Surface Reactivity of Tooth Enamel with Dyes, Oxidizing Agents and Magnesium Ions and Its Effect on Tooth Color

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December 2012

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Dental Sciences.

DEDICATED TO
My parents
for their endless support and love

"Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us."

Marianne Williamson

Abstract

Enamel, the outer protective layer of human teeth, is constantly interacting with its surrounding harsh environment. These interactions play a major role in many mechanisms that affect the dental health such as tooth caries, tooth discoloration and plaque formation. Accordingly, most treatments used to maintain and/or restore dental health focus first on the enamel surface. Even though most of the enamel structure has been thoroughly studied, many of the enamel surface features, and the chemical reaction that occur on it, are not well understood.

Extrinsic tooth discoloration poses a major problem to many patients and dentists. Most causes of extrinsic tooth staining have been well identified and are usually treated with hydrogen peroxide. However, the mechanism of extrinsic tooth discoloration and tooth bleaching using peroxide oxidizers are not fully understood. It is unknown why certain staining molecules attach to the enamel surface, whether peroxide radicals make teeth whiter by removing these stains, and why sometimes hydrogen peroxide does not work. Also, treating darkened teeth with peroxide agents has its limitations and disadvantages. This has pushed us to look for better and less harmful whitening agents. Recent studies have revealed that there is a correlation between crystallographic characteristics of enamel and its physical properties, such as tooth shade and microhardness. This discovery predicts that tooth properties can be changed by modifying its crystallographic structure. Since magnesium ions are known to react with synthetic hydroxyapatite and affect its crystallographic properties, it is possible that magnesium ions could react with dental enamel and induce changes in dental enamel crystallographic, optical and mechanical properties.

To address the aforementioned research problems, we divided this thesis into three parts. The first part of this thesis investigates how teeth get stained, the second part investigates how hydrogen peroxide whitens teeth, and the subject of the third and last part of this thesis is to find an alternative way to whiten darkened teeth using magnesium ions.

After obtaining the McGill University Health Center Ethical Committee approval and the signed informed consent from the patients, two hundred and four sound teeth were collected from adult patients attending McGill Undergraduate Dental Clinic for tooth extractions. In the first part of our study, teeth specimens were treated with different dyes to assess how teeth get stained. In the second part, specimens were treated with de-proteinizing, de-mineralizing and oxidizing agents to assess how bleaching works. In the third part, teeth specimens were treated with solutions saturated with magnesium ions to investigate an alternative method to whiten teeth. In each of the three parts of this thesis, tooth shade and enamel physicochemical properties were assessed before and after the chemical treatments using shade spectrophotometry, X-ray photoelectron spectroscopy (XPS), energy dispersive x-ray spectroscopy (EDS), Raman spectroscopy, x-ray diffraction (XRD) and Vickers microhardness test (VMH). The data obtained was analyzed using parametric and non-parametric statistical tests, and statistical significance was set at $P < 0.05$.

In the first part, our results indicated that the outer surface of tooth enamel is covered by a carbon rich layer that is susceptible to be stained with anionic dyes (i.e. tannins present in coffee, tea and wine). The second part showed that bleaching did not influence the shade parameters in de-proteinized teeth, but increased the lightness in de-mineralized teeth. This indicates that hydrogen peroxide oxidizes the organic material in dental

enamel. In the third part, we showed that magnesium ions reacted with enamel surface resulting in a significant decrease in the size of enamel hydroxyapatite nanocrystals that induced significant changes in tooth shade lightness and enamel hardness.

Several significant conclusions and achievements are presented in this thesis. We demonstrated the presence of a carbon rich layer on the surface of enamel that contains relatively high amounts of calcium. This layer might explain the high staining ability of certain types of anionic staining agents that are among the most common causes of external tooth discoloration. Moreover, we showed that hydrogen peroxide does not induce significant changes in tooth enamel organic and inorganic relative contents, and it whitens teeth just by oxidizing their organic matrix. This finding is of great clinical significance since it explains the mechanism of tooth bleaching and the reasons behind the limited predictability of the treatment outcomes. Finally, we showed for the first time that magnesium ions can react with tooth enamel and induce a reduction in the size of enamel hydroxyapatite nanocrystals. This change in crystallography affected the enamel optical and mechanical properties; making the enamel harder and whiter. This is a new method that can be exploited to whiten teeth without using peroxide-based bleaching agents. We concluded that crystallographic ultrastructure plays a key role in defining the tooth enamel properties which can be tailored through ionic substitution for improvement of optical and mechanical properties without causing the possible negative effects of peroxide agents.

Résumé

L’émail est la couche protectrice externe des dents humaines en constante interaction avec son environnement hostile. Ces interactions jouent un rôle important dans de nombreux mécanismes qui influencent la santé dentaire, tels que les caries dentaire, la coloration dentaire et la formation de plaque. Par conséquence, la majorité des traitements utilisés pour maintenir et/ou restaurer la santé dentaire se concentre principalement sur la surface de l'émail. Tandis que la structure de l'émail a été largement étudiée, les caractéristiques de sa surface, ainsi que les réactions chimiques se produisant à son niveau ont rarement été décrites.

La coloration extrinsèque des dents pose un problème majeur pour certains patients et dentistes. La majorité des causes de colorations extrinsèques des dents ont été bien identifiées et sont habituellement traitées avec du peroxyde d'hydrogène. Cependant, le mécanisme de coloration extrinsèque des dents et de blanchiment des dents à l'aide des oxydants du peroxyde ne sont pas entièrement décrits. Les raisons pour lesquelles certaines molécules colorantes s’attachent à la surface de l'émail, comment les radicaux de peroxyde peuvent blanchir les dents en enlevant ces colorations, ainsi que la raison pour laquelle le peroxyde d'hydrogène n’est pas optimal en terme de blanchiment des dents, restent toujours méconnus. De plus, le traitement avec des agents de peroxyde de dents noircies présente certaines limitations et inconvénients. Ce qui nous a motivés à intensifier les recherches pour de meilleurs agents de blanchiment avec le moins d’effets nuisibles.

Des études récentes ont montré qu'il existait une corrélation entre les caractéristiques cristallographiques de l'émail et ses propriétés physiques, comme la couleur de la dent et la microdureté. Cette découverte prédit que les propriétés de la dent peuvent être changées par la modification de sa structure cristallographique. Comme les ions du magnésium sont connus de leur capacité de réagir avec l'hydroxyapatite synthétique et d'affecter ses propriétés cristallographiques, il est possible que ces ions puissent réagir avec l'émail dentaire et induire des changements cristallographiques à son niveau ainsi que des changements de ses propriétés optiques et mécaniques.

Afin d'adresser les problèmes de recherche mentionnés ci-dessus, nous avons divisé cette thèse en trois parties. La première partie de la thèse étudie le mécanisme par lequel les dents deviennent colorées, la deuxième partie examine comment le peroxyde d'hydrogène blanchit les dents et enfin pour la troisième et dernière partie de cette thèse est de trouver une voie alternative de blanchiment des dents noircies à l'aide d'ions magnésium.

Après avoir obtenu l'approbation du comité d'éthique du Centre universitaire de santé McGill et le consentement éclairé signé par les patients, 204 dents saines ont été recueillies auprès des patients adultes qui fréquentent la clinique dentaire du premier cycle de l'Université McGill pour des extractions dentaires. Dans la première partie de notre étude, les échantillons ont été traités avec des différents colorants pour évaluer la décoloration des dents. Dans la deuxième partie, les échantillons ont été traités avec des agents pour enlever le contenu protéique et minéral ainsi que des agents oxydants afin d'évaluer le mécanisme de blanchiment. Dans la troisième partie, les échantillons des dents ont été traités avec des solutions saturées en ions du magnésium pour étudier une méthode alternative pour blanchir les dents. Dans chacune des trois parties de cette thèse,

la teinte des dents et les propriétés physico-chimiques de l'émail ont été évaluées avant et après les traitements chimiques par ombre spectrophotométrie (shade spectrophotometry), rayon-x photoélectrons spectroscopie (X-ray photoelectron spectroscopy , XPS), microscopie électronique à balayage microscope à dispersion d'énergie des rayons X spectroscopie (scanning electron microscope energy dispersive x-ray spectroscopy SEM-EDS), spectroscopie Raman, diffraction des rayons X (x-ray diffraction , XRD) et de test de microdureté Vickers (Vickers microhardness test , VMH). Les données obtenues ont été analysées à l'aide des analyses statistiques paramétriques et non paramétriques avec un niveau de signification statistique $p < 0,05$.

Dans la première partie, nos résultats indiquent que la surface extérieure de l'émail des dents est couverte par une couche riche en carbone qui est susceptible d'être teinté avec des colorants anioniques (p. ex les teintes présentes dans le café, le thé et le vin). La deuxième partie a montré que le blanchiment n'a pas influencé les paramètres de couleur des dents sans contenu protéique, mais il a augmenté la luminosité des dents sans contenu minéral. Ceci indique que le peroxyde d'hydrogène oxyde la matière organique dans l'émail dentaire. Dans la troisième partie, nous avons montré que les ions du magnésium ont réagi avec la surface de l'émail, ainsi qu'ils ont entraîné une diminution significative de la taille des nano-cristaux d'hydroxyapatite et ont induit des changements significatifs dans la luminosité des dents et la dureté de l'émail.

Plusieurs conclusions et réalisations importantes sont présentées dans cette thèse. Nous avons montré la présence d'une couche riche en carbone sur la surface de l'émail qui contient des quantités relativement élevées de calcium. Cette couche pourrait expliquer la capacité colorante élevée de certains types d'agents anioniques, qui sont parmi les causes

les plus communes de la coloration extrinsèque des dents. De plus, nous avons montré que le peroxyde d'hydrogène ne provoque pas de changements significatifs dans le contenu organique et inorganique relatif à l'émail des dents, ainsi qu'il blanchit les dents uniquement en oxydant leur matrice organique. Cette conclusion est d'une valeur clinique importante car elle explique le mécanisme de blanchiment des dents et les raisons de la prévisibilité limitée des résultats du traitement. Enfin, nous avons montré pour la première fois que les ions du magnésium puissent réagir avec l'émail des dents et provoquent une réduction de la taille des nano-cristaux d'hydroxyapatite. Ce changement dans la cristallographie affecte les propriétés optiques et mécaniques de l'émail et le rend plus dur et plus blanc. Cette nouvelle méthode peut être exploitée pour blanchir les dents sans avoir recours à l'utilisation d'agents de blanchiment à base du peroxyde. Nous avons conclu que l'ultrastructure cristallographique joue un rôle clé dans la définition des propriétés de l'émail des dents, ce qui peut être ajusté par la substitution ionique afin d'améliorer les propriétés optiques et mécaniques et sans causer des effets potentiels nocifs des agents basés sur le peroxyde.

Acknowledgements

First and foremost, my greatest appreciation goes to my supervisor Dr. Faleh Tamimi who has been a mentor and like a big brother. His insightful guidance and good source of advice shaped my academic development throughout my master studies. The achievements of this thesis would not have been possible without his tireless support, continuous motivation and vast knowledge. I would like also to express my sincerest gratitude to my co-supervisor Dr. Marta Cerruti for always having her door open for my questions and concerns. Dr. Cerruti provided me her consistent support and taught me that we all make mistakes and that we should learn from these mistakes.

Thanks to my lab members (Hazem Eimar, Omar Alageel, Khadijeh Al-Abedalla, Elnaz Ghadimi, Xixi Wu) for their help and support through this project and for always having someone around to share my thoughts and ideas. A special gratitude goes to Hazem Eimar and his family, Zeeshan Sheikh, Laura Boghian and Zaher Jabbour for being like a close family in Montreal. Moreover, I would like to thank Zaher Jabbour for helping me with the French translation.

My thanks also extend to Dr. Rubens Albuquerque, Dr. Samer Abi Nader and Mr. Michael McHugh for their technical support and helpful feedbacks. Ms. Linda Harrison deserves a special appreciation for lending me lab equipment and materials at a moment's notice. I would also like to thank the biointerface lab members for helping with the preliminary experiments and showing me how to work safely in the lab.

I am grateful for the encouragement and support from my whole family and friends. My parents and brother encouraged my endeavours throughout my whole life in every way possible. They always helped me to stand up on my feet again whenever I encountered a setback or a fall and constantly reminded me that I should always believe in myself. Whatever I write would not be enough to express my eternal indebtedness to them.

Last and by no means least, I give my deepest gratitude to my loving wife, Mai, for her endless love and care. Thank you for always standing beside me in the bad and good moments and for accepting me the way I am. Thank you for adding a meaning to my journey and making it worthwhile.

Author Contribution

Some sections of this thesis include parts of an already published article. Permission has been taken from the Journal of Dentistry and co-authors to reform the article and include it within this master thesis (see Appendix II and III). A brief summary of the work and a statement of the involvement of each co-author are as follows: Preparation and execution of the experiments, in particular Raman spectroscopy and data analysis have been performed by the candidate; Dr. Hazem Eimar and Ryan Siciliano also performed parts of the experiments and data analysis; the consulting expertise and technical advice of Dr. Samer Abi Nader, Dr. Wala M. Amin, Dr. Pedro-Pablo Martinez and Dr. Alicia Celemin have been most helpful during the project; Dr. Marta Cerruti and Dr. Faleh Tamimi planned and supervised the research project.

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Chapter 1: Introduction

3.1. Thesis Outline

This thesis is prepared in a traditional format and is divided into ten chapters. Chapter 1 is a brief general introduction and research rationale, whereas Chapter 2 entails a literature review in which the enamel structure, composition and associated properties are reviewed. Current knowledge about tooth discoloration and peroxide based bleaching agents are also discussed. Chapter 4 describes the materials and methods used in this project. Chapter 5, 6 and 7 present the three studies that were designed to test our hypotheses: Chapter 5 explores how teeth get stained; Chapter 6 investigates how hydrogen peroxide whitens teeth; Chapter 7 presents an alternative method to whiten teeth using magnesium ions. Chapter 8 summarizes the overall findings of the three studies and draws general conclusions. Chapter 9 encompasses the comprehensive list of literature references cited in this thesis.

3.2. Research Rationale

The composition of the outer layer of a material is usually very different from the bulk composition [1, 2], and dictates its behavior with the surrounding environment determining important characteristics such as: adhesion, wettability, chemical activity, corrosion resistance, abrasion resistance [1-6]. Dental enamel is the outer protective layer that envelopes the tooth and it is the first layer that comes into contact with the surrounding oral environment. Enamel surface is constantly interacting with the various species of molecules and ions found in saliva, ingested food and beverages and even

substances used to maintain the tooth healthy such as tooth pastes, mouth rinses or other likely treatments applied by the dentist.

Interestingly, interaction of enamel surface with the surrounding environment varies among people. It has been observed that some individuals are more likely to develop tooth discolorations caused by ingested food and beverages [7, 8]. In addition, some patients respond better to whitening agents and others respond better to treatment with fluoride [9-11].

Tooth discoloration (or staining) is a problematic subject to both patients and dentists since some stains are more difficult to remove and require more invasive restorative treatments [7, 8]. External tooth staining mechanism involves the interaction of enamel surface with certain molecules present in the ingested food and drinks or present in the breathed air through the mouth, which later on attaches to the tooth surface and are difficult to remove. However the exact nature of this interaction and why some individuals develop stains easier than others is not fully understood.

Dentists have been using bleaching agents based on peroxide molecules to remove stains and lighten dark teeth [12-15]. Despite their wide spread usage, the effect of these bleaching agents on tooth shade is often temporary and lacks stability [16-19]. In addition, there were some reported cases of increased tendency to develop stains immediately after bleaching. Bleaching agents show variable efficacy among people for reasons still unknown and the mechanism of tooth bleaching and the interaction between the peroxide molecules with tooth enamel surface is not fully understood. A better understanding of the underlying mechanism of tooth discoloration and tooth bleaching is

fundamental in order to implement better preventive measures and treatment approaches that maintain the teeth in a healthy bright condition with the least possible harmful effect.

This study focused on understanding enamel surface interactions with the surrounding environment, more specifically with stains, hydrogen peroxide molecules and explores alternative approaches to treat tooth discoloration. Accordingly, we investigated the underlying mechanism of tooth discoloration and bleaching in order to provide possible explanations for their variable effects and outcomes among people; we also explored a new alternative method to lighten the dark teeth through ionic interactions on the enamel surface.

Chapter 2: Background and Literature Review

2. Human Teeth

There are usually 32 teeth in the adult human which are separated equally into two arches: the upper arch (maxilla) and the lower arch (mandible). Depending on their position in the arch, teeth are divided into either anterior or posterior teeth. Normally in each arch, the anterior teeth consists of incisors and canines which aid in cutting and shearing the food, while the posterior consists of premolars and molars which aid in the mastication of food [20, 21]. Each human tooth is anatomically separated into a crown and one or more roots. The anatomical crown is composed of an inner soft tissue (pulp chamber) enveloped with a relatively harder tissue called dentine that is further surrounded by one of the hardest tissues in the human body known as enamel [20-23]. The junction between the pulp chamber and dentine is called dentinopulpal junction (DPJ), whereas the junction between the dentine and enamel is known as dentino-enamel junction (DEJ) [20, 21].

2.1. Human Tooth Enamel

Dental enamel is the outer protective layer that aids the tooth in bearing the masticatory forces and endure the harsh oral environment [24]. Mature dental enamel is a complex structure mainly made of inorganic minerals (96% w/w) and a small fraction of organic material and water (4% w/w) and contains no cells and no collagen [25-28]. The enamel structure is divided it into highly organized seven different levels ranging from nanoscale to microscale: hydroxyapatite nanocrystals (*~15-30nm*), nanofibrils (*~30–40nm*), fibrils

(*~80-130nm*), fibres (*~80nm*), prisms (*~1μm*), key-hole shaped structures *(~6-8 μm)* and prism bands *(~100 μm)* [29].

2.1.1. Hierarchical Structure of Human Enamel

Hydroxyapatite crystals, the basic structural blocks of human tooth enamel, elongate along a preferential axis (c-axis) and aggregate to form nanofibrils [29]. These fibrils are unique structures in human tooth enamel that always run lengthways with respect to each other and aggregate into thicker fibrils. These fibrils are well packed together and form enamel fibers that also aggregate into bigger structure called enamel rods or prisms [29]. The highest level of organization are prisms' bands that run parallel to each other and are arranged in different patterns along the entire thickness of enamel [29, 30]. In a longitudinal sectional view, prism bands in the outer third of enamel "radial enamel" are oriented radially and intercept the outer enamel surface perpendicularly. These bands are responsible for the Retzius lines phenomena [29]. On the other hand, in the inner regions the prism bands are arranged in a more perpendicular pattern and are responsible for the Hunter-Shreger bands phenomena [29, 30]. When cut cross-sectionally, prism bands appear to be made of well packed key-hole shaped structures (~6-8 μm in size) that encompass multiple packed prisms and inter-prisms separated by an organic rich sheath containing no crystallites [29]. Enamel prisms project perpendicularly from the dentino-enamel junction (DEJ) to the surface of enamel and run somehow parallel to each other [31]. The long c-axis of crystallites forming the prism is parallel to the long axis of the prisms, while crystallites around each prism tilt about 40^{o} to 65^{o} relative to the direction of the prism and form inter-prisms [32].

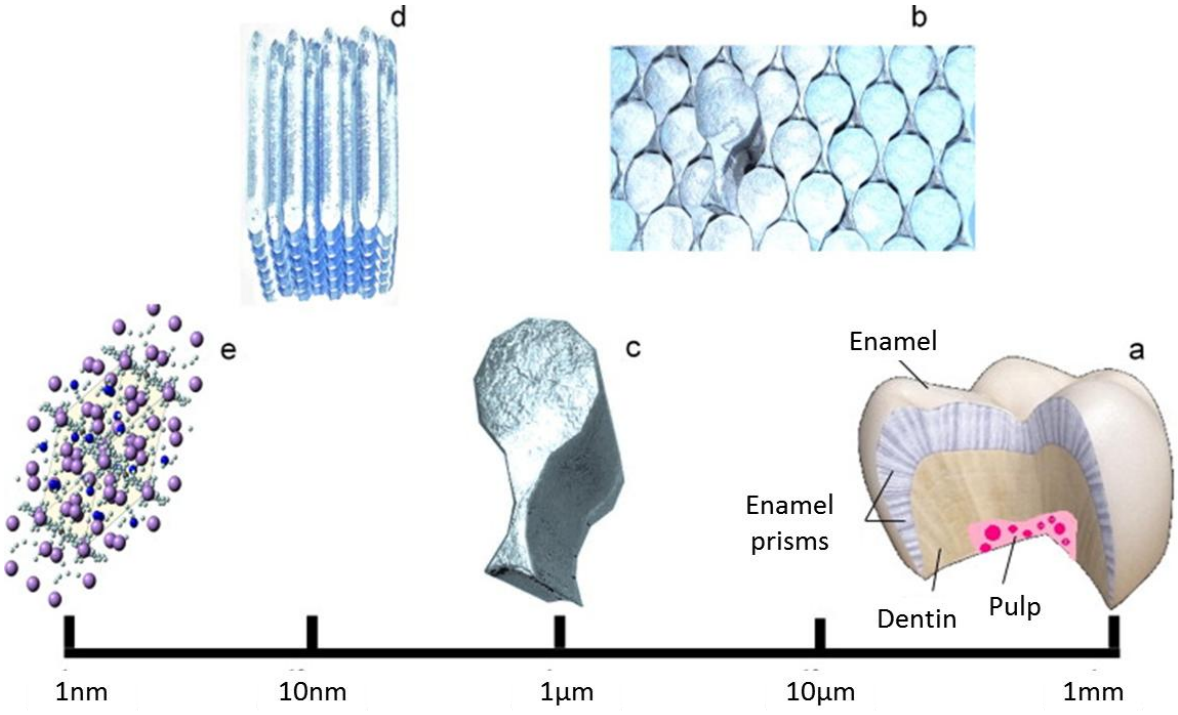

Fig. 1.1 - Computer generated images of a posterior tooth showing the hierarchical structure of tooth enamel on the micro- to nanoscale levels. (a) Enamel is the external tooth layer protecting the softer underlying structures (dentin and pulp). (b) Enamel consists of prisms bands, "keyhole"-shaped structures, packed together. (c) A single "keyhole"-shaped structure is made up of enamel prisms. (d) Each enamel prism is composed of multiple CHA nanocrystals. (e) The atomic composition of a single CAP nanocrystal. (Reproduced with permission from Elsevier)

2.1.2. Organic Component of Human Enamel

The organic component of mature enamel consists of 60% proteins and 40% lipids [33]. The enamel proteins are non-collagenous phosphorylated proteins: amelogenin, enamelin, amelin, ameloblasin and proteinases [34-38]. Immature enamel contains higher amounts of proteins which have an important role in enamel development [39]. As the enamel matures, the total amount of proteins decreases and the organic portions of the mature enamel become concentrated mainly at the prism sheath and to lesser amounts between the crystallites [39, 40]. Due to the semipermeable nature of the organic part of enamel,

it has been assumed that it acts as a channel that allows small molecules such as bleaching agents to penetrate enamel [41].

2.1.3. Mineral Component of Human Enamel

Enamel mineral is composed of well packed calcium-phosphate apatite nanocrystals. The mineral phase of enamel is compatible to that of pure stoichiometric hydroxyapatite (HA) with the general formula $Ca_{10}(PO_4)_6(OH)_2$ [42]. Hydroxyapatite crystals are hexagonal structures that belong to the space group $P6_3m$ and have two cell lattice parameters: a (= b) = 0.942 A^o, and c = 0.688 A^o. The 10 calcium ions (Ca^{+2}) are located in two distinct non-equivalent crystallographic sites in the apatite structure [42, 43] (Fig.1.1) : four of these calcium ions are located at a position known as Ca(1) and are aligned in a columnar manner surrounded by nine oxygen (O) atoms that belong to six phosphate tetrahedral $(PO_4)^{3-}$. The other six Ca ions are located at a position known as Ca(2) and are arranged in two sets of triangles surrounded by six O atoms that belong to four $(PO_4)^{3-}$ and one O atom that belongs to a hydroxyl ion (OH^-). The two triangles are arranged in a way that they form a channel along the c-axis called (anion channel) and are occupied by two OH^- ions per crystal unit.

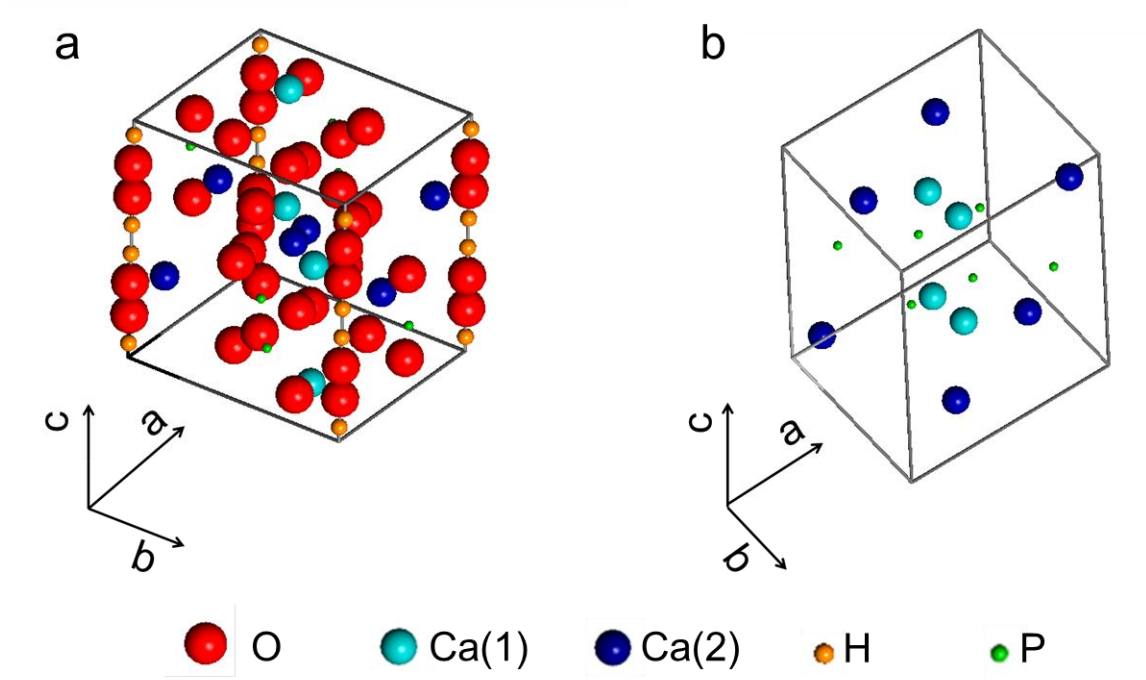

Fig. 1.2 - a) Scheme of the hydroxyapatite (HA) unit cell structure showing the following atoms: oxygen (O), columnar calcium (Ca1), triangular (Ca2), hydrogen (H), phosphorus (P); b) scheme of the HA unit cell illustrating the two distinct crystallographic sites of calcium atoms: columnar (Ca1) and triangular (Ca2). (The graphs were generated by the candidate using Xtaldraw software (version: Aug 1, 2003) and the online American Mineralogist Crystal Structure Database)

Similarly to pure hydroxyapatite, dental enamel crystals are also hexagonal structures but with larger cell lattice parameters (a = b = 9.433 A^o, 6.896 A^o) [42], and contain relatively large amounts of carbonate ions $(CO_3)^{2-}$ and small concentrations of incorporated trace element ions such as magnesium (Mg^{2+}), fluoride(F^{1-}), chloride (Cl^{1-}), Sodium (Na^{1+}) and potassium (K^{1+}) [44] . Therefore, enamel crystals are better described as carbonated hydroxyapatite (CHA) crystals [45].

2.1.4. Ionic Substitutions in the Enamel Apatite Crystal

Enamel apatite, similarly to other biological apatites, is characterized by being non-stoichiometric and poor crystalline material which enables enamel to accommodate certain chemical (or ionic) substitution [43, 46-50]. The crystal structure of apatite has several sites where ionic exchange can occur allowing the incorporation of many different ions [51]. However, the allowed ionic substitutions must fulfill certain criteria; they must maintain the overall charge balance of the crystal lattice and the substituting ions should possess certain geometric parameters that allows them to fit in the crystal lattice [51]. Among the possible ionic substituting in apatite crystal are $(CO_3)^{2-}$, F^{1-}, Cl^{1-}, Na^{1+}, iron ions (Fe^{2+}) and Mg^{2+} [29, 51-53] . These relatively small ionic substitutions in the apatite structure change the minerals characteristics and have key effects on apatite's physical and chemical properties, such as hardness, solubility, thermal stability [43, 46-50].

Carbonate and fluoride

Dental enamel has a relatively high carbonate $(CO_3)^{2-}$ content of about 2-5 wt. % [54, 55]. Carbonate is relatively a large anion and approximately 90-95% of the $(CO_3)^{2-}$ replaces phosphate tetrahedral $(PO_4)^{3-}$ (type B substitution), while the remaining remaining 5-10% replaces OH^{1-} (type A substitution) [54, 55]. It has been reported that increased carbonate content in dental enamel leads to increased solubility and caries susceptibility [56], decreased microhardness [57] and crystallinity [54, 57]. On the other hand, fluoride replaces OH^- and is known to have the best fit in the anion channel site [58]. Fluoride incorporation in dental enamel tends to increase its resistance to

demineralization [59] and restores the hardness of enamel to its initial values after being decreased by the application of bleaching agents [60, 61].

Magnesium

Magnesium (Mg) is one of the most abundant ions in mineralized tissues of the human body and it constitutes about 0.72, 1.23 and 0.44 wt. % of bone, dentine and enamel, respectively [43, 62]. In mature human enamel, magnesium concentrations vary from 0.4% near the dentino-enamel junction (DEJ) and goes down to about 0.1% near the enamel surface [63]. With increasing age, magnesium levels tend to drop in the body stores and Mg deficiency has a major impact on mineralized tissues; it causes defects in their metabolism, growth and physical properties [64-66]. Moreover, the content of Mg ions in the enamel fluid surrounding the forming crystals plays an important role during tooth enamel development [67] and the degree of Mg ions uptake regulates enamel mineralization [68-70].

The effect of Mg ions has been extensively investigated in synthetic hydroxyapatites prepared by precipitation, hydrolysis or wet-chemical methods [43, 48, 49, 62, 71-77]. Even though the amount of Mg ions that can be incorporated in synthetic apatites is small, it induces changes in the crystallographic structure and influences the crystal size, crystallinity, cell lattice parameters and stability of the apatite structure [78, 79]. When Mg content increases in synthetic apatites, the crystallites become smaller and more irregular [43]. It has been shown that Mg ions compete with Ca ions on the growth sites of synthetic apatite crystals and inhibit further crystal growth [76]. Similar observations

were reported in animal studies where Mg ions were added to immature tooth enamel and inhibited the crystal growth rendering it less mineralized and less crystalline [70].

2.1.5. Dental Enamel Hardness

Hardness of a material is a property used to describe its resistance to plastic deformation, scratching or indentation. Dental enamel is considered the hardest biological structure in the human body and this property allows it to endure the harsh oral environment [24]. Tooth enamel hardness has been widely investigated in the literature and has been utilized as an indicator to assess other tooth properties, such as enamel demineralization, caries susceptibility, wear resistance and crack propagation [80-85]. It has been observed that teeth with lower hardness profile are more susceptible to demineralization and more prone to develop enamel caries and cracks [80-85]. Tooth enamel hardness ranges from approximately 3 GPa to 5 GPa [57, 86]. It varies between different individuals and between different teeth within the same individual. It has been postulated that age, prism orientation, crystal size, crystallinity, organic matrix content, water content and inorganic carbonate content might have an effect on enamel hardness as well [24, 61, 80, 87-89].

2.2. Human Tooth Color

Teeth play a major role in your overall facial appearance which affects individual's self-confidence and social life [8, 10]. Teeth appearance depends on their shape, alignment in the arch and color. Therefore, understanding the tooth color is essential for many aspects in dental practice, in particular importance in restorative and cosmetic dentistry.

2.2.1. Perception of Color

The perception of color is a complex phenomenon which is determined by a combination of three factors: light source, the viewed object and the observer [90-93]. Depending on the wavelength of the light source and object properties, four phenomena occur when light hits an object, which: i) specular reflection at the object surface, ii) diffuse reflection at the object surface, iii) absorption and scattering of light within the object and iv) transmission through the object. The color perceived by the observers eye is a combinations of the light reflected and scattered by the object.

2.2.2. Color Space Systems

The color of an object can be described using a color space system. Among the most widely and routinely used color systems in dental research and clinical applications are the Munsell's color system and the Commission International de l'Eclairage (CIE) Lab color system [10, 94].

2.2.2.1. CIE Lab Color Space System

This system describes the object's shades as perceived by the observer's eye and is based on three dimensional axes: (L^*, a^*, b^*) [10, 90, 95]. The L^* value represents the lightness of the object where it is quantified on black-to-white scale (perfect black is zero, perfect white is 100). The a^* value quantifies the shade along a red-to-green scale (red being positive ($+a^*$), while green being ($-a^*$)). The b* value quantifies the shade along a yellow-to-blue scale (yellow being positive (+b*), while blue being negative ($–b^*$)).

2.2.2.2. Munsell's Color Space System

Munsell's color space system describes the object's shade based on three dimensional parameters: "Lightness", "Chroma" and "Hue". Lightness (or value) describes color brightness along the white-black axis colour, Chroma is the degree of colour saturation and describes the strength of a colour, while Hue is a descriptive term used to distinguish between colours (purple, blue, green, yellow and red) [8, 96, 97]. Most of the tooth shade guides used in dental practices are based on the Munsell's system [96, 98, 99].

2.2.3. Tooth Shade Measurement Methods

A wide range of tooth shade measurement methods has been in clinical practice and dental research and they can be divided into subjective (visual) and objective (instrumental) methods [10, 92].

Subjective Method

This is the most widely used method in the dental clinical practice where visual examination of the tooth shade is compared with a specific shade guide [95, 100, 101]. Visual examination requires trained and experienced individuals [10, 102], since it is a subjective method that relies on the observer's judgment. Some of the factor that must be considered and should be controlled and standardized as much as possible are: the light source, time of the day, surrounding colors and patients make up, fatigue of the human eye and color blindness [8, 10, 92].

Instrumental Methods

Colorimeters or spectrophotometers are instruments that are designed to measure the color surface of an object [100]. These instruments are more commonly used in dental research because of their ease of use, ability to detect small changes and unbiased (do not depend on the observer's judgement) [100]. However, these instruments have several drawbacks; they can only assess small areas at a time and they are designed to be used on flat surfaces (teeth normally are not flat) [100].

2.2.4. Natural Tooth Shade

Tooth shade is a distinct optical phenomenon that occurs due to a combination of interactions between the light hitting the tooth surface and tooth tissues before reaching the observer's eye [100, 103]. The overall tooth shade represents a combination of the inherit shade of dental tissues (enamel, dentine, pulp) and the extrinsic stains that are adsorbed on the dental enamel surface [8, 100]. Shade of human teeth are made of a combination of colors that vary from light yellow to grayish white and graduate from the incisal edge to the gingival margin of the tooth [8, 10]. The gingival margin tends to be more yellowish and darker, while the incisal edge tends to be more grayish and lighter [8]. Teeth are lighter in younger people and primary dentition [7]; however, with increasing age, teeth become darker. This may be caused by various reasons: accumulation of extrinsic stains, gradual tooth wear and increased amounts of secondary dentine [7, 8].

Even though dentine is the major determinant of the tooth shade [10], many properties of dental enamel have been reported to influence tooth shade, such as enamel thickness,

chemical composition and crystallographic properties [104]. However, it has been suggested that the crystal size of CHA of enamel has a major effect on tooth shade parameters by influencing the scattering of light that occurs on the tooth surface: smaller crystals scatter more light making the teeth appear whiter [90, 103, 104].

2.2.5. Tooth Discoloration

Tooth discoloration still poses a major problem in aesthetic dentistry and is a chief concern for many patients and dentists [7, 105]. Tooth discoloration varies in aetiology, severity and adherence to tooth structure [106]. It can be generally classified either to intrinsic staining (changes within the tooth), extrinsic staining (changes on the outer surface of the tooth) or a combination of both [7, 105].

2.2.5.1. Intrinsic Tooth Staining

Intrinsic stains occur during tooth formation or after tooth eruption due to one or more of the following causes: i) changes in the structural composition of dental hard tissues, ii) change in thickness of dental hard tissues, or iii) incorporation of chromogenic molecules into dental tissues [8, 106]. Pre-eruptive causes that might lead to intrinsic tooth discoloration include inherited developmental disorders (i.e. amelogenesis imperfecta and dentinogenesis imperfecta), tetracycline medication usage, excessive fluoride ingestion and trauma to the developing tooth [106]. Pulp necrosis, certain dental restoration and iatrogenic causes are among the main causes of post-eruptive intrinsic discoloration [7, 106].

2.2.5.2. Extrinsic Tooth Staining

Extrinsic enamel stains have been described as superficial tooth stains caused by the adsorption of chromogens within the dental biofilm or dental plaque and may also be retained on the tooth surface via ion interactions forming a stain-enamel complex [8, 107, 108]. Certain factors predispose to the accumulation of extrinsic stains on the outer surface of enamel; these include poor oral hygiene and plaque formation, pH, saliva flow rates and composition and tooth enamel roughness [7, 105, 109, 110]. Extrinsic staining can be further divided into direct staining and indirect staining [8].

Direct Extrinsic Staining

Direct staining is caused by compounds that are directly incorporated to the dental biofilm on the enamel surface and produce a stain as their basic color. Among the direct staining compounds are tobacco and certain types of beverages (e.g. coffee, tea, red wine) that contain dietary chromogens called tannins or polyphenols [7, 8, 105, 110, 111]. It is presumed that these anionic polyphenols adhere to the tooth surface via salivary proteins that are selectively adsorbed on the enamel surface and form the pellicle [112]. However, there is no scientific evidence supporting these presumptions and it is still not clear how teeth get stained extrinsically.

Indirect Extrinsic Staining

Indirect extrinsic staining is caused by compounds that are typically without color or possess a color that is different from the extrinsic stain produced on the tooth surface [8]. Such compounds are usually cationic (i.e. chlorhexidine antiseptics) or heavy metals (i.e. iron, tin, copper and silver) [7, 10].

2.3. Tooth Bleaching (Tooth Whitening)

Treatment modality varies according to the cause, type, and extent of tooth discoloration [106]. The treatment might be simple polishing and scaling, whitening toothpastes, microabrasion, tooth bleaching or even more invasive approaches such as fillings, veneers and crowns [10]. However, tooth bleaching has become a prevailing treatment modality for discoloured teeth and is among the top aesthetic services demanded by patients [10], since it is a minimally invasive, low cost mean to treat discoloured teeth when compared to veneers or crowns [113, 114].

Tooth bleaching can be further classified into internal (non-vital) bleaching and external (vital) bleaching [10]. Internal bleaching involves the placement of the bleaching agent inside the pulp chamber of teeth that had undergone root canal treatment [7, 106, 115]. Unlike external bleaching, internal bleaching whitens the teeth from inside and is used to treat certain cases of intrinsic staining [7, 106, 115]. However, internal bleaching has some potential negative effects, such as internal resorption that might lead eventually to tooth loss [106, 115]. External tooth bleaching is a procedure where certain chemicals are applied on the outer surface of tooth enamel to treat certain types of discoloration and /or to improve tooth aesthetics [7, 10]. External bleaching is more commonly used than internal bleaching because it is a less invasive and less expensive procedure, and it causes less harmful effects [106].

Diverse forms of bleaching agents are currently used for tooth whitening and can be mainly separated into two major groups: peroxide-based bleaching agents or non-peroxide-based bleaching agents [10]. The majority of bleaching procedures utilize peroxide-based bleaching agents that generally contain hydrogen peroxide or one of its

reactive precursors (sodium perborate or carbamide peroxide) as an active whitening ingredient [10]. Peroxide-based bleaching procedures can be carried out in the dental office "in office bleaching", at home "home bleaching" by the patients, or by a combination of both techniques [116]. More recently, non-peroxide based bleaching agents, such as sodium chlorite ($NaClO_2$), have been used; however the clinical efficacy of these products remains questionable [117, 118].

2.3.1. Peroxide-based Bleaching Agents

Hydrogen peroxide, the most commonly used oxidizing agent for tooth bleaching, can be applied directly on the tooth, or produced locally in a chemical reaction from precursors such as sodium perborate or carbamide peroxide [106, 119]. Hydrogen peroxide can diffuse through tooth enamel to reach the dentino-enamel junction and dentin regions [120-130]. Under alkaline conditions hydrogen peroxide acts as a strong oxidizing agent through the formation of free radicals, reactive oxygen molecules, and hydrogen peroxide anions [130]. It has been speculated that these radicals may eliminate chromophores by either cleaving double bonds in organic molecules or oxidizing their chemical moieties, resulting in soluble molecules, or less heavily pigmented constituents that reflect less light thus creating a "whitening effect" [10, 123, 130-132]. However, there is no scientific evidence supporting any of these claims and it is still not clear how bleaching whitens teeth.

2.3.2. Effects of Peroxide-based Bleaching Agents on Tooth Enamel

Bleaching using peroxide agents can be considered a conservative method of treatment and has been used widely to lighten dark teeth, and to treat cases of mottled enamel,

dental fluorosis, tetracycline as well as for other external and internal stains [12-15]. The final outcome of the peroxide-based bleaching procedures depends on the concentration of the bleaching agent, as well as the duration and number of bleaching cycles [10, 106]. Even though bleaching procedures give mostly initial satisfactory results, the final outcome is often temporary and lacks stability [16-19]. Moreover, there have been some concerns about the safety of using peroxides and some studies have reported they may have negative effects on the hard and soft tissues in the oral cavity [106].

The impact of peroxide-based bleaching agents on tooth enamel hardness has been extensively investigated in the literature [61, 114, 116, 133-150]. However, there is still controversy whether bleaching agents have a negative effect on enamel microhardness. Some studies have reported a significant decrease in enamel microhardness after bleaching [61, 114, 116, 133-150], while other studies showed no significant change [116, 148, 151-155]. Moreover, several studies investigated the change of enamel elemental composition after bleaching and reported a decrease in the concentrations of calcium (Ca) [156-158] that could be clinically significant [156-162].

Chapter 3: Thesis Hypothesis and Objectives

3.1. Study Hypothesis

We hypothesize in this thesis that stains develop through chemical interactions between the stain molecules and the tooth enamel surface. However, the removal of these stains by hydrogen peroxide has limitations since hydrogen peroxide only affects a minor part of tooth enamel (organic component). Therefore, developing an alternative technique that works on the major part of tooth enamel (mineral component) to whiten teeth, could overcome these limitations.

3.2. Study Working Hypotheses

Our study hypotheses were addressed by following these working hypotheses:

i. Chemical composition of the outer enamel surface determines the adherence affinity of stains to dental enamel.
ii. Hydrogen peroxide molecules make the teeth whiter by oxidizing their outer enamel surface.
iii. Modifying the crystallographic properties of tooth enamel can be an alternative method for tooth whitening.

3.3. Objectives

In order to test our working hypotheses, our research targeted the following objectives:

Working Hypothesis 1

i. Characterize the elemental composition of the outer enamel surface and determine the functional groups available on the outer enamel surface.

ii. Determine the adherence of two dyes with opposite charges to the outer enamel surface.

Working Hypothesis 2

i. Determine the effects of de-proteinizing, de-mineralizing and oxidizing solutions on the chemical and optical properties of human tooth enamel.

ii. Determine the effects of hydrogen peroxide on human teeth with de-proteinized enamel and human teeth with de-mineralized enamel.

Working Hypothesis 3

i. Determine whether magnesium ions can react with dental enamel.

ii. Determine the effect of magnesium ions on the enamel crystallographic and physical properties.

Chapter 4: Materials and Methods

4. Materials and Methods

4.1. Materials

4.1.1. Sample Collection and Preparation

A sample of 204 sound anterior teeth (free of caries, areas of demineralization, cracks, cavities, restorations, severe or atypical intrinsic stains, and/or a history of tooth bleaching) from the upper jaw were collected from adult patients with dental conditions that require tooth extraction. The extraction procedure was performed in McGill Undergraduate Dental Clinics after obtaining approval from McGill University Health Center Ethical Committee and the signed informed consent from the patients (see Appendix IV, V and VI). Upon extraction, teeth were immersed in 10% formalin solution (BF-FORM, Fisher Scientific, Canada) for 1 week. The samples were then cleaned in an ultrasonic bath (FS20D Ultrasonic, Fisher Scientific, Canada) filled with DW for 60 min at 25^{o}C and polished for 1 min with a low-speed dental handpiece (M5Pa, KAB-Dental, USA) using SiC cups (Pro- Cup, sdsKerr, Italy) and dental prophylaxis pumice of low abrasive capability (CPRTM, ICCARE, USA). Then, teeth were rinsed in an ultrasonic bath before storing them in labelled Eppendorf tubes with 10% formalin solution for further analysis. The specific sample preparation and treatment agents used will be mentioned in detail in the following chapters.

4.2. Methods

4.2.1. X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) (also known as Electron Spectroscopy for Chemical analysis (ESCA)) is currently considered the standard tool for characterizing solid surfaces. It relies on the concept that the absorption of X-rays by the atoms in the sample leads to ejection of core electrons (photoelectrons) and these photoelectrons have energies that are unique to each element and sensitive to the chemical state of the element. It is a highly sensitive non-destructive surface analytical technique which is widely used to investigate the most superficial ～1 to 10 atomic layers (～0.5 to 5.0 nm) of a material with a detection limit of 0.1-1.0 at% and a relative error of 20 % [163-166]. XPS is used for the identification and quantification of all elements, except hydrogen and helium since they do not have core electrons [167, 168], and it provides information about the chemical environment of the detected elements [167, 168]. It can also provide information about the spatial distribution of the surface components through depth profiling and 3D mapping [167, 169]. Some of the disadvantages of XPS are that it works only under high vacuum pressure and it may cause some surface charge build-up on non-conducting or poorly conducting materials, which eventually affects the accuracy of the results [167, 170].

In our experiments, a monochromatic X-ray photoelectron spectrometer K Alpha (Thermo Fischer Scientific Inc, East Grinstead, UK) was used for determining the relative quantities and chemical environments of the constituent elements on the surface of tooth enamel samples. The setup was equipped with an Al $K\alpha$ X-Ray radiation source (1486.6 eV, 0.834 nm), a micro-focused monochromator and an ultrahigh vacuum

chamber (10^{-9} torr). For all the samples, survey scans were obtained over the range of 0 – 1350 eV with pass energy of 200 eV at a step of 1.0 eV and high resolution scans were collected with pass energy of 50 eV at a step of 0.1 eV. A flood gun was used to neutralize the surface charge build-up. The binding energy (BE) scale for the samples was determined by setting the value of the carbon bonded to hydrogen or carbon (C- (H, C) as a reference at 285.0 eV. Data analysis and peak fitting were performed using Avantage (4.60v) chemical surface analysis software.

4.2.2. Raman Spectroscopy

Raman spectroscopy is a non-destructive analytical method that enables us to obtain the vibrational spectra of the molecules or compounds found in a material with a spectral resolution up to 1 cm^{-1} and a spatial resolution up to 1 μm [56]. It generates spectra by inducing inelastic scattering "Raman effect" through focusing a monochromatic laser light onto the sample. The exact position and numbers of peaks in the obtained spectra are very characteristic to each material, especially in the finger print region (400 – 1800 cm^{-1} range) and the intensity of the peaks is in relative proportion to the prevalence of that molecule or compound which permits their quantitative estimations [54].

The Raman spectra of tooth enamel tissue provide us with valuable information about the structure and composition of its mineral constituent [56, 171-176]. Raman spectra were recorded using a Raman spectrometer (Senterra, Bruker, Karlsruhe, Germany) coupled with an Olympus microscope and equipped with a 785 nm (diode) laser source. The microscope was set to a 10X objective with a spot size of approximately 20 μm. The machine was set at a resolution of 2 m^{-1}. Collection times were set to 30 s with 2 co-additions. The collected spectra were baseline corrected using the Raman

spectrophotometer's software (OPUS9 7.0.0, Bruker, Karlsruhe, Germany). The crystallinity index was quantified using the full width at half maximum intensity value (FWHM) of the phosphate symmetric stretch peak $(\nu_1 PO_4)^{3-}$ at ~ 960 cm^{-1} [177-180], and the carbonate-to-phosphate ratio was estimated from the relative intensity of carbonate type B (at 1070 cm^{-1}) to main phosphate peak (at 960 cm^{-1}) [54, 171].

4.2.3. X-ray Diffraction (XRD)

X-ray Diffraction (XRD) is a non-destructive analytical technique used to investigate the crystallographic ultrastructure and physical properties of a material by means of observing the intensity of diffracted X-ray beam as a function of wavelength and scattered angle. It provides valuable information about the crystalline phase and the mean size of the crystallite particles present in a sample. XRD has been widely used to assess the tooth enamel ultrastructure [52, 87, 181-190].

X-ray diffraction (D8-DISCOVER/GADDS, Bruker, Germany) was used to characterize the crystallographic structure of enamel powder samples. The XRD parameters were calibrated according to earlier work: 40 kV and 40 mA Cu-Ka radiation, parallel graphite monochromator, 10–608 scanning angle, 0.028 step size and 1800 s scan step time [191]. The data of each XRD spectrum was analyzed using DIFFRAC plus EVA software (AXS, Bruker, Germany). [191]. The average CHA crystal dimensions along the *c*-axis and *a*-axis were calculated by applying Scherrer's formula to the (0 0 2) and (3 1 0) Bragg peaks for each enamel sample XRD spectrum [192]:

$$D = \frac{K\lambda}{\beta \cos\theta}$$

D: the average diameter; *K*: the shape factor; λ: the X-ray wavelength; β: the line broadening at half the maximum intensity (FWHM) and θ: the Bragg angle.

The enamel crystal cell lattice parameters *a*-axis and *c*-axis were calculated from the XRD (0 0 2) and (1 1 2) Bragg peaks using the equation [42]:

$$\frac{1}{d^2} = \left(\frac{4}{3}\right).\left(\frac{h^2 + hk + k^2}{a^2}\right) + \frac{l^2}{c^2}$$

d: the spacing between adjacent planes (interplanar spacing) in the crystal; hkl: the miller indices that are the reciprocal intercepts of the plane on the unit cell axes; a: *a*-axis and c: *c*-axis.

4.2.4. Energy Dispersive X-ray Spectroscopy (EDS)

Energy Dispersive X-ray Spectroscopy (EDS) is a non-destructive analytical technique used for the chemical characterization of a material. It relies on the characteristic X-rays emitted from the sample when irradiated with a beam of electrons which penetrates 0.5 – 10 μm into the specimen [193]. EDS has been used as a semi-quantitative technique to estimate the elemental concentration in tooth enamel [160, 185, 186, 194-205].

An EDS probe (Oxford INCA 300, Berkshire, UK) installed in an SEM (Hitachi S - 3000N VP, Wokingham, Berkshire, UK) was used to determine the elemental composition of the tooth enamel samples. The SEM-EDS probe analysis was adjusted at 15 kV with 5 nm spot size. The working distance used was 15 mm and the counting time was 300 seconds. For each measurement the EDS analysis was carried out by scanning 1 mm^2 area to improve the representativeness of the data. For each sample, the elemental composition was analyzed and the following elemental ratios were calculated: carbon-to-

phosphate (C/P), calcium-to-phosphate (Ca/P), chloride-to-phosphate (Cl/P), oxygen-to-phosphate (O/P), sodium-to-phosphate (Na/P) and magnesium-to-phosphate (Mg/P).

4.2.5. Tooth Enamel Microhardness

Vickers microhardness tester and Knoop microhardness tester are among the most common methods used to evaluate dental enamel hardness, since they are suitable to test very thin materials and measure individual microstructures within a larger structure [9, 46, 64-69, 71-93]. Both testers use a diamond indenter (pyramid shape in Vickers tester and rhombus shaped in Knoop tester) with a certain range of loads and the resulting indentations on the enamel surface can be used to calculate the hardness value. There is no significant difference between the two methods when they are used to measure enamel microhardness [11]. However, Vickers microhardness tester was used in this thesis since it is more convenient than Knoop tester [206].

The mechanical properties of tooth enamel samples were measured using a Vickers microhardness indenter machine (Clark CM100 AT, HT-CM-95605, Shawnee Mission, KS). The indentation load was adjusted to 100 g per 10 s. Computer software (Vision PE 3.5, Clemex Technologies Inc., Shawnee Mission, KS) was used to measure the microhardness value at the site of indentation from images captured by a built-in camera. Percentage change in hardness was calculated from the initial and final hardness measurements.

4.2.6. Tooth Shade Measurements

The tooth shades were measured using a spectrophotometer (Easy shade®, Vita Zahnfabrik, Germany) since spectrophotometry is considered the most reproducible and accurate method for tooth shade measurements [207, 208]. The shade data was collected based on the Munsell's color system (Lightness, Chroma, Hue) since most tooth shade guides used in dental clinics are based on it [96, 98, 99]. The shade registration process was carried out in a dark room and teeth were kept wet at all times in order to avoid its dehydration. Measurements were repeated three times on the mid-labial surface for each tooth and the mean as well as the standard deviation for each shade parameter was calculated.

4.2.7. Statistical Analysis:

All data were tested for normality using Shapiro-Wilk test. For data following a normal distribution (Gaussian distribution) paired-sample t-test was used to test for changes within the same sample and two-sample t-test to compare between different groups. For data not following a normal distribution, non-parametric tests were used; paired Samples Wilcoxon signed Rank test was used to test changes within the same sample and Mann-Whitney test was used to compare between different groups. All the statistical analyses were performed using Origin 7.0 software (OriginLab, Northampton, MA) and SPSS 19 software (IBM, Chicago, IL). The significance level was set below 0.05 ($p < 0.05$).

Chapter 5: How Do Teeth Get Stained?

5.1. Introduction

The appearance of teeth, particularly their color, is a major concern to a large number of people, and for the past decades, there has been an increase on demand for treating tooth staining and discoloration [8]. Certain beverages (e.g. coffee, tea, red wine) have been proven to cause these discolorations [8, 105, 110, 111]. It is presumed that these beverages have the ability to stain teeth because they contain anionic polyphenols that essentially provide color to these beverages [8, 105, 110, 111]. Some studies suggested that the polyphenol molecules adhere to enamel surface through interactions with the tooth surface or by attaching to the cationic salivary proteins [7, 112, 209-211]. These salivary proteins are found in the biofilm coating the tooth and some of them have been identified as being non-phosphorylated basic Proline-rich proteins (bPRPs) [110, 211-213]. However, the mechanism of how the aforementioned beverages stain the enamel surface is still not fully understood and the question remains as to why stains occur.

Enamel surface has been postulated as a contributing factor to many processes, such as caries formation, tooth staining, bio-film buildup and crack propagation [214-220]. Characterization of the enamel surface is fundamental for understanding these processes that take place between the tooth and the surrounding environment. Surprisingly, little is known about the distribution of elements and functional groups on tooth enamel surface and it is still unclear why certain molecules have higher affinity to adhere to the outer enamel surface.

The aim of this study was to characterize the elemental and functional groups across the surface of tooth enamel to estimate the relative concentration of these constituents and assess the chemical interactions between them and the surrounding environment. We hypothesize that the outer enamel surface is different than the rest of enamel and has more cationic groups that have the ability to bind the anionic polyphenol molecules. In order to test our hypothesis we designed an *ex vivo* study and characterized the surface of outer enamel and compared it to the surface of inner enamel and acetone-treated outer enamel. Polishing and cleaning was done to remove any remnant of the dental biofilm and any possible surface contaminants. On the other hand, acetone was used to alter the structure of outer enamel by eliminating the hydrophobic molecules adsorbed on it. Later on, we assessed the ability of two dyes with different charges (one cationic and one anionic) to stain the different surfaces.

5.2. Materials and Methods

5.2.1. Sample Collection

A sample of 58 sound anterior teeth (free of caries, stains, cracks, demineralization or pitting) were used in this study and were prepared as mentioned in Chapter 4. Thirty teeth were used to characterize the surface elemental composition, while the other twenty-eight teeth were used in the staining procedure.

5.2.2. Surface Elemental Composition

A sample of 30 teeth was cut into thin sections of enamel (1-1.5 mm thick) using a carbide bur (FG56, SDS Kerr, Orange, CA) adapted to a high speed dental hand piece (TA98LW, Synea, Canada) cooled with DW. Ten sections were assigned to the outer

enamel surface group where the outer surface was analyzed, ten sections were assigned to the bulk enamel surface group where the inner surface of the section was analyzed and 10 sections were assigned to the acetone-treated outer enamel surface where the sections were ultrasonicated in a bath filled with acetone for 30 minutes and washed with DW for 30 minutes before analyzing the outer surface of the section.

X-ray photoelectron spectroscopy (XPS) was used for determining the relative quantities and chemical environments of the constituent elements on the different enamel surfaces. For each sample, the elemental composition and chemical environment was analysed and the following ratios were calculated: carbon-phosphate, oxygen-to-phosphate, nitrogen-to-phosphate, calcium-to-phosphate, carbon-to-calcium, oxygen-to-calcium, nitrogen-to-calcium.

5.2.3. Staining Procedure and Tooth Shade Measurements

All dyes in this study were obtained from Fisher Scientific (Montreal, QC). Methylene blue (MB) (0.1 %) and acid fuchsin (AF) (0.1%) were prepared to perform the staining experiments on the different enamel surfaces (outer enamel, bulk enamel and outer acetone-treated enamel) in order to visualize possible surface reactions. Twenty eight teeth were randomly assigned to four groups (n= 7 per group): MB outer enamel, MB acetone-treated outer enamel, AF outer enamel, AF acetone-treated outer enamel. The staining procedure was performed by immersing the tooth in the specific dye solution for 30 seconds. The tooth sample was then rinsed with copious amounts of DW and subsequently cleaned ultrasonically for 20 min in DW. Tooth shades were measured using the spectrophotometer and the test specimens were carefully handled with clean forceps and were kept hydrated in distilled water between measurements.

5.3. Results

5.3.1. Composition of the Tooth Enamel Surface

XPS analysis showed that the three types of enamel surfaces (sound outer, acetone-treated outer and sound bulk) had different elemental composition (Fig.5.1 a, b and c). A total of 10 elements were found on the enamel surfaces, including sodium (Na), potassium (K), oxygen (O), nitrogen (N), calcium (Ca), carbon (C), phosphorus (P), zinc (Zn), magnesium (Mg), copper (Cu), silicone (Si) and sulphur (S) (Fig.5.1f). Elements present in the highest concentrations were C, O, N, Ca and P and they were present in all samples, while the remaining elements were present in minor quantities and were not present in all samples. Zn was present in all samples of out enamel, but was present in only half the samples of the bulk enamel and acetone treated enamel. In the outer enamel surface group, four samples showed some traces of Si, one sample showed Cu and one samples showed S. Mg was observed only in one sample in the bulk enamel and acetone treated enamel.

Compared to bulk enamel and acetone treated enamel, the sound outer enamel surface showed relatively the highest carbon concentration and the lowest concentrations of oxygen (Fig.5.1d), calcium and phosphate (Fig.5.1e). In comparison with the outer enamel surface, the intact bulk surface showed lower concentrations of carbon and higher concentrations of oxygen, nitrogen, calcium and phosphate (Fig.5.1 d and e). The acetone-treated outer enamel surface showed relatively the lowest carbon and nitrogen concentrations, and the highest oxygen concentration (Fig.5.1e). The sound outer enamel surface showed relatively the highest carbon-to-phosphate, oxygen-to-phosphate, nitrogen-to-phosphate, calcium-to-phosphate, carbon-to-calcium, and oxygen-to-calcium

and nitrogen-to-calcium concentrations (Fig.5.1. g, h and i). The acetone-treated outer enamel surface showed relatively the lowest carbon-to-phosphate, nitrogen-to-phosphate, carbon-to-phosphate, nitrogen-to-calcium (Fig.5.1. g, h and i). Both bulk enamel and acetone-treated outer enamel surfaces showed lower oxygen-to-phosphate, calcium-to-phosphate and oxygen-to-calcium concentrations compared to outer enamel surface (Fig. 5.1 g, h and i).

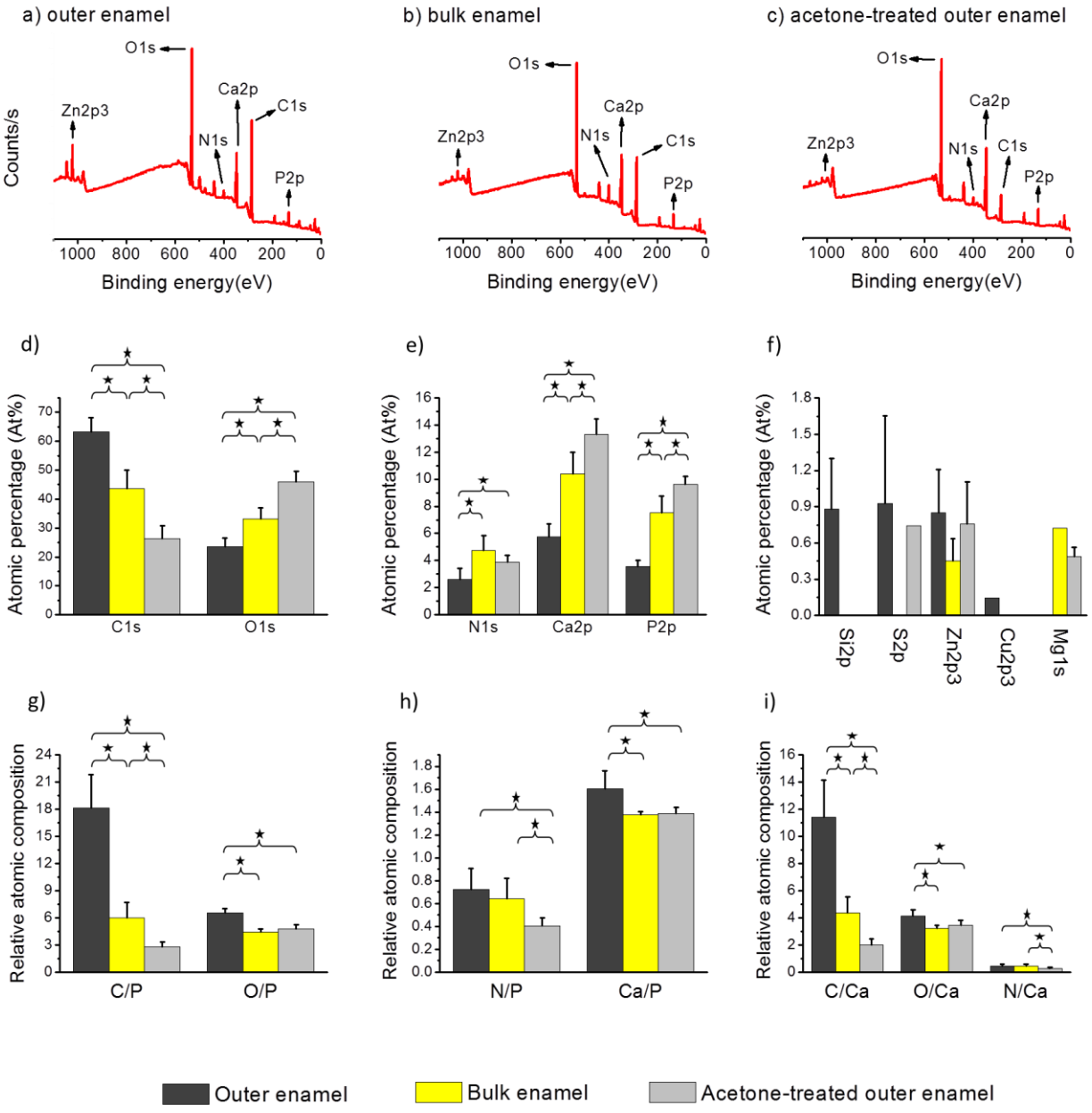

Fig 5.1 - a-c) XPS survey spectra of the three different surfaces: outer enamel (a), inner enamel (b), acetone treated outer enamel (c). Peaks of the elements: carbon (C1s), oxygen (O1s), nitrogen (N1s), calcium (Ca2p), phosphate (P2p) and zinc (Zn2p3) are shown. d-i) Bar charts illustrating the elemental composition of the different surfaces. * indicates significant difference between the different groups ($p < 0.05$).

High resolution XPS scans of the C1s peak were deconvoluted into five different peaks. These peaks represent five different chemical states of the carbon atom which probably originated from the specific composition of enamel and any common carbon contamination in surplus (Fig.5.1). The separation between these peaks allowed differentiation of five carbon species: hydrocarbon (C-(C,H)) at 285 eV; carbon single bonded to oxygen or nitrogen (C-(O, N)) at ~ 286.23 ($\pm$ 0.1) eV; carbon double bonded to oxygen (C=O) at ~ 287.23; ($\pm$ 0.1) eV; carboxylate (COO^-) at ~ 288.73 ($\pm$ 0.1) eV; and carbonate $(CO_3)^{2-}$ at ~ 289.9 ($\pm$ 0.1) eV (Fig.5.2d) as used in previous work [221]. The area under the peaks allows quantification of the relative amounts of each carbon species present, while the position of the peaks represents the different chemical states. The outer enamel surface showed relatively the highest hydrocarbon concentration and the lowest concentrations of C- (O, N), C = O, COO^- and carbonate (Fig.5.2d). The acetone-treated outer enamel surface had relatively the lowest concentration of hydrocarbons and the highest concentrations of C-(O, N), C=O, COO^- and carbonate.

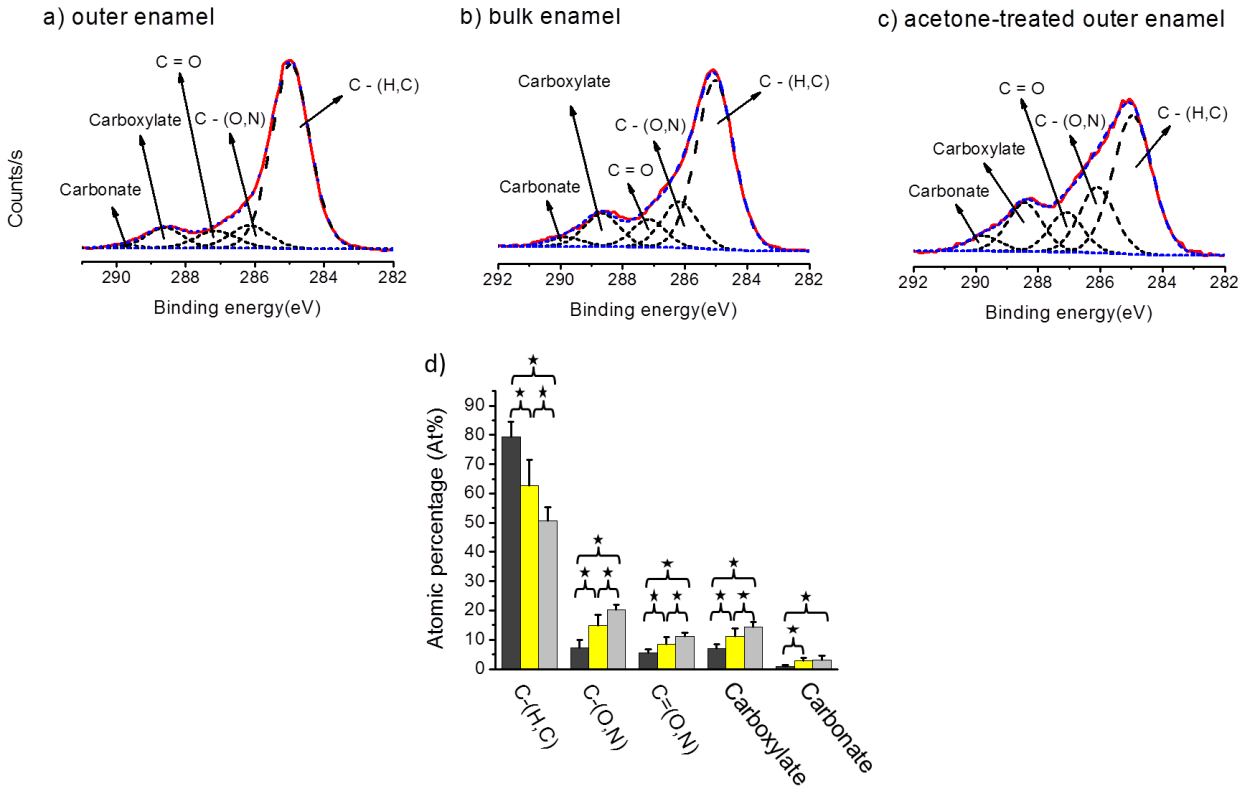

Fig. 5.2 - a-c) Peak fitting of the XPS high resolution (C1s) carbon spectra in the surface of: outer enamel (a), inner enamel (b), acetone treated outer enamel (c). d) Bar charts illustrating the different chemical states of carbon in the three different surfaces. * indicates significant difference between the different groups ($p < 0.05$).

5.3.2. Effects of Staining Procedure on Tooth Shade Measurements

Staining procedure was performed to visualise the possible surface reactions. Methylene blue had a significant effect on tooth hue ($p < 0.05$), in both outer enamel surface and acetone-treated outer enamel surface; however its effect was stronger in the acetone-treated outer enamel surface ($p < 0.05$) (Fig.5.3 a). Acid fuchsin had a significant effect on tooth hue only in outer enamel surfaces and it was significantly different than the acetone-treated outer enamel surfaced ($p < 0.05$) (Fig.5.3 b).

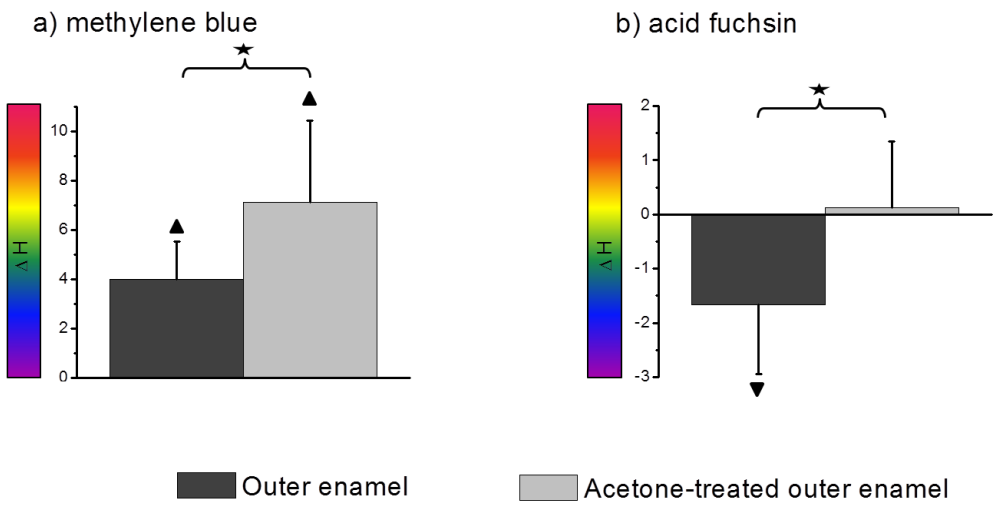

Fig.5.3 - Bar graphs illustrating the change in tooth shade hue (H) after staining the specimens with methylene blue (a), acid fuchsin (b) among the two groups of enamel. ▲ indicates significant change within the same specimens after each staining procedure; * indicates significant difference between the two groups ($p < 0.05$).

5.4. Discussion

In this study we characterized the compositions of different surfaces of enamel. The results of this study indicate that the outer surface of tooth enamel is covered by a carbon rich layer that plays an important role in extrinsic tooth staining. Underneath we discuss in details these findings.

5.4.1. Composition of the Tooth Enamel Surface

The outer most surface of the erupting tooth is said to be covered by enamel cuticle which is an acellular organic layer, that mediates the adherence of bacteria even without the presence of the acquired pellicle, however due to abrasion and attrition factors it is replaced by a thin film of precipitated glycoproteins from saliva [222-224]. Even though our samples were polished and brushed with pumice, persistence of an organic layer can be observed on the surface of the erupted teeth. Similar observations were reported and it

was suggested that this layer could be residual pellicle adsorbed on the surface [225, 226]. However, the fact that it is washed away by acetone indicates that it is hydrophobic in nature.

There was high carbon content in all the analyzed surfaces but there was more carbon in the outer enamel surface than the bulk enamel. Although part of the Carbon could be of atmospheric contamination [26, 164], significant differences were observed between the three surfaces of enamel confirming that the surfaces were different. Moreover, peak fitting of high resolution C1s spectra indicated that most of the carbon was bound to either hydrogen or carbon (C–(C, H)). Previous analysis with another technique, elastic recoil detection analysis (ERDA), has also shown that the hydrogen-to-carbon ratio in outer enamel surface is 2:1 indicating a surface saturated with an organic material [26].

Similar decrease in carbon and nitrogen content was observed in enamel treated with potassium hydroxide, sodium lauryl sulphate and etching with benzoic acid in acetone [221, 226, 227]. Acetone is a hydrophobic organic solvent, therefore it can remove hydrophobic molecules, but it cannot dissolve charged molecules such as carboxylate and carbonate, this might explain the observed relative decrease in hydrocarbon and the relative increase of carboxylate and carbonate after treating the outer surface of enamel with acetone. These findings indicate that acetone removed hydrophobic organic molecules from the superficial surface of outer enamel and made it more negatively charged.

The stoichiometric ratio of calcium-to-phosphate (Ca/P) in pure hydroxyapatite (HA) is approximately 1.67; however dental enamel, like other biological apatites, has been

described as being a non-stoichiometric with a Ca/P ratio of 1.48±0.09 [228-230]. This decrease in ratio has been attributed to: substitutions of Ca by Na, Mg and other trace elements, or excess phosphates on the crystal surface [228-230]. Calcium and phosphate relative contents were the lowest in outer enamel because of the attenuation effects of the outermost carbon rich layer, as reported previously [226]. However, the Ca/P was the highest indicating that there is more Ca available on the outer surface. Previous studies also have suggested that there is a calcium rich layer on the outer surface of enamel and presumed calcium might be bound within an organic layer, most probably within the adsorbed glycoproteins on enamel surface [219]. This layer has been reported to be more acid resistant and might explain why incipient caries progress beneath an unaffected Ca rich layer [219]. Our results confirm these assumptions and show that the outer layer of enamel is both rich in calcium and carbon that is most probably hydrophobic and of organic origin.

5.4.2. Interaction of the Tooth Enamel Surface with Dyes

From enamel surface analysis, we identified the available atoms and functional groups available for binding sites on the three different enamel surfaces. The outer enamel surface had higher calcium content indicating that it might have more calcium available for binding to anionic groups. In contrast, the acetone-treated outer enamel showed higher $(PO_4)^{3-}$ and negative functional groups (carbonate and carboxylate) available for binding cationic groups. The fact that acetone treated teeth were stained by MB more than intact teeth, confirms our XPS findings and indicates that teeth have relatively higher anionic groups on the surface when treated with acetone. Moreover, the intact teeth were stained by AF more than the acetone-treated ones, which also confirms our

XPS findings and indicates that the surface of intact teeth is rich in cationic groups that are removed when washed with acetone.

5.4.3. Interaction of the Tooth Enamel Surface with Extrinsic Stains

Extrinsic enamel stains have been described as superficial molecules adsorbed within the dental biofilm or dental plaque and may also be retained on the tooth surface through ion exchange reactions and/or form a stain-enamel complex [8, 107, 108]. Certain types of beverages (e.g. coffee, tea, red wine) have the ability to cause extrinsic staining because they contain anionic polyphenols to provide color to these beverages [8, 105, 110, 111]. Many mechanisms has been suggested to explain extrinsic staining; some studies have suggested that it might occur due to surface reactions between the staining molecule and tooth surface [7, 112], while other studies assumed that it is due to interaction between these anionic molecules and cationic salivary proteins [209-211]. However, the mechanism of how these molecules form extrinsic stains on the tooth surface is still not fully scientifically proven and no clear consensus has been reached over the relative importance of each mechanism. Our XPS and staining findings show a possible explanation for this phenomenon; it seems that the observed calcium rich organic layer plays an important role in facilitating the adherence of the anionic polyphenols and integrating them into the tooth structure forming a stain-enamel complex. This might also explain why extrinsic stains cannot be removed by simple brushing methods and more aggressive methods are needed (i.e. scaling, polishing, microabrasion, surface active materials, bleaching agents and laser) which either remove the superficial layer of enamel and/or oxidizes the organic molecules [231-237].

5.5. Selective Absorbance of Salivary Proteins

Despite the fact that the present study was not designed to investigate the selective absorbance of salivary proteins on enamel surface, our findings support the observations and assumptions of previous studies on the adherence of salivary proteins to enamel surface. It is known that the acquired enamel pellicle (AEP) formation is a complex interaction between its constituent (mainly salivary proteins) and the enamel surface [214, 216], and even small changes in the chemical properties of the surface may influence the binding of these proteins [217, 238-240]. The initial stage of pellicle formation is selective binding of certain molecules called pellicle precursors that have a higher affinity to adhere to hydroxyapatite and the outer surface of enamel [214-216, 241]. Pellicle precursors includes the following phosphorylated salivary proteins; acidic-Proline-rich proteins (aPRPs), statherins and histatins [211, 220, 241-245]. All these precursors, except histatins, are negatively charged [211, 220, 241-245]. It was suggested that these pellicle precursors adhere to the enamel surface by binding with calcium ions available on the surface [110, 218], since anionic groups (carboxylate and phosphoester) bind to Calcium sites while cationic groups bind to phosphate [246]. In summary, the selective absorbance of the anionic precursors on enamel surface suggests that more calcium binding sites are available on outer enamel which was proven by our XPS findings and staining experiments.

5.6. Clinical Significance and Future Work

Our study provides a comprehensive view of the chemical interactions that occur on the tooth enamel surface and gives a better understanding of the mechanism of extrinsic tooth staining. Further investigations are required to evaluate the exact role of saliva proteins and biofilm in extrinsic stain formation.

Chapter 6: How Hydrogen Peroxide Whitens Teeth?

6.1. Preface

This Chapter includes parts of an already published article "*Hydrogen peroxide whitens teeth by oxidizing the organic structure.* Journal of Dentistry, 2012. **24**(12): p. 00226-6 " (see Appendix II). Permission has been taken from the Journal of Dentistry and co-authors to reform the article and include it within this master thesis (see Appendix III).

6.2. Introduction

For over a century, dentists have been using oxidizing agents such as hydrogen peroxide and carbamide peroxide to lighten dark teeth, and treat cases of mottled enamel, dental fluorosis, tetracycline stained teeth as well as for other external and internal stains [12-15]. Despite its wide spread usage, the effect of bleaching on tooth shade is often temporary and lacks to stability and predictability [16-19]. This has encouraged the overuse of bleaching products, resulting in harmful effects such as: tooth demineralization and sensitivity, gingival irritation, and damage to restoration materials [247-249].

The mechanism of tooth bleaching using peroxide oxidizers is not fully understood. It is still unknown whether peroxide radicals make teeth whiter by deproteinizing, demineralizing, or oxidizing tooth tissues. Previous studies have determined that tooth mineral is the main factor affecting tooth shade, while tooth organic content is mainly transparent and has no significant effect on tooth shade [104, 250-252]. Accordingly, we hypothesize that hydrogen peroxide whitens teeth by oxidizing the transparent organic matrix in enamel into an opaque whiter material. In order to test our hypothesis, we

designed an *ex vivo* study to understand the exact mechanism by which hydrogen peroxide, the most widely used oxidizing agent, whitens teeth. In this chapter we will show a comparison between the effects of de-proteinizing, de-mineralizing and oxidizing solutions on human teeth enamel, and reveal the specific effect of each chemical on the tooth enamel composition and properties.

6.3. Materials and Methods

6.3.1. Sample Collection and Preparation

A sample of 60 sound freshly extracted teeth (free of caries, demineralization areas, cracks, cavities, restorations, severe or atypical intrinsic stains, and/or a history of tooth bleaching) were used in this section of the study and were collected and prepared as mentioned in Chapter 4.

6.3.2. Chemical Solutions

All reagents in this study were obtained from Fisher Scientific (Montreal, QC) unless stated otherwise, and used without further purification. The pH of the reagents was measured by pH test paper (Alkacid Test Paper, Fisher Scientific, Montreal, QC). Four solutions were prepared for treating the teeth.

- Deproteinizing solution: 1M sodium hydroxide (NaOH) solution, pH 13.3, was employed to de-proteinize teeth and remove the organic components.
- Demineralizing solution: 0.5M ethylenediaminetetraacetic acid (EDTA), pH 8, was employed to decalcify the teeth. Four protease inhibitors were added to stabilize dissolved substances and prevent their redeposition (mmol/L): benzamidine HCl (2.5), ε-amino-n-caproic acid (50), N-ethylmaleimide (0.5), and phenylmethylsulphonyl fluoride (0.3).

- Oxidizing solution: 30% hydrogen peroxide (H_2O_2), pH 7, solution was employed to oxidize tooth tissue.
- Control solution: de-ionized distilled water was employed to serve as control.

6.3.3. Chemical Treatments

The collected teeth were randomly divided into 6 assigned groups of 10 teeth each. The teeth were placed in separate test tubes and subjected to various chemical treatments at 25°C with the different solutions. The treatment solutions (8ml/tooth) were renewed every 24 hours for 4 days. The 4 days' timeline for this study was chosen based on our previous observations obtained from series of pilot experiments (data not shown), in which we found that the NaOH and EDTA solutions induced significant changes in tooth shade only after 4 days of treatment. For consistency, all teeth were treated for four days, even though hydrogen peroxide treatment resulted in faster changes in tooth shade. Group 1 was treated with the deproteinizing solution. Group 2 was treated with the demineralizing solution. Group 3 was treated with the oxidizing solution. Group 4 was treated with de-ionized distilled water and acted as control group.

In order to determine which tooth component (organic or inorganic) was affected by bleaching; further assessment of the effect of the oxidizing solution was made on partially deproteinized teeth, and on partially demineralized teeth. Accordingly, teeth of group 5 and 6 were treated with the deproteinizing and demineralizing solutions, respectively, for 4 days. Then, these teeth were cleaned with de-ionized distilled water for 5 minutes, and immersed in the oxidizing solution for 4 days.

6.4. Measurements

The teeth were submitted for elemental, crystallinity index and shade analyses before and after each specific treatment. Tooth enamel elemental analysis was performed using Scanning Electron Microscope Energy Dispersive Spectroscopy (SEM-EDS). The atomic percentage of the following elements was recorded: carbon (C), calcium (Ca), phosphorus (P) and oxygen (O). The following elemental ratios were calculated for each tooth: C/Ca and C/P, O/C and Ca/P.

Tooth enamel crystallinity index analysis was conducted by means of Raman spectroscopy. Crystallinity index was calculated based on the bandwidth at the half peak intensity of the ν_1PO_4 band at 960 cm^{-1}. Tooth shade analysis was conducted by means of Easy shade® spectrophotometer. During shade measurements, each tooth was kept wet at all times in order to avoid its dehydration, because dehydration induces changes in tooth shade [88]. The teeth were momentary taken out of the solutions at specific time points to analyze the changes in the shade with the spectral photometer. Before each measurement the teeth were washed in distilled water for 5 minutes.

6.5. Results

6.5.1. Effect of the deproteinizing, demineralizing and oxidizing solutions on sound teeth

Tooth enamel elemental analysis with SEM-EDS was used to assess changes in C/Ca, C/P, O/C and Ca/P ratios after each specific treatment. The deproteinizing solution (group 1) decreased the enamel organic content ($\delta C/Ca=-0.42\pm0.57$, $p=0.018$; and $\delta C/P=-0.23\pm0.23$, $p=0.018$) (Fig.6.1 a, b). The demineralizing solution (group 2) relatively increased the enamel organic content ($\delta C/Ca=1.21\pm0.80$, $p=0.027$; and $\delta C/P=0.76\pm0.27$,

p=0.018) due to the decrease in the mineral component (Fig.6.1a, b). The oxidation solution (group 3) did not induce significant changes in the enamel organic and inorganic relative contents, but it increased the oxygen relative content (δO/C=0.20±0.14, p=0.018) (Fig.6.1c). Control teeth (group 4) did not exhibit any changes in their enamel elemental composition (Fig.6.1).

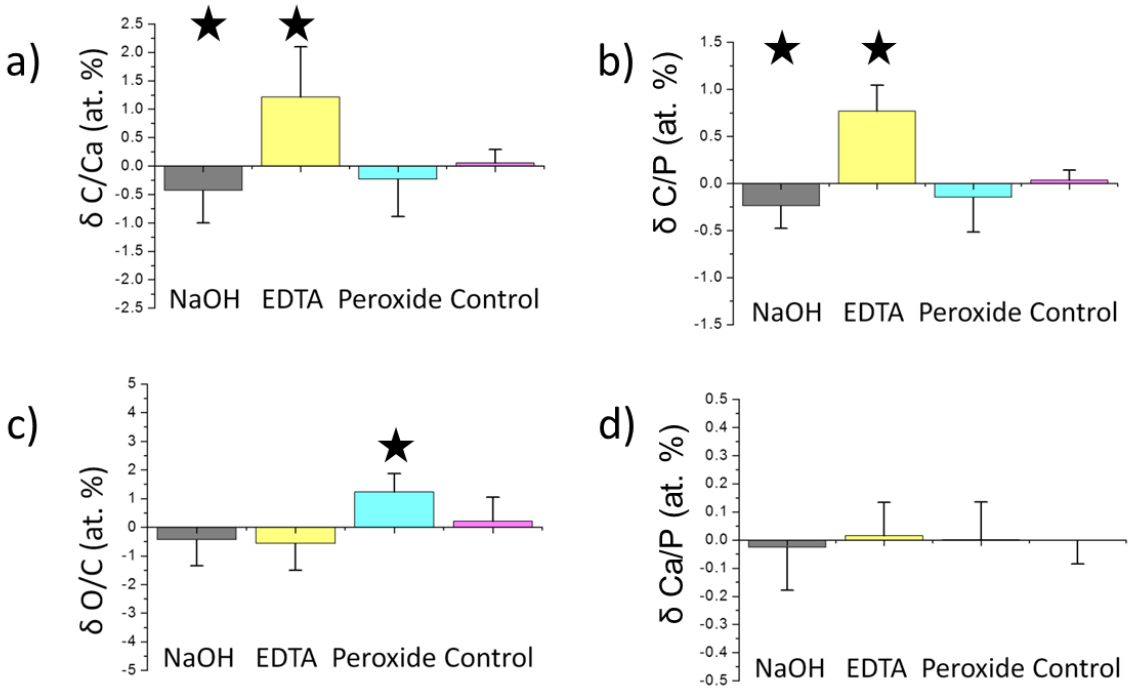

Fig.6.1. Bar graphs illustrating the elemental analysis obtained by EDS analysis of the labial surfaces of teeth treated with NaOH (group 1), EDTA (group 2), H_2O_2 (group 3) or distilled water as control (group 4), respectively. * indicates statistically significant changes in elemental constitutes after specific treatment.

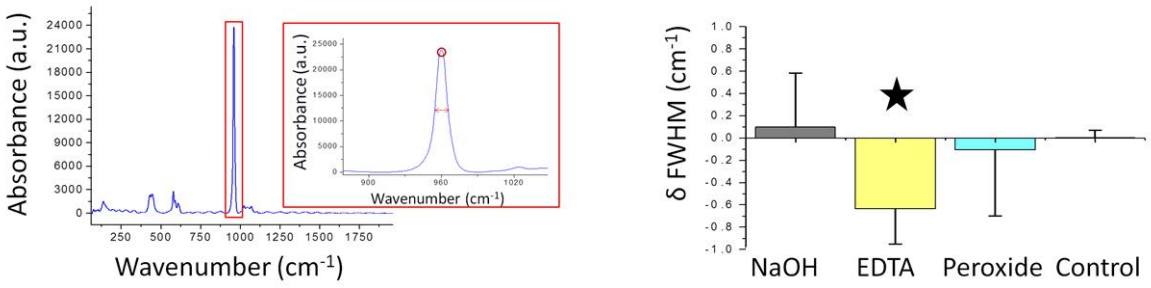

Fig.6.2 - (a) Raman absorbance spectrum of tooth enamel showing the PO_4 peak at 960 cm^{-1} and its full width of half height (FWHH) that was used to determine enamel crystallinity. (b) Bar graph depicting the crystallinity analysis for the teeth treated with NaOH (group 1), EDTA (group 2), H_2O_2 (group 3) or distilled water as control (group 4), respectively. * indicates statistically significant differences in crystallinity index after specific treatment.

In summary, our results indicated that the deproteinizing solution was able to decrease enamel organic relative content without changing their mineral component (deproteinization). The demineralizing solution decreased enamel inorganic relative content (decalcification). The oxidizing solution increased enamel oxygen content, an indication of the oxidization reaction. The demineralization solution (group 3) decreased significantly teeth enamel crystallinity (p=0.026) in comparison to the other solutions (Fig.6.2b). This indicated that the demineralizing solution reduced the total crystalline volume of tooth enamel apatite crystals. The deproteinization, demineralization, and oxidation solutions induced significant changes in teeth shades (Fig.6.3). Teeth deproteinization increased the lightness (δL=4.8±2.7 degrees, p=0.018) and the chroma (δC=2.7±2.6 degrees, p=0.045). Teeth demineralization decreased the lightness (δL=8.5±5.6 degrees, p=0.028) and the hue (δH=6.1±6.2 degrees, p=0.049), and increased the chroma (δC=5.6±3.8 degrees, p=0.028). Teeth oxidization increased the lightness (δL=19.9±6.5 degrees, p=0.001) and the chroma (δC=3.7±2.2 degrees,

p=0.049). Control teeth did not exhibit any significant changes in their shade parameters (δL=1.8±1.0 degrees, δC=-1.6±2.7 degrees and δH=1.2±2.8 degrees).

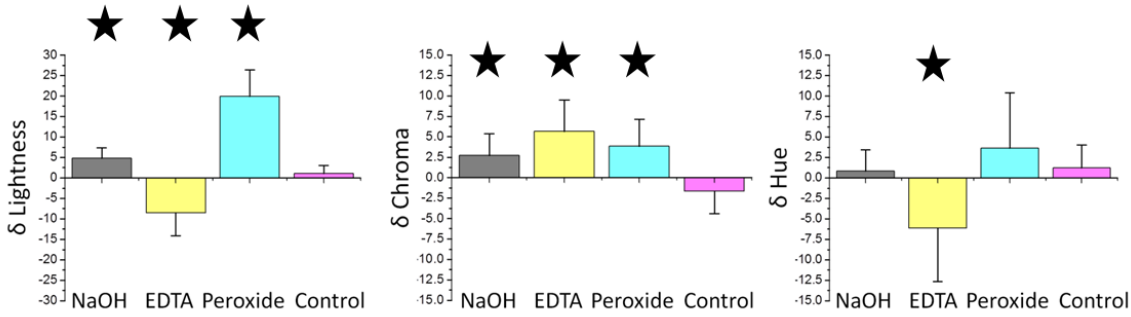

Fig.6.3 - Bar graphs illustrating the changes in tooth shade parameters (lightness (a) , chroma (b) and hue (c)) among the treated groups: NaOH (group 1), EDTA (group 2), H_2O_2 (group 3) or distilled water as control (group 4), respectively. * indicates statistically significant differences in shade parameter after specific treatment.

6.5.2. Effect of the oxidizing solution on the deproteinized teeth and on the demineralized teeth

Elemental analysis of the deproteinized (Group 5) and demineralized (Group 6) teeth determined that no significant changes occurred in tooth enamel organic and inorganic contents following their treatment with the oxidizing solution (Fig.6.4a, b, d). The O/C ratio was significantly increased in the demineralized teeth (δO/C=0.20±0.14, p=0.018), but not in the deproteinized teeth (Fig.6.4c), indicating that the oxidation reaction was more intense in teeth of high organic content (demineralized). Oxidation of the deproteinized or the demineralized teeth did not significantly influence enamel crystallinity (Fig.6.5), confirming that peroxides active radicals do not affect the mineral structure.

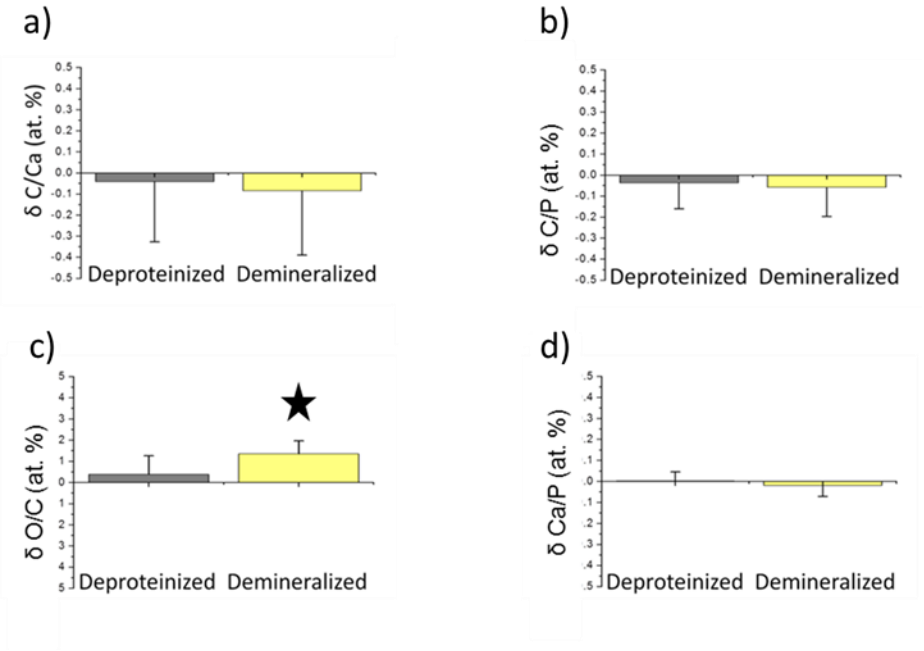

Fig.6.4 - Bar graphs demonstrating the elemental analysis obtained by EDS analysis of the labial surfaces of the deproteinized teeth (group 5) and the demineralized teeth (group 6) after four days of H_2O_2 treatment. * indicates statistically significant changes in elemental constitutes after specific treatment.

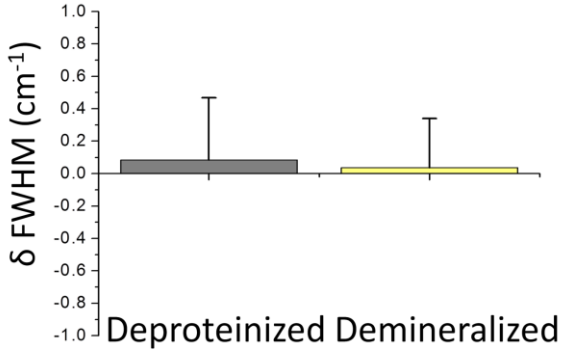

Fig.6.5 - Bar graph depicting the crystallinity analysis of the deproteinized teeth and demineralized teeth enamel following their treatment with H_2O_2.

Shade analysis indicated that unlike the deproteinized teeth, oxidation of the demineralized teeth resulted in a significant improvement in shade lightness (δL=10.7±5.8 degrees, p=0.018) and chroma (δC=5.5±3.0 degrees, p=0.043) (Fig. 6.6a, b). These results indicated that H_2O_2 was effective only in the presence of organic structure within tooth enamel.

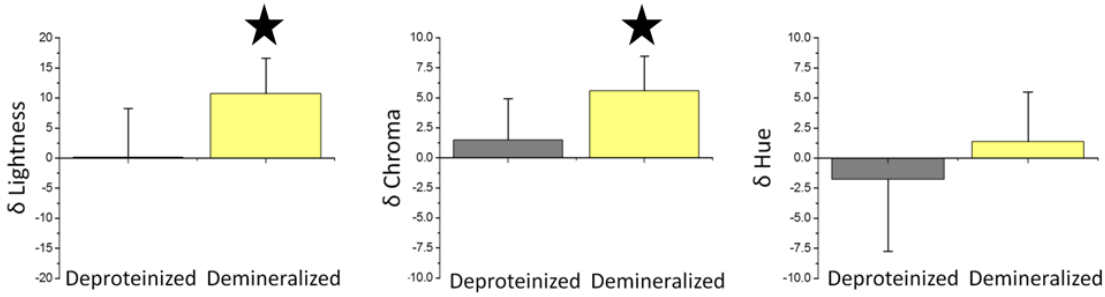

Fig.6.6 - Bar graphs illustrating the changes in tooth shade parameters (lightness (a) , chroma (b) and hue (c)) among the deproteinized teeth and demineralized teeth after four days of H_2O_2 treatment. * indicates statistically significant differences in shade parameter after specific treatment.

6.6. Discussion

In this study, teeth were treated with different chemical solutions. Teeth enamel elemental and crystallographic changes following their specific chemical treatment were quantified and correlated to changes in teeth shade. The findings of the above experiments helped us explain teeth bleaching mechanism by peroxides**.**

Deproteinizing solution

NaOH, a strong alkali caustic metallic base, is often used as ingredient in toothpaste and endodontic irrigant solution to remove the organic debris, thus improving their cleanness efficacy [253, 254]. NaOH is capable of removing the organic and protein structures in

both tooth enamel and dentin without affecting tooth mineral composition [255]. Our study confirmed the above observations by demonstrating that teeth treated with NaOH (the deproteinizing solution) exhibited a decrease in their enamel organic relative content (expressed by C/Ca and C/P ratios) without inducing any changes in their mineral component (Fig.6.1 and Fig.6.2b). Tooth shade analysis determined that the deproteinized teeth exhibited a minor improvement in their shade lightness and chroma (Fig.6.3 a, b). The above results confirmed our previous observations indicating that tooth organic content plays a minor role in the overall tooth shade [104].

Demineralizing solution

Demineralizing agents such as EDTA are often used as ingredients in endodontic irrigant solutions to remove the inorganic debris (smear layer) from the internal surface of the tooth root canal without affecting the organic matrix [256, 257]. The results of the present study confirmed the above observations by demonstrating that teeth treated with the EDTA (the demineralizing solution) exhibited a relative increase in their enamel organic content due to decrease in calcium and phosphate contents (Fig.6.1a, b). Moreover, crystallinity analysis demonstrated that tooth demineralization decreased enamel crystallinity, confirming the mineral loss observed with SEM-EDS analysis (Fig.6.2 b). Shade analysis conducted on these teeth determined that tooth demineralization highly deteriorated the overall shade (Fig.6.3a, b) [258]. The above results confirmed our previous observation indicating that tooth enamel mineral content plays the most important role in the overall tooth shade while the organic matrix, mainly transparent, has limited effect on tooth shade [90, 104, 258, 259].

Oxidizing solution

Hydrogen peroxide is commonly used for cleaning (i.e. cloths), bleaching (i.e. hair and teeth) and disinfection (i.e. skin wounds). Its oxidizing capability is controlled by the substrate and reaction environment (temperature, pH and light) [10]. Peroxide produces highly reactive oxygen species able to oxidize or remove a wide variety of organic and inorganic structures [10]. In this study, hydrogen peroxide treatment resulted in a great increase in teeth lightness and chroma shade parameters compared to what is usually observed in clinical tooth bleaching procedures [260]. This could be related to the fact that the teeth used in this study were treated for longer period of time compared to real clinical scenario [261].

In this study, unlike NaOH and EDTA solutions, the H_2O_2 solution did not induce any changes in enamel organic or inorganic relative content, or in its crystallinity (Fig.6.1 and Fig.6.2b). H_2O_2 treatment can cause minimum demineralization if the solution is acidic, however, in our study, we applied neutral pH H_2O_2 solution, and it did not demineralize the enamel [9, 262-266]. Interestingly, the only noticed chemical change was the increase in oxygen relative content in tooth enamel structure, which reflects an oxidizing process (Fig.6.1c). Teeth treated with H_2O_2 oxidizing solution were much whiter than those treated with NaOH or EDTA solutions (Fig.6.3a, b). The above results indicated that the whitening effect of peroxide was obtained by oxidizing tooth tissues without causing any deproteinization or demineralization effect.

In order to determine which tooth component (organic or inorganic) was oxidized, we conducted further experiments. We treated a group of deproteinized teeth and another group of demineralized teeth with oxidizing solutions to determine the effect of peroxide

on tooth shade after removing their organic and inorganic component, respectively. Our results indicated that the application of oxidizing solution on the deproteinized teeth or the demineralized teeth did not cause further changes in their enamel organic or mineral content and crystallinity (Fig.6.4 and Fig.6.5). An increase in relative oxygen content was noticed in the demineralized teeth, but not in the deproteinized teeth, indicating that an oxidation reaction is more intense in teeth with high organic content (Fig.6.4c). H_2O_2 significantly affected tooth shade in the demineralized teeth compared to the deproteinized teeth (Fig.6.6). In summary, three observations indicated that H_2O_2 whitens teeth by oxidizing their organic structures: i) the absence of changes in tooth enamel organic and inorganic relative contents after oxidations; ii) deproteinized teeth did not get oxidized; iii) deproteinized teeth did not get whiter with bleaching.

Previous studies claimed that tooth shade is affected by the intrinsic organic chromophores present in enamel [267, 268]. Organic chromophores are colorful chemical molecules which consist of either conjugated pi systems such as aromatic compounds or bioinorganic metallic complexes such as chelates [269]. These chemical compounds can be easily identified with Fourier Transform Infra-Red (FTIR) and Raman spectroscopies [270, 271]. However, studies on tooth enamel using FTIR or Raman spectroscopies were never able to detect any of these potential chromophores [104, 272-274].

It has been hypothesized in the past that peroxide whitens teeth by destructing the chromophores modifying them into more translucent molecules that reflect less light [10, 123, 130]. However, this hypothesis is very weak since: i) the organic chromophores concentration, if they exist, in tooth enamel is extremely low (below the detection limit of many spectroscopy techniques), and ii) several studies have shown that following tooth

bleaching, the translucency of tooth enamel decreased significantly, making it more opaque [275-277]. Accordingly, hydrogen peroxide whitening effect cannot be attributed to the destruction of chromophores because their presence in tooth enamel is yet to be proven.

Several studies have shown that tooth enamel organic matrix is mainly composed of amide groups that represent enamel proteins [104, 270-272]. In our study, we have found that hydrogen peroxide does not modify the organic nor inorganic relative contents of dental enamel (Fig.6.1 and 6.2), but it oxidizes their enamel organic matrix (Fig.6.1 and 3). Therefore, oxidation of enamel protein (Fig.6.1) and the increase in enamel opacity following peroxide treatment seem to indicate that the peroxide whitens teeth by oxidizing its transparent organic matrix into an opaque whiter material [87, 273, 275]. Accordingly, our results provided the first comprehensive explanation of the mechanism by which peroxide whitens teeth.

6.7. Clinical Implications

The results of this study provided a valuable insight into the variability in bleaching efficacy among people. We have shown that tooth bleaching by peroxides is controlled by the tooth organic content. Therefore, the contrasted variability in tooth organic content among people [104] could be one of the reasons behind the wide variation in results obtained following tooth bleaching treatment.

Despite the fact that the present study was not designed to investigate the influence of age on tooth bleaching, the results of this study could explain why bleaching works better in young patients than in older patients. The organic component of the permanent teeth gets

reduced with age as a result of the ongoing remineralization in the oral environment [278, 279]. Therefore, the age related decrease in the protein content might be one of those reasons behind the lower bleaching efficacy in teeth of elder individuals [280]. However, future studies will have to be performed in order to confirm these predictions.

In this study, we described the mechanism behind tooth bleaching by hydrogen peroxide. These findings are of great relevance in dentistry since they improve our understanding of tooth aesthetics, and will help develop new possible bleaching treatments. Also, this study provides a better understanding of the mechanism by which current bleaching treatments affect teeth, and could help address their limitations and disadvantages.

Chapter 7: Alternative Way to Whiten Teeth

7.1. Introduction

Teeth are mineralized tissues made almost entirely of inorganic carbonated hydroxyapatite (CHA) nanocrystals arranged in a unique and highly organized three dimensional architecture that contributes to their remarkable features [29, 43, 46, 48-51]. It has been recently shown that tooth crystallographic ultrastructure has a major role in determining the tooth physical and optical properties [87, 281]. Tooth enamel microhardness and fracture resistance has an inverse correlation with the size of its CHA crystals along the c-axis that follows the Hall-Petch model [85, 87]. Moreover, teeth with smaller enamel crystals have a lighter shade than teeth with bigger crystals [281]. Therefore, we hypothesized that inducing a reduction in the size of apatite nanocrystals in enamel would result in teeth with whiter appearance and more resistant to the harsh wear environment of the oral cavity.

The human body regulates the crystal structure of its mineralized tissues by incorporating ions, thus controlling their physical, chemical and biological properties [51]. The CHA crystals of tooth enamel are capable of undergoing multiple ionic substitutions and incorporate relatively large amounts of carbonate ions as well as a small amount of trace elements such as Cl, K, F, Fe and Mg [29, 52, 53]. Magnesium has been reported to influence crystallographic structure and mechanical properties in synthetic apatites [43, 48, 49, 62, 71-74], and it inhibits the crystals growth of mineralized tissues limiting their crystals size [70, 282]. However, to the best of our knowledge, the effect of incorporating Mg ions directly on biological apatites has not been investigated yet.

Accordingly, this study was designed to assess if Mg ions can react with enamel apatite and investigate the possible changes in crystallographic and physical properties of dental enamel that might occur after treatment with saturated solutions of magnesium.

7.2. Materials

7.2.1. Chemicals and Solution Preparation

All reagents were obtained from Sigma Aldrich (Oakville, Ontario), and used without further purifications in order to prepare three solutions for treating the teeth: i) de-ionized distilled water (DW; control); ii) saturated magnesium chloride ($MgCl_2$) solution; and iii) saturated magnesium nitrate ($Mg(NO_3)_2$) solution.

7.2.2. Sample Collection and Preparation

A sample of 86 anterior teeth from the upper jaw was used in this section and were collected and prepared as mentioned earlier. Five analyses were performed in order to assess the effect of Mg ions on enamel: X-ray diffraction (XRD), scanning electron microscope energy dispersive spectroscopy (SEM-EDS), Raman spectroscopy, Vickers microhardness (VMH) and tooth shade spectroscopy. The preparation of enamel specimens varied according to the type of analysis as follows: enamel powder for XRD, tooth sections for SEM-EDS, resin embedded cross sections of enamel for Raman and VMH, and whole teeth for tooth shade measurements.

For tooth shade measurements, 30 whole teeth were used. For XRD, enamel powder was collected from 24 teeth by trimming the tooth crown with a carbide bur (FG56, SDS Kerr, Orange, CA) attached to a high-speed dental hand-piece cooled with DW (TA-.98LW, Synea, Canada). The enamel powder was dried at 37°C before storage for XRD

experiments. For EDS, 10 teeth were each cut into 3 coronal sections of enamel as described earlier and each one of the sections was randomly assigned to one of the three treatments. For VMH and Raman analysis, 11 teeth were sectioned as described earlier and each coronal section was resin-embedded in separated methyl methacrylate blocks (DP-Ortho-F, DenPlus, Montreal, Canada). The blocks were trimmed and polished by means of water-cooled trimmer and 240- to 1200-grit silicon carbide papers (Paper-c wt, AA Abrasives, Philadelphia, PA) to obtain a flat surface. The specimens were placed in an ultrasonic device containing distilled water for 5 minutes to remove polish debris and smear layer.

7.3. Methods

7.3.1. Tooth Enamel Treatment

Enamel samples (powder, resin blocks, sections and whole teeth) were randomly assigned into one of the three treatment solutions (DW, $MgCl_2$ and $Mg(NO_3)_2$). The number of samples per group varied for each experimental technique as follows: SEM-EDS (n = 10), XRD (n = 8), Raman (n=11), microhardness (n=11) and tooth shade (n =10). Each tooth enamel specimen was immersed (enamel section in 1 ml; whole tooth in 10 ml; resin-block in 15 ml) or mixed thoroughly (100 mg of enamel powder in 1ml) in one of the three treatment solutions and left to react for 14 hours. After treatment, all specimens except enamel powder were sonicated in distilled water for 30 minutes and left to dry. Enamel powder was separated from the treatment solution through 4 cycles of centrifugation at 10 000 rpm and washing in DW, before finally drying at 37 °C prior to XRD analysis.

7.3.2. Measurements

Enamel samples were analyzed before and after each specific treatment with SEM-EDS, XRD, Raman spectroscopy, VMH and tooth shade spectroscopy. Measurements were obtained in triplicates, except for Raman, where eight measurements were obtained.

EDS was used to determine the elemental composition of the tooth enamel samples. For each sample, the elemental composition was analyzed and the following elemental ratios were calculated: carbon-to-phosphate (C/P), calcium-to-phosphate (Ca/P), chloride-to-phosphate (Cl/P), oxygen-to-phosphate (O/P), sodium-to-phosphate (Na/P), magnesium-to-phosphate (Mg/P) and (calcium-and-magnesium)-to-phosphate ((Ca+Mg)/P).

XRD was performed to characterize the crystallographic ultrastructure of enamel powder, Raman was used to measure the crystallinity index and the carbonate-to-phosphate ratio The crystallinity index was quantified using the full width at half maximum intensity value (FWHM) of the phosphate symmetric stretch peak (ν_1PO_4) at ~ 960 cm^{-1} [177-180]. The relative intensity of type B carbonate peak (at 1070 cm^{-1}) to the main phosphate peak (at 960 cm^{-1}) was used to calculate the carbonate-to- phosphate ratio [54, 171]. VMH was used to measure the mechanical properties of dental enamel, while the spectrophotometer was used assess the tooth shades.

7.4. Results

Prior to treatment, there were no significant differences between the assigned groups of tooth specimens in all measurements, but the changes that occurred after the treatment are detailed underneath.

7.4.1. Reaction of Mg Ions with Dental Enamel

EDS analysis revealed that elemental composition of enamel changed after treatment with Mg solutions but not with DW (Fig.7.1). Both Mg solutions ($MgCl_2$ and $Mg(NO_3)_2$) increased the concentration of magnesium ([Mg]) ($p< 0.05$), and the magnesium-to-phosphate ratio ([Mg]/[P]) ($p< 0.05$) in enamel, while decreasing the calcium-to-phosphate ratio ([Ca]/[P]) ($p< 0.05$), without affecting the (calcium-and-magnesium)-to-phosphate ratio ([Ca+Mg]/[P]) (Fig.7.1.b). Treatment with $MgCl_2$ increased the concentration of chloride ([Cl]) ($p< 0.05$).

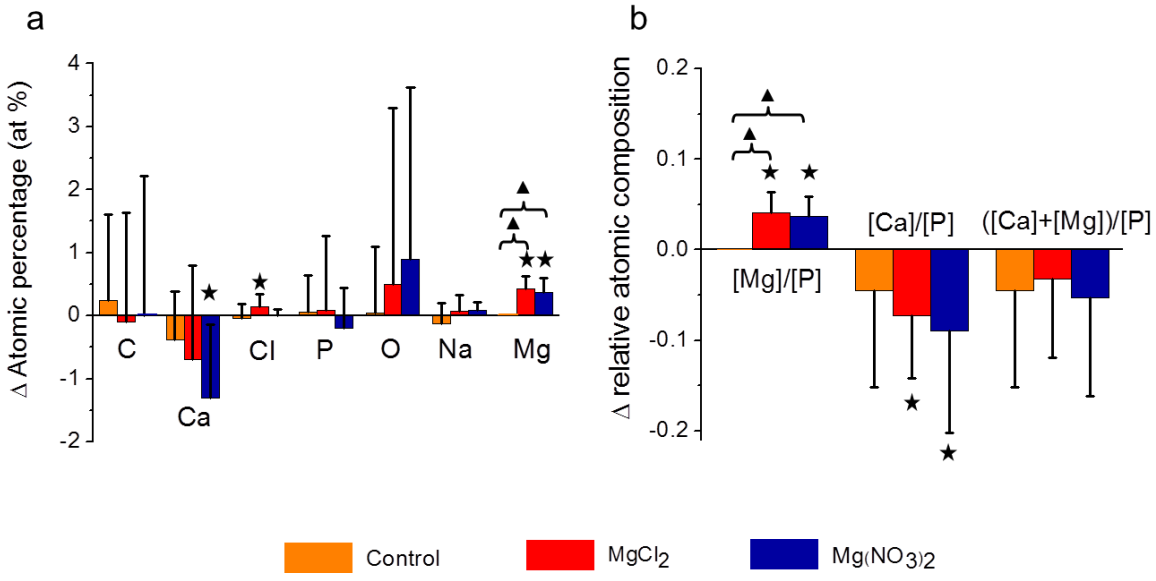

Fig.7.1 - a) Bar graphs of EDS data demonstrating the change in elemental atomic percentage (Δ at %) of the enamel surfaces after treatment. b) Bar graph illustrating the changes in ratios among the 3 different groups of specimens. * indicates significant change within the same specimens after each treatment; ▲ indicates significant difference between the different groups ($p< 0.05$).

The XRD patterns revealed that enamel apatite phase and cell lattice were preserved after treatment and did not show any new phases. However, the XRD peaks became broader and less resolved after treatment with Mg solutions (Fig.7.2.a). Unlike DW, Mg solutions induced a decrease in enamel crystal size along the c-axis of apatite nanocrystals (Fig.7.2. g). Neither DW nor Mg solutions had any effect on crystal dimension along the a-axis (Fig.7.2. h).

Upon Raman analysis, all samples, before and after treatment, demonstrated phosphate (ν_1, ν_2, ν_3, ν_4) and carbonate bands (type A and type B) characteristic of carbonated apatite (Fig.7.3.d-f). The strongest peak at 960 cm^{-1} was attributed to the symmetric stretching mode of phosphate ($\nu_1PO_4^{3-}$) in carbonated apatite. Peaks at 1045 cm^{-1} and 1024 cm^{-1} were assigned to $\nu_3PO_4^{3-}$, peaks at 610 cm^{-1} and 580 cm^{-1} to $\nu_4PO_4^{3-}$, and peak at 430 cm^{-1} to $\nu_2PO_4^{3-}$. Furthermore, peaks at 1070 cm^{-1} and 1103 cm^{-1} were assigned to B type and A type CO_3^{2-}, respectively.

The position of the main $\nu_1PO_4^{3-}$ peak (at 960 cm^{-1}) and the carbonate-to-phosphate ratio (type B CO_3^{2-} / $\nu_1PO_4^{3-)}$ remained constant in all samples after treatment. This indicates that neither DW nor Mg solutions induced changes in enamel carbonate and phosphate content. FWHM measurements revealed that both Mg solutions had a different effect on enamel crystallinity (1/FWHM) than DW (Fig.7.2.i). The Mg solutions decreased crystallinity ($p< 0.05$), while DW did not induce any change.

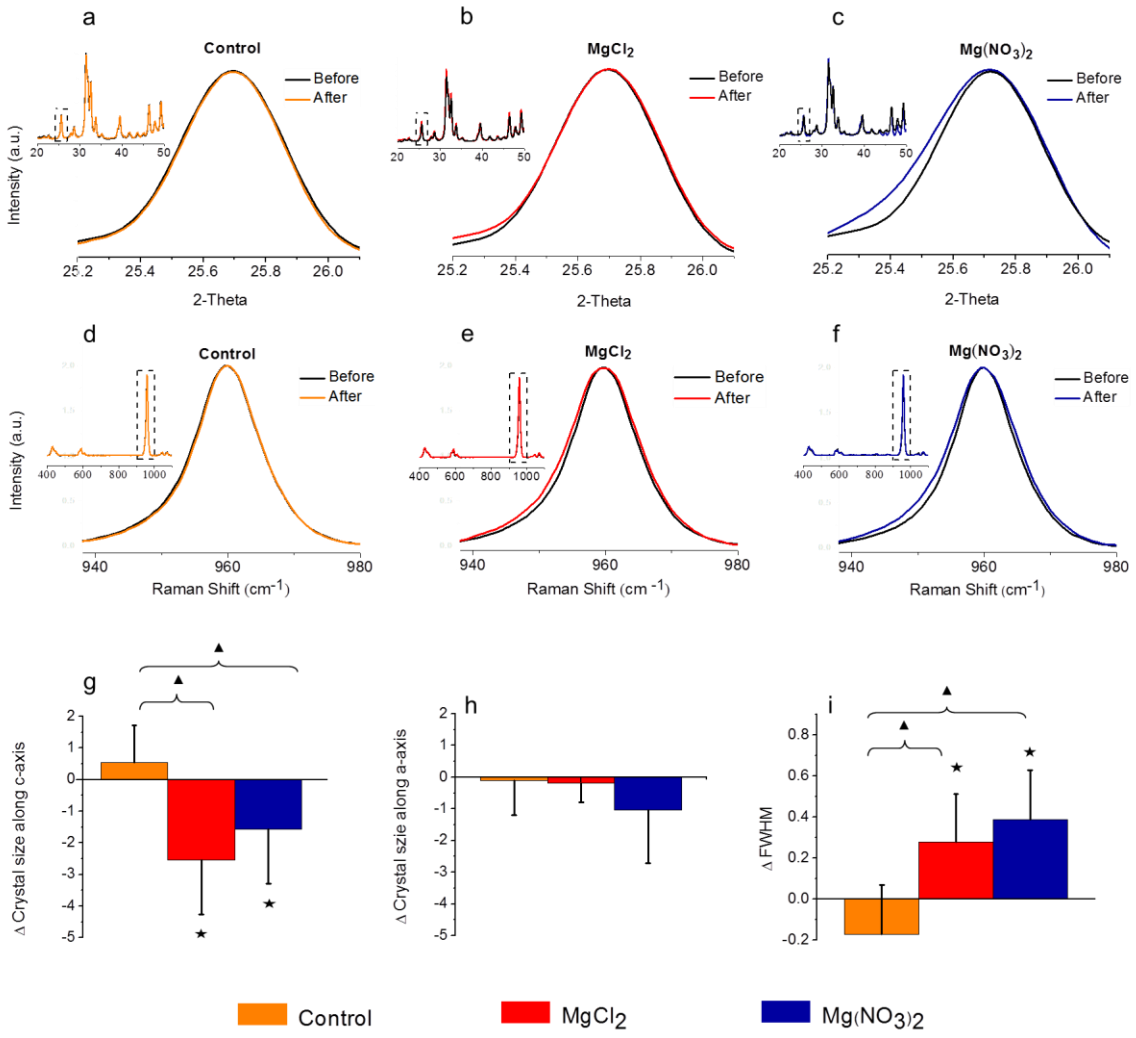

Fig.7.2 - a-c) XRD spectra of representative enamel powder before and after treatment with the 3 different solutions. d-f) Raman spectra of representative enamel powder before and after treatment with the 3 different solutions. g-i) bar graphs illustrating changes in crystals size along the c-axis (g), crystal size along the a-axis (h) and full width at half maximum of the $\nu_1PO_4^{3-}$ Raman peak (at 960 cm^{-1}) (FWHM) among the 3 different groups of specimens (i). * indicates significant change within the same specimens after each treatment; ▲ indicates significant difference between the different groups ($p< 0.05$).

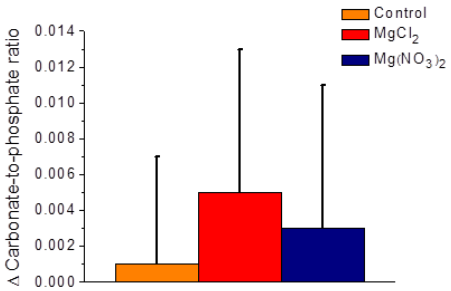

Fig.7.3 - Bar graphs demonstrating the changes in carbonate-to-phosphate ratio among the 3 different groups of specimens. There were no significant changes within each treated group or across the different groups ($p \geq 0.05$).

7.4.2. Effect of Mg Ions on Mechanical and Optical Properties of Enamel

$MgCl_2$ and $Mg(NO_3)_2$ had a comparable effect on enamel hardness ($p > 0.05$) and it was different than DW ($p < 0.05$ (Fig.7.4a). Surface hardness did not change significantly after treatment with (DW) ($p > 0.05$) but treatment with $MgCl_2$ and $Mg(NO_3)_2$ increased surface hardness ($p < 0.05$). Unlike DW, $MgCl_2$ and $Mg(NO_3)_2$ solutions induced changes in microhardness that were proportional to changes in crystallinity ($p < 0.05$ (Fig.7.4b).

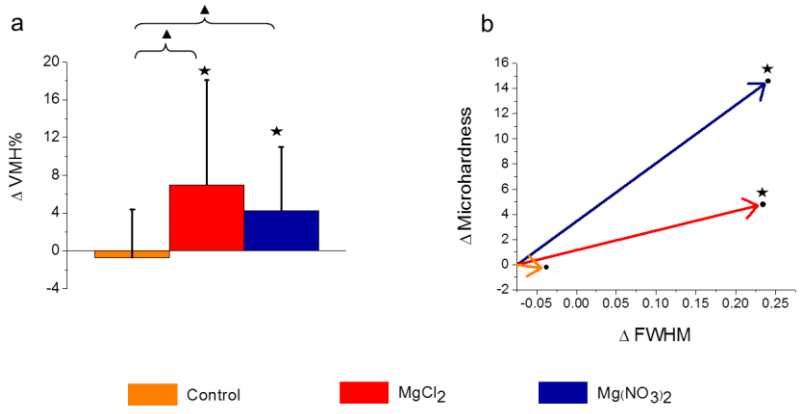

Fig.7.4 - a) Bar graphs illustrating percentage changes in Vickers microhardness values (ΔVMV %) among the 3 different groups of specimens. b) 2D vector plot of change in microhardness over change in crystallinity (Δ1/Raman). * indicates significant change within the same specimens after each treatment; ▲ indicates significant difference between the different groups ($p < 0.05$).

Unlike DW, magnesium solutions had a significant effect on tooth shade "Lightness" and "Chroma" ($p < 0.05$) (Fig.7.5 and Fig.7.6). Mg treatments induced an increase in Lightness and Chroma ($p < 0.05$), while DW did not. $MgCl_2$ had a stronger effect on Chroma than $Mg(NO_3)_2$. Also, treatment with $MgCl_2$ had a different effects on the tooth Hue than DW and $Mg(NO_3)_2$; $MgCl_2$ treatment increased Hue ($p < 0.05$), while treatment with DW and $Mg(NO_3)_2$ did not induce any significant change in Hue.

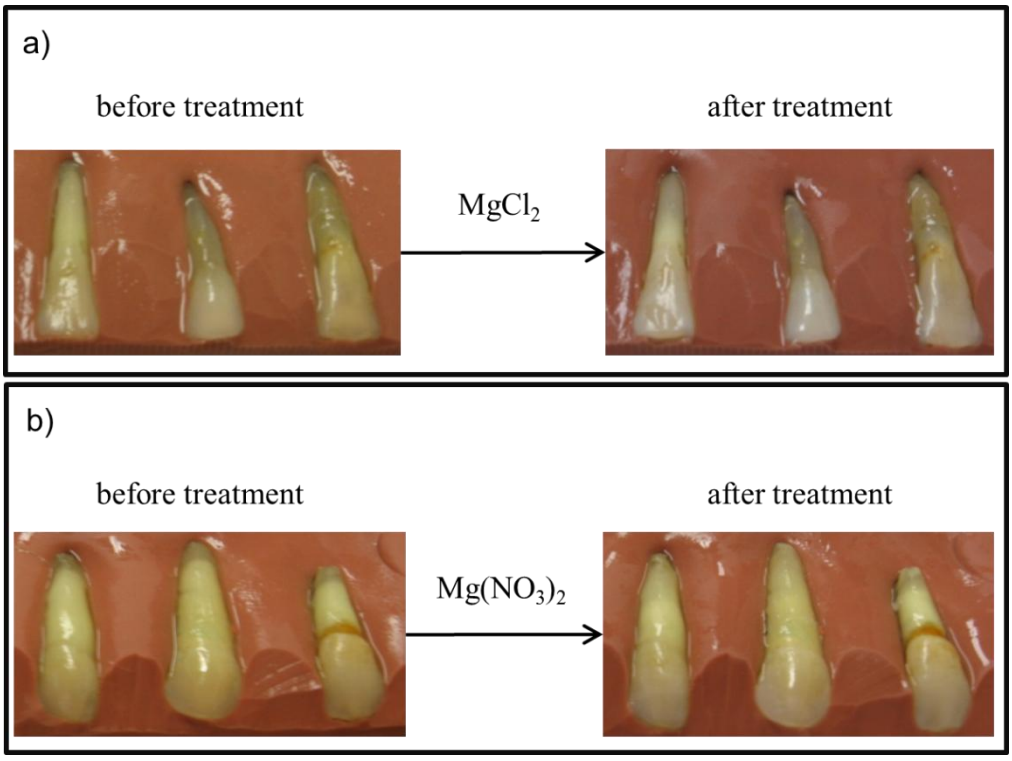

Fig.7.5 - Photographs showing teeth shades before and after treatment with (a) magnesium chloride $MgCl_2$ and (b) magnesium nitrate $Mg(NO_3)_2$.

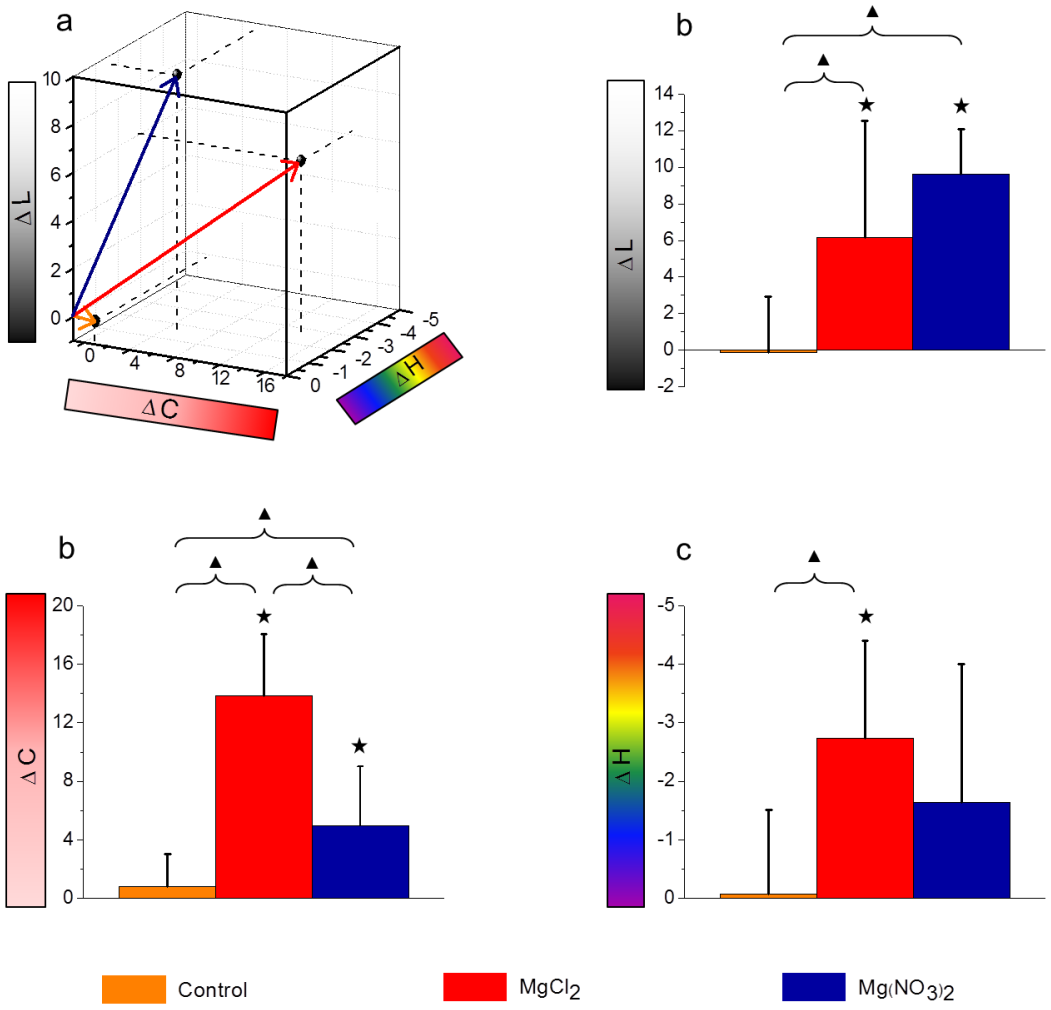

Fig.7.6 - a) 3D Vector plot illustrating the change on tooth shade parameters lightness (ΔL), chroma (ΔC) and hue (ΔH). b-c) Bar graphs illustrating the change in tooth shade parameters ((ΔL) change in lightness, (ΔC) change in chroma, (ΔH) change in hue) among the 3 different groups of specimens. * indicates significant change within the same specimens after each treatment; ▲ indicates significant difference between the different groups ($p < 0.05$).

7.5. Discussion

In this study we showed that Mg ions can react with enamel probably by exchanging with calcium ions and induce crystallographic changes in enamel carbonated hydroxyapatite (CHA) which affect dental enamel properties. This study confirms the evident role

crystallographic structure of dental enamel has on the optical and mechanical properties of teeth and exploits this information to tailor tooth properties.

7.5.1. Reaction of Mg Ions with Dental Enamel

Previous studies investigated the effect of incorporating Mg ions in synthetic HA [43, 48, 49, 62, 71-77]. However, to best of our knowledge, this is the first study to investigate the direct effect of Mg ions on biological HA. The amount of Mg ions that can be included in synthetic HA is usually small which is most probably limited to its surface and sometimes forms a less crystalline superficial layer [78, 79]. Nonetheless, incorporation of small amounts of Mg ions in synthetic apatites can induce changes in the crystallographic structure and influence the crystal size, crystallinity, morphology and stability of the apatite structure [78, 79].

Our Raman and XRD results revealed that even though the amount of Mg ions incorporated in dental enamel was small, it was sufficient to induce a substantial decrease in the crystals size of enamel apatite. XRD analysis showed that enamel crystals along the c-axis were smaller after treatment with Mg solutions. This is also confirmed by the broadening observed in the main phosphate peak in Raman spectra; that is invariant for the a-axis of CHA crystals and depends solely on their c-axis [174]. Similarly to what has been reported in the literature on synthetic hydroxyapatites doped with Mg ions [77], we observed that Mg ions had no significant effect on the crystals lattice of enamel hydroxyapatite.

Synthetic HA can grow through the fusion of smaller crystals either by sintering HA or by ion deposition (Fig.7.7a) [283]. Mg ions competes with Ca ions on CHA, in fact their

incorporation causes inhibition of crystal growth by ion deposition [76]. Moreover, their presence on the crystal boundaries also inhibits crystal growth fusion with neighbouring crystals. It is known that during development, enamel apatite crystals grow in the direction of the c-axis [40] and that Mg ions can be incorporated in immature tooth enamel rendering it less crystalline and less mineralized by inhibiting the crystal growth in this specific direction [70]. Accordingly, our data suggest that it is possible that Mg substitution for Ca is occurring on the crystal surface causing break-up of the crystals into smaller crystals along the c-axis reversing the crystal fusion process (Fig.7.7.b).

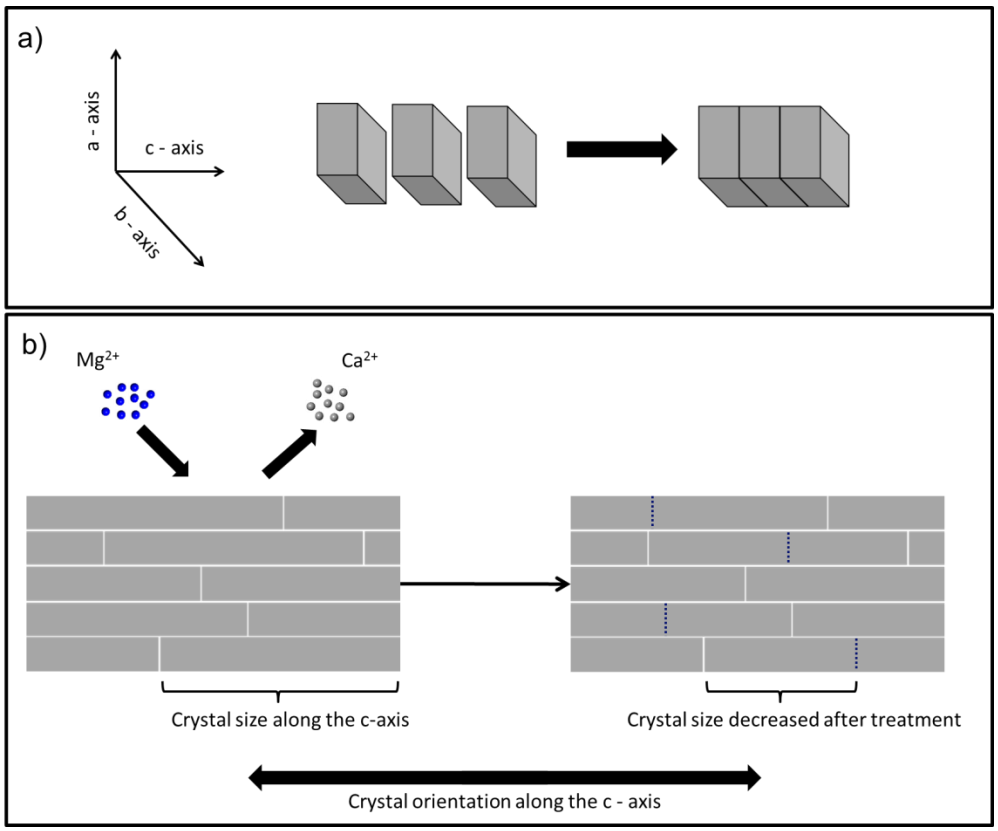

Fig.7.7 - a) Scheme illustrating the fusion of smaller crystals along their c-axis into bigger crystals in synthetic apatites, b) Scheme illustrating the break-up of hydroxyapatite crystals of tooth enamel along their c-axis into smaller crystals after treatment with Mg solutions.

7.5.2. Effect of Mg Ions on Enamel Physical Properties

Shade and microhardness of dental enamel are influenced by its macrostructure, crystallography, hydration and chemical composition [24, 61, 80, 87-89, 281]. Teeth with smaller enamel crystals along the c-axis are harder and have a lighter shade than teeth with bigger enamel crystals [87, 281]. Our study showed that treatment with Mg solutions increased microhardness of tooth enamel and this change was proportional to the change in crystal size.

Direct observational methods could not be applied to confirm the changes in crystallographic structure caused by Mg solution that was detected indirectly with XRD and Raman. However, crystal size effect on enamel's microhardness is known to follow the Hall-Petch model for polycrystalline materials [87]. The observed increase in microhardness confirms that the crystals decrease in size, since microhardness is inversely proportional to crystal size only and not to other crystallographic parameters (i.e. crystallinity) [87, 284, 285]. This increase may be attributed to the decrease in crystal size along the c-axis observed by XRD and Raman, since the crystallinity index of a material can decrease by either reduction in crystallite size or decrease in the perfection of atoms ordering [51].

7.5.3. New Tooth Whitening System

For over a century, tooth whitening has been focused on the use of oxidizing agents such as hydrogen peroxide and carbamide peroxide [10]. However, these substances can be hazardous and may have a negative effect on enamel hardness [106, 126, 127, 139, 147, 158, 159, 286-297]. Moreover, these substance only affect a small portion of the tooth enamel (the organic component) [237]. In our study, by using Mg solutions to alter the

crystallographic structure of tooth enamel, we have induced changes in the larger portion of tooth enamel (mineral component). This is the first study ever to use a new approach for whitening teeth based on the control of the crystallographic structure of enamel and without using oxidizing agents. This approach avoids the potential harmful effects of oxidizing agents while providing additional benefits such as increased enamel hardness.

7.6. Clinical Implications and Significance

Even though crystallographic composition of mineralized tissues has been associated to their mechanical and optical properties [87, 281], this is the first study ever to show a cause-effect relationship that can be exploited to improve the properties of teeth. Mineralized tissues in young individuals are usually richer in magnesium and have smaller crystals compared to older people [298]. However, during the aging process magnesium levels drop in the body, especially in mineralized tissues and saliva [64-66]. Our study suggests that this might be one the factors that explains why mineralized tissues in older people have bigger crystals and are more brittle [85]. Adding Mg ions to tooth enamel, not only improves the shade of the teeth, but might be also making the teeth virtually younger by re-introducing magnesium previously lost from its structure.

7.7. Limitations and Future Studies

All the analyses were done on extracted (non-vital) teeth due the unfeasibility of performing the experiments on teeth in patients' mouth. Even though it is known that there are no large differences in tooth shade before and after tooth extraction [299], the present study did not simulate the oral environment. Future studies are required to investigate the potential effects of saliva or salivary pellicle while treating tooth enamel with Mg solutions. Enamel crystals are the building blocks of larger enamel structures

(prisms or rods) [29], and it would be logical to expect that changes in crystal size could result in alterations in these larger structures, however, future studies are needed to investigate this possibility.

The size of bone crystals influences the mechanical properties; larger crystals make bone more brittle and less able to withstand normal loads [300, 301]. It is known that bone tissues with larger crystals have lower magnesium content [301, 302] and magnesium ions have a very important role in decreasing bone loss, fragility and hip fracture risk while increasing bone strength in the body [282, 303-306]. This study opens new modalities of treatment for improving mechanical properties of mineralized tissues, such as bone, enamel and dentine, and perhaps decreasing risk of fracture through controlling the crystal structure of teeth and other mineralized tissues.

Chapter 8: General Conclusions

From the results of this thesis we can arrive at the following conclusions:

1. Even though 96% of enamel is composed of HA (mainly calcium and phosphate), our results indicate that the tooth enamel surface seems to be covered by a carbon rich layer which is mainly composed of hydrocarbons and has a relatively high calcium content**.** This layer explains the higher affinity of certain anionic molecules to adhere to the outer surface of dental enamel and stain the teeth.

2. Bleaching does not remove substantial amounts of neither the organic nor the inorganic material from tooth, and it whitens teeth by mere oxidation of its transparent organic matrix into a whiter material. These findings are of great relevance in dentistry since they provide the first complete explanation on the mechanism of tooth bleaching by peroxides. In addition, they also help understand the limitations and disadvantages of applying peroxide on human teeth.

3. Our data shows that magnesium ions can react with tooth enamel and induce changes in its crystallographic and physical properties. Incorporation of magnesium ions into tooth enamel results in a significant decrease in crystals size along the c-axis, which in turn increased the tooth microhardness, and made the teeth whiter. Not only we prove a new alternative method to whiten teeth, this study is the first to show the direct effects of Mg ions on the physical properties of biological apatites and introduces a new mechanism to control their physical properties.

List of References

1. Kasemo, B. and J. Lausmaa, *Material-tissue interfaces - The role of surface properties and processes.* Environmental Health Perspectives, 1994. **102**: p. 41-45.

2. Castner, D.G. and B.D. Ratner, *Biomedical surface science: Foundations to frontiers.* Surface Science, 2002. **500**(1-3): p. 28-60.

3. El-Ghannam, A., *Bone reconstruction: from bioceramics to tissue engineering.* Expert Review of Medical Devices, 2005. **2**(1): p. 87-101.

4. Kasemo, B. and J. Lausmaa, *Biomaterial and implant surfaces - On the role of cleanliness, contamination, and preparation procedures.* Journal of Biomedical Materials Research-Applied Biomaterials, 1988. **22**(A2): p. 145-158.

5. Stanford, C.M., *Surface Modification of Biomedical and Dental Implants and the Processes of Inflammation, Wound Healing and Bone Formation.* International Journal of Molecular Sciences, 2010. **11**(1): p. 354-369.

6. Wilson, C.J., et al., *Mediation of biomaterial-cell interactions by adsorbed proteins: A review.* Tissue Engineering, 2005. **11**(1-2): p. 1-18.

7. Hattab, F.N., M.A. Qudeimat, and H.S. al-Rimawi, *Dental discoloration: an overview.* J Esthet Dent, 1999. **11**(6): p. 291-310.

8. Watts, A. and M. Addy, *Tooth discolouration and staining: a review of the literature.* Br Dent J, 2001. **190**(6): p. 309-316.

9. Joiner, A., *Review of the effects of peroxide on enamel and dentine properties.* Journal of Dentistry, 2007. **35**(12): p. 889-96.

10. Joiner, A., *The bleaching of teeth: A review of the literature.* Journal of Dentistry, 2006. **34**(7): p. 412-419.

11. Attin, T., et al., *Effect of bleaching on restorative materials and restorations - a systematic review.* Dental Materials, 2004. **20**(9): p. 852-861.

12. CP, F., *Etiology of the discoloration of teeth.* Dental Cosmos, 1861. **3**: p. 133-6.

13. JD, W., *Bleaching.* Dental Register of the West, 1861(15): p. 576-7.

14. AW, H., *Hydrogen dioxide (in the treatment of alveolar abscess, pyorrhea and the bleaching of teeth).* Dental Cosmos, 1882. **(10)**(24): p. 515-23.

15. HB, Y., *Bleaching mottled enamel.* Texas Dental Journal, 1942(60): p. 467-70.

16. Lin, C.H., et al., *Evaluation of the effect of laser tooth whitening.* International Journal of Prosthodontics, 2008. **21**(5): p. 415-418.

17. Gerlach, R.W., R.D. Gibb, and P.A. Sagel, *Initial color change and color retention with a hydrogen peroxide bleaching strip.* American Journal of Dentistry, 2002. **15**(1): p. 3-7.

18. Ishikawa-Nagai, S., et al., *Prediction of optical efficacy of vital tooth bleaching using regression analysis.* Color Research and Application, 2004. **29**(5): p. 390-394.

19. Kugel, G., et al., *Clinical Trial Assessing Light Enhancement of In-office Tooth Whitening.* Journal of Esthetic and Restorative Dentistry, 2009. **21**(5): p. 336-347.

20. Cate, A.r.T., *Oral Histology: Development, Structure, and Function*. 7th ed2008: Mosby Year Book Inc.

21. Berkovitz B, H.G., Moxham BJ, *Oral anatomy, histology and embryology*. 4 edition ed2009: Mosby.

22. Hu, J.C.C., et al., *Enamel formation and amelogenesis imperfecta.* Cells Tissues Organs, 2007. **186**(1): p. 78-85.

23. Cui, F.-Z. and J. Ge, *New observations of the hierarchical structure of human enamel, from nanoscale to microscale.* Journal of Tissue Engineering and Regenerative Medicine, 2007. **1**(3): p. 185-191.

24. Cuy, J.L., et al., *Nanoindentation mapping of the mechanical properties of human molar tooth enamel.* Arch Oral Biol, 2002. **47**(4): p. 281-91.

25. Hsu, C.C., et al., *Influences of ionic concentration on nanomechanical behaviors for remineralized enamel.* Journal of the Mechanical Behavior of Biomedical Materials, 2011. **4**(8): p. 1982-1989.

26. Glauche, V., et al., *Analysis of Tooth Surface Elements by Ion Beam Analysis.* Journal of Hard Tissue Biology, 2011. **20**(2): p. 99-105.

27. Fincham, A.G., J. Moradian-Oldak, and J.P. Simmer, *The structural biology of the developing dental enamel matrix.* J Struct Biol, 1999. **126**(3): p. 270-299.

28. Reitznerova, E., et al., *Determination of some trace elements in human tooth enamel.* Fresenius Journal of Analytical Chemistry, 2000. **367**(8): p. 748-754.

29. Cui, F.Z. and J. Ge, *New observations of the hierarchical structure of human enamel, from nanoscale to microscale.* Journal of Tissue Engineering and Regenerative Medicine, 2007. **1**(3): p. 185-191.

30. Popowics, T.E., J.M. Rensberger, and S.W. Herring, *Enamel microstructure and microstrain in the fracture of human and pig molar cusps.* Archives of Oral Biology, 2004. **49**(8): p. 595-605.

31. Anderson, P. and J.C. Elliott, *Rates of mineral loss in human enamel during in vitro demineralization perpendicular and parallel to the natural surface.* Caries Research, 2000. **34**(1): p. 33-40.

32. Cevc, G., et al., *The caries resistance of human-teeth is determined by the spatial arrangement of hydroxyapatite micro-crystals in the enamel.* Nature, 1980. **286**(5771): p. 425-426.

33. Girija, V. and H.C.Y. Stephen, *Characterization of lipid in mature enamel using confocal laser scanning microscopy.* Journal of Dentistry, 2003. **31**(5): p. 303-311.

34. Simmer, J.P. and J.C. Hu, *Dental enamel formation and its impact on clinical dentistry.* Journal of dental education, 2001. **65**(9): p. 896-905.

35. Hu, J.C.C., et al., *Localization of EMSP1 expression during tooth formation and cloning of mouse cDNA.* Journal of Dental Research, 2000. **79**(1): p. 70-76.

36. Krebsbach, P.H., et al., *Full-length sequence, localization, and chromosomal mapping of ameloblastin - A novel tooth-specific gene.* Journal of Biological Chemistry, 1996. **271**(8): p. 4431-4435.

37. Cerny, R., et al., *A novel gene expressed in fiat ameloblasts codes for proteins with cell binding domains.* Journal of Bone and Mineral Research, 1996. **11**(7): p. 883-891.

38. Bartlett, J.D., et al., *Molecular cloning and mRNA tissue distribution of a novel matrix metalloproteinase isolated from porcine enamel organ.* Gene, 1996. **183**(1-2): p. 123-128.

39. Fattibene, P. and F. Callens, *EPR dosimetry with tooth enamel: A review.* Applied Radiation and Isotopes, 2010. **68**(11): p. 2033-2116.

40. Simmer, J.P. and A.G. Fincham, *Molecular mechanisms of dental enamel formation.* Critical Reviews in Oral Biology and Medicine, 1995. **6**(2): p. 84-108.

41. Miranda, C.B., et al., *Evaluation of the bleached human enamel by Scanning Electron Microscopy.* J Appl Oral Sci, 2005. **13**(2): p. 204-11.

42. Hong, H.L., L.Y. Tie, and T. Jian, *The crystal characteristics of enamel and dentin by XRD method.* Journal of Wuhan University of Technology-Materials Science Edition, 2006. **21**(1): p. 9-12.

43. Ren, F., et al., *Synthesis, characterization and ab initio simulation of magnesium-substituted hydroxyapatite.* Acta Biomaterialia, 2010. **6**(7): p. 2787-2796.

44. Curzon, M.E.J. and D.C. Crocker, *Relationships of trace-elements in human tooth enamel to dental-caries.* Archives of Oral Biology, 1978. **23**(8): p. 647-653.

45. Elliott, J.C., D.W. Holcomb, and R.A. Young, *Infrared determination of the degree of substitution of hydroxyl by carbonate ions in human dental enamel.* Calcified Tissue International, 1985. **37**(4): p. 372-375.

46. Gross, K.A. and C.C. Berndt, *Biomedical application of apatites*, in *Phosphates: Geochemical, Geobiological, and Materials Importance*, M.J. Kohn, J. Rakovan, and J.M. Hughes, Editors. 2002. p. 631-672.

47. Elliott, J.C., *Calcium phosphate biominerals*, in *Phosphates: Geochemical, Geobiological, and Materials Importance*, M.J. Kohn, J. Rakovan, and J.M. Hughes, Editors. 2002. p. 427-453.

48. Bertoni, E., et al., *Nanocrystals of magnesium and fluoride substituted hydroxyapatite.* Journal of Inorganic Biochemistry, 1998. **72**(1-2): p. 29-35.

49. Bigi, A., et al., *The role of magnesium on the structure of biological apatites.* Calcified Tissue International, 1992. **50**(5): p. 439-444.

50. Bigi, A., et al., *Magnesium influence of hydroxyapatite crystallization.* Journal of Inorganic Biochemistry, 1993. **49**(1): p. 69-78.

51. Wopenka, B. and J.D. Pasteris, *A mineralogical perspective on the apatite in bone.* Materials Science & Engineering C-Biomimetic and Supramolecular Systems, 2005. **25**(2): p. 131-143.

52. Xie, R.Q., et al., *EDTA-Assisted Self-Assembly of Fluoride-Substituted Hydroxyapatite Coating on Enamel Substrate.* Crystal Growth & Design, 2011. **11**(12): p. 5206-5214.

53. Sydneyzax, M., I. Mayer, and D. Deutsch, *Carbonate content in developing human and bovine enamel.* Journal of Dental Research, 1991. **70**(5): p. 913-916.

54. Penel, G., et al., *MicroRaman spectral study of the PO4 and CO3 vibrational modes in synthetic and biological apatites.* Calcified Tissue International, 1998. **63**(6): p. 475-481.

55. Demul, F.F.M., et al., *Micro-Raman Line Broadening in Synthetic Carbonated Hydroxyapatite.* Journal of Dental Research, 1986. **65**(3): p. 437-440.

56. Xu, C., et al., *Chemical/molecular structure of the dentin-enamel junction is dependent on the intratooth location.* Calcif Tissue Int, 2009. **84**(3): p. 221-8.

57. Xu, C., et al., *The distribution of carbonate in enamel and its correlation with structure and mechanical properties.* Journal of Materials Science, 2012: p. 1-9.

58. Mathew, M. and S. Takagi, *Structures of biological minerals in dental research.* Journal of Research of the National Institute of Standards and Technology, 2001. **106**(6): p. 1035-1044.

59. Wefel, J.S. and J.D. Harless, *The effect of topical fluoride agents on fluoride uptake and surface-morphology.* Journal of Dental Research, 1981. **60**(11): p. 1842-1848.

60. Chen, H.P., et al., *Effect of fluoride containing bleaching agents on enamel surface properties.* Journal of Dentistry, 2008. **36**(9): p. 718-725.

61. Attin, T., et al., *Effect of fluoride treatment on remineralization of bleached enamel.* J Oral Rehabil, 1997. **24**(4): p. 282-286.

62. Kim, S.R., et al., *Synthesis of Si, Mg substituted hydroxyapatites and their sintering behaviors.* Biomaterials, 2003. **24**(8): p. 1389-1398.

63. Robinson, C., J.A. Weatherell, and A.S. Hallsworth, *Distribution of magnesium in mature human-enamel.* Caries Research, 1981. **15**(1): p. 70-77.

64. Bales, C.W., et al., *Zinc, magnesium, copper, and protein concentraions in human saliva - age-related and sex-related differences.* American Journal of Clinical Nutrition, 1990. **51**(3): p. 462-469.

65. Barbagallo, M., M. Belvedere, and L.J. Dominguez, *Magnesium homeostasis and aging.* Magnesium Research, 2009. **22**(4): p. 235-246.

66. Tsuboi, S., et al., *Magnesium distribution in human bone.* Calcified Tissue International, 1994. **54**(1): p. 34-37.

67. Aoba, T., S. Shimoda, and E.C. Moreno, *Labile or surface pools of magnesium, sodium, and potassium in developing porcine enamel mineral.* Journal of Dental Research, 1992. **71**(11): p. 1826-1831.

68. Tomazic, B., M. Tomson, and G.H. Nancollas, *Growth of calcium phosphates on hydoxyapatite crystals - effect of magnesium.* Archives of Oral Biology, 1975. **20**(12): p. 803-808.

69. Feenstra, T.P., J. Hop, and P.L. Debruyn, *The influence of small amounts of magnesium on the formation of calcium phosphates in moderately supersaturated solutions.* Journal of Colloid and Interface Science, 1981. **83**(2): p. 583-588.

70. Spencer, P., et al., *Incorporation of magnesium into rat dental enamel and its influence on crystallization.* Archives of Oral Biology, 1989. **34**(10): p. 767-771.

71. Bigi, A., et al., *Magnesium influence on hydroxyapatite crystallization.* Journal of Inorganic Biochemistry, 1993. **49**(1): p. 69-78.

72. Fadeev, I.V., et al., *Synthesis and structure of magnesium-substituted hydroxyapatite.* Inorganic Materials, 2003. **39**(9): p. 947-950.

73. Suchanek, W.L., et al., *Preparation of magnesium-substituted hydroxyapatite powders by the mechanochemical-hydrothermal method.* Biomaterials, 2004. **25**(19): p. 4647-4657.

74. Zyman, Z., et al., *Magnesium-substituted hydroxyapatite ceramics.* Materialwissenschaft Und Werkstofftechnik, 2006. **37**(6): p. 474-477.

75. LeGeros, R.Z., et al., *Synergistic effects of magnesium and carbonate on properties of biological and synthetic apatites.* Connective Tissue Research, 1995. **32**(1-4): p. 525-531.

76. Aoba, T., E.C. Moreno, and S. Shimoda, *Competitive adsorption of magnesium and calcium-ions onto synthetic and biological apatittes.* Calcified Tissue International, 1992. **51**(2): p. 143-150.

77. Kolmas, J., et al., *Incorporation of carbonate and magnesium ions into synthetic hydroxyapatite: The effect on physicochemical properties.* Journal of Molecular Structure, 2011. **987**(1-3): p. 40-50.

78. Mayer, I., R. Schlam, and J.D.B. Featherstone, *Magnesium-containing carbonate apatites.* Journal of Inorganic Biochemistry, 1997. **66**(1): p. 1-6.

79. Terpstra, R.A. and F.C.M. Driessens, *Magnesium in tooth enamel and synthetic apatites.* Calcified Tissue International, 1986. **39**(5): p. 348-354.

80. Buchalla, W., et al., *Relationship between nanohardness and mineral content of artificial carious enamel lesions.* Caries Research, 2008. **42**(3): p. 157-163.

81. Honorio, H.M., et al., *Cross-Sectional Microhardness of Human Enamel Subjected to Erosive, Cariogenic or Combined Erosive/Cariogenic Challenges.* Caries Research, 2010. **44**(1): p. 29-32.

82. Markitziu, A., et al., *Modulatory effect of fluoride and irradiation on rat molar rate of wear.* Bull Group Int Rech Sci Stomatol Odontol, 1991. **34**(3-4): p. 139-44.

83. Huang, T.T.Y., et al., *Nano-Indentation Characterisation of Natural Carious White Spot Lesions.* Caries Research, 2010. **44**(2): p. 101-107.

84. Maupome, G., et al., *In vitro quantitative microhardness assessment of enamel with early salivary pellicles after exposure to an eroding cola drink.* Caries Research, 1999. **33**(2): p. 140-7.

85. Ghadimi, E., Eimar, H, Song, J, Marelli, B, Ciobanu, O, Nazhat, SN, Vali, H, Tamimi, F., *Regulated Fracture in Tooth Enamel: a Nanotechnological Strategy from Nature.* Submitted.

86. Habelitz, S., et al., *Mechanical properties of human dental enamel on the nanometre scale.* Archives of Oral Biology, 2001. **46**(2): p. 173-183.

87. Eimar, H., et al., *Regulation of enamel hardness by its crystallographic dimensions.* Acta Biomaterialia, 2012. **8**(9): p. 3400-10.

88. Baldassarri, M., H.C. Margolis, and E. Beniash, *Compositional determinants of mechanical properties of enamel.* Journal of Dental Research, 2008. **87**(7): p. 645-649.

89. Kielbassa, A.M., et al., *Correlation of transversal microradiography and microhardness on in situ-induced demineralization in irradiated and nonirradiated human dental enamel.* Archives of Oral Biology, 1999. **44**(3): p. 243-251.

90. Joiner, A., *Tooth colour: a review of the literature.* Journal of Dentistry, 2004. **32**: p. 3-12.

91. Hovis, J.K. and P. Neumann, *Colorimetric analyses of various light-sources for the d-15 color-vision test.* Optometry and Vision Science, 1995. **72**(9): p. 667-678.

92. Jahangiri, L., et al., *Relationship between tooth shade value and skin color: An observational study.* Journal of Prosthetic Dentistry, 2002. **87**(2): p. 149-152.

93. Obrien, W.J., W.M. Johnston, and F. Fanian, *Double -layer color effects in porcelain systems.* Journal of Dental Research, 1985. **64**(6): p. 940-943.

94. Kuehni, R.G., *The early development of the Munsell system.* Color Research and Application, 2002. **27**(1): p. 20-27.

95. Yap, A.U.J., *Color attributes and accuracy of vita-based manufacturers' shade guides.* Operative Dentistry, 1998. **23**(5): p. 266-271.

96. Paravina, R.D., et al., *Teaching of color in predoctoral and postdoctoral dental education in 2009.* Journal of Dentistry, 2010. **38**: p. E34-E40.

97. Tyler, J.E. and A.C. Hardy, *An analysis of the original Munsell Color System.* Journal of the Optical Society of America, 1940. **30**(12): p. 587-590.

98. Della Bona, A., et al., *Visual and instrumental agreement in dental shade selection: Three distinct observer populations and shade matching protocols.* Dental Materials, 2009. **25**(2): p. 276-281.

99. Spear, F. and J. Holloway, *Which all-ceramic system is optimal for anterior esthetics?* Journal of the American Dental Association, 2008. **139**: p. 19s-24s.

100. Brook, A.H., R.N. Smith, and D.J. Lath, *The clinical measurement of tooth colour and stain.* Int Dent J, 2007. **57**(5): p. 324-330.

101. Preston, J.D., *Current status of shade selection and color matching.* Quintessence international (Berlin, Germany : 1985), 1985. **16**(1): p. 47-58.

102. Ragain, J.C. and W.M. Johnson, *Color acceptance of direct dental restorative materials by human observers.* Color Research and Application, 2000. **25**(4): p. 278-285.

103. ten Bosch, J.J. and J.C. Coops, *Tooth color and reflectance as related to light scattering and enamel hardness.* Journal of Dental Research, 1995. **74**(1): p. 374-80.

104. Eimar, H., et al., *The role of enamel crystallography on tooth shade.* Journal of Dentistry, 2011. **39, Supplement 3**(0): p. e3-e10.

105. Azer, S.S., A.L. Hague, and W.M. Johnston, *Effect of pH on tooth discoloration from food colorant in vitro.* Journal of Dentistry, 2010. **38**: p. E106-E109.

106. Dahl, J.E. and U. Pallesen, *Tooth bleaching - A critical review of the biological aspects.* Critical Reviews in Oral Biology & Medicine, 2003. **14**(4): p. 292-304.

107. Addy, M. and J. Moran, *Mechanisms of Stain Formation on Teeth, in Particular Associated with Metal Ions and Antiseptics.* Adv Dent Res, 1995. **9**(4): p. 450-456.

108. Tantbirojn, D., et al., *Spatial chemical analysis of dental stain using wavelength dispersive spectrometry.* European Journal of Oral Sciences, 1998. **106**(5): p. 971-976.

109. Azer, S.S., A.L. Hague, and W.M. Johnston, *Effect of bleaching on tooth discoloration from food colorant in vitro.* Journal of Dentistry, 2011. **39**: p. E52-E56.

110. Bennick, A., *Interaction of plant polyphenols with salivary proteins.* Critical Reviews in Oral Biology & Medicine, 2002. **13**(2): p. 184-196.

111. Omata, Y., et al., *Staining of hybrid composites with coffee, oolong tea, or red wine.* Dent Mater J, 2006. **25**(1): p. 125-131.

112. Nathoo, S.A., *The chemistry and mechanisms of extrinsic and intrinsic discoloration.* Journal of the American Dental Association, 1997. **128**: p. S6-S10.

113. Haywood, V.B., *History, safety, and effectiveness of current bleaching techniques and applications of the nightguard vital bleaching technique.* Quintessence international (Berlin, Germany : 1985), 1992. **23**(7): p. 471-88.

114. Sulieman, M., et al., *A safety study in vitro for the effects of an in-office bleaching system on the integrity of enamel and dentine.* Journal of Dentistry, 2004. **32**(7): p. 581-90.

115. Plotino, G., et al., *Nonvital tooth bleaching: A review of the literature and clinical procedures.* Journal of Endodontics, 2008. **34**(4): p. 394-407.

116. Attin, T., et al., *Influence of study design on the impact of bleaching agents on dental enamel microhardness: A review.* Dental Materials, 2009. **25**(2): p. 143-157.

117. Wang, X., et al., *Side effects of a non-peroxide-based home bleaching agent on dental enamel.* Journal of Biomedical Materials Research Part A, 2009. **88A**(1): p. 195-204.

118. Zantner, C., et al., *Randomized clinical trial on the efficacy of 2 over-the-counter whitening systems.* Quintessence International, 2006. **37**(9): p. 695-706.

119. Price, R.B., M. Sedarous, and G.S. Hiltz, *The pH of tooth-whitening products.* Journal, 2000. **66**(8): p. 421-6.

120. Thitinanthapan, W., P. Satamanont, and N. Vongsavan, *In vitro penetration of the pulp chamber by three brands of carbamide peroxide.* Journal of esthetic dentistry, 1999. **11**(5): p. 259-64.

121. Joiner, A. and G. Thakker, *In vitro evaluation of a novel 6% hydrogen peroxide tooth whitening product.* Journal of Dentistry, 2004. **32 Suppl 1**: p. 19-25.

122. Gokay, O., A. Mujdeci, and E. Algin, *In vitro peroxide penetration into the pulp chamber from newer bleaching products.* International Endodontic Journal, 2005. **38**(8): p. 516-520.

123. Fuss, Z., S. Szajkis, and M. Tagger, *Tubular permeability to calcium hydroxide and to bleaching agents.* Journal of Endodontics, 1989. **15**(8): p. 362-4.

124. Pohjola, R.M., et al., *Sensitivity and tooth whitening agents.* Journal of esthetic and restorative dentistry : official publication of the American Academy of Esthetic Dentistry ... [et al.], 2002. **14**(2): p. 85-91.

125. Anagnostou, M., et al., *Effect of tooth-bleaching methods on gloss and color of resin composites.* Journal of Dentistry, 2010. **38 Suppl 2**: p. e129-36.

126. Attin, T., et al., *Subsurface microhardness of enamel and dentin after different external bleaching procedures.* American Journal of Dentistry, 2005. **18**(1): p. 8-12.

127. Rodrigues, J.A., et al., *Microhardness evaluation of in situ vital bleaching on human dental enamel using a novel study design.* Dental Materials, 2005. **21**(11): p. 1059-1067.

128. Azer, S.S., et al., *Effect of home bleaching systems on enamel nanohardness and elastic modulus.* Journal of Dentistry, 2009. **37**(3): p. 185-90.

129. Goldberg, M., M. Grootveld, and E. Lynch, *Undesirable and adverse effects of tooth-whitening products: a review.* Clinical Oral Investigations, 2010. **14**(1): p. 1-10.

130. Bowles, W.H. and Z. Ugwuneri, *Pulp chamber penetration by hydrogen peroxide following vital bleaching procedures.* Journal of Endodontics, 1987. **13**(8): p. 375-7.

131. Sulieman, M., *An overview of bleaching techniques: history, chemistry, safety and legal aspects (part 1).* SADJ : journal of the South African Dental Association = tydskrif van die Suid-Afrikaanse Tandheelkundige Vereniging, 2006. **61**(7): p. 304-10, 312.

132. Hermans, N., et al., *Challenges and pitfalls in antioxidant research.* Current medicinal chemistry, 2007. **14**(4): p. 417-30.

133. Attin, T., et al., *Influence of different bleaching systems on fracture toughness and hardness of enamel.* Operative Dentistry, 2004. **29**(2): p. 188-195.

134. Potocnik, I., L. Kosec, and D. Gaspersic, *Effect of 10% carbamide peroxide bleaching gel on enamel microhardness, microstructure, and mineral content.* Journal of Endodontics, 2000. **26**(4): p. 203-206.

135. Navimipour, E.J., et al., *In Vitro Evaluation of the Effect of Delaying Toothbrushing With Toothpaste on Enamel Microhardness Subsequent to Bleaching the Teeth With 15% Carbamide Peroxide.* Operative Dentistry, 2012. **37**(1): p. 87-92.

136. Abouassi, T., M. Wolkewitz, and P. Hahn, *Effect of carbamide peroxide and hydrogen peroxide on enamel surface: an in vitro study.* Clin Oral Investig, 2011. **15**(5): p. 673-80.

137. Attin, T., et al., *In situ evaluation of different remineralization periods to decrease brushing abrasion of demineralized enamel.* Caries Research, 2001. **35**(3): p. 216-222.

138. Nakashima, S., et al., *Effect of a test dentifrice containing nano-sized calcium carbonate on remineralization of enamel lesions in vitro.* Journal of oral science, 2009. **51**(1): p. 69-77.

139. da Costa, J.B. and R.F. Mazur, *Effects of new formulas of bleaching gel and fluoride application on enamel microhardness: an in vitro study.* Operative Dentistry, 2007. **32**(6): p. 589-94.

140. Faraoni-Romano, J.J., C.P. Turssi, and M.C. Serra, *Concentration-dependent effect of bleaching agents on microhardness and roughness of enamel and dentin.* American Journal of Dentistry, 2007. **20**(1): p. 31-4.

141. Murchison, D.F., D.G. Charlton, and B.K. Moore, *Carbamide peroxide bleaching - effects on enamel surface hardness and bonding.* Operative Dentistry, 1992. **17**(5): p. 181-185.

142. Rodrigues, J.A., et al., *Effects of 10% carbamide peroxide bleaching materials on enamel microhardness.* American Journal of Dentistry, 2001. **14**(2): p. 67-71.

143. Justino, L.M., D.R. Tames, and F.F. Demarco, *In situ and in vitro effects of bleaching with carbamide peroxide on human enamel.* Operative Dentistry, 2004. **29**(2): p. 219-225.

144. Basting, R.T., A.L. Rodrigues Junior, and M.C. Serra, *The effect of 10% carbamide peroxide bleaching material on microhardness of sound and demineralized enamel and dentin in situ.* Operative Dentistry, 2001. **26**(6): p. 531-9.

145. White, D.J., et al., *Effects of Crest Whitestrips bleaching on subsurface microhardness and ultrastructure of tooth enamel and coronal dentin.* American Journal of Dentistry, 2004. **17**(1): p. 5-11.

146. de Menezes, M., et al., *Susceptibility of bleached enamel and root dentin to artificially formed caries-like lesions.* American Journal of Dentistry, 2007. **20**(3): p. 173-6.

147. Wiegand, A., M. Schreier, and T. Attin, *Effect of different fluoridation regimes on the microhardness of bleached enamel.* Operative Dentistry, 2007. **32**(6): p. 610-615.

148. Rodrigues, J.A., G.P.F. Oliveira, and C.M. Amaral, *Effect of thickener agents on dental enamel microhardness submitted to at-home bleaching.* Braz Oral Res, 2007. **21**(2): p. 170-5.

149. Zhang, C., et al., *Effects of KTP laser irradiation, diode laser, and LED on tooth bleaching: A comparative study.* Photomed Laser Surg, 2007. **25**(2): p. 91-95.

150. Araujo, E.M.J., et al., *In situ effect of 10% carbamide peroxide on microhardness of human enamel: function of time.* J Esthet Restor Dent, 2003. **15**(3): p. 166-174.

151. Unlu, N., et al., *Effect of home bleaching agents on the microhardness of human enamel and dentin.* J Oral Rehabil, 2004. **31**(1): p. 57-61.

152. White, D.J., et al., *Peroxide interactions with hard tissues: effects on surface hardness and surface/subsurface ultrastructural properties.* Compendium of continuing education in dentistry (Jamesburg, N.J. : 1995), 2002. **23**(1A): p. 42-50.

153. Lewinstein, I., et al., *Effect of hydrogen peroxide and sodium perborate on the microhardness of human enamel and dentin.* J Endod, 1994. **20**(2): p. 61-3.

154. Leonard, R.H., et al., *Effect on enamel microhardness of two consumer-available bleaching solutions when compared with a dentist-prescribed, home-applied bleaching solution and a control.* J Esthet Restor Dent, 2005. **17**(6): p. 343-351.

155. Zantner, C., et al., *Surface microhardness of enamel after different home bleaching procedures.* Dental Materials, 2007. **23**(2): p. 243-250.

156. Lee, K.H., et al., *Mineral loss from bovine enamel by a 30% hydrogen peroxide solution.* J Oral Rehabil, 2006. **33**(3): p. 229-233.

157. Goo, D.H., et al., *The efficiency of 10% carbamide peroxide gel on dental enamel.* Dent Mater J, 2004. **23**(4): p. 522-527.

158. McCracken, M.S. and V.B. Haywood, *Demineralization effects of 10 percent carbamide peroxide.* Journal of Dentistry, 1996. **24**(6): p. 395-398.

159. Al-Salehi, S.K., D.J. Wood, and P.V. Hatton, *The effect of 24h non-stop hydrogen peroxide concentration on bovine enamel and dentine mineral content and microhardness.* Journal of Dentistry, 2007. **35**(11): p. 845-50.

160. Souza, R.O.A., et al., *Analysis of Tooth Enamel After Excessive Bleaching: A Study Using Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy.* International Journal of Prosthodontics, 2010. **23**(1): p. 29-32.

161. Bizhang, M., et al., *Demineralization effects of 2 bleaching procedures on enamel surfaces with and without post-treatment fluoride application.* Operative Dentistry, 2006. **31**(6): p. 705-709.

162. Tezel, H., et al., *Effect of bleaching agents on calcium loss from the enamel surface.* Quintessence International, 2007. **38**(4): p. 339-347.

163. McArthur, S.L., *Applications of XPS in bioengineering.* Surface and Interface Analysis, 2006. **38**(11): p. 1380-1385.

164. Ruse, N.D. and D.C. Smith, *Adhesion to bovine dentin - surface characterization.* Journal of Dental Research, 1991. **70**(6): p. 1002-1008.

165. Kasemo, B. and J. Lausmaa, *Surface science aspects on inorganic biomaterials.* Crc Critical Reviews in Biocompatibility, 1986. **2**(4): p. 335-380.

166. Mine, A., et al., *Spectroscopic characterization of enamel surfaces irradiated with Er : YAG laser.* Dent Mater J, 2006. **25**(2): p. 214-218.

167. Rouxhet, P.G. and M.J. Genet, *XPS analysis of bio-organic systems.* Surface and Interface Analysis, 2011. **43**(12): p. 1453-1470.

168. Sennou, H.E., A.A. Lebugle, and G.L. Gregoire, *X-ray photoelectron spectroscopy study of the dentin-glass ionomer cement interface.* Dental Materials, 1999. **15**(4): p. 229-237.

169. Tilinin, I.S., A. Jablonski, and W.S.M. Werner, *Quantitative surface analysis by Auger and x-ray photoelectron spectroscopy.* Progress in Surface Science, 1996. **52**(4): p. 193-335.

170. Vereecke, G. and P.G. Rouxhet, *Surface charging of insulating samples in X-ray photoelectron spectroscopy.* Surface and Interface Analysis, 1998. **26**(7): p. 490-497.

171. Spizzirri, P.G., et al., *A Comparative Study of Carbonate Determination in Human Teeth Using Raman Spectroscopy.* Caries Research, 2012. **46**(4): p. 353-360.

172. Duschner, H., et al., *Effects of hydrogen peroxide bleaching strips on tooth surface color, surface microhardness, surface and subsurface ultrastructure, and microchemical (Raman spectroscopic) composition.* J Clin Dent, 2006. **17**(3): p. 72-8.

173. Leroy, G., et al., *Human tooth enamel: A Raman polarized approach.* Applied Spectroscopy, 2002. **56**(8): p. 1030-1034.

174. Tsuda, H. and J. Arends, *Orientational micro-raman spectroscopy on hydroxyapatite single-crystals and human enamel crystallites.* Journal of Dental Research, 1994. **73**(11): p. 1703-1710.

175. Tsuda, H. and J. Arends, *Raman spectroscopy in dental research: a short review of recent studies.* Adv Dent Res, 1997. **11**(4): p. 539-47.

176. He, L.H. and M.V. Swain, *Enamel--a functionally graded natural coating.* Journal of Dentistry, 2009. **37**(8): p. 596-603.

177. Yerramshetty, J.S. and O. Akkus, *The associations between mineral crystallinity and the mechanical properties of human cortical bone.* Bone, 2008. **42**(3): p. 476-482.

178. Freeman, J.J., et al., *Raman spectroscopic detection of changes in bioapatite in mouse femora as a function of age and in vitro fluoride treatment.* Calcified Tissue International, 2001. **68**(3): p. 156-162.

179. Mandair, G.S., T.A. Bateman, and M.D. Morris, *Raman Spectroscopy of Murine Bone in Response to Simulated Spaceflight Conditions.* Optics in Bone Biology and Diagnostics, 2009. **7166**.

180. Akkus, O., F. Adar, and M.B. Schaffler, *Age-related changes in physicochemical properties of mineral crystals are related to impaired mechanical function of cortical bone.* Bone, 2004. **34**(3): p. 443-453.

181. Wilson, R.M., J.C. Elliott, and S.E.P. Dowker, *Rietveld refinement of the crystallographic structure of human dental enamel apatites.* American Mineralogist, 1999. **84**(9): p. 1406-1414.

182. Baig, A.A., et al., *Relationships among carbonated apatite solubility, crystallite size, and microstrain parameters.* Calcif Tissue Int, 1999. **64**(5): p. 437-49.

183. Bottero, M.J., J. Yvon, and J. Vadot, *Multimethod analysis of apatites in sound human tooth enamel.* European Journal of Mineralogy, 1992. **4**(6): p. 1347-1357.

184. Fu, B.P., et al., *Evidence of chemisorption of maleic acid to enamel and hydroxyapatite.* European Journal of Oral Sciences, 2004. **112**(4): p. 362-367.

185. Mahoney, E.K., et al., *Mechanical properties and microstructure of hypomineralised enamel of permanent teeth.* Biomaterials, 2004. **25**(20): p. 5091-100.

186. Oliveira, M., et al., *Microstructure and Mineral Composition of Dental Enamel of Permanent and Deciduous Teeth.* Microscopy Research and Technique, 2010. **73**(5): p. 572-577.

187. Kantola, S., E. Laine, and T. Tarna, *Laser-induced effects on tooth structure .6. X-ray-diffraction study of dental enamel exposed to a co2-laser.* Acta Odontol Scand, 1973. **31**(6): p. 369-379.

188. Sakae, T., *X-ray-diffraction and thermal studies of crystals from the outer and inner layers of human dental enamel.* Archives of Oral Biology, 1988. **33**(10): p. 707-713.

189. Yoshioka, M., et al., *Adhesion/decalcification mechanisms of acid interactions with human hard tissues.* J Biomed Mater Res, 2002. **59**(1): p. 56-62.

190. Zhao, W., et al., *The crystallographic properties of the mineral phases of enamel and dentin in normal deciduous and permanent teeth.* Zhonghua Kou Qiang Yi Xue Za Zhi, 2002. **37**(3): p. 219-21.

191. Xue, J., et al., *High-resolution X-ray microdiffraction analysis of natural teeth.* J Synchrotron Radiat, 2008. **15**(Pt 3): p. 235-8.

192. P.Scherrer, *Bestimmung der Grösse und der inneren Struktur von Kolloidteilchen mittels Röntgenstrahlen.* Nach Akad Wiss Göttingen II Math Phys Klasse, 1918. **2**: p. 98.

193. Stefaniak, E.A., et al., *Molecular and elemental characterisation of mineral particles by means of parallel micro-Raman spectrometry and scanning electron microscopy/energy dispersive X-ray analysis.* Spectrochimica Acta Part B-Atomic Spectroscopy, 2006. **61**(7): p. 824-830.

194. Wiegand, A., et al., *Comparison of the Effects of TiF4 and NaF Solutions at pH 1.2 and 3.5 on Enamel Erosion in vitro.* Caries Research, 2009. **43**(4): p. 269-277.

195. Kinoshita, H., et al., *Functional mapping of carious enamel in human teeth with Raman microspectroscopy.* Journal of Raman Spectroscopy, 2008. **39**(5): p. 655-660.

196. Arnold, W.H. and P. Gaengler, *Quantitative analysis of the calcium and phosphorus content of developing and permanent human teeth.* Annals of Anatomy-Anatomischer Anzeiger, 2007. **189**(2): p. 183-190.

197. Arnold, W.H., et al., *Effect of fluoride toothpastes on enamel demineralization.* BMC oral health, 2006. **6**: p. 8-8.

198. Austin, R.S., et al., *A qualitative and quantitative investigation into the effect of fluoride formulations on enamel erosion and erosion-abrasion in vitro.* Journal of Dentistry, 2011. **39**(10): p. 648-655.

199. Emma Rodriguez-Vilchis, L., et al., *Morphological and Structural Changes on Human Dental Enamel After Er:YAG Laser Irradiation: AFM, SEM, and EDS Evaluation.* Photomed Laser Surg, 2011. **29**(7): p. 493-500.

200. Iwai-Liao, Y., et al., *Fine structure study and energy dispersive spectroscopy (EDS) X-ray microprobe (EDX) analysis of organic elements in the human enamel.* Journal of Trace and Microprobe Techniques, 1997. **15**(4): p. 673-682.

201. Smidt, A., O. Feuerstein, and M. Topel, *Mechanical, morphologic, and chemical effects of carbamide peroxide bleaching agents on human enamel in situ.* Quintessence International, 2011. **42**(5): p. 407-412.

202. Weerasinghe, D.D.S., et al., *Scanning electron microscopy and energy-dispersive X-ray analysis of self-etching adhesive systems to ground and unground enamel.* Journal of Materials Science-Materials in Medicine, 2007. **18**(6): p. 1111-1116.

203. He, B., et al., *Mineral densities and elemental content in different layers of healthy human enamel with varying teeth age.* Archives of Oral Biology, 2011. **56**(10): p. 997-1004.

204. De Menezes Oliveira, M.A.H., et al., *Microstructure and mineral composition of dental enamel of permanent and deciduous teeth.* Microscopy Research and Technique, 2010. **73**(5): p. 572-7.

205. Rominu, R.O., et al., *The Effect of Surface Treatment on Human Enamel - a SEM/EDAX Study*. Revista De Chimie, 2011. **62**(9): p. 937-940.

206. Westbrook JH, C.H., *The science of hardness and its research applications*1973, Metals Park, Ohio: American Society for Metals.

207. Chu, S.J., R.D. Trushkowsky, and R.D. Paravina, *Dental color matching instruments and systems. Review of clinical and research aspects.* Journal of Dentistry, 2010. **38**: p. E2-E16.

208. Kim-Pusateri, S., et al., *Reliability and accuracy of four dental shade-matching devices.* Journal of Prosthetic Dentistry, 2009. **101**(3): p. 193-199.

209. Sheen, S., N. Banfield, and M. Addy, *The effect of unstimulated and stimulated whole saliva on extrinsic staining in vitro - a developmental method.* Journal of Dentistry, 2002. **30**(7-8): p. 365-369.

210. Joiner, A., et al., *Adsorption from black tea and red wine onto in vitro salivary pellicles studied by ellipsometry*. European Journal of Oral Sciences, 2003. **111**(5): p. 417-422.

211. Proctor, G.B., et al., *Salivary proteins interact with dietary constituents to modulate tooth staining.* Journal of Dental Research, 2005. **84**(1): p. 73-78.

212. Hagerman, A.E. and L.G. Butler, *The specificity of proanthocyanidin-protein interactions.* Journal of Biological Chemistry, 1981. **256**(9): p. 4494-4497.

213. Wroblewski, K., et al., *The molecular interaction of human salivary histatins with polyphenolic compounds.* European Journal of Biochemistry, 2001. **268**(16): p. 4384-4397.

214. Siqueira, W.L., et al., *Identification of protein components in in vivo human acquired enamel pellicle using LC-ESI-MS/MS.* Journal of Proteome Research, 2007. **6**(6): p. 2152-2160.

215. Zhang, J.L., et al., *The measurement and research of surface potentials of human tooth in vitro,* in *Proceedings of the 23rd Annual International Conference of the Ieee Engineering in Medicine and Biology Society, Vols 1-4: Building New Bridges at the Frontiers of Engineering and Medicine*2001. p. 3009-3011.

216. Siqueira, W.L., et al., *Quantitative Proteomic Analysis of the Effect of Fluoride on the Acquired Enamel Pellicle.* Plos One, 2012. **7**(8).

217. Rykke, M., et al., *Protein adsoption to hydroxyapatite and to calcium-fluoride invitro and amino-acid analyses of pellicle formed on normal enamel and on calcium-fluoride-covered enamel invivo.* Acta Odontol Scand, 1989. **47**(4): p. 245-251.

218. Juriaanse, A.C., et al., *The adsorption invitro of purified salivary proteins on bovine dental enamel.* Archives of Oral Biology, 1981. **26**(2): p. 91-96.

219. Preoteasa, E.A., et al., *Broad-beam PIXE and mu-PIXE analysis of normal and in vitro demineralized dental enamel.* X-Ray Spectrometry, 2008. **37**(5): p. 517-535.

220. Stenudd, C., et al., *The association of bacterial adhesion with dental caries.* Journal of Dental Research, 2001. **80**(11): p. 2005-2010.

221. Es-Souni, M., H. Fischer-Brandies, and V. Zaporojshenko, *On the interaction of polyacrylic acid as a conditioning agent with bovine enamel.* Biomaterials, 2002. **23**(14): p. 2871-2878.

222. Farina, M., et al., *Atomic force microscopy study of tooth surfaces.* J Struct Biol, 1999. **125**(1): p. 39-49.

223. Newman, H.N., *The pre-eruptive cuticle of human-enamel.* Journal of Dental Research, 1980. **59**: p. 1828-1828.

224. Newman, H.N., *Ultrastructural observations on the human pre-eruptive enamel cuticle.* Archives of Oral Biology, 1980. **25**(1): p. 49-&.

225. Kuboki, Y., K. Teraoka, and S. Okada, *X-ray photoelectron spectroscopic studies of the adsorption of salivary constituents on enamel.* Journal of Dental Research, 1987. **66**(5): p. 1016-1019.

226. Es-Souni, M., R. Zimehl, and H. Fischer-Brandies, *Microscopic and electron spectroscopic characterization of dental enamel surfaces.* Colloid and Polymer Science, 1999. **277**(4): p. 382-387.

227. Lubarsky, G.V., et al., *The Role of Enamel Proteins in Protecting Mature Human Enamel Against Acidic Environments: A Double Layer Force Spectroscopy Study.* Biointerphases, 2012. **7**(1-4).

228. Ziglo, M.J., et al., *Argon laser induced changes to the carbonate content of enamel.* Applied Surface Science, 2009. **255**(15): p. 6790-6794.

229. Busscher, H.J., et al., *Xps determination of the thickness of adsorbed mouthrinse components on dental enamel.* Surface and Interface Analysis, 1990. **15**(5): p. 344-346.

230. Lou, L., et al., *Surface chemical composition of human maxillary first premolar as assessed by X-ray photoelectron spectroscopy (XPS).* Applied Surface Science, 2008. **254**(21): p. 6706-6709.

231. Sarrett, D.C., *Tooth whitening today.* Journal of the American Dental Association, 2002. **133**(11): p. 1535-1538.

232. Lima, D.A.N.L., et al., *In vitro assessment of the effectiveness of whitening dentifrices for the removal of extrinsic tooth stains.* Braz Oral Res, 2008. **22**(2): p. 106-11.

233. Dawson, P.L., et al., *Dental stain prevention by abrasive toothpastes: A new in vitro test and its correlation with clinical observations.* Journal of Cosmetic Science, 1998. **49**(4): p. 275-283.

234. Croll, T.P., *Enamel microabrasion: Observations after 10 years.* Journal of the American Dental Association, 1997. **128**: p. S45-S50.

235. Kamp, A.A., *Removal of white spot lesions by controlled acid-pumice abrasion.* Journal of clinical orthodontics : JCO, 1989. **23**(10): p. 690-3.

236. Schoenly, J.E., et al., *Near-UV laser treatment of extrinsic dental enamel stains.* Lasers in Surgery and Medicine, 2012. **44**(4): p. 339-345.

237. Eimar, H., et al., *Hydrogen peroxide whitens teeth by oxidizing the organic structure.* Journal of Dentistry, 2012. **24**(12): p. 00226-6.

238. Hay, D.I., *Adsorption of salivary proteins by hydroxyapatite and enamel.* Archives of Oral Biology, 1967. **12**(8): p. 937-&.

239. Mayhall, C.W., *Amino-acid composition of enamel cuticle of human teeth.* Journal of Dental Research, 1977. **56**: p. B76-B76.

240. Sonju, T. and G. Rolla, *Chemical-analysis of acquired pellicle formed in 2 hours on cleaned human teeth in-vivo - rate of formation and amino-acid analysis.* Caries Research, 1973. **7**(1): p. 30-38.

241. Jensen, J.L., M.S. Lamkin, and F.G. Oppenheim, *Adsorption of human salivary proteins to hydroxyapatite - a comparison between whole saliva and glandular salivary secretions.* Journal of Dental Research, 1992. **71**(9): p. 1569-1576.

242. Lamkin, M.S., A.A. Arancillo, and F.G. Oppenheim, *Temporal and compositional characteristics of salivary protein adsorption to hydroxyapatite.* Journal of Dental Research, 1996. **75**(2): p. 803-808.

243. Alhashimi, I. and M.J. Levine, *Characterization of invivo salivary-derived enamel pellicle.* Archives of Oral Biology, 1989. **34**(4): p. 289-295.

244. Lendenmann, U., J. Grogan, and F.G. Oppenheim, *Saliva and dental pellicle--a review.* Adv Dent Res, 2000. **14**: p. 22-8.

245. Peng, J.M. and S.P. Gygi, *Proteomics: the move to mixtures.* Journal of Mass Spectrometry, 2001. **36**(10): p. 1083-1091.

246. Hay, D.I. and E.C. Moreno, *Differential adsorption and chemical affinities of proteins for apatitic surfaces.* Journal of Dental Research, 1979. **58**: p. 930-942.

247. Haywood, V.B., et al., *Effectiveness, side effects and long-term status of nightguard vital bleaching.* Journal of the American Dental Association, 1994. **125**(9): p. 1219-26.

248. Nathanson, D., *Vital tooth bleaching: sensitivity and pulpal considerations.* Journal of the American Dental Association, 1997. **128 Suppl**: p. 41S-44S.

249. Efeoglu, N., D.J. Wood, and C. Efeoglu, *Thirty-five percent carbamide peroxide application causes in vitro demineralization of enamel.* Dental Materials, 2007. **23**(7): p. 900-904.

250. Ragain, J.C. and W.M. Johnston, *Accuracy of kubelka-munk reflectance theory applied to human dentin and enamel.* Journal of Dental Research, 2001. **80**(2): p. 449-52.

251. Zijp, J.R. and J. TenBosch, *Theoretical model for the scattering of light by dentin and comparison with measurements.* Applied Optics, 1993. **32**(4): p. 411-415.

252. Zijp, J.R., J.J. TenBosch, and R.J. Groenhuis, *HeNe-Laser light scattering by human dental enamel.* Journal of Dental Research, 1995. **74**(12): p. 1891-1898.

253. Clarkson, R.M. and A.J. Moule, *Sodium hypochlorite and its use as an endodontic irrigant.* Australian Dental Journal, 1998. **43**(4): p. 250-256.

254. Joiner, A., et al., *Enhanced enamel benefits from a novel fluoride toothpaste.* International Dental Journal, 2009. **59**(4): p. 244-253.

255. Toyoda, S., et al., *Toward high sensitivity ESR dosimetry of mammal teeth: the effect of chemical treatment.* Journal of radiation research, 2006. **47 Suppl A**: p. A71-4.

256. O'Connell, M.S., et al., *A Comparative Study of Smear Layer Removal Using Different Salts of EDTA.* Journal of Endodontics, 2000. **26**(12): p. 739-743.

257. Nishitani, Y., et al., *Tensile strength of mineralized/demineralized human normal and carious dentin.* Journal of Dental Research, 2005. **84**(11): p. 1075-8.

258. Tenbosch, J.J. and J.C. Coops, *Tooth color and reflectance as related to light-scattering and enamel hardness.* Journal of Dental Research, 1995. **74**(1): p. 374-380.

259. Lynch, C.D., et al., *Hunter–Schreger Band patterns and their implications for clinical dentistry.* Journal of Oral Rehabilitation, 2011. **38**(5): p. 359-365.

260. Llambes, G., et al., *In vitro evaluation of the efficacy of two bleaching procedures.* Medicina Oral Patologia Oral Y Cirugia Bucal, 2011. **16**(6): p. E845-E851.

261. Putt, M.S., et al., *Clinical validation and calibration of in vitro peroxide tooth whitening.* The Journal of clinical dentistry, 2009. **20**(3): p. 79-86.

262. Lee, K.H., et al., *Mineral loss from bovine enamel by a 30% hydrogen peroxide solution.* Journal of Oral Rehabilitation, 2006. **33**(3): p. 229-33.

263. Li, Q., et al., *Quantitative evaluation of colour regression and mineral content change of bleached teeth.* Journal of Dentistry, 2010. **38**(3): p. 253-60.

264. Pretty, I.A., W.M. Edgar, and S.M. Higham, *The effect of bleaching on enamel susceptibility to acid erosion and demineralisation.* British Dental Journal, 2005. **198**(5): p. 285-90; discussion 280.

265. Shannon, H., et al., *Characterization of enamel exposed to 10% carbamide peroxide bleaching agents.* Quintessence International, 1993. **24**(1): p. 39-44.

266. Park, H.J., et al., *Changes in bovine enamel after treatment with a 30% hydrogen peroxide bleaching agent.* Dental Materials Journal, 2004. **23**(4): p. 517-521.

267. Sulieman, M., M. Addy, and J.S. Rees, *Development and evaluation of a method in vitro to study the effectiveness of tooth bleaching.* Journal of Dentistry, 2003. **31**(6): p. 415-422.

268. Watts, A. and M. Addy, *Tooth discolouration and staining: a review of the literature.* British Dental Journal, 2001. **190**(6): p. 309-316.

269. McNaught, A.D. and A. Wilkinson, *I{UPAC}. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book")*: WileyBlackwell; 2nd Revised edition edition.

270. Ashraf, S.M., S. Ahmad, and U. Riaz, *A Laboratory Manual of Polymers*2009: I.K. International Publishing House Pvt. Limited.

271. Anderson, R.J., et al., *Organic Spectroscopic Analysis*2004: Royal Society of Chemistry.

272. Fattibene, P., et al., *A comparative EPR, infrared and Raman study of natural and deproteinated tooth enamel and dentin.* Physics in medicine and biology, 2005. **50**(6): p. 1095-1108.

273. Darchuk, L.A., et al., *Infrared investigation of hard human teeth tissues exposed to various doses of ionizing radiation from the 1986 Chernobyl accident.* Spectroscopy-an International Journal, 2008. **22**(2-3): p. 105-111.

274. Eimar, H., et al., *Regulation of enamel hardness by its crystallographic dimensions.* Acta biomaterialia, 2012.

275. Vieira, G.F., Y. Arakaki, and T.M. Caneppele, *Spectrophotometric assessment of the effects of 10% carbamide peroxide on enamel translucency.* Brazilian oral research, 2008. **22**(1): p. 90-5.

276. Ma, X., et al., *Effects of tooth bleaching on the color and translucency properties of enamel.* American journal of dentistry, 2009. **22**(6): p. 324-8.

277. Ma, X., et al., *Separate contribution of enamel and dentine to overall tooth colour change in tooth bleaching.* Journal of Dentistry, 2011. **39**(11): p. 739-745.

278. He, B., et al., *Mineral densities and elemental content in different layers of healthy human enamel with varying teeth age.* Archives of Oral Biology, 2011. **56**(10): p. 997-1004.

279. Fujikawa, H., et al., *Influence of Salivary Macromolecules and Fluoride on Enamel Lesion Remineralization in vitro.* Caries Research, 2008. **42**(1): p. 37-45.

280. Gerlach, R.W. and X. Zhou, *Vital bleaching with whitening strips: summary of clinical research on effectiveness and tolerability.* The journal of contemporary dental practice, 2001. **2**(3): p. 1-16.

281. Eimar, H., et al., *The role of enamel crystallography on tooth shade.* Journal of Dentistry, 2011. **39**: p. E3-E10.

282. Kenney, M.A., H. McCoy, and L. Williams, *Effects of magnesium-deficiency on strength, mass, and composition of rat femur.* Calcified Tissue International, 1994. **54**(1): p. 44-49.

283. He, Z.M., J. Ma, and C. Wang, *Constitutive modeling of the densification and the grain growth of hydroxyapatite ceramics.* Biomaterials, 2005. **26**(14): p. 1613-1621.

284. Bala, Y., et al., *Modifications of bone material properties in postmenopausal osteoporotic women long-term treated with alendronate.* European Journal of Endocrinology, 2011. **165**(4): p. 647-655.

285. Wang, N., et al., *Effect of grain-size on mechanical-properties of nanocrytalline materials.* Acta Metallurgica Et Materialia, 1995. **43**(2): p. 519-528.

286. Lopes, G.C., et al., *Effect of bleaching agents on the hardness and morphology of enamel.* J Esthet Restor Dent, 2002. **14**(1): p. 24-30.

287. Lewinstein, I., et al., *Effect of different peroxide bleaching regimens and subsequent fluoridation on the hardness of human enamel and dentin.* Journal of Prosthetic Dentistry, 2004. **92**(4): p. 337-342.

288. Basting, R.T., A.L. Rodrigues, and M.C. Serra, *The effects of seven carbamide peroxide bleaching agents on enamel microhardness over time.* Journal of the American Dental Association, 2003. **134**(10): p. 1335-1342.

289. de Oliveira, R., A.F. Paes Leme, and M. Giannini, *Effect of a carbamide peroxide bleaching gel containing calcium or fluoride on human enamel surface microhardness.* Braz Dent J, 2005. **16**(2): p. 103-6.

290. Pinto, C.F., et al., *Peroxide bleaching agent effects on enamel surface microhardness, roughness and morphology.* Braz Oral Res, 2004. **18**(4): p. 306-11.

291. Park, H.J., et al., *Changes in bovine enamel after treatment with a 30% hydrogen peroxide bleaching agent.* Dent Mater J, 2004. **23**(4): p. 517-521.

292. Lewinstein, I., et al., *Effect of hydrogen-peroxide and sodium perborate on the microhardness of human enamel and dentin.* Journal of Endodontics, 1994. **20**(2): p. 61-63.

293. Basting, R.T., A.L. Rodrigues, and M.C. Serra, *The effect of 10% carbamide peroxide, carbopol and/or glycerin on enamel and dentin microhardness.* Operative Dentistry, 2005. **30**(5): p. 608-616.

294. Walsh, L.J., *Safety issues relating to the use of hydrogen peroxide in dentistry.* Aust Dent J, 2000. **45**(4): p. 257-269.

295. Li, Y., *Tooth bleaching using peroxide-containing agents: current status of safety issues.* Compendium of continuing education in dentistry (Jamesburg, N.J. : 1995), 1998. **19**(8): p. 783-6, 788, 790, passim; quiz 796.

296. Hanks, C.T., et al., *Cytotoxicity and dentin permeability of carbamide peroxide and hydrogen-peroxide vital bleaching materials, invitro.* Journal of Dental Research, 1993. **72**(5): p. 931-938.

297. Benetti, A.R., et al., *In vitro penetration of bleaching agents into the pulp chamber.* Int Endod J, 2004. **37**(2): p. 120-124.

298. Legeros, R.Z., J.A. Piliero, and L. Pentel, *Comparative properties of deciduous and permanent (yound and old) human-enamel.* Journal of Dental Research, 1983. **62**: p. 259-259.

299. Goodkind, R.J. and W.B. Schwabacher, *Use of a fiberoptic colorimeter for invivo color measurements of 2830 anterior teeth.* Journal of Prosthetic Dentistry, 1987. **58**(5): p. 535-542.

300. Burnell, J.M., et al., *Effects of dietary alteration of bicarbonate and magnesium on rat bone.* American Journal of Physiology, 1986. **250**(2): p. F302-F307.

301. Cohen, L. and R. Kitzes, *Infrared-spectroscopy and magnesium content of bone-mineral in osteoporotic women.* Israel Journal of Medical Sciences, 1981. **17**(12): p. 1123-1125.

302. Boskey, A.L., et al., *Effect of short-term hypomagnesemia on the chemical and mechanical-properties of rat bone.* Journal of Orthopaedic Research, 1992. **10**(6): p. 774-783.

303. Salimi, M.H., J.C. Heughebaert, and G.H. Nancollas, *Crystal-growth of calcium phosphates in the presence of magnesium-ions.* Langmuir, 1985. **1**(1): p. 119-122.

304. Rude, R.K. and H.E. Gruber, *Magnesium deficiency and osteoporosis: animal and human observations.* Journal of Nutritional Biochemistry, 2004. **15**(12): p. 710-716.

305. Wallach, S., *Effects of magnesium on skeletal metabolism.* Magnesium and Trace Elements, 1990. **9**(1): p. 1-14.

306. Yaegashi, Y., et al., *Association of hip fracture incidence and intake of calcium, magnesium, vitamin D, and vitamin K.* European Journal of Epidemiology, 2008. **23**(3): p. 219-225.

Appendices

I. Abbreviations

II. Published Article

I. **Abbreviations**

Abbreviation	**Meaning**
AEP	Acquired enamel pellicle
AF	Acid fuchsin
aPRPs	Acidic Proline-rich proteins
at%	Atomic percentage
bPRPs	Basic Proline-rich proteins
C	Carbon
Ca	Calcium
CHA	Carbonated hydroxyapatite
CIE	Commission international de l'Eclairage
Cl	Chloride
C- (C,H)	Hydrocarbon
C = O	Carbon double bonded to oxygen
COO^-	Carboxylate
CO_3^{-2}	Carbonate
C - (O, N)	Carbon single bonded to oxygen or nitrogen
Cu	Copper

DEJ	Dentino-enamel junction
DPJ	Dentino-pulpal junction
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
F	Fluoride
Fe	Iron
FTIR	Fourier Transform Infra-Red
FWHM	Full width at half maximum
H	Hydrogen
HA	Hydroxyapatite
H_2O_2	Hydrogen peroxide
K	Potassium
MB	Methylene blue
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$Mg(NO_3)_2$	Magnesium nitrate
N	Nitrogen
Na	Sodium
NaOH	Sodium hydroxide
O	Oxygen

P	Phosphorus
PO_4^{3-}	Phosphate tetrahedral (phosphate)
S	Sulphur
SEM-EDS	Scanning electron microscope energy dispersive x-ray spectroscopy
Si	Silicone
VMH	Vickers microhardness
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction
Zn	Zinc

II. Published Article

JOURNAL OF DENTISTRY 40S (2012) e25–e33

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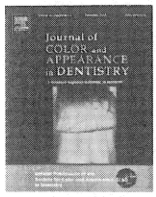

Hydrogen peroxide whitens teeth by oxidizing the organic structure

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ARTICLE INFO

Article history:
Received 16 May 2012
Received in revised form
24 July 2012
Accepted 14 August 2012

Keywords:
Tooth bleaching
Tooth shade
Hydrogen peroxide
Organic matrix
Oxidization

ABSTRACT

Objectives: The mechanism of tooth bleaching using peroxide oxidizers is not fully understood. It is unknown whether peroxide radicals make teeth whiter by deproteinizing, demineralizing, or oxidizing tooth tissues. This study was designed to define the mechanism of tooth bleaching and determine which of tooth enamel chemical components is/are affected by bleaching.

Methods: Sixty sound teeth were collected from adult patients. The teeth were divided into 6 equal groups ($n = 10$). Groups 1, 2, 3 and 4 were treated for 4 days with one of the following solutions: deproteinizing (NaOH) that removes organic content, demineralizing (EDTA) that decalcifies the mineral content, oxidizing (H_2O_2) and distilled water (control). Group 5 and 6 were pre-treated with either deproteinizing or demineralizing solutions before treating them with oxidizing solutions for 4 days. Changes in enamel elemental ratios, crystallinity index and tooth shade parameters of the treated teeth were examined by means of EDS, Raman spectroscopy and shade-spectrophotometry. The data obtained was analysed with Wilcoxon Signed-Ranks Test, and the statistical signicance was set at $p < 0.05$.

Results: Tooth deproteinization increased the lightness by $4.8 \pm 2.7^{\circ}$, tooth demineralization resulted in $8.5 \pm 5.6^{\circ}$ decrease in the lightness and tooth oxidization induced $19.9 \pm 6.5^{\circ}$ increase in the lightness. Oxidization of the deproteinized teeth did not influence shade parameters, but oxidation of the demineralized teeth resulted in $10.7 \pm 5.8^{\circ}$ increase in the lightness.

Conclusion: Hydrogen peroxide does not induce significant changes in tooth enamel organic and inorganic relative contents, and it whitens teeth just by oxidizing their organic matrix. These findings are of great clinical significance since they explain the mechanism of tooth bleaching, and help understanding its limitations and disadvantages.

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0300-5712/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jdent.2012.08.008