Universitat Autònoma de Barcelona

A novel approach for tooth whitening: liposome-encapsulated reductant. In vitro efficacy studies and characterization of the treated teeth including synchrotron techniques

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Tesi Doctoral

Programa de Doctorat en Química

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2020

This PhD thesis has been carried out in the Centre GTS (Grup de Tècniques de Separació en Química) at the Chemistry Department of the Universitat Autònoma de Barcelona, under the supervision of Professor Manuel Valiente and Dr. Maria Jesús Sánchez Martín.

Professor Manuel Valiente Malmagro

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العِلمُ نور والجَهلُ ظَلام

"La ciencia es luz y la ignorancia es oscuridad"

Abdelhamid Ben Badis

AGRAÏMENTS

Estava fent una llista per no deixar-me a ningú i m'acabo d'adonar que en aquest 5 anys he conegut a moltissima gent, i que realment han sigut molt intensos. Crec que he aprofitat al màxim cada segon, absrobint coneixements i vivències una darrere l'altre, i he après i he canviat molt més del que mai m'hagués esperat. Tot gràcies a vosaltres.

Començaré els agraïments pels memebres del Grup de Tècniques de Separació (GTS), "Gordos Tragones Sebosos (GTS)" para los amigos.

Manolo (catedrático, asignador de rombos): muchas gracias por darme la oportunidad de formar parte de este grupo y por todo el apoyo como director en este largo camino. Es mérito tuyo que, después de muchos "dental Hubs", al final la Farmacéutica no se conforme con una explicación simple sobre las cosas, sinó a que piense un poco más allá. Bueno, ¡y no solo he aprendido de química! Como todo el que pasa por GTS, me voy mi título de máster en cortar jamón.

Cris (Professora, "Que nice!"): va ser molt xulo fer experiments juntes al laboratori d'INTEMA, i per cert, he integrat la teva tènica per enrasar els matrassos aforats. També em ve al cap que vam compartir una de les experiències més impressionants que he viscut, que va ser quan ens van portar en aquella barqueta a posar-nos a sota d'una de les cascades del Iguazú; em vaig quedar per uns moments sense respiració.

Montse (Professora, especialista en cálculo -renal-): trobaré a faltar els dinars de tupper al menjador, quan sempre no sé per què acabavem parlant d'anècdotes absurdes. Gràcies per tota l'ajuda amb els dubtes de les classes durant els primers anys, també amb els articles i a prende decisions importants.

Mari (ex-postdoc, gestora de proyectos y de minions de dientes): de las primeras personas que conocí en GTS y con la que seguramente he compartido más cosas. Sería el destino, porque cuando llegué ya te enchufaron el marrón de tener que ser mi guía los primeros días; y luego resulta que acabaste siendo mi co-directora de tesis, y yo tu primer doctorando! Cuantos inventos locos hemos hecho y lo bien que han salido :P Gran parte de este trabajo es gracias a ti, a tus ideas, a todo el tiempo que has dedicado a resolverme mil dudas y a corregirme cientos de proposals y papers. Y no nos dejemos lo más improtante... que hemos sido compañeras eternas de cervezas, desde el minuto 1!! Cuando salimos juntas no se que pasa pero siempre tengo una birra llena en la mano... Por cierto, nos ha faltado irnos juntas de estancia! Y esto de la cerveza me hace pensar en vino...

Gus (gestor de proyectos, guía espirtirual y un poco Nostradamus): Muy serio pareces al principio... y luego resulta que eres la persona con la que más ataques de risa me han dado en GTS. Creo que la más emblemática fué la del Mortadelo. Otra cosa bastante característica es que te metieras con mi comida, o conmigo en general, para que me pusiera roja y llamarme "gusiluz". En fin, a parte de las bromas, puedo decir que me has dado siempre mucho apoyo y muy buenos consejos en momentos de crisis. Espero que no perdamos la buena tradición de ir de vinos con Mari (y Vero!).

Vero (ex-PhD student, now Dra. Verdugo, la chica que a veces necesita "un whiskazo"): cuando pienso en ti también pienso en cerveza... pero sobretodo en conciertos! Y en Hijos de Caín, y en las locuras con Ana! Y en cuando me quedaba en vuestro piso a dormir si salíamos por Cerdanyola. Me acuerdo que tu tambíen te quedaste una vez a dormir en mi casa de Poblenou, aunque poco dormimos porque nos tocó levantarnos a las 7 de la mañana para ir a colgar pósters en el ISMEC, y no fué tarea fácil las condiciones en las que estábamos. Bueno, y también me viene a la memoria que siempre venia al lab A a preguntarte cosas, dudas de problemas, que si donde está este reactivo, que si como funciona la centrífuga... en fin, gracias por todo!!

JingJing (former PhD student "just for fun", now dr. Zhao): since the very first moment I knew we will be very good friends! We started the PhD together and this gave us this special complicity in the first moment. And then you always signed up for every plan we made, concerts, hikings, travels... you even came to help me paint my house when I moved! We have experienced a lot together, we have also laughed a lot because I think we like the same jokes, and I have always thought that if we would speak the same language we would realize that we are a lot alike. I feel also very lucky because of you I started to learn about Chinese culture, customs, food (making dumplings!).... which are amazing, and now I can't wait to visit you there.

Albert (ex-postdoc en la UAB, ahora postdoc pero con un croissant de mantequilla en la mano y una boina): Sort que estaves tu al principi a Biomat i ens feiem companyia, perquè allò estava més desert que un seminari del departament sense assistència obligatòria. Em venen al cap bastantes anècdotes concretes com per

exemple el rot, o allò de que m'acosto a preguntar-te una cosa i anaves sense pantalons. O quan fugies del lab perquè t'ofegava amb el dissolvent de la resina i amb la olor de mandíbules podrides. Tonteries a part, m'has donat moltissims consells i m'has resolt moltissims dubtes, i sempre tenies una estona per ajudar-me.

Mon (Safety and Quality Manager, profesional de los secondments, organizadora de welcomes): Quantes hores hem passat juntes??? Les nostres aventures a Argentina/Chile son impagables, podria mencionar mil anècdotes: "eso es lo que venimos a ver?", el restaurante japonés con Olga, happy hour en Antares, el mercadillo de San Telmo, el ajedrez gigante, también alfajores gigantes. I les nostres converses... han sigut vitals! Tots aquests anys m'has ensenyat moltes coses, tant professionals com personals.

Sandra (PhD student, madre de unicornios, reina de Biomat): ¡qué ilusión cuando me dijeron que venía "una de Burgos" a cepillar dientes! Lo que no podia imaginar era encontrarme con tal personaje temerario. Siempre tentando a la muerte, como cuando fuimos a Estocolmo y a la que me despisto un momento estabas caminando sobre el mar, y yo pensando en cómo sacarte si te hundías en el hielo. Aunque bueno, visto que puedes nadar en medio del hielo como si estuvieras en el jakuzzi del SAF, pues igual no me habria preocupado tanto. Menos mal que no te vi tirándote del puente. Pero suerte que apareciste por aquí, porque qué habría hecho sin ti cortando mandíbulas, tantas horas en el SEM, o sin canciones como "sandra enfría sandra enfría" en MIRAS, o bueno, en mil situaciones más. Por cierto, quiero una copia de la libreta de frases.

Jorge (PhD student, "¡madre miaaaa...!"): muchas cosas podría decir, pero creo que la mejor es "sodium chloride". También puedo decir que eres una persona muy sincera, cosa que se aprecia mucho, sobretodo jugando al "yo nunca" ;) Creo que te sabes esta tesis mejor que yo, porque diría que la mayor parte de los experimentos que hay aquí los hemos hecho juntos. ¿Y lo bien que nos lo hemos pasado haciendo liposomas hasta las 11 de la noche, puliendo y cepillando dientes, y midiendo con el colorímetro (que en paz descanse)?

Iris (PhD student, fan de las "Humanstones"): siempre me acordaré de que te conocí mientras pesabas palomitas. También de tu célebre frase "Albert! Sal de la zona del caos!!!" Y de las míticas sesiones musicales de heavy en el tiempo que compartimos despacho en el subzulo. Muchas gracias por todos los consejos y brainstormings, y por todos los problemas que me has resuelto :)

Ting Ting (ex-PhD student, now doctor Xiao, always hungry): you have given us so many funny moments that I don't even know which one to name. I really love the way you are, always really active and happy, and always making funny ironic jokes. And you are the best cooking tea eggs, they are so delicious. I can't wait to see you again!

Mangi (ex-PhD student, now Dr. Subirana, coleccionista de rombos): quan vaig arribar estaves d'estància, però la gent m'anava repetint que ens portariem bé perquè teniem interessos comuns (crec que es referien a la cervesa) :P i no s'equivocaven! Em venen al cap conçerts, l'Olut i tot els cops hem envaït casa teva a Gavà. També recordo molts sincrotrons, sort de la teva ajuda amb els papeleos i les proposals, i amb el test infernal aquell online de seguretat tant divertit xD.

Lou (PhD student, Arsenal fan): it has been a pleasure to share this PhD with you. I have really nice memories, for example in that "welcome party" when Sandra made Zurracapote, and when they ask you if you wanted more, you said "just one finger", but with the finger pointing down...

Dong (PhD student, smoking partner): we meet each other for a really short time, but we managed to have some nice conversations in the faculty entrance. I look forward to keep doing things together with the guys and get to know you better!

Manu (técnico perenne, encantador de ICPs, forofo de Eurovisión): mientras pienso en qué escribir me estoy partiendo sola por momentos como el de "lefosomas", o estoy "en el ICP con King Kong", o "qué cadáver?". Espero que hagamos más excursiones a la costa brava con tus imprescindibles hinchables, más tardes de croquetas en la pizzarrita de Juanlu y cenas en restaurantes chinos.

Sergi (técnico con garantía juvenil, sanitario, "avui no m'he dutxat"): de quan estaves al "OGTS" recordo molt els cafès del matí on aprofitaves per explicar-me aventures de la nit abans. També quan ja m'havia mudat amb l'Albert i la Iris al "subzulo", i venies a emprenyar tota l'estona. I com no... "la portaaaaaaaaaaaaaaaa". Gràcies per posar ordre a Biomaterials, la veritat es que feies una gran tasca a base de les teves "pullitas".

Víctor (técnico en permanente lucha con un tal AF4): voy a echar de menos los tes en el despacho de Mon mientras contábamos cotilleos, tus galletas sanas de plátano, y tus consejos sobre decoración de interiores. Muchas gracias por la máster class en AF4!

Laia (PhD Student, reina del disfraz): no vaig poder venir al carnaval de Aro, però gràcies per guanyar el concurs de disfresses i omplir-nos de vi. I per ser la meva compi de foto al calendari!

Roberto (Post-doc, XRay-Man): tengo que confesar que no prové las hormigas que trajiste para comer en la welcome :P Y ha costado, pero al final conseguimos sacarle partido a todo ese trabajo en MIRAS! Gracias por la paciencia con las reuniones de planificación y las correcciones.

OGTs: gracias Dani por el baile del gusano, Alejandro por tus muelas del juicio y... por ser como eres, Anna por secuestrar a Gorka, Paula por instruirnos en nuevas tendencias como el "hàmster" y Javi por tus grandes anécdotas sobre ciborgs.

Moltes gràcies a la **Maria Dolors** i l'**Alba** per totes les calçotades i sopars de Nadal que hem compartit, i per suposat per tota la seva feina aconseguint-nos tot el que demanem!

También a los chicos que han venido de estancia y con los que también he compartido muchos buenos momentos: **Lorenzo, Fa, Giorgios i Andreas.**

I want to thank **Dr. Li** and **Dr. Kwon** from the **Center For Dental Research** in Loma Linda, for giving me the opportunity to do a stay in their Research group. Also to all the people I had the pleasure to work with, Dr. Wu, Gina, Wang, Mehta, Omaima, Marilou, Dr. Oyoyo, Dr. Perry and Elvin.

A toda la gente de **INTEMA**: Lucía, Adrián, Carla, Laura, Gonzalo, John, Natalia, Mariela, Mariano y Raúl, por toda la ayuda en el laboratorio, con todas las gestiones, y por preocuparos por buscar planes geniales y hacer que mi estancia en Argentina fuera como estar a casa.

I also want to thank **Professor Moustapha Hassan** for the opportunity to do a stay in the **KFC group**, and also to Dr. Zhao Ying for her time during the training. Also to all the other people in the staff: Rui, Wenji, Ibrahim and Risul, for all the "fika" we had together.

També a la resta de gent de la **planta d'Analítica**: Rober, Ana, Arnau, Dioni, Marta, Diego i Dong. I en especial al Professor Maspoch, per haver compartit els seus coneixements en les pràctiques d'ADP, i per totes les estones que ha dedicat a explicar-me anàlisi de dades de FTIR.

A tots els **Inorgs i Orgànics:** Creus, Quim, Jose, Nacho, Nuria, Marc, Jordi GA, Alba. En realitat les gràcies les hauria de donar a la Biennal de Química a Sitges, que és quan us vaig començar a conèixer a tots. Espero que tingueu un record d'aquells 4 díes tant bo com el meu. I vola agraïr especialment al Sergio, a la Momo i al Rodri (ya se que eres de anlítica, pero me cuadras más en este grupo), per haver estat companys en el corredor de la muerte dels doctorands durant l'últim sprint.

A les noies de **secretaria**, en especial a l'Elena, pel seu suport excel·lent i rapidíssim amb tota la burocràcia, que no es poca.

A la gent del **SEM**: Emma, Mònica, Vicenç, Àlex, Núria, Pau i Martí, per aconseguir fer unes fotos magnífiques a les dents (que no es fàcil, ja sabeu...). Especial agraïment als dos últims per haver participat en el sexy scientific calendar amb la millor foto de totes (que a més va tenir el seu risc).

Thank you to the people of the **ALBA Synchrotron**: Ibraheem, Martin, Tanja and Marta, for all their help and support during and after the experiments. An extra thanks to Ibraheem for the really interesting arabic-spanish tandems we made for a while.

A **Jose Manuel Amigo**, porque no sé qué habría hecho sin tu ayuda con los datos. Te debo muchas cervezas.

A **José Manuel** por el impecable trabajo y toda la ayuda en las prácticas de laboratorio.

A **Abril**, por las pequeñas charlas diarias a primera hora.

A los del **bar de Ingenieria**, por servirnos cada dia con una sornisa y muy buen humor los cafés con leche en vaso, las bombas de chocolate y los bocadillos dietéticos.

Al **Pau Solsona**, per fer-nos uns màsters del polit i per les nanoindentacions. I per moltes altres coses: els pitis, les barbacoes a casa teva, els partits de tenis, les tardes a la piscina (més aviat a la sauna), els festivals de música...

A **Dani** por los viajes en tren, por esa minuciosa investigación sobre la distribución de Poisson de los gajos de mandarina, por los conciertos de cantoautor, por las palizas (que me pegabas) al tenis, por venir a verme cuando estaba en Estocolmo, y por hacerme de guía por Buenos Aires! Eso sí que fué suerralista...

També gràcies a la gent de fora de la universitat que indirectament ha format part de tota aquesta aventura perquè no he parat d'inflar-los el cap amb el tema de la tesi.

Als **Churretes** (Alba, Clara, Marta, Diana, Heura, Laura, Uri, Paco, Ato, Luna, Boski, Orts, Palo, Paola, Augus, Roberto, Joan), els meus amics de tota la vida que sempre estàn allà per fer un afterwork quan calgui. A los **ex-GTS & outsiders** (Javi, Olga, Jun, Wei), y en especial a Elena por aguantarme un año y medio como compi de piso. A **Les Oques** (Cris, Anna, Katia), que no tinc paraules per descriure tot el que hem liat entre Pintors i Viñas, a la meva vida hi ha hagut un abans i un després de conéixe-us. A l'**Erik** per posar música tant bona a les classes de body pump. Als **DosDé** (Juan, Lex, Edu, Guille, Sara, Albert) per les sempre mítiques DosDecoas, i també als de la **JOSO** (Santi, Jodri, Àlex, i en especial al Dani). Als de **Vela** (Bàrbara, Tomeu, Arnau, Iván), per animar-me de tant en tant a treure un J per desconnectar. A les **farmacèutiques** (Cris i Anna) per els nostres sopars entre setmana per posar-nos al dia. Ai miei **Amici di Bologna** (Emilio, Michele, Andrea, Mauro, Sabri, Kavi, Jesús, Roberto, Anna, Pasquale, Angelica e Cris) che sono quasi tutti venuti a vivere a Barcellona e quelli che no, sono venuti comunque a rompere le palle in qualche momento; un ringraziamento speciale a Emilio, Loris, Marilena e "Federico", e ai tortellini in brodo. To the friends I made in the **US** (Jason, Ben, Juan Carlos, Ahlam, Fred, Anthony) for showing me some amazing places and making my weekends special. To the friends I made in **Sweden** (Andre, Felix, Linde, Marloes, Elena, Ana, Mufasa, Marija, Nora, Vassilis) for sharing amazing moments between snow and ice; specially to Andre, for showing me the Akkurat, best bar in Stockholm. A mi profe y a mi compañero de **Árabe** (Mou y Skandar) por las clases en la Emeroteca. A los amigos de **Argentina** (Osval, Nacho, Stefano, Franco) por iniciarme en la cerveza artesana y llevarme siempre de conciertos; especialmente **Any**, por venir tantas veces a vernos a Barcelona, Guadalajara o donde haga falta, por cuidarnos tanto y por hacer unos asados tan espectaculares. Al la gent d'**Excelvit** (Kiku, Òscar, Joan, Sònia, Elena) i **Fitonat**, i en especial a l'Àngel ja que també l'he embolicat en tot això. Als meus companys de **Zenement** (Llorenç, Gonçal -i Jordi-) que n'estan patint la recta final.

Jordi, m'enrecordo que el primer cop que vam parlar va ser a l'ISMEC. Després ens saludàvem de tant en tant pel passadís. Un any vam coincidir en un quinto de Nadal a la sala de reunions II i poc després en una calçotada. Com que ja hi havia una mica més de "confi", un día que em vaig quedar sense dihidrogenfosfat de potassi em vaig atrevir a demanar-te'n (sempre va bé tenir contactes). D'això fa més de dos anys. I com son les coses, que aquell petit favor ara no es pot comparar amb tot el que has fet per mi en aquest últim any. I agafo amb moltes ganes tots els que estan per venir.

Finalment, a la meva **família**, començant pels Babot (i Pomar): els Ramons, el Guille, la tieta, la iaia i l'Emèlia, que son els que m'aguanten sempre perquè no tenen més remei (és bromaaaa). No ens ho diem però en realitat ens estimem molt ;) I sempre m'agrada molts veure-us i que ens expliquem coses, i especialment amb la iaia, que tenim molta complicitat en tot. Als Marquillas: Carmina, Toto, Tito, Miki, Reyes, Edu, Luisa, Toni, Marta, Cris, Rubén, Paula, Alberto i Edu petit, per els nostres sopars de Nochebuena que sempre espero amb moltes ganes; i als Marquillas que viuen a Chile: Lali, Dani, Ernesto, que no ens podem veure cada any però vaig tenir la sort de passar uns dies amb vosaltres a Viña del Mar, i vaig estar tant bé que després no volia marxar! I per acabar, vull dedicar el meu agraïment més especial a dues persones que els hagués fet fins i tot més il·lusió que a mí que acabés aquesta tesi: al meu avi, **Francisco Marquillas**, que m'explicava amb molt d'entusiasme coses de química avançada quan jo només tenia un any; i a la meva mare, **Mercè Marquillas**, que em va convèncer per estudiar ciències perquè sempre sabia millor que jo el que jo volia.

RESUM

La demanda de blanqueig de dents ha anat augmentant en les darreres dècades, generant importants ingressos per a la indústria dental. Tot i això, els tractaments no han evolucionat significativament i el peròxid d'hidrogen (HP) o peròxid carbamida (CP) continuen sent els principals agents actius de blanqueig que es troben al mercat. És àmpliament acceptat que el seu efecte es basa en l'oxidació dels dobles enllaços conjugats de les molècules que causen les taques dentals, trencant-los i, per tant, eliminant el seu color. Els peròxids necessiten concentracions altes i temps d'aplicació llargs (aproximadament d'1 hora) per aconseguir resultats observables, cosa que provoca efectes secundaris i resulta incòmode per al pacient. Trobar ingredient blanquejador més ràpid que permeti concentracions més baixes i temps d'aplicació més curts podria resoldre aquest problema, i generar un gran interès tant per als usuaris com per a les empreses dentals.

En aquesta tesi, es conceptualitza, desenvolupa i optimitza un tractament blanquejant per a la seva aplicació a la cavitat oral, basat en un enfocament diferent a la oxidació. També s'estudien els seus efectes sobre l'estructura dental.

Es va plantejar la hipòtesi que l'agent reductor metabisulfit de sodi (MBS) podia tenir una acció blanquejant sobre les taques de les dents, per saturació dels dobles enllaços conjugats. Com a prova de concepte, les reaccions entre una molècula colorant, l'àcid tànnic (TA) i peròxid de carbamida (CP), MBS o un fort oxidant, persulfat de potassi (PS), es van monitoritzar durant 48 hores mesurant la seva absorbància, per tal de comparar els seus efectes blanquejants. Es va demostrar que l'efecte de blanquejant del MBS era més ràpid i superior el del CP. El PS va produir un efecte d'enfosquiment de la solució del TA després de la tercera hora a causa de la forta absorbància del metabòlit d'oxidació. La reacció entre TA i MBS es va estudiar mitjançant voltametria cíclica; es va observar un augment progressiu de la intensitat del pic anòdic de TA en presència de MBS, demostrant que es produïa una reacció de reducció. A més, es va fer una prova *in vitro* amb dents de bovines tenyides amb TA per observar si el MBS exercia també un efecte de blanquejant també sobre les dents. L'efecte blanquejant de CP i MBS (mesurat com a ΔE) es va monitoritzar i comparar al llarg del temps (als 3, 9, 14 i 20 minuts) mitjançant colorimetria de superfície; el MBS va produir un blanqueig superior i més ràpid el CP, especialment als 3 minuts.

Per evitar l'oxidació del MBS fins al seu contacte amb la dent, es va encapsular en liposomes de DPPC (liposomes MBS). En el model *in vitro*, es va observar que amb l'encapsulació els resultats de blanqueig milloraven als 3 minuts. Es va aplicar microscòpia confocal fluorescent per observar la interacció dels liposomes amb les dents; això va posar de manifest que, més enllà de protegir el MBS de l'oxidació, els liposomes formaven una capa que envoltava la superfície de l'esmalt, millorant l'eficàcia del tractament afavorint la difusió del reductor cap a l'interior del mateix. Posteriorment, es va aplicar un disseny experimental (DoE) per optimitzar les tres variables rellevants de la fomulació, és a dir, les concentracions de MBS i DPPC, i del pH. Per quantificar-ne els efectes, es va fer servir la colorimetria de superfície per mesurar l'eficàcia blanquejant, juntament amb tècniques de profilometria i nanoindentació per avaluar els seus efectes sobre la superfície de l'esmalt. Els models lineals obtinguts es van utilitzar per obtenir la nova formulació optimitzada, anomenada NewT101. Aquesta es va comparar amb els tractaments estàndard (HP 35% i CP 16%) seguint les "directrius ISO 28399 - Productes per blanquejar dents externs". NewT101 va presentar un efecte blanqueajant comparable al de

35% d'HP; també va produir, després de 3 sessions de tractament, un augment de la rugositat superficial, però dins dels límits estàndard acceptats.

Finalment, es van estudiar les interaccions químiques entre el mineral de l'esmalt, hidroxiapatita (HAP), amb els liposomes MBS i amb el CP. Per fer-ho, en primer lloc es va delimitar l'àrea d'estudi fent servir mesures de rigidesa contínua (CSM) per determinar la profunditat assolida pels tractaments. Després, es va aplicar la tècnica de Micro-Espectroscòpia d'Infraroig amb Transformada de Fourier acoplada a Llum Sinctrotró (SR-μFTIR) per tal d'obtenir una imatge química de la zona tractada. L'anàlisi de components principals (PCA) dels espectres va mostrar que les principals diferències entre tractaments es van trobàven en la intensitat del pic de fosfats v3 PO_4^3, degut a una desmineralització de les dents produïda per la naturalesa àcida del MBS; en el cas dels liposomes MBS, aquests canvis es van observar en regions més profundes de l'esmalt, suggerint que els liposomes afavoreixen la difusió de MBS a l'esmalt. També es va observar que els tractaments oxidant i reductor induïen desplaçaments contraris d'aquest pic, posant de manifest que CP i MBS promouen alteracions diferents en la estructura de la HAP: el MBS va induir un desplaçament batocròmic, suggerint l'allargament de l'enllaç PO del grup fosfat, mentre que el CP va produïr un desplaçament hispocròmic, suggerint-ne l'escurçament.

Com a conclusió, l'MBS encapsulat amb liposomes ha demostrat ser un ingredient prometedor per al blanquejament dental, mostrant efectes visibles en 3 minuts i una amb aplicació segura per a l'esmalt. Els resultats també han mosrat un mecanisme d'acció i d'interacció amb les dents que és diferent al dels tractaments actuals. S'espera que aquest treball pugui obrir una nova etapa en la investigació i en el mercat del blanquejament dental.

ABSTRACT

Tooth whitening demand has been increasing over the last decades, bearing great revenue for the dental industry. Even so, treatments have not evolved significantly, and hydrogen peroxide (HP) or carbamide peroxide (CP) continue to be the main whitening active agents found in the market. It is widely accepted that its effect is based on the oxidation of the tooth satins' conjugated double bonds, breaking them and therefore cancelling their color. Peroxides need high concentrations and long application times (of about 1 hour) to achieve observable results, leading to secondary effects and being inconvenient for the patient. A faster whitening ingredient which permits lower concentrations and shorter application times could potentially overcome this problem, being of high interest for both users and dental companies.
In this thesis, a treatment based on a different approach is conceptualized, developed and optimized for its application in the oral cavity. Also, its effects over the tooth structure are studied.
It was hypothesized that the reducing agent sodium metabisulphite (MBS) had whitening action over the tooth stains, by saturation of the conjugated double bonds. As an example for the proof of concept, the reactions between a staining molecule tannic acid (TA) and carbamide peroxide (CP), MBS, or the strong oxidant potassium persulfate (PS), were monitored for 48 hours by measuring its absorbance, in order to compare their different whitening effects. It was shown that MBS bleaching effect over time was faster and higher than CP's. PS produced a darkening effect after the 3rd hour because of the strong absorbance of the oxidation metabolite. The reaction between TA and MBS was studied by cyclic voltammetry; a progressive increase in the intensity of the TA anodic peak when MBS was present was observed, demonstrating that a reduction was taking place. In addition, an in vitro test using bovine teeth stained with TA was used to observe if MBS had whitening effects also on teeth. CP and MBS whitening performance (measured as ΔE) were monitored and compared over time (at 3, 9, 14 and 20 minutes) by surface colorimetry; MBS produced faster and higher whitening than CP, specially at 3 minutes.

To avoid the oxidation of MBS until its contact with the tooth, it was encapsulated on DPPC liposomes (MBS liposomes). In the *in vitro* model, it was observed that the whitening results at 3 minutes were enhanced by the encapsulation. Fluorescent confocal microscopy was applied to observe the interaction of liposomes with teeth; it showed that, beyond protecting MBS from oxidation, liposomes formed a layer surrounding the enamel surface, enhancing the treatment efficacy by favoring the diffusion of the reductant towards the enamel. Afterward, an experimental design (DoE) was applied to optimize the three main variables of the formula, i.e., MBS and DPPC concentrations, as well as pH. To quantify its effects, surface colorimetry was used to evaluate the whitening effectiveness, along with profilometry and nanoindentation techniques to evaluate its effects on the enamel surface. The obtained linear models were used to obtain the optimized new formulation, named NewT101. This was compared with standard treatments (HP 35% and CP 16%) following the "*ISO 28399 guidelines - Products for external tooth whitening*". NewT101 presented a whitening performance comparable to HP 35%; also produced an increase in the surface roughness after 3 treatment sessions, but within the standard accepted limits.

Finally, the chemical interactions between the enamel's mineral hydroxiapatite (HAP) with MBS liposomes and with CP were studied. To delimitate this area, Continuous Stiffness Measurements (CSM) were used to determine the depth reached by the treatments in order to delimitate the area of study. Subsequently, chemical imaging was performed using Synchrotron-based micro Fourier Transformed Infrared Spectroscopy (SR-μFTIR) measurements in reflectance mode. Principal component analysis (PCA) of the collected spectra showed that the main differences between treatments were found in the intensity of the ν_3 PO_4^{3-} peak, due to a tooth demineralization produced by the acidic nature of MBS; this changes were observed in deeper regions of the enamel when MBS was encapsulated in liposomes, proposing that liposomes favour the diffusion of MBS into the enamel. It was also observed that the oxidant and reducing treatments induced counter shifts of this peak, meaning that CP and MBS promote different alterations in the HAP mineral strucure: MBS induced a bathocromic shift, pointing an elongation of the P-O bond of the phosphate group, while CP produced an hypsochromic shift, suggesting its shortening.

As conclusion, liposome encapsulated MBS showed to be a promising ingredient for tooth whitening, having significant effects within 3 minutes while being safe for the enamel. The results also showed a different mechanism of action and tooth interaction than current treatments. It is expected that this work could open a new stage in tooth whitening research and market.

ABBREVIATIONS

CLSM	Confocal Laser Scanning Microscopy
CP	Carbamide Peroxide, $CH_6N_2O_3$
CSM	Continuous Stiffness Measurement
DEJ	Dentino-Enamel Junction
DHS	Dental Hypersensitivity
DoE	Design of Experiments
FTIR	Fourier Transformed Infra Red
HAP	Hydroxiapatite
HP	Hydrogen Peroxide, H_2O_2
IR	Infra Red
ISO	International Organization for Standarization
MBS	Sodium Metabisulphite, $Na_2S_2O_5$
OTC	Over the Counter
PC	Principal Component
PCA	Principal Component Analysis
PS	Potassium Persulfate, $K_2S_2O_8$
SNR	Signal-to-Ratio
SR-µFTIR	Synchrotron based Micro-FTIR
TA	Tannic Acid, $C_{76}H_{52}O_{46}$
TEM	Transmission Electron Microscopy
UV-Vis	Ultraviolet-Visible Spectroscopy

TABLE OF CONTENTS

AGRAÏMENTS I
RESUM V
ABSTRACT VII
ABBREVIATIONS IX

1. INTRODUCTION 1

1.1. History and economical relevance of tooth whitening 3
1.2. Tooth physiology 4
1.2.1. Composition and structure of enamel 5
1.2.2. Bovine teeth as an *in vitro* model 7
1.3. Teeth color 8
1.3.1. Measurement of teeth color 8
1.3.2. Tooth staining, whitening and bleaching 8
1.4. The mechanisms of tooth whitening 9
1.4.1. Chemistry of stains: tannic acid as model staining molecule 9
1.4.2. Chemistry of whitening 10
1.5. Whitening treatments 12
1.5.1. Commercialized treatments 12
1.5.2. Problems and side effects of current whitening treatments 13
1.6. Innovative solution proposed 14
1.6.1. Whitening by means of reduction 14
1.6.2. Encapsulation in liposomes 16
1.7. Techniques used 17
1.7.1. Design of experiments (DoE) 17
1.7.2. UV-Vis Spectrophotometry 19
1.7.3. Surface colorimetry 19
1.7.4. Cyclic voltammetry 21
1.7.5. Cryo-EM 22
1.7.6. Confocal microscopy 24
1.7.7. Nanoindentation 25
1.7.8. Profilometry 27
1.7.9. Micro FTIR coupled to synchrotron light 27
1.8. Objectives 32
1.9. References 34

2. RESULTS: COMPILATION OF ARTICLES 43
2.1. Breaking the rules: tooth whitening by means of a reducing agent 44
2.2. Flash tooth whitening: a friendly formulation based on a nanoencapsulated reductant 52

3. DISCUSSION 65
3.1. Treatments effects and optimization 67
3.1.1. *In vitro* proof of concept: whitening and hardness 67
3.1.2. Formula optimization 68
3.1.3. ISO 28399: in vitro test with the optimized formula 69

3.2. Treatment mechanisms 70
3.2.1. Tannic acid reduction by MBS 70
3.2.2. Comparison of the whitening outcome of MBS and CP 71
3.2.3. Liposomes adhesion to enamel surface 72
3.2.4. Chemical changes in enamel structure and depth extension 74
3.3. References 77

4. CONLUSIONS 81

ANNEXES 85

ANNEX I. Pending paper: Tooth whitening, oxidation or reduction? Study of physicochemical alterations in bovine enamel using Synchrotron based Micro-FTIR 87

ANNEX II. Patent application EP19382958.7/ 1916023.3: Tooth whitening composition 105

1. INTRODUCTION

Everybody desires a Hollywood-star smile with perfect, aligned, shine and white teeth. But few people know why teeth get dark and how tooth whitening works. It is a widely studied topic, so hundreds of research publications and patents about tooth whitening can be found. In first instance, because the chemistry of tooth whitening mechanisms and the changes induced in the tooth structure are questions still not fully understood; so every new contribution to these questions have scientific-knowledge value. In second instance, due to economical interests; the demand for dental aesthetics is increasing, hence leading dental companies are focusing on R&D, searching improved or novel whitening technologies that give them a competitive advantage in a market that has not evolved significantly for decades.

This introductory chapter presents some historical and economical facts regarding tooth whitening, and introduces key concepts that will appear all along the work. For a better understanding of the contents, also describes the basics of all the techniques used. Finally, the objectives of the work are presented, as well as how these objectives have been accomplished through the works presented.

1.1. History and economical relevance of tooth whitening

The first part we notice of a person is the face. It is considered the most important part of the body with regard to attraction and interpersonal communications. The mouth, together with the teeth, are important elements in facial aesthetic evaluations, since they occupy a considerable area of the face. From the observer point of view, dento-facial appearance has a direct impact on the individual, since it can affect perceived friendliness, social class and popularity. Furthermore, persons with ideal smiles are considered more intelligent and have a greater chance of finding a job when compared with persons with non-ideal smiles [1]. On the other hand, self perceived image of dental aesthetics can remarkably affect an individual's social and psychological well-being, which is reflected in their behavior and can affect their self confidence [2, 3].

Tooth whitening is not a new tendency. Ancient civilizations were already worried about mouth aesthetics, as demonstrated by Mesopotamian cuneiform tablets (dating back to 3000 B.C.) that collect the earliest registered recommendations known about tooth whitening [4]. This "formula" recommended to "*rub the teeth with a mixture of 'salt of Akkad', Lolium and pine turpentine",* and afterwards *"clean the mouth and nostrils and wash with honey, oil and kurunnu-beer"*; another recommendation was to *"scrape the teeth"*. It is also said that in Egypt whitened teeth were considered a standard of beauty, but evidences seem to reveal that dental treatments were extremely limited [5].

Looking at contemporary times, the first published dental report on tooth bleaching is from the past century (1877), reporting the use of oxalic acid; few years later, in 1884, it was published the first report using hydrogen peroxide [6]. Nowadays, tooth whitening has become highly demanded. In the benchmarking survey "Cosmetic Dentistry State of the industry" published by American Academy of Cosmetic Dentistry (AACD) in 2015 [7] 32% of the dentist answered that whitening was the most popular cosmetic procedure in its practice; in the survey of 2019 dentist reported a decrease in whitening procedures [8], suggesting that the consumption of products intended to be used at home, named over-the-counter (OTC), had increased.

According to a 2019 new research report [9], the worldwide market for Tooth Whitening Products is expected to grow at a compound annual growth rate (CAGR) of roughly 5.2% over the next five years; from 13300 million US$ in 2019 will reach 18000 million US$ in 2024. Prices of tooth whitening products have a wide window. OTC products are the least expensive option; e.g., whitening strips can be found for 30-80€ per box, containing enough product to treat teeth for a week or two. Also, OTC products consisting of trays that are filled with a whitening solution placed over the teeth can be acquired, typically under 50€. A more expensive but usually more effective option, is to perform a whitening treatment in the dentist office, with a custom-fitted tray to use at home, with a total cost of about 400-500€.

1.2. Tooth physiology

Teeth are the structures found in the jaws of most vertebrates used for biting and chewing. They consist of three layers: enamel, dentin and pulp (Figure 1). The enamel is the outer layer and is the hardest tissue in the body; mature enamel is composed in 96% of carbonated hydroxyapatite (HAP), a hard mineral packed at high density, and 4 wt% of soft organic matrix and water [10]. This HAP has a characteristic hierarchical crystalline organization made of parallel aligned nanocrystals, which is the key to its exceptional resilience and its mechanical performance [11] (this part is developed more extensively in section 1.2.1). When light is reflected off an enamel surface longitudinally sectioned and polished, an alternating series of dark and light bands may be seen; these are called Hunter-Schreger Bands (HSBs), and are related to the synchronous crossing of enamel prisms in the horizontal plane; this optical phenomenon is probably caused by reflection of light by inter-prismatic material [12].

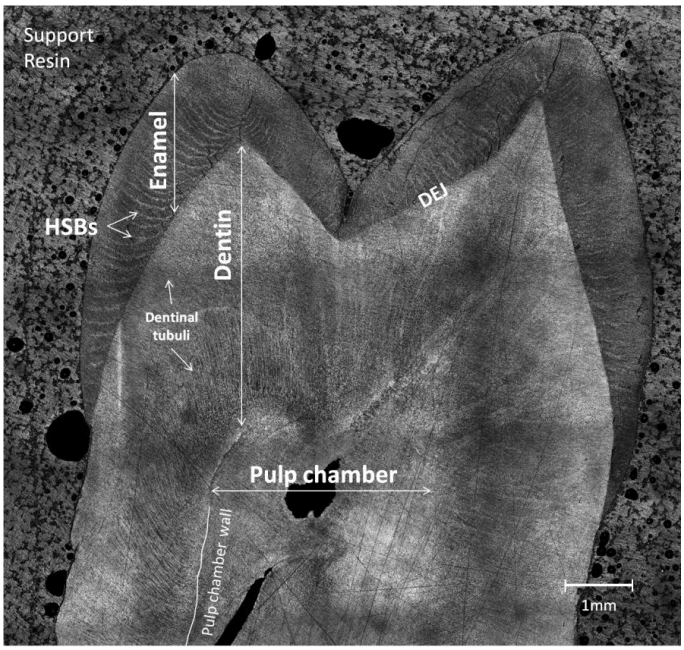

Figure 1. Human molar observed at confocal microscope. Objective 5x, white light, projected image.

Immediately below the enamel we find an interfacial region called Dentino-Enamel Junction (DEJ); as an interface between two mineralized tissues with different compositions and biomechanical properties (enamel and dentine), its function is to prevent crack propagation

from enamel to dentin [13]. The tooth middle layer is composed of dentine, a porous structure less hard than enamel and with a similar composition to bone; its most characteristic feature are the dentinal tubules, microscopic channels that radiate from the DEJ and travel through all the dentin to the pulp wall. The dentine forms the main bulk of each tooth and extends almost the entire length of the tooth, being covered by enamel on the crown portion and by cementum on the roots. In the most internal part it is found the pulp, which nourishes the dentine; it is a cavity located in the center of the tooth that consists of cells, tiny blood vessels and nerves. These nerve fibers delve into the dentin but do not traverse its entire thickness, being restricted to the innermost 10–15% of the tubule length. The pulp canal extends almost the whole length of the tooth and communicates with the body's circulatory and nervous systems.

Another important feature of tooth is the acquired enamel pellicle (AEP). It is a cuticular material formed on the enamel surface after eruption, consisting of adsorbed proteins and other macromolecules from the oral environment, saliva, crevicular fluids (inflammatory exudate derived from the periodontal tissues) and bacteria. In the first stage of its formation, which has a duration between 30 seconds and 3 minutes, only discrete proteins from the saliva (mucin, aPRPs, statherin, and histatins) are adsorbed on the enamel due to electrostatic interactions with the tooth surface ionic charges, coming from calcium and phosphate from the hydroxiapatite. The second stage consists of a continuous adsorption of biopolymers (aggregates) from saliva by protein-protein interactions with the already adsorbed proteins. Within 60 min, the thickness of the *in situ*-formed pellicle further increase between 100 and 1000 nm [14]. These biopolimers include parotid proteins, which have a globular structure with a diameter of 100–200nm, explaining the knotted, globular surface of the pellicle formed over periods of 30–120 min [15]. This second stage is a dynamic process, since this film disappears when chewing or brushing. The AEP provides lubrication to the tooth, enhancing mastication and speech, and providing protection against abrasive damage. It regulates mineral homeostasis, protecting against acid-induced demineralization by bacterial or chemical agents. Finally, it modulates the bacterial adherence; the overall flora depends on the complex interplay of AEP constituents (more than 100 proteins identified in the in vivo AEP) that may promote or inhibit colonization [16].

1.2.1. Composition and structure of enamel

Enamel has an acellular and avascular structure without the ability to regenerate or repair itself. As has been already introduced in section 1.1, enamel is composed approximately 96% wt inorganic mineral and 4% wt of organic matter and water. The inorganic dominating part is made of non-stoichiometric calcium hydroxiapatite [$Ca_{10}(PO_4)_6(OH)_2$] (HAP) with an hexagonal crystalline organization, and CO_3^{2-} impurities (~ 4.0%). Carbonate can be a substitute into two anionic sites of the hydroxyapatite structure: at PO_4^{3-} sites giving B-type carbonated apatite (the dominant type in biological apatites), and OH^- sites giving A-type carbonated apatite [17]. These substitutions modify the lattice parameters of the structure as a consequence of the different size of the substituting ions. The carbonate content increases across outer to inner enamel, which is associated with decreased crystallinity and increased solubility [18].

HAP crystals are tightly packed in three levels of hierarchical organization (Figure 2), a

structure that provides to the enamel its mechanical properties: extreme hardness, stiffness and exceptional resilience. In the first level (nanoscale) consist of a tightly packed mass of thousands of hexagonal nanocrystals of carbonated hydroxiapatite, which are 70 nm wide (a-axis) and that can grow to lengths (along the c-axis) that can extend across the full width of enamel [19]. These crystals abut parallel to one another in a "ribbon like" structure, and there exists a discontinuous organic meshwork in between these ribbons. These packed crystals form the basic unit of the enamel, a cylindrical structure called 'rod' which measures 5 µm in diameter [11]. In cross section, enamel rods have a keyhole-like pattern. Rods run continuously through the enamel width from the DEJ to the enamel surface. Close to the DEJ, they become thinner and organized in a tortuous course, and its diameter progressively grows to almost double in the enamel surface. The next level (microscale) is formed by enamel rods (prisms) and interrods, (organic interprismatic substance). At the largest structural level, bundles combining dozens of enamel rods conform the Hunter-Schreger bands; these bands are approximately 50 µm wide and are visible because of the different directions that adjacent bands of prisms reflect or transmit the light [12].

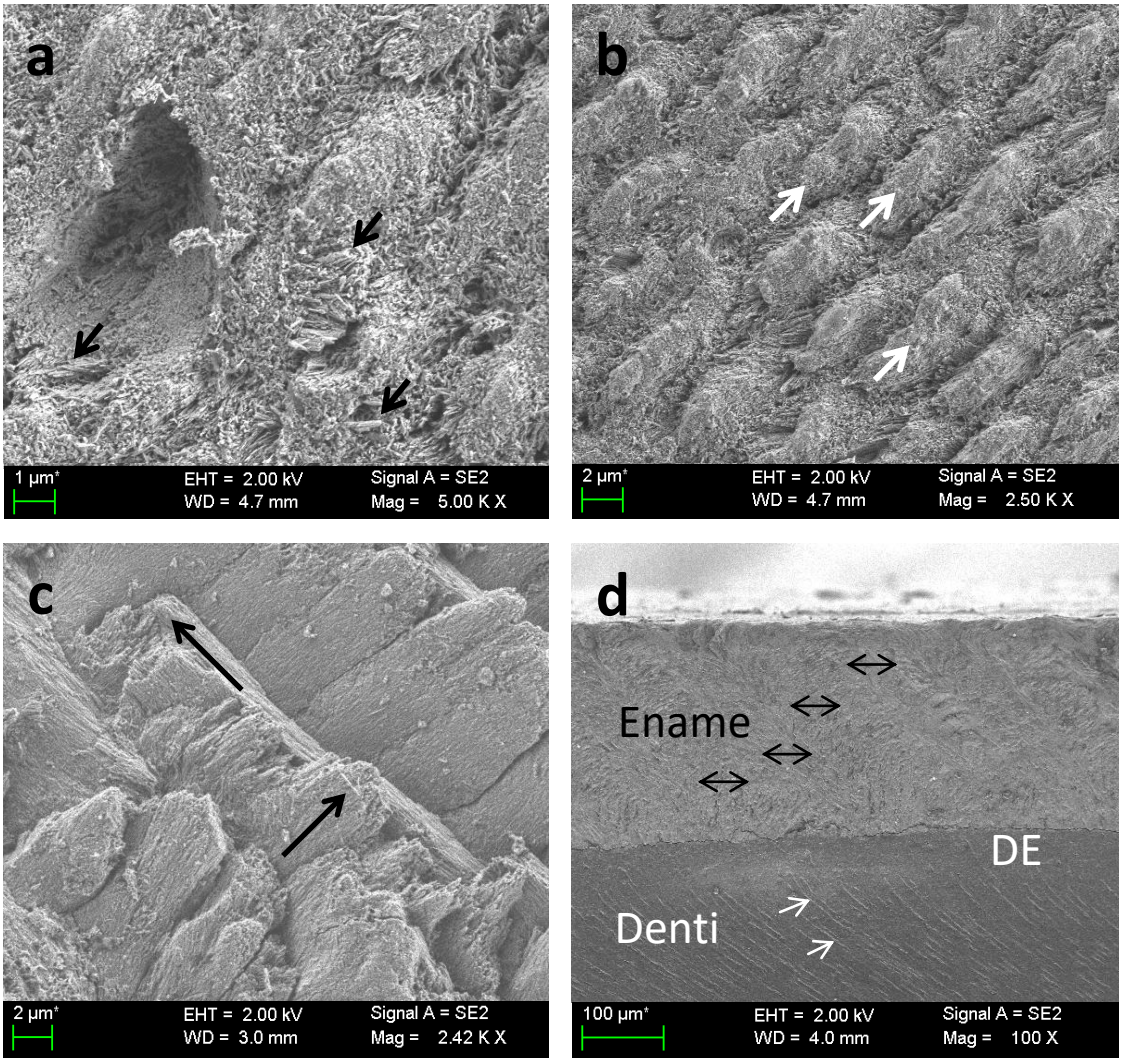

Figure 2. SEM images of a phosphoric acid etched bovine tooth. a) Tooth surface, showing HAP nanocrystals (black arrows). b) Tooth surface showing rods (white arrows); the "keyhole" shape can be only slightly appreciated since its orientation is not in perfect cross section. c) Tooth longitudinal cut, showing enamel rods running in different direction (black arrows). d. Tooth longitudinal cut showing enamel Hunter-Schreger bands (black arrows). Dentinal tubules can be appreciated in the dentin (grey arrows).

Although mammalian enamels are similar, the specific enamel microstructures appear to vary between classes and species. Bovine enamel contains a larger number of fibril-like interprisms than human enamel; the average diameter of human enamel crystals is smaller than bovine enamel crystals. Also it has different decussating patterns; in bovine enamel HAP crystallites within a rod are not simply parallel but twisting as fibers in a thread of wool [20].
It is important to consider this characteristic composition and organization of the enamel when performing chemical or physical analysis of tooth samples.

1.2.2. Bovine teeth as an *in vitro* model

To carry out *in vitro* tooth whitening studies, the "ideal" samples, non-carious and free of other defects human teeth, with a flat surface big enough to enable the tooth color measurements by a colorimeter, will be naturally stained. Thus, the best teeth are the human two central incisors. These teeth are really difficult to obtain, since extracted tooth are normally molars with carious lesions or endodontically treated. Other usually extracted are wisdom teeth with no lesions, but they are still molars, thus difficult to measure.
Bovine mandibular incisors are frequently used as a substitute for human teeth. These teeth are easy to obtain directly from a slaughterhouse and have a relatively large flat surface without caries lesions and defects. In addition, ethical review is not needed. Human and bovine incisors have some slight differences that have been described, for example the content and the distribution of calcium (~1% more in bovine enamel), or the fracture resistance (lower in bovine teeth) because of the different organization of the HAP crystal planes [20]. These morphological, chemical or physical differences are the reason why some authors consider that the model is not suitable for some kind of research, like bonding/adhesive strength tests, microleakage or dental caries studies [21]. But, as said, bovine incisors have been used in numerous tooth whitening studies for many years (as reflected in much of the bibliography provided in this work); besides, the results are easily comparable with data in the literature. Furthermore, they are considered as a valid model in the ISO 28399:2020: Products for external tooth bleaching. Thus, in this work, bovine incisors (Figure 3) have been used as an *in vitro* model in some of the studies.

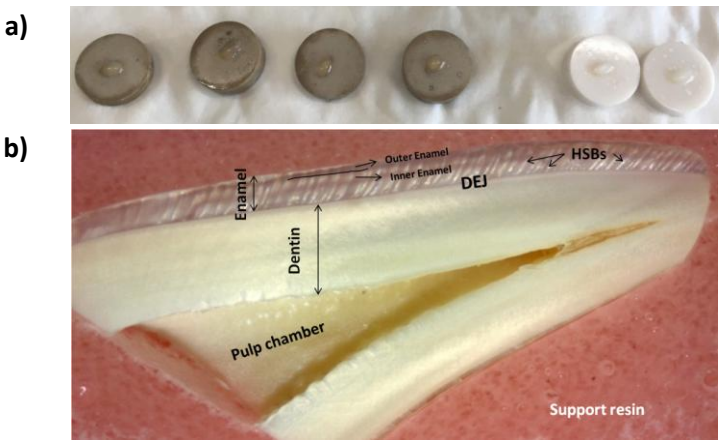

Figure 3. a) Stained (left) and non stained (right) bovine incisors embedded in a support resin, with the enamel surface exposed and polished. A drop of a whitening treatment solution can be observed in the surface. b) Bovine incisor horizontally cut and specular polished (for micro FTIR measurements).

1.3. Teeth color

1.3.1. Measurement of teeth color

Physiological teeth color depends on the intrinsic pigmentation of the tooth, which is naturally/genetically controlled, and it's determined by the paths of light inside the tooth and absorption along these paths [22]. Since enamel absorption is small (it is relatively transparent), the majority of physiological (natural) tooth color is determined by the dentine, which is yellow to brown. Enamel contributes through the light scattering at wavelengths in the blue range, bringing blue, green and pink tints of the overall color [23].
Teeth color measurement can be performed visually or instrumentally. The most frequently used technique in the shade matching of teeth; it is done manually and visually with *Dental Shade Guides,* which consist of a set of simulated teeth made of plastic or porcelain. The standard and the tooth are observed at the same time under the same lighting conditions [24]. Although shade guides have been improved along the years [25], the method still lack of consistency, because visual color assessment is subjective. The observer deals with a multilayered translucent material (tooth), which overall color is influenced by its shape, structure, texture, gloss and dissimilarities between the center and the sides [26]. In the last years, instruments to measure color (colorimeters) have been adopted in both research and clinics; this technique is explained later in section 1.7.3.

1.3.2. Tooth staining, whitening and bleaching

Teeth staining or teeth discoloration is abnormal tooth color, hue or translucency, caused by stains. Even if it differs in etiology, appearance, composition and severity, normally is classified according to the location of the stain, which may be either intrinsic, extrinsic or internalized [27]. Intrinsic discoloration is a consequence of genetic disorders (e.g., alkaptonuria, amelogenesis imperfecta), systemic factors (e.g. fluorosis, tetracycline staining, ageing) or local injuries (e.g. pulpal hemorrhagic products). Extrinsic discoloration takes place when colored substances (stains) lie on the tooth surface or in the acquired pellicle; stains can be metallic (e.g. iron, calcium of magnesium ions), or nonmetallic (dietary components, beverages, tobacco or mouth rinses) [28, 29]. Internalized discoloration is the incorporation of extrinsic stains within the tooth substance through enamel interprismatic spaces, enamel defects (enamel cracks, dental caries or restorative materials) or through the porous surface of exposed dentine (dental wear or gingival recession). The overall discoloration can be also a combination of both intrinsic and extrinsic factors. Because of the method used to stain the teeth for the experiments performed, this work is focused on nonmetallic extrinsic/internalized staining (the staining solution is in contact with the teeth long enough to penetrate thorough the enamel and reach the dentin).
In literature it can be found that the terms of tooth whitening and tooth bleaching have been used interchangeably. The International Organization for Standardization (ISO) 28399:2020 [30] indicates that *'External tooth bleaching products are used in dentistry for changing the color of natural teeth towards a lighter or whiter shade'*. Afterwards, it defines tooth bleaching as '*removal of intrinsic or acquired discolorations of natural teeth, or changing their color towards a lighter or whiter shade using chemicals, sometimes in combination with the*

application of auxiliary means'. According to this, bleaching refers to the chemical transformation of the stains performed by the application of bleaching products. Y.Li and L.Greenwall [31] defined whitening as the final outcome that is achieved in the material being treated, in this case, teeth, regarding to a white standard. Thus, tooth whitening is an outcome of tooth bleaching. When, for example, abrasive methods are used, there is no chemical reaction with the stains but its physical removal, and the outcome is also a whitening.

1.4. The mechanisms of tooth whitening

1.4.1. Chemistry of stains: tannic acid as a model staining molecule

To understand tooth whitening, stains need to be understood firstly. Tannic acid (TA) is a natural molecule found in coffee, tea, wine, all beverages widely known as tooth staining.

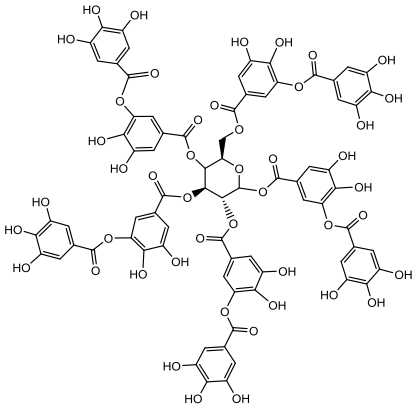

Figure 4. Tannic acid molecule

TA (Figure 4) is a huge molecule formed by esters of gallic acid (GA) dimmers with a glucose moiety (mw = 1701.2 g/mol). It is classified as an hydrolysable tannin, non-flavonoid polyphenol. It is soluble in water, producing a brown colored solution (due to the trace oxidation of the TA solubilized molecules) with astringent properties and a bitter taste. TA has a pKa_1 of 4.1 [32].

Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens, through mechanisms such as lipid peroxidation inhibition or metal chelation. Polyphenols react with oxygen more readily than most other organic compounds, protecting them from oxidation. They can neutralize free radicals by donating an electron or hydrogen atom [33], what makes them powerful antioxidants. As all polyphenols, TA has also antioxidant properties. The mechanism of oxidation on gallic acid (GA) has been studied by cyclic voltammetry, and it has been observed that, in acidic conditions, 4-hydroxyl groups of GA are oxidized/deprotonated irreversibly to form semiquinone radicals, followed by oxidation/deprotonation to form quinone [34] (two-electron reduction potential of GA quinone/catechol redox couple, E^0 = 0.794 V) [35] (Figure 5).

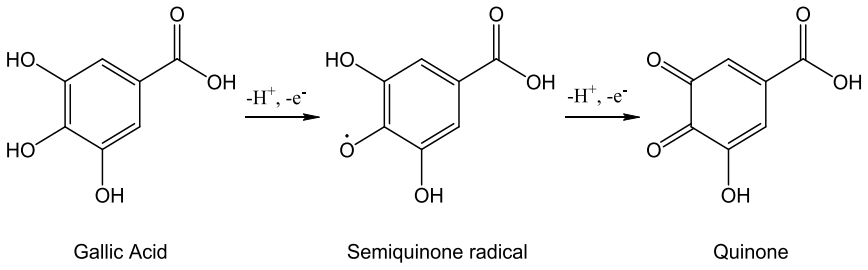

Figure 5. Gallic acid oxidation

When it is solubilized in water, tannic acid is initially almost transparent but by exposure to atmospheric oxygen ($E^0_{O_2/H_2O}$=1.23 V), part of the gallic acids undergo what is called auto-oxidation [36], giving its brown characteristic color in the visible spectra. TA can also go through chemical oxidation, or enzymatic oxidation mediated by polyphenoloxidases (PPO) [37]; the quinones formed are highly reactive electrophilic molecules, which can covalently modify and crosslink a variety of cellular constituents like proteins [33], or get further oxidized to brown melanin pigments [37].

Quinones have a darker color than its hydroxyl forms because of the substitution with bathochromic groups (which produce displacement to longer wavelengths, deepening the color of the chromogen) in benzene rings [38]. But, as we will see in the next section, further oxidation can break these chromophore groups and decrease its color.

Normally, in literature, the staining of teeth to perform *in vitro* tooth whitening assays is carried out with a solution consisting of a mixture of coffee and/or tea [39]. But, these solutions have a complex composition, including melanoidins formed during the roasting process of coffee which structure is already not well known [40]. For a better understanding of the chemistry of the process, in this work, TA has been chosen as a staining model molecule, because it is a commercial reagent with a well-known composition and purity, its concentration in solution can be controlled accurately, and represents well extrinsic stains since it can be found in both tea and coffee.

1.4.2. Chemistry of whitening

One of the main characteristics of the chromophores is that they are oxidizable. The best way to remove oxidizable stains is through oxidizing agents, that are able to break double bonds into colorless single bonds between carbons bearing corresponding oxygen, e.g. by dihydroxilation of conjugated double bonds [41], or breaking them into smaller molecules that are colorless as well. A well-known example of an oxidizer is sodium hypochlorite (bleach), used in every house to whiten clothes. All oxidants have a different “bleaching capacity” based on their redox potential (E^0, given in Volts) [42]. The higher the value of the potential, the more oxidant is the agent.

Hydrogen peroxide (HP), H_2O_2, is another widely used oxidant. In the paper industry is used for bleaching the pulp, or in wastewater remediation since it is a green reagent [43]. Also, as we have seen previously, is the most widely used dental bleaching agent. It is a colorless liquid, slightly more viscous than water, with a molar mass of 34.01 g/mol. These properties make it suitable to be used as a tooth whitener, especially because of its low molecular weight that allows it to penetrate tooth structures. Carbamide peroxide (also named urea hydrogen peroxide) is also widely used, especially in products intended for at home dental bleaching. It is a water-soluble, white crystalline solid, composed of approximately 3.5 parts of H_2O_2 and 6.5 parts of urea, so that a bleaching gel of 10% carbamide peroxide provides around 3.5% H_2O_2. As it contains solid and water-free hydrogen peroxide, offers a higher stability and better controllability than liquid hydrogen peroxide when used as an oxidizing agent.

A whitening agent needs at least two steps to perform its action; in first instance, to penetrate the tooth structure to reach both the extrinsic and the internalized stains, and secondly, to interact chemically with the stain [44]. Dental tissues behave as a semipermeable membrane

allowing the diffusion of substances according to Fick's laws. The first law state that a specie diffuses in the direction of decreasing concentration; Fick's second law (Eq. 1) can be derived from the first law of diffusion, and predicts how diffusion causes the concentration of diffusing species to change with time [45]. As seen in Eq.1, the concentration (*C)* depends on time (t), is proportional to the diffusion coefficient (D) and is inversely proportional to the distance from the surface (x) [44].

$$\frac{\delta C}{\delta t} = D\,\frac{\delta^2 C}{\delta x^2} \qquad \text{(Eq. 1)}$$

Molecules are taken up from the interprismatic spaces into the dentinal tubules, which seem to act as channels to lead the HP molecules into the pulp cavity [46]. It has been demonstrated that HP can reach the pulp chamber within 1 minute when applied to the external surface of the tooth, and that the amount diffusing it's influenced by the HP concentration, the application time or the application of heat [47–49].

Unlike the diffusion step, which is well known, the basic mechanism underlying the chemistry of the whitening process remains unclear. The dominant theory in whitening mechanism is that, as in dye chemistry, stain molecules are oxidized into colorless compounds. HP is an amphoteric compound, it can act as an oxidant or as a reductant. When behaves as an oxidant, it gets reduced as seen in Eq. 2 ($E^0_{H_2O_2/H_2O}$ = 1.776 V), while its oxidation occurs through equation 3 ($E^0_{O_2/\,H_2O_2}$ = 0.682 V).

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \qquad \text{(Eq. 2)}$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \qquad \text{(Eq. 3)}$$

It is important to take in account that the reduction potentials can be influenced by pH, according to Nernst Equation $E_{pH} = E^0 - 0.059\ pH$. Thus, at pH=4 the reduction potential of H_2O_2 is 1.52, and at pH=7 is 1.30. Nevertheless, HP is a highly unstable molecule and decomposes according to a sequence of reactions (Eq.4-6), which can be influenced not only by pH, but incident light, temperature or interactions with transition metals. In solution, HP molecules exist in equilibrium with protons and perhydroxyl ions through Eq. 4, giving rise to an acidic pH around 4 (pKa=11.62); the perhydroxyl anion interacts with another peroxide molecule resulting in the formation of the free radicals hydroxyl ($HO^{\bullet}$) and perhydroxyl ($HO_2^{\bullet}$), also called oxygen active species (Eq. 5); the hydroxyl radicals react with more peroxide and result in the formation of more perhydroxyl radicals and water (Eq. 6) [50].

$$H_2O_2 \leftrightarrow H^+ + HOO^- \qquad \text{(Eq. 4)}$$

$$HOO^- + H_2O_2 \rightarrow HO_2^{\bullet} + HO^{\bullet} + H_2O \qquad \text{(Eq. 5)}$$

$$HO^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O \qquad \text{(Eq. 6)}$$

At basic pH, Eq. 4 reaction is driven towards completion and large concentrations of perhydroxyl ions (HOO^-) are generated, and thus do the hydroxyls. The last ones have higher oxidizing properties than its precursor H_2O_2 ($E_{pH7\ OH\bullet/H_2O}$=2.32 [51], $E^0_{OH\bullet/H_2O}$=2.80 V [52])[1], which

[1] For radicals, the potentials are given as one electron potential, while non-radical species values are two electron potentials.

explains why whitening is accelerated at basic pH [53] (in fact, whitening products are commercialized at pH 8).

When a whitening treatment is applied in the tooth surface, it is hypothesized that H_2O_2 produces free radicals while diffusing through enamel and dentin [31, 44]. These radicals interact with stains breaking their double bonds, thus changing the size or configuration of the chromophores, reducing their light absorption and turning it into a lighter compound. All the literature about tooth whitening agrees with this theory, and some examples are proposed with carotenes oxidation [41], but no references can be found explaining the actual mechanism of how the radicals break the double bonds in the structures of polyphenols.

The oxidation of polyphenols, as it has been seen in section 1.4.1, leads to its quinone form (Figure 5). These quinones can experience further oxidation at alkaline pH by H_2O_2 or by attack of hydroxyl ions [54] to form carboxylic acids (Figure 6), which could be an explanation for the whitening mechanism of HP and CP.

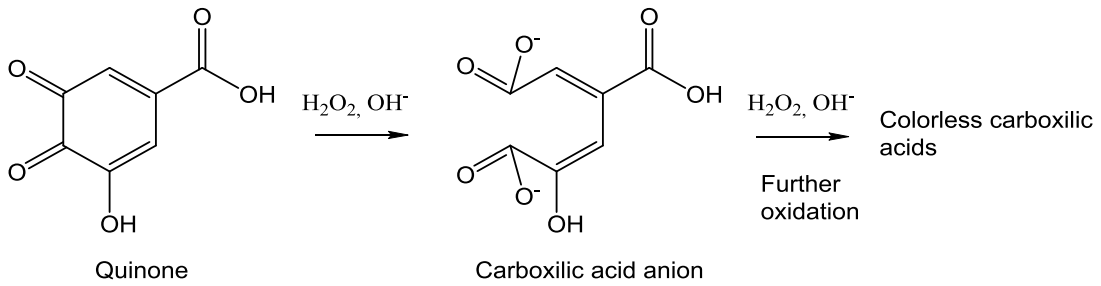

Figure 6. Oxidation of quinone to carboxylic acid by H_2O_2

Summarizing, the whitening process by H_2O_2 needs several steps, .i.e. radical formation, polyphenol oxidation to quinone, and quinone breakdown to smaller colorless carboxilic acid molecules.

1.5. Whitening treatments

1.5.1. Commercialized treatments

Looking into the available products on distributors, pharmacies, supermarkets or the internet, hundreds of products intended for tooth whitening can be found in different formats: gels, strips, toothpastes, mouthwashes, pencils, syringes and trays, dental floss and even chewing gums. These products can be classified in three groups: *in-office*, just available for professional use in dental office; *at-home* or professionally administered, the dentist makes custom trays in the clinic and the patient follows the treatment at home; or *Over The Counter* (OTC), self-administered low-cost alternative without dentist administration nor supervision.

Even if in the past reagents as oxalic acid or sodium perborate were used by dentists, from the early 1900's the main ingredient used in *in-office* whitening is the oxidizing agent hydrogen peroxide (HP), because of its efficacy and safety [55]. Products used in dental office are generally characterized by high concentrations of HP, between 25% to 40% [31]. In average, a complete treatment needs 3 appointments; in each appointment the dentist protects the

gums applying a gingival barrier to avoid tissue burns (ulceration) and the whitening gel is applied and left 15-60 min, summing a total of 1.5 to 2 hours of chair time [56].

At-home bleaching, currently the most common tooth whitening procedure dispensed by dentists to their patients, was first described by Haywood and Heymann in 1989 as "Nightguard Vital Bleaching". It consists in the application of 10% carbamide peroxide (CP) in a customized tray that is worn at night normally from one to two weeks, although can be extended to treat difficult stains [57].

Higher concentrations of HP lead to faster results [58], hence in-office whitening is effective in few sessions, while at-home procedure needs longer applications times. In fact, it seems to be no categorical difference in the results obtained with one or another technique [59]. Still, both application methods are uncomfortable for the patient.

It was not until 1990 that the first over-the-counter (OTC) products were introduced in the market to be used at home; since then, they have been commercialized under lots of different formats like toothpastes, mouthrinses, chewing gums, dental floss or strips [60]. Some of these products are also based on HP but in low concentrations (0.1-6%). However, we can also find a wider variety of other ingredients with a whitening action. The most common ingredients used are abrasives, like hydrated silica, calcium carbonate, calcium pyrophosphate, alumina, perlite, sodium bicarbonate or plastic microbeads [61]. These ingredients mechanically remove pigmented biofilms and chromophores on the surface of the dental enamel, but the abrasion needs to be moderate to avoid excessive wear of the teeth. Besides of CP, other adducts of hydrogen peroxide can be also found: magnesium peroxide, calcium peroxide or sodium percarbonate. Proteolytic enzymes as papain or bromelain can help to remove the extrinsic stains incorporated into the enamel pellicle [62, 63]. Some tooth whitening pastes contain blue covarine (a blue pigment), which deposits a thin, semitransparent film of bluish pigment on the pellicle-coated tooth surface, providing an instant whiteness perception; blue opposes yellow in the color spectrum, creating the visual appearance of whiter and brighter teeth by shifting yellow-blue color axis towards white [64]. Finally, one of the last trends in tooth whitening products is the use of activated charcoal. Some product information claims that it binds to all tooth surface deposits, 'holding' plaque, bacteria and stained material in the pores, leaving the tooth surfaces free of any deposits when it is brushed away; but this still lacks of scientific evidence [54]. OTC products are potentially harmful if they are not used correctly, and the results are not as good as the ones offered by dentists. In addition, frequently there is a lack of clinical trials that provide substantial scientific background [60].

1.5.2. Problems and side effects of current whitening treatments

The process of stains oxidation by H_2O_2 is slow since it needs several steps (see section 1.4.2), and is even slower for CP since it needs an extra step for the dissociation of the HP from the urea [65] (this is why whitening treatments based on peroxides need long application times). Because of its very high reactivity, oxygen radicals have a short live; thus, to produce its whitening effect, it is necessary that they are formed *in situ,* once they have reached the stains. The radical formation can be accelerated by the presence of a catalyst (Fenton reaction with metal ions [66], Foto-Fenton (UV light) or by heating with lasers [67]). Therefore, photo-activation may be an ideal vehicle for the generation of radicals in the place where are needed, since visible light can penetrate deep into the tooth. In the last years, LED or laser have been

introduced to overcome these problems, trying to improve comfort, safety, and to decrease the procedure time. But there is discrepancy in the literature about the use of lasers, since no improvement in the efficacy of bleaching or decrease in dental sensitivity have been observed [68, 69].

Tooth whitening treatments have associated secondary effects. The most common adverse effect of tooth whitening is dental hypersensitivity (DHS). It is a short, sharp and rapid pain, arising from exposed dentin in response to different stimuli (thermal, evaporative, tactile, osmotic, chemical or electrical), lasting through the period of contact with this stimulus. This pain can affect the quality of life of the person suffering it [70]. The incidence has a direct relation with the time of contact and with the concentration of HP, being between 15-65% or higher (67-78%) when used in combination with heat [65]. Whitening-induced DHS has a complex etiology. It is a reversible inflammatory response produced when HP and its degradation products reach the pulp chamber [71]. It is also well established that urea and ammonium ions (NH_4^+) cause structural degradation of the interprismatic proteins as amelogenin or collagen, and the removal of these components is associated with the loss of some hydroxyapatite crystals [65]. This effect modifies enamel and dentin permeability, facilitating the arrival of HP to the pulp and causing an increased dentinal sensibility. The symptomatology is commonly resolved after two or three days, but may be prolonged and evolve in reversible pulpitis, characterized by pain localized in one or more teeth. DHS can be treated with a desensitizing agent like potassium nitrate, or prevented by blocking the dentinal tubules with the use of sodium fluoride [72].

Another factor to consider is the physical/mechanical changes that external bleaching can cause on the enamel surface, more specifically on its hardness and its roughness. These two factors are normally tested *in vitro* by surface microhardness and surface profilometry. Both are described in ISO28399 and must accomplish a marked range of values in order to the bleaching product be considered safe. The majority of studies indicate that current products containing HP or CP have no significant deleterious effects on enamel [73].

1.6. Innovative solution proposed

1.6.1. Whitening by means of reduction

Previously, the oxidation of polyphenols to quinones have been exposed. It was also described that, in solution, quinones have a darker color (brown) than its catechol forms (yellow, colorless). A characteristic and important reaction of quinones is their reduction to the corresponding hydroxyquinone, which can be achieved with a variety of reducing agents (metals in aqueous acid, catalytic hydrogenation).

Sulfites are used in the food industry to control the browning in foods. The hydrogen sulfite ion (HSO_3^-) is a reducing agent ($E^0_{SO_4^{2-}/HSO_3^-}$ = -0.520V [51]), and its oxidation takes place as shown in Eq. 7.

$$HSO_3^- + H_2O \rightarrow SO_4^{2-} + 3H^+ + 2e^- \quad \text{(Eq. 7)}$$

The mechanism of browning control is based on the reduction of *o*-quinones of the oxidized polyphenols back to phenol groups (displacing the oxidation reaction shown in Figure 7

towards the left), forming stable colorless products and preventing the accumulation (and further polymerization) of *o*-quinones [74].

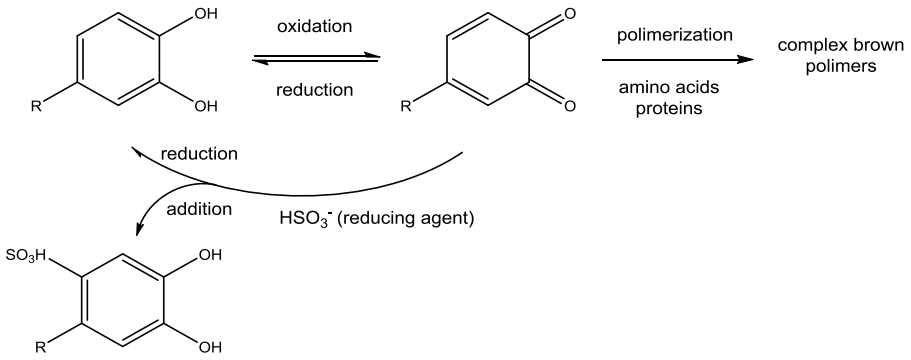

Figure 7. Oxidation and reduction reaction of 1,2-dihydroxybenzene to 1,2-benzoquinone, adapted from [74, 75].

Danilewicz *et. al.* [75] studied the oxidation of 4-methylcatechol in the presence of HSO_3^-, and it was seen that ~ 38% of the quinone formed reacted with bisulphite to produce a sulphonic acid adduct (addition) and most of the remainder was reduced back to the catechol.

As it has been previously exposed, polyphenols from foods can be adsorbed in the tooth structure. In the oxidizing atmosphere, they can undergo further autooxidation, becoming darker, producing internalized discoloration. A novel approach using Sodium Metabisulphite ($Na_2S_2O_5$), from now on referred as MBS, as a source of HSO_3^-, has been hypothesized to have a whitening action over the oxidized internalized tooth stains. MBS is a water soluble molecule with a low molecular weight (190,107 g/mol), which is expected to be able to diffuse through the enamel, reaching the internalized oxidized stains and reducing them to colorless molecules, through the mechanisms described in Figure 7. In solution, MBS leads to acid pH, which is not suitable to be applied in the tooth, since at a pH lower than 5.5, the enamel dissolves [76]. But, MBS has the potential to be used as a reductant in the oral environment, since HSO_3^- is still present in a range of pH from 2 to 7 according to the MEDUSA species distribution diagram (Figure 8).

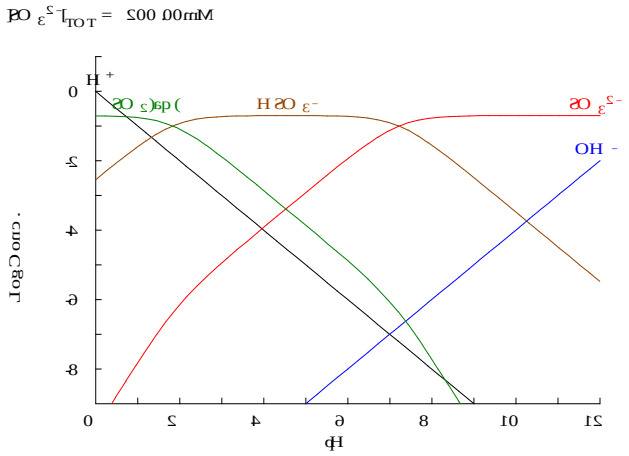

Figure 8. Distribution diagram of sulfite (SO_3^{2-}) species in aqueous solution. Obtained with MEDUSA.

MBS is very well-known and described. It is widely used in the food industry as a preservative (E-223). It has also known toxicological data (DL_{50} in rats of 1310 mg/Kg) and is already an approved ingredient for cosmetic applications (Annex V, REGULATION (EC) No 1223/2009). A

limitation presented by MBS is that in aqueous solution is not stable to air and reacts with oxygen, forming Na_2SO_4 and SO_2; to obtain whitening formulas based on MBS in aqueous solution, could be of great interest to find a strategy to stabilize it.

1.6.2. Encapsulation in liposomes

Liposomes are small structures of microscopic size and spherical shape, that can be generated from phospholipids, cholesterols and surfactants [77].
Phospholipids are a class of lipids that are a major component of all cell membranes. They are amphiphilic, since they have a polar water-soluble (hydrophilic) phosphate group, called the "polar head", attached to two water-insoluble (hydrophobic) fatty acid chains, the "tail"; the two components are usually joined together by a glycerol molecule. In aqueous solution, the hydrophobic tails of phospholipids are repelled by water molecules resulting in self-assembly forming liposomes (Figure 9), that can consist of single (unilamelar liposomes) or multiple (multilamelar liposomes) lipid bilayers and an hydrophilic compartment.

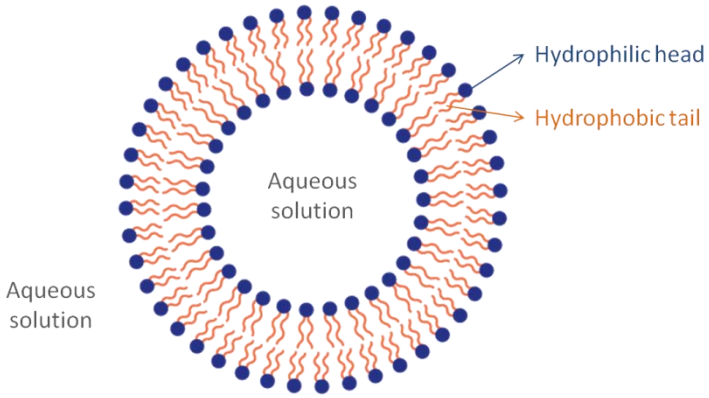

Figure 9. Structure of a unilamelar (single bilayer) Liposome

Liposome properties differ considerably with lipid composition, surface charge, size, and the method of preparation. Furthermore, the choice of bilayer components determines the 'rigidity' or 'fluidity' and the charge of the bilayer. Unsaturated phosphatidylcholine (PC) species from natural sources (egg or soybean) give much more permeable and less stable bilayers, whereas the saturated phospholipids with long acyl chains like dipalmitoylphosphatidylcholine (DPPC), form a rigid, quite impermeable bilayer structure [78]. Phospholipids can be combined in different proportions, also with cholesterol, in order to tune this property. Liposomes can encapsulate hydrophobic compounds in the bilayer, and hydrophilic substances in the core. Substances can be loaded in the liposomes passively (during the liposome formation) or actively (after liposome formation). In this work, a hydrophilic substance, MBS, solubilized in the aqueous phase has been passively encapsulated.
The interaction of liposomes with biological targets, mainly cells, is by adsorption (by specific interactions with cell-surface components or by electrostatic forces), endocytosis (forming an endosome) or, much rarely, fusion with the plasma cell membrane [78]. The liposome delivers its content by directly into the cytoplasm, by facilitated diffusion or by lysosomal degradation of the endosomes [79]. Liposomes are biocompatible and biodegradable, and have low toxicity and immunogenicity [80].

All the above-mentioned characteristics, make liposomes a very interesting drug carrier and delivery system. Furthermore, their surface can be modified to enhance its function; for example, conjugation with poliethylenglycol (PEG) increase its blood circulation time, also functionalization with small molecules or immunoglobulins enhances the efficiency of delivery at the target tissue [81]. Another interesting application is to use liposomes as a barrier that can protect its cargo from rapidly breakdown *in vivo*, enabling to use lower doses without losing efficacy [82].

1.7. Techniques used

1.7.1. Design of Experiments (DoE)

A DoE is a statistical model that establishes an experimentation strategy to maximize the amount of information that can be obtained from a process given an experimental effort and costs [83]. The DoE permits to estimate the influence and detect the interactions of the individual factors (independent variables) upon a response (dependent variable), within an established limits (levels of the factors). This allows to gain knowledge about the best operating conditions of a system, process or product, normally to optimize its effectiveness and costs [84].

To perform a DoE it is needed to select the factors in the system that are significantly influencing the studied response. In the case of this thesis, the whitening performance (response) of a formulation is optimized, and the significant factors are the reagents concentrations and the pH (is not significant, for example, if there is natural or artificial light). Another important point is that limits have to be established, and this is done based on previous experience or knowledge about the system studied. For example, in the case of MBS, it is logic to take a concentration range that includes efficient but non-toxic or harmful concentrations, and a pH range suitable for the oral cavity.

There are different approaches to perform a DoE, but when the goal is to measure how *n* factors influence a process, a 2^n full factorial design is the optimal experimental strategy [85], since it gives cause-effect relations and detects whether they interact with each other. In these designs, each factor is studied at two levels, and the experiments contemplate all combinations of each level of one factor with all levels of the other factors; the interactions between factors (how their combination affect the response) can be calculated. The number of experiments to run depends on how many factors we want to study; thus, to study 3 factors, 2^3=8 runs will be needed, and they are organized in a matrix as shown in, where the signs - and + are referred to the upper and lower limit we want to study for each factor

a)

	Factor			Interaction			
Run	A	B	C	AB	AC	BC	ABC
1	-	-	-	+	+	+	-
2	+	-	-	-	-	+	+
3	-	+	-	-	+	-	+
4	+	+	-	+	-	-	-
5	-	-	+	+	-	-	+
6	+	-	+	-	+	-	-
7	-	+	+	-	-	+	-
8	+	+	+	+	+	+	+

b)

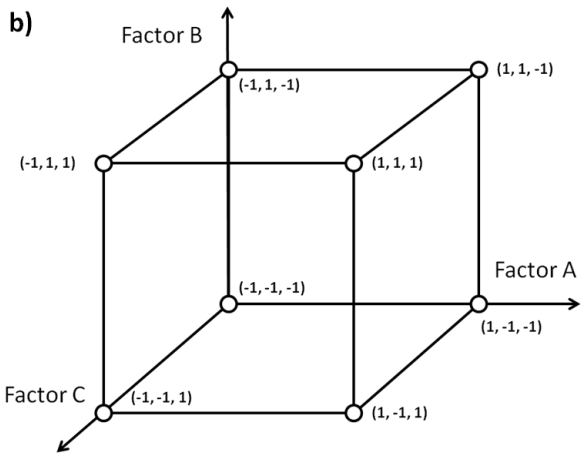

Figure 10. (a) Experimental design 2^2; (b) spatial limits and cubical distribution. Vector = (A, B, C)

Once the experimental results are obtained, an equation (linear or quadratic) that describes the system within the imposed limits can be defined; this equation can predict the response when varying one of the factors within the limits studied (Figure 10b). To obtain this model, the eight responses are combined to obtain eight coeficients (as many as experiments): the mean value of the responses (b_0), three main effects (b_A, b_B, b_B), three two-factor interaction effects (b_{AB}, b_{AC}, b_{BC}), and a three-factor interaction effect (b_{ABC}). To calculate them, mean response of the four experiments at level +1 (Y^+) and the mean response of the four experiments at level -1 (Y^-) are rested. For example, $b_A = y_A^+ - y^{A-}$. Finally, a model like Eq. 8 is obtained.

$$Y= b^0+b_AX_A+ b_BX_B + b_CX_C + b_{AB}X_{AB} + b_{AC}X_{AC} + b_{BC}X_{BC} + bA_{BC}XA_{BC} \qquad \text{(Eq. 8)}$$

In this work, a 2^3 full factorial design was used to optimize both MBS and phospholipid concentration as well as pH of the novel whitening ingredient. Factor A was MBS concentration, with the limits at 0.01 M and 0.1 M; factor B the pH from 5.5 to 6.5; factor C DPPC concentration, at 2mM and 20 mM; the measured responses were whitening effect (ΔE) and enamel surface hardness (GPa). The 8 experiments needed are listed in Table 2.

Table 1. 2^n experimental design for the novel whitening formula

Run	[MBS] (M)	pH	[DPPC] (mM)	ΔE	GPa
1	0.01	5.5	2	-	-
2	0.1	5.5	2	-	-
3	0.01	6.5	2	-	-
4	0.1	6.5	2	-	-
5	0.01	5.5	20	-	-
6	0.1	5.5	20	-	-
7	0.01	6.5	20	-	-
8	0.1	6.5	20	-	-

1.7.2. UV-Vis Spectrophotometry

When a light source passes through a solution with molecules absorbing that light, a decrease in its intensity can be measured as "absorbance". As more molecules present in the solution, more radiation will be absorbed, being absorbance proportional to the solution concentration. Using discrete wavelengths, this method is widely used to quantitative determine concentrations using the Lambert-Beer law (Eq. 9) [86].

$$A = \log_{10} \frac{I_0}{I} = \varepsilon c L \qquad \text{(Eq. 9)}$$

A is the measured absorbance, I_0 is the intensity of the incident light at a given wavelength, *I* is the transmitted intensity (to the detector), *L* the path length through the sample (which depends on the container of the solution), and *c* the concentration of the absorbing species. *ε* is a constant known as the molar absorptivity or extinction coefficient, and is characteristic for each specie at a given wavelength.
To undergo this analysis, a UV-Vis spectrophotometer is used (Figure 11). It consists of UV-Vis light source (Deuterium and Tungsten halogen lamps) that emits the UV or visible light, which then goes through a monochromator to select the desired wavelength. This discrete wavelength radiation within an intensity I_0 crosses the sample with a concentration *c*, placed in a bucket of a known wide length *L*. The outgoing radiation with an intensity *I* travels to the photodiode array detector, giving a value of absorbance *A*.

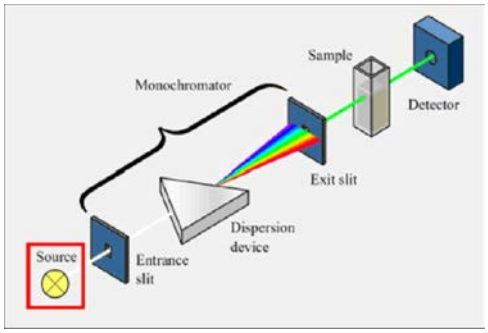

Figure 11. Spectrophotometer scheme

Colored molecules (with enough concentration in solution) absorb light in the visible range. Each one has a maximum of absorbance in a determined wavelength, corresponding to a color that is the complementary (opposite) to the observed one.
In this work, the first approach to compare the whitening performance of different treatments was performed in TA solution avoiding the complexity of the diffusion into the tooth structure. Spectrophotometry was applied to monitor the color change of the solution (brown colored), at 450 nm (blue light) over time in presence of different whitening treatments. The instrument used was a UNICAM, model UV-2 200 (USA) double-beam spectrophotometer.

1.7.3. Surface Colorimetry

A color is defined by 3 parameters: hue, saturation and lightness. Hue is the property of light by which the color of an object is classified as red, blue, green, or yellow in reference to the spectrum. Adjacent colors in these series can be mixed to obtain a continuous variation from

one color to another. Saturation (also called chroma) measures the degree to which a color differs from a gray of the same brightness or lightness, in other words, refers to how pure or intense a given hue is. Lightness (also called value) is the relative degree of black or white mixed with a given hue. Adding white makes the color lighter and adding black makes it darker [87].

Colorimetry is the measurement of color; it permits to communicate colors more accurately, quantifying and expressing them numerically [87]. To assign a number to a color, it is needed to define a "color space", which is a specific organization of colors. For example, RGB or CMYK are wide used color spaces in the graphic design field.

The L*a*b* color space (CIELAB), is one of the uniform color spaces defined by the *Commission International de* l'Éclairage (CIE) (Figure 12) [88].

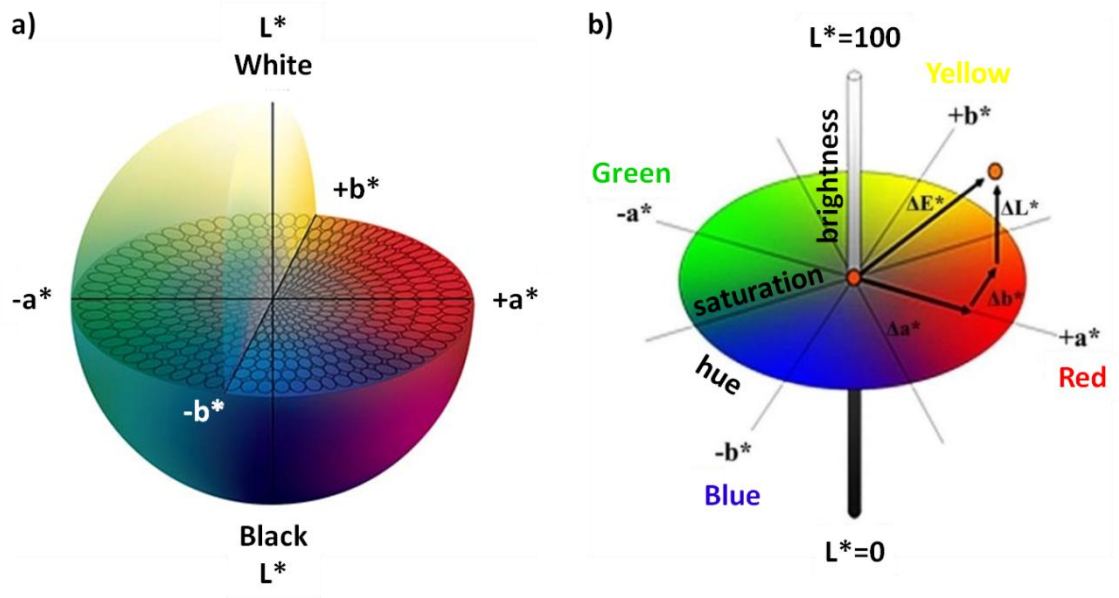

Figure 12. a) CIELAB color space represented as a sphere. b) Graphical representation of hue, saturation, brightness and ΔE*ab in the CIELAB space. Adapted from http://cie.co.at/

In this space, L* indicates lightness and a* and b* are the chromaticity coordinates that indicate color directions, being +a* the red direction, -a* green, +b* yellow and -b* blue. The center is achromatic, and as a* and b* values increase and the point moves out from the center, the color saturation increases. Color difference can be expressed as a single numerical value, ΔE*ab, which is most widely used in color research in dentistry, calculated by Eq. 10 [89].

$$\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad \text{(Eq. 10)}$$

To measure the color of a surface (surface colorimetry) Reflectance Spectrophotometers are used. In this work, we have used the Minolta CR-231 spectrophotometer (Figure 13a). Its optical system for the measurement of glossy objects consists of a diffuse light source (D65 and/or C), an illuminating and viewing geometry of 45°/0° and an optical fibre cable for measuring the specimen [90]. The sample surface is irradiated with the light source at a controlled angle of 45°. Only the reflected light perpendicular to the surface reach the optical

fiber (placed right above the sample at 0°), which collects and calculates the light flux data into color parameters (CIELAB). These can be used to determine the color values before and after a process (e.g. staining or whitening) and calculate color differences. The surface measured is of 3 mm of diameter, and the measuring time is of 3 seconds.

Colorimetric instrumentation has been introduced in the modern dental practice, using portable models of these instruments (Figure 13b). There are, however, a number of problems in the color measurement of human teeth: the reproducibility of the measurement itself is very poor due to the irregular surface of the teeth and translucency influences the measurement accuracy [87]. However, *in vitro*, some of this variability is overcome, since specimens are polished to obtain a flattened surface, and are marked to measure the specimen always in the same point.

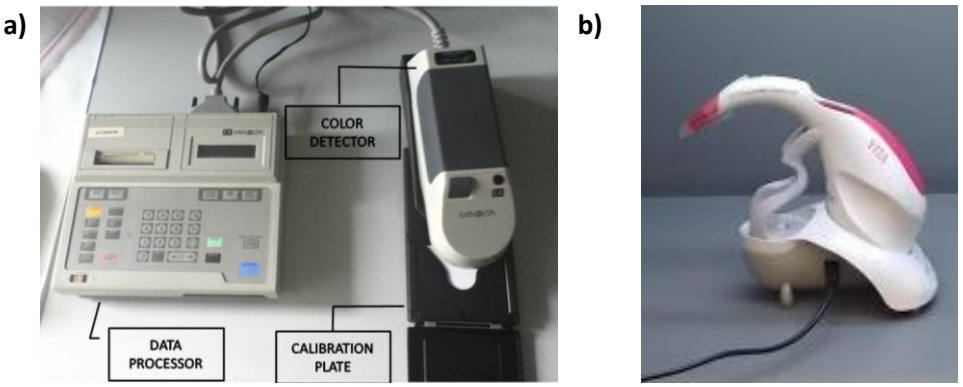

Figure 13. a) Minolta chroma meter CR-231 (equipment used in this work). b) Vita Easyshade Compact system, colorimeter adapted for its use in clinics

1.7.4. Cyclic voltammetry

Cyclic voltammetry (CV) is widespread electrochemical technique commonly employed to investigate the reduction and oxidation processes of molecular species, (performing electrochemical reduction/oxidation). In an electrochemical reduction, a compound (in solution) is reduced via heterogeneous electron transfer from an electrode (solid-state). Using an external power source (potentiostat), voltage can be applied to the electrode to modulate the energy of the electrons. When this electrodes are at higher energy than the lowest occupied molecular orbital (LUMO) of an atom, an electron from this electrode is transferred to this atom [91], giving the reduction. Also, CV can give quick and adequate information related to Faradaic and capacitive processes taken place at the surface of electrodes.

An electrochemical cell for CV measurements consists of 3 electrodes. Firstly, a *working electrode* that is connected to the potentiostat to control the applied potential, which is composed of redox-inert material in the potential range of interest; secondly, a *reference electrode* with a stable and well-known electrode potential, by which the potential of other electrodes can be measured; finally, a *counter electrode* that completes the electrical circuit. The three electrodes are immersed in the solution containing the molecular specie to be scanned, and the current is recorded as electrons flow between the working electrode and the counter electrode. The conductivity of the solution is dependent on the concentrations of a

dissolved salt called supporting electrolyte, that migrates to the electrodes where electron tranfer is taking place to balance the charge.

The electrochemical cell scans are performed to obtain a voltammogram (Figure 14), a graph where the x-axis represents the applied potential (E) and the y-axis the resulting current (i) passed. A typical cyclic voltammogram is a trace with two out of face opposite peaks. In first instance, the potential is scanned negatively (cathodically) (from high potentials to low potentials) to a determined scan rate, υ (mV/s). During this process, the reductable molecular species diffuse from the bulk solution to the electrode, where they are reduced; this process goes on until it reach a peak (reduction peak or cathodic peak, Epc), and then is slowed due to the slowdown of the diffusion of the oxidized molecule. When the switching potential is reached, the scan direction is reversed, and the potential is scanned in the positive (anodic) direction to return to the initial potential; the reduced species present at the electrode are oxidized as the applied potential become more positive, and an oxidation peak is observed (anodic peak, Epa). The two peaks (reduction and oxidation) are separated due to the diffusion of the analyte to and from the electrode. At halfway potential between the two observed peaks ($E_{1/2}$), the concentrations of the reduced and oxidized species are equal, and correspond to E°' for a reversible electron transfer at the experimental conditions because of Nernst Equation (Eq 11) [42]:

$$E = E^0 + \frac{RT}{nF} ln \frac{[ox]}{[red]} \quad \text{(Eq. 11)}$$

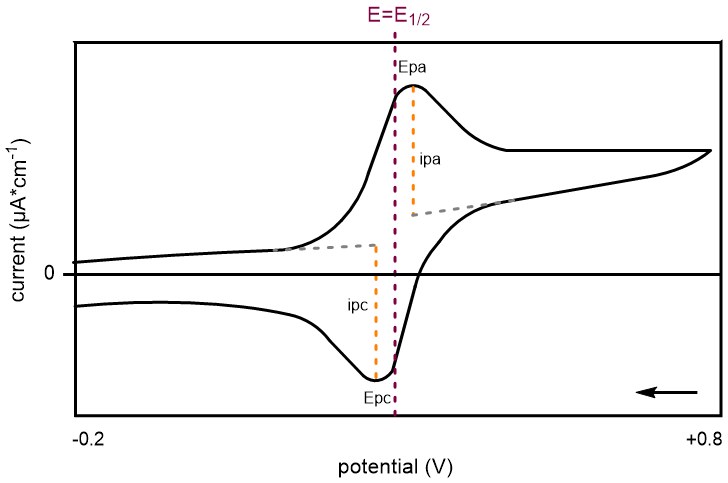

Figure 14. Typical voltammogram in the IUPAC convention

In this work, current and potential were monitored by a Voltalab PGP 201 potentiostat with an Au working electrode. A saturated calomel electrode (SCE; 0.244 V *vs.* SHE) was used as the reference electrode, and a Pt wire of large enough area as auxiliary electrode. Na_2SO_4 was used as a supporting electrolyte.

1.7.5. Cryo-EM

In light microscopy, visible light is used as the source of radiation; photons pass through the specimen and they are refracted through glass optical lenses to form an image. In transmission

electron microscopy (TEM), the radiation source is a beam of electrons which are focused by electromagnetic lenses. Resolution is directly influenced by the wavelength of the imaging radiation source: the shorter the wavelength, the higher the attainable resolution. This makes the most significant difference between optical microscopy and electron microscopy: the resolving power of the two methods. The resolution achieved with visible light (wavelengths ~ 400–700 nm), is significantly less than that achieved with electron sources in a typical transmission electron microscope (wavelength of ~ 0.002 nm for operation at 300 kV) [92]. Thus, the resolution of an electron microscope is theoretically unlimited for imaging cellular structure or proteins even if, in practice, the resolution is limited to ~0.1 nm due to the objective lens system.

Electrons are emitted by an emission source (tungsten filament or a lanthanum hexaboride single crystal) and then accelerated down the microscope column at accelerating voltages of typically 80–300 kV. The penetration depth of electrons depends on the section thickness of the specimen, and by applying a high voltage driving force, electrons are able to penetrate through tissue sections with very little scattering. For example, a ~100-150 nm is the thickest sample that can be observed using a 100 keV. Since electrons are negatively charged particles, they are repelled by electrons surrounding the nucleus of atoms. When electrons interact with the sample, this interaction causes propagating electrons to scatter and a contrast is generated by electron-dense materials in the path of the electron beam. The higher the atomic number of the atom the more electrons possess around their nucleus, and thus more incident electrons will be scattered obtaining a better contrast.

TEM lenses are grouped into 3 systems (Figure 15). A condenser lens system, which are electromagnetic converging lenses that gather the electrons and focus them onto the specimen to illuminate only the area being examined. Right after the sample, an objective lens system is used to focus the scattered electrons and initially magnify the image. An objective aperture is used to enhance specimen contrast. Finally, projector lenses further magnify the image lens and project it on to the detector.

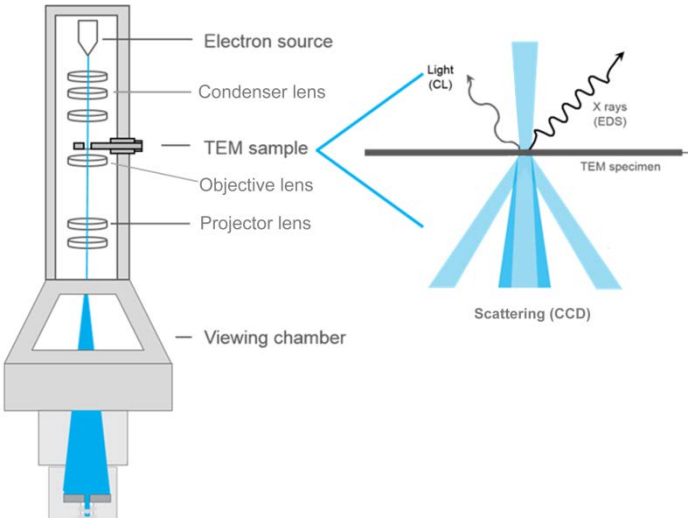

Figure 15. TEM schematic representation. In the right part of the image, a representation of all the possible trajectories of the electrons resulting of the interactions with the sample; in brackets, the detector for measurement.

Finally, a detector (e.g. charge-coupled device, CCD; energy-dispersive spectrometer, EDS; cathodoluminescence, CL) uses the information contained in the electron waves exiting from the sample to form an image.

Since electrons are very small and easily deflected by hydrocarbons or gas molecules, it is necessary to use the electron beam in a vacuum environment. For this reason, specimens must be 'fixed' to avoid structure alterations caused by dehydration. This fixation can be done chemically (glutaraldehyde or osmium tetroxide) or by rapid freezing, done in liquid nitrogen to avoid the formation of water crystals that will destroy the sample structure (hence the name cryo-EM). Samples are held in a thin vitrified aqueous film and kept at cryogenic temperatures [93].

In this work, cryo-EM has been used in order to observe liposomes. It is the standard technique for liposome imaging, since they can be imaged directly and their structure is preserved very close to their native state in solution [94].

1.7.6. Confocal microscopy

Fluorescence is the emission of electromagnetic radiation, usually visible light, caused by excitation of the atoms in a material when applying a light source, which ceases as soon as the exciting source is removed. The emitted light has a longer wavelength (and therefore lower energy) than the absorbed radiation, because some of the energy from the absorbed photon is lost internally.

Confocal microscopy or confocal laser scanning microscopy (CLSM) is an optical imaging technique that overcome some limitations of traditional wide-field fluorescence microscopes. In a conventional fluorescent microscope, light travels through the sample as far into the specimen as it can penetrate (~ 2-3 µm); all parts of the sample are excited at the same time and the resulting fluorescence is detected by the microscope's detector, including a large unfocused background part. A confocal microscope (Figure 16) uses a smaller beam of light (point illumination) that focuses only at one narrow depth level at a time (~ 0.5 µm), and also uses a filter (pinhole) to eliminate out-of-focus signal [95]. The filter is in an optically conjugate plane in front of the detector, and the name "confocal" stems from this configuration.

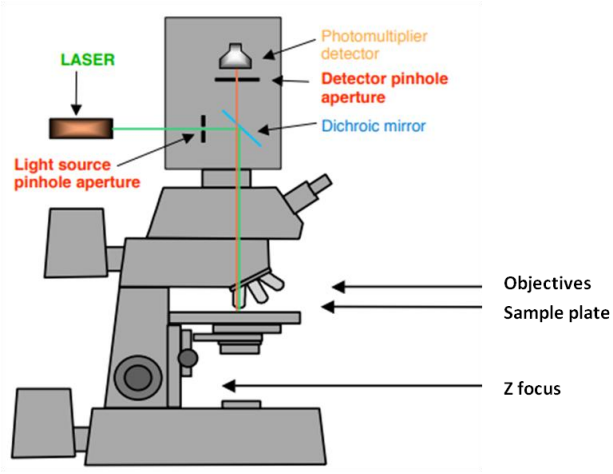

Figure 16. Confocal microscope scheme

As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution is much better than that of wide-field microscopes. However, as much of the light from sample fluorescence is blocked at the pinhole, the signal intensity is decreased, and long exposure time is often required. Biological samples are often treated with fluorescent dyes (e.g. Rhodamine B) to make selected objects highly visible [96].
CLSM provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick specimens. The capture of multiple two-dimensional (x,y planes) images at different depths (z plane) in a sample (optical sectioning) enables the reconstruction of three-dimensional structures within it. The achievable thickness of the focal plane is defined mostly by the wavelength of the used light, the aperture of the objective lens and by the optical properties of the specimen. As only one point in the sample is illuminated, to obtain an image (2D or 3D) the beam is scanned across the sample in the horizontal plane by using an oscillating dichronic mirror. The scan speed can be varied; slower scans provide a better signal-to-noise ratio, resulting in better contrast [96].
In CLSM the illumination intensity has to be very high, so it uses lasers sources (e.g. Argon, Argon-Krypton, Helium-Neon) that generate monochromatic light of a discrete wavelength. The detectors used are avalanche photodiode (APD) or Photomultiplier tubes (PMTs).
In this work, confocal microscopy has been used to observe the interaction of Rhodamine B marked liposomes with the tooth.

1.7.7. Nanoindentation

The ability of a material to resist permanent indentation, wear, abrasion or scratch, is known as hardness. Several tests to quantify hardness can be performed, also at different scales: macroindentation, e.g. Brinell test; microindendation, e.g. Vickers test. But, when the objective is to study small samples and/or thin layers, nanoindentation can be used.
Traditional nanoindentation used a tip, normally made of a hard material like diamond or sapphire with known mechanical properties and shape, to press it into a sample in order to obtain its mechanical properties. A load was placed on the indenter tip, and was increased as the tip penetrates further into the specimen until it reached a user-defined value. This operation left a mark (indentation) in the material, and the hardness can be measured as in Eq. 12, where H is the hardness, Pmax is the maximum load and Ar is the residual area.

$$\boldsymbol{H = \frac{P_{max}}{A_r}} \qquad \text{(Eq. 12)}$$

As can be seen from the equation, the hardness of a material is inversely proportional to the indentation; hence, given the same load, a soft material will be pierced easier. This technique requires imaging techniques as atomic force microscopy or scanning electron microscopy (like the image shown in Figure 17) to perform the measurement of the indentation area.

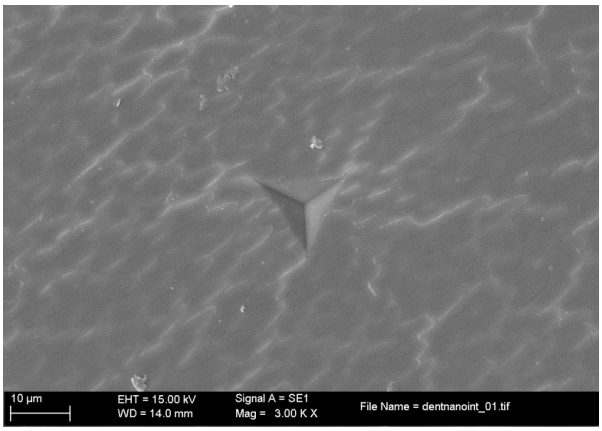

Figure 17. Scanning electron microscopy (SEM) image of a nanoindentation performed on the polished enamel of a bovine tooth

Currently, instrumentalized nanoindentation is used. It permits testing the hardness and Young's modulus of materials by high-precision instrumentation in the nanometer scale. A tip with a geometry known to high precision, usually pyramidal with square base (Vickers) or pyramidal with triangular base (Berkovich) is employed, and either the force or the displacement of the indenter is controlled by an instrumented nanoindenter, with a capacitative depth sensor o LVDT, and the force is plotted as a function of the indentation depth [97].

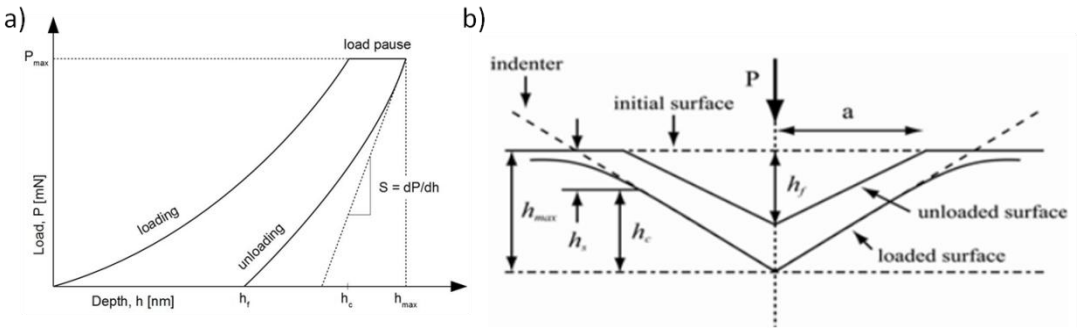

Figure 18. a) Typical load-displacement nanoindentation curve. b) Schematic illustration of the indenter and specimen surface at full load and unload and the parameters characterizing the contact geometry [98]

The slope of the curve (dP/dh) upon unloading is indicative of the stiffness (*S*) of the contact, used to calculate Young Modulus (*E*). The hardness (*H*) can be calculated by a software, which starts by searching for the maximum displacement (h_{max}) point and a maximum load (P_{max}). The displacement is used to calculate the contact surface area A_s based on the indenter geometry. For a perfect Berkovich indenter the relationship is $A_s=24.5h_{max}^2$. In addition, instrumentalized nanoindentation permits to perform continuous stiffness measurements (CSM) test, which is the measurement of depth-dependent properties of materials. The method involves applying a dynamic load on the top of the static load while loading.

Nanoindentation is a widely used technique in the dental research field to characterize tooth mechanical properties. Both human and bovine enamel hardness and stiffness as well as its alteration under different environments (e.g., acid etching) have been studied by this technique [99–101], even the salivary pellicle [102], since porosity and roughness of the enamel surface have an important effect in these measurements at the nanometric scale. In this work, nanoindentation has been used to measure the enamel surface hardness, as well as the hardness at different depths of the outer enamel using CSM test.

1.7.8. Profilometry

Profilometry is a technique used to extract topographical data from a surface, like surface morphology, heights or surface roughness. This information can be obtained with contact profilometers (using a stylus) or with non-contact profilometers (using light).
Stylus profilometry (Figure 19a) requires force feedback and physically touching the surface, so it is extremely sensitive and provides high Z resolution. The height position of the stylus while travelling along the surface (in the X direction) generates a signal which is converted into a digital signal. Since it is a direct technique, is not sensitive to surface reflectance or color, and no modelling is required. This technique can be destructive for soft surfaces and the probe can become contaminated. Its main limitation is that it needs a lot of time of measuring only one profile, thus it's difficult to obtain data from a whole surface (XY).

a) b)

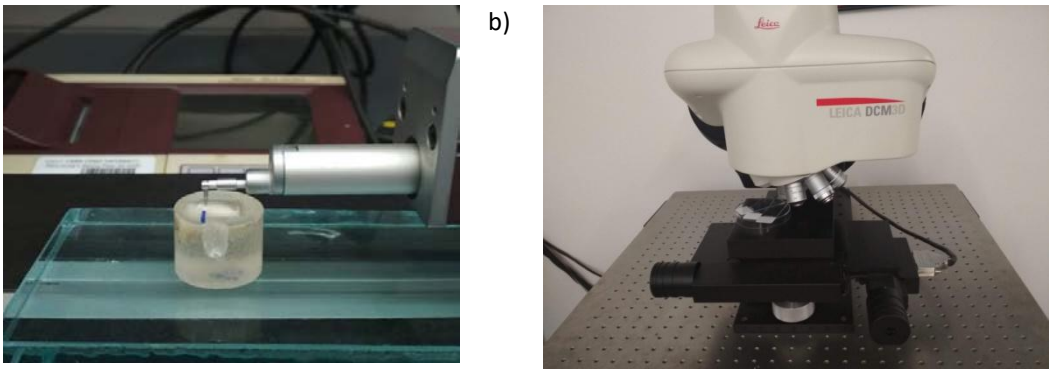

Figure 19.a. Stylus profilometer. b. Optical profilometer

Optical profilometry (Figure 19b) uses light to compare the optical path difference between the test surface and a reference surface. The reference mirror is of a "perfect flatness", so the optical path differences are due to height variances into the surface, measured by a digital camera. The advantage of this technique is that surface measurements are obtained in seconds. Non-contact profilometry has been previously used in dental research [103].
In this work, teeth surface is measured with a Leica DCM 3D optical profilometer, before and after the whitening treatment, to obtain ΔRa according to Eq. 13.

$$\Delta Ra = Ra_f - Ra_i \qquad \text{(Eq. 13)}$$

1.7.9. Micro FTIR coupled to synchrotron light

Fourier Transformed Infrared Spectroscopy

Infrared Spectroscopy (IR) is the study of the interaction of infrared light with matter. It is used to identify the vibrational signatures and therefore the chemical composition of materials. Measurements are performed with infrared spectrometers, to obtain an infrared spectrum. The peak positions in an infrared spectrum correlate with molecular structure and with the molecule concentration.

Fourier Transformed Infrared Spectroscopy (FTIR) is a specific type of infrared spectrometer which uses IR spectra, which corresponds to wavenumbers range from 14000 to 400 cm^{-1}. The core of a FTIR spectrometer is an optical device called an interferometer (most common type of interferometer in use today is Michaelson interferometer, Figure 20a), that measures the interference pattern between two light beams. The light from an infrared source enters the interferometer and reach a *splitter* (which is placed at 45° from the source of light) splits the single light beam into two light beams. One travels to a stationary mirror, and the other to a moving mirror. The two beams, that have traveled different distances, are reflected back to the splitter where are combined again, and a single beam leave the interferometer. The difference in the optical path length between the two arms to the interferometer is known as the *optical path difference* (δ), which is measured in terms of the wavelength used. Changing the distance of the moving mirror, constructive or destructive interferences from the two beams are obtained. An interferogram is the fundamental measurement obtained by an FTIR Figure 20b. To obtain an interferogram using an interferometer the mirror is moved back and forth once. This is called a *scan*.

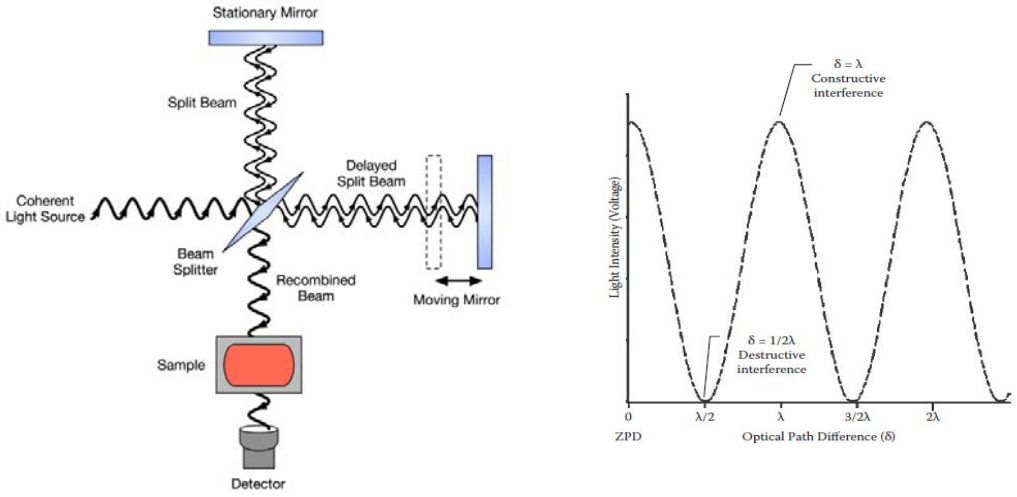

Figure 20. a. Michaelson interferometer. b. Interferogram for a single wavelength of light passing through the interferometer (in real measurements, the interferogram obtained corresponds to many wavenumbers of light passing through the interferometer together).

The beam exiting from the interferometer arrives to a pyroelectric Mercury Cadmium Telluride (MCT) detector that responds to changes in temperature produced by IR radiation intensity, converting it to an electrical signal. Through a computer software, the interferogram is converted to a spectrum of the signal at a series of discrete wavelengths by Fourier transformation, hence the term Fourier Transform Infrared (FTIR) spectroscopy.

The amplitude of a Fourier frequency is proportional to the amount of light hitting the detector at that wavenumber. If we put a sample in the infrared beam that absorbs at that wavelength, the amount of light hitting the detector will be reduced, thus the amplitude of this interferogram will go down. A reduction in the intensity (due to the interaction with a sample) upon Fourier transformation, gives rise to a peak in the absorption spectrum of the sample, obtaining the characteristic FTIR spectra of each molecule.

FTIR can be coupled to a microscope (μFTIR). In this work, we have used μFTIR used in specular reflection mode. The principle used is the same, but specular reflectance techniques involve a mirror-like reflection from the sample surface, which occurs when the reflection angle equals

the angle of incident radiation, reflecting the IR beam to the detector. It is used for samples that are reflective (smooth surfaces), thus the sample needs a specular polishing.

a) **b)**

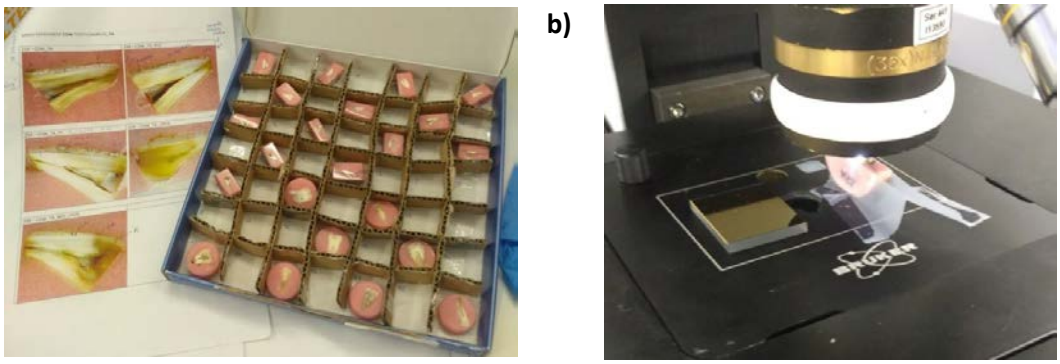

Figure 21. a) Tooth samples with specular polishing. b) FTIR microscope objective pointing on the sample; at its side, a golden mirror (perfect mirror) used as a reference to acquire the reflectance baseline.

Specular techniques provide a reflectance measurement for reflective materials [104], or a reflection–absorption (transflectance) measurement for translucent samples (e.g. enamel), where the tissue reflectance is influenced by a surface layer of a thickness in the order of the light wavelength. Therefore, surface changes localized to the outer 10 µm of tissue are probed [105].

Synchrotron light source

A synchrotron light facility is a very bright source of electromagnetic radiation covering from infrared to hard X-rays (mostly X-rays). The emission of synchrotron radiation is a relativistic effect. Any charged particle which is accelerated emits radiation. In a synchrotron facility, electrons travelling at relativistic velocities (close to the speed of light) emit synchrotron radiation when their linear trajectory bends by the effect of magnetic fields which impose a centripetal acceleration on the electrons. The synchrotron radiation is then emitted tangentially to the orbit described by the curved trajectory and in the forward direction as a narrow-collimated fan of radiation. This makes the light to be highly collimated (the brilliance of synchrotron light is trillions of times greater than conventional laboratory X-ray sources).
To illustrate more in detail how a synchrotron works, we have selected ALBA synchrotron as a representative example [106]. In every synchrotron the electrons are generated by an *electron gun*. In ALBA, a 90kV DC thermionic electron gun generates bunches of electrons of a length of 2ns. These electrons are extracted from a metal (tungsten impregnated of BaO) heated at 1200 degrees. Then, the electrons pass a bunching region that reduces their bunch length (groups them) and increases their energy using microwaves (radio frequency cavities). After the bunching system the beam energy is 16 MeV (1 eV is the amount of energy that an electron gains when it moves through a potential difference of 1 volt in a vacuum). Further downstream the LINAC (linear accelerator) two additional accelerating structures increase the beam energy up to more than 100 MeV. Electrons travel from the LINAC to the Booster Ring where a specifically designed Radio Frequency Cavities further accelerate the electrons from 100 MeV to 3000 MeV as they circulate in the Booster Ring. At this energy, 3 GeV, the electrons are travelling at 99.9999986% of the speed of light (3.0×10^8 m/s). Following this, the electrons are injected into the Storage Ring in discrete pulses (typically, one or two hundred bunches distributed around the whole ring for top-up mode operation). The storage ring is not

a true circle, but a polygon, made of straight sections. At the junction, the dipole magnets curve the trajectory of the electrons and synchrotron light is emitted. A radio-frequency cavity feeds energy to the electron bunches circulating using microwaves, to compensate for their energy losses during their emission of radiation. The electrons (and later the photons) bunches travel always under ultra-high vacuum conditions (10^{-10} - 10^{-12} torr) to avoid colliding with particles.

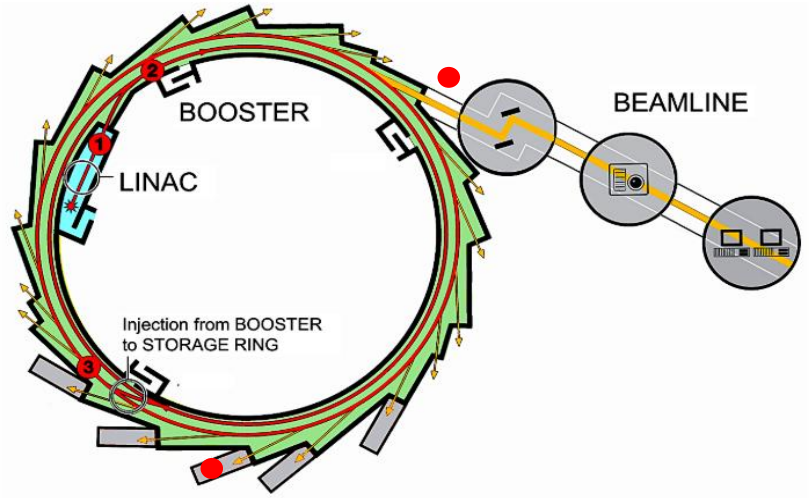

Figure 22. ALBA synchrotron scheme. 1. LINAC, 2. Booster ring, 3. Storage ring 4. Beamline [107]

The raw X-ray stored in the ring is provided to a beamline, where they pass through specialized mirrors and crystal optics to focus the beam and to select the wavelength, or energy, desired. This is used as a light source by the equipment installed in the experimental cabin, in the case of this work, a µFTIR.

Synchrotron-based FTIR microspectroscopy

Synchrotron-based FTIR microspectroscopy (SR-µFTIR) is emerging as a powerful technique that combines FTIR with the high brightness of the synchrotron source. The advantage of using the synchrotron light as an IR source is that the beam, as already exposed before, is highly collimated, which is translated in two main benefits. Firstly, this property leads to better signal-to-noise ratio (SNR) compared to the traditional IR globar sources [108] (Figure 23). SNR of a spectral region is proportional to the square root of the amount of time (t) spent observing the intensity of light in that region and is a measure of the quality of a peak. It can be improved both increasing the time observing the measuring point (performing more scans) and/or increasing the light in the measuring point. A better SNR means lower noise and cleaner peaks, permitting the better observation of the singular vibrations. Secondly, the highly collimated beam allows to narrow the aperture of the diaphragm to have a thinner beam, achieving high spatial resolution (3µm), which allows access to small biological targets [109]. In other words, this technique permits to point the light in very precise spots, allowing to observe changes in very small areas with high peaks resolution.

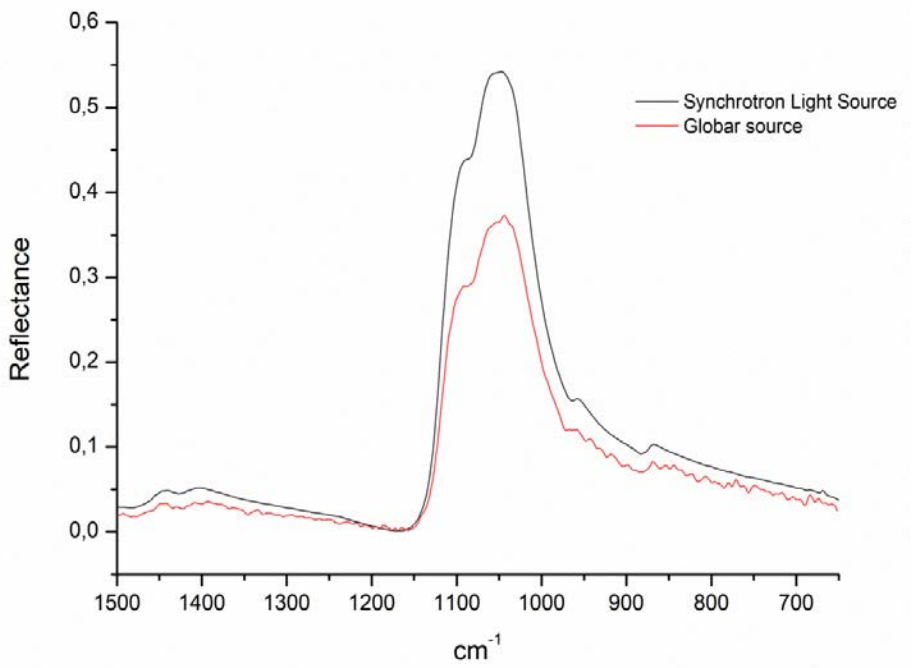

Figure 23. Specular reflectance spectra from a point in the enamel. Red: spectra obtained with globar internal source, at 10x10 µ aperture and 256 scans. Blue: spectra obtained with synchrotron light source, at 5x15 µm aperture and 256 scans. Data obtained in MIRAS beamline (ALBA synchrotron).

SR-µFTIR in specular reflectance mode is well suited for resolving chemical changes on the surface of enamel. It has been previously used to study the effects of the application of pulsed CO_2 lasers (used in dental clinics) [110], obtaining significant differences in phosphate and carbonate peaks of the enamel.

In this work, tooth samples were analyzed by SR-FTIR using the mid IR spectra (4000 to 600 cm^{-1}) in MIRAS beamline from ALBA Synchrotron, with the objective to study with high precision the chemical changes produced by different whitening treatments in the outer enamel.

Figure 24. MIRAS beamline in ALBA Syncrotron (Source: https://www.cells.es/en/beamlines/bl01-miras)

1.8. Objectives

The starting hypothesis of this work is to use a reducing agent as a tooth whitening ingredient instead of the most common oxidizing agents, to reduce (instead of oxidize) chromophores to less light-absorbance forms. This paradigm shift aims to bring innovation in the tooth whitening field, and to establish a new basis on which to develop innovative and more effective and safe products.

To this purpose we employ MBS, a very well known reducing agent used in sectors including food industry and cosmetic industry.

The main objective of this thesis is to study and to understand the whitening effect of the reducing agent sodium metabisulphite (MBS) on teeth. This objective is planned to be accomplished by performing the following specific tasks:

1. To evaluate the whitening effect of MBS in a staining solution using a well-known molecule, facilitating accurate color change measurements over time by spectrophotometry, as well as the study of the redox process through voltammetric measurements.
2. To validate the whitening effect of using a tooth whitening *in vitro* model. This lays similar diffusion parameters that the ingredient will find in its application in human teeth, and at the same time permits to evaluate secondary effects as changes in the surface hardness or roughness.
3. To enhance the whitening effect of MBS by means of its encapsulation in liposomes and to optimize this new formula (MBS-liposomes) to be used in the oral cavity, using the same *in vitro* model to evaluate the results and evaluate if it is safe to be applied in humans.
4. To observe the interaction of liposomes with the enamel surface and the effects of MBS in the first layers of the outer enamel, to better understand the physical behavior and the chemical effects of the MBS-liposomes on the hydroxyapatite.
5. To learn and understand the fundamentals of the techniques used in order to draw realistic and adequate conclusions.

The stated tasks objectives have been achieved through two works published, enclosed in Chapter 2, and a third work pending of publication enclosed in ANNEX I.

The first work published was focused on finding a solution to overcome the main problems of currently commercialized tooth whitening treatments (oxidizing agents), i.e. the needed use of high concentrations and long exposure times. A novel approach was proposed, under the hypothesis that a whitening effect can also be achieved by chemical reduction. The whitening capabilities of the reducing agent sodium metabisulphite (MBS) were explored.
After demonstrating the effective bleaching ability of MBS both in solution and *in vitro,* further work was developed with the objective of finding a highly effective novel whitening ingredient. It was hypothesized that the encapsulation of MBS in liposomes would both avoid its interaction with other oral structures and would facilitate its delivery on the enamel surface, enhancing its performance. The shape of the MBS charged liposomes and its interaction with

the tooth surface were explored, and its whitening performance *in vitro* was measured. Experimental design was applied to optimize its concentrations and pH for the oral environment, modeling whitening and surface hardness.
To understand the effects the whitening treatments had into the enamel (beyond changes in the surface hardness), a SR-µFTIR study was performed in ALBA synchrotron's MIRAS beamline, to observe the chemical changes induced over the HAP structure with high precision. A third work was written with the data obtained.

Finally, from this thesis, a novel whitening ingredient has been developed. Since it accomplished the "novelty" characteristic, a patent application was done under the project 2018-LLAV-00052 from the "IMPULSA LLAVOR" program. This patent has potential interest for those companies that want to include in their product catalog a bleaching treatment that differs from the existing ones, making it much more competitive. The text submitted for the patent application is enclosed in ANNEX II.

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2. RESULTS: COMPILATION OF ARTICLES

2.1 Breaking the rules: tooth whitening by means of a reducing agent

In this paper, it was hypothesized that, otherwise to peroxides (the most common whitening ingredients used in the dental industry, which perform their whitening action by oxidation) the reducing agent sodium metabisulphite (MBS) will perform a whitening action over the tooth stains by saturation of the conjugated double bonds.

To study this hypothesis, the absorbance of a tannic acid solutions in contact with MBS, carbamide peroxide (CP), or with the strong oxidant potassium persulfate (PS), were monitored for 48 hours by measuring its absorbance. It was shown that MBS bleaching effect over time was faster and higher than CP's, and that PS produced a darkening effect after the 3rd hour because of the strong absorbance of the oxidation metabolite.

To confirm the event of a reduction process, a control solution of TA and the reaction between TA and MBS were studied by cyclic voltammetry. For the first, continuous decrease in the TA anodic peak was observed, as the TA molecules were oxidized at the electrode surface. Instead, there was a progressive increase in the intensity of this peak when TA reacted with MBS since more reduced tannic acid was available in order to be oxidized, demonstrating that a reduction was taking place.

Finally, an *in vitro* test was used to compare the effect MBS and CP over stained teeth, a closer model to real application. Both agents were able to perform the whitening visible effect when applied on the enamel surface within the 20 minutes studied, with the difference that MBS reached the same ΔE values than CP but in just 3 minutes.

The results obtained may lead to the development of novel formulas in the tooth whitening market, making it evolve beyond peroxides

Clinical Oral Investigations

Volume 24, Issue 8, August 2020

DOI: 10.1007/s00784-019-03140-3

Breaking the rules: tooth whitening by means of a reducing agent

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Clinical Oral Investigations (2020) 24:2773–2779
https://doi.org/10.1007/s00784-019-03140-3

ORIGINAL ARTICLE

Breaking the rules: tooth whitening by means of a reducing agent

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Received: 25 February 2019 / Accepted: 30 October 2019 / Published online: 20 November 2019

Abstract

Objectives It is widely accepted that current tooth whitening treatment effect is based on the oxidizing action of peroxides, even if the mechanism of action remains still unclear. Treatments are claimed to be safe, but several secondary effects have been described, since long application times and high concentrations are needed. A faster whitening ingredient which permits the use of lower concentrations and shorter application times could potentially overcome this problem. In this work, a different approach based on a reducing agent, sodium metabisulfite (MBS), is explored.

Materials and methods The reaction between tannic acid (TA) with carbamide peroxide (CP), MBS, and potassium persulfate (PS), as an oxidizing agent, was monitored for 48 hours by measuring its absorbance, comparing their different whitening effects. The reduction process between TA and MBS was confirmed by cyclic voltammetry. An in vitro test was used to observe if MBS whitens also stained teeth.

Results It is shown that MBS bleaching effect is faster and higher than CP's effect over time. PS produced a darkening effect after the 3rd hour because of the strong absorbance of the oxidation metabolite. Cyclic voltammetry showed a progressive increase in the intensity of the TA anodic peak when MBS was present, demonstrating that a reduction is taking place. In vitro, MBS showed a faster whitening performance than CP, using lower concentrations.

Conclusions Using a TA solution as a staining model, it was possible to show that MBS has a visible bleaching effect through a reduction reaction, faster than CP, both in solution and in vitro. Low concentrations of MBS are effective in whitening.

Clinical significance This work shows MBS as a promising candidate to develop novel whitening treatments, which is acting by reducing mechanism instead of oxidation.

Keywords Tooth whitening · Sodium metabisulfite · Reducing agent · Tannic acid · Tooth bleaching

Introduction

Nowadays, teeth whitening is the most popular cosmetic treatment [1], becoming a buoyant industry that generates millions of dollars per year. Commercial whitening products are based on hydrogen peroxide or carbamide peroxide (CP) (which is composed of urea and hydrogen peroxide) at different concentrations (0.1–35%) [2]. Even if they provide good results, no model fully integrating the details of the mechanism to the final outcome of color change has been developed [3]. The dominant explanation is the chromophore theory: it is known that conjugated double bonds provide color to molecules, and if these bonds are broken, the molecule becomes transparent. Thus, hydrogen peroxide oxidizes the colored compounds adsorbed on the tooth yielding colorless molecules [4]. Current whitening treatments are claimed to be safe, but high concentrations of peroxides are needed to obtain a faster rate of whitening [5], and/or long application times are required in order to obtain the desired effect [6]. This can trigger some of the secondary effects that have been described, like gingival irritation and tooth hypersensitivity [7]. Several studies have been carried out to overcome the secondary effects, with the aim to reduce the concentration of the peroxides or to shorten the treatment times [8–12]. But these treatments do not solve completely the secondary effects [13–16].

In this work, we propose a different approach based on a reducing agent, sodium metabisulfite (MBS). It potentially can saturate the double bonds breaking the aromaticity of the staining molecules, making them colorless. When MBS

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is dissolved in water, the reactions described in Eq. 1–3 take place [17].

$$Na_2S_2O_{5(s)} + H_2O \rightarrow 2NaHSO_{3(aq)} \quad (1)$$

$$2NaHSO_{3(aq)} \rightarrow 2SO_{2(g)} + H_2O + 2Na^{+}{}_{(aq)} \quad (2)$$

$$SO_{2(g)} + H_2O \rightarrow H_2SO_{3(aq)} \rightarrow 2HSO_3{}^{-} + 2H^{+} \quad (3)$$

MBS is a well-known food additive (E-223) [18], and some research listed it as a tooth-bleaching agent [19, 20], even as an ingredient for caries prevention [21].

Testing the whitening effect in a tooth model implies, besides the stain whitener interaction, the diffusion phenomena through the enamel. To avoid the diffusion step, the action of whitening agents upon the absorbance of tea and coffee stain solutions has been previously used as a model [22]. However, these solutions contain not only polyphenols but different types of compounds (e.g., melanoidins or sugars) that could be interacting with the whitening agent complicating the spectrophotometric and voltammetry measurements and results interpretation and making it difficult to control the stain concentrations as well as the reaction's stoichiometry. To avoid that, a single molecule, tannic acid (TA), was chosen as a model to prepare the staining solution used to test the whitening effect of MBS as a first approach and to stain the bovine teeth to carry out an in vitro test. TA is a water-soluble polyphenol formed by a glucose moiety substituted by gallic acid dimmers which produces a brown colored solution when solved. It is found in both tea and coffee, and it is an important component of polluted water, so it has been previously used as a model in studies for water remediation [23]. Polyphenols are susceptible to oxidation when they are in solution, since they require oxidation for most of their ecological activity (e.g., antimicrobial) [24] and they act also as antioxidants thanks to their radical scavenging and metal chelating activity [25]. When oxidized, their phenol groups become quinones, which are darker than in their reduced form [26]. Thus, when polyphenols interact with tooth, they are already high-colored molecules. Nevertheless, it is well-known that quinones can be back reduced to phenols (quinone/catechol redox couple for gallic acid $E^0 = 0{,}794$ V [27]) with a reductant compound like $NaHSO_3$ (H_2SO_3, $E^0 = -0{,}172$ V [28]) as shown in the reaction in Fig. 1 [29].

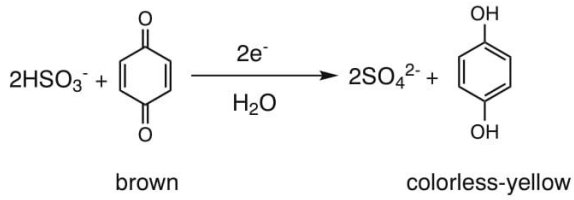

Fig. 1 Reduction of a brown-colored quinone molecule to a colorless-yellow hydroquinone molecule

The main aim of this work was to test a different tooth whitening approach, based on a reducing mechanism of action of MBS, both in solution and in vitro, comparing its performance with the most used ingredient in the market, carbamide peroxide.

Materials and methods

Reagents

Tannic acid was purchased from Sigma-Aldrich, sodium metabisulfite and potassium persulfate from Riedel-de Haën, all in powder form. Carbamide peroxide was purchased from Aros, in 1 g stabilized tablets (contains 35% H_2O_2). All chemicals were of analytical grade. Deionized water was purified through a Millipore purification system from Millipore (Milford, MA, USA).

UV-Vis spectrophotometry

The whitening effect study in solution was carried out using a UV-Vis spectroscopy with a UV double-beam spectrophotometer from UNICAM, model UV-2 200 (USA). Measurements were performed at 450 nm, bandwidth 2 nm, and a scanning speed of 1200 nm*min^{-1}. For the metabisulfite whitening efficacy and the reductant concentration studies (sections 3.1 and 3.2), the absorbance of the tannic acid solutions was measured after 20 minutes of reaction with the corresponding reagent. In order to follow the whitening effect over time (section 3.3), the solutions were monitored for 48 hours, measuring its absorbance at different times (0, 0.3, 1.5, 3.5, 16, 20, 24, 30, 40, 48 hours). For the spectrophotometric assays, a concentration of tannic acid solution of 4.7 x 10^{-2} M was needed to obtain good absorbance values. All the experiments were performed at room temperature.

Cyclic voltammetry

A conventional three-electrode cell was used to carry out the electrochemical measurements. Current and potential were controlled by a Voltalab PGP 201 potentiostat with an Au working electrode, a SCE as reference electrode (244 mV vs. SHE) and a Pt wire of large enough area as auxiliary electrode. The experimental parameters after optimization were as follows: initial potential, −200 mV; sweep rate, 50 mVs^{-1}; and final potential, +800 mV. The potential values cited in this paper are referred to the $Ag/AgCl/KCl_{(sat)}$ electrode (199 mV vs. SHE). Na_2SO_4 0.5 M was used as a supporting electrolyte when measuring the solutions. All the experiments were performed at room temperature.

In vitro model

Specimen preparation

A total of 30 specimens of bovine teeth (n = 10 per group; NC, CP, MBS) were cleaned of gross debris, the roots sectioned using a diamond saw and then preserved in 0.5% chloramine solution until needed. The teeth were embedded in self-curing polyacrylic cylinders (2 cm wide and 3 cm high) to expose a flat window of enamel surface needed for the colorimetric measurements. The surface was ground using silicon carbide paper, starting at P400 grain size and sequentially increasing to P4000 under a constant flow of water. A slurry of aluminum oxide with a mean particle size of 0.3 μm was used for the final polishing. The specimens were stained for 5 days using a tannic acid staining solution 0.47 M. Afterwards the surfaces were abundantly rinsed with MilliQ water using an electric tooth brush in order to eliminate all the surface stain remains, leaving only the stains adsorbed in the enamel.

Whitening treatments

MilliQ water was used as negative control (NC); carbamide peroxide 16% was freshly prepared dissolving the tablets in MiliQ water, at a concentration of 1.7 M; sodium metabisulfite (MBS) powder was dissolved in MilliQ water at a concentration of 0.47 M. The treatments were applied for a total of 20 minutes above the flattened surface of the specimens, which let the reaction occur during the determined time and removed before each measurement. An electric tooth brush was used for 30 seconds while rinsing the tooth with MilliQ water in order to prepare the surface for the colorimetric measurements. The measurements were performed after 3, 6, 9, 14, and 20 minutes in order to monitor the whitening effect over time.

Colorimetry

For color measurements, a contact-type spectrophotometer Minolta CR-231 obtaining color coordinates, L*, a*, and b*, was used. The overall color change is expressed as ΔE^*_{ab}, from the Commission Internationale de l'Eclairage, relative to the baseline color parameters, and using the following equation:

$$\Delta E^*_{ab} = \left[\left(\Delta L^*\right)^2 + \left(\Delta a^*\right)^2 + \left(\Delta b^*\right)^2 \right]^{1/2} \quad (4)$$

Measurements were performed at baseline and after 3, 6, 9, 14, and 20 minutes of treatment.

Statistics

One factor ANOVA was used to determine significant differences between the different molar ratios of MBS:TA used and Turkey's post hoc test to identify the significance between them. For the in vitro test, Kruskal-Wallis procedure was used to determine significant differences in color change among the groups at different times, followed by a Dunn-Bonferroni pairwise comparison. Tests of hypotheses were two-sided with an alpha level of 0.05. Analysis was conducted with IBM® SPSS® Statistics 20.0.0.

Results

Metabisulfite whitening efficacy

A first test to demonstrate that MBS can accomplish a bleaching effect was performed. An aqueous solution of tannic acid (0.08 mg/mL, 0.047 M) was mixed with CP with a final concentration of 16% (1.7M) (which is the concentration used in commercial products) as well as with the same concentration of MBS (1.7M). After 20 minutes of reaction, the absorbance of the two solutions was measured and compared with a control (TA solution).

A decrease in the absorbance value is related to a loose of color (i.e., to a whitening effect), and an increase corresponds to a darkening effect. The values presented in Table 1 show how CP doesn't achieve any whitening effect after 20 minutes, while the absorbance decreases by 16.2% when MBS is mixed with the TA solution.

Reductant concentration study

For future applications, lower concentrations of the bleaching agent will be preferable, since they can lead to cheaper and safer products. For this reason, different MBS:TA molar ratios were studied with the aim to select the most convenient reductant concentration for a visible whitening. In addition to the equimolar (MBS:TA, 1:1) concentration, a battery of lower relations, 1:2, 1:4, and 1:8, was tested. To explore the effect of increasing the reductant concentration, the ratios 2:1 and 4:1 were also tested.

Table 1 Absorbance of tannic acid (TA) control solution measured 20 minutes after the reaction with: carbamide peroxide(CP), sodium metabisulfite (MBS), and alone. [TA]: 0,08 g/mL; [MBS]: 1,7M; [CP]: 16% (1,7M)

ABS (450 nm)		
TA	TA + CP	TA + MBS
0.779 ± 0.002	0.707 ± 0.031	0.630 ± 0.011

As shown in Fig. 2 for the higher concentrations tested (1:1, 2:1, and 4:1), the whitening effect after 20 minutes of reaction was increased about 10% every time the MBS concentration doubled the TA's. Comparing the lower concentrations (1:2, 1:4, 1:8), we observe similar results. According to statistical analysis, all the samples differ from the control, and all the proportions tested differ from the equimolar experiment. In the former section, the proportion MBS:TA used for the test was almost 30:1, obtaining a 16.2% of whitening, which shows that further increase in the concentration of MBS leads to lower effectiveness.

Study of the whitening effect over time

To compare the MBS with the commercially available product effect over time, the whitening reaction was monitored for 48 hours. MBS was used in a molar ratio of 1:1 MBS:TA , since in the previous study, this concentration demonstrated a good whitening performance in the first 20 minutes (25%) and still left at room temperature to observe further changes over time. CP was kept in the standard commercial concentration (16% CP). In order to observe the effects of a strong oxidizing agent, potassium persulfate ($K_2S_2O_8$) (PS) ($S_2O_8^{2-}$, E^0 = 2.010 V) was also tested in a molar ratio of 0.5:1 PS:TA, since 1:1 was not possible due to the low solubility of the compound (4.49 g/100 mL).

In Fig. 3, there can be observed a faster and higher whitening effect of the MBS when compared with the one obtained from the CP, even if the used concentration of the reductant (4.7 x 10^{-2} M) is 36 times lower. In contrast, the reaction with the potassium persulfate shows how the TA gets dark when it is oxidized. No color variations were observed in the control.

Cyclic voltammetry

Cyclic voltammetry was used to induce an electrochemical redox process in the TA molecule, in order to study both its reduction and oxidation potential and whether a reduction process is taking place when it reacts with MBS. For this purpose, TA solution, MBS solution, and a mixture of both were analyzed.

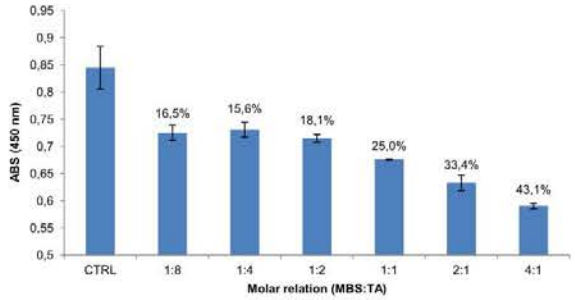

Fig. 2 Solution absorbance after 20 minutes of reaction at different molar ratios of TA and MBS. At the top of the bar, the corresponding bleaching effect with respect to the TA control (in %). (a) Statistically different from control. (b) Statistically different from 1:1 ratio

Figure 4 shows the cyclic voltammetry for the tannic acid solution, where an anodic peak (oxidation) can be observed at 400 mV; the results are consistent with those described in the literature [30]. The apparent reduction peak at 256 mV can be attributed to the discharge of the electric double layer, meaning that the electrochemical reaction of TA is irreversible. A continuous decrease in the anodic peak can be observed, as the TA molecules are being oxidized at the electrode surface. Due to non-hydrodynamic control of the experiment, the 1st cycle is observed for higher current density values than subsequent cycles owing to the initial massive oxidation of TA molecules close to the Au surface of working electrode. Once TA molecules near working electrode react, the current quickly drops off because TA must migrate from the center of the solution to the double layer boundary to continue reacting.

The measurement of a blank solution of MBS (Fig. 5) shows a unique anodic peak at 500 mV, which is decreasing with time, meaning that the sulfite ion is being oxidized to sulfate.

In Fig. 6, the blue curve corresponds to TA in the first 4 cycles where in the 5th cycle MBS was added. A continuous increase in the current intensity (from a to b) for the anodic peak was observed. The red curve corresponds to the TA and MBS solution previously mixed and measured after 5 minutes of stabilization time. Also, in this case, a progressive increase in the intensity of the anodic peak was observed. In both cases, the increase in the current intensity may indicate the effect of the MBS as a reductant, showing an increased oxidation of the TA on the Au electrode since more reduced species are present, thus indicating that a reduction reaction is taking place. A cyclic voltammetry for TA solution with 16% CP was also performed, but no relevant peaks were observed.

In vitro proof of concept

This experiment was carried out in order to see if MBS can achieve a tooth whitening effect on real teeth. To achieve it, a diffusion of the MBS from the surface of the enamel to the dentin needs to take place, since the enamel is translucent [31], and, to observe a bleaching effect, deeper stains need to be whitened as well. To favor this diffusion process, the MBS concentration used was 10 times higher (0.47 M) than the one used for the experiments in solution. Comparing the ΔE of the MBS and the CP solutions at the same molar concentration in Fig. 7, it can be observed how the MBS performs a significant faster bleaching during the first 3 minutes, achieving 5 points

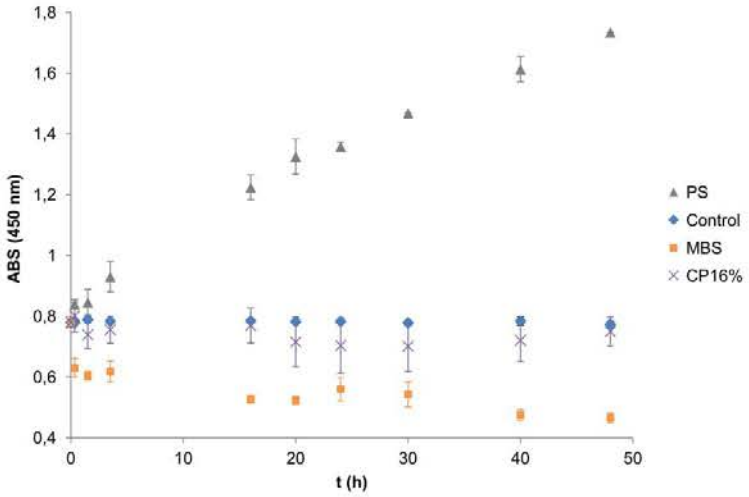

Fig. 3 TA reaction with $Na_2S_2O_5$ (0.047 M) and CP (1.7 M) measured as ABS versus time

of ΔE versus the 2 of the CP. After the 9th minute, the bleaching reaction performed by the MBS seems to be stabilized, reaching similar ΔE values as CP at 20 minutes treatment. The MBS showed a higher whitening effect when compared to CP, being significantly higher at all the time points measured but at 20 (Two-Sample Assuming Unequal Variance, $p < 0.05$). No previous publication of sodium metabisulfite applied to teeth whitening could be found on the literature, in order to compare the achieved result.

Discussion

Tooth whitening effect of MBS, a reducing agent, has been studied using tannic acid solution as a model staining solution and compared with an oxidant and to the regular tooth whitening ingredient carbamide peroxide. A visible bleaching effect, spectrophotometrically measured, occurs within the first 20 minutes when the reaction between the polyphenol and the reductant takes place in aqueous solution while it is not happening with CP (Table 1). CP is composed by hydrogen peroxide and urea, and the whitening agent (H_2O_2) needs to be released before it can perform its action. According to Haywood and Sword [32], 50% of the peroxide release occurs during the first 2 hours; thus carbamide peroxide products need long application times. Hence, it is not surprising that CP did not perform any whitening effect in the first 20 minutes, and the rapid effect of MBS could cover the goal of finding a whitening agent that permits short application times. Regarding the reductant concentration screening (Fig. 2), no significant differences were observed between 1:2, 1:4, and 1:8 molar ratios, an observation to take into account when optimal whitening secondary effects will be studied in future works, as lower concentrations will imply less damage on the teeth. If we consider the molar concentrations needed from

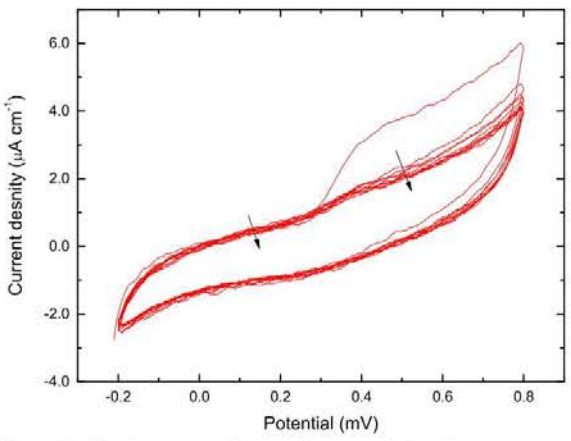

Fig. 4 Cyclic voltammetry of TA solution (1 mg/mL), pH

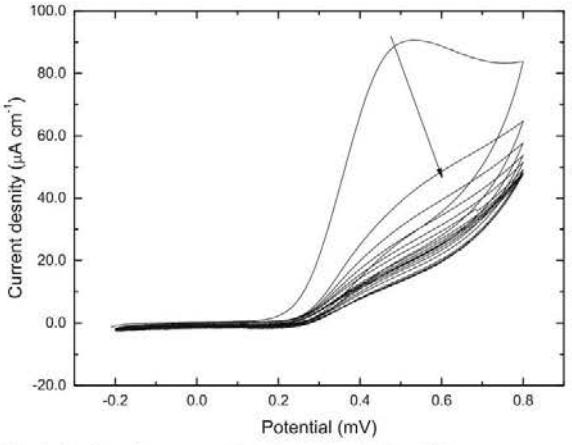

Fig. 5 Cyclic voltammetry of MBS solution ($1.88*10^{-5}$ M)

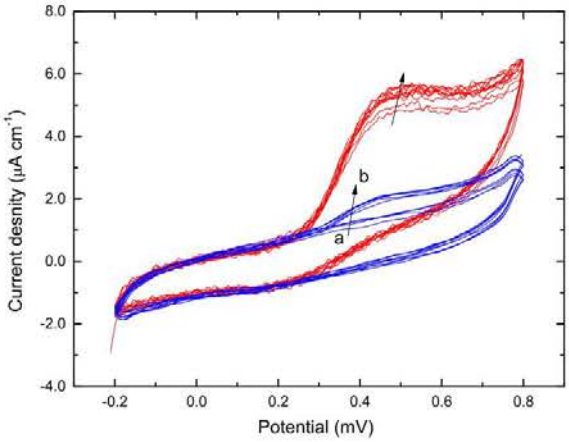

Fig. 6 Cyclic voltammetry of TA solution (1 mg/mL), Na_2SO_4 (0.5M), and MBS ($1.88*10^{-5}$ M)

each reagent, we notice that MBS acts with lower concentrations than CP, which could lead to cheaper products, also due to the fact that food grade MBS is cheaper than CP. While the mechanism by which CP is producing its bleaching effect is not well described, in this work we have studied the new whitening candidate by cyclic voltammetry, observing that the oxidation peak of tannic acid is being increased when MBS is added to the solution (Fig. 6). Due to the reduction process taking place, more reduced tannic acid is available in order to be oxidized, thus increasing the oxidation peak in the next cycle, behavior that cannot be observed in the control TA solution or when mixed with CP. Another point to highlight is the darkening effect observed when an oxidant is reacting with the tannic acid, probably due to the oxidation of the remaining phenol groups into quinones [33]. This result clashes with the theory that tooth whitening is due to oxidation, since we are obtaining the opposite effect. Finally, we have seen that MBS is able to perform the whitening effect also when applied on teeth. Since, as previously said in section 3.5, the penetration of the treatment into the enamel is necessary in order to obtain a whitening effect, this observation lead us to think that it exists a good diffusion of the reductant into the enamel within the 20 minutes studied. Furthermore, MBS treatment reached the same ΔE values than CP but in a shorter time (Fig. 7), an observation similar to the one made in tannic acid solution study (see section 3.3). These are promising results that can potentially lead to novel and enhanced products, since visible effects are obtained in faster times and lower concentrations than the one's needed for CP. Further studies to optimize MBS concentration, pH, and application time are needed, as well as the development of a pharmaceutical form (e.g., gel or stripes) which facilitate professional/user application.

Conclusions

The whitening effect of a reducing agent, MBS, was studied and compared with the widely used oxidant CP. MBS has shown a good whitening effect, reaching better (in solution) or equal (in vitro) ΔE values than CP but in shorter times and with lower concentrations. Even if this study is a proof of concept and treatment optimization is necessary, the results obtained may lead to the development of novel formulas in the tooth whitening market, making it evolve beyond peroxides.

Acknowledgments This work was supported by the Spanish Project CTM 2015-65414-C2-1-R and by the Universitat Autònoma de Barcelona with the "Personal d'Investigació en Formació" scholarship.

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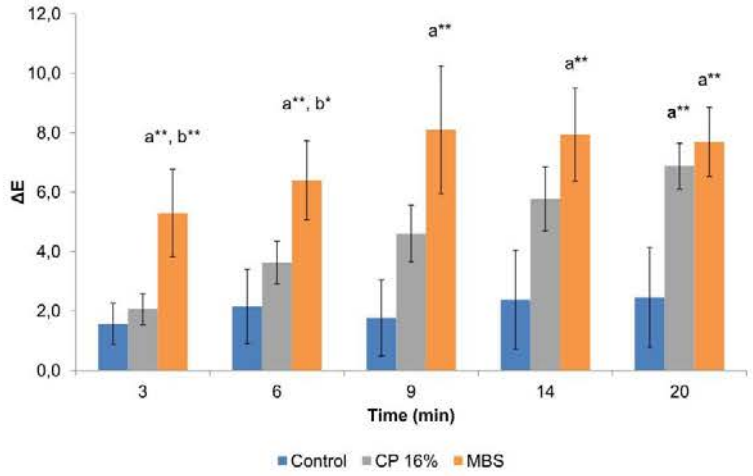

Fig. 7 Whitening effect of water (control), carbamide peroxide (CP 16%; 1.7 M), and sodium metabisulfite 0.47 M (MBS) in tannic acid stained teeth. N = 10. (a) Significant differences compared to control. (b) Significant differences compared to CP; ** $p < 0.01$; *$p < 0.05$

Compliance with ethical standards

Conflict of interest Clara Babot Marquillas declares that she has no conflict of interest. Maria-Jesús Sánchez-Martín declares that she has no conflict of interest. Raúl Procaccini declares that he has no conflict of interest. Manuel Valiente Malmagro declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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2.2 Flash tooth whitening: A friendly formulation based on a nanoencapsulated reductant

This paper was focused on the encapsulation of the whitening ingredient sodium metabisulphite (MBS) in liposomes, to enhance its performance by avoiding its oxidation until its contact with the tooth. It was reported how the liposomes interact with teeth, as well as the whitening efficacy of the encapsulated MBS. Finally, to go one step further, a formula with an adequate safety/ efficacy ratio for its application in humans was obtained and tested.

MBS was encapsulated using a saturated phospholipid, dipalmitoylphosphatidylcholine (DPPC), to avoid possible reactions between them. The liposomes were observed by Electron Transmission Microscopy, showing a size between 75 and 125 nm predominantly.

The interaction between the MBS liposomes and the tooth surface was studied by confocal microscopy. Liposomes formed a layer surrounding the enamel surface showing how, beyond protecting MBS from oxidation, they enhance the treatment efficacy favoring diffusion of the reductant towards the enamel.

The whitening effect over time studied by surface colorimetry using an in vitro model, comparing MBS liposomes with DPPC liposomes, MBS and CP. The encapsulated MBS showed a faster and more efficient whitening effect when compared with the other treatments, especially in the first 3 minutes. Moreover, the ΔE values obtained with MBS liposomes were higher than the sum of the values obtained with MBS and DPPC liposomes, suggesting a synergistic effect.

With the objective of formulate a suitable product suitable for the oral cavity, MBS and DPPC concentrations, as well as pH, were chosen as variables to be optimized by a two-level 2^3 factorial experimental design (DoE). The two effects studied - color (ΔE), surface roughness (Ra) and Hardness (*H*) - were measured by surface colorimetry, along with surface profilometry and nanoindentation techniques. The obtained linear models were used to obtain the optimized new formulation, named NewT101.

NewT101 was compared with standard treatments (HP 35% and CP 16%) following the "*ISO 28399 guidelines - Products for external tooth whitening*". NewT101 presented a whitening efficacy comparable to HP 35%, and produced an increase in the surface roughness after 3 treatment sessions, but within the standard accepted limits.

The better efficiency of this formulation encourages its use as an alternative to current oxidative treatments.

Colloids and surfaces B: Biointerfaces

Volume 195, November 2020

DOI: 10.1016/j.colsurfb.2020.111241

Flash tooth whitening: A friendly formulation based on a nanoencapsulated reductant

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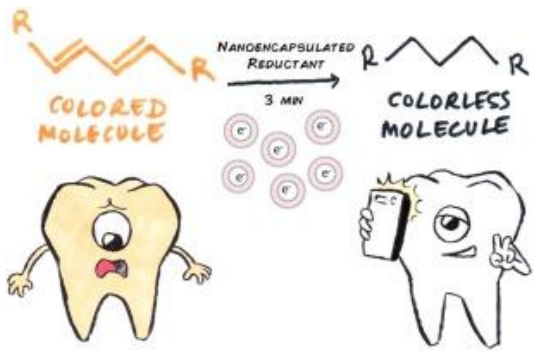
Graphical abstract



Colloids and Surfaces B: Biointerfaces 195 (2020) 111241

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Flash tooth whitening: A friendly formulation based on a nanoencapsulated reductant

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ARTICLE INFO

Keywords:
Tooth whitening
Reducing agent
Nanoencapsulation
DPPC liposomes
Sodium metabisulfite

ABSTRACT

Tooth whitening materials have not undergone relevant advances in the last years. Current materials base their action on the oxidant activity of peroxides, which present the disadvantage of requiring long application times, along with unpleasant side effects of dental hypersensitivity (*e.g.* sharp pain). In this work, a novel tooth whitening formulation based on the encapsulation of a reducing agent (sodium metabisulfite) in liposomes is developed. An experimental design was applied to optimize the formulation in terms of whitening action and safety, using bovine teeth as *in vitro* model. Results were obtained by colorimetry, profilometry and nanoindentation techniques. The comparison with standard whitening treatments showed a similar whitening action of the optimized formulation but in remarkable shorter application times. Moreover, teeth roughness values obtained with the presented formulation conformed with ISO 28399. As mechanism of action, results obtained from fluorescent confocal microscopy showed the liposomal formulation to form a layer surrounding the enamel surface, enhancing the treatment efficacy in terms of diffusion of the protected reductant towards the enamel. The better efficiency of this formulation encourages its use as an alternative to current oxidative treatments.

1. Introduction

Tooth bleaching has gained popularity over the last few years as a cosmetic treatment. It generates a market of millions of dollars per year, not only because of the treatments at the dentist office but especially since it has become more accessible to the customer thanks to the over the counter (OTC) products, which can be applied by the user himself at a lower price [1]. Everyone is exposed to tooth extrinsic staining; it occurs when organic colored molecules like polyphenols or flavonoids, coming from foods like coffee, tea or wine, are adsorbed in the tooth structure *via* hydrogen bridges [2,3]. Conjugated double bonds present in the structure of these molecules increase visible light absorption, darkening the teeth. Stains stay in the tooth surface but also diffuse to the enamel inner parts through its interprismatic spaces as well as to the dentin through the dentinal tubules and [4], since enamel is translucent, tooth color depends mostly on dentin color [5]. Surface stains can be physically removed using abrasive products or whitening toothpastes, while the inner stains need to be reached by a chemical reagent capable to diffuse to the discolored spots. This reagent must modify the light-absorbing nature of the staining molecules, increasing its perception of whiteness [6]. Commercialized bleaching products are based on hydrogen peroxide (HP) or carbamide peroxide (CP) at different concentrations (0.1–35 %); they act oxidizing the colored compounds yielding colorless molecules, leading to the desired whitening effect [7]. Generally, higher concentrations of peroxides result in a faster rate of whitening [8]. In addition to the high concentrations, long application times are required in order to obtain the desired effect [9]. Whitening treatments are claimed to be safe, but several side effects have been reported, such as gingival irritation and tooth hypersensitivity [10,11]. To overcome this problems, some studies explore faster and more efficient whitening results using metal complexes or nanoparticles in order to catalyze the oxidizing reaction [12,13], using products with optical properties like blue covarine [14], or even enzymes [15].

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https://doi.org/10.1016/j.colsurfb.2020.111241
Received 16 March 2020; Received in revised form 12 June 2020; Accepted 2 July 2020
Available online 02 July 2020
0927-7765/

In a former work [16], we explored a different approach based on a reducing agent, sodium metabisulfite (MBS), demonstrating a good *in vitro* performance within 20 min of application. MBS is a white yellowish crystalline powder with sulphurous odor and an acid saline taste. It is used as a food additive (E-223) [17]. and was already employed as a tooth whitening agent in the past [18,19]. It has been also studied as an ingredient for caries prevention [20]. In contrast to the oxidizing agents like peroxides, which act by oxidizing the double bonds, the reductant acts by saturating them, breaking off the aromaticity of the colored molecules, thus making them transparent. The results showed a faster whitening performance than carbamide peroxide, both in a tannic acid solution as well as in bovine stained teeth having visible whitening effects in just 3 min.

On exposure to air and moisture, MBS is slowly oxidized to sulphate. In water solution, it hydrolyses to hydrogen sulfite anion (HSO3-) with acidic pH [21]. Because of these characteristics, MBS needs to avoid contact with other substances before reaching the enamel stains, which can be achieved by incorporating the substance into a protecting drug delivery system. In this concern, liposomes were selected as encapsulating system able to release the MBS content when getting in contact with the teeth. Liposomes present the ability to encapsulate hydrophobic substances in the lipid bilayer [22,23] and/or water-soluble substances enclosed in the aqueous interior [24]. The interaction of liposomes with biological targets, mainly cells, is by adsorption (by specific interactions with cell-surface components or by electrostatic forces), endocytosis or much rarely fusion with the plasma cell membrane [25] and also by mimicking intrinsic cell structures [26]. The liposome delivers its content by facilitated diffusion, by lysosomal degradation of the endosome or directly into the cytoplasm [27]. Because of its biocompatibility, biodegradability, low toxicity and immunogenicity and targeting capability [28], liposomes are used in commercialized anticancer, vaccine and antibiotic formulations [29]. Their surface can be modified with multiple molecules (proteins, peptides, antibodies, enzymes, magnetic nanoparticles) to achieve different clinical applications [30,31], as well as the interior can be filled for clinical purposes, *e.g.* with magnetic fluid as a contrast agent for magnetic resonance imaging [32]. Liposomes can also be used as a barrier to protect its cargo from rapidly breakdown *in vivo*, enabling to use lower doses without losing efficacy [33]. Over the last years, the potential application of liposomes as a drug delivery system in the oral cavity has been studied, *e.g.* as a delivery vector for oral cancer therapy or for periodontitis treatment [34,35]. They have been applied as well in the tissue regeneration field; bioactive dentin growth factors and matrices can be incorporated and released from liposomes to recruit and differentiate dental pulp stem cells, promoting dentinogenesis *in vitro* Recent studies found the goodness of liposome as delivering system of a diversity of reagents for oral treatments, e,g, an alkali solution able to release its contents in a sustained way along 3 h, being effective to neutralize the acidification to combat the dentine erosion and demineralization in oral cavity [36], also a smooth release of not invasive anesthesia for periodontal treatment [37]. Regarding its interaction with teeth, it has been seen that liposomes can mimic the vesicular structures of salivary proteins which can be adsorbed onto enamel surfaces to provide a protective barrier [38]. Thus, it is expected that liposomes will provide appropriate carrying and release of inorganic HSO_3^- ions to the enamel surface [39]. To our knowledge, the use of liposomes in tooth whitening is unknown. The rationale for using liposomes is to accomplish a double function, as a carrier of the whitening compound to its target (*i.e.* stains adsorbed on the enamel) as well as to protect the compound until it reaches the tooth surface, allowing, thus, to employ lower concentrations. A saturated phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was chosen because it is not susceptible to further reductions, avoiding this way to react with the encapsulated MBS. DPPC is a zwitterionic phospholipid which forms neutral or lightly negatively charged liposomes [40]. *in vitro* studies of Nguyen et al. have demonstrated that liposomes having DPPC as the main lipid had the highest adsorption to hydroxyapatite (HA) powder (HA is the main component of the enamel) [38]. Liposomes adsorption on the enamel is reported 50 % and the attachment remains for at least 60 min under water flow [41], accomplishing a carrier function to point the enamel surface as a target. Moreover, Smistad et al. concluded that DPPC showed one of the lower toxicity in cell culture, when compared to other liposomes [42].

The present study develops a tooth whitening formulation consisting of DPPC liposomes encapsulating MBS buffered solution. A design of experiments (DoE) has been used for an efficient optimization of the formulation. DoE is extensively used in pharmaceutical field, and it has been previously used in formulating liposomes as a drug delivery system [43].

The main aim of this work is to develop a friendly novel whitening formulation, and to analyze the possible mechanism of action. On the other hand, the study includes the influence of the different components on the formulation performance by means of a DoE, in order to optimize its efficiency and to improve its safety.

2. Results

2.1. Metabisulfite encapsulation in DPPC

Transmission electron microscopy captures (Fig. 1) revealed DPPC liposomes obtained as described in section 5.2.1. They led to multilamellar vesicles presenting different sizes in a range of 260 to 20 nm,

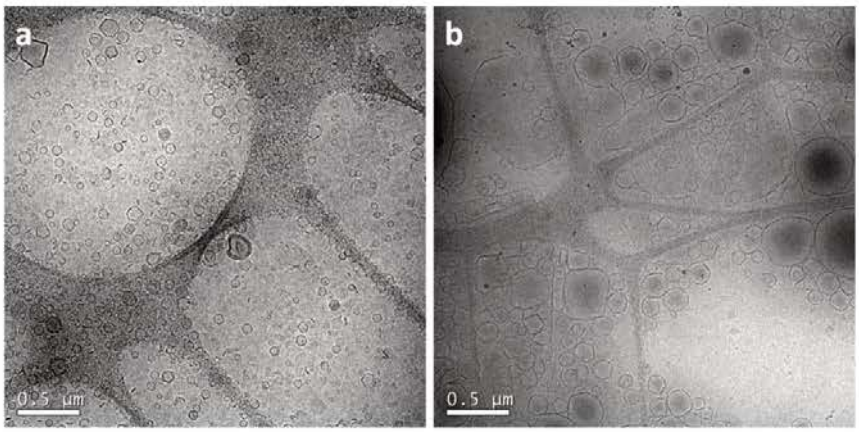

Fig. 1. Liposomes observed by Cryo-TEM at 6000 × . (a) DPPC liposomes; (b) MBS liposomes.

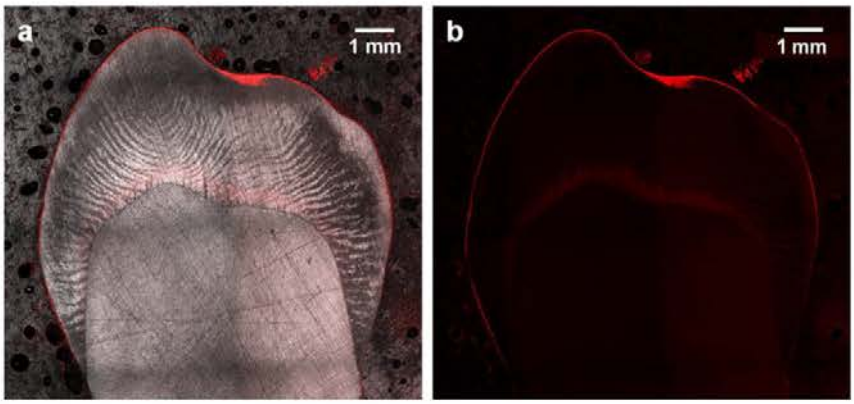

Fig. 2. Confocal images taken using excitation light from the laser line 561 nm, superposed to the one measured by reflected light 488 nm (a) and confocal images taken using excitation light from the laser line 561 nm (b) of tooth embedded in liposomes marked with Rhodamine B solution.

but with the highest size distribution between 40 and 60 nm (**Fig. S2a**), with both rounded and/or faceted polyhedral morphology. This transformation can occur when cooling a dispersion originally prepared above its phase transition temperature (Tm) (Fig. 1a). [44,45] Liposomes encapsulating MBS solution presented a more spherical shape (Fig. 1b), higher size distribution and bigger sizes in a range from 10 to 1000 nm being the maximum concentration between 75 and 125 nm (**Fig. S2b**), with a darkest color in the central part of the biggest liposomes due to the protrusion out of the vitrified film [46].

2.2. Liposome adhesion to the tooth surface

To reveal the behavior of the DPPC liposomes when contacting the enamel surface, fluorescent confocal microscopy was used.

Fig. 2 shows the liposomes attached to the tooth surface forming a thin fluorescent layer which is not present in the former tooth samples, in agreement with the studies of Nguyen et al. [38]. This layer was not observed in the control teeth, treated with water (*See supporting information Fig. S1a*) or RhodB (*Fig.S1b*). Control tooth showed autofluorescence probably coming from the collagen degradation already observed in non-fresh extracted teeth. [47] The RhodB tooth showed fluorescence emitted by the compound accumulated in the imperfections of the enamel, as well as in the organic parts surrounding the root, but either presented the layer surrounding the enamel.

2.3. Nanoencapsulated MBS as a whitening treatment

Previous studies showed the MBS capability for tooth whitening. [16] In this study, a comparison of the whitening effect of different treatments including MBS encapsulated in liposomes was conducted.

Fig. 3 shows how MBS performs a significantly faster whitening than CP during the first 3 min, achieving 7.3 points of ΔE in front of the 2 points of the CP. After the 9th minute the bleaching reaction performed by the MBS seems to stabilize, reaching significantly higher ΔE values than CP at 20 min of treatment. The encapsulated MBS showed a faster and more efficient whitening effect when compared with MBS. Moreover, the values obtained with MBS liposomes are higher than the sum of MBS and DPPC liposomes, showing a synergistic effect.

To evaluate the damage caused to the enamel by the different treatments, nanoindentation tests were carried out to measure the enamel surface hardness (H). The results reported in Table 1 show no differences between control, DPPC liposomes and CP, but a significant decrease in the MBS treatments.

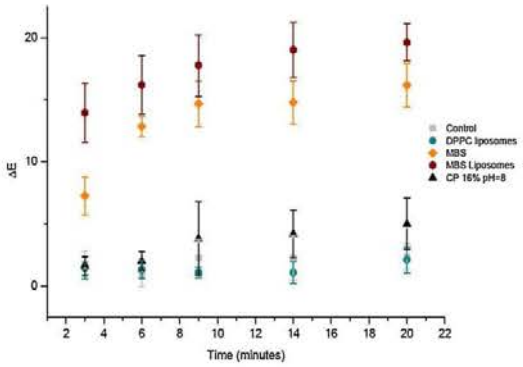

Fig. 3. Whitening effect over tannic acid stained teeth of water (control), DPPC liposomes (20 mM), carbamide peroxide (CP 16 %; 1.7 M; pH = 8), sodium metabisulfite 0.47 M (MBS) and encapsulated MBS (n = 8).

Table 1
Hardness values of the enamel surface after 20 min treatment.

Sample	pH	H (GPa)
H_2O	6.5	4.2 ± 0.3
DPPC liposomes (20 mM)	5.7	3.4 ± 0.4
MBS (0.47 M)	2.9	2.4 ± 0.2
MBS liposomes	2.4	2.6 ± 0.2
CP	8.0	4.1 ± 0.1

2.4. Formulation optimized by design of experiments

Observing the results of the previous section, it was determined that the formulation should be improved in order to find an acceptable compromise between its whitening performance and its side effects on enamel surface. To perform it, a factorial design was carried out, modeling color change (ΔE, Y_1) and enamel surface hardness (H, Y_2) responses. The variables studied were pH, MBS and DPPC concentrations at two levels. The results are shown in Table 2.

The obtained experimental responses were linearly modeled and expressed by the following equations (Eq. 3 and 4):

$$Y_1 = 6.40 + 1.19A - 1.56B + 1.74C - 2.10AB + 0.74AC - 1.08BC - 1.39ABC \quad (3)$$

$$Y_2 = 3.47 - 0.50A + 0.15B + 0.11C + 0.04AB - 0.04AC + 0.03BC + 0.11ABC \quad (4)$$

Table 2
Two-level factorial design of 3 factors (2^3) for the Liposomal Whitening Treatment (LWT) optimization. Independent Variables: MBS concentration (A), pH (B) and DPPC concentration (C), and response values, ΔE (Y_1) and H (Y_2).

Liposomal Whitening Treatment	Factor A [MBS] (M)	Factor B pH	Factor C [DPPC] (mM)	Y_1 (ΔE) (Mean ± SD)	Y_2 (H) (GPa) (Mean ± SD)
LWT1	0.01	5.5	2	4 ± 1	3 ± 1
LWT2	0.10	5.5	2	6 ± 1	2.8 ± 0.3
LWT3	0.01	6.5	2	4.4 ± 0.6	4 ± 1
LWT4	0.10	6.5	2	3.5 ± 0.9	2.9 ± 0.3
LWT5	0.01	5.5	20	6 ± 1	4.0 ± 0.7
LWT6	0.10	5.5	20	16 ± 2	2.4 ± 0.9
LWT7	0.01	6.5	20	7 ± 2	4.1 ± 0.2
LWT8	0.10	6.5	20	3.8 ± 0.6	3.3 ± 0.2

The error table for the linear model is shown in supporting information **Table S1**.

Observing the models, it can be said that whitening effect Y_1 response was deeply influenced by the three variables studied, being all of them as well as its interactions significant ($p < 0.05$) (see **Table S2** in supporting information).

Whitening effect increases when MBS and/or DPPC concentrations increase (note the positive coefficient in front of A and C in the equation modeling the system) and at lower pH values (factor B). It can be observed how some interactions are affecting the Y_1 response even more than its factors separately (note AB coefficient in Equation 3, **-2.1**). Hardness (Y_2) response is significantly influenced by all the variables but not by its dual interactions. It is clearly decreased when the formula gains MBS, and positively influenced when DPPC or pH are increased. Interaction ABC is significant in both Y_1 and Y_2, reflecting how all the variables are playing a key role in the formula.

Intermediate combinations within the studied space were explored for the different factors, being 0 the medium value of each factor, *i.e.* 0.05 mM for MBS, 6.0 for pH and 10 mM for DPPC (theoretical outcomes in Table 3), to find the formulation with the highest whitening effect but with the lowest effect on *H*.

Combination 101 (A = 1 B = 0 C = 1) predicted a good whitening effect while leaving the hardness over 3 GPa. Treatment 101 was synthesized and tested, and also two other randomly treatments (1 − 0-0 and 0 − 0-0) were chosen to check the predictability of the model.

Experimental outcomes (Table 3) showed that the linear model predicts better hardness outcome than whitening. Although the predicted values for ΔE are far from the experimental ones, a tendency is observed, *i.e.* the combination predicting higher values, also experimentally is the one that gives the highest value. This experiment confirmed a good performance with low side effects for the combination 1 − 0-1, thus it was chosen as the enhanced formula, from now on called NewT 101.

2.5. Enhanced formula applied to an in vitro model following ISO 28399

2.5.1. In vitro whitening test

In former sections, the test duration time for whitening formulations application was 20 min. However, *in vitro* whitening treatments require to conform the ISO 28,399 regulation that sets specific conditions closer to *in office* treatment. This standard was followed to test NewT 101.

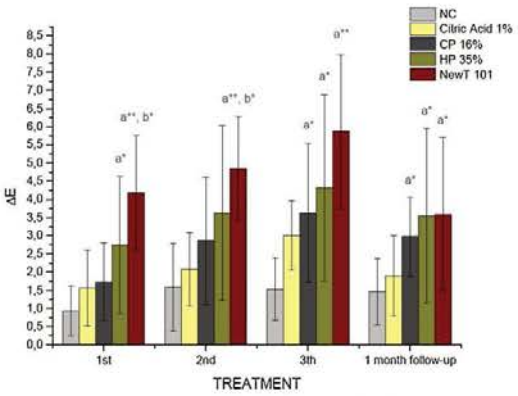

Fig. 4. Whitening effect (ΔE) over tannic acid stained teeth treated with: milliQ water (NC), citric acid 1% at pH 3.9 as a positive control, carbamide peroxide (CP 16 %; pH = 8), hydrogen peroxide (HP, 35 %, pH 8), NewT 101. a: compared to control; b: compared to citric; **: $p < 0.01$; *: $p < 0.05$.

Fig. 4 shows the whitening results obtained from the different treatments. After the first treatment, both HP and NewT 101 showed a significant increase of ΔE when compared to control without significant differences between them; NewT 101 differs also from the positive control. In the second treatment only NewT 101 showed statistically significant whitening effect, differing from the third treatment where the three of them have a statistically whiter color when compared to control. The one-month follow-up shows how the color of all groups are darker than after the third treatment, but still the bleached groups showed a whiter color than control.

2.5.2. Profilometry studies

Profilometry was applied to evaluate the surface roughness, related with the damage caused by the treatment. 11 specimens per group were analyzed.

The surface roughness of the specimens after the different treatments is shown in Fig. 5. The observed roughness effect of NewT 101, similar to citric acid, is attributed to the acid media affecting the enamel hydroxyapatite. This effect is absent in CP and HP because of the higher basicity of the media.

3. Discussion

3.1. Nanoencapsulated MBS as a whitening treatment

Results collected in Fig. 3, suggest a synergism of liposomes and MBS, in particular after 3 min of contact time. Such enhanced effect may be interpreted, firstly, as a higher MBS concentration reaching the enamel surface due to the liposome encapsulation protection that prevents the MBS degradation by oxidation (supporting information on **Table S3**). Secondly, liposomes located on the tooth surface [41] forming a layer as observed in Fig. 2c, favors the diffusion of the MBS

Table 3
Theoretical outcomes from the modeling.

	Theoretical outcomes from the modeling																	Experimental outcomes		
Factors Setting	A	0	1	1	0	0	1	−1	−1	0	0	−1	0	−1	0	1	−1	1	0	1	1
	B	0	0	1	1	0	0	0	−1	0	−1	0	−1	0	1	−1	1	0	0	0	0
	C	0	0	0	0	1	1	0	0	−1	−1	−1	0	1	−1	0	0	−1	0	0	1
Response	Y_1	6.40	7.59	3.93	4.84	8.14	10.06	5.22	4.67	4.66	5.14	6.22	7.96	6.22	4.19	11.24	5.76	5.11	5 ± 1	6 ± 1	5.9 ± 0.9
	Y_2 (GPa)	3.46	2.96	3.16	3.61	3.57	3.03	3.96	3.84	3.35	3.23	3.81	3.31	4.10	3.47	2.77	4.07	2.90	3.2 ± 0.4	2.4 ± 0.1	3.0 ± 0.2

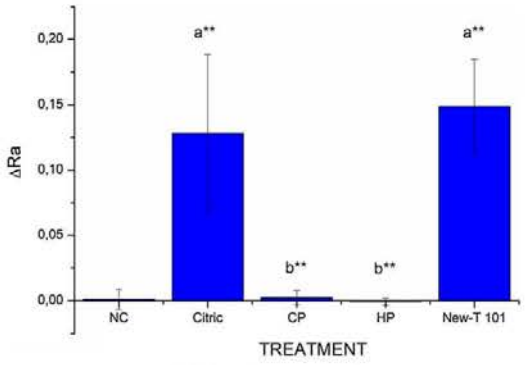

Fig. 5. Surface roughness (Ra) after 3 treatments of 1 h. Treatments: water as negative control (NC), citric acid 1% at pH 3.9 (citric) as a positive control (citric acid 1 %), carbamide peroxide (CP 16 %; pH 8), hydrogen peroxide (HP, 35 %, pH 8), NewT 101. a: compared to control; b: compared to citric; **: $p < 0.01$; *: $p < 0.05$.

into the enamel. When contacting tooth surface, liposomes release their cargo into the enamel and MBS diffuses towards dentine, otherwise observed whitening will not occur, since dentin highly contributes to the tooth color. [48]

Hardness results in Table 1, can be understood because of the high concentration of MBS (0.47 M) and low pH 2.9 employed, since the critical pH for enamel demineralization is generally considered of 5.5. [49,50] The MBS solution applied was fairly below this critical value, which explain the etching-like damage that it caused on the enamel.

3.2. Metabisulfite encapsulation in DPPC

The faceted shape presented by DPPC liposomes have been previously described and could be attributed to the physical gel state of DPPC at room temperature, which is under its liquid-crystalline phase transition temperature (41 °C). [44,45] As we underlined before, MBS liposomes (Fig. 1b) showed larger size and more rounded shape than the control ones (Fig. 1a) on in absence of MBS, showing that the presence of the metabisulfite affects the gel state of DPPC. This noticeable variation in the shape is understood by the influence of ionic strength, [51] pH and the presence of specific ions that can alter the surface charge and thus the interactions of the phospholipid headgroups, [52,53] modifying liposomes membrane properties. In our case, the three factors (MBS 0.47 M, pH 2.9 and the presence of ions HSO_3^-, H^+ and Na^+) contribute to the observed variation in the liposomes shape.

3.3. Liposomes adhesion to the tooth surface

As shown in Fig. 2, liposomes remained attached to the enamel surface. As indicated in previous sections, Nguyen et al. made similar observations, concluding that liposome's surface charge seems to play a highly important role on their significant bio-adhesion. In this concern, both calcium-dependent electrostatic and hydrophobic interactions could be involved in such strong liposomes adsorption phenomenon. [41] It is known that enamel surface is negatively charged when no divalent counterions are present, due to the negatively charged phosphate ions that dominate the HA surfaces; in contact with a solution, an hydration layer is formed, containing calcium ions acting as counter ions [54]. Consequently, acidic proteins or anionic species can be attached to calcium sites on the HA surface or to the hydration layer [55]. This phenomenon is related to the initial stage of the dental pellicle formation, characterized by an almost instantaneous adsorption of salivary proteins (amphiphilic phosphoproteins that have the ability to associate into micelle-like globules) on the enamel surface [56,57]. It needs to be noticed that in this experiment no enamel pellicle could be present in teeth specimens because of their etching treatment performed previously to liposomes contact, and no contact with human or artificial saliva occurred during the entire process. When treating the tooth with the liposomal solution, the interaction was taking place directly between liposome and the exposed enamel surface, thus liposomes could be behaving like salivary proteins forming the initial enamel pellicle.

3.4. Formulation optimized by design of experiments

It could generally be drawn from Table 2 that the experiments with higher MBS concentration lead to higher ΔE and lower GPa values. Experiment LWT6 had the highest whitening performance and the lowest GPa value, which seems reasonable being the one with the most extreme conditions (higher concentrations of reagents and lower pH).

The effect of the interactions between the different components is quantified in the linear modeling (Eq. 3 and 4). AB coefficient in Eq. 3 (–2.10) reflects how the MBS reducing activity is favored at acidic pH. This is consistent with the fact that sodium metabisulfite is not the actual reducing agent but its acidic product in aqueous solution, HSO_3^- [21] species that are favored at lower pH. Instead, the positive coefficient of the interaction AC (0.73) supports the previously indicated synergy between MBS and the liposomes. The linear models obtained were able to predict better the hardness value, but not the whitening. The explanation could be found in the colorimetric technique itself. Even if teeth are biological samples, bovine teeth give good reproducibility in terms of hardness, since all of them were obtained from cows from the same area, sharing the same age and diet. Even doing treatments at different times, the reproducibility of the nanoindentation was good. On the other hand, when measuring color, an extra step in sample preparation (staining) was added. The staining process may influence the final result of the bleaching treatment [58], and even if a pure molecule (tannic acid) was chosen in order to make the staining process more reproducible, a lot of factors can change the final outcome. For example, oxidation of the staining solution starts immediately after being prepared and it gets darker over time. This oxidation also leads to changes in the tannic acid solubility and tend to form a pellicle in the flattened tooth surface while being stained, which needs to be carefully cleaned every day during the staining process to facilitate the solution penetration into the teeth. Both described processes may influence the final staining level and, if the baseline measurements are different in between experiments, the whitening treatments will also show different outcomes. However, the tendency observed is the same when comparing results within the same experiment.

The enhanced formula NewT101 provides a whitening (ΔE) of 5.90 ± 0.9 after 20 min application, less than half of its non-optimized former version (14.0 ± 2.4), but this new formulation is safer as its pH is comprised in the mouth pH range; NewT101 contains 25 % less reducing agent than the first formulation and reduces the enamel hardness. Results of the optimized conditions are clearly superior that commercial treatments with CP 16 % (3.64) or even HP 35 % (4.32).

3.5. Optimized NewT101 applied to an in vitro model following ISO 28399

The *in vitro* comparison of HP, CP and NewT101 treatments by one-hour application following ISO 28,399, showed no significant differences between HP and NewT101 in both individual sessions and the 1-month follow-up measurements. However, observed differences between CP and both HP and NewT101 in sessions 1 and 2 are due, on the one hand, to the difference of oxidant concentration between CP(16 %) and HP(35 %) and, on the other hand, to the higher efficacy of reducing NewT101 activity against the oxidant CP action. Moreover, it is consistent with CP slower mechanism [59]. NewT101 had a similar whitening performance than HP, with lower standard deviation, meaning that it provides whitening results that are more homogeneous

than HP. Even if ISO 28399 requires one-hour application of the treatment, as we showed in Section 3.4 (Table 3), NewT101 whitened 5.9 points of ΔE in 20 min, which presents an advantage in terms of treatment time for both the patient and the dentist.

Profilometry results showed that NewT101 increased the surface roughness of the treated teeth, but no statistical difference when compared with the positive control (citric acid treatment) was observed. The standard ISO 28,399 considers a safe treatment when it does not exceed more than three times the level of roughness caused by the positive control (tested in accordance with annex B of the ISO document). Thus, NewT101 accomplishes with the standard and it is safe for the enamel.

4. Conclusions

This is the first study that evaluates the whitening effect of an enhanced formula composed by a reducing agent incorporated into liposomes. Experimental results lead to following conclusions:

- The protection of the reducing agent within liposomes leads to higher whitening values against current commercial dental whiteners, in particular within the first 3 min of contact.
- Liposomes form a pellicle surrounding the enamel surface, which may contribute to the improved effect observed.
- The optimized formula, NewT101, presents a comparable whitening performance to the widely known hydrogen peroxide at a concentration of 35 %, while preserving the standard of surface roughness after 3 treatment sessions. It has also the potential to maintain the same results within the first 20 min of application.

This new formulation has potential to become an alternative to the existing whitening treatments based on peroxides. Spite of the need for further studies including safety tests, the friendly chemical nature of NewT101 components, individually used in both food and cosmetic industries, assure this formulation (patent pending) to an easy way for clinical trials.

5. Experimental section

5.1. Materials

Tannic acid, sodium citrate, citric acid, calcium chloride, magnesium chloride hexahydrate, potassium dihydrogen phosphate, potassium chloride and HEPES sodium salt (buffer) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium hydroxide was purchased from VWR BDH Chemicals (Oud-Heverlee, Belgium). Sodium metabisulfite and potassium persulphate were purchased from Riedel-de Haën (Seelze, Germany). Carbamide peroxide was purchased from Acros Organics (New Jersey, USA) (it contains 35 % H_2O_2). A 16 % (w/v) solution was prepared before its use with deionized water and adjusting it with NaOH 3 M to pH 8.0. Hydrogen peroxide 35 % solution was purchased from Scharlau (Sentmenat, Spain). The solution was adjusted to pH 8.0 with NaOH 3 M. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar (Sigma-Aldrich), NaOH was purchased from Panreac (Barcelona, Spain). All chemicals were of analytical grade. Rhodamine B (RhodB) was purchased from Sigma-Aldrich. Deionized water was purified through a Millipore purification system from Millipore (Milford, MA, USA).

5.2. Methods

5.2.1. Preparation of DPPC liposomes

Liposomes were obtained by hydration and further mechanical dispersion. Briefly, DPPC was dissolved in chloroform/methanol (2:1, v/v) at 100 mM. 0.4 mL of the solution were introduced in a bottom round flask and evaporated using a rotary evaporator (Büchi, Flawil, Switzerland). When a thin layer of DPPC was obtained on the inside surface of the flask, 2 mL of purified water were added to the flask and then the suspension was mechanically downsized with an ultrasound bath for 15 min. When liposomes contained sodium metabisulfite (MBS liposomes), 2 mL of a sodium metabisulfite solution (0.47 M) were added to layer of phospholipid and then, the suspension was treated as above. When liposomes contained whitening treatments (Liposomes LWT 1–8), 2 mL solution of sodium metabisulfite (0.1 or 0.01 M) in water or in citrate buffer solution (pH 5.5 or 6.5, adjusted with NaOH 3 M) were added to phospholipid as indicated for plain liposomes. To prepare liposomes with RhodB, the phospholipid was mixed with a chloroform solution of RhodB. The final solution contained a 1% (w/w) of RhodB. All liposomal preparations were freshly prepared the day before the application and stored at 4 °C until their use.

5.2.2. Cryo transmission Electron mycroscopy (Cryo-TEM)

Liposome dispersions were characterized by Cryo-transmission electron microscopy using a JEM-2011, JEOL, JAPAN. Japan Samples for Cryo-TEM studies were prepared by depositing a small drop (~3 μL) of the sample on a copper grid covered with a carbon reinforced holey polymer film. Thin sample films (30 − 300 nm) were prepared by blotting the grid with a filter paper for 1.1 s. After blotting the grid was immediately plunged into liquid ethane kept just above its freezing point. Samples were kept below −179 °C and protected from atmospheric conditions during both transfer from the preparation chamber to the microscope and during examination. All sample preparations were performed in a custom-built climate chamber at 25 °C and 70 % relative humidity. Images were analyzed manually using Image J (https://imagej.nih.gov/ij/); a total surface of about 12 μm^2 has been analyzed for each sample (DPPC liposomes and MBS liposomes), using 2 images at 5000x and 2 images at 8000x.

5.2.3. Specimen preparation

5.2.3.1. Specimens for colorimetry. Bovine teeth where used for the colorimetric tests, since they have a flat surface that allows the correct measurement with the surface colorimeter, and they are considered a validated model in the ISO 28,399 products for external tooth bleaching tests [60]. Specimens were cleaned of gross debris and the root was sectioned using a diamond saw and preserved in sodium azide solution (0.2 %) until the experiment performance. The crowns were stained for 5 days immersing them in an aqueous solution of tannic acid (0.047 M), subjected to 37 °C and under stirring [61]. The stained teeth were embedded in self-curing polyacrylic cylinders. The surface was polished to expose a window of at least 3 × 3 mm of the enamel surface which permitted the roughness and colorimetric measurements. The surface was polished using a sequence of silicone carbide paper starting at grit size P400 and sequentially increasing to P4000, under a constant flow of water. Then, a sequence of diamond pastes with a mean particle size of 3 and 1 μm followed by a slurry of aluminum oxide with a mean particle size of 0.3 μm were used. Finally, they were stored in artificial saliva for 24 h prior to the experiment. Artificial saliva was prepared according to the modified Shellies solution [62], and adjusted to pH 7.0 with KOH pellets.

For 20 min whitening applications, the different treatments -water as negative control MBS (0.47 M), DPPC liposomes (20 mM), MBS encapsulated in liposomes and carbamide peroxide (16 %) as the commercial treatment- were freshly prepared and applied on the flattened surface of the stained specimens. Colorimetric measurements at 3, 6, 9, 14 and 20 min were performed, followed by treatments removal from the specimens, using an electric toothbrush while rinsing with milliQ water for 30 s.

For ISO 28,399 whitening test, treatment regimens -water as control, citric acid 1% as positive control, HP (35 %) and CP (16 %) as commercial treatments and NewT 101- consisted in three applications (1 h each) of the freshly prepared solutions, at 5-days intervals. 50 μL of the corresponding treatment solution were applied covering the

exposed enamel surface, refilling with 25 µL of the same solution every 20 min during the 1 h session, thus, the final volume of the treatment used was 100 µL. The negative (NC) and positive control (PC) groups were treated according to the ISO regulation, with grade 3 water and citric acid (1.0 %) adjusted to pH 3.9 for 60 min at 35 °C, respectively. In all cases, after each treatment, teeth were stored in artificial saliva.

5.2.3.2. Specimens for nanoindentation and profilometry. Bovine teeth specimens were prepared in the same way as in the previous section but avoiding the staining step. These specimens were used to evaluate their Roughness (Ra) before and after the treatment by means of profilometry, and their hardness (GPa) after the treatment applications through nanoindentation.

5.2.3.3. Specimens for confocal microscopy. In order to study the interaction of the liposomes with teeth, whole human molars, preserved in 0.5 % chloramine solution, were used. After treating the samples for 30 s with HCl (1M) in order to remove any organic parts to expose the enamel surface, the specimens were submerged for 24 h in the corresponding solution: milliQ water as control, rhodamine B (RhodB) solution 2×10^{-3} M and rhodamine-labeled liposomes prepared as described in section 5.2.1, ensuring that only the tooth crown remained in contact with the solution, to avoid its penetration through the root. Whole teeth, including roots, were embedded in self-curing polyacrylic cylinders. Before the treatment, the exposed surface was polished as described in section Specimens for whitening treatment and measured the same day to avoid dehydration. After the treatment, the embedded specimen was cut along the vertical plane, in order expose all the interior parts of the teeth, from the enamel to the dentin, with the aim to observe any penetration pattern through this structure during the treatment, specially through the enamel. The exposed surface was polished as described in section *Specimens for whitening treatment* and measured the same day to avoid dehydration.

5.2.4. Confocal microscopy

The samples were mounted on a glass slide. Mapping images were taken with a Leica SP5 (Carl Zeiss Microimaging GmbH, Jena, Germany), using reflected light (488 nm) for the specimens and light from the laser line 561 nm for the chromophore, with an objective of 5x and a step size of 12 µm. All images were further processed with Leica Application Suite Advanced Fluorescence 2.6.0.7266, 2011 Leica Microsystems CSM GMBH.

5.2.5. Tooth color measurement

For color measurements, a contact type spectrophotometer Konica Minolta CR-321 Chroma Meter Colorimeter Bundled W/ DP-301 Data Processor was used, obtaining the CIELAB color space. It expresses color as three values, L*, a*, and b*. The overall color change is expressed as ΔE^*_{ab}, from the *Commission Internationale de l'Eclairage*, relative to the baseline color parameters, according to Eq. (5)

$$\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (5)$$

5.2.6. Profilometry test

The profilometry tests were performed following the international standard ISO 28399. The equipment used for the roughness measurements was a Leica confocal microscope, model DCM-3D, using the acquisition software Leica SCAN DCM3D version 3.3.2.4. Two areas of 0.95 × 1.27 mm were measured in each sample, at a speed factor of 1x and a threshold of 5. The data were analyzed using Leica Map Premium software version 6.2.6409. The data processing was performed applying a filtering with a threshold of 250 µm was applied to the data acquisition. Roughness (Ra) of every sample was obtained as a mean of the two measurements and roughness change (ΔRa) was calculated by subtracting post-treatment values from baseline values. The treatment regimen was applied in the same way as for the efficacy study, but in no stained teeth.

5.2.7. Nanoindentation

Enamel surface modulus of elasticity (Young modulus) and hardness of two specimens of each treatment were determined by nanoindentation test using a Berkovich tip mounted on a nanoindenter (Nanoindenter XP-MTS). Before every test, the Berkovich diamond indenter was calibrated on a standard fused silica specimen. Hardness is given by Eq. (6).

$$H = \frac{P_{max}}{A_r} \quad (6)$$

5.2.8. Statistical analysis

For the *in vitro* test, Kruskal Wallis test was used to determine significant differences in color change among the groups at every time point, followed by a Dunn-Bonferroni pairwise comparison. Tests of hypotheses were two-sided with an alpha level of 0.05. Analysis was conducted with IBM® SPSS® Statistics 20.0.0. One factor ANOVA was used to determine significant differences among treatments' roughness output, and a Turkey's post-hoc test was used to identify the significance between them.

5.2.9. Experimental design

A two-level factorial design of 3 factors (2^3) was used to study the effects of the amount of MBS, the lipid concentration, and the pH. Every factor was studied at two levels, lower (-) and upper (+), and two responses, whitening (ΔE) and surface roughness (ΔRa) were measured, as shown in Table 2 in section 3.4.

Ethical statement

Human teeth were obtained from adult donors that got extractions for different clinical reasons in the health department of the Autonomous University of Barcelona. An informed and written consent was given by the donors to the health department and given anonymously to the research group.

CRediT authorship contribution statement

Clara Babot-Marquillas: Conceptualization, Investigation, Writing - original draft. **María-Jesús Sánchez-Martín:** Conceptualization, Methodology, Writing - review & editing. **Jorge Rodríguez-Martínez:** Investigation, Writing - review & editing. **Joan Estelrich:** Resources, Validation, Writing - review & editing. **Maria-Antonia Busquets:** Validation, Writing - review & editing. **Manuel Valiente:** Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Spanish Project CTM 2015-65414-C2-1-R (Spanish Ministry for Education and Science) and the *Secretaria d'Universitats i Recerca* of the *Departament d'Empresa i Coneixement* from *Generalitat de Catalunya* through the project 2018 LLAV 00052, this project has been co-financed by the European Union through the Regional Development European Fund (FEDER) .

Clara Babot is a recipient of "*Personal d'Investigació en Formació*" scholarship and "*Ajuts per a estades breus a l'estranger*" from Universitat

Autònoma de Barcelona. She wants to acknowledge Prof Yiming Li for receiving her at the Center for Dental Research in Loma Linda University, and for all the knowledge acquired during her stay under the supervision of Dr. So Ran Kwon.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2020.111241.

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Supplementary Material

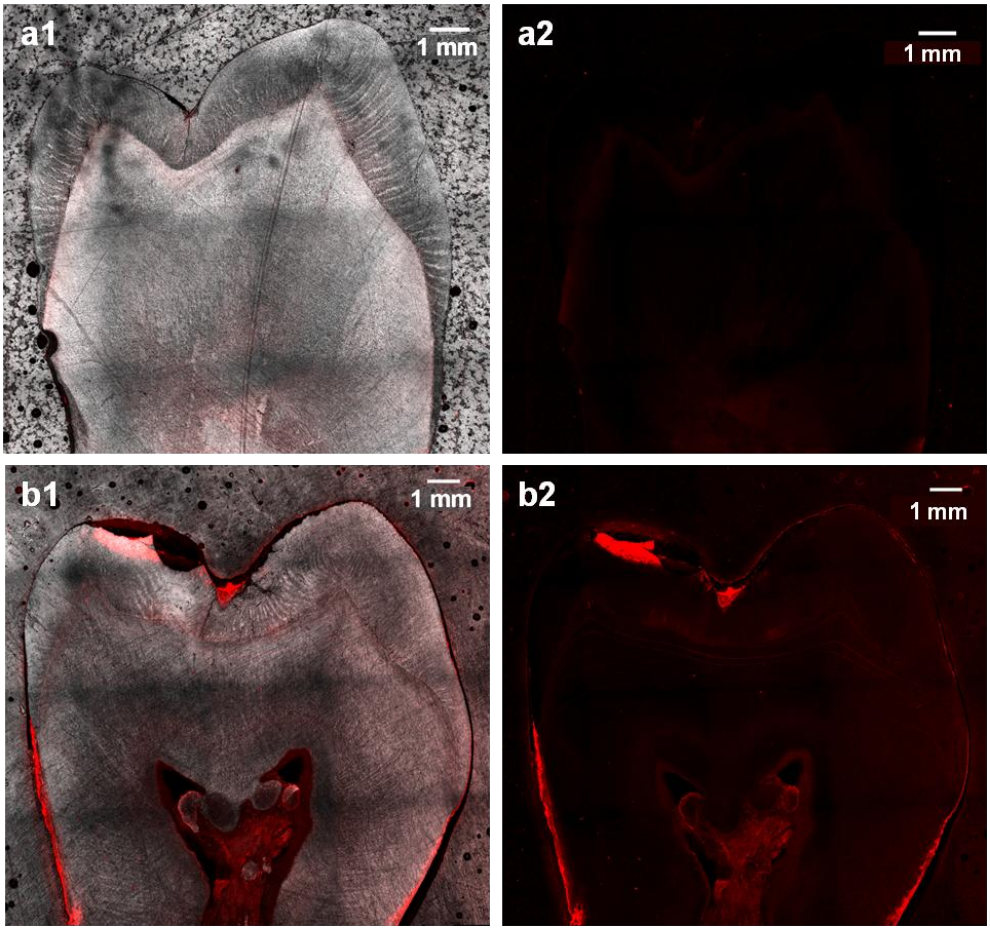

Figure S1. Confocal images taken using excitation light from the laser line 561 nm, superposed to the one measured by reflected light 488 nm (1) and confocal images taken using excitation light from the laser line 561nm (2) of tooth embedded in water (a) and RhodB solution (b).

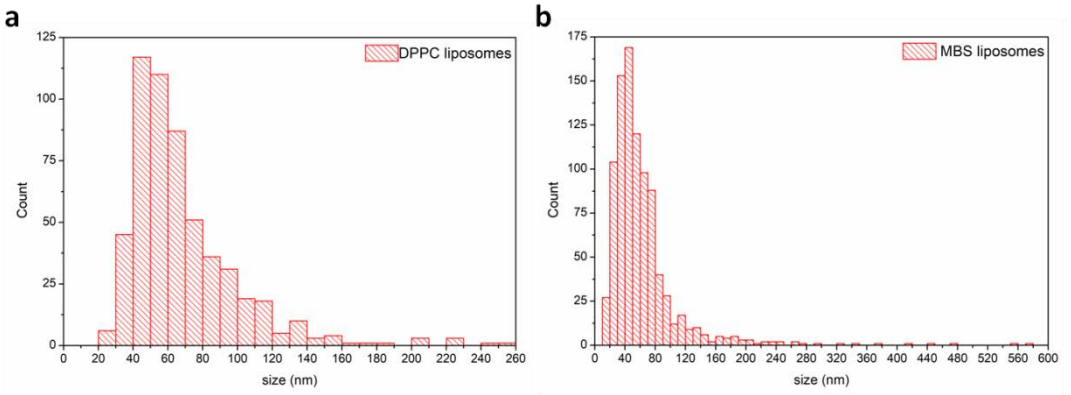

Figure S2. (a) DPPC liposomes size distribution. (b) MBS liposomes size distribution

Table S1. Error table for linear model

	Y_1 (ΔE)	Y_2 (H)
S	1	0.2
Mean	6	3.6
C.V. in %	21	6.1
R^2	0.9225	0.9082
Adjusted R^2	0.8886	0.8680
Predicted R^2	0.8256	0.7935

Table S2. Coefficients for the variables studied and statistical values

	Y_1 (ΔE)			Y_2 (H)		
	B-coefficient	p-value	t-value	B-coefficient	p-value	t-value
B_0	6.40			3.47		
A	1.19	0.00	4.24	-0.50	0.00	-11.45
B	-1.56	0.00	-5.57	0.15	0.00	3.50
C	1.74	0.00	6.22	0.11	0.02	2.50
AB	-2.10	0.00	-7.50	0.04	0.37	0.92
AC	0.73	0.02	2.64	-0.04	0.37	-0.91
BC	-1.08	0.00	-3.86	0.03	0.45	0.77
ABC	-1.39	0.00	-4.97	0.11	0.02	2.54

Table S3. *In vitro* whitening effect of the different treatments at different time points.

TREATMENT/MINUTES	3’	6’	9’	14’	20’
MBS	7±2	12.9±0.8	15±2	15±2	16±2
DPPC LIPOSOMES	1.5±0.9	1.3±0.7	1.1±0.4	1.1±0.9	2±1
MBS LIPOSOMES	14±2	16±2	18±3	19±2	20±2
SUM OF MBS + DPPC LIPOSOMES EFFECT	8±2	14±1	16±2	16±2	18±2

3. DISCUSSION

In this work a novel tooth whitening treatment consisting on an encapsulated reducing agent has been studied. Its development was based in two hypothesis; the first one, that the use of a reducing agent (MBS) would have a whitening effect over the extrinsic tooth stains; the second, that the encapsulation of the reducing agent in liposomes (DPPC) will carry and protect the reductant, improving its effectiveness.

For its discussion, the results obtained have been divided in two parts. In first place, the results related to the effectiveness of the new ingredient, in terms of whitening performance and its effects over the enamel hardness and surface roughness, compared with the current commercialized oxidizing treatments. This part goes from the proof of concept to the optimization of the whitening formula, to transfer its applicability from the laboratory to the market. In second place, the results related to the mechanisms of action of the reductant and the liposomes, in terms of its physico-chemical interactions with the stains and the enamel.

3.1 Treatment effects and optimization

3.1.1 In vitro proof of concept: whitening and hardness

Bovine tooth were used as *in vitro* model to evaluate the whitening effects of the MBS encapsulated in DPPC liposomes. This model also permitted to measure the enamel surface hardness, in order to quantify the secondary effects of the treatments. Table 2 shows the whitening effect (ΔE) over time, and the Surface Hardness (GPa) obtained after 20 minutes of the different treatments.

Table 2. *In vitro* study of the whitening effect (ΔE) over tannic acid stained teeth of: water (control), DPPC liposomes (20 mM), carbamide peroxide (CP, 16%; 1.7 M), sodium metabisulfite (MBS, 0.47 M) and encapsulated MBS (MBS liposomes 0.47M MBS/20 mM DPPC); n=8. Surface hardness (H) after 20 minutes treatments over teeth without staining.

TREATMENT/Time (min)	Whitening (ΔE)					pH	*H* (GPa)
	3’	6’	9’	14’	20’		20'
Control	1.8±1.1	0.9±0.9	2.3±1.4	2.3±1.8	2.4±1.0	6.5	4.2±0.3
CP	1.7±0.8	2.0±0.8	3.9±3.0	4.2±1.9	5.0±2.1	8.0	4.1±0.1
MBS	7±2	12.9±0.8	15±2	15±2	16±2	2.9	2.4±0.2
DPPC LIPOSOMES	1.5±0.9	1.3±0.7	1.1±0.4	1.1±0.9	2±1	5.7	4.0±0.2
MBS LIPOSOMES	14±2	16±2	18±3	19±2	20±2	2.4	2.6±0.2
SUM OF MBS + DPPC LIPOSOMES EFFECT	8±2	14±1	16±2	16±2	18±2	-	-

Firstly, it can be seen how, as was observed in solution, the whitening effect of MBS is much faster than the one obtained with CP, showing a ΔE increase of 7 in only 3 minutes. In a recent review, it is concluded that an overall color change of ΔE 2.872 is already perceptible [1], meaning that MBS performed a perceptible whitening in just 3 minutes, while CP needed 9 minutes to cross the threshold. Since this experiment was performed with actual (bovine) teeth, it is important to consider the diffusion rate (D) of the whitening agents. *D* is a function of several factors including molecular weight of the diffusing species, temperature, and

viscosity of the medium in which diffusion occurs. For ions in aqueous solutions, the ion charge density influences the size of the hydration shell around the ion which, in turn, influences the value of the diffusion coefficient for that ion. In water and at 25ºC, *D* it is reported to be $1.33*10^{-5}$ cm^2 s^{-1} for HSO_3^- [2], and $1.19*10^{-5}$ cm^2 s^{-1} for HP [3]. Being both molecules of the same size, their diffusion in water is similar. Thus, the difference observed for the whitening effect is not related to its diffusion but to its *k*, as discussed in section 0.

On the other hand, the whitening effect obtained with MBS liposomes was bigger than the sum of the whitening obtained by DDPC liposomes and MBS, specially at 3 minutes of contact time, suggesting a synergism of liposomes and MBS. Such enhanced effect may be interpreted, firstly, as a higher HSO_3^- concentration reaching the enamel surface due to the liposome encapsulation protection that prevents it from oxidation. Moreover, when approaching tooth surface, liposomes attach to the enamel surface forming a layer, as will be seen in section 0. Afterwards they release their cargo (MBS solution), and the formed layer prevents its diffusion towards the outside, favoring the diffusion of the MBS into the enamel.

Nevertheless, this initial formula had a pH of 2.9. The solubility of HA increases about 10-fold for each unit decrease in pH [4], hence, in acidic MBS liposomes conditions, the enamel experienced erosion [5], leading to a low value in the enamel surface hardness of 2.6 GPa. The formula needed to be optimized in order to make it suitable to be used in the oral cavity.

3.1.2 Formula optimization

The next step was to perform a 2^n DoE to study the influence of the components in the formula, [MBS] (**A**), [DPPC](**C**) and pH (**B**) over the whitening (ΔE) and enamel surface hardness (*H*). The pH range (5.5-6.5) was chosen considering the mouth pH, which is generally in the range of 6.5-7.0 [6], and the critical value for HAP dissolution, which is 5.5 [4]. Considering the acidic nature of MBS, its concentration was studied in the range 0.1-0.01 M (reduced when compared to the proof of concept formulation, where was of 0.47 M). Finally, DPPC, originally 20 mM, was studied between 2-20 mM in order to observe if the amount of phospholipid could be reduced without losing effectiveness, since it is the most expensive ingredient of the formula, therefore a limiting factor in future commercialization of the whitener. Linear modeling was calculated from the experimental values of *AE* and *H*, which permitted to quantify the influence of each factor, and to explore intermediate points in the system. Equation 14 permits to obtain the theoretical whitening yield that will be obtained with the different values of A, B and C, and equation 15 calculates the hardness.

$$Y_{\Delta E} = 6.40+1.19A-1.56B+1.74C-2.10AB+0.74AC-1.08BC-1.39ABC \quad \text{(Eq. 14)}$$

$$Y_{H} = 3.47-0.50A+0.15B+0.11C+0.04AB-0.04AC+0.03BC+0.11ABC \quad \text{(Eq. 15)}$$

Equation X coefficients showed, not surprisingly, that MBS (+1.19) and DPPC (+1.7) increasing concentration and a pH reduction (-1.56) had a positive effect in the whitening; also that a strong influence of the interaction between MBS and DPPC exists (-2.10), confirming the synergism between these two factors. Equation Y showed how an increase in the [MBS] and a

decrease of pH lead to a reduction in the hardness, while an increase of the [DPPC] helped to avoid or reduce this effect.

A formula with a good whitening-hardness balance was obtained and was named NewT101, whose composition was 0.1M MBS and 20 mM DPPC, at pH 6. Treatment 101 was synthesized and tested in a 20 minutes application. The predicted and obtained values are compared in Table 3.

Table 3. Theoretical and experimental outcome of NewT101, after 20 minutes treatment.

		Theoretical outcome	Experimental outcome
Response	Y_1 (ΔE)	10.06	5.9 ± 0.9
	Y_2 (GPa)	3.03	3.0 ± 0.2

The model predicted well the Hardness but not the whitening; this was probably because when staining tooth for the whitening tests, even if using the same staining days, the final color obtained is not constant. Nevertheless, a perceptible whitening effect was obtained in only 20 minutes while improving the surface hardness value (3.0 GPa) when compared to the proof of concept (2.4 GPa).

3.1.3 ISO 28399: in vitro test with the optimized formula

Finally, and as a first step for a future market launch, an *in vitro* experiment was performed following the whitening protocols described in *ISO 28399: products for external whitening.* They consist in applying the whitening treatments as they are actually applied in the dental clinics, evaluating the whitening effect (ΔE) after 3 sessions of 1 hour of application, and the enamel roughness variation (ΔRa) before and after the whole treatment. The results obtained are shown in Figure 27.

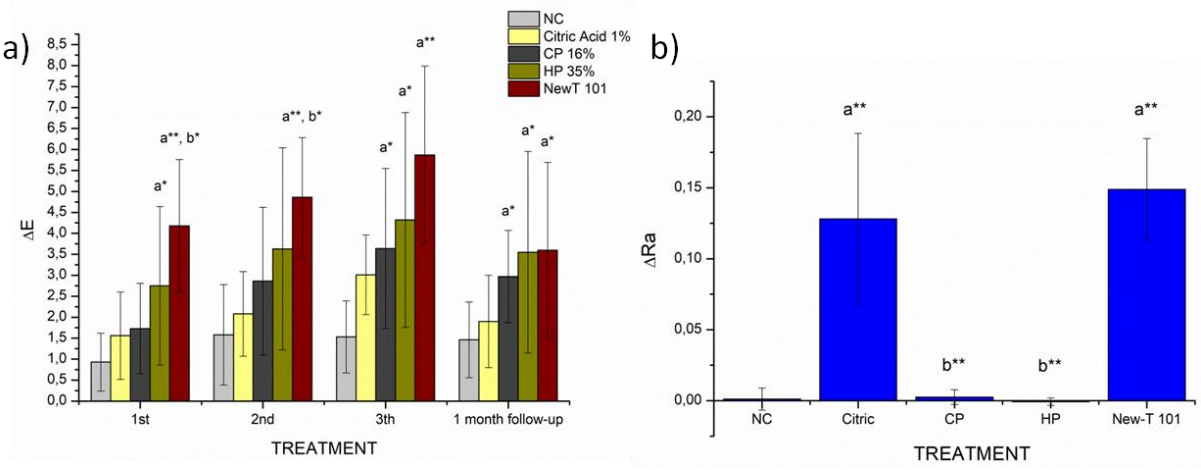

Figure 27. a) Whitening (ΔE) effect of the different treatments after 1, 2 and 3 sessions and one month after the treatment; b) Roughness (Ra) variation before and after the whole treatment. The different treatments are: Negative Control (NC, water), Positive control (citric acid, pH=4), HP 35% (pH=8.0), CP 16% (pH=8.0), and NewT101 (pH=6.0). Statistics (KW test, Turkey's post-hoc test); a: compared to control; b: compared to citric; **: p<0.01; *: p<0.05.

Figure 27 shows the whitening results obtained from the different treatments. After the first treatment, HP and NewT101 showed a significant increase of ΔE when compared to control without significant differences between them, while CP didn't show any significant change. NewT101 differed also from the positive control. In the second session only NewT101 showed statistically significant whitening effect, while in the third treatment, the three of them have a statistically whiter color than control. The one-month follow-up shows how the color of all groups are darker than after the third treatment, but still the bleached groups showed a whiter color than control. The observed differences between CP and HP are due to the difference in the oxidant concentration between CP (contents 5.6% of HP) and HP (35%), and is also consistent with CP slower mechanism [7]. NewT101 had a similar whitening performance than HP, with lower standard deviation, meaning that it provided more homogeneous whitening than HP.

NewT101 leads to a surface roughness similar to citric acid. This is attributed to the acid nature of both compounds, since the acid can dissolve the enamel hydroxyapatite leading to erosion [5]. This effect is absent in CP and HP because of the higher basicity of the media. The standard ISO 28399 considers that the product is applicable for tooth whitening when the roughness obtained does not exceed more than three times the level of roughness caused by the positive control (tested in accordance with annex B of the ISO document). The increased roughness of the NewT101 treated teeth shows no statistical difference when compared with the positive control (citric acid treatment), thus it accomplishes with the standard and it is safe for the enamel.

Even if ISO 28399 requires 1 hour application of the treatment, in section 3.1.2 it was seen that NewT101 has the ability to whiten in just 20', which may reduce the secondary effects intended as surface roughness. It also presents an advantage in terms of treatment time for both the patient and the dentist.

3.2 Treatment mechanisms

3.2.1 Tannic acid reduction by MBS

To observe the reduction of the stains by the reducing agent, solutions of tannic acid, MBS and its interaction were studied by cyclic voltammetry.

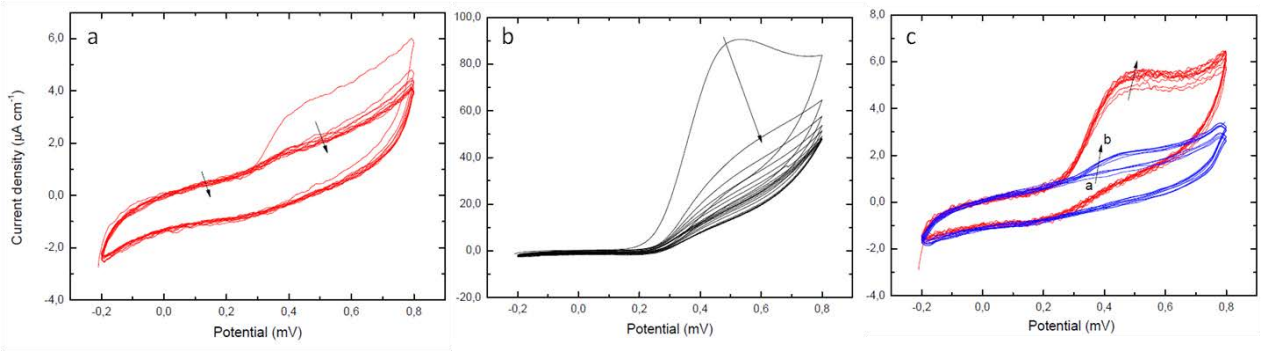

Figure 18. a) Cyclic voltammetry of TA solution (1 mg/mL) and Na_2SO4 (0.5 M); b) Cyclic voltammetry of MBS (1.88*10-5 M) and Na_2SO_4 (0.5 M); c) Cyclic voltammetry of TA solution (1 mg/mL), Na_2SO_4 (0.5 M), and MBS (1.88*10-5 M); blue curve: TA in the first 4 cycles, MBS added in the 5th cycle; red curve: TA and MBS solution previously mixed and measured after 5 minutes of stabilization.

Control solutions of TA (Figure 28a) and MBS (Figure 28b) were firstly measured, observing a single anodic peak, indicating a non-reversible electrochemical oxidation in both species. The anodic peak of TA at ~0.4V is coincident with the data reported in literature [8–10].
On the other side, in Figure 28c it can be observed an increase in the current intensity of the anodic peak of TA (from a to b) when it is mixed with MBS, either when MBS is added after some cycles of electrochemical oxidation are performed or when is mixed previously to the measurements. This fact indicates that a reduction reaction is taking place, regenerating the oxidation substrate, which can be further oxidized at the Au electrode increasing the intensity of the TA peak. In fact, Comuzzo *et al*. reported similar increase of the anodic peak of catechin form when adding SO_2 as an antioxidant [11], explaining this result by considering the ability of sulfites to react with quinones and reduce them back to catechols.

3.2.2 Comparison of the whitening outcome of MBS and CP

As said before, the first hypothesis of this work was that a reduction of stains would lead to a whitening effect. To observe the whitening yield obtained by two different mechanisms, oxidation and reduction, MBS was compared with the commercial treatment (CP) and potassium persulphate (PS) as a strong oxidant, monitoring the absorbance of TA solutions mixed with the different reagents by spectrophotometry for the first 20 minutes of reaction and along 48 hours (Figure 29).

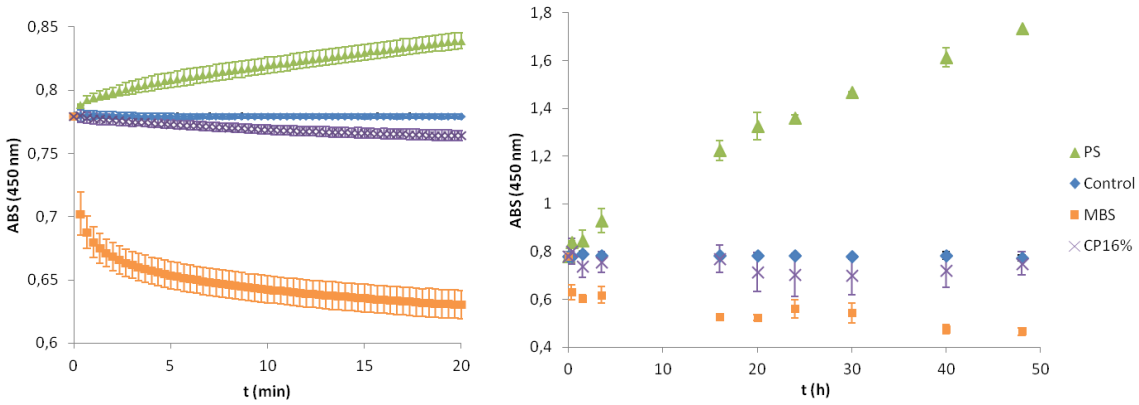

Figure 29. TA (0.047M) reaction with MBS (0.047 M; equivalent to 0.094M of HSO_3^-), CP (16%, equivalent to 5.7% H_2O_2, 1.67 M H_2O_2) and PS (0.024M) followed by UV-vis spectrophotometry, measuring ABS in front of time. Initial pH of the solutions: 3.6.

No color variations were observed in the control. The commercial whitening ingredient, CP, showed a low but progressive whitening effect over the TA during the first 20 minutes, and afterwards a tendency to go back to the original color. According to Haywood and Sword [7], 50% of the peroxide release occurs during the first two hours; also, after the release from the urea molecule, peroxide undergoes a series of decompositions and reactions that lead to oxygen active species [12] being those that perform the whitening action [13]. Hence, it is not surprising that CP did perform a poor whitening effect in the first 20 minutes, since it has a slow mechanism; the pseudo-first order rate constants *k* for degradation of quinones by H_2O_2 have been reported to be of the order of 10^{-4} min^{-1} [14], at a H_2O_2 concentration of $2.7*10^{-3}$ M and pH 4.0.

It was expected that the reaction with an strong oxidizing agent as PS (E^0=2.010 V [15]) lead to the whitening of the solution as HP does. Instead, TA got darker in contact with PS, probably due to the oxidation of the tannic acid phenol groups into quinones [16]. H_2O_2 and hydroxyl ions ($E^0_{OH\bullet/H_2O}$=2.80 V [17]) also oxidize hydroxyl groups to quinone, and in basic conditions are able to further oxidize the quinone groups to form carboxylic acids [18], leading to the whitening effect. The browning observed after the 30th hour may be due to the fact that this process was performed in at pH 3-4, thus the oxidation of quinones is not totally favored, while the remaining catechol groups are continuously oxidized to quinones [19], darkening the solution.

A very fast whitening effect was obtained with MBS. It is understood that the bleaching effect is mediated by the hydrogen sulfite ion (HSO_3^-) released on the dissolution of the MBS in water [20]. As can be observed, this is a much faster reaction. It has been reported that a rapid reversible complexation of p-benzoquinone (colored) with bisulfite ion takes place at a rate constant of the order of 10^2 min^{-1} (0.1 M HSO_3^-, pH 5.75) or 10^1 (at pH 4.5), giving the hydroquinone monosulphonate product (uncolored) [21]. Also, as can be seen, the whitening obtained is stable over time because of the formation of the sulfite carbonyl adduct, which quinone/catechol couple has a higher reduction potential than the original catechol system, and then should not experience further oxidation [22]. Furthermore, comparing the reduction and oxidation reaction of the quinones thermodynamically, it can be seen how the reduction reaction is more favorable. Given the equations $\Delta G=-nFE^0_{rxn}$, being $E^0_{rxn}= E^0_{cathode} - E^0_{anode,}$ and knowing the following values: $E^0_{GA\ quinone/catechol\ redox\ couple}$ = 0.794 V, $E^0_{H_2O_2/H_2O}$ = 1.776 V (2 electron), $E^0_{OH\bullet/H_2O}$ = 2.80 V (1 electron) and $E^0_{SO_4^{2-}/HSO_3^-}$ = -0.520V (2 electron), it can be deduced that $\Delta G_{HSO_3^-\ rxn} < \Delta G_{OH\cdot\ rxn} < \Delta G_{CP\ rxn}$, meaning that the final products of the reaction with HSO_3^- is the most stable.

3.2.3 Liposomes adhesion to enamel surface

To study the interaction of DPPC liposomes with the enamel, teeth were embedded in a solution of RhodB-marked DPPC liposomes, and afterwards observed by confocal microscopy.

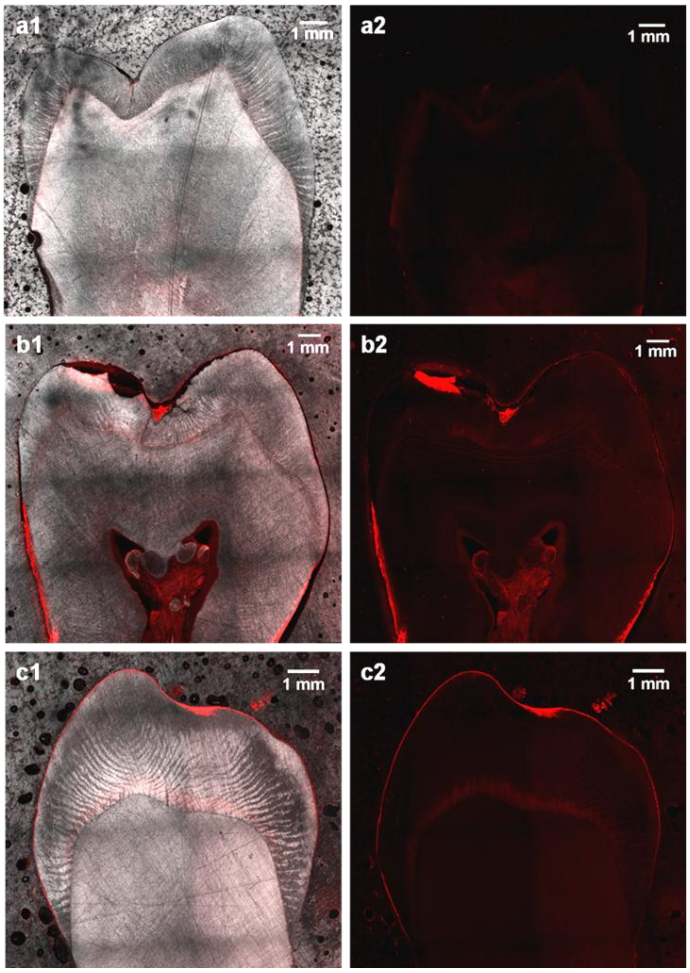

Figure 30. Confocal images taken using excitation light from the laser line 561 nm, superposed to the one measured by reflected light 488 nm (1) and confocal images taken using excitation light from the laser line 561nm (2) of: a) Control tooth, b) tooth embedded in Rhodamine B solution and c) tooth embedded in liposomes marked with Rhodamine B solution.

Figure 30a shows the auto-fluorescence of the control tooth, coming from the collagen degradation already observed in non-fresh extracted teeth [23]. Figure 30b shows the fluorescence emitted by the RhodB accumulated in the imperfections of the enamel as well as in the organic parts surrounding the root. Figure 30c shows the liposomes attached to the tooth surface forming a thin fluorescent layer which is not present in the former samples. This is in agreement with the studies of Nguyen *et al*, which found that liposomes are able to retain on the enamel surfaces resisting even a flow of water [24]. Liposome's surface charge seems to play a highly important role on their significant bio-adhesion, and even if DPPC is a zwitterionic phospholipid with an overall neutral charge, neutral liposomes exhibit a zeta potential (usually negative) in water due to adsorption of hydroxyl ions [25]. Naked enamel surface is negatively charged due to the phosphate ions that dominate the HAP crystals surface; in contact with a solution, an hydration layer is formed, containing calcium ions acting as counter ions [26] and acidic proteins or anionic species can be attached to calcium sites [27]. This phenomenon takes place in the initial stage of the dental pellicle formation, which consists in an almost instantaneous adsorption of salivary proteins (amphiphilic phosphoproteins that associates into micelle-like globules) on the enamel surface [28, 29]. In this experiment, an etching treatment was performed in the tooth to remove all the organic material, leaving the enamel

surface totally exposed. Hence, when applying the treatment with the liposomal solution, the interaction was taking place directly between the HAP crystals and the solution, leading to the creation of an hydration layer containing the cations present in the solution; afterwards, due to its tendency to be negatively charged and its globular structure, DPPC liposomes behave like salivary proteins forming the initial enamel pellicle, getting retained in the enamel surface as observed.

3.2.4 Chemical changes in enamel structure and depth extension

SR-µFTIR was used to study the chemical changes induced in HAP by either oxidizing (CP) or reducing (MBS) whitening treatments in the bovine incisor's enamels, down to 50 µm depth from the surface. PCA was performed to compare the effect of these changes all over the surface analyzed. The five whitening treatments were clearly identified from principal component (PC) 1, 2 and 3 (accounting for a total of 99% of the variance) (Figure 31a).

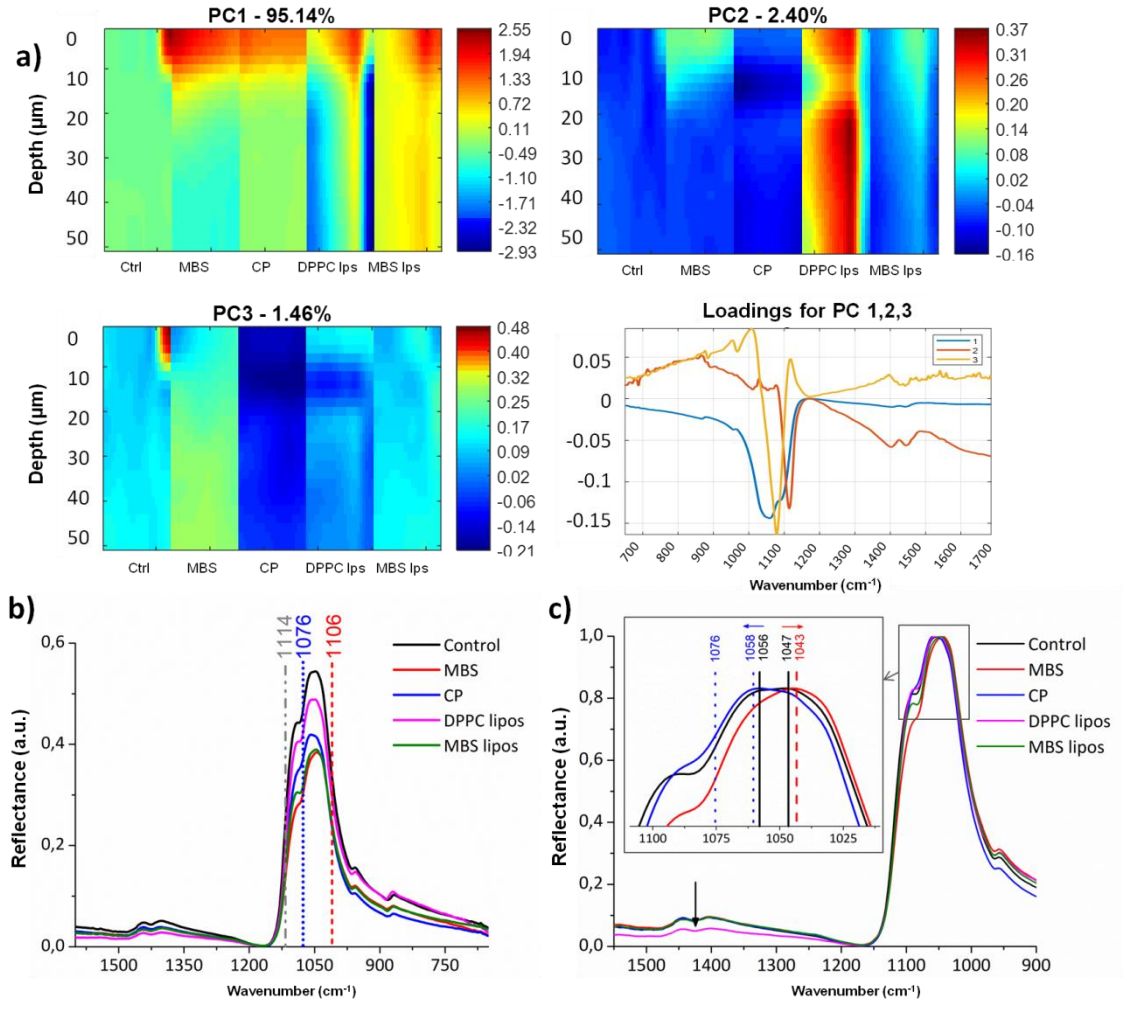

Figure 31. a) Scores (distributed across the enamel depth, starting in the enamel surface at 0 µm to 50 µm deep), and loadings for the PCA treatments. b) Average of the spectra corresponding to the first 10 µm for each treatment (n=12). c) Plot b normalized to maximum. Red dash line: MBS shift. Blue dots line, CP shift. Dash and dots grey line: characteristic shoulder of the HAP peak.

To better relate the observed PCs with the spectra, Figure 31b represents the average of the 12 spectra corresponding the first 10 µm of enamel of each treatment, since are the most affected. Additionally, Figure 31c presents the spectra normalized to maximum height to facilitate the appropriate comparison of relative peak intensities and their shifts (Figure 31c).

As can be observed in the loadings representation in Figure 31a, PC1 represents 95.14% of the variation, comprising the whole spectra, especially the phosphate peak. In the scores plot, it can be seen that PC1 (which is negative and thus correspond to the blue parts of the image) did not change for the whole depth analysed in the control sample; for MBS and CP there was a lack of this component in the surface, meaning that the intensity was lower for the first 10 µm deep when compared to control. For MBS liposomes, PC1 showed a high influence in the mantel enamel (most outer part) and then in a thin line entering the inner parts; besides, it can be observed how the whole space is yellow, meaning that the treatment reached the whole surface analysed. In Figure 31b, the groups MBS, CP, and MBS liposomes showed a reduced reflectance when compared to control, and thus PC1 is related to the reflectance peak intensity. According to literature, intensity reduction in the v3 phosphate peak can be related to a loss of mineral content (erosion) [30]. In addition, MBS showed a decrease of the 1058 cm^{-1} intensity, and the contribution of the 1091 cm^{-1} shoulder had almost disappeared. This points out to a clear distortion of the calcium phosphate structure. For DPPC liposomes, it was observed that this treatment also affected the first 10 µm of the enamel, and some of the inner parts were affected by a thin line (as if some liposomes have penetrated the enamel); a higher reflectance intensity in some points of the sample explains the intense blue observed in some parts in the scores map.

PC2 (2.40% of the variance) loading profile presented a peak at 1114 cm^{-1}, placed in the negative part of the loadings. It corresponds to the Ca-O-P secondary vibration of v_1 PO_4^{3-} (reflects changes in the bandwidth) and to the whole v_2 CO_3^{2-} band. Its presence is related to the blue parts in the corresponding scores map. It can be noticed how the lack of this component was highly significant in DPPC liposomes, and, although with less intensity, in the first microns of the samples treated with MBS and MBS with liposomes. It is mainly related with a change in the relative intensity of the carbonates since, as indicated in Figure 4c with a black arrow, for DPPC liposomes it was lower than in the other treatments. Also, all the surface of the CP treated tooth appears blue, meaning that this compound did not affect the carbonates. The phosphate peak corresponding to CP is slightly tightened when compared to the other treatments, that could explain the intense blue in some points of the PC2 scores map since it is also related with the bandwidth. These observations may point that the mechanism by which MBS and CP interact with the enamel is chemically different.

PC3 (1.46%) loading was negative at 1076 cm^{-1}, i.e. blue in the PC3 scores map, and positive at 1006 cm^{-1} (red in the score map). The first is related to a hypsochromic shift of CP (from 1058 to 1056 cm^{-1}), and the second related to a bathochromic shift of MBS, as can be observed in the specific part of the HAP peak in Figure 4c. This shift was no observed with the other whitening treatments and represents different chemical interactions with the HAP structure.

Kim *et al.* [31] also observed that acid-eroded enamel presented an hypsochromic shift of v_3 PO_4^{3-} peak. They related these observations to changes in the local structure; in apatite, each P atom is linked to four Ca atoms via a shared oxygen atom (P–O–Ca atomic bridges), and the exposure to acid breaks the Ca–O bonds and consequently reduces the P–O bond length, due to the redistribution of the electron density in the vicinity of the bridging oxygen. According to

that, under the acidic conditions of MBS treatments, a decrease in the reflectance due to the acidic nature of MBS (PC1) was observed, but a bathochromic shift, i.e., an elongation took place instead of a shortening of the P-O bond. This could be explained by a SO_4^{2-} substitution of the PO_4^{3-} group; the lattice parameter along the c-axis increases with sulphur substitution because of the length of the atomic bond, as well as an hypsochromic shift happens in the phosphate peak [32]. While, in PC1, MBS affected only the outer layers of the tooth, MBS liposomes affected the whole analysed surface, especially in the middle part; we relate this effect to the increased diffusion promoted by the liposomes [33]. Instead, the bathochromic shift affected only the tooth treated with MBS. Vasluianu *et al.* [34], studying the changes induced in HAP by HP whitening treatments, observed a decrease in the reflectance and an hypsochromic shift for the main FITR peaks of HAP; they related it, again, to the shortening of P-O, even if the pH of the treatments was 7.4. In our experiment, CP's pH was 8 as in the commercial treatments, since its effectiveness is increased at this pH [35]. Accordingly, a decrease in the reflectance (PC1) and a hypsochromic shift (PC3) were observed. At basic pH, HAP dissolution should not occur, but protein degradation in the tooth organic matrix might be happening. Indeed, the urea in CP along with the ammonium ions (NH_4^+) formed in contact with water act on the hydrogen bonds of the proteins weakening their structure [36]. Since the components of the organic matter form a support structure that stabilizes the crystalline enamel layer [37], any change in the organic matter could cause an effect in the reflectance in the first 30 µm, as observed in PC1. According to Pleshcko *et al.* [38], a narrowing of the phosphate peak is related with increased crystallinity; this effect was observed for CP treatment, probably because the crystallinity was better detected after the loose of organic matter, because of its better reflective properties.

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4. CONCLUSIONS

- This thesis was focused on the search for a new approach to tooth whitening, based on the understanding of the chemistry behind its mechanism. A new ingredient has been developed and its effects on the dental enamel have been studied, bringing new knowledge to the field of tooth whitening and a valuable product to the market.

- The reducing agent MBS presented a significant whitening effect, reaching better (in solution) or equal (in vitro) ΔE values than CP, but in shorter times and at lower concentrations.

- The fast whitening effect achieved with the reductant is due to the rapid complexation reaction of the colored molecules (benzoquinones) with the bisulfite ion (HSO_3^-) that gives colorless molecules (hydroquinones monosulfonate adducts) as a product, with pseudo-first order rate constant in the order of 10^2 min^{-1}. Instead, the degradation of benzoquinones to uncolored carboxylic acids by H_2O_2 has a slower mechanism, with a pseudo-first order rate constant in the order of 10^{-4} min^{-1}.

- The reduction reaction has been confirmed by cyclic voltammetry. MBS regenerated the oxidation substrate (TA) producing an increase in the anodic peak at every cycle.

- The whitening effect in solution is maintained over time, since the hydroquinone monosulfonate adducts are less susceptible of oxidation than the hydroquinone molecules.

- MBS is able to perform a whitening effect also in the bovine teeth *in vitro* model, meaning that the molecule is able to diffuse through the tooth structures reaching the dentin. Compared to the commercial ingredient CP, MBS whitening effect is faster, in particular within the first 3 minutes of contact.

- The encapsulation of the reducing agent within DPPC liposomes leads to higher *in vitro* whitening values against MBS alone. Moreover, the whitening effect obtained with MBS liposomes is higher than the sum of the whitening obtained by DDPC liposomes and MBS separately, specially at 3 minutes of contact time, suggesting a synergism between liposomes and MBS.

- Liposomes contribute to the observed improved effect through two mechanisms:
    - Acting as a carrier and protecting the reductant from the environment until it reaches the tooth surface.
    - Forming a pellicle surrounding the enamel surface, which favors the diffusion of the reductant towards the inner structures of the tooth.

- The SR-μFTIR studies showed that MBS treatments softened the first 10 μm of enamel, as happens in the initial states of tooth decay. But, since saliva acts as a buffer and promotes remineralisation once acid contact has been removed, MBS treatments applied with enough separation in time should be totally safe for human application.

- CP and MBS promoted different changes in the HAP mineral; CP shortened the P-O bond while MBS seems to elongate it. Moreover, MBS promoted the loose of carbonates while CP doesn't, which is probably related to the solution's pH.

- When comparing MBS and MBS Liposomes, it was observed how liposomes favoured the diffusion of MBS to inner layers, since the effects of MBS were observed in deeper enamel. Thus, the encapsulated MBS whitening effect is highly improved in terms of time when compared to MBS alone or CP.

- The encapsulated MBS tooth whitening formula was optimized to adapt it to be used in the oral cavity. The optimized formula was named NewT101, and consisted of 0.1M of MBS, 20 mM of DPPC and a pH of 6.0.

- NewT101 was tested versus HP 35 % and CP 16%, according to the ISO28399. It presented whitening effects comparable to the commercial treatments at all the sessions tested. It showed a higher increase in the enamel surface roughness than HP and CP, but still preserving the values that the ISO standard considers safe.

- A novel tooth whitening ingredient has been successfully developed, leading to a patent application. Because of its effectiveness in short times, this new formulation has potential to become an alternative to the existing whitening treatments based on peroxides. Spite of the need for further studies including safety tests, the friendly chemical nature of NewT101 components, individually used in both food and cosmetic industries, assures this formulation an easy approval for clinical trials. This provides, in addition to the scientific value provided by the scientific publications, an economic value to the work developed.

ANNEXES

ANNEX I. Pending paper: Tooth whitening, oxidation or reduction? Study of physicochemical alterations in bovine enamel using Synchrotron based Micro-FTIR

This work aimed to observe if any chemical interaction took place between enamel, composed of carbonated hydroxiapatite (HAP) mineral, and the whitening agents carbamide peroxide (CP), dipalmitoylphosphatidylcholine (DPPC) liposomes, sodium metabisulphite (MBS) and MBS liposomes after treatment application. Also, if any chemical change was taking place, to track its extent.

Synchrotron-based micro Fourier Transformed Infrared Spectroscopy (SR-μFTIR) in reflectance mode was used for this purpose. It can provide very precise chemical information in small areas, therefore it was a very suitable technique carry out this study with precision. Bovine teeth were used as *in vitro* model for the experiment.

In first instance, the whole enamel width of the control was mapped, revealing its mineral distribution. Phosphates showed higher concentration in the outer enamel, oppositely to the carbonates, more abundant near the dentin. This was in accordance with the characteristic HAP crystals physiological organization in the enamel: highly packed and organized in the surface but presenting thinner crystals with more carbonate impurities in the deeper parts.

To follow the extension of the potential treatment-induced chemical modifications on the HAP, Continuous Stiffness Measurements (CSM) were firstly used in order to delimitate the depth of study. It was observed that MBS treatments affected the hardness of the 2 μm depth analyzed, but a with tendency to recover hardness as depth was gained.

An area of 50 x 50 μm of each treated tooth was mapped. Principal component analysis (PCA) of the collected spectra showed that the main differences between treatments were found in the intensity of the ν_3 PO_4^{3-} peak related with tooth demineralization, produced by the acidic nature of MBS. This changes were observed in deeper regions of the enamel when MBS was encapsulated in liposomes, proposing that liposomes favour the diffusion of MBS into the enamel. It was also observed that the treatments induced counter shifts of this peak, meaning that CP and MBS promote different changes in the HAP mineral: MBS induced a bathocromic shift, pointing an elongation of the P-O bond of the phosphate group, while CP produced an hypsochromic shift, suggesting its shortening.

Tooth whitening, oxidation or reduction? Enamel physicochemical alterations study

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ABSTRACT

While tooth whitening demand has been increasing over the last decades, the formulations have not evolved significantly. Hydrogen peroxide (HP) or carbamide peroxide (CP) continue to be the main active agents used in the market. These oxidizing compounds need high concentrations and long application times (of about 1 hour) to achieve the desired whitening results, leading to secondary effects and being inconvenient for the patient. A novel approach based on a reducing agent, sodium metabisulphite, encapsulated in liposomes has demonstrated significant whitening results in just 3 minutes. The chemical interactions between the tooth mineral hydroxiapatite (HAP) with liposomes encapsulated metabisulphite and with CP have been studied. Chemical imaging was performed by synchrotron-based micro Fourier transformed infrared spectroscopy (SR-μFTIR) measurements in reflectance mode. Continuous Stiffness Measurements (CSM) were used to determine the depth reached by the treatments in order to delimitate the area of study. The principal component analysis (PCA) showed that the main differences between treatments were found in the intensity of the ν_3 PO_4^{3-} peak related with tooth demineralization produced by the acidic nature of MBS; this changes were observed in deeper regions of the enamel when MBS was encapsulated in liposomes. It was also observed that the treatments induced counter shifts of this peak; MBS induced a bathocromic shift indicating an elongation of the P-O bond of the phosphate group while CP produced an hypsochromic shift, indicating its shortening. The obtained results indicated that CP and MBS promote different changes in the HAP mineral, and that liposomes favour the diffusion of MBS into the enamel.

Keywords: tooth whitening, sodium metabisulphite, liposomes, synchrotron, microFTIR, PCA

1. INTRODUCTION

Tooth whitening is a widely practiced aesthetic treatment around the world. The main goal is to change teeth colour, to obtain a bright and white smile, hence its effects are widely studied mainly using colourimetric techniques [1]. Besides the desired final aesthetic result, tooth whitening also has undesirable effects due to its mechanism of action. The whitening effect is

based on the oxidizing action of peroxides, e.g. hydrogen peroxide (HP), or carbamide peroxide (CP), the latter composed by urea and hydrogen peroxide molecules. HP decomposes in reactive oxygen species (ROS) that are highly oxidative [2]. It performs its action by penetrating through the different tooth structures of the discoloured tooth, reaching the adsorbed stains in the enamel and dentin; eventually, the ROS break the double bonds of the staining molecules making them unable to absorb light [3]. In the case of CP, the urea groups degrade the organic parts of the enamel, leaving empty minute spaces that favour the diffusion of hydrogen peroxide throughout the whole enamel thickness [4]. Higher concentrations of peroxides and longer application times are associated with better results [5], but also with a higher incidence of side effects like hypersensitivity (the most common secondary effect of dental whitening) [6], pulp damage [7], tooth demineralization (leading to changes in roughness and hardness) and gingival irritation [4]. Besides, the need to apply these products for hours, either at the dentist or at home, is not convenient for the user.

Efforts have been made to find a solution to reduce HP concentrations and exposure times [8]. Various methods to accelerate the decomposition of HP in ROS are currently used. The addition of sodium hydroxide (NaOH) to whitening formulas to increase its pH, photo oxidation methods by applying UV or blue light [9] or methods applying heat with LEDs lamps or lasers (e.g. NdYAG) are common. However, alkaline pH can cause tissue burning leading to gingival irritation, the use of UV is not recommended since it can cause skin burns [11] and the increase in temperature caused to the pulp by the lasers can lead to pulpitis or even pulp necrosis [12].

Novel strategies avoiding the use of peroxides are also being explored. For instance, whitening based on piezocatalysis using $BaTiO_3$ nanoparticles and vibration showed significant results, but after 10 hours application [13]. Natural compounds like proteolytic enzymes (bromelain, papain and ficin) also showed visible results after 4 applications of 15 minutes each [14]. These solutions may solve the secondary effects derived from the peroxides, but not the inconvenience of long application times.

In previous works, we reported an outstanding whitening achieved by using a reducing agent, sodium metabisulphite (MBS), as bleaching agent when compared with the traditional oxidizing ones [15]. In addition, we found that the whitening effect increases when MBS is encapsulated in liposomes, obtaining significant whitening results in 3 minutes [16]. Indeed, the purpose of these nanostructures is twofold, they act as a carrier of the MBS and, on the other hand, they create a layer over the enamel surface that improves the diffusion of the reductant towards the tooth. It was also seen that the encapsulated MBS had physical effects over the enamel, increasing the surface roughness within safe limits; nevertheless, the enamel-treatment chemical interaction was still pending to study.

When analyzing the side effects of whitening treatments on enamel, most of the studies are focused on determining the changes in its mechanical properties, like surface hardness or roughness; however, these techniques are not able to provide information about the chemical changes taking place in neither the inorganic nor the organic structures. Fourier transformed infrared (FTIR) spectroscopy is a molecular vibrational technique that has been shown to be advantageous to detect alterations in mineralized tissues like teeth, providing information on the chemical structure at the molecular scale. For example, it has been previously used to study enamel erosion, correlating the demineralization with an intensity decrease in the phosphate peak [17]. The addition of a microscope to FTIR microspectroscopy (μFTIR) has led

to the possibility of combining biochemical and spatial information, i.e., chemical imaging, with the potential to examine tissues at cellular resolution [18]. Coupling it to synchrotron radiation light source, synchrotron-based FTIR microspectroscopy (SR-µFTIR), provides a much better signal to noise ratio [19], and the possibility of using a smaller beam size without losing signal efficiency [18] than when using traditional globar sources. Actually, it has been previously used to study enamel ablation caused of pulsed CO_2 lasers, allowing to detect ablations of 9 µm [20].

SR-µFTIR in specular reflectance mode can provide very precise chemical information in small areas, therefore it is a very suitable technique to study the changes induced by whitening treatments and to characterize the extent of its effects with precision. Here we report a SR-µFTIR study on the chemical changes induced in HAP by either oxidizing (CP) or reducing (MBS) whitening treatments in bovine incisors enamel, down to 50 µm depth from the surface. Besides, the chemical distribution of inorganic and organic species in the enamel of bovine incisor were mapped and studied by using multivariate analysis (principal component analysis, PCA). To our knowledge and up to date, no study has been reported using SR-µFTIR to determine the effects of MBS as a whitening treatment in terms of chemical HAP modifications, and the extent in deep of these effects. Neither the bovine incisor enamel has been chemically mapped using synchrotron light.

2. MATERIALS AND METHODS

2.1. REAGENTS

Sodium metabisulfite was purchased from Riedel-de Haën (Seelze, Germany) in powder form. Carbamide peroxide was obtained from Acros Organics (New Jersey, USA), in 1 g stabilized tablets (35% w/w H_2O_2). Dipalmitoylphophatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Alabama, USA). All chemicals were of analytical grade. Deionized water was purified through a purification system from Millipore (Milford, MA, USA).

2.2. WHITENING TREATMENTS

Deionized water was used as control. Sodium metabisulphite solution was at a concentration of 0.47 M. Carbamide peroxide solution was prepared at 16% (w/v). DPPC liposomes solution was obtained by lipid film hydration [21] at a concentration of 20 mM of DPPC encapsulating water. MBS liposomes solution was obtained by monolayer technique at 20 mM of DPPC encapsulating a solution of metabisulphite at 0.47 M.

2.3. SAMPLE PREPARATION

2.3.1. Samples for Continuous Stiffness Measurement

Ten specimens of bovine incisors were cleaned of gross debris, and the root was sectioned using a diamond saw and preserved in sodium azide solution (0.2%) until the experiment performance. Teeth were embedded in self-curing polyacrylic cylinders. Their surface was polished to expose a window of at least 3x3 mm of the enamel surface, which permitted the Continuous Stiffness Measurements (CSM). The polishing was done using a sequence of silicon carbide paper starting at grit size P400 and sequentially increasing to P4000, under a constant flow of tap water, and afterwards with a sequence of diamond pastes with a mean particle size of 3 and 1 µm followed by a slurry of aluminium oxide with a mean particle size of 0.3 µm. After every polishing step, the samples were submerged in deionized water and sonicated for

30 seconds. The different whitening treatments were freshly prepared and applied for 20 minutes on the flattened surface of the specimens.

2.3.2. Samples for SR-µFTIR

Bovine incisors were treated with the freshly prepared whitening treatments for 20 minutes making sure that only the enamel was in contact with the solution, avoiding the penetration of the treatment through the root cut part where the pulp is exposed, and then brushed for 30 seconds while rinsed with distilled water. Afterwards, the specimens were embedded in an acrylic resin, and cut vertically along the central lobe to obtain a mesial view from the inside, The exposed surface was polished up to 1 µm particle size as described in section 2.3.1, in order to obtain proper reflection properties for the measurements.

2.4. Continuous Stiffness Measurement

The dynamic continuous stiffness method (CSM) permits investigate the dependence of the dynamic hardness as a function of the penetration depth during a nanoindentation. CSM was performed with a XP-MTS Nanoindenter using a Berkovich diamond tip. Nine measurements per sample were performed. Before every measurement, the diamond indenter was calibrated on a standard fused silica specimen.

2.5. Specular reflectance SR-µFTIR measurements

Specular reflectance spectra were acquired using the FTIR microscope in the reflection mode at MIRAS beamline of ALBA synchrotron light source (Cerdanyola del Vallès, Spain) [22], using a Hyperion 3000 microscope coupled to a Vertex 70 spectrometer (Bruker, Germany), and equipped with a Mercury-Cadmium-Telluride (MCT) detector. The microscope is utilizing a 36x Schwarzschild objective (NA = 0.52) coupled to a 36x Schwarzschild condenser to focus the synchrotron IR light on the sample. OPUS 7.5 (Bruker, Germany) was used to collect the spectra, taking a minimum of 256 co-added scans per spectrum in the 650-4000cm^{-1} Mid-IR range and with a 4 cm^{-1} spectral resolution, using a 5x5 μm^2 masking aperture size. A gold mirror reference was used as reference to collect the background.

2.6. DATA TREATMENT

Principal component analysis (PCA), a multivariate method, widely used in hyperspectral images [23] aims at extracting the major sources of variance (variability) in the map of the sample. This variance correlates, to some extent, with the chemical compounds conforming the sample, giving an indication of the compounds distribution along the surface in an unsupervised and straightforward manner. In the present work, PCA was applied by using HYPER-Tools version 2 [24].

In the spectral data obtained for this study, the most intense peak is the ν_3 PO_4^{2-}. Although, it has been already reported that when dentin is treated with acids, its intensity is insufficiently constant for normalization [25], causing artefacts in the normalized spectra. Therefore, raw reflectance spectra were used to perform the PCA analysis. The Unscrambler X® (2016, CAMO Software AS, Norway) was used to assign peak spectra numbers and to perform maximum normalizations when needed to observe the peak shifts. The chemical mapping was performed

by integrating the peak area, bordered by the spectrum curve, the x-axis and the two wavenumber limits defined, using the software OPUS 7.5 (Bruker, Germany).

Principal component analysis (PCA), a multivariate method, widely used in hyperspectral images [23] aims at extracting the major sources of variance (variability) in the map of the sample. This variance correlates, to some extent, with the chemical compounds conforming the sample, giving an indication of the compounds distribution along the surface in an unsupervised and straightforward manner. In the present work, PCA was applied by using HYPER-Tools version 2 [24].

3. RESULTS

3.1. PEAKS ASSIGNMENT

Enamel is composed of approximately 96% wt. of inorganic mineral and 4% wt of organic matter and water. The inorganic dominating part is made of carbonated calcium-deficient hydroxyapatite (HAP), which consists of non-stoichiometric calcium hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and CO_3^{2-} impurities (~ 4.0%). Carbonate can substitute two anionic sites of the hydroxyapatite structure: at PO_4^{3-} sites giving B-type carbonated apatite (the dominant type in biological apatite), and OH^- sites giving A-type carbonated apatite [26].
The bands corresponding to carbonates and phosphates of the HAP forming the enamel were identified (Figure 1) in a control sample.

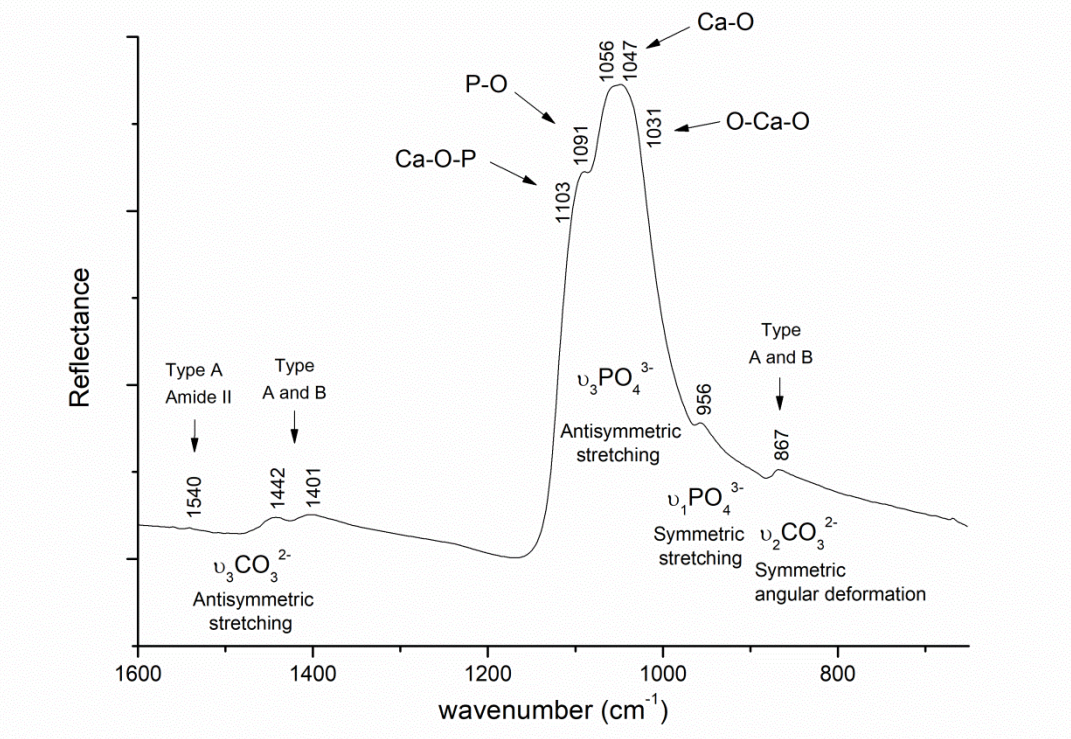

Figure 1. Specular reflectance FTIR (carbonated hydroxiapatite) spectrum of enamel and band assignment numbers (original figure).

The broad absorption bands in the 1170–965 cm^{-1} region correspond to the apatitic phosphate groups coming from a triply degenerated asymmetric stretching mode ($v_3PO_4^{3-}$) [27]. Its deconvolution is described in the literature as secondary phase vibrations of Ca-O-P at 1103 cm^{-1}, P-O at 1091 cm^{-1}, Ca-O at 1047 cm^{-1} and O-Ca-O at 1031 cm^{-1} [28] (typically occurring in stoichiometric apatite with a relation $Ca_{10}(PO_4)_6(OH)_2$ [29]). The band at 965 cm^{-1} corresponds to the symmetric stretching mode of phosphate ($v_1PO_4^{3-}$) [27]. The region between 1580-1320 cm^{-1} corresponds to $v_3CO_3^{2-}$ antisymmetric stretching; in this band two maximums can be observed, at 1401 and 1442 cm^{-1}, which intensities are slightly different, being the peak at 1401 cm^{-1} a little higher; also a peak at 1540 cm^{-1} (region 1535-1565 cm^{-1}), corresponding to $v_3CO_3^{2-}$ and organic material (amide II), was detected [17]. Another carbonate band arises at 880-830 cm^{-1}, corresponding to the out-of-plane bending mode of the C–O bond ($v_2CO_3^{2-}$ group) [17,30–32]. When the carbonate substitutes the hydroxyl group it is designated as type A apatite and when it substitutes the phosphate group it is designated as type B apatite, and it is described that there are equal amounts of A and B in dental enamel [33]. According to Brangule *et.al.* [34] peaks' deconvolution, there is a peak doublet at 1540 and 1455 cm^{-1} corresponding to type A carbonates and a doublet at 1475 and 1416 cm^{-1} corresponding to type B; for the $v_2CO_3^{2-}$ band, a peak at 880 cm^{-1} is assigned to type A and other at 871 cm^{-1} to type B. This means that the peaks in the regions 1580-1320 cm^{-1} and 880-830 detected in the reflection mode are showing a mixture of type A and B carbonates, except for the peak at 1540 cm^{-1} that reflects type A as well as organic matter.

3.2. CHEMICAL DISTRIBUTION IN INCISOR BOVINE ENAMEL

To evaluate the chemical distribution of carbonates and phosphates, an area of non-treated bovine tooth enamel was fully mapped by SR-µFTIR from the surface to the dentino-enamel junction (DEJ) along a cross-section of 500 µm long and 60 µm wide (Figure 2) in the mid crown (labial side). For the sake of clarity, the intensity of the colour scale is adjusted to the intensity of the corresponding highest peak.

Vibration	$\nu_3\ PO_4^{3-}$	$\nu_1\ PO_4^{3-}$	$\nu_3\ CO_3^{2-}$		$\nu_2\ CO_3^{2-}$
Range (cm^{-1})	1160-965	965-935	1530-1550	1580-1320	880-830

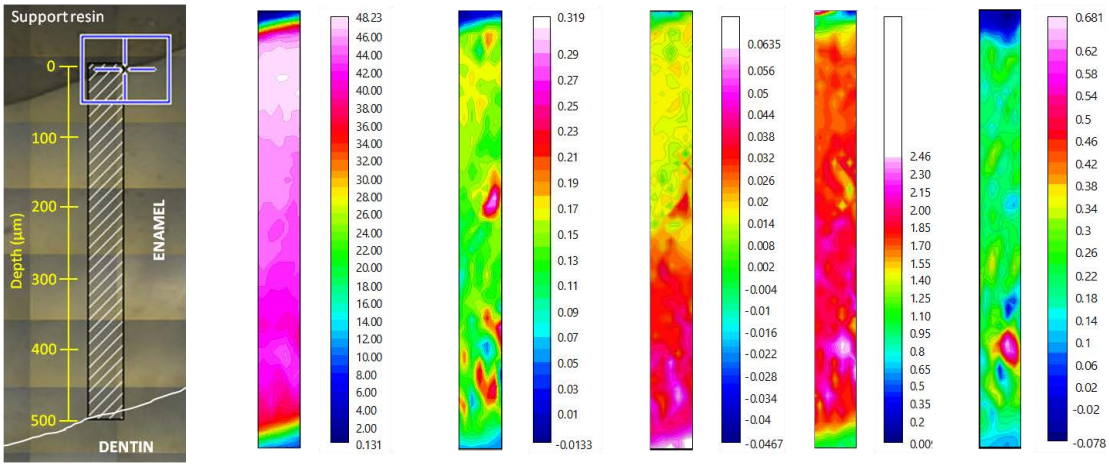

Figure 2. Chemical distribution (calculated by peak integration) of carbonates and phosphates in the middle crown of a bovine incisor control tooth (vertical section). The mappings where obtained using OPUS by performing chemical imaging, using peak integration mode A.

Phosphate **(PO_4^{3-})** is the principal molecular species that gives rise to the HAP absorbance in the 900-1200 cm^{-1} region, and its distribution can be seen from the integration of ν_3 antisymmetric and ν_1 symmetric stretching vibrations. For $\nu_3\ PO_4^{3-}$, the peak intensity is higher (white area) in the mantel of the enamel. Moreover, it is progressively reduced as it goes deeper in the enamel (Figure 2), and a dramatic reduction of the intensity is observed in the dentin since its phosphate content is lower and its crystallinity decreases [38].

For $\nu_1\ PO_4^{3-}$, high intensity spots were observed in several places along the enamel; Pleshko N. *et al.* found a direct relationship between the percentage area of the ν_1 band (near 960 cm^{-1}) and the crystal size [39], therefore those regions could correspond to discrete areas with larger crystals. Carbonates showed complementary intensity distribution to the phosphates, with the highest intensity close to the dentin and lower in the outer part of the enamel. This is in accordance with previous works reporting that the carbonate content increases from outer to inner enamel in bovine incisors [40]. Comparing the regions 1580-1320 cm^{-1} and 880-830 cm^{-1}, corresponding to the two types of vibration of the carbonate group (antisymmetric stretching and symmetric angular deformation), similar intensity patterns can be observed (note the similar intense point at around 400 µm depth) revealing its presence in the enamel. In the region 1530-1550 cm^{-1}, the highest intensity was found in the dentin region at the bottom of the map, which corresponds to type A carbonates, but also to amide II vibrations, which are found in higher proportion in the dentin.

3.3. TREATMENT EFFECTS

3.3.1. Continuous Stiffness Measurements (CSM)

It is known that the acid contact with teeth cause partial loss of mineral that results in a reduction of enamel surface hardness [41]. In our work, CSM was used to probe the demineralization caused by MBS as well as the depth reached by this effect, measuring the hardness variations of the enamel from the tooth surface to deeper parts.

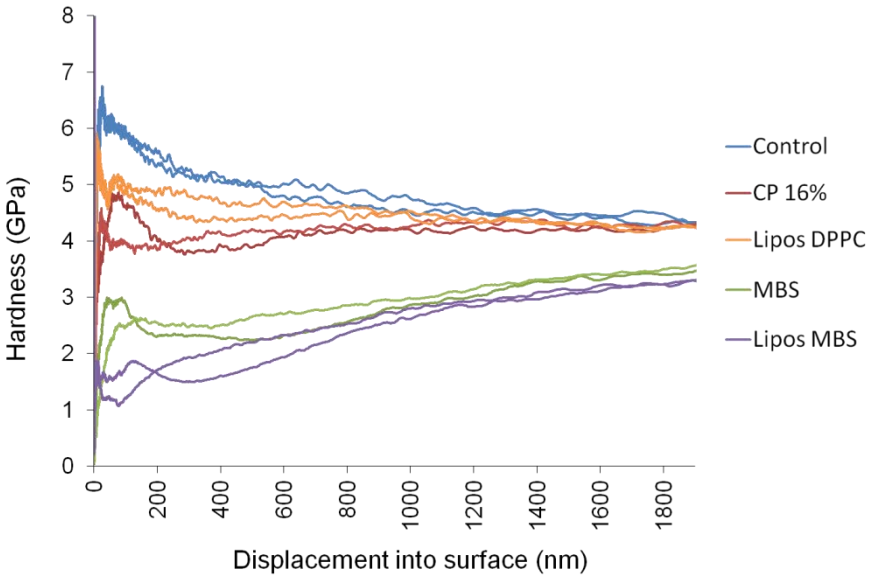

Figure 3. Continuous stiffness measurement for the different treatments tested

CSM analysis (Figure 3) showed a hardness of about 5 GPa along the depth analyzed (2 µm) for the control. Similar behaviour was observed for CP and DPPC liposomes with a hardness value at around 4.5 GPa. MBS treatment decreased the hardness dramatically especially in the first nanometers, although, a recovery of hardness is observed as the nanoindenter is deepened in the enamel. MBS liposomes also decreased the hardness initially to almost 2 GPa, but also the hardness was increased as depth was gained. Although the analysis has a depth limit set at 2 µm, the observed trend for the last two treatments is that the hardness is progressively recovered. The values observed in the first nanometers of the measurements correspond to the size effect, caused by geometrical dislocation of the HAP crystals when the tip is pressed into the sample [42].

3.3.2. Treatment effects analyzed by PCA

Since whitening treatments are applied directly on the enamel surface, the most affected parts in terms of chemical transformations are the surface itself and the enamel immediately below, since these parts are exposed to the highest concentration of the active ingredient. According to this, the results in section 3.3.1 showed how the first 2 µm were affected by the treatments but a tendency to recover the hardness was observed. Nevertheless, a study revealed that in

vitro application of 10% CP on enamel for two weeks caused demineralization of the enamel extending to a depth up to 50 µm below the enamel surface [43].

For this reason, and in order to follow the treatment penetration up to 50 µm deep, mappings of the outer enamel region, of 50 x 50 µm, were performed also in the mid crown (labial side), starting in the enamel surface and moving towards the dentin direction. Spectra measurements were taken in spots separated by 10 µm each, resulting in a matrix of 50x6 points in the control and 6x6 points in the treated samples.

PCA was performed to compare the effect of these changes all over the surface analyzed. The five whitening treatments were clearly identified from principal component (PC) 1, 2 and 3 (accounting for a total of 99% of the variance) (Figure 4a). To better relate the observed PCs with the spectra, Figure 4b represents, the average of the 12 spectra corresponding the first 10 µm of enamel of each treatment, since are the most affected. Additionally, Figure 4c presents the spectra normalized to maximum height to facilitate the appropriate comparison of relative peak intensities and their shifts (Figure 4c).

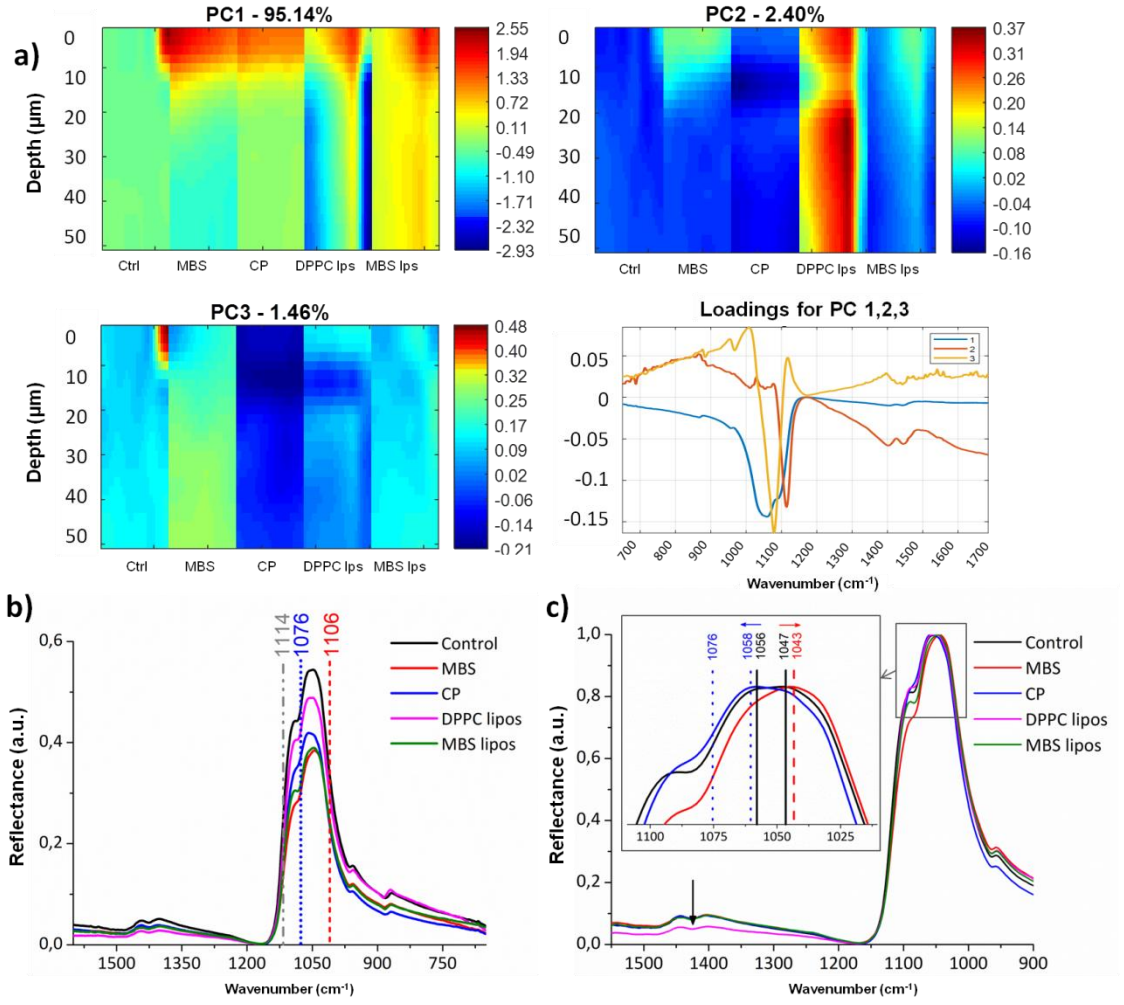

Figure 4. (a) Scores (distributed across the enamel depth, starting in the enamel surface at 0 µm to 50 µm deep), and loadings for the PCA treatments. (b) Average of the spectra corresponding to the first 10 µm for each treatment (n=12). (c) Plot b normalized to maximum. Red dash line: MBS shift. Blue dots line, CP shift. Dash and dots grey line: characteristic shoulder of the HAP peak.

As can be observed in the loadings' representation in Figure 4a, PC1 represents 95.14% of the variation, comprising the whole spectra, especially the phosphate peak. In the scores plot, it can be seen that PC1 (which is negative and thus correspond to the blue parts of the image) did not change for the whole depth analysed in the control sample; for MBS and CP there was a lack of this component in the surface, meaning that the intensity was lower for the first 10 µm deep when compared to control. For DPPC liposomes, it was observed that this treatment also affected the first 10 µm of the enamel, and some of the inner parts were affected by a thin line (as if some liposomes have penetrated the enamel); a higher reflectance intensity in some points of the sample explains the intense blue observed in some parts in the scores map. For MBS liposomes, PC1 showed a high influence in the mantel enamel (most outer part) and then in a thin line entering the inner parts; besides, it can be observed how the whole space is yellow, meaning that the treatment reached the whole surface analysed. In Figure 4b, the groups MBS, CP, and MBS liposomes showed a reduced reflectance when compared to control, and thus PC1 is related to the reflectance peak intensity. In addition, MBS showed a decrease of the 1058 cm^{-1} intensity, and, in addition, the contribution of the 1091 cm^{-1} shoulder had almost disappeared. This points out to a clear distortion of the calcium phosphate structure.

PC2 (2.40% of the variance) loading profile presented a peak at 1114 cm^{-1}, placed in the negative part of the loadings. It corresponds to the Ca-O-P secondary vibration of v_1 PO_4^{3-} (reflects changes in the bandwidth) and to the whole v_2 CO_3^{2-} band. Its presence is related to the blue parts in the corresponding scores map. It can be noticed how the lack of this component was highly significant in DPPC liposomes, and also, although with less intensity, in the first microns of the samples treated with MBS and MBS with liposomes. It is mainly related with a change in the relative intensity of the carbonates since, as indicated in Figure 4c with a black arrow, for DPPC liposomes it was lower than in the other treatments. Also, all the surface of the CP treated tooth appears blue, meaning that this compound did not affect the carbonates. The phosphate peak corresponding to CP is slightly tightened when compared to the other treatments, that could explain the intense blue in some points of the PC2 scores map since it is also related with the bandwidth.

PC3 (1.46%) loading was negative at 1076 cm^{-1}, i.e. blue in the PC3 scores map, and positive at 1006 cm^{-1} (red in the score map). The first is related to a hypsochromic shift of CP (from 1058 to 1056 cm^{-1}), and the second related to a bathochromic shift of MBS, as can be observed in the specific part of the HAP peak in Figure 4c. This shift was no observed with the other whitening treatments and represents different chemical interactions with the HAP structure.

4. DISCUSSION

CSM results showed that both treatments, MBS and MBS with liposomes, decreased the enamel hardness beyond 2 µm depth, although the trend revealed that the hardening was partially recovered at larger depth. We related it to erosive demineralization, a process that takes place when the tooth is in contact with a solution with a pH lower than 5.5 (HAP critical pH). Such damage is characterized by an initial softening of the enamel surface, which extent is between 0.2 and 3 µm, depending on the immersion time and the acids under study [41], which in our case was MBS.

The enamel mapping of the non-treated bovine incisor showed an heterogeneous distribution of the different inorganic species. Physiologically, enamel is an anisotropic material, showing spatial variations in its chemical composition and density [35]. HAP crystals are organized in a basic structural unit called 'rod'. Rods run continuously through all the width of enamel, and close to the dentin become thinner [36]; this creates a mineral gradient, in which the HAP is more packed in the outer enamel, leaving less space for organic matter. The frequencies and the intensity ratio between the HAP spectrum bands depend on the local environment, and they also vary depending on the modifications on the crystallinity degree [44]. Accordingly, phosphates showed higher reflectance in the outer enamel, where their concentration is higher and the crystal framework is neater, while the reflectance decreased since close to the dentin, where the crystals become thinner. Instead, in the inner parts of the enamel carbonates showed higher and the intensity of the HAP peak was reduced. In fact, carbonates modify the lattice parameters of the structure as a consequence of the different size of the substituting ions; the carbonate content increases across outer to inner enamel, which is associated with decreased crystallinity (thus decreased reflectance) and increased solubility [37].

Considering the sample thickness (around 0.5 cm thick), and the area of the analyzed spots (5x5 µm), the presence of the treatment components should not be significant over the signal emitted by the enamel itself when measuring in reflection mode. Therefore, no contribution of liposomes, CP or MBS vibration peaks were expected to be observed, but only the induced modifications in the HAP structure. The treatments applied produced different effects on the FTIR HAP reflectance spectra. The most important change observed was the decrease in the v_3 PO_4^{3-}, which can be related to a loss of mineral content in the tooth surface [45] (erosion). Kim *et al.* [25] also observed a decrease in the absorbance, but also an hypsochromic shift of v_3 PO_4^{3-} peak in the acid-eroded enamel. They related these observations to changes in the local structure; in apatite, each P atom is linked to four Ca atoms via a shared oxygen atom (P–O–Ca atomic bridges), and the exposure to acid breaks the Ca–O bonds and consequently reduces the P–O bond length, due to the redistribution of the electron density in the vicinity of the bridging oxygen. According to that, in MBS treatments, a decrease in the reflectance due to the acidic nature of MBS (PC1) was observed, which is consistent with the results obtained by CSM. But a bathochromic shift, i.e., an elongation of the P-O bond took place instead of a shortening. This could be explained by a SO_4^{2-} substitution of the PO_4^{3-} group; the lattice parameter along the c-axis increases with sulphur substitution because of the length of the atomic bond, as well as an hypsochromic shift happens in the phosphate peak [46]. While, in PC1, MBS affected only the outer layers of the tooth, MBS liposomes affected the whole analysed surface, especially in the middle part; we relate this effect to the increased diffusion promoted by the liposomes [16].

Vasluianu *et al.* [44], studying the changes induced in HAP by HP whitening treatments, also observed a decrease in the reflectance and an hypsochromic shift for the main FITR peaks of HAP; they related it, again, to the shortening of P-O, even if the pH of the treatments was 7.4. In our experiment, CP pH was 8 as in the commercial treatments, since its effectiveness is increased at this pH [9]. Accordingly, a decrease in the reflectance (PC1) and an hypsochromic shift (PC3) were observed. At basic pH, HAP dissolution should not occur, but protein degradation in the tooth organic matrix might be happening. Indeed, the urea in CP along with

the ammonium ions (NH_4^+) formed in contact with water act on the hydrogen bonds of the proteins weakening their structure [4]. Since the components of the organic matter form a support structure that stabilizes the crystalline enamel layer [47], any change in the organic matter could cause an effect in the reflectance in the first 30 µm, as observed in PC1. According to Pleshcko *et al.* [39], a narrowing of the phosphate peak is related with increased crystallinity; this effect was observed for CP treatment, probably because the crystallinity was better detected after the loose of organic matter, because of its better reflective properties.

Regarding the carbonates, MBS treatments promoted the loose of this component at the same extent as it affected the PO_4^{3-} peak (PC1), while CP did not affect the content at all, according to the results observed in the scores map of PC2. This confirms the previous observation, that the mechanism by which the compounds interact with the enamel is different: the first produces an acid attack to the mineral content, the second by promoting the organic matrix decomposition.

5. CONCLUSIONS

From the reported results, the following conclusions can be extracted:

MBS treatments soften the first 10 µm of enamel, as happens in the initial states of tooth decay. But, since saliva buffers acid and promotes remineralization once acid contact has been removed MBS treatments applied with enough separation in time, should be totally safe for human application.

CP and MBS promote different changes in the HAP mineral; CP shorten the P-O bond while MBS seems to elongate it. Moreover, MBS promotes the loose of carbonates while CP doesn't, which is probably related to the solution's pH.

When comparing MBS and MBS Liposomes, it is observed how liposomes favours the diffusion of MBS to inner layers, since the effects of MBS can be observed in deeper enamel. Thus, the encapsulated MBS whitening effect is highly improved in terms of time when compared to MBS alone or CP.

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ANNEX II. Patent application EP19382958.7/ 1916023.3: Tooth whitening composition

TOOTH WHITENING COMPOSITION

Technical Field

The present invention belongs to the field of compositions for tooth whitening.

Background art

The natural colour of teeth is opaque to translucent white or slightly off-white. However, the use of certain foods and tobacco, the process of aging, diseases, trauma, medications, some congenital conditions, and environmental effects can cause teeth to become discoloured. Because whiter teeth are considered to be aesthetically superior to stained or discoloured teeth, there has been a large demand for dental bleaching compositions.

Typical tooth bleaching agents release active oxygen radicals. Such bleaching agents include peroxides, such as hydrogen peroxide, percarbonates and perborates of the alkali and alkaline earth metals, or complex compounds containing hydrogen peroxide, such as carbamide peroxide. Also, peroxide salts of the alkali or alkaline earth metals and peroxyacetic acid are known to be useful in whitening teeth, as disclosed in EP-A-0545594. Peroxyacetic acid may be generated *in situ* from a precursor comprising labile acyl groups, as disclosed in US-A-2001/0021374. Sodium chlorite is also disclosed as tooth whitening agent, for example, in WO-98/04235.

Current bleaching agents contain both active and inactive ingredients. The active ingredients include usually hydrogen peroxide or carbamide peroxide in a wide range of concentrations (5-35% or 10-35% respectively). However, the major inactive ingredients may include thickening agents, carrier, surfactant and pigment dispersant, preservative, and flavouring.

Patients who have desired to have their teeth whitened have typically done so by applying a bleaching composition to the teeth by means of a dental tray for repeated treatments, or they have had to submit to conventional in-office bleaching techniques that required from 4 to 10 visits to the dental office before clinically significant results were achieved. Clinically significant results are quantifiable by means of colour measurement.

Reducing agents have been used scarcely as tooth whitening agents. In WO-A-95/05148 it is disclosed a composition for reducing or removing surface deposited stains from natural teeth and dental prostheses comprising an effective amount of an orally acceptable sequestering agent and an orally acceptable reducing agent, such as Vitamin C, vitamin E, BHA, BHT, and propyl gallate, in an orally acceptable carrier. In WO-A-01/64175 it is disclosed a “multi-stage” whitening procedure for teeth, wherein different types of whitening agents are used consecutively. One of the agents is papain enzyme activated by additional co-ingredients, such as thiol containing groups, such as cysteine hydrochloride, mercaptoethanol, and

dithiotreitol, or metabisulfite salts. In WO-A-99/07818 it is disclosed an ultrasonic water-soluble dental tablet cleansing composition comprising scrubbing particles, a bleaching agent, a bleaching enhancer, a tablet aid, a tablet disintegrator, a tartar control agent, a crystallization inhibitor, a chelating agent, and a pH adjusting agent. As bleaching enhancers are mentioned sodium sulphite, thiosulfate, thiourea and metabisulfite.

Different technical solutions have been disclosed in prior art to improve the tooth whitening effect.

For example, to accelerate the whitening effect of peroxygen compounds, in WO-A-97/02805 it is disclosed the use of a manganese coordination complex compound such as manganese gluconate in combination with the bleaching compound.

Another approach is the formulation of the tooth whitening compound with a gelling compound. In US5290566 it is disclosed a tooth whitening formulation comprising urea peroxide in combination with a gelling agent, which keeps the formulation in contact with the teeth for a period of time sufficient to cause whitening thereof. An anhydrous dental bleaching gel composition comprising propylene glycol, polyethylene glycol, glycerine, neutralized carboxypolymethylene, hydroxypropyl-cellulose, and carbamide peroxide, is disclosed in US5718886. Hydrogen peroxide has also been gelled to improve the bleaching effect by combining it with polyacrylic acid, as disclosed in US-A-2003/0170592. In EP-A-0511782 it is disclosed a sustained-release film-forming polymer composition comprising a specific water-soluble cellulosic polymer; a pharmaceutically or veterinary acceptable oxidising agent; and a pharmaceutically or veterinary acceptable vehicle.

A further approach is the use of an abrasive together with the tooth whitening agent. In EP-A-0535816 it is disclosed an abrasive dentifrice composition containing a peroxide compound, a dicalcium phosphate compound and a metal ion free peroxide compound. Further tooth whitening abrasive compositions are disclosed, for example, in WO-A-97/11675, WO-A-97/21419, and WO-A-99/02126. In CN-A-103356396 it is disclosed a whitening tooth powder, which comprises silicon dioxide, sodium hydrogen carbonate, sodium tripolyphosphate, flavouring agents, sodium metabisulfite and sodium carbonate.

However, in an attempt to increase the efficiency of bleaching agents, such as carbamide peroxide, higher concentrations are used, which leads to the occurrence of most common adverse effects, such as mild to moderate tooth sensitivity and/or gingival irritation, as disclosed in Desai *et al*., The effect of a chemical activator on tooth bleaching with two different concentrations of carbamide peroxide: An *in vitro* study, Int. J. Appl. Dental Sci., 2018, 4(1), 286-289.

The use of oxygen-based tooth whitening treatments is associated to controversy over the effects on tooth structure (physical properties of enamel and dentin), such as increased porosity of the superficial enamel structure, demineralization and decreased protein concentration, organic matrix degradation, modification in the calcium: phosphate ratio, calcium loss, reduction of dentin microhardness, and decrease in the flexural strength and flexural modulus of dentin, as disclosed in M. Q. Alqahtani, Tooth-bleaching procedures and their controversial effects: A literature review, The Saudi Dental J., 2014, 26, 33-46.

Despite the numerous proposals available in the state of the art, there is still a need to have new compositions for tooth whitening showing improved efficiency and reduced secondary effects.

Object of the invention

The object of the present invention is a method for preparing a composition for tooth whitening.

It is another aspect of the invention the composition for tooth whitening obtainable according to that method.

It is another aspect of the invention the non-therapeutic cosmetic use of that composition for tooth whitening.

Figures

Figure 1

Figure 1 discloses the colour change obtained in the *in vitro* tooth whitening treatments described in Example 3. In ordinates there is the colour change (increase of whiteness) expressed as ΔE for each treatment, wherein treatment 1 represents the negative control (water), treatment 2 comprises aqueous liposomes, treatment 3 comprises sodium metabisulfite, treatment 4 represents 16 wt.% carbamide peroxide, and treatment 5 comprises the composition of the invention according to Example 1, and in abscises there are the different times (3', 6', 9', 14' and 20'), when the whitening increase was measured. It was observed that the composition of the invention showed a faster and more effective whitening effect than the single components.

Figure 2

Figure 2 discloses the colour change obtained in *in vitro* tooth whitening treatments according to international standard ISO 28399 of Example 12. In ordinates there is the colour change (increase of whiteness) expressed as ΔE for each treatment, wherein treatment 1 represents the negative control (water), treatment 2 represents the positive control (citric acid 1 wt.%), treatment 3 represents 16 wt.% carbamide peroxide, treatment 4 represents 35 wt.% hydrogen peroxide, and treatment 5 represents the treatment with the composition disclosed in Example 9. In abscises there are the results for the first, second and third treatment, each at 5-days intervals, and the follow up after one month of the last treatment. It was observed the high efficacy of the tooth whitening treatment with the composition of the invention in comparison to prior art treatments.

Figure 3

Figure 3 discloses the increase in roughness of the tooth, in ordinates as ΔRa, measured in compliance with ISO 28399, for the treatments disclosed in Example 12, which are in abscises, wherein treatment 1 represents the negative control (water), treatment 2 represents the positive control (citric acid, 1 wt.%), treatment 3 represents 16 wt.% carbamide

peroxide, treatment 4 represents 35 wt.% hydrogen peroxide, and treatment 5 represents the treatment with the composition disclosed in Example 9. It was observed that the result for the composition of the invention is substantially similar to that of the positive control, citric acid solution, which is considered safe for dental treatments. ISO 28399 considers tested treatment safe in terms of roughness, if its value is lower than three times the value of the positive control.

Detailed description of the invention

The object of the present invention is a method for preparing a tooth whitening composition, which comprises:

1) the step of mixing at least one membrane-forming lipid in an organic solvent in a vessel, and

either

2a) dispersing the mixture obtained in step 1) in an aqueous solution comprising a reducing agent, and

3a) removing the organic solvent to obtain a composition comprising liposomes comprising the solution of the reducing agent,

or

2b) removing the organic solvent of the mixture obtained in step 1) to obtain a layer of the membrane-forming lipid on the inner surface of the vessel, and

3b) adding an aqueous solution comprising a reducing agent to the layer of step 2b) to obtain a composition comprising liposomes comprising the solution of the reducing agent.

The authors of the present invention have developed a method for preparing a composition suitable for tooth whitening, which show improved efficiency due to the rapid action, which can lead to reduced secondary effects, such as the weakening of tooth, by means of a reduced time of treatment application. The composition comprises a solution of a reducing agent, partially encapsulated in liposomes. Without being bound to any theory, it is considered that the liposome fraction of the composition remains attached on the surface of the tooth improving the whitening effect of the reducing agent. The composition of the invention shows a synergistic effect in comparison with the use of a reducing agent alone, or liposomes alone.

The term "approximately" or “about” refers to a deviation of plus/minus 10%, preferably plus/minus 5%.

In the present description, as well as in the claims, the singular forms "a", "an" and "the" include the plural reference unless the context clearly indicates otherwise. The ranges defined by the preposition "between" include also the two ends thereof.

According to the American Dental Association, restoring the natural colour of teeth is called whitening, and whitening teeth beyond their natural colour is called bleaching. However, teeth whitening and teeth bleaching are used interchangeably in prior art. In the

present invention a whitening process for teeth covers both the process for restoring the natural colour of teeth by removing stains from the tooth surface and the process for whitening teeth beyond their natural colour.

Method

In the method of the invention, the at least one membrane-forming lipid is dispersed in an aqueous solution comprising a reducing agent, to obtain a composition comprising liposomes comprising the solution of the reducing agent.

The composition obtained in step 3a) or 3b) of the method comprises:

i. liposomes formed by at least one membrane-forming lipid, which comprise an aqueous solution of the reducing agent, optionally comprising also a buffer system, and

ii. an aqueous solution of the reducing agent, optionally comprising also a buffer system, which is not entrapped in the liposome membrane.

In both cases, a) and b), the solution of the reducing agent stays both inside and outside the membrane-forming lipid.

Liposomes are spontaneously formed when phospholipids are dispersed in aqueous medium, as disclosed in Bangham *et al*., in Korn, E.D. (Ed.) Methods in Membrane Biology, Vol.1, Plenum Press, New York, 1975, pp. 1-68.

The preparation of liposomes resulting from the dispersion of the at least one membrane-forming lipid in the aqueous solution of a reducing agent, can be carried out by well-known techniques. In general, the preparation method involves mixing the membrane-forming lipids in an organic solvent, removal of the organic solvent to form a layer of the membrane-forming lipid, subsequent dispersion of the membrane-forming lipid in an aqueous solution, and further size reduction by mechanical treatment.

Alternatively, the preparation method involves mixing the membrane-forming lipids in an organic solvent, subsequent dispersion of the membrane-forming lipid containing phase in an aqueous solution, removal of the organic solvent, and further size reduction by mechanical treatment.

In an embodiment of the invention, the method for preparing a tooth whitening composition comprises:

1) the step of mixing at least one membrane-forming lipid in an organic solvent in a vessel, and

either

2a) dispersing the mixture obtained in step 1) in an aqueous solution comprising a reducing agent, and

3a) removing the organic solvent to obtain a composition comprising liposomes comprising the solution of the reducing agent,

In an embodiment of the invention, the method for preparing a tooth whitening composition comprises:

1) the step of mixing at least one membrane-forming lipid in an organic solvent in a vessel,

2b) removing the organic solvent of the mixture obtained in step 1) to obtain a layer of the membrane-forming lipid on the inner surface of the vessel, and

3b) adding an aqueous solution comprising a reducing agent to the layer of step 2b) to obtain a composition comprising liposomes comprising the solution of the reducing agent.

The solution of the reducing agent comprises optionally a buffer system.

Usually, the membrane-forming lipid is dissolved or dispersed in the at least one organic solvent, preferably dissolved. The organic solvent is usually selected from the group comprising chloroform, dichloromethane, methanol, ethanol, and mixtures thereof. In a preferred embodiment the organic solvent is selected from chloroform and a combination of chloroform and methanol.

In a preferred embodiment, the method further comprises a step of treating mechanically the liposomes obtained in step 3a) or 3b) to reduce their size.

The mechanical treatment may be carried out by known methods, such as sonication, extrusion or homogenization. In a preferred embodiment, the size of liposomes is reduced by sonication, preferably in an ultrasound bath.

Membrane-forming lipid

The membrane-forming lipid suitable to be used in the method of the invention comprises a phospholipid.

The phospholipid may be selected from a group consisting of a natural phospholipid, a synthetic phospholipid, and combinations thereof. Lecithin is one of the natural resources for phospholipid. Lecithin is a mixture found in egg yolk and soy. It comprises a number of phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). Natural phospholipids also include, e.g. hydrogenated soy PC (HSPC), sphingomyelin, and phosphatidylglycerol (PG).

Synthetic phospholipids include, but are not limited to, derivatives of phosphocholine (for example, DDPC (1,2-didecanoyl-*sn*-glycero-3-phosphocholine), DLPC (1,2-dilauroyl-*sn*-glycero-3-phosphocholine), DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine), DOPC (1,2-dioleoyl-*sn*-glycero-3-phospho-choline), POPC (1-dalmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), DEPC (1,2-die-rucoyl-*sn*-glycero-3-phosphocholine)), derivatives of phosphoglycerol (for example, DMPG (1,2-dimyristoyl-*sn*-glycero-3-phosphorylglycerol), DPPG (1,2-dipalmitoyl-*sn*-glycero-3-phosphorylglycerol), DSPG (1,2-distearoyl-*sn*-glycero-3-phosphorylglycerol), POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol)), derivatives of phosphatidic acid (for example, DMPA (1,2-dimyristoyl-*sn*-glycero-3-phosphate), DPPA (1,2-dipalmitoyl-*sn*-glycero-3-phosphate), DSPA (1,2-distearoyl-*sn*-glycero-3-phosphate)), derivatives of phosphoethanolamine (for example, DMPE (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine), DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine), DSPE (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine), DOPE (1,2-

dioleoyl-*sn*-gly-cero-3-phosphoethanolamine)), derivatives of phosphoserine (for example, DOPS (1,2-dioleoyl-*sn*-glycero-3-phosphoserine)), PEG derivatives of phospholipid (for example, PEG-phospholipid, polyglycerin-phospholipid, functionalized-phospholipid, and terminal activated-phospholipid), or salts thereof.

In one embodiment of the present invention, the phospholipid is a hydrogenated phospholipid, specifically, dipalmitoylphosphatidylcholine (DPPC).

The concentration of the at least one membrane-forming lipid in the composition comprising liposomes obtained in step 3a) or step 3b) is usually comprised between 1 mM and 100 mM, preferably between 5 mM and 50 mM, more preferably between 10 mM and 40 mM, yet more preferably between 15 mM and 30 mM, and yet more preferably between 15 mM and 25 mM. In a preferred embodiment, the concentration is 20 mM.

The properties of the membrane may be modified by combining the phospholipid with further components. In an embodiment of the invention, the membrane-forming lipid comprises a phospholipid and a further component. Further components may be incorporated to the membrane as disclosed in, for example, He *et al*., Acta Pharm. Sinica B, 2019, 9(1), 36-48; Fonseca *et al*., Biochim. Biophys. Acta Biomembranes, 1996, 1279 (2), 259-265; and Milla *et al*., Current Drug Metabol., 2012, 13(1), 105–119. In the context of the invention, further components that may be incorporated to the membrane are, for example, cholesterol; stearylamine; soybean-derived sterols; bile salts, such as sodium glycocholate, sodium taurocholate and sodium deoxycholate; polymers, such as polysaccharides, collagen, chitosan, chitosan derivatives, such as *N*-trimethyl chitosan and methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan, polyethylene glycols, polyethylene glycol derivatives, such as PEG-distearoylphosphatidylethanolamine; and non-ionic surfactants, such as Tween 80.

In a preferred embodiment, the membrane-forming lipid comprises a phospholipid and a further component selected from cholesterol, stearylamine, soybean-derived sterol, bile salt, polysaccharide, collagen, chitosan, chitosan derivatives, polyethylene glycol, polyethylene glycol derivatives, non-ionic surfactant, and mixtures thereof.

In a more preferred embodiment, the membrane-forming lipid is combined with cholesterol. The presence of cholesterol as a component of the membrane-forming lipid improves the fluidity of the bilayer membrane, reduces the permeability of water-soluble molecules through the membrane, and improves the stability of bilayer membrane in the presence of biological fluids.

Reducing agent

A reducing agent (also called a reductant or reducer) is an element or compound that loses (or "donates") an electron to another chemical species in a redox chemical reaction.

In the context of the present invention, a reducing agent is selected from the group of sulphur containing compounds such as dithionite, metabisulfite, sulphite, bisulfite, and alkaline metal salts thereof. Preferably the reducing agent is selected from sodium dithionite, potassium dithionite, sodium metabisulfite, potassium metabisulfite, sodium sulfite, potassium sulfite, sodium bisulfite, and potassium bisulfite, and more preferably from sodium metabisulfite and potassium metabisulfite.

The concentration of the reducing agent in the aqueous solution of step 2a) or step 2b) of the method is usually comprised between 0.01 M to 0.5 M, preferably between 0.01 M and 0.4 M, more preferably between 0.05 M and 0.3 M, and yet more preferably between 0.1 M and 0.2 M. In one preferred embodiment, the concentration is 0.1 M.

The aqueous solution of reducing agent usually has a pH value comprised between 2 and 8, preferably between 3 and 7.5, more preferably between 4 and 7, yet more preferably between 5.5 and 6.5, and more preferably 6.0. In one preferred embodiment the aqueous solution has the pH resulting from the dissolution of the reducing agent in water. For example, a 0.47 M solution of sodium metabisulfite has a pH value of about 2.9.

Buffer system

The aqueous solution comprising the reducing agent comprises, in a preferred embodiment, a buffer system.

In that embodiment, the aqueous solution of reducing agent comprises a buffer system to adjust the pH to a value comprised between 4 and 7, preferably between 5.5 and 6.5, and more preferably 6.0. In a preferred embodiment the aqueous solution of reducing agent is buffered to pH 5.5, and in another preferred embodiment to pH 6.5.

The buffer system is selected by the skilled person among known buffer systems. Preferably the buffer system comprises citric acid and disodium citrate. If necessary, pH value may be further adjusted by adding any acid, such as hydrochloric acid, or base, such as sodium hydroxide.

Liposomes

Liposomes comprising the membrane-forming lipid and reducing agent are obtained in step 1) of the method of the invention. In a preferred embodiment, liposomes also comprise an aqueous solution comprising a buffer system.

As used in this description, "liposome" is used to describe oligo-lamellar lipid vesicles comprising one or more natural or synthetic lipid bi-layers surrounding an internal aqueous phase.

Liposomes are commonly used in cosmetic formulations for improving dermal penetration of actives. As is well known in the art, liposomes are spherical vesicles with sizes generally in the range between about 60 nm and 300 nm and are most often composed of phospholipids, such as phosphatidylcholine, which form at least one phospholipid bilayer, but may also include other lipids, such as egg phosphatidylethanolamine. Liposomes contain hydrophilic cores in which hydrophilic actives may be encapsulated, while hydrophobic actives are incorporated in the bilayer, so liposomes are suitable carriers for both hydrophilic and lipophilic actives (Knoth *et al*., Nanocarrier-Based Formulations: Production and Cosmeceutic Applications, in: Cosmetic Formulation. Principles and Practice, Benson H.A.E., Roberts M.S., Rodrigues Leite-Silva V. and Walters K.A., editors, CRC Press, 2019).

Liposomes usually are classified either by the method of their preparation or by the number of bilayers present in the vesicle, or by their size.

According to the method of the invention, the obtained liposomes usually show a particle size from 20 nm to 500 nm, and they are formed by a mixture of unilamellar

vesicles and multilamellar vesicles. By high-pressure extrusion through, for example, very small pore polycarbonate it is possible to reduce the average diameter of the liposomes to about 60 nm – 80 nm after several passes. It is known that when reducing the size of liposomes, they tend to become unilamellar.

Tooth whitening composition

It is an aspect of the invention a tooth whitening composition obtainable according to the process of the invention.

The tooth whitening composition of the invention comprises an aqueous solution comprising a reducing agent, and liposomes, comprising at least one membrane-forming lipid and an aqueous solution comprising a reducing agent.

In a preferred embodiment, the tooth whitening composition consists essentially of an aqueous solution comprising a reducing agent, and liposomes, comprising at least one membrane-forming lipid and an aqueous solution comprising a reducing agent.

In a preferred embodiment the tooth whitening composition further comprises a buffer system.

In a more preferred embodiment, the tooth whitening composition consists essentially of an aqueous solution comprising a reducing agent, a buffer system, and liposomes comprising at least one membrane-forming lipid, and an aqueous solution comprising a reducing agent, and a buffer system.

Preferably the reducing agent is selected from the group of sulphur containing compounds such as metabisulfite, sulphite, bisulfite, and alkaline metal salts thereof. Preferably the reducing agent is selected from sodium metabisulfite, potassium metabisulfite, sodium sulfite, potassium sulfite, sodium bisulfite, and potassium bisulfite, and more preferably from sodium metabisulfite and potassium metabisulfite.

Preferably the membrane-forming lipid comprises a phospholipid, more preferably a hydrogenated phospholipid, and yet more preferably dipalmitoylphosphatidylcholine (DPPC).

Usually the tooth whitening composition comprises further flavouring agents, sweeteners, preservatives, stabilizers or mixtures thereof to improve the acceptance by the user.

Use

It is another aspect of the invention the non-therapeutic cosmetic use of that composition for tooth whitening.

A cosmetic effect relates to beautify and/or improve the appearance of teeth. A cosmetic effect does not involve any therapeutic effect, i.e., cosmetics are not intended to prevent or ameliorate any disease. A cosmetic effect is, for example, the whitening of teeth.

The composition of the invention may be used for whitening vital teeth, non-vital teeth, and prosthesis.

Tooth whitening trials with the composition of the invention show surprisingly an improved whitening effect, much higher than expected for the components alone as shown

in Figure 1. The composition of the invention comprising a reducing agent partially encapsulated in liposomes show a better result than that obtained using only the reducing agent, or only liposomes. It is observed that liposome encapsulation provides stability to the reducing agent and creates a layer on the enamel to favour penetration of the reducing agent to carry out the whitening activity.

Tooth whitening experiments may be carried out with bovine incisors or extracted human permanent maxillary central incisor, for example, as disclosed in Desai *et al*., *op. cit*. These specimens are usually cleaned of gross debris and the root cut using a diamond saw, and then may be preserved in 0.2% sodium azide solution until the experiment performance. The discolouration may be obtained by staining the tooth surface using a tannic acid solution. The whitening treatments may be carried out following a short laboratory treatment up to 20 minutes, performing colorimetric measurements at different times in order to monitor the whitening effect over time, or treatments according to ISO 28399 with longer treatments at 5-day intervals, preserving the tooth between times in artificial saliva, prepared according to the modified Shellis solution, as disclosed in R. P. Shellis, Effects of a Supersaturated Pulpal Fluid on the Formation of Caries-Like lesions on the Roots of Human Teeth, Caries Res., 1994, 28, 14–20. In both cases, the tooth whitening composition of the invention shows a fast and higher whitening performance in comparison to single components (reducing agent or liposomes) and to prior art treatments (hydrogen peroxide or carbamide peroxide).

Surprisingly, the composition of the invention is characterized by a fast and high whitening performance. The efficiency of the composition allows short treatment times and a reduction of secondary effects derived from the long application times of actual treatments.

The present invention comprises the following embodiments:

1.- A method for preparing a tooth whitening composition, characterized in that it comprises:

1) the step of mixing at least one membrane-forming lipid in an organic solvent in a vessel, and

either

2a) dispersing the mixture obtained in step 1) in an aqueous solution comprising a reducing agent, and

3a) removing the organic solvent to obtain a composition comprising liposomes comprising the solution of the reducing agent,

or

2b) removing the organic solvent of the mixture obtained in step 1) to obtain a layer of the membrane-forming lipid on the inner surface of the vessel, and

3b) adding an aqueous solution comprising a reducing agent to the layer of step 2b) to obtain a composition comprising liposomes comprising the solution of the reducing agent.

2.- The method according to embodiment 1, characterized in that it comprises:

1) the step of mixing at least one membrane-forming lipid in an organic solvent in a vessel,

2a) dispersing the mixture obtained in step 1) in an aqueous solution comprising a reducing agent, and

3a) removing the organic solvent to obtain a composition comprising liposomes comprising the solution of the reducing agent,

3.- The method according to embodiment 1, characterized in that it comprises:

1) the step of mixing at least one membrane-forming lipid in an organic solvent in a vessel,

2b) removing the organic solvent of the mixture obtained in step 1) to obtain a layer of the membrane-forming lipid on the inner surface of the vessel, and

3b) adding an aqueous solution comprising a reducing agent to the layer of step 2b) to obtain a composition comprising liposomes comprising the solution of the reducing agent.

4.- The method according to any of embodiments 1 to 3, characterized in that the organic solvent is selected from the group comprising chloroform, dichloromethane, methanol, ethanol, and mixtures thereof.

5.- The method according to embodiment 4, characterized in that the organic solvent is selected from chloroform and a combination of chloroform and methanol.

6.- The method according to any one of embodiments 1 to 5, characterized in that it further comprises a step of treating mechanically the liposomes obtained in step 1) to reduce their size.

7.- The method according to embodiment 6, characterized in that the step of mechanically treating the liposomes is carried out by sonication, extrusion or homogenization.

8.- The method according to embodiment 7, characterized in that the step of mechanically treating the liposomes is carried out by sonication.

9.- The method according to any one of embodiments 1 to 8, characterized in that the at least one membrane-forming lipid comprises a phospholipid.

10.- The method according to embodiment 9, characterized in that the phospholipid is selected from the group consisting of a natural phospholipid, a synthetic phospholipid, and combinations thereof.

11.- The method according to embodiment 10, characterized in that the natural phospholipid is selected from phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), hydrogenated soy PC (HSPC), sphingomyelin, phosphatidylglycerol (PG), and mixtures thereof.

12.- The method according to embodiment 10, characterized in that the synthetic phospholipid is selected from derivatives of phosphocholine, derivatives of phosphoglycerol, derivatives of phosphatidic acid, derivatives of phosphoethanolamine, derivatives of phosphoserine, PEG derivatives of phospholipid, and mixtures thereof.

13.- The method according to embodiment 12, characterized in that the synthetic phospholipid is selected from DDPC, DMPC, DPPC, DSPC, DOPC, POPC, DEPC, DMPG, DPPG, DSPG, POPG, DMPA, DPPA, DSPA, DMPE, DPPE, DSPE, DOPE, DOPS, and mixtures thereof.

14.- The method according to embodiment 13, characterized in that the synthetic phospholipid is dipalmitoylphosphatidylcholine (DPPC).

15.- The method according to any one of embodiments 1 to 14, characterized in that the concentration of the at least one membrane-forming lipid in the composition comprising liposomes obtained in step 3a) or step 3b) is comprised between 1 mM and 100 mM, preferably between 5 mM and 50 mM, more preferably between 10 mM and 40 mM, yet more preferably between 15 mM and 30 mM, yet more preferably between 15 mM and 25 mM and most preferably is 20 mM.

16.- The method according to any one of embodiments 1 to 14, characterized in that the membrane-forming lipid comprises a phospholipid and a further component.

17.- The method according to embodiment 16, characterized in that the further component is selected from cholesterol, stearylamine, soybean-derived sterol, bile salt, polysaccharide, collagen, chitosan, chitosan derivatives, polyethylene glycol, polyethylene glycol derivatives, non-ionic surfactant, and mixtures thereof.

18.- The method according to embodiment 17, characterized in that the further component is cholesterol.

19.- The method according to any one of embodiments 1 to 18, characterized in that the reducing agent is selected from the group of dithionite, metabisulfite, sulphite, bisulfite, and alkaline metal salts thereof.

20.- The method according to embodiment 19, characterized in that the reducing agent is selected from sodium dithionite, potassium dithionite, sodium metabisulfite, potassium metabisulfite, sodium sulfite, potassium sulfite, sodium bisulfite, and potassium bisulfite, and more preferably from sodium metabisulfite and potassium metabisulfite.

21.- The method according to embodiment 20, characterized in that the reducing agent is selected from sodium metabisulfite and potassium metabisulfite.

22.- The method according to any one of embodiments 1 to 21, characterized in that the concentration of the reducing agent in the aqueous solution of step 2a) or step 2b) of the method is comprised between 0.01 M to 0.5 M, preferably between 0.01 M and 0.4 M, more preferably between 0.05 M and 0.3 M, yet more preferably between 0.1 M and 0.2 M, and most preferably is 0.1 M.

23.- The method according to any one of embodiments 1 to 22, characterized in that the aqueous solution has the pH value comprised between 2 and 8, preferably between 3 and 7.5, more preferably between 4 and 7, yet more preferably between 5.5 and 6.5, and more preferably 6.0.

24.- The method according to embodiment 23, characterized in that the aqueous solution has the pH resulting from the dissolution of the reducing agent in water.

25.- The method according to any one of embodiments 1 to 23, characterized in that the aqueous solution of reducing agent comprises a buffer system.

26.- The method according to embodiment 25, characterized in that the aqueous solution of reducing agent comprises a buffer system to adjust the pH to a value comprised between 4 and 7, yet more preferably between 5.5 and 6.5, and more preferably 6.0.

27.- A tooth whitening composition obtainable according to the process of any one of embodiments 1 to 26.

28.- The tooth whitening composition according to embodiment 27, characterized in that it comprises an aqueous solution comprising a reducing agent, and liposomes, comprising at least one membrane-forming lipid and an aqueous solution comprising a reducing agent.

29- The tooth whitening composition according to embodiment 28, characterized in that it further comprises a buffer system.

30.- The tooth whitening composition according to embodiment 28, characterized in that it consists essentially of an aqueous solution comprising a reducing agent, and liposomes comprising at least one membrane-forming lipid and an aqueous solution comprising a reducing agent.

31.- The tooth whitening composition according to embodiment 30, characterized in that it consists essentially of an aqueous solution comprising a reducing agent, a buffer system, and liposomes comprising at least one membrane-forming lipid, and an aqueous solution comprising a reducing agent and a buffer system.

32.- The tooth whitening composition according to any of embodiments 27 to 31, characterized in that it further comprises flavouring agents, sweeteners, or mixtures thereof.

33.- Non-therapeutic cosmetic use of composition according to any one of embodiments 27 to 32 for tooth whitening.

34.- The use according to embodiment 33, characterized in that the composition is used for whitening vital teeth, non-vital teeth, and prosthesis.

In the following examples, particular embodiments of the composition of the invention are shown.

Examples

Colorimetric measurements

For colour measurements, a contact type spectrophotometer Konica Minolta CR-321 Chroma Meter Colorimeter Bundled W/ DP-301 Data Processor, obtaining colour coordinates, L*, a*, and b*, was used.

The overall colour change is expressed as ΔE*ab, from the Commission Internationale de l'Eclairage, relative to the baseline colour parameters, and using the following equation:

$$\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

For 20 minutes whitening, measurements were performed at baseline, and after 3, 6, 9, 14 and 20 minutes of treatment.

For clinic-like whitening, measurements were performed at baseline (T1), 1-day post 1st application session (T2), 1-day post 2nd session (T3), 1-day post 3rd session (T4), and 1-month post whitening follow-up (T5).

The measurements were taken in the flat polished surface of the specimens.

Nanoindentation measurements

Enamel surface Young modulus of elasticity and hardness of two specimens of each treatment were determined by nanoindentation test using a Berkovich tip mounted on a nanoindenter (Nanoindenter XP-MTS). Before each test, the Berkovich diamond indenter was calibrated on a standard fused silica specimen, as disclosed in Nanoindentation – Ensuring Accuracy and Reliability Using Standard Specimens, Fischer-Cripps Labs., 2010 (https://www.azom.com/article.aspx?ArticleID=5415). Hardness is given by the equation below:

$$H = \frac{P_{max}}{A_r}$$

To convert the Vickers hardness number to SI units the hardness number in kilograms-force per square millimeter (kgf/mm²) has to be multiplied with the standard gravity (9.806 65) to get the hardness in MPa (N/mm²) and furthermore divided by 1000 to get the hardness in GPa.

Preparative example 1: Preparation of specimens for whitening treatments

Bovine teeth (specimens) were cleaned of gross debris and the root was cut using a diamond saw, then preserved in 0.2 wt.% sodium azide solution until the experiment performance. The crowns were stained for 5 days using a tannic acid staining solution with a concentration of 80 g/L, at 37ºC and under stirring. The stained teeth were embedded in self-curing polyacrylic cylinders. The surface was polished to expose a window of 3 mm x3 mm enamel surface which will permit the surface roughness and colorimetric measurements. Specimens were ground using a sequence starting at P400 and sequentially increasing to P4000 silicon carbide paper under a constant flow of water. A diamond polishing suspension with a mean particle size of 1 µm followed by a slurry of aluminium oxide with a mean particle size of 0.3 µm were used for the final polishing. Finally, they were placed in artificial saliva, prepared according to the modified Shellis solution, and adjusted to pH 7.0 for 24 h prior to initiating the experiment.

The whitening treatments used in this description were performed under different conditions:

a) Tooth whitening treatments at 20 minutes according to 2^2 factorial design

The treatments were applied above the flattened surface of the specimens for a total of 20 minutes, performing colorimetric measurement at 3, 6, 9, 14 and 20 minutes in order to follow-up the kinetics of the whitening effect. To stop the whitening reaction during the colorimetry, the treatment material was removed from the specimens using an electric toothbrush while rinsing with miliQ water for 30 seconds.

b) Tooth whitening treatments at 20 minutes according to 2^3 factorial design

The treatments were applied above the flattened surface of the specimens for a total of 20 minutes.

c) Whitening treatments according to ISO 28399

The treatment regimens were performed in three applications (1h each) at 5-day intervals for 3 repetitions, using 25 µL of the product in order to cover the exposed surface. The whitening solutions were replenished with 50 µL once during the 20-minute application session. The negative (NC) and positive control (PC) groups will be treated according to the International Organization for Standardization (ISO) 28399 protocol with miliQ water and 1.0 wt.% citric acid adjusted to pH 3.9 for 60 minutes, respectively. In all cases, after each treatment repetition teeth will be stored in artificial saliva.

Treatments carried out on the above disclosed specimens are able to provide relative values (i.e. provision of comparative results within the experiments set), but they do not provide reproducible absolute values, because the result depends on the staining process.

Preparative example 2: Preparation of hydrogen peroxide solution

A 35 wt.% H_2O_2 (HP) aqueous solution was adjusted to pH 8.0 with NaOH. That solution was freshly prepared immediately before its use.

Preparative example 3: Preparation of carbamide peroxide solution

A 16 wt.% carbamide peroxide (CP) aqueous solution was adjusted to pH 8.0 with NaOH. The solution was freshly prepared immediately before its use.

Preparative example 4: Preparation of sodium metabisulfite solution for 2^2 factorial design tooth whitening treatments

A solution of sodium metabisulfite 0.47 M was prepared by dissolving 8.9 g of sodium metabisulfite in 100 mL of deionized water. The pH value of this solution was 2.9.

Preparative example 5: Preparation of sodium metabisulfite solution for 2^3 factorial design tooth whitening treatments

Solutions of sodium metabisulfite were prepared in a citric/citrate buffer solution adjusted to pH 5.5 or 6.5, according to the following procedure:

1) Preparing a 0.5 M citric acid solution (CAS)
2) Preparing a 0.5 M sodium citrate solution (SCS)
3) Mixing 7.2 mL of CAS and 42.8 mL of SCS
4) Using the solution in step 3 to solubilize the specific amount of sodium metabisulfite for the tooth whitening test
5) Adjusting pH with NaOH 3M to the desired pH (between 5.5 or 6.5)
6) Adjusting the final volume to 100 mL using deionized water.

For preparing a 0.1 M sodium metabisulfite solution, 1.9 g of sodium metabisulfite was weighed and solubilized in a mixture of 7.2 mL of CAS and 42.8 mL of SCS.

The pH value was adjusted to 5.5 using 3M NaOH. The solution was adjusted to 100 mL with deionized water.

Preparative example 6: Preparation of DPPC liposomes

A 20 mM liposomal solution was prepared according to the following procedure:

1) 0.4 mL of a 100 mM DPPC chloroform solution were pipetted into a round rotary flask.
2) A thin layer was created in the flask, by evaporating all the chloroform while applying rotation to the flask, using a rotary evaporator.
3) 2 mL of water was added to the flask, and then mixed for 15 minutes with an ultrasound bath.

The 20 mM liposomal solution was freshly prepared before the application and stored at 4ºC until its use.

Example 1: Preparation of the tooth whitening composition (sodium metabisulfite 0.47 M partially encapsulated in liposomes)

A 20 mM liposomal solution of sodium metabisulfite 0.47 M was prepared according to the following procedure:

1) 0.4 mL of a 100 mM DPPC chloroform solution were pipetted into a round rotary flask.
2) A thin layer was created in the flask, by evaporating all the chloroform while applying rotation to the flask, using a rotary evaporator.
3) 2 mL of a 0.47 M sodium metabisulfite solution prepared according to the Preparative example 4, was added to the flask, and then mixed for 15 minutes with an ultrasound bath.

The 20 mM liposomal solution of sodium metabisulfite 0.47 M was freshly prepared before the application and stored at 4ºC until its use. pH value was 2.9, and the concentration of DPPC was 20 mM.

Example 2: Preparation of the tooth whitening composition (sodium metabisulfite 0.1 M partially encapsulated in liposomes)

A 20 mM liposomal solution of sodium metabisulfite 0.1 M was prepared according to the following procedure:

1) 0.4 mL of a 100 mM DPPC chloroform solution were pipetted into a round rotary flask.
2) A thin layer was created in the flask, by evaporating all the chloroform while applying rotation to the flask, using a rotary evaporator.
3) 2 mL of a 0.1 M sodium metabisulfite solution prepared according to the Preparative example 4, was added to the flask, and then mixed for 15 minutes with an ultrasound bath.

The 20 mM liposomal solution of sodium metabisulfite 0.1 M was freshly prepared before the application and stored at 4ºC until its use. pH value was 6.0, and the concentration of DPPC was 20 mM.

Example 3: Tooth whitening treatments according to 2^2 factorial design

Tooth whitening trials were carried out with a composition of the invention (Example 1) and three comparative compositions, according to a 2^2 factorial experimental design, which is suitable to quantify the effect of the components and interactions between the factors, i.e. synergistic effect.

Trials were carried out on the specimens following a treatment of 20 minutes, as disclosed in Preparative example 1.

Factors and levels are shown in Table I:

TABLE I

Factor	Low level	High level
Sodium metabisulfite (M)	0	0.47
DPPC (mM)	0	20.0

The response was the increase in the whiteness of the tooth, ΔE, measured as explainer earlier in this Examples section. The experiments were carried out in 8 specimens for each treatment.

Table II shows the experiments:

TABLE II

Example	MBS	DPPC
Comparative 1 (control)	0	0
Comparative 2 (only MBS) (Preparative example 4)	0.47	0
Comparative 3 (only liposomes) (Preparative example 6)	0	20
Example 1 (invention)	0.47	20

The responses were measured at different times. In Table III are shown the results for the increase in the whiteness of the tooth, ΔE ± standard deviation, at 3, 6, 9, 14 and 20 minutes:

TABLE III

Example	3’	6’	9’	14’	20’
Comparative 1 (control)	1.8±1.1	0.9±0.9	2.3±1.4	2.3±1.8	2.4±1.0
Comparative 2 (only MBS)	7.3±1.5	12.9±0.8	14.7±1.8	14.8±1.7	16.2±1.7

Comparative 3 (only liposomes)	1.5±0.9	1.3±0,7	1.1±0.4	1.1±0.9	2.1±1.0
Example 1 (invention)	**14.0±2.4**	16.2±2.3	17.8±2.5	19.0±2.2	19.6±2.1

In Figure 1 can be seen the kinetic of the tooth whitening for these four treatments and in comparison with the treatment using the composition of Preparative example 3, a 16 wt.% aqueous solution carbamide peroxide.

It can be clearly observed that the composition of the invention shows a synergistic effect, in particular at short times. The whiteness' increase of the composition of the invention (Example 1) at 3 minutes was 14, whereas the effect of MBS alone is only 7.3, and the liposomes alone did not produce any whitening effect.

Further, the whitening effect is faster than that of the standard prior art product, 16% aqueous solution of carbamide peroxide. Surprisingly, it was observed that at 3 minutes the whitening effect was substantially similar to that obtained at the end of the experiment, i.e. 20 minutes, showing a fast and high whitening performance, as shown in Figure 1. The efficiency of the composition of the invention allows short treatment times and a significant reduction of secondary effects.

Examples 4 - 11: Tooth whitening treatments according to a 2^3 factorial design

Tooth whitening trials were carried out according to a 2^3 factorial experimental design on the specimens disclosed in Preparative example 1, and following a treatment of 20 minutes, as disclosed earlier. Compositions were prepared following substantially the method disclosed in Example 2 (adapting concentrations of MBS and DPPC, as well as pH value). The experiments were carried out in triplicate.

Factors and levels are shown in Table IV:

TABLE IV

Factor	Low level	High level
Sodium metabisulfite (M)	0.01	0.1
pH	5.5	6.5
DPPC (mM)	2.0	20.0

Responses were the increase in the whiteness of the tooth, ΔE, and the enamel surface hardness of the tooth, expressed in GPa, both parameters measured after 20 minutes of treatment, as explained earlier in this Examples section.

Table V shows the treatments and the results:

TABLE V

Example	MBS	pH	DPPC	ΔE	Hardness (GPa)
4	0.01	5.5	2.0	4.0 ± 1.1	3.5 ± 1.2

5	0.1	5.5	2.0	6.3 ± 1.1	2.8 ± 0.3
6	0.01	6.5	2.0	4.4 ± 0.6	4.0 ± 1.2
7	0.1	6.5	2.0	3.5 ± 0.9	2.9 ± 0.3
8	0.01	5.5	20.0	5.7 ± 1.1	4.0 ± 0.7
9	0.1	5.5	20.0	16.2 ± 2.4	2.4 ± 0.9
10	0.01	6.5	20.0	7.1 ± 2.3	4.1 ± 0.2
11	0.1	6.5	20.0	3.8 ± 0.6	3.3 ± 0.2

The responses were measured at 20 minutes. It was observed an increase of whiteness of the tooth in all treatments.

Modelling the response from these results, an enhanced formula was obtained, as described in example 2.

Example 12: Tooth whitening treatments according to ISO 28399

Tooth whitening treatments according to ISO 28399 were carried out as disclosed in Preparative example 1.

The tested products were:

1) Negative control (water)
2) Citric acid aqueous solution (1 wt.%, pH 3.9), positive control
3) 16 wt.% carbamide peroxide solution (Preparative example 3)
4) 35 wt.% hydrogen peroxide solution (Preparative example 2)
5) 0.1 M sodium metabisulfite liposome solution (Example 2)

Figure 2 shows the results obtained regarding the increase in the whiteness for those products, represented as ΔE at 20 minutes of treatment. The effectiveness of the product of the invention is better than the prior art treatments (carbamide peroxide and hydrogen peroxide) after the first and second treatment, and comparable to HP and better than CP after the third treatment and after one month of follow up.

The roughness of the teeth after the treatments was measured according to ISO 28399. The increase in roughness is represented as ΔRa in Figure 3 for each one of the treatments. It can be observed that the composition according to the invention showed an effect comparable to that of citric acid, used as positive control, being consequently suitable for dental use.

CLAIMS

1.- A method for preparing a tooth whitening composition, characterized in that it comprises:

1) the step of mixing at least one membrane-forming lipid in an organic solvent in a vessel, and

either

2a) dispersing the mixture obtained in step 1) in an aqueous solution comprising a reducing agent, and

3a) removing the organic solvent to obtain a composition comprising liposomes comprising the solution of the reducing agent,

or

2b) removing the organic solvent of the mixture obtained in step 1) to obtain a layer of the membrane-forming lipid on the inner surface of the vessel, and

3b) adding an aqueous solution comprising a reducing agent to the layer of step 2b) to obtain a composition comprising liposomes comprising the solution of the reducing agent.

2.- The method according to claim 1, characterized in that the organic solvent is selected from the group comprising chloroform, dichloromethane, methanol, ethanol, and mixtures thereof.

3.- The method according to claim 1 or 2, characterized in that it further comprises a step of treating mechanically the liposomes obtained in step 1) to reduce their size.

4.- The method according to any one of claims 1 to 3, characterized in that the at least one membrane-forming lipid comprises a phospholipid.

5.- The method according to claim 4, characterized in that the phospholipid is selected from the group consisting of a natural phospholipid, a synthetic phospholipid, and combinations thereof.

6.- The method according to claim 5, characterized in that the natural phospholipid is selected from phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), hydrogenated soy PC (HSPC), sphingomyelin, phosphatidylglycerol (PG), and mixtures thereof.

7.- The method according to claim 5, characterized in that the synthetic phospholipid is selected from derivatives of phosphocholine, derivatives of phosphoglycerol, derivatives of phosphatidic acid, derivatives of phosphoethanolamine, derivatives of phosphoserine, PEG derivatives of phospholipid, and mixtures thereof.

8.- The method according to any one of claims 1 to 7, characterized in that the concentration of the at least one membrane-forming lipid in the composition comprising liposomes obtained in step 3a) or step 3b) is comprised between 1 mM and 100 mM.

9.- The method according to any one of claims 1 to 8, characterized in that the reducing agent is selected from the group of dithionite, metabisulfite, sulphite, bisulfite, and alkaline metal salts thereof.

10.- The method according to any one of claims 1 to 9, characterized in that the concentration of the reducing agent in the aqueous solution of step 2a) or step 2b) of the method is comprised between 0.01 M to 0.5 M.

11.- The method according to any one of claims 1 to 10, characterized in that the aqueous solution has the pH value comprised between 2 and 8.

12.- The method according to any one of claims 1 to 11, characterized in that the aqueous solution of reducing agent comprises a buffer system.

13.- A tooth whitening composition obtainable according to the process of any one of claims 1 to 12.

14.- The tooth whitening composition according to claim 13, characterized in that it comprises an aqueous solution comprising a reducing agent, and liposomes, comprising at least one membrane-forming lipid and an aqueous solution comprising a reducing agent.

15.- Non-therapeutic cosmetic use of composition according to claim 13 or 14 for tooth whitening.

FIGURES

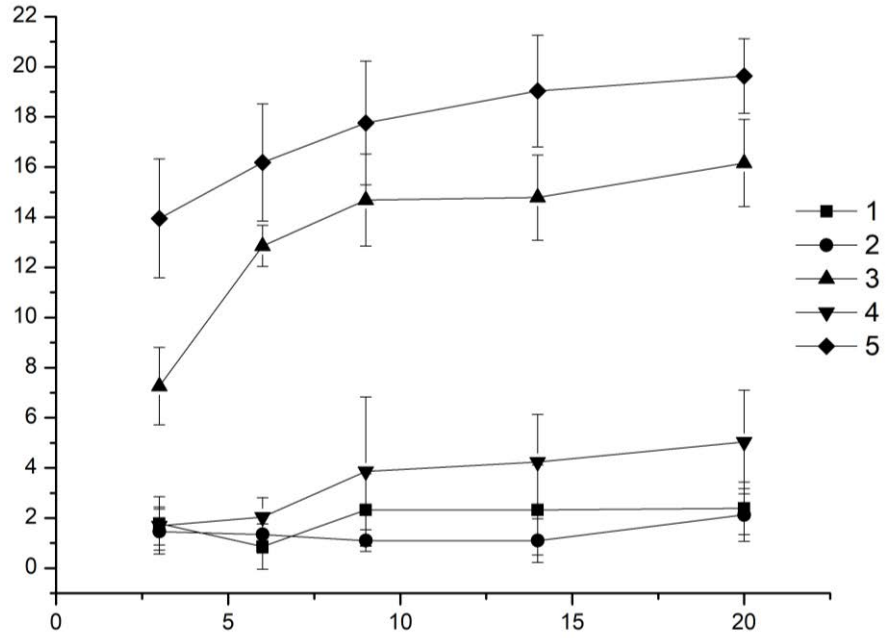

Figure 1

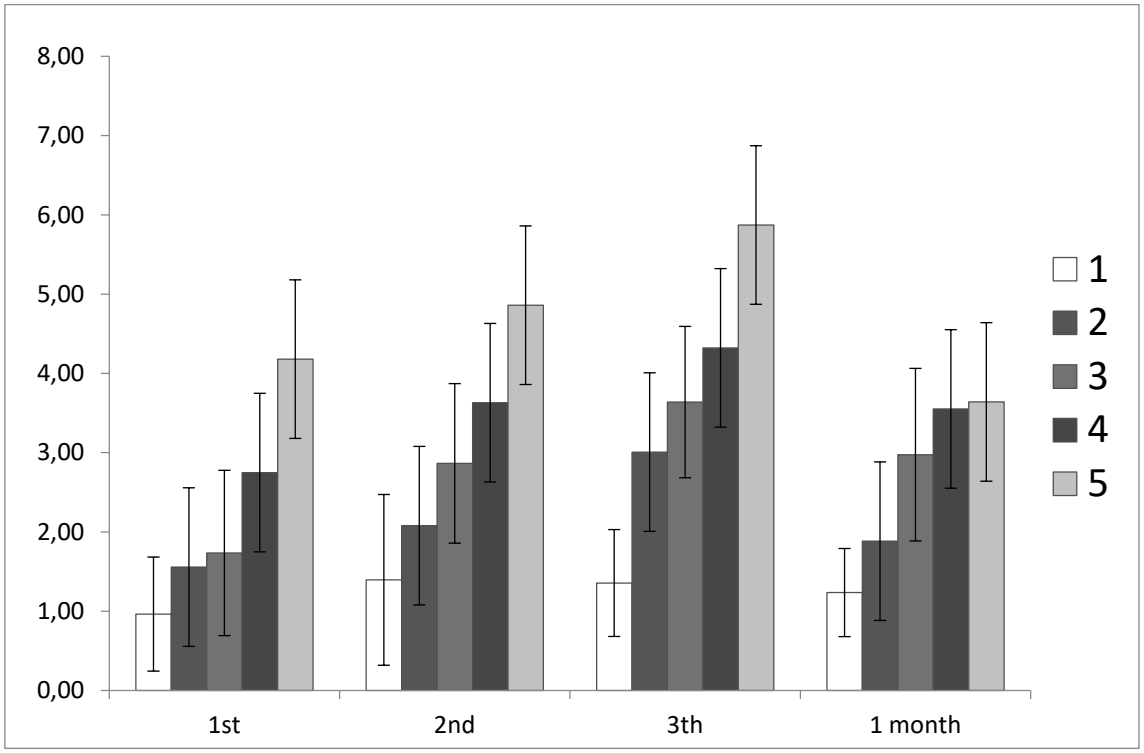

Figure 2

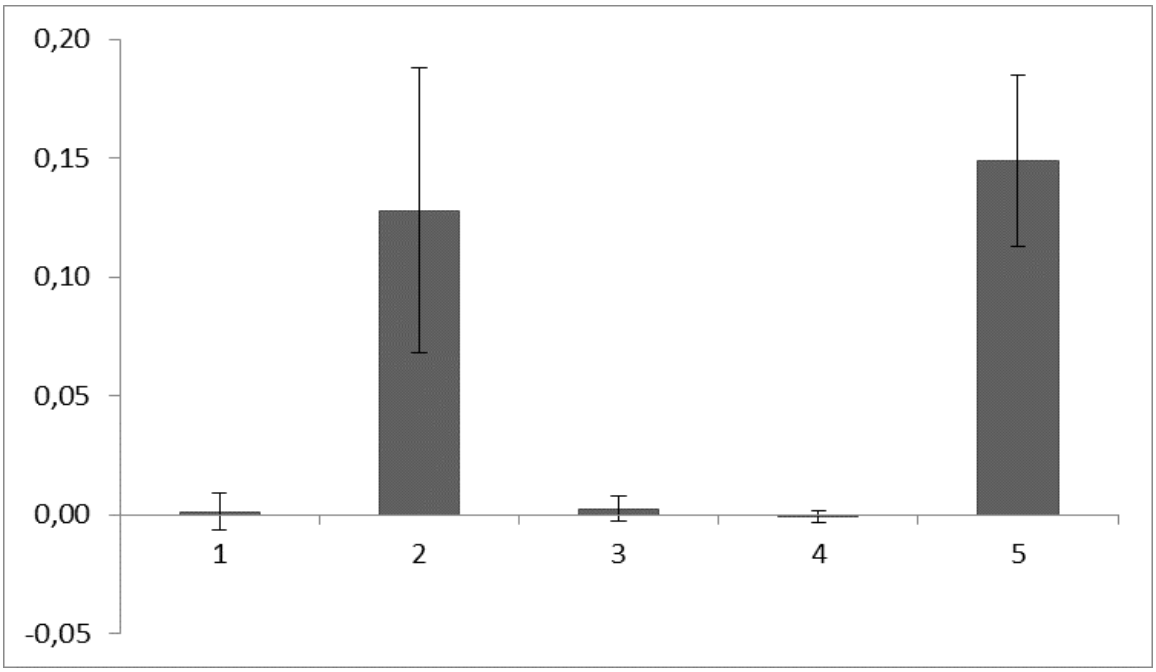

Figure 3

SUMMARY

The present invention relates to a tooth whitening composition, which comprises a reducing agent partially encapsulated in liposomes. It relates also to a method for preparing that tooth whitening composition and to the non-therapeutic cosmetic use of that composition for tooth whitening.

RESUMEN

La presente invención se refiere a una composición para el blanqueo de dientes, que comprende un reductor parcialmente encapsulado en liposomas. También se refiere a un procedimiento para obtener dicha composición, y al uso no terapéutico cosmético de la misma para el blanqueo de dientes.