Non-oxidative Tooth Whitening Effect of Hydroxyapatite on Bovine Enamel

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In Memory of My Dear Father

Guangwei Shang

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Chapter 1: General introduction of tooth esthetics

1.1 Tooth appearance

The stereotype of "what is beautiful is good" (Dion et al., 1972) still influences our judgment of people. Judging people by their appearances can be seen every day and everywhere in this economically competitive society. For instance, better-looking persons are considered to be more intelligent and more likely to get higher-paying jobs and promotions (Henson et al., 2011). An attractive facial appearance was reported to be the most influential characteristic for the achievement of popularity among American teenagers (Henson et al., 2011). The current social media also reinforce in us a high standard of facial attractiveness. A perfect smile is considered to be a prominent feature of beauty (Samorodnitzky-Naveh et al., 2007).

Subsequently, dental esthetics has become a worldwide significant issue since it plays a key role in facial esthetic. According to the "Whitening Survey, Summer 2012" of the American Academy of Cosmetic Dentistry (2012), 99.7% of Americans considered a smile an important social asset. In the survey, participants were asked what they noticed as the first thing in a person's smile. The top three responses were straightness, whiteness, and cleanliness of the teeth. In a Chinese urban population (405 participants), 48.9% of enrolled patients suffered from tooth discoloration and 52.6% of them were not satisfied with their tooth appearances (Xiao et al., 2007). It was also reported that 35% of a population in Nigeria (410 patients) showed dissatisfaction with their dental appearance (Ajayi et al., 2021).

The satisfaction of tooth esthetic is associated with age, gender and educational background (Demirel and Tuncdemir, 2019). Women tend to have lighter teeth than men. Higher educated people were reported to have brighter teeth than those lower educated. The older age group seemed more satisfied with their tooth esthetics. About 12% of an elderly German population (225 subjects) were dissatisfied with tooth color, shape or position (Hassel et al., 2008).

1.1.1 Nature of human tooth color

Human tooth color is influenced by the combination of intrinsic and extrinsic colorations (Watts and Addy, 2001, Hattab et al., 1999). On one hand, intrinsic tooth coloration is associated with the optical properties of dental hard tissue. When light falls on the tooth surface, it is subjected to a series of optical phenomena including specular transmission through tooth tissue, specular and diffuse reflection on enamel surface and absorption and scattering inside tooth tissue (Jahangiri et al., 2002, Joiner and Luo, 2017). The optical phenomena are affected by the thickness and translucency of dental tissue (van der Burgt et al., 1990) (***Figure 1.1***)

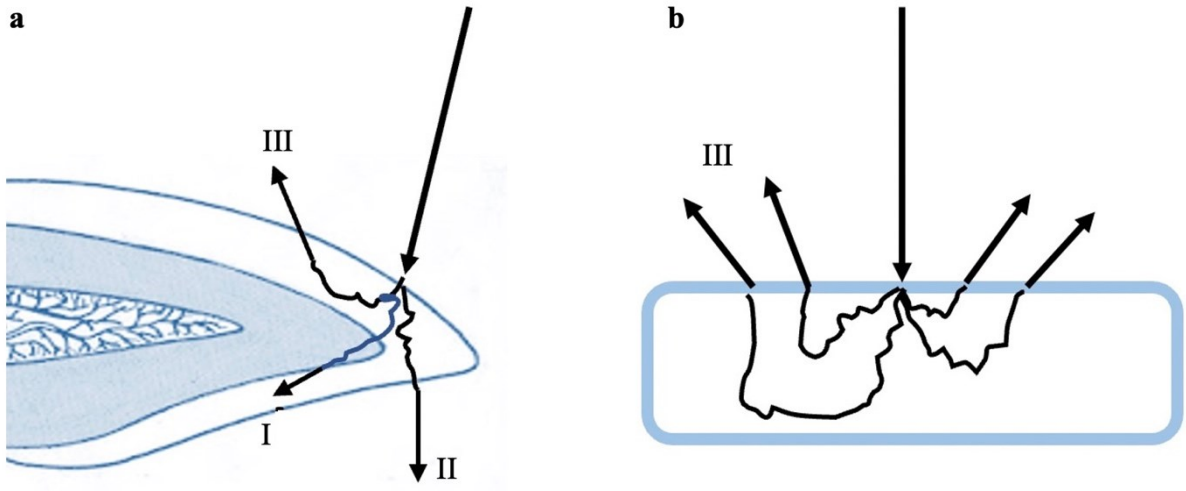

Figure 1.1 Optical phenomena of tooth appearance. a: enamel with thickness of less than 1 mm; b: enamel thicker than 1 mm; I: light scattering and absorption; II: light scattering and transmission; III: light scattering and reflection.

Regarding enamel with a thickness of less than 1 mm, photons can pass through it and lead to a translucent appearance of teeth. Other photons are scattered backward, resulting in volume reflection. Regarding thicker tooth tissue, photons cannot pass through it, which makes enamel appear opaque. Before the light emerges from the tooth surface and reaches the eyes of observers, the light follows highly irregular paths through enamel and dentine.

On the other hand, extrinsic coloration is linked with the absorption by colored compounds (chromophores) into the acquired pellicle, biofilm or calculus on the enamel surface (Epple et al., 2019). Chromophores influence the tooth color by absorbing light in visible range and thereby make teeth appear darker (Epple et al., 2019).

1.1.2 Etiology of tooth discoloration

1.1.2.1 Intrinsic tooth discoloration

Intrinsic tooth discoloration can happen during tooth development and after tooth eruption. The causes of intrinsic discoloration can be metabolic, inherited, iatrogenic, traumatic or aging (***Table 1.1***) (Sulieman, 2005). The chromophores are located in enamel or the underlying dentin. The removal of intrinsic stain is nearly impossible from outside of teeth (Epple et al., 2019). Conventional bleaching methods are considered effective for many types of intrinsic discoloration. For instance, tetracycline-stained teeth could be lightened by at-home bleaching with an application period of three months (Botelho et al., 2017). The bleaching effect was found to be long-lasting and could be observed even after 90 months (Leonard et al., 2003). Moreover, regarding the severe cases of amelogenesis imperfecta and fluorosis, which both can lead to porosities of the dental hard tissue, conventional bleaching methods could also provide patients with an improvement of esthetic of teeth. For example, the organic stain that adheres to the porous enamel caused by dental fluorosis can be easily removed by conventional bleaching agents. Although the white spots on the enamel from fluorosis cannot be bleached, the overall tooth color and shade can be lightened. Therefore, the contrast between the tooth color and the white spots will be diminished. As a result, the tooth esthetic is improved. Resin infiltration, composite fillings, veneers, crowns and microabrasion are good complementary treatments and are applied as a dental routine for severe intrinsic tooth discolorations (Di Giovanni et al., 2018).

Aging alone does not cause tooth discoloration. The combination of the accumulation of pigment molecules on tooth surfaces and tooth abrasion over the whole lifetime makes the teeth of elder patients appear darker. As the transparent enamel gradually wears away, the color of the less transparent dentin underneath is revealed.

Table 1.1 Causes of intrinsic tooth discoloration (Sulieman, 2005).

Causes	Diseases
Metabolic causes	Cogenital erythropoietic porphyria
Inherited causes	Amelogenesis imperfecta, Dentinogenesis imperfecta
Iatrogenic causes	Tetracycline, Fluorosis, Amalgam staining
Traumatic causes	Enamel hypoplasia, Pulpal haemorrhage products, Internal resorption
Aging causes	Aging

1.1.2.2 Extrinsic tooth discoloration

Organic extrinsic stains can derive from the consumption of chromophore-containing food and beverages, such as coffee, tea, red wine, herbs, fruits, acetic salad dressings or anything which is colored and can be eaten (Eriksen and Nordbo, 1978). The chromophore molecules can bind to the enamel surface and be mediated by inorganic divalent cations including iron and tin (Flotra et al., 1971). The etiology of extrinsic staining was also reported to be associated with oral hygiene environment, adequate toothbrushing, tobacco consumption and application of chlorhexidine-containing mouthrinses (Li, 2017).

The role of the acquired pellicle in the formation of extrinsic staining has been well understood (Eriksen and Nordbo, 1978). Chromophore molecules can incorporate into the acquired pellicle. If not being brushed away on time, the chromophore would stay in the biofilm or even in calculus, making tooth whitening more difficult. Conventional whitening dentifrices that

contain abrasives are effective in improving the tooth color by removing extrinsic stain. Professional tooth cleaning with ultrasonic technique is efficient for the removal of chromophore-containing biofilm and calculus. Besides that, tooth bleaching with peroxide-containing dental care products can chemically break down extrinsic pigment molecules and thereby whiten teeth.

To date, scientists tend to stain samples with chromophore-containing liquids in the studies which focused on the evaluation of the whitening effect of different whitening products. In these studies, coffee, cola, red wine, lime juice, and tea were used as the staining solutions. Coffee solution seemed to be able to cause the greatest color changes compared with cola and tea (Gross and Moser, 1977) .

1.2 Oxidizing tooth whitening

1.2.1 Mechanism of tooth bleaching

Hydrogen peroxide is the most commonly used active agent in commercial oral whitening products (Dahl and Pallesen, 2003). Hydrogen peroxide is a strong oxidizing agent and can diffuse through the enamel and reach the organic chromogens that exist in dentine. The "chromophore theory" is the dominant mechanism of tooth bleaching (Kwon and Wertz, 2015). Hydrogen peroxide releases free radicals and reactive oxygen radicals, which interact with stain molecules by breaking the chromophore molecules mostly at the C-C double bonds. This results in shorter molecules and changes in their optical properties, which do not absorb visible light anymore. Therefore, the oxidizing process of bleaching causes a change of tooth color and creates a whitening effect.

1.2.2 Dentist-supervised bleaching: in-office and at-home bleaching

In-office and at-home bleaching are the two main bleaching techniques that should be operated under the supervision of dentists. The in-office bleaching applies high concentrations of

hydrogen peroxide (35-38%) (Bortolatto et al., 2014). Patients usually need three bleaching appointments with dentists and the time of each appointment ranges from 30 to 50 minutes. An immediate whitening outcome can be achieved after in-office bleaching. At-home bleaching requires patients to wear bleaching trays that are filled with low concentrations of carbamide peroxide (10-20%) for hours daily. The treatment lasts generally several weeks. Compared with in-office bleaching, the whitening efficacy of at-home bleaching can be only achieved after a longer period of daily application.

The advantages and disadvantages of the in-office and at-home bleaching techniques remain controversial. The controversy centered on the tooth sensitivity after bleaching. The tooth sensitivity of at-home bleaching is considered milder and the intensity is lower than in-office bleaching (Maran et al., 2020). It was reported that the mean value intensity of bleaching sensitivity was 0.5 for at-home bleaching and 2.8 for in-office bleaching according to a pain scale that has five verbal rating scales (0 = none; 1 = mild; 2 = moderate, 3 = considerable; 4= severe) (Rezende et al., 2016). On the contrary, Basting et al. (2012) found that in-office bleaching with 38% hydrogen peroxide has a lower prevalence of tooth sensitivity than at-home bleaching with 20% carbamide peroxide. A systematic review and meta-analysis compared the whitening effectiveness, risk and intensity of tooth sensitivity of in-office and at-home bleaching and found no significant difference between them (de Geus et al., 2016).

1.2.3 Self-directed bleaching: over-the-counter bleaching

The success of dentist-supervised bleaching techniques stimulated the marketing of over-the-counter (OTC) bleaching products. These products served as alternatives to dentist-prescribed bleaching systems since they have advantages in financial and time costs. Barrier-free bleaching dentifrices, mouthrinses, paint-on gels, strips that contain low concentrations of hydrogen peroxide or carbamide peroxide are available in the dental cosmetic market. However, the current clinical evidence seems not to be able to confirm the whitening effects of the OTC

bleaching products because of the small number of clinical trials (Demarco et al., 2009). Theoretically, the whitening efficacy of OTC systems should not be as good as the professional-guided ones because of the low concentration of active peroxide agents. A randomized controlled trial (Bizhang et al., 2009) was performed to compare the efficacy, post-bleaching sensitivity and gingival irritation of at-home bleaching gel and an OTC whitening strip. The study confirmed that commercial whitening strips that contained 6% hydrogen peroxide were less effective than at-home bleaching with 10% carbamide peroxide. After three months of application, the mean value of ΔE in the at-home bleaching group was 4.49, whereas it was 2.99 in the OTC group. However, long-term clinical trials that evaluate the whitening efficacy of OTC products are still lacking.

1.2.4 Safety concerns of bleaching products

Tooth sensitivity, oral mucosa irritation, the alteration of enamel microstructure are the common post-bleaching symptoms (Mounika et al., 2018, Lin et al., 2019, Orilisi et al., 2021, Monterubbianesi et al., 2021). Besides that, general toxicity, genotoxicity and carcinogenicity that can be caused by bleaching, are under cautious discussion. Reactive oxygen species (ROS) can destroy cellular macromolecules in the etiology of human aging and diseases (Ames and Gold, 1991). The bleaching process can also produce ROS, resulting in damages in cells including DNA-protein cross-links, bulky adducts and strand breaks (Moller and Wallin, 1998). The damages cause failure in the DNA repair system and lead to carcinogenic consequences. An *in vivo* study evaluated the genotoxicity and oxidative damage in patients who used OTC whitening strips. An increased number of nuclear abnormalities (NAs) and 8-hydroxy-2$^{'}$-deoxyguanosine in oral mucosa was found after the exposure to 10% hydrogen peroxide, which suggested the existence of oxidative DNA damages (Del Real Garcia et al., 2019). Therefore, it is recommended that bleaching products should not be used without gingival protection and should be avoided in patients with damaged tissues (Tredwin et al., 2006).

According to the response of the American Food and Drug Administration (FDA) to the American Dental Association (ADA) on the bleaching products in 2014, most of the OTC bleaching products meet the definition of a cosmetic and should not be restricted unless more evidence could be provided by ADA. Until this year, the regulation on hydrogen peroxide-containing products has not been changed by FDA. However, the Council Directive 2011/84/European Union (2011) regulated that only the products that contain a limit of 0.1% hydrogen peroxide are considered safe and can be sold directly to consumers. The concentrations of dentist-supervised bleaching agents should range from 0.1% to 6%. The products with a concentration of hydrogen peroxide of more than 6% are not safe and cannot be used for patients anymore.

1.3 Non-oxidizing tooth whitening

1.3.1 Physical removal of stain with abrasives

Conventional non-oxidizing tooth whitening products are formulated by physically removing extrinsic stain (Joiner, 2010). Abrasives, such as hydrated silica, calcium carbonate, and dicalcium phosphate dihydrate, are incorporated into toothpastes and serve as the primary physical stain removal ingredient. Other ingredients, such as surfactants, polyphosphates and enzymes, were also shown to be able to remove and prevent extrinsic discoloration (Epple et al., 2019). A mechanical abrasive process can break up accumulated stain on enamel and thereby make teeth brighter without changing their natural colors. However, one must consider the potential risk of undesired wear of natural enamel after a long-term application of abrasive toothpastes. *In vitro* studies showed that tooth brushing with abrasive toothpastes produced wear to dentine (Kodaka et al., 2001, Sexson and Phillips, 1951). It was reported that whitening dentifrices that contain abrasives significantly enhance enamel and dentin wear in comparison with commercial regular ones (Mosquim et al., 2017, Vertuan et al., 2020). Accordingly,

professional awareness of the abrasiveness of whitening dentifrice has been raised. Abrasion of a whitening dentifrice can be assessed and calculated by radioactive dentine (RDA) and enamel abrasion (REA) (Hefferren, 1976). RDA is considered the gold standard for evaluating the abrasion of whitening products in the dental market for a long period of time (Gonzalez-Cabezas et al., 2013). However, the RDA test has been also questioned since the data obtained by different laboratories differed a lot (Dörfer, 2011). The classification of the abrasivity was suggested in ***Table 1.2***. Theoretically, whitening products should be designed to maximize the whitening performance and minimize tooth wear. To seek a balance between the two is the main goal of developing commercial whitening products.

Table 1.2 The classification of abrasion of commercial toothpastes (Hamza et al., 2020).

Classification	Description	Values
RDA-1	Very low abrasion	< 20
RDA-2	Low abrasion	20–40
RDA-3	Moderate abrasion	40–60
RDA-4	Strong abrasion	60–80
RDA-5	Very strong abrasion	> 80

1.3.2 Mechanism of tooth whitening with HAP

Hydroxyapatite (HAP) is a natural form of calcium apatite mineral. Human bones and teeth consist of HAP. It is widely used as a biomimetic material in various fields of dentistry. They are commercially available for the treatment of dentin hypersensitivity, prevention of post-bleaching sensorial reactions and repair of early caries lesions (Souza et al., 2015, Vano et al., 2015, Bordea et al., 2020). The HAP ingredient can be applied in both leave-on and rinse-off oral cosmetic products, including toothpaste, mouthwash and gel, with different concentrations and specifications (Scientific Committee of Consumer Safety and Bernauer, 2020).

A seamless fixing process of early caries without prior excavation was reported in Nature (Yamagishi et al., 2005). The authors prepared a biomimetically modified HAP toothpaste and applied it to an early caries lesion. The HAP crystals of the original enamel dissolved but rapidly grew again and formed a layer that contained nanosized elongated crystals on enamel. This finding confirmed that the HAP could integrate into the original enamel structure. The new regrown layer covered the entire enamel surface and reconstructed the early caries lesion without excavation. This discovery laid the theoretical foundation for explaining the whitening effect of HAP.

To date, two main hypotheses explain the whitening mechanism of HAP. Niwa et al. (2001) favored the mechanism of light specular reflection. They believed that the remineralization effect that conducted by HAP particles could make enamel smoother and glossier and thereby increase the specular reflection rate of light. However, Roveri et al. (2009) found that the adhered HAP layer made the teeth rougher and the increased roughness could lead to a measurable enhancement in diffuse reflection rate of light, which could make teeth appear whiter.

1.3.3 Previous research that focused on the whitening effect of HAP

Dabanoglu et al. (2009) analyzed the whitening effect of three HAP suspensions and two HAP mixtures in dissolvable polymer films. The concentrations of the HAP suspensions ranged from 39.3 to 44.41%, while the concentrations of the HAP in the polymer film were 1% and 3%, respectively. The HAP materials were applied to bovine enamel three times. Each HAP application was followed by a 24-hours incubation in distilled water. The color changes were measured before and after each HAP application. They found that all the five groups exhibited significant color changes between baseline and after the HAP application with the ΔE values ranging from 1.0 to 3.3. A linear numeral increase of ΔE values was observed after each HAP application. However, the increase was not statistically significant. HAP crystallites and

agglomerates were identified on enamel in all groups, which increased the surface roughness of enamel and thereby changed the light reflectance on it.

The experimental methodology of the Dabanoglu et al. (2009) was used by Jin et al. (2013) in their *in vitro* research. Three HAP formulations with three concentrations (10, 20 and 30 wt%) were prepared and applied to bovine enamel. They found the mean values of tooth color changes ranged from 0.91 to 2.20, which were smaller than those in the study of Dabanoglu et al. (2009). The authors believed that the selection of teeth before experiment might explain the smaller color changes. Only the teeth, which were darker than A3 were chosen in this study. Neither a dose-dependency nor a dependency of repeated application was observed. The concept of the maximal load of adhering was firstly raised, which means once the maximal load is reached, further HAP application will not change tooth color.

Since the whitening effect of HAP in the above two studies seemed to be limited because of the thinness of the adhered HAP layer, scientists sought to modify the HAP particles to enhance their affinity to tooth surfaces. In 2018, a self-assembling peptide P11-4 Matrix was mixed with HAP particles by Bommer et al. (2018). According to the authors, P11-4 peptides were used as scaffolds for the nucleation of calcium phosphate by providing both positively and negatively charged nucleation sites. As a result, a multi-layered HAP adhesion was observed on enamel. The authors declaimed that the multi-layered structure could lead to a long-lasting whitening effect since the P11-4 Matrix could strongly bind HAP particles to enamel. This hypothesis was confirmed with higher tooth color changes with the mean ΔE value of 4.9. Bidirectional light reflectance was measured by using an angular-resolved goniometer. The gloss measurement showed that the whitening effect of HAP was due to the enhanced diffuse light reflection.

After two years, Hojabri et al. (2020) compared the whitening effect of P11-4 peptide and HAP suspensions with a commercial bleaching agent that contained 6% hydrogen peroxide. The

experimental groups were designed according to exposure time (1, 5 and 10 minutes, 24 hours) and application frequency (1 and 5 times). It was reported that the peptide-HAP suspension showed a mild whitening ability compared with the commercial bleaching gel. With the exposure time of 24 hours and 5 times of application of the HAP suspension, the mean ΔE value reached 6.42, while the mean ΔE value of the commercial bleaching group was 9.01. The exposure time and the repeated application had no significant influence on the whitening performance of the peptide-HAP suspension.

Recently, clinical questionnaires were filled out by patients, who were asked to brush teeth twice a day for four weeks with a commercial HAP toothpaste and gel (Steinert et al., 2020b, Steinert et al., 2020a). The questions concerning tooth hypersensitivity, tooth smoothness, tooth color, gingiva bleeding and feeling of freshness were asked before and after the daily application. The patients felt that their teeth became smoother, less sensitive, whiter, and fresher after the application of HAP toothpaste and gel for 28 days.

1.3.4 Advantages of HAP as a whitening agent

According to the above previous studies (Dabanoglu et al., 2009, Jin et al., 2013, Bommer et al., 2018, Hojabri et al., 2020, Steinert et al., 2020b, Steinert et al., 2020a), HAP materials have a series of advantages in comparison with the bleaching agents. First, HAP products have no irritation effect on oral hard and soft tissue because of their unbeatable biocompatibility, which makes them ideal for patients who suffered from dentin hypersensitivity and burning mouth syndrome. Second, HAP products do not chemically change the enamel morphology. Therefore, they do not affect the bonding effect of composite and resin-based cement. Third, the adhered HAP layer could protect the enamel surface from acidic attack since the acid will touch the HAP layer firstly instead of human enamel. Fourth, the promising tooth-whitening performance (the mean ΔE value reached 6.42 (Hojabri et al., 2020)) makes HAP products a promising alternative to the conventional bleaching agents. Moreover, the high acceptance and

satisfaction among the patients, who used HAP products daily, make the non-oxidizing tooth-whitening effect of HAP a hotspot in tooth-whitening research field.

1.3.5 Safety concerns of HAP-containing products

The European Commission's Scientific Committee of Consumer Safety and Bernauer (2020) stated the opinion on HAP (nano). According to the opinion, the genotoxic potential of HAP remains unknown. After concerning the available information in scientific literature, the Scientific Committee of Consumer Safety could not conclude on the safety of rod-shaped HAP nanoparticles for application in dental care products. The needle-shaped form of HAP should not be integrated into dental cosmetic products because of its potential toxicity.

1.4 Visual and instrumental methods of tooth color measurement

The accurate color measurement of tooth and restorative dental materials is becoming increasingly significant along with the increased awareness of confident smiles and whiter teeth. The commercial VITA classical shade guide is most widely applied when determining tooth color for prosthodontic purposes (Joiner and Luo, 2017, Chu et al., 2010). Besides that, other devices of color measurement are spectrophotometers, colorimeters and digital cameras (Pan and Westland, 2018).

1.4.1 VITA classical shade guide

The Vita shade range can be divided into four groups from A (lightest) to D (darkest), which is arranged according to the difference in hue. Each hue group can be divided into subgroups numerically order according to the difference in chroma (such as A1 to A4). Although being routinely applied in dental practice, the inherent weaknesses of commercial color shades should not be forgotten. First, the color differences between tabs are often considered neither uniform nor systematic (Olms et al., 2013, Joiner and Luo, 2017). The difference in metric shade between two shade levels (for example the difference between A1 and A2, B1 and B2, etc.) is

not the same, which has a particular influence on evaluating the tooth-whitening performance. Second, the visual tooth color determinations that are conducted by commercial color shades is subjective and can be influenced by a series of variables such as age, gender, individual color perception, experience, eye fatigue of observers or wide range of tones, translucency and opacities of natural human teeth (Lehmann et al., 2011, Derdilopoulou et al., 2007). Third, the disinfection after each use might also lead to color changes in shade guides, which could lead to inconsistent shade determinations and unsatisfied esthetic restorations (Alshethri, 2014). Finally, the color patterns of the Vita shade guide are multi-layered, which consist of composite or ceramic layers with different transparencies and colors. Therefore, the Vita shade guide cannot exactly reflect the true color of natural teeth, even if there is a good visual match between the two.

1.4.2 Colorimeter

Spectrophotometers produce a full range of wavelengths of the visible spectrum, whereas colorimeters use LED light sources and three broadband color filters. Accordingly, they can only provide fixed wavelengths. The XYZ tristimulus values were measured by filtering the reflected light into red, green and blue regions of the visible spectrum and transforming the data to $L^*a^*b^*$ values (Joiner and Luo, 2017). ***Figure 1.2*** shows the working principles of spectrophotometers and colorimeters.

Colorimeters are less expensive than spectrophotometers. They are considered technically sensitive and less accurate than spectrophotometers in the hand of untrained and inexperienced users (Borse and Chaware, 2020). Colorimeters are designed for color measurement on flat objects. Human teeth are not totally flat and varied in morphology, which could result in an undesirable light loss at the edge of the measured areas (Joiner, 2004). To get a reliable result, the colorimeter should be calibrated on the docking station after individual measurement. Once the images are captured, the Shade Vision system transfer the images to a connected computer

for processing. The accurate shade of a tooth is then selected from an online database that provides CIE *L*a*b** values (Kim-Pusateri et al., 2009). It is reported that the accuracy rate could reach 92.6% (Kanawati and Richards, 2009, Sarafianou et al., 2012). Therefore, the photocolorimetric method can serve as an acceptable substitute to visual shade guide for dentists who have trouble with shade selection (Dancy et al., 2003).

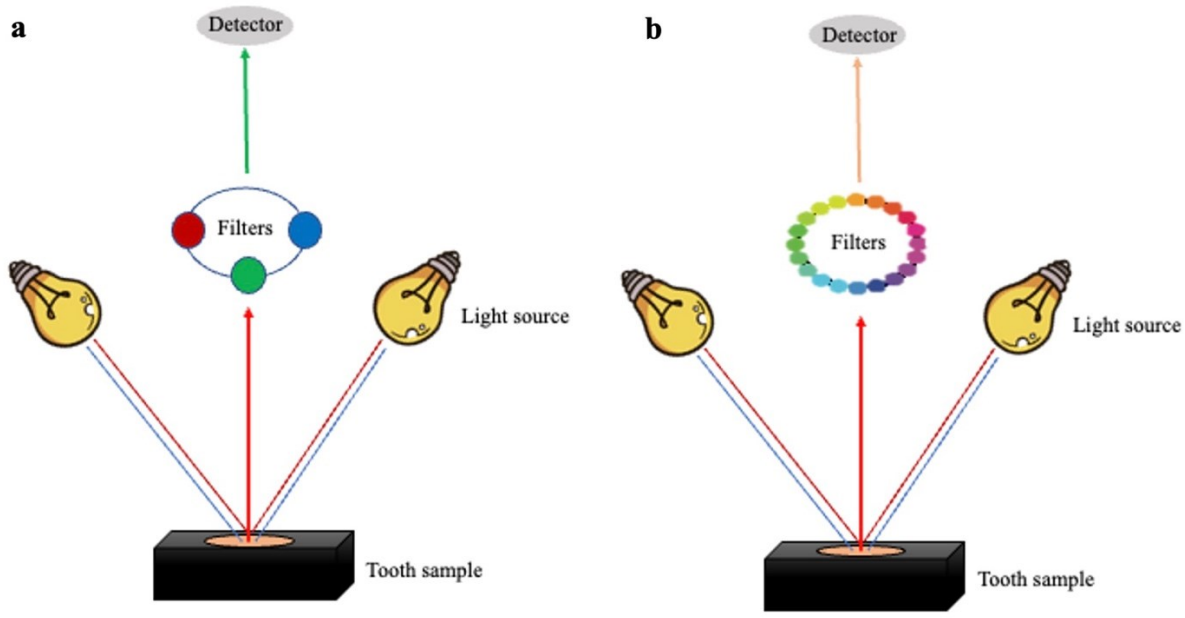

Figure 1.2 The working principles of colorimeters (a) and spectrophotometers (b).

1.4.3 Spectrophotometer

Spectrophotometers measure the spectral reflectance and/or transmittance curve of samples. Commercial reflectance spectrophotometers can measure reflectance on sample surfaces at an interval of 1 to 25 nm along the visible spectrum (Pan and Westland, 2018, Kielbassa et al., 2009). According to a systematic review, a spectrophotometer was the most accurate device and showed greater reproducibility than the Vitapan tooth shades, colorimeters and digital cameras (Chen et al., 2012). The Gretag Macbeth spectrophotometer Color Eye 7000A has widely been used *in vitro* studies, in which the colors of tooth, restorative materials or

prosthetic restorations were measured (Hojabri et al., 2020, Alaqeel, 2020, Hasssija et al., 2014, Silami et al., 2019). This spectrophotometer has a spectral range of 360 to 750 nm with a wavelength interval of 10 nm. D/8 (diffuse) optical geometry configuration and pulsed Xenon illumination are installed (***Figure 1.3***).

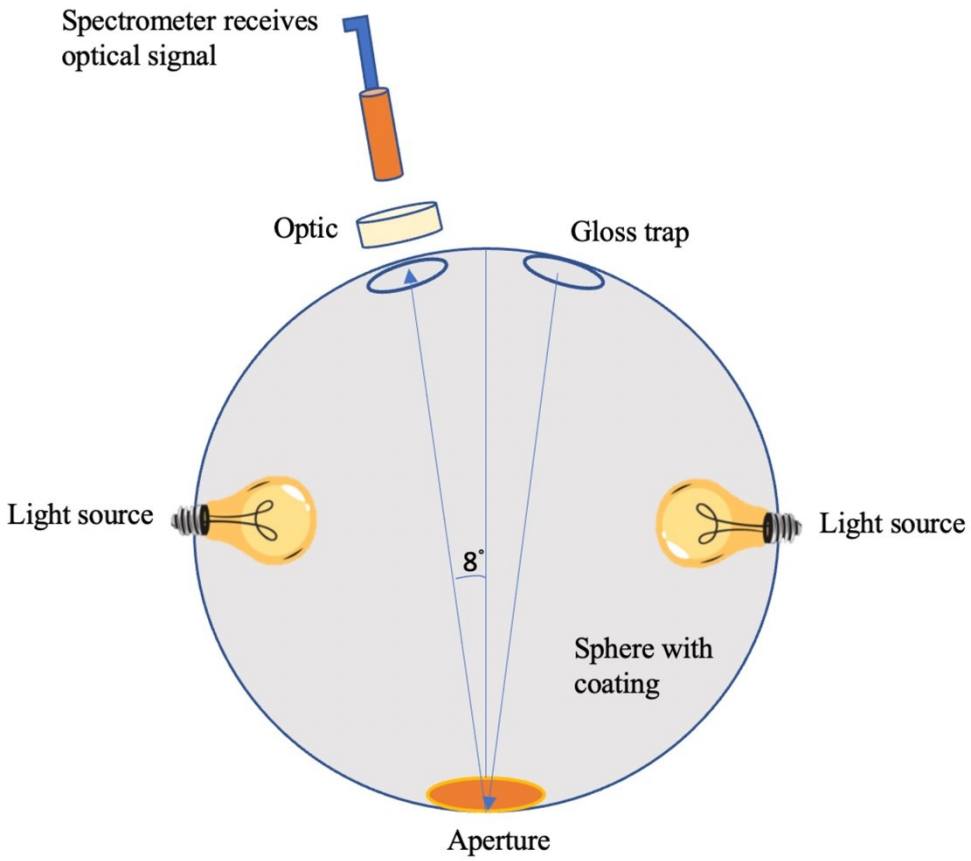

Figure 1.3 The working principle of the Gretag Macbeth Color Eye 7000A spectrophotometer.

According to the manufacturer, the Gretag Macbeth spectrophotometer Color Eye 7000A has two spectral analyzers. This dual-beam design could provide both single and multiple flashes and thereby ensure the stability of measurement over time. The light source is fixed on the rear of the sphere which can provide diffuse illumination.

Since spectrophotometers are usually massive, complex and expensive, their applications are restricted among patients. The VITA Easyshade is an intraoral and visible-range color

measureing device, which enables reproducible shade matching during dental practices. The Vita Easyshade measuring device was specially developed for color measurement in the mouth. The tooth is centrally illuminated with an LED light source via a light guide. The satellite fibers of the light guide transmit the reflected light from the tooth to the photodiodes, which are connected to each other. The measured values are assigned to tooth colors by a neural network (Wayne D. Jung, 2009), which correspond to the Vita ceramic shade guides. The weighting of the individual photodiodes is unknown. The topology of the neural network is also a company secret. In addition to assigning the measured values to ceramic shade guides, the Vita Easyshade measuring device also calculates colorimetric values, for example, CIE $L^*a^*b^*$ values. How exactly these values are calculated and whether they are valid remains unknown. However, it is certain that the Vita Easyshade measuring device is not a full spectrophotometer. After the collection of data, the data are then transformed to shade tab equivalents. The inter-observer reliability of VITA Easyshade was evaluated by Knezovic et al. (2016). Four well-trained observers were asked to assess the colors of the central region of the labial surfaces. The intraclass correlation coefficients showed no significant difference in the presented data of the four observers, which indicated that the inter-observer reliability of VITA Easyshade was acceptable.

1.4.4 Digital camera

The spectrophotometers and colorimeters with a defined aperture have an inherent shortcoming that only the color within the aperture can be captured. Subsequently, the color information of the whole tooth surface cannot be evaluated. The non-contact digital cameras can overcome this shortcoming and provide the entire color information of a tooth (Tam and Lee, 2012). Comparing to spectrophotometers and colorimeters, digital cameras are more suitable for the measurement of translucent and non-flat objects. The digital measurement can minimize the systematic errors which are caused by surface curvature and translucency of teeth (Guan et al.,

2005). Digital images can also reduce misunderstanding between dentists and dental technicians (Tam and Lee, 2012).

However, uncontrolled environmental variables, such as illumination and shooting position, can influence the digital information of tooth color. Besides that, the results of color measurement depend on the device itself. The accuracies of color measurement of three digital single-lens reflex cameras Nikon D100, Canon D60 and Sigma SD9 were compared with a spectroradiometer (Wee et al., 2006). It was found that every camera and calibration differed from another in tooth color, with the ΔE values ranging from 1.79 to 5.25. The accurate calibration is the prerequisite for the accurate color measurement of the commercial digital SLR cameras (Wee et al., 2006). Provided that the calibration was well controlled, digital imaging could be considered as a reliable alternative to colorimeters in dentistry (Caglar et al., 2010).

1.5 CIELAB and CIEDE2000 formulas

The International Commission on Illumination recommended a color difference in 1976: the CIELAB formula. The CIELAB formula is described as follows:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}$$

where ΔL (L_2^*-L_1^*), Δa (a_2^*-a_1^*) and Δb (b_2^*-b_1^*) are the differences in lightness, greenness-redness and blueness-yellowness, respectively. The L^* values range from 0 (black) to 100 (white) and a^* and b^* values indicate the chromaticity coordinates.

Human eyes are more sensitive to subtle color changes in some areas of color wheel but less sensitive in others. Considering that ΔE value of 1.0 is the lower limit of color perception threshold, it reveals sometimes a small visible difference in one area and sometimes a large

visible difference in another area. From this perspective, CIELAB presents a poor uniform color space regarding subtle color changes. Therefore, improvement of the color-difference formula is needed. In 2001, the CIEDE2000 formula was officially adopted as follows:

$$\Delta E_{00}^{*} = \sqrt{\left(\frac{\Delta L'}{k_L S_L}\right)^2 + \left(\frac{\Delta C'}{k_C S_C}\right)^2 + \left(\frac{\Delta H'}{k_H S_H}\right)^2 + R_T \frac{\Delta C'}{k_C S_C} \frac{\Delta H'}{k_H S_H}}$$

where ΔL^*, ΔC^* and ΔH^* represent for the differences in lightness, chroma and hue, respectively. The S_L, S_C and S_H are the weighting factors for lightness, chroma and hue, respectively (Wee et al., 2007).

The accuracies in color perception of the two formulas were compared by Gomez-Polo et al. (2016). In their work, a nonmetric multidimensional scaling was applied to the results which were obtained by calculating with the two formulas. The coordinates of the samples were then expressed in a Euclidean space. They found that the CIEDE2000 formula showed the color differences that perceived by naked human eyes better than the CIE76 formula. Compared with the old formula, the CIEDE2000 formula provides a better fit to the color difference and better indicators of color perceptibility and acceptability (Wee et al., 2007). Therefore, some believe that the CIEDE2000 formula should be used in the future to evaluate tooth color instead of the CIELAB formula.

However, Kuehni (2002) stated that the CIEDE2000 formula might not be the final word for evaluating the small color differences. The experimental data on which both formulae are based are far from perfect. If we take a further investigation of both formulae, the CIEDE2000 formula only offers a mathematical correction for the CIELAB formula. Neither of them could provide us with absolutely accurate color measurements. Both are designed to prove the

existence of color changes. From this perspective, it does not matter which one is used to determine tooth color changes.

To investigate the prevalence of the application of the two formulas, we searched PubMed for original studies in the last ten years, in which either of the two formulas was applied. The search results are shown in ***Table 1.3***. We found that the number of studies that used the CIELAB formula was twice as many as the studies that used the CIEDE2000 formula.

Table 1.3 The number of hits of the two formulas in PubMed

Formula	Hits	Search strategy
CIELAB	94	((CIELAB[Title/Abstract]) OR (CIE76[Title/Abstract]) AND (y_10[Filter])) AND ((tooth color[MeSH Terms] AND (y_10[Filter])) OR (((tooth color[Title/Abstract]) OR (tooth shade*[Title/Abstract])) OR (teeth color[Title/Abstract]) AND (y_10[Filter])) AND (y_10[Filter])) Filters: in the last 10 years
CIEDE2000	49	((CIEDE2000[Title/Abstract]) OR (CIE2000[Title/Abstract]) AND (y_10[Filter])) AND ((tooth color[MeSH Terms] AND (y_10[Filter])) OR (((tooth color[Title/Abstract]) OR (tooth shade*[Title/Abstract])) OR (teeth color[Title/Abstract]) AND (y_10[Filter])) AND (y_10[Filter])) Filters: in the last 10 years

1.6 Main objectives of the thesis

This thesis has five main aims:

1. To evaluate the impact of human saliva and enamel morphology on the HAP whitening performance;

2. To investigate the whitening effect of an experimental toothpaste that contains HAP nanoparticles;

3. To investigate the influence of concentration of HAP on its tooth-whitening effect;

4. To achieve further understanding of the impact of particle size of HAP on its tooth-whitening effect;

5. To evaluate the tooth-whitening effect of HAP mouthrinses after prolonged application time.

The main purposes and the structure of the current thesis are shown in ***Figure 1.4***.

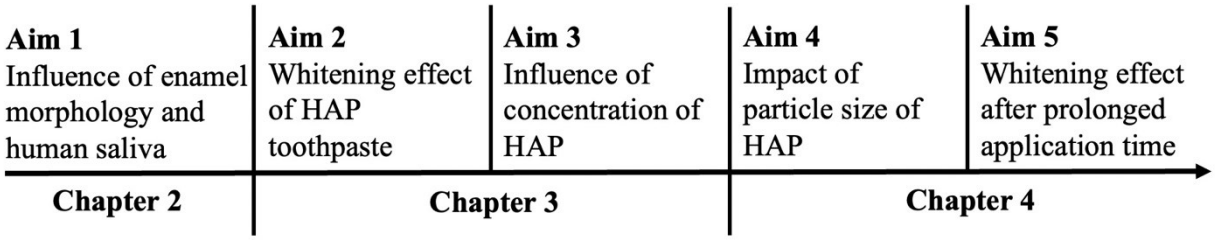

Figure 1.4 The aims and structure of the thesis.

1.7 Structure of the thesis

The second chapter of the thesis analyzed the impact of human saliva and enamel morphology on the HAP whitening performance. A total of 60 bovine incisors were randomly assigned to 6 groups (n=10 for each group): HAP-treated native enamel with the storage in the human saliva or in the mineral water, HAP-treated ground enamel with the storage in the human saliva or in the mineral water, bleaching mouthrinse-treated native surface with the storage in mineral water and non-treated native surface with the storage in mineral water. Each sample was treated with 10 wt% HAP aqueous suspension three times. The HAP-treated enamel was observed by SEM. The tooth color was measured and average color changes ($\triangle E$ values) were calculated.

The third chapter of the thesis evaluated the post brushing tooth-whitening effect of toothpaste that contained hydroxyapatite nanoparticles (nano-HAPs). The impact of the concentration on the whitening performance of nano-HAP toothpaste was also investigated. Two concentrations of nano-HAP (10 wt% and 1 wt%) were incorporated in nonabrasive toothpastes. Forty bovine incisors were randomly assigned into four groups: 10 wt% nano-HAP, 1 wt% nano-HAP, toothpaste without nano-HAP as a negative control and water as a blank control. Each tooth

was treated with the toothpaste three times and hydrodynamic shear force (HSF) once. The nano-HAP-treated enamel was observed by SEM after each application. Tooth color was measured by using a spectrophotometer and color changes were calculated.

In the fourth chapter of the thesis, the tooth-whitening effect of mouthrinses that contained HAP particles of different sizes was investigated after a prolonged period of application and compared with a commercially available whitening mouthwash**.** Fifty bovine incisors were stained and randomly distributed into five groups: the HAP groups with the particle sizes of 3 μm, 200 nm and 50 nm, respectively, the commercial whitening mouthwash group and the distilled water group. The teeth underwent prolonged mouthwash applications that equivalent to 3- and 6-month mouthrinsing. Tooth color was measured and calculated before and after mouthrinsing.

In the fifth chapter, the abstract and conclusion of each chapter was summarized.

Chapter 2: The influence of enamel morphology and human saliva on the tooth-whitening performance of hydroxyapatite

2.1 Background and significance

Hydroxyapatite (HAP) is a promising biomimetic tooth-whitening agent and is considered to be an ideal alternative to conventional peroxide-based bleaching systems (Dabanoglu et al., 2009, Jin et al., 2013, Yamagishi et al., 2005, Hojabri et al., 2020). Compared with the bleaching agents, the whitening effect of HAP was shown to be mild (Hojabri et al., 2020) and non-cytotoxic (Coelho et al., 2019). Moreover, due to its extraordinary biocompatibility, HAP could not cause hard or soft tissue irritations, such as post-treatment dentin hypersensitivity, oral mucosa burning, and change of enamel microstructure or microhardness (Jin et al., 2013, Dabanoglu et al., 2009). It is reported that HAP dental products are suitable for all age groups including patients under the age of 18 (Steinert et al., 2020a).

In most early *in vitro* studies, HAP was applied on sound enamel surfaces and exhibited the whitening effect (Dabanoglu et al., 2009, Bommer et al., 2018, Jin et al., 2013). However, these findings could only confirm the whitening effectiveness of HAP on natural enamel. To date, it remains unknown about the whitening effect of HAP on worn enamel. The term of tooth wear refers to the loss of enamel tissue without the existence of dental caries and trauma. Erosion, attrition and abrasion are the three main mechanisms of tooth wear (Bishop et al., 1997), making tooth wear an ever-increasing and sometimes unavoidable problem in current society, especially in the elderly age group (Jaeggi et al., 2006, Liu et al., 2014). The prevalence of tooth wear in the aging population of northwest China was reported as high as 100% (Liu et al., 2014). Increased teeth sensitivity and yellowing appearance of teeth are the two main symptoms of tooth wear. Considering the adverse effect of bleaching agents, the patients with worn enamel are contraindicated to dental bleaching. Therefore, the whitening effect of HAP

on worn and ground enamel should be urgently evaluated.

Laboratory tests should simulate clinical conditions as closely as possible to generate results relevant to the clinical situation. Within a few minutes after tooth-brushing, tooth enamel will be covered with an acellular layer, namely acquired pellicle, which is only 0.1 to 1 mm thick and consists of proteins and peptides that derived from the complex composition of organic components in human saliva. Therefore, HAP dental products firstly come into the contact with the acquired pellicle instead of with enamel. Understanding the pellicle-HAP interaction is essential to discover the mechanism of the tooth-whitening effect of HAP in the oral environment. However, the HAP-treated teeth in early studies were either stored in water (Jin et al., 2013, Dabanoglu et al., 2009, Bommer et al., 2018) or artificial saliva (Hojabri et al., 2020). The role of human saliva in the HAP tooth-whitening process remains unknown.

To bridge these gaps, the whitening effect of HAP on ground enamel was evaluated by comparing it with that on natural enamel. The impact of human saliva on the whitening performance of HAP mouthrinse was also investigated. A commercial bleaching over-the-counter (OTC) mouthrinse served as the control group. The mouthrinses used in our study were applied to tooth surfaces by simulating oral rinsing. The application was repeated three times. Tooth color was measured before and after each HAP application. The null hypothesis of this chapter was that there was no influence of human saliva and enamel morphology on the whitening effect of HAP.

2.2 Materials and Methods

2.2.1 Specimen preparation

A total of 60 bovine teeth were divided equally into 6 groups. Each group had 10 samples. The groups were described as follows (N = native enamel, G = ground enamel, S = saliva, W = water):

Group 1: HAP treatment + native enamel + the storage in the human saliva (HAP-N-S);

Group 2: HAP treatment + ground enamel + the storage in the human saliva (HAP-G-S);

Group 3: HAP treatment + native enamel + the storage in the mineral water (HAP-N-W);

Group 4: HAP treatment + ground enamel + the storage in the mineral water (HAP-G-W);

Group 5: bleaching treatment + native enamel + the storage in the mineral water (BL-N-W);

Group 6: mineral water treatment + native enamel + the storage in the mineral water (W-N-W).

Firstly, the bovine teeth were immersed in the coffee solution (Nescafe Espresso, Nestle AG, Germany) for 72 h. According to the recipe of the manufacturer, 100 g prepares 55 cups of coffee solution, with 60 ml hot water required for each individual dose. Considering that 3.2 servings are consumed daily by typical coffee drinkers, 5.8 g coffee (3.2 × 100 / 55) in 192 ml boiling water was considered as the recipe of the staining solution. After staining, the teeth were cleaned ultrasonically to remove the loose extrinsic colorants. Then the stained teeth were embedded into 3D-printed sample holders (***Figure 2.1***), which were designed to match exactly with the position of the measuring window of the spectrophotometer (Color Eye 7000 Gretag MacBeth, X-Rite-, Germany).

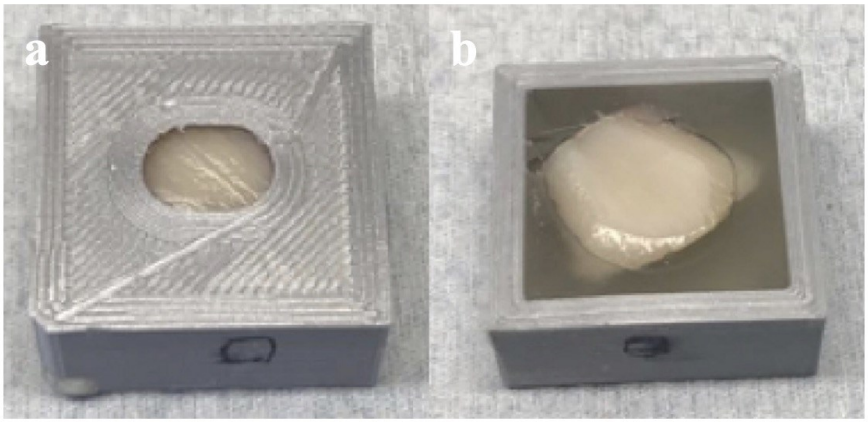

Figure 2.1 Enamel morphology of the bovine teeth that embedded in 3D-printed sample holders. a: native enamel; b: ground enamel.

2.2.2 Enamel morphology

To evaluate the influence of enamel morphology on the whitening effect of HAP, a half number of samples were ground flat. The native enamel is highly glossy and polarizes the light. A natural tooth has an outer layer of crystalline enamel (amorphous enamel) and this layer is removed after grinding. This might affect the optical properties of the tooth. Moreover, the natural formation of acquired pellicle on enamel might also be influenced.

The labial superficial enamel of the samples was ground on an abrasive polishing machine (Struers Pedemin DAP-7, Roper Technologies Inc., USA) with 180 grit SiC abrasive paper (Buehler, ITW, USA) to form an ellipse flat surface of 10 mm in the vertical axis and 8 mm in the horizontal axis. The specimens were then polished serially with 600-, 800- and 1200-grit SiC papers under water cooling. After that, the samples were carefully ultrasonically washed.

2.2.3 Repeated HAP application

2.2.3.1 Preparation of mouthrinses

To determine the whitening effect of HAP products as well as possible, chemically, and morphologically suitable raw material should be used as it contains no additives such as flavors, preservatives and rheology modifiers, which might affect the HAP whitening ability. The pure HAP powder (DP-PC-BIO-2018-004, Budenheim, Germany) that was used in our study was provided by the company Budenheim. The pure powder was mixed into the water to form an aqueous suspension with a concentration of 10 wt%.

According to the manufacture, the commercial bleaching mouthwash (Colgate Plax Whitening, Colgate, USA) contained 1.5% hydrogen peroxide, glycerin, propylene glycol, sorbitol, tetrapotassium pyrophosphate, tetrasodium pyrophosphate, zinc citrate, PVM/MA copolymer, sodium fluoride and sodium saccharin. This commercially available bleaching OTC product has a totally different tooth whitening mechanism comparing with the HAP products.

2.2.3.2 Simulated mouthrinsing process

To simulate the mouthrinsing process, the embedded samples were mounted on the inner wall of a 500 ml glass beaker and exposed to the HAP or commercial bleaching mouthrinse, which was applied to samples continuous stirring at the speed of 100 rpm for 1 minute. This application process was repeated three cycles.

After each mouthrinse application, the samples were stored in human saliva or in mineral water (Evian, Danone Waters Deutschland, Germany) for 24 hours at 37 °C before the next application. The acquired pellicle will form on the enamel surface in human saliva, which is not possible when the samples were stored in water.

2.2.4 Storage in whole human saliva

To evaluate the impact of human saliva on the whitening effect of HAP, a half number of samples was stored in human saliva after each application of mouthrinses. Saliva samples were collected from caries-free volunteers aged between 25 and 35 years. The donors received a thorough oral hygiene procedure, followed by rinsing mouths with distilled water. The whole human saliva was collected in the morning (8 - 10 a.m.) without stimulation by draining methods: the donors sat quietly with the head slightly bend down and mouth open to allow the saliva to drip passively into the sterile tubes. After centrifuging for 20 minutes, the supernatant of the pooled saliva was stored at -4 °C before use. The human saliva was freshly collected before use.

2.2.5 Color measurement

To ensure the accuracy of repositioning, the samples were placed on a position locator, which was mounted in the measuring area of the spectrophotometer. Before measurement, we calibrated the spectrophotometer with a ceramic calibration tile. The color measurement was performed in a dark room, which could eliminate associated errors (Hojabri et al., 2020).

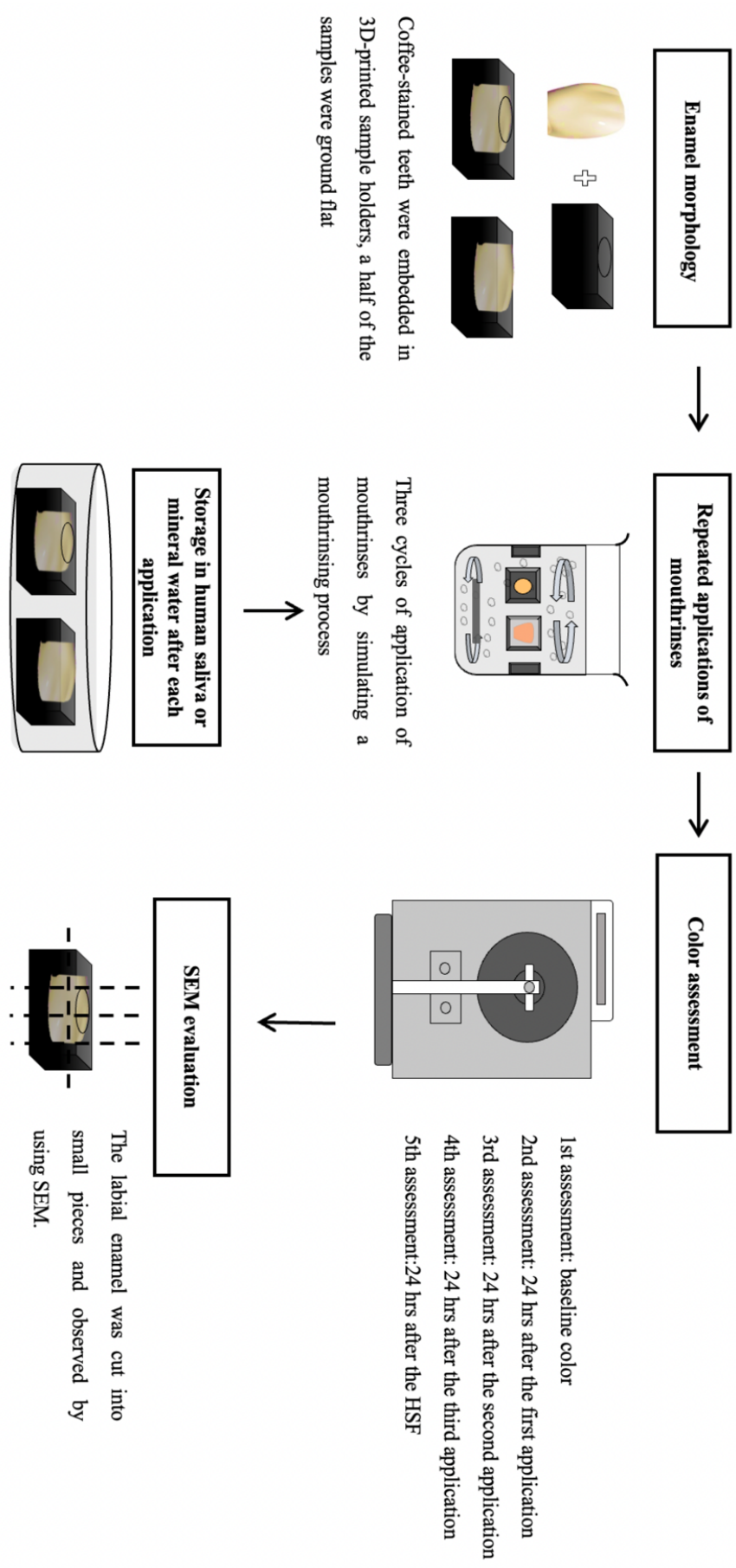

Figure 2.2 The workflow of the second chapter.

The baseline tooth color before mouthrinse treatment and the color after each cycle of application were measured according to the CIE $L^*a^*b^*$. For color analysis, the average color change ΔE values were defined by the formulas: $\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}, \Delta L = L - L_0, \Delta a = a - a_0, \Delta b = b - b_0$.

2.2.6 SEM evaluation

After the third application of mouthrinses, the samples were randomly selected from each group and were cut by the low-speed saw (Isomet, Buehler, Germany) into small slices followed by careful rinsing. One ground tooth and one native tooth without the HAP treatment served as the blank controls. The samples were dehydrated by immersing in ascending concentrations of ethanol (50, 75, 85, 95, 100%). After that, the samples were air dried for 24 hours. Then the samples were sputter-coated and observed using the SEM (Supra 55vp, Zeiss, Germany). The workflow is presented in ***Figure 2.2***.

2.2.7 Statistical analysis

The data were analyzed in R (R 3.1.0, R Foundation for Statistical Computing). A nonparametric analysis of longitudinal data using the nparLD package with the F1-LD-F1 design was performed to determine the enamel morphology, storage medium and their interactions. When an interaction between the two main effects was found, a Kruskal-Wallis test was applied to confirm the group effect. A Friedman test was applied to evaluate the influence of repeated application. Pairwise Wilcoxon rank-sum tests with BH correction were performed to compare the tooth color changes of individual groups. Statistical significance was set for all tests at $P < 0.05$.

2.3 Result

2.3.1 Influence of enamel morphology and storage medium

The results revealed significant main effects of group and application time for the ΔE, ΔL, Δa and Δb values ($P < 0.00001$) (***Table 2.1***). The group effect described the combination effect of enamel morphology and storage medium. Significant interactions were found between the two main effects for the ΔE, ΔL, Δa and Δb values ($P < 0.05$). The Kruskal-Wallis test and Friedman test confirmed the group effect and time effect on the ΔE and most of ΔL, Δa and Δb values ($P < 0.05$), indicating enamel morphology, storage medium and repeated application significantly influenced the whitening effect of HAP.

Table 2.1 Statistical results of ANOVA-type statistic, Kruskal-Wallis and Friedman test.

ANOVA-Type Analyze			
Effect	Statistic	df	*P* value
Group	13.88	4.11	1.45E-11*
Time	87.96	1.57	3.14E-31*
Group and time	4.33	5.22	4.90E-04*
Kruskal-Wallis rank sum test (time effect)			
First application	30.07	5	1.43E-05*
Second application	28.58	5	2.81E-05*
Third application	24.20	5	1.99E-04*
Friedman rank sum test (group effect)			
BL-N-W	20.00	2	4.54E-05*
W-N-W	8.60	2	0.01*
HAP-G-S	13.40	2	1.23E-03*
HAP-G-W	15.17	2	5.09E-04*
HAP-N-S	16.80	2	2.25E-04*
HAP-N-W	18.17	2	1.11E-04*

* $P < 0.05$

The Δ*E* values of each group were shown in ***Figure 2.3***. Within the four HAP groups, the HAP-G-S group exhibited the highest Δ*E* mean values (2.20 after the first application, 2.63 after the second application, and 3.21 after the third application), whereas the HAP-N-W group showed the lowest Δ*E* mean values (1.11 after the first application, 1.23 after the second application, and 2.04 after the third application). The commercial bleaching mouthrinse showed no significant difference in Δ*E* values comparing with the HAP-G-S groups throughout the observational period. The W-N-W group had the lowest color changes throughout the experiment.

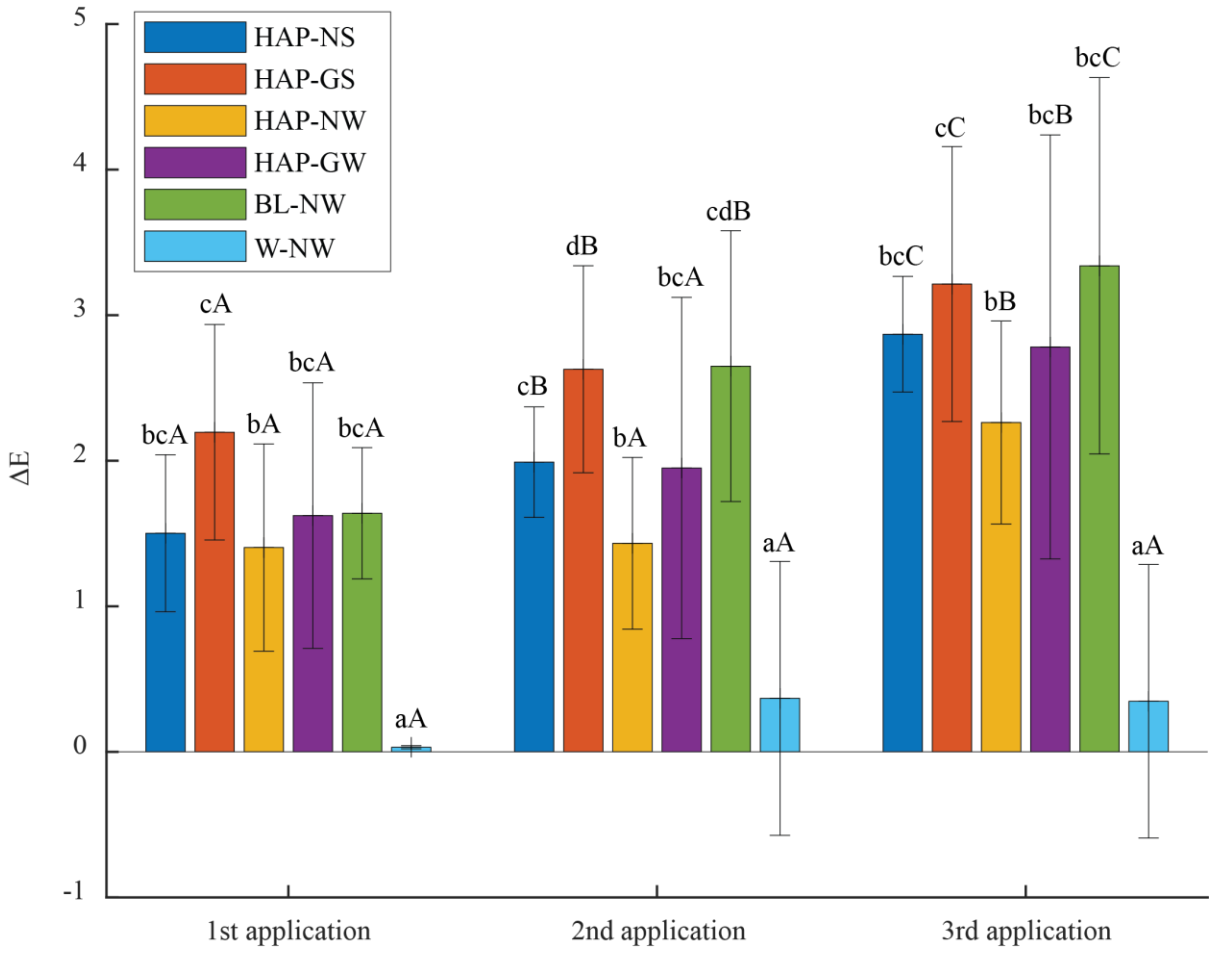

Figure 2.3 The average color changes (expressed as Δ*E* values) after HAP 1-3. Different lowercase letters indicate the statistical differences between different groups with the same application time; * indicates the statistical differences of the same group between individual application; different capital letters indicate groups that statistically differed from any other group ($P < 0.05$); same letter suggests that groups showed no statistical differences ($P > 0.05$).

Table 2.2 The changes in L^*, a^* and b^* axes (expressed as ΔL, Δa, and Δb values). Results are shown as mean value (standard deviation).

Groups	**HAP 1**	**HAP 2**	**HAP 3**
ΔL			
HAP-N-S	0.33 (0.65)^{bA}	0.23 (0.76)^{aA}	0.55 (0.74)^{aA}
HAP-N-W	1.22 (0.71)^{cdA}	0.97 (0.76)^{aA}	1.97 (0.70)^{bA}
HAP-G-S	1.19 (1.21)^{bdA}	0.87 (0.81)^{abA}	0.72 (0.67)^{aA}
HAP-G-W	0.32 (1.06)^{bA}	0.36 (0.71)^{aA}	0.67 (0.63)^{aA}
BL-N-W	1.20 (0.67)^{cA}	2.14 (1.07)^{bB}	2.92 (1.53)^{cC}
W-N-W	0.003 (0.02)^{aA}	0.33 (0.95)^{abA}	0.31 (0.95)^{aA}
Perception	Brighter	Brighter	Brighter
Δa			
HAP-N-S	-0.29 (0.24)^{abA}	-0.29 (0.24)^{acA}	-0.29 (0.28)^{abA}
HAP-N-W	-0.07 (0.14)^{bB}	0.02 (0.10)^{bC}	-0.14 (0.14)^{bA}
HAP-G-S	-0.39 (0.22)^{aA}	-0.48 (0.27)^{acA}	-0.40 (0.24)^{aA}
HAP-G-W	-0.21 (0.39)^{abA}	-0.17 (0.28)^{bcA}	-0.50 (0.54)^{abA}
BL-N-W	-0.49 (0.45)^{aA}	-0.61 (0.43)^{aA}	-0.67 (0.43)^{aA}
W-N-W	0.00 (0.02)^{abA}	0.00 (0.03)^{bA}	0.00 (0.03)^{cA}
Perception	Greener	Greener	Greener
Δb			
HAP-N-S	-1.27(0.57)^{aC}	-1.78 (0.48)^{bB}	-2.71 (0.38)^{aA}
HAP-N-W	-0.57 (0.39)^{bA}	-0.85 (0.41)^{cA}	-0.98 (0.49)^{cA}
HAP-G-S	-1.33 (0.82)^{aA}	-2.29 (0.71)^{aA}	-1.81 (0.83)^{bA}
HAP-G-W	-0.95 (1.14)^{abC}	-1.74 (1.21)^{abB}	-2.48 (1.54)^{abA}
BL-N-W	-0.25 (0.75)^{bB}	-0.94 (0.93)^{cA}	-0.86 (0.88)^{cA}
W-N-W	0.00 (0.02)^{cA}	0.01 (0.03)^{dA}	0.01 (0.02)^{dA}
perception	Bluer	Bluer	Bluer

Different lowercase letters suggest the differences between different groups at same experiment time point; different capital letters indicate the differences of the same group between different experiment time points; same letter suggests that groups showed no significant differences ($P > 0.05$);from a (A) to c (C), the mean value is increased.

After the first application, the HAP-G-S group exhibited statistically higher Δ*E* values than the HAP-N-W group ($P = 0.014$). The difference between the two groups remained statistically significant after the second ($P = 0.002$) and third application ($P = 0.03$). After the second application, the HAP-G-S group showed statistically higher Δ*E* values than those of the HAP-N-S group ($P = 0.03$). These findings suggested that the attached HAP particles on ground enamel behaves differently compared with those on the native enamel. We also observed that the whitening performance increased with the repeated application.

The tooth color changes of the HAP groups and commercial mouthwash group were contributed by the increased *L** values and decreased *a** and *b** values. The mean value and standard deviation of the Δ*L,* Δ*a* and Δ*b* values of each group were summarized in ***Table 2.2***. Expect the W-N-W group, all the other groups showed enhancement in the *L* values and decrease in the *a,* and *b* values.

2.3.2 SEM evaluation

The enamel morphology of the samples was shown in ***Figure 2.4*****.** After the third HAP application, the adhesion of HAP particles could be clearly identified on the enamel surfaces in all HAP groups (***Figure 2.5***). The HAP aggregations consisted of round HAP crystallites, which connected to each other and to the enamel surface with small solid bridges, resulting in the three-dimensionally multilayered structures. In the HAP-N-S and -G-S groups, a mixture of bacteria, plaque and HAP agglomerates were observed. Comparing with the HAP-N-W and HAP-G-W groups, more adhering HAP aggregations were observed in the HAP-N-S and HAP-G-S groups, suggesting that human saliva influences the adhesive behavior of HAP particles. More HAP attachments were observed in the HAP-G-W group than that of the HAP-N-W group. In the HAP-G-S and HAP-N-S groups, the enamel surfaces were almost entirely covered with HAP.

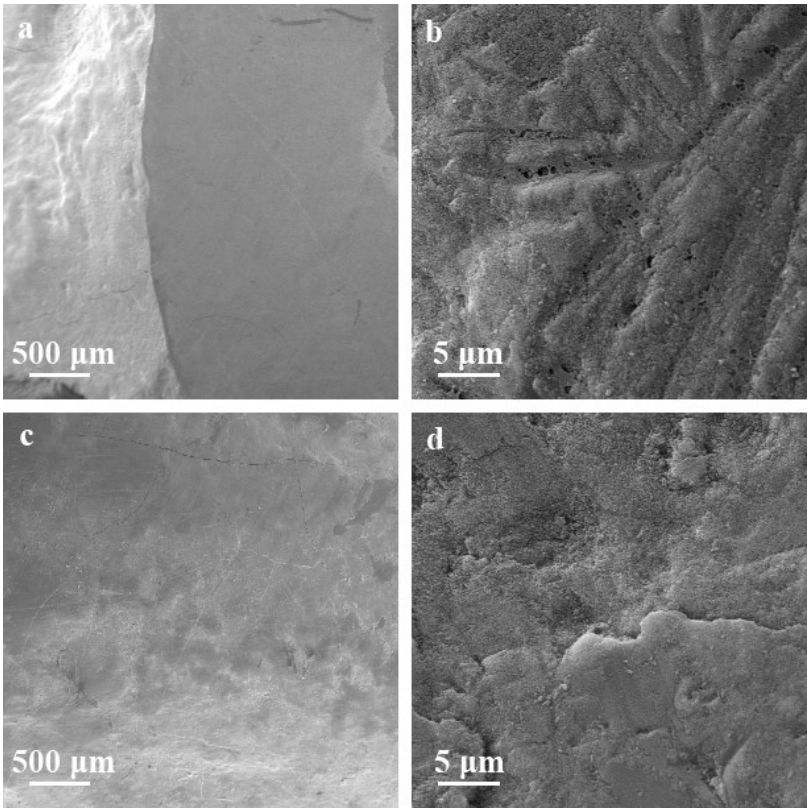

Figure 2.4 Enamel morphological surfaces were visualized at 20 × and 2000 ×. a and b: ground enamel surface; c and d: natural intact enamel surface.

2.4 Discussion

2.4.1 Tooth-whitening effect of HAP mouthrinse

In the present study, we found that the Δ*E* mean values of the HAP mouthrinses ranged from 1.40 to 3.21, whereas the Δ*E* mean value of the water group ranged from 0.03 to 0.37, which confirmed the whitening effect of HAP. Alghazali et al. (2012) evaluated the perceptibility threshold of tooth color difference. In their work, a total of 80 observers were enrolled and divided into groups of technicians, dental nurses, dentists and researchers. They found that 50% of all observers could detect a tooth color change with Δ*E* values ranging from 1.7 and 2.1, indicating that the changes in tooth color caused by the HAP mouthrinses could be detected with the naked eyes.

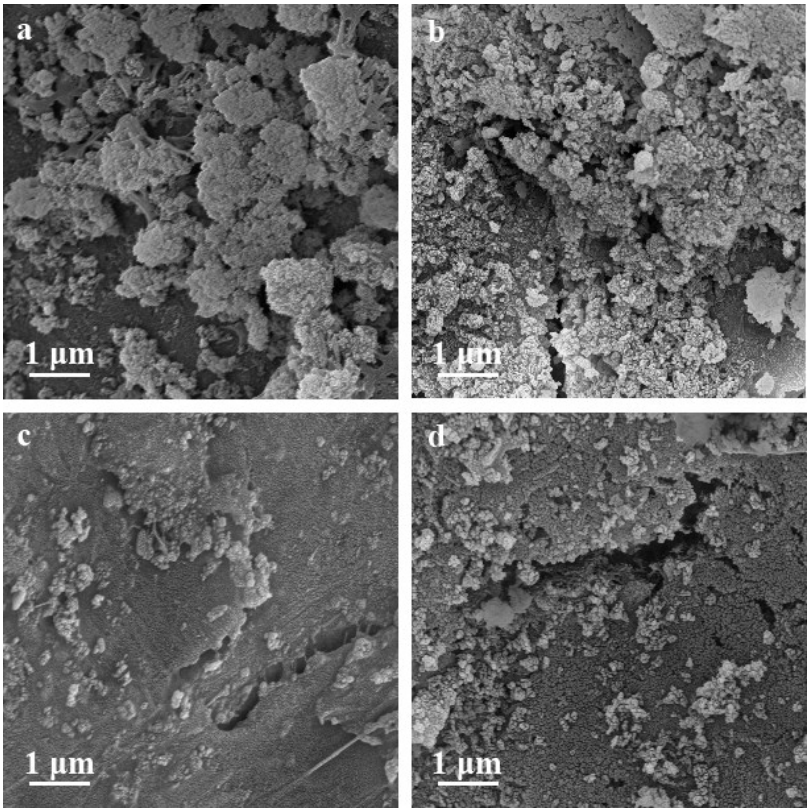

Figure 2.5 The HAP mouthrinse-treated enamel surfaces were visualized at 5000 × after the third application. a: HAP-N-S group; b: HAP-G-S group; c: HAP-N-W group; d: HAP-G-W group.

Several *in vitro* studies have attempted to investigate the tooth-whitening effect of HAP products. In their studies, HAP was either mixed into a dental gel (Sarembe et al., 2020) and water (Jin et al., 2013), or incorporated with dissolvable polymer films (Dabanoglu et al., 2009) and self-assembling peptides (Bommer et al., 2018, Hojabri et al., 2020). The Δ*E* values in these studies fluctuated over a wide range, which varied from 1.41 to 12.73. The high variation might be caused by different modifications to HAP particles, which were designed to enhance the attachment of HAP to enamel surfaces (Hojabri et al., 2020). In our study, no modification at the molecular level was performed, which could explain the relatively limited whitening ability of the raw HAP particles.

We found that the commercial bleaching mouthrinse which contained 1.5% hydrogen peroxide exhibited similar Δ*E* mean values (1.63 to 3.34) to those of HAP mouthrinse. This encouraging

finding illustrated that under the same experimental conditions and protocol, a HAP mouthrinse could be considered as an effective alternative to a conventional peroxide-based bleaching mouthrinse for the purpose of tooth whitening. It is worth to mention that the whitening effects of both HAP mouthrinse and the commercial bleaching mouthrinse that used in our study did not reach the level of color acceptance with a ΔE mean value of 4.2 (Alghazali et al., 2012). The low concentration of hydrogen peroxide in the OTC dental mouthrinse that used in our study might explain the limited whitening effect. It was reported that the OTC bleaching mouthrinses should be applied for 12 weeks to get similar whitening results to those of two-week at-home bleaching with 10% carbamide peroxide (Torres et al., 2013). Whether a prolonged application of HAP would result in more effective whitening performance remains largely unknown. In Chapter 4 of the thesis, the whitening effect of HAP after a prolonged application period was investigated and discussed.

2.4.2 Influence of enamel morphology

The null hypothesis that enamel morphology and storage medium has no influence on the tooth-whitening effect of HAP was rejected since we found that all the variables had statistically significant influences on the whitening ability of HAP.

In our study, ground enamel tended to appear brighter than the natural one. The light might be reflected differently on enamel with different morphology. The HAP whitening experiments that performed on the untreated, native enamel surfaces could provide us with clinically relevant results. However, the surface curvature and gloss of native enamel could result in the randomly distributed light reflection, leading to an undesirable great scattering of measured values. Ground enamel could reduce the random light reflection which occurs on intact enamel (Hojabri et al., 2020). Accordingly, more light might reflect from the ground enamel and reach human eyes. On the other hand, the prism direction and the crystallite orientation differ between natural enamel surfaces and deeper enamel (Poole and Johnson, 1967), which might

lead to different adhesion behavior of HAP particles. In the present study, more HAP attachment was observed in the HAP-G-W group than that of the HAP-N-W group. We believed that the ground enamel facilitates HAP adhesion, which is probably the most important explanation for the differences in whitening effect between ground and native enamel. It is worth to mention that the comparison between ground and enamel is only relevant for future laboratory investigations.

2.4.3 Influence of human saliva

Considering that the HAP tooth-whitening effect is due to the enhanced light reflection on enamel that caused by adhesion of HAP particles, it is reasonable to assume that the human saliva could even further improve the HAP attachment to tooth surfaces instead of disturbing it. This assumption was confirmed by SEM photos. In the saliva groups, a mixture of bacteria, plaque and HAP agglomerates was observed. A greater amount of HAP agglomerates was found in saliva groups comparing with the in-water stored groups. Studies have shown that some proteins in the acquired pellicle, such as statherin, histatins and proline-rich glycoproteins, have an affinity for enamel surface (Oppenheim et al., 2007). Therefore, these proteins may occupy the binding sites for HAP particles and thereby increase their adhesion. Moreover, the calcium-binding peptides in the acquired pellicle provide new binding sites to the calcium ions (Baumann et al., 2017) and may attract the HAP attachment.

In the saliva-treated groups, microsized mineralized bridges between enamel and HAP agglomerates were discovered. This finding is in accordance with the study of Nobre et al. (2020), in which the adhesion of HAP nanoparticles to titanium, ceramics and polymethyl-methacrylate under simulated oral conditions was investigated. They also found small bridges between HAP nanoparticles and material surfaces in the presence of acquired pellicle. In the present work, microsized HAP particles were used. Therefore, we assumed that the human

saliva could act as bridges that connect the HAP particles and enamel, regardless of the particle size.

2.4.4 Influence of repeated application

The HAP mouthrinse was applied on three consecutive days. Each application was followed by 24-hours storage in either human saliva or mineral water. This experimental protocol was described and used in most of the *in vitro* studies, in which the whitening effect of HAP was investigated (Jin et al., 2013, Bommer et al., 2018, Dabanoglu et al., 2009). To compare the whitening results with these early approaches, the same protocol was chosen in our research. It was concluded by these early studies that tooth color increased numerically with reapplication without statistical significance (Dabanoglu et al., 2009), "time" factor was not significant and had no influence on the outcome (Jin et al., 2013), and repeated application would not change tooth color anymore after the maximum whitening effect had been reached (Bommer et al., 2018). In the current study, tooth color changes increased significantly with repeated application. The tendency has reached the statistical level. The simulated mouth rinsing could prevent the undesired gravity-induced HAP deposition, which might slow down the achievement of the maximal adhering load.

2.4.5 Strengths

We bridged the gap of understanding the role of human saliva in the whitening performance of HAP and took a further step toward clinical reality. The whitening performance of HAP on ground enamel surfaces was confirmed in our study, indicating that HAP whitening products are also suitable for patients who suffer from teeth wear.

Chapter 3: Tooth whitening with an experimental toothpaste containing hydroxyapatite nanoparticles

3.1 Background and significance

The color of a tooth is a complex optical phenomenon. When the incident light falls on the tooth surface, it undergoes a process of reflection, absorption and scattering (ten Bosch and Coops, 1995, Jahangiri et al., 2002). Additionally, morphological tooth characteristics, such as curvature and texture, can affect the direction of the light reflection and thereby influence the tooth appearance (Cal et al., 2006). There are also age-related changes in tooth color, for example, the reduction in enamel thickness, which makes the teeth appear darker since the dentin shines through more. In addition to this physiological change, tooth color is also affected by a combination of intrinsic and extrinsic discoloration. Intrinsic discoloration, such as tetracycline teeth or dental fluorosis, is caused by the incorporation of chromophores into enamel and dentin, while extrinsic discoloration refers to discoloration on the tooth surface, usually caused by the ingestion of colorants (such as tobacco, coffee and tea.) into the oral cavity. It was reported that 28% of adults in the United Kingdom, 34% of adults in the United States, and 56% of adults in China are dissatisfied with the esthetic of their teeth (Joiner, 2006, Xiao et al., 2007). According to the American Academy of Cosmetic Dentistry, tooth whitening is one of the most popular and common dental procedures in cosmetic dentistry ((AACD), 2015).

Conventional OTC whitening approaches include physical removal of stains with abrasives and detergents and chemical degradation of chromophores with peroxide-based agents (Carey, 2014). However, these all have potential adverse effects, such as abrasion, posttreatment hypersensitivity, mucosal irritation and genotoxicity. This limits their use in patients with worn enamel, erosion, exposed dentin or during pregnancy (Goldberg et al., 2010, Tredwin et al.,

2006). In addition, the Council Directive of European Union (2011) stated that OCT bleaching products that contain higher than 0.1% hydrogen peroxide cannot be sold to patients without the supervision of dentists. Therefore, alternative nonoxidative tooth-whitening formulations are needed.

Alternative whitening products should not only be gentle and suitable for a considerable range of consumers but also efficient for daily at-home dental care. Hydroxyapatite (HAP) is considered one of the most biocompatible and biomimetic materials, since it has a high similarity to the apatite crystal of human tooth enamel in terms of its morphology and crystal structure (Coelho et al., 2019, Tschoppe et al., 2011). In addition to its wide application in preventive dentistry, oral surgery, periodontology and implantology (Yamagishi et al., 2005, Vano et al., 2018), the whitening effect of HAP has gradually attracted the attention of dental researchers.

In 2001, Niwa et al. (2001) found that toothpaste containing HAP had a tooth-whitening effect, which was not caused by their polishing effect. Four years later, Yamagishi et al. (2005) reported that HAP could adhere to the enamel surface after pretreatment with phosphoric acid, forming a layer that covers the entire tooth surface. This newly formed layer was then proven to be able to increase the reflection of light (Bommer et al., 2018). In the following decade, some *in vitro* studies (Jin et al., 2013, Dabanoglu et al., 2009, Hojabri et al., 2020) confirmed the tooth-whitening effect of aqueous HAP suspensions, which could be used clinically as nonoxidizing whitening mouth rinses. However, the problem is that a patient has to buy an additional oral care product in the form of a mouth rinse to fulfill their desire for whiter teeth. To make it easier for patients, it would be nice if a HAP toothpaste exhibits similar tooth-whitening ability. This would allow a patient to achieve whitening and cleaning purposes with a single product. Recently, Vorleitner (2021) mixed HAP materials into a commercial toothpaste and found that the incorporation of HAP did not result in any tooth color changes.

However, the interaction between the HAP material and the ingredients in the toothpaste is unknown because neither the ingredients nor their amount was reported. Some ingredients may influence the whitening performance of commercial toothpastes with HAP additives such as fluoride or metaphosphates.

To date, there is no consensus on the influence of the nano-HAP concentration on the tooth-whitening effect. A quantitative analysis confirmed a positive correlation between the HAP concentration and adhesion efficiency (Fabritius-Vilpoux et al., 2019). Thus, increasing the HAP concentration may positively influence its whitening outcome. However, this assumption was not observed by two previous studies, in which nano-HAP was mixed into different substrates (such as a dissolvable polymer film and a self-assembling peptide) to achieve an enhancement of adhesion (Dabanoglu et al., 2009, Hojabri et al., 2020). The unique spatial molecular structures of the substrates may interfere with revealing the impact of the HAP concentration on its whitening effect. Studies addressing the role of its concentration in the whitening process of nano-HAP toothpastes with known composition are still lacking.

To fill these gaps, we prepared nonabrasive nano-HAP toothpastes and applied them to the enamel surface with a real toothbrush to simulate the daily toothbrushing process. Our objective was to evaluate the potential of self-administered use of HAP toothpaste. The null hypothesis states that the HAP concentration does not influence the whitening effect of HAP toothpaste.

3.2 Materials and methods

3.2.1 Preparation of toothpaste slurries

Abrasive-free toothpaste slurries were prepared as described by Wiegand et al. (2009). Briefly, the toothpaste slurries consisted of artificial saliva (Pharmacy of the LMU Munich, Germany), glycerine, sodium bicarbonate, and carboxymethylcellulose. Nano-HAP (NanoXIM CarePaste,

Fluidinova, Portugal) was then added to prepare the toothpaste slurries, which contained two different concentrations of nano-HAP (1 wt% and 10 wt%). The toothpaste slurries were stirred before each application to ensure the homogeneity of the nano-HAP.

3.2.2 Preparation of tooth samples

After careful removal of connective tissue from the bovine teeth, the teeth were rinsed with distilled water. Then, they were stained artificially. The staining solution was prepared by dissolving six grams of instant coffee (Nescafe Espresso, Nestle AG, Germany) in 200 ml of boiling water. The coffee solution was centrifuged at 2000×g/min for 10 minutes (ROTIXA/A, Hettlich, Germany). The tooth samples were stored in the supernatant at 37°C for three days. The samples were then polished with a polishing paste and ultrasonically cleaned for 10 minutes to remove all extrinsic stains. Then, all coffee-stained teeth were embedded into 3D-printed specimen holders using self-curing polyester material (Technovit 4000, Kulzer Technik, Germany) (***Figure 3.1a*** and ***b***). The specimen holders had a measurement window that allowed color measurements to be made on the middle third of the labial enamel surface (***Figure 3.1c, d*** and ***e***).

3.2.3 Application of toothpaste slurries

A total of 40 tooth samples were randomly assigned to four groups: toothpaste slurry with 10 wt% nano-HAP (n = 10), toothpaste slurry with 1 wt% nano-HAP (n = 10), toothpaste slurry without nano-HAP as a negative control (n = 10), and water as a blank control (n = 10). Each sample was immersed in the toothpaste slurry and brushed with a soft-bristle toothbrush for 30 seconds. Assuming that a person has 28 teeth and one toothbrushing cycle at home takes two minutes, the duration of 30 seconds of toothbrushing during the experiment corresponds to the duration of seven toothbrushing cycles. After brushing, the samples were gently rinsed with distilled water and stored in artificial saliva at 37°C for 24 hours before the next application. We applied the nano-HAP three times to the tooth surfaces, which were described as HAP 1,

HAP 2 and HAP 3. This protocol is well accepted in previous studies, which focused on the whitening performance of HAP aqueous suspensions and a gel (Dabanoglu et al., 2009, Jin et al., 2013, Sarembe et al., 2020). To compare the whitening performance of HAP toothpaste with these HAP formulations, this protocol was also used in the current study.

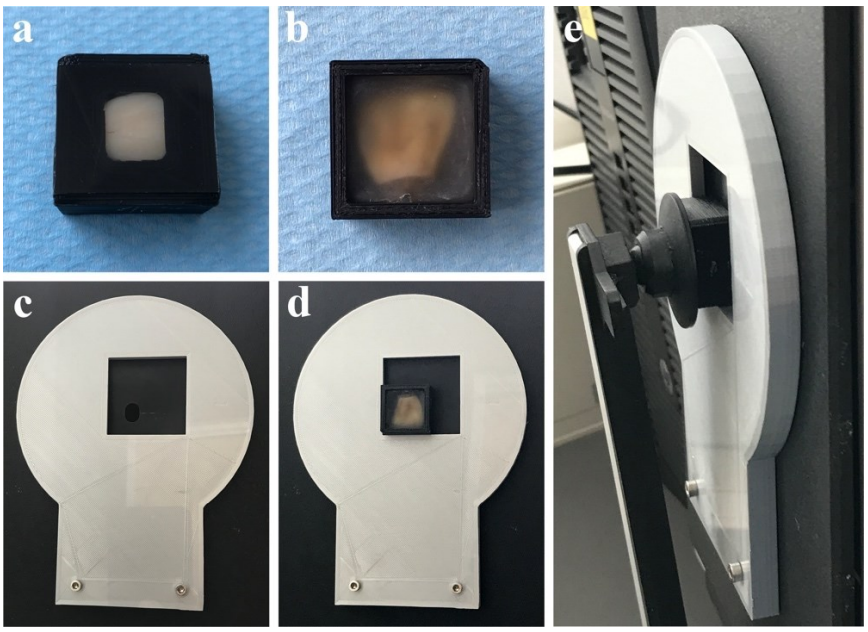

Figure 3.1 The 3D-printed repositioning system for the color measurement. A bovine tooth was embedded in the sample holder (a and b); the size of the measuring window of the sample holder is identical to that of the spectrophotometer (c); the embedded tooth was placed on the lower-left corner of the position locator (d), which was fixed on the spectrophotometer; the sample was held firmly by the movable arm during the color measurement (e).

3.2.4 Application of hydrodynamic shear force

A hydrodynamic shear force (HSF) was generated by an electric toothbrush and applied to each sample for 2 minutes to simulate mechanical forces in the patient's mouth after HAP 3. The tips of the bristle were mounted at 1 mm from the enamel surfaces. The bristle motion of the toothbrush generated a turbulent fluid flow that directly caused a hydrodynamic effect on the tooth surfaces (Digel et al., 2020).

3.2.5 Color measurement

The color of each tooth was measured with a spectrophotometer (Color-Eye 7000, Gretag MacBeth X-Rite-, Germany). To ensure the repositioning and reproducibility of the color measurement, the samples were placed on the lower-left corner of a 3D-printed position locator, which was fixed firmly to the spectrophotometer (***Figure 3.1c, d*** and ***e***), and the measurements were performed in darkness. To avoid enamel dehydration, we placed a cover glass on the measuring side of the embedded samples and added a few drops of water between the tooth and the cover glass. The color assessment was defined by the Commission Internationale de I'Eclairage (CIE) LAB color system. The baseline color of the teeth (L_0, a_0 and b_0) was obtained before all applications, and the color (L, a and b) at each experimental time point (HAP 1- 3 and HSF) was measured 24 h after each application. The average color changes (ΔE values) were determined as the Euclidean distance of the measuring points in the three-dimensional Lab color space $\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}, \Delta L = L - L_0, \Delta a = a - a_0, \Delta b = b - b_0$.

3.2.6 SEM evaluation

Sixteen additional bovine incisors were again randomly selected and assigned to the four groups (n = 4 for each group). Nano-HAP toothpaste and HSF were applied as described above. The samples were cut along the median line into two parts. The labial part was chosen for SEM evaluation. After dehydration in increasing concentrations of ethanol, the specimens were sputter-coated with a thin (25 nm) layer of a gold-palladium alloy (SC 7620, Polaron, Quorum Technologies, Kent, UK). Subsequently, the sample was imaged with a field-emission scanning electron microscope (FE-SEM, Supra 55 vp, Zeiss, Germany) at a magnification of 5000 ×. The workflow of the present chapter is shown in ***Figure 3.2***.

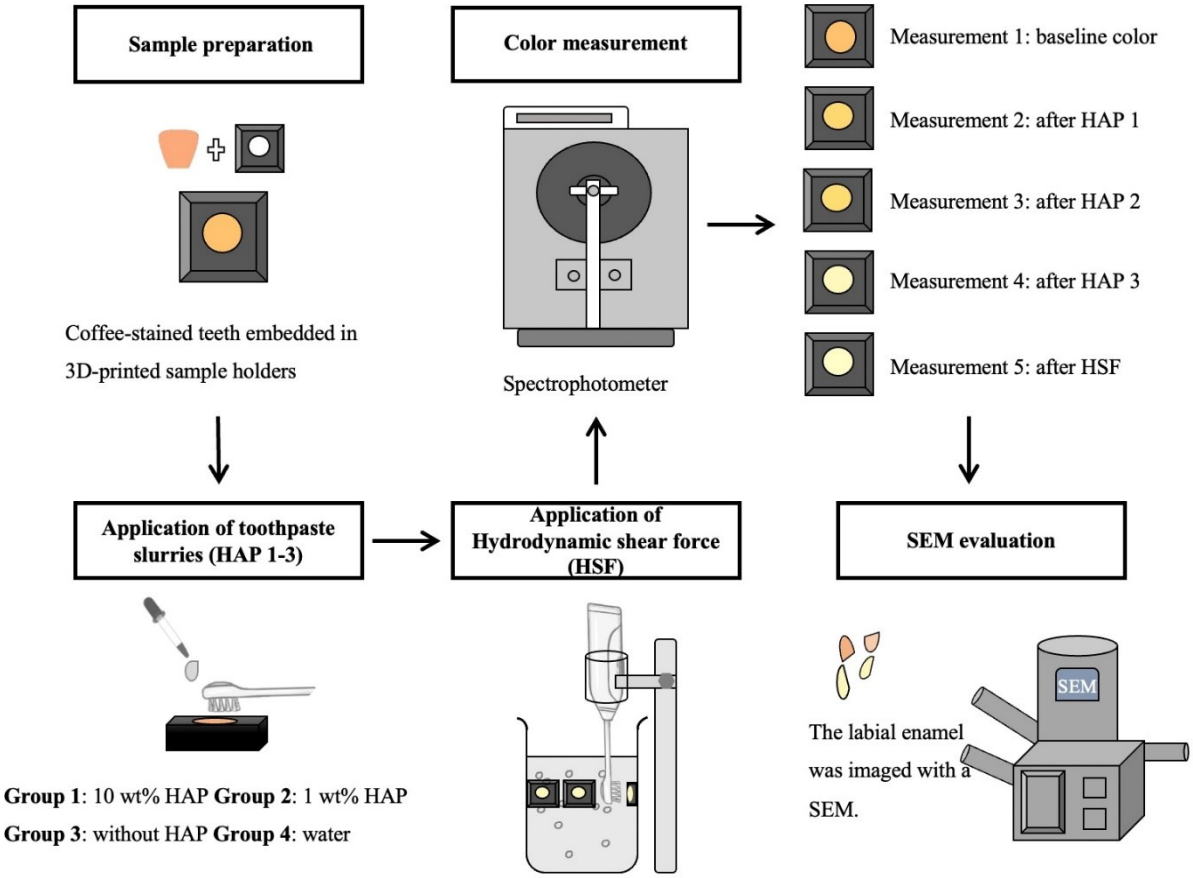

Figure 3.2 The workflow of the third chapter.

3.2.7 Statistical analysis

The data were analyzed in R. Tests of normality (Shapiro-Wilk test), homoscedasticity (Levene's test) and homogeneity of covariance (Box's M-test) were applied to the data. Two-way mixed analysis of variance (ANOVA) was performed to determine the effects of the concentration and repeated application on the tooth-whitening effect of nano-HAP. A Box–Cox transformation was applied to the data before the ANOVA test to meet all of the statistical assumptions. Tukey post hoc pairwise comparisons were used to compare individual groups. Paired t-tests were employed to investigate the impact of the hydrodynamic condition on the whitening effect of nano-HAP, where HAP 3 and HSF were compared. Statistical significance was identified at $P < 0.05$.

Power analysis was performed by software G*Power 3 software(Faul et al., 2007) to evaluate whether the evidence of the small sample size was strong enough to detect the whitening effect of nano-HAP toothpaste.

3.3 Results

3.3.1 Influence of concentration and repeated application

3.3.1.1 Two-way mixed ANOVA analysis of the two main factors

The results of the two-way mixed ANOVA are shown in ***Table 3.1***. A significant main effect of concentration was found on the ΔE, ΔL, Δa and Δb values ($P < 0.05$). A significant main effect of repeated application on the ΔE, Δa and Δb values was also observed ($P < 0.05$). No significant interaction between the two main effects was found ($P > 0.05$). The power analysis showed 100% power of the data evidence.

3.3.1.2 The ΔE values of each group

The ΔE values of each group are shown in ***Figure 3.3***. The 10 wt% nano-HAP group showed the highest ΔE mean values throughout the observation period, followed by the 1 wt% nano-HAP group, the tooth slurry group without nano-HAP (negative control group), and then the water group. This finding suggested a positive association between the concentration and the whitening effect. A Tukey post hoc test revealed significant pairwise differences ($P < 0.05$) in the ΔE values between the 10 and 1 wt% nano-HAP groups (HAP 1 - 3), between the 10 wt% nano-HAP and negative control groups (HAP 1 - 3), and between the 1 wt% nano-HAP and negative control groups (HAP 2 and 3). With nano-HAP reapplication, the ΔE mean values of the 10 wt% nano-HAP group increased from 2.76 (HAP 1) to 4.28 (HAP 2) and then to 4.47 (HAP 3), whereas those values changed from 1.62 (HAP 1) to 2.17 (HAP 2) and then to 2.55 (HAP 3) in the 1 wt% nano-HAP group. The ΔE values increased statistically with repeated

HAP application ($P < 0.05$), which indicated that reapplication of HAP toothpaste could lead to more obvious tooth color changes.

Table 3.1 Two-way mixed ANOVA analysis of the two main factors of concentration and application on the tooth-whitening effect of nano-HAP toothpaste.

Factor	**Sum of squares**	**DF**	**Mean square**	**F**
ΔE				
Concentration	38.71	2.00	19.35	37.96*
Application	4.86	1.37	3.56	34.74*
Interaction	0.28	2.73	0.10	1.01
ΔL				
Concentration	27.68	2.00	13.84	20.60*
Application	0.21	1.43	0.15	1.07
Interaction	0.35	2.86	0.12	0.87
Δa				
Concentration	260.24	2.00	130.12	4.22*
Application	37.36	1.39	26.96	5.39*
Interaction	37.92	2.77	13.68	2.74
Δb				
Concentration	879.38	2	439.69	14.41*
Application	12.64	1.05	11.99	1.77
Interaction	54.32	2.11	25.75	3.80*

* $P < 0.05$

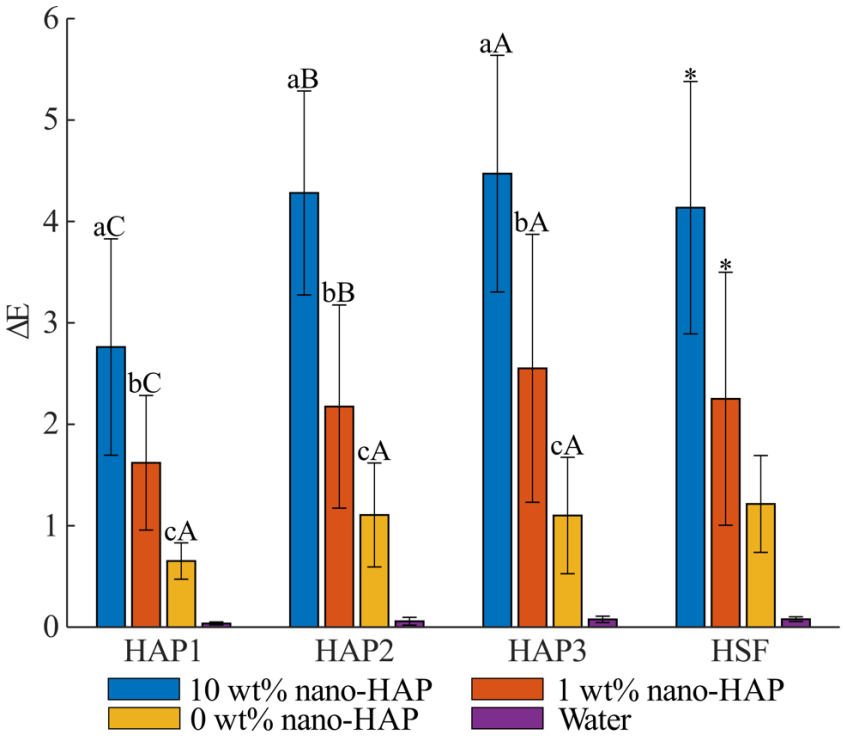

Figure 3.3 The average color changes (expressed as Δ*E* values) after HAP 1-3 and HSF. HAP 1: the first circle of nano-HAP application; HAP 2: the second circle of nano-HAP application; HAP 3: the third circle of nano-HAP application; HSF: application of hydrodynamic shear force; different lowercase letters indicate the differences between different groups at the same experiment time point; different capital letters indicate the differences of the same group between different experiment time points ($P < 0.05$); same letter suggests that groups showed no significant differences ($P > 0.05$); from a (A) to c (C), the mean value is decreased; * indicates the significant differences between HAP 3 and HSF ($P < 0.05$).

3.3.1.3 The Δ*L*, Δ*a* und Δ*b* values of each group

The increased Δ*E* values of both nano-HAP groups were contributed by the increased *L** values and decreased *a** and *b** values. The Δ*L,* Δ*a,* and Δ*b* values of each group are shown in ***Table 3.2***. The 10 wt% nano-HAP group showed statistically higher Δ*L* values than the 1 wt% nano-HAP group after HAP 1 and 2 ($P < 0.05$). Meanwhile, the 10 wt% nano-HAP group exhibited statistically lower Δ*b* values than the 1 wt% nano-HAP group (HAP 2 and 3). This finding suggested that the higher concentration of nano-HAP changed the tooth color to a lighter and bluer tone than the lower concentration.

Table 3.2 The changes in L^*, a^* and b^* axes (expressed as ΔL, Δa, and Δb values) at each experimental time point (HAP 1 – 3 and HSF). Results are shown as mean value (standard deviation).

Group	Experimental time point			
	HAP 1	**HAP 2**	**HAP 3**	**HSF**
ΔL				
10 wt% nano-HAP	2.30 (1.12)^{aA}	2.70 (1.12)^{aA}	2.76 (1.36)^{aA}	2.09 (1.72)*
1 wt% nano-HAP	1.13 (0.73)^{bB}	1.51 (1.20)^{bAB}	1.78 (1.70)^{aA}	1.30 (1.67)*
Without HAP	-0.01 (0.42)^{bA}	-0.13 (0.68)^{cA}	-0.08 (0.73)^{bA}	-0.14 (0.87)
Water	0.01 (0.01)	0.01 (0.02)	0.00 (0.04)	0.00 (0.03)
Δa				
10 wt% nano-HAP	-0.21 (0.21)^{aA}	-0.75 (0.90)^{bB}	-0.95 (0.86)^{bB}	-0.82 (0.63)
1 wt% nano-HAP	0.05 (0.47)^{aA}	-0.20 (0.72)^{abA}	-0.31 (0.83)^{abA}	-0.25 (0.85)
Without HAP	0.05 (0.33)^{aA}	0.09 (0.40)^{aA}	0.12 (0.36)^{aA}	0.12 (0.37)
Water	0.01 (0.02)	-0.04 (0.04)	-0.03 (0.06)	-0.03 (0.05)
Δb				
10 wt% nano-HAP	-1.34 (0.64)^{bA}	-2.55 (1.84)^{bB}	-2.66 (1.90)^{cB}	-2.71 (1.86)
1 wt% nano-HAP	-0.84 (0.63)^{abA}	-0.96 (0.83)^{aA}	-0.10 (0.83)^{bA}	-0.88 (0.94)
Without HAP	0.03 (0.47)^{aA}	0.21 (0.96)^{aA}	0.25 (0.96)^{aA}	0.25 (0.93)
Water	0.00 (0.03)	-0.01 (0.04)	0.02 (0.04)	0.01 (0.05)

HAP 1: the first circle of nano-HAP application; HAP 2: the second circle of nano-HAP application; HAP 3: the third circle of nano-HAP application; HSF: application of hydrodynamic shear force; different owercase letters indicate the differences between different groups at same experiment time point; different capital letters indicate the differences of the same group between different experiment time points; same letter suggests that groups showed no significant differences ($P > 0.05$); from a (A) to c (C), the mean value is decreased;* indicates the significant differences between HAP 3 and HSF ($P < 0.05$).

3.3.2 Influence of hydrodynamic condition

The tooth color changes caused by the application of hydrodynamic shear force were compared with those after HAP 3. For both nano-HAP groups, the HSF led to statistical reductions in the ΔE and ΔL values ($P < 0.05$). The Δa and Δb values were not significantly influenced by the hydrodynamic effect ($P > 0.05$).

3.3.3 SEM evaluation

The nano-HAP adhesion on the enamel surface is shown in ***Figure 3.4***. The photos of the two control groups exhibited the normal microstructure of enamel (***Figure 3.4a* to *h***). For both nano-HAP groups, HAP single crystallites and agglomerates were observed throughout the experimental period (HAP 1 – 3, and HSF). After HAP 1, more nanosized agglomerates were identified in the 10 wt% nano-HAP group than in the 1 wt% nano-HAP group (***Figure 3.4i* and *m***). After HAP 2 and 3, the nanosized crystallites and agglomerates grew larger and became microsized (***Figure 3.4j, k, n* and *o***). Compared with HAP 1, more enamel surfaces were covered. Especially in the 10 wt% nano-HAP group, the tooth surface was almost completely covered. After HSF, most of the microsized agglomerates were removed, leaving the nanosized agglomerates to adhere relatively more firmly to the enamel surfaces (***Figure 3.4l* and *p***).

3.4 Discussion

3.4.1 The tooth-whitening effect of HAP toothpastes

The main goal of this study was to investigate the tooth-whitening effect of nano-HAP, used as an ingredient in toothpaste. Compared with the negative control group, the nano-HAP groups showed significantly higher ΔE values due to the increased L^* values and the decreased a^* and b^* values, indicating that the nano-HAP toothpaste could make teeth appear brighter, less red and less yellow. These optical alterations can be explained by the increased diffuse light reflection and reduced light transmission through the tooth caused by the HAP particles adhering to the enamel (Hojabri et al., 2020, Roveri et al., 2009). After three applications, the ΔE mean values of the 10 wt% and the 1 wt% nano-HAP groups were 4.47 and 2.55, respectively. Considering that $\Delta E > 3.7$ can be recognized by human eyes (Johnston and Kao, 1989), the color changes of the 10 wt% nano-HAP group were visually perceivable

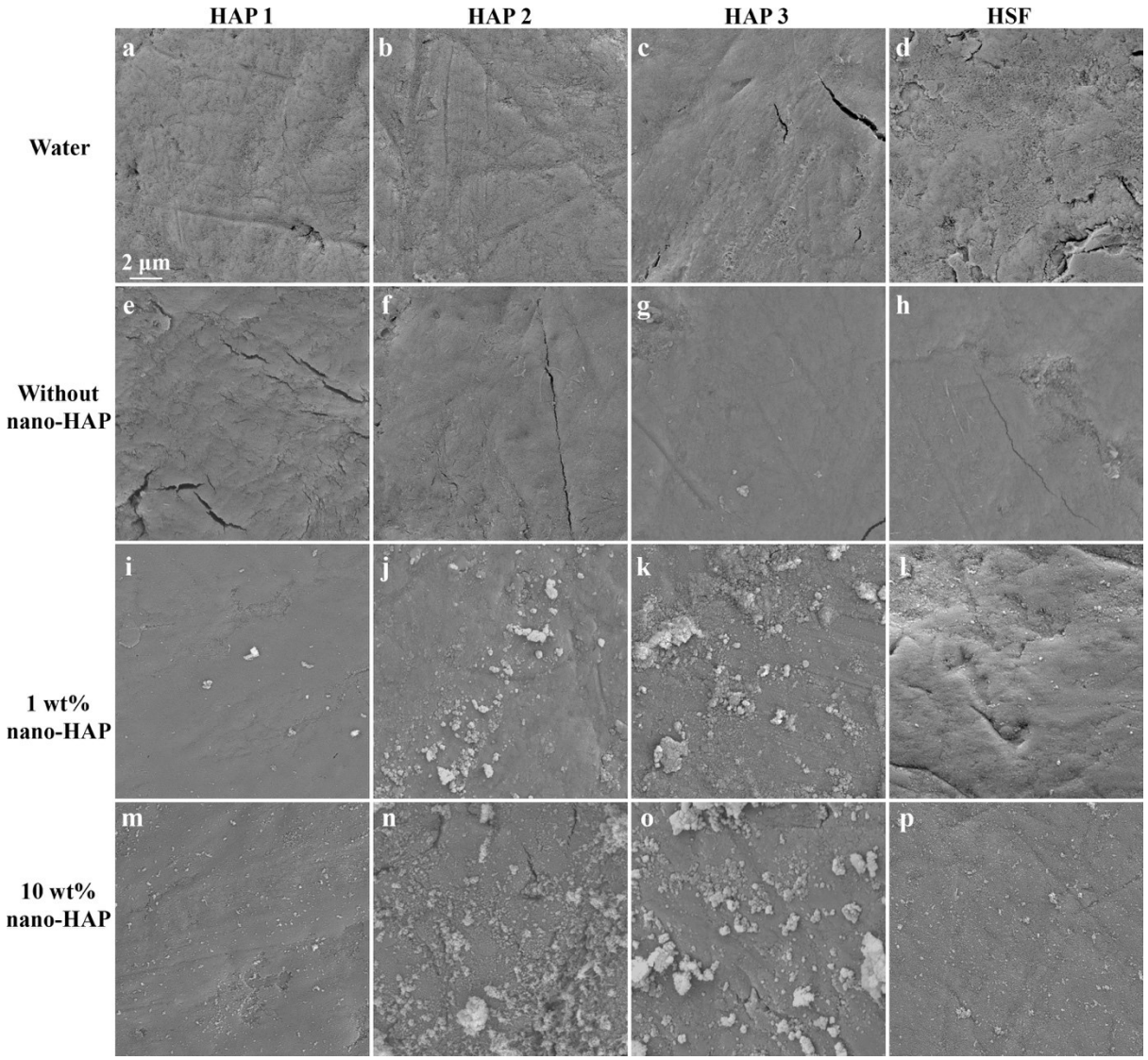

Figure 3.4 The nano-HAP toothpaste-treated enamel surfaces were visualized at 5000× after HAP 1 - 3 and HSF. a-d: water group; e-h toothpaste without nano-HAP; i-l: 1 wt% nano-HAP toothpaste; m-p: 10 wt% nano-HAP toothpaste.

The whitening performance of the 10 wt% nano-HAP toothpaste seemed better than that of a 44.4 wt% nano-HAP aqueous suspension, of which the Δ*E* value was 3.30 after the third use (Dabanoglu et al., 2009). Several randomized clinical trials reported that the Δ*E* values of commercial abrasive toothpastes and peroxide-based toothpastes ranged from 2.25 to 4.46 with a clinical application period of up to 90 days (de Moraes Rego Roselino et al., 2018, Kim et al., 2020). Therefore, it is considered that the 10 wt% nano-HAP toothpaste in our study had a satisfying postbrushing tooth-whitening effect. It is worth mentioning that the negative control

group exhibited a slight whitening effect compared with the water group, with the mean values of ΔE fluctuating around approximately 1. The abrasive particles adhering to the enamel surface could also contribute to light scattering to some extent.

3.4.2 Influence of concentration

Understanding the relationship between the nano-HAP concentration and tooth-whitening effect is crucial for optimizing the efficiency of nano-HAP toothpaste. To analyze the role of the concentration more accurately, we did not make any structural modifications to the nano-HAP. To avoid interference from the ingredients of commercial toothpastes, none of them were incorporated into the toothpaste. Nano-HAP has been applied to some commercial toothpastes at concentrations up to 10 wt% (Scientific Committee of Consumer Safety and Bernauer, 2020), which was considered optimal for remineralization of early enamel caries (Huang et al., 2010). Within this concentration, nano-HAP would not have any significant systemic exposure via the oral mucosa or cytotoxicity after a 48-hour exposure (Scientific Committee of Consumer Safety and Bernauer, 2020). Therefore, a concentration of 10 wt% was also chosen as the upper limit in the present study.

The null hypothesis that the HAP concentration does not influence the whitening effect of HAP toothpaste could be rejected, as we found a significant main effect of the concentration on the ΔE, ΔL, Δa, and Δb values. Compared with the 1 wt% nano-HAP group, the 10 wt% nano-HAP group exhibited significantly higher ΔE values throughout the observation period. This finding could be explained by the SEM images. After HAP 1 – 3, more adhered HAP crystallites and agglomerates were observed in the 10 wt% nano-HAP group than in the 1 wt% group. The enamel coverage area was quantitatively calculated and shown to be increased from 10 to 30% with the HAP concentration increasing from 1 to 10 wt% (Fabritius-Vilpoux et al., 2019). More coverage may result in an increase in the reflection of light on the HAP layer and thereby lead to the increase in the ΔL values. This finding appeared to be well substantiated by

a previous study (Niwa et al., 2001), in which the increase in the amount of HAP in toothpaste resulted in the enhancement in the degree and rate of brightness. At the same time, more coverage could decrease the light transmission through enamel and dentin, which could lower the a^* and b^* values (Hojabri et al., 2020).

3.4.3 Influence of repeated application

A significant main effect of the repeated application on the color changes was found in the current study. For both nano-HAP groups, the ΔE values increased significantly with the reapplication, which could be caused by the increased enamel coverage and the size change of the adherent particles from nanosized to microsized. These changes in the particle size appeared to be well substantiated by the previous studies, in which it was confirmed that the photoelectric characteristics and maturation time enabled the nano-HAP to be gathered within microsized conglomerates (Bystrov V, 2009, Roveri and Iafisco, 2010). A previous study reported that there was a maximal adhering load on enamel (Jin et al., 2013). The tooth color does not change much when adhering saturation has been achieved. However, we did not find this saturation within three HAP reapplications. In our study, nano-HAP was applied to the enamel by tooth brushing. The loosely attached nano-HAP particles may be brushed away by mechanical friction.

3.4.4 Influence of hydrodynamic condition

From a clinical view of point, the adherent HAP agglomerates are exposed to mechanical forces in the patient's mouth after brushing. HSF is often used to create force comparable to the mechanical stress caused by the movement of the lips and cheeks (Sharma et al., 2005, Purevdorj et al., 2002). For both nano-HAP groups, statistical reductions in the $\triangle E$ values were observed after the HSF application. Nevertheless, the ΔE mean value of the 10 wt% was still higher than 4, suggesting that a tooth whitening effect of the nano-HAP toothpaste is still to be expected under a loaded condition. After HSF application, most of the microsized agglomerates

were removed from the enamel surfaces, while the nanosized agglomerates remained in place, which could be explained by their higher surface charges and stronger electrostatic forces (Bystrov V, 2009, Fabritius-Vilpoux et al., 2019). The primary goal was to apply the singular primary particles to the tooth surface. However, we could not avoid the formation of agglomerates. A more sophisticated experimental protocol is needed to limit the number of agglomerates. It is the task of the university to prove the feasibility of a hypothesis. The task of companies is to realize these ideas in a technically optimized way.

3.4.5 Strengths and limitations

Our study has some particular strengths. First, we took a further step toward real clinical situations. We applied nano-HAP toothpaste to enamel by tooth brushing. The whitening effect of HAP toothpaste has been confirmed. Second, the interference factors were strictly controlled. For instance, tooth dehydration could decrease enamel translucency and increase luminosity, making the tooth falsely appear whiter (Kugel et al., 2009, Hatirli et al., 2020). Compared with previous studies (Dabanoglu et al., 2009, Jin et al., 2013, Sarembe et al., 2020), in which the samples were air-dried before the color measurement, we measured the tooth color in a liquid environment, which could avoid the interference of dehydration in tooth color assessment. Accurate repositioning is a key factor for color measurement, as the teeth are multilayered, translucent and exhibit color transitions in all directions (Chu et al., 2010). However, the previously published studies did not address how the repositioning was achieved in their work (Dabanoglu et al., 2009, Bommer et al., 2018, Hojabri et al., 2020). To bridge this information gap, we invented a repositioning system by using 3D printing technology to enable accurate color measurement (***Figure 3.1***).

Within the framework of laboratory tests, one tries to select the conditions in such a way that they can be transferred as well as possible to the clinical situation. Ideally, the tests would be carried out with human teeth. However, due to the success of prevention and modern filling

materials, hardly any teeth are extracted in industrialized nations today that can be used for laboratory examinations. Although bovine enamel is somewhat more porous than human enamel, its chemical composition and surface properties are identical to those of human teeth. Therefore, we used bovine teeth in our study.

In 2001, the CIE developed a more complex formula, CIEDE2000. It has been reported that the CIEDE2000 formula reflected the color changes that were perceived by observers better than the CIELAB formula (Gomez-Polo et al., 2016). After literature research with the keyword "CIEDE 2000 dental" in PubMed, we found that only 11 publications used this method to interpret color changes in dentistry over the last 10 years, while 266 results were found when we used the keyword "CIE LAB dental". We found that the most frequently used formula for analyzing color changes is still the CIE LAB color system. To compare our findings with previous studies, which focused on tooth-whitening products, the CIE LAB color system was applied in the present study.

We used artificial saliva instead of human saliva. The statherin and proline-rich glycoproteins in human saliva could bond strongly to HAP and thereby might influence its tooth whitening effect (Oppenheim et al., 2007). Moreover, human saliva has high variability of individual factors and complexity of its components (J et al., 2017). Therefore, human saliva was not chosen.

The findings of this study have to be seen in light of some limitations. First, although we confirmed for the first time the whitening effect of nano-HAP toothpaste and the significant main effect of its concentration on tooth color changes, the whitening effect of nano-HAP after a prolonged application period needs to be further proven. Second, our *in vitro* study cannot reflect the complexity of the oral environment. Oral pH fluctuations and temperature changes also influence nano-HAP adhesion behavior (Yan Deng, 2014).

Considering the limitations of this study, nano-HAP toothpaste showed a postbrushing whitening effect and good resistance to mechanical forces. It could be considered a promising alternative to over-the-counter oxidizing bleaching products. The whitening effect is significantly affected by the concentration. Nano-HAP could be added to commercial toothpastes at a concentration of up to 10%, as this concentration showed visually perceptible tooth whitening performance in our study while being considered biologically safe.

Chapter 4: Biomimetic tooth-whitening effect of different-sized hydroxyapatite-containing mouthrinses after long-term simulated oral rinsing

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Chapter 5: Summary and conclusions

This thesis aimed to evaluate the non-oxidizing whitening effect of HAP. The HAP materials used in the study were incorporated either in water or in non-abrasive toothpaste. Factors such as concentration, application period, particle size, enamel morphology and storage medium which might influence the whitening effect of HAP were evaluated. We hope that our findings can help dental manufacturers to develop effective HAP whitening products and provide patients with an easy self-administered application regime.

This thesis consisted of three parts. The first part compared the tooth-whitening effect of a HAP mouthrinse to that of a commercial bleaching OTC mouthrinse. The influences of enamel morphological structure and human saliva on the whitening performance of HAP were investigated. Sixty bovine incisors were randomly assigned into six groups according to enamel morphology (ground or native) and storage medium (human saliva or mineral water). The commercial bleaching mouthrinse and water groups served as control groups. In conclusion, the HAP mouthrinse has a similar whitening effect to that of the OTC bleaching mouthrinse. The whitening effect of HAP was observed both on native and on ground surfaces. Therefore, we believe that HAP whitening products are also suitable for patients who have worn enamel and sensitive teeth. In future *in vitro* studies, the whitening effect of HAP could be performed on native enamel which has a natural spherical structure of teeth. If no integrating sphere is available, one must be aware that the HAP whitening outcomes will be higher than those on native enamel. Human saliva has a positive influence on the whitening effect of HAP, indicating the whitening performance might be even better in the mouth. However, some disadvantages limit the application of human saliva in future experiments. First, human saliva is not easy to collect. Second, there are large individual differences in the physical and chemical properties of saliva among participants. Moreover, bacterial colonization and contamination of saliva can affect the results of experiments.

Chapter 5

The second part of the study evaluated the post-brushing tooth-whitening effect of toothpaste containing hydroxyapatite nanoparticles (nano-HAPs). The impact of the concentration on the whitening performance of nano-HAP toothpaste was also investigated. Forty bovine incisors were randomly assigned into four groups: 10 wt% nano-HAP, 1 wt% nano-HAP, toothpaste without nano-HAP as a negative control and water as a blank control. We concluded that nano-HAP toothpaste showed a postbrushing whitening effect and good resistance to mechanical forces. It could be considered a promising alternative to over-the-counter oxidizing bleaching products. The whitening effect is significantly affected by the concentration. Nano-HAP could be added to commercial toothpastes at a concentration of up to 10%, as this concentration showed visually perceptible tooth whitening performance in our study while being considered biologically safe.

The third part of investigated the tooth-whitening effects of mouthwashes containing different sizes of HAP particles after prolonged application time and compare them with a commercial whitening mouthwash. Fifty bovine incisors were stained and randomly distributed into five groups: The HAP groups with 3 μm, 200 nm and 50 nm particle size, the commercial whitening mouthwash group and the distilled water group. The teeth underwent prolonged mouthwash applications that equivalent to simulated 3- and 6-month mouthrinsing. In summary, this part of study confirmed for the first time the tooth-whitening effect of prolonged application of HAP mouthrinses. After the 3- and 6-month-equivalent mouthrinsing, the HAP mouthrinses exhibited similar tooth-whitening effects to the commercial whitening mouthrinse. It was also noticed that the tooth-whitening performance of HAP was dependent on the particle size and application time. After the 6-month-equivalent application, the 50 nm HAP mouthrinse showed significantly higher color changes than the 3 μm one. This finding should be taken into consideration by dental manufacturers for their HAP containing dental products. To achieve a

better outcome in tooth-whitening, the patients should apply the mouthrinse regularly for a longer period.

The adhered HAP layers observed in the present study might protect the tooth surface from acidic attacks as the layers would interact initially with the acid (Jin et al., 2013). From a chemical point of view, the adhered HAP layers would be dissolved in the acidic environment, resulting in the reduction of the whitening effect. However, researchers observed the HAP particles could adhere to enamel even in an acidic solution (Yamagishi et al., 2005), which suggested that the acidic condition might not affect the whitening ability of HAP materials. Studies, which are designed to evaluate the HAP whitening effect with complicated pH value changes in the oral environment, are needed in the future. To further augment the whitening effect of HAP mouthrinse, non-oxidizing, blue-toned agents could be combined with HAP products (Tao et al., 2017). If the adhesion of HAP can be observed in real time, its whitening mechanism would be understood more profoundly.

Zusammenfassung

Ziel dieser Arbeit war es, die nichtoxidierende aufhellende Wirkung von HAP zu bewerten. Die in der Studie verwendeten HAP-Materialien wurden entweder in Wasser oder in nicht-abrasive Zahnpasta eingearbeitet. Faktoren wie Konzentration, Anwendungsdauer, Partikelgröße, Schmelzmorphologie und Lagermedium, welche die aufhellende Wirkung von HAP beeinflussen könnten, wurden bewertet. Wir hoffen, dass unsere Ergebnisse den Herstellern von Zahnarzneimitteln dabei helfen können, wirksame HAP-Aufhellungsprodukte zu entwickeln und den Patienten eine einfache, selbst anzuwendende Methode zu bieten.

Diese Arbeit bestand aus drei Teilen. Im ersten Teil wurde die zahnaufhellende Wirkung einer HAP-Mundspülung mit der einer handelsüblichen bleichenden OTC-Mundspülung verglichen. Die Einflüsse der morphologischen Struktur des Zahnschmelzes und des menschlichen Speichels auf die Aufhellungsleistung von HAP wurden untersucht. Sechzig Rinderschneidezähne wurden nach dem Zufallsprinzip je nach Schmelzmorphologie (geschliffen oder nativ) und Speichermedium (menschlicher Speichel oder Mineralwasser) in sechs Gruppen eingeteilt. Die Gruppen mit kommerzieller Bleichspülung und Wasser dienten als Kontrollgruppen. Zusammenfassend lässt sich sagen, dass die HAP-Mundspülung eine ähnliche aufhellende Wirkung hat wie die OTC-Bleaching-Mundspülung. Die aufhellende Wirkung von HAP wurde sowohl auf nativen als auch auf geschliffenen Oberflächen beobachtet. Daher glauben wir, dass HAP-Bleaching-Produkte auch für Patienten mit abgenutztem Zahnschmelz und empfindlichen Zähnen geeignet sind. In künftigen *In vitro* Studien könnte die aufhellende Wirkung von HAP auf nativem Zahnschmelz, der eine natürliche kugelförmige Zahnstruktur aufweist, untersucht werden. Wenn keine Ulbricht-Kugel zur Verfügung steht, muss man sich darüber im Klaren sein, dass die Aufhellungsergebnisse von HAP höher sein werden als die auf nativem Zahnschmelz. Der menschliche Speichel hat einen positiven Einfluss auf die Aufhellungswirkung von HAP, was

darauf hindeutet, dass die Aufhellungsleistung im Mund noch besser sein könnte. Einige Nachteile schränken jedoch die Verwendung von menschlichem Speichel in zukünftigen Experimenten ein. Erstens ist menschlicher Speichel nicht einfach zu sammeln. Zweitens gibt es große individuelle Unterschiede in den physikalischen und chemischen Eigenschaften des Speichels zwischen den Teilnehmern. Außerdem können die bakterielle Besiedlung und Verunreinigung des Speichels die Versuchsergebnisse beeinträchtigen.

Im zweiten Teil der Studie wurde die zahnaufhellende Wirkung von Zahnpasta mit Hydroxylapatit-Nanopartikeln (nano-HAPs) nach dem Zähneputzen untersucht. Außerdem wurde der Einfluss der Konzentration auf die Aufhellungsleistung von Nano-HAP-Zahnpasta untersucht. Vierzig Rinderschneidezähne wurden nach dem Zufallsprinzip in vier Gruppen eingeteilt: 10 Gew.-% nano-HAP, 1 Gew.-% nano-HAP, Zahnpasta ohne nano-HAP als Negativkontrolle und Wasser als Blindkontrolle. Wir kamen zu dem Schluss, dass die nano-HAP-Zahnpasta nach dem Zähneputzen einen Aufhellungseffekt und eine gute Beständigkeit gegenüber mechanischen Kräften aufweist. Sie könnte als vielversprechende Alternative zu frei verkäuflichen oxidierenden Bleichmitteln betrachtet werden. Der Aufhellungseffekt wird erheblich von der Konzentration beeinflusst. Nano-HAP könnte handelsüblichen Zahnpasten in einer Konzentration von bis zu 10 Gew.-% zugesetzt werden, da diese Konzentration in unserer Studie eine visuell wahrnehmbare Zahnaufhellung bewirkte und gleichzeitig als biologisch sicher gilt.

Der dritte Teil der Studie untersuchte die zahnaufhellende Wirkung von Mundspülungen, die HAP Partikel unterschiedlicher Größe enthalten, nach längerer Anwendungszeit und verglich sie mit einer kommerziellen aufhellenden Mundspülung. Fünfzig Rinderschneidezähne wurden gefärbt und nach dem Zufallsprinzip in fünf Gruppen aufgeteilt: Die HAP-Gruppen mit 3 µm, 200 nm und 50 nm Partikelgröße, die Gruppe mit dem kommerziellen Whitening-Mouthwash und die Gruppe mit destilliertem Wasser. Die Zähne wurden einer verlängerten Mundspülung

unterzogen, die einer simulierten 3- und 6-monatigen Mundspülung entsprach. Zusammenfassend lässt sich sagen, dass dieser Teil der Studie zum ersten Mal die zahnaufhellende Wirkung einer verlängerten Anwendung von HAP-Mundspülungen bestätigt. Nach der 3- und 6-monatigen äquivalenten Mundspülung zeigten die HAP-Mundspülungen ähnliche zahnaufhellende Wirkungen wie die kommerziellen Whitening-Mundspülungen. Es wurde auch festgestellt, dass die zahnaufhellende Wirkung von HAP von der Partikelgröße und der Anwendungszeit abhängig war. Nach der 6-monatigen äquivalenten Anwendung zeigte die 50-nm-HAP-Mundspülung deutlich stärkere Farbveränderungen als die 3 μm-Spülung. Dieses Ergebnis sollte von Dentalherstellern für ihre HAP-haltigen Dentalprodukte in Betracht gezogen werden. Um ein besseres Ergebnis bei der Zahnaufhellung zu erzielen, sollten die Patienten die Mundspülung regelmäßig und über einen längeren Zeitraum anwenden.

Die in der vorliegenden Studie beobachteten haftenden HAP-Schichten könnten die Zahnoberfläche vor Säureangriffen schützen, da die Schichten zunächst mit der Säure interagieren würden (Jin et al., 2013). Aus chemischer Sicht würden sich die anhaftenden HAP-Schichten in der sauren Umgebung auflösen, was zu einer Verringerung des Aufhellungseffekts führen würde. Forscher beobachteten jedoch, dass die HAP-Partikel auch in einer sauren Lösung am Zahnschmelz haften können (Yamagishi et al., 2005), was darauf hindeutet, dass die saure Umgebung die Aufhellungsfähigkeit von HAP-Materialien nicht beeinträchtigt. Für die Zukunft sind Studien erforderlich, die den Aufhellungseffekt von HAP bei komplizierten pH-Wert-Änderungen in der Mundhöhle bewerten sollen. Um die aufhellende Wirkung von HAP-Mundspülungen weiter zu verstärken, könnten nicht-oxidierende, blau gefärbte Mittel mit HAP-Produkten kombiniert werden (Tao et al., 2017) Wenn die Adhäsion von HAP in Echtzeit beobachtet werden kann, würde der Aufhellungsmechanismus besser verstanden werden.

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List of publications since 2018

Accepted:

1. Impact of hyperglycemia on the rate of implant failure and peri-implant parameters in patients with type 2 diabetes mellitus: Systematic review and meta-analysis.

Journal of the American Dental Association, 2021 Mar;152(3):189-201.e1.

2. Biomimetic tooth-whitening effect of different-sized hydroxyapatite-containing mouthwashes after long-term simulated oral rinsing.

American Journal of Dentistry, 2021 Dec;34(6):307-312.

3. Tooth whitening with an experimental toothpaste containing hydroxyapatite nanoparticles

BMC Oral Health: accepted in 2022

In submission

1. The influence of enamel morphology and human saliva on the tooth-whitening performance of hydroxyapatite

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