exposed major changes in spectral zones corresponding to the aromatic ring but none in those for the hydrocarbon chain. The LG oxidation mechanism proposed here involves the following steps (Fig. 9-7): (1) Laccase catalyses the oxidation of LG by removing electrons and hydrogen ions from phenolic hydroxyl groups to produce phenoxy radicals (Widsten and Kandelbauer, 2008) (2) which are further oxidized to quinones (3), thereby causing cleavage of the aromatic ring and producing hydrophilic carboxyl groups in the form of muconic acid derivatives (Gess and Dence, 1971; Österberg and Lindström, 2009) (4). The next step is pictured as consisting of alternative pathways that account for condensation of hydroxyl groups in the alcohol and carboxyl groups (–COOH) to form a lactone (Gierer and Imsgard, 1977) (5) or decarboxylation and further oxidation (6, 7). According to some authors, laccase–mediator systems can form activated oxygen species such as hydroxyl radical (HO•) and superoxide anion radical (O_2•–) (Guillén *et al.*, 2000). Such radicals can also boost oxidation of the LG product by breaking C–C bonds and causing cleavage of aromatic rings and scission of conjugated double bonds as a result (Gierer, 1997).

Formation of the dimer by regeneration of hydroquinones (after step 3) must be made difficult by steric hindrance from the long hydrocarbon chain of LG. As a result, the reaction with laccase produces LG molecules with open-ring acid products anchored to the alkyl chain via the original ester (Fig. 9-7). As hypothesized in previous work (chapters 5 and 6), the strong anionic character of such molecules must be the main agent enhancing retention on cationic surfaces.

The dark colour of the Lacc–LG reaction products can be ascribed to the formation of quinone structures as in step (3) of Fig. 9-7. However, such colour decreased as the reaction developed, which suggests that further oxidation increased the likelihood of obtaining increasing amounts of the colourless molecule formed in step (7) of Fig. 9-7 and hence a lighter coloured reaction product.

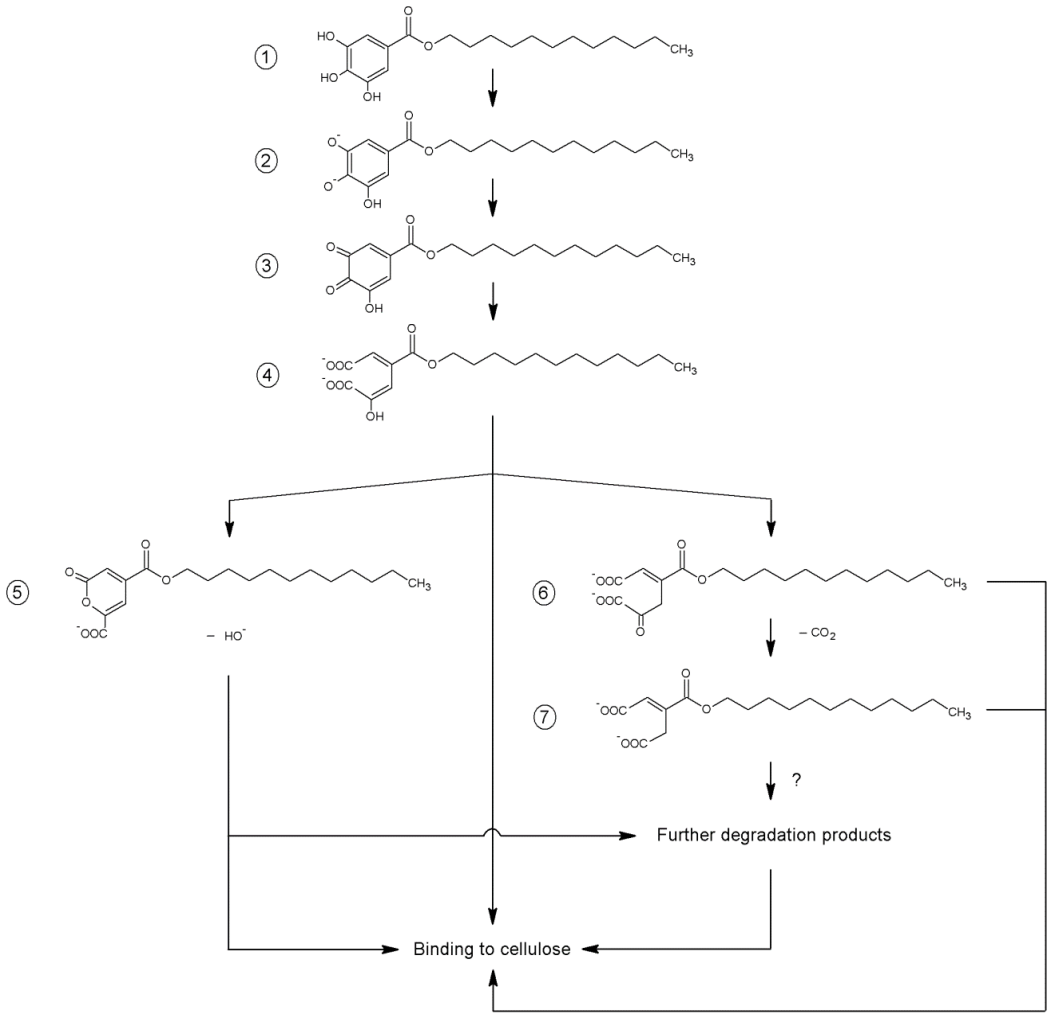

Figure 9-7 Proposed oxidation mechanism for the LG

The formation of LG polymerized species such as dimers or trimers was limited owing to the above-described potential steric hindrance and confirmed by the decreased turbidity of the reaction medium containing the enzyme-treated LG aqueous product. Dynamic light scattering and turbidity measurements showed particle size in LG to be decreased by effect of the enzyme (viz. by its reducing the turbidity of the reaction product as assessed with a Turbiscan®) (chapter 6); such an effect was present after long treatment — as long as some enzymatic activity remained. These results suggest clarification of the solution by effect of particle size reduction and quinone destruction, as well as the formation of none of the above-mentioned condensation adducts.

As confirmed by the IR data, the results species (Fig. 9-7) can establish hydrogen bonds trough the carboxyl groups formed (Fig. 9-8).

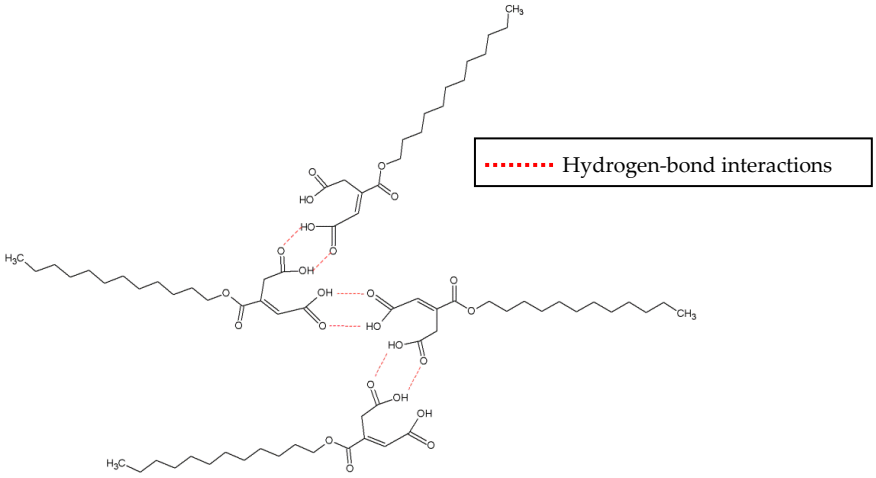

Figure 9-8 Hydrogen bonding interactions between enzyme-modified LG molecules.

9.3.3. Bonding of enzyme-modified LG to cellulose

Previous studies revealed that interactions between enzyme-modified LG and cellulosic materials increase cellulose hydrophobization (Cusola *et al.*, 2013b, chapters 4, 5 and 6). The enzyme-modified LG molecule possesses a hydrophilic head consisting of the acid products formed and a hydrophobic tail containing the alkyl chain. Because open-ring carboxyl groups (Fig. 9-7) confer enzyme-modified LG a strong anionic character, they must have been the main agent facilitating stronger retention onto cationized cellulosic material —and increasing hydrophobicity— as found in previous work (chapters 5 and 6).

Based on previous considerations of Gess (1995) on paper sizing, effective hydrophobization of cellulosic materials with LG requires (a) the hydrophilic head of LG to be oriented towards fibres and the hydrophobic tail outwards; (b) LG to evenly distribute throughout the material; and (c) the spacing between LG molecules on fibres to allow water to be efficiently repelled. Since the most likely scenario for enzyme-modified LG is one where all the molecules in Fig. 9-7 coexist, we propose several mechanisms for the grafting or functionalization of enzymatically treated LG to cellulosic materials.

8.3.3.1. Mechanism 1. Esterification reactions

The oxidized form of LG obtained in step (7) of Fig. 9-7 would react with cellulose and water by esterification as depicted in Fig. 9-9. Under drying conditions, an ester can be formed between carboxyl groups in LG and hydroxyl groups in cellulose surfaces. Oxidized LG could even form a highly reactive cyclic anhydride (Fig. 9-9) which would facilitate grafting without heat treatment via a reaction mechanism similar to that for alkenyl succinic anhydrides (ASAs) used as sizing agents for cellulosic materials (Roberts, 1991; Eklund and Lindström, 1991). Minimizing hydrolysis of the enzyme-modified LG molecule to promote the grafting reactions entails efficiently removing water in the medium by drying. Oxidized LG moieties may attach to cellulose surfaces via Fischer's esterification reaction between hydroxyl surface groups in cellulose and carboxyl groups in enzyme-modified LG (Fig. 9-9). In this mechanism, the sodium tartrate buffer used in the enzymatic reaction, which derives from tartaric acid, would act as a strong acid catalyst for the reaction by promoting protonation of carboxyl groups (a reversible reaction); the composition of the reaction mixture or equilibrium position would be thermodynamically governed. Obtaining the ester in good yields would require efficient removal of water by drying. In fact, the presence or addition of water here is of some concern as the water can shift the reaction equilibrium away from the ester formation and towards the carboxylic acid.

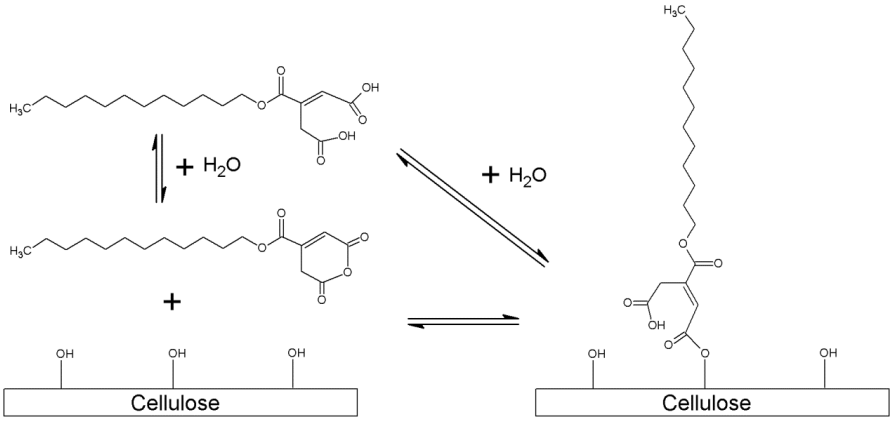

Figure 9-9 Attachment of enzyme-modified LG moieties to the surface of cellulose via Fischer's esterification reaction and drying.

By effect of the esterification reaction, the hydrophobic tail of LG would adopt an appropriate orientation —which, as noted earlier, is a prerequisite for becoming hydrophobic. A previous study (Cusola *et al.*, 2013b) revealed that heat treatments

increase hydrophobicity; this effect can be ascribed to the removal of water, which is therefore essential to promote the esterification reactions.

8.3.3.2. Mechanism 2. Physicochemical attraction

As found in previous chapters (chapters 5 and 6), significantly increased hydrophobicity in cellulosic materials was obtained by increasing their cationic character with compounds such as polyamidoamine–epichlorohydrin (PAAE), cationic starch (CSt) or methyl glycol chitosan (MGCh) before treatment with enzyme-modified LG. The strong anionic character of enzyme-modified LG conferred by its carboxyl groups would facilitate interaction with cationic groups in cellulose and hence its functionalization (see Fig. 9-10).

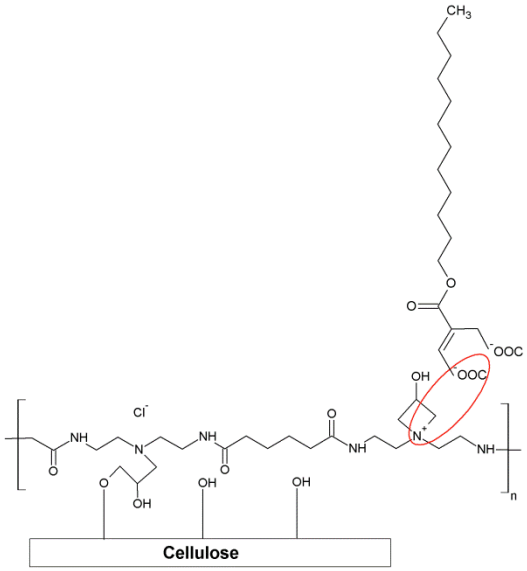

Figure 9-10 Physico-chemical interaction between PAAE-treated cellulose and an enzyme-modified LG moiety.

8.3.3.3. Mechanism 3. Grafting by ring opening polymerization

Ring opening polymerization (ROP) is an effective technique for polymerizing cyclic monomers such as lactones and other strained cyclic monomers such as lactide. A wide variety of aliphatic and cyclic monomers including esters (lactones), amines, sulphides, olefins and cyclotriphosphazenes have been successfully polymerized by ROP. The ROP mechanism involves a monomer, an initiator and a catalytic system. Since an alcohol (or hydroxyl group) is generally used as ROP initiator, cellulose in its native form acts as a

multifunctional trigger for the polymer modification of cellulose or cellulose derivatives (Carlmark, Larsson and Malmström, 2012).

The cyclic product obtained in step (5) of Fig. 9-7 is a lactone that can be grafted to cellulose via the ROP mechanism as shown with ε-caprolactones and lactic acids elsewhere (Teramoto *et al.*, 2004; Lönnberg *et al.*, 2008; Lönnberg *et al.*, 2006). The ROP technique uses a metal catalyst (usually stannous 2-ethylhexanoate, SnOct2); this makes the resulting cellulose products useless for biomedical materials and food packaging purposed, and has promoted the use of alternative, non-toxic organocatalysts since 2001 (Najemi *et al.*, 2010; Thomas, Peruch and Bibal, 2012; Carlsson *et al.*, 2012). For example, Hafrén and Córdova (Hafrén and Córdova, 2005) developed a method for the surface-initiated ROP of ε-caprolactone from a cellulose substrate using tartaric acid as catalyst for grafting. It should be noted that the buffer solution used to obtain enzyme-modified LG was prepared from tartaric acid (Cusola *et al.*, 2013b), which may allow the enzyme-modified LG lactone formed in (5) of Fig. 9-7 to be grafted via ROP onto the cellulosic material under heating conditions. Heating is in fact required for efficient ROP of lactones onto cellulosic materials; as shown in previous work (Cusola *et al.*, 2013b), heat treatments boost grafting of enzyme-modified LG onto cellulose. Figure 9-11 depicts the proposed mechanism for the ROP grafting of the enzyme-modified LG lactone.

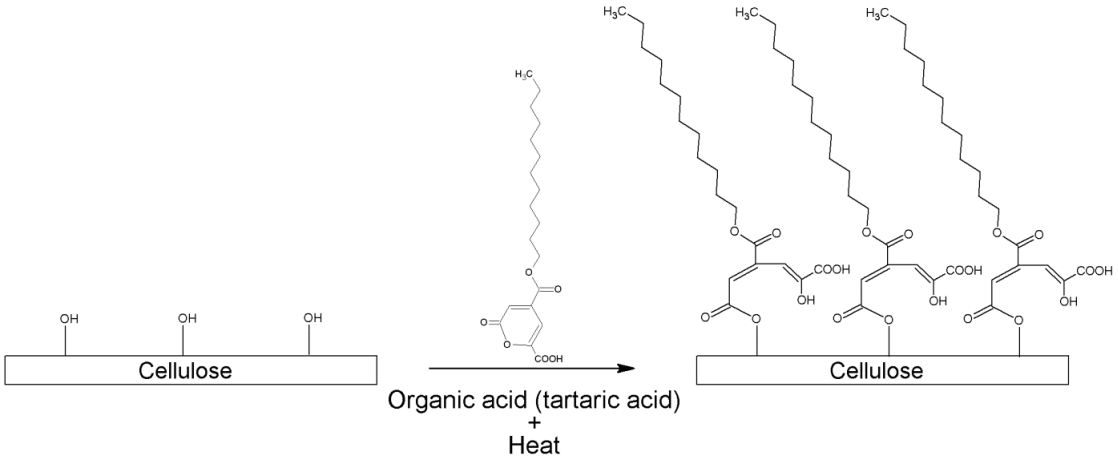

Figure 9-11 Tartaric acid-catalyzed ROP of the enzyme-modified LG lactone from cellulose fibers.

8.3.3.4. Mechanism 4. Physicochemical attraction + Esterification or ROP

Although the above-described physico–chemical interaction resulting from electrochemical attraction between compounds may appear to be weak, the system can be

further reacted to achieve grafting (e.g. by Fischer esterification or ROP). A combined mechanism is also possible, however. Thus, functionalization would occur in two steps, namely: (1) electrochemical attraction, which would increase retention/attraction of LG moieties on the surface of the cellulosic material; and (2) subsequent esterification or ROP. The bonding strength between cellulose and enzyme-modified LG was assessed in previous work and found to be quite high. Even if grafting occurs by covalent bonding or electrochemical interaction, the bonds remain very strong even after Soxhlet extraction with acetone and washing with hot water (chapter 4).

9.3.4. Residual enzyme activity and role of the SL

The residual activity of laccase in the FS and control treatments (KFS) was measured after 3, 21 and 52 h reaction. The original enzyme activity (before treatment) was about 588 U/mL; as can be seen in Fig. 9-12, it decreased slightly with time in all solutions.

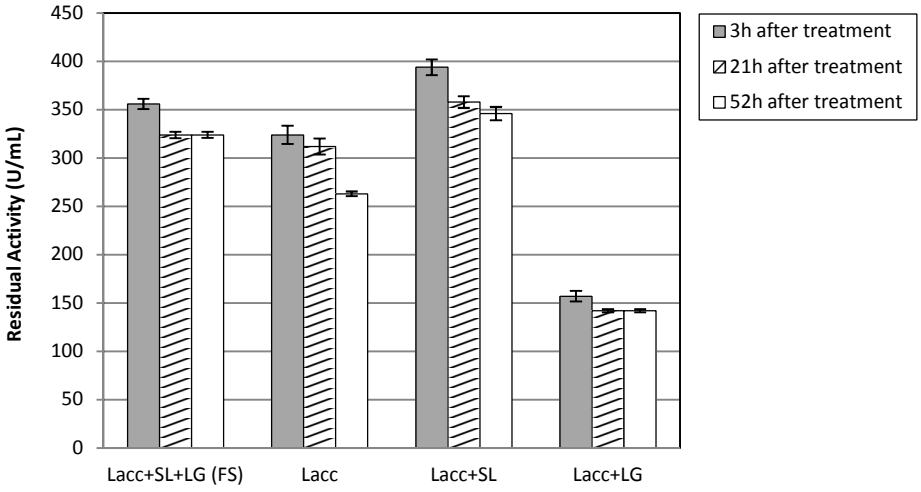

Figure 9-12 Residual enzyme activity in FS and control solutions after 3, 21 and 52h reaction.

The FS (Lacc+SL+LG) and Lacc+SL treatments retained the residual enzymatic activity at similar levels as the ones of pure laccase (Lacc), but the Lacc+LG control treatment reduced it considerably after 3, 21 and 52 h reaction. These results testify to the prominent role of SL in maintaining the enzyme activity or the oxidative ability of SL-containing products. Even the enzyme control (Lacc) reduced the activity after 52 h, the reduction being less pronounced with the SL-containing controls (FS and Lacc+SL).

Considering the oxidation of the LG compound catalyzed by the SL at high redox potentials (higher than 478 mV) proposed in the voltammetric study, there's also the possibility that in the residual activity measurements the enzyme-oxidized SL species may have boosted oxidation of ABTS. This phenomenon would have led to spurious measurements of enzyme activity by effect of ABTS oxidation being partly the result of reduction of previously formed SL radicals rather than the enzyme itself.

9.3.5. SEM analysis of cellulosic material treated with FS and controls

The surface of cellulose and FS-treated cellulose specimens was examined by scanning electron microscopy (SEM). Figure 9-13 shows scanning electron micrographs of eucalyptus ECF sheets before and after treatment with FS. The images on the left are macroscopic views of the paper specimens (2000×) and those on the right close views of the surface of a single fibre (17,000× and 20,000×). Figures 9-13a and 9-13b show the bare paper without treatment, and Figs. 9-13c and 9-13d PAAE-treated paper. There was no significant difference in fibre morphology and no deposition of material onto fibre surfaces by effect of the PAAE treatment. Figures 9-13b and 9-13d show smooth, clean fibre surfaces; also, there is no indication of a potential effect of PAAE resin. By contrast, treatment with FS (Figs. 9-13e and 9-13f) caused appreciable morphological changes in the fibres. Thus, FS caused small (nanosized) particles to appear on the paper surface. Such particles consisted of enzyme-modified LG and were responsible for the hydrophobicity of the paper sheets. The particles are barely distinguishable in the macroscopic image of FS-treated paper (Fig. 9-13e) —and only a few of the larger ones can clearly be seen—, but apparent in the magnified view of the surface in Fig. 9-13f. Worth special note is the wide range of LG particle sizes present in the image (aggregates from 100 to more than 500 nm in size), and so is the fact that gold-sputtering the fibre surfaces —a requirement for SEM analysis of non-conductive materials such as cellulose— coated the sample with a thin (10–30 nm) layer of gold which may have completely covered small LG particles and hidden them from view.

Since FS was an enzymatic product obtained by reacting the enzyme laccase (Lacc) with the hydrophobic compound lauryl gallate (LG) in the presence of a lignosulfonate (SL), PAAE-treated sheets were also impregnated with several control treatments to elucidate the effect of each individual compound on paper fibres after impregnation (Fig. 9-14). Figure 9-14a shows the surface of fibres treated with the Lacc control; the surface was smooth and clean, which suggests that the control treatment did not alter fibre morphology. However, as can be seen from Figs. 9-14b and 9-14c, the treatments using the LG and Lacc+LG controls caused deposition of LG particles onto fibre surfaces.

Although the LG and LG+Lacc treatments had the same visual effect on the samples, a significant difference between them in terms of attachment of LG particles was apparent. Thus, the specimen treated with the LG control consisted of LG-deposited particles whereas that treated with Lacc+LG contained firmly attached particles on the fibre surface, consistent with the results of previous work (chapter 6). Unlike the surface particles produced by the Lacc+LG treatment, those resulting from the LG treatment were easily removed by washing with water. The effects of the enzyme were thus quite clear: altering the chemical nature of LG and facilitating its anchoring onto fibre surfaces.

The difference between FS and the Lacc+LG control was the presence of lignosulfonate (SL) during the enzymatic reaction. SL was introduced in the reaction because it had some favourable effects in terms of LG dispersion, enzyme stability and hydrophobicity (Cusola *et al.*, 2013b) (chapters 4, 5 and 6). The SL and Lacc+SL controls caused no morphological changes in the fibres (Figs. 9-14d and 9-14e). As a result, the SEM images of the FS-treated (Fig. 9-13f) and (Lacc+LG)-treated fibres (Fig. 9-14c) exhibited no appreciable differences; in both, fibre surfaces contained attached LG particles.

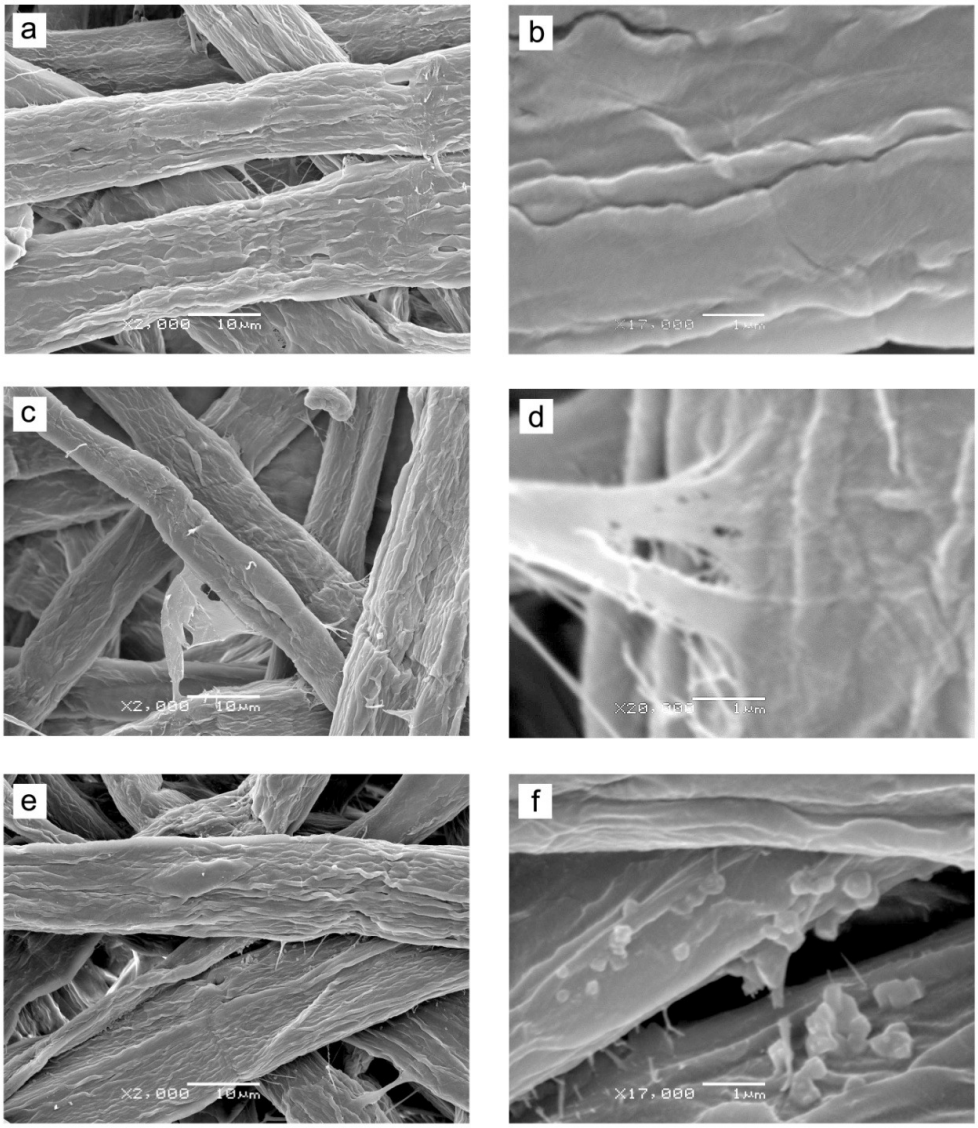

Figure 9-13 SEM images of untreated eucalyptus sheets (a: 2000x, and b: 17000x), eucalyptus sheets treated with PAAE (c: 2000x, and d: 20000x) and PAAE-treated sheets impregnated with FS (e: 2000x, and f: 17000x).

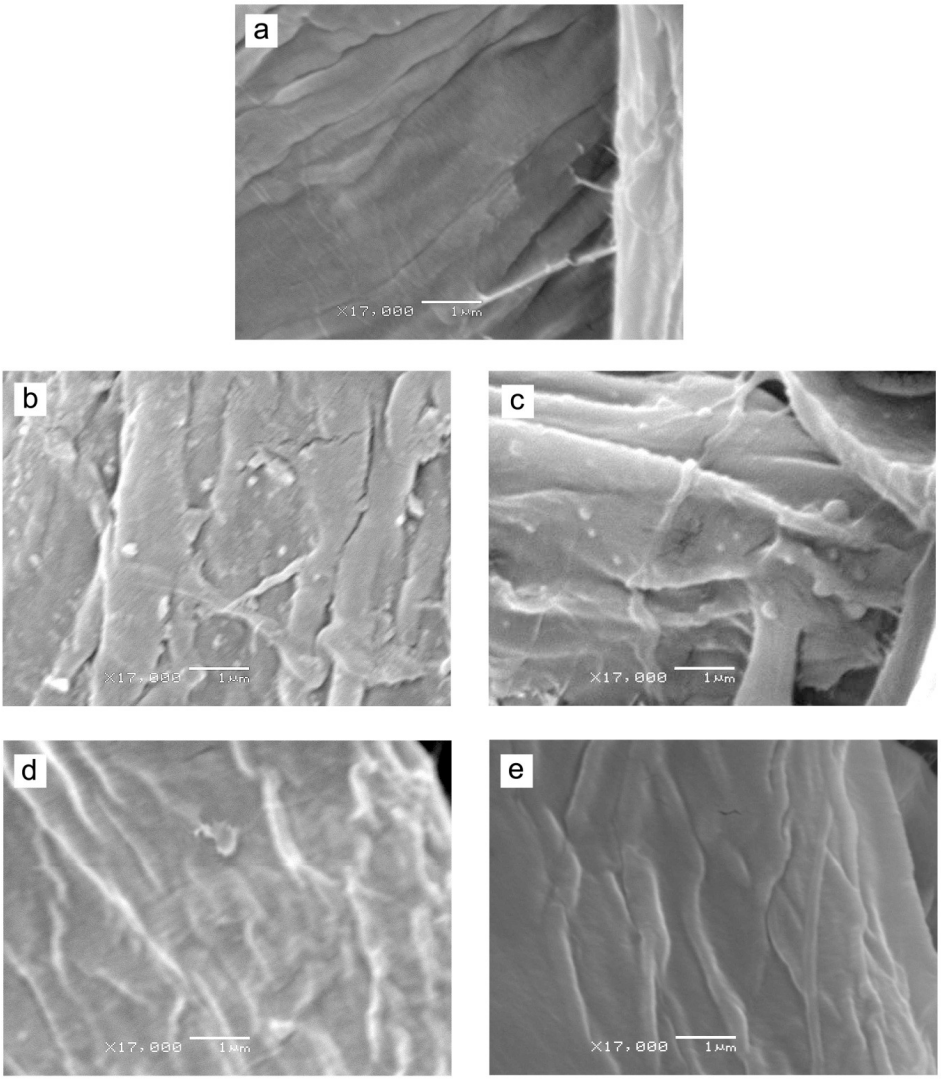

Figure 9-14 SEM images of PAAE-treated eucalyptus sheets impregnated with various control treatment solutions (KFS) as viewed at 17000x. Key: Lacc (a); LG (b); LG+Lacc (c); SL (d); SL+Lacc (e).

9.4. Conclusions

Chemical oxidation of GA, EG, PG, OG and LG by cyclic voltammetry exposed a well-defined relationship between the intensity of the anodic peak and the chain length of the gallate. The cyclic voltammograms for GA, EG, PG and OG in the presence of SL revealed that SL is less electroactive than the gallate; therefore, the gallate is the species acting as enhancer and SL is the oxidized species (CE at 700 mV). However, the main electroactive species in the LG+SL system at 700 mV was SL, which suggests that LG was oxidized through SL reduction at the electrode surface. The fact that the voltammetric curves for SL and LG intersected at 478 mV suggests that SL boosts the oxidation of LG at high potentials (above 478 mV). Chemically, enzyme-modified LG consists mainly of open-ring acid products that can be oxidized via various pathways; by contrast, the hydrocarbon chain of LG remains unaltered; this was confirmed by FTIR analysis, which also revealed strong hydrogen bonding in the resulting molecules. A plausible oxidation mechanism was developed, and several pathways for the grafting or functionalization of enzymatically treated LG to cellulosic materials were proposed. Residual enzyme activity measurements confirmed the positive role of SL in the treatments; thus, SL helped preserve the enzyme activity, which confirms the hypothesis based on the CV measurements that the enzyme-oxidized SL species enhances oxidation of LG —or ABTS in residual activity measurements. The SEM images of paper specimens treated with FS and controls (KFS) revealed the presence of LG particles attached to fibre surfaces.

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Chapter 10

General Discussion

Resum

En aquest capítol es realitza una discussió general dels resultats obtinguts en la present tesi. En un primer estudi s'analitza l'ús de les ciclodextrines per a la funcionalització no-enzimàtica de papers i teixits, obtenint suports capaços d'alliberar principis actius. Seguidament es posa de manifest la viabilitat de la funcionalització enzimàtica superficial de suports cel·lulòsics base paper, fent èmfasi en el desenvolupament de les propietats hidrofòbica i antioxidant. Es realitza l'optimització del procés superficial mitjançant la introducció de lignosulfonats i ultrasons. Seguidament, s'estudien els fenòmens fisicoquímics que tenen lloc en la funcionalització mitjançant diverses tècniques d'anàlisi. A més a més, es profunditza en l'estudi de l'alteració química soferta pel lauril gal·lat (LG) i catalitzada per la lacasa, i en l'estudi dels mecanismes químics de reacció del lauril gal·lat oxidat amb la cel·lulosa. Finalment, es realitzen una sèrie d'estimacions de tipus econòmic per al tractament de suports base paper mitjançant el producte funcionalitzador (FS) i es du a terme una prova en planta pilot per a l'aplicació del producte desenvolupat.

10.1. Introducció

La present tesi centra l'atenció en els processos per a l'obtenció de suports cel·lulòsics amb propietats avançades. Aquest tipus de materials tenen aplicacions molt diverses en els camps del packaging, mèdic, comunicació i higiene personal. El terme "suport cel·lulòsic" engloba materials en base paper, tèxtil, i no-teixits. En termes paperers, aquells materials que presenten propietats avançades s'anomenen papers especials o "specialty papers". Primerament es posa de manifest un novedós mètode no-enzimàtic de funcionalització per, a posteriori, centrar tota l'atenció en els processos de funconalització utilitzant enzims. Concretament, la present tesi es centra en cinc aspectes: **1)** La funcionalització no-enzimàtica de suports cel·lulòsics mitjançant ciclodextrines, **2)** l'obtenció d'un procediment per a la funcionalització enzimàtica superficial de suports cel·lulòsics, **3)** l'optimització d'aquest procediment per fer-lo més viable de cara a la implementació industrial, **4)** l'extensió del procediment per al desenvolupament de la propietat antioxidant, i **5)** la caracterització fisicoquímica del "grafting" i dels canvis soferts pels compostos degut a l'acció de l'enzim. En aquest capítol es realitza una síntesi i una discussió general dels resultats, i es realitza una estimació del cost de la tècnica de funcionalització.

10.2. Funcionalització no-enzimàtica de suports cel·lulòsics mitjançant ciclodextrines

La funcionalització de suports cel·lulòsics consisteix en dotar aquests materials de propietats avançades, que tradicionalment no se'ls hi adscriuen, i que permeten revaloritzar-los proporcionant-los un cert valor afegit. Generalment, aquesta funcionalització s'aconsegueix a través de l'alteració química de la cel·lulosa, acoblant grups químics funcionals en la seva superfície mitjançant tècniques d'empelt o "grafting", ja sigui a partir de mètodes físics (làser, acció mecànica...) o químics (reaccions d'empelt, tècniques electrolítiques...). Així doncs, en un primer estudi es va optar per un procés de funcionalització no-enzimàtic utilitzant unes molècules cícliques anomenades ciclodextrines. Les ciclodextrines (CDs) presenten l'habilitat de poder formar complexes del tipus hoste-convidat i poder així encapsular compostos químics en el seu interior gràcies a interaccions del tipus hidrofòbica-hidrofílica (Dodziuk, 2008; Bender i Komiyama, 1978). Les ciclodextrines s'han utilitzat amb èxit per a la funcionalització de membranes de polifluorur de vinilidè, teixits, no-teixits, hidrogels, polièster, entre altres, però la seva utilització per a la funcionalització de suports cel·lulòsics acabats (comercials) de base paper no ha estat reportada fins el moment. Així doncs, el capítol 3 centra l'atenció en la modificació de suports cel·lulòsics comercials (2 base paper i un

tèxtil) mitjançant ciclodextrines, amb la intenció de carregar-les amb els compostos químics necessaris per a que puguin alliberar un principi actiu (Fig. 10-1), obtenint d'aquesta manera suports amb propietats bactericides que podrien ser utilitzats en l'àmbit mèdic.

10.2.1. Fixació de les ciclodextrines

El primer aspecte a tenir en compte ha estat la reacció d'empelt de les ciclodextrines en els diferents suports cel·lulòsics mitjançant una reacció de poliesterificació a través d'un àcid policarboxílic (Martel *et al.*, 2002). Aquesta reacció té lloc a elevada temperatura entre l'àcid i les funcions –OH de les ciclodextrines i la cel·lulosa, per tant, en primer lloc s'han realitzat una sèrie de cinètiques temps-temperatura per a cadascun dels suports amb la finalitat d'optimitzar les condicions de reacció per aconseguir fixar el màxim de molècules de ciclodextrina a la superfície del material cel·lulòsic. Les cinètiques es van realitzar en els intervals de temps entre 5 i 45 min i temperatures entre 130 i 160°C per a cadascun dels suports considerats. Els resultats de les cinètiques temps-temperatura per a l'empelt de les ciclodextrines en els diferents suports (capítol 3), mostren que, per al suport consistent en un paper no-estucat, el màxim valor en l'augment de pes del suport degut a la fixació de les ciclodextrines es produeix a partir dels 30 minuts utilitzant temperatures superiors als 150°C; per tant, com que és preferible utilitzar les condicions de procés més suaus que impliquin el menor temps possible, es selecciona un temps de 30 minuts a 150°C com a paràmetres òptims de reacció per al paper no-estucat. Utilitzant aquesta mateixa temperatura, s'han dut a terme les cinètiques corresponents als altres suports (paper crepat i vena mèdica), corroborant que el temps òptim de reacció era 30 minuts. Pel que fa al guany de massa degut a la fixació de les ciclodextrines, l'augment va ser del 13% per al paper no-estucat, 9% per a la vena, i 8% per al paper crepat. Per tant, s'arriba a la conclusió que, utilitzant les mateixes condicions de reacció la morfologia del suport és un paràmetre clau en el nombre de molècules de ciclodextrina que es poden fixar en un suport determinat.

Es poden relacionar les propietats físiques d'un suport amb la capacitat d'aquest per a ser funcionalitzat amb èxit mitjançant aquesta tècnica?

Donat que la deposició de les ciclodextrines previ a les reaccions d'esterificació (i empelt) s'ha realitzat mitjançant la impregnació dels suports en una solució aquosa que contenia CDs, àcid cítric i catalitzador ("pad-dry-cure finishing"), s'ha relacionat l'augment de pes amb el valor de $Cobb_{60}$ dels suports. El $Cobb_{60}$ és un paràmetre que indica la capacitat d'un suport determinat per a retenir aigua; intuïtivament s'ha considerat la hipòtesi consistent en que com més alt el valor de $Cobb_{60}$, més elevada la possibilitat de provocar

l'empelt d'un major nombre de molècules de CD i, per tant, més elevat l'augment de massa. Així doncs, els assajos experimentals han confirmat que existeix una relació entre la capacitat d'un suport per a retenir aigua i la fixació d'una quantitat més gran de CDs.

Es pot corroborar que realment es produeix una reacció d'esterificació?

La utilització de la microscopia SEM ha permès visualitzar els canvis en la morfologia dels suports deguts al procés de funcionalització. Tot i així, aquesta tècnica no permet esbrinar quin tipus de reacció té lloc durant la funcionalització.

Els assajos FTIR dels suports funcionalitzats i sense funcionalitzar han proporcionat evidències de l'empelt de les CDs en els suports. La principal evidència ha consistit en comparar el pic a 1726 cm^{-1} corresponent al doble enllaç C=O que presenten les mostres funcionalitzades; l'esmentat doble enllaç apareix com a conseqüència de la reacció d'esterificació, i no està present en els suports no-funcionalitzats. Tots els suports funcionalitzats presenten un pic visible a 1726 cm^{-1}, a diferència dels suports no-funcionalitzats.

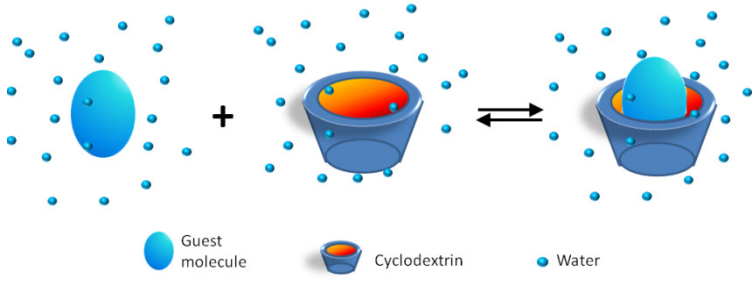


Figura 10-1 Fenomen d'inclusió d'una molècula invitada a l'interior de la ciclodextrina en un medi aquós.

10.2.2. Encapsulació del principi actiu

Un cop aconseguida la fixació de les molècules de CD en la cel·lulosa, el següent pas ha consistit en carregar les CDs amb un principi actiu (Fig. 10-1). Existeixen gran varietat de molècules amb propietats bactericides susceptibles de ser encapsulades en el sí de les ciclodextrines: povidona iodada, alcohol etílic, hipoclorit sòdic, permanganat de potassi, hexamidina, entre altres. No obstant, cal que les dimensions de la molècula siguin les òptimes per a que aquesta pugui entrar a la cavitat de la ciclodextrina. En aquests termes, el digluconat de clorhexidina (digCHX) és un bon candidat per a la formació del complex hoste-convidat. Per tal que els suports funcionalitzats siguin aptes per ser utilitzats en

aplicacions mèdiques, cal que les CDs siguin capaces d'encapsular el digCHX, per tal d'alliberar-lo de forma mantinguda durant un cert període de temps. Així, la tècnica utilitzada per a caracteritzar aquest alliberament ha consistit en submergir els suports funcionalitzats amb CDs i carregats amb digCHX en medi aquós, extraient alíquotes en intervals de temps prefixats, per tal d'analitzar-los mitjançant espectrometria UV. Com que la molècula de digCHX presenta dos pics d'absorbància marcats entre 200 i 300 nm, s'ha escollit el pic a 254 nm per relacionar la intensitat del pic amb la concentració de digCHX en l'alíquota mitjançant la llei de Beer Lambert.

El màxim nombre de molècules de CD fixades en el suport es tradueix en un millor comportament en termes d'alliberament de principi actiu?

Per tal d'assajar l'efecte del nombre de CDs fixades en el suport en la quantitat de digCHX alliberada en el temps, s'ha funcionalitzat el paper no-estucat amb dos nivells de "grafting" (5 min i 30 min a 150ºC) i s'han considerat també dos suports no-funcionalitzats, però que han estat tractats amb diferents condicions de procés a mode de control (paper sense cap tractament i paper assecat a 150ºC durant 30 min).

Els resultats obtinguts mostren que el suport que ha estat capaç de carregar un major nombre de molècules de digCHX ha estat el funcionalitzat amb les condicions de 5 min a 150ºC, a diferència del que intuïtivament cabia esperar, que era que el suport que carregaria més principi actiu seria el que ha estat funcionalitzat amb les condicions òptimes trobades anteriorment (30 min, 150ºC), i amb les quals s'aconseguia la fixació del major nombre de molècules de CD en el suport. Fins i tot les mostres sense cap tractament han estat capaces de retenir quantitats interessants de digCHX; no obstant, el millor suport en termes de càrrega ha estat el funcionalitzat amb les condicions de 5 min a 150ºC.

La raó per la qual els suports funcionalitzats utilitzant les condicions òptimes no són els millors en termes de càrrega rau en l'estructura dels suports; després de l'empelt amb CDs, l'estructura del suport es tanca, i les porositats superficials del suport s'omplen de CDs. D'altra banda, l'àcid cítric provoca una reticulació intra i inter-fibra, resultant en un substrat amb menor capacitat d'absorció d'agent actiu. Cal remarcar, no obstant, que la capacitat de càrrega inicial de digCHX no és la propietat que s'està perseguint en els suports; el que es persegueix és la capacitat d'alliberar quantitats determinades de digCHX de forma mantinguda en el temps.

Un altre factor clau a tenir en compte és el fet que la càrrega de digCHX en el suport funcionalitzat es produeix principalment per la inclusió d'aquesta molècula en el sí de la ciclodextrina, degut a interaccions del tipus hidròfila-hidrofòbica, però cal considerar que

també hi poden haver interaccions de tipus iònic. La reacció entre l'àcid cítric i les CDs resulta en una xarxa polimèrica tridimensional, l'estructura de la qual es basa en CDs lligades a través de residus d'àcid cítric esterificats que porten grups carboxílics lliures. Aquests grups carboxílics apareixen en forma de carboxilat i poden experimentar una interacció iònica amb els grups amino presents en les molècules de digCHX.

Els resultats obtinguts mostren que per a tots els suports de paper no estucat (tant funcionalitzats com no-funcionalitzats) l'alliberament de gran part del digCHX carregat té lloc en la primera immersió en medi aquós. Aquest fet no és sorprenent, ja que la major part de les molècules de digCHX carregades estan adsorbides dèbilment a la superfície, i una petita part encapsulada per les CDs. No obstant, el que és interessant per a aquest estudi és l'alliberament de la petita part de principi actiu encapsulada per les CDs. De fet, aquestes quantitats poden semblar molt petites, però s'ha demostrat que són efectives i interessants des del punt de vista de la inhibició i destrucció bacteriana (Woodcock, 1988; do Amorim, Aun i Mayer, 2004; Odore, Colombatti i Re, 2000).

Els resultats mostren com, a diferència dels suports no-funcionalitzats, els suports tractats amb CDs són capaços de retenir certes quantitats de digCHX i alliberar-les progressivament en el temps. S'ha trobat que el suport que més temps allibera principi actiu d'una forma mantinguda no ha estat el que va ser funcionalitzat amb les condicions òptimes (major nombre de CDs en la superfície), sinó que ha estat el funcionalitzat amb unes condicions que provocaven la fixació d'un menor nombre de CDs. Els resultats mostren que els fulls funcionalitzats a l'òptim (30 min, 150ºC) retenen una quantitat interessant de digCHX, però retenen fortament aquesta quantitat, sense alliberar-la. Potser per a determinades aplicacions mèdiques, com per exemple apòsits, aquest digCHX atrapat a l'interior de la molècula de CDs ja podria ser efectiu, sense necessitat d'alliberar-la. En el present estudi s'ha caracteritzat l'alliberament en medi aquós, ja que la quantificació amb espectrofotometria UV és un mètode pràctic; no obstant, en futurs estudis es podrien considerar altres condicions d'alliberament.

En els tres suports analitzats, la funcionalització amb CDs ha proporcionat un alliberament mantingut de principi actiu, a diferència dels controls sense funcionalitzar. Els millors resultats s'han obtingut amb el paper no-estucat, amb el que s'aconsegueix un suport que és capaç d'alliberar de forma progressiva quantitats interessants de principi actiu durant 20 dies.

10.3. Funcionalització enzimàtica superficial de suports cel·lulòsics. Part 1: hidrofobicitat.

La funcionalització de la cel·lulosa mitjançant tractaments enzimàtics és un camp que ha atret l'interès dels investigadors en els darrers anys (Aracri *et al.*, 2010; Fillat *et al.*, 2012; Garcia-Ubasart *et al.*, 2012; Liu, Qin i Li, 2013; Saastamoinen *et al.*, 2012; Gaffar Hossain *et al.*, 2010). Els enzims són catalitzadors proteics produïts pels organismes vius, que acceleren la velocitat de les reaccions químiques i són altament específics del tipus de reacció que catalitzen. La utilització d'enzims aporta una sèrie d'avantatges respecte als processos químics tradicionals, pel fet que es deriven de recursos renovables, són biodegradables, treballen en condicions relativament suaus de temperatura i pH, i tendeixen a oferir elevada selectivitat tant en reactiu com en estereoquímica del producte (Bajpai, 1999; Witayakran i Ragauskas, 2009; Eriksson, 1998; Call i Mucke, 1997; Virk, Sharma i Capalash, 2012; Demuner, Junior i Antunes, 2011). Actualment la majoria dels treballs que reporten l'ús de tractaments enzimàtics per tal de funcionalitzar un suport cel·lulòsic són dissenyats de tal manera que, el tractament amb l'enzim es du a terme sobre les fibres i abans de la fabricació del suport. En alguns casos de tractaments enzimàtics en teixits o fusta, el tractament es du a terme sobre el suport ja finalitzat (Kudanga *et al.*, 2008), però fins al moment no s'han reportat estudis de tractaments enzimàtics superficials del paper amb l'objectiu de funcionalitzar. Així doncs, hem de diferenciar entre dos tipus de tractaments enzimàtics:

- **Tractaments enzimàtics en suspensió fibrosa (o en massa)**: consisteixen en aquells tractaments que es duen a terme sobre les fibres cel·lulòsiques quan aquestes es troben individualitzades en suspensió aquosa. La biomodificació té lloc en la superfície de la fibra individual.
- **Tractaments enzimàtics superficials**: entendrem per tractaments enzimàtics superficials aquells que són aplicats en la superfície d'un suport cel·lulòsic ja format, és a dir, en el qual les fibres ja no es troben individualitzades, sinó formant part d'una estructura en forma de làmina o teixit. Exemples de tractaments enzimàtics superficials serien aquells aplicats sobre paper, cartró, teixits, "nonwovens" o fusta, entre altres.

La present tesi es centra única i exclusivament en els tractaments enzimàtics superficials.

En aquests termes el capítol 4 té com a objectiu principal el desenvolupament d'un mètode per a la funcionalització enzimàtica superficial del paper. El mètode es basa en els treballs realitzats per Garcia-Ubasart *et al.*, (2012; 2011) on es va reportar un mètode per a

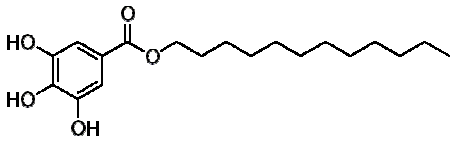
la funcionalització de fibres cel·lulòsiques en suspensió aquosa per tal de funcionalitzar-les i conferir-los hidrofobicitat.

En una sèrie de proves preliminars s'han utilitzat les mateixes condicions de reacció que les utilitzades per Garcia-Ubasart, *et. al* amb la diferència que en aquest cas, el substrat a funcionalitzar no són fibres a una consistència determinada, sinó fulls del suport ja format. Els resultats obtinguts han demostrat que és possible hidrofobitzar el paper mitjançant aquesta tècnica, però amb una pèssima distribució del producte hidrofòbic (lauril gal·lat) en la superfície, donant lloc a una hidrofobicitat superficial no-homogènia.

Cóm es pot millorar la distribució superficial del producte hidrofòbic per a la funcionalització de superfícies?

La mala distribució del producte (lauril gal·lat, taula 10-1) es deu en gran part a les característiques fisicoquímiques d'aquest. El lauril gal·lat (LG) és un compost insoluble en aigua, que presenta una molt baixa energia lliure de superfície, i que té gran tendència a formar agregats. El compost es sintetitza a partir de l'alcohol làuric i l'àcid gàl·lic; aquest últim s'obté a partir dels tanins de certes plantes. Es presenta en format de pols blanca, lleugerament groga.

Taula 10-1 Propietats del lauril gal·lat.



Nom IUPAC	Dodecyl 3,4,5-trihydroxybenzoate
Numero CAS	1166-52-5
Fórmula Molecular	$C_{19}H_{30}O_5$
Pes Molecular	338,44 g/mol
Solubilitat	Insoluble en aigua. Soluble en etanol i èter.
Rang de fusió	95°-98° després d'assecar.
Altres noms	Lauryl gallate; Dodecyl gallate; Gallic acid n-dodecyl ester

A major pes molecular de l'ester del gal·lat, més soluble és en els greixos, disminuint la seva solubilitat en aigua. Per tant, el LG és el més soluble en lípids dins de la família d'antioxidants, però és altament hidrofòbic. Aquest fet provoca que no es distribueixi de forma homogènia en el còctel de reacció amb l'enzim, i conseqüentment, no es distribueix de forma homogènia en la superfície dels fulls.

Així doncs, per tal d'aconseguir integrar millor el lauril gal·lat en el medi aquós, s'ha optat per la introducció d'un surfactant amb l'objectiu de disminuir la tensió superficial de l'aigua. De tot el ventall de possibles surfactants susceptibles de ser utilitzats, s'ha escollit un lignosulfonat, ja que aquests són compostos naturals, sense perjudici per al medi ambient. Els lignosulfonats són subproductes del procés de producció de la cel·lulosa al bisulfit. La capacitat dels lingosulfonats per reduir la tensió superficial de l'aigua ve determinada per l'estructura difílica de les seves molècules que contenen tant un marc hidròfob com un component hidròfil d'una macromolècula: grups ionogènics funcionals. La introducció del surfactant a una concentració igual a la de lauril gal·lat en el còctel de reacció ha resultat en una millora substancial en la distribució del producte (capítol 4).

La medició de l'activitat enzimàtica residual (capítols 4 i 9) ha demostrat que el lignosulfonat actua com a agent preservador de l'activitat enzimàtica, de forma que les solucions resultants del tractament presenten una activitat residual elevada en el cas d'utilitzar el lignosulfonat en comparació als tractaments sense lignosulfonat. Els resultats de voltametria cíclica del capítol 9 han permès realitzar la hipòtesi que, en realitat, com que el lignosulfonat ajuda a oxidar les espècies del lauril gal·lat que s'oxiden a potencials més elevats, per analogia aquest efecte podria produir-se amb l'ABTS que és el compost que s'utilitza per a mesurar l'activitat enzimàtica; d'aquesta manera, no es tractaria d'una preservació real de l'activitat de l'enzim, sinó que es tractaria d'un efecte catalitzador del lignosulfonat a l'hora d'oxidar compostos com el lauril gal·lat o l'ABTS.

Les mesures de dispersió dinàmica de llum (Dynamic Light Scattering, DLS) efectuades en el capítol 6, demostren que el lignosulfonat ajuda a reduir la mida de les partícules de lauril gal·lat, arribant pràcticament a solubilitzar-lo en el medi aquós, obtenint un producte aquós que permet el pas de la llum, a diferència del tractament en el que no s'ha utilitzat lignosulfonat, el qual continua presentant terbolesa degut a la presència de partícules en suspensió.

Degut a la millora de la distribució del producte lauril gal·lat en la superfície del suport cel·lulòsic, la propietat hidrofòbica també s'ha vist uniformement repartida. Els nivells d'hidrofobicitat assolits també es veuen incrementats gràcies a la introducció del lignosulfonat, tal com s'exposa en els capítols 4 i 5. Així doncs, l'addició del lignosulfonat en el tractament enzimàtic amb lauril gal·lat s'ha demostrat que proporciona una sèrie d'avantatges interessants.

D'altra banda, per tal de desintegrar els agregats de LG i integrar-lo en el medi de reacció, en el capítol 5 s'ha introduït, a més a més, una etapa d'ultrasons. A través de

l'energia adequada durant un cert temps, els ultrasons provoquen la reducció de les partícules de LG integrant-lo en el medi aquós en forma de suspensió de partícules. La suspensió obtinguda no és estable i tendeix a precipitar ràpidament (Fig. 10-2), però si es manté en constant agitació es distribueix d'una forma homogènia en el medi aquós. En el capítol 5 es mostra l'efecte positiu d'aquesta etapa d'ultrasons, augmentant el nivell d'hidrofobicitat dels suports obtinguts. La destrucció d'agregats i la major distribució del LG, potenciat a més a més per l'efecte del lignosulfonat, fa que l'enzim tingui major accessibilitat per a oxidar el LG i aquest fet es tradueix en un augment de la dissolució del LG i de la hidrofobicitat. Al finalitzar la reacció, degut a l'etapa d'ultrasons en combinació amb la utilització del SL, el LG es veu pràcticament solubilitzat del tot, resultant en un producte totalment estable.

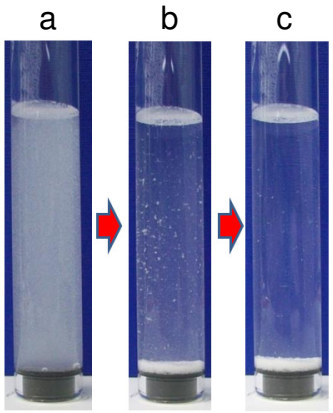


Figura 10-2 Imatges del lauril gal·lat ultrasonicat (a), i després de deixar la suspensió en repòs durant 60 (b) i 140 (c) minuts.

Es pot optimitzar el paràmetre "temps de reacció"?

Els tractaments preliminars per tal d'assajar si és possible hidrofobitzar els fulls cel·lulòsics s'han dut a terme utilitzant 1h de temps de reacció. Per tal d'optimitzar el paràmetre temps amb l'objectiu d'augmentar el nivell d'hidrofobicitat assolit, s'han tractat papers utilitzant diferents temps de reacció i posteriorment se n'ha assajat la seva hidrofobicitat. Els resultats (capítol 4) mostren com la propietat hidrofòbica va augmentant de forma progressiva a mesura que ho fa el temps de reacció, fins que a partir de les 4h s'assoleix una estabilització. Així doncs, el nou temps de reacció òptim s'ha establert a les 4h.

10.3.1. Optimització del mètode de funcionalització enzimàtica superficial de suports cel·lulòsics

El mètode de funcionalització superficial desenvolupat en el capítol 4 ha resultat efectiu per a la hidrofobització dels suports cel·lulòsics base paper. No obstant, el mètode ha estat assajat a escala de laboratori, i presenta dos grans inconvenients per a que pugui ser aplicat a escala industrial:

- La totalitat de la superfície del paper necessita estar submergida en el medi de reacció. Aquest fet limita les dimensions del suport que es poden tractar.
- El suport necessita estar submergit durant 4h en el medi de reacció mentre l'enzim actua. Aquest fet és molt limitant tenint en compte les actuals velocitats de fabricació de suports cel·lulòsics.

La funcionalització enzimàtica dels suports cel·lulòsics, ja sigui superficial o no, es produeix gràcies a que l'enzim és capaç d'oxidar el compost funcionalitzador (en aquest cas el LG) modificant-lo químicament, i activant-lo de tal manera que es pot enllaçar i/o adsorbir fortament amb la cel·lulosa, modificant-ne les seves propietats superficials, tal com es mostra esquemàticament en la Fig. 10-3, per al cas d'una sola fibra cel·lulòsica.

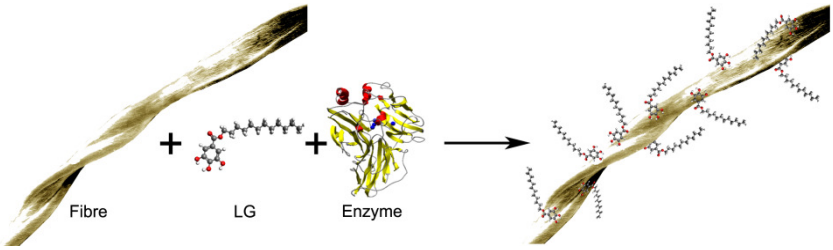


Figura 10-3 Esquema de la funcionalització catalitzada per l'enzim d'una fibra cel·lulòsica.

D'aquesta manera, el compost funcionalitzador necessita estar accessible per l'enzim en el medi adequat (pH, temperatura, temps, agitació) per a que l'enzim pugui oxidar-lo correctament. No obstant, no sabem fins a quin punt el substrat cel·lulòsic necessita estar present en el medi d'actuació de l'enzim per tal que es produeixi el "grafting", i no està clar que sigui l'enzim el responsable d'aquest empelt. Així doncs, ens hem plantejat la següent hipòtesi: si el compost funcionalitzador ha d'ésser oxidat per l'enzim per a que es produeixi el "grafting", però creiem que l'enzim no és en sí mateix el responsable de la unió amb la cel·lulosa d'aquest compost oxidat, potser es podria oxidar el compost

mitjançant l'enzim, i a posteriori intentar la unió d'aquest compost bio-modificat amb la cel·lulosa mitjançat altres tècniques.

En aquests termes, en el capítol 5 s'ha obtingut un producte post-enzimàtic resultant de la reacció de l'enzim amb el LG durant 4 h. D'aquesta manera s'ha aconseguit un producte aquós consistent en LG oxidat per l'enzim, i en el qual la reacció enzimàtica ha tingut lloc sense la presència de material cel·lulòsic de cap tipus durant el temps de la reacció. Posteriorment, aquest producte s'ha utilitzat per a impregnar el suport cel·lulòsic (que en un principi ha estat paper comercial de filtre de laboratori), i després d'un assecat complet, se n'ha assajat la propietat hidrofòbica. Els fulls tractats amb aquest sistema, han presentat nivells d'hidrofobicitat tant o més elevats als obtinguts en el capítol 4 en termes del Water Drop Test (WDT), indicant la viabilitat d'aquest mètode.

Com que aquest producte és capaç de conferir hidrofobicitat mitjançant una simple impregnació del material cel·lulòsic a partir d'una aplicació molt ràpida sobre el suport, els factors limitadors del mètode exposat en el capítol 4 (temps i mida de la mostra) es veuen superats. Aquest sistema ja permetria una utilització a nivell industrial d'aquest producte. En el cas del procés de fabricació del paper, aquest producte podria ser aplicat mitjançant qualsevol dels sistemes actuals per al tractament superficial de la fulla de paper en màquina, com per exemple immersió-impregnació, esprai, size-press, entre altres. Aquest sistema suposa un avenç molt important en quant a la viabilitat dels tractaments enzimàtics per a la funcionalització.

El producte post-enzimàtic funcionalitzador, o tal com s'ha anomenat a la present tesi "functionalization solution" (FS) conté tres elements principals en la seva composició; aquests elements són: l'enzim (Lacc), el lauril gal·lat (LG) i el lignosulfonat (SL). Així doncs, amb l'ànim de determinar la contribució de cadascun d'aquests elements en el desenvolupament de la propietat hidrofòbica, s'ha preparat una bateria de productes control que posteriorment hem aplicat superficialment per impregnació sobre el suport cel·lulòsic (en aquest cas paper comercial de filtre de laboratori), per tal de determinar-ne la hidrofobicitat. Aquests productes control han consistit en solucions dels productes individuals i diverses combinacions entre ells. Les condicions de pH, temperatura i concentració sempre han estat les mateixes que les utilitzades en la preparació del FS. Els resultats obtinguts (capítol 5) han demostrat que els dos únics productes capaços de conferir hidrofobicitat han estat el FS, i el control consistent en Lacc+LG. Tot i que el control Lacc+LG ha proporcionat nivells d'hidrofobicitat interessants, l'augment substancial d'aquesta propietat es produeix en el cas del FS; per tant, com que la única diferència entre el control Lacc+LG i el FS és la introducció del SL en el sistema, es

conclou que la utilització del SL produeix un efecte clarament positiu en el desenvolupament de la propietat hidrofòbica mitjançant aquest mètode.

Es pot aplicar aquest sistema a altres suports cel·lulòsics?

Per tal d'entendre la naturalesa de la funcionalització produïda mitjançant el mètode d'impregnació del substrat cel·lulòsic, s'ha assajat la seva aplicació sobre altres suports base paper per tal de veure si el mètode era efectiu amb altres tipus de suports. S'ha aplicat el FS sobre fulls de paper d'eucaliptus, lli, i sisal realitzats en el laboratori, i s'ha observat que el mètode no era efectiu amb aquests suports, obtenint uns nivells molt baixos d'hidrofobicitat.

Per què el sistema funciona amb el paper de filtre del laboratori però no amb altres suports cel·lulòsics base paper?

Per tal de poder respondre a aquesta pregunta ens hem plantejat quines eren les principals diferències entre el paper de filtre utilitzat i els altres papers (eucaliptus, lli, sisal) produïts en el laboratori. Les principals característiques del paper de filtre comercial que el diferencien dels papers realitzats en el laboratori són:

- Està compost d'una mescla de fibres, és a dir, no totes les fibres provenen de la mateixa font.
- Les fibres que el composen han passat per un procés de refinat.
- En la seva formulació s'han utilitzat una sèrie d'additius, essent els més importants els agents de resistència en humit. La resistència en humit dels papers de filtre és un requisit indispensable per al seu ús.

Tal com es mostra en el capítol 5, l'efecte del tipus de matèria primera (tipus de fibra) utilitzat en la composició del paper en la òptima hidrofobització del suport utilitzant FS, ha quedat demostrat pel fet d'haver utilitzat papers fets a partir de diferents fonts de fibres. Entre els fulls d'eucaliptus, lli i sisal s'observen lleugeres diferències en la hidrofobicitat després de la impregnació amb FS, però els nivells assolits estan molt lluny dels que s'aconsegueixen amb el paper de filtre. Seguidament s'ha volgut avaluar l'efecte del refí en la funcionalització mitjançant impregnació amb FS. S'han refinat les pastes d'eucaliptus, lli i sisal i s'han obtingut fulls de laboratori a partir d'aquestes pastes. Un cop formats els fulls, aquests s'han impregnat utilitzant FS, i se n'ha assajat el nivell d'hidrofobicitat. Els resultats mostren que el refí afavoreix la retenció del FS, ja que els nivells d'hidrofobicitat assolits són més elevats comparats amb els dels papers a partir de pastes no-refinades. No obstant, els valors assolits segueixen sent molt inferiors als obtinguts per als papers de filtre de laboratori, i no es pot afirmar que el refinat de la

pasta sigui el factor determinant per a una òptima funcionalització. S'observen lleugeres diferències en la hidrofobicitat dels fulls fets a partir de pasta refinada i tractats amb FS, però aquestes lleugeres diferències segurament són degudes a la diferent morfologia de les fibres.

Per últim, s'ha considerat la utilització d'additius per al tractament de les fibres cel·lulòsiques per a la fabricació del paper utilitzant agents de resistència en humit. S'ha escollit una resina de poliamidoamina-epiclorhidrina (PAAE) ja que és un additiu de resistència en humit utilitzat habitualment en la industria paperera. S'han obtingut fulls d'eucaliptus a partir de pasta que ha estat tractada amb resina PAAE, i els fulls obtinguts han estat impregnats utilitzant FS. Els resultats obtinguts (capítol 5) mostren que els nivells d'hidrofobicitat per a aquests fulls es situen en els mateixos ordres de magnitud que aquells obtinguts per al paper de filtre comercial de laboratori, indicant que són els additius de resistència en humit (en aquest cas una resina PAAE) els causants de la òptima funcionalització dels suports cel·lulòsics utilitzant FS. Per tal de confirmar aquesta hipòtesi, s'han obtingut fulls d'eucaliptus de laboratori a partir de fibres tractades amb un altre additiu de resistència en humit habitualment utilitzat, en aquest cas un midó catiònic (CSt). Després de la impregnació dels fulls tractats amb CSt utilitzant el FS, els nivells d'hidrofobicitat d'aquests s'han situat també en els mateixos valors que pel cas del paper de filtre i els fulls a partir de fibres tractades amb PAAE.

Així doncs, s'ha pogut confirmar que aquest tipus d'additius són els responsables de la òptima funcionalització dels suports cel·lulòsics per mitjà de la impregnació amb FS.

Per quin motiu la resina PAAE i el midó catiònic són els responsables de la òptima funcionalització dels suports cel·lulòsics?

Donat que la funcionalització es produeix amb una simple impregnació, d'una forma molt expeditiva i a temperatura ambient, resultaria molt difícil que tinguessin lloc enllaços de tipus covalent entre els elements presents en el FS i el suport cel·lulòsic. En una primera hipòtesi s'ha cregut que la funcionalització té lloc gràcies a interaccions del tipus fisicoquímic. Observant l'estructura química dels compostos PAAE i CSt (capítol 5) ens adonem que la principal característica comú que presenten són els grups químics que confereixen caràcter catiònic al compost. En la resina, el caràcter catiònic prové dels grups "azetidinium" presents en la cadena de la PAAE reticulada, mentre que en el CSt, la cationicitat prové dels grups amino terciaris i quaternaris que s'han afegit a la molècula de midó com a substituents catiònics.

Així doncs, per tal de verificar que el caràcter catiònic dels fulls (donat pels agents de resistència en humit PAAE i CSt) són els responsables de la interacció entre el suport i el

producte FS, s'han tractat els fulls amb un altre compost capaç de conferir cationicitat als suports cel·lulòsics, però que no està relacionat amb cap additiu de resistència en humit del paper. El compost utilitzat ha estat el metil-glicol quitosan (MGCh), ja que és un derivat de la quitina i es tracta d'un producte natural que no generaria cap tipus d'efluent tòxic en el processament del paper. En el MGCh, el caràcter catiònic prové dels grups "amino" de la seva estructura. S'ha aplicat una fina capa d'estucat de MGCh a la superfície dels papers d'eucaliptus ECF i posteriorment, s'ha utilitzat el FS per tal de funcionalitzar el paper. Els elevats nivells d'hidrofobicitat obtinguts (capítol 5) demostren que efectivament, la cationicitat és el factor clau que provoca la retenció del FS en els fulls cel·lulòsics.

Quina és la influència conjunta dels paràmetres "temps de reacció" i "concentració" en el desenvolupament de la capacitat hidrofòbica mitjançant el mètode d'impregnació?

Per tal de determinar l'efecte conjunt que els paràmetres "temps de reacció" i "concentració" tenen en el desenvolupament de la capacitat hidrofòbica, s'han realitzat una sèrie de tractaments amb l'objectiu d'obtenir una bateria de productes enzimàtics funcionalitzadors utilitzant diversos temps de reacció i concentració dels productes de reacció. La taula 10-2 mostra el rang de concentracions i els diferents temps utilitzats per a l'obtenció dels diferents productes. La concentració dels productes LG, SL i Lacc s'ha modificat a partir de les dosis utilitzades en els capítols 4 i 5. Els diferents productes obtinguts s'han aplicat sobre fulls d'eucaliptus tractats amb resina PAAE i posteriorment se n'ha assajat la hidrofobicitat mitjançant el WDT i l'angle de contacte.

Taula 10-2 Rang de concentracions i temps de reacció utilitzats en l'obtenció dels diferents productes.

Producte	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Concentració [g/L]	x0,5	x1	x2	x5	x10	x15	x20
Temps de reacció	30′	1h	1h30′	2h	2h30′	3h	3h30′
Producte	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Concentració [g/L]	x20	x15	x10	x5	x2	x1	x0,5
Temps de reacció	30′	1h	1h30′	2h	2h30′	3h	3h30′

La Fig. 10-4a mostra l'evolució de la propietat hidrofòbica dels fulls tractats amb productes obtinguts utilitzant dosis i temps creixents, mentre que la Fig. 10-4b mostra l'evolució de la propietat utilitzant dosis decreixents i temps creixents. Els resultats mostren que els temps de reacció curts (entre 1 i 2 hores) no són efectius en termes de desenvolupament de la propietat hidrofòbica, ja sigui a baixes dosis o dosis elevades.

Aquest fet pot ser degut a que l'enzim no té temps de completar l'oxidació del LG i produir la bio-modificació necessària per a que aquest es pugui unir de manera efectiva al suport cel·lulòsic.

Tenint en compte els temps de reacció llargs (entre 2 i 4 hores), la utilització de baixes concentracions dels productes (LG, SL i Lacc) resulta més efectiva. Es pot observar com el nivell d'hidrofobicitat més elevat s'obté utilitzant la dosi que s'havia considerat inicialment en els capítols 4 i 5 (1,2 g/L de LG i SL, i 1,2 U/mL d'enzim) i un temps de reacció de 3 h. Aquestes observacions confirmen que tant les dosis com els temps de reacció considerades en els capítols 4 i 5 ja es troben a prop de l'òptim per a aquest sistema enzimàtic en termes de desenvolupar la propietat hidrofòbica. La utilització de dosis molt elevades (x24) ja sigui amb temps de reacció llargs o curts no produeix una millora de la hidrofobicitat.

La següent qüestió que s'ha plantejat ha estat si seria possible realitzar la preparació del producte a elevada concentració per tal de diluir-lo posteriorment en el moment de l'aplicació. Aquest fet és d'especial rellevància de cara a la implementació industrial del producte, ja que permetria reduir considerablement els costos de transport i emmagatzematge. Per tal d'analitzar aquest aspecte, cadascun dels productes preparats anteriorment a diferents temps i concentracions s'han diluït tots en aigua destil·lada i aplicat sobre els fulls d'eucaliptus a la concentració de 0,6 g/mL. Els resultats obtinguts es mostren en la Fig. 10-5a i 10-5b. De forma general, es pot observar que els nivells d'hidrofobicitat tendeixen a augmentar respecte el cas anterior, on els productes s'aplicaven directament sense cap dilució. Per tant, el producte pot ser preparat a elevada concentració per tal de ser diluït posteriorment abans de l'aplicació obtenint valors inclús més elevats d'hidrofobicitat.

S'observa que el rang de concentracions i temps de tractament que proporcionen hidrofobicitat augmenta quan aquests es dilueixen a la concentració de 0,6 g/mL (x 0,5) abans de l'aplicació. En el cas d'aplicar els lleixius diluïts a 0,6 g/mL, la dosi òptima de preparació i temps de reacció amb els quals s'assoleix la màxima hidrofobicitat són conc. x15 i 3h respectivament. En la Fig. 10-5 també s'observa que és preferible utilitzar un temps de reacció llarg que permeti l'acció de l'enzim, que no pas una elevada dosificació dels reactants.

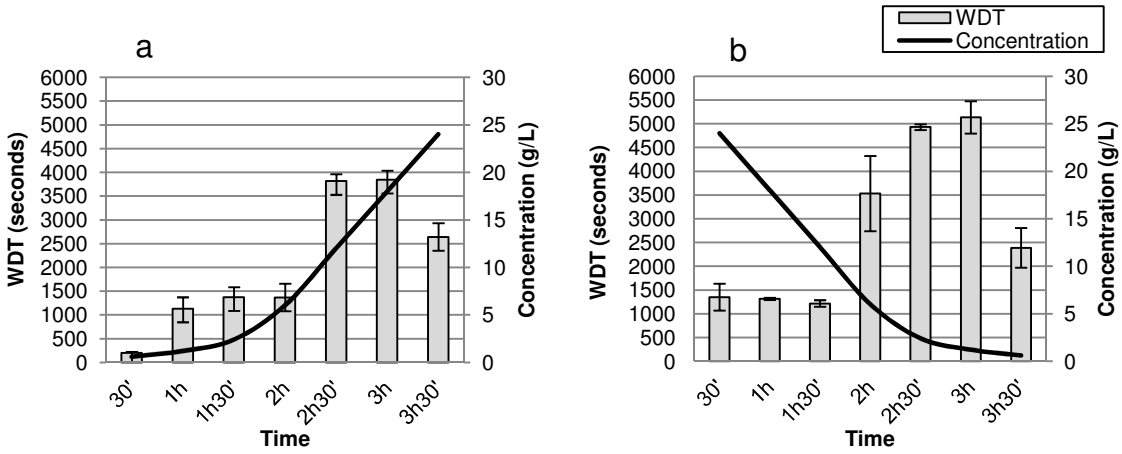


Figura 10-4 Hidrofobicitat mesurada segons el WDT de fulls d'eucaliptus tractats amb diferents productes preparats a diferents dosis i temps de reacció. Concentració de productes creixent amb el temps (a), i concentració decreixent amb el temps (b).

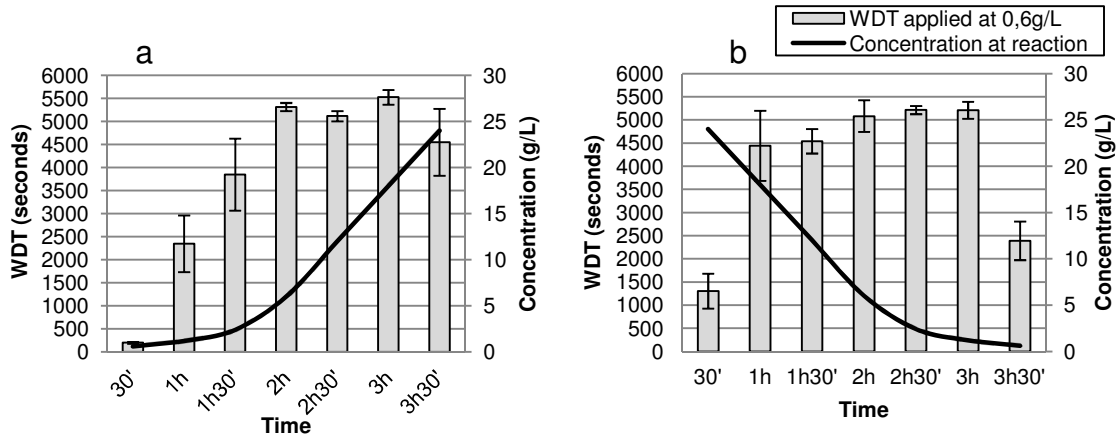


Figura 10-5 Hidrofobicitat mesurada segons el WDT de fulls d'eucaliptus tractats amb productes preparats a diferents dosis i temps de reacció, i aplicats tots sobre els fulls després d'una dilució a la concentració de 0,6 g/L. Concentració de productes creixent amb el temps (a), i concentració decreixent amb el temps (b).

És possible utilitzar altres compostos de la família dels gal·lats per a desenvolupar la hidrofobicitat mitjançant aquesta tècnica enzimàtica? Quina és la influència de la longitud de la cadena hidrocarbonada en el nivell d'hidrofobicitat assolit?

Els bons resultats en termes de la hidrofobicitat assolida en els suports cel·lulòsics han permès efectuar una hipòtesi en la manera com es produeix la hidrofobització d'aquest

tipus de materials. L'estructura química del LG presenta un anell aromàtic unit a tres grups hidroxil (-OH) i a una cadena hidrocarbonada de 12 carbonis (d'aquí prové el nom del compost) unida a l'anell aromàtic a través d'un ester. Aquesta estructura suggereix que el caràcter polar o hidròfil resideix en l'anell aromàtic, mentre que la part hidrofòbica o dispersiva es trobaria localitzada en la cadena alquílica. Els possibles mecanismes d'unió entre la molècula de LG biomodificada i la cel·lulosa es discuteixen en el capítol 9. El LG pertany a la sèrie homòloga dels gal·lats, però aquesta sèrie està formada per un grup de compostos similars amb longitud de cadena variable, els quals serien susceptibles de ser utilitzats per a la modificació enzimàtica proposada.

En el capítol 8 s'ha assajat l'evolució de la propietat hidrofòbica de suports cel·lulòsics de paper de filtre els quals han estat tractats utilitzant una sèrie de compostos funcionalitzadors (FS), preparats a partir de compostos de la sèrie homòloga dels gal·lats. D'aquesta manera s'ha pogut determinar l'efecte de la longitud de la cadena hidrocarbonada (susceptible de ser la causant de la hidrofobització de la cel·lulosa) en la propietat hidrofòbica aconseguida. Els resultats han demostrat com el nivell d'hidrofobicitat augmenta a mesura que ho fa la longitud de la cadena del gal·lat utilitzat, però fins a un cert nombre d'àtoms de carboni en la cadena. D'aquesta manera, s'ha observat com la hidrofobicitat comença a decréixer a partir del lauril gal·lat (12 àtoms de carboni en la cadena). Així doncs, el LG és el compost òptim a utilitzar per tal d'obtenir els màxims nivells d'hidrofobicitat mitjançant aquesta tècnica. En el capítol 8 s'ha assajat també l'efecte que produeixen els tractaments de curat a temperatura dels suports tractats. S'ha observat que quan es realitzen aquests curats, i tal i com ja s'havia avançat en el capítol 4, els nivells d'hidrofobicitat assolits es veuen incrementats significativament. També s'ha vist que aquests tractaments eviten la caiguda de la propietat a partir del compost LG, mantenint el nivell d'hidrofobicitat constant. Una possible explicació d'aquesta millora substancial degut al tractament tèrmic seria que l'elevada temperatura afavoreix les reaccions químiques entre el LG i la cel·lulosa (tipus esterificació o ROP) que només tenen lloc a elevada temperatura. Aquest tipus de reaccions provocarien una major orientació del gal·lat degut a l'enllaç produït, de forma que la part hidrofòbica (cadena hidrocarbonada) s'orientaria cap a l'exterior de la superfície cel·lulòsica modificada, i la part hidròfila (anell aromàtic modificat enzimàticament) seria la que s'enllaçaria o interaccionaria amb la cel·lulosa. Aquesta interacció es discuteix en el capítol 8, i una discussió més extensa de les possibles vies d'unió es realitza en el capítol 9.

És possible la utilització d'altres enzims per al desenvolupament de la tècnica enzimàtica de funcionalització proposada?

En la present tesi s'ha treballat bàsicament amb un enzim del tipus oxidoreductasa, concretament una lacasa de *Trametes villosa* proporcionada per l'empresa Novozymes®. No obstant, s'han fet proves amb altres lacases, una lacasa de *Cerrena Unicolor* subministrada per l'empresa Fungal Bioproducts®, i una lacasa de *Myceliphthora Thermophila* subministrada també per l'empresa Novozymes®, obtenint comportaments molt similars en termes d'hidrofobicitat. Per aquest motiu, els resultats d'hidrofobicitat amb ambdós últims enzims no han estat inclosos, i els resultats que es mostren en la present tesi corresponen als obtinguts amb l'enzim de *Trametes villosa*. En el capítol 7, on s'analitza la capacitat antioxidant desenvolupada en els suports utilitzant la tècnica enzimàtica, sí que s'han observat diferències substancials en aquesta propietat en funció del tipus d'enzim.

10.3.2. Caracterització dels suports cel·lulòsics tractats

El mètode utilitzat inicialment en els capítols 4 i 5 per tal de caracteritzar el nivell d'hidrofobicitat assolit en els suports cel·lulòsics ha estat el "water drop test" (WDT). No obstant, aquest mètode dóna més aviat informació del comportament del suport en termes d'absorció o penetració d'aigua, però no necessàriament del nivell d'hidrofobicitat de la superfície en termes energètics. Tot i que l'absorció i l'energia lliure de superfície estan relacionades, no es poden caracteritzar ambdues amb el WDT. És per aquest motiu que en els capítols 4, 5, 6 i 8 s'utilitza també la tècnica de l'angle de contacte per tal de determinar la hidrofobicitat. L'angle de contacte proporciona millor informació sobre la hidrofobicitat del suport cel·lulòsic en termes energètics. De fet, amb l'aparell OCA15 de Dataphysics® utilitzat, també és possible monitoritzar l'evolució de l'angle de contacte a mesura que la gota d'aigua es va absorbint en el suport, i obtenir així informació en termes d'absorció.

El caràcter hidrofòbic depèn de l'energia lliure de superfície (SFE) del suport. Per a que un líquid mulli un sòlid és necessari que l'energia lliure de superfície del sòlid sigui menor que la tensió superficial del líquid; és possible que el tractament utilitzant la FS, estigui disminuint la SFE dels suports cel·lulòsics. D'altra banda, per tal de caracteritzar superfícies sòlides en termes d'hidrofobicitat generalment s'assumeix que si l'angle de contacte entre l'aigua i el sòlid és menor a 90°, aquest es considera hidròfil, i en cas contrari, hidrofòbic. En el capítol 5 s'ha mesurat l'energia lliure de superfície dels suports tractats amb FS i els corresponents controls (KFS). Els resultats mostren que tan sols els tractaments LG+Lacc i FS aconsegueixen disminuir substancialment l'energia lliure de

superfície dels suports cel·lulòsics, aconseguint una major reducció en el cas de la FS. Els resultats obtinguts confirmen la hipòtesi de l'augment d'hidrofobicitat degut a la reducció de l'energia lliure de superfície dels suports.

Els suports d'eucaliptus ECF tractats i sense tractar s'han analitzat mitjançant microscòpia electrònica de rastreig (scanning electron microscopy, SEM) (capítol 9). Els resultats mostren com el producte FS i el control LG+Lacc provoquen l'aparició de partícules de dimensions nanomètriques a la superfície de les fibres (partícules que s'han caracteritzat en el capítol 6). Aquestes partícules de lauril gal·lat modificat per l'enzim són les responsables de l'augment d'hidrofobicitat de les fibres del suport cel·lulòsic mitjançant dos mecanismes: **1)** la seva baixa energia lliure de superfície i **2)** les seves dimensions nanomètriques que fa que augmenti la rugositat de la fibra a la nanoescala.

En el capítol 6 s'ha realitzat la mesura de l'angle de contacte de superfícies de sílice. La sílice representa un bon model de la cel·lulosa, presentant en la seva superfície –OH lliures, evitant la naturalesa tova i rugositat superficial dels materials cel·lulòsics. S'han tractat oblies de sílice amb la FS i els respectius controls KFS, per tal de mesurar-ne, a posteriori, la hidrofobicitat. S'han utilitzat oblies de sílice ja que la superfície dels sensors de la microbalança de cristall de quars (QCM) també consisteixen en sílice, i d'aquesta manera es poden comparar les dades de QCM amb les d'angle de contacte. Els sensors tractats mitjançant QCM no s'han utilitzat per a la mesura de l'angle de contacte ja que la seva superfície és molt reduïda, i és necessari dipositar diverses gotes per a que la mesura de l'angle de contacte sigui fiable. A més a més, com que la superfície dels sensors de QCM s'analitzen mitjançant microscòpia de força atòmica (AFM), no s'ha volgut mesurar-hi l'angle de contacte per tal de no alterar-ne la morfologia entre l'anàlisi QCM i la mesura utilitzant AFM. El procediment emprat en els tractaments, així com els temps de tractament es mostren en la Fig. 10-6.

Per tal de poder relacionar la hidrofobicitat obtinguda en la sílice amb les dades de QCM, el tractament de les oblies s'ha realitzat en dinàmic (fluid en moviment) per així reproduir l'anàlisi QCM. En el QCM el fluid es desplaça contínuament a través de la càmera del sensor, per tant, un tractament de les oblies per simple impregnació no seria comparable al que està passant en l'anàlisi QCM on el fluid es renova constantment en la superfície del sensor. Per tal de tractar les oblies de sílice amb una renovació constant del líquid en la superfície del sensor, aquestes s'han col·locat en l'interior de recipients que contenen la FS i els diferents controls KFS, i al seu torn els recipients s'han col·locat en una plataforma giratòria (Fig. 10-7); d'aquesta manera el líquid a l'interior dels recipients es troba en constant moviment.

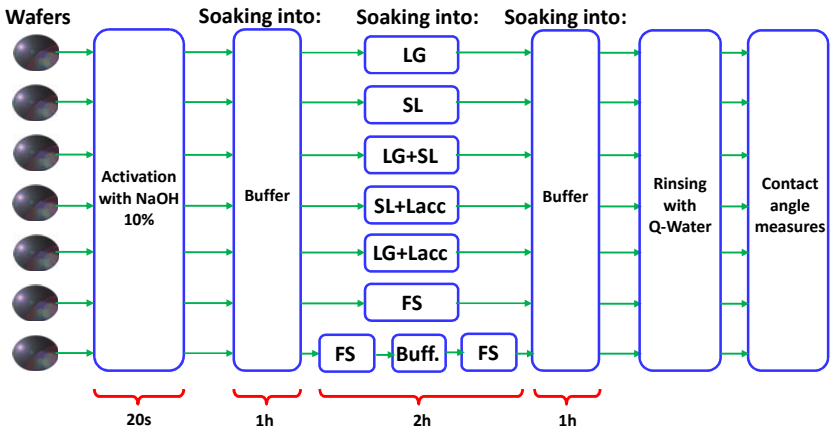


Figura 10-6 Esquema i durada del tractament de les superfícies de sílice mitjançant la FS i els controls KFS.

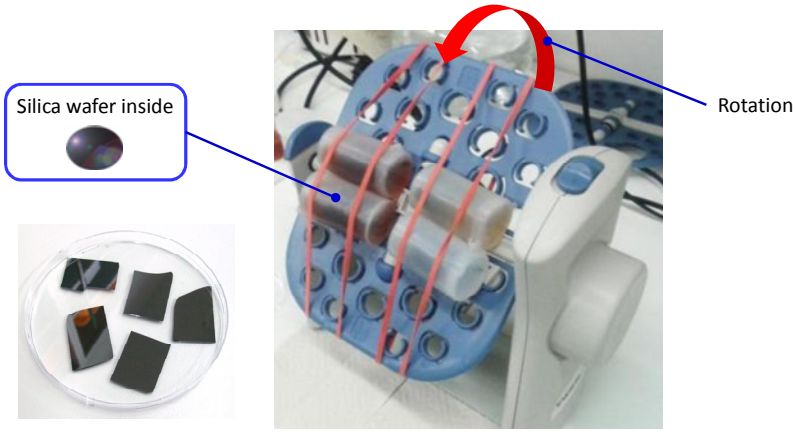


Figura 10-7 Esquema del muntatge realitzat per tal de tractar les oblies de sílice de forma dinàmica mitjançant la FS i els controls KFS.

Els resultats de l'angle de contacte mostren com l'angle de contacte inicial de la sílice (15°) es veu lleugerament incrementat fins als 20° pel fet de tractar-la a partir de la solució "buffer". La FS i el control SL+Lacc produeixen un increment similar de l'angle de contacte fins un valor de l'ordre dels 35°, mentre que el control LG+Lacc produeix un augment més significatiu, arribant fins a un valor de l'ordre dels 85°. Aquests resultats contrasten amb els resultats de WDT i energia superficial obtinguts en el capítol 5, on el millor tractament en termes d'absorció i disminució de l'energia lliure de superfície era el FS. No obstant, sí que s'observa un increment substancial de l'angle de contacte de la

sílice pre-cationitzada tractada amb FS, arribant en aquest cas a un valor de l'ordre dels 50°; així doncs, es confirma l'efecte positiu del caràcter catiònic del substrat en la hidrofobicitat mitjançant la FS exposat en el capítol 5. La pre-cationització i el tractament amb el control LG+Lacc també proporciona resultats molt elevats d'hidrofobicitat, assolint valors de l'ordre dels 88°.

A continuació s'han comparat els angles de contacte assolits sobre les superfícies de sílice i superfícies de sílice que han estat recobertes amb una fina capa de cel·lulosa nanofibril·lar. Els resultats mostren com el comportament d'ambdues superfícies és molt similar, aconseguint angles de contacte molt semblants després del tractament amb la FS i els controls. L'efecte positiu de la pre-cationització en l'adsorció de la FS també s'observa en el cas de les nanofibres.

Els resultats suggereixen que existeix una relació entre la quantitat de material (partícules de LG) dipositades en la superfície de la sílice o les nanofibres, i l'angle de contacte. Per tal de discernir de quin tipus de relació es tracta, s'ha traçat un gràfic on es representa el valor de l'angle de contacte (WCA) vs la caiguda de la freqüència del sensor (ΔF) provocada per la deposició de material (Fig. 6-10). Per la situació dels punts en la gràfica, sembla que a mesura que augmenta la quantitat de material dipositat en la superfície dels sensors, augmenta també la hidrofobicitat mesurada a través de l'angle de contacte; no obstant, sembla que a partir d'una certa quantitat de material en la superfície del sensor, la tendència s'estabilitza i un augment de la massa dipositada ja no es tradueix en augments significatius de la hidrofobicitat.

És possible que la tècnica enzimàtica desenvolupada confereixi, a més a més de la hidrofobicitat, propietats barrera al vapor d'aigua en els papers?

En els capítols 4, 5 i 6 s'ha mesurat la propietat hidrofòbica dels papers mitjançant les tècniques de WDT i WCA obtenint uns valors elevats d'hidrofobicitat en els suports cel·lulòsics assajats; així, els papers tractats mitjançant aquesta tècnica presenten unes propietats barrera excel·lents respecte a l'aigua. No obstant, ens hem plantejat si aquesta tècnica era també capaç de conferir propietats barrera al vapor d'aigua en els suports.

A priori, podria semblar que donada l'elevada hidrofobicitat dels papers tractats, aquests podrien presentar també propietats barrera al vapor d'aigua, però tenint en compte l'aspecte superficial dels papers un cop tractats amb la FS, visualment no s'apreciava una alteració de la seva porositat ni aspecte superficial (a part del canvi de color). Per tant, la propietat barrera al vapor d'aigua no es feia tan evident, donat que possiblement les molècules d'aigua en estat vapor podrien difondre a través de la porositat del paper.

Per a tal efecte s'ha determinat l'índex de transmissió del vapor d'aigua (WVTI) en fulles de laboratori (fetes a partir de pasta d'eucaliptus), tractades i sense tractar amb la FS. Per a la determinació del WVTI, s'ha utilitzat el mètode gravimètric, segons UNE 53097. Els índexs de transmissió obtinguts han estat de 2737 g/(m²·d) per al paper sense tractar i de 2866 g/(m²·d) per al paper tractat; per tant, els resultats demostren que els índexs de transmissió són molt similars. Les corbes de transmissió de vapor d'aigua mostren una taxa d'absorció lineal, amb una pendent pràcticament idèntica entre el paper tractat i el no-tractat (Fig. 10-8).

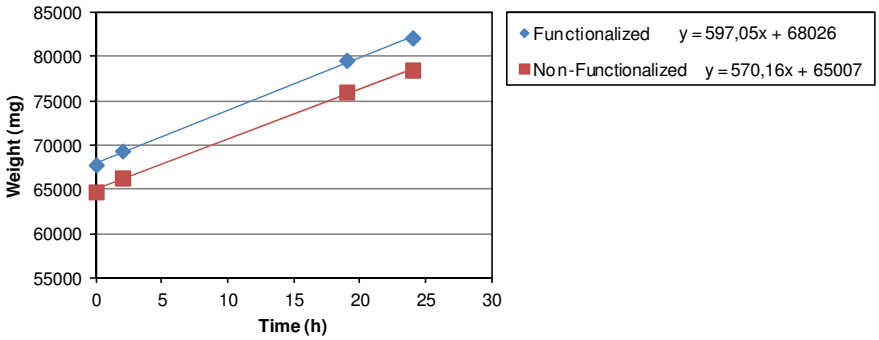


Figura 10-8 Corbes de transmissió de vapor d'aigua per al paper funcionalitzat i sense funcionalitzar amb la FS, expressat com a guany de pes (mg).

Així doncs, es pot concloure que el tractament amb la FS utilitzant com a compost hidrofòbic el lauril gal·lat, no proporciona propietats barrera al vapor d'aigua als suports cel·lulòsics; el vapor d'aigua pot difondre a través de l'estructura porosa del paper tractat amb la FS, de la mateixa manera que ho fa en el paper no-tractat.

10.4. Funcionalització enzimàtica superficial de suports cel·lulòsics. Part 2: capacitat antioxidant.

Fins ara s'ha parlat del desenvolupament d'un mètode de funcionalització superficial de suports cel·lulòsics en el qual la funcionalitat a desenvolupar ha estat principalment la hidrofobicitat d'aquests suports. No obstant, en la funcionalització química de substrats, es poden conferir un gran nombre d'altres propietats depenent de la naturalesa dels grups químics que s'aconsegueixin unir a la superfície que es funcionalitza. Capacitat antioxidant, capacitat antimicrobiana, capacitat de retenció i alliberament de principis actius, indicadors químics, són exemples d'altres funcionalitats que es poden conferir a les superfícies. En aquests termes, en la present tesi s'ha volgut assajar si és també

possible l'obtenció de suports cel·lulòsics amb capacitat antioxidant a partir de la mateixa funcionalització enzimàtica amb la qual s'ha desenvolupat la hidrofobicitat.

La capacitat antioxidant és una de les propietats d'una substància que consisteix en la capacitat per a inhibir la taxa d'oxidació d'un radical lliure. Un antioxidant és una molècula capaç d'alentir o prevenir l'oxidació d'altres molècules. L'oxidació és una reacció química de transferència d'electrons d'una substància a un agent oxidant; les reaccions d'oxidació poden produir radicals lliures que comencen reaccions en cadena que danyen les cèl·lules. Els antioxidants acaben aquestes reaccions eliminant intermedis del radical lliure i inhibeixen altres reaccions d'oxidació oxidant-se ells mateixos. Per aquest motiu els antioxidants són sovint agents reductors tals com tiols o polifenols. Els antioxidants són àmpliament utilitzats en la indústria alimentària, mèdica i de la cosmètica. En aquest sentit, el desenvolupament de substrats cel·lulòsics amb capacitat antioxidant és interessant des del punt de vista del packaging alimentari i mèdic.

En el capítol 7 s'han considerat una sèrie de 25 compostos (entre els quals es trobava també el LG) susceptibles de desenvolupar la capacitat antioxidant mitjançant la tècnica enzimàtica descrita en la present tesi. Els compostos han estat seleccionats donat el seu poder antioxidant com a compost pur, és a dir, sense modificació enzimàtica de cap tipus expressat segons el seu valor TEAC (Trolox equivalent antioxidant capacity). Seguidament s'ha preparat una bateria de productes post-enzimàtics funcionalitzadors, o tal com s'ha anomenat a la present tesi "functionalization solutions" (FS), utilitzant el mateix procediment i les mateixes condicions de reacció que es van utilitzar en el cas del LG en els capítols 5 i 6, però amb la diferència que el compost modificat enzimàticament era cadascun dels 25 candidats. Una altra diferència ha estat que en aquest "screening" inicial de compostos no s'ha utilitzat lignina sulfonada (SL) en el tractament enzimàtic. Posteriorment s'han tractat diversos suports cel·lulòsics amb cadascuna d'aquestes solucions post-enzimàtiques i se n'ha avaluat la seva capacitat antioxidant. Tenint en compte la capacitat antioxidant dels suports cel·lulòsics sense cap tractament i la capacitat antioxidant que produeix en els suports el tractament amb el control enzima (Lacc), els resultats han demostrat que tots els compostos estudiats eren capaços de conferir capacitat antioxidant als substrats utilitzant la tècnica descrita. Els productes que feien augmentar més significativament aquesta propietat en els papers tractats han estat els preparats a partir dels compostos FA, OG, LG, TRP i RV.

Quin és el paper que juga l'enzim en termes de la capacitat antioxidant?

Donat que la majoria dels compostos utilitzats en l'"screening" inicial ja presenten capacitat antioxidant per sí mateixos (de fet, molts d'ells s'utilitzen habitualment com a

additius alimentaris i de la cosmètica per a prevenir l'oxidació d'aliments o l'envelliment de la pell) s'ha volgut veure quin és el paper que juga l'enzim en aquesta propietat. Cal tenir en compte que encara que el compost inicial presenti capacitat antioxidant, el compost modificat enzimàticament no té perquè presentar-ne, ja que l'enzim produeix canvis en l'estructura química d'aquest.

Per tal d'assajar l'efecte de l'enzim s'han preparat productes FS, i productes control utilitzant els compostos lauril gal·lat (LG) i àcid ferúlic (FA). Aquests productes control consisteixen en dissolucions dels compostos en el medi de reacció enzimàtica sota les condicions de temps i temperatura de reacció però sense l'addició de l'enzim. Posteriorment aquests productes van ser utilitzats per a la impregnació dels substrats cel·lulòsics. Els resultats han demostrat que tant els productes preparats utilitzant compostos purs com els preparats a partir de modificacions enzimàtiques són capaços de desenvolupar la capacitat antioxidant dels substrats. No obstant, s'ha comprovat que els compostos modificats enzimàticament poden incrementar lleugerament la capacitat antioxidant dels suports que són tractats mitjançant aquesta tècnica. L'efecte més rellevant de la utilització de l'enzim per al desenvolupament de la capacitat antioxidant és el que s'observa en termes d'enllaç químic entre el producte i el substrat. Així, les mostres de paper tractades amb els diversos productes van ser sotmeses a rentats severs a elevada temperatura (80 ºC durant 30 minuts) i posteriorment se'n va tornar a assajar la seva capacitat antioxidant. Els resultats mostren com aquelles mostres tractades amb els productes preparats a partir dels compostos modificats enzimàticament, mantenen una elevada capacitat antioxidant després dels rentats, respecte a aquelles tractades utilitzant els productes control (compostos purs). Aquest fet és indicatiu de que l'enzim produeix canvis en l'estructura química dels compostos que afavoreixen la unió d'aquests amb la cel·lulosa.

Influeix el tipus de lacasa que s'utilitza en la reacció?

El tipus de lacasa utilitzat en la preparació dels productes post-enzimàtics té un efecte rellevant en la posterior capacitat antioxidant. En el capítol 7 s'han preparat diverses solucions post-enzimàtiques utilitzant tres lacases de diferent origen i posteriorment s'han tractat fulls de paper utilitzant aquests productes. Els resultats demostren que hi ha diferència en el nivell d'inhibició assolit en els diferents suports. És possible que depenent de l'enzim utilitzat, la modificació química produïda en el compost sigui diferent, i en conseqüència també ho siguin les propietats (hidrofobicitat i antioxidant) que se'n deriven posteriorment als substrats tractats.

Quin és el paper que juga l'addició de la lignina sulfonada (SL) en la capacitat antioxidant dels productes post-enzimàtics?

Tal com ja s'ha comentat en els paràgrafs precedents, en els capítols 4 i 5 de la present tesi s'ha utilitzat lignina sulfonada (SL) en la preparació dels productes post-enzimàtics per al desenvolupament de la hidrofobicitat dels substrats cel·lulòsics. En el capítol 7 s'ha volgut assajar l'efecte de la utilització d'aquest compost en la propietat antioxidant dels substrats tractats. De forma general, s'ha vist que l'addició del SL en la preparació dels productes té un efecte molt variable depenent del compost utilitzat, però en la majoria dels casos la seva addició no té un efecte negatiu. S'ha observat com la utilització del SL té un efecte positiu en la retenció dels productes post-enzimàtics en els suports cel·lulòsics ja que quan es sotmeten els fulls tractats a un rentat sever (80ºC durant 30 min) la capacitat antioxidant dels fulls que han estat tractats amb productes que contenien SL era més elevada. Cal indicar que els substrats per si mateixos, el SL i l'enzim no han presentat una capacitat antioxidant significativa, la qual cosa indica que aquesta propietat es manifesta en els suports tractats degut a la presència dels compostos utilitzats, estiguin o no modificats enzimàticament.

Quina és la capacitat antioxidant del producte post-enzimàtic FS abans de ser aplicat sobre el paper i quin paper juga cadascun dels elements que el composen (Lacc, LG i SL) en la capacitat antioxidant?

En les primeres experiències referents a la capacitat antioxidant s'ha analitzat aquesta propietat en papers o substrats cel·lulòsics que havien estat prèviament tractats amb les corresponents FS. Per a tal efecte s'ha utilitzat un mètode per a components insolubles que determinava el percentatge d'inhibició dels fulls de paper a través de la decoloració del radical catiònic $ABTS^{\bullet+}$, tal com s'explica en el capítol 7. No obstant, el producte FS i els seus respectius controls (KFS) són productes aquosos en forma de dissolucions parcials o suspensions col·loïdals, i el seu poder antioxidant s'ha calculat mitjançant el TEAC (Trolox Equivalent Antioxidant Capacity), que és un mètode àmpliament utilitzat per a la determinació de la capacitat antioxidant de components solubles. El procediment és descrit en el capítol 2, i consisteix en obtenir rectes d'inhibició en funció de la concentració del component soluble, i comparar la pendent d'aquestes rectes amb la recta corresponent al compost Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), que és un compost antioxidant anàleg a la vitamina E.

Així doncs, en el capítol 7 s'ha determinat el TEAC per a la FS i les corresponents KFS (LG, SL, LG+SL, SL+Lacc, LG+Lacc). Un valor del TEAC superior a la unitat és indicatiu d'una elevada capacitat antioxidant. En la Fig. 10-9 es mostra una gràfica resum on es

poden veure cadascuna de les rectes dels productes aquosos FS i KFS i també la recta corresponent al Trolox, la qual cosa permet detectar ràpidament com existeixen alguns productes que presenten una elevada capacitat antioxidant i altres que no. És interessant observar com el control LG+Lacc pràcticament no presenta capacitat antioxidant per sí mateix, mentre que el mateix control aplicat sobre el substrat cel·lulòsic sí que proporcionava capacitat antioxidant sobre el paper, tal com s'ha comentat en els paràgrafs anteriors. De la mateixa manera, el producte FS no presenta una capacitat antioxidant significativa, i es veu com la pendent de la recta és clarament inferior a la pendent de la recta corresponent al Trolox.

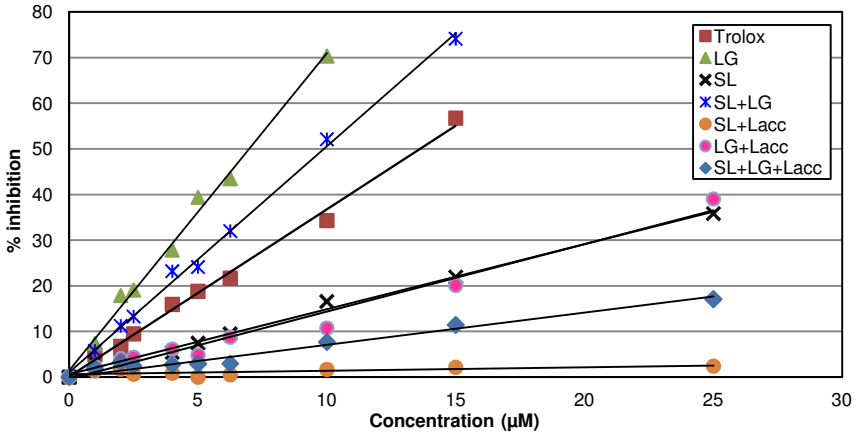


Figura 10-9 Rectes de percentatge d'inhibició (%) vs. concentració (µM) corresponents al Trolox, LG, SL, LG+SL, SL+Lacc, LG+Lacc, i FS. El Trolox s'utilitza com a referència.

Per quin motiu els papers tractats amb la FS i el control LG+Lacc presenten elevada capacitat antioxidant mentre que en els productes aquosos per sí mateixos aquesta propietat és molt feble?

Per tal de respondre a aquesta qüestió s'han plantejat dues hipòtesis o possibles explicacions a aquests fenòmens.

La primera hipòtesi consisteix en assumir que el producte LG+Lacc no posseeix capacitat antioxidant degut a la modificació que sofreix l'estructura química del compost LG a través de l'acció de l'enzim, fet que proporciona una molècula de LG modificada amb una capacitat antioxidant reduïda. Cal remarcar que el compost LG sí que posseeix capacitat antioxidant per sí mateix, tal com es pot observar en la recta corresponent al control LG de la Fig. 10-9. El fet que quan aquest control LG+Lacc és aplicat sobre el

suport cel·lulòsic sí que presenti capacitat antioxidant, seria degut a un re-ordenament de l'estructura química del compost LG a través de la reacció o interacció química amb la cel·lulosa. Aquesta interacció proporcionaria novament una molècula de LG (modificat) que presentaria novament capacitat antioxidant.

La segona hipòtesi fa referència a les quantitats de producte LG+Lacc que es dipositen sobre el paper quan són tractats per impregnació abans de la mesura de la capacitat antioxidant. Encara que la recta d'inhibició del producte LG+Lacc sigui inferior a la del Trolox, no es coneixen les quantitats de producte que es dipositen sobre els fulls de paper quan son tractats. Per tant, encara que la pendent del producte LG+Lacc sigui baixa, és possible que les quantitats de LG-modificat que es fixen sobre el paper siguin molt elevades, i per aquest motiu els papers tractats presentarien una capacitat antioxidant més elevada que el producte en sí en el que es considera la pendent de la recta. En altres paraules, la mesura d'antioxidant del paper proporciona un % d'inhibició, però desconeixem quina és la concentració de LG+Lacc sobre el paper que està produint aquesta inhibició.

Per tal d'establir quina de les dues hipòtesis és més plausible, s'ha realitzat una sèrie d'assajos amb l'objectiu d'avaluar la distribució i les quantitats de LG+Lacc que es dipositen sobre el suport mitjançant la tècnica d'impregnació (veure capítols 7, 9 i annex A). Un cop conegudes aquestes quantitats es pot determinar si són les causants de la major capacitat antioxidant observada en els suports respecte al propi producte. Els resultats han demostrat que les quantitats de FS i LG+Lacc retingudes sobre el suport cel·lulòsic no poden ser les causants d'una major capacitat antioxidant en els suports, donat que els percentatges d'inhibició que produeixen quan es considera el producte per ell mateix són molt febles. D'aquesta manera el percentatge d'inhibició dels fulls tractats es situa a l'entorn del 73%, mentre que les quantitats de producte aquós (FS o LG+Lacc) equivalents que es troben sobre el suport proporcionen tan sols un 15% d'inhibició.

La primera de les hipòtesis formulades és la que pren més força. Els possibles mecanismes d'interacció que s'esmenten en la primera hipòtesi s'han discutit en el capítol 9 de la present tesi.

Existeix una relació entre el valor del TEAC d'un compost pur i la capacitat antioxidant que es pot acabar obtenint mitjançant la tècnica enzimàtica?

Finalment, en el capítol 7 s'ha determinat si existeix una relació entre el valor del TEAC que proporciona un determinat compost i la habilitat d'aquest per a desenvolupar la capacitat antioxidant dels suports cel·lulòsics quan són tractats amb el producte post-enzimàtic corresponent, obtingut a partir d'aquest compost. En cas d'existir tal relació,

aquesta seria útil ja que es podria assajar la viabilitat d'un compost per a desenvolupar la capacitat antioxidant mitjançant la tècnica enzimàtica, però sense necessitat de dur a terme la reacció, simplement mesurant el valor TEAC del compost. Els resultats obtinguts a partir dels sis compostos seleccionats denoten que no existeix una relació clara entre els paràmetres considerats, però s'observa una lleugera tendència a augmentar la capacitat antioxidant dels papers, a mesura que augmenta el valor TEAC dels compostos. Seria necessària una ampliació de l'estudi amb nous compostos, per tal de confirmar aquesta tendència. D'aquesta manera es podria obtenir una recta de compostos amb un valor de TEAC creixent que es correspondria amb un valor creixent del % d'inhibició desenvolupat en el suports cel·lulòsics.

És possible utilitzar altres compostos de la família dels gal·lats per tal de desenvolupar la capacitat antioxidant? Quina és la influència de la longitud de la cadena hidrocarbonada dels compostos gal·lats en el nivell de capacitat antioxidant assolit?

En el capítol 8, i de forma anàloga al que s'ha fet per a la propietat hidrofòbica, s'ha estudiat l'efecte de la longitud de la cadena hidrocarbonada dels compostos de la família dels gal·lats en la capacitat antioxidant. En el cas de la hidrofobicitat, l'estructura de la molècula de LG es pot relacionar molt bé amb el desenvolupament de la propietat a través de les seves parts hidròfila i hidrofòbica. No obstant, pel que fa a la capacitat antioxidant, la relació entre la longitud de la cadena i aquesta propietat no sembla a priori tan òbvia; semblaria, basant-nos en el que es reporta en la literatura, que el desenvolupament de la capacitat antioxidant estaria més relacionada amb la part fenòlica dels gal·lats (Kuskoski *et al.*, 2005). Donat que tots els gal·lats presenten una estructura fenòlica idèntica, a priori s'esperaria no observar massa diferències entre els compostos d'aquesta família en termes d'activitat antioxidant.

En el capítol 8 s'ha assajat l'evolució de la propietat antioxidant de suports cel·lulòsics de paper de filtre els quals han estat tractats utilitzant una sèrie de compostos funcionalitzadors (FS), preparats a partir de compostos de la sèrie homòloga dels gal·lats. Els resultats mostren com la propietat antioxidant dels fulls augmenta gradualment amb l'augment de la longitud de la cadena del gal·lat, i s'estabilitza a partir del compost octil gal·lat (OG, 8 carbonis en la cadena hidrocarbonada). La longitud de la cadena sembla ser un paràmetre molt important en termes de la capacitat antioxidant. En l'estudi s'ha inclòs el compost àcid tànic (TA), donat que es volia veure si el fet de posseir un gran nombre d'anells aromàtics en l'estructura era determinant en el desenvolupament de la capacitat antioxidant. Els resultats mostren que, en termes de la capacitat antioxidant, el fet de tenir una cadena alquílica llarga sembla ser més rellevant en un compost que el fet de posseir un gran nombre d'anells aromàtics en la seva estructura.

Donat que els tractaments tèrmics dels suports tractats jugaven un paper important en la propietat d'hidrofobicitat, també s'ha volgut analitzar l'efecte dels tractaments de curat a elevada temperatura en el desenvolupament de la capacitat antioxidant. Els resultats han demostrat que els tractaments tèrmics no han produït efectes significatius en la propietat antioxidant.

10.5. Estudi de les interaccions entre la FS i els suports cel·lulòsics

Tal com s'ha demostrat en els capítols 4, 5, 6 i 7 el producte FS és capaç de desenvolupar la propietat hidrofòbica i antioxidant dels suports cel·lulòsics amb una simple impregnació. Al llarg de la tesi s'han plantejat diversos anàlisis amb l'objectiu de determinar el mecanisme d'unió dels compostos presents en la FS i els suports, així com la fortalesa d'aquesta unió. Degut a les condicions de temperatura (temperatura ambient) i temps (instantani, amb posterior assecat) en que té lloc la funcionalització dels suports, i l'efecte positiu en la funcionalització si aquests són tractats amb agents químics capaços de conferir cationicitat, tot sembla indicar que es produeix una interacció del tipus fisicoquímic entre els suports i la FS.

Es tracta d'una simple deposició, o bé d'una adsorció físico-química?

En el capítol 4 s'ha volgut assajar la força de l'enllaç entre la FS i els suports cel·lulòsics sotmetent aquests últims a condicions severes de rentat i extraccions Soxhlet. S'han sotmès els fulls tractats a rentats amb aigua destil·lada a 80ºC durant 30 minuts sota agitació constant, i també a extraccions Soxhlet amb acetona. Posteriorment a l'assecat, s'ha assajat la propietat hidrofòbica d'aquests fulls, observant reduccions en l'angle de contacte de només el 4% en el cas dels rentats amb aigua a temperatura i del 5% en el cas de les extraccions Soxhlet respecte als fulls sense rentar i extrets. Aquest resultat posa de manifest que la propietat hidrofòbica no es confereix als fulls tractats mitjançant una simple deposició del material present en la FS, sinó que hi ha algun altre tipus d'interacció bastant forta. Un enllaç covalent, a priori, semblaria difícil que es produís, degut a les condicions de baixa temperatura i rapidesa en que es du a terme la impregnació dels fulls. Podria tractar-se d'algun tipus d'enllaç iònic mitjançant la interacció electrostàtica que es produiria degut a la gran diferència d'electronegativitat entre els grups catiònics dels suports cel·lulòsics (preparats a partir de fibres tractades amb compostos capaços de conferir cationicitat) i les partícules de LG presents en la FS, que podrien portar càrrega negativa.

Com ja s'ha comentat, i tal com s'ha analitzat en el capítol 5, és necessari un caràcter catiònic per tal que la funcionalització i la hidrofobicitat dels suports assoleixi valors

interessants. Per tal de poder caracteritzar millor aquest tipus d'interacció, en el capítol 6 s'han realitzat una sèrie d'anàlisis d'absorció de la FS i els corresponents controls KFS utilitzant una microbalança de cristall de quars (QCM). Aquest dispositiu permet detectar deposicions de material a escala molecular sobre un petit sensor, i en el nostre cas s'han utilitzat sensors de sílice com a model de la cel·lulosa. Els motius per a l'elecció dels sensors de sílice han estat els següents:

- La sílice presenta en la seva superfície silanols lliures i silanols geminals que simulen els grups hidroxil lliures de la cel·lulosa.
- Utilitzant la sílice és possible evitar les distorsions de la matèria tova (com la cel·lulosa) que no tenen a veure amb l'adsorció, per exemple inflament per hidratació, difusió, etc.
- El sensor de sílice presenta una estructura totalment plana, evitant la rugositat i porositat que presenta el paper. D'aquesta manera, el producte s'adsorbirà per interacció electrostàtica, i no física a través dels intersticis del paper.

En uns primers assaigs (capítol 6) utilitzant la FS amb l'objectiu de comprovar si s'adsorbeix o no sobre la superfície de la sílice, s'observa com es produeix una disminució en la freqüència de vibració del cristall, fet que es relaciona directament amb un augment de pes degut a la deposició de material a la superfície del sensor. Per tal de veure si aquest augment de massa és degut a una simple deposició, posteriorment al pas de la FS a través de la cavitat del sensor, s'ha injectat solució "buffer"; d'aquesta manera, en cas d'una simple deposició, el "buffer" eliminaria les partícules dipositades en la superfície, fet que es correspondria amb una augment de la freqüència de vibració. No obstant, s'ha observat que la freqüència de vibració es mantenia en els nivells baixos assolits, indicant que el material estava fortament adsorbit en la superfície de la sílice. Partint de la hipòtesi d'una interacció del tipus electrostàtic entre les partícules de la FS i la sílice (per analogia al que s'havia observat en el cas del paper), s'ha realitzat un segon assaig que ha consistit en la injecció d'un electròlit (0,1 M NaCl) al final de l'adsorció enlloc d'utilitzar la solució "buffer". La finalitat ha estat la d'augmentar la conductivitat del medi, i afavorir així la dissipació de càrregues elèctriques amb una hipotètica eliminació del material adsorbit en la superfície dels sensors de sílice; el resultat obtingut ha estat que la freqüència de vibració s'ha mantingut en nivells baixos, indicant que la introducció de l'electròlit no ha produït la desorció del material. No obstant, analitzant la corba de dissipació en aquest cas s'ha observat que a partir de la introducció de l'electròlit la dissipació ha disminuït significativament; aquesta disminució pot ser

deguda a la compactació de la capa de material dipositat, que inicialment es trobava més hidratada.

Per tal de corroborar l'efecte de la cationicitat del substrat en la interacció amb la FS mitjançant QCM, s'ha dipositat una fina capa de quitosan a la superfície del sensor, i posteriorment s'ha introduït la FS. Per dipositar el quitosan, aquest s'ha introduït en la cavitat del sensor en forma de solució, seguit d'un rentat posterior amb solució "buffer" per tal de comprovar que la capa de quitosan està fortament adsorbida. La disminució de la freqüència degut a l'adsorció del quitosan ha estat molt feble, la qual cosa indica que la capa adsorbida és molt fina. El rentat posterior amb "buffer" ha eliminat una part molt insignificant del quitosan adsorbit, la qual cosa és indicativa de que, a pesar de tractar-se d'una capa molt fina, el quitosan està fortament adsorbit en la superfície del sensor. Un cop recoberta la superfície de sílice del sensor amb la fina capa de quitosan, s'ha procedit a la introducció de la FS a la cavitat del sensor de forma anàloga als assajos realitzats prèviament. Els resultats mostren un increment espectacular de la quantitat de material de la FS que s'adsorbeix en la superfície del sensor, respecte al sensor sense cationitzar; aquest increment (de l'ordre del 250%), confirma, mitjançant QCM, la forta interacció entre els substrats catiònics i la FS, que ja s'havia observat en el capítol 5 en el cas del paper.

També s'han realitzat cinètiques d'absorció en QCM dels controls KFS. En el cas del lignosulfonat (SL) i el lignosulfonat modificat per l'enzim (SL+Lacc) s'observa com ambdós productes s'adsorbeixen de manera efectiva en la superfície de la sílice, però les quantitats adsorbides no són tan elevades com les que s'havien observat per a la FS. Aquesta observació és interessant, ja que cal tenir en compte que la FS conté també LG. En el cas del control LG, no s'aprecia absorció de material a la superfície del sensor; cal tenir en compte que tant la FS com els controls KFS han estat filtrats utilitzant filtres amb unes dimensions de porus de 450 nm abans de ser introduïts en la cavitat del sensor. Aquest filtrat és necessari, per tal d'evitar la presència de partícules molt grans que proporcionarien una mesura errònia. Així doncs, el que ha passat en realitat amb el control LG és que tot el material ha quedat atrapat en el procés de filtrat, i no s'ha detectat l'adsorció de cap partícula; aquest fet confirma els resultats obtinguts amb DLS, on s'afirma que tot el LG queda atrapat amb un filtrat utilitzant filtres de 450 nm. No obstant, pel que fa a l'absorció del control LG+Lacc s'observa una disminució espectacular de la freqüència de vibració (la qual cosa indica un augment important de la massa dipositada); en aquest cas, degut al tractament utilitzant l'enzim, i tal com ja s'ha vist en els resultats de DLS, el LG+Lacc ha estat capaç de passar a través del filtre dipositant-se en quantitats molt significatives al ser injectat a la cavitat del sensor. L'acció

de l'enzim reduint la mida de partícula del LG, ha quedat també demostrada a partir dels assajos amb QCM. L'adsorció de l'enzim sobre la sílice (control Lacc) també s'ha assajat mitjançant QCM, obtenint un augment de massa en la superfície del sensor. L'enzim també és adsorbit fortament, ja que una quantitat de massa molt significativa roman adherida després de rentar la superfície injectant solució "buffer" a la cavitat del sensor.

Els resultats obtinguts han demostrat que els tres compostos presents en la FS (LG, SL i Lacc) són adsorbits d'una forma efectiva a la superfície de la sílice.

Es pot adsorbir la FS sobre la cel·lulosa nanofibril·lar?

L'adsorció de la FS i els controls SL+Lacc i LG+Lacc també s'ha analitzat mitjançant anàlisi QCM. En aquest cas, l'estratègia ha consistit en recobrir la superfície del sensor de sílice amb una capa de cel·lulosa nanofibril·lar, per posteriorment analitzar les interaccions d'aquesta superfície amb els productes esmentats.

Els resultats mostren que la FS s'adsorbeix sobre la cel·lulosa nanofibril·lar de manera molt similar a com ho feia sobre la sílice; després d'un rentat amb solució "buffer", una quantitat important de material roman fortament adherida a la superfície del sensor. Seguidament s'ha dut a terme el mateix experiment que en el cas de la sílice, que consisteix en la pre-adsorció d'una fina capa de quitosan en la superfície del sensor (en aquest cas recobert de cel·lulosa nanofibril·lar) per tal de conferir-li caràcter catiònic. Posteriorment s'introdueix la FS i es comprova l'efecte que el caràcter catiònic del quitosan provoca en l'adsorció de la FS, per comparació amb l'experiment sense la prèvia cationització. Els resultats mostren un espectacular augment de la quantitat de FS adsorbida en la nanocel·lulosa cationitzada, respecte a la no-cationitzada; aquests resultats són similars als que ja s'han observat en el cas de les superfícies de sílice, però les quantitats de FS adsorbides sobre la cel·lulosa cationitzada són lleugerament inferiors a les observades en el cas de la sílice. Els resultats mostren com el control SL+Lacc s'adsorbeix d'una forma molt similar al cas de la sílice sobre la cel·lulosa nanofibril·lar, mentre que el control LG+Lacc s'adsorbeix en quantitats molt més elevades. En la sílice, la disminució en la freqüència de vibració del cristall deguda a la deposició de material era de l'ordre dels -389 Hz, mentre que en el cas de la cel·lulosa nanofibril·lar ha estat dels -564 Hz. Aquest increment de la massa adsorbida podria ser degut al fet que el sensor recobert amb cel·lulosa nanofibril·lar presenta una major rugositat superficial que la sílice, augmentant la superfície específica disponible per a la deposició de material.

Per tal de visualitzar l'efecte de l'adsorció dels diferents productes sobre la sílice, en el capítol 6 s'han dut a terme anàlisis mitjançant microscòpia de força atòmica (AFM). Les imatges mostren com la sílice presenta una superfície completament llisa i sense la

presència de cap partícula adsorbida. Seguidament, el control amb l'enzim (Lacc) revela la presència de petites partícules adsorbides de forma irregular en la superfície; aquestes poden ser degudes a dos factors: al tractar-se d'un enzim comercial, podrien ser additius o altres compostos presents en el còctel enzimàtic, o bé tractar-se d'agregats del propi enzim. La imatge corresponent a l'adsorció del control SL+Lacc mostra una superfície bastant llisa, amb alguna partícula puntual que es correspon a agregats del lignosulfonat. El control LG+Lacc mostra en la superfície la presència de grans partícules de LG distribuïdes de forma homogènia al llarg de tota la superfície. Aquestes partícules només poden ser el LG modificat per l'enzim, ja que la imatge corresponent al control amb l'enzim no revela la presència d'aquestes partícules. La imatge corresponent a la FS adsorbida mostra també la presència de partícules homogèniament distribuïdes, però a diferència del control LG+Lacc aquestes són de menors dimensions. Aquestes partícules es corresponen al LG modificat per l'enzim, ja que les poques partícules detectades observant les imatges del control amb enzim (Lacc) i el control amb lignosulfonat (SL+Lacc), no expliquen la presència de la gran quantitat de partícules que es poden veure en la imatge de la FS adsorbida. Aquest resultat, com ja s'ha comentat anteriorment, confirma les hipòtesis fetes a partir dels resultats de DLS, que afirmaven que el SL potencia l'efecte de l'enzim en la reducció de la mida de partícula del LG. Finalment, la imatge corresponent a l'adsorció de la FS sobre la sílice pre-cationitzada mitjançant quitosan revela la enorme quantitat de partícules que es dipositen en el cas que el substrat presenti caràcter catiònic; en aquest cas, la densitat de partícules per unitat d'àrea augmenta dràsticament, provocant el conseqüent augment de massa en la superfície del sensor, que es tradueix en una disminució de la freqüència de vibració.

10.6. Caracterització del producte post-enzimàtic funcionalitzador (FS)

Durant la reacció per tal de preparar la FS, l'enzim (Lacc) provoca canvis evidents en els productes presents en la reacció (LG i SL). D'aquesta manera, al final de la reacció enzimàtica, el producte aquós resultant presenta una marcada coloració fosca, la qual cosa fa evident l'oxidació soferta pel LG i el SL. Aquests canvis es van detectar mitjançant espectroscòpia UV-VIS tal com es mostra en el capítol 4.

Una observació detallada dels productes aquosos resultants de la reacció és un primer indicador de l'activitat de l'enzim i l'efectivitat d'aquest. En el capítol 5, es veu el color dels controls LG i SL amb i sense tractament enzimàtic. Els resultats oxidats d'ambdós productes experimenten una forta coloració (veure Fig. 5-7).

A part de la coloració, un fet molt remarcable és la terbolesa. Si tenim en compte el producte corresponent al control LG (que consisteix únicament en LG sonicat), aquest presenta una pèssima estabilitat; tot i que mitjançant la sonicació s'aconsegueix integrar-lo en el medi aquós, aquest tendeix a agregar-se i precipitar ràpidament si es deixa en repòs. Aquest fenomen és degut a la seva insolubilitat en aigua, i la presència de partícules en suspensió és fàcilment perceptible a simple vista. No obstant, també a simple vista es pot comprovar com després de la reacció enzimàtica el producte LG+Lacc presenta terbolesa (degut a la presència de partícules en suspensió), però aquestes ja no tendeixen a precipitar, mantenint-se en el medi aquós en forma de suspensió col·loïdal. D'altra banda, el producte FS, el qual també conté LG en la seva formulació, no presenta terbolesa al final de la reacció enzimàtica, suggerint una completa solubilització del compost LG. Aquests resultats ens han permès realitzar la hipòtesi que, l'efecte de l'enzim sobre el LG ha estat el de causar la seva oxidació, però també reducció de la mida de partícula. Aquesta reducció de la mida de partícula es veuria potenciada per la presència del lignosulfonat (en el cas de la FS), provocant inclús una solubilització del compost LG.

Per tal de comprovar la hipòtesi de la reducció de la mida de les partícules de LG a través de l'enzim, en el capítol 5 es mesura el potencial Z de la FS i els diversos controls. La mesura del potencial Z és indicativa de l'estabilitat de les suspensions col·loïdals; com més gran és la mesura del potencial (ja sigui positiu o negatiu) més gran és l'estabilitat. Els resultats obtinguts mostren com el producte LG, incrementa la seva estabilitat després del tractament amb l'enzim (LG+Lacc). També s'observa l'efecte de la presència del lignosulfonat (control LG+SL) augmentant l'estabilitat del conjunt, i confirmant la hipòtesi del capítol 4, on es posa de manifest aquest efecte positiu augmentant la distribució del LG i els nivells d'hidrofobicitat assolits en els suports. Tant l'aigua com el producte control SL+Lacc tendeixen a potencials menors ja sigui degut a la no-presència de partícules en suspensió o bé a una pràctica dissolució; en el cas del SL+Lacc, l'enzim hauria dissolt les partícules de SL i el resultat final en les propietats electrocinètiques del producte seria que aquestes s'estarien aproximant a les de l'aigua (no-presència de partícules, o dissolució total).

L'efecte de l'enzim en la terbolesa de la FS i els productes control (KFS) s'observa clarament a partir de l'estudi de difusió de llum realitzat en el capítol 5. L'experiment ha consistit en fer passar un feix de llum coherent a través dels productes continguts en un vas de precipitats tot observant els fenòmens de difusió que aquest feix de llum experimentava. El control LG, que consisteix en LG sonicat, presenta gran quantitat de fenòmens de difusió i una pèssima estabilitat, tal com s'ha indicat anteriorment. No

obstant, quan el LG es combina amb el lignosulfonat (LG+SL), l'estabilitat augmenta, i no s'observen tants fenòmens de difusió (la difusió és més homogènia), fet que indicaria una menor tendència del LG a formar partícules agregades de majors dimensions. L'efecte de l'enzim en el LG s'observa en el control LG+Lacc; es pot veure com l'efecte obtingut en termes de comportament de la llum és similar al que provoca l'addició del SL en el control LG+SL, i s'aprecien fenòmens de difusió similars, i reduint la tendència del LG a formar agregats. El resultat més significatiu en termes de comportament de la llum és el que s'observa en el producte FS, on es pot veure com la llum no experimenta cap tipus de fenomen de difusió, la qual cosa indicaria una completa dissolució del compost LG, o bé una dramàtica reducció de la mida de partícula.

Aquestes observacions respecte a les dimensions de les partícules de LG i la hipòtesi de la seva reducció a través del tractament enzimàtic s'han corroborat en el capítol 6, mitjançant mesures de difusió dinàmica de la llum (DLS). Els resultats obtinguts han demostrat la dràstica reducció del diàmetre mig de les partícules de LG (inicialment entorn els 5 μm), fins a unes dimensions de 300-200 nm gràcies a l'efecte de l'enzim. En el cas del lignosulfonat sembla que aquest també veu reduïda la seva mida de partícula després del tractament amb l'enzim. Les dimensions de les partícules de LG del producte FS també es veuen dràsticament reduïdes per l'efecte de l'enzim, i els resultats han demostrat que gràcies a l'addició del lignosulfonat, s'aconsegueix una major reducció de la mida de les partícules, arribant a obtenir-ne de l'ordre dels 80 nm. Els anàlisis DLS s'han efectuat abans i després del filtrat utilitzant filtres de xeringa amb unes dimensions de porus de 450 nm, per tal de visualitzar millor l'efecte de reducció de mida en els casos de mostres amb una elevada polidispersió, com és per exemple el cas del producte FS. Es pot concloure que l'enzim provoca la reducció de la mida de partícula del compost LG, i que la presència del lignosulfonat potencia aquesta reducció, assolint dimensions de partícula menors.

Els resultats obtinguts amb DLS corroboren les hipòtesis fetes anteriorment mitjançant l'anàlisi amb potencial Z i l'observació del comportament d'un feix de llum coherent a l'incidir en els productes.

Els resultats de l'estudi mitjançant microscòpia de força atòmica (AFM) sobre suports de sílice que es mostren en el capítol 6, confirmen els anàlisis anteriors sobre la mida de les partícules amb DLS. En aquest estudi es diposita la FS i alguns dels productes control (KFS) a la superfície d'uns petits sensors en forma d'oblies de sílice, analitzant-ne posteriorment la seva topografia per tal de determinar les dimensions de les partícules dipositades. Els resultats mostren la presència de partícules entorn als 200-300 nm de diàmetre en la superfície de la sílice tractada amb el control LG+Lacc, mentre que en el

cas de la sílice tractada amb la FS aquestes partícules són només de l'ordre dels 100 nm. Aquests resultats confirmen també que la presencia del lignosulfonat (SL) augmenta l'acció de l'enzim reduint encara més la mida de les partícules de LG.

Una altra tècnica d'anàlisi que permet caracteritzar la terbolesa i l'estabilitat de les suspensions aquoses consisteix en l'anàlisi mitjançant l'aparell Turbiscan®. Aquest aparell mesura la transmissió (TR) i la retrodifusió (BS) de les suspensions aquoses a través d'un cilindre de cristall, proporcionant informació sobre la sedimentació, coalescència, floculació, escumat, entre altres fenòmens. Els resultats que es mostren en el capítol 6 indiquen que el producte control LG presenta una pèssima estabilitat, fet que es fa evident a través de l'augment de la transmissió de la llum en el temps a mesura que les partícules es van agregant i precipitant. El tractament del LG amb l'enzim (control LG+Lacc) provoca un augment de l'estabilitat, ja que totes les corbes de transmissió se superposen, però uns percentatges de transmissió molt baixos degut a la forta coloració del producte de reacció i la presència de partícules. En el cas de la FS, s'observa una lleugera inestabilitat, però un augment substancial en el percentatge de transmissió. Aquest augment de la transmissió s'atribueix al fet que, a diferència del control LG+Lacc, en aquest cas les partícules són més petites i permeten el pas de la llum. Els controls SL, SL+Lacc i Lacc presenten una gran estabilitat com es pot veure per la superposició de totes les corbes de transmissió.

Així doncs, s'han observat una sèrie d'efectes que l'enzim provoca sobre el compost LG: reducció de la mida de partícula, estabilització de la suspensió de LG, i augment de la coloració de la suspensió aquosa degut a l'oxidació del LG. A més a més, la presència del lignosulfonat potencia l'efecte de l'enzim en la reducció de la mida de partícula del compost LG, augmentant la transparència de la solució aquosa de la FS respecte el control LG+Lacc.

L'anàlisi mitjançant l'aparell Turbiscan® s'ha dut a terme a partir dels productes aquosos FS i KFS un cop ja havia tingut lloc la reacció enzimàtica (4 h, 50ºC). No obstant, per tal de veure l'evolució de la mida de partícula a mesura que té lloc la reacció enzimàtica, s'han realitzat anàlisis mitjançant el Turbiscan On-Line®. Aquest aparell mesura també la transmissió i la retrodifusió, però en aquest cas a partir de la captació i anàlisi de petites alíquotes a mesura que té lloc la reacció enzimàtica. Tal com es mostra en els resultats obtinguts en el capítol 6, després de la introducció de l'enzim, la FS i els controls SL+Lacc, LG+Lacc, presenten comportaments molt diferents en l'evolució de la terbolesa i la mida de partícula. En el cas del control SL+Lacc es pot observar com després de la introducció de l'enzim, els valors de transmissió i retrodifusió es mantenen constants, fet que és indicatiu que l'enzim no produeix grans canvis en el lignosulfonat (SL) en termes de

terbolesa i mida de partícula. Observant els resultats per al control LG+Lacc es pot veure com en les primeres 7-8 h es produeix una dràstica disminució en els percentatges de TR i BS, moment a partir del qual ja no es produeix recuperació d'aquests valors durant les 27 h de reacció, mantenint-se en valors molt baixos (per sota del 10%). Aquests valors indiquen la presència de partícules fosques en el producte aquós, donat que la llum no pot travessar la cel·la de mesura, i tampoc es reflexa en el medi, absorbint-se en el sí d'aquest, degut a la forta coloració de les partícules. Pel que fa a la FS, durant les primeres 7-8 h s'observa la mateixa disminució en els percentatges de TR i BS que en el cas del control LG+Lacc, però a diferència d'aquest últim, a partir d'aquest període els percentatges de TR i BS augmenten significativament, i en el cas de la TR arribant fins i tot a valors significativament superiors als dels moments previs a la introducció de l'enzim. Aquest fet és indicatiu de la reducció de la mida de partícula del LG, disminució de la forta coloració, i augment de l'estabilitat. Donat que la única diferència entre el tractament FS i el control LG+Lacc és la presència en el primer del SL, es posa de manifest un cop més l'efecte potenciador de l'enzim d'aquest compost (SL) en l'oxidació, la reducció de la mida de partícula, i l'augment de l'estabilitat. Coneixent la fracció en volum de les partícules en el medi, les mesures de TR i BS es poden relacionar amb el diàmetre mig de les partícules a través de la teoria de Mie; els resultats obtinguts han estat que, al cap de 27 h de reacció, el diàmetre mig de les partícules del tractament LG+Lacc ha estat de l'ordre dels 177 nm, mentre que en el cas del tractament FS aquest diàmetre s'ha vist reduït fins als 81 nm.

10.7. Anàlisi de l'oxidació enzimàtica i l'estructura química del LG modificat per l'enzim

En el capítol 9 de la tesi s'ha volgut profunditzar en el mecanisme d'oxidació enzimàtica i els canvis que la lacasa produeix sobre el compost LG. El compost LG forma part d'una família de compostos anomenats "gal·lats", que són esters derivats de l'àcid gàl·lic. Aquesta família de compostos es caracteritzen per presentar una estructura cap-cua on el cap consisteix en un àcid trihidroxibenzoic amb caràcter polar, i la cua consisteix en una cadena hidrocarbonada amb caràcter apolar de diferent nombre de carbonis, depenent del tipus de gal·lat que es tracti; la diferència entre els gal·lats tan sols ve determinada per la longitud de la cadena hidrocarbonada.

10.7.1. Voltametria cíclica

Per tal de guanyar comprensió sobre l'oxidació enzimàtica del lauril gal·lat i l'efecte del lignosulfonat en aquesta oxidació, s'ha utilitzat la tècnica de la voltametria cíclica en una

sèrie de representants de la família dels gal·lats, incloent el lauril gal·lat. Els compostos estudiats han estat: l'àcid gàl·lic (GA), l'etil gal·lat (EG), el propil gal·lat (PG), l'octil gal·lat (OG) i el lauril gal·lat (LG). L'avantatge d'utilitzar aquesta tècnica és que es pot oxidar els compostos de forma controlada, obtenint-ne informació del voltatge i la corrent generada en els processos d'oxidació-reducció.

Tenint en compte els resultats obtinguts, en primer lloc s'observa (Fig. 9-1) com tots els gal·lats experimenten oxidacions irreversibles, presentant un marcat pic anòdic degut a l'oxidació, però sense la presència de pic catòdic, la qual cosa indicaria reaccions de reducció de les espècies oxidades. L'absència de pics catòdics és una evidència de que les espècies oxidades formades són molt inestables i desapareixen ràpidament a través de reaccions d'acoblament radicalari o bé de la formació de quinones o productes acídics per obertura de l'anell aromàtic (Gess i Dence, 1971; Österberg i Lindström, 2009). Els resultats mostren també com la intensitat del pic anòdic (relacionada amb l'oxidació del compost) decreix de forma gradual a mesura que augmenta la longitud de la cadena hidrocarbonada del gal·lat. Aquest fet podria ser degut a que els gal·lats de cadena llarga presenten una elevada hidrofobicitat i tenen tendència a formar agregats, dificultant-ne l'oxidació a través de l'elèctrode i per tant, disminuint-ne el corrent. Una altra possible raó podria ser l'impediment estèric que presentarien els gal·lats de cadena llarga per a que el cap polar de la molècula (que és el que s'oxida) s'aproximi a la superfície de l'elèctrode, augmentant la resistivitat del medi, i per tant, la transferència d'electrons. Observant els voltamogrames dels gal·lats es pot veure com el GA, EG i PG, presenten un únic pic anòdic, mentre que el OG i el LG en presenten 2. El potencial al que apareix el primer pic anòdic es desplaça cap a l'esquerra a mesura que augmenta la longitud del gal·lat, la qual cosa suggereix que els gal·lats amb cadena llarga s'oxiden a menors potencials.

Per tal de veure si les espècies radicals formades en l'oxidació són estables durant períodes de temps més curts, s'han dut a terme voltamogrames a una velocitat més alta (velocitat d'escaneig de 200 mV/s) per comprovar si a major velocitat es produeix regeneració de les espècies radicals. Els resultats mostren com a major velocitat, tots els gal·lats sofreixen també processos irreversibles, indicant la poca estabilitat de les espècies oxidades.

A continuació s'ha estudiat l'oxidació dels gal·lats en presència del lignosulfonat. En primer lloc, s'observa com el corrent d'oxidació del SL és molt més feble que el corresponent als gal·lats, amb la qual cosa es considera que el SL és electroinactiu en els rangs de potencial estudiats. Per aquest motiu, l'augment del pic anòdic dels compostos PG, OG, i LG es deu a la regeneració (reducció) del gal·lat en la superfície de l'elèctrode

degut a que aquest, està provocant l'oxidació del SL. Encara que els compostos del LG oxidats presenten molt poca estabilitat, tenen un temps de vida suficient com per causar l'oxidació del SL reduint-se ells mateixos.

Encara que l'estudi de voltametria s'ha dut a terme analitzant el comportament d'una sèrie de gal·lats, el major interès se centra en el LG i el sistema LG+SL. En els capítols 4, 5 i 6 es considera que la molècula que s'uneix al suport cel·lulòsic i que en produeix la funcionalització són els productes oxidats del LG, i que el SL ajuda a l'obtenció d'aquests productes oxidats. Observant amb atenció les corbes voltamètriques del LG i del LG+SL (Fig. 9-2) es pot veure a simple vista que el compost LG presenta unes corbes diferents de les de la resta de gal·lats, amb uns corrents d'oxidació inferiors. En la resta de gal·lats (GA, EG, PG i OG), es pot veure com els seus corresponents corrents d'oxidació estan en ordres de magnitud molt diferents, per tant el SL es pot considerar electroinactiu per a aquests compostos. No obstant, per al cas del LG, es pot veure com la corrent d'oxidació d'aquest compost i el corresponent al SL estan en el mateix ordre de magnitud, i ja no és tan evident que per al LG, el SL sigui electroinactiu. L'observació clau consisteix en adonar-se que els corrents d'oxidació del SL i el LG es creuen quan s'arriba a un valor de potencial de 478 mV. Per sota dels 478 mV, el corrent d'oxidació del LG està per sobre del SL, i per sobre d'aquest valor de potencial la tendència s'inverteix. Aquesta observació és clau per a entendre el rol que el SL juga en el procés d'oxidació del LG.

El procés oxidatiu del sistema LG+SL es detalla en el capítol 9, i es podria resumir de la següent manera: per a potencials entre els 0 i els 478 mV, la major espècie oxidada és el LG, catalitzant al seu torn l'oxidació del SL amb una petita regeneració; a partir dels 478 mV, l'augment de la corrent anòdica és degut a l'oxidació del LG a través de la reducció del SL, donat que per aquests potencials el LG és electroinactiu respecte al SL.

L'efecte global sobre el compost LG és que per a potencials baixos, l'oxidació del LG és produïda bàsicament a través de l'elèctrode, amb una petita regeneració causada pel SL; mentre que per a potencials elevats l'elèctrode no és capaç de seguir oxidant les primeres espècies oxidades del LG, i llavors aquesta tasca l'assumeix el SL. L'efecte global és que el SL potencia l'oxidació del LG, actuant precisament en aquells rangs de potencial on l'elèctrode ja no pot seguir oxidant el LG.

A pesar que l'estudi voltamètric ha ajudat a entendre els processos oxidatius del sistema LG+SL, cal tenir present que en aquest estudi es treballa controlant l'evolució del potencial incrementant-lo i disminuint-lo de forma gradual, mentre que l'enzim treballa a un potencial fix. Diversos estudis han reportat el potencial d'oxidació de la lacasa, situant-se aquest a l'entorn dels 700 mV (Morozova *et al.*, 2007; Shleev *et al.*, 2004). Tenint

en compte aquest fet, es pot constatar que la diferència en la forma d'actuar de la lacasa respecte a l'elèctrode de la cel·la voltamètrica és que aquesta aplica directament un potencial de 700 mV, cosa que desencadena tots els processos comentats anteriorment però d'una forma molt més ràpida. En tot cas, com que en els 700 mV la corrent anòdica del SL és superior a la del LG, tindrà tendència a predominar el procés consistent en l'oxidació del LG catalitzada pel SL.

L'estudi voltamètric dels gal·lats i els gal·lats en combinació amb el lignosulfonat s'han dut a terme també a 5 mV/s efectuant dos escanejos consecutius. Els resultats mostren com es produeix una evident disminució de la corrent anòdica en el segon escaneig, probablement degut a la polimerització de les espècies oxidades tant del LG com del SL en la superfície de l'elèctrode, disminuint així la transferència d'electrons. També s'observa que en el segon escaneig el pic anòdic es desplaça cap a la dreta, la qual cosa indica que es necessiten potencials majors per a oxidar el LG, probablement degut a la capa de polímer a la superfície de l'elèctrode.

Els resultats de voltametria cíclica mostren que a mesura que la longitud de la cadena hidrocarbonada del gal·lat augmenta, disminueix el potencial que es requereix per a que aparegui la primera espècie oxidada (Taula 9-1).

10.7.2. Anàlisi FTIR de la biomodificació del LG

Els canvis que l'enzim provoca en l'estructura química del compost LG durant la seva oxidació s'han analitzat mitjançant un estudi de FTIR, complementat amb la informació existent en la bibliografia. Per a tal efecte, s'ha dut a terme l'oxidació enzimàtica del compost LG (en absència de SL) i se n'ha efectuat el corresponent espectre infraroig; així mateix, s'ha obtingut l'espectre infraroig del compost LG sense cap tipus de modificació.

Els resultats de l'anàlisi FTIR (capítol 9) mostren, en primer lloc, que l'aspecte global de l'espectre IR del LG i el del LG modificat enzimàticament és molt diferent. Aquesta diferència és indicativa dels canvis químics que l'enzim provoca durant la reacció. La comparació dels espectres permet afirmar que la zona corresponent als grups metil i metilè de la cadena hidrocarbonada romanen inalterats, la qual cosa indica que l'enzim no provoca la destrucció o modificació de la cadena, mantenint-se aquesta intacta després de la reacció. S'observa com el pic característic de l'ester corresponent al LG no-modificat es veu lleugerament desplaçat cap a freqüències més baixes del que és habitual; aquest fet pot ser degut a la connexió del grup amb l'anell aromàtic, i les interaccions del tipus pont d'hidrogen que la molècula de LG pot experimentar amb altres molècules o dins de la mateixa molècula.

Segons la literatura existent sobre l'oxidació enzimàtica de compostos fenòlics utilitzant enzims, l'acció d'aquest es centra en els fenols (Barreca *et al.*, 2003; Kobayashi, Uyama i Kimura, 2001). D'aquesta manera és probable que l'acció de l'enzim produeixi l'obertura del grup fenòlic del LG produint àcids carboxílics; possiblement aquests àcids carboxílics causin l'aparició del pic que s'observa per al LG modificat a 1718 nm. El pic corresponent a l'ester i el corresponent als àcids carboxílics es mesclen a aquesta longitud d'ona. Una altra evidència de l'obertura de l'anell aromàtic són les alteracions produïdes en l'interval de longituds d'ona de 1600-1450 nm. Els àcids carboxílics formats són grups que tenen una gran tendència a formar enllaços del tipus pont d'hidrogen i estructures dimèriques; aquest fet s'ha detectat gràcies a la reducció, l'eixamplament, i lleuger desplaçament cap a freqüències menors de la corba IR corresponent al LG modificat per l'enzim.

Prenent com a base el mecanisme proposat per Tulyathan, *et al.*, (1989) conjuntament amb la literatura existent, i els resultats de les medicions FTIR, en el capítol 9 es proposen una sèrie de possibles vies de reacció per a l'obtenció dels diversos compostos oxidats del LG. Les possibles espècies químiques resultants de l'oxidació enzimàtica del LG consistirien en productes derivats de l'obertura de l'anell aromàtic (derivats de l'àcid mucònic), lactones, entre altres productes de degradació. En qualsevol cas, tot sembla indicar que l'acció de l'enzim es centra en el grup aromàtic, mentre que la cadena hidrocarbonada es veuria inalterada.

En el producte aquós resultant de l'acció de l'enzim (Lacc+LG) l'escenari més probable és que coexisteixin tant els productes finals de degradació com els productes intermedis. En aquest sentit se sap que les quinones, formades a partir de l'oxidació dels radicals fenoxi, provoquen una forta coloració dels compostos que les contenen (Widsten i Kandelbauer, 2008; Chakar i Ragauskas, 2001); l'observació del producte de reacció (Lacc+LG) evidencia una forta coloració del producte resultant, fet que seria indicatiu de la formació de quinones. L'aspecte visual del producte de reacció, i l'estudi de la interacció de la llum efectuat en el capítol 5 indiquen una gran diferència entre el control Lacc+LG i la FS en termes de coloració. Mentre que el control Lacc+LG presenta una forta coloració, sembla que en la FS aquesta coloració tendeixi a revertir-se o disminuir. Després d'analitzar l'efecte positiu del SL (capítols 4, 5 i 6) potenciant l'oxidació del compost LG, és possible que la reducció de la coloració en el cas de la FS sigui deguda a una major oxidació del compost LG (afavorida per la presència del SL), a través de la reducció de la quantitat d'espècies intermèdies que presenten quinones en la seva estructura.

Quines són les possibles vies d'unió (grafting) entre el LG biomodificat i els suports cel·lulòsics?

Un cop analitzades les possibles vies de modificació química del compost LG a través de l'oxidació enzimàtica, en el capítol 9 s'ha efectuat una sèrie d'hipòtesis relacionades amb les possibles vies d'unió entre el LG bio-modificat i els suports cel·lulòsics. Aquestes hipòtesis s'han realitzat també en base als anàlisis efectuats en els capítols 4, 5 i 6.

A partir de les deduccions obtingudes mitjançant l'anàlisi FTIR, cal tenir en compte que la molècula de LG biomodificada exhibeix un cap hidròfil degut als grups acídics generats en el fenol durant l'oxidació, i una cua hidrofòbica consistent en la cadena hidrocarbonada. Els grups –COOH presenten un marcat caràcter aniònic, la qual cosa provoca una elevada predisposició de la molècula de LG biomodificada per a experimentar interaccions del tipus electroquímic.

D'aquesta manera, una primera possibilitat d'unió seria a través de reaccions d'esterificació. Aquest mecanisme implica necessàriament l'eliminació d'aigua ja sigui per assecat natural a temperatura ambient, o assecat forçat mitjançant algun element calefactor. En condicions de deshidratació existirien dues possibilitats: i) formació directa de l'ester entre els àcids carboxílics del LG bio-modificat i les funcions –OH de la superfície de la cel·lulosa, o bé ii) la formació de l'anhídrid cíclic, obtenint una molècula altament reactiva que provocaria l'esterificació sense necessitat d'aportar calor, actuant de forma similar als anhídrids succínics alquenils (ASAs) utilitzats en l'encolat del paper. La deshidratació és un factor clau per a que tingui lloc la funcionalització a través d'aquest mecanisme. En cas que es produís una esterificació de Fischer, el tampó d'àcid tartàric utilitzat en el tractament enzimàtic actuaria com a catalitzador d'àcid fort en la reacció, potenciant la protonació dels àcids carboxílics. En cas de produir-se aquest tipus d'unió, el cap hidròfil del LG bio-modificat quedaria unit al material cel·lulòsic, mentre que la cua hidrofòbica quedaria orientada cap a l'exterior, la qual cosa provocaria la hidrofobització del material.

Existeix la possibilitat que la molècula de LG bio-modificat i el suport cel·lulòsic sofreixin una interacció del tipus fisicoquímic. No obstant, degut al fort caràcter aniònic del producte LG bio-modificat, és necessari modificar el material cel·lulòsic amb algun tipus de compost que li pugui conferir caràcter catiònic per tal que es produeixi la interacció tal com s'exposa en els capítols 5 i 6. Aquest tipus d'interacció podria semblar a priori feble, però tal com s'ha demostrat en els capítols 4 i 5, després de rentats en condicions severes de temperatura i extraccions amb Soxhlet utilitzant acetona, la propietat hidrofòbica de la cel·lulosa modificada es manté.

Una altra possibilitat per al "grafting" seria a través d'una polimerització a partir de l'obertura de l'anell o "ring opening polymerization (ROP)", en el cas que la molècula de LG resultant del tractament enzimàtic consistís en una lactona, tal com s'exposa en el capítol 9. La polimerització de compostos alifàtics cíclics com les lactones a partir de la ROP ha estat àmpliament descrita en la literatura (Thomas, Peruch i Bibal, 2012; Najemi *et al.*, 2010), així com la funcionalització o "grafting" de materials cel·lulòsics de lactones a través d'aquesta tècnica (Teramoto *et al.*, 2004; Lönnberg *et al.*, 2008; Lönnberg *et al.*, 2006). Per a que aquest tipus d'empelt tingui lloc és necessària la presència d'un monòmer, un iniciador, i un sistema catalític. Donat que generalment en aquest tipus de polimeritzacions s'utilitza un alcohol (o grup hidroxil) com a iniciador, la cel·lulosa en la seva forma nativa és un iniciador multifuncional donada la presència de grups hidroxil en la seva superfície. Generalment, en l'empelt mitjançant la tècnica ROP s'utilitzen catalitzadors metàl·lics, però diversos treballs han reportat que l'àcid tartàric és també un bon catalitzador d'aquest tipus de reaccions. Donat que en el tractament enzimàtic el tampó es prepara precisament utilitzant l'àcid tartàric, és probable que el medi catalític ja sigui l'òptim per a que es produeixi aquest tipus de reacció. L'aplicació de calor també és necessària per a que es produeixin reaccions d'empelt a partir de la ROP; tal com s'ha demostrat en el capítol 4, l'aplicació d'un tractament tèrmic augmenta favorablement la hidrofobicitat del material cel·lulòsic, fet que podria ser explicat a través de l'afavoriment d'aquest tipus de reaccions d'empelt (ROP), entre la lactona derivada del LG bio-modificat i la cel·lulosa.

Una darrera possibilitat consistiria en una combinació dels mecanismes que s'han proposat prèviament, i que estaria caracteritzada per una primera atracció de tipus electroquímic entre les molècules de LG bio-modificat i el suport cel·lulòsic cationitzat, amb una posterior fase on tindrien lloc les reaccions d'esterificació o ROP.

10.8. Anàlisi econòmic i proves en planta pilot

S'han inclòs dos annexes en la present tesi. En el primer es realitza un càlcul orientatiu de les implicacions econòmiques relacionades amb els costos dels productes que intervenen en la preparació de la FS, i en el segon s'analitzen els resultats obtinguts en planta pilot, en els que s'ha utilitzat una size-press per a l'aplicació del producte. En l'Annex A s'han utilitzat una sèrie de suports cel·lulòsics amb diferent morfologia i s'han impregnat amb el producte funcionalitzador FS. L'objectiu ha consistit en determinar les quantitats de FS absorbides per cada tipus de suport i, conegudes les concentracions, poder establir les quantitats de LG, SL i enzim necessàries per tal de funcionalitzar una unitat d'àrea de paper. S'ha estimat la producció diària en fàbrica de paper i seguidament el consum diari

de cada un dels productes utilitzats en funció del tipus de paper. Els resultats obtinguts indiquen que la funcionalització dels papers utilitzant el producte FS incrementaria de l'ordre d'un 10% el cost normal del paper pel que fa al cost dels productes necessaris per a preparar la FS. No s'ha realitzat cap estimació en termes energètics.

Al llarg dels capítols de la tesi s'especula sobre les diverses possibilitats d'aplicació del producte funcionalitzador (FS) sobre els suports cel·lulòsics. Per aquest motiu, en l'annex B s'ha assajat l'aplicació de la FS sobre suports paper comercials utilitzant una size-press en planta pilot. Els resultats han demostrat l'efectivitat del mètode, de forma que s'ha incrementat significativament la hidrofobicitat dels papers en termes de l'angle de contacte i absorció d'aigua. Tant la morfologia dels suports (en termes de porositat i llisor superficial) com la quantitat de FS absorbida per cada suport són factors que afecten al nivell d'hidrofobicitat assolit.

10.9. Conclusió

En la present tesi s'han estudiat mètodes de funcionalització de suports cel·lulòsics ja formats. Els suports més estudiats han estat papers comercials, o obtinguts a nivell de laboratori. En primer lloc s'ha aconseguit la funcionalització no-enzimàtica de suports cel·lulòsics mitjançant ciclodextrines, obtenint suports capaços d'alliberar molècules de principi actiu de forma mantinguda. Seguidament s'ha desenvolupat, amb èxit, un mètode enzimàtic per al tractament superficial de suports cel·lulòsics amb l'objectiu de conferir-los propietats avançades. Les propietats conferides als suports han estat la hidrofobicitat i la capacitat antioxidant. S'ha profunditzat en la caracterització dels suports funcionalitzats, en l'estudi del producte funcionalitzador obtingut, i en les interaccions fisicoquímiques entre el producte i la cel·lulosa mitjançant diverses tècniques d'anàlisi, a partir dels resultats de les quals s'han proposat possibles vies de reacció i/o interacció química. També s'ha realitzat una estimació dels costos de la tècnica i una prova d'aplicació en planta pilot.

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Chapter 11

Main Conclusions

The primary purpose of this doctoral work was to functionalize the surface of cellulose-based substrates with a view to developing high-value products via environmentally friendly processes. A non-enzymatic approach and an enzymatic approach to substrate functionalization were used, albeit with emphasis on the latter.

The non-enzymatic approach involved developing a method to use β-cyclodextrins (β-CD) to confer bactericidal properties to cellulose-based materials (specifically, to finished commercial paper substrates of variable morphology). The method proved effective with all studied substrates and resulted in a weight gain of 7–15% upon β-CD grafting. Also, the β-CD-functionalized substrates exhibited an increased ability to release antiseptic agents over longer periods.

The enzymatic approach involved treating previously formed substrates and was found to increase the hydrophobicity and antioxidant capacity of the substrates. Treatments evolved towards the preparation of a functionalization solution (FS) consisting of an enzymatic product that was subsequently applied to the surface of finished paper. This method was also effective and provided major advantages with a view to its industrial implementation; in fact, it is the subject of an international patent application.

The WDT, WCA, SEM, AFM and SFE techniques were used to characterize treated cellulosic substrates and analytical methods based on DLS, QCM, FTIR, UV-VIS spectra, Z-potential, Turbiscan® and cyclic voltammetry provided a better understanding of the reaction mechanisms and physico–chemical interactions behind the laccase treatments, and also of FS–substrate interactions.

The most relevant results concerning the characteristics of the treated substrates were as follows:

- Surface application of the previously prepared functionalization solution (FS) to cellulosic substrates provided an effective method for developing hydrophobic and antioxidant properties.
- The proposed cellulose-functionalization technique consisting in the obtention of a FS performed similarly well with laccase from different microorganisms.
- Lauryl gallate (LG) was efficiently oxidized by the enzyme —which also oxidized SL, albeit only slightly. Including SL in the treatments had favourable effects such as increased dispersion and hydrophobicity, and enzyme activity preservation. Time, dose and the use of a heat treatment were found to be the key variables for the development of the hydrophobicity in the substrates. Soxhlet extraction and heavy

washing tests confirmed the high bonding strength between enzyme-modified LG and paper fibres.

- Sonication prior to the enzymatic reaction allowed LG aggregates to be efficiently disintegrated, and resulted in improved dispersion of the compound and interaction with the enzyme.
- The cationic character of the sheets was the key to achieving a high level of functionalization by effect of the electrical attraction between negatively charged hydrophobic compounds present in FS and the positively charged sheets.
- Silica and nanofibrillar cellulose (CNF) surfaces were found to provide good model surfaces for assessing FS adsorption by QCM. Adsorption onto model surfaces was enhanced by pre-adsorbing a chitosan layer on the substrates —which confirmed physico–chemical interactions between LG and cationized surfaces. A direct relationship between the amount of LG adsorbed onto silica or nanofibres and WCA values was observed.
- SEM and AFM images of silica and paper sheets treated with FS and the control systems (KFS) revealed the presence of LG particles attached to those surfaces.
- The enzymatic method based on a functionalization solution (FS) is also effective for developing antioxidant properties in cellulosic materials. The resulting antioxidant capacity can be adjusted by using a specific phenolic compound in the enzymatic reaction.
- Hydrophobicity in treated paper sheets was related to the length of the gallate alkyl chain; thus, it increased with increasing length and peaked between octyl gallate (OG) and lauryl gallate (LG). The curing treatments substantially increased hydrophobicity irrespective of chain length.
- Antioxidant capacity in treated PS also increased with increasing chain length of the gallate —this variable appears to be more influential than the presence of many aromatic rings in the phenolic compound. The curing treatments had no significant effect on the antioxidant capacity of treated PS.

The most relevant results regarding the studies on the characterization of the functionalization solution were as follows:

- In the FS preparation process, the enzyme reduced the particle size of LG from several microns down to 300 nm; also, the presence of SL enhanced the effect by reducing

particle size even further (to 80 nm). All effects of the enzyme on LG particle size were confirmed by light transmission and backscattering measurements.

- Residual enzyme activity measurements confirmed the favourable effect of including SL in the treatments —it helped preserve enzyme activity.
- Chemical oxidation of GA, EG, PG, OG and LG by cyclic voltammetry revealed that SL enhanced the oxidation of LG at high potentials (above 478 mV). Chemically, enzyme-modified LG consisted mainly of open-ring acidic products susceptible to oxidation via various oxidation pathways; however, the hydrocarbon chain of the phenolic compound remained unaltered as confirmed by FTIR analysis.
- As confirmed by FTIR-ATR analysis, the alkyl chain of the gallates remained unaltered upon enzymatic modification, which, however, caused major changes in the peaks corresponding to the phenolic structure.
- FS and the LG+Lacc treatment proved effective in developing antioxidant capacity in PS; however, neither solution exhibited antioxidant properties by itself.
- Once the possible chemical structure of the resulting enzyme-oxidized molecules was analyzed, three potential grafting mechanisms of enzyme-modified compounds to cellulose were proposed.

Annex A

Cellulose sheets FS Uptake Determination

Summary

The functionalization of cellulose-based materials by means of the enzymatic product FS is produced upon surface treatment of cellulosic sheets. Several industrial devices such as size presses, metering bars or sprayers are available for this purpose. The following rough approximations aim to estimate the product costs of the functionalization of paper products using the FS, by means of impregnation. The objective is to acquire an idea about the increase in price produced by the treatment of paper materials with the FS.

Annex A.1. Materials and methods

Annex A.1.1. Paper, enzyme and chemicals

Filter paper sheets (62 g/m^2) were supplied by FILTERLAB® (Sant Pere de Riudebitlles, Barcelona, Spain). Eucalyptus paper sheets (77 g/m^2) were obtained from eucalyptus ECF pulp supplied by ENCE® (Spain) using a Rapid-Köten lab former according to ISO 5269-2:2004. Kraft liner paper (148 g/m^2) was obtained from CARTISA® (Castellbisbal-Barcelona, Spain). Office paper (76 g/m^2) from HP (Hewlett Packard) was purchased from stationery store. The enzyme used in this work was a laccase from *Trametes villosa* (Tv) with an activity of 588 U/mL, supplied by Novozymes (Bagsvaerd, Denmark. Lauryl gallate (LG) was purchased from Sigma–Aldrich. Soluble sulfonated kraft lignin (SL) was supplied by Borreegard (Sarpsborg, Norway), and used as received.

Annex A.1.2. FS product preparation

The enzymatic reaction was performed in the absence of paper sheets (PS) and the resulting functionalization solution (FS), used to impregnate paper sheets by dipping. Enzymatic treatments were performed using 250 mL beakers containing final concentrations equivalent to 50 mM sodium tartrate buffer (pH 4), 1.2 g/L LG, 1.2 g/L lignosulfonate and 1.2 U/mL laccase. LG was used as a colloid suspension obtained by sonication. Reaction conditions were 50 ℃, 4h reaction time, and pH 4.

Annex A.1.3. FS uptake calculation

The considered papers were an office paper, a filter paper, a Kraft liner paper and laboratory-made paper from eucalyptus ECF pulp. The quantity of FS absorbed by papers (Functionalization Solution Uptake = FSU) was calculated by mass difference. Tests were conducted as follows:

1. Cut papers into 10x10 cm^2 specimens
2. Record the weight of the specimen before FS uptake (g1)
3. Impregnate the specimen by immersion in the FS for 3 seconds.
4. Remove quickly the specimen from the FS, place it between two blotting papers, and press the set with two passes using a 2kg weight roller.
5. Record the weight of the specimen after the impregnation (g2).

The tests were conducted under a normalized atmosphere (23℃, 50% RH). 10 specimens of each paper grade were tested. A scheme of the procedure is shown in the Fig. Annex A-1.

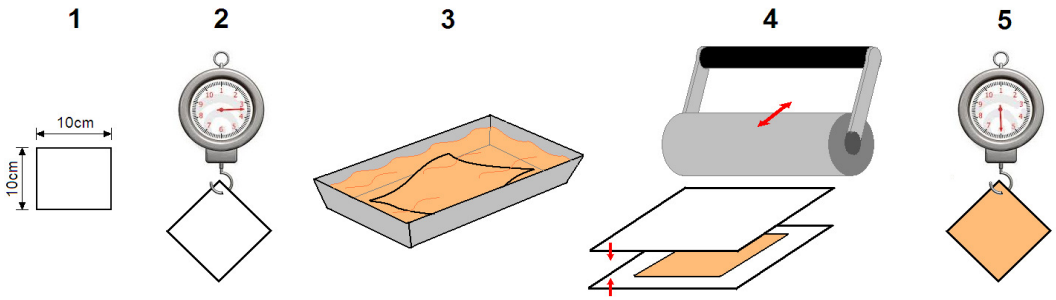

Figure Annex A-1 Schematic representation of the procedure to determine the weight of the specimen before (g1) and after (g2) the FS uptake.

The mass gain was calculated by the difference g2-g1, and divided by the area (A=0.01 m^2) of the sheet to calculate the FSU in g/m^2:

$$FSU = (g2 - g1)/A \qquad \text{[Annex A-1]}$$

Annex A.2. Results and discussion

Annex A.2.1. FS uptake

The initial grammage and the mass gain due the uptake of FS for each paper grade are shown in Fig. Annex A-2. As it can be seen the FS uptake is strongly dependent on the type of paper considered.

The parameters affecting the FSU are the same ones that those which affect the absorption of water, since the FS presented a liquid consistency with rheological properties similar to water. In this sense the pore distribution and bulk are more important than the grammage. For example, if papers show a coated layer which clogs the fibrous structure it is not possible to the FS to completely impregnate the structure, as it is shown for the case of the office paper, which shows the lowest FSU. The paper which showed the highest FSU was the Eucalyptus paper. Comparing the Kraft liner and the Eucalyptus papers, we can realize that the FSU was higher for the eucalyptus paper although its grammage is lower.

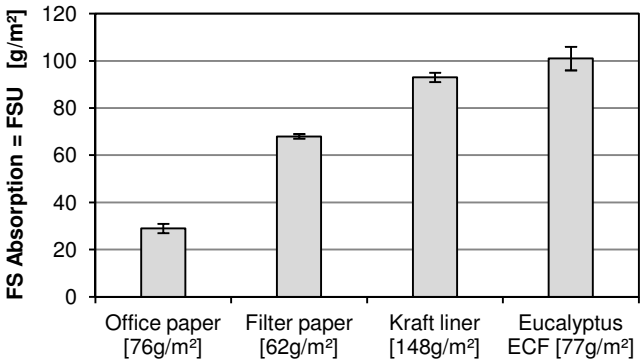

Figure Annex A-2 Initial grammage and mass gain due to the uptake of FS for each paper grade (in g/m²).

Annex A.2.2. FS consumption and costs

The following calculations are done considering the filter paper as example, and analogous proceeding was used for the other paper grades. First, a bibliographic study was conducted in order to estimate the commercial prices of the compounds. The approximate prices of the products are detailed in the table Annex A-1.

Table Annex A-1 Commercial prices of the compounds used to prepare the FS.

Product	Price	Supplier
LG	~ 20 €/Kg	Shangqiu Kangmeida Bio-Technology Co., Ltd.
SL	~ 0.3 €/Kg	Taian Yonghe Chemical Co., Ltd.
Enzyme (*T.Villosa*)	~ 2.5x10-5 €/U	Novozymes®

Although the LG compound is not soluble in aqueous mediums, after sonication and treatment with the enzyme it is completely solubilized. The FS results in a completely stable and homogeneous liquid, which density was calculated using a micropipette and a precision balance resulting in a density of 1.009±0.006 g/mL.

Taking into account the total uptake of the FS product and the concentration of each compound, the surface distribution of the products can be calculated as follows:

$$Sd = FSU \times \frac{1}{\delta_{FS}} \times C \qquad \text{[Annex A-2]}$$

Where:

Sd = Surface distribution [g/m²]

FSU = Functionalisation Solution Uptake [g/m²]

δ_{FS} = Density of the FS [g/mL]
C = Concentration of the product in the FS [g/mL]

The resulting surface distribution of the compounds is:

For LG and SL:

$$Sd = 68\frac{g}{m^2} \times \frac{1mL}{1g} \times \frac{1.2x10^{-3}g}{mL} = 0.0816\frac{g}{m^2} \quad \text{[Annex A-3]}$$

For the enzyme:

$$Sd = 68\frac{g}{m^2} \times \frac{1mL}{1g} \times \frac{1.2U}{mL} = 81.6\frac{U}{m^2} \quad \text{[Annex A-4]}$$

Considering a paper mill with a paper machine which runs at 1000m/min, with 3 working shifts, manufacturing a 2 m web, the daily consumption of compounds can be calculated as follows:

$$Daily\ consumption = \frac{V \times 60 \times Wh \times PWW \times Sd}{1000} \quad \text{[Annex A-5]}$$

Where:
V = Speed of the paper machine [m/min]
Wh = Working hours x day [h/day]
PWW = Paper web width [m]
Sd = Surface distribution [g/m^2]

The resulting daily consumption of the compounds is:

For LG and SL:

$$Daily\ consumption = 1000\frac{m}{min} \times 60\frac{min}{1h} \times \frac{24h}{day} \times 2m \times 0.0816\frac{g}{m^2} \times \frac{1Kg}{1000g} = 235\frac{Kg}{day} \quad \text{[Annex A-6]}$$

For the enzyme:

$$Daily\ consumption = 1000\frac{m}{min} \times 60\frac{min}{1h} \times \frac{24h}{day} \times 2m \times 81.6\frac{U}{m^2} = 235008000\frac{U}{day} \quad \text{[Annex A-7]}$$

The daily costs of each product and the product price per kilogram of manufactured paper for the all the considered paper grades are summarized in the table Annex A-2.

Table Annex A-2 Daily consumption, daily costs and costs per Kg of manufactured paper of each compound present in the FS for each one of the studied papers.

	Daily consumption	Daily cost	Cost per Kg of manufactured paper
Office paper			
LG	100.2 Kg/day	2004.5 €/day	0.0092 €/Kg
SL	100.2 Kg/day	30.1 €/day	0.0001 €/Kg
Enzyme	100.2 Kg/day	2505.6 €/day	0.01 €/Kg
Filter paper			
LG	235.0 Kg/day	4700.2 €/day	0.0263 €/Kg
SL	235.0 Kg/day	70.5 €/day	0.0004 €/Kg
Enzyme	235.0 Kg/day	5875.2 €/day	0.03 €/Kg
Kraft liner			
LG	321.4 Kg/day	6428.2 €/day	0.0151 €/Kg
SL	321.4 Kg/day	96.4 €/day	0.0002 €/Kg
Enzyme	321.4 Kg/day	8035.2 €/day	0.02 €/Kg
Eucalyptus ECF			
LG	349.1 Kg/day	6981.1 €/day	0.0005 €/Kg
SL	349.1 Kg/day	104.7 €/day	0.04 €/Kg
Enzyme	349.1 Kg/day	8726.4 €/day	0.0005 €/Kg

The most expensive paper to be functionalized using the FS was the Eucalyptus ECF paper, followed by the kraft liner paper, the filter paper, and finally the office paper which was the cheapest one. Results show how the FSU of the papers is directly related to the final costs; higher the FSU, higher the cost. However the grammage of the substrate seems not to be related to the final cost.

Taking into account the FOEX® indexes about the commercial prices of the printing and writing grade papers, the approximate price is located around the 0.7 €/Kg. Therefore, since the cost of the functionalization of papers (in terms of the costs of the products needed to prepare the FS) using the FS is approximately 0.06 €/Kg, we can conclude that the increase in the price of paper produced by the treatment with the FS is located around the 9%. On the other hand, the cost of the energy and the equipment needed to prepare the FS was not calculated, but needs also to be considered to acquire the whole picture.

The increase in the common paper price produced by the treatment with the FS may appear to be high, but it is important to take into account that this treatment produces a high value-added product (specialty paper) that could be sold at higher prices in the market. Additionally, the present study was conducted by treating the PS by an impregnation method, but there exist other ways to apply the FS product that may reduce the FS amounts to treat a given area of substrate, reducing therefore the costs.

Annex A.3. Conclusions

The present study estimates the product costs of the functionalization of paper products using the FS, by means of impregnation. Several cellulosic supports (papers) presenting different grammages were treated, and the costs per square meter (related to the costs of the products needed to prepare the FS) was estimated for each support. Results showed that the functionalization of papers using the FS produces an increase on the common paper price around the 9%. However, this increase would be balanced due the higher selling prices of the specialty papers.

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Annex B

Size Press Pilot Plant Trials

Summary

In the present thesis, the procedure to apply the FS to the paper sheets consisted in a manual impregnation method. However, possibility to apply the FS using industrial devices is mentioned elsewhere. The size-press device is an interesting technique since it is widely used in the paper processing industry. In order to validate the feasibility to implement the FS product in industrial processes, the R&D centre of TORRASPAPEL® was involved in some pilot trials. The goal was to explore the actual potential of the FS product to effectively hydrophobize paper substrates when applied to the sheets using industrial equipment. A commercial filter paper and two printing and writing grade papers were used in the experiments. The FS product was prepared using the same doses and conditions as the ones found in the present thesis to be the optimum to obtain the highest hydrophobic response.

Annex B.1. Materials and methods

Annex B.1.1. Paper, enzyme and chemicals

Filter paper (62 g/m^2) was supplied by FILTERLAB® (Sant Pere de Riudebitlles, Barcelona, Spain). Printing and writing grade papers Torrasp-1 (67 g/m^2) and Torrasp-2 (46 g/m^2) were both supplied by TORRASPAPEL®. The enzyme used in this work was a laccase from *Trametes villosa* (Tv) with an activity of 588 U/mL, supplied by Novozymes (Bagsvaerd, Denmark). Lauryl gallate (LG) was purchased from Sigma–Aldrich. Soluble sulfonated kraft lignin (SL) was supplied by Borreegard (Sarpsborg, Norway), and used as received.

Annex B.1.2. FS product preparation

The enzymatic reaction was performed in the absence of paper sheets (PS) and the resulting functionalization solution (FS), used to treat the paper sheets by size-press. Enzymatic treatments were performed using a 5000mL agitated reactors containing final concentrations equivalent to 50 mM sodium tartrate buffer (pH 4), 1.2 g/L LG, 1.2 g/L lignosulfonate and 1.2 U/mL laccase. LG was used as a colloid suspension obtained by sonication. Reaction conditions were 50 ℃, 4h reaction time, and pH 4.

Annex B.1.3. FS uptake calculation

The quantity of FS absorbed by papers (Functionalization Solution Uptake = FSU) was calculated by mass difference. Tests were conducted as follows:

6. Cut paper sheets and record the area of the specimens (A).
7. Record the weight of each specimen before FS uptake (g1)
8. Treat the specimen by size-press using the FS.
9. Record the weight of each specimen after the size-press FS uptake (g2)
10. Remove quickly the specimen from the size-press and dry the sheet using a stainless steel drum drier at 60 ℃.

The mass gain was calculated by the difference g2-g1, and divided by the area (A=0.01 m^2) of the sheet to calculate the FSU in g/m^2:

$$FSU = (\mathrm{g2} - \mathrm{g1})/\mathrm{A} \qquad \text{[Annex B-1]}$$

Annex B.1.4. Size-press treatments

Size-press treatments were performed using pilot scale size-press equipment in the R&D centre of TORRASPAPEL®. The size press machine was operated at a velocity of 5m/min with a pressure between the cylinders of 1.5 bar. The sheets were introduced to the size press nip and collected for drying using a stainless steel drum drier at 60℃.

Annex B.1.5. Hydrophobicity assessment

Contact angles (WCA) were measured with a Dataphysics® OCA15 contact angle goniometer, using a 4 μL water drop for delivery to the sample surface in each measurement. The evolution of the contact angle was monitored until complete absorption of each water drop. The $Cobb_{60}$ value was calculated following the ISO 535 standard.

Annex B.2. Results and discussion

Annex B.2.1. Size-press treatments

A schematic representation of the size-press technique is shown in Fig. Annex B-1. The purpose of a size press on a paper machine is to apply a liquid solution onto the surface of the dry paper, after which the paper is dried again. The machine runs by flooding the entire nip between the two rollers of the size press with the liquid solution; the paper absorbs some of the solution and the balance is removed in the nip.

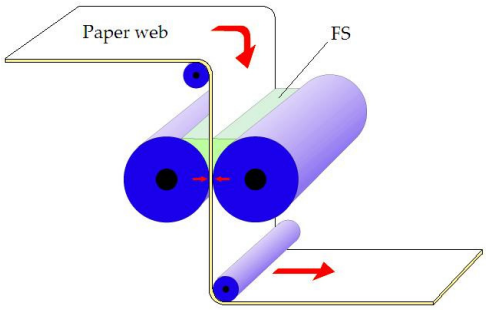

Figure Annex B-1 Schematic representation of the industrial size-press technique used to apply liquids to the surface of already formed paper sheets.

The rolls are dynamically balanced on demanded speed and can be covered with different coats (rubber, ceramics) depending on the produced kind of paper and the rheological properties of the liquid agent. The paper surface treatment by size press is

commonly used to achieve the paper sizing by applying a solution of starch or other material onto the surface of paper. The main objectives are to increase the surface strength, improve coating and ink holdout, reduce dusting tendency, increase the stiffness, or to reduce the air-permeability.

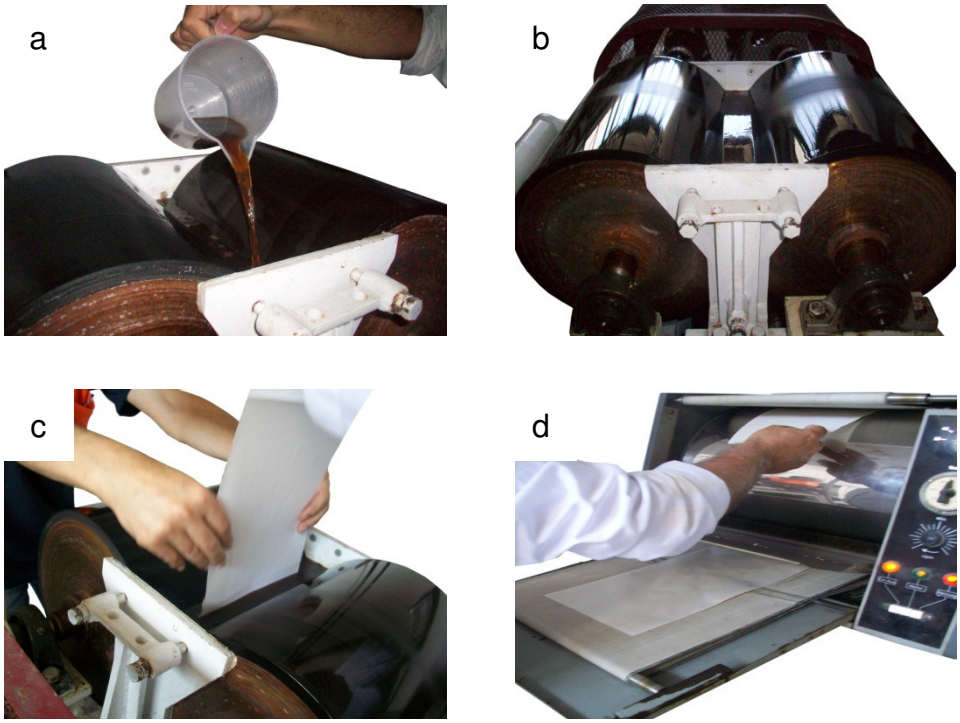

Figure Annex B-2 Several images describing the size press process. Loading the nip with the FS (a and b), introducing the paper sheet to the device for the treatment (c), and drying the sheet with the stainless steel drum drier (d).

The parameters affecting the FSU are the same ones that those affecting the absorption of water, since the FS presented similar rheological properties. In this sense, the pore distribution and bulk are more important than the grammage. For example, if papers present a coated layer which clogs the fibrous structure it is not possible for the FS to completely impregnate the structure, as it is shown for the case of the office paper, which shows the lowest FSU. The paper which showed the highest FSU was the Eucalyptus paper. Comparing the Kraft liner and the Eucalyptus papers, we can realize that the FSU was higher for the eucalyptus paper although its grammage is lower (see Annex A).

Annex B.2.2. Size-press treated sheets characterization

The size-press-treated paper sheets (Fig. Annex B-2), were characterized in terms of hydrophobicity by measuring its initial contact angle, the $Cobb_{60}$ value, and monitorizing the evolution of the contact angle trough time. Figure Annex B-3 shows the differences in terms of the initial contact angle and $Cobb_{60}$ between the non-treated and the size-press-treated papers.

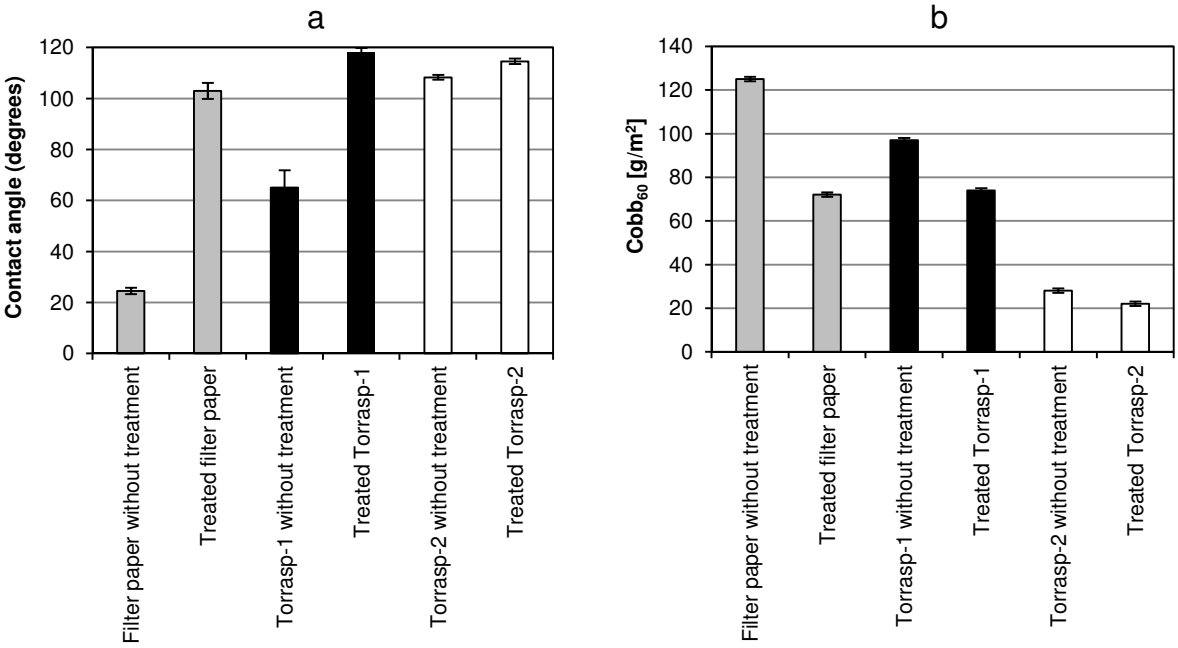

Figure Annex B-3 Contact angles (a) and $Cobb_{60}$ values (b) of the non-treated and the size-press-treated papers.

The three papers presented different initial contact angles (25°, 65° and 108°) when they were not treated with the FS. However, after the size-press treatment the contact angles of all papers raised up to above the 100°. All the substrates increased their contact angle upon treatment, being the filter paper the one in which the increase was more significant. Results demonstrated that the FS was able to develop the hydrophobic property of commercial-grade papers when it was applied using a size-press device at pilot scale. The $Cobb_{60}$ value is more related to the sizing of the papers rather the surface energy, which is better characterized by the water contact angle. Since the $Cobb_{60}$ value is related to the sheet's water absorption ability, the lower the $Cobb_{60}$ value, the higher the sizing of paper. The $Cobb_{60}$ value follows the same tendency as observed for the WCA. The filter

paper is the most absorptive paper, with a $Cobb_{60}$ value of 125 g/m², followed by the Torrasp-1 (97 g/m²) and Torrasp-2 (28 g/m²). All the substrates decreased their $Cobb_{60}$ upon treatment. In these terms, the major differences between the non-treated and treated papers were observed for the filter paper, followed by the Torrasp-1 and finally the Torrasp-2. The increase in the contact angle upon treatment for each paper can be related to the FS uptake, as it is shown in Fig. Annex B-4. It can be seen how the higher the absorbed amounts of FS, the higher the increase in the contact angle. The Torrasp-2 was the paper which presented the highest contact angle without treatment (108°), while the non-treated filter paper presented the lowest contact angle (25°). However, the FS absorption was much higher for the filter paper, producing an increase on the WCA around the 325% compared to the 5% for the Torrasp-2.

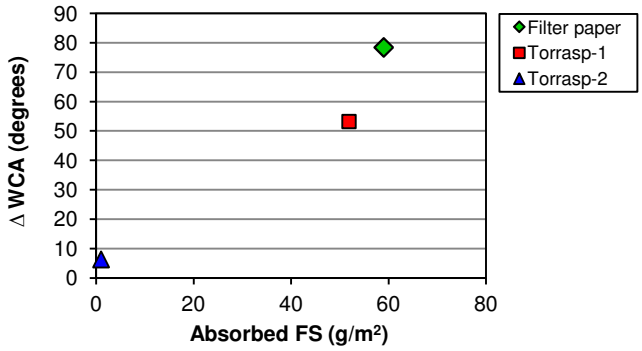

Figure Annex B-4 Increase in the contact angle vs. the amounts of FS absorbed by the three paper substrates.

The WCA measurements provide information on the surface energy of the substrates rather than the water absorption behavior. In order to better understand the effect of the FS-size-press treatment on the absorption behavior, the evolution of the contact angle was measured in each paper. Figure Annex B-5 shows the evolution of the WCA for the non-treated and treated papers. It can be seen how the non-treated sheets presented differences in the absorption rate; the absorption was very fast for the filter paper sheets (less than 1 s) while for the Torrasp-2 it took up to 30 minutes to the drop to be completely absorbed. This result was obvious taking into account the morphology of the surface of the papers. The filter paper presented an open-pore structure while the Torrasp-2 presented a closed structure with a fine coating layer which clogs the pores and hinders the water absorption. Considering the absorption curves for the size-press-FS-treated sheets, the absorption time was significantly increased in all the substrates and

the absorption rate was slowed-down. The absorption time was increased by a 79% for the filter paper, by a 240% for the Torrasp-1 paper, and by a 27% for the Torrasp-2 paper. The FS uptake upon the size-press treatment seems not to be related with the decrease in the water absorption rate, since the paper which rendered the highest absorption times (Torrasp-1) was not the one which absorbs the highest amounts of FS.

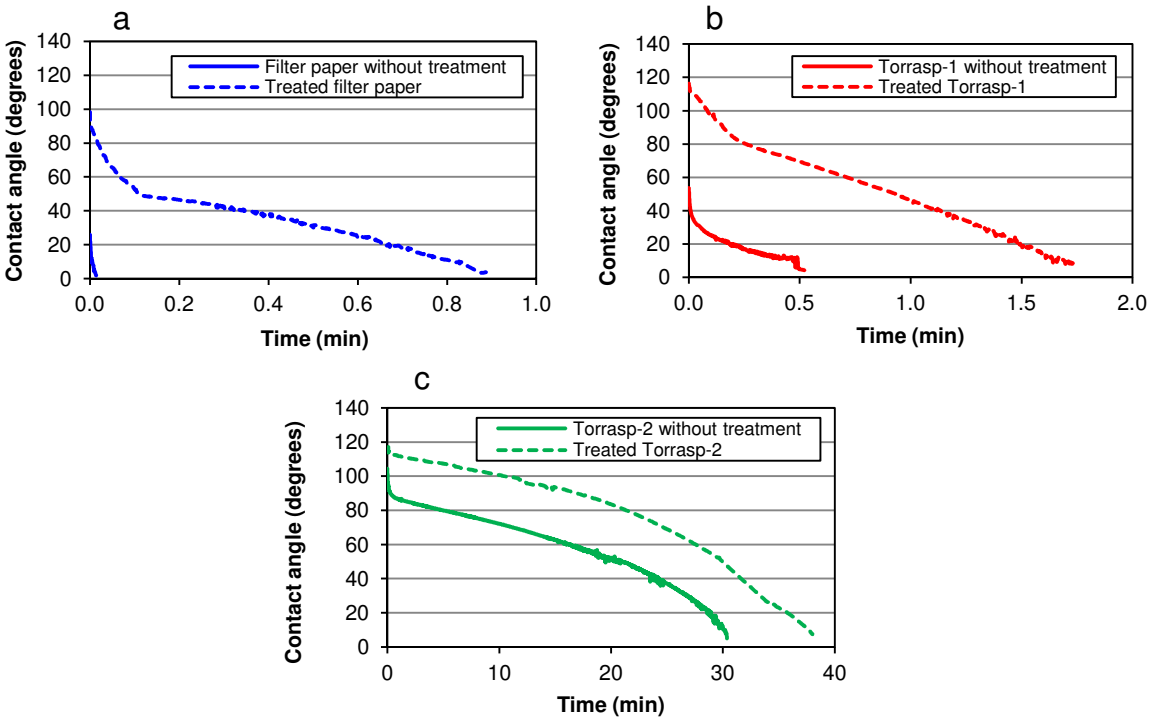

Figure Annex B-5 Evolution of the WCA for the non-treated and treated papers. Key: Filter paper (a), Torrasp-1 (b) and Torrasp-2 (c).

Annex B.2.3. Cost of the size-press treated sheets

The product costs of the functionalization of papers by size-press with the FS were also calculated. The costs were calculated using the grammage and the FSU, and considering the same approximations as in Annex A. The obtained costs are shown in the table Annex B-1.

Table Annex B-1 Costs per Kg of manufactured paper of the FS-functionalizaton by size-press applied to the filter and Torraspapel® papers.

Paper	Grammage [g/m²]	FSU [g/m²]	Total product costs [€/kg]
Filter paper	58	59	0.0553
Torrasp-1	67	52	0.0422
Torrasp-2	46	1	0.0012

Annex B.2.4. Comparison between impregnation and size-press in terms of $Cobb_{60}$

The $Cobb_{60}$ value of the manually-impregnated and size-pressed filter paper sheets was compared. The FS-treated filter paper sheets when the FS was applied by impregnation provided a $Cobb_{60}$ value of 22 g/m², while the $Cobb_{60}$ of the sheets when the FS was applied by size-press achieved a value of 72 g/m². Since the FSU is a key parameter in the development of the hydrophobicity of the treated sheets, this difference may be due to the divergence on the FS amounts absorbed by the filter paper by the two methods. The FSU for the impregnated sheets was 68 g/m², while for the size-pressed sheets it was 59 g/m². However, in the size-press technique there are a lot of parameters that could affect the final hydrophobic property of papers. In this sense the speed or the pressure between the rollers, are parameters that would need further study.

Annex B.3. Conclusions

The results indicated that the application of the FS using the size-press device was effective in developing the hydrophobic property of the treated surfaces. The hydrophobic effect (in terms of contact angle and absorption) of the size-press-treated substrates was dependent on the amounts of absorbed FS, and it was strongly dependent on the surface morphology of the paper sheet. Results indicated that the industrial implementation of the FS product would be effective.

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Standard Methods

ISO 5269-2:2004	Preparation of laboratory sheets for physical testing - Part 2: Rapid-Köthen method.
ISO13321:1996	Particle size analysis - Photon correlation spectroscopy.
ISO 187:1990	Paper, board and pulps - Standard atmosphere for conditioning and testing and procedure for monitoring the atmosphere and conditioning of samples.
ISO 5636-3:1992	Paper and board - Determination of air permeance (medium range) - Part 3: Bendtsen method.
ISO 2758:2001	Paper - Determination of bursting strength.
ISO 1974:1990	Paper - Determination of tearing resistance (Elmendorf method).
ISO 5626:1993	Paper - Determination of folding endurance.
ISO 1924-2:2008	Paper and board - Determination of tensile properties - Part 2: Constant rate of elongation method (20 mm/min).
ISO 3781:2011	Paper and board - Determination of tensile strength after immersion in water.
ISO 535:1991	Paper and board - Determination of water absorptiveness - Cobb method.
ISO 15361:2000	Pulps - Determination of zero-span tensile strength, wet or dry.
ISO 5264-1:1979	Pulps - Laboratory beating - Part 1: Valley beater method.
T835 om-08	Water absorption of corrugating medium: water drop absorption test.