Evaluation of the Whitening Effect of a Mixture of Self-assembling Peptide and Hydroxyapatite on Bovine Enamel

vorgelegt von

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To My Husband and My Mother

In Memory of My Dear Father

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Chapter 1: General introduction and literature review

1.1 Historical background of cosmetic dentistry

A number of social and psychological studies have indicated that a person's physical appearance considerably influences the quality of his/her interactions with others. How people look is an important dimension of nonverbal communication and it can play a crucial role in the impression they make on others. According to Thornton and Ryckman (1991), physical attractiveness and self-esteem are significantly correlated. Moreover, it has been shown that individuals, who perceived by society to be attractive, experience notable advantages over less attractive people (Goldstein, 1993). To enhance the psychological attractiveness and its positive consequences, a powerful and easy solution is use of cosmetics (Graham and Jouhar, 1983). As a result, several cosmetic products and procedures are marketed to the public as to enhance personal appearance.

The importance of self-perceived attractiveness in the context of health care has been recognized and understood (Craig, 1999). Attempts have shown that improving patients' appearance brings positive impacts on illness and recuperation times. In particular, numerous studies have confirmed the relation between dental esthetics and the perceived levels of self-confidence (see e.g. Jenny et al., 1990). Broadly speaking, esthetic dentistry includes any action that makes the appearance of a person's teeth and gums better (e.g. improvement of tooth color). It mainly deals with color, position, shape and size of teeth. Traditional dentistry deals with prevention, diagnosing and treating oral diseases, while cosmetic dentistry deals with improving and enhancing the shape and color of a person's teeth. However, it is important to note that there is not a clear border between cosmetic dentistry and restorative dentistry. Dental fillings, for instance, may fall into cosmetic dentistry, depending on the filling materials. One may use composite materials that match the color of the teeth better than amalgam that left visible dark spots on the teeth. There are various cosmetic dentistry procedures. Figure 1-1 presents the most common procedures used in cosmetic dentistry.

Tooth whitening and reaching an ideal esthetic result is becoming a widespread increasingly important issue in modern cosmetic dental practice. Tooth whitening is defined as "any process that lightens the color of enamel & dentin" (Carey, 2014; P.72). According to the American Academy of Cosmetic Dentistry (AACD), whitening, which may be accomplished by physical removal of teeth stains or a chemical reaction that lightens the teeth color, is one of the most demanding dental treatments by the public (AACD, 2015). Kim et al. (2011)

reported that 28% of UK adults, 34% of USA adults and 56% of Chinese adults are dissatisfied with their teeth color and appearance.

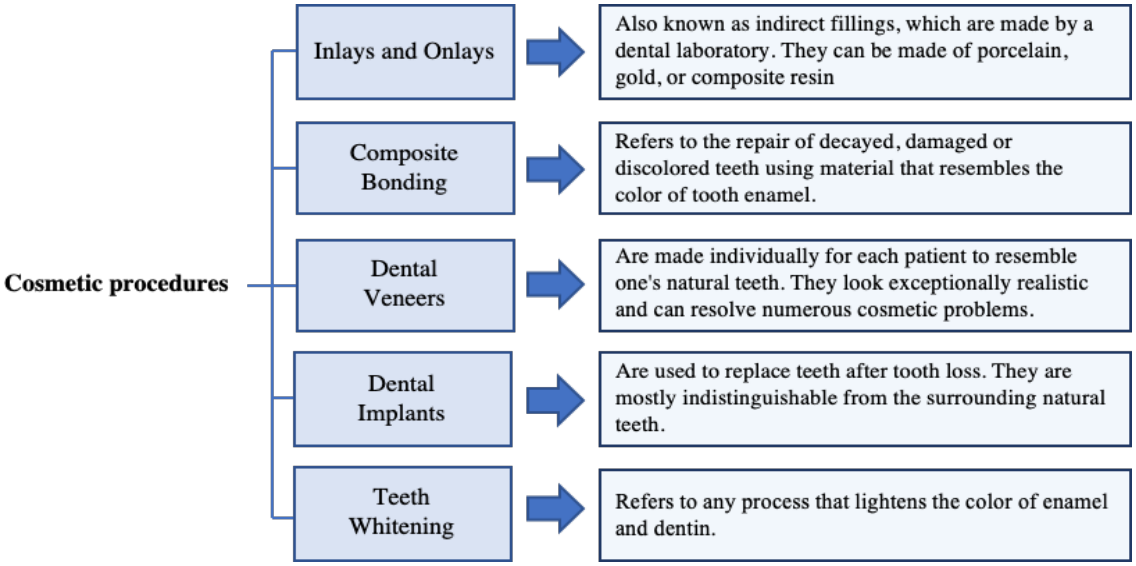


Figure 1-1 An overview of common dental cosmetic procedures

1.2 Etiology of tooth discoloration

There are various reasons for tooth discoloration. Since the treatment procedure may vary depending on the cause(s) of the discoloration, it is important to detect the cause(s) of tooth staining (Freccia et al., 1982). In fact, a careful assessment of cause of discoloration facilitates the treatment procedure and may promote the degree to which it improves the tooth color (Haywood and Heymann, 1989; Jordan and Boksman, 1984). Tooth discoloration can be located surficial or deeper. The major cause of tooth discoloration is the accumulation of chromogenic particles on the tooth surface. As stated by Karadas and Seven (2014), tooth discoloration occurs at the outer layer of the enamel by adhering metallic or non-metallic stain particles on the tooth surface. This type of discoloration occurs due to use of foods, drugs and tobacco. In particular, the most common staining substances are black tea, coffee, cola and red wine (Côrtes et al., 2013), as well as curry and berries. The mentioned stains which are mainly incorporated in the pellicle, are produced by the reaction between amino acids and sugars. They may also be acquired by the retention of external chromophores in the pellicle (Viscio et al., 2000). Dental professional cleaning can help removing such stains (Macpherson et al., 2000). Furthermore, brushing the teeth after every meal with an effective toothpaste and rinsing the mouth with water after drinking wine or coffee can help preventing surface stains (Joiner, 2004; Colgate, 2018).

Another type of tooth discoloration occurs when the deeper structure of the tooth discolors. This type of discoloration is often more resistant against treatments. Burgt et al. (1986), Davis et al. (2002) and Haywood (2002) discussed tooth discoloration associated with endodontic and restorative treatments. According to their studies, some root canal sealers that contains metallic components and iodoform-based medicaments in the pulp chamber, remained tissue in the pulp chamber and certain dental fillings materials are capable of causing measurable coronal tooth discoloration. In addition, small defects, located on enamel surface, may change optical properties of the tooth and at the same time attracts stains in the oral cavity, which consequently result in darker teeth (Li, 2017). In some cases, teeth may discolor due to some systemic diseases and congenital disorders such as thalassemia, sickle cell anemia, severe dental fluorosis, porphyria, amelogenesis imperfecta and dentinogenesis imperfecta (Chaudhary et al., 2009; Rotstein, 2002; Livingston and Dellinger, 1998). According to Watts and Addy (2001), dentinogenesis imperfecta may lead to blueish, brown, purple and amber discoloration.

Certain medications used locally and systematically can cause teeth to discolor. The antibiotic minocycline, when the permanent teeth have erupted, and tetracycline, during teeth development, are known to discolor teeth in children (Raymond et al., 2015). Consumption of antibiotics during pregnancy can also lead to pronounced tooth discoloration (Kossatz et al., 2012). It is noteworthy that excessive use of potassium permanganate mouthwash, which is used by patients with oral candidiasis and stannous fluoride mouthwash, which is used as a desensitizing agent can produce tooth stains (Thomas and Denny, 2014; West et al., 2012; Vogel, 1975). Furthermore, some specified treatment methods like chemotherapy and radiation may also be associated with increased risk of tooth discoloration (Busenhart et al., 2018).

According to Pindborg (1970) and Watts and Addy (2001), childhood diseases with oral manifestations, trauma in a permanent tooth and internal bleeding can lead teeth to discolor. Based on a case report by Bussell et al. (2010), some oral microorganisms, such as Pseudomonas aeruginosa in chronic pulmonary infected children, may be chromogen and may cause generalised navy-blue stains. Some widespread disorders with structural dental changes and discolorations were reported by Rashid and Anca (2011). The patients suffering from celiac disease, vitamin D deficiency and dental fluorosis develop enamel hypoplasia and patches of white, brown or yellow discolorations. It is noteworthy that the correction of tooth discoloration associated with the mentioned systemic disorders need specific actions and fundamental treatments by physicians and dentists. In some cases, treating the initial disease and eliminating the cause(s) may reverse the tooth discoloration without taking any additional action. However,

regular dental prophylaxis is recommended as a standard and effective method to remove dental stains located on the tooth surface.

Natural ageing is another common explanation of yellowish-brownish tooth discoloration. As time goes by, the underlying dentin becomes darker because of the formation of secondary dentin which is darker compared to the primary dentin (Watts and Addy, 2001). On the other hand, over time, the overlying enamel tends to thin owing to abrasion associated with incorrect and too vigorously tooth brushing techniques or erosion resulted from use of fruit juice, sport drinks and carbonated drinks (e.g. sparkling water), acid reflux disease, as well as certain eating disorders, in which frequent self-induced vomiting exposes teeth to stomach acids (e.g. bulimia nervosa). Regular and prolonged consumption of high acidic supplements or drugs such as Aspirin or Vitamin C, wine and imbalanced eating pattern can also erode enamel (Picos et al., 2018; Dynesen et al., 2008; Rosten and Newton, 2017; Moazzez et al., 2000). As a result, the underlying dentin shimmers through the thinned enamel. The result of this tooth loss and exposure of dentin is teeth seem darker (Alqahtani, 2014). Table 1-1 presents some common examples of tooth staining causes.

Table 1-1 Examples of tooth staining causes – Based on Nathoo (1997) and Alqahtani (2014)

Tooth staining causes		
Chlorhexidine	Red wine	Amelogenesis imperfecta
Coffee and tea	Tobacco	Tetracycline medication
Cola	Dental caries	Dentinogenesis imperfecta
Berries	Spices (e.g. curry)	Excessive fluoride ingestion

1.3 Current measures for tooth whitening and stain removal

The history of tooth whitening can be traced back to more than 150 years ago. Over the past three decades, tooth whitening has become one of the most requested esthetic dental treatments (American Dental Association, 2018). In response to the considerably high demand for whiter teeth, several alternative methods and products for this purpose have been made available. These methods use different whitening agents, concentrations, time and frequency of application, and application modes. Table 1-2 presents a review of historical tooth whitening agents.

Table 1-2 A review of historical whitening agents

Whitening agent	Source
Cyanide of potassium	Kingsbury (1961)
Chloride of lime	Dwinelle (1850)
Solution of calcium hydro chlorite and acetic acid	Kirk (1889)
Oxalic acid	Bogue (1872)
Sulfurous acid	Kirk (1889)
Aluminum chloride	Harlan (1891)
Pyrozone	Atkinson (1892)
Hydrogen peroxide	Kirk (1893)
Sodium peroxide	Kirk (1893)
Superoxol (30% aqueous solution of hydrogen peroxide)	Pearson (1951)

Tooth whitening includes any treatment or process which removes or covers teeth stains and lightens the teeth at the same time. It may be accomplished by chemical actions (e.g. bleaching) or physical stain removal techniques. According to American Academy of Cosmetic Dentistry (2014), whitening systems can be categorized as demonstrated in the approach below.

1.3.1 Whitening Toothpastes

A wide range of whitening toothpastes are developing constantly by various manufactures of oral care products all over the world. According to the global marketplace, sales of whitening toothpastes alone were almost $3.2 million in 2016 and are expected to exceed $3.7 million in 2021 (Technavio, 2016).

While most of these products contain peroxide-based agents which influence the teeth color by chemical reactions, there are some products, containing specific solid agents, improve physical stain removal (Joiner, 2006a). The main functional ingredient of the whitening toothpastes is the solid abrasive (Farago et al., 2011). The abrasives are capable of removing the surface stain during tooth brushing due to their physical hardness compared to tooth stain. Table 1-3 illustrates that the whitening toothpastes may also contain surfactants, enzymes and polyphosphates. There are studies (e.g. Joiner, 2010 and Joiner et al. 2004) that claim some formulations of toothpastes prevent the formation and accumulation of stains. Enzymes have been widely used in whitening dentifrices to promote the stain removal. The stains are firstly incorporated into the salivary protein layer so-called pellicle which forms on the surface of the tooth. The enzymes are able to act as a protease and help proteolysis which leads to removing

stain film (Joiner, 2010). According to Shellis et al. (2005) and Baig et al. (2005), phosphate containing toothpastes (e.g. sodium triphosphate and sodium hexametaphosphate) are known as effective agents for removing and preventing stain. In addition, they could prohibit stain adherence to the enamel. Joiner et al. (2008) and Philpotts et al. (2017) claimed that the blue covarine, as an optical agent incorporated into silica whitening toothpastes, can deposit onto the surface of enamel and giving a yellow to blue color shift. This results in enhancement of tooth whitening. However, this is a temporary color improvement. Besides, some studies (e.g. Tao et al., 2017) reported that covarine-containing toothpastes reduce the tooth yellowness (b*) and significantly improve the tooth whiteness immediately after tooth brushing (Tao et al., 2017b).

Table 1-3 Various whitening toothpastes ingredients – Based on Joiner (2010), Hoic et al. (2004) and Ayad et al. (1999)

Ingredients	Examples
Abrasives	Hydrated silica
	Calcium carbonate
	Dicalcium phosphate dihydrate
	Calcium pyrophosphate
	Alumina
	Perlite
	Sodium bicarbonate
Chemical	Hydrogen peroxide
	Citrates
	Sodium chlorite
	Calcium peroxide
	Sodium citrate
	Sodium pyrophosphate
	Sodium tripolyphosphate
	Sodium hexametaphosphate
	Papain
Optical	Blue covarine

However, most of the traditional whitening toothpastes mainly obtain their whitening efficacy through the abrasive components which contribute to remove the stains from tooth surface. There are some other toothpastes available on the market claiming to promote the remineralization process and improve the surface smoothness (Niwa et al., 2001). Smoothing the surface results in increasing the whiteness and brightness of the teeth due to increasing the light

reflection rate from the surface. Pedreira de Freitas et al. (2011; P.6) argued that "changes on surface morphological wavelengths, on the same order of magnitude of the visible light wavelengths, are responsible for the visual changes, like changes in gloss".

Toothpastes that contain hydroxyapatite (HAP) have been developed more recently. Some studies (e.g. Niwa et al., 2001) claimed that HAP-containing toothpastes can increase the whiteness and brightness of the teeth. However, the intensity of their effectiveness is depends on the amount of HAP in the toothpaste. Roveri et al. (2009) reported that after the treatment of a demineralized enamel for 10 minutes, a new homogenous thick hydroxyapatite layer covers the tooth surface. This contributes to an increasing diffuse reflection of the light, leading to a measurable enhancement of lightness.

1.3.2 OTC Whitening products

Over the counter (OTC) bleaching and whitening products used for the first time in the USA (Donly KJ et al., 2007). This technique allows patients to do treatment without supervision of dentists. It typically uses relatively low concentrations of carbamide peroxide or hydrogen peroxide (3-5%) in products that are sold directly to consumers. These include whitening strips, pens, paint-on gels, powders, toothbrushes, whitening dental floss and whitening chewing gums with sodium hexametaphosphate (Demarco et al., 2009; Zantner et al., 2007). Furthermore, special battery-powered toothbrushes that remove more extrinsic tooth stain during brushing are also considered as OTC whitening products. Although this method may not offer the same degree of whiteness that one can get from the discussed professional methods, it is more widely used by people than in-office bleaching therapy. This is mainly because OTC bleaching is usually cheaper and gives the convenience of doing the whiting procedure yourself with no restrictions (Serraglio et al., 2016; Demarco et al., 2009). Moreover, In-office whitening is not a permanent solution and many dentists recommend home maintenance follow-up with whitening gels at a lower concentration.

1.3.3 Whitening Rinses

While some rinses are just antiseptic and reduce the microbial load of oral cavity or focus on getting rid of halitosis, others have special qualities that help reducing stain accumulations as patients are swooshing the liquid around (Gasparri et al., 2018). Furthermore, a considerable number of mouthwashes contain hydrogen peroxide (HP), which penetrates the tooth and break apart the chromophore bonds. This results in changes in tooth whiteness. Despite their high demand in the market, there is only few evidence showing the effectiveness of the whitening

mouthwashes (Karadas and Hatipoglu, 2015). It is important to mention that these HP-containing whitening mouthwashes available in the market may decrease the enamel microhardness (Fernandes et al., 2017).

1.3.4 Tooth Bleaching

It is important to note that the terms “whitening” and “bleaching” have been often used in the literature, interchangeably. However, they do not refer to the same meaning. Bleaching is defined by the International Organization for Standardization (2011), as the “removal of acquired discolorations of natural teeth through the use of chemicals, sometimes in combination with the application of auxiliary means.” In other words, tooth bleaching is technically an oxidative chemical process that leads to increasing tooth’s perception of whiteness by changing light-reflecting or light-absorbing nature of a tooth (Li, 2017).

In contrast, tooth whitening is the process of making the teeth’s perceived color seems whiter, regardless of the agents used. It includes not only bleaching with peroxides but also mechanically removing surface stains by means of abrasive dental prophylaxis pastes and tooth brushing with a whitening dentifrice (Li, 2017).

From a general perspective, bleaching procedures can be divided into “non-vital tooth bleaching” and “vital tooth bleaching” (Demarco et al., 2013). While non-vital tooth bleaching must be supervised by a dentist, vital tooth bleaching can be carried out both at home and at office.

1.3.4.1 Vital tooth bleaching

Vital tooth bleaching was introduced in the year 1864 (Latimer, 1868). Cyanide of potassium has been considered as the main vital tooth bleaching agent (Kingsbury, 1961). According to the American Dental Association (2010), there are two general approaches for professional bleaching of vital teeth: in-office tooth bleaching, dentist-supervised night-guard or at-home bleaching.

In-office tooth bleaching

In-office teeth whitening is performed by dentists or dental professionals using highly concentrated bleaching agents such as products containing hydrogen-peroxide (H_2O_2) with a concentration of 25-40% (Kihn, 2007). In fact, in-office whitening takes place under carefully supervised conditions which allow for a safe and effective use of bleaching agents. In this approach, the agent may be further activated by exposing the teeth to a laser light or heat light

during the bleaching process (Sulieman, 2004). In order to avoid gum irritations and protect oral mucosa, a rubber dam, protecting gel or Vaseline can be placed carefully on the gums without covering the teeth's surface (Wiesel, 2000). In most cases, in-office approach requires a short treatment period and makes the patient's teeth whiter immediately after one treatment visit (Kugel and Ferriera, 2006; Gurgan et al., 2010; Henry et al., 2013). However, for a better result, it may require multiple treatment appointments.

Tray-based Whiteners; home bleaching products

At-home or night-guard bleaching, introduced by Haywood and Heymann (1989), typically uses whitening agents at a low concentration (10 to 20% carbamide peroxide that equals 3.5 to 6.5% hydrogen peroxide). These agents are applied to the teeth's surface, using a mouth tray during the night for one or two weeks (Alqahtani, 2014). This therapy is a do-it-yourself bleaching method, but it must be controlled by dentists sometimes during the treatment period. According to Sulieman (2005), this method has been widely used for many decades.

As explained, most of the common commercial bleaching agents are based on the direct application of hydrogen-peroxide-contained agents that may be released from carbamide peroxide (Jiang et al., 2008). These free radical producible agents have a characteristic that let them penetrate the tooth structure. They attack and break down the chromophore bonds, which consequently results whiter teeth (Joiner, 2010). Hydrogen peroxide as an oxidizing bleaching agent diffuses into enamel and dentin and decomposes into unstable free radicals (Figure 1-2). It works by attacking exogenous organic pigmented molecules which are organic, in the spaces between the inorganic molecules in the enamel and interrupting the double-bond conjunction in the dark-colored molecules.

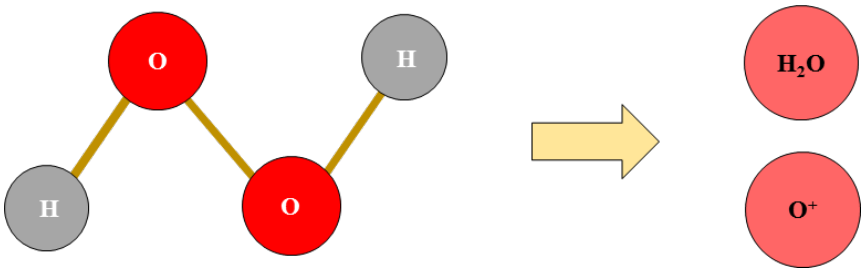


Figure 1-2 Hydrogen peroxide decomposition into unstable free radicals

1.3.4.2 Non-vital tooth bleaching

Non-vital bleaching is carried out for a non-vital endodontically treated tooth that has

become discolored due to the deposition of blood degradation products in the dentinal tubules (Wray and Welbury, 2001). The first non-vital tooth bleaching was performed in 1848 by using chloride of lime (Dwinelle, 1850). In 1864, the most effective procedure for non-vital tooth bleaching was introduced by Truman, using a mixture of calcium hydro chlorite & acetic acid (Kirk, 1889). Later, in the late 19th century, a large number of other bleaching agents and procedures have successfully introduced (Haywood, 1992). Harlan (1885) reported the first application of a hydrogen peroxide containing agent in the pulp chamber as a bleaching agent. Prinz (1924) proposed using heated mixtures containing sodium perborate and Superoxol in order to cleaning the pulp chamber.

Prior to starting a non-vital bleaching, a well-condensed, fully extended root canal filling must be performed. There are several non-vital bleaching methods performed today. Most of these techniques are performed at dental offices, using a combination of external stain removal and internal procedures (Joiner, 2006b). Examples include, walking bleach, modified walking bleach, internal non-vital power bleaching, and inside-outside bleaching methods.

The walking bleach approach introduced by Spasser (1961), places a paste of sodium perborate mixed with water into the pulp cavity of a non-vital tooth and sealing it in. This technique was later modified by Nutting and Poe (1967) who combined the sodium perborate containing paste with 30% hydrogen peroxide.

The internal power bleaching of non-vital teeth uses hydrogen peroxide gel (30–35%) in the pulp cavity and activates it either by using a light or a heat lamp. At the end of each visit the bleaching agent is left in the tooth until the next visit in (at least) two weeks (Sulieman, 2005). This technique increases the effectiveness of bleaching agent (Freccia et al. 1982).

Finally, in the inside-outside technique, originally described by Settembrini et al. (1997), the bleaching gel must be applied into the pulp cavity and also on the external aspects of a non-vital, root filled discolored tooth (Leith et al., 2009). The access cavity is left open during treatment so that the whitening agent (e.g. 10% Carbamide peroxide) can be easily and regularly changed (Poyser et al., 2004).

1.4 Disadvantages of existing bleaching processes/agents

There are a large number of tooth bleaching products available today. The most of these bleaching agents are based on hydrogen peroxide or its ancestor, carbamide peroxide. In essence, hydrogen peroxide has various industrial applications, including bleaching or decarbonizing textiles, hair and foods. It is also used as a seed disinfectant and neutralizing agent in

wine distillation (Tredwin et al., 2006). According to Deliperi (2008), low-concentrated hydrogen peroxide can be found in human and plant tissues, water surface, rain, as well as several foods and beverages. Hydrogen peroxide has been used as a tooth bleaching agent for more than 100 years (Walsh, 2000).

However, a large number of clinical studies (e.g. Auschill et al., 2005) have discussed negative effects and disadvantages of the use of this tooth bleaching agent. The studies encompass cellular, human and animal experiments for both vital and non-vital bleaching therapies. In fact, hydrogen peroxide is a member of highly reactive oxygen substances. These are superoxide, hydroxyl, peroxyl along with alkoxyl radicals (Walsh, 2000). Reactive oxygen radicals can cause severe cell damages via several mechanisms, including DNA stand breaks, genotoxicity, and cytotoxicity (Tredwin et al., 2006; Hanks et al., 1993). That is to say, when the level of hydrogen peroxide goes beyond the tolerance of cellular defense mechanisms, it causes a critical health hazard (DeSesso et al., 2000).

On the other hand, tooth sensitivity and gingival irritations like erosions of the gingiva are widely reported as the most discussed side effects of using hydrogen peroxide (Firat et al., 2011). Therefore, to apply the hydrogen peroxide solution on a patient's teeth, it is necessary to prevent the patient's gums from contacting this agent. To this end, usually dentists use rubber dam or some isolation materials (e.g. Vaseline). However, these methods suffer from various problems and setbacks. For instance, due to a poor placement of the rubber dam, hydrogen-peroxide-containing agent may leak around the rubber dam, endangering the gingiva tissues (Wray and Welbury, 2001). Furthermore, as earlier discussed, to accelerate the bleaching process, mostly heat lamp is used. However, using heat lamp for a great deal of time can cause "extreme inconvenience" to the patient (Wiesel, 2000, P.2). However, Cunha et al. (2011) claimed that the use of light during bleaching does not accelerate the bleaching procedure.

In non-vital tooth bleaching, cervical root resorption has been expressed as the main concern of the application of hydrogen peroxide. According to a review of case studies, presented by Tredwin et al. (2006), the existence of a correlation between the bleaching of non-vital tooth and cervical root resorption has been widely observed. Based on an experiment conducted by Rotstein et al. (1996), when hydrogen peroxide is placed in the pulp cavity, it penetrates the tooth's dentine and makes it mechanically weaker.

Rotstein et al. (1996) and Bitter (1992) argued that using carbamide or hydrogen peroxide may dissolve enamel minerals (Calcium and Phosphate ions). This affects enamel surface integrity and increase surface roughness. Moreover, it leads to porosity (Figure 1-3) and

increased depth of enamel grooves (Ben-Amar et al., 1995; Abouassi et al., 2010). Moreover, the released oxygen radicals may inhibit with the composite polymerization and consequently reduce bond strength of the composite (Garcia-Godoy et al. 1993 ; Lai et al., 2002).

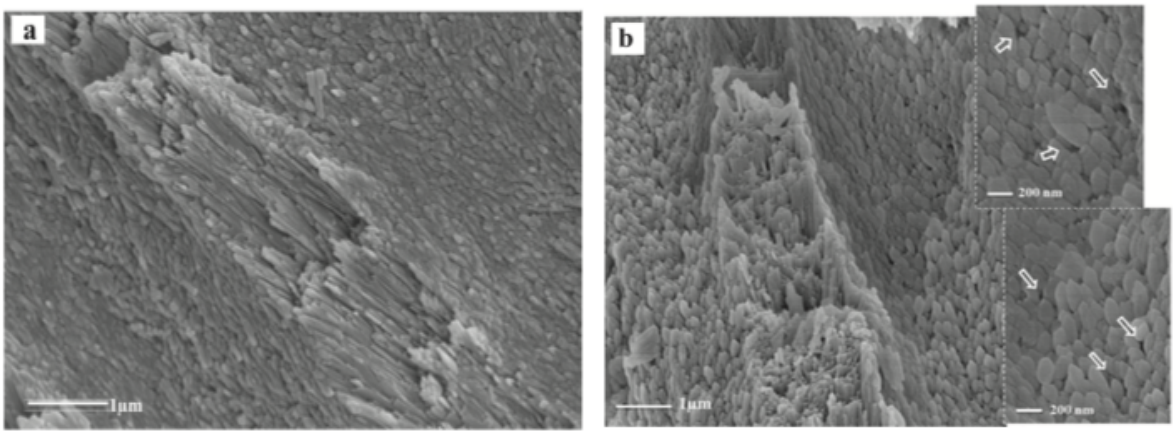


Figure 1-3 Higher magnification of sound and bleached enamel structure , (a) demonstrates the packed HAP crystals, rod structure surrounded by the inter rod enamel (b) demonstrates treated enamel with bleaching agent, increased porosity and increased spaces between the crystals –Elfallah et al. (2015). Reuse of the image is with written permission by Prof. Michael Swain (2019)

Another disadvantage of hydrogen peroxide is concerning its effectiveness in whitening teeth. While clinical observations have confirmed the whitening effect of hydrogen-peroxide on yellow hued teeth caused by foods and drinks, stains resulting from certain drugs may be unresponsive to this agent (Kihn, 2007). Furthermore, there are several other side effects of tooth whitening with hydrogen peroxide or carbamide such as tooth sensitivity, pain and loss of taste while eating and drinking after bleaching (Dahl and Pallesen, 2003; Carey, 2014; Goldberg et al., 2010). The disadvantages of the available tooth bleaching agents are summarized in the Table 1-4.

Table 1-4 Disadvantages of commercial bleaching agents

Gum irritation	Increasing tooth surface porosity
Tooth hypersensitivity	Dissolution of enamel
Cell damage	Impaired taste
Gingival erosion	Cervical root resorption
Reduction of dentin microhardness	Pain during chewing

Suffice to say that the current tooth whitening and tooth bleaching agents on the market suffer from a number of adverse side effects. This highlights the necessity of developing a new

whitening agent that remedies the mentioned side effects.

1.5 Color assessment in dentistry

The term “color” is classified into “physical color” and “perceptual color”(Robertson, 1992). According to Figure 1-4, in some cases the teeth have an identical physical color, while they have different perceptual color. It is important to mention that the perceptual color is always more crucial to patients (Pan and Westland, 2018).

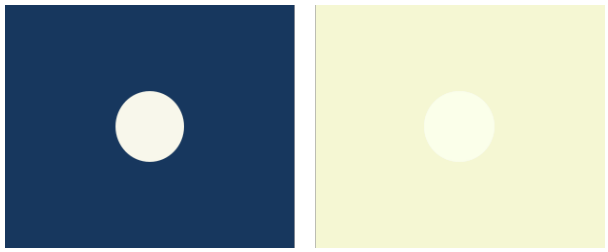


Figure 1-4 The two points are physically identical in color but the left one appears lighter than the right one due to the contrast effect with the background (perceptual color) – Based on Pan and Westland (2018)

The human eye`s retina includes three types of color receptors. To perceive a color, the receptors are stimulated by the spectrum of visible light entering the eye which subsequently transmits impulses to the brain via optic nerve (Johnston, 2009; Hecht, 2017).

Munsell’s system was the first that defined color as independent three dimensions and specified that based on three properties: hue, value and chroma. This system was introduced by Professor Albert H. Munsell in 20th century, who illustrated the colors in three-dimensional space (Kuehni, 2002). Hue is the correct word for the pure spectrum (color name e.g. blue, red, yellow), which distinguishes one family of color from another. Basically, three basic hues can be mixed and result in all other hues. The value describes the whiteness or blackness of a color and chroma is the attribute by which the object appear to be more saturated (Chang et al., 2012; Wetter et al., 2004). In tooth color detection an increase in whiteness is associated with increasing the lightness and reducing the chroma, whereas a decrease in whiteness is the result of changes in scattering of dentin and enamel and absorption or when stains attach to the tooth’s surface (Watts and Addy, 2001).

The tooth color is specified by a combination of its optical characteristics. In dentistry there are several methods described in the literature to assess the tooth color and evaluate the efficacy of tooth whitening approaches. Among a number of color measurement techniques

exist today, some (e.g. tooth shade guides, portable spectrophotometers and digital images) are frequently used in clinical studies (Pan and Westland, 2018). Since visual assessment of color has inherent inaccuracies and it may be affected by the lighting and the environment of the tooth and on the other hand it requires highly experienced and trained observer, the digital color detection is a more preferred method (Hammad, 2003; Paravina, 2009). However, the digital color detection (e.g. by means of spectrophotometers) suffers from difficulties associated with calibration and setup of the devices (Jahangiri et al., 2002). Based on a clinical study in 2014, the shade matching quality via spectrophotometer was significantly better than conventional visual method. According to this study, the adequate shade was chosen only 36.3% by visual techniques and 80.4% via a spectrophotometer (Bahannan, 2014).

1.5.1 Digital measurement of tooth whitening efficacy; Spectrophotometric assessment

One of the widely used spectrophotometers is the Ocean Optics (USB4000 VIS-NIR). This device is a fiber-optics spectrophotometer (Figure 1-5), which is responsive from 350 to 1000 nm. The spectrophotometer operating software, Ocean View is a software that has the ability to perform spectroscopic measurements such as absorbance, reflectance and emission. To perform a color measurement, the spectrophotometer must be connected to a halogen light source (e.g. HL-2000-Cal, Ocean Optics, Ostfildern, Germany). After 30 minutes of warm up, the light transmits through an optical fiber to the sample. Before measurement initiation, it is important to calibrate the device using calibration standards. Subsequently, the light interacts with the sample´s surface during measurement and it is collected from another optical fiber to transfer the outcome to the spectrophotometer due to measure the amount of reflected light and convert the measured spectral data (e.g. reflectance) to color coordinates (CIELAB). The processed data presents spectral information such as reflection and L*a*b* values. Reflection is characterized by the return of radiation by a surface, without change in wavelength (Merkus, 2009). Reflection is expressed as a percentage relative to the reflection from the white standard (WS-1-SS), which is used during calibration. After calibration with the white standard, the light path to the spectrophotometer should be blocked to take the dark spectrum before the measurement initiation (Optic, 2017).

The diffuse reflectance from the surface is measured via Lab-grade Reflection Probes (e.g. R200-7-UV/VIS) with 6-around-1-fiber bundle design. The 6-fiber leg for illumination, which must be linked to the light source and the single-read fiber leg to spectrophotometer. The probe ferrule is fixed in black Reflection Probe Holder (e.g. RPH-1) at 45° (Figure 1-6).

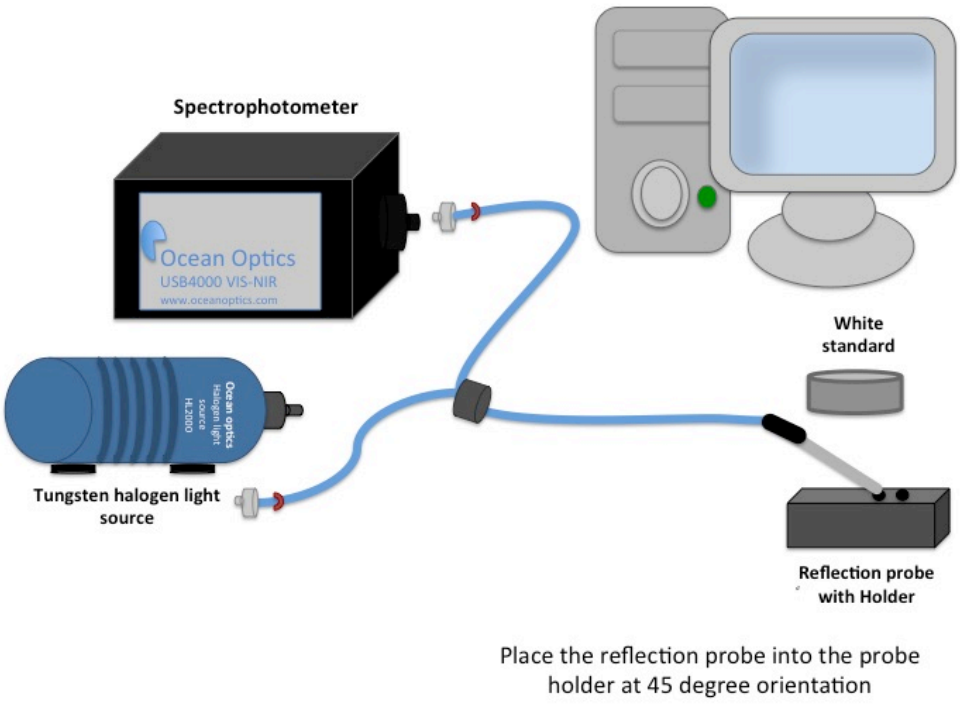


Figure 1-5 Schematic drawing representing Ocean optics spectrophotometer, modified after https://oceanoptics.com

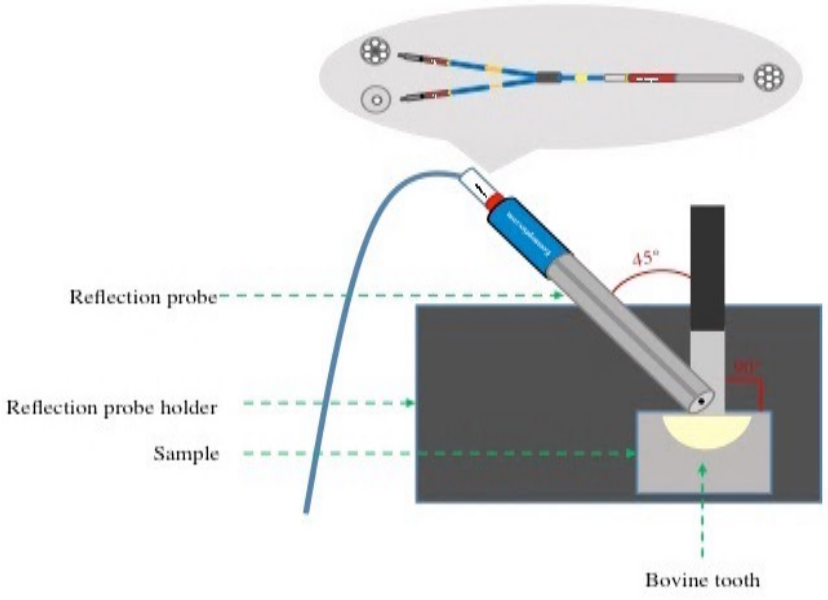


Figure 1-6 Scheme of the Ocean Optics Probe holder and reflection probe, modified after https://oceanoptics.com

In many studies, dealing with tooth color changes, Gretag Macbeth Color Eye 7000A spectrophotometer has been employed (Al-Nahedh and Awliya, 2013; Swift et al., 2009). The

Figure 1-7 presents a schematic drawing of Color Eye spectrophotometer. This device is an 8° diffuse sphere instrument, which is lined with a diffuse white reflecting coating and has a spectral range from 360 to 750 nm. The light source is placed on the rear of the sphere providing diffuse illumination. This hollow spherical cavity contains a small aperture in the front part. The spectrophotometer must be calibrated before the measurement using a black trap and white calibration tile as the standard before measurement. To eliminate the associated errors, the experiment was carried out without any light. To this end, the whole room was darkened during measurement, as recommended by the spectrophotometer's instruction.

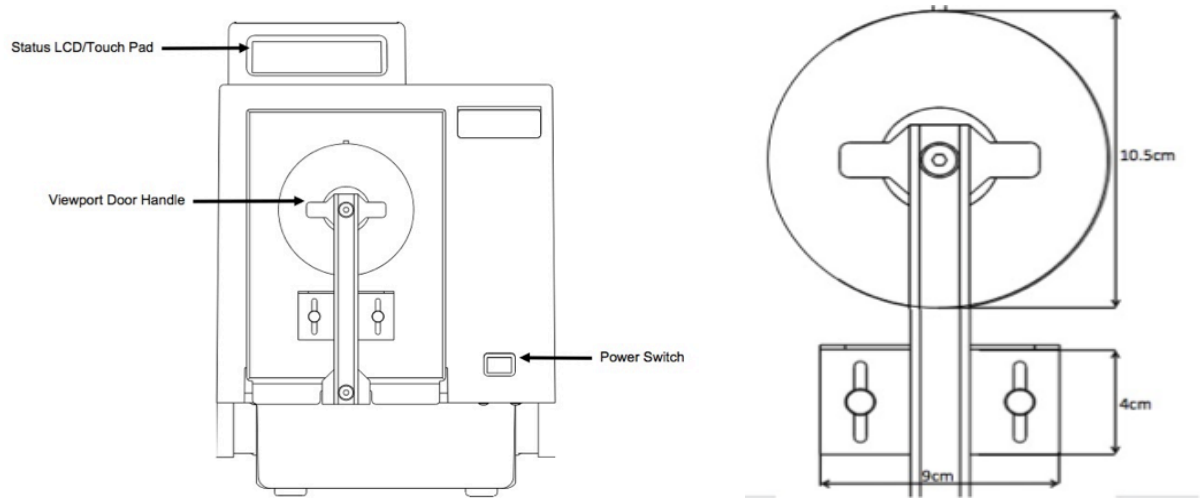


Figure 1-7 Schematic drawing representing Color Eye 7000A spectrophotometer

1.5.2 CIE L*a*b* color system

CLELAB is a color system adopted by CIE in 1976 and is deployed for color management as a "device independent model". This color scale is based on the opponent-colors theory of color vision.

The International commission on illumination or the commission international de l´eclairage (CIE), defined a three-axis color space (L*a*b*) in which L* represent lightness. The L* values begin from 0 (black or total absorption) to 100 (white), a* and b* indicate the chromaticity coordinates. +a indicates amount of red and -a represents amounts of green. On the b-b', The +b value indicates the level of yellowness and -b shows the amount of blueness, while zero is for both a and b axes neutral gray (Figure 1-8).

When the L*a*b* is considered as vector, the total differences can be stated as a single value, known as ΔE*, by using vector mathematics to compare the sample color before and after treatment (Eq. 1). Therefore, ΔE* quantifies the difference between two colors. It was

claimed that ΔE > 2 is always detectable for the trained eyes, whereas ΔE* value almost equal to 1 is assumed visually detectable 50% of the time. ΔE* values less than 1 are perceived identical (Yannikakis et al., 1998).

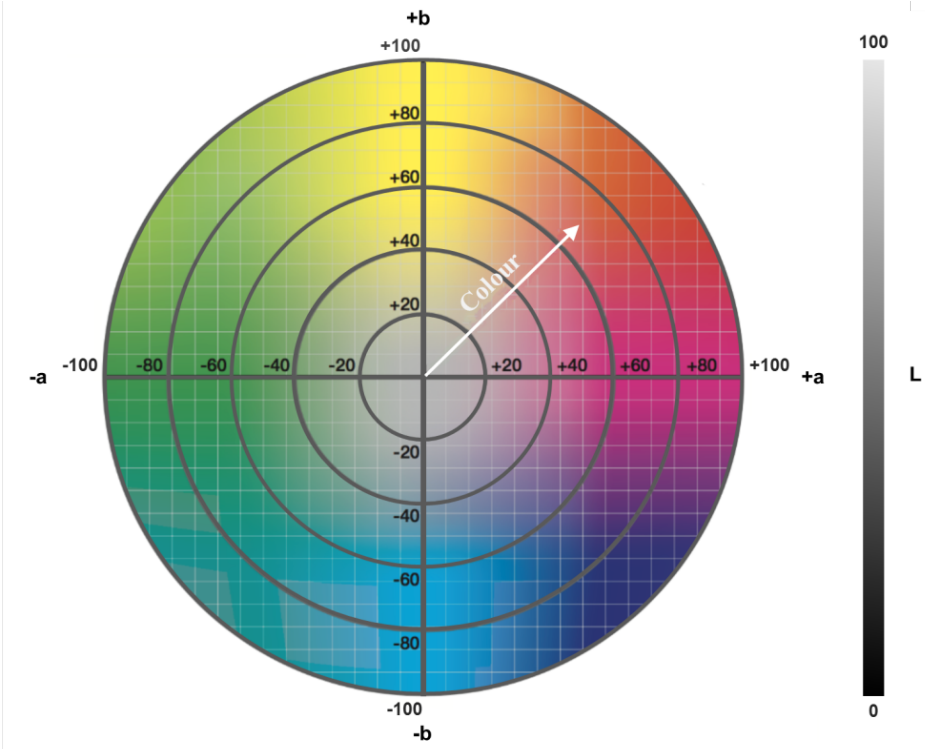


Figure 1-8 L*a*b* color system – modified after (https://www.xrite.com)

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \textbf{Eq. 1.1}$$

L^*: Lightness

a^*: Redness-Greenness

b^*: Yellowness-Blueness

1.6 Main objectives of thesis

Adolescents are increasingly concerned about their tooth color and appearance. They seek enhanced tooth shape and brighter tooth color to improve their self-confidence. On the other hand, the already-introduced whitening methods often suffer from various drawbacks and side-effects. Therefore, this dissertation aims at proposing a new whitening formulation which is safer, less expensive and less time-consuming than the existing products.

According to the previous section, we hypothesized that the interaction between the calcium phosphate (in our case, hydroxyapatite = HAP) mixed with solved P11-4 self-assembling

peptide and the surface of the tooth could enhance the adhesion of the hydroxyapatite particles to the tooth surface and improve the whitening efficacy by causing defuse reflection from the new generated layer.

This study aimed at:

1. Evaluating the whitening efficacy of different calcium phosphate-based formulations.
2. Achieving better whitening results and improving the treatment stability by using self-assembling peptide as a matrix for HAP nucleation.
3. Removing the smear layer from the surface and promoting the adherence of HAP layer on the sample's surface by using a pre-treatment agent.
4. Visualizing the new formed HAP layer and analyzing SEM pictures.

1.7 Structure of thesis and approach

The second chapter of this study compared a low-concentrated suspension of P11-4 self-assembling peptide and HAP with a commercial home-bleaching method so as to assess the whitening effectiveness of the proposed suspension. Further, it evaluated whether exposure time, treatment frequency and storage in saliva had any significant impact on the whitening effect of the suspension.

The third chapter compared aqueous low-concentrated P11-4 peptide, HAP suspension with polymeric high-concentrated P11-4 peptide and HAP suspension gel. In addition, it discussed the effect of saliva on the color of samples after the treatments. Finally, it evaluated if re-staining after the treatment and storing in saliva contributed to significant color changes.

The next chapter analyzed the whitening effect of a high concentration of P11-4 Peptide in combination with HAP after using 3% sodium hypochlorite as the pre-treatment. To provide a detailed assessment, we assessed the whitening effect of each of the components of the proposed suspension.

The fifth chapter mainly provided a qualitative assessment of the surface structure changes after treatment with the P11-4 peptide -HAP suspension, analyzing SEM images.

Finally, the last chapter presented our study summarized, following by conclusions and outlook for future studies.

Chapter 2: Analysis of the effect of application frequency, exposure time and saliva on color change after the treatment with aqueous low-concentrated P11-4 HAP suspension

2.1 Background and significance

2.1.1 Hydroxyapatite as a new whitening agent

One of the proposed whitening agents that has been recently suggested by a number of studies is hydroxyapatite (HAP). Yamagishi et al. (2005a) and Onuma et al. (2005) demonstrated that by using a low-pH (acidic) solution with fluoride-substituted hydroxyapatite (F-HAP), nano HAP-crystals (approximately 100 to 400 nm long and 20 to 80 nm wide) seamlessly grow and adhere as a thin new layer on the enamel. The low pH of the solution causes dissolving the enamel's surface and dissociating of calcium phosphate clusters ($Ca_9(PO_4)_6$). Based on their findings, phosphoric acid can roughen and clean the enamel surface. Subsequently, the dissociated ions can lead to a more rapid growth of HAP crystals compared to calcium phosphate clusters. In addition, the crystallographic orientation was in line with the primary apatite crystals. However, it is also possible that HAP particles become nucleated with a random orientation. The nucleation occurs by using calcium phosphate clusters, which are undissociated. This approach can contribute to tooth whitening because of two possible reasons. First, HAP itself is a white agent. Second, it can be assumed that the newly generated HAP layer leads to diffuse reflection on the surface of the tooth, causing the tooth appear brighter. However, because of possible soft tissue damages, it is necessary to apply this method under the supervision of a dentist.

In a study, Dabanoglu et al. (2009) used a non-acidic hydroxyapatite suspension as a whitening material. Based on this study, non-acidic hydroxyapatite suspension indicates more obvious results and dose dependency than a hydroxyapatite-containing dissolvable film. They also demonstrated that this material has a self-adhering characteristic. Hence, the generated HAP layer cannot be removed completely by using hydrodynamic shear force.

2.1.2 Self-assembling peptide P11-4

Peptide self-assembly is a relatively new approach for building synthetic super molecular architectures (Liu et al., 2017). According to Hug and Lysek (2015), it refers to the organization of peptides with other peptides having similar structure into multimeric assemblies by non-

covalent interactions, which occurs spontaneous and reversible. having similar structure into multimeric assemblies by non-covalent interactions. These interactions create the multimeric assemblies encompassing van-der-Waals force, pi-stacking and hydrogen bond force, polar force and also ionic interactions, which occur between the amino acid backbones and/or the side chains of amino acid.

The P11-4 peptide that comprises the sequence Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln, is an embodiment of self-assembling peptide, which is preferred. In response to pH triggers, the rationally designed peptides construct assemblies in the hierarchical order as follow: tapes, ribbons, fibrils and fibers. In particular, for a pH lower than 7.5, P11-4 peptide is able to assemble in one dimension to form ß-sheet, tape-like assemblies (Aggeli et al., 2003). When pH decreases, the assemblies of peptide can transfer from a fluid phase to a nematic gel.

In general, there are several self-assembling peptides such as P11-4, which can be applied as templates or scaffolds in tooth remineralization. This scaffolds support *in situ* the nucleation of calcium phosphate (Hug and Lysek, 2015). A negative charged residue (Glu) which is acidic, can serve nucleation sites for Ca^{2+}, while an acidic residue that is positively charged, such as Arg, may be able to interact with phosphate ions. If the side chains of glutamic acid and arginine are able to interact with solved calcium and phosphate to enhance the nucleation of calcium phosphate combinations, they may also interact not only with calcium phosphate particles but also with the enamel surface.

Teeth are re-mineralized mainly by the delivery of phosphate and calcium ions into tooth cavities. According to Hug and Lysek (2015), the phosphate and calcium ions may be presented in different crystalline forms (e.g. HAP-based materials) or as amorphous calcium phosphate (e.g. Recaldent-based materials). Recently, an alternative approach for tooth re-mineralization has been introduced, which is based on rationally-designed self-assembling peptides (Brunton et al., 2013).

We hypothesized that it may be possible to enhance the adhesion of the HAP layer and the chance of re-mineralization at the same time, we used a self-assembling peptide, which may hold and stabilize the HAP particles like a glue for a longer period of time on the teeth's surface and acts as a matrix for nucleation.

2.2 Main objectives of this chapter

The current chapter aimed at evaluating the impact of application frequency, exposure time and storage in saliva on the degree of color changes caused by a suspension based on Curolox

technology, An aqueous suspension of 0.02mg/ml P11-4 peptide and 0.5wt% HAP. To evaluate the competitiveness of the new formulation, one of the existing commercial home bleaching agents that is based on peroxide (Viva Style Paint on Plus- 6% hydrogen peroxide), served as the control treatment.

More precisely, this chapter answered following questions:

- How did the peptide-HAP suspension whiten the samples' surface after 1 time of application and after 5 times of application?
- How did the suspension whiten the samples' surface after 1 min of exposure time and after 5 min of exposure time?
- Did storage the treated samples in artificial saliva for 24 hours have any considerable effect on their color?
- Was the whitening effect of the suspension competitive with the home bleaching Vivastyle Paint on Plus, which contains 6% hydrogen peroxide?

2.3 Material and methods

To answer the aforementioned questions, the materials demonstrated in Table 2-1 were used as treatment agents in the present chapter.

Table 2-1 The treatment agents used in the treatment groups of Chapter 2

Group name	Treatment agent	Company
Group 1	Aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP	Curodont, Credentis AG, Windisch, Switzerland
Group 2	Vivastyle Paint On Plus	Ivoclar Vivadent AG, FL-9494 Schaan/Liechtenstein

2.3.1 Sample preparation

To prepare the samples, 16 extracted healthy bovine incisors without root were collected. The teeth were previously examined in order to validate the absence of fractures and defects in their structure. The roots were cut off under the cementoenamel junction. The samples were kept in Evian water until the application of the material at 7°C. It is noteworthy that the teeth were used within one month after the extraction.

Before samples preparation, the teeth were cleaned carefully using a rubber cup in a dental hand piece for 30 seconds with a fine prophylactic polishing paste (Proxyt, RDA 7, fine, Ivoclar Vivadent AG, Schaan, Liechtenstein). Then, the teeth were embedded in Technovit 4004

transparent embedding kits (Kulzer, Germany), using the same-sized cuboid molds which we made out of silicon. The molds' dimensions were 2.4 mm × 2 mm × 1.5 mm (Figure 2-1).

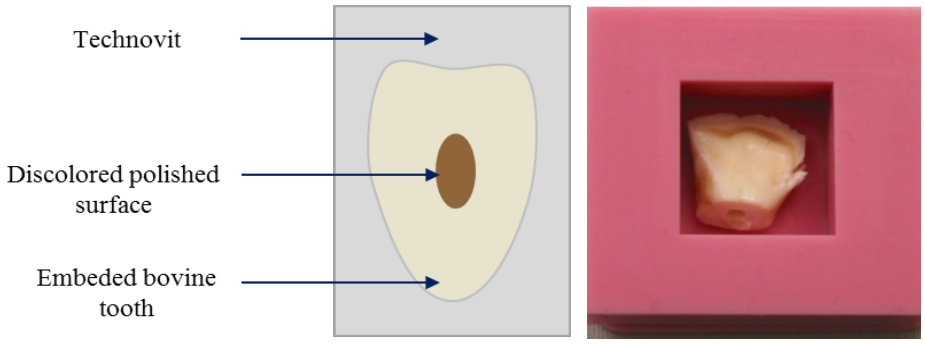


Figure 2-1 Sample preparation; bovine tooth within the silicon mold (right) and schematic drawing of embedded sample after surface polishing and staining (left)

In order to standardize the color measurement area, the vestibular surface of each sample was polished in a way that an ellipse of enamel with vertical major axis of approximately 10 mm and horizontal minor axis of approximately 6 mm was exposed (Figure 2-1, Figure 2-2). The cervical region and incisal ridge were not exposed. The samples were polished with 600- and 1200-grit SiC abrasive papers (Leco corporation, St. joseph, USA) under water-cooling. The 600 grit SiC and 1200 grit SiC correspond to polishing particles with an average size of 16 µm and 6.5 µm, respectively. Before the treatments were initiated, the samples were stored in a standard mineral water (Evian; Danone waters Deutschland, Frankfurt, Germany) at room temperature.

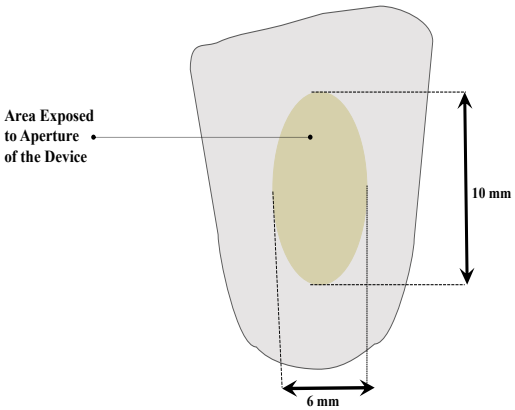


Figure 2-2 Exposed stained enamel area of the tooth after embedding and polishing, where color measurements were made

Table 2-2 shows a summary of the materials used in chapters 2, 3 and 4 for sample preparation.

Table 2-2 Materials used in Chapter 2, 3 and 4 for samples preparation

Material	Company
Proxyt Prophylactic polishing paste, RDA 7, fine	Ivoclar Vivadent AG, Schaan, Liechtenstein
Technovit 4004 transparent embedding kits	Kulzer, Germany
600- and 1200-grit SiC abrasive papers	Leco corporation, St. joseph, USA
Brand Micro-brush	Grafton, USA

2.3.2 Preparation of the staining solution

Côrtes et al. (2013) used coffee and wine as pigment solution in their study. Wetter et al. (2004) made a pigmenting solution containing tobacco, black tea, coffee, Coca Cola and red wine. In contrast, Attin et al. (2003) stained the samples only with black tea. Watts and Addy (2001) introduced also black tea and coffee as two potential causes for teeth discoloration.

The staining solution that we used in this study contained 5 g black tea (TEEKANNE GmbH, Düsseldorf, Germany), 5 g premium dark soy sauce (a Chinese brand), 5 g espresso (Nestle AG, Frankfurt am Main, Germany) and 5 g Maggi sauce (Maggi GmbH, Germany) (Figure 2-3). 5 g of black tea was weighed into a container and 100 ml of water at a temperature of 95°C was added (Shellis et al., 2005). The other ingredients demonstrated in Table 2-3 are prepared in the same way as tea. Then, all the solutions were mixed together. In order to cool down, the solution was left to stand for 20 minutes before using.

Figure 2-3 Components of the staining solution

Table 2-3 Materials used in chapters 2, 3, 4 and 5 for preparing the staining solution

Material	Company
Black tea	TEEKANNE GmbH, Düsseldorf, Germany
Premium dark soy sauce	a Chinese brand
Nescafe type espresso	Nestle AG, Frankfurt am Main, Germany
Maggi Würze	Maggi GmbH, Germany

2.3.3 Study Design

A total of 16 extracted caries-free bovine teeth were prepared (as described in the section 2.3.1). The samples were randomly assigned to two groups (n=8 for each group) and subsequently were immersed into the staining solution for 72 hours at room temperature.

After staining procedure, the samples in each group were named randomly from 1 to 8. The baseline color of all samples in both groups were measured, using the Color Eye 7000 spectrophotometer (Gretag MacBeth, X-Rite- Munich, Germany) and recorded with 0 index. Each sample in the first group was treated as below:

1. Low-concentrated aqueous suspension of 0.02mg/ml P11-4 peptide and 0.5wt% HAP was applied once. After 1 minute of exposure time, the sample was rinsed with water and blot dried with a soft absorbent paper towel (half dry) and the color was recorded. The second measurement was taken after 5 minutes of exposure time.
2. In this step, we applied the solution four more times (after each time of application, we rinsed the sample for 5 seconds with water and was blot dried with a soft paper towel). After 1 minute of the last application the samples color was measured. The second measurement was taken after 5 minutes, same as the previous step.
3. All of the samples were immersed in artificial saliva (Pharmacy University of Munich, Munich, Germany) in incubator at 37°C for 24 hours. Subsequently the sample's color was measured again.

The treatment steps for the samples in the first group are demonstrated in Figure 2-4.

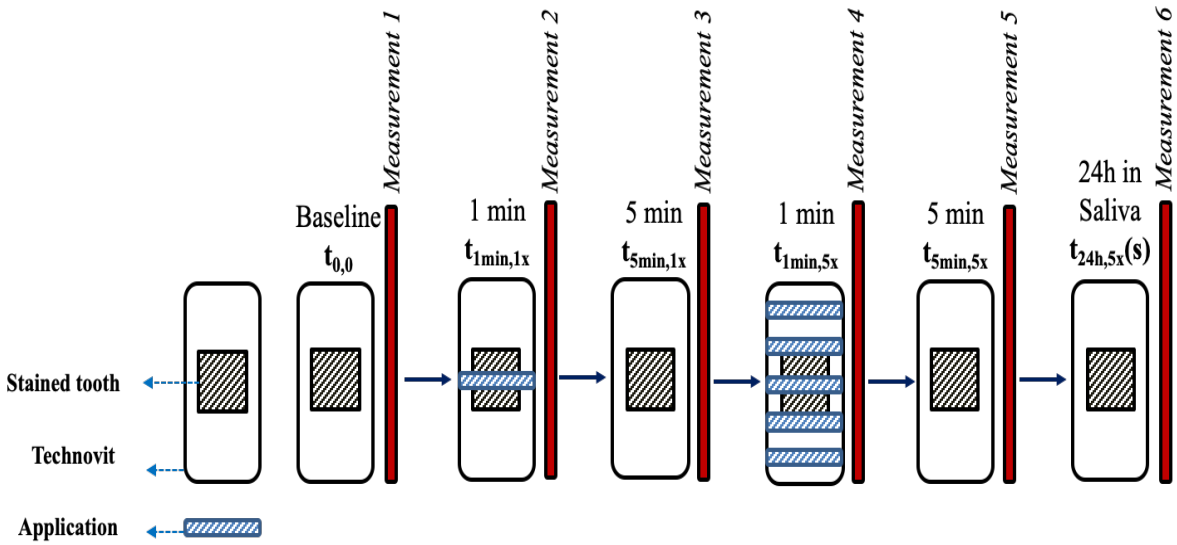


Figure 2-4 The schematic figure of the treatment steps for the samples in the first group (t: Treatment with low-concentrated aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP, S: Saliva, x: Application frequency)

To treat each sample in the second group, we followed the steps below.

1. A thin layer of the Vivastyle varnish (Vivastyle Paint On Plus contacting 6% hydrogen peroxide) was directly applied to the sample's surface with a micro brush.
2. The varnish is left for 10 minutes on the sample´s surface to allow undisturbed interactions with the surface (based on manufacture's instruction).
3. After 10 minutes of exposure time, the dried varnish was removed accurately by scalpel, then by brushing the surface with a soft toothbrush without tooth paste and rinsed thoroughly with water and was blot dried with a soft paper towel.
4. Subsequently the color of the samples was measured with the spectrophotometer and the results were collected.

The treatment steps of the samples in the second group are demonstrated in Figure 2-5.

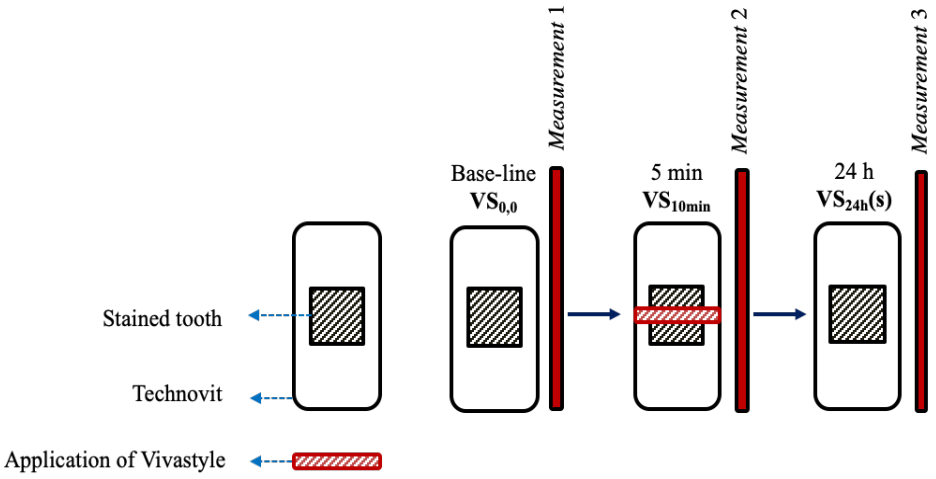


Figure 2-5 The schematic illustration of the treatment steps for the samples in the second group (VS: Treatment with Vivastyle, S: Saliva)

A summary of the described treatment conditions for both groups is given in Table 2-4.

Table 2-4 Characteristics of the treatment conditions of the samples in both groups (S: Saliva, x: Application frequency, t: treatment with Aqueous suspension*, VS: Treatment with Vivastyle Paint On Plus)

Treatment condition	Treatment agent		Number of received treatments		Exposure time			Storage in saliva for 24h
	Aqueous suspension*	Vivastyle	1 x	5 x	1 min	5 min	10 min	
Group 1								
$t_{1min,1x}$	✓		✓		✓			
$t_{5min,1x}$	✓		✓			✓		
$t_{1min,5x}$	✓			✓	✓			
$t_{5min,5x}$	✓			✓		✓		
$t_{24h,5x}(s)$	✓			✓		✓		✓
Group 2								
VS_{10min}		✓					✓	
$VS_{24h}(s)$		✓					✓	✓

* Low-concentrated aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP

Note that all of the performed measurements were conducted with the Color Eye 7000A spectrophotometer under D65 illuminant and 10° observer (Color-Eye 7000A; X-Rite). The samples' color was measured according to the CIE L*a*b* color scale. The obtained data were collected. To reduce errors associated with the light, the measurements were carried out in the dark. The results were calculated according to the following equations:

$$\Delta L^* = L^*(t) - L^*(0) \quad \textbf{Eq. 2.1}$$

$$\Delta a^* = a^*(t) - a^*(0) \quad \textbf{Eq. 2.2}$$

$$\Delta b^* = b^*(t) - b^*(0) \quad \textbf{Eq. 2.3}$$

$L^*(t)$ indicates the measured lightness of the sample after treatment, while $L^*(0)$ represents the lightness of the samples in baseline. The $a^*$is the green-red component and b^* is the blue-yellow component. a^* (t) and $b^*(t)$ are the obtained values after treatment, whereas $a^*(0)$ and $b^*(0)$ indicate values in the baseline.

To analyze whether the color changes were significant, statistical tests were performed. To this end, SPSS statistical program version 18.0 was used.

2.4 Results

The pH of the staining solution was measured with an electronic pH meter (WTW bench pH/mV meters Routine meter pH 526, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and it was 5.5 at 23°C. The average color changes (ΔE) of the various treatment agents were determined relative to the baseline values. The data from repeated applications were pooled and analysed.

2.4.1 Comparison of the reflections and CIELAB values associated with the aqueous peptide-HAP formulation after different treatment conditions

To see how the application of the suspension changed the reflections, Figure 2-6 compares the mean reflections over the wavelengths from 360 nm to 750 nm for $t_{0,0}$ and $t_{1min,1x}$. It can be observed that for all the wavelengths, $t_{1min,1x}$ presented higher reflection than $t_{0,0}$.

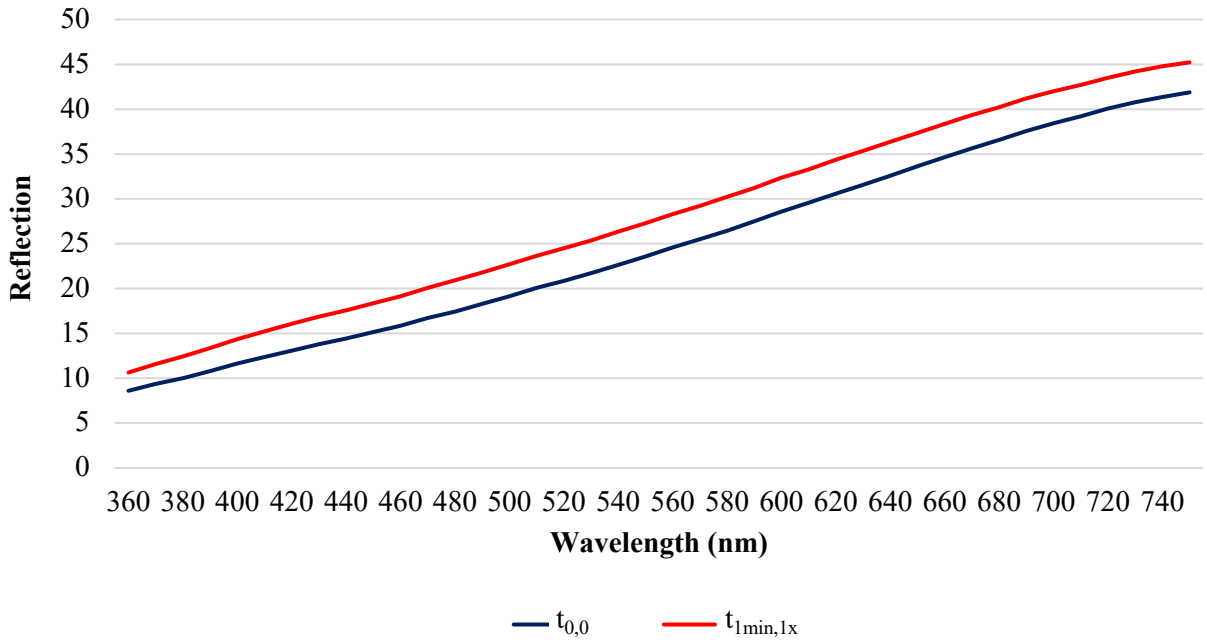


Figure 2-6 Mean reflection spectra of $t_{0,0}$ and $t_{1min,1x}$ over the wavelengths from 360 to 750 nm

Figure 2-7 provides the comparison of the calculated ΔL^* mean values or lightness of all the conditions in Group 1, which were calculated relative to the baseline values. It can be seen that $t_{1min,1x}$ had the lowest ΔL^* mean value (2.86). While the mean lightness in $t_{1min,5x}$ increased by about 16% compared to $t_{1min,1x}$, the ΔL^* mean values of $t_{5min,1x}$ and $t_{5min,5x}$ were almost 40% higher than that of $t_{1min,1x}$. On the other hand, although $t_{24h,5x}$(S) presented the highest lightness effect, the ΔL^* mean value of this condition was only 9% higher than $t_{5min,5x}$.

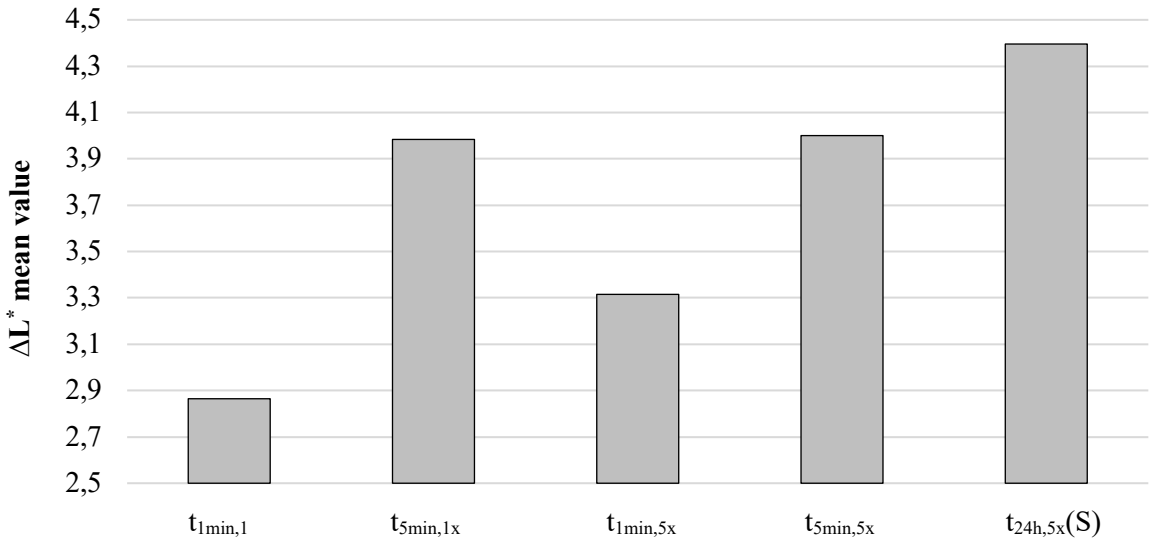


Figure 2-7 Comparison of ΔL^* mean values of the conditions in Group 1, relative to the baseline values

Figure 2-8 reflects the comparison of the Δa^* mean values or green-red effect of all the conditions in the first group, which were calculated relative to the baseline values. The Δa^* mean value in $t_{1min,1x}$ was - 0.74, meaning that this condition presented the weakest green-red effect. The absolute Δa^* mean values in the $t_{5min,1x}$ and $t_{1min,5x}$ were about 35% and 25% higher than $t_{1min,1x}$, respectively. The strongest effect was shown by $t_{5min,5x}$ whose absolute Δa^* mean value was more than 70% higher than that in $t_{1min,1x}$. Therefore, it is seen that the exposure time had a notable impact of the green-red effect of the solution. Finally, $t_{24h,5x}(s)$ resulted in negligibly (0.06) different Δa^* mean value compared to $t_{5min,5x}$.

Figure 2-9 illustrates the comparison of the calculated Δb^* mean values or blue-yellow effect of all the conditions in Group 1, which were calculated relative to the baseline values. It can be seen that $t_{1min,1x}$ had the lowest absolute Δb^* mean value. The absolute Δb^* mean values in the $t_{5min,1x}$ and $t_{1min,5x}$ were about 29% and 24% higher than $t_{1min,1x}$, respectively. Similar to what we observed in Figure 2-8, the strongest effect was shown by $t_{5min,5x}$ whose absolute Δb^* mean value was more than 80% higher than that in $t_{1min,1x}$. Finally, $t_{24h,5x}(s)$ resulted in only 0.22 higher Δa^* mean value compared to $t_{5min,5x}$. This means that similar to what we observed for the ΔL^* and Δa^* mean values, storing in saliva for 24 hours had a negligible impact on the blue-yellow color component of the samples treated by the low-concentrated aqueous suspension for 5 times and with 5 minutes of exposure time.

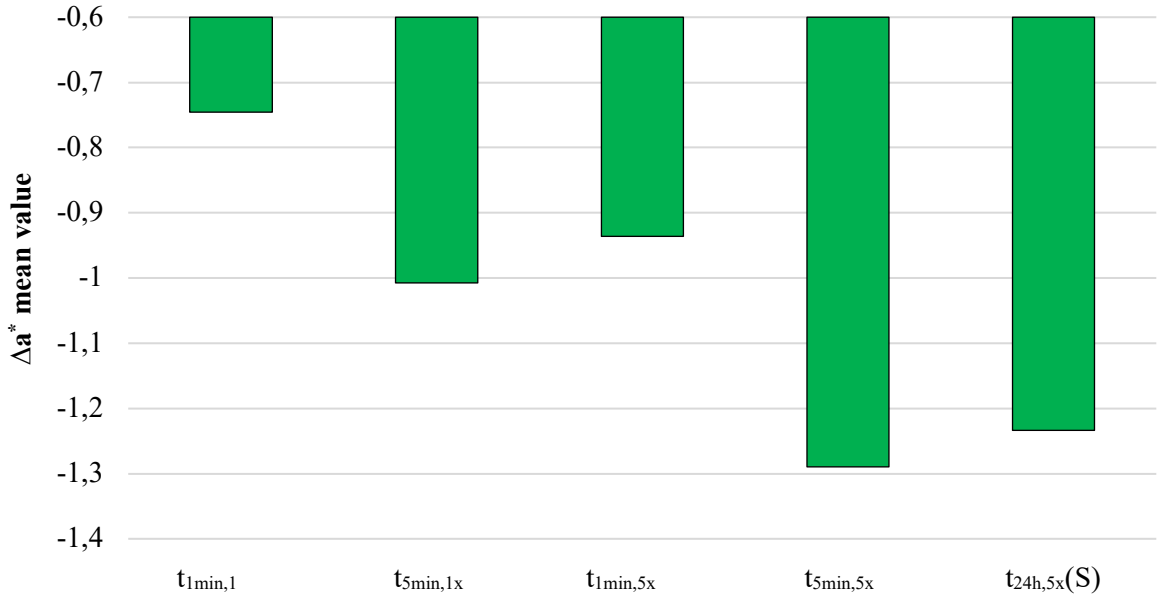


Figure 2-8 Comparison of Δa^* mean values of the conditions in Group 1, relative to the baseline values

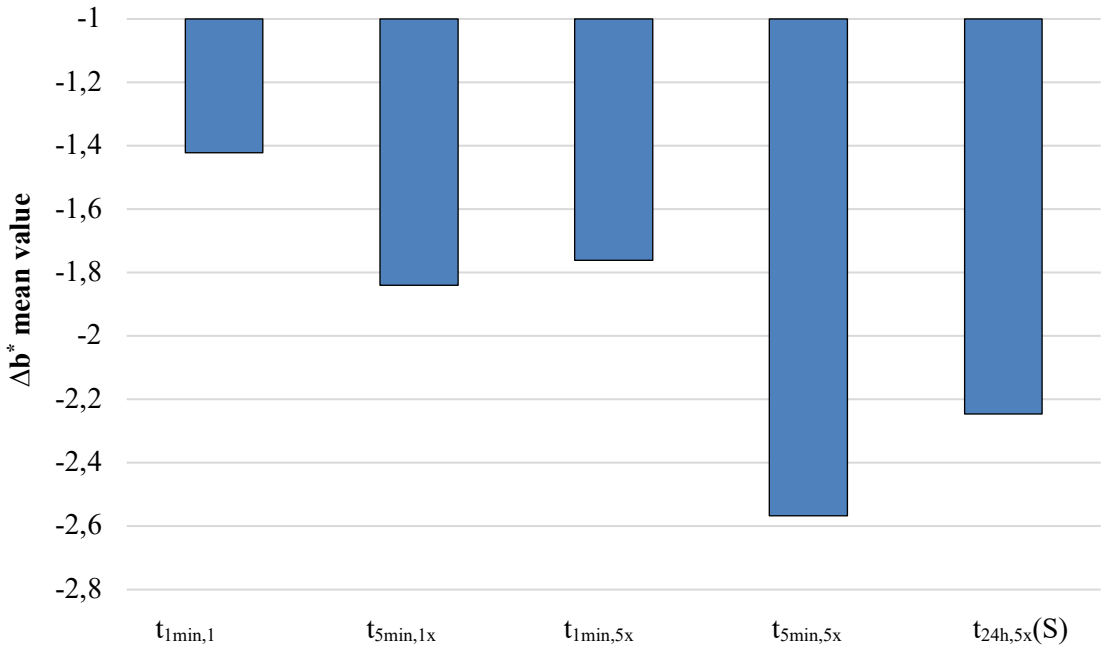


Figure 2-9 Comparison of Δb^* mean values of the conditions in Group 1, relative to the baseline values

Table 2-5 summarizes CIELAB delta mean values and the associated color perception for all the conditions in Group 1.

Table 2-5 CIELAB delta mean values and the associated color perception for all conditions in the first group, relative to the baseline values

CIELAB	$t_{1min,1x}$	$t_{5min,1x}$	$t_{1min,5x}$	$t_{5min,5x}$	$t_{24h,5x}$(S)
ΔL^*	2.864	3.984	3.3141	4.001	4.396
Color perception	Lighter	Lighter	Lighter	Lighter	Lighter
Δa^*	-0.745	-1.007	-0.936	-1.289	-1.234
Color perception	Less red	Less red	Less red	Less red	Less red
Δb^*	-1.423	-1.841	-1.762	-2.567	-2.246
Color perception	Less yellow	Less yellow	Less yellow	Less yellow	Less yellow

2.4.2 Overall color changes caused by the aqueous peptide-HAP formulation after different treatment conditions

To determine whether the application frequency of the formulation, exposure time and storage in saliva played any major role in changing samples' color in Group 1, we compared ΔE values of the $t_{1min,1x}$, $t_{5min,1x}$, $t_{1min,5x}$, $t_{5min,5x}$ and $t_{24h,5x}$(s) conditions, which were calculated relative the baseline values.

Table 2-6 shows descriptive statistics including mean, standard deviation and standard error of ΔE values of the five conditions. It is interesting to see that $t_{1min,1x}$ had the lowest mean (4.210), the lowest standard deviation (3.984) and the lowest standard error mean (1.409), while $t_{24h,5x}(s)$ presented the highest mean (6.421), the highest standard deviation (6.313) and the highest standard error mean (2.232). Figure 2-10 reflects the differences in the ΔE mean values.

Table 2-6 Descriptive statistics of the ΔE values of the conditions in the first group, relative to the baseline values

Treatment condition	Mean	Standard Deviation	Standard Error Mean	Number of sample
$t_{1min,1x}$	4.120	3.984	1.409	8
$t_{5min,1x}$	5.217	5.350	1.891	8
$t_{1min,5x}$	4.724	4.840	1.712	8
$t_{5min,5x}$	5.813	5.424	1.918	8
$t_{24h,5x}(s)$	6.421	6.313	2.232	8

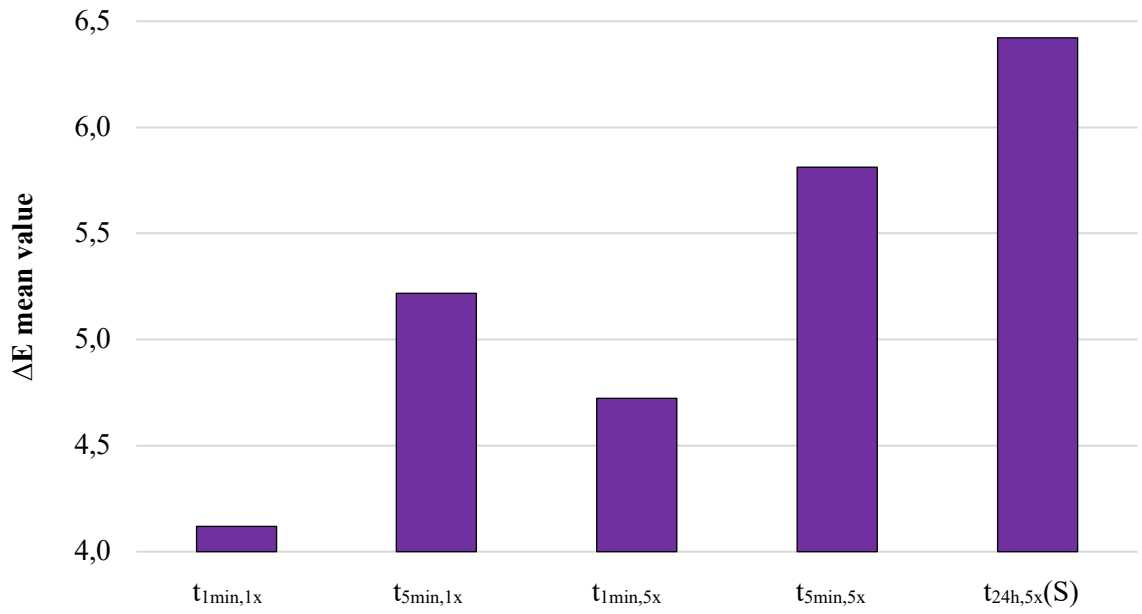


Figure 2-10 Comparison of the ΔE mean values of the conditions in the first group, relative to the baseline values

To determine if the differences between the ΔE mean values of the conditions were significant, a statistical test should be performed. Since the participants were the same in each

condition, an ANOVA with repeated measures was found appropriate. According to Laerd Statistics (2014), this test is usually used in two situations: (1) when samples are measured multiple times to see changes to an intervention; or (2) when samples are subjected to more than one condition and the response to each condition should to be compared. However, repeated measures ANOVA is particularly susceptible to violating the assumption of sphericity, referring to the condition where the variances of the differences between all combinations of the groups must be equal (Laerd Statistics, 2014). Therefore, the initial necessary step for carrying out the repeated measures ANOVA was to test the sphericity assumption. Mauchly's test of Sphericity is a formal test for this purpose.

Mauchly's test of sphericity tested the null hypothesis that the variances of the differences were equal. The significance level was set to (α =0.05). Results from Mauchly's test of Sphericity are shown in Table 2-7.

Table 2-7 Mauchly's test of Sphericity for within subjects' effect

Mauchly's W	**Approx. Chi-Square**	**df**	**Sig.**	**Epsilon**		
				Greenhouse-Geisser	Huynh-Feldt	Lower-bound
0.044	16.910	9	0.060	0.437	0.571	0.250

The results of the test indicated that we could not reject the hypothesis and, therefore, the assumption of sphericity was not violated, $\chi^2(9) = 16.910$ and p=0.06. Not violating this assumption meant that we did not need to make any corrections in order to determine statistical significance.

To test if there was an overall significant difference between the means at the different conditions, the test of Within-Subjects Effects for $\alpha = 0.05$ was applied. Table 2-8 shows the *F* value of the factor "Condition" and its associated significance. Since our data seemed spherical, we ignored the Greenhouse-Geisser, Huynh-Feldt and lower bound results and simply interpreted the uncorrected results denoted as "Sphericity Assumed". The significance value of Sphericity Assumed was 0.037 < 0.05. Therefore, the difference between the conditions was statistically significant.

Table 2-8 Tests of Within-Subjects Effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Condition	Sphericity Assumed	25.929	4	6.482	2.967	0.037
	Greenhouse-Geisser	25.929	1.75	14.817	2.967	0.094
	Huynh-Feldt	25.929	2.29	11.344	2.967	0.075
	Lower-bound	25.929	1.00	25.929	2.967	0.129
Error (Condition)	Sphericity Assumed	61.171	28	2.185		
	Greenhouse-Geisser	61.171	12.25	4.994		
	Huynh-Feldt	61.171	15.99	3.823		
	Lower-bound	61.171	7.00	8.739		

The results presented in the previous table show that there was an overall significant difference between the means ΔE values, but we did not know where the difference(s) occurred. Table 2-9 illustrates the results of the Bonferroni post hoc test that discovered which specific means differed. The significance level was set to 0.05 ($\alpha = 0.05$). This table provides a number of information regarding the differences between each of two conditions. In this table, "Mean Difference" refers to the difference between the means of the two conditions. "Standard error" identifies the precision of the mean difference. "95% Confidence Interval of the Difference" represents that with 95% confidence the actual mean difference between the two conditions is between lower and upper values.

It is interesting to note that although the test of Within-Subjects Effects (Table 2-8) showed that there was an overall significant difference between the conditions, according to the Table 2-9, the differences between individuals were not significant. This can be explained by the fact that Bonferroni post hoc is a more conservative test. Therefore, we can argue that the application frequency, exposure time and storage in saliva did not have any significant impact on the samples' color.

Table 2-9 Pairwise comparisons by Bonferroni post hoc test

(I) Condition	(J) Condition	Mean Difference (I-J)	Standard Error	Sig.	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
$t_{1min,1x}$	$t_{5min,1x}$	-1.097	0.634	1.000	-3.652	1.458
	$t_{1min,5x}$	-0.604	0.426	1.000	-2.321	1.113
	$t_{5min,5x}$	-1.693	0.915	1.000	-5.380	1.994
	$t_{24h,5x}(s)$	-2.300	1.179	0.920	-7.051	2.450
$t_{5min,1x}$	$t_{1min,1x}$	1.097	0.634	1.000	-1.458	3.652
	$t_{1min,5x}$	0.493	0.321	1.000	-0.803	1.788
	$t_{5min,5x}$	-0.596	0.510	1.000	-2.652	1.459
	$t_{24h,5x}(s)$	-1.203	0.807	1.000	-4.457	2.050
$t_{1min,5x}$	$t_{1min,1x}$	0.604	0.426	1.000	-1.113	2.321
	$t_{5min,1x}$	-0.493	0.321	1.000	-1.788	0.803
	$t_{5min,5x}$	-1.089	0.578	1.000	-3.419	1.241
	$t_{24h,5x}(s)$	-1.696	0.901	1.000	-5.328	1.935
$t_{5min,5x}$	$t_{1min,1x}$	1.693	0.915	1.000	-1.994	5.380
	$t_{5min,1x}$	0.596	0.510	1.000	-1.459	2.652
	$t_{1min,5x}$	1.089	0.578	1.000	-1.241	3.419
	$t_{24h,5x}(s)$	-0.607	0.699	1.000	-3.423	2.209
$t_{24h,5x}(s)$	$t_{1min,1x}$	2.300	1.179	.920	-2.450	7.051
	$t_{5min,1x}$	1.203	0.807	1.000	-2.050	4.457
	$t_{1min,5x}$	1.696	0.901	1.000	-1.935	5.328
	$t_{5min,5x}$	0.607	0.699	1.000	-2.209	3.423

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

2.4.3 Comparison of the aqueous peptide-HAP formulation with the Vivastyle Paint On Plus commercial home bleaching agent

To assess the competitiveness of the new formulation, this section compares the perceived color changes of this formulation with the commercial home bleaching Vivastyle. In this regard, Table 2-10 provides the comparison of the calculated CIELAB mean values and the associated color perception. Both agents presented perceptible color differences. It can be seen that in terms of lightness, the Vivastyle agent treated more effectively. While the mean ΔL^*

value in the $t_{5min,5x}$ was 4.00, this in the VS_{10min} was 5.73. After storage in Saliva, the mean ΔL^* value resulted from Vivastyle was more than 50% higher than the mean ΔL^* value obtained from the peptide-HAP formulation (4.40).

Table 2-10 The mean values of ΔL^*, Δa^* and Δb^* and the associated color perception

CIELAB	$t_{5min,5x}$	VS_{10min}	$t_{24h,5x}(s)$	$VS_{24h}(s)$
ΔL^*	4.00	5.73	4.40	6.73
Color perception	lighter	lighter	lighter	lighter
Δa^*	-1.29	-0.99	-1.23	-2.51
Color perception	less yellow	less yellow	less yellow	less yellow
Δb^*	-2.57	-1.36	-2.25	-3.99
Color perception	less red	Less red	Less red	les red

Figure 2-11 compares the overall color changes (mean ΔE values) caused by the agents before and after storage in saliva for 24h, which were calculated relative to the corresponding baseline values. In both conditions (i.e. before and after storage in saliva), the Vivastyle presented a stronger overall color change effect. While before storage in saliva, it resulted in 16% stronger overall color change effect than the new agent, after saliva, it showed even stronger effect, showing 40% stronger overall color change than the new agent.

To determine whether there was a statistically significant difference between the ΔE values in the $t_{5min,5x}$ and $VS_{10min,}$ an independent t-test was initially selected. However, a necessary condition of this test is that the dependent variable is (approximately) normally distributed within each group (Mordkoff, 2000). Violation of this assumption may yield biased results. Table 2-11 shows the results from the mostly applied normality tests, the Kolmogorov-Smirnov test and the Shapiro-Wilk test for $t_{5min,5x}$ and VS_{10min}. However, since the Shapiro-Wilk test is more appropriate for limited number of samples (Razali and Wah, 2011), here we relied on the Shapiro-Wilk test to assess the normality assumption.

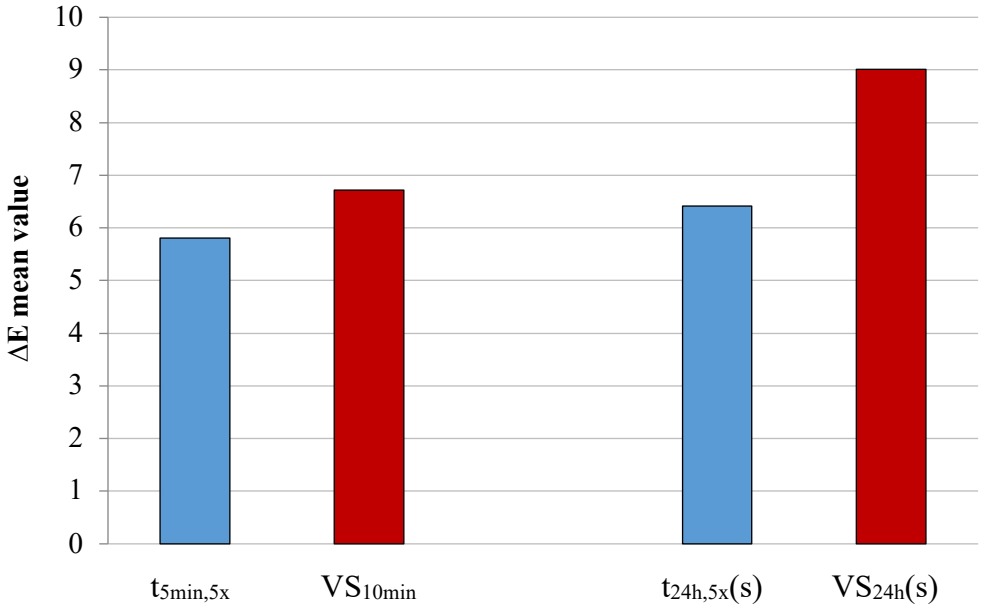


Figure 2-11 Comparison of the mean ΔE values of the new formulation and the Vivastyle agent, before and 24h after storage in saliva

Table 2-11 The results of tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk) for $t_{5min,5x}$ and VS_{10min}

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
$t_{5min,5x}$	0.289	8	0.048	0.828	8	0.056
VS_{10min}	0.346	8	0.005	0.677	8	0.001

a. Lilliefors Significance Correction

If both Sig. values of the Shapiro-Wilk test are upper than 0.01, we can argue that the data is approximately normally distributed. However, it can be observed in the Table 2-11 that the Sig. value of $t_{5min,5x}$ (0.056) was not higher than 0.01. This means that the data distribution deviated from a normal distribution.

Since we found that the data was not approximately normally distributed, the Mann-Whitney U test was carried out. This test is a non-parametric test and does not require the normality assumption (Shier, 2004). Table 2-12 shows the results of this test for $t_{5min,5x}$ and VS_{10min} groups.

It can be seen that the ΔE values of VS_{10min} and $t_{5min,5x}$ were not statistically significantly different ($U = 25.00$, $p = 0.487$).

Table 2-12 The results of Mann-Whitney test for $t_{5min,5x}$ and VS_{10min}

	ΔE value
Mann-Whitney U	25.000
Wilcoxon W	61.000
Z	-0.736
Asymp. Sig. (2-tailed)	0.462
Exact Sig. [2*(1-tailed Sig.)]	0.505[b]
Exact Sig. (2-tailed)	0.487
Exact Sig. (1-tailed)	0.244
Point Probability	0.015

a. Grouping Variable: Group
b. Not corrected for ties.

In order to determine if there was a statistically significant difference between the ΔE values of $t_{24h,5x}$(s) and VS_{24h}(s), we first tested the normality assumption. Table 2-13 reflects the results of the Kolmogorov-Smirnov and Shapiro-Wilk tests for these groups (α =0.01).

Table 2-13 The results of tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk) for $t_{24h,5x}$(s) and VS_{24h}(s)

	Kolmogorov-Smirnov[a]			**Shapiro-Wilk**		
	Statistic	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
$t_{24h,5x}$(s)	0.232	8	0.200	0.860	8	0.119
VS_{24h}(s)	0.248	8	0.160	0.800	8	0.029

a. Lilliefors Significance Correction

Since the Sig. values of Shapiro-Wilk test were both higher than 0.01, we concluded that the distribution was approximately normalized, meaning that we could use the independent t-test. The null hypothesis of this test was that there were no significant differences between two groups. The significance level was set to 0.05 ($\alpha = 0.05$). Table 2-14 illustrates the results of independent t-test for ΔE values of $t_{24h,5x}$(s) and VS_{24h}(s) groups.

Table 2-14 The results of independent t-test for ΔE values of $t_{24h,5x}$(s) and VS_{24h}(s)

Group name	**Kolmogorov-Smirnov**		**Shapiro-Wilk**		
	F	**Sig.**	**t**	**df**	**Sig. (2-tailed)**
Equal variances assumed	0.716	0.412	-0.535	14	0.601
Equal variances not assumed			-0.535	12.992	0.602

According to the Table 2-14, we could not reject the null hypothesis. In other words, the applications of Vivastyle and low-concentrated aqueous peptide-HAP suspension after 24h storage in saliva did not lead to significantly different color changes.

2.5 Discussion

L^* value represents the measure of reflection on HAP particles, while a* and b* values are the measures of attenuation of the inherent color of the tooth under HAP layer. Increasing the L^* value arises through the reflection of the light on HAP layer and decreasing the light transmission through HAP of the tooth (Figure 2-12).

The absorption of certain wavelengths in the tooth determines the tooth's color and thus the a^* and b^* values (Figure 2-13). We assumed that in presence of HAP layer on the tooth surface, the light is also scattered on the HAP underside. This may lead to less light transmission through enamel and dentin and also reduction in the a^* and b^* values measured by the spectrophotometer.

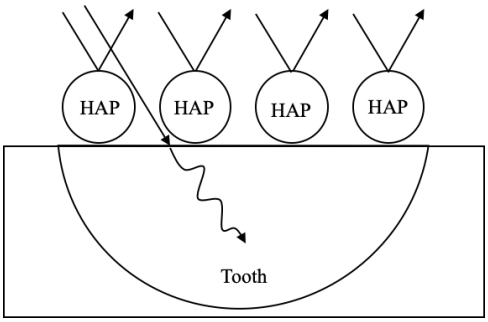


Figure 2-12 Schematic drawing of the light's behavior on the tooth surface in the presence of HAP particles

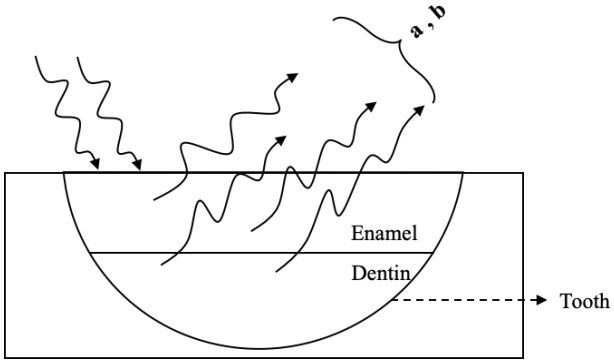


Figure 2-13 Schematic drawing of the light's behavior on the tooth surface; the light is scattered on the HAP underside

2.5.1 Bovine tooth enamel

Bovine teeth have been chosen as the most common alternative for human teeth among several types of non-human teeth (Tanaka et al., 2008; Attin et al., 2007; Soares et al., 2010; Yassen et al., 2011). The bovine tooth enamel is a highly-mineralized substance. The superficial and the deeper layers of bovine dentin have almost the same density of tubules. According to Schilke et al., (2000), bovine teeth have a greater diameter of the dentin tubules than the human teeth. However, the difference is not statistically significant (Tanaka et al., 2008). It can be argued that human and bovine teeth have similar structure. Reis et al. (2004) used Scanning Electron Microscope (SEM) observation to show the similarities. Davidson et al. (1973) claimed that there are negligible differences in chemical compositions and physical properties between bovine enamel and human enamel. They also reported in the same study that the distribution of calcium in bovine enamel is slightly more homogenous compared to human enamel. Moreover, bovine enamel contains 37.9% calcium, while human enamel is composed of 36.8% calcium.

White et al. (2001), Xie et al., (2008) and Hannig and Hannig, (2010) confirmed that the size of hexagonal human enamel crystals ranges from 50 to 100 nm in length and 68 to 500 nm in diameter. Wang et al. (2012) claimed that the average diameter of human enamel crystals is a bit smaller than bovine enamel crystals.

Since the aforementioned differences between bovine enamel and human enamel are in tolerable area, we used bovine teeth in this study. On the other hand, due to three main reasons, we conducted our study *in vitro* by using extracted bovine teeth. First, extracted bovine incisors are more accessible than extracted human incisors. Second, fogging of the optical lens of the intraoral spectrophotometer may occur during clinical color measurements, leading to inaccurate results. Third, instrumental color detections may cause patient discomfort (Chu, 2003).

2.5.2 Dental enamel

The enamel prisms are the units of the tooth enamel. They follow a slightly curved pattern and are mostly made up of minerals, primarily HAP. The color of tooth enamel is semi-translucent and it can vary from light yellow to greyish white. At the thin areas, the enamel appears yellow-white reflecting underlying dentin. This is due to this fact that the outer layer of human enamel is gradually worn off due to aging and also as a consequence of mastication. In order to minimalize individual enamel surface variations, simulate a possible condition (e.g. erosion) in the human oral cavity and standardize and flatten the specimen's surface, we polished the

surface with a polishing paper (Torres et al., 2010; Poggio et al., 2013). However, it is noteworthy that natural erosion of tooth surfaces occurs more slowly than polished surfaces. The polishing was carried out to remove enamel aprismatic layer, which contains higher amount of minerals than enamel subsurface. This prism-free layer is less permeable to treatment agents and acidic solutions (Badr and Al Ibrahim, 2010). Therefore, eliminating the aforementioned layer may have a positive impact of penetrating our peptide-HAP suspension.

The specific structure of enamel may make it possible that P11-4 self-assembling peptide, designed by Aggeli et al. (1997) and Kyle et al. (2010), binds the enamel in its assembled form. The anionic groups of the P11-4 peptide side chains attract calcium ions of HAP of the enamel (Kirkham et al., 2007). Furthermore, the p11-4 peptide fluoresces. As a consequence, it can cause color changes without the presence of HAP. As comprehensively discussed in section 4.9.4, this mechanism is comparable to blue covarine (Vaz et al., 2019).

2.5.3 Hydroxyapatite

Hydroxyapatite (HAP) has been reported to be effective in removing dental plaque, promoting remineralization of tooth surfaces, increasing brightness and whiteness of the teeth and arresting the progress of incipient caries (Kim et al., 2006). HAP-based formulations have illustrated efficacy in filling and smoothening the irregular surface porosity, preventing and treating hypersensitivity and improving periodontal diseases (Niwa et al., 2001).

HAP-containing agents adhere to the tooth surface without chemically affecting the deeper tooth tissue (Yamagishi et al., 2005b). Forasmuch as the enamel tissue is not regenerable in the natural way because of its characteristics, the proposed suspension in this study aimed at improving the esthetic of the teeth by changing the color and enhancing regeneration of the enamel micro cavities at the same time (Brunton et al., 2013; Alkilzy et al., 2018).

In order to achieve a better adhesion of HAP layer on the teeth's surface, we evaluated the adherence and the corresponding whitening effect of an aqueous suspension of HAP in combination with polymeric P11-4 peptide in a low concentration.

2.5.4 Staining solution

As mentioned earlier, black tea and coffee, which contain tannins and chromogens, are capable of causing tooth discoloration and they have been frequently used in the literature to stain teeth (Attin et al., 2003; HUG et al., 2016; Côrtes et al., 2013). While several studies have used either of them, there are a number of studies used both as a mixture. In the present study,

we used a staining solution containing black tea and coffee because of their intense staining potential. In addition, to enhance the staining effectiveness and making it more compatible with daily life, we used two other dark-colored solutions (e.g. Chinese soy sauce and Maggi Würze). To prepare the staining solution, we used only 5 g of each of the ingredients to simulate what an average person consumes every day.

2.5.5 Tooth dehydration

Tooth dehydration results in increasing visible and measurable tooth brightness by increasing enamel opacity (Burki et al., 2013; Stevenson, 2009). Burki et al. (2013) claimed that the rehydration of dehydrated teeth takes more than 30 minutes. Interestingly, it is possible to return to the baseline color of a tooth after sufficient rehydration. According to Suliman et al. (2019), color measurement procedures should be conducted fast enough before teeth dehydrate by means of isolation.

Therefore, during the entire experiments of the present study, dehydration of the samples was prevented by continuous storage the samples in water. We kept the lead time[1] before each measurement as short as possible to avoid unacceptable results and the errors associated with dehydration of samples.

2.5.6 Color detection

To measure the tooth whitening efficacy various methods have been introduced in the literature. One of the frequently used methods is color assessment by using dental shade guides and image analysis of digital teeth photograph. Although such clinical methods are more accessible and relatively cost-effective, they usually suffer from lack of sufficient accuracy and require a skilled performer. In contrast, the instrumental methods, including various spectrophotometers and colorimeters, are considered to be more reliable and objective in determining the tooth color (Chang et al., 2012). There are several spectrophotometers available which are directly employed intraoral in dental clinics. Using such devices makes it possible to measure the visible spectra of both vital and Non-vital teeth (Paul et al., 2002; Russell et al., 2000). However, the performance of intraoral color measurements may depend on the measurement environment and performer's experience. In contrast, there are a number of laboratory

[1] The time needed to put the sample out of water, prepare it for the treatment and apply the treatment

spectrophotometers that are limited to research purposes (mostly due to their dimensions) but are capable to provide more precise results.

Although each of the available dental color-detecting devices has its own limitations, they are thought to be more accurate than the alternatives. Therefore, in this study, to have a more reliable quantitative assessment and to eliminate the errors associated with human eye perception, we used a laboratory spectrophotometer to measure the samples' color.

To facilitate positioning of the samples and to ensure the reproducibility of samples' position during the measurements, we developed a probe holder which was cut out of plastic. As illustrated in Figure 2-14, the probe holder was attached at the front side of the Color Eye 7000 spectrophotometer. The exposed stained enamel area was adjusted according to the size of the device's aperture. To obtain reliable and reproducible results, it was necessary to place the same side of the samples on the same side of the sample holder at each measurement. In addition, as a quality control, the test-retest reliability for two repeated measurements was performed.

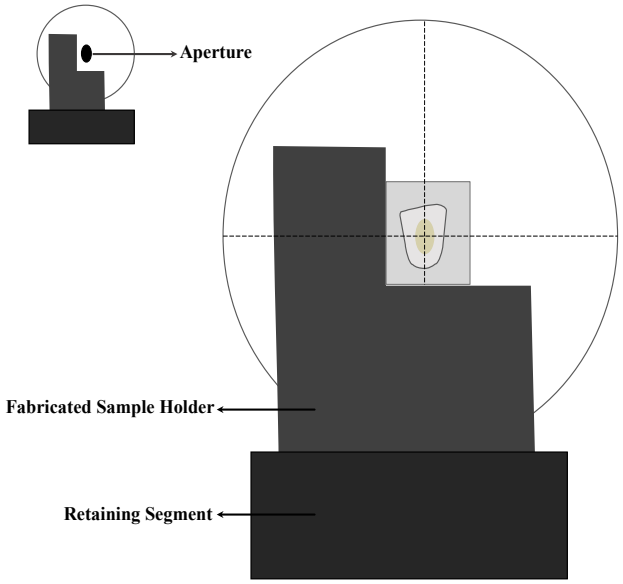


Figure 2-14 Front side of the Color Eye 7000 spectrophotometer; the reproducible positioning of the sample in the sample holder in front side of the aperture

After collecting and analyzing the L^*, a^*, b^* values, following color measurements, and calculating the corresponding ΔE mean values, it was seen that all the ΔE mean values for all the conditions were higher than 4, meaning that the overall color changes were perceptible (Table 2-6).

2.5.7 Exposure time

We intended to evaluate whether the time that the tooth surface exposes the low-concentrated aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP has any significant influence on the whitening effect of the agent. To this end, we designed the study in a way that the effectiveness of the agent after two different exposure times were compared. As Farah et al. (2009) argued, mouthwashes should only be used for short periods of time to be well tolerated by patients. Since we took into account that the final product will be used by patients on a regular basis, the exposure times should have been short enough. In this regard, we compared the color changes after one minute and after five minutes of exposure time.

Although we observed improvement in tooth color after longer exposure time, it was seen that the exposure time did not have statistically significant impact on the color changes caused by the aqueous suspension.

Based on the results showed in Table 2-9, we can argue that a possible clinical consequence is that one-minute application of the peptide- HAP suspension is a rational minimum for treatment period. Although longer exposure time may improve the whitening effect, this effect was not found statistically significant. Longer application time and a long-term treatment is because of the other potential benefits of peptide-HAP suspension (e.g. remineralization potential) could be recommended.

2.5.8 Frequency of application

Several common bleaching methods recommend a more frequent treatment to achieve better and more stable results (S. S. Lee et al., 2005). For instance, most of home bleaching methods must be performed once a day for a week. In this regard, we evaluated the impact of application frequency on the whitening effectiveness of the aqueous peptide/HAP suspension. We measured and compared the samples' color after one-time application and five-time application of the aqueous suspension. The samples were rinsed off with water after each time of application to simulate the frequent use of the proposed formulation by patients during a day or a week. It was shown that while one-time application of the suspension caused significant overall color change of the samples, increasing the number of applications to five did not lead to significantly different results. In other words, the results of the present *in vitro* study did not show any association with the number of applications.

The physical explanation of our results is the saturation of the surface with HAP particles. It can be concluded that after the first application, the samples' surface was already almost

entirely covered with the maximal adhering particle load. Therefore, more frequent applications showed only slight changes. It was seen that the additional applications did not indicate any statistically significant impact (Figure 2-15). However, since the conditions *in vivo* are different than those in our study, a more frequent application of the proposed agent may have different results intraoral. In addition to whitening effect, the adhering HAP particles may have additional beneficial effects as they can act as a solid buffer. In fact, the HAP layer can interact with protons during acidic attack and protect the enamel.

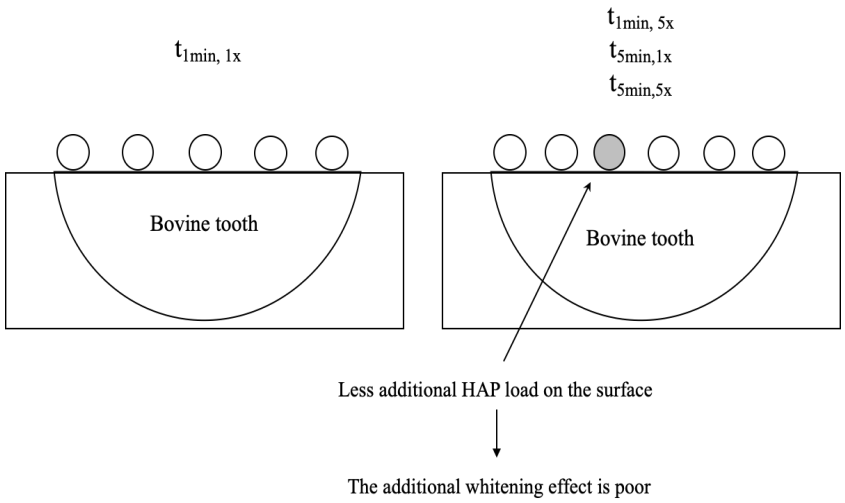


Figure 2-15 Schematic drawing of the physical explanation of the results after more frequent treatment with peptide-HAP suspension

2.5.9 Artificial saliva

Tooth enamel is considered as a highly mineralized tissue in the human body (Mann and Dickinson, 2006). It is relatively stable in a non-pathological oral condition, where saliva plays role in promoting the balance between dissolution and deposition of minerals. In fact, saliva contains a wide range of proteins, antibodies and inorganic components like calcium ions and phosphate ions, which can promote the remineralization of enamel. According to Zaharia et al. (2017), saliva is crucial for tooth remineralization as it supplies calcium and phosphate ions to build HAP blocks into crystal voids. However, the rate and level of dissolution depends on many factors such as the concentration of calcium and phosphate ions in saliva and its pH (Zaharia et al., 2017). Besides providing mineral components that help re-mineralizing teeth, saliva contains a wide range of organic compounds that protect and maintain the tooth structure (Humphrey and Williamson, 2001).

Some *in vitro* studies have used artificial saliva or natural saliva before treatment to simulate the clinical situation (Zeczkowski et al., 2015). However, in the present study, we did not store the samples in saliva before treatment so as to avoid its interference in the whole experiment. Instead, we used artificial saliva in a separate experiment step after applying the treatment agent to investigate whether it had any significant impact on the color changes.

The artificial saliva that we used includes potassium chloride (KCl, 1.20 g), sodium chloride (NaCl, 0.84g), di-potassium hydrogen phosphate (K2HPO4, 0.26 g), calcium chloride (CaCl2, 0.14 g) and water (1000 g).

In the current chapter, we treated the samples with the P11-4 peptide-HAP suspension to promote ion deposition onto tooth enamel. We assumed also that storage in artificial saliva for 24 hours after treatment with the proposed suspension may improve the whitening process. This was due the fact that artificial saliva is able to mimic the intraoral environment and supply the bioavailable ions which enhance the remineralization of enamel. However, the ion clusters that precipitate onto the tooth surface from the supersaturated saliva, are weakly soluble. Hence, the concentration of the available calcium phosphate ions intraoral was not sufficient and the remineralization occurs only poorly.

Due to the presence of a wide range of salivary proteins (e.g. statherin), the precipitation of calcium and phosphate ions in saliva *in vivo* does not happen in an ideal manner. In contrast, the intraoral calcium phosphate precipitations mostly occur within the organic biofilm, which is rich in microorganisms and remnants of the food (Akcalı and Lang, 2018). It is interesting to mention that the formation of calculus does not happen with an identical rate in the mouth cavity. The salivary glands that encompass more calcium and phosphate (e.g. sublingual gland), can develop more mineralized organic matrix (calculus). Therefore, it can be assumed that the slight remineralization capacity of the saliva is not equally effective in other sites of the mouth cavity.

Based on our results, storage in artificial saliva slightly enhanced the mean lightness of the samples. However, the observed overall color changes after storage in saliva were not statistically significant. It was seen that storage in artificial saliva for 24h reduced the redness of the teeth treated by the low-concentrated aqueous suspension for 5 times and with 5 minutes of exposure time. Similar to what we observed for the ΔL^* and Δa^* mean values, storing in saliva for 24h reduced negligibly the yellowness of the samples treated by the low-concentrated aqueous suspension.

As it can be observed in the Figure 2-11, the mean overall color change in the Vivastyle group was increased after storage in saliva for 24 hours. It can be argued that after the application of Vivastyle, the oxidizing agent diffused in the tooth. On the other hand, considering that the chemical process and the interaction of this agent with tooth required time, storage in saliva for 24 hours was a sufficient period of time to have further oxidation of chromophores.

Furthermore, we compared the overall color changes of the treated samples in both experimental groups (peptide-HAP suspension and Vivastyle) after storage in artificial saliva for 24h. Despite the observed differences given in the Figure 2-11, the applications of Vivastyle and the low-concentrated aqueous peptide-HAP suspension after 24h storage in saliva did not lead to significantly different color changes. The crystal growth which may occur due to the presence of calcium phosphate ions in artificial saliva, may explain the slight color changes of the samples treated with peptide-HAP suspension, after storage in saliva (Chapter 5).

2.5.10 Comparison with a commercial agent

Vivastyle Paint On (6% carbamide peroxide varnish) is one of the commercial home-bleaching agents. According to Betke et al. (2006), the bleaching agents which contain glycerine have a significant dehydrating effect on bovine teeth. Based on the information given by the manufacturer, Vivastyle Paint On is not a glycerine-based agent. However, it includes ethanol as a solvent. It can be assumed that ethanol, similar to glycerine, may cause dehydration of dentin. Besides, Vivastyle contains as an active agent carbamide peroxide. Some studies showed that in comparison with carbamide peroxide, pure hydrogen peroxide is released and decomposed in a faster way (Hannig et al., 2003).

It is important to note that after application of Vivastyle gel the patients should be recommended to rinse the mouth with water and to spit out the initially released H_2O_2 to decrease contamination of the oral soft tissues with peroxide which can cause gum burning and erosion of the gingiva. The study showed that the daily exposure time of the oral cavity to peroxide-based whitening agent (Vivastyle) was higher compared to what we proposed for the P11-4-HAP suspension.

As discussed, according to the presented results, the differences between the observed color changes caused by the proposed formulation and Vivastyle, before and after storage in saliva, were not statistically significant. Nevertheless, in absolute terms Vivastyle resulted in a stronger color change than the new formulation. One of the reasons for the observed differences can be different exposure times of the treatment agents; while the exposure time for Vivastyle

was 10 minutes (based on the manufacture's instruction), this for the peptide-HAP suspension was only 5 minutes. We did not increase the exposure time of the new formulation because we aim at developing a new method which is not time consuming and can be daily used. On the other hand, our proposed suspension does not contain any oxidizing ingredient., whereas Vivastyle is a H_2O_2-based agent and is a more invasive method with potential side effects as discussed in Chapter 1. Therefore, considering the advantages of the proposed suspension, its slightly less effectiveness compared to Vivastyle can be neglected and this method is preferable.

Chapter 3: Comparison of whitening effect of two different concentrations of hydroxyapatite-protein-containing suspensions

3.1 Background and significance

Owing to its considerable applicability potential, promising biocompatibility and resemblance with proteins, the well-ordered nano-sized structures of P11-4 self-assembling peptide has drawn significant attention in recent years. As in previous studies proofed, P11-4 peptide is a pH responsive agent (Aggeli et al., 2003). Owing to the ionisable arginine and glutamate side chains, it adopts a hydrogel state at acidic pH and remains soluble at neutral pH.

After formation of anti-parallel ß-sheet tapes, the tapes can stake together and form fibrils. The peptide in hydrogel form was discovered initially to be used as potential application in biology and tissue engineering such as enamel remineralisation. The peptides could self-assemble into various nanostructures such as nanofibers, nanotubular structures or vesicular structures and hydrogels. The various nanostructures of the peptide could entrap some bioactive molecules and release them with a controlled pattern. The process of creating self-assembled nanostructures is a spontaneous organization of chaotic units and is mediated through the non-covalent molecular interactions. The process of assembly is controlled by the balance of attractive and repulsive forces (Parang, 2014).

Due to the suitable characteristics and inherent biocompatibility of the P11-4 peptide, we used this material to promote and stabilize the whitening effect of HAP on the samples. In fact, the formation of the 3D scaffold may contribute to a better adhesion of HAP particles on the tooth surface.

3.2 Main objectives of the present chapter

This chapter evaluated the whitening effect of a mixtures containing P11-4 peptide based on Curolox technology in combination with two different concentrations of the HAP suspension.

Furthermore, it evaluated the impact of storage in artificial saliva and the interactions between storage in saliva and the aforementioned agents on sample's color. Finally, this part of the study aimed at exploring the effect of re-staining on teeth's color after the treatment with the agents.

More precisely, this chapter aimed at answering following main questions.

- How was the whitening effect of the aqueous low-concentrated peptide-HAP suspension in comparison with the high-concentrated P11-4-HAP suspension?
- Was the whitening effectiveness of the high-concentrated suspension statistically more significant than the low-concentrated suspension?
- How did the results change after 24 hours of storage in saliva at 37°C?
- How was the teeth's color affected by re-staining after the treatment with the different concentrations of protein-HAP suspension?

3.3 Material and methods

The active ingredients that we used in this chapter are summarized in Table 3-1.

Table 3-1 The treatment agents used in Chapter 3

Treatment agent	Company
Aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP	Curodont, Credentis AG, Windisch, Switzerland
Curodont Protect HAP suspension (6.25%)	Curodont, Credentis AG, Windisch, Switzerland

The devices used in the present chapter are presented in Table 3-2.

Table 3-2 The devices used in Chapter 3

Device	Company
Color Eye 7000 spectrophotometer	Gretag MacBeth, X-Rite- Munich, Germany
Vortex mixer	NeoLab, Heidelberg, Germany
Electronic pH meter	WTW bench pH/mV meters Routine meter pH 526, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany

3.3.1 Study design

24 freshly extracted bovine incisors without any stain and cracks or caries were selected randomly and polished, prepared and stained in the same way as described in chapter 2. Subsequently, they were stored in Evian water at 7°C until the experiment is conducted.

The prepared samples were assigned randomly to three experimental groups, each with eight samples (n = 8). The first group was treated with a high-concentrated peptide-HAP

suspension (Curodont protect and 6.25% HAP) suspension. The second group received aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP. The samples in the last group (control group) did not get any treatment. Table 3-3 shows the treatment agent that the samples in each group received.

Table 3-3 Treatment agents used in the experimental groups of Chapter 3

Group name	Treatment agent
Group 1	Aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP
Group 2	High-concentrated protein suspension (P4-11 peptide + 6.25% HAP)
Group 3	No treatment

3.3.1.1 Treatment of the first group with aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP

For each sample in the first group, we performed the treatment according to the following steps:

1. The baseline color of the sample was measured, using Color Eye7000 spectrophotometer (Gretag MacBeth, X-Rite- Munich, Germany).
2. The prepared suspension was applied continuously to the sample's surface with a micro brush (Brand Micro-brush, Grafton, USA) for 30 seconds. After five minutes of exposure time, the sample was rinsed with water.
3. The sample was dried with a towel paper (half dry) and its color was recorded.
4. It was stored after treatment in artificial saliva at 37°C in incubator for 24 hours.
5. The specimen was dried with a towel paper and the color was measured again.
6. The sample was kept in the staining solution for 72 hours.
7. It was rinsed thoroughly with water and dried with a soft absorbent paper towel (half dry) and the color was measured.

3.3.1.2 Treatment of the second group with high-concentrated protein-HAP suspension (5 ml P11-4 + 6.25% HAP)

For each sample in this group, we treated according to the steps below.

1. The baseline color of the sample was measured, using ColorEye7000 spectrophotometer.

2. To prepare the treatment suspension, 5 ml of Curodont Protect was mixed with 10 ml of 6.25% HAP[2], using a Vortex mixer (neoLab, Heidelberg, Germany).
3. The prepared suspension was applied continuously on the tooth's surface with a micro brush (Brand Micro-brush, Grafton, USA) for 30 seconds.
4. After five minutes of exposure time, the sample was rinsed thoroughly with water.
5. The sample was dried with a towel paper (half dry) and its color was recorded.
6. The sample was stored in artificial saliva at 37°C in incubator for 24 hours.
7. The specimen was dried with a towel paper and the color was measured.
8. Subsequently, the sample was kept in the staining solution for 72 hours.
9. It was rinsed thoroughly with water and dried with a soft absorbent paper towel (half dry) and the color was measured.

3.3.1.3 Treatment of the third group (no treatment)

The samples in this group which served as the control group, did not receive any special treatment. For each sample, the following steps were conducted:

1. The baseline color of the sample was measured, using ColorEye7000 spectrophotometer (Gretag MacBeth, X-Rite- Munich, Germany).
2. The sample was only rinsed with Evian water and left for 5 minutes.
3. The sample was dried with a towel paper (half dry) and its color was recorded.
4. The specimen was stored subsequently in artificial saliva at 37°C in incubator for 24 hours.
5. It was dried with a towel paper and the color was measured again.
6. The sample was kept in the staining solution for 72 hours.
7. It was rinsed thoroughly with water and dried with a soft absorbent paper towel (half dry) and the color was measured.

Figure 3-1 illustrates the treatment steps of the samples in the third group. As explained,

[2] The initial concentration was 25%

application of treatment agent in this group refers to rinsing with water.

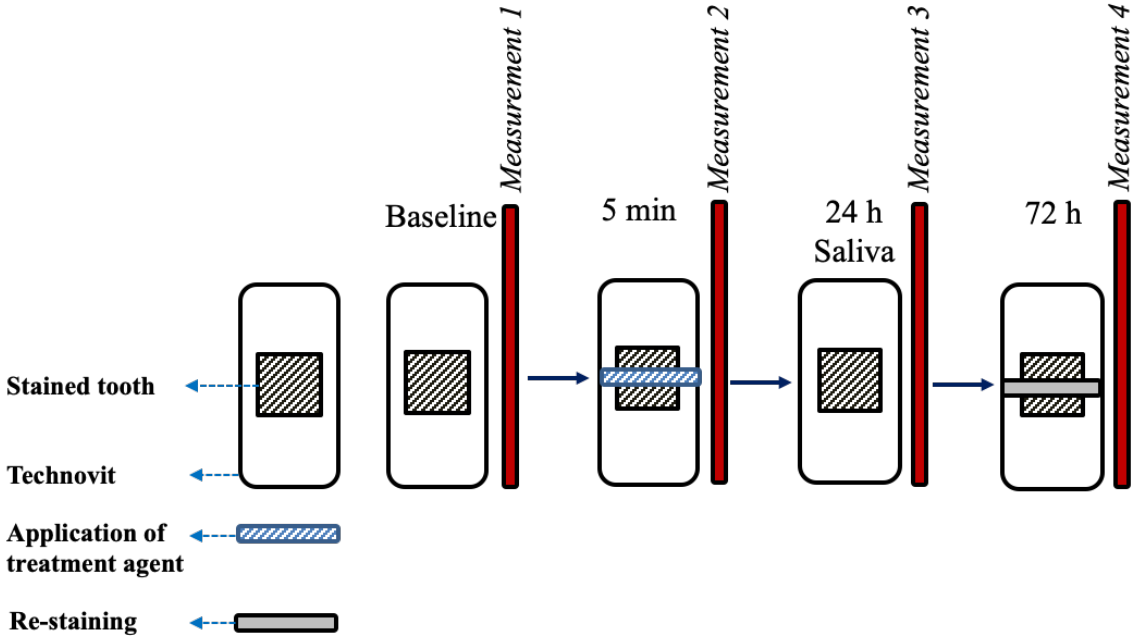


Figure 3-1 Treatment and measurement procedure of the samples in each group

3.4 Results

To determine the CIELAB values of follow-up-applications, the ColorEye 7000A spectrophotometer was set to 10° observer and D65 illumination. The measurements were performed, L*a*b* values were collected and the associated ΔE values were calculated.

3.4.1 Comparison of the CIELAB values

Figure 3-2 presents the mean and standard deviation (SD) of absolute CIELAB values for all three groups in baseline, after treatment with agent, after storage in saliva for 24h and after re-staining for 72h. It is interesting to see that in all groups, storage in saliva led to the highest mean L^* value and the lowest mean a^* value. In contrast, the re-staining resulted in lowest mean L^* value and the highest mean a^* value.

Table 3-4 summarizes the means and standard deviation of the ΔL^*, $\Delta a^*$and Δb^*values of the groups after the experiment steps, which were calculated relative to the baseline values. It is seen that the mean ΔL^* value after treatment in the Group 1 (0.24) was notably higher than that in the Group 2 (0.04). The mean Δa^* and Δb^* values in the Group 1 were -0.10 and -0.39, respectively, while there in the Group 2 equalled 0.30 and 0.83, respectively.

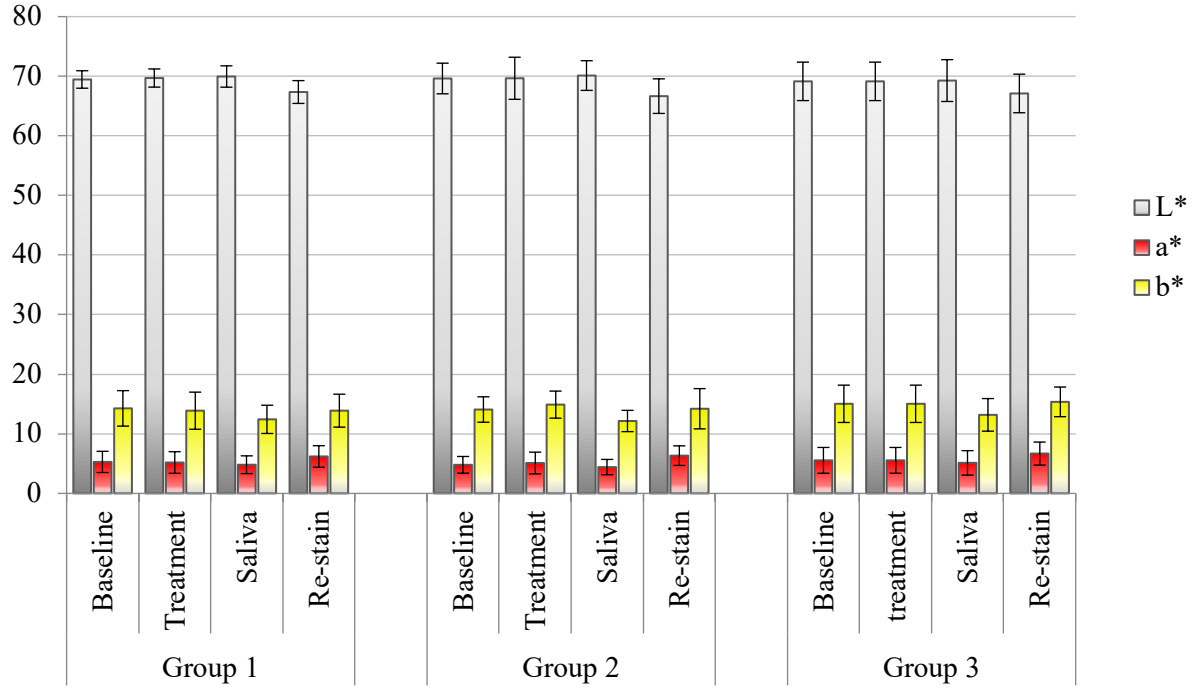


Figure 3-2 The mean and SD of L*, a* and b* values for each group at different conditions

Table 3-4 The Mean and Standard Deviation of ΔL^*, $\Delta a^*$and Δb^* values for each group after each experiment step

Group name		**ΔL^***		**Δa^***		**Δb^***	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Group 1	Treatment	0.24	0.09	-0.10	0.09	-0.39	0.26
	Saliva	0.50	0.51	-0.47	0.45	-1.84	0.95
	Re-stain	-2.11	0.76	0.92	0.51	-0.38	1.38
Group 2	Treatment	0.04	1.11	0.30	0.68	0.83	1.90
	Saliva	0.49	0.31	-0.38	0.48	-1.92	0.96
	Re-stain	-2.96	1.97	1.54	1.75	0.13	2.48
Group 3	Treatment	0.00	0.00	0.00	0.00	0.00	0.00
	Saliva	0.13	0.83	-0.42	0.23	-1.86	0.68
	Re-stain	-2.03	0.89	1.14	0.42	0.33	2.08

Table 3-5 shows that while the application of "Aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP" led to a lighter, less red and less yellow color perception, the treatment with "P11-4 + 6.25% HAP" yielded a lighter, redder and yellower color perception.

Storage in saliva for 24h led to a lighter**,** greener and less yellow color perception in all three groups. However, the lightness effect of storage in saliva was considerably lower in the

Group 3 (control group) than the other two groups. While after re-staining the mean ΔL^* values in the Group 1 and Group 2 were 0.50 and 0.49, respectively, this in the Group 3 was 0.13. The Δa^* values in Group 1, Group 2 and Group 3 after re-staining were equal to -0.47, -0.38 and 0.42, respectively, whereas the Δb^* values of these groups were -1.84, -1.92 and -1.86, respectively.

The re-staining resulted in darker, redder and less yellow color perception in Group 1, while it led to darker, redder and yellower color perception in Group 2 and Group 3. It is noteworthy that the mean ΔL^* values in the Group 1 (-2.11) and the Group 2 (-2.96) were higher than the mean ΔL^*value in the Group 3 (-2.03). The Δa^* values in Group 1, Group 2 and Group 3 after re-staining were equal to 0.92, 1.54 and 1.14, respectively, while the Δb^* values of these groups were -0.38, 0.13 and 0.33, respectively.

Table 3-5 The color perception of each group after the experiment steps

Group name		**ΔL^***	**Δa^***	**Δb^***
		Color perception	**Color perception**	**Color perception**
Group 1	Treatment	Lighter	Less red	Less yellow
	Saliva	Lighter	Less red	Less yellow
	Re-stain	Darker	Redder	Less yellow
Group 2	Treatment	Lighter	Redder	Yellower
	Saliva	Lighter	Less red	Less yellow
	Re-stain	Darker	Redder	Yellower
Group 3	Treatment	-	-	-
	Saliva	Lighter	Less red	Less yellow
	Re-stain	Darker	Redder	Yellower

3.4.2 Impact of the suspension concentration on the overall color change

To provide a more comprehensive assessment of the color changes, the ΔE values of the groups were calculated relative to the baseline values. Table 3-6 summarizes the mean and standard deviation (SD) of ΔE values in each group after the experiment steps.

Table 3-6 Mean and Standard Deviation (SD) of ΔE values for each group after each experiment step

Group name		ΔE	
		Mean	**SD**
Group 1	Treatment	0.51	0.20
	Saliva	2.05	1.00
	Re-stain	2.65	0.94
Group 2	Treatment	1.81	1.57
	Saliva	2.06	1.03
	Re-stain	4.04	2.68
Group 3	Treatment	0.00	0.00
	Saliva	2.06	0.72
	Re-stain	3.07	0.93

The table shows that after treatment, the mean ΔE values in Group 2 (1.81) was much stronger than that in Group 1 (0.51). Group 2 exhibited minimal color difference before and after 24 hours of storage in artificial saliva. While before storage in artificial saliva the mean ΔE value of this group was 1.81, this increased slightly to 2.06 after the storage. In contrast, the mean total color change in Group 1 dramatically increased from 0.51 before saliva to 2.06 after saliva. It is also noteworthy that saliva noticeably affected the color of the samples in Group 3. The mean ΔE value in this group after storage in saliva was 2.06.

The samples color in Group 2 was affected the most by re-staining for 72h. The ΔE mean value of this group after re-staining was 4.04, while for the other two groups this was below 3.08. Furthermore, the standard deviation of Group 2 (2.68) was also noticeably higher than the other groups. By looking at the Figure 3-3 it can be seen that the color of one of the samples (sample 4) in Group 2 significantly changed by re-staining. Despite this “out of range” behavior after re-staining, we cannot conclude that re-staining always caused significant color change on the samples that were already treated by the high-concentrated P11-4 + 6.25% HAP suspension.

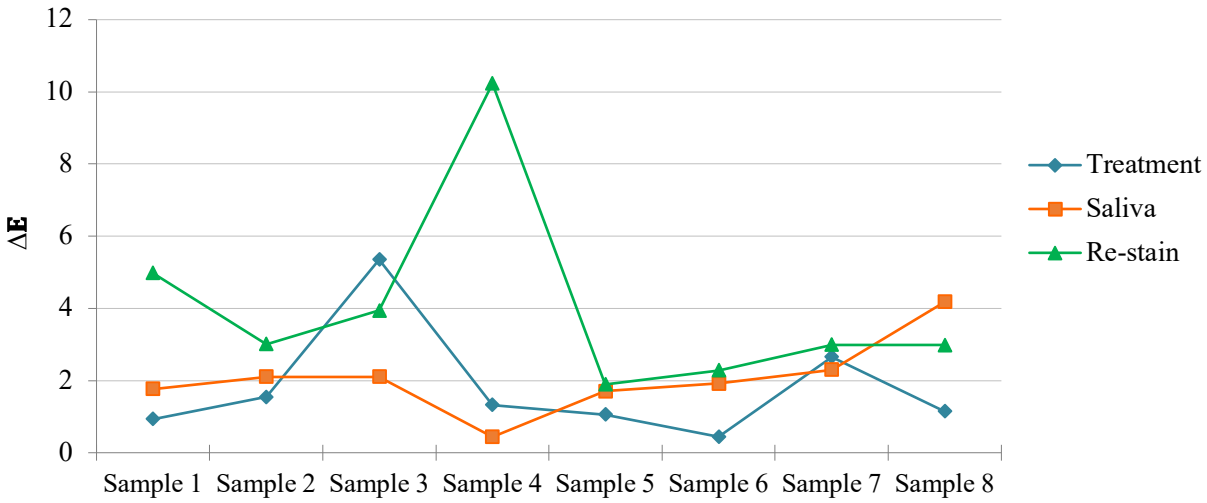


Figure 3-3 **ΔE** values of the samples in Group 2 after treatment with P4-11 peptide + 6.25% HAP, storage in saliva and re-staining

To have a more detailed insights, we compared the mean reflection spectra of the group with the new mean reflection after discarding the outlier (Figure 3-4). It is seen that discarding the outlier increased the mean reflection spectra of the Group 2 for the wavelengths between 360 nm to 680 nm.

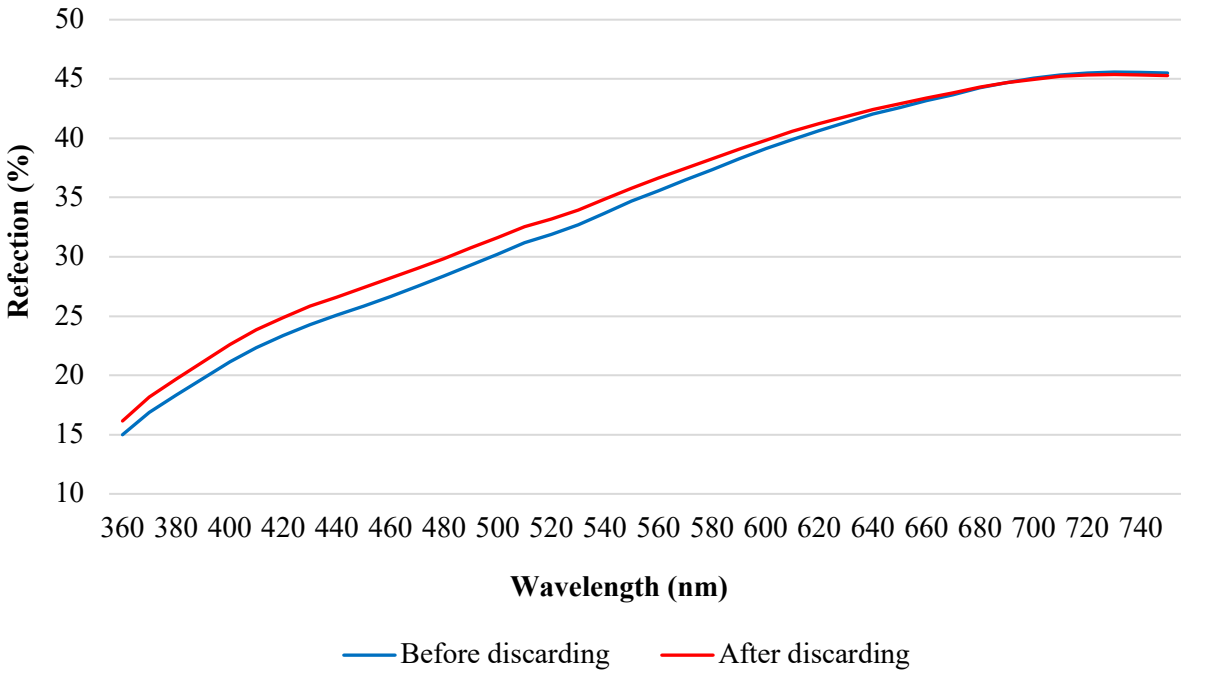


Figure 3-4 Mean refection spectra of Group 2 after the re-staining step, before discarding the outlier sample and after discarding the outlier sample

Table 3-7 compares the mean ΔE values of the treatment groups after re-staining and discarding the outlier sample in Group 2. Eliminating the sample 4 in the Group 2 reduced the mean ΔE value of this group from 4.04 to 2.85. This means that the new mean ΔE value of the Group 2 was lower than the mean ΔE value of the Group 3.

Table 3-7 The mean **ΔE** values of the treatment groups after the re-staining step and discarding the outlier sample in Group 2

Group Name	Mean ΔE
Group 1	2.65
Group 2	2.85
Group 3	3.07

To determine whether the differences between two solutions after treatment were statistically significant, a statistical test should be performed. To this end, first we compared the overall color changes (ΔE values), caused by treatment Group 1 and Group 2, using an independent t-test. The reason for choosing this test is that the groups are unrelated. However, to implement this test, it is essential that the dependent variable is approximately normally distributed within each group and the variances of the two groups are equal in the population. Violation of these assumptions may lead to wrong results. Table 3-8 shows the results from the Kolmogorov-Smirnov test and the Shapiro-Wilk test for these groups. Since the Shapiro-Wilk test is more appropriate for small sample sizes (Razali and Wah, 2011), here we used the Shapiro-Wilk test to assess the normality assumption. Since approximate normality was sufficient, the α value was set to 0.01.

Table 3-8 The results of normality tests (Kolmogorov-Smirnov and Shapiro-Wilk) for Group 1 and Group 2 after the treatment

Group name	Kolmogorov-Smirnov			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Group 1	0.252	8	0.143	0.903	8	0.308
Group 2	0.316	8	0.018	0.757	8	0.009

According to the Table 3-8, since one of the Sig. values of Shapiro-Wilk test (i.e. 0.009) was not higher than 0.01, we concluded that the assumption of normality was violated. Therefore, the Mann-Whitney U test as a non-parametric test that does not require the assumption of normality (Shier, 2004) was carried out. Table 3-9 provides data regarding the output of the actual Mann-Whitney U test and presents mean rank and sum of ranks for the both groups.

Table 3-9 The comparison of the mean Rank and sum of Ranks of ΔE values of Group 1 and Group 2 after the treatment

	Group name	N	Mean Rank	Sum of Ranks
ΔE	Group 1	8	5.25	42.00
	Group 2	8	11.75	94.00

This table illustrates which group was considered as presenting the higher overall color change; namely, the group with the higher mean rank. In this case, Group 2 after treatment presented higher Mean Rank (11.75) than Group 1. However, to see if this difference is significant, Table 3-10 shows the actual significance value of the test. Specifically, this table reflects the test statistic, *U* statistic, as well as the asymptotic significance (2-tailed) *p*-value. From the results of this test it can be concluded that after treatment, the overall color change in Group 2 was statistically significantly higher than that in Group 1 ($U = 6$, $p = .005$). Increasing the concentration of the suspension significantly promoted the overall color change effect on the samples.

Table 3-10 The results of Mann-Whitney test for ΔE values of Group 1 and Group 2, after the treatment

	ΔE
Mann-Whitney U	6.000
Wilcoxon W	42.000
Z	-2.731
Asymp. Sig. (2-tailed)	0.006
Exact Sig. [2*(1-tailed Sig.)]	0.005[a]
Exact Sig. (2-tailed)	0.005
Exact Sig. (1-tailed)	0.002
Point Probability	0.001

a. Not corrected for ties.

3.4.3 Impact of saliva on the overall color change in each group

To analyze the effect of artificial saliva on the overall color change of the samples in the groups, a paired t-test was applied. The null hypothesis of the test is that the mean difference between ΔE values before and after storage in saliva is zero. As already discussed, this test requires the assumption of normality of distribution. Table 3-11 shows the results of the

Kolmogorov-Smirnov and Shapiro-Wilk tests for these groups. As already explained, here we used the Shapiro-Wilk test to assess the normality assumption ($\alpha = 0.01$).

Table 3-11 The results of tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk) for ΔE values of Group 1 and Group 2, before and after storage in saliva

Group	Kolmogorov-Smirnov			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Group 1	0.190	8	0.200	0.955	8	0.758
Group 2	0.250	8	0.149	0.907	8	0.331

According to the Table 3-11, both of the Sig. values of the Shapiro-Wilk test (0.758 and 0.331) were higher than 0.01. Therefore, we could conclude that the assumption of normality was not violated and the paired t-test was conducted.

Table 3-12indicates the mean, standard deviation and standard error mean of ΔE values of the of the three groups before and after storage in saliva, while Table 3-13 presents the results of the paired t-test for the differences in ΔE of each group before and after storage in saliva.

Table 3-12 Paired samples statistics of ΔE values of Group 1 and Group 2, before and after storage in saliva

Group name		Mean	N	Std. Deviation	Std. Error Mean
Group 1	Before	0.507	8	0.200	0.071
	After	2.048	8	1.001	0.354
Group 2	Before	1.806	8	1.568	0.554
	After	2.063	8	1.029	0.364

In Group 1, the mean ΔE values before and after storage in saliva were 0.507 and 2.048, respectively. Therefore, the mean difference between ΔE values before and after storage in saliva was -1.541. The Table 3-13 shows that the difference is significant (0.004 < 0.05). In other words, storage in saliva for 24h led to a statistically significant different overall color change in Group 1.

In Group 2, the mean ΔE values before and after storage in saliva were 1.806 and 2.063, respectively. This means that the mean difference between ΔE values of this group before and after storage in saliva was -0.258. As indicated in the Table 3-13, this difference was not statistically significant (0.704 > 0.05). Therefore, storage in saliva for 24h did not cause a

significant color change on the samples in this group.

Table 3-13 The results of paired t-test for differences in ΔE values of the groups, before and after storage in saliva (Before – After)

Group name		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Group 1	(Before – After)	-1.541	1.045	0.369	-2.415	-0.668	-4.171	7	0.004
Group 2	(Before – After)	-0.258	1.843	0.651	-1.798	1.283	-0.395	7	0.704

In Group 2, the mean ΔE values before and after storage in saliva were 1.806 and 2.063, respectively. This means that the mean difference between ΔE values of this group before and after storage in saliva was -0.258. As indicated in the Table 3-13, this difference was not statistically significant (0.704 > 0.05). Therefore, storage in saliva for 24h did not cause a significant color change on the samples in this group.

3.4.4 Impact of re-staining on the overall color change in each group

To determine the effect of re-staining on the overall color of the samples in all groups, a paired t-test was conducted. The null hypothesis of this test is that the mean difference between ΔE values before and after re-staining is zero. As already discussed, this test requires that the differences in the dependent variable between the groups should be approximately normally distributed. Table 3-14 illustrates the results of the normality tests.

Table 3-14 The results of normality tests (Kolmogorov-Smirnov and Shapiro-Wilk) for ΔE values of the groups, before and after re-staining

Group	Kolmogorov-Smirnov			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Group 1	0.219	8	0.200*	0.930	8	0.520
Group 2	0.265	8	0.102	0.763	8	0.011
Group 3	0.183	8	0.200*	0.930	8	0.520

* This is a lower bound of the true significance

The Sig. values of Shapiro-Wilk test in the Table 3-14were higher than 0.01. Therefore, we concluded that the assumption of normality was not violated and the paired t-test could be conducted.

Table 3-15 indicates the mean, standard deviation and standard error mean of ΔE values of the groups, before and after re-staining, while the results of paired t-test for the differences in ΔE values of each group before and after re-staining are given in Table 3-16.

Table 3-15 Paired samples statistics of ΔE values of the groups, before and after re-staining

Group		**Mean**	**N**	**Std. Deviation**	**Std. Error Mean**
Group 1	Before	2.048	8	1.001	0.354
	After	2.653	8	0.940	0.332
Group 2	Before	2.063	8	1.029	0.364
	After	4.039	8	2.678	0.947
Group 3	Before	2.0576	8	.71872	.25411
	After	3.0696	8	.92648	.32756

Table 3-16 The results of paired t-test for the differences in ΔE values of the groups, before and after re-staining (Before – After)

Group		**Paired Differences**					**t**	**df**	**Sig. (2-tailed)**
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Group 1	(Before – After)	-0.604	1.034	0.366	-1.4690	0.260	-1.653	7	0.142
Group 2	(Before – After)	-1.975	3.408	1.205	-4.824	0.873	-1.640	7	0.145
Group 3	(Before – After)	-1.012	1.1482	0.406	-1.972	-0.052	-2.493	7	0.041

It is observed that the mean ΔE values of Group 1 before and after re-staining were 2.048 and 2.653, respectively, meaning that the mean difference between ΔE values was -0.604. This difference was not statistically significant (0.142 > 0.05). It can be concluded that re-staining did not cause a significant overall color change in this group.

In Group 2, the mean ΔE values before and after re-staining were 2.063 and 4.039, respectively. As given in the Table 3-15, the mean difference between ΔE values before and

after re-staining in this group equaled -1.975 which was not considered statistically significant (0.145 > 0.05). Therefore, similar to Group 1, re-staining did not lead to a significant overall color change in this group.

The mean ΔE values of Group 3, before and after re-staining were 1.029 and 2.678, respectively and the corresponding mean difference between ΔE values was -1.012. In contrast to the other groups, the difference in this group was found to be statistically significant and therefore re-staining significantly changed the overall color of the samples in the Group 3.

3.4.5 Comparison of the overall color changes in the groups, after each experiment step

In this section, overall color changes of all three groups after the experiment steps are compared. Table 3-17 presents the descriptive statistics of ΔE values of the groups after Treatment, Saliva and Re-staining steps, which were calculated based on the baseline values. It can be observed that after each experiment step, Group 2 represented the highest Mean ΔE values. However, the difference after storage in saliva was not very pronounced.

Table 3-17 Descriptive statistics of the ΔE values of the "P11-4 + 6.25% HAP", "Aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP" and "No treatment" groups after Treatment, Saliva and Re-staining

Experiment step	Group	Mean	Std. Deviation	N
Treatment	Group 1	0.507	0.200	8
	Group 2	1.806	1.568	8
	Group 3	0.000	0.000	8
	Total	0.771	1.168	24
Saliva	Group 1	2.048	1.001	8
	Group 2	2.064	1.029	8
	Group 3	2.058	0.719	8
	Total	2.056	0.886	24
Re-staining	Group 1	2.653	0.940	8
	Group 2	4.039	2.678	8
	Group 3	3.070	0.926	8
	Total	3.254	1.751	24

To detect any overall differences between the related ΔE mean values, the ANOVA with repeated measures (within-subject factors) test was initially selected. This test particularly requires the assumption of sphericity (Lane, 2016). This assumption refers to the condition

where the variances of the differences between all possible pairs of related groups are equal. Violation of sphericity may cause the test to produce biased results. Therefore, it was crucial to determine whether sphericity condition was violated.

Mauchly's test is a well-known statistical test for validating the sphericity assumption. The null hypothesis of this test is that the variances of the differences were equal. Thus, if the test was statistically significant ($p < 0.05$), we could reject the null hypothesis and accept that the variances of the differences are not equal. The results of Mauchly's Test of Sphericity are given in Table 3-18.

Table 3-18 The results of Mauchly's Test of Sphericity for the experiment steps

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Experiment steps	0.722	6.516	2	0.038	0.782	0.915	0.500

This table presents that the assumption sphericity was violated, $\chi^2(2) = 6.516$, $p = 0.038$. To overcome this issue, the Greenhouse-Geisser correction was applied to the degrees of freedom (*df*) (Grieve, 1984). Table 3-19 indicates if there was an overall significant difference between the mean ΔE values after the experiment steps and shows if the was a significant interaction between the experiment steps and the treatment agent in each group (i.e. Aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP in Group 1 and high-concentrated P11-4 + 6.25% HAP in Group 2,). It is seen that the was no significant interaction ($p = 0.302$). However, there was a significant difference between the experiment steps ($p < 0.0005$).

The results presented in the Table 3-19 denotes that we had an overall significant difference in the mean ΔE values of the experiment steps. Table 3-20 simply provides important descriptive statistics of these steps.

Table 3-19 Tests of Within-Subjects Effects for experiment step and interaction between experiment step and treatment agent

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Experiment step	Sphericity Assumed	73.999	2	37.000	21.795	0.000
	Greenhouse-Geisser	73.999	1.565	47.287	21.795	0.000
	Huynh-Feldt	73.999	1.830	40.446	21.795	0.000
	Lower-bound	73.999	1.000	73.999	21.795	0.000
Experiment step × Agent	Sphericity Assumed	8.615	4	2.154	1.269	0.297
	Greenhouse-Geisser	8.615	3.130	2.752	1.269	0.302
	Huynh-Feldt	8.615	3.659	2.354	1.269	0.299
	Lower-bound	8.615	2.000	4.307	1.269	0.302
Error (Experiment steps)	Sphericity Assumed	71.300	42	1.698		
	Greenhouse-Geisser	71.300	32.863	2.170		
	Huynh-Feldt	71.300	38.421	1.856		
	Lower-bound	71.300	21.000	3.395		

Table 3-20 The descriptive statistics of the ΔE values of the experiment steps

Experiment step	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Treatment	0.771	0.186	0.384	1.158
Saliva	2.056	0.189	1.663	2.450
Re-staining	3.254	0.352	2.522	3.985

Since we did not know where those differences occurred, it was necessary to carry out the Bonferroni post hoc test which allowed us to discover which specific means were different. Table 3-21 shows the results of this test. The mean difference is significant at the 0.05 level.

The “Mean Difference (I-J)” column of the Table 3-21 shows that the mean ΔE value of Saliva was 1.286 higher than that of Treatment, while it was 1.197 lower than the mean ΔE value of Re-staining. Further, the mean ΔE value of Re-staining was 2.483 higher than that of Treatment. It is seen that there were significant differences in the mean ΔE values between treatment and Saliva ($p < 0.005$), between Treatment and Re-staining ($p < 0.005$), and between Saliva and Re-staining ($p = 0.039$).

Table 3-21 The results of Pairwise Comparisons of the mean ΔE values of the experiment steps

(I) Step	(J) Step	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Treatment	Saliva	-1.286	0.264	0.000	-1.971	-.600
	Re-staining	-2.483	0.401	0.000	-3.525	-1.441
Saliva	Treatment	1.286	0.264	0.000	.600	1.971
	Re-staining	-1.197	0.441	0.039	-2.344	-0.050
Re-staining	Treatment	2.483	0.401	0.000	1.441	3.525
	Saliva	1.197	0.441	0.039	0.050	2.344

a. Adjustment for multiple comparisons: Bonferroni.

To see whether the treatment agents presented statistically different overall color change, Table 3-22 shows the ANOVA results for ΔE values of the between-subjects variable, group. The null hypothesis of this test was that there was no significant difference between the treatment agents ($\alpha = 0.05$).

Table 3-22 The results of tests of Between-Subjects Effects

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	295.847	1	295.847	233.436	0.000
Group	13.364	2	6.682	5.272	0.014
Error	26.614	21	1.267		

Since the Sig. value (0.014) in the table was smaller than 0.05, we concluded that the overall color changes in the groups were significantly different. In order to discover where the differences between the mean ΔE values of the groups occurred, the Bonferroni post hoc test was conducted. Table 3-23 illustrates the results of pairwise comparisons for the mean ΔE values of the groups.

The table shows that the mean ΔE value of Group 2 was 0.9 higher than that of Group 1 and this difference was statistically significant (p = 0.034). Furthermore, Group 2 presented 0.927 higher mean ΔE value than the Group 3, which was considered as a significant difference (p = 0.029). In contrast, it can be seen that the difference between the mean ΔE values of Group 1 and Group 3 equaled 0.027 which was not statistically significant (p = 1.000).

Table 3-23 The results of Pairwise Comparisons of the mean ΔE values of the groups

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Group 1	Group 2	-0.900	0.325	0.034	-1.746	-0.055
	Group 3	0.027	0.325	1.000	-0.819	0.872
Group 2	Group 1	0.900	0.325	0.034	0.055	1.746
	Group 3	0.927	0.325	0.029	0.082	1.772
Group 3	Group 1	-0.027	0.325	1.000	-0.872	0.819
	Group 2	-0.927	0.325	0.029	-1.772	-.082

a. Adjustment for multiple comparisons: Bonferroni.

3.5 Discussion

Many studies (e.g. Cougot et al., 2018) used teeth after removing aprismatic enamel layer. In all chapters of the present study, the aprismatic layer of enamel's surface was eliminated in order to remove the exogenous sediments on the surface and achieve a flat measurement surface. The native tooth surface which is slightly curved, shines strongly. Due to its shine, the proportion of specular reflection of light is high. On the other hand, the curvature makes the direction in which the light is reflected, unpredictable. Although we used an integrating sphere to measure the reflections, to avoid the unreliability due to the strong specular light reflection from the curved surface, we ground the surface flat.

Furthermore, the grinding process also makes the surface slightly rough. This reduces slightly the specular reflection. This, however, is really positive that the exogenous discolorations can be completely removed by surface grinding.

During sample preparation, we also unintendedly simulated some possible conditions in the oral cavity, in which superficial enamel is missed. These include for example erosion and abrasion, which result in aprismatic and prismatic enamel losses (Wu et al., 2017; Mullan et al., 2017). However, the pattern of aprismatic enamel dissolution is different from prismatic enamel loss (Meurman and ten Cate, 1996). The surface enamel removal may positively influence the behavior of the treatment suspension and its adherence to the surface. This is because the aprismatic outer layer of enamel has less permeability than the underlying enamel (Torres et al., 2010; Amaechi et al., 1999).

Therefore, it can be assumed that polished samples respond differently from sound enamel to the exposure to treatment agents.

In this chapter, we performed the color measurements, using ColorEye spectrophotometer with the aid of the sample holder described in chapter 2.

Furthermore, during all the measurements, we used the transparent embedding material without light protection or covering holder. All the measurements were carried out in the dark to avoid the influence of the room's light which may distort out results.

3.5.1 Curodont Protect: the P11-4 self-assembling peptide in polymeric form (gel consistency)

Curodont Protect (Curodont, Credentis AG, Windisch, Switzerland) includes P11-4-based on Curolox technology, which are self-organizing molecules. These molecules have effective protective characteristics due to combination and stabilization with calcium phosphate.

This product is presented as a gel in polymeric form. The peptides are polymerized to small fibers by the manufacture. Bröseler et al. (2013) and Schlee et al. (2014) argued that by applying the gel onto the tooth surface, the peptide diffuses into the micro pores of the subsurface and forms a 3D scaffold from the fibers and it enhances the crystallization of HAP. Since the P11-4 self-assembling peptide has a considerable affinity to the calcium ions of HAP as the fundamental component of the tooth enamel, it acts similar to bonding glue between HAP particles and enamel (Figure 3-5).

Self-assembling peptide P11-4 was originally introduced for treating initial caries. Nevertheless, in the present study, we used the P11-4 peptide for another purpose. We tried to benefit from its characteristics to enhance whitening efficacy and stability of HAP. To this end, bovine teeth without any caries were used. This means that we needed to modify the manufacture structure. Since we treated sound enamel, we skipped etching the enamel surface before treatment. Our goal was to prove that the P11-4 peptide acts as a glue or trap to bond the HAP particles to the enamel, due to its high affinity for HAP.

Calcium phosphate formulations can contribute to precipitation of inorganic crystals onto the enamel surface. The remineralization property of this material is widely described in the literature (e.g. Tung and Eichmiller, 2004; Neel et al., 2016). Due to this property; we used in our study HAP as a whitening agent, which may boost mineral gain and strengthening of the natural enamel during the whitening process (Xu, 2015).

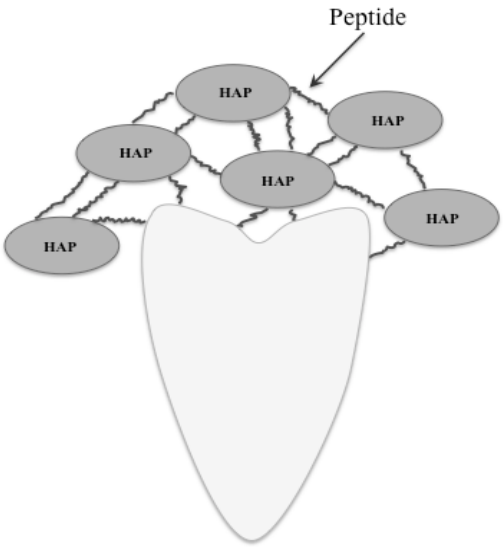


Figure 3-5 The schematic demonstration of the bonding mechanism of P11-4 peptide and HAP to the enamel surface

Based on our results, the adherence of HAP particles to the tooth surface may happen just in case that the binding sites of the surface are not blocked through the organic smear layer caused by polishing the samples after embedding. To this end, in next chapter, we used a pre-treatment to eliminate the smear layer resulted from surface polishing.

3.5.2 Staining Solution

The pH of water slightly decreases when it boils, because with increase in temperature (95°C) the rate of forward ionization increases. As we already know, a solution is acidic only if it contains more hydrogen ions than hydroxide ions. The pH of boiling water is around 6.14 and if its temperature decreases to 25°C then it is close to 7 (Kulthanan et al., 2013; Clark, 2017).

However, this does not mean that the hot water is more acidic because the concentration of hydrogen ions and hydroxide ions are always the same. We used the boiling water to prepare the staining solution which was more acidic than boiling water and it has a more acidic pH at the room temperature (pH = 5).

Furthermore, usually increasing the temperature accelerates the reaction, while decreasing the temperature slows it down. Water solubility[3] may increase also with temperature, depending on the substance properties. High temperature results in faster movement of water molecules

[3] The ability of substances to go into solution in water

and increasing the water's energy. This energy may be used to break bonds between the protein layer and the solid surface for which colder water will not have enough energy. This means that the boiling water may be effective as a solvent and be able to weaken or dissolve the newly formed protein-HAP layer from the sample surface. As mentioned, many physical and chemical reactions are affected by changing in temperature. Therefore, we left the staining solution after preparation to cool down. To avoid biased results due to high temperature of water, we used the staining solution for re-staining at the room temperature.

Shellis et al. (2005) concluded that salivary proteins may influence the adsorption of stain to apatite surfaces. However, we stored the samples in staining solution after immersing in artificial saliva, it can be assumed that if we have used natural saliva as the storage medium, the salivary proteins could increase the stain accumulation on the surface and could result in decreasing mean L^* value. But since we stored our samples in artificial saliva that did not contain any protein, the results obtained after re-staining (lowest mean L^* value) cannot be explained by this explanation.

Furthermore, due to the discussed findings, the formed protein-HAP layer on the surface after treatments with two different concentrated suspensions may be affected by the pH of the staining solution during re-staining. This acidic pH of the solution may lead to dissolving the HAP layer and remove it from the surface, which can be the main reason for slight darkening the treated samples after re-staining (Badr and Al Ibrahim, 2010). Our results pointed out that re-staining of the samples that received a treatment with peptide-HAP suspensions led to slightly darkening of the samples in comparison to re-staining of the samples that did not received any treatment.

As explained in the result section, one of the samples (sample number 4) in the Group 2 was an outlier. To provide more insight, we discarded this sample and recalculated the ΔL^*, Δa^*, Δb^* and ΔE values for this group and subsequently carried out again statistical analysis. Interestingly, the new values in Group 2 were similar to the corresponding values in Group 1 and the reflection was improved (Figure 3-4) (Table 3-7). After a detailed assessment of the results, it was seen that the water group showed the strongest color change after re-staining compared to the other two groups. It can be assumed that peptide may offset tooth discoloration after re-staining. However, a correlation between the concentration of the suspension and the intensity of discoloration of the organic matrix of the peptide after re-staining was not observed.

It was observed that excluding one of the samples in Group 2 that showed an outlier behavior, confirmed that the suspension played a role on the impact that re-staining had on the

samples' color. Since we analyzed only a limited number of samples in each group (n = 8), it can be argued that to have a more robust and reliable assessment, it is necessary to increase the number of samples.

According to our observations, it can be argued that the peptide is able to fluoresce in the similar way as blue covarine (Collins et al., 2008).

3.5.3 Medium solution

Several storage mediums have been used and suggested in the previous published studies. Examples include purified and mineral water, ethanol, glutaraldehyde, formalin, methanol, chloramine T, salt solution, mineral oil and etc. (Secilmis et al., 2011). Most of the publications used artificial saliva and natural saliva. The storage period varies from a few minutes to months. Zeczkowski et al. (2015) evaluated the effect of these storage mediums on the physical characteristics of bleached enamel. While both natural saliva and artificial saliva have the ability to maintain the hardness after treatment (Aliping-McKenzie et al., 2003), Zeczkowski et al. (2015) argued that natural saliva acts more effectively to protect the damages caused by bleaching and it is a suitable storage medium for *in vitro* studies. In several studies, fluoride-containing products have been used as storage medium to simulate the clinical situation (Cao et al., 2014).

Artificial saliva consisting only of inorganic calcium and phosphate components, has not always reversed the demineralization effect (Yui et al., 2010; Sundfeld et al., 2012; Furlan et al., 2017). This might be due to the fact that artificial saliva suffers from a lack of standard formulation. in As argued by Pytko-Polonczyk et al. (2017), there is no universal artificial saliva model has been introduced and different types of saliva may inhibit different characteristics (e.g. remineralization potential) and it is very difficult to create an artificial formula identical to natural saliva. Nevertheless, several studies (e.g. Pinto et al., 2006; Cavalli et al., 2004) used artificial saliva as storage medium.

On the other hand, a number of studies (e.g. Ben-Amar et al., 1995; Yeh et al., 2005) stored their samples between bleaching steps in distilled water. Due to ion imbalances, distilled water has the potential to demineralize teeth during the storage period. Although the use of distilled water as storage condition does not accurately reflect the clinical situation, it prevents any (unwanted) remineralization effect of salivary factors. In all parts of the current study, we used Evian water as storage medium before treatments and also as a rinse. It was due to its optimal characteristics. First, Evian water is not deionized. Second, it is a highly standardized water which has a close to natural pH value (7.18). Finally, since Evian water is considered not to be

saturated with ions, in contrast to saliva, it could prevent the crystal growth that was not attributed to the treatment. However, we stored the samples in chapter 2 and 3 in artificial saliva after treatments in a separate step, to see how the natural re-mineralization potential of saliva may influence our results. The period of storage that we chose in our study was 24 hours.

The third group of the present chapter aimed to identify how driving forces (e.g. water, saliva) other than the treatment agents, influence the main findings. In particular, since saliva contains a wide range of proteins, antibodies and inorganic components like calcium and phosphate ions, which may have an effect on the whitening process, this identified whether storage in saliva have any impact on the samples' color. It was observed that storage in artificial saliva slightly changed the samples' color. In other words, saliva did not lead to statistically significant color changes.

We also evaluated the possible impact of the P11-4 peptide and Nano-HAP concentration on the whitening and remineralization of enamel. Huang et al. (2009) claimed that 10% Nano-HAP agent may have an optimal effect on re-mineralization of enamel. In this study, we aimed at investigating how the concentration of the suspension influences the whitening efficacy. Therefore, we tested both HAP suspension and peptide with two different concentrations. It can be argued that increasing the concentration of the suspension may significantly promote the color change and dampens the effect of storage in saliva on the color change. However, more studies should be conducted to develop a more effective and more convenient concentration of the suspension for an ideal at-home tooth whitening and remineralization.

According to the presented results the mean lightness of the samples treated by the low-concentrated suspension was slightly higher than that of the samples treated by high-concentrated suspension. However, a more detailed analysis of L^* values of the samples showed that one of the samples treated with the high-concentrated suspension, was very dark-colored in baseline. Surprisingly, this sample became even darker after treatment. This can be explained by a hypothesis. It might be because the proposed suspension was not effective enough for the teeth which were intensively stained. In other words, we can claim that our treatment agent had a more desirable effect on the teeth that were lightly stained.

Based on Table 3-6, the overall color changes caused by both treatment agents was not very pronounced and not perceivable.

Chapter 4: The evaluation of whitening effect of a suspension containing P11-4 self-assembling peptide and hydroxyapatite after using NaOCl as pre-treatment

4.1 Background and significance

In the previous chapter, we used the peptide in already polymeric form (Curodont Protect) in gel consistency. In the present chapter we tried to achieve a more significant whitening effect of self-assembling P11-4 suspension in monomeric form mixed with calcium-phosphate suspension, using 3% sodium hypochlorite (NaOCl) as a pre-treatment.

Curodont Repair (Credentis AG, Windisch, Switzerland), used in the present chapter, contains a self-assembling biomimetic β-sheet peptide that generates a fibrous, supramolecular three-dimensional matrix in the acidic environment and causes the nucleation of hydroxyapatite (HAP) crystallites (Firth et al., 2006; Brunton et al., 2013). The self-assembling peptide has been used in clinical studies and treatments for the purpose of enamel and dentin remineralization.

The rehydrated peptide in its initial monomeric form has a low viscosity. After Polymerization (e.g. in presence of lactic acid) the peptide forms a scaffold-like structure and switches its form to an elastomeric gel (Figure 4-1), which can help by mineral deposition, nucleation and also crystal growth (P. . Brunton et al., 2013).

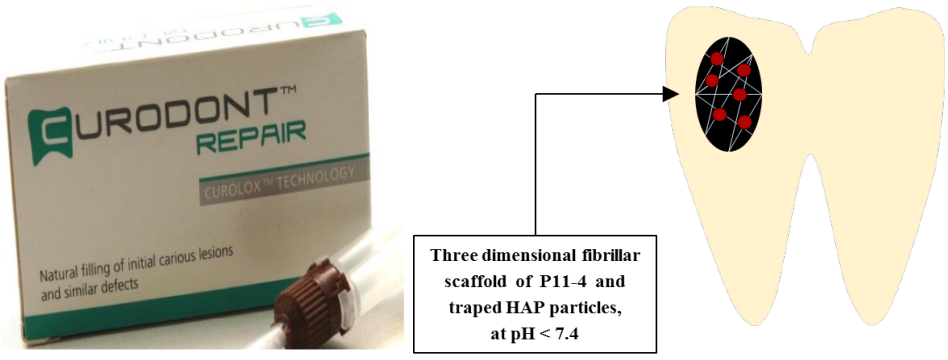


Figure 4-1 Curodont Repair (left). Schematic drawing of three-dimensional scaffold of P11-4 (right)

The main and initial effect of the P11-4 peptide was conducting a re-mineralization based on increasing mineral gain and inhibiting mineral loss. The effect of this patent was tested by Jablonski-Momeni and Heinzel-Gutenbrunner (2014) on initial enamel lesions identified at

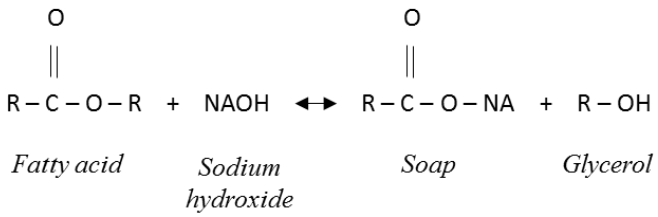
their early stage of development.

Since the main ambition of this study was to whiten healthy bovine teeth, we had to modify the manufacture's instruction and skip the etching of enamel surface with phosphoric acid (Curodont ™ Application). Instead of using phosphoric acid for removal of inorganic materials, we performed our experiment by using NaOCl as a pre-treatment.

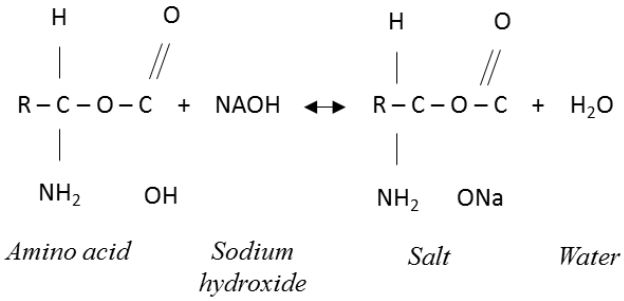
In essence, NaOCl is able to remove any organic layer from the surface. It is well-known worldwide for its considerable antimicrobial and tissue dissolution capacity. It is an effective proteolytic agent (Kuruvilla and Kamath, 1998) and excellent organic tissue solvent (Ohara et al., 1993) with a quick effect. NaOCl is considered as a hydrolyzing and oxidizing agent at the same time. A low concentrated NaOCl has acceptable biological compatibility. Due to this fact, it is widely used in dentistry, especially in endodontic treatments as a root canal irrigant (Hülsmann and Hahn, 2000). Kandil et al. (2014) reported that NaOCl acts as a fat and organic solvent for vital, necrotic and fixed tissues and also neutralizes amino acids and transforms them into salt and water. With the presence of hydroxyl ions, the pH is reduced. The chemical reactions of NaOCl with fatty acid and amino acid are demonstrated in Figure 4-2.

According to the aforementioned characteristics of NaOCl, we used in this chapter of our study an aqueous low-concentrated (3%) NaOCl solution (Hendinger, Aug. Hedinger GmbH & Co. KG, Stuttgart, Germany) with a pH of 12.2, as a pre-treatment agent.

Scheme 1. Saponification reaction.



Scheme 2. Amino acid neutralization reaction.



Scheme 3. Chloramination reaction.

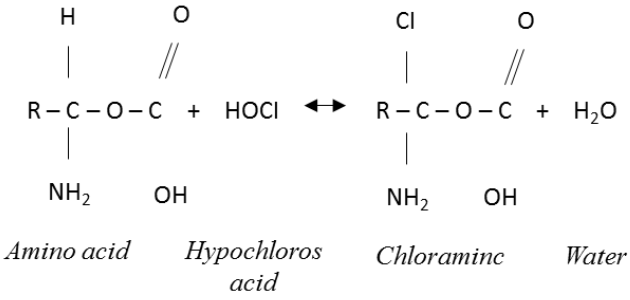


Figure 4-2 The chemical reactions of NaOCl with fatty acid and amino acid (Estrela et al., 2002) – Reuse of the image is with the written permission by Prof. Estrela (2019)

4.2 Objectives of the chapter

This chapter aimed at assessing the whitening efficacy of the suspension containing Curodont repair combined with HAP after using NaOCl as the pre-treatment agent. One of our aims was to determine how removing the smear layer before treatment with the peptide-HAP suspension changes our results. In addition, this chapter evaluated the effectiveness of each of the components of the suspension. Similar to the Chapter 2 and Chapter 3, in order to eliminate errors and limitations associated with human color perception and provides a more accurate quantitative assessment, this chapter used spectroscopy to measure color differences. However, to identify if the type of spectrophotometer could influence main findings of the experiment,

the presence chapter employed two different types of spectrophotometer, namely Ocean Optics and Color Eye, and compared the results given by these devices.

4.3 Material and methods

The materials that we used in chapter 4 are listed in Table 4-1.

Table 4-1 The materials used in chapter 4

Material	Company
Curodont Repair	Curodont, Credentis AG, Windisch, Switzerland
HAP suspension (6.25%)	
Lactic acid 0.1 mol- pH 4,0	Pharmacy University of Munich, Munich, Germany
NaOCl 3%	Hendinger, Aug. Hedinger GmbH & Co. KG, Stuttgart, Germany

4.3.1 Study design

In this chapter, 50 samples were prepared and stained in accordance with chapter 2. The samples were assigned randomly to five groups, each with ten samples (n = 10). Each group aimed at providing different insights in this context (Table 4-2). the main suspension group evaluated the whitening effectiveness of the mixture of HAP, peptide and lactic acid, using NaOCl as a pre-treatment agent. While the HAP, peptide and NaOCl groups aimed at evaluating the whitening efficacy of each of the components of the main suspension. The water group served as the control group.

The color measurements were done with Ocean Optics spectrophotometer under D65 illuminant and 2° observer. However, in order to identify if the measurement device had a significant impact on the main findings of the experiment, the reflections and the associated CIELAB values of the samples in the main suspension group were additionally measured by the Color Eye 7000A spectrophotometer under D65 illuminant and 10° observer. Before starting the measurements, calibrations were performed for both devices according to the manufacturer's protocol with the use of black trap and white reflectance standard (e.g. WS-1-SS).

In this chapter, paired t-test and one-way ANOVA statistical tests were performed to identify if significant differences in perceived color changes existed among the groups and to determine if the measurement device had any significant effect on the main finding of the experiment. If the test confirmed the existence of significant differences, Tukey's Honestly Significant Difference (HSD) Post-Hoc test was applied to define differences between two specific

groups. In essence, this test is one of the preferred tests for conducting post hoc analyses on a one-way ANOVA (Ruxton and Beauchamp, 2008). The significance level for this the tests was set to 0.05 (α =0.05). The follow-up analyses were conducted with SPSS statistical program version 18.

Table 4-2 The designed group in chapter 4

Group name	Treatment agent		Pre-treatment	Spectrophotometer	
	Protein	HAP (6.25%)	NaOCl (3%)	Color Eye 7000A	Ocean Optics
Main suspension	✓	✓	✓	✓	✓
HAP		✓			✓
NaOCl			✓		✓
Peptide	✓				✓
Water					✓

4.3.1.1 Main suspension group (peptide and HAP)

The samples in this group were treated with the main suspension containing both peptide and HAP, after using NaOCl as the pre-treatment agent. As already discussed, to see if the measurement device had any significant impact on the results of the study, in addition to the Color Eye 7000A spectrophotometer, we used Ocean Optics spectrophotometer (Ocean Optics, USB4000-VIS-NIR-ES, Ostfieldern, Germany) to measure the color of the samples in this group.

For each of the samples in this group, we performed according to the following steps:

1. The sample was initially rinsed with water.
2. NaOCl (3%) was applied continuously for 30 seconds.
3. NaOCl was rinsed thoroughly with water and then the sample was put within the sample holder (Figure 4-3).
4. The baseline color was measured with the Ocean Optics spectrophotometer.
5. The baseline color was immediately measured with the Color Eye 7000A spectrophotometer.
6. To prepare the protein solution, Curodont repair crystals were dissolved and

rehydrated with 0.05 ml of sterile water and mixed with 10 ml of 6.25% HAP[4], using a Vortex mixer (neoLab, Heidelberg, Germany).

7. To initiate the polymerization of the peptide, the lactic acid was added to the mixture using a Eppendorf pipette (Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany) in order to adjust the pH at 24°C. The pH of the suspension must be optimized (slightly acidic).
8. The suspension was directly applied to the sample's surface for continuous 30 seconds, using a fine disposable micro-brush (BRAND Micro-brush, Grafton, USA).
9. The sample was rinsed with water. Subsequently, soft absorbent paper towels were used to blot dry the sample´s surface (half dry).
10. After 90 seconds of air exposure time, the color was measured again, using both devices.

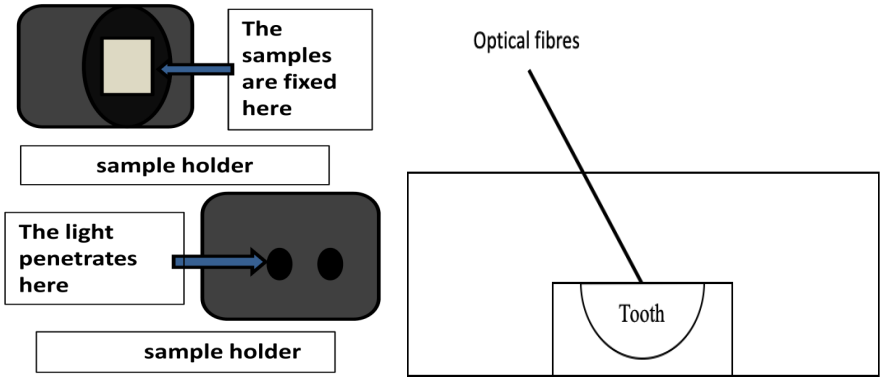


Figure 4-3 Schematic drawings of sample holder used to measure the color of the samples with Ocean Optics spectrophotometer; the sample within the sample holder, the optical fibre fixed in the associated aperture of the sample holder (right)

4.3.1.2 HAP group

For the samples in the second group, we assessed the whitening effectiveness of HAP (6.25%) without any pre-treatment and also without the presence of protein. While for the first group we used both Color Eye 7000A and Ocean Optics spectrophotometer, for this group, we only employed the Ocean Optics device. For each sample in this group, we performed according to the following steps:

[4] The initial concentration was 25%

1. The baseline color of the sample was measured using the Ocean Optics spectrophotometer.
2. The HAP (6.25%) suspension was applied continuously on the sample's surface for 30 seconds.
3. The sample was rinsed thoroughly with water.
4. After 90 seconds of air exposure time, the color was measured, using Ocean Optics.

4.3.1.3 NaOCl group

Third group discovered if the application of 3% NaOCl had any considerable effect on the samples' color. For color measurement, we only used the Ocean Optic spectrophotometer. For each sample in this group, we performed according to the following steps:

1. The baseline color of the sample was measured using Ocean Optics.
2. The NaOCl (3%) was applied continuously to the sample's surface for 30 seconds.
3. The sample was rinsed thoroughly with water.
4. After 90 seconds of air exposure time, the color was measured with Ocean Optics.

4.3.1.4 Peptide group

This group identified the whitening effect of peptide without any pre-treatment with NaOCl. Same as the second and third groups, for this group we only used the Ocean Optics spectrophotometer. The treatment process of each sample was conducted according to the following steps:

1. The baseline color of the sample was measured with Ocean Optics.
2. The peptide was applied continuously to the sample's surface for 30 seconds.
3. The sample was rinsed thoroughly with water.
4. After 90 seconds of air exposure time, the color was measured, using Ocean Optics.

4.3.1.5 Water group

This group served as the control group. The samples in this group received neither any treatment, nor any pre-treatment. In fact, we applied only mineral water (Evian) with the Micro brush to the samples in this group. The colors were measured with the Ocean Optics spectrophotometer. For each sample, we performed according to the following steps:

1. The baseline color of the sample was measured with Ocean Optics.
2. Water was applied to the sample's surface continuously for 30 seconds.
3. After 90 seconds of air exposure time, the color was measured, using Ocean Optics.

4.4 Results

4.5 Impact of spectrophotometer on color measurement

In order to identify if spectrophotometer had any significant impact on the main findings of the experiment, we compared the CIELAB values of the samples in the main suspension group containing both peptide and HAP, calculated by Ocean Optics and Color Eye. Figure 4-4 and Figure 4-5 show the distribution of CIELAB values.

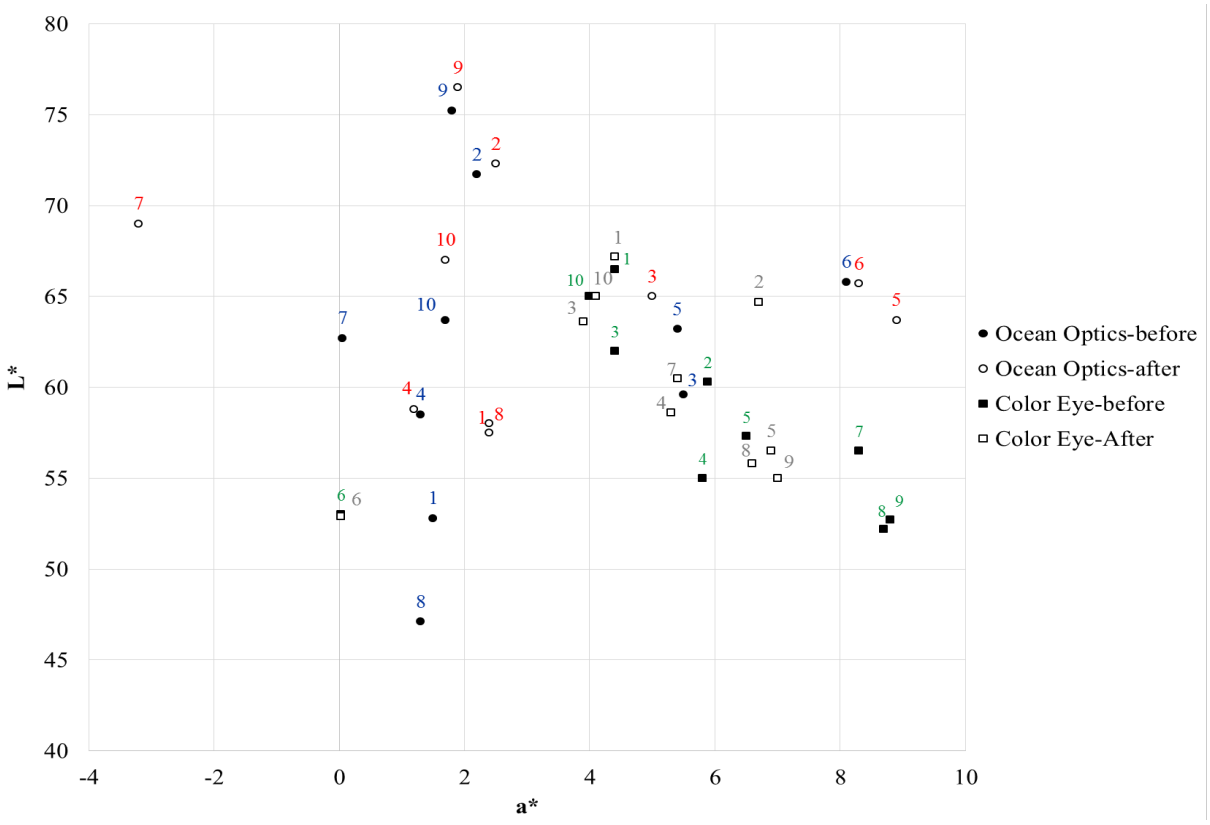


Figure 4-4 Distribution of CIE L* and a* values obtained from the Ocean Optics and Color Eye spectrophotometers, before and after treatment with the main suspension containing both peptide and HAP; the samples are labeled 1 to 10.

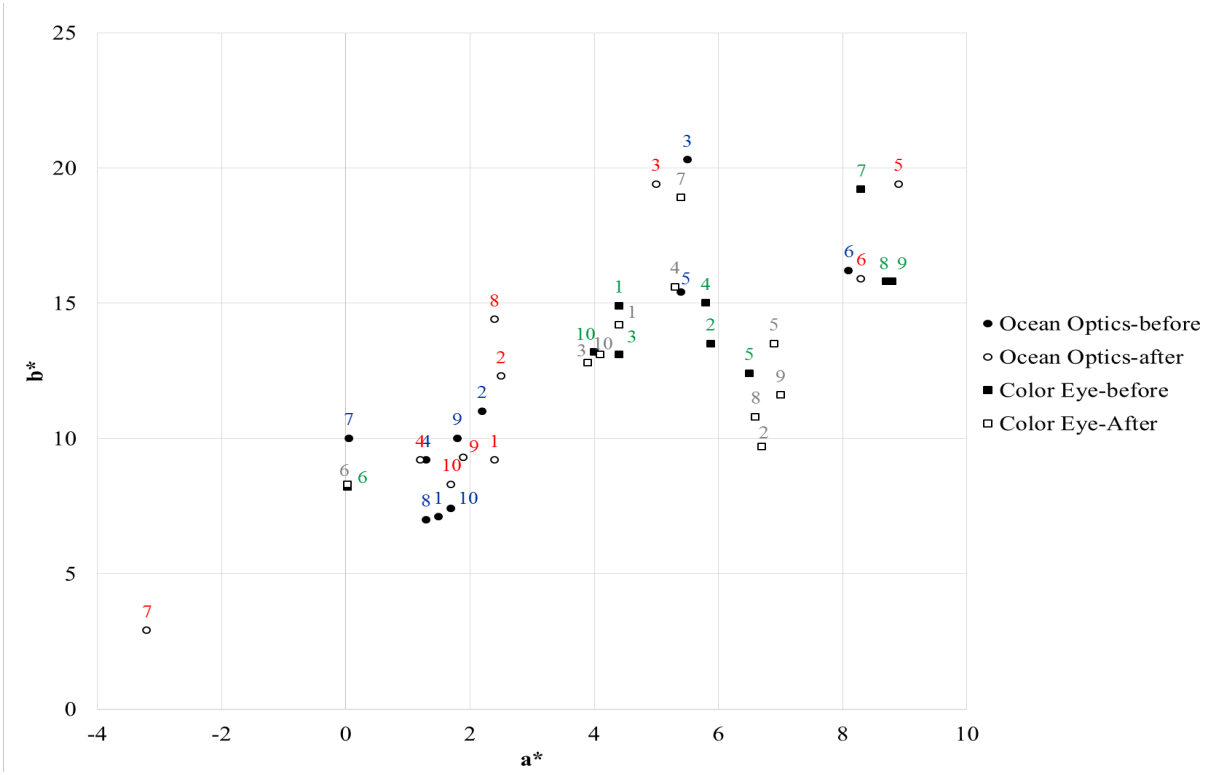


Figure 4-5 Distribution of CIE b* and a* values obtained from the Ocean Optics and Color Eye spectrophotometers, before and after treatment with the main suspension; the samples are labeled 1 to 10.

Figure 4-6 demonstrates the mean reflections that were determined by the spectrophotometers, before and after the treatment, for the samples in the main suspension group containing peptide and HAP.

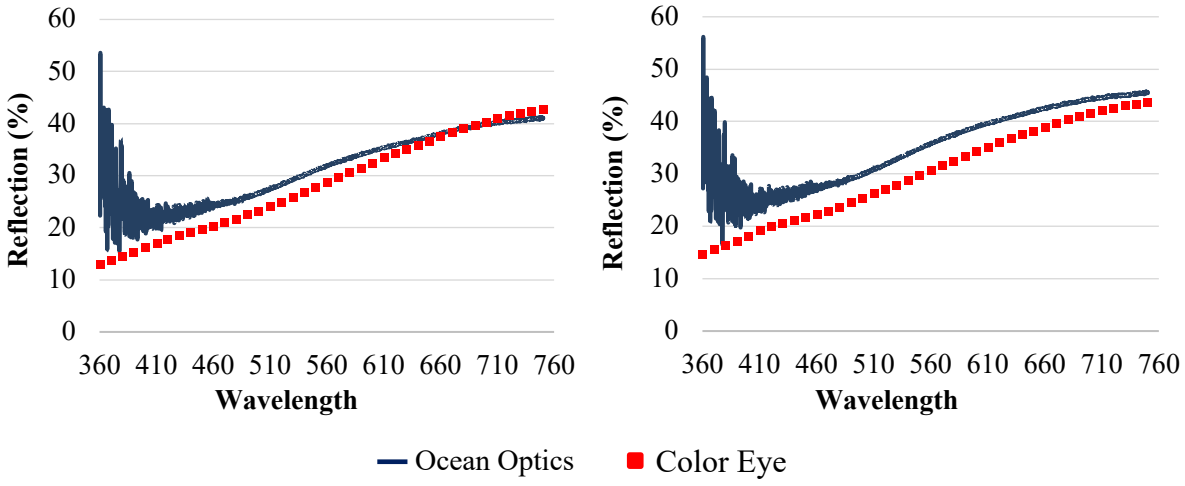


Figure 4-6 Mean reflection spectra of the mean suspension before treatment (left) and after treatment (right), obtained from the Ocean Optics and Color Eye spectrophotometers, before and after treatment with the main suspension containing P11-4 and HAP

It can be observed that for some samples such as number 6 and number 7, the spectrophotometers showed noticeably different values. However, to determine whether there were statistically significant differences between the results obtained from the spectrophotometers, it was necessary to conduct a statistical test. Since the same samples were measured by the devices, a paired t-test could be carried out. However, this test requires that the differences in ΔE values calculated by the devices are approximately normally distributed. Table 4-3 shows the result of normality tests. Considering the fact that the Shapiro-Wilk test leads to more reliable results in the presence of small number (< 50) of observations, here we only used the results of this test.

Table 4-3 The results of normality tests (Kolmogorov-Smirnov and Shapiro-Wilk) for differences in ΔE values measured by Color Eye and Ocean Optics

Group name	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Color Eye – Ocean Optics	0.262	10	0.051	0.869	10	0.097

a Lilliefors Significance Correction

It can be observed that p-value = 0.097 > 0.05 = α. Therefore, we could not reject the null hypothesis and concluded with 95% confidence that the differences between ΔE values calculated by the devices, were normally distributed. Hence, a paired t-test could be carried out. The null hypothesis of the test was that the mean difference between the ΔE values of the spectrophotometers is zero.

Table 4-4 shows the descriptive statistics of the ΔE values measured by each device. While the mean ΔE value of Color Eye was 4.637, this value for Ocean Optics was 1.584 lower.

Table 4-4 Paired samples statistics of the ΔE values measured by Ocean Optics and Color Eye

	Mean	N	Std. Deviation	Std. Error Mean
Ocean Optics	3.053	10	2.446	0.774
Color Eye	4.637	10	4.252	1.345

To see whether the mean difference was significant or not, Table 4-5 indicates the results of the paired t-test. As the *p*-value (0.248) was higher than 0.05, we can conclude that the there was no statistically significant difference between the mean ΔE values measured by Ocean Optics and Color Eye.

Table 4-5 Results of the paired t-test on the mean ΔE values calculated by the Ocean Optics and Color Eye devices

Group (I) – Group (J)	Paired Differences					t	df	Sig. (2-tailed)
	Mean (I-J)	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Ocean Optics – Color eye	-1.584	4.060	1.284	-4.488	1.320	-1.23	9	0.248

Once it was confirmed that the overall color changes measured by Ocean Optics and Color Eye spectrophotometers were not significantly different, we used only Ocean Optics to compare the mean ΔE values of the experimental groups.

4.6 Comparison of the reflections in the experiment groups

The box-and-whisker plots in Figure 4-7 compare the range of reflections over the wavelengths from 360 nm to 750 nm for each group before the treatment (i.e. baseline) and after the treatment. In the main suspension group (peptide and HAP), noticeable differences between the baseline and after the treatment were observed. The median of the reflections in this group increased from 32.4 before the treatment, to 36.3 after the treatment. In contrast, the reflections of the samples in the HAP group did not considerably change between the baseline and after the treatment. The median of this group decreased only slightly from 19.8 to 19.5 after the treatment. Applying peptide reduced the median value of the samples' reflections from 29.2 to 28.4, whereas using NaOCl increased the median value from 33.1 to 34.1. It is not surprising that the reflections of the samples in the water group did not noticeably change after applying water.

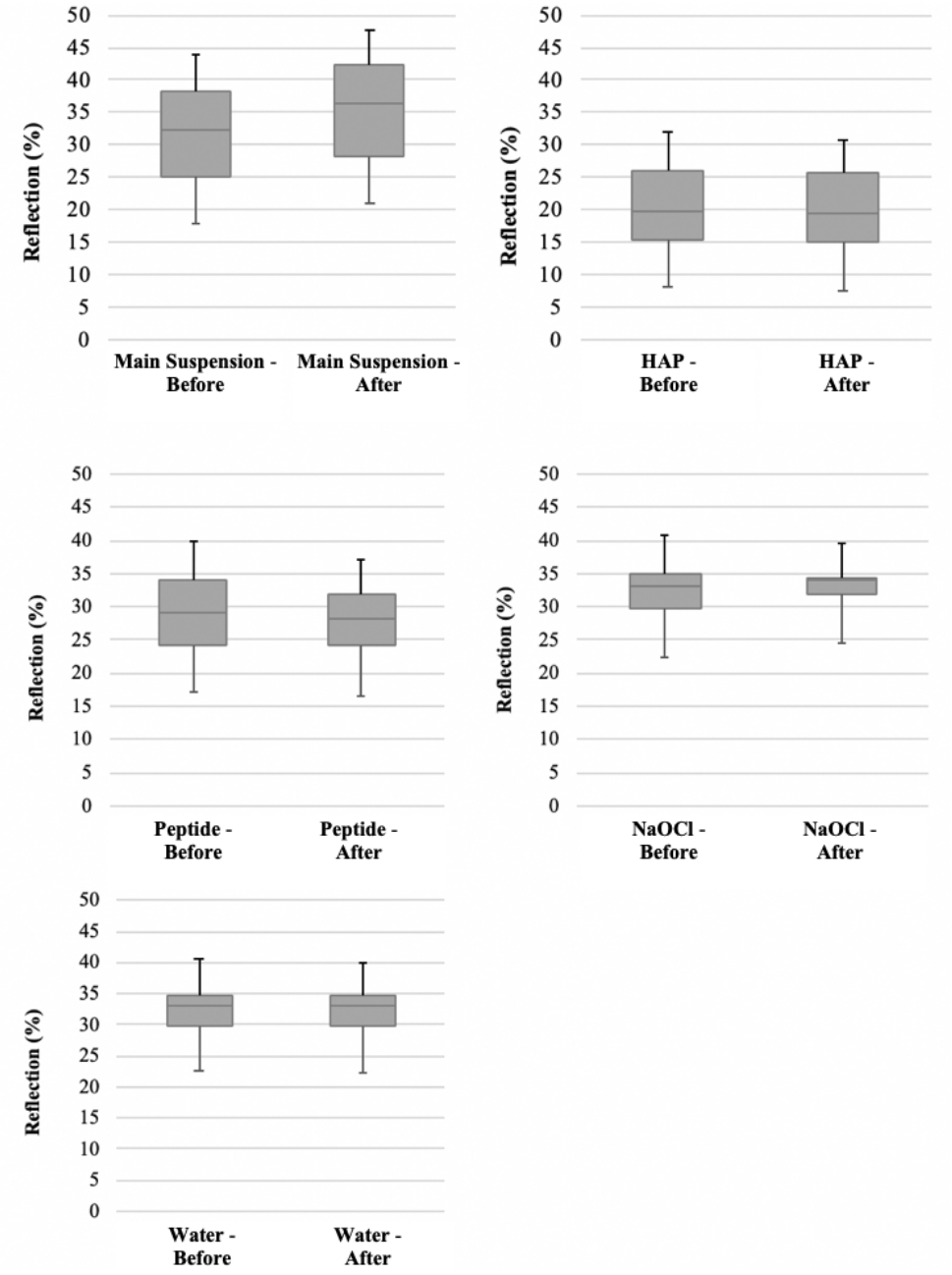


Figure 4-7 Box-and-whisker plot of the mean reflections of each group before treatment (i.e. baseline) and after treatment

The mean reflection spectra of the HAP, peptide and main suspension groups before and after treatment are given in Figure 4-8, Figure 4-9 and Figure 4-10, respectively. The visual assessment of the reflection spectra of the main suspension group showed that for almost all wavelengths there was a parallel shift of spectra, while the mean reflection changes in the peptide group and HAP groups presented a wavelength dependence. However, in both HAP and peptide groups, for the wavelengths from 360 nm to almost 500 nm, we observed more fluctuating differences between mean reflections before and after treatment.

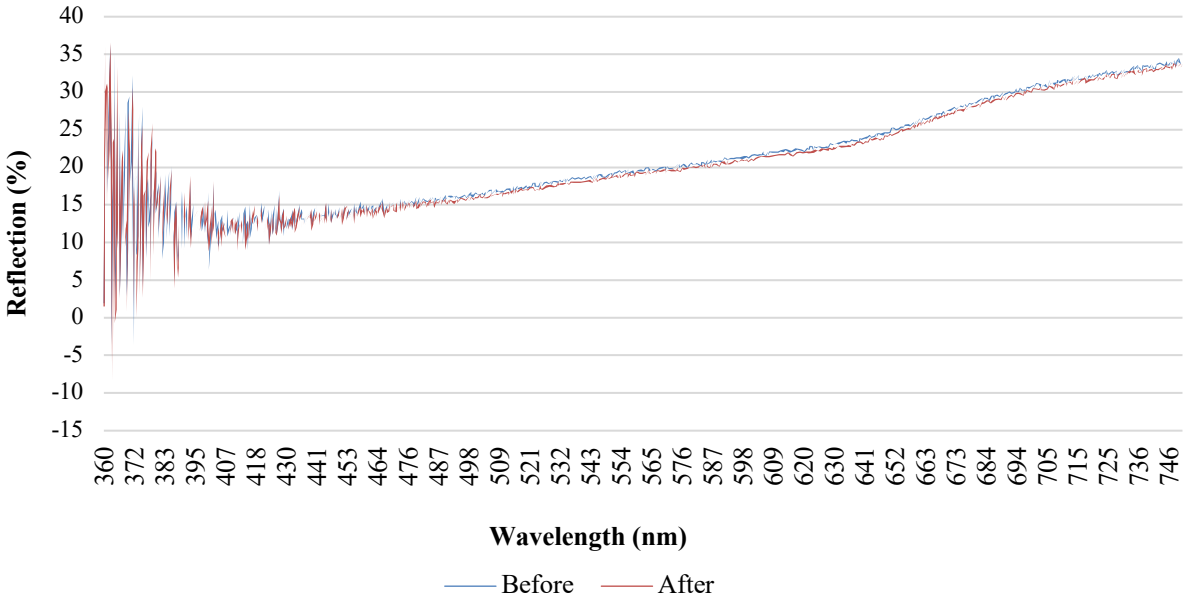


Figure 4-8 Mean reflection spectra of the HAP group before and after treatment for the wavelengths between 360 nm and 750 nm

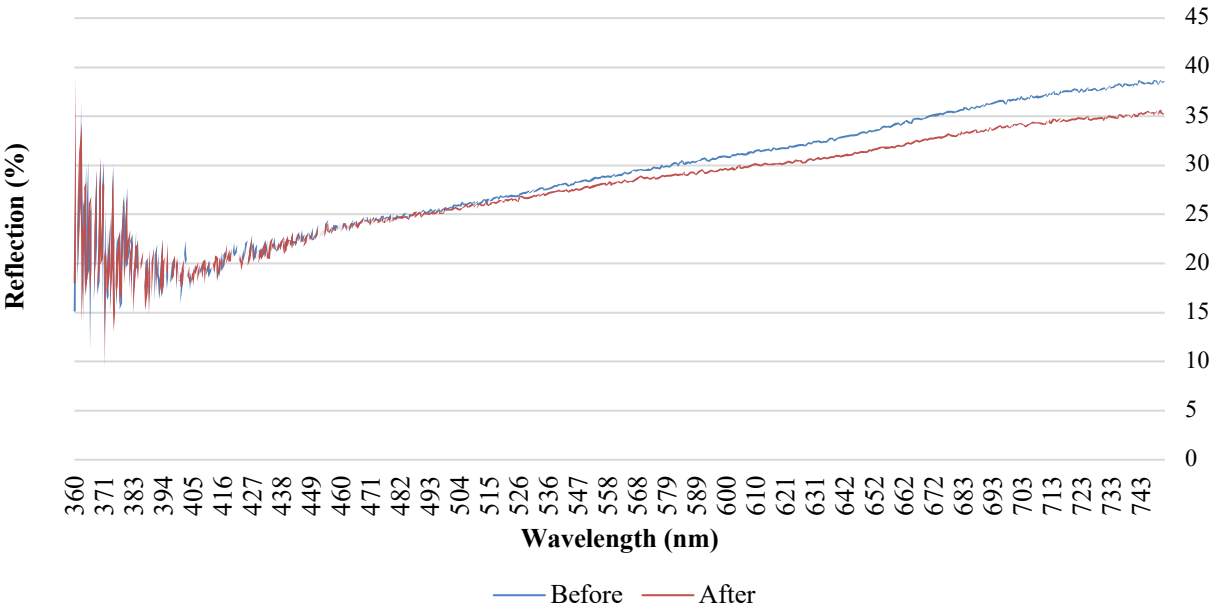


Figure 4-9 Mean reflection spectra of the peptide group before and after treatment for the wavelengths between 360 nm and 750 nm

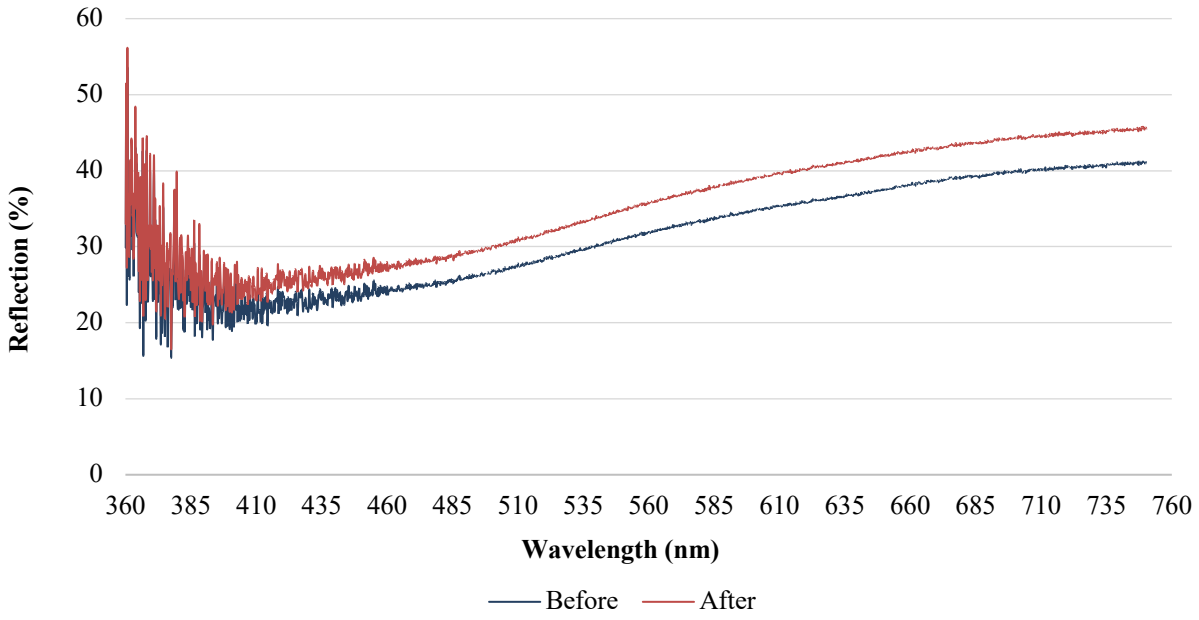


Figure 4-10 Mean reflection spectra of the main suspension group before and after treatment for the wavelengths between 360 nm and 750 nm

4.7 Comparison of the overall color changes in the experiment groups

To assess the overall differences between the groups, we compared the mean ΔE values of the groups, which were calculated relative to the baseline values. Table 4-6 presents relevant descriptive statistics, including the mean value, standard deviation, 95% confidence intervals, minimum and maximum for the ΔE values of each treatment group, as well as of all groups together. It can be seen that the main suspension group, with the mean equal to 4.64, showed the most pronounced overall color change compared to the other groups. In contrast, as expected, the water group represented the lowest mean with 0.99. On the other hand, it is interesting to note that the main suspension presented the largest standard deviation compared to the other groups. In fact, the perceived color changes of the samples in this group varied substantially from one to the other. As given in Table 4-6, the gap between minimum (0.32) and maximum (13.22) of this group was the most significant.

Next to water, HAP presented the weakest color change ($\Delta E = 1.27 \pm 1.32$). Furthermore, peptide, with ΔE equal to 2.33 ± 1.71, also showed a relatively weak color change. Although NaOCl was used as a pre-treatment agent, it caused a notable overall color change ($\Delta E = 3.90 \pm 2.79$).

Table 4-6 Descriptive statistics of ΔE values of each group, as well as for all groups

Group	Paired Differences					N	Min	Max
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Main suspension	4.64	4.25	1.34	1.59	7.68	10	0.32	13.22
Peptide	2.33	1.71	0.54	1.11	3.55	10	0.22	5.50
HAP	1.27	1.32	0.42	0.32	2.21	10	0.11	4.14
NaOCl	3.90	2.78	0.88	1.91	5.89	10	1.36	10.80
Water	0.99	1.17	0.37	0.15	1.83	10	0.30	4.20
Total	**2.62**	**2.82**	**0.40**	**1.82**	**3.43**	**50**	**0.11**	**13.22**

To explore whether there were significant differences between the overall color changes in the groups, a one-way ANOVA was carried out. The results of the test are shown in Table 4-7. The null hypothesis of the test was that the mean ΔE values of the studied groups are not significantly different ($\alpha = 0.05$). It is seen that the significance value between groups was 0.007 (i.e., $p = .007$), which is below 0.05. This rejected the null hypothesis and confirmed that there were statistically significant differences between the mean ΔE values of the groups.

Table 4-7 Results of the one-way ANOVA test for the ΔE values of the groups

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	102.75	4	25.69	4.03	0.007
Within Groups	286.92	45	6.38		
Total	389.66	49			

To identify which specific groups were significantly different, it was necessary to carry out a Post-Hoc test. It this case a Tukey's HSD test was employed. Table 4-8 provides the results of the test. The null hypothesis of this test was that there was no significant difference between the mean ΔE values of two specific groups ($\alpha = 0.05$).

According to this table, the mean ΔE difference between main suspension and peptide was 2.31 and the corresponding Sig. value was 0.26. Furthermore, the mean ΔE difference between main suspension and NaOCl was 0.74 and the corresponding Sig. value was 0.97. Therefore, although the main suspension group presented more obvious overall color change than peptide and NaOCl, the observed differences between these groups were not significant. In contrast,

main suspension presented significantly stronger overall color change in comparison with HAP ($p = 0.04$) and Water ($p = 0.02$).

On the other hand, the Table 4-8 indicates that the difference of the water group with the peptide, HAP and NaOCl groups was not statistically significant. Suffice to say that although neither peptide nor HAP nor NaOCl resulted in significantly different overall color change compared to the water group, the main suspension group that combined peptide and HAP and used NaOCl as a pre-treatment agent, presented significantly stronger overall color change than the water group.

Table 4-8 Results of the Tukey's HSD test for the ΔE values of the groups

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Main suspension	Peptide	2.31	1.13	0.26	-0.90	5.52
	HAP	3.37	1.13	0.04	0.16	6.58
	NaOCl	0.74	1.13	0.97	-2.47	3.95
	Water	3.65	1.13	0.02	0.44	6.85
Peptide	Main suspension	-2.3	1.13	0.26	-5.52	0.90
	HAP	1.06	1.13	0.88	-2.15	4.27
	NaOCl	-1.57	1.13	0.64	-4.78	1.64
	Water	1.34	1.13	0.76	-1.87	4.55
HAP	Main suspension	-3.37	1.13	0.04	-6.58	-0.16
	Peptide	-1.06	1.13	0.88	-4.27	2.15
	NaOCl	-2.63	1.13	0.15	-5.84	0.58
	Water	0.28	1.13	1.00	-2.93	3.49
NaOCl	Main suspension	-0.74	1.13	0.97	-3.95	2.47
	Peptide	1.57	1.13	0.64	-1.64	4.78
	HAP	2.63	1.13	0.15	-0.58	5.84
	Water	2.91	1.13	0.09	-0.30	6.12
Water	Main suspension	-3.67	1.13	0.02	-6.85	-0.44
	Peptide	-1.34	1.13	0.76	-4.55	1.87
	HAP	-0.28	1.13	1.00	-3.49	2.93
	NaOCl	-2.91	1.13	0.09	-6.12	0.30

4.8 CIELAB values of the main suspension

Once we recognized the main suspension had a significant overall color change effect on the samples, to provide a more detailed assessment, we compared the CIELAB values of the samples in this group before and after the treatment. As illustrated in Figure 4-11, the treatment with this agent increased all three CIELAB values.

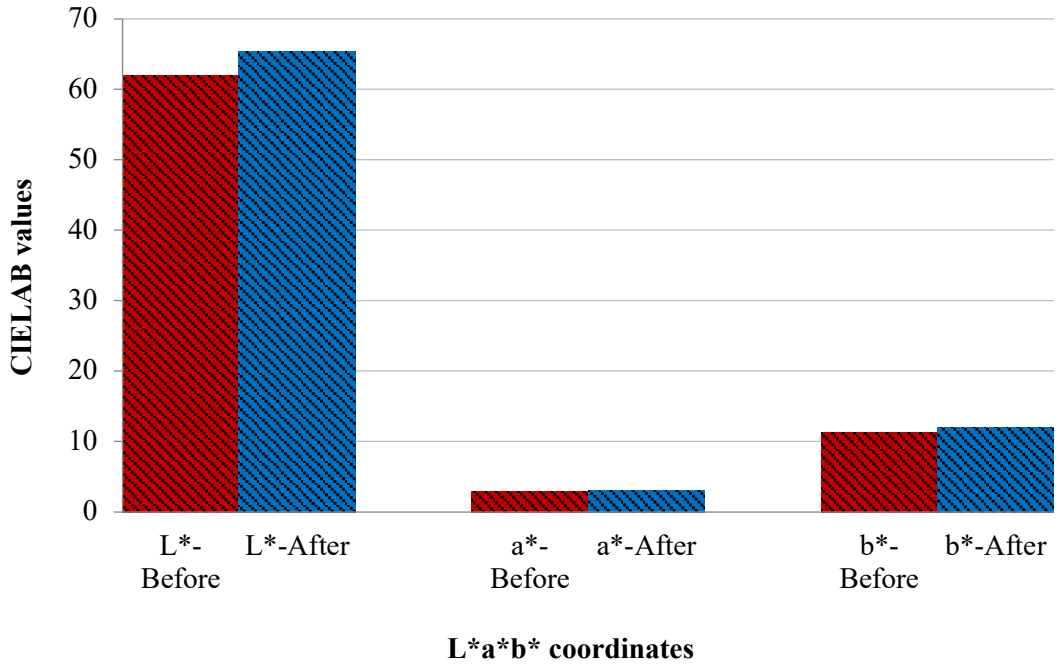


Figure 4-11 The mean CIELAB values before and after the treatment with the main suspension

In absolute terms, L^* experienced the strongest growth ($\Delta L^* = 3.22$), followed by b^* ($\Delta b^* = 0.67$). The red-green factor (a^*) increased from 2.89 before the treatment to 3.11 after the treatment ($\Delta a^* = 0.22$) (Table 4-9).

Table 4-9 The ΔL^*, $\Delta a^*$and Δb^* mean values and the associated color perception for the main suspension

	ΔL*	Δa*	Δb*
Mean value	+3.22	+0.22	+0.67
Color perception	Lighter	Redder	Yellower

4.9 Discussion

4.9.1 Spectrophotometer

Visual color assessment is unfortunately neither accurate nor reproducible due to various subjective and objective factors. Since tooth color assessment is one of the most challenging aspects of esthetic dentistry, it is of crucial importance to use a reliable, reproducible and above all accurate and precise device for color measurement.

Dozic et al. (2007) concluded that digital tooth color measuring devices (e.g. spectrophotometer) is one of the most reliable color assessment methods in both *in vitro* and *in vivo* circumstances.

We used in the present chapter two different spectrophotometers in order to see if the type of spectrophotometer had any impact on the main findings. The Ocean Optics device was used in all parts of the present chapter, while Color Eye 7000A spectrophotometer was employed only in the first section of this chapter. The Color Eye spectrophotometer is a highly standardized integrating (Ulbricht) sphere in the industry.

One of the main characteristics of the Color Eye spectrophotometer is its wide measurement window, while the Ocean Optics possess a small measurement aperture with less light scattering. To reduce errors associated with the light, all the measurements were carried out in the dark. Furthermore, to ensure the quality of measures, test-runs were performed. Although for some samples the spectrophotometers showed different color values, according to the results of the statistical test, the type of spectrophotometer did not have any significant impact on the results of the experiment.

One of the challenges of this part of study was the lack of standard measurement conditions (e.g. measurement window). The Ocean Optics has been used with very small-sized apertures, while Color Eye has a measurement window of approximately 8 mm. A wider measurement window detects a larger area of the samples. This may result in more accurate measurement because by using a small measurement window, a spot on the measured surface or a limited surface inconsistency in the detected area may bias the measurement results. On the other hand, a small measurement window may cause difficulties to obtain reproducible results. However, the small-sized aperture of Ocean Optics may induce less light scattering than Color Eye.

Furthermore, the measurements with Ocean Optics were taken at 2-degree standard observer, whereas the Color Eye could only be set at 10-degree standard observer.

The negligible variations in results can be explained by different constructions of measurement devices and methods. As demonstrated in Figure 1-6, the reflection probe of Ocean Optics measures the reflection only at a certain angle, the so-called specular reflection. While the Gretag MacBeth includes an integrated sphere coated with a highly smooth and reflective white layer (e.g. barium sulfate), a light source and a baffle (Figure 4-12). According to Sanderson,(2015), the inner surface of the integrated sphere makes it possible that both specular and diffuse reflections are detected by the sensor. Sanderson claimed that although the textured surface (treated surface) cause more diffuse reflection and scatter, it is not possible to suppress the specular reflection completely by using Color Eye spectrophotometer.

Even though the reasons for these findings cannot be readily clarified, it seems most likely that a same-sized measurement window would have more similar results from the devices.

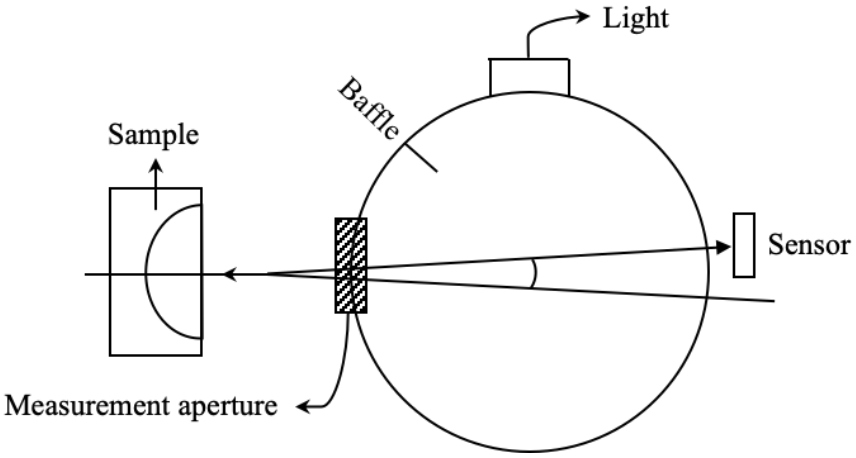


Figure 4-12 Schematic drawing of Gretag MacBeth; integrated sphere

As recommended by Tiznado-Orozco et al. (2009) and Baldassarri et al. (2008), during all shade measurements, each sample was kept humid to avoid its dehydration which may have an effect on samples 'color. According to the discussion of chapter 2, humidity and dehydration induce changes in tooth color. The level of humidity or dehydration could not be accurately adjusted for all of the samples. This might also have an influence on our results showed in table Table 4-6.

4.9.2 HAP-based agents

HAP-containing formulations have been recently proposed as a whitening agent. They may be seen as a potential alternative for oxidizing whitening chemicals. Further, the experiment of Yamagishi et al. (2005b) presented that a new HAP layer could be grown and adhered

on the enamel surface after treatment with fluoride-substituted HAP. They showed that using phosphoric acid increases enamel surface roughness and dissociation of ions, which can contribute to more aggressive growth of HAP crystals compared to calcium phosphate clusters. It is interesting to note that the crystals of the new nucleated HAP layer had a similar orientation as the original apatite crystals.

By using non-dissociated calcium phosphate clusters, we can reach a HAP layer nucleated with a random orientation. This can contribute to tooth whitening because of the diffuse reflection from the new generated HAP layer on the tooth surface, yielding the tooth seems brighter. Using phosphoric acid can cause severe soft tissue injuries. Hence, this method should be performed under dentist's supervision.

Many studies argued that using HAP nanocrystals makes it possible to t reat the tiny early lesions of enamel (Ahmadian et al., 2018) . These studies concluded that Nano-HAP promotes remineralization of the superficial enamel caries lesion (e.g. white spots). The white spots are caused by porosity in underlying enamel, which is resulted from acid dissolution of HAP crystals structure. In order to trigger the remineralization of this lesion, a stable scaffold is required to template HAP formation (Kirkham et al., 2000). This scaffold requires to be able to permeate into the enamel surface.

It is important to note that full remineralization is not plausible under non-acidic conditions. As pH decreases, Nano-HAP can accelerate the depth and the rate of penetration (Deyhle et al., 2015; Huang et al., 2011). This method could contribute to the reduction of lesion depth without drilling a hole in the tooth, which leads to removing a considerable amount of healthy tissue. This fact shows another benefit of using HAP-containing agents.

Calcium phosphate nanoparticles are applied recently in many toothpastes and dental health care products (Enax and Epple, 2018). The potential exposure ways of these nanoparticles include oral uptake, inhalation, skin and mucosal contact. There have been some concerns about their potential negative biological impacts and possible health risks associated with long-term uses of these products. Epple (2018) discussed in his study the potential health risks associated with oral exposure to calcium phosphate nanoparticles. It should be note that milk also contains a considerable amount of calcium phosphate nanoparticles which are stabilized by casein (Cross et al., 2005). In case during tooth brushing these particles are accidentally swallowed, the highly acidic pH of stomach helps to dissolve them completely (Peitsch et al., 2010).

In a nutshell, adverse health consequences by an oral intake of calcium phosphate nanoparticles can be neglected because they have no chance to survive the acidic condition in the

stomach, unless the swallowed amount is more than ten grams. Epple concluded that the potential health risk is limited to notably high exposition levels and particle doses. Therefore, calcium phosphate nanoparticles can be assumed as safe for human body.

4.9.3 Sodium hypochlorite as the pre-treatment

The smear layer caused by polishing the samples surface after embedding and also the chromogenic particles located on the surface after staining are the organic layers which block the surface and may interfere with the adherence of the P11-4-HAP layer.

Considering the chemical properties of sodium hypochlorite, it is known to be strongly effective at removing organic materials. Sodium hypochlorite can remove the organic layer by oxidizing it. Hence, the smaller degraded molecules could be simply washed away.

Penumatsa and Sharanesha (2015) used 5% NaOCl as an effective agent for bleaching of fluorosis stains. They reported that sodium hypochlorite should be applied continuously for 25-30 minutes in order to achieve an acceptable bleaching result.

Our aim was to use sodium hypochlorite as pre-treatment to discard the smear layer and expose the binding sites of the enamel surface to the main suspension containing peptide and HAP in order to gain a better adherence of the P11-4-HAP layer on the enamel surface.

Since NaOCl gets in contact with the chromogenic particles of the samples for a considerable period of time, it can also be removed in the same way as organic smear layer, which leads to bleaching the samples. We observed a notable overall color change after treatment with NaOCl (Table 4-6). This bleaching effect of NAOCl is caused by oxidation. Therefore, in order to avoid bleaching the samples after pre-treatment before the application of the main suspension, we used a low concentration of sodium hypochlorite for a very short period of time to dissolve and remove only the organic components of the smear layer caused by polishing. In addition, it is noteworthy that the baseline colors of the samples in main suspension group (Peptide + HAP) were measured after pre-treatment.

The use of sodium hypochlorite for a prolonged period of time may have some adverse side effects such as mucosa ulcerations and allergic reactions (Hülsmann and Hahn, 2000). Notwithstanding, due to the high tissue dissolution capacity and aggressiveness of NaOCl, it should be applied to the tooth surface using a delicate applicator and the teeth should be isolated before treatment for example with a rubber dam and each tooth have to be ligated to protect the oral mucosa. Consequently, it cannot be used by patients as an ingredient of their

mouthwash. This open roads for future research to find an alternative safe agent as a pre-treatment solution removing organic components of the smear layer.

Due to the reasons discussed earlier, our target was not to use NaOCl intraoral, rather we aimed at evaluating whether an organically contaminated surface (e.g. polished surface or pellicle) after removing the organic components of the layer may influence the results of the treatment.

4.9.4 Self-assembly P11-4 peptide

The P11-4, known as a small molecule, is capable of forming a 3D biocompatible matrix with enamel surface characteristics mimicking the enamel matrix. In the same way as for natural intraoral tooth remineralization, the formed 3D matrix may be surrounded by calcium and phosphate ions that are available in saliva.

Peptide can develop fibrillar scaffold after self-assembling in response to specific environmental triggers. By simply changing pH can the peptide switch to soft like and solid like materials such as liquid crystals, nematic gels or organogels. It is able to template de novo hydroxyapatite crystal nucleation. On the other hand, it supports mineral crystal growth in a process of biomimetic mineralization

After increasing in pH, The P11-4 self-assembling peptide switches from the nematic hydrogel state to fluid (Hug and Lysek, 2015). After applications of the nematic gel, β-sheet fibrillar networks with biological functions form. They include scaffolds for cell growth and templates for mineral growth. At low pH showed the P11-4 peptide a nematic texture, it was monomeric in solution at high pH (7 < pH). The fibrils form nematic gel within 1 < pH < 3. While within 3 < pH < 5 flocculation occurs (Aggeli et al., 2001).

Each fibril of new formed P11-4 network encompasses 4 ribbons. The acidic glutamate residues which are arrayed along the fibrillar surface and have an adverse charge domain, is able to contribute to calcium ion binding by providing nucleating sites for HAP crystals. Whereas, the hydrophobic residues (e.g. Phenyl) may contribute to the peptide assembly (Palmer and Stupp, 2008). He et al. (2003) also reported that the self-assembled β-sheet cationic domains of dentin matrix protein. The peptide-amphiphile makes a nanostructured fibrous scaffold in response to changing the pH. According to Hartgerink et al. (2001), the nanofibers can be cross-linked through the formation of intermolecular bonds (disulfide bonds) resulting in robust fibers which are capable of nucleate HAP on their surface .

The formation of the 3D scaffolds which can promote the deposition of HAP on their surface is induced by specific environmental conditions. The peptide switches from a low viscosity state to a gel state at pH values which are lower than 7.4 (Ceci et al., 2016). The lateral chains of bioactive peptide scaffold are able to pull calcium ions. It causes the precipitation of hydroxyapatite de novo (Kirkham et al., 2007). In many *in vivo* and also *in vitro* and studies, the assembled P11-4 fibers have been proven to be highly temperature stable and biocompatible (Aggeli et al., 1997).

To support the described hypothesis, some studies have shown that peptide does not move the equilibrium toward demineralization, rather it moves it toward remineralization. Accordingly, it results in a net mineral gain after application of peptide to artificial lesions of enamel following 5 days of cycling in an oscillating pH model (Kirkham et al., 2007). In our study, we suggested the application of the peptide mixed with the HAP suspension can also be effective in improving tooth whitening. Our results showed that the whitening efficacy of the main suspension was better than the HAP group. This is in line with the results obtained by the Kirkham.

Moreover, in this chapter, the main suspension was applied only one time to the samples. Based on the findings of chapter 2, the desired whitening effect was achieved after only one application. This can be justified by the following reason. After one application, the maximum whitening efficacy has already been achieved by maximum surface loading.

P11-4 is a pH responsive peptide, which exists as monomer of random coil configuration at pH 9 and switches to β-sheet conformation upon reducing the pH to 7.4 (Kyle et al., 2010).

The pH value of the suspension that we used as the main suspension was approximately equal to 6.5 (Figure 4-13), which was slightly acidic. According to Aggeli et al., (2003), at pH 6.6 the viscosity exhibits a decrease and consequently the peptide switches in the fluid nematic state (viscoelastic fluid). We measured the pH value of the suspension by using a pH paper. The pH value of the suspension could not be measured with an electrical pH device because of its viscosity. Besides, the electrodes of these devices are too large and it could be damaged during measurement.

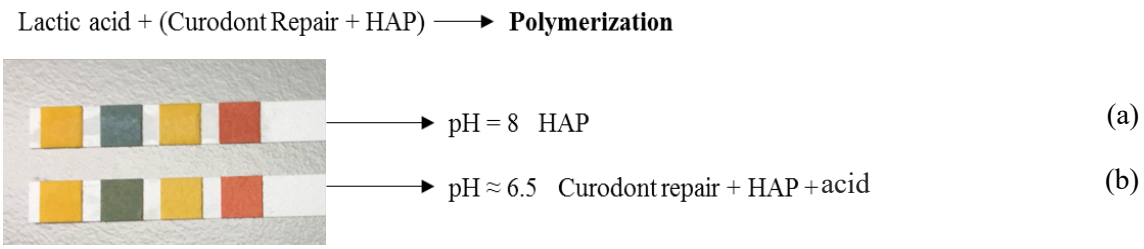


Figure 4-13 The alkaline pH of HAP (a). The acidic pH of the main suspension (b) in which P11-4 self-assembling peptide is in the fluid nematic state

In the previous studies that dealt with P11-4 for caries therapy, the teeth received an acid treatment in order to open the micropores and to remove the organic material before the application of the peptide (P. A. Brunton et al., 2013). Based on these studies, after preparing the tooth surface with acid, the monomeric peptide was applied to the initial enamel lesion. They showed that peptide penetrates the lesion in a better way after a pre-treatment with an acidic agent and assembly occurs within the enamel lesion. The matrix can operate as nucleator and increase net mineral gain resulting in tissue regeneration from within.

Bailey et al. (2009) argued that the remineralization of a more porous enamel surface is much easier. This fact is due to a better penetration of the minerals into the enamel, which are required for remineralization process. Beside in the literature introduced common approaches for surface scratches such as acid etching, the tooth bleaching is also an effective method to increase enamel roughness and porosity of the surface. This damaged dental tissue could be protected by using a nano-carbonate apatite agent which is additionally able to maintain the same whiteness achieved after the bleaching (Kim et al., 2011).

In this experiment, a pre-treatment with an etching solution was not performed. We skipped this step because etching the enamel increases the porosity and roughness. Our aim was to make the teeth appear whiter without causing enamel damages.

A number of studies (e.g. Gerlach et al., 2000) dealing with tooth whitening, claimed that the yellow-blue value is the most crucial factor for the perception and interpretation of tooth color. According to their researches, the b^* value reduces faster and stronger than the L^* value (Gerlach et al., 2002). This Finding was also used in developing the blue covarine whitening toothpastes. After treatment with blue covarine, the presence of the thin blueish layer on the surface should induce a reduction in the b^* value of the samples (Dantas et al., 2016; Joiner et al., 2008). This is in accordance with our results that b^* value was reduced after treatment in both peptide and HAP group, decreasing the yellow appearance of the samples. Our results

indicated that P11-4 without the presence of HAP has also an influence on samples color. One can argue that peptide can fluoresce similar to blue covarine. The pigments that show an optical effect (e.g. blue covarine), are able to modify the perception of yellowish color of the tooth. They act by depositing an almost transparent blue and thin layer on the tooth. By shifting the tooth color toward white, it appears visually brighter and whiter (Vaz et al., 2019; Collins et al., 2008).

The analysis of the Figure 4-8, Figure 4-9 and Figure 4-10 showed that only in the main suspension group that received the pre-treatment, treatment noticeably increased the mean reflection spectrum for almost all the wavelengths. This is in line with our hypothesis that the presence of a barrier on the surface may have negative impact on the adhesion of peptide and HAP particles. The qualitative assessment of the figures clearly demonstrated that in the peptide and HAP groups some wavelengths were reflected more strongly than others.

It is known from dental composites that the opalescence effect can be affected by the particle size. The same behavior could be expected from the HAP particles on the surface (Y. K. Lee et al., 2005). In accordance to Lee et al., (2005), the light scattering may be affected by the size and number of the HAP particles on the surface. For example, if the size of the HAP particle is similar to the wavelength of green or blue light, the reflected light from the particles on the surface would be green-blue or blue-violet.

We determined the size of HAP particles with the aid of SEM pictures (Chapter 5). The HAP particles were notably smaller than the wavelengths of visible light (380 nm – 740 nm). Therefore, the results in Figure 4-7, Figure 4-8 and Figure 4-9 may also be explained by the Rayleigh's scattering law (Young, 1982). The Rayleigh scattering introduced by Lord Rayleigh, describes elastic scattering of light or any electromagnetic radiation by particles. It applies only if the particles have a noticeably smaller size than the wavelengths of the visible light. However, the HAP particles might be also agglomerated to build bigger HAP particles.

On the other hand, since the samples were randomly assigned to the studied groups, we did not control the samples' baseline color before the experiment started.

To find a rational explanation for our results, we re-evaluated the baseline color of each sample entered the experimental groups. We observed that some samples in the HAP and peptide groups were accidentally relatively dark in the baseline (low L^* values). This may be responsible for the lack of an ideal and apparent whitening efficacy after treatment in peptide group.

Based on our results, we can claim that the peptide group led to noticeable color changes. This might also be explained by the phenomenon that describes how proteins absorb the electromagnetic wavelengths from the visible light spectrum, transform the light and emit it with a different reflection, as a different color. However, the wavelength of the light that is emitted has a lower level of power.

The presented results confirmed that the samples received the pre-treatment and subsequently treated with the main suspension showed increasing in lightness. Moreover, the overall color change of this group was statistically significant compared to the water group. In contrast, the NaOCl, HAP and peptide groups did not show statistically significantly stronger overall color change than the water group.

It was also observed that the main suspension group which contained both peptide and HAP and received pre-treatment presented the highest standard deviation. This might be explained by the reason that the effectiveness of the treatment agent may depend on the staining intensity of the teeth before the treatment.

The proposed suspension can provide the ambitious users a new additional daily dental care, which may restore tiny enamel decays and enhance the whiteness and smoothness of the teeth, simultaneously.

We used in our study NaOCl in order to remove the organic layer (e.g. smear layer, pellicle, etc.) from the surface before the treatment with the proposed suspension. The proposed suspension can be introduced on the market as a toothpaste or a mouthwash. The effect of a mouthwash is, however, mostly chemical. Due to its high tissue dissolution capacity and aggressiveness, NaOCl cannot be used by patients as an ingredient of their mouthwash or toothpaste. Besides, a mouthwash cannot destroy and remove the pellicle during rinsing. This is due to the fact that by using a mouthwash, perhaps there is a slight mechanical mixing of peptide-HAP and pellicle possible. This may be slightly possible owing to the activity of intraoral muscles during rinsing, while we could speculate that during tooth brushing with a peptide- HAP-based toothpaste, it may be mix with pellicle mechanically. The effectiveness of this mechanical action, however, depends on whether a manual toothbrush or a powered one is used. According to Rosema et al. (2008), the powered toothbrush was found to be more effective.

Chapter 5: The visualization of the newly formed hydroxyapatite layer on the bovine enamel by using Scanning Electron Microscopy (SEM)

5.1 The aims of the field-emission scanning electron microscopy analysis

This chapter consists of two main parts. The first part aimed at illustrating aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% on glass slides without storage and after storage in natural and artificial saliva for 24 hours at 37°C. The second part evaluated the structure changes of bovine enamel surface after treatment with the P11-4-HAP suspension introduced in the previous chapter.

5.2 Material and method

The materials and devices used in the present chapter are summarized as follow (Table 5-1):

Table 5-1 the Materials and devices used in chapter 5

Material	Company
Curodont Repair	Curodont, Credentis AG, Windisch, Switzerland
Curodont protect	Curodont, Credentis AG, Windisch, Switzerland
Aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP	Curodont, Credentis AG, Windisch, Switzerland
NaOCl 3%	Hendinger, Aug. Hedinger GmbH & Co. KG, Stuttgart, Germany
HAP suspension (6.25%)	
Lactic acid 0.1 mol- pH 4,0	Pharmacy University of Munich, Munich, Germany
Dentobuff strips “saliva kit”	Orion Diagnostics Ltd
Scanning electron microscope	ZEISS Supra 55vp; Zeiss, Oberkochen, Germany
vacuum evaporator	SC7620, Polaron, Quorum Technologies, Kent, UK
Diamond saw	Leica SP 1600-Saw Microtome, Leica Microsystems Nussloch GmBH, Nussloch, Germany

5.2.1 Demonstration of the aqueous HAP-P11-4 suspension on the glass

5.2.1.1 Study design

The aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP was applied on three microscopic slides with identical size (5 mm*5 mm), using a micro-brush (n = 3). The samples were air-dried for

24 hours at the room temperature. The first sample was observed employing a field-emission scanning electron microscope (ZEISS Supra 55vp; Zeiss, Oberkochen, Germany) at 10 kV and the working distance of 3 mm and with the magnification of 35.000x. The image was recorded and is given in Figure 5-1.

Two other samples treated with the same concentration of peptide-HAP suspension, were immersed in two different containers of natural saliva and artificial saliva. The second sample was treated with the suspension and was immersed in natural saliva for 24h at 37°C. The sample was air-dried at the room temperature and subsequently the image was taken with SEM at 35.000x magnification, 10 kV and a working distance of 5 mm (Figure 5-2). The third sample received also the same treatment and stored in artificial saliva for 24h at 37°C. Then, the sample was exposed to air to dry at the room temperature before the observation. Finally, the image was detected at 35.000x magnification, 10 kV and 5 mm working distance (Figure 5-3).

5.2.1.2 SEM Results and Analysis

The SEM micrographs of glass slides after treatment and the slides which were treated and immersed in natural and artificial saliva are presented in Figure 5-1, Figure 5-2 and Figure 5-3. obvious variation between three samples can be observed. The specimens immersed in pooled human saliva showed a formed pellicle film on the surface of the slide. The treatment agent located on the slide, seemed to be covered with saliva proteins. In contrast, the slide treated with P11-4-HAP suspension and immersed in artificial saliva did not show a pronounced growth of HAP particles. The saliva-coated particles were not as obvious as the group stored in natural saliva. It was observed that the sample without any storage showed small, mostly single particles on the glass. In contrast, after storage in the natural saliva, we did not observe single particles, rather paste-like structure. Furthermore, the sample stored in the artificial saliva showed cloudy aggregates of particles.

The analysis and comparison of the samples showed that there is a definitive difference between artificial saliva and natural saliva as the storage media, which was a crucial point in our study. This fundamental difference may refer to the high protein and high microbial content of natural saliva.

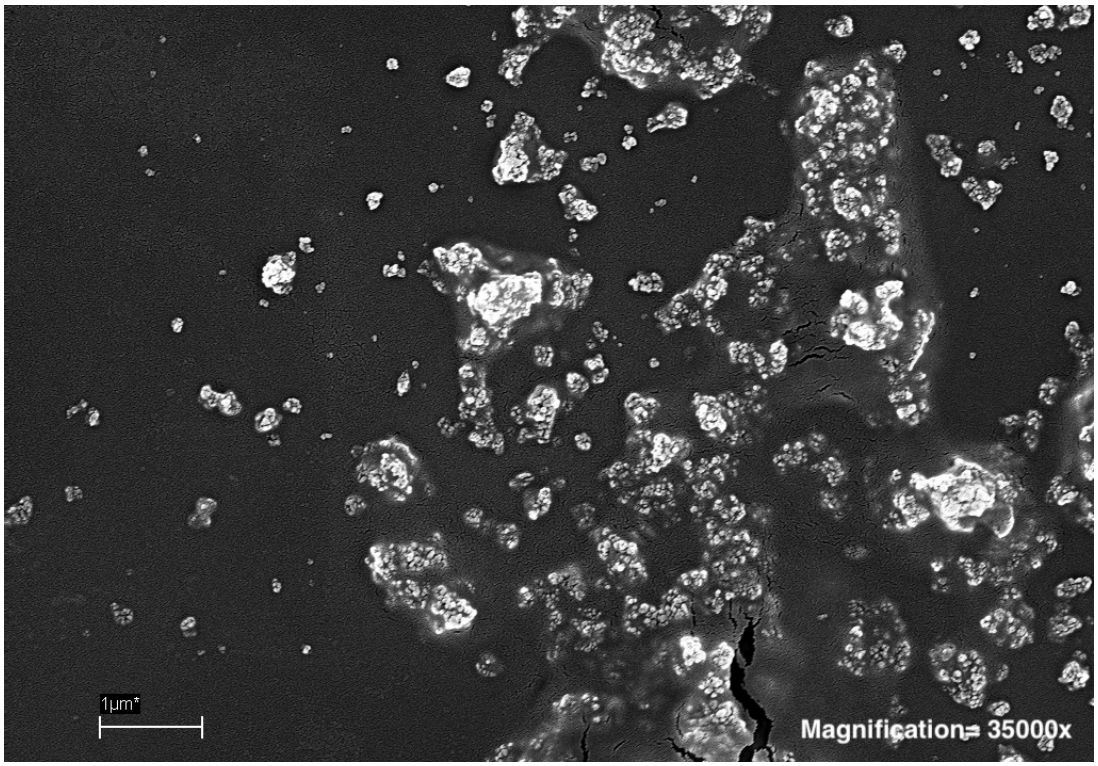


Figure 5-1 SEM micrograph, demonstration of aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP on the glass without storage

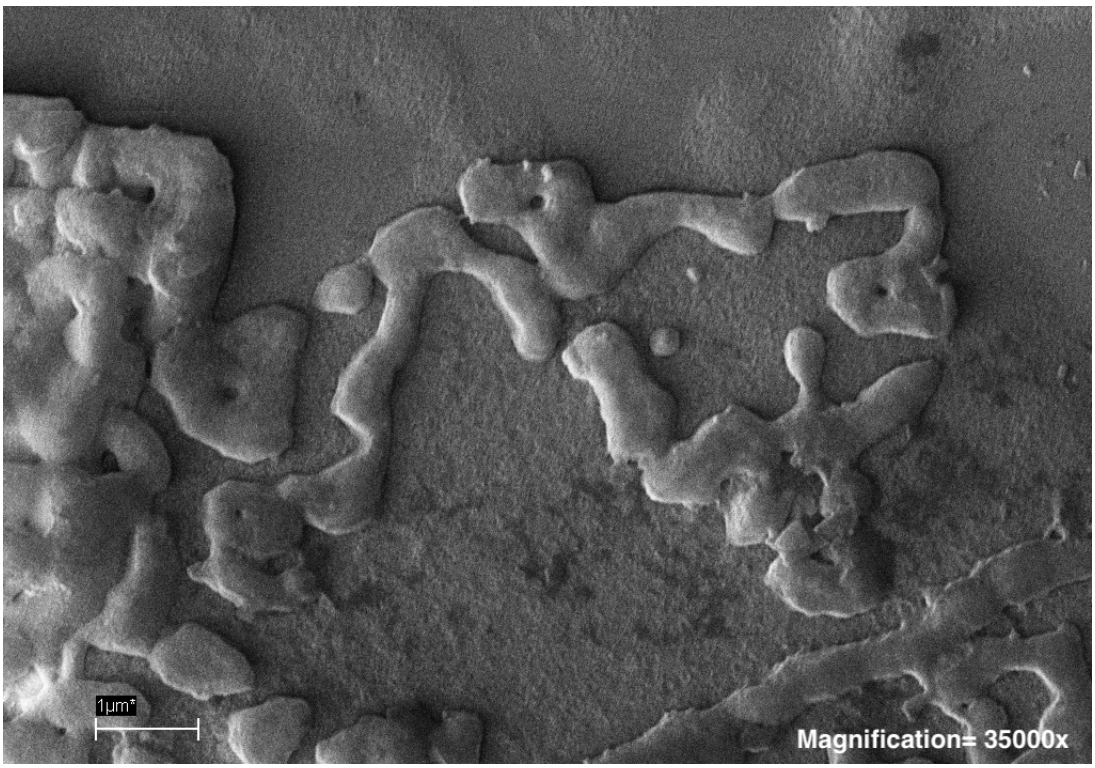


Figure 5-2 SEM micrograph, demonstration of aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP on the glass after 24 hours of storage in natural saliva at 37 °C

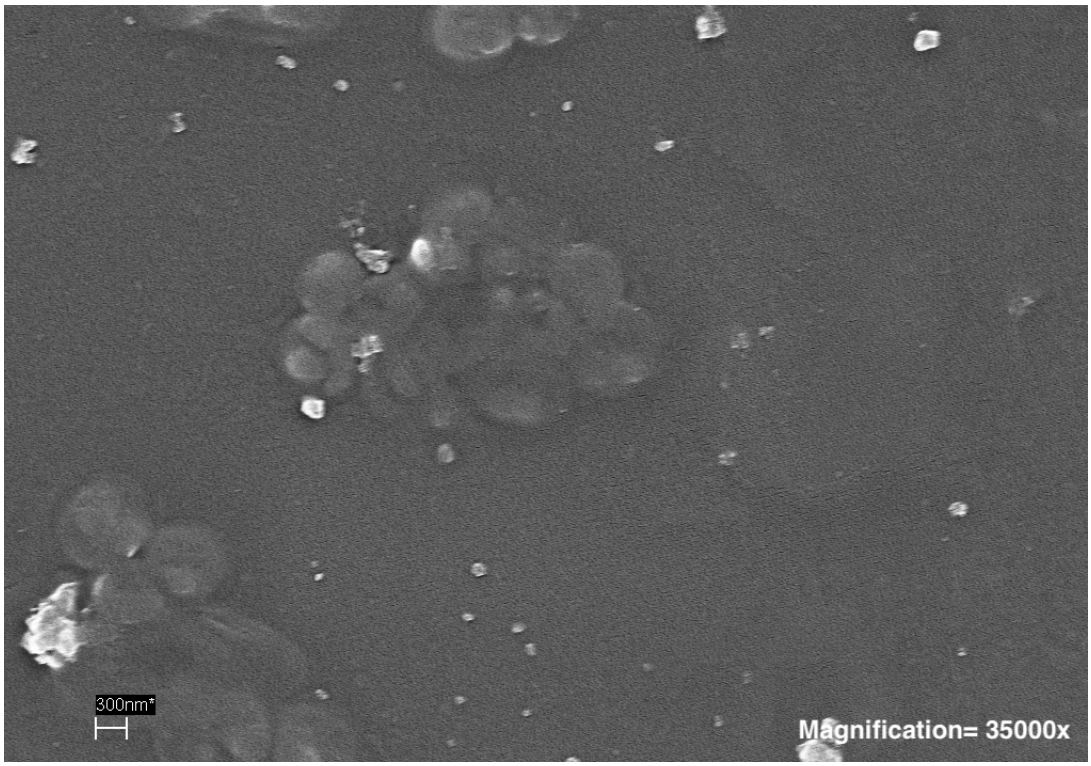


Figure 5-3 SEM micrograph, demonstration of aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP on the glass after 24 hours of storage in artificial saliva at 37 °C

5.2.2 Demonstration of the HAP layer on the bovine enamel after treatment with different concentrations of peptide-HAP suspension, using 3% NaOCl as the pre-treatment

5.2.2.1 Sample preparation for SEM

Six freshly extracted bovine mandibular incisors without caries and roots were selected randomly and stored in Evian water at 7°C. After polishing with Proxyt fine polishing paste, the samples were stored in the staining solution (explained in Chapter 2) for 72h at the room temperature.

The surface of the bovine teeth was polished with 1200-grit SiC abrasive paper under water-cooling. Each tooth was cut approximately into 5 mm*5 mm*1 mm pieces, using a low speed diamond saw (Leica SP 1600-Saw Microtome, Leica Microsystems Nussloch GmBH, Nussloch, Germany) under water-cooling.

According to Wang et al.(2012) and as demonstrated in Figure 5-4, the bovine teeth were first cut into nine fragments. As given in the figure (a), line 1 represents the line of mesial and middle one-third, while line 2 points out the line of middle and distal one-third. Line 3 and line 4 indicate the line of occlusal and middle one-third, and the line of middle and cervical one-third respectively. As illustrated in the figure (b), each fragment was trisected equally. The figure (c) shows that we

achieved the tangential sections by cutting through the middle of bovine enamel thickness. While L denotes the longitudinal direction of the bovine incisors, H indicates the horizontal direction of them. T shows the tangential line which is parallel to the bovine enamel surface. We chose our samples from a comparable area of each tooth among the sectioned enamel fragments. The small tooth fragments were assigned into three groups (n = 2) and mounted onto glass slides for easier handling during the experiment.

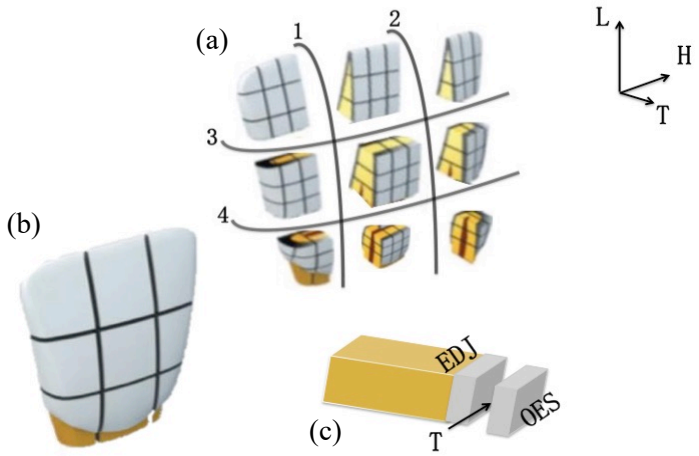


Figure 5-4 Illustration of the sample preparation of bovine incisors for SEM analysis – Reproduced based on Wang et al. (2012). (EDJ: Enamel–dentin junction, OES: Outer enamel surface)

5.2.2.2 Treatment of the samples

- All of the samples received a pre-treatment with 3% NaOCl for 3 minutes and then they were rinsed with water.
- To treat the groups 1, the P11-4 self-assembling peptide in monomeric form (Curodont Repair) and HAP suspension (6.25wt%) were mixed in the same way as described in chapter 4 and the mixture was applied to the surface.
- To treat the group 2, the P11-4 self-assembling peptide in polymeric form (Curodont Protect) and HAP suspension (6.25wt%) were mixed the same way as described in chapter 3 and the mixture was applied.
- To treat the group 3, an aqueous suspension of 0.02mg/ml P11-4 and 0.5wt% HAP was applied to the surface.

It is important to mention that the suspensions were applied to the enamel surface in a thin and homogenous layer with a micro-brush for 30 seconds continuously, and then were left for 5 minutes to allow undisturbed interactions between these agents and the sample's surface. One of the samples

of each group was rinsed off with water after 5 minutes of exposure time. The other samples were not rinsed off after treatment. All the samples were air-dried for 24h at room temperature (25°C).

After treatment, the samples were examined at different magnifications (1000x, 5000x, 20,000x). The uncoated samples were observed using a scanning electron microscope (ZEISS Supra 55vp, Zeiss, Oberkochen, Germany) and the microphotographs were collected. The specimens were sputter-coated with an ultrathin layer of electric conducting material like gold-Palladium (AuPd) alloy, of approximately 25 nm thickness, in a mini sputter coater, vacuum evaporator (SC7620, Polaron, Quorum Technologies, Kent, UK) for the second examination with the same detector.

The images were taken at 10 kV and with a working distance of 8-12 mm. The digital images were obtained and stored from secondary electrons (SE) and back-scattered electrons (BSE). The HAP particles were observed at different magnifications.

Table 5-2 provides the designed groups for the SEM analysis.

Table 5-2 The groups designed for the SEM analysis

Group name	Treatment agent					Pre-treatment	After treatment	
	Polymeric peptide (2%)	Monomeric peptide	Polymeric peptide	HAP (6.25%)	HAP (0.5wt%)	NaOCl 3%	Rinsed off	Not rinsed off
Group 1		✓		✓		✓	✓	
		✓		✓		✓		✓
Group 2			✓	✓		✓	✓	
			✓	✓		✓		✓
Group 3	✓				✓	✓	✓	
	✓				✓	✓		✓

5.2.2.3 SEM images Analysis

Each sample was examined two times in SEM before and after sputter coating to evaluate the effect of sputter coating and to differentiate the sputter artifacts from the HAP layer.

In the groups that the samples were rinsed off after the application of the agent, the formed HAP layer was partially removed from the surface. The taken images confirmed that some parts of the enamel surface were exposed after rinsing with water (Figure 5-5 and Figure 5-6). In contrast, in the samples that were not rinsed off, the HAP layers were more seamless on the surface (Figure 5-7).

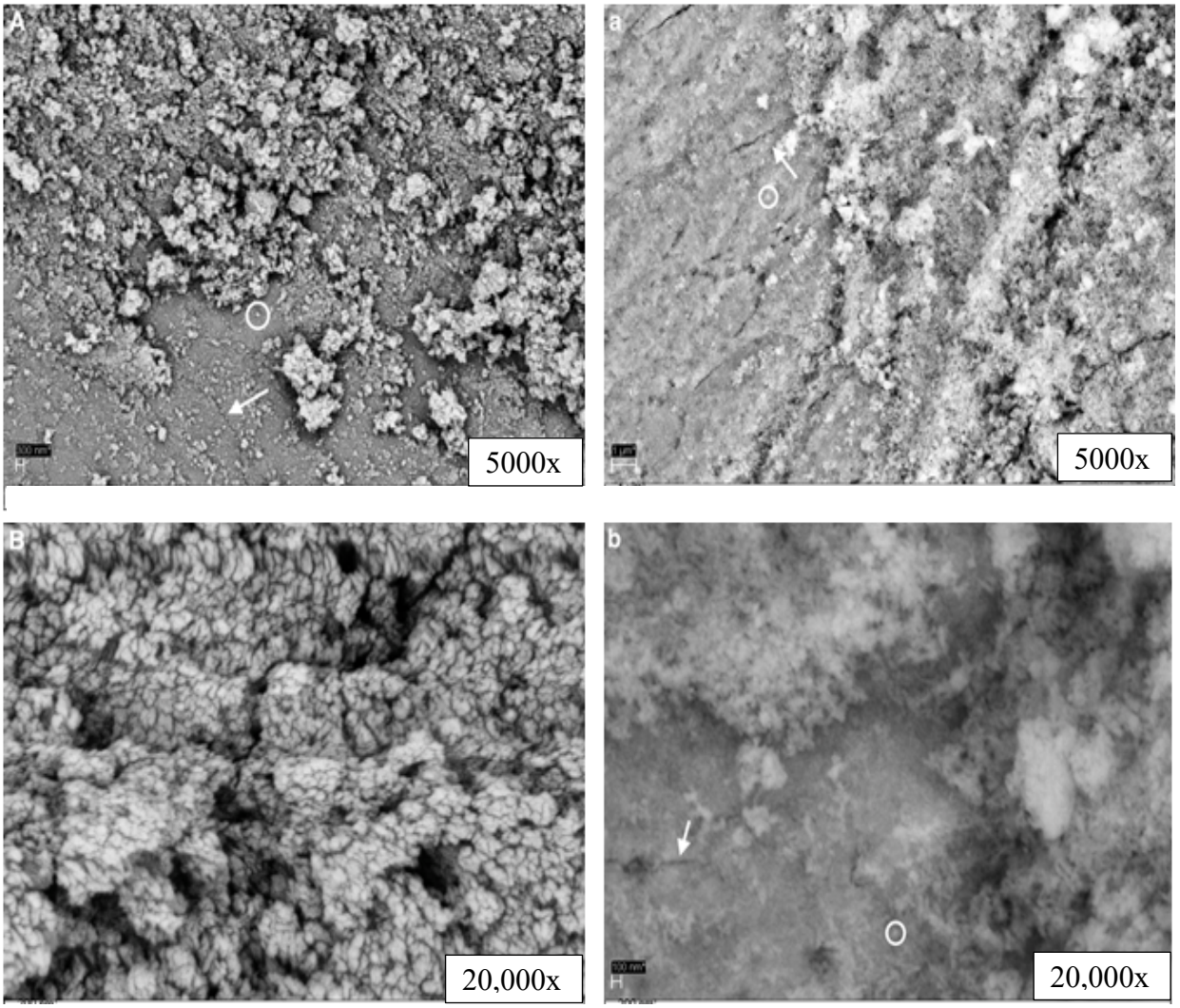


Figure 5-5 Enamel surface treated with P11-4-HAP suspension (Curodont Repair) after rinsing with water, visualized at 5000x magnification (A, a), 20,000x magnification (B, b). The circles surround adhered HAP particles & the arrows point to exposed enamel scratches. The size of the particles is at the nanometer scale. (A, B) = Sputter-coated surface, (a, b) = uncoated enamel surface

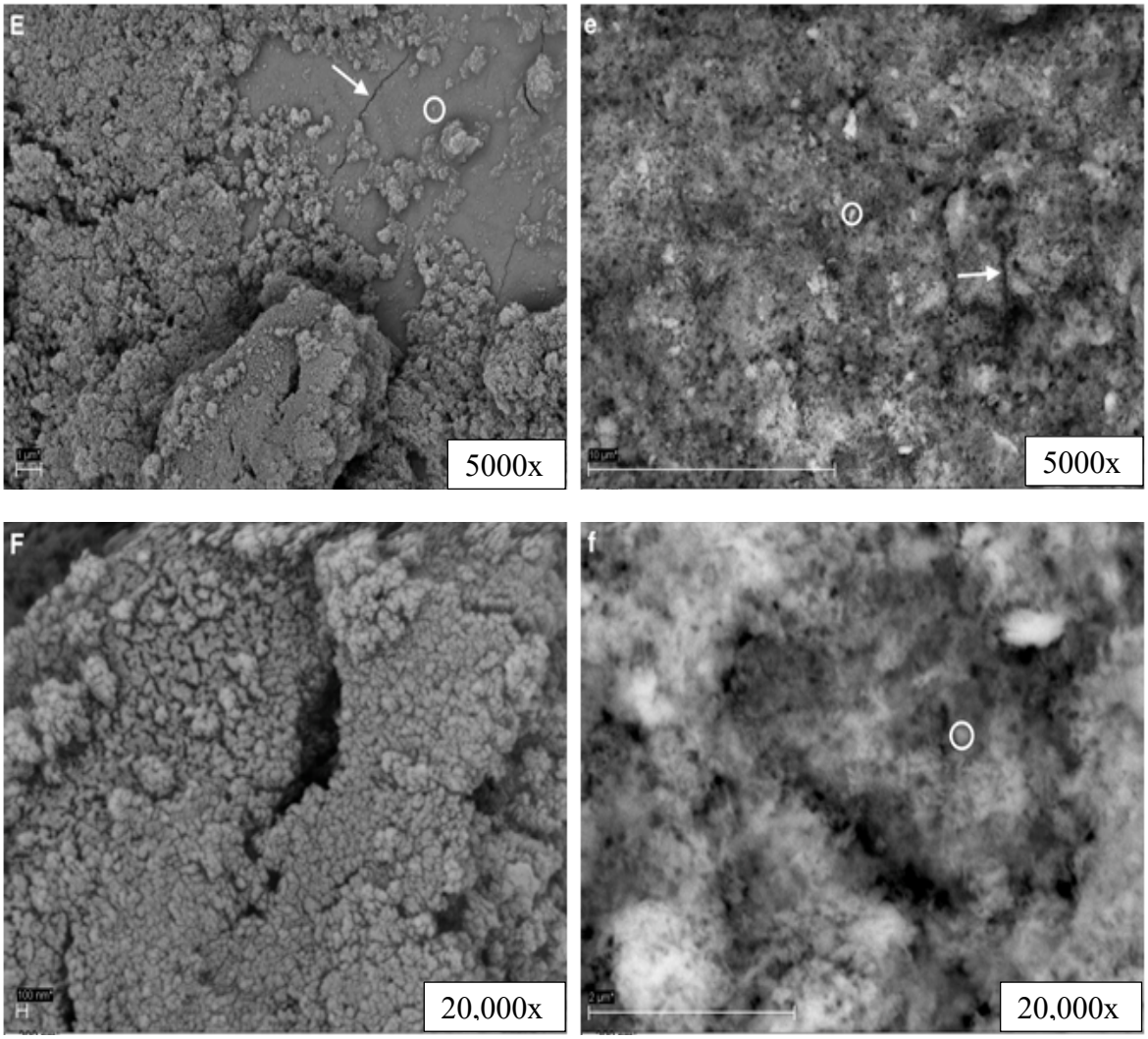


Figure 5-6 The enamel surface treated with P11-4-HAP suspension after rinsing with water (Curodont Protect), visualized at (5000x, 20,000x) magnifications. The circles surround adhered HAP particles and the arrows point to exposed enamel scratches. Small dispersed particles at nanometer level can be seen. They may form a thick layer on a considerable area of surface. (E, F) = Sputter-coated surface, (e, f) = uncoated enamel surface.

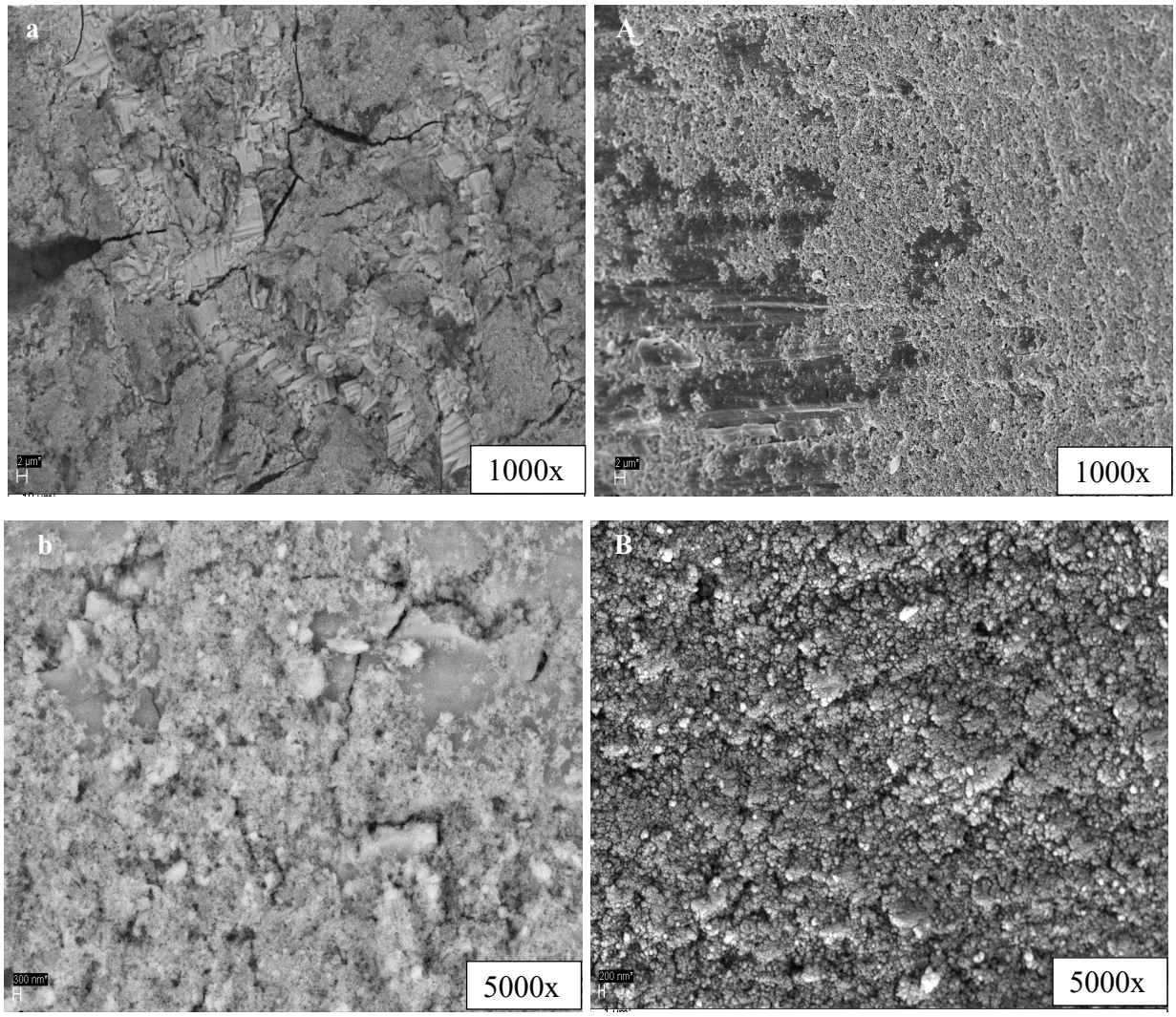


Figure 5-7 The enamel surfaces after treatment with P11-4-HAP suspension (Curodont Repair) before rinsing with water, visualized at 1000x magnification (a, A) and 5000x magnification (b, B). (A, B) = Sputter-coated surface, (a, b) = uncoated enamel surface. New formed HAP layer and the small particles can be observed on some parts of the enamel surface.

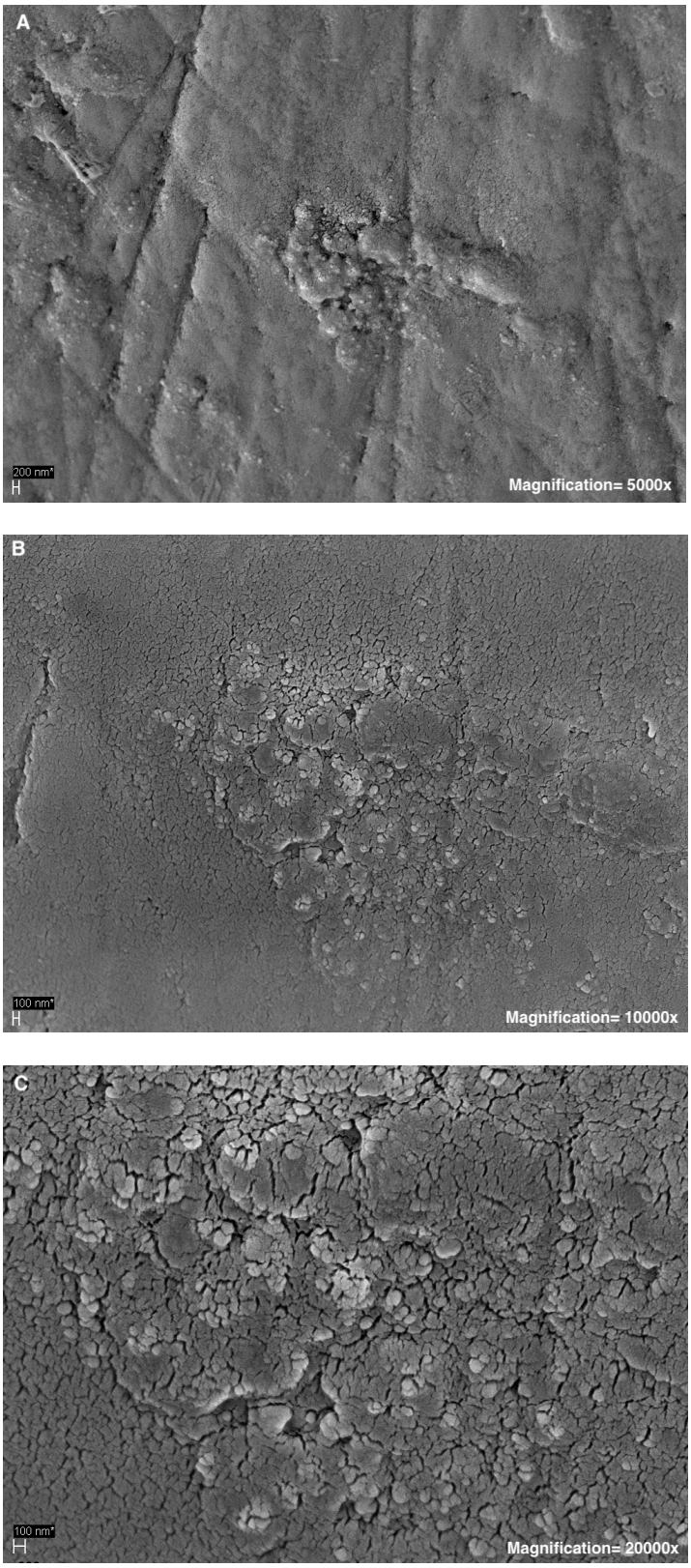


Figure 5-8 Sputter-coated enamel surface treated with an aqueous suspension of 2wt% polymeric peptides mixed with 0.5wt% HAP before rinsing, visualized at 5000x magnification (A), 10,000x magnification (B) and 20,000x magnification (C). Small dispersed particles can be seen on the sample's surface. The size of the illustrated particles is at the nanometer level.

5.3 Discussion

The present SEM images allowed us to have a qualitative understanding of the process of diffuse reflection of the bovine enamel surface after treatment with peptide-HAP containing suspension through the observation of obtained morphological and structural changes, which may lead to whitening the teeth.

5.3.1 Natural saliva collection

For the first part of this chapter, we collected natural saliva. In fact, due its lack of stability outside the oral environment, the use of natural saliva in large scale in both *in vivo* and *in vitro* studies is very challenging. Furthermore, the colonization of natural saliva by bacteria that can result in changes in its composition, and the sterilization may lead to the degradation of organic salivary components (Mandel, 1974). Based on Zeczkowski et al. (2015) to use natural saliva properly, it is necessary to carry out enzyme activity tests at different time steps to make sure that the salivary enzyme peroxidase in natural saliva remained active during experiment. This can be seen as a barrier for using natural saliva as the storage medium in an experiment. In this study we did not need to collect a considerable amount of natural saliva and we collected all the required natural saliva from one donator. Moreover, we did not manipulate the saliva after collection and used it immediately.

Stimulated saliva was acquired from a single donor. To stimulate and collect human natural saliva, the mouth was first rinsed with tap water for 30 seconds. Subsequently, a strip of Dentobuff "saliva kit" (Orion Diagnostica Oy, Espoo, Finland) was steadily chewed for 2 minutes. Based on Shellis et al. (2005), saliva collected in the first minute was discarded. After that, 10 ml of paraffin stimulated saliva was collected into a container, based on method introduced by Frostell (1980).

5.3.2 Preparation the enamel surface for SEM evaluation

It is important to note that we had to use different sizes of samples from the samples in other chapters of our study for the SEM investigation, because the samples' size must be small in order to achieve high vacuum in the SEM.

We observed the samples after treatment with the main suspension introduced in chapter 4 (Curodont Repair/HAP- using 3% NaOCl as the pre-treatment), using a field-emission scanning electron microscopy to visualize the P11-4-HAP layer adhered to the surface. The SEM investigation indicated that a better adhesion of the proposed suspension to the surface was obtained by using sodium hypochlorite as pre-treatment. This is because of its ability to dissolve and remove the organic

smear layer caused by polishing of the surface. In this investigation, the color changes of the samples were not of interest. In fact, the exposure time to the pre-treatment in the present chapter had not any impact of the outcome and cannot misrepresent the results.

5.3.3 Sputter coating

Usually, the calcium phosphate nanocrystals are platelet-shaped. While their width is several tens of nm, their thickness is a few nm (Epple, 2018). The gold coating usually helps to get a better resolution and higher magnification, which usually leads to more detailed observations. However, we observed nano-sized HAP particles at higher magnifications; it was hard to identify whether a HAP particle was observed or sputter coating droplets (Figure 5-5, Figure 5-6 and Figure 5-8).

Analysis of the SEM digital images showed that the sputter-coated samples were difficult to assess because of gold sputter artifacts under higher magnification (20,000x).

Even though sputter coating improves the image qualities and simplifies the analysis of sample structure and composition, on sputter coated enamel surface under 20,000x magnification, the HAP layer was not recognizable from enamel surface. Because the main constituent of dental enamel has similarities to the HAP, which we used in this study, and the observed sputtered particles and hydroxyapatite particles are both in nanometer range.

Regardless of whether the samples were rinsed with water or not, a complete coverage of the samples surface by HAP particles was not observed. Furthermore, according to the scanning electron microscopy images, the small particles seemed to adhere to irregularities and scratches of the surface caused by the polishing procedure. SEM images of uncoated enamel surface in both treatment groups (Curodont Repait and Curodont Protect) revealed that the HAP layer was well-formed on the surface and covered a considerable surface area.

There were observed sputter artifacts. However, under all of the lower magnifications the small HAP particles can be seen at the same field of view of enamel surface. To ensure that the detected small particles of the sputter-coated samples are adhered Hydroxyapatite particles; the images of reproducible areas were taken from uncoated specimens and were compared with them. As demonstrated in images small particles of HAP at nanometer scale can be observed, which may form a thick layer on the whole surface. Because of the limitation in current study, since SEM is only a qualitative analysis, we cannot quantify the extension of the formed HAP layer. Further analytic and more precise methods should be used in the future studies.

Based on hypotheses of Niwa et al. (2001) can we explain the mechanism of tooth whitening with our proposed suspension containing P11-4 and HAP. According to his observations tooth

whiteness and brightness increase after the treatment with HAP-based toothpastes. He assumed that remineralization of enamel surface made the surfaces smoother resulting in an enhancement of brightness. Roveri et al. (2009) observed a surface layer of HAP which can cause an increased diffuse reflection of light. These findings may result in a considerable increase in whiteness and lightness.

In this *in vitro* study, according to the SEM images, we observed a new formed layer of HAP particles on the surface. However, the obtained layer seemed not to be seamless. As discussed earlier, the aforementioned layer is adhered to the surface of enamel after treatment of the samples' surface with the P11-4-HAP-containing suspension. The obtained layer may improve diffuse reflection from the surface and is also potentially capable of forming a barrier against acid attacks at the same time.

Notwithstanding that the proposed suspension has no cleaning properties to remove and prevent extrinsic tooth stain, its application in the case of high concentration of protein and HAP lead to perceivably and measurably whiter teeth through covering tooth's surface immediately after the treatment. This is achieved by the deposition of HAP particles onto the tooth surface.

When making judgments on the effect of the peptide-HAP containing suspension against commercial bleaching products, it is important to consider, how often and for how long the suspension is used by the patients. Most of the peroxide-based commercial bleaching products are used for 1 to 2 hours continuously and some others during the whole night (Approx. 8 hours). As previously mentioned, they certainly cause tissue damages. Nevertheless, the positive and intensive results obtained after treatment with these types of agents satisfy the patients. The proposed suspension that we tested, was in contact with teeth's surface noticeably shorter. A short exposure time is of course more realistic for daily usage of a mouthwash or a toothpaste. Although it seems not argumentative to expect the same results, we observed that the overall color changes caused by the proposed suspension were not statistically significantly weaker than that caused by one of the commercial bleaching agent (Vivastyle Paint on Plus).

The tooth enamel is composed of minerals and it does not grow back. Therefore, in addition to their high costs and time consuming, this is not beneficial to use the common oxidizing whitening agents frequently, because they cause irrecoverable damages to the teeth by excessive using. In contrast, the proposed suspension of this study is beneficial to the enamel structure. Hence, without any concern, it can be easily used as a daily mouthwash or a toothpaste several times a day to achieve brighter and more robust teeth.

It is clear that the smear layer caused by polishing and the pigments of the staining solution were composed of protein that can bind to the calcium phosphate of the tooth and occupy the binding sites. These binding sites should have been occupied by peptide. Moreover, the pre-treatment with sodium

hypochlorite aimed at removing the organic layer from the binding sites and make them available to the P11-4 peptide to bind. However, in order to avoid the potential bleaching effect of sodium hypochlorite (Amaechi and Higham, 2002), the exposure time after this step had to be adequately short.

There are many various possible methods to utilize the proposed suspension. For instance, it can be used in fabricated deep drawn splints. However, it costs almost a lot of money and the production of splints in the laboratory takes some time. The P11-4-HAP suspension with a sufficient concentration could be also used in toothpastes with an integrated pre-treatment agent as a non-prescription drug. Nevertheless, it is difficult to be sold successfully in the market because of the huge number of competing products. Therefore, we assume that a P11-4-HAP-based mouthwash is a better idea, if we have a more soft-tissue-friendly alternative instead of sodium hypochlorite as a pre-treatment agent.

Chapter 6: Summary, conclusions and outlook

Due to the discussed disadvantages of hydrogen peroxide-based and OTC whitening products, these are not a safe and appropriate method for the long-term uses. Achieving a well-tolerated, cost effective and easy-to-apply treatment agent (Figure 6-1) is beneficial not only for patients with high tendency of cosmetic dentistry, but also for dentists. Use of a peptide-HAP-based mixture as a daily whitening agent results in a painless tooth whitening and healing of initial lesions prior to cavitation and hypersensitivity at the same time.

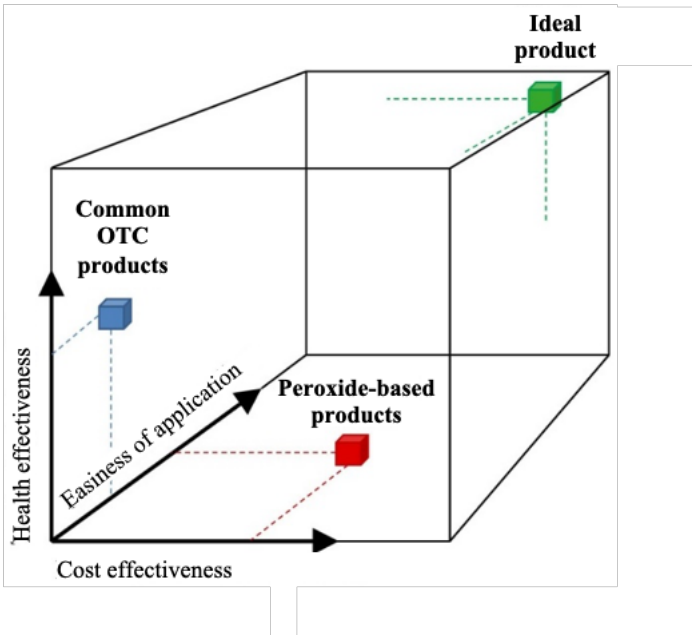


Figure 6-1 specification of an ideal agent versus common whitening agents

In this study, we used the P11-4 self-assembling peptide in combination with calcium phosphate suspension (HAP) to enhance the stability of HAP layer on the enamel surface. Our aim was to determine if the coverage of the teeth' surface with the HAP layer can be improved by using P11-4 peptide as a fibrillar scaffold, yielding the teeth seem whiter. This is not only because of the HAP's whiteness, but also because the new generated HAP layer is supposed to contribute to the diffuse reflection of light from the tooth surface (Jin et al., 2013).

The P11-4-HAP-containing suspension were used in this study in various concentrations, with different exposure times and different application frequency in order to evaluate whether these factors improve our results. This study was conducted in four parts.

The first part of this study compared a low-concentrated suspension of P11-4 and HAP with a commercial home-bleaching method so as to assess the whitening effectiveness of the

proposed suspension. Further, it evaluated whether exposure time, treatment frequency and storage in saliva had any significant impact on the whitening effect of the suspension.

The second part compared aqueous low-concentrated P11-4- HAP suspension with polymeric high-concentrated P11-4- HAP suspension. In addition, it discussed the effect of saliva on the color of samples after the treatments. Finally, it evaluated if re-staining after the treatment and storing in saliva contributed to significant color changes.

The third part analyzed the whitening effect of a high concentration of P11-4- HAP suspension after using 3% NaOCl as the pre-treatment. To provide a detailed assessment, we assessed the whitening effect of each of the components of the proposed suspension.

And the fourth part mainly provided a qualitative assessment of the surface structure changes after treatment with the P11-4 peptide -HAP suspension, analyzing SEM images.

Finally, the last chapter presented a summary of the study, following by conclusions and outlook for future studies.

In addition, to make sure that the use of P11-4 peptide contributes to form a new HAP-containing layer, we benefited from SEM micrographs to visualize the newly-formed HAP-layer. SEM results showed evidences for the presence of the peptide and HAP on the surface. They also confirmed that P11-4 peptide can enhance the formation of the HAP containing layer after using pre-treatment agent. Based on the given results, we can claim that the P11-4 peptide underwent self-assembly and acts as a template between the HAP particles and the tooth enamel.

The study resulted in perceptible whitening effect on stained bovine teeth after treatment with peptide-HAP containing solution. However, the magnitude of the effect was influenced by the use of NaOCl as a pre-treatment, concentration of the peptide-HAP suspension, exposure time and the baseline color.

In conclusion, this study demonstrated that:

- Under the limitations of the present *in vitro* study, the treatment with P11-4 peptide and HAP containing agent was effective in whitening the stained samples. However, the whitening efficacy depended on concentration of the suspension.
- The P11-4 peptide could be adsorbed onto the surface of the enamel crystals by electrostatic interaction to provide the matrix.
- The treatment with P11-4 and HAP suspension without using an acidic solution (e.g. phosphoric acid), as a pre-treatment, was also effective in tooth whitening.

- Even if the whitening effectiveness of the proposed suspension was not fully permanent and stable, the treatment with such an agent can be repeated more frequently, because, in contrast to high concentration peroxide-based whitening agents, it does not have harmful effects.
- Although all the chapters of this study were conducted carefully and strictly according to the previous protocols introduced in the literature, all *in vitro* studies have some limitations. Therefore, their results cannot be extrapolated to clinical conditions.
- The results of the treatment with P11-4-HAP suspension were more satisfying when we used NaOCl as a pre-treatment.

Since bleaching the teeth may also alter the enamel surface in the same way as etching solution, this new formulation may also be used to reverse the enamel damages after tooth bleaching. However, to confirm this hypothesis, more investigations are needed.

Furthermore, future studies should be conducted to develop a more effective and convenient form of application of the proposed agent (e.g. toothpaste, mouthwash, lack etc.). This open roads for future work to find a rational and cost-effective form and concentration of the suspension for achieving an ideal at-home tooth whitening and remineralization.

Zusammenfassung

Aufgrund der beschriebenen Nachteile von Produkten auf Wasserstoffperoxid Basis und OTC-Bleichmitteln sind diese keine sichere und geeignete Methode für den langfristigen Gebrauch. Das Erreichen eines gut verträglichen, kostengünstigen und einfach anzuwendenden Behandlungsmittels (Abbildung 6 1) ist nicht nur für Patienten mit hoher Tendenz zur kosmetischen Zahnmedizin, sondern auch für Zahnärzte von Vorteil.

In dieser Studie haben wir das selbst montierende Peptid P11-4 in Kombination mit Calciumphosphatsuspension (HAP) verwendet, um die Stabilität der HAP-Schicht auf der Schmelzoberfläche zu erhöhen. Unser Ziel war es, festzustellen, ob die Abdeckung der Zahnoberfläche mit der HAP-Schicht durch den Einsatz von P11-4 Peptid als fibrilläres Gerüst verbessert werden kann, so dass die Zähne heller erscheinen. Dies liegt nicht nur an dem Weißen des HAPs, sondern auch daran, dass die neu erzeugte HAP-Schicht zur diffusen Reflexion des Lichts von der Zahnoberfläche beitragen soll (Jin et al., 2013).

Die P11-4-HAP-haltige Suspension wurde in dieser Studie in verschiedenen Konzentrationen, mit unterschiedlichen Expositionszeiten und unterschiedlicher Anwendungsfrequenz verwendet, um zu beurteilen, ob diese Faktoren unsere Ergebnisse verbessern. Diese Studie wurde in vier Teilen durchgeführt.

Der erste Teil dieser Studie verglich eine niedrig konzentrierte Suspension von P11-4 und HAP mit einer kommerziellen Home-bleaching-Methode, um die Bleichwirkung der vorgeschlagenen Suspension zu beurteilen. Darüber hinaus wurde untersucht, ob die Expositionszeit, die Behandlungshäufigkeit und die Speicherung im Speichel einen signifikanten Einfluss auf die Aufhellungswirkung der Suspension haben.

Der zweite Teil verglich wässrige niedrigkonzentrierte P11-4- HAP-Suspension mit polymerischer hochkonzentrierter P11-4- HAP-Suspension. Darüber hinaus wurde die Wirkung des Speichels auf die Farbe der Proben nach den Behandlungen diskutiert. Schließlich wurde bewertet, ob die Rückfärbung nach der Behandlung und die Lagerung im Speichel zu signifikanten Farbveränderungen beigetragen haben.

Der dritte Teil analysierte den Aufhellungseffekt einer hohen Konzentration von P11-4-HAP-Suspension nach Verwendung von 3% NaOCl als Vorbehandlung. Um eine detaillierte Bewertung zu ermöglichen, haben wir die Aufhellungswirkung jeder der Komponenten der vorgeschlagenen Suspension untersucht.

Der vierte Teil lieferte vor allem eine qualitative Bewertung der

Oberflächenstrukturveränderungen nach der Behandlung mit der P11-4 Peptid -HAP-Suspension, die REM-Bilder analysiert.

Schließlich präsentierte das letzte Kapitel eine Zusammenfassung der Studie, gefolgt von Schlussfolgerungen und Ausblicken auf zukünftige Studien.

Darüber hinaus, um sicherzustellen, dass die Verwendung von P11-4 Peptid dazu beiträgt, dass sich ein neues HAP Schicht auf dem Zahn bildet, REM Bilder wurden verwendet.

Mit Hilfe von REM-Mikroskop Aufnahmen konnten wir die neu gebildete HAP-Schicht visualisieren. Die REM-Ergebnisse zeigten Beweise für das Vorhandensein des Peptids und von HAP auf der Oberfläche. Sie bestätigten auch, dass das P11-4-Peptid die Bildung der HAP-haltigen Schicht nach Verwendung eines Vorbehandlungsmittels verstärken kann. Basierend auf den vorliegenden Ergebnissen können wir behaupten, dass das P11-4-Peptid eine Selbstmontage durchlaufen hat und als Vorlage zwischen den HAP-Partikeln und dem Zahnschmelz dient.

Die Studie ergab einen spürbaren Aufhellungseffekt bei gefärbten Rinderzähnen nach der Behandlung mit peptidischer HAP-haltiger Lösung. Das Ausmaß des Effekts wurde jedoch durch die Verwendung von NaOCl als Vorbehandlung, die Konzentration der Peptid-HAP-Suspension, die Expositionszeit und die Grundfarbe beeinflusst.

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Ich erkläre hiermit an Eides statt,

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Evaluation of the Whitening Effect of a Mixture of Self-assembling Peptide and Hydroxyapatite on Bovine Enamel

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