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New Approaches to Tooth Whitening Based on Changing the Optical Properties with Calcium Phosphate Containing Suspensions

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To My Husband and My Daughters

In Memory of My Dear Mother

Contents

Chapter 1

General Introduction

Tooth esthetics, which depends mainly on tooth color, is an important topic in dentistry. As reported, between 17.9–21.3 % (Alkhatib et al., 2005) and up to 34 % (Odioso et al., 2000) of adults in the U.K., as well as in the U.S.A., are dissatisfied with their tooth color. Thus, there is a huge demand for products and research related to tooth whitening.

1.1 Etiology of tooth discoloration

The mechanism of how the tooth discoloration was caused may have an effect on the outcome of treatment and may also influence the treatment options which can be offered to patients (Watts and Addy, 2001). The coronal portion of the tooth consists of enamel, dentine, and pulp. Light transmitting and reflecting properties within the enamel and dentine give rise to the intrinsic color of the tooth. Since the enamel is relatively translucent (Muia, 1982), the properties of the dentine play a major role in determining the overall tooth color (Joiner et al., 2008a).

Tooth discoloration is normally classified into three categories: intrinsic discoloration, extrinsic discoloration, and internalized discoloration (Watts and Addy, 2001). Intrinsic discoloration occurs after the structural composition of the tooth has changed. A series of metabolic diseases and systemic factors which influence the developing dentition result in intrinsic discoloration. Watts and Addy summarized the following factors for intrinsic discoloration: alkaptonuria, congenital erythropoietic porphyria, congenital hyperbilirubinaemia, amelogenesis imperfecta, dentinogenesis imperfecta, tetracycline staining, fluorosis, enamel hypoplasia, pulpal haemorrhagic products, root resorption and ageing (Watts and Addy, 2001).

The origins of the extrinsic stain may be classified into two classifications: organic stains and inorganic stains. The possible etiological agents for the organic extrinsic stains

include dietary components, beverages, tobacco, mouthrinses and other medicaments (Watts and Addy, 2001). The chromogens of these agents can be adsorbed onto tooth surface deposits, such as plaque or the acquired pellicle, and form extrinsic stains. On the other hand, the extrinsic staining of teeth may also be associated with occupational exposure to metallic salts such as tins and irons and a number of medicines containing metal salts (Addy and Roberts, 1981). Over the years, chromogens can diffuse into the teeth and cause intrinsic discoloration with extrinsic reasons.

Finally, internalized discoloration is the incorporation of extrinsic stain within the tooth defects following dental development. Pigments may become internalized at some developmental defects and acquired defects such as tooth wear, gingival recession, dental caries and restorative materials (Watts and Addy, 2001).

1.2 Tooth whitening methods

Considering the reported consumer's and patient's dissatisfaction with their perceived tooth color, manufacturers have developed various contemporary products that can be used at home or in the dentist's office to address the problem of tooth discoloration. With the exception of professional cleaning to remove stain and tartar or with the placement of crowns and veneers, the majority of whitening methods work in two mechanisms: internal/external bleaching of non-vital/vital teeth and the removal and control of extrinsic stains with whitening toothpastes. In this thesis, the former mechanism is called "bleaching" which is a procedure involves using oxidizing agents to bleach teeth; and the latter mechanism is called "whitening," which means that a physical method was used to increase mainly "brightness," a human sensation of tooth light reflection. According to the latter mechanism, another possible method of tooth whitening is by preparing a thin white surface cover with hydroxyapatite (Dabanoglu et al., 2009).

1.2.1 The bleaching of teeth

1.2.1.1 Mechanism of tooth bleaching

The mechanism of tooth bleaching by peroxide (hydrogen peroxide or carbamide peroxide) is by the diffusion of peroxide through the enamel, leading to the oxidation of

the colored species, especially within the dentinal regions. The oxidants cleave the double bonds in the chromophore molecules into smaller fragments, which absorb shorter wavelengths of light (such as ultraviolet light) rather than visible light so that the teeth appear to be bleached (Dadoun and Bartlett, 2003; Nathanson and Parra, 1987).

1.2.1.2 The methods of teeth bleaching

Three fundamental bleaching approaches have been described in the literature: dentist-supervised night guard bleaching, in-office or power bleaching and over-the-counter (OTC) bleaching products (Heymann, 2005). The most important factors of the efficacy of each bleaching treatment are dependent on the concentration of the bleaching agents and the duration of the exposure time (Heymann, 2005).

Night guard vital bleaching using 10% carbamide peroxide in a custom-fitted tray has proven to be one of the most ecomomic, safe and effective treatments for tooth bleaching (Sulieman, 2005a). Until now, only a 10 ± 1% carbamide peroxide concentration has received the seal of acceptance by the American Dental Association (ADA), which assures its safety and efficacy for at-home tooth bleaching (ADA, 2006)

In-office bleaching generally uses relatively high levels of whitening agents, such as 25–35% hydrogen peroxide containing products which can be further activated by heat or light (Joiner, 2006).

OTC products first appeared in the USA in the beginning of the 2000's, as a less expensive alternative to other costly methods (Donly et al., 2007). OTC bleaching products normally contain lower levels of the oxidizing agent (e.g. 3 – 6% hydrogen peroxide), which can be self-applied to the teeth via gum shields, strips or paint-on product formats (Collins et al., 2004; Gerlach, 2002; Slezak et al., 2002).

Dentist-supervised night guard bleaching and OTC bleaching products normally require at least a two-week application to get the optimum efficacy. Although in-office bleaching can obtain the obvious whitening efficacy after only one treatment, multiple

treatments are necessary to achieve optimum whitening efficacy (Al Shethri et al., 2003; Sulieman, 2005b).

1.2.2 The whitening of teeth - whitening toothpastes

Whitening toothpastes are based on formulations with enhanced physical and chemical cleaning abilities, including abrasives, chemical agents, and optical agents.

1.2.2.1 Abrasives

One of the main functional ingredients in whitening toothpastes is the abrasive, which can effectively remove extrinsic stains and prevent tooth stains from reforming without undue abrasivity towards the dental hard tissues (Joiner, 2010). The history of abrasives used in toothpaste can be traced back over 2000 years when preparations using bones and ground shells have been described (Forward, 1991). Nowadays, hydrated silica, calcium carbonate, dicalcium phosphate dihydrate, calcium pyrophosphate, alumina, perlite and sodium bicarbonate are used as abrasives in commercial toothpaste in order to aid in the physical removal of stains, plaque and food debris (Hefferren, 1998). These abrasives can remove the stains during tooth brushing because they are physically harder than the stain. Therefore, abrasive cleaning primarily influences only extrinsic stains and does not greatly influence any underlying intrinsic discoloration or the natural shade of the tooth (Joiner, 2010).

In order to improve whitening efficacy while preventing unwanted tooth wear, toothpaste abrasion towards the dental hard tissues is considered an important factor for whitening toothpaste design. Relative Dentine Abrasion (RDA) and Relative Enamel Abrasion (REA) were used to measure the erosive effect of the abrasives in toothpaste on the enamel (Hefferren, 1976; 1998). An improved “soft silica” abrasive (White, 2001), perlite (an amorphous glassy silicate, (Joiner et al., 2002) and a dual silica system consisting of 17% high cleaning silica and 17% polishing silica (Nathoo et al., 2008) have been described. It was concluded that they were shown to give a significant improvement in stain removal and/or prevention properties and did not give rise to a concomitant significant increase in enamel wear compared to other control silica

toothpastes.

1.2.2.2 Chemical agents

For augmenting the abrasive cleaning by aiding the removal and/or prevention of extrinsic stains, whitening toothpastes may contain additional agents such as peroxides, enzymes, citrates, pyrophosphates and hexametaphosphates (Joiner, 2010). 1% hydrogen peroxide (Hoic et al., 2004; Kleber et al., 1998), 0.5% calcium peroxide (Ayad et al., 1999) and sodium chlorite ($NaClO_2$) have been used in whitening toothpaste formulations. In previous studies, toothpastes containing these additional agents significantly impacted the removal of more extrinsic stains and gave better whitening efficacy than whitening toothpastes containing only silica.

Enzymes have also been used to enhance the removal of extrinsic stains because extrinsic stains are primarily incorporated into the pellicle, which is a salivary protein film that forms on the tooth's surface. It is possible that enzymes, such as proteases, could degrade the stained films and therefore remove it (Joiner, 2010).

Phosphate materials tend to have a strong binding affinity to enamel, dentine and tartar, and during adsorption, they could desorb stain components (Shellis et al., 2005; White, 2002). According to this principle, sodium pyrophosphate, sodium tripolyphosphate (STP) or the combination of these two compounds have been utilized in whitening toothpastes and have proven to have good stain prevention efficacy (Ayad et al., 2002; Rice et al., 2001). Sodium hexametaphosphate (HMP) is a longer chain molecule containing 10–12 repeating pyrophosphate subunits which work at multiple binding sites. Therefore, HMP could increase its retention and substantivity to tooth surfaces compared to pyrophosphate and as a result, prohibit stain-chromogen adhering to the enamel (Baig et al., 2005).

1.2.2.3 Optical agents

An optical agent, such as blue covarine, was incorporated into silica whitening toothpaste. The blue covarine can deposit onto the enamel surface and give a yellow to

blue color shift resulting in an improvement in measurable and perceivable tooth whitening (Joiner et al., 2008b). Furthermore, it has been reported that blue covarine containing toothpastes can give a significant and immediate reduction in tooth yellowness (reduction in b*) and an increase in tooth whiteness (Collins et al., 2008). Therefore, it was concluded that the silica-based toothpaste containing blue covarine not only removes extrinsic stains effectively, but also whitens the intrinsic color of tooth significantly (Joiner, 2010).

1.2.2.4 Toothpaste containing hydroxyapatite (HA-toothpaste):

The foregoing compositions of the whitening toothpastes mainly obtained the whitening efficacy through the abrasion of the extrinsic stain on the tooth's surface. Toothpastes have traditionally been used as a cleanser rather than as a preventive and curative agent (Niwa, 1994). Niwa (Niwa et al., 2001a) reported that toothpastes containing hydroxyapatite (HA-toothpaste) can increase the brightness and whiteness of teeth by promoting the process of remineralization, rather than changing the polishing properties. Since brightness and whiteness depend on the reflection rate of light, the whitening mechanism might be explained by a remineralization process which could turn the tooth surface smoother and glossier. They also found that brightening and whitening effects were improved while using a higher concentration of hydroxyapatite in the toothpaste (Niwa et al., 2001a). Aoki also gave the following reasons for observing whiter and brighter teeth: the polishing properties of HAP-toothpastes were lower than those of the toothpastes without HAP and remineralization could make the tooth surface smoother and glossier so as to increase the reflection rate of light. Changes on the surface's morphological wavelengths on the same order of magnitude of the visible light wavelengths are responsible for visual changes, similar to changes in gloss (Pedreira De Freitas et al., 2011). Roveri *et. al* observed a thick and homogeneous apatite layer covering the surface of the demineralized enamel after treatment with hydroxyapatite nanocrystals (Roveri N, 2009). This apatite layer can cause an increased diffuse reflection of light, resulting in a measurable increase of lightness (Roveri N, 2009). Dabanoglu and others (Dabanoglu et al., 2009) also reported that it was possible to whiten teeth with non-acidic HAP aqueous suspensions. The HAP suspension demonstrated a more

noticeable dose dependency and better effects compared to HAP in a dissolvable polymer film. Hydrodynamic shear force could remove some, but not all, of the material, indicating that the material is adhering to the enamel surface (Dabanoglu et al., 2009).

1.2.3 The measurement of tooth whitening efficacy

The methods used to measure tooth color include subjective clinical determinations and objective instrumental methods. In some clinical studies, the overall tooth color change was measured using techniques such as Vita shade guides, colorimeters, spectrophotometers, and image analysis of digital photographs of teeth.

1.2.3.1 Shade guides - subjective method

Shade guides for prostheses normally serve as the color standard in the clinical work since it is a quick and economic method for tooth color measurement. Also, they have been widely applied for a large number of tooth whitening studies where longitudinal changes in tooth color have been measured (Kihn et al., 2000; Mokhlis et al., 2000). However, several disadvantages of this method have been described. For example, it is a subjective method, whereby the process can be influenced by a number of factors such as lighting conditions, experience, room decor, and etc. The range of available shades is limited and cannot cover the whole spectrum of natural tooth color (Joiner, 2004). The results cannot be transformed into the CIE Lab color scale (van der Burgt et al., 1985) and furthermore, it is difficult to get consistent results among and within individual dentists in matching colors (Culpepper, 1970; Donahue et al., 1991; Okubo et al., 1998).

A new shade guide system (3DMaster, Vita, Bad Säckingen, Germany), which has been specifically designed for the selection of teeth, contains shade tabs that are uniformly arranged in the color space of natural teeth (Priest and Lindke, 2000). This shade guide has been shown to significantly improve consistency of tooth shade measurement compared to the traditional shade guide for a group of general practitioners (Hammad, 2003).

1.2.3.2 Colorimeters - objective method

Colorimeters are instruments designed to measure the color of objects. They have color filters that simulate the spectral function of the standard observer's eye. The result of colorimeters is often expressed in terms of the Commission Internationale de L'Eclairage (CIE) Lab color space. When colorimeters are used, it is required of custom positioning jigs to ensure reproducible positioning of the instrument's aperture onto the tooth's surface (Douglas, 1997). However, a further criticism of colorimeters is that systematic errors are difficult to manage and can be expected to adversely affect instrument accuracy in spite of the degree of precision or control of environment. Regardless of these issues, colorimeters have been shown to be highly sensitive when measuring tooth color differences and changes.

1.2.3.3 Spectrophotometers - objective method

Spectrophotometers measure the whole spectrum of light at a time from the reflection or transmittance of an object.

1.2.3.4 Image analysis of digital photographs - objective method

Image analysis of digital photographs of teeth can also be used to measure the tooth color. An image of the anterior teeth is captured under controlled lighting conditions by a digital camera together with suitable calibration tiles or standards and then subsequently analyzed by computer software (Gerlach et al., 2000). The color is often expressed in terms of CIE Lab values.

Regardless of the methodology employed, important factors in stain assessments include the calibration and standardization of objective measures and/or the demonstration of reproducibility and sensitivity in subjective indices.

1.2.4 The mechanism of the action of self-assembling peptides

Non-covalent interactions are responsible for forming the multimeric assemblies, which include van-der-Waals, pi-stacking, hydrogen bonds, polar and ionic interactions between the amino acid backbones and/or the amino acid side chains of the peptides

(Michael and Amadeus, 2014). In the presence of Non-covalent interactions，self-assembling peptides refers to the spontaneous and reversible organization of peptides with other peptides of their own kind (or peptides having a similar structure) into multimeric assemblies. The P11-4 self-assembling peptide comprises the sequence Glu-X2-Glu-X2-Glu, wherein X2 is an amino acid with a hydrophobic side chain selected from the group consisting of alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, and tryptophan (Michael and Amadeus, 2014). The sequence Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln is another preferred embodiment of P11-4 peptide. The peptides were designed to form assemblies in the following hierarchical order: tapes, ribbons, fibrils and fibres when pH work as a trigger (Aggeli et al., 2003). P11-4 peptide can assemble in one dimension to form beta-sheet, tape-like assemblies when pH is under 7.5. The peptide assemblies can switch from a fluid to a nematic, stiffer gel state in response to pH triggers.

P11-4 peptides have been described that can be used as templates or scaffolds for in situ nucleation of calcium phosphate (Michael and Amadeus, 2014). The acidic residue Glu, which has a negative charge, can provide nucleation sites for Ca^{2+}, at the meanwhile, the positively charged acidic residues, for example Arg, maybe have interaction with phosphate. If the Glu and Arg side chains can interact with solved calcium and phosphate to promote the nucleation of calcium phosphate combinations then the side chains can also interact with calcium phosphate particles and also the enamel surface. Based on this interaction, it was our hypothesis, that the interaction with the calcium phosphate particles, in our case hydroxyapatite, and the tooth surface could promote the adhesion of the HAP particles to the tooth surface and enhance the whitening effect.

1.3 The aim of this study

1. To evaluate a potential self-administered application regimen and obtain the whitening efficacy. The present study evaluated the performance of different calcium phosphate based material mixed into a toothpaste formulation.

2. To refine whitening methods so as to improve whitening efficacy, a self-assembling

peptide which can form a biomimetic matrix for nucleation and mineralization was tested.

3. To ensure the existence of a newly formed hydroxyapatite layer, a type of lanthanum (La) labeled HAP was employed. The hypothesis is that La should be detected by EDX if HAP forms a new layer on the intact enamel.

4. To ensure that the self-assembling peptides can effectively adhere to the enamel surface. ATR-FTIR was used to detect the amide bond of peptides. The hypothesis is that the amide bond should be detected after all treatments with the peptides.

Chapter 2

Efficacy of tooth whitening with different calcium phosphate-based formulations

2.1 Background and significance

Tooth color is the product of both the intrinsic tooth color, which is mainly determined by the properties of the dentin because the enamel is relatively translucent (PJ., 1985), and the presence of any extrinsic stain, which is often caused by dietary intake of substances such as coffee, tea or red wine. Although tooth brushing often does not remove extrinsic stains that form in less-accessible areas of the dentition (Macpherson et al., 2000), professional oral-hygiene measures can remove most external discoloration. Both slight discoloration, which is resistant to cleaning, and intrinsic discoloration can be aesthetically improved by the application of chemical agents that contain one or several compounds, such as sodium hypochlorite, sodium perborate, hydrogen peroxide or carbamide peroxide. Different concentrations of these tooth-whitening agents are available; higher concentrations must be used under professional supervision, whilst products containing between 0.1 and 6% hydrogen peroxide are permitted for use at home after medical education and supervision (The Cosmetic Products (Safety) (Amendment) Regulations 2012 No. 2263. , 2012).

Although chemical bleaching is very popular, this method can have detrimental effects. For example, bleaching agents can cause damage to dental hard tissues, especially when applied at high concentrations; in addition, alterations to the microstructure of the dental surface, decreases in surface microhardness, and fracture toughness changes have been reported (Akal et al., 2001; Bitter, 1992; Perdigao et al., 2004; Wandera et al., 1994). Furthermore, in restored teeth, chemical bleaching agents can influence the bonding strength between resin composites and the dental hard tissue, whether applied before or after restoration. For example, the shear bond strength of resin composites will be reduced after applying chemical bleaching agents (Garcia-Godoy et

al., 1993; McGuckin et al., 1992), and the bonding strength will be weaker on bleached hard tissues than on untreated tissues (Onuma et al., 2005; Titley et al., 1988; Torneck et al., 1990). Additionally, chemical bleaching agents (H_2O_2) can diffuse into the pulp chamber and produce H_2O and O_2 by catalase. O_2 may increase the pressure in pulp chamber and cause more pain.

Recently, hydroxyapatite (HAP), instead of oxidizing chemicals, has been proposed as a whitening agent. Yamagishi *et al.* (Onuma et al., 2005; Yamagishi et al., 2005) used an acidic solution with fluoride-substituted HAP (F-HAP) to regrow a new HAP layer, and the result of this experiment indicated that a new layer of HAP could be grown on the original enamel and that it adhered seamlessly. This acidic solution had two functions: dissolving the surface of the enamel and dissociating calcium phosphate clusters. These findings indicated that phosphoric acid can render the surface of the enamel clean and rough and that the dissociated ions can contribute to faster growth of HAP crystals than calcium phosphate clusters. Moreover, the crystallographic orientation, using ionic nucleation, was in accordance with the orientation of the original apatite crystals. In contrast to this finding, HAP can be nucleated with a random orientation using undissociated calcium phosphate clusters. This method can also be used for tooth whitening, not only because HAP itself is white, but also because the newly grown thin layer of HAP is assumed to contribute to diffuse reflection on the tooth surface, which causes the teeth to appear brighter. This method can only be applied under professional supervision because the acidic solution can damage the oral soft tissues.

Dabanoglu *et al.* reported that it was possible to whiten teeth with non-acidic HAP aqueous suspensions (Dabanoglu et al., 2009). The HAP suspension demonstrated a more noticeable dose dependency and better effects in comparison to HAP in a dissolvable polymer film. Hydrodynamic shear force could remove some, but not all, of the material, which implied that the material was self-adhering to the enamel surface (Dabanoglu et al., 2009). To evaluate a potential self-administered application regimen, the present study evaluated the performance of a similar material mixed into a toothpaste formulation.

2.2 Materials and methods

2.2.1 Treatment preparations

Three different HAP preparations were compared. In two, the concentration of HAP was varied, resulting in formulations containing 10, 20, and 30 wt% HAP. A commercial toothpaste and a topical fluoride agent, based on amine fluoride, served as control treatments.

The details of the materials are summarized in Table 2.1. Microrepair is an aqueous suspension of zinc-carbonate-apatite ($ZnCO_3$/Ap), which contains carboxymethylcellulose (CMC) as a "thickener". The IF 000062-N-1 group contained the same active ingredients as the Microrepair groups. In addition, the toothpaste contained all the ingredients of a complete toothpaste formulation, such as tensides, cleaning silica, and flavoring.

Table 2.1 Materials used in the different treatment groups

Material	Active Ingredient	Lot No.
Microrepair, 30 wt% (suspension)*	Zinc-carbonate-apatite	43-29.09.2011
Microrepair, 20 wt% (suspension)*	Zinc-carbonate-apatite	43-29.09.2011
Microrepair, 10 wt% (suspension)*	Zinc-carbonate-apatite	43-29.09.2011
C-73-33, 30 wt% (suspension)*	Tricalcium phosphate	TDS-15.10.2011
C-73-33, 20 wt% (suspension)*	Tricalcium phosphate	TDS-15.10.2011
C-73-33, 10 wt% (suspension)*	Tricalcium phosphate	TDS-15.10.2011
IF000062-N-1 (paste)*	Zinc-carbonate-apatite	30.11.2011
Colgate Sensation White (paste)	Unknown (probably abrasives of different sizes and types)	CP(L)1344PL1124
Elmex Fluid (liquid)	Amine fluoride	115712 (03-2014)

* Material supplied by Wolff Pharma, Bielefeld, Germany.

2.2.2 Study design

In this study the tooth color was measured using a dental spectrophotometer (Easyshade, Vita, Bad Säckingen, Germany). Tooth colors that were brighter than A3 were excluded from the study. A total of 90 extracted caries-free human teeth were randomly assigned to nine groups (n = 10 for each group) and were fixed onto glass microscopic slides. Before treatment, the teeth were polished with a rubber cup in a dental hand piece for 1 min with a prophylactic polishing paste (Stain Removal, NuproSensodyne; Dentsply, Konstanz, Germany). The teeth were then cleaned with running tap water and sonicated in distilled water for 3 min. The teeth had been stored in distilled water, with sodium azide as a disinfectant, from the time of extraction until application of the material. The study was approved by the University Ethical Committee (LMU, München, Germany) and was conducted according to the principles of the Helsinki Declaration for biomedical research. Informed consent was obtained from all donors. All teeth were used innominately.

The materials were directly applied to the buccal or lingual enamel surfaces with a cotton pellet and then agitated for 3 min. Afterwards, the suspensions/pastes were left for 5 min to allow undisturbed interactions of these materials with the enamel surface. Then, the teeth were stored in mineral water (Evian, Danone Waters Deutschland, Frankfurt, Germany) for 24 h at 37°C. This procedure was repeated three times.

To detect the stability of the interaction between the applied materials and the enamel surface, hydrodynamic shear force, produced by a sonic toothbrush (Sonicare PL-1, Philips Oral Healthcare, Hamburg, Germany), was applied to all teeth for 2 min. According to the work of Hope et al., the brush head vibrates at a high frequency and is accompanied by oscillation of the bristles (Hope et al., 2003). When water was used as an immersion liquid, hydrodynamic shear force was generated around the bristle tip by movement of liquid and applied to the enamel surface. The brush head was kept at a fixed distance of 1 mm from the tooth surface to ensure reproducible conditions.

2.2.3 Field-emission scanning electron microscopy study

After applying hydrodynamic shear forces, two randomly selected teeth from each group, as well as two untreated teeth serving as the blank control group, were examined using a field-emission scanning electron microscope (FE-SEM, ZEISS Supra 55vp, Zeiss, Oberkochen, Germany) at 10 kV and a working distance of 3 - 5 mm. The samples were dehydrated then observed using scanning electron microscopy. In brief, the teeth were immersed in ascending concentrations of ethanol (50, 75, 87.5, and 94%) for 15 min at each concentration. Then, the teeth were placed in 97% ethanol for 1 h before being transferred to hexamethyldisilizane (HMDS) for 5 min and allowed to air-dry for 20 min, according to the methods of Perdigao et al. (Perdigao et al., 1995). Next, the specimens were sputter-coated with a film of gold palladium alloy, of approximately 30 nm thickness, in a vacuum evaporator (SC7620 Mini Sputter Coater, Polaron, Quorum Technologies, Kent, UK). Digital images were obtained from secondary electrons. Three images of representative areas were stored at different magnifications (500×, 5,000×, and 20,000×).

2.2.4 Color assessment and statistical analysis

The dental spectrophotometer was calibrated after each power-on cycle with its built-in ceramic calibration reference block, with the probe covered with an infection control shield, as suggested by the manufacturer. To standardize the measurements, silicon attachments were created (Optosil Comfort Putty, Hereaus Kulzer, Wehrheim, Germany). The teeth were mounted onto a microscopic slide using hot glue (Pattex Hot Sticks, Henkel, Düsseldorf, Germany) for ease of handling during the experiment. The silicon attachments ensured exact repositioning of the light guide in terms of position and angulation. In addition, the ambient illumination conditions were standardized by performing these measurements inside of a dark box. Before measurement of color, the enamel surface was gently dried with an air syringe and then contacted perpendicularly with the probe, which was passed through the holes in the silicon attachments. In the present studies, color was expressed within the L*a*b* color space. The L* value indicates lightness and is measured on a scale from 0 (black) to 100 (white); the a* value

indicates redness (+) or greenness (-); and the b* value is a measurement of yellowness (+) or blueness (-).The L*a*b* value represents the three axes in this three-dimensional color space. The L*a*b* values were recorded at five time points (t1, without any treatment; t2, 24 h after the first application; t3, 24 h after the second application; t4, 24 h after the third application; and t5, 24 h after application of hydrodynamic shear forces). Three measurements were obtained after each test stage, and the mean of the three measurements was subjected to further analysis (Sulieman et al., 2003). The color changes between the different measurements and the baseline measurement in each group were calculated using Eqn (1) (CIE publication No.15.2. Commission Internationale de L'Eclairage. Colorimetry 1986.).

$$\Delta E = \sqrt{(L_1 - L_2)^2 + (a_1 - a_2)^2 + (b_1 - b_2)^2}\,. \qquad \text{Eqn (1)}$$

To determine whether significant differences in color changes existed among the time points and groups, two-factor ANOVA was performed. If the outcome presented significant differences, Tukey's post-hoc test, corrected with Bonferroni adjustment, was used to identify differences between two groups or two time points. Significance was set at $P < 0.05$. All aforementioned statistical analyses were performed using the R statistical program (R, 2008).

2.3 Results

2.3.1 Color assessment

The average color changes (ΔE) following application of the various treatments were calculated relative to the baseline values. Two-factor ANOVA was performed to analyze the differences using the following equation (Model 1):

ΔE ~ material + time +material*time. (Model 1)

As the 'time' factor and the interaction between the factors 'material' and 'time' were not significant, model 1 was refined to Model 2, as follows:

ΔE ~ material. (Model 2)

There were significant differences between all materials.

Table 2 summarizes the means and SD values of the average color changes for each

treatment group. The data from repeated applications were pooled, as the ‘time’ factor had no influence on the outcome. There were no significant differences among the groups that were in the same subsets, but the color changes were significantly different between the two different subsets. From a to b subsets, the color changes are more and more pronounced. C-73-33 (10 wt%) belonged to d subset and showed the most strongest color change ($\Delta E = 2.20 \pm 0.90$), whilst Microrepair (20 wt%) belonged to a subset and showed the weakest color change ($\Delta E = 0.91 \pm 0.50$). No significant difference was found among the Microrepair 20 wt%, Microrepair 10 wt%, and C-73-33 20 wt% groups, which displayed only weak changes and were therefore included in subset a. Also, there were no significant differences among Microrepair 30 wt%, C-73-33 10 wt%, IF000062-N-1, and Elmex Fluid, which showed more pronounced whitening effects and were therefore included in subset d. Moderate color changes were observed in b or c subset for C-73-33 30 wt% and Colgate Sensation White. In addition, significant differences were displayed between subsets a and d.

Table 2.2 Color changes (expressed as ΔE values) after application of different materials

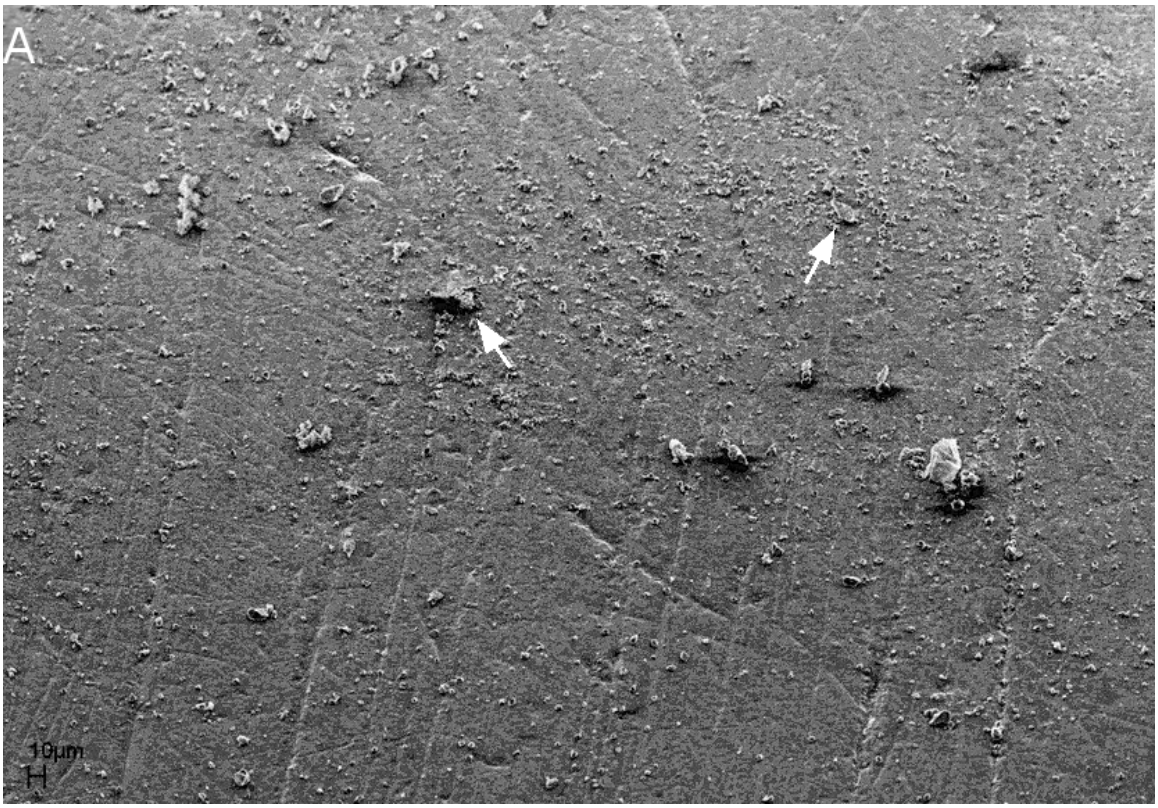
Material	n	ΔE
		Mean ± SD
Microrepair, 30 wt%	40	1.70 ± 1.60[b,c,d]
Microrepair, 20 wt%	40	0.91 ± 0.50[a]
Microrepair, 10 wt %	40	1.03 ± 0.76[a]
C-73-33, 30 wt%	40	1.06 ± 0.75[a,b]
C-73-33, 20 wt%	40	1.03 ± 0.67[a]
C-73-33, 10 wt%	40	2.20 ± 0.90[d]
IF000062-N-1	40	1.93 ± 0.99[c,d]
Colgate Sensation White	40	1.43 ± 0.81[a,b,c]
Elmex Fluid	40	1.80 ± 1.10[c,d]

Δ E values are given as mean ± SD. Different superscript letters indicate statistically significant differences for ΔE in different groups ($P < 0.05$). The same superscript letter denotes subsets in which values were not significantly different for different treatments ($P > 0.05$). From a to d, the mean value of Δ E is increased.

Among the materials containing $ZnCO_3$/Ap as the active ingredient, the water-based suspension with the highest concentration (30 wt%) achieved a better effect compared with the water-based suspension containing lower $ZnCO_3$/Ap concentrations (20 and 10 wt%, $P < 0.05$). In addition, the toothpaste, which also contained $ZnCO_3$/Ap, showed a better whitening effect compared with the suspensions. The 10 wt% tricalcium phosphate suspension demonstrated the best whitening effect.

Both the commercial toothpaste and the topical fluoride (based on amine fluoride) also displayed a certain degree of whitening effect.

2.3.2 Field-emission scanning electron microscopy



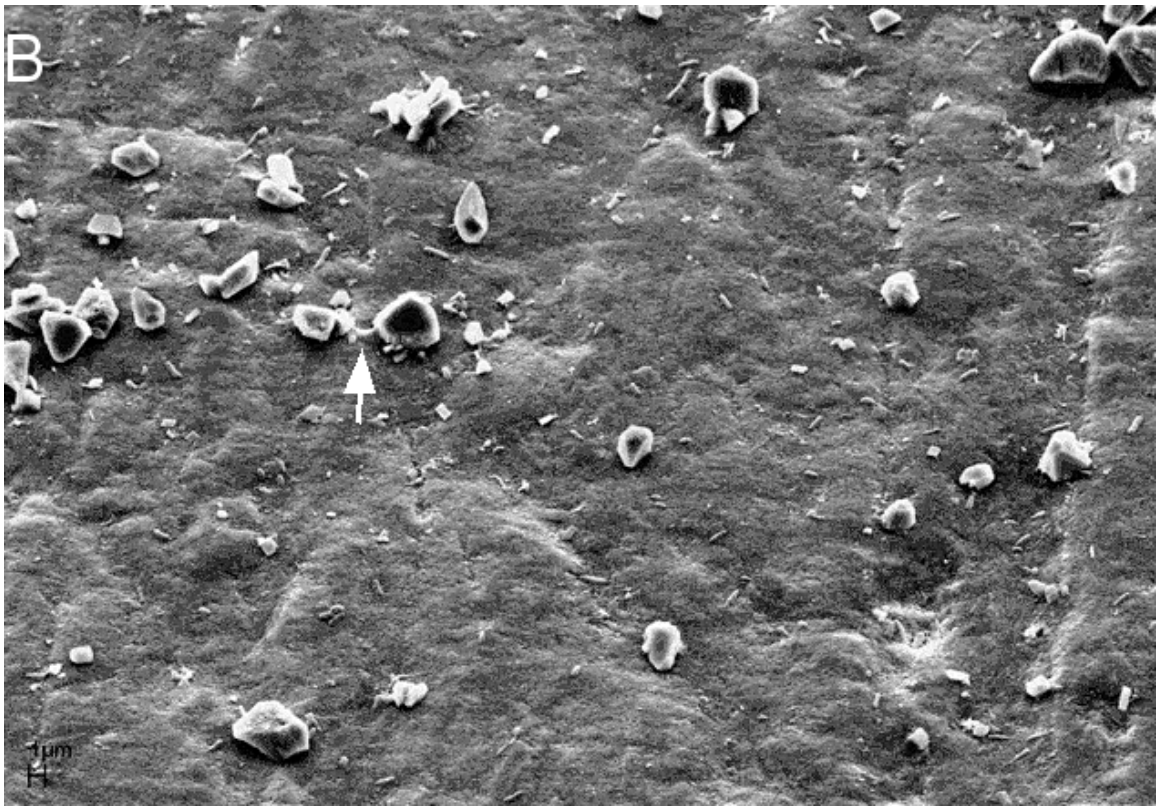
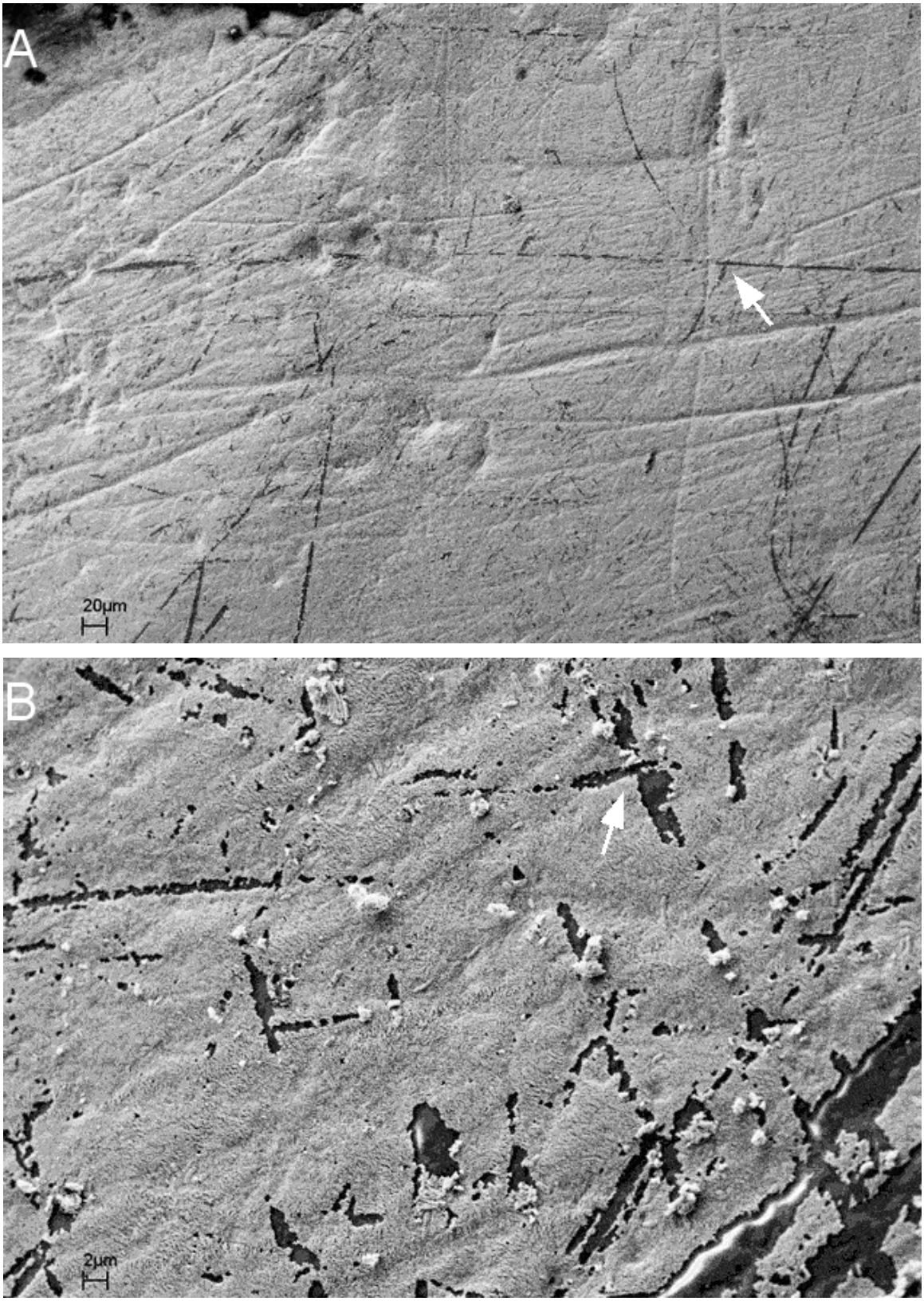


Fig.2.1 Enamel surface treated with Microrepair ($ZnCO_3$/Ap) and visualized at 500× magnification (A), 5,000× magnification (B), and 20,000× magnification (C). The arrows point to hydroxyapatite (HAP) crystals that adhered to the scratches.


A
20µm
B
2µm

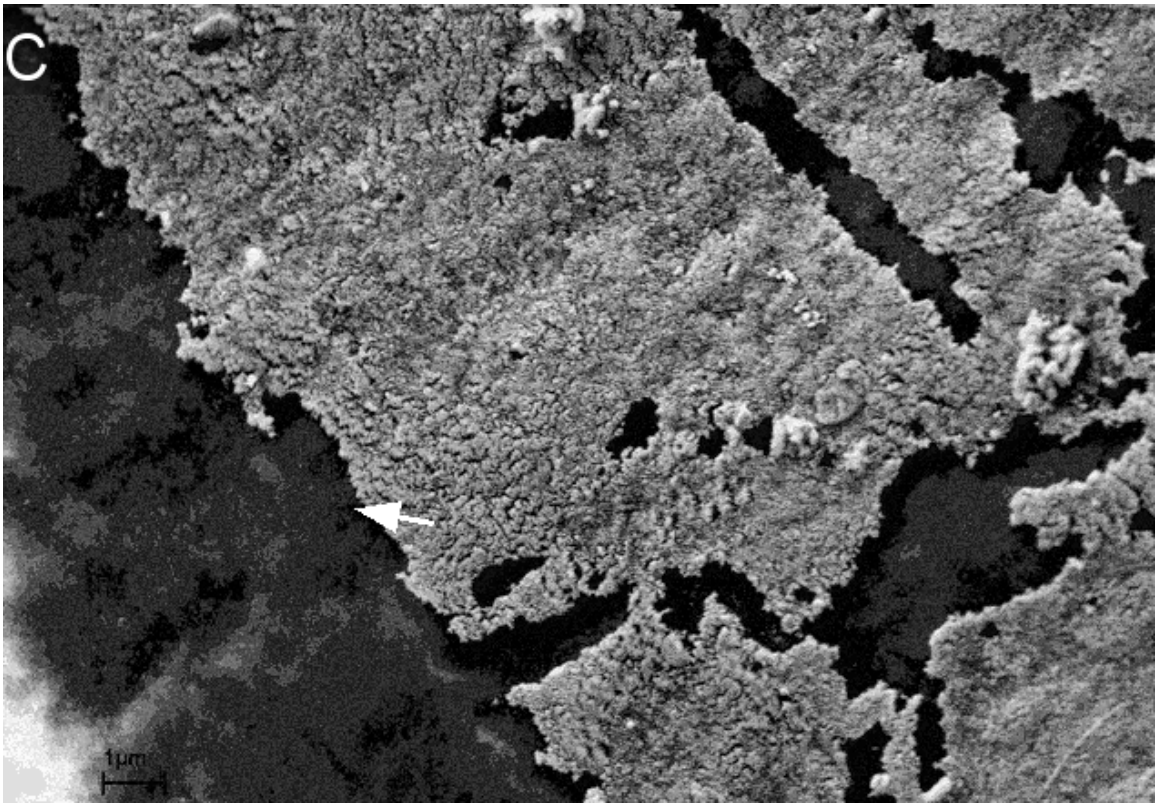
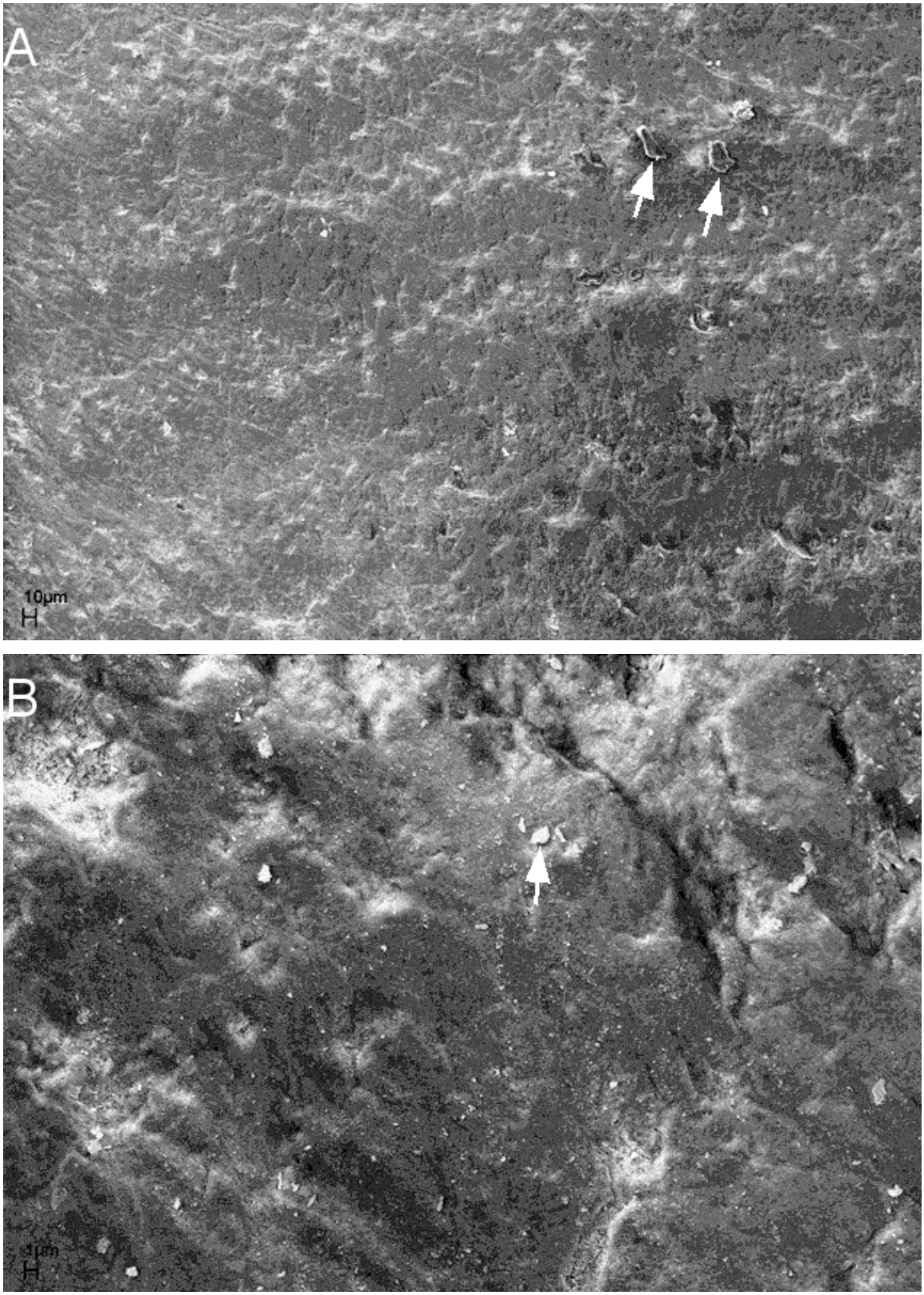


Fig.2.2 Enamel treated with C-73-33 (tricalcium phosphate) and visualized at 500× magnification (A), 5,000×magnification (B), and 20,000× magnification (C). The arrows identify areas where the film, which covered the surface, was scratched and the underlying enamel surface exposed.


A
10µm
H
B
1µm
H

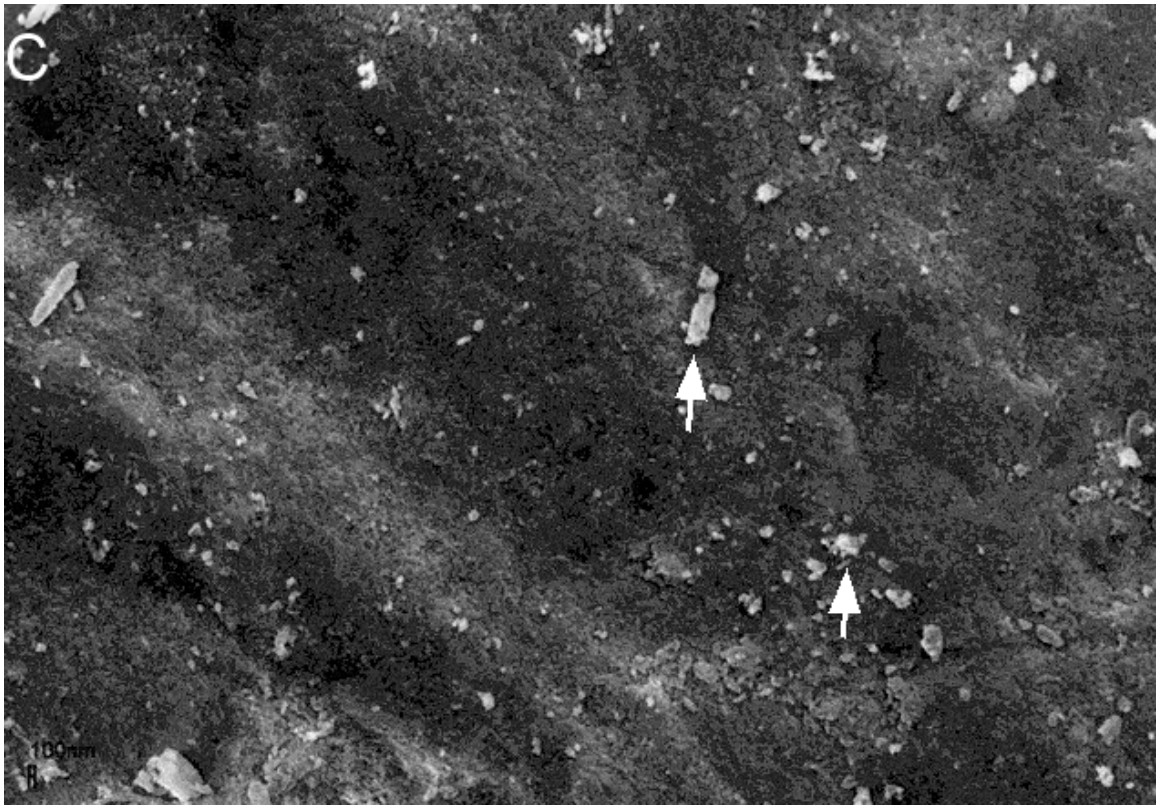


Fig.2.3 Enamel treated with IF 000062-N-1 ($ZnCO_3$/Ap) and visualized at 500× magnification (A), 5,000× magnification (B), and 20,000× magnification (C). The arrows show hydroxyapatite (HAP) crystals that adhered to the enamel surface.

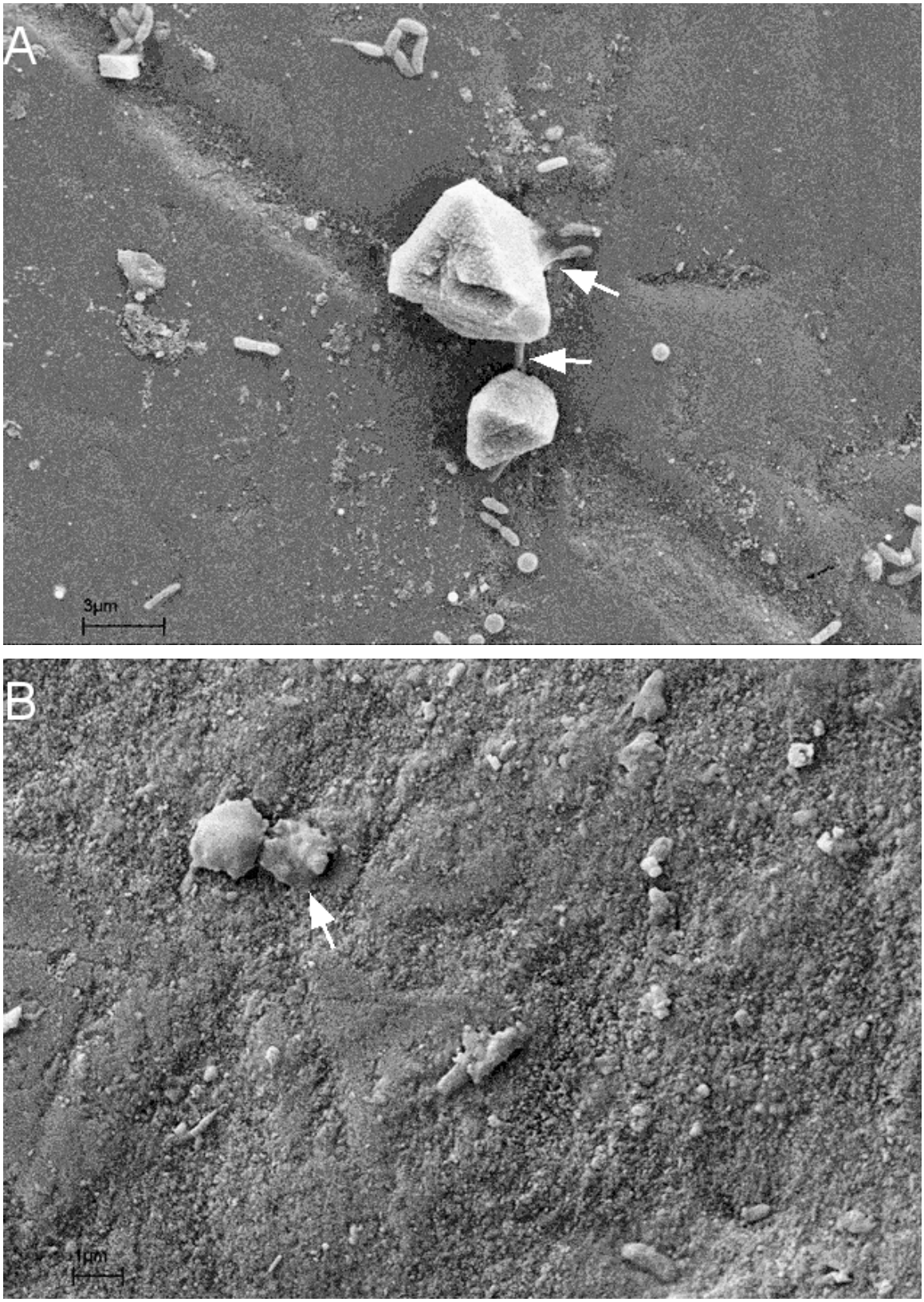


Fig.2.4 Enamel surface after treatment with Microrepair 10 wt% (11,000 ×

magnification) (A) and C-73-33 20 wt% (20,000× magnification) (B). There was a well-formed mechanical connection between the particles and the enamel surface (arrows).

Based on the scanning electron microscopy images, the Microrepair particles were 1–10 μm in size (Fig. 2.1), whilst the C-73-33 particles were much smaller (<1μm; Fig. 2.2). In addition, the scanning electron microscopy images suggested that the particles, particularly the small particles, tended to adhere preferably to surface irregularities (e.g. to scratches) as a result of the polishing procedure. We did not observe complete coverage of the surface by the large Microrepair particles, regardless of whether they were applied in suspension or paste formulations (Figs 2.1 and 2.3), whereas the smaller particles of C-73-33 seemed to cover the entire surface area (Fig. 2.2). Both Microrepair particles and C-73-33 particles showed a well-formed mechanical connection between the particles and the enamel surface (arrows in Fig. 2.4).

2.4 Discussion

To obtain reproducible results, the probe tip of the spectrophotometer had to be placed on exactly the same site of the tooth at the same angle at each measurement. Khurana et al. (Khurana et al., 2007) showed that Easyshade only produced repeatable results in 50% of the measurements in a clinical study. However, Dozic et al. (Dozic et al., 2007) concluded that Easyshade was one of the most reliable color-measuring devices on the market, in both in-vitro and in-vivo circumstances. In the current study, a silicon attachment to the light guide was used for each tooth to ensure a reproducible position for each measurement. At the same time, ambient light was carefully controlled with the black box. Therefore, the background of the sample could be kept constant during the measurement. These two measures resulted in very small variations in the measurements as a result of the measurement process itself.

Distilled water and deionized water can demineralize samples during the storage period because of ion imbalances. In some in-vitro studies, artificial saliva or fluoride products have been used between or after treatments to simulate the clinical situation

more closely. However, the aim of the current study was to investigate the whitening effects of HAP with the growth of a new HAP crystallite layer. Therefore, to avoid interference with other remineralization factors, artificial saliva and fluoride were not present in the storage solution. In the current study, Evian water was used for three reasons. First, because it is not deionized, it could prevent the HAP particles in suspensions from dissolving before they reacted with the enamel surface. Second, unlike artificial saliva, Evian water is not saturated with ions, so it can prevent crystal growth not attributed to the treatment. Third, Evian water is highly standardized, with the quality being nearly the same worldwide, as well as having a pH value of 7.18, which is close to neutral. Therefore, Evian water should have had a neglectable influence on crystal growth.

Two published hypotheses have been proposed to explain the mechanism of tooth whitening with HAP materials. Niwa et al. (Niwa et al., 2001b) observed increased tooth brightness and whiteness after the application of a HAP-containing toothpaste, and he postulated that remineralization processes made the surfaces smoother, which resulted in an increase in brightness. Whilst the hypothesis of Niwa et al. (Niwa et al., 2001b) would favor specular light reflection, which can make teeth appear brighter in the eyes of observers, the surface layer, as observed by Roveri et al. (Roveri N, 2009), can cause an increased diffuse reflection of light, which results in a measurable increase in lightness. In our study, we observed a surface layer and particles that both favored diffuse reflection, although small particles were more effective, as they covered the entire surface. Even if this outcome was not a permanent effect, the application of such HAP materials can be repeated without adverse effects, in contrast to peroxide-based whitening agents.

Dabanoglu et al. reported, in 2009, that a HAP suspension demonstrated more noticeable dose dependency, and these authors observed more pronounced whitening than we did in the present study (Dabanoglu et al., 2009). Moreover, in our study, the application time did not seem to have any influence on the whitening effect and the ΔE value was smaller than in this previous study. One possible reason for this difference is that, with the method of Dabanoglu et al. (Dabanoglu et al., 2009), the teeth were not

preselected according to their color, whereas teeth that were brighter than A3 were excluded from our study. Moreover, the concentrations of HAP and the pH values were different between the two studies because of the use of different solvents (Evian drinking water in the present study) and also a thickener (CMC) in the present study. If the surface was already covered with the maximal adhering particle load after the first application, our regimen would not have demonstrated any association with the number of applications. In the mouth, however, with repeated de- and remineralization changes, repeated applications should have a positive effect. In addition to tooth whitening, the adhering HAP particles should have an additional positive effect as they can serve as a solid buffer, which means that during acid attack, the protons will interact not only with the enamel surface but also with the HAP layer.

In our study, the Microrepair particles were rather small (100–300 nm), but according to the scanning electron microscopy images, they clearly agglomerated during the mixing process (Fig. 2.1). It has been reported that carbonate-hydroxyapatite nanocrystals can aggregate in microsize crystal clusters, the dimensions of which increase with increasing maturation time in the mother solution at a constant temperature and stirring rate (Gazzaniga et al., 2013). In our study, a similar agglomeration occurred, resulting in the application of Microrepair particles that were approximately 1–10μm in size, whilst the C-73-33 particles were much smaller (<1μm) and formed a uniform film on the enamel surface (Fig. 2.2). According to the scanning electron microscopy images, it seemed plausible that increasing concentrations of the larger particles caused a greater whitening effect as a result of their increased density on the enamel surface. In contrast, the lack of any dose dependency of the small C-73-33 particles might be explained by the observation that the surface was already completely covered at the lowest concentration; as a result, increasing concentrations could not increase the coverage.

The concentrations of Ca^{2+} and PO_4^{3+} are key factors during remineralization, and calcium and phosphate are known to exist in two forms: ions and clusters. Onuma et al. (Onuma et al., 2005; Yamagishi et al., 2005) reported that HAP could be nucleated with a random orientation using the calcium phosphate cluster. In contrast to this finding, the

crystallographic orientation using ionic nucleation, which was induced by an acidic solution, was in accordance with the orientation of the original apatite crystals; as a result, a homogeneous and stable apatite layer was formed, with crystal sizes smaller than the wavelength of light. In another study of the remineralization of early enamel caries, a 10 wt% suspension proved to be an optimal concentration, whereas a concentration higher than 15 wt% caused a certain level of unavoidable aggregation (Huang et al., 2009). This finding is also in accordance with the present results. Furthermore, Roveri et al. observed similar coating with a non-acidic solution as an initiator, but these authors stated that this coating was less crystalline in comparison with native enamel apatite and consisted of a new apatite mineral deposition, which progressively filled the scratches and pits (Roveri N, 2009). This finding is also in accordance with the scanning electron microscopy images of the present study.

When present in toothpaste formulations, $ZnCO_3$/Ap results in a more obvious whitening effect than when present in suspension formulations containing the same whitening ingredients (Fig. 2.3). It may be that surfaceactive tensides enhance adsorption to the enamel surface, although this is currently purely speculative.

Elmex fluid also demonstrated a whitening effect. Although this finding was unexpected, amine fluoride has a low pH of 3.9, which can acid-etch the enamel surface and increase the surface roughness. Rougher surfaces reflect more light, which is scattered by the surface irregularities, and the diffuse reflection makes the surface appear whiter. This phenomenon is well known in dentistry, in which phosphoric acid causes a frosty appearance of the enamel surface during preparation for adhesive restorations. During the measurements, the tooth surfaces were dry, which enhanced the diffuse reflection after the application of Elmex fluid. Elmex fluid was never intended to be a whitening agent and it is not recommended for this purpose because there may be a long-term risk of erosion with the frequent application of acidic products. Elmex fluid was included in this study because the manufacturers of the HAP-containing products made an additional claim about their ability to remineralize enamel lesions, which was not addressed in the current study protocol. Elmex fluid showed similar effectiveness,

which was not surprising because it is already an accepted product for remineralization. For reasons of completeness, we also included Elmex fluid as a control in the current study.

Colgate Sensation White toothpaste displayed a whitening effect to a certain extent. Based on its composition, it is not clear how this bleaching action can be explained. The initial hypothesis was that Colgate Sensation White only contains effective abrasives, which remove extrinsic stains and thereby contribute to the whiter appearance of teeth. In our in-vitro study, the teeth were thoroughly cleaned before testing, and on scanning electron microscopy images the surface appearance of these treated teeth was comparable with that of the other samples, which would exclude excessive abrasive action as an explanation. Moreover, the individual ingredients listed on the toothpaste label could not explain an oxidation-based bleaching effect. However, it could be speculated that the combination of the ingredients contributed to the observed tooth whitening.

In previous de-/remineralization studies, some researchers have assumed that the connection between a new apatite deposition coating and the demineralized area of an enamel surface was caused by chemical bonding (Roveri N, 2009). In our study, this connection was observed in some, but not all, samples (Fig. 2.4). Therefore, it is necessary to investigate this mechanism of adherence in future, more detailed, studies.

The results of the present study identified positive trends, although the dose and time relationships were not easy to interpret. The whitening effect was not very pronounced, but it was statistically significant. We should perform more research on how to augment the whitening effect. It also should be investigated, in greater detail, how a significantly higher number of applications would affect color changes over time and how pH cycles would influence the final outcomes. In addition, it should be investigated, in greater detail, how the size of the HAP particles influences their interaction with light.

Nevertheless, the current results are promising because HAP additives have more advantages than bleaching agents. They were shown to have no adverse effects on

toothpaste formulations. From a clinical viewpoint, HAP additives consist of small particles with a low hardness, resulting in weak relative dentin abrasion (RDA) of toothpaste; from a chemical viewpoint, HAP additives are chemically the same as tooth or bone tissue and non-corrosive at all concentrations. Moreover, these additives adhered to the enamel surface and contributed to remineralization and tooth whitening, and it is possible that they may also protect the tooth surface from acidic attacks.

Chapter 3

Improve the Whitening Efficacy with Biomineralization Peptide and Nano-Calcium Phosphate Formulation

3.1 Background and significance

In the study provided in chapter 2, although the efficacy is not very apparent, we were indeed able to get a whitening effect using calcium phosphate-based formulations. Mineralization and whitening efficacy can be obtained simultaneously, both of which are meaningful for current preventive dentistry and esthetic dentistry. In this chapter, we aim to refine the methods in order to improve whitening efficacy and enhance the chance of biomineralisation at the same time. For this aim, a self-assembling peptide, which can form a biomimetic matrix for nucleation and mineralization, was used in this study.

3.1.1 Enamel formation during odontogenesis

Generally, the enamel formation undergoes three steps: in the beginning, the organic amelogenin matrix forms and induces crystal-nucleation and regulation of the crystal growth. This control of hydroxyapatite crystal deposition and growth is a key process in enamel growth mediated by amelogenin (Akita et al., 1992; Robinson et al., 1995). Subsequently, the matrix degrades and the enamel crystals mature.

Enamel tissue cannot be regenerated naturally. There are three main reasons: First, enamel does not contain any cells, which could mediate the formation of new tissue. Second, matrix formed by amelogenin, which could support de novo biomineralisation, has already been degraded in the matured enamel. Third, especially initial caries, which occur underneath the hypermineralised plate, are difficult to be recovered by remineralization.

3.1.2 Amelogenin

Amelogenin, which is secreted by the ameloblasts, is the predominant protein of the enamel's protein matrix. This protein can be divided into three domains (Toyosawa et al., 1998). The first domain, called tyrosine-rich amelogenin peptide (TRAP), is a 45 amino acid N-terminal domain with high tyrosine content. Amelogenin cannot self-assemble if the TRAP was been removed (Ravindranath et al., 1999). The second domain contains hydrophobic character and is constructed of X-Y-Pro repeating motifs where X and Y are often glutamine. This is the largest segment and comprises the central segment. The third domain is the C-terminal region which contains negatively charged acidic residues, providing nucleation sites for calcium phosphate. Because of these three domains, amelogenin has amphiphilic characteristics and the ability of self-assemble (Margolis et al., 2006).

The amelogenin protein is mostly hydrophobic but dose contain a hydrophilic C terminus. Recent data demonstrate that hydrophobic collapse can drive amelogenin assembly (Lakshminarayanan et al., 2007). Amelogenin has also been found to self-assemble into nanospheres (Fincham et al., 1994; Fincham et al., 1995; Moradian-Oldak and Goldberg, 2005). Nanospheres can give rise to higher order structures such as chains and ribbons (Ecarot-Charrier et al., 1983) (Veis, 2005) which can direct apatite growth (Du et al., 2005; Fincham et al., 1994; Moradian-Oldak et al., 1998).

3.1.3 Casein phosphopeptides (CPP)

According to the principles of the enamel formation during odontogenesis, many studies have been performed to mimic the enamel formation during the biomineralisation of enamel or for the remedy of initial caries. Some studies have indicated that CPP, which contain multiple phosphoseryl residues, are also capable of nucleating hydroxyapatite (Chang et al., 2006; Hartgerink et al., 2001).

CPP is a hotspot in mineralization research. The sequence –Ser(P)–Ser(P)–Ser(P)–Glu–Glu– is the active center of CPP, where Ser(P) represents a

phosphoseryl residue. The ability of the CPPs to stabilize calcium and phosphate ions in supersaturated solution depends on the length and sequence of the peptides and the specific number of phosphoseryl residues present (Cross et al., 2005; Cross et al., 2007; Reynolds et al., 1982). Furthermore, the binding of the CPPs to the pellicle and plaque appears to be associated with the hydrophobic residues of the peptides (Cross et al., 2007). The positively charged surface of the calcium and phosphate ion clusters primarily interacts with the CPP through the negatively charged residues of the peptides (Cross et al., 2005). With modern peptide synthetic approaches, it is possible to incorporate additional phosphoseryl residues and to alter other residues for better stabilization and delivery of bioavailable calcium and phosphate ions.

3.1.4 Self-assembling peptide P11-4

With the exception of CPP, a kind of self-assembling peptide (Curodont^{TM}, credentis ag, Windisch, Switzerland) for enamel and dentin remineralisation has already been produced and used in clinical treatments. Curodont Repair contains P11-4 as a self assembling peptide. It can be used as an enamel remineralization matrix to regenerate enamel.

This peptide was originally designed for initial caries or demineralization. In the current studies, we tried to use this peptide to induce better biomineralization and to enhance whitening efficacy. Because we wanted to whiten healthy teeth, the introduction of this peptide had to be modified. That is to say, we tried to form a new HAP layer by using P11-4 peptide and nano-calcium phosphate without etching the enamel surface before treatment. Because the P11-4 peptide has a high affinity for HAP, which is the main component of enamel and suspensions/pastes used in the current study, the hypothsis is that P11-4 peptide should act like a glue to bond the HAP particles to the enamel (Fig. 3.1).

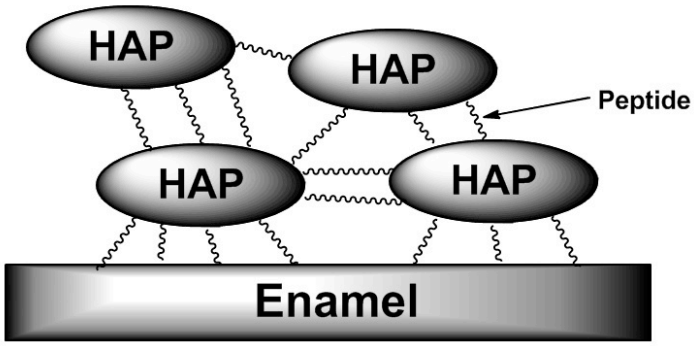


Fig.3.1 The schematic of the whitening mechanism of P11-4 peptide. P11-4 peptide act like a glue to bond the HAP particles to the enamel.

3.1.5 Calcium phosphate for the remineralization of enamel

Remineralization has been used previously to achieve mineral gain, including precipitation of minerals onto the enamel surface (Tung and Eichmiller, 2004). Precipitation is the formation of a solid phase of ion clusters from supersaturated solutions. The problem of enamel remineralization is due to the poor solubility of calcium phosphate. Thus, the concentration of calcium and phosphate ions are too low for remineralization to occur.

Tricalcium phosphate (TCP) has recently been added to dental products to remineralize white-spot lesions. Interestingly, TCP is referred to as being "functionalized," since it has been altered by ball milling with sodium lauryl sulfate (Karlinsey et al., 2009; Karlinsey et al., 2010).

3.2 Materials and methods

3.2.1 Treatment preparations

The active ingredient in the calcium phosphate-paste (Budenheim, Germany) used in the current study is tricalcium phosphate (TCP). The diameter of the TCP primary particles is approximately 50-100 nanometers.

The formula of the artificial saliva contains potassium chloride (KCl, 1.20 g), sodium chloride (NaCl, 0.84g), di-potassium hydrogen phosphate (K_2HPO_4, 0.26 g), calcium chloride ($CaCl_2$, 0.14 g) and water (1000 g). This artificial saliva was used as the

storage solution in the current study.

3.2.2 Sample preparation

A total of 20 adult bovine incisor teeth without roots and stains were selected. The teeth were mounted onto a microscope slide using hot glue (Pattex Hot Sticks; Henkel, Düsseldorf, Germany) for ease of handling during the experiment. To standardize the measurements, silicon attachments were created (Optosil Comfort Putty; Hereaus Kulzer, Wehrheim, Germany). The silicon attachments ensure exact repositioning of the light guide in terms of the position and angulation. The teeth were then polished with a rubber cup in a dental hand piece for 1 min with a prophylactic polishing paste (Stain Removal, NuproSensodyne; Dentsply, Konstanz, Germany). Subsequently, the teeth were cleaned with running tap water and sonicated in distilled water for 3 min. The teeth were stored in distilled water, containing sodium azide as a disinfectant, from the time of extraction until application of the material.

3.2.3 Study design

First of all, we did the pre-test: 5 bovine teeth were selected and prepared as above described. After enamel polishing, the original color of tooth was measured as baseline. Then, the P11-4 peptide solution was applied on the enamel surface. After 5 min, the enamel surface was gently dried, and color was measured again. After the second measurement, the bovine teeth were stored in the artificial saliva for 24h, and then the tooth color was measured thirdly. The ΔE between every measurement was calculated with the same method as Chapter1. Finally, we found that all of ΔE were less than 1. According to the analysis in chapter1, we speculated that the small ΔE is due to random error rather than color changes. P11-4 peptide has no influence on tooth color in itself.

Afterwards, 20 bovine teeth were randomly assigned into two groups (n = 10 for each group). Before treatment, the buccal enamel surfaces were gently dried. For group 1: HAP suspension (TCP-MV 500) was directly applied to the buccal enamel surfaces with a cotton pellet and then agitated for 3 minutes. Afterwards, the suspensions/pastes were

left for 5 minutes to allow undisturbed interactions of these materials with the enamel surface. For group 2: Enamel remineralisation matrix (Curodont Repair, Credentis Ag, Windisch, Switzerland) was reconstituted with 50µl of water (B. Braun Melsungen AG, Melsungen, Germany). A drop of peptide solution was applied onto air dried buccal enamel. The drop was evenly spread onto the surface with a syringe needle and was left on the tooth for up to 5 minutes. The remainder of the peptide solution was not rinsed off, or wiped away. Then HAP suspension (TCP-MV 500) was applied to the enamel surface and left for an additional 5 minutes without agitating. After all treatments, group 1 and 2 were stored in artificial saliva at pH 6.9 (Pharmacy University of Munich, Munich, Germany) for 24 h at 37°C. These procedures were repeated two more times.

After the third treatment and storage, to detect the stability of the interaction between the applied materials and the enamel surface, a hydrodynamic shear force, produced by a sonic toothbrush (Sonicare PL-1; Philips Oral Healthcare, Hamburg, Germany), was applied to all teeth for 2 minutes. According to the work of Hope et al. (Hope et al., 2003), the brush head vibrates at a high frequency and is accompanied by oscillation of the bristles. When water was used as the immersion liquid, hydrodynamic shear force was generated around the bristle tips by the movement of liquid and applied to the enamel surface. The brush head was kept at a fixed distance of 1 mm from the tooth surface to ensure reproducible conditions.

3.2.4 Field-emission scanning electron microscopy study

After applying hydrodynamic shear force, two randomly selected teeth from each group, were examined using a field-emission scanning electron microscope (ZEISS Supra 55vp; Zeiss, Oberkochen, Germany) at 10 kV and a working distance of 3–5 mm. The samples were dehydrated and sputter-coated with a film of gold palladium alloy, of approximately 30 nm thickness, in a vacuum evaporator (SC7620 Mini Sputter Coater, Polaron; Quorum Technologies, Kent, UK). Then the specimens were observed using scanning electron microscopy and digital images were obtained from secondary electrons. Three images of representative areas were stored at three different magnifications (10000×, 20000×, and 35000×).

3.2.5 Color assessment and statistical analysis

In this study, the tooth color was measured using a dental spectrophotometer (Easyshade; Vita, Bad Säckingen, Germany) as described in chapter 1. Before the color was measued, the enamel surface was gently dried with an air syringe for 1 minute and then contacted perpendicularly with the probe, which was passed through the holes in the silicon attachments. In the present studies, color was also expressed within the L*a*b* color space as described in chapter 1. The L*a*b* values were recorded at four time points (t1, before treatment; t2, 24 hours after the first application; t3, 24 hours after the second application; t4, 24 hours after the third application and after hydrodynamic shear force was applied). Three measurements were obtained after each test stage, and the mean of the three measurements was subjected to further analysis (Sulieman et al., 2003). The color changes between the different measurements and the baseline measurement in each group were calculated using Eqn (3.1) (CIE publication No.15.2. Commission Internationale de L'Eclairage. Colorimetry 1986.).

$$\Delta E=\sqrt{(L_1-L_2)^2+(a_1-a_2)^2+(b_1-b_2)^2} \qquad \text{equation (3.1)}$$

The ΔE was analyzed with a two-way ANOVA. The treatment (with or without protein) and time (three level) were considered as two factors. In order to analyze the time factor further, we use a one-way ANOVA to analyze each group, respectively. Significance was set at $P < 0.05$. All aforementioned statistical analyses were performed using the SPSS 16.0 statistic software.

3.3 Results

3.3.1 Color assessment

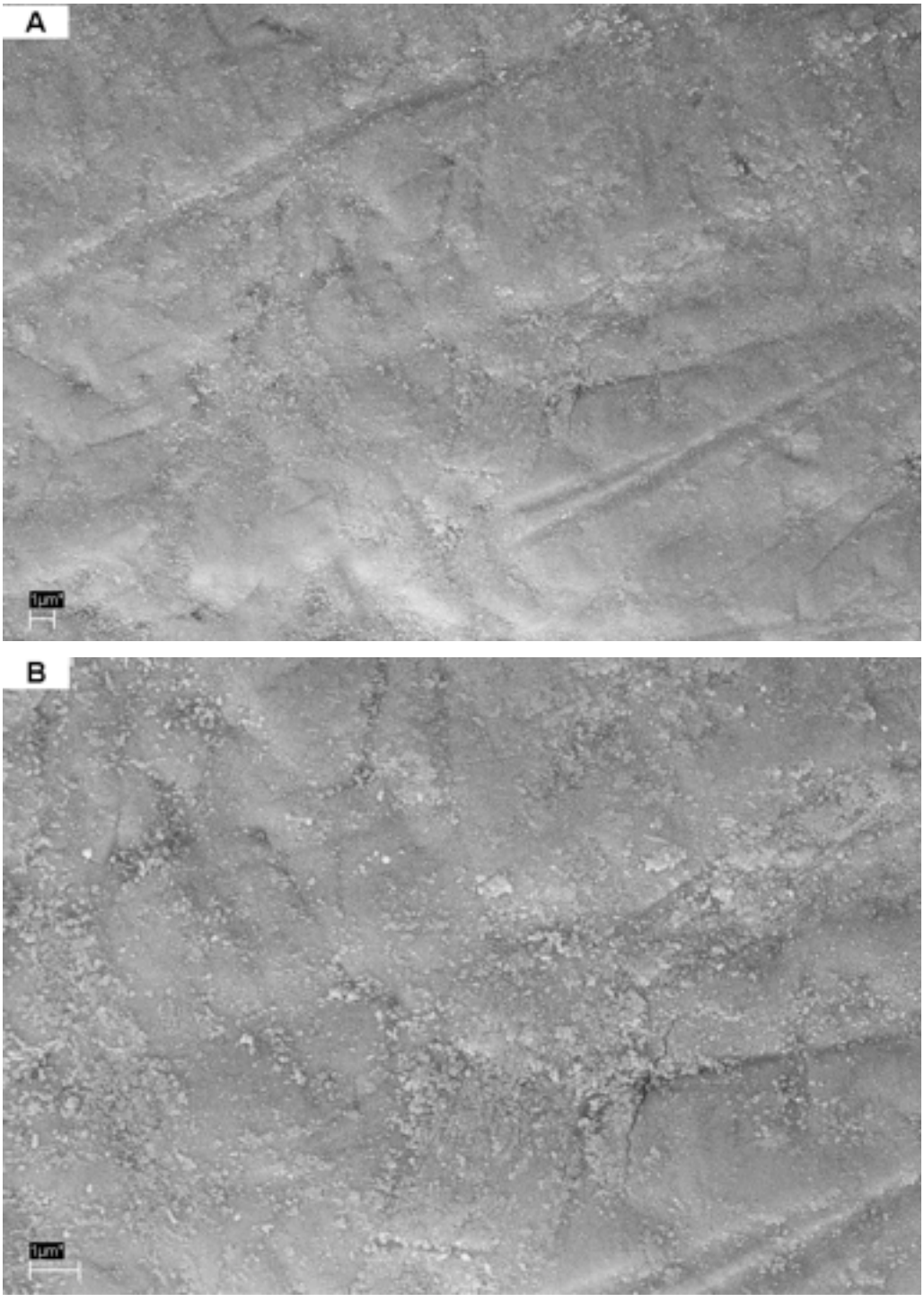
Table 3.1 Color changes (expressed as ΔE values) after different treatment and application times

Treatment	Application times		
	ΔE (t2-t1)	ΔE (t3-t1)	ΔE (t4-t1)
HAP (n=10)	1.76 ± 0.73^{a}	2.42 ± 0.10^{b}	3.31 ± 1.46^{c}
Peptide and HAP (n=10)	3.62 ± 1.82^{c}	4.46 ± 1.91^{d}	4.32 ± 1.85^{cd}

According to repeated measurements of ANOVA results, with respect to within-subjects factors (application times), we can see significant differences among ΔE of the three application times (P=0.000), and there are also significant interactions between ΔE and different treatment groups (p=0.013). ΔE (t2-t1) is lower than ΔE (t3-t1) and ΔE (t4-t1). However, no significant difference is observed between ΔE (t3-t1) and ΔE (t4-t1). Considering the between-subjects factors, the treatment factor has a significant influence on the whitening effect. The whitening effect with peptide and HAP is better than the treatment group using only HAP (P=0.022).

Considering the significant interaction effects, we have to analyze the tendency of color changes according to different treatment groups. For the HAP treatment group (the control group), ΔE increases following the application time (ΔE (t2-t1) < ΔE (t3-t1) < ΔE (t4-t1)) ($p<0.05$), indicating the whitening efficacy becomes pronounced after three times of application. In the peptide and HAP treatment group, which was the experimental group, ΔE (t3-t1) is higher than ΔE (t2-t1) ($p<0.05$), however it shows no difference when compared with ΔE (t4-t1).

3.3.2 Field-emission scanning electron microscopy



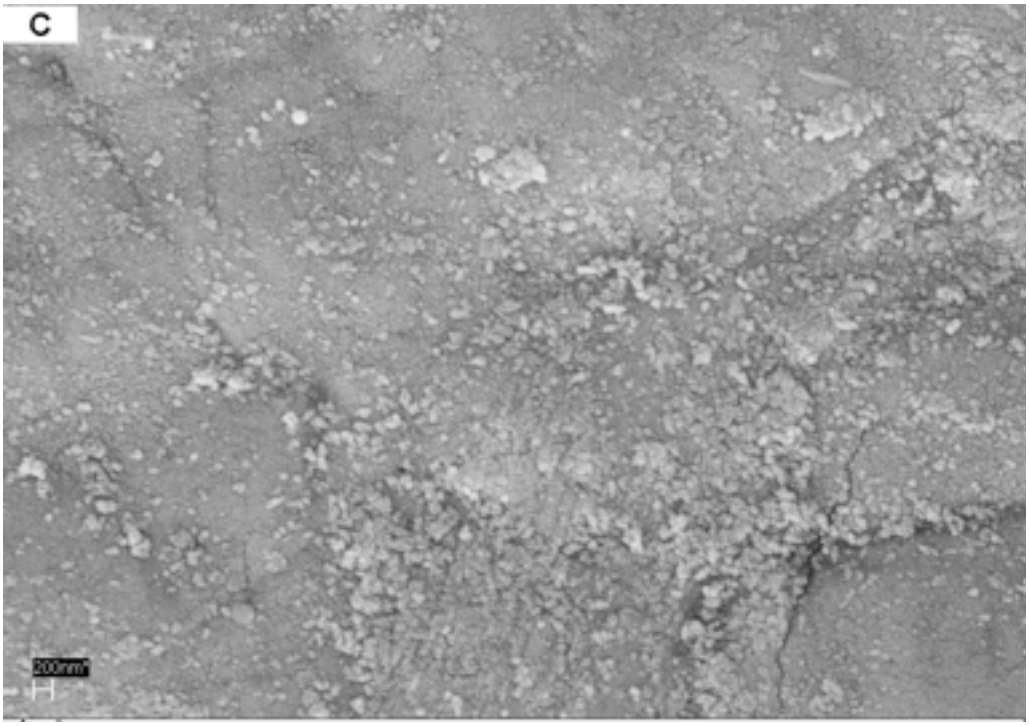


Fig.3.2 The enamel surfaces after treatment only with HAP (A: 10000×, B: 20000×, C: 35000×). Small particles can be seen on parts of the enamel surface.

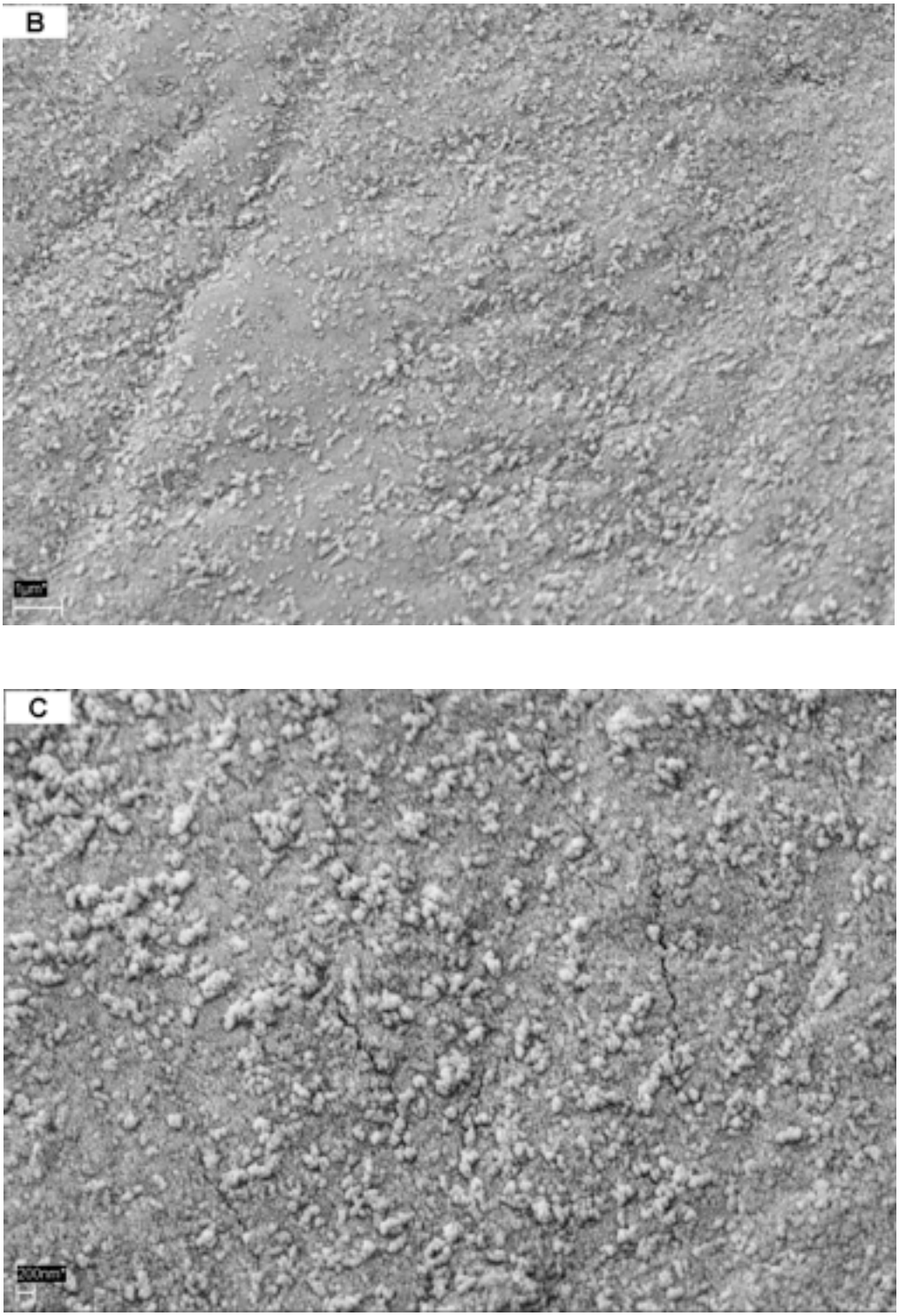


Fig.3.3 The enamel surface after treatment with protein and HAP suspension (A:10000×, B: 20000×, C: 35000×). Small particles can be seen on the entire enamel surface and

form a uniform layer.

3.4 Discussion

The spontaneous folding of proteins is one of the best examples of self-assembly in nature. The biomimetic approach to enamel remineralization has recently been extended to the use of self-assembling peptide P11-4 scaffolds (Kirkham et al., 2007). P11-4, which was developed by the University of Leeds, is an 11 amino acid peptide that undergoes a triggered self-assembly to form a self-supporting hydrogel (Kyle et al., 2010). This peptide was considered as a key for the revolution in initial caries therapy. In the current study, we try to use biomineralization to form a new HAP layer on the enamel in order to finally achieve the whitening effect. Based on the evidence provided in chapter 2, that remineralization layer on the enamel could make the tooth whitening, we have the hypothesis that P11-4 can enhance the remineralization, then can also make an improvement of tooth whitening.

3.4.1 Self-assembly P11-4 peptide

The P11-4 peptide spontaneously forms a biocompatible, three-dimensional matrix that mimics the enamel matrix. Around the newly formed matrix, de novo enamel-crystals are formed from calcium phosphate existed in saliva. Similar to the natural remineralization of a healthy tooth, the process is in equilibrium with demineralization. P11-4 peptide is constituted of CH_3CO-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH_2. Amino acids self-assemblingly form into dimeric antiparallel β-sheet tape and then further form into individual oligopeptides. Each fibrils of newly formed P11-4 fibrillar network is comprised of 4 ribbons, which is made by above-mentioned individual oligopeptides (Kirkham et al., 2007). The acidic residue Glu, which has a negative charge, may contribute to calcium binding. The hydrophobic residues (Phe, Trp) probably contribute to the assembly of the peptide (Palmer et al., 2008). The physicochemical properties of the amino acids in the P11-4 peptide are shown in Table 3.2.

Table 3.2 Physicochemical properties of amino acids in P11-4 peptide

Amino Acid	3-Letter	Side-chain charge (pH 7.4)	Hydropathy index
Arginine	Arg	Positive, alkalinous	−4.5
Glutamic acid	Glu	Negative, acidity	−3.5
Glutamine	Gln	Neutral, hydrophilicity	−3.5
Phenylalanine	Phe	Neutral, hydrophobicity	2.8
Tryptophan	Trp	Neutral, hydrophobicity	−0.9

Hartgerink et al. reported that a kind of synthetic amphipathic self-assembling polymer is able to nucleate hydroxyapatite de novo (Hartgerink et al., 2001). Chang et al. described a biomimetic peptide based upon human phosphophoryn which also displays a similar ability in vitro (Chang et al., 2006). He et. al. also reported the nucleation of hydroxyapatite crystals by self-assembled β-sheet cationic domains of dentine matrix protein 1 in vitro (He et al., 2003). P11-4 peptide, a kind of β - sheet - forming peptide, can spontaneously assemble to form 3-D biomimetic scaffolds, which are capable of nucleating hydroxyapatite de novo. Kirkham et. al. investigated the effect of P11-4 on the re- and de-mineralisation of carious lesions in human enamel in successive trials and days and found the total mineral gain as well as mineral gain on 4 out of 5 days is significantly higher with P11-4 than the mineral gain of the control group (Kirkham et al., 2007).

In general, the peptide is believed to move the equilibrium toward remineralization rather than demineralization, resulting in a net gain of mineral. Kirkham et al. used the application of the P11-4 peptide for enamel repair. In the current study, we suggest the application of this peptide can be further used for whitening the teeth. We found that the whitening efficacy in experimental (peptide and HAP) group is much better than the HAP group. This is in accordance with the remineralization result which was obtained by the Kirkham group.

Furthermore, in the peptide and HAP group, the ideal whitening efficacy was achieved after only one application. There might be two reasons for this result: one is, after the second treatment, the whitening efficacy has already been achieved up to the

maximum. It is also possible that the whitening effect would become better after the third application, but later decrease slightly by the hydrodynamic shear force. That means that the factor time is important in the control group, and the repetition contributes to the whitening efficacy. However, in the P11-4 experimental group, only one application achieves a good result.

3.4.2 Bovine teeth

In the current study described in Chapter 3, we used bovine teeth instead of human teeth. There are some researchers who concluded that, for color measurement, the bovine teeth can substitute the human teeth (Yassen et al., 2011). The bovine teeth have a flatter enamel surface which is more suitable for color measurement because the principle for Easyshade measurement is light reflection and additionally the probe of Easyshade is flat. We found that the angle between enamel surface and the probe of Easyshade had some influence on the color measurement result. The color measurement will be more stable if the probe can contact the enamel surface very well. Considering these factors, we chose bovine teeth for this study.

3.4.3 Artificial saliva

The term ‘remineralization’ has been used previously by researchers to describe the mineral gain, including precipitation of mineral onto enamel surfaces (Tung and Eichmiller, 2004). Precipitation can be defined as ionic clusters that form in a supersaturated solution as a solid phase. In the studies described in Chapter 1, we use this principle to obtain the remineralization layer on the enamel surface for whitening. In the current study, we use P11-4 peptide and artificial saliva to promote ion deposition onto enamel. Artificial saliva can mimic the oral environment better and supply bioavailable calcium and phosphate ions to the tooth, promoting the remineralization of enamel. We achieved ideal effectiveness with remineralization and whitening using artificial saliva in vitro. However precipitation of calcium phosphate normally does not occur in saliva in vivo, due to the presence of salivary proteins, particularly statherin (Schlesinger and Hay, 1977) and proline-rich phosphoproteins (Oppenheim et al., 1971). The segments of the

proteins containing phosphoseryl residues, in particular the statherin sequence, which bind to calcium and phosphate ion clusters, prevent the growth of the ion cluster to the critical size required for precipitation and transformation into a crystalline phase (Hay and Moreno, 1989). Therefore, more research should be done to promote remineralization in vivo.

P11-4 exists as monomer of random coil conformations in water above pH 7.5, but at a reduced pH, it adopts an antiparallel β-sheet conformation (Kyle et al., 2010). The pH of the artificial saliva used in our research is 6.9, which is suitable for P11-4 self-assembly to form the matrix.

3.4.4 Acid etching pre-treatment

In the study of Kirkham and Aggeli, before being treated with the P11-4 self-assembling peptide, human teeth were subjected to an acid treatment to open the pores of the hypermineralized plate. The P11-4 was then applied to the initial caries lesions to form the matrix, which mimic the enamel matrix. In our study, we did not carry out this pretreatment because our purpose was to whiten the tooth without damaging to the hard tissue.

Some studies presumed that a more porous surface layer is much easier to be remineralized because it allows for better penetration of the ions required for remineralization (Bailey et al., 2009). Therefore, some approaches have been suggested for surface scratches such as microabrasion (Ardu et al., 2007), acid etching (Flaitz and Hicks, 1994), bleaching/deproteination (Ng and Manton, 2007; Robinson et al., 1990), and a combination of bleaching and etching (Milnar, 2007). Bleaching appears to be an effective method for deproteinating the lesion surface to increase porosity inter-prismatically without the need of acid etching. Kim et. al. investigated the effect of nano-HAP on the prevention of re-staining after dental bleaching and found that using a 10% nano-carbonate apatite (n-CAP) additive could significantly maintain the initial color and protect the damaged enamel structure after bleaching (Kim et al., 2011). Bleaching can promote the whitening process, and whitening can decrease the side

effects which are caused by bleaching.

In conclusion, whitening efficacy can be obtained with the application of P11-4 peptide and HAP without etching the enamel surface. Maybe this method in our study can also be used to restore the damage on the enamel after bleaching.

Chapter 4

Using EDX and ATR-FTIR to characterize the peptide and the newly formed HAP-layer

4.1 Background and significance

In the work of this chapter, we used energy-dispersive X-ray spectroscopy (EDS, EDX, or XEDS) and attenuated total reflectance fourier transform Infrared Spectroscopy (ATR-FTIR) for the characterization of the peptide and the newly formed HAP-layer.

EDX is an analytical technique used for the elemental analysis or chemical characterization of a sample. Under X-ray irradiation, each element in the sample gives a set of unique peaks in its X-ray spectrum. Thus, the element type and content of a sample can be measured (Goldstein, 2003).

Fourier transform infrared spectroscopy (FTIR) is a technique which is used to obtain an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid, liquid or gas. An FTIR spectrometer simultaneously collects spectral data in a wide spectral range. FTIR can detect the chemical bonds which exist in the object. Each chemical bond gives unique absorption peaks in the infrared spectrum. Therefore according to the infrared spectrum, we can gather the information about chemical bonds and functional groups. Attenuated total reflectance (ATR) is a sampling technique used in conjunction with infrared spectroscopy which enables samples to be examined directly in the solid or liquid state without further preparation (http://shop.perkinelmer.com/content/TechnicalInfo/TCH_FTIRATR.pdf, 2005). ATR uses the property of total internal reflection resulting in an evanescent wave. The penetration depth into the sample is typically between 0.5 and 2 micrometers (The Cosmetic Products (Safety) (Amendment) Regulations 2012 No. 2263. , 2012). The accessibility of ATR-FTIR has led to substantial use by dental researchers.

In the last two chapters, we have provided evidence for the whitening effect and the

HAP layer on the enamel surface can be observed in the SEM. In this chapter, we have attempted to characterize the newly formed HAP-layer and peptide. For the elemental analysis, it is difficult to identify the newly formed HAP- layer because the constituent of enamel is also HAP. Thus we used La-labeled HAP (B000507，C73-33-La-1000) to identify the newly formed HAP-layer.

4.2 Materials and methods

4.2.1 Energy dispersive X-ray spectroscopy (EDX) and scanning electron microscopy (SEM) analysis.

4.2.1.1 Sample preparation for SEM and EDX

Three human third molars were selected from subjects between the age of 18 and 30 years old. The enamel of the teeth was matured without caries, cracks or other defects. The teeth were stored in distilled water, with sodium azide as a disinfectant, from the time of extraction until the application of the material. The study was approved by the University Ethical Committee (LMU, Muenchen, Germany) and was conducted according to the principles of the Helsinki Declaration for biomedical research. Informed consent was obtained from all donors.

The roots were removed, and the enamel was prepared using a low-speed diamond saw (Leica SP 1600-Saw Microtome, Leica Microsysterms Nussloch GmBH, Nussloch Germany) to obtain two enamel samples from each tooth. One was assigned to the La-HAP group, the other was assigned to the peptide-La-HAP group. Therefore, each group has 3 samples from a tooth. The samples were mounted onto microscope slides and polished the same way as described in chapters 2 and 3.

4.2.1.2 Study design

In the La-HAP group, after drying with an air syringe, the La-HAP paste was directly applied onto the enamel surface with a cotton pellet and then agitated for 3 min. Afterwards, the paste was left undisturbed for 5 minutes to allow interactions of the

materials with the enamel surface. In the peptide-La-HAP group, the peptide solution was applied first using the same procedure as in chapter 3. After 5 minutes, the La-HAP paste was applied to the enamel surface and left for an additional 5 minutes without agitating. After all treatments, these two groups were stored in artificial saliva (pH=6.9, Pharmacy University of Munich, Munich, Germany) for 24 h at 37 °C, respectively. This procedure was repeated two more times. After the third storage phase, a hydrodynamic shear force was applied as in chapter 2 and 3.

4.2.1.3 SEM and EDX analysis.

After applying hydrodynamic shear force, morphological changes of these specimens were also observed using a field-emission scanning electron microscope (FE-SEM, ZEISS Supra 55Vp, Zeiss, Oberkochen, Germany) at 10 kV and a working distance of 5 to 10 mm when secondary electrons (SE2) were used. Three images of representative areas were stored at three different magnifications (10000×, 20000× and 35000×). At a magnification of 5000×， each sample was analyzed for Lanthanum content using Energy-Dispersive X-ray Spectroscopy (EDS, AMETEK, 55Vp, A POLLOX, USA) operated at 20 KV and a working distance of 10 mm. An aliquot of La-HAP paste was also centrifugated, dried and detected with EDX to clarify the peak position of Lanthanum.

4.2.2 Sample preparation for attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR)

Eight human permanent central incisors without caries, cracks or other defects were chosen because their enamel surface is flatter than other teeth. The roots were removed, and the enamel was cut into approximately 2mm*2mm*1mm plates using a low speed diamond saw (Leica SP 1600-Saw Microtome, Leica Microsysterms Nussloch GmBH, Nussloch, Germany) with water cooling. The enamel surface was kept intact and the bottom of each specimen was ground flat for the ATR-FTIR measurements. In total, we prepared 20 samples which were assigned to 5 groups (Table 4.1)

Table 4.1 The treatment for different groups of ATR-FTIR

Group	Treatment
Group 0 (N = 4)	Control group, intact enamel without any treatment
Group 1 (N = 4)	Reference group, one drop of peptide solution applied on enamel and air dried
Group 2 (N = 4)	Peptide solution applied (5 min), then HAP suspension applied (5 min) on the enamel, then samples stored in artificial saliva for 24 h at 37 °C
Group 3 (N = 4)	Peptide solution applied (5 min), then HAP powder applied (5 min) on the enamel, then samples stored in artificial saliva for 24 h at 37 °C
Group 4 (N = 4)	Peptide solution and HAP powder mixed to get HAP-peptide suspension, then applied onto the enamel, then samples stored directly in it for 24 h at 37 °C.

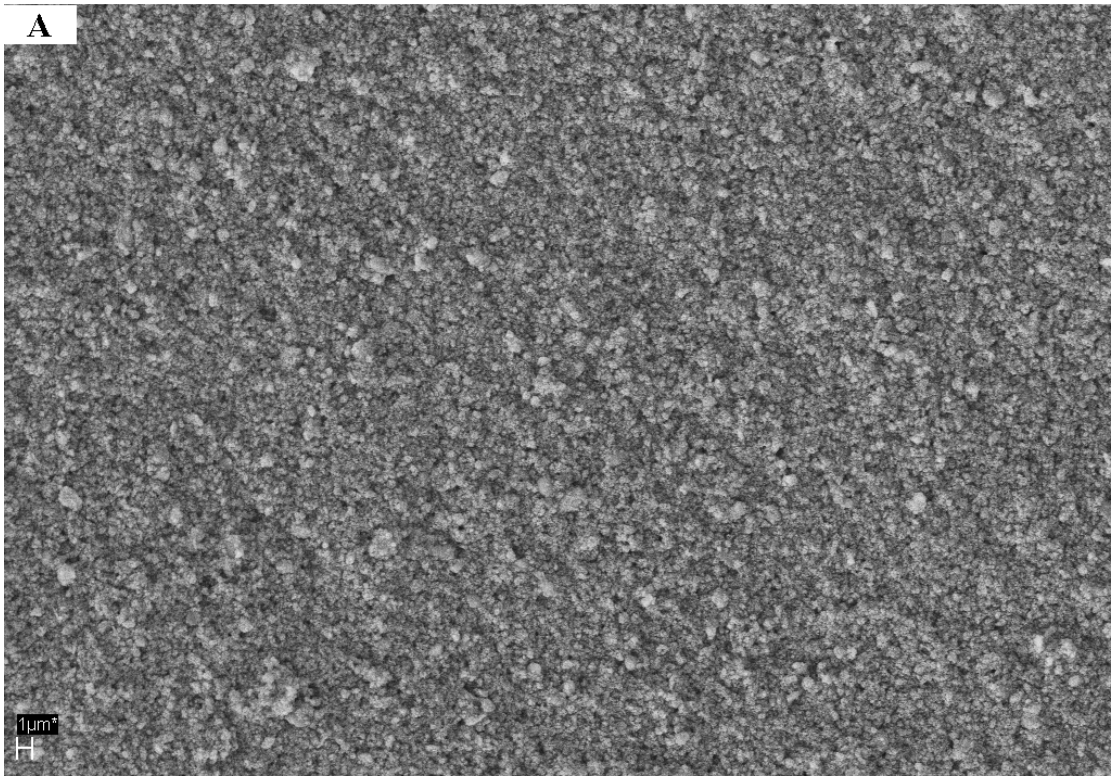
In groups 2 - 4, the treatments were administered a total 3 times, respectively. After the third storage, a hydrodynamic shear force was applied for 1 min as in chapter 2 and 3. All samples in group 0 - 4 were dried with an air syringe and then measured by ATR-FTIR. We also added a drop of peptide solution onto the ATR-FTIR's crystal reflection plate and dried it directly to detect the typical peak of this peptide as a reference.

4.3 Results

4.3.1 Results from SEM and EDX

We can observe that the size of the HAP particles in the La-HAP suspension is at the nanometer level and the Lanthanum's characteristic peak appears clearly at about 4.6-4.8 keV. The weight percentage (Wt %) and atomic percentage (At %) of La can be achieved

to more than 15.74% and 3%, respectively. When only La-HAP was applied to the enamel, fewer particles are observed on the enamel surface after hydrodynamic shear force application than on the specimen treated with both peptide and La-HAP. Also, in the EDX energy spectrum, La cannot be detected on the enamel treated only with La-HAP, while the La peak can be observed clearly after the treatment with both the peptide and the La-HAP. Wt % and At % of La are 9.21% and 1.58%, respectively.



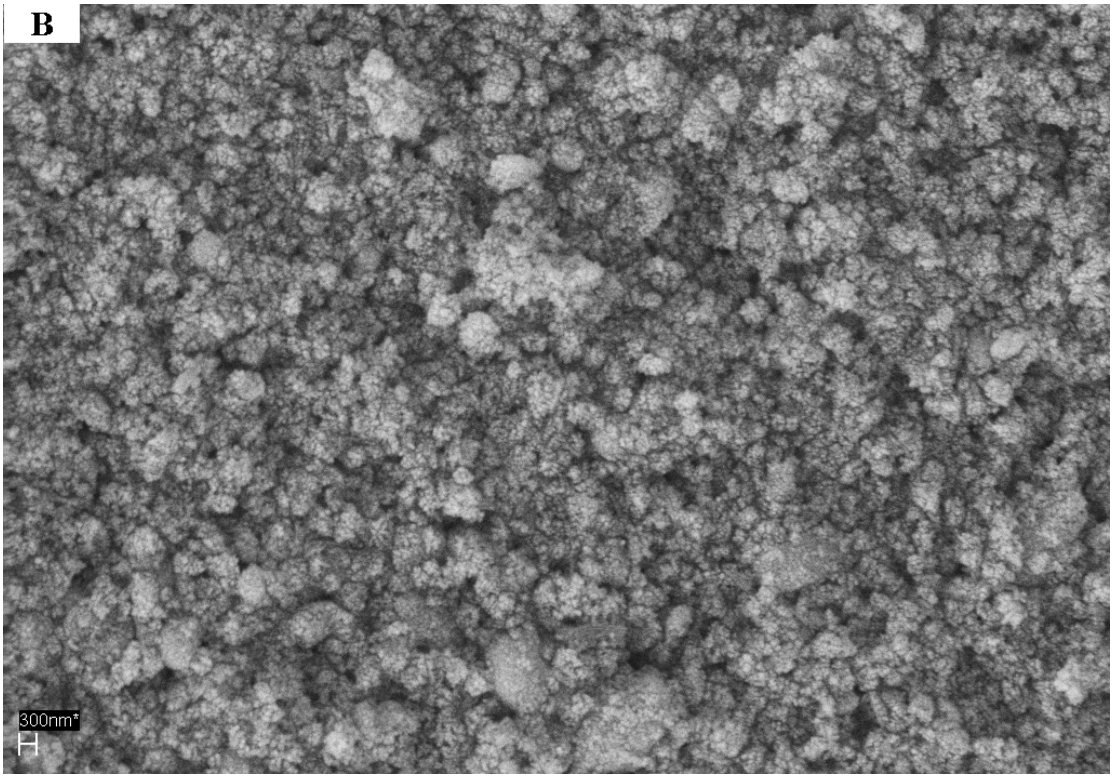


Fig.4.1 SEM picture of La-HAP powder. A and B show the particles in the La-HAP suspension (A: 5000×, B: 20000×). The size of the particles is at the nanometer level.

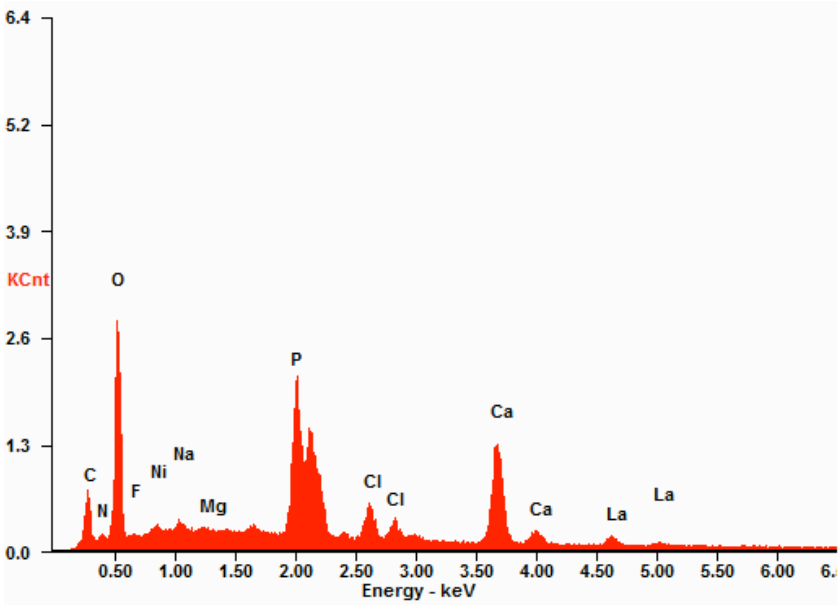
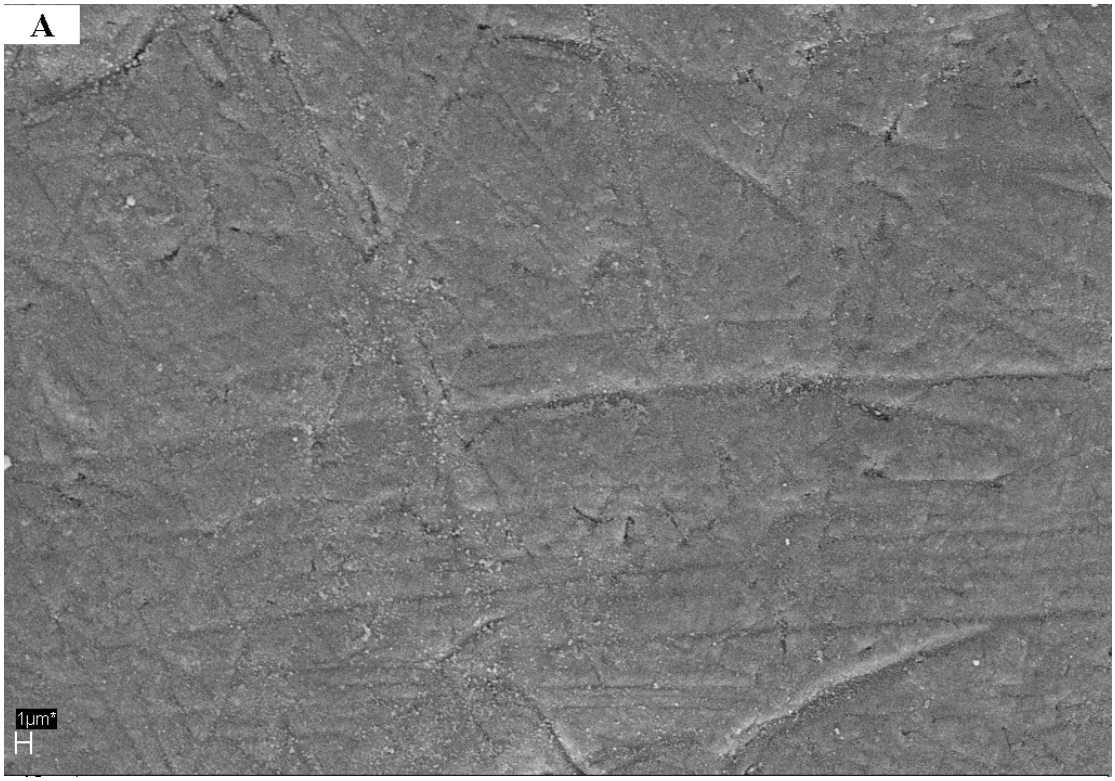


Fig.4.2 EDX analysis spectrum of La-HAP powder. The La-peak appears at about 4.6-4.8 keV.


A
1µm*

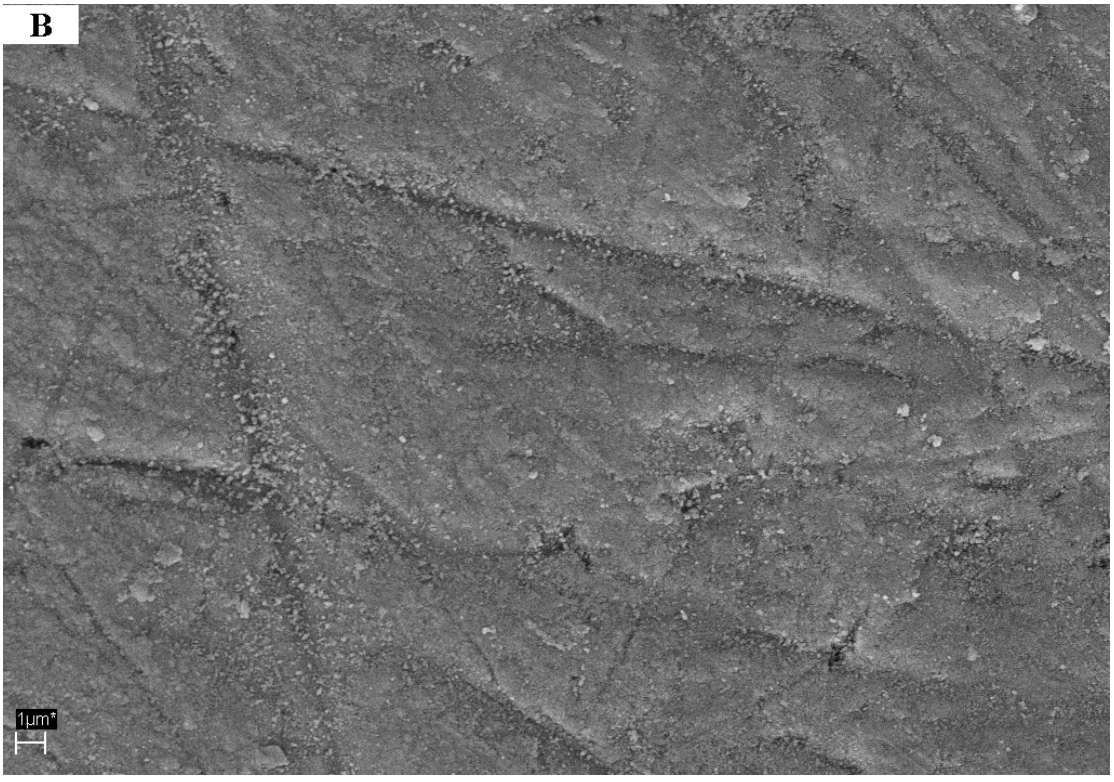

B
1µm*

Fig.4.3 SEM pictures of the enamel surface after treatment with only La-HAP suspension (A: 5000×, B: 10000×,C: 35000×). Small particles can be seen on the enamel surface, especially along the scratches.

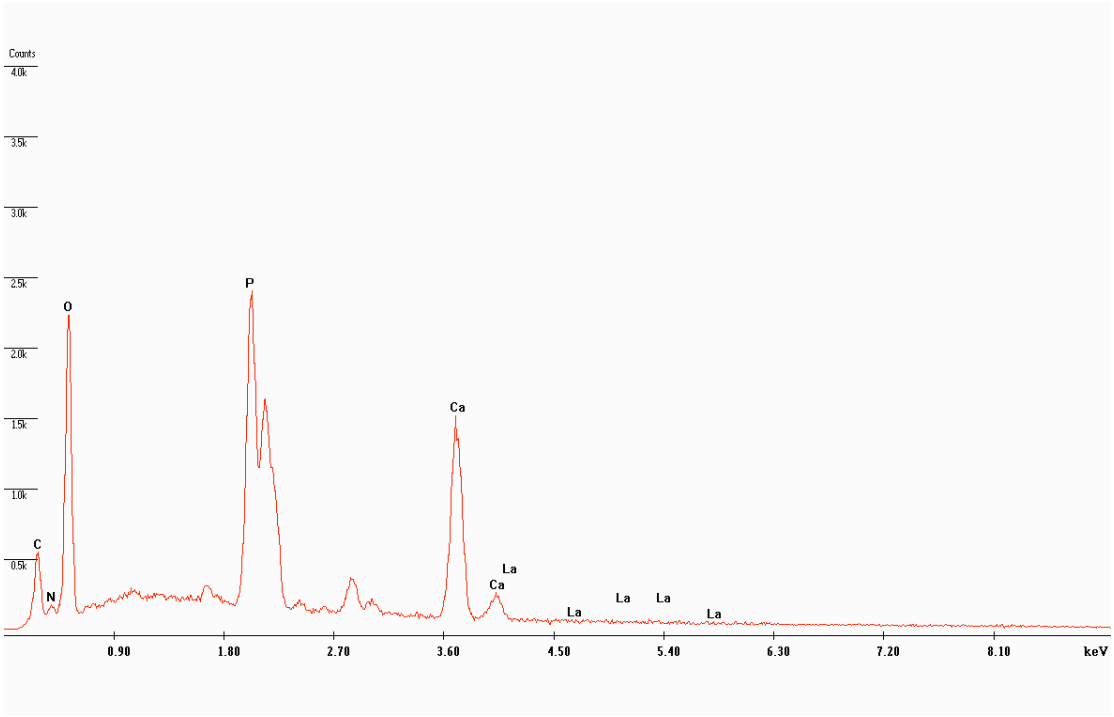
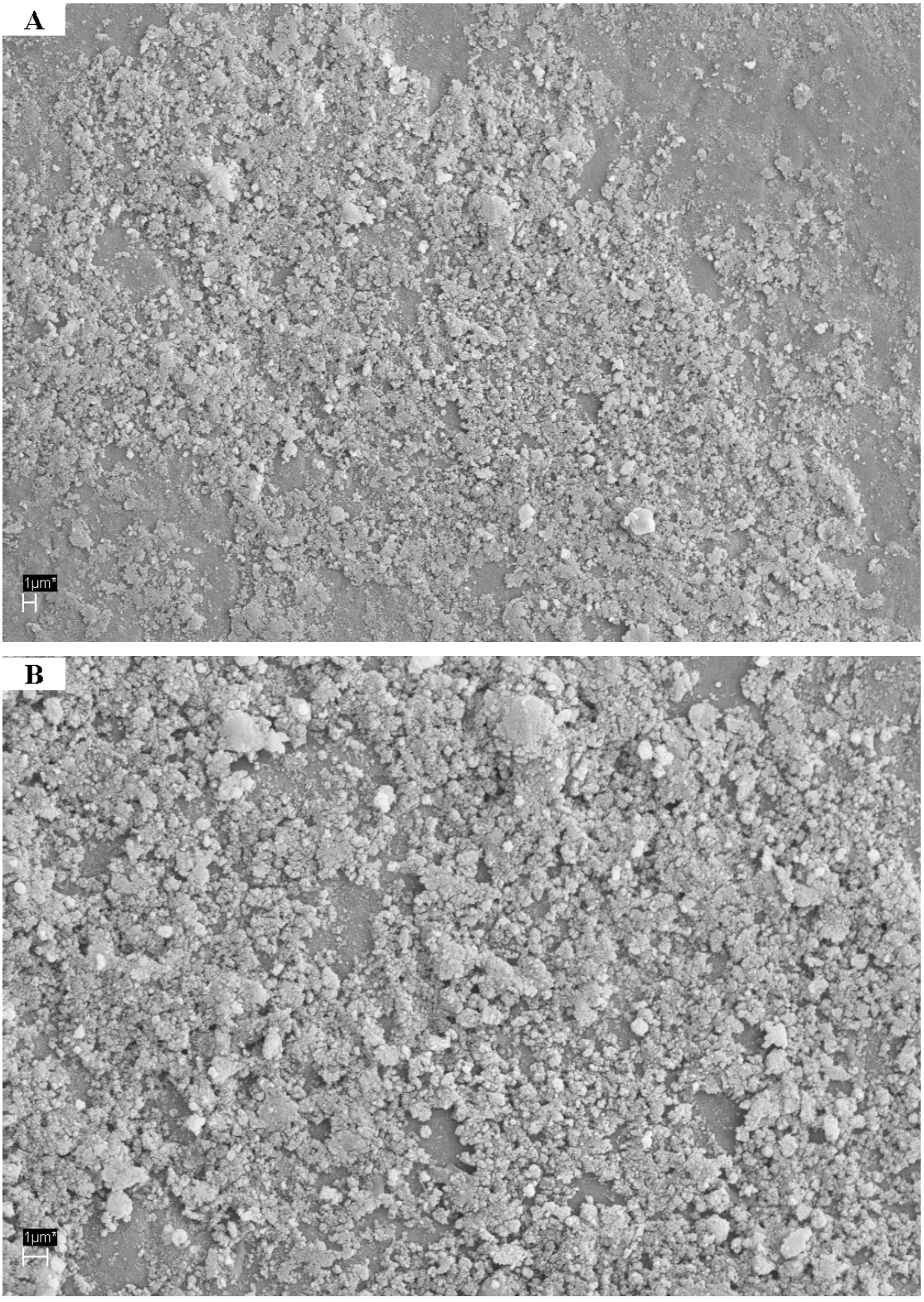


Fig.4.4 EDX energy spectrum of the enamel surface after treatment with only La-HAP suspension (5000 ×). The La peak cannot be observed on this EDX energy spectrum.


A
1µm*
B
1µm*

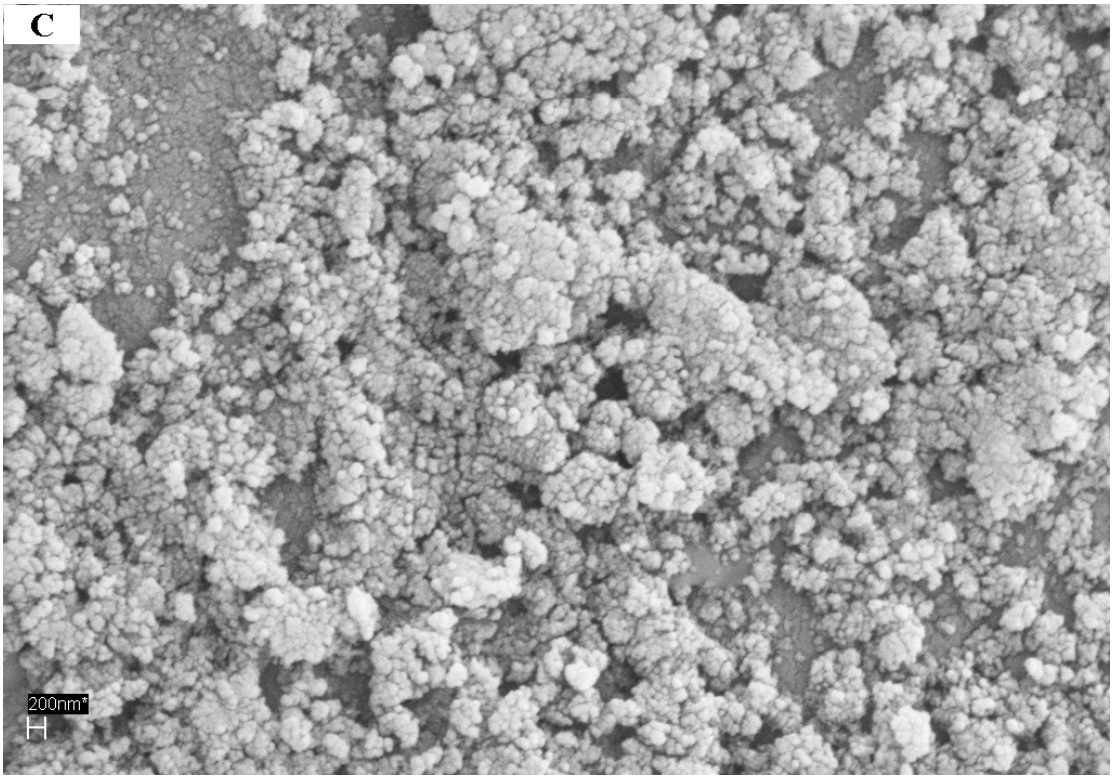


Fig.4.5 SEM pictures of the enamel surface after treatment with the peptide and La-HAP suspension (A: 5000 ×, B: 10000 ×, C: 30000 ×). Small particles at nanometer level can be observed and they can form a thick layer on the whole surface.

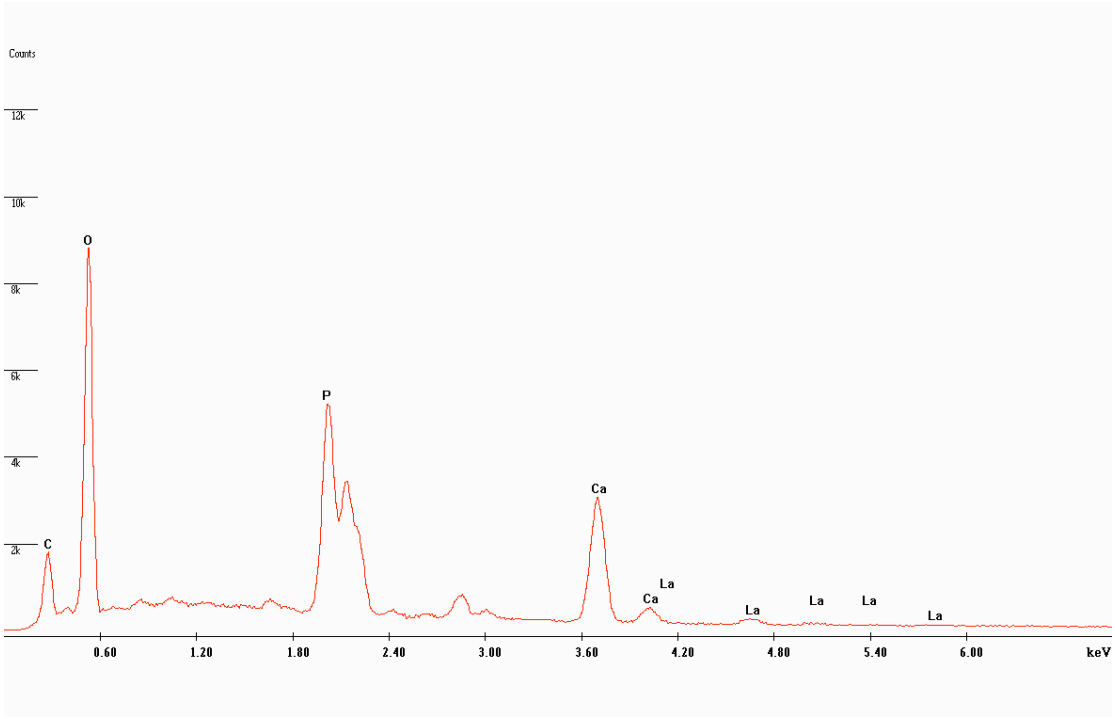


Fig.4.6 EDX energy spectrum of the enamel surface after treatment with peptide and La-HAP suspension (5000 ×). The La peak can be seen on this EDX energy spectrum at 4.6-4.8 keV.

4.3.2 ATR - FTIR results

In the infrared spectrum, we can identify the PO_4^{3-} , CO_3^{2-}, OH^- and amide groups easily. The amide band, which is the characteristic band of remineralization peptide, presented itself only after the peptide solution was applied. There are PO_4^{3-} and CO_3^{2-} bands but no amide bands in the infrared spectrum of the "intact enamel" (Group 0). After applying the peptide solution (Group 1), the amide band appeared. The amide band appears on all samples, Groups 1-4, however, we cannot observe a difference in peak intensity among them.

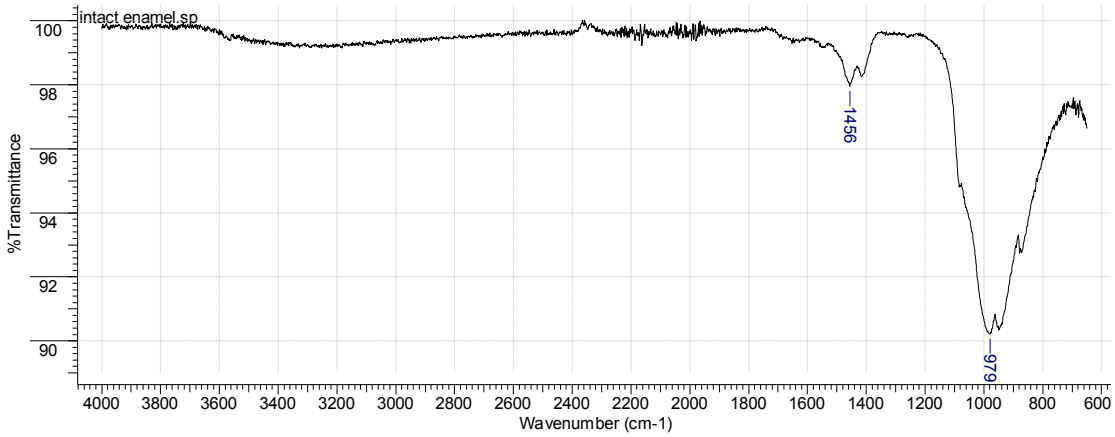


Fig.4.7 The ATR-FTIR spectrum of Group 0. Intact enamel without any treatment (samples in Group 0). The band of PO_4^{3-} (around 1000 cm^{-1}) and CO_3^{2-} (around 1450 cm^{-1}) can be seen in the infrared spectrum.

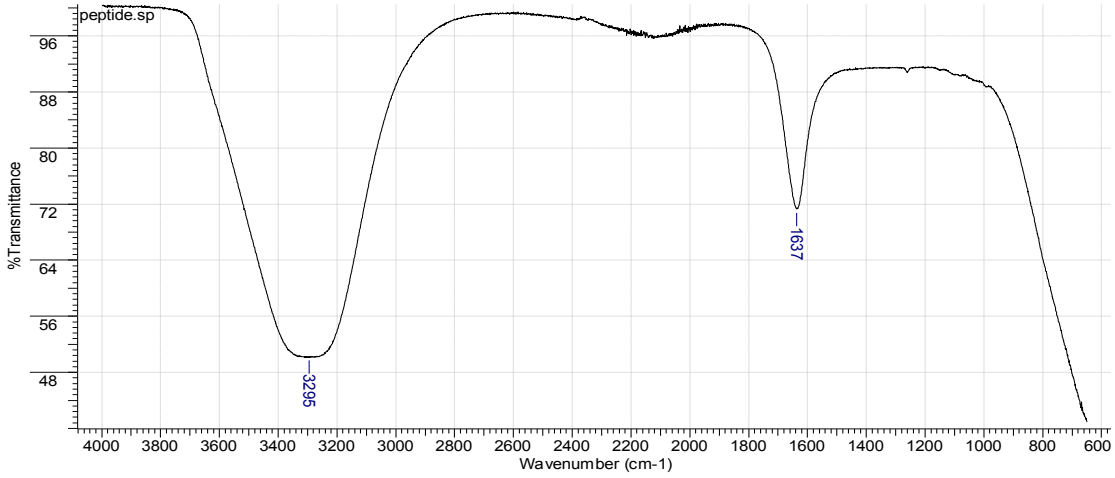


Fig.4.8 The ATR-FTIR spectrum of the peptide solution. The amide band appears at 1637 cm^{-1} and the OH^- band appears at approximately 3300 cm^{-1}. The band of PO_4^{3-} and CO_3^{2-} are not detected.

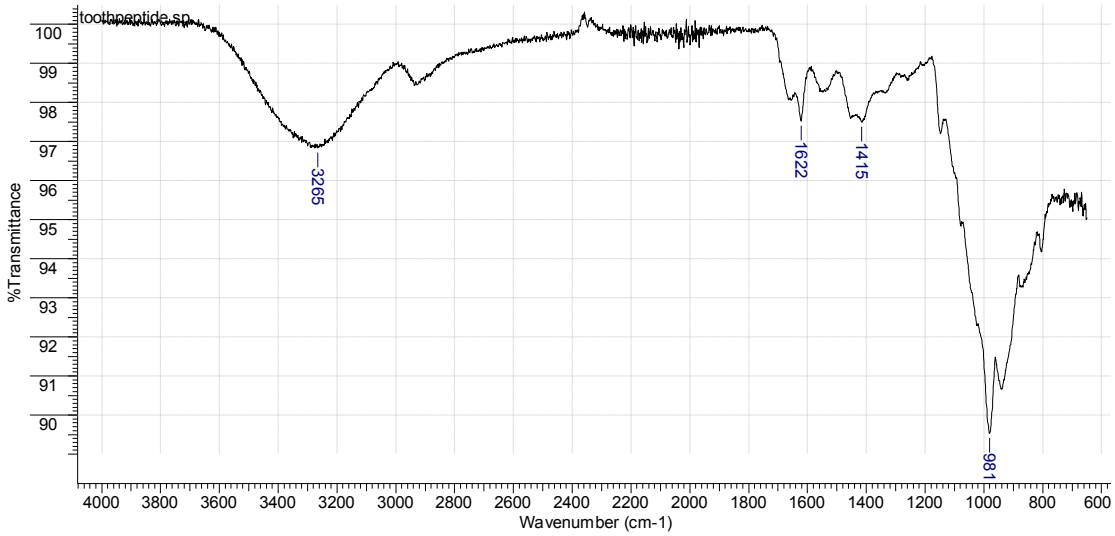


Fig.4.9 ATR-FTIR spectrum of Group 1**.** The band of PO_4^{3-}, CO_3^{2-}, OH^- and amide can be detected with ATR-FTIR.

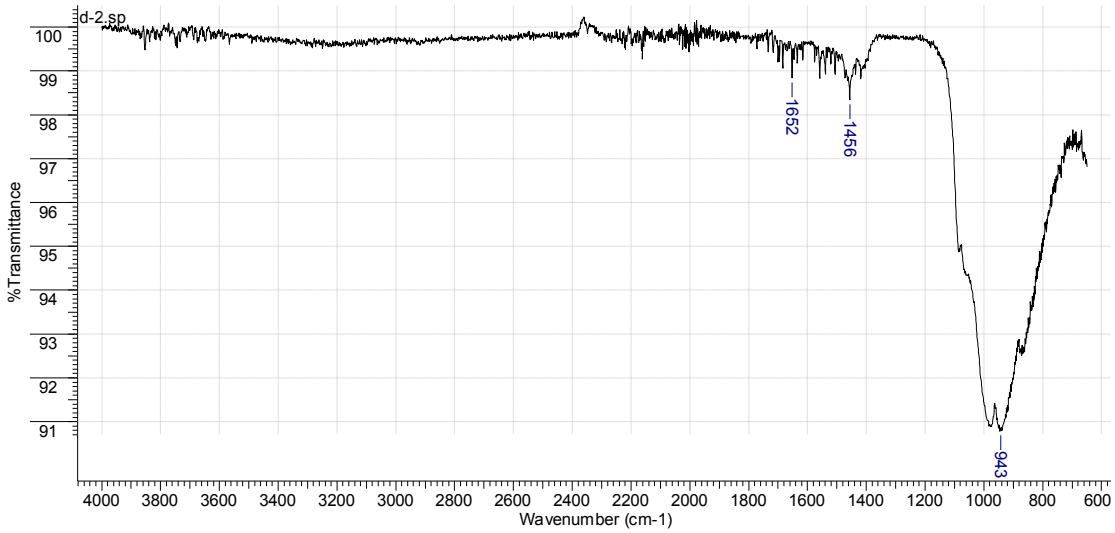


Fig.4.10 ATR-FTIR spectrum of Group 2**.** The band of PO_4^{3-}, CO_3^{2-} and amide can be detected with ATR-FTIR.

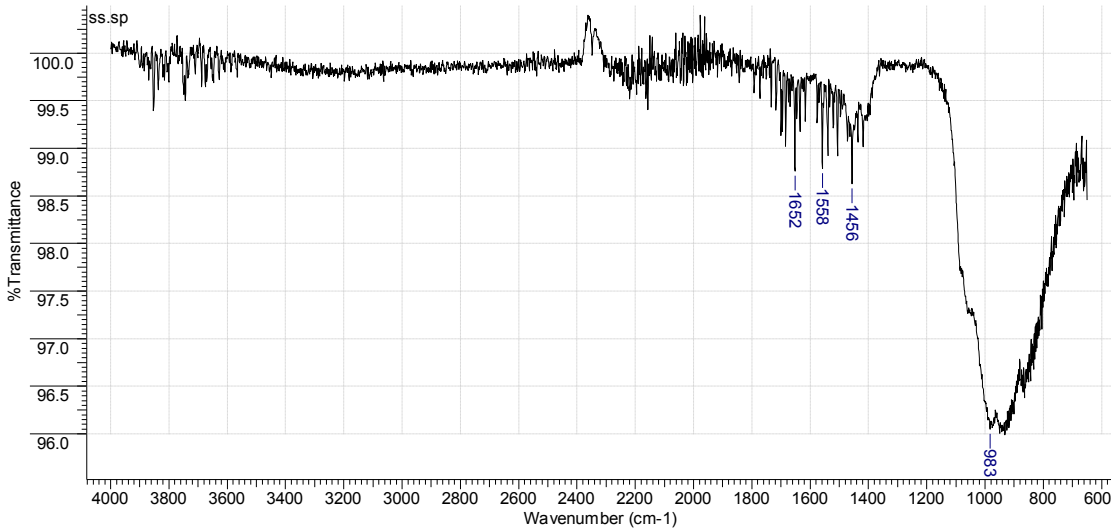


Fig.4.11 ATR-FTIR spectrum of Group 3. The band of PO_4^{3-}, CO_3^{2-} and amide can be detected with ATR-FTIR.

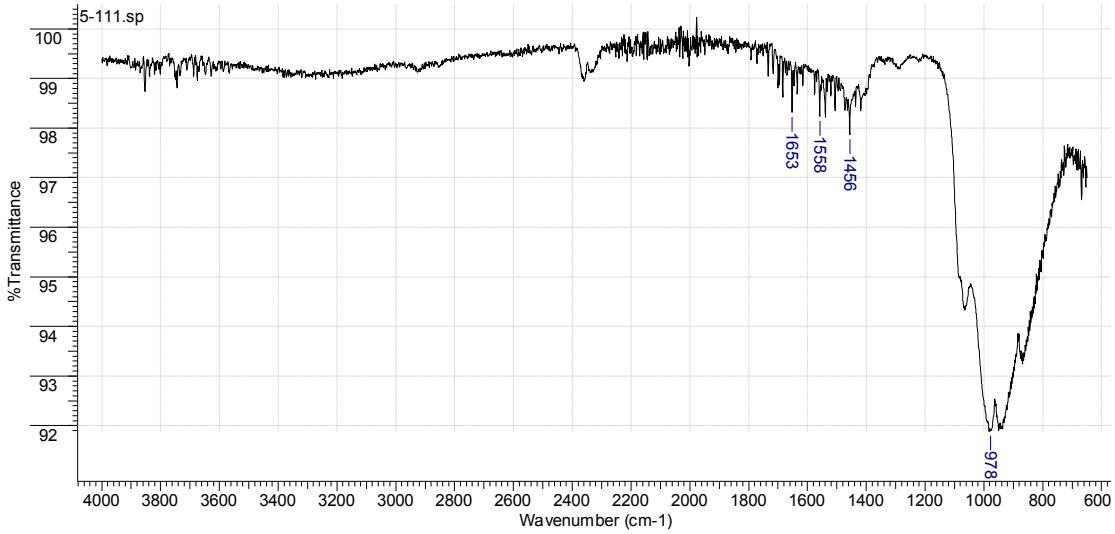


Fig.4.12 ATR-FTIR spectrum of Group 4. The band of PO_4^{3-}, CO_3^{2-} , OH^- and amide can be detected with ATR-FTIR.

4.4 Discussion

4.4.1 Characterization of the peptide

Peptides are short chains of amino acid monomers linked by peptide (amide) bonds. The covalent chemical bonds are formed when the carboxyl group of one amino acid

reacts with the amino group of another. Chen et al. described that PAMAM self-assemblies act as an organic template between the regrown crystals and etched enamel, based on the observation of peptides on enamel by ART- FTIR (Chen et al., 2013). In the current study, the amide band, which is the characteristic absorption band of a peptide, appeared at about 1637 cm^{-1} when the pure peptide solution was analyzed. The PO_4^{3-} ion (around 1000 cm^{-1}) dose not exist in the peptide solution. Mature enamel is composed of 95–97% carbonated HA by weight with less than 1% organic material. Therefore, the band of PO_4^{3-} (around 1000 cm^{-1}) and CO_3^{2-} (around 1450 cm^{-1}) is seen in the infrared light spectrum while the amide band disappears in ATR-FTIR spectrum of untreated intact enamel. We can also detect the OH^- band which may come from HAP or H_2O.

In conclusion, the amide band can be detected on treated enamel surfaces from groups 2-4. This result demonstrates the presence of P11-4 peptide within the newly formed HAP containing layer. However, we cannot estimate the residual amount of P11-4 on the treated tooth because ART-FTIR is only used for qualitative analysis of chemical bond.

4.4.2 Proof of the newly formed HAP-layer

Because the main constituent of enamel is similar to the remineralization material which we used in the studies of chapter 2 and 3, it is difficulty to identify the newly formed HAP-layer by EDX. In this study, we modified the remineralization material by using lanthanum labeled HAP to identify the newly formed HAP-layer.

In the EDX analysis, first, we analyzed the La-HAP suspension just to make sure La can be detected，however its quantity can only be estimated. Then we compared the specimens treated only by La-HAP with the ones treated by both P11-4 peptide and La-HAP. La can be detected on peptide and La-Hap treated enamel surface. However, on the enamel which was only treated with La-Hap, the peak of La is not so obvious. Therefore, in the current study, we demonstrated that with the aid of the P11-4 peptide, the density of biomimetic crystals adhering to the enamel surface is more pronounced in a

shorter time.

EDX is only precise for qualitative analysis, however it is very imprecise for quantitative analysis. Because of this limitation, in current study, we cannot quantify the quantitation of La either in the La-HAP suspension or in the newly formed HAP layer. Other analysis method should be used in the future study.

4.4.3 The dosage forms of HAP

The effect of the nano-hydroxyapatite concentration on the remineralization of enamel has been investigated by some researchers. Huang et al. found that 10% nano-hydroxyapatite may be optimal for the remineraliztion of enamel (Huang et al., 2009). In this study, we also tried to find a suitable dosage for HAP remineralization and whitening. Therefore we tested both HAP suspension and HAP powder. With the ATR-FTIR, the amide peak was detected and was similar in both cases, therefore, we believed the dosage forms of HAP will not influence the P11-4 self-assembly to the matrix and adhere to the enamel surface in our study. More research should be done to develop an effective and convenient dosage form for home tooth whitening and remineralization.

Conclusively, we found that the P11-4 peptide can undergo self-assembly and perform as an organic template between the regrown HAP-layer and enamel to regulate both mineral nucleation and crystal growth in a HAP suspension or paste. In the beginning, the P11-4 self-assembled peptide could be adsorbed onto the surface of the enamel crystals by electrostatic interaction between their carboxylic terminus and calcium cations in enamel crystals to provide the matrix. Then, in the HAP suspension or its saturated solution (HAP powder combined with P11-4 peptide solution), the P11-4 matrix serves as a nucleation site by catching calcium ions bound to its carboxylic acid terminus. The interaction leads to a high local concentration of calciums, and the deposition of HAP occurs when the phosphate ions arrive. As a result, tooth whitening and remineralization could be possible to achieve at the same time.

Summary

In general, there are two main methods for tooth whitening: one method involves using oxidizing agents to bleach teeth, and the other involves using calcium phosphate-based formulations to remineralize the enamel to obtain the whitening effect. It is well known that tooth bleaching has many adverse effects, therefore, there is a huge demand for products and related research in remineralization for tooth whitening as an alternative. This study is a gradual research on whitening efficacy of calcium phosphate-based formulations.

The study was divided into three parts:

1.Three different hydroxyapatite preparations at three concentrations were compared to two control preparations. The color assessment results indicated significant differences between the materials, but neither dose- nor time-dependent associations were found. Microrepair ($ZnCO_3$/Ap), the highest concentration (30 wt%) achieved a better effect than the lower concentrations. The toothpaste, which also contained $ZnCO_3$/Ap, showed a better whitening effect than the suspensions. The 10 wt% tricalcium phosphate suspension demonstrated the best whitening effect. The whitening effect was not very pronounced, but it was statistically significant. We can conclude that calcium phosphate based formulations that can adhere to the enamel surface and contribute to tooth whitening have promising tooth-whitening potential. More research on how to augment the whitening effect should be performed.

2. We wanted to refine the methods to improve the whitening efficacy and enhance the chance for biomineralisation. For this aim, a self-assembling peptide, which can form a biomimetic matrix for nucleation and mineralization, was used in this study. The result showed that the specimens in the peptide and HAP treatment group (experimental group) achieved a better whitening effect than the HAP treatment group (control group). Furthermore, ΔE increases following the application time in the HAP treatment group, indicating the whitening efficacy becomes more pronounced after three applications. In

the peptide and HAP treatment group, the whitening effect achieved a maximum only after the second treatment. Conclusively, we proposed that P11-4 peptide can self-assemble and operate as an organic template for the HAP particles. The matrix together with the HAP particles influence the immediate whitening effect and may also contribute to further remineralization.

3. Furthermore, to ensure that the P11-4 peptide contributes to form a new HAP containing layer, we used EDX and La-HAP to characterize the newly-formed HAP-layer and ATR–FTIR to show evidence for the presence of the peptide. The SEM and EDX results confirm that P11-4 peptide can promote the formation of the HAP containing layer since the La can be detected after the treatment with the peptide and La-HAP. According to the ATR–FTIR results, we can deduce that the P11-4 peptide underwent self-assembly and acts as an organic template between the HAP and the enamel.

Zusammenfassung

Es besteht eine große Nachfrage nach Zahnpflegeprodukten, die zu einem helleren Erscheinungsbild der Zähne führen. Man kann Produkte mit oxidativer Aufhellung von Produkten mit abrasiver Aufhellung unterscheiden. Im ersten Fall werden Chromophore in der Zahnhartsubstanz oxidativ zerstört, wodurch die Zähne heller erscheinen. Im zweiten Fall werden auf die Zahnoberfläche ausgelagerte Beläge entfernt. Zu diesen weit verbreiteten Methoden gibt es außerdem weniger bekannte Alternativen. Covarine Blue kann beispielsweise durch Absorption von blauem Licht über Fluoreszenzeffekte den Eindruck einer helleren Zahnfarbe erwecken. Eine weitere Alternative ist die Auflagerungen von Hydroxylapatitpartikeln auf die Zahnschmelzoberfläche mit dem Ziel, die diffuse Lichtreflexion an der Zahnoberfläche zu erhöhen. Als Folge der stärker reflektierten Lichtintensität erscheint der Zahn ebenfalls heller.

Die vorliegende Untersuchung war in drei Abschnitte unterteilt:

1. Drei unterschiedliche Apatit-Präparate wurden in vitro jeweils in drei unterschiedlichen Konzentrationen mit zwei Kontrollgruppen verglichen. Die Farbänderung der Zähne nach jeder Applikation dieser Präparate wurde mit einem dentalen Spektralphotometer gemessen. Die Ergebnisse weisen auf einen signifikanten Unterschied zwischen den verwendeten Apatit-Präparationen hin. Es konnte jedoch keine Dosis- und auch keine Anwendungshäufigkeitsassoziation nachgewiesen werden. Bei Microrepair ($ZnCO_3$/Ap) erzielte die höchste Konzentration (30 Gew%) einen besseren Effekt als die niedrigeren Konzentrationen. Die Formulierung, die $ZnCO_3$/Ap enthielt, hinsichtlich der Zusammensetzung aber wie eine Zahnpasta aufgebaut war, erzielte einen besseren Aufhelleffekt als die wässrige Subvention mit $ZnCO_3$/Ap. Innerhalb der Gruppe der Tri-Kalziumphosphat-Suspensionen wies die zehnprozentige Suspension die besten Aufhellergebnisse auf. Der Aufhelleffekt war nicht sehr ausgeprägt, die Unterschiede waren statistisch jedoch signifikant. Wir können daher zusammenfassen, dass Kalziumphosphat Formulierungen, deren Bestandteile an die Schmelzoberfläche anhaften, ein viel versprechendes Potenzial haben, Zahnaufhellung zu bewirken. Weitere

Untersuchungen sollten prüfen, wie man den Aufhelleffekt weiter steigern kann.

2. Mit dem Ziel, die die Aufhellung zu verbessern, wurde eine zweite Versuchsserie durchgeführt. Hierfür wurde experimentell ein selbstorganisierendes Peptid mit Hydroxylapatit kombiniert. Das selbstorganisierende Peptide stellt eine biomimetische Matrix dar, in die Hydroxylapatitkeime eingelagert wurden. Die Ergebnisse zeigen, dass sowohl die Peptidgruppe als auch die Vergleichsgruppe auf Basis einer wässrigen Suspension die Zahnfarbe aufhellen können. Die ΔE-Werte nahmen in der Gruppe der wässrigen Suspension kontinuierlich zu. In der Peptidgruppe wurde der maximale Aufhelleffekt bereits nach zwei Anwendungen erreicht. Wir können daraus ableiten, dass das selbstorganisierende P11-4 Peptid als organische Matrix für Hydroxylapatit-Partikel dient. Die Matrix und die Hydroxylapatitpartikel fördern die sofortige Aufhellung und könnten gleichzeitig potentiell zu Remineralisation von oberflächlichen Defekten beitragen.

3. Zum Nachweis, dass das P11-4 Peptid zusammen mit den Hydroxylapatit Partikeln an der Aufhellung beteiligt ist, wurde Lanthan dotiertes Hydroxylapatit verwendet. Da Lanthan im Zahnschmelz nur in geringen Spuren vorhanden ist, konnte mit Hilfe einer EDX Analyse über eine erhöhte Lanthankonzentration in der Zahnoberfläche nachgewiesen werden, dass die anhaftenden Partikel aus der P11-4 Peptid/Hydroxylapatit- Mischung stammen. Mit ATR–FTIR wurde außerdem das P11-4 Peptide auf die Schmelzoberfläche nachgewiesen.

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Eidesstattliche Versicherung

Xu Xiaohui

Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

New Approaches to Tooth Whitening Based on Changing the Optical Properties with Calcium Phosphate Containing Suspensions

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