Strategies against acid induced hydroxyapatite dissolution of human dental enamel

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INDEX OF CONTENT

ABBREVIATIONS

AA	ascorbic acid
AFM	atomic force microscopy
CA	citric acid
CAS	citric acid solution
CLSM	confocal laser scanning microscopy
EU	European Union
GA	gum arabic
HA	hydroxyapatite (also used for human enamel)
HP	highly esterified pectin
INS	International numbering system (E-number = European classification)
LA	lactic acid
LP	low esterified pectin
MA	malic acid
Na-CMC	Na-carboxymethyl cellulose
NI	nanoindentation
PA	phosphoric acid
PGA	propylene glycol alginate
PMCAS	polymer modified citric acid solution
SEM	scanning electron microscopy
TA	tartaric acid
wt	weight
XPS	X-ray photoelectron spectroscopy

MOTIVATION

The lifestyle and the quality of life of industrialized western countries have reached a high standard. The most obvious standards are the personal lifestyle, as well as a health-oriented perspective in terms of physical fitness and nutrition. Besides healthy mental and physical condition also adequate external representation plays an important role in life. In addition to these personal goals in life there are more and more aesthetic aspects important today. This is reflected in a fresh outlook as well as a healthy and excellent cosmetic condition of the teeth. The dental health is thereby strongly influenced by external circumstances from the consumption of harmful food and the inner personal attitude. From both the inner and outer factors the health of the teeth is affected and dental diseases such as caries, gingivitis and periodontitis occur. Moreover, the consumption of beverages such as coffee, tea or wine leads to optical changes of the tooth surface, such as discoloration. In modern times, known dental diseases are increasingly replaced since a new disease is obtained: dental erosion. This relatively new, not yet fully understood disease of the tooth surfaces moved in the focus of scientific investigations. The prevalence of this disease is already rated very high and an increasing number of patients, mainly children show a destruction of the dental hard tissue caused by the process of erosion.

The focus of this work is to study the process of tooth erosion with an *in vitro* model and the development of new strategies against the process of dental erosion. By modification of the influencing factors and systematically testing the erosion effects on *in vitro* enamel was investigated quantitative and qualitative. New insights are presented to understand the process of erosion much better and to possibly reduce and minimize the erosion on human dental enamel.

1 INTRODUCTION

This chapter gives brief insights into the macro and micro anatomical characteristics of teeth and the composition of human dental enamel. The following section gives insights about the definition and the background of human dental enamel erosion, as well as the mechanisms that are known for human dental erosion. The last part of this chapter states the most important mechanisms to reduce erosion.

1.1 Macro and micro anatomy of teeth and composition of human dental enamel

The main function of teeth is to take, crush and grind food. In addition, they play an important role in the articulatory processes of the human speech and have a major social function (aesthetics) [1]. They are integrated in the oral cavity of vertebrates and movable connected with the upper (*maxilla*) and lower (*mandible*) jaw. The teeth and the mouth are supplied with saliva by three big glands (*parotid gland, submandibular gland, sublingual gland*). The saliva is responsible for maintaining healthy teeth, as the ingredients contain important digestive and protective systems, like the secretion of enzymes for digestion (such as ptyalin) or wound-healing and antimicrobial substances (such as lysozymes, immunoglobulin A, lactoferrin and histidine) [2]. Besides this the saliva has a buffering function for the neutralization of acids formed by bacteria *(e.g. Streptococcus mutans*) or the acids contained in food [3-5].

The human tooth consists of a crown (*corona dentis*), the neck of tooth *(cervix dentis*) and at least one root (*radix dentis*). Depending on the tooth type, teeth differ in size, function and their external shape [1]. The exterior is usually whitish in colour, but varies depending on age and lifestyle factors such as diet, genetic condition and smoking [6]. The teeth are innervated and vascular supplied by the jaws via the tooth roots. The interior of the tooth consists of a cavity (*cavum dentis*) in which the dental pulp (*pulpa dentis*) is located **(Figure 1-1)**. The teeth consist of hard and soft tissue. The

dental hard tissue is formed by the enamel (*enamelum*), dentin (*dentinum*) and the dental cement (*cementum*). The dental soft tissue mainly consists of the pulp (*pulpa dentis*), which can be found in the interior parts of the tooth, and the *periodontium*. The periodontal ligament connects the tooth moveable with the jaw. Adults have 38 teeth in their permanent denture, plus up to 4 third molars (wisdom teeth). These are slightly larger than the back teeth and very often still hidden in the jaw and covered by the gingiva. They may break through later in adolescence or adulthood [1]. This deferment provides a relatively long additional protection external influences so that the molars can be used preferably for *in vitro* studies [7].

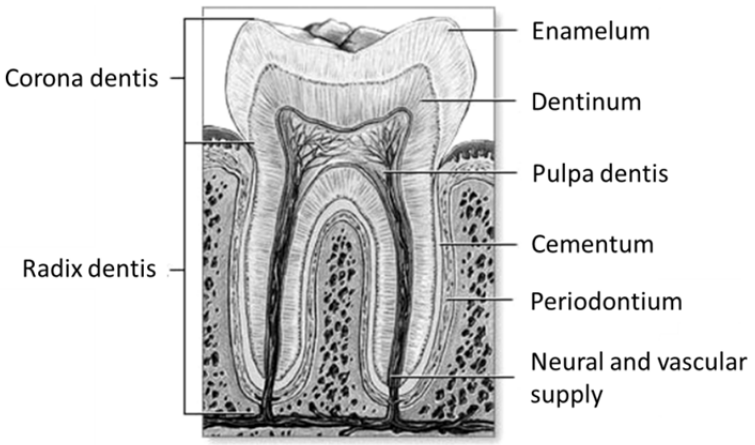

Figure 1-1: Cross-section of a human tooth (according to [8]) that shows the hard tissues (enamel and dentin) as well as the inner organic part (*pulpa dentis*) and the neural and vascular supply.

Contrary to the strong differences in the macroscopic structure, function and size of the human teeth, they differ only slightly in their microscopic structure. The two hard tissues dentin and enamel are quantitatively the main components. The dental crowns are covered by an approximately 2.5 mm thick enamel layer that becomes thinner toward the roots [1]. The translucent enamel encloses the dentin above the neck of tooth completely and forms a natural barrier against external mechanical and chemical influencing factors. The enamel is organized in prism rods that are built from bundles of hydroxyapatite (HA) crystallites. The prism rods have a diameter of around 6 µm and form a honeycomb-like pattern [9]. The prisms consist of hexagonal crystallites of $25 \times 40 \times 160$ nm (thickness $\times$ width $\times$ length) in a very strong long-range order. The

crystallites inside the rods are always perpendicular to the surface of the tooth. The prisms are surrounded by the non-prismatic enamel, which is characterized by a lower long-range order [9]. The typical enamel surface shows always the typical prism rods with a diameter of 6 µm (**Figure 1-2 A**). These rods consist of crystallites with a hexagonal cross section (**Figure 1-2 B**) of a width of about 70 nm [10] and a thickness of about 35-59 nm [11]. These crystallites belongs to the hexagonal crystal system that is shown in **Figure 1-2 C.**

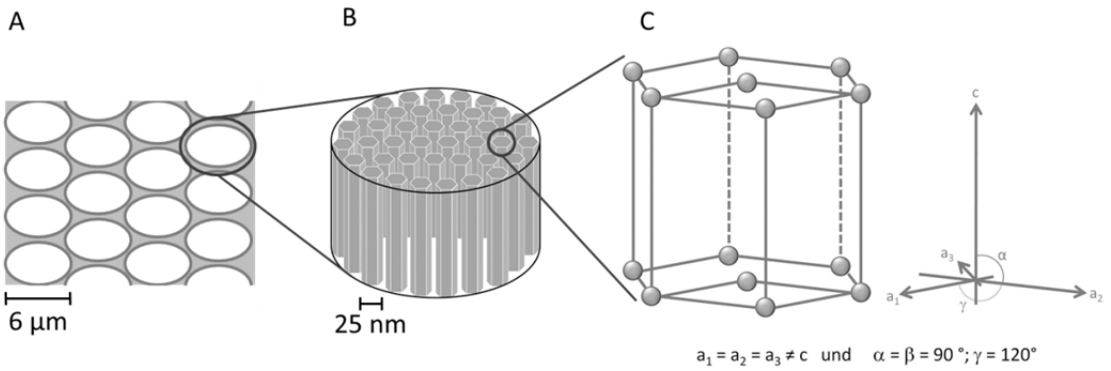

Figure 1-2: Schematic surface of a human dental enamel surface (A) with the typical prism rods that look honeycomb-like. Between the prism rods aprismatic enamel is shown in grey colour. The prism rods (B) consist of perpendicular to the surface structured hydroxyapatite crystals. These hydroxyapatite crystals belong to the hexagonal crystal system (C).

During the formation of the teeth human dental enamel is formed by ameloplasts. They initially form a protein-rich tissue, which subsequently mineralizes almost entirely by the incorporation of calcium and phosphate. This hierarchical structure gives the tooth their final shape and mechanical properties [1]. The teeth are embedded in the jaw under the overlaying gingiva. Before the teeth break through the gingiva the mineralization process is largely completed. Remineralisation with new formed enamel is only possible for the enamel-dentin border [8]. But it is possible that fluoride can be incorporated during the tooth mineralization [12]. In addition, an external application of fluoride shows, that fluoride diffuse into the enamel surface [12-14]. Fluoridated enamel has a higher hardness [15,16] and shows a stronger resistance against acids than not fluoridated enamel [17,18]. Dental enamel is formed exclusively during the

amelogenesis. Until now, no biological process is known, that regenerates the destroyed or degraded enamel [1,19].

Enamel is the hardest and most mineralized tissue of the human body and consists of > 96 % inorganic hydroxyapatite [$Ca_{10}(PO_4)_6OH_2$] and < 4 % of constituents such as proteins (amelogenin, enamelin), water and fats [8]. Hydroxyapatite belongs to the hexagonal crystal system and occurs in nature as a yellowish-grey-white, opaque mineral. In the human body the stoichiometric hydroxyapatite **Figure 1-3** shows a non-stoichiometric[1] composition. Hydroxyl groups can be replaced by fluoride ions and phosphate partially by carbonate.

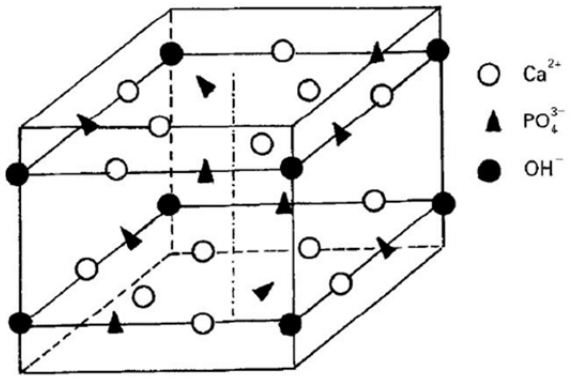

Figure 1-3: The unit cell perspective of hydroxyapatite in a stoichiometric composition (according to [20]). In case of non-stoichiometric hydroxyapatite cationic Ca^{2+} can be substituted by Na^+, Mg^+ or K^+ and negatively PO_4^{3-} by CO_3^{2-} and OH^- by F^- ions, respectively.

Enamel can therefore be described as highly substituted, calcium reduced, carbonate-rich hydroxyapatite [21]. In addition, small amounts of sodium (0.6 wt %), magnesium (0.2 wt %) and potassium (0.03 wt %) may be present. The composition of the human hydroxyapatite (HA) follows the formula: $Ca_{10-x}Na_x[(PO_4)_{6-x}(CO_2)_x(OH)_{2-y}F_y]$, with $0.3 \leq x \leq 0.6$ und $0 \leq y \leq 0.3$ [9]. Experiments showed that enamel is a four-layer arranged structure (**Figure 1-4**) with a varying chemical composition. Fluoride is

[1] In nonstoichiometric compounds, the average number of atoms per unit cell is not equivalent to the number of sites and in one of the sublattice (anion or cation), there is a deficiency or an excess of the species. [New Directions in Solid State Chemistry. Rao CNR, Gopalakrishnan J. *New Directions in Solid State Chemistry*. Cambridge University Press (1997).

incorporated, especially in the outer regions of the dental enamel and forms a flour apatite [12].

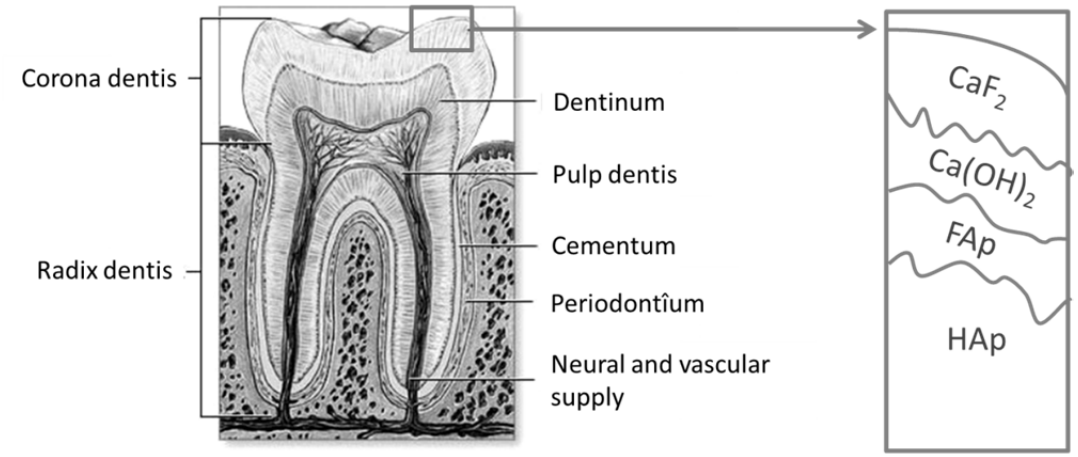

Figure 1-4: Chemical four-layer structure of fluoridated dental enamel: an external calcium fluoride layer (CaF_2), underneath a calcium hydroxide layer ($Ca(OH)_2$), followed by flour apatite (FAp) and last a non-stoichiometric hydroxyapatite (HAp) layer (according to [12].

Hydroxyapatite is the basis of all hard, inorganic tissues of the human body and therefore primarily found in bone and teeth. It is structurally almost exclusively the non-stoichiometric poorly crystallized Mg-, Na-, and Carbonate-hydroxyapatite, as described before.

Enamel is characterized by a high hardness and elastic modulus and a good resistance against external physical and chemical influences [8]. It has a low breaking strength and a high reduced elastic modulus. In this way and by the very flexible underlying dentin the likelihood of fractures and breaks is minimized [10]. The main properties of hydroxyapatite found in human tissues differ from tissue to tissue and are given in **Table 1-1**. The hardness and reduced elastic modulus are subject to large fluctuations, depending on various external and endogenous factors, such as the geographic region [22] and the social environment [6]. Moreover, the hardness and elastic modulus decrease from the outer layers toward the enamel-dentin junction [23].

Table 1-1: Comparative structural parameters of inorganic phases of adult human calcified tissues [8].

	Enamel	Dentin	Bone	hydroxyapatite (HA, stoichiometric)
Crystallinity index, (HA = 100)	70 - 75	33 - 37	33 - 37	100
Elastic modulus (GPa) [24]	80	15	0.34 - 13.8	10
Tensile strength (MPa)	10	100	150	100
Hardness (GPa)	3.5 - 4.5 [25,26]	1.0 [27]	0,5 - 1 [28]	1.17 - 6.08 [29]

At pH values ≤ 5.5, the enamel is dissolved by chemical processes [30]. Also influences of alkaline substances on the enamel surface were described [31]. A high substitution by other ions in the stoichiometric HA can cause a destruction of the crystal lattice [21,32]. Acids formed by endogenous and exogenous processes change the pH in the mouth and react with the HA surface of the teeth. When human enamel is treated strongly and for prolonged times with acids, the so-called dental human erosion occurs, that is characterised by degeneration and irreversible destruction of the tooth tissue.

1.2 Definition, mechanism and pathology of human dental enamel erosion

Dental erosion was first scientifically described in 1892 by Darby, in 1907 and 1923 by Miller Pickerill [33]. Since the mid-1990s, erosion as dental disease has been examined more frequently. Erosion is the dissolution of human dental hard tissue without the involvement of bacteria [34]. This dissolution is mainly caused by acids found in food and beverages (“soft drinks”). These acids are added during the production [35]. Erosion is one of the most important dental diseases [36,37]. More and more, this disease is described and investigated focused on effects for children [38-40] and first case reports were published [41,42].

In general all acids can lead to erosion [34], while the most important acids in the process of erosion are citric, phosphoric and ascorbic acid. These acid are quantitatively the most common acids used in soft drinks [43]. Acids dissociate in water in two parts: the protons and the corresponding acid radicals. Both parts of the dissolved acid play an important role in the erosion process [44]. The interaction of the acids and the dental enamel causes a dissolution process that is characterized by two processes: (I) softening of the dental enamel and (II) loss of enamel material [45]. This process is strongly influenced by the treatment time. **Figure 1-5** shows the time dependency of the erosive process. After short acid exposure times (in the range of few seconds) a softening of the enamel occurs. Prolonged acid exposure times (in the minute range) to human dental enamel lead to a loss of material.

The interaction of acids with the enamel leads to the dissolution of the HA crystallites [46] from the enamel surface that subsequently be dissolved to Ca^{2+}, PO_4^{3-} and OH^- ions [45]. During this dissolving a softening of the enamel occurs [9,25]. Prolonged treatment of enamel with acid and therefore, a continuing process of erosion lead to massive loss of enamel material [47,48].

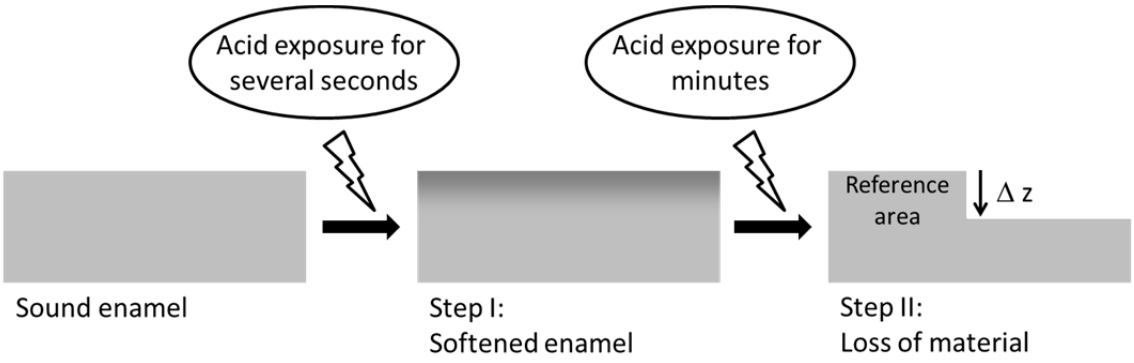

Figure 1-5: Representation of the time-dependent and acid-induced process of human dental erosion: Step I softening of human dental enamel and step II loss of enamel material (according to [45])

The consumption of acidic beverages is the main reason for erosion-caused diseases of adults, young people and children [49]. Especially children show strong erosive caused changes of the enamel surface of both, the deciduous and the permanent teeth

[50,51]. The increasing consumption of acidic drinks, like sports drinks and soft drinks is one main cause of erosion [49]. The per capita consumption of soft drinks in Germany in 2008 of 117.0 liter [52,53] clearly shows, that acidic beverages play an important role in erosion-caused diseases. In addition to acidic drinks that cause erosion [54] more and more other foods are known to cause erosion of human dental enamel. Especially for children, significant erosion damage are caused by the consumption of chewing gums [55] and sour sweets [56]. For adolescents and adults, other drinks such as alcopops [57] and wine consumption [58-60] lead to severe erosion damages. In addition, the erosion potential of certain teas [18,61,62] and yogurt products [63,64] was investigated. Also fruits, vegetables and juices can contain significant amounts of acid and so lead to erosion effects as well [35,65].

The process of erosion is influenced by a large number of risk factors [66] and different biological [3], intrinsic [67,68] and extrinsic factors [65,67]. **Figure 1-6** shows an overview of intrinsic and extrinsic factors that affect human dental enamel erosion. Especially the chemical properties of the beverages and acids as extrinsic factors are important (pH, type of acid, acidity). In addition, the influences of biological factors are important, too [3]. These include for example salivary flow and salivary buffering capacity, dental anatomy, gingiva diseases or physical stress like daily gnashing of teeth. But until today these biological factors are greatly underestimated; however, they decisively contribute to the process of erosion. Next to this also socio-economic factors play an increasing role. Especially the socio-economic status, like income and occupation or the personal level of education, seems to influence the process of erosion.

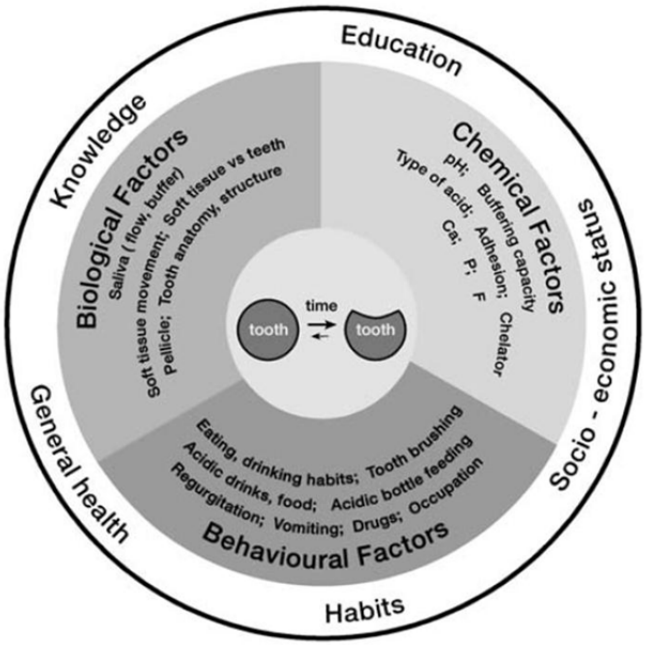

FIGURE 1-6: Examples of intrinsic and extrinsic factors are shown that affect human dental enamel erosion. (according to [67]).

As consequence of the influence of all these factors is the pathological and irreversible damage of the human dental hard tissue, known as erosion. Usually patients and also dentists recognize erosion late in an advanced stage. Erosion only cause unspecific symptoms such as pain, inflammation of the pulp [69] or unspecific lesions [70]. Therefore, the diagnosis and the management of erosion is very difficult [71,72]. Erosion can be classified based on clinical surveys or personal knowledge of the dentists [73]. Because of the high number of influencing factors and the problems in diagnostic it is difficult to identify the prevalence of human erosion [74]. But, 50 % of the 6-years old and 32 % of 14-years old children in the UK showed strong, partly dentin affecting erosion [51]. The pathological side of erosion can be macroscopic classified by the dentists [75]. Due to the erosion-caused damage of the dental enamel and the resulting loss of stability of the enamel prisms first macroscopic lesions [9] and changes in the natural enamel colour [76,77] can be identified.

1.3 Strategies against human dental enamel erosion

Since the 1990's some strategies to reduce or prevent dental enamel erosion caused by acids have been developed. In most studies the approach was to modify the acid-containing beverage itself and thus to reduce its erosive potential [78].

One of the first efforts to reduce erosion was to change the degree of saturation of Ca^{2+} and PO_4^{3-} ions with respect to hydroxyapatite in the soft drinks [79,80]. In this way, the gradient of ions (Ca^{2+} and PO_4^{3-}) between the hydroxyapatite surface and the soft drink can be reduced to the extent that a softening of the enamel can be prevented completely. The disadvantage of this method is the considerable change in the sensory properties of the beverages caused by the addition of the calcium and phosphate salts. Further investigations found concentrations that were sensory acceptable, and nevertheless led to a reduced enamel softening [80]. *In situ* studies investigated a soft drink that demonstrably causes less erosion when Ca^{2+} and PO_4^{3-} ions were added compared to the same drink without the addition of these ions [81-83].

Furthermore, other compounds have been tested for their capability to reduce dental enamel erosion. Some beverage modifying agents, such as citrate [84] and fluoride [85,86], have been added to acidic solutions to reduce the dissolution of human enamel. In addition, the effect of proteins in the saliva which bind to the enamel surface has been investigated with respect to the reduction of erosive effects [87-89]. Next to this also milk-proteins were tested for their binding capability to human enamel and thus for their ability to reduce dental enamel erosion [90-92]. In all these studies reduced erosive enamel damages were demonstrated.

One actual and important scientific approach is the use of polymers as additives to acidic soft drinks to reduce dental enamel erosion. Food approved polymers can affect the dissolution of the HA surface [93-95]. It could be shown that polymers can prevent or reduce the dissolution of Ca^{2+} and PO_4^{3-} ions from the HA prisms [94,96]. Therefore,

such polymers may be useful to reduce erosion or to study enamel remineralisation mechanisms [97]. So for example the food-approved polymers xanthan gum and carboxymethyl cellulose reduce the dissolution of Ca^{2+} and PO_4^{3-} ions caused by citric acid solution [94]. The results of a decreased enamel loss were also reported *in situ* for xanthan gum [98] and the combination of xanthan gum with calcium or polyphosphate [93,95].

1.4 Aims and Objectives

The main aim of this study was to investigate the erosion effects caused by acidic solutions on human dental enamel in more detail. Citric acid solutions were modified with food-approved polymers to reduce erosion and to develop polymer based strategies against human dental erosion induced by citric acid. Parts of this work were supported and accompanied by an industrial partner and industrial specifications.

Concerning the above described approaches the following main objectives were:

I to investigate if tactile profilometry measurements of eroded enamel alter the surface of the enamel and thus lead to damages of the surface, which result in an increased material loss, compared to non-tactile optical measurement methods.

II to investigate the suitability of the polymers propylene glycol alginate (PGA), highly esterified pectin (HP) and gum arabic (GA) as additives to citric acid solutions to reduce erosion of human dental enamel after short time treatments.

III to investigate the interaction of these polymers with the *in vitro* hydroxyapatite surface of human dental enamel as well as artificial hydroxyapatite.

IV to investigate the thickness and morphology of polymer layers that were deposited on *in vitro* enamel surfaces as protective barriers against citric acid.

V to investigate the interaction between the polymers and the enamel surface with different chemical and physical methods and to develop adequate models.

VI to investigate if acids that were adjusted to an identical acidic taste lead to different erosion damages and that fruit acids lead to less erosion enamel softening than citric or phosphoric acid.

In this study two important main methods were used. First, profilometry, that is a well-established technique in surface science and dental research [7,99,100]. Profilometry was used to determine the loss of material of enamel surfaces treated with erosive citric acid solutions compared to untreated samples. Profilometry is suitable for long time erosion treatments, that can be prolonged from few minutes [101,102] up to hours [48] and days [83]. The another often used method was atomic force microscope based nanoindentation (AFM-based NI) [25,30]. This method is usable to investigate the nano mechanical properties of treated enamel compared with untreated enamel surfaces [103-105]. With AFM-based NI it is possible to investigate enamel softening caused by erosion after short time treatment in the range of seconds [106,107].

2 OVERVIEW OF MANUSCRIPTS

MANUSCRIPT I (PUBLISHED)

Quantification of dental erosion - A comparison of stylus profilometry and confocal laser scanning microscopy (CLSM)

Erik Heurich, Markus Beyer, Klaus D. Jandt, Jörg Reichert, Volker Herold, Matthias Schnabelrauch, Bernd W. Sigusch

Dental Materials 2010; 26 (4): 326-336.

CONDENSED ABSTRACT

The paper deals with the comparison of tactile (profilometer) and non-tactile (confocal laser scanning microscopy) measurement systems to investigate the time dependent citric acid induced material loss of human dental enamel. The different methods were compared to each other. Special focus was provided to the evaluation of the physical damage by tactile measurement systems on softened and eroded human enamel.

INFORMATION ABOUT OWN CONTRIBUTION

EH was responsible for measurements and data analysis, as well as writing of the manuscript; MB was responsible for supervision of the *in vitro* study, sample handling and preparation and for conducting the statistically analyses; KDJ for funding and supervising; JR supported discussion and proof reading; VH supported the profilometer measurement and their analysis; MS supported the data interpretation; BWS supported the clinical interpretation of the data.

MANUSCRIPT II (PUBLISHED)

Pectin, alginate and gum arabic polymers reduce citric acid erosion effects on human enamel

Markus Beyer, Jörg Reichert, Erik Heurich, Klaus D. Jandt, Bernd W. Sigusch

Dental Materials 2010; 26 (9): 831-839

CONDENSED ABSTRACT

Three different food-approved polymers were tested for their inhibiting effects of human dental erosion. The study compared the erosion behaviour of polymer modified citric acid solutions and not modified citric acid solutions on human dental enamel. The study focused on changes of the nano mechanical properties of human dental enamel when it was treated with citric acid solutions containing polymers. When polymers were added as additives to citric acid solutions the decrease of the nano mechanical properties of human dental enamel was reduced.

INFORMATION ABOUT OWN CONTRIBUTION

MB was responsible for sample handling and preparation, measurements and statistical data analysis, as well as writing of the manuscript; JR was responsible for supervision of the *in vitro* study and supported discussion; EH supported data interpretation; KDJ for funding and supervising; BWS supported the clinical interpretation of the data.

MANUSCRIPT III (IN PREPARATION)

Morphology and structure of polymer layers protecting dental enamel against erosion

Markus Beyer, Jörg Reichert, Bernd W. Sigusch, Klaus D. Jandt

CONDENSED ABSTRACT

The study focused on the investigation of polymer layers, which were formed when human dental enamel was treated with polymer modified citric acid solutions (PMCAS). With AFM it was shown, that the polymers were deposited on human dental enamel. Using profilometry and XPS measurements insights were gained into the morphology and the structure of the deposited layers. A model was developed that describes the structure of the layer as two opposing gradients of polymer molecules and dissolved HA enamel particles.

INFORMATION ABOUT OWN CONTRIBUTION

MB was responsible for sample handling and preparation, measurements and statistical data analysis, as well as writing of the manuscript; JR was responsible for supervision of the *in vitro* study and supported discussion; BWS supported the clinical interpretation of the data; KDJ for funding and supervising.

MANUSCRIPT IV (PUBLISHED)

Acids with an equivalent taste lead to different erosion of human dental enamel

Markus Beyer; Jörg Reichert; Jörg Bossert; Bernd W. Sigusch; David C. Watts;
Klaus D. Jandt

Dental Materials 2011; 27 (10): 1017-1023.

CONDENSED ABSTRACT

Five different acids were used in a concentration that gave an equivalent acidic taste for all acids. *In vitro* enamel samples were treated with these acid solutions for 60 s and the erosion caused damages were analysed with AFM-based nanoindentation and with scanning electron microscopy. The results showed that there were two groups of acids, whereas the group I (malic, tartaric and citric acid) caused statistically significant lower enamel softening than the acids of group II (lactic, ascorbic and phosphoric acid).

INFORMATION ABOUT OWN CONTRIBUTION

MB was responsible for sample handling and preparation, measurements and statistical data analysis, as well as writing of the manuscript; JR was responsible for supervision of the *in vitro* study and supported discussion; BWS supported the clinical interpretation of the data.

3 MANUSCRIPTS

MANUSCRIPT I (PUBLISHED)

Quantification of dental erosion - A comparison of stylus profilometry and confocal laser scanning microscopy (CLSM)

Erik Heurich, Markus Beyer, Klaus D. Jandt, Joerg Reichert, Volker Herold, Matthias Schnabelrauch, Bernd W. Sigusch

Dental Materials 2010; 26 (4): 326-336

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Quantification of dental erosion—A comparison of stylus profilometry and confocal laser scanning microscopy (CLSM)

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ABSTRACT

Objectives. Since stylus profilometry applies a force on the sample surface, it is logical to hypothesize that the profilometer penetrates the surface of the enamel softened by acid solutions. The aims of the present study were, therefore, to test the hypothesis that surface profilometry measurements of eroded enamel alter the surface of the enamel, to quantify the potential effect of the surface alteration (scratches) on the measured values of enamel erosion by atomic force microscopy and to compare the values of enamel loss caused by erosion as measured by profilometry and non-contact confocal laser scanning microscopy (CLSM).

Methods. Enamel samples, cut from unerupted human third molars were treated with Volvic Mineral Water and citric acid solutions of different pH values. The enamel material loss was measured by two different contact profilometers and a reflection mode CLSM. The scratches depth was analyzed by atomic force microscopy.

Results. Our study demonstrated that the tip of the profilometer penetrated the surface of eroded enamel during the profilometry measurements, leading to clearly visible surface scratches on the enamel samples. The profilometers created surface scratches of a depth ranging from 57.6 (47.1) nm to 577.1 (157.6) nm on the surface of the eroded enamel and led, therefore, to a larger measured value of erosion. It was shown that the depth of the scratches depends on the pH value, the erosion time and the profilometer used.

Significance. With few exceptions profilometers deliver reliable values of erosive enamel material loss, although they create surface scratches on eroded enamel. Reflection mode CLSM is a non-tactile, fast and precise method for analyzing enamel erosion quantitatively in vitro.

1. Introduction

Human dental enamel is one of the hardest biological materials in the body. While fluorapatite is highly resistant against acidic dissolution, hydroxyapatite can be dissolved by several

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acids commonly found in beverages. Beverages like soft drinks frequently contain citric acid or phosphoric acid. The chemical dissolution of dental enamel without the involvement of bacteria has been defined as enamel erosion [1].

It has been proposed that dental erosion progresses in three different stages of enamel erosion [2]. The first stage of erosion is called initial demineralization. Acids which dissolve Ca^{2+} and PO_4^{3-} ions soften the enamel surfaces significantly [3]. At this stage the enamel is susceptible to further damage, as different studies have shown [4,5]. The softened enamel collapses in the second stage of enamel erosion because of further exposure to acids and mechanical stress caused by tooth brushing, mastication or bruxism [6]. As a result, the collapsed enamel is removed in the third stage, the underlying unaffected enamel is exposed and the enamel erosion process starts over [2]. Hence, enamel loss should increase with prolonged erosion times.

In general, acidic solutions with low pH values are able to dissolve higher concentrations of calcium and phosphate ions from dental enamel [6]. Therefore, low pH value (pH 2–4) solutions should increase enamel softening and enamel loss of up to several micrometers [7,8]. Several studies showed that softened enamel is very susceptible to scratching [5,9,10]. It was found that the enamel loss caused by tooth brushing depends on the duration of time between enamel erosion and tooth brushing [9]. Softened enamel is highly unstable and can be easily removed by short and relatively gentle physical action [5]. Tooth brushing on eroded enamel leads to minor changes in surface morphology and mechanical properties [10].

It has been suggested that profilometry measurements may affect the surface eroded enamel [2,11]. During scanning, the profilometer applies a force onto the enamel surface. Depending on this force and the contact area of the profilometer tip there is a compressive stress which may plastically deform the affected enamel surface. If the hardness of the softened enamel is too low, the enamel could collapse and the profilometer tip would, therefore, create a linear scratch on the surface, since the profilometer scans across the enamel surface. The scratches potentially created by the profilometer should be visible as straight lines on the eroded enamel surface. Optical microscopy would only allow qualitative observations of the scratches. It is necessary to use a quantitative technique such as atomic force microscopy to measure the depth of the scratches created on the enamel surface.

Profilometry, also called surfometry is a well-established technique often applied in surface science and dental research [6,12]. The profilometer can be used to measure the contour of the surface, the profile and roughness, quantitatively creating a path-height diagram. It uses a probe consisting of a stylus with a sharp diamond tip which scans a line on the surface of the sample [13]. To the best of our knowledge, it was used for the first time in dental research in 1972 to study the abrasion of dentin caused by different toothpastes [14]. In a study, three different instruments for measuring enamel erosion – quantitative light-induced fluorescence (QLF), transverse microradiography (TMR) and optical surface profilometry – were evaluated. The authors stated disadvantages, such as the destructive nature of most methods, like for example contact profilometry. However, they neither quantified the damage of eroded enamel nor did they cite studies which corroborate this hypothesis [15]. To our best knowledge no study has so far quantified how profilometry measurements affect or change the surface of eroded enamel.

Another method for analyzing eroded enamel surfaces is confocal laser scanning microscopy (CLSM). This method produces images of a sample surface by scanning the surface with a laser and by using the principle of confocal imaging. Thus, it is possible to measure the height difference between an eroded enamel surface and a given reference area. The effect of erosion [16] or abrasion [17] on dentin has been studied by confocal laser scanning microscopy previously. So far only a few studies describe the use of optical profilometry, laser scanning microscopy or white light microscopy for investigating enamel erosion [4,18,19]. Two different studies have been carried out using CLSM for detecting dental enamel erosion either qualitatively [18] or by quantitative measurements [19].

A third method to quantify demineralization and erosion of dental tissue is atomic force microscopy (AFM), which uses the attractive and repulsive forces between the surface and a tip to detect different surface characteristics [20–23]. The high resolution and the ability to measure the surface hardness by AFM nanoindentation [4,22,24] make the AFM a very useful device for analyzing dental erosion [10,25,26].

The aims of the present study were (i) to test the hypothesis that surface profilometry measurements of eroded enamel alter the surface of the enamel, e.g. by penetration of the profilometer tip into the softened enamel surface, (ii) to quantify the potential effect of such surface alteration on the values of enamel erosion as measured by atomic force microscopy, and (iii) to compare the values of enamel loss caused by erosion as measured by profilometry and non-contact confocal laser scanning microscopy (CLSM).

2. Materials and methods

2.1. *Sample preparation*

Samples were prepared from non-erupted human third molars which had been extracted for medical reasons. The teeth were disinfected in 1%-thymol solution (Merck, Darmstadt, Germany) until preparation. For cleaning, teeth were stored in sodium hypochloride (Carl Roth, Karlsruhe, Germany) for 24 h. Organic remains were removed by carefully using a dentist's set of instruments.

The enamel surface of each tooth was sectioned by a low speed diamond saw (Bühler Isomet, Bühler, Düsseldorf, Germany). The enamel pieces, which had a size of 3 mm × 2 mm × 1.5 mm, were embedded in epoxy resin (Stycast 1266 Emerson & Cumming, ICI Westerlo, Belgium). After a polymerization time of 12 h, samples were ground with SiC paper and polished with monocrystalline diamond suspension with a particle size ranging from 6 μm to 1 μm. The samples were stored in deionized water after preparation. One half of the polished surface was covered with PVC adhesive tape (Tesapack ultra strong, Tesa, Hamburg, Germany) to create a reference area unaffected by erosion [8]. The uncovered part of the enamel surface was subsequently cleaned with a cotton swab and ethanol. Since some glue could remain at the

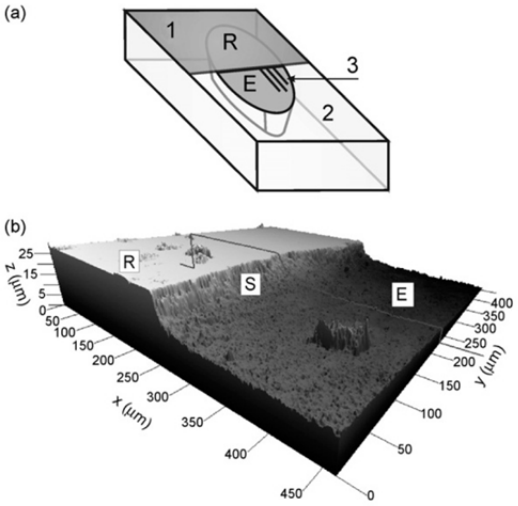

Fig. 1 – (a) Scheme of a tooth sample covered by PVC adhesive tape (1). The cut tooth sample is embedded in a resin (2). After covering half of the surface with adhesive tape, the tooth was eroded. The eroded surface of the tooth is labeled with (E), the reference area is labeled with R, and the scratches are labeled with (3). (b) CLSM micrograph of an eroded enamel surface. After treatment with citric acid solution there is a step (S) between reference area (R) and eroded enamel surface (E).

reference area after removing the adhesive tape, this part of the tooth surface was cleaned in the same way after enamel erosion. Fig. 1a shows a sketch of such a sample.

2.2. *Erosion of the enamel samples*

From 60 teeth obtained, each tooth was cut into manageable samples. From each of these cut samples a random piece was taken from each tooth and divided into three groups (A–C), 9 samples per group. Two aqueous (deionized water) solutions of citric acid (Merck Schuchardt, Hohenbrunn, Germany) were prepared for the erosion test of the samples. Citric acid (CA) was dissolved in deionized water in two different concentrations: CA 1 was a 0.1 M CA solution with pH 2.3, whereas CA 2 was a 0.01 M CA solution with pH 3.3. The samples of the control group were treated with Volvic Mineral Water (VMW) (Volvic Naturell, Danone Waters Deutschland, Wiesbaden, Germany). The mineral water pH was 7.1. The pH values of all solutions were measured with a pH-meter (Knick pH-meter 765 Calimatic, Germany).

Samples were treated in 25 ml of CA 1 (group A), CA 2 (group B) and VMW (group C) at room temperature. To prevent an increased concentration of dissolved Ca^{2+} and PO_4^{3-} ions at the sample surface during the erosion process, the solutions were agitated with an automatic shaker (Heidolph Polymax 2040, Heidolph Instruments, Schwabach, Germany) at 50 rpm. Three samples from each group were removed from the solution after 10, 20 and 40 min of erosion time, respectively. The sample surfaces were rinsed with deionized water for 10 s and subsequently dried with compressed air.

After removing the tape, all sample surfaces were examined using an optical light microscope (Leica MZ8, Leica Microsystems, Wetzlar, Germany) at a 5× magnification. Any adhesive remnants from the tape found on the reference area were removed carefully with ethanol and a cotton swab. All samples from each group, arranged by erosion time, were fixed in rows onto a single glass slide and stored in Petri dishes, in air, until use.

2.3. *Calibration of the profilometers, the CLSM and the AFM*

The profilometers, the CLSM, and the AFM were calibrated in z-direction using a standard for profilometer calibration (Carl Zeiss Jena, Jena, Germany) with rectangular trenches which have a rectangular cross-section. The height of the calibration standard profile was 4.2 (±0.1) µm. A line scan was made to check the accuracy of the profilometers, whereas the section mode for 3D images was used to calibrate the CLSM and the AFM. With these methods the height of the standard was measured with each instrument at ten different positions of the height standard. After the measurements the average height and the calibration standard deviation of the standard were calculated for each instrument.

2.4. *Profilometry*

Prior to taking measurements, a glass slide with the samples was fixed onto a mount with double-sided adhesive tape to prevent the samples from moving during the measurements. Two profilometers were used to compare the effect of two different tip radii, loads, and sensor detection technologies on the eroded enamel surfaces.

The first instrument is a profilometer with an laser interferometric transducer (Taylor Hobson FormTalysurf Series 2, Taylor Hobson, Leicester, England). The cone shaped diamond tip has a tip radius of 2.00 µm. The testing force applied to the enamel surface was 0.87 mN. Line scans of a length of approximately 4 mm were performed with the FormTalysurf Series 2. After each line scan, the sample was moved slightly to prevent a repetition of the measurements on the scan line of the previous measurement. The lateral distance between each recorded profile was approximately 50 µm. Each sample was measured three times, scanning from the reference surface area to the eroded surface area.

The second profilometer has an inductive transducer to record height measurements (Hommeltester T 1000, Hommel-Etamic, Schwenningen, Germany). The cone shaped diamond tip of this profilometer has a tip radius of 1.95 µm. The testing force applied to the enamel surface was 1.60 mN. With the Hommeltester T 1000 each sample was also measured three times, scanning from the reference surface to the eroded surface.

After the profile measurements, the enamel samples were examined with an optical microscope in order to examine if the profilometry measurements had created visible surface scratches. The height profiles of the eroded enamel surface recorded by both profilometers were analyzed by the software

according to the profilometer FormTalysurf Series 2 (Taly μltra V.5.11.6.17, Taylor & Hobson, Leicester, England). The curves were filtered by a primary filter (cut off = 0.025 mm) to eliminate the signal noise. Lastly, the fitted curves were adjusted to the baseline.

2.5. Confocal laser scanning microscopy (CLSM)

The CLSM (Zeiss 510 Meta, Carl Zeiss MicroImaging, Jena, Germany) is equipped with a helium/neon-laser with a wavelength $\lambda = 488$ nm. A 20× dry lens objective lens (Zeiss NEONFLUAR 20×, 0.5 HD) with a basic field of view of 450 μm × 450 μm was used in reflection mode. The transition zone between the eroded sample area and the reference area was positioned in the center of the field of view. The eroded enamel surface was imaged by the CLSM and three height profiles were measured on the surface. The data from CLSM were analyzed by the additional software Zeiss LSM 510 Meta. The surface image was plane fitted into a 3D image (Fig. 1b). Noise was filtered with a 7 × 7 Gaussian filter. The profiles at three different positions were measured and exported to ASCII files for evaluation.

2.6. Atomic force microscopy (AFM)

Topographic images of the profilometer traces were recorded with an atomic force microscope (Digital Instruments Dimension 3100, Veeco Instruments, Santa Barbara, CA, USA) operating in tapping mode and using silicon cantilevers (OMCL AC160TS-W, Olympus, Tokyo, Japan). The AFM was used because of its ability to measure the depth of the surface scratches caused by the profilometers and its extremely high resolution in x-, y- and z-directions (<1 nm) [27]. It was necessary to capture the AFM images in tapping mode to avoid damaging the softened enamel. In this AFM operation mode lateral forces applied to the enamel surface by the AFM tip are minimized by the tapping movement of the tip. After being captured, the height images were fitted by an x–y-fit, similar to the fit used for the CLSM micrographs but without filtering. For depth measurements, the "average section tool" of the AFM software (Veeco NanoScope V. 5.12r5, Digital Instruments, Santa Barbara, CA, USA) was used. With this software tool, a line A was drawn along the bottom of each scratch of the enamel and a second line B was drawn parallel to the first one on the eroded surface (Fig. 6c). The software integrated the surface structure within the marked area and calculated the means $\bar{z}$ of the height values z(A) and z(B) on the tooth surface along each line A and B. The height of the line B was set to 0 and the difference Δz_{AFM} of these two mean values (Eq. (1)) was computed.

$$\Delta z_{AFM} = \bar{z}(B) - \bar{z}(A) \tag{1}$$

To analyze the influence of profilometric scratches on the measured enamel loss by profilometer, the depth of the scratches Δz_{AFM} was subtracted from the enamel loss $z_{profilometer}$ measured by the profilometers (Eq. (2)).

$$\Delta z_{profilometer} = z_{profilometer} - \Delta z_{AFM} \tag{2}$$

The subtracted values $\Delta z_{profilometer}$ were compared to the tactile measured original values of enamel loss ($z_{profilometer}$) by a one-way ANOVA.

2.7. Statistical analysis

A statistics software (StatGraphics Centurion XV Version 15.2.00, StatPoint, Herndon, VA, USA) was used to perform all statistical analyses. To determine the influence of the used instrument, the pH value and the erosion time on the measured enamel loss, a multifactor ANOVA and a Bonferroni test were performed for the eroded samples.

The depths of the scratches on the enamel surface caused by the profilometer were also measured. A multifactor ANOVA with the factors pH value, erosion time and profilometer as well as a Bonferroni test were used to investigate the influence of these factors on the depth of the scratches on the eroded samples.

In addition, a one-way ANOVA and Bonferroni test were performed to compare the statistical significance of the original values of the enamel loss recorded by the profilometers to the corrected values.

All statistical analyses were performed with a 95% confidence interval.

The measurements from the two profilometers and the CLSM were compared to each other using a correlation test. In this test, the correlation factor ρ quantifies how similar two datasets are. If the correlation factor is 1 or −1, the datasets are identical. If the correlation factor is 0, the datasets are not similar. The correlation factor is defined by

$$\rho = \frac{KOV(X, Y)}{S_x \cdot S_y} \tag{3}$$

with

$$KOV(X, Y) = \frac{1}{n} \cdot \sum_{i=1}^{n} [(\bar{x} - \bar{x}_i) \cdot (\bar{y} - y_i)]. \tag{4}$$

When comparing the measurements of two different instruments, n is the number of compared values, x_i and y_i are the values of the first instrument and the second instrument, respectively. $\bar{x}$ and $\bar{y}$ are the means of the two datasets. S_x and S_y are the standard deviations of the compared values of each instrument.

The correlation test was performed by a spreadsheet software (Excel 2007 Enterprise Edition, Microsoft, Redmond, WA, USA). The correlation factor was calculated by a correlation function from Excel.

To show the correlation of the two datasets graphically, the results were plotted in a diagram and a regression line was fitted. The coefficient of determination was also calculated by Excel 2007. If the regression line has a slope of 45°, the datasets are similar. Therefore, the calculated correlation could be verified graphically. The coefficient of determination R^2 shows how well the regression line fits to the dataset. It is defined by the difference of the measured values and the drawn regression line.

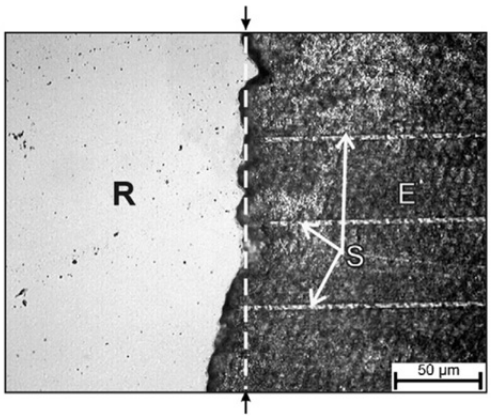

Fig. 2 – Image recorded with an optical microscope showing an eroded surface of a tooth sample with scratches from the profilometer FormTalysurf Series 2 (white arrows). R: reference area, E: eroded surface. The step between the eroded and the reference area is marked by a dashed line between the two black arrows. The white box in the right corner below has a length of 50 µm.

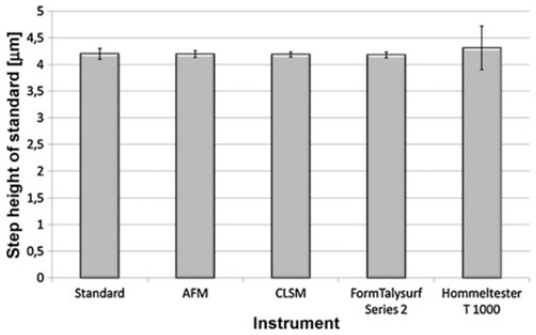

Fig. 3 – Graph showing the step height of a glass standard measured by AFM, CLSM and the stylus profilometers FormTalysurf Series 2 and Hommeltester T 1000. "Standard" shows the height (4.2 ± 0.1 µm) given by the manufacturer.

3. Results

3.1. *Enamel loss*

After the profilometric measurements, the samples were examined with an optical light microscope (Leica DMR-XE, Leica Microsystems, Wetzlar, Germany). The eroded surface appeared as a dark area, which seems to be opaque. Straight scratches were found on the eroded surface treated with the solutions CA 1 (pH 2.3) and CA 2 (pH 3.3), whereas the reference area exhibited no scratches (Fig. 2).

For calibrating the profilometers, the CLSM and the AFM, a height standard of 4.20 (±0.1) µm was used. The FormTalysurf Series 2 measured a height of 4.31 (±0.05) µm. The height measured by the Hommeltester T 1000 was 4.18 (±0.41) µm. The CLSM measured a height of 4.19 (±0.05) µm. The tapping mode AFM measured a height of 4.20 (±0.06) µm for the calibration sample (Fig. 3). The FormTalysurf Series 2 measured the highest values and the Hommeltester T 1000 had the highest standard deviation of the measurements. Therefore, the accuracy of the four different instruments was as follows:

AFM > CLSM > FormTalysurf Series 2 > HommeltesterT 1000.

The enamel losses measured with the profilometers and the CLSM are shown in Fig. 4 as a function of the erosion time for different pH values. Fig. 4a shows the results from the samples in group A (CA 1, pH 2.3), the results from the samples in group B (CA 2, pH 3.3) are shown in Fig. 4b. The graphs show that the enamel loss increased with increasing time of exposure to the acid. Furthermore, the measured enamel loss depended on the instrument used. The enamel losses of the samples treated with Volvic Mineral Water (VMW, Control)

Table 1 – Statistical analysis of the mean enamel loss.

Instrument	Erosion time		
	10 min	20 min	40 min
pH 2.3			
FormTalysurf Series 2	×	×	×
Hommeltester T 1000	×	×	×
CLSM	×	×	×
pH 3.3			
FormTalysurf Series 2	×	×	×
Hommeltester T 1000	×	×	×
CLSM	×	×	×
pH 7.1			
FormTalysurf Series 2	×	×	×
Hommeltester T 1000	×	×	×
CLSM	×	×	×

Homogeneous groups of pH value, erosion time, and instrument are displayed by crosses (×). If the crosses are beneath each other, there is no statistical significance of difference. If the crosses are moved towards the right, there is a statistically significant difference.

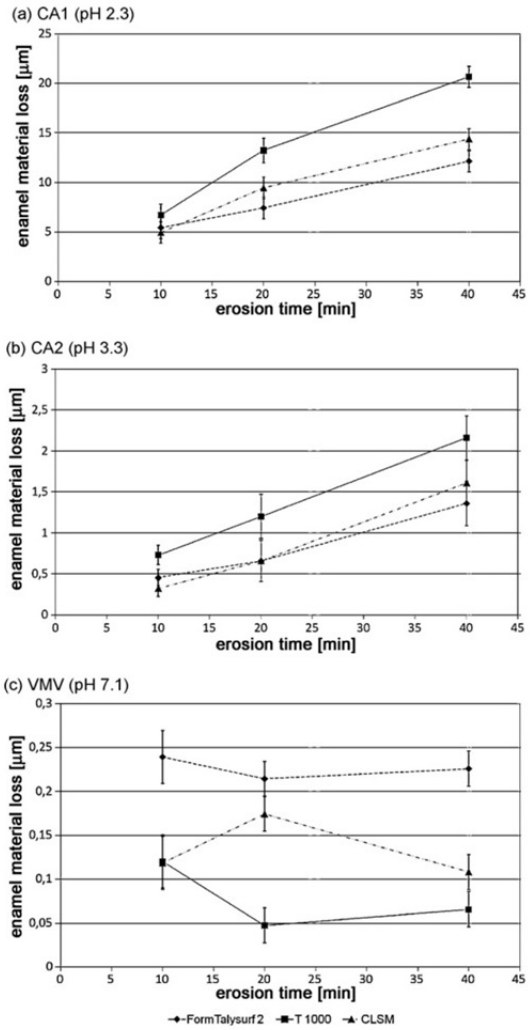

Fig. 4 – Three graphs showing the enamel material loss vs. erosion time as measured by the profilometers FormTalysurf Series 2 (♦), the Hommeltester T 1000 (■), and the confocal laser scanning microscope (CLSM) (▲). In group A (pH 2.3) and B (pH 3.3) the Hommeltester T 1000 measured the highest values (a) and (b). (c) shows the results of enamel loss after treatment with VMW. The enamel loss is not depending on the erosion time. Thus, it can be assumed that (c) only shows the surface roughness of the polished tooth samples.

are shown in Fig. 4c. No statistically significant enamel loss was observed after treatment with VMW, as the values of the samples in this group were not dependent on erosion time or used instrument. Whereas Fig. 4a and b shows that the Hommeltester T 1000 measured the highest enamel loss values for CA 1 and CA 2, Fig. 4c indicates that this device measured the smallest values for water.

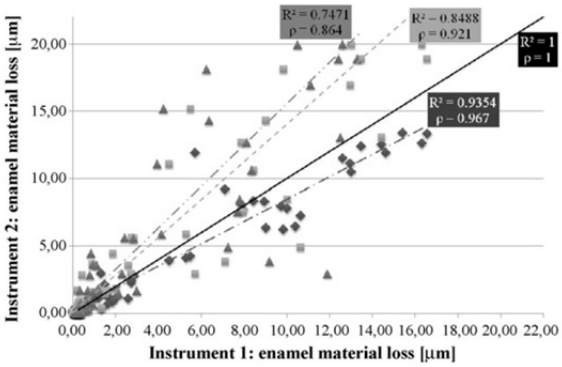

Fig. 5 – Enamel material loss measured by the three different instruments compared to each other. Diamonds and dash-dotted line: CLSM vs. FormTalysurf Series 2; squares and dashed line: CLSM vs. Hommeltester T 1000; triangles and dashed double dotted line: FormTalysurf Series 2 vs. Hommeltester T 1000; black solid line: 45°-regression line with $R^2 = 1$ which represents perfect correlation.

The results of the multiway ANOVA of the measured enamel material loss are shown in Table 1. The enamel loss depended on pH value as well as on erosion time. The values of enamel loss depended strongly on the instrument used. There were statistically significant differences between the measured values of enamel material loss obtained using all instruments.

In Fig. 5, the enamel material loss measured by the profilometers and the CLSM was compared to each other graphically. A regression line was fitted to the values (Fig. 5). If the coefficient of determination, R^2, is equal to 1, the regression line fits the data perfectly. If it is below 0.9, the line does not fit well to the dataset. The coefficient of determination for the results comparing CLSM and FormTalysurf Series 2 was $R^2 = 0.9354$. The R^2 for the results comparing CLSM and Hommeltester T 1000 was $R^2 = 0.8488$, and for FormTalysurf Series 2 and the Hommeltester T 1000 $R^2 = 0.7471$. The correlation factor ρ of the values measured by the profilometer FormTalysurf Series 2 and those of the CLSM was 0.967. The correlation factor of the data measured by the FormTalysurf Series 2 and those of the Hommeltester T 1000 was 0.864. The correlation factor of the enamel loss measured by the Hommeltester T 1000 and the values measured by the CLSM was 0.921. The manually calculated correlation factors were 0.968 (FormTalysurf Series 2–CLSM), 0.800 (FormTalysurf Series 2–Hommeltester T 1000) and 0.853 (Hommeltester T 1000–CLSM).

3.2. *AFM*

AFM images of eroded tooth samples which exhibited surface scratches created by the profilometry measurements are shown in Fig. 6. The depth of the enamel scratches depended on pH value and erosion time. The enamel samples in group A (Fig. 6a) have a relatively rough surface compared to the

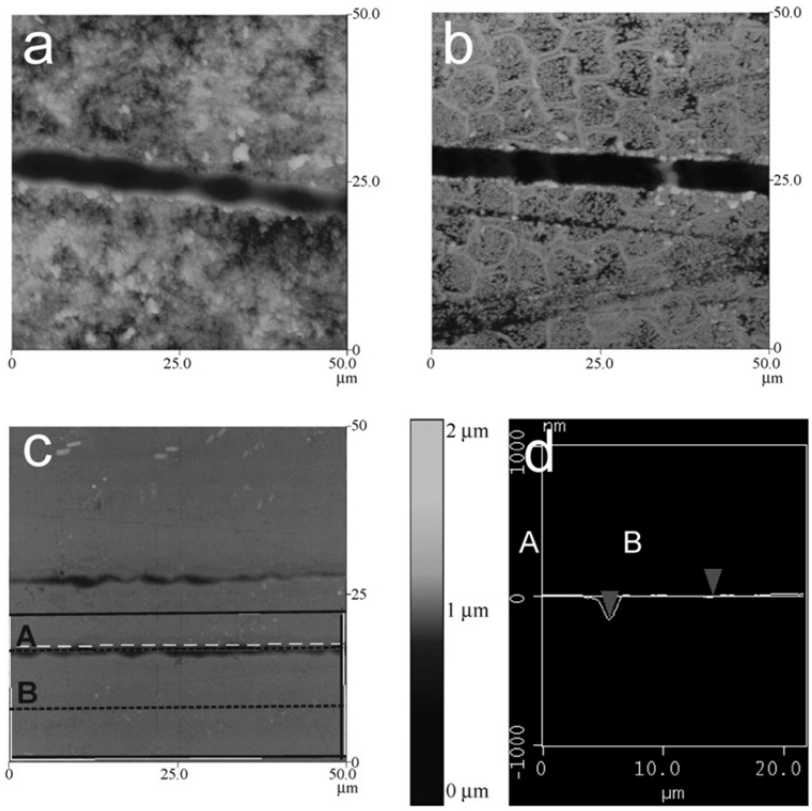

Fig. 6 – AFM micrographs of scratches on the enamel surface caused by the profilometer. The size of each image is 50 µm × 50 µm, the height scale bar shows 2.0 µm. The samples were treated with CA 1 (pH 2.3) (a) and CA 2 (pH 3.3) (b). (c) shows the surface of a sample treated with CA 2 while measured with the average section tool (black frame with dashed lines A and B). (d) shows the depth profile of the scratch in (c) measured by the average section tool. The scratches on the enamel surface caused by the profilometer appear as dark straight lines across the images. The prism structure of the enamel can be seen in (b). The scratch in (a) is 459.65 nm deep. The scratch in (b) is 477.10 nm deep.

smooth surfaces of the enamel in group B (Fig. 6b and c). Surface scratches caused by the profilometer measurements can be seen as dark lines in the AFM images (Fig. 6a–c). No surface scratches on the enamel surface were found on the samples of group C (control) treated with VMW measured by AFM.

Table 2 shows the values of the depth of the scratches as measured by AFM. It can be seen that the depth of the scratches changed depending on all three parameters tested, the pH value, the erosion time and the measuring device used. The statistical significance of this dependence was analyzed by a multiway ANOVA (Table 3). In almost all cases, the scratches on the samples eroded by CA 1 (pH 2.3) were statistically significantly deeper than the scratches of the samples eroded by CA 2 (pH 3.3). Only the scratches caused by the Hommeltester T 1000 on the samples eroded by CA 2 for 40 min were deeper than the scratches on the samples treated for

Table 2 – Depth of the scratches on the enamel surface caused by the profilometer (in nm scale) at different pH values and erosion times as measured by AFM.

Erosion time (min)	Average scratch depth measured by AFM (nm)			
	FormTalysurf Series 2		Hommeltester T 1000	
	CA 1	CA 2	CA 1	CA 2
10	152.5 (24.5)	57.6 (47.1)	275.0 (140.4)	100.4 (46.1)
20	303.7 (177.3)	245.5 (41.3)	461.3 (171.9)	308.5 (35.9)
40	577.1 (157.6)	472.2 (185.9)	543.8 (363.8)	607.6 (225.7)

Table 3 – Significant influence of the factors pH value, erosion time, and instrument, on the depth of the scratches on the enamel surface caused by the profilometer.

Factor	Homogeneous group			p value
pH value				
2.3	×			0.0180
3.3		×		
7.1			×	
Erosion time				
10	×			0.0000
20		×		
40			×	
Instrument				
FormTalysurf Series 2	×			0.0000
Hommeltester T 1000		×		

The depth depends on the erosion time as well as on pH value and the chosen device. The crosses (×) mark homogenous groups. If the depth of the scratches on the enamel surface caused by the profilometer is significant, the crosses are moved towards the right.

40 min with CA 1. The depths of the scratches caused by both profilometers also increased with increasing erosion times. Interestingly, there was a statistically significant difference in the scratches' depth for each sample group depending on the type of the profilometer. The scratches caused by the T 1000 were almost always deeper than the scratches caused by the FormTalysurf Series 2 (Table 2). The multifactor ANOVA showed that the factors pH value ($p = 0.0180$), erosion time ($p = 0.0000$) and used profilometer ($p = 0.0000$) had a statistically significant influence on the depth of the scratches (Table 3).

To analyze the effect the surface scratches caused by profilometry had on the values of enamel loss measured by profilometry, the mean values of the depth of the scratches (Table 2) were subtracted from the measured data (Eqs. (2) and (3)). The result is shown in Fig. 7. These adjusted values of the enamel loss were then compared to the original values measured by FormTalysurf Series 2 and Hommeltester T 1000. Fig. 7 shows this comparison depending on the erosion time and pH value. The original results and the corrected data were compared statistically using a one-way ANOVA. This statistical analysis showed that there was no statistically significant difference between original and corrected data ($p = 0.8910$).

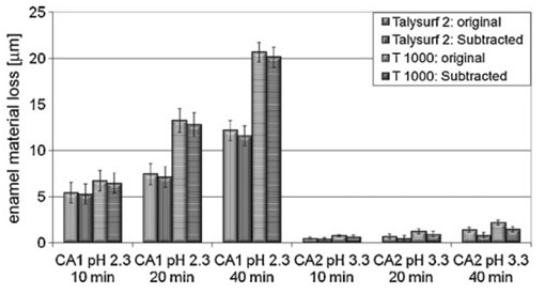

Fig. 7 – The enamel loss measured by the profilometers FormTalysurf Series 2 and Hommeltester T 1000 (labeled as "Original") was compared to the real enamel loss calculated by subtracting the depth of the scratches (labeled as "Subtracted"). There is no correction of the values of the CLSM because this non-tactile method induces no scratches.

4. Discussion

As stated earlier, enamel erosion leads to a softening followed by a collapse of the enamel. To investigate this erosion in vitro, the enamel surface was partially covered by an adhesive tape. The enamel loss, based on in vitro enamel erosion, results in a step between the eroded enamel surface area and an enamel surface area which was protected against erosion by the adhesive tape (Fig. 1a). The height of this step represents the value of the enamel loss.

Several studies have shown that eroded human enamel can be further damaged by mechanical stress, such as tooth brushing. It was found that the enamel loss caused by tooth brushing depends on the duration of time between enamel erosion and tooth brushing [9]. Abrasion increases not only by erosion. Additionally, abrasive effects have been observed at subsurface lesions, caused by a cariogenic challenge [28]. For our samples, however, there was no evidence of subsurface lesions. Furthermore, it was concluded that softened enamel is highly unstable and can be easily removed by short and relatively gentle physical action [5]. It has been shown that eroded human enamel can be damaged further by tooth brushing [10]. Enamel samples were eroded with citric acid solutions and afterwards these samples were brushed using an electric toothbrush. The resulting enamel loss caused by erosion and the followed abrasion caused by tooth brushing was measured by AFM. All these studies demonstrated that brushing further increases the enamel loss caused by erosion.

It has been suggested that profilometry measurements also may have a destructive effect on the surface eroded enamel since this tactile method applies stresses to the enamel surface [11,15]. Therefore, we hypothesized that surface profilometry measurements of eroded enamel alter the surface of the enamel, e.g. by the penetration of the profilometer tip into the softened enamel surface. To test this hypothesis, tooth samples were divided into three different groups A–C. Group A and B were eroded with different citric acid solutions (CA 1 and CA 2), and group C (control) was treated with Volvic Mineral Water (VMW) to compare the effect of citric acid with that of a non-erosive drink.

To the best knowledge of the authors, this is the first study that systematically investigates the potential altering of the eroded enamel surface by profilometry measurements.

There were no scratches found on the enamel surface of the samples in group C (Control) and on the reference areas (R) of the samples in groups A and B. On the eroded enamel surface (E) distinct scratches (marked by white arrows in Fig. 2) caused by the profilometer measurements were identified by optical microscopy, CLSM and AFM. These results demonstrate that the softened enamel surface was altered by profilometry measurements. The hypothesis that surface profilometry measurements of eroded enamel alter the surface of the enamel is therefore accepted. However, there was no statistically significant influence on the values of enamel erosion.

The profilometers, the CLSM and the AFM were calibrated with a height standard. The standard had a height of 4.2 (±0.1) μm. The AFM and the CLSM measured very exact height values. The standard deviation of the values measured by the CLSM was smaller than the standard deviation of the values measured by AFM. The FormTalysurf Series 2 measured higher values with a low standard deviation. But it is still within the tolerance of the height value given by the manufacturer of the calibration standard. The Hommeltester T 1000 measured lower values than the FormTalysurf Series 2 and higher height values than AFM and CLSM. The standard deviation is very high. Therefore, this instrument is less accurate than the other devices.

As shown in Fig. 4, the enamel loss of the samples in group A (treated with CA 1) and B (treated with CA 2) increased with increasing erosion time. This behavior was also shown previously by several other authors [7,8,29,30]. For samples from the same group, the stylus profilometer Hommeltester T 1000 measured the highest values of enamel loss, whereas the values from the FormTalysurf Series 2 did not differ statistically significantly from the values measured by CLSM (Fig. 4a and b, Table 1). The stress applied to a surface is defined by the applied force divided by the contact area. Since the Hommeltester T 1000 applies a higher force to the enamel surface than the FormTalysurf Series 2 and both profilometers have the same tip size, it is assumed that the stylus of the Hommeltester T 1000 indented the eroded enamel deeper and, therefore, measured a higher enamel material loss than the FormTalysurf Series 2. In the current study and in several other studies it was shown that the enamel loss depends on the erosion time [5,7,8]. In Fig. 4c it can be seen that the enamel loss of the tooth samples treated with Volvic Mineral Water (control group C) is not dependent on the erosion time within the period investigated. This was shown by measurements of the FormTalysurf Series 2, the Hommeltester T 1000 as well as by the measurements of the CLSM. In fact, the observed enamel material loss for this control group of samples is very small and ranges from 0.05 μm to 0.25 μm. The values are larger than the standard deviation of the calibration measurements. It is assumed that the height values shown in Fig. 4c are not real enamel loss. The tooth samples were ground and polished mechanically. Therefore, the data could be interpreted as the height of scratches, cracks, and other shape deviations of the sample surfaces, for example created during sample preparation. Furthermore, the surface of the tooth samples did not show the typical prismatic pattern which can be seen on eroded enamel surfaces (Fig. 6b) [31]. These results show that there was no statistically significant enamel erosion affecting the enamel surface of this control group.

The second aim of this study was to quantify the potential effect of such surface alteration on the measured values of enamel erosion by AFM. Table 2 shows the depth of the profilometer scratches measured by AFM. The Hommeltester T 1000 almost always produced deeper scratches than the FormTalysurf Series 2. The assumption that the Hommeltester T 1000 indents deeper into the eroded enamel surface than the FormTalysurf Series 2 (Table 2) is therefore confirmed. Fig. 7 shows that the profilometer measurements $z_{profilometer}$ tend to give slightly larger values of enamel loss than the corrected values $\Delta z_{profilometer}$. A one-way ANOVA showed that there was no statistical significance between these two results (Table 3). Therefore, scratching the surface by profilometer measurements has no statistically significant effect on the measured results of the enamel loss under the conditions of the current study. The significance of this finding is that the measurements of enamel material loss with profilometers give accurate values within the accuracy level (FormTalysurf Series 2: 14 nm, Hommeltester T 1000: 20 nm) of the instrument. It was reported in Section 2 of this paper that the Hommeltester T 1000 applies a larger testing force than the FormTalysurf Series 2. Since the stress applied on the eroded enamel surface is dependent on the force, the Hommeltester T 1000 applies higher compressive stresses and, therefore, indents deeper into the enamel surface than the FormTalysurf Series 2.

The third aim of the present study was to compare the enamel material loss as measured by confocal laser scanning microscopy (CLSM) and two different profilometers in order to assess the accuracy of these methods. CLSM is a non-tactile technology for analyzing a surface, which can be used to scan a surface area in approximately 10 min. Few previous studies have used CLSM, white light microscopes or optical profilometers to investigate dental enamel erosion. CLSM was also used to perform qualitative measurements of enamel erosion [18]. They investigated the erosive effect of oral care products and orange juice and assessed the microstructure of eroded enamel by CLSM. White light microscopy was used to measure the enamel loss of enamel erosion and abrasion [19]. The authors used a resin for embedding the tooth samples as reference area and measured the enamel loss by performing three profile line scans.

To the authors' best knowledge there are no studies where the 3D-topography in the reflection mode of the CLSM was used to measure the enamel loss quantitatively. The advantage of this method is the high resolution (less than 300 nm in x and y direction and 20 nm in z-direction) and the fast recording of the surface topography. The disadvantage of using the CLSM is that the laser may penetrate the translucent enamel leading to background noise. Nevertheless, it was possible to generate a highly detailed image of the enamel surface. Comparing the results of the CLSM with the results generated by the FormTalysurf Series 2 (Fig. 5), it can be seen that there was a good correlation between the profilometer FormTalysurf

Series 2 and the non-tactile CLSM. Profilometry is a common technology in measuring enamel loss. The good correlation of the CLSM and FormTalysurf Series 2 data ($\rho = 0.967$) demonstrates that the values measured by CLSM compare well to those measured by the FormTalysurf Series 2. This is verified by Fig. 5 which shows that the values of the CLSM and the profilometer FormTalysurf Series 2 correlate well to each other. If the values measured by instrument 1 and instrument 2 are perfectly identical, then the regression line has a slope of 45°. This is plotted as a black solid line on the graph to compare the regression lines of the results. The regression line of the scatter plot comparing the CLSM and the profilometer FormTalysurf Series 2 has a slope which is close to the 45° regression line. The coefficient of determination of the regression line (CLSM–FormTalysurf Series 2) is $R^2 = 0.9354$. The profilometers, the CLSM and the AFM were calibrated with a calibration standard. AFM and CLSM were found to generate the most accurate height measurements of the calibration standard with the lowest standard deviation. Furthermore, the data obtained by the FormTalysurf Series 2 had a small standard deviation and measured a height which is within the tolerance of the calibration standard. The Hommeltester T 1000 measured exaggerated values of the enamel material loss. It is described in the previous paragraph that this instrument measured higher values, because of its higher testing force. However, the difference between the accurate values and the data measured by the Hommeltester T 1000 was not statistically significant. The results show that the CLSM measures reliable values of enamel material loss. Therefore, it is possible to quantify the enamel loss by reflection mode confocal laser scanning microscopy.

In summary, we demonstrated that measuring enamel loss by stylus profilometry will affect the softened enamel surface. Scratches were found on the enamel surface of eroded enamel after profilometry measurements and were characterized quantitatively by AFM. The depth of the scratches depends on the erosion time and the pH value of the citric acid. There was a statistically significant difference between the values of enamel loss measured by the CLSM and those measured by the profilometer Hommeltester T 1000 but no statistically significant difference between the values measured by CLSM and those measured by the profilometer FormTalysurf Series 2. AFM measurements have shown that there are statistically significant differences between the values of the depth of the scratches caused by the Hommeltester T 1000 and those caused by the FormTalysurf Series 2. After subtracting the depth of the scratches from the values measured by profilometry and CLSM the result of the subtraction showed no statistically significant difference to the measured values. Thus, the profilometer can be used for analyzing eroded enamel with respect to the erosion time, the pH value and the applied testing force. However, care has to be taken if profilometers which apply larger forces are used, since it has been demonstrated that such profilometers penetrate the eroded enamel surface deeper than those with lower forces. A good alternative for measuring the erosion enamel loss is CLSM. 3D-imaging can be used to generate topographical images of the enamel surface and non-tactile measurements of the profile are possible. The CLSM is a very fast and exact method for analyzing enamel erosion quantitatively.

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Pectin, alginate and gum arabic polymers reduce citric acid erosion effects on human enamel

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ABSTRACT

Objectives. Consumption of acidic soft drinks may lead to the dissolution and softening of human enamel, known as erosion. The first aim of this in vitro study was to test the hypothesis that food-approved polymers added to citric acid solutions (CAS) decrease the erosion of human dental enamel compared to citric acid solutions without these polymers. The second aim was to test the hypothesis that these polymers added to CAS form a polymer layer directly on the eroded enamel surface.

Methods. Enamel samples were obtained by embedding pieces of non-erupted human third molars in resin, grinding, and polishing them. CAS with pH values (pH: 2.3, 3.3 and 4.0) of typical soft drinks were prepared and modified by adding one of the following food-approved polymers (1%, w/w): highly esterified pectin (HP), propylene glycol alginate (PGA) and gum arabic (GA). The enamel samples were exposed to these polymer-modified citric acid solutions (PMCAS) or CAS not containing polymers, respectively, for different time periods (30, 60 and 120 s). Atomic force microscopy (AFM)-based nanoindentation was used to analyze the nanomechanical properties of the treated enamel samples and the control samples. The enamel nanohardness and the reduced elastic modulus of the samples treated with PMCAS were statistically analyzed (ANOVA, *t*-test) and compared to the mechanical properties of the samples treated with unmodified CAS. Thus treated enamel samples were imaged by scanning electron microscopy (SEM) to investigate the surface morphology of the different enamel samples.

Results. Enamel samples treated with PMCAS containing GA or PGA showed statistically significantly higher nanohardness ($p < 0.05$) compared to samples treated with CAS. PMCAS containing HP did not reduce the enamel nanohardness loss significantly compared to the CAS treated enamel samples. The enamel samples eroded with PMCAS show generally a smoother surface compared to the enamel surfaces of samples treated only with CAS as detected by SEM. Therefore, it is hypothesized that the polymers possibly adsorb on the eroded enamel surface.

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The present in vitro erosion study shows that some of the polymers used in this study may possibly adsorb like a protective layer directly onto the human enamel surface. For GA and PGA this possibly formed polymer layer reduces the erosive effects of citric acid solutions as shown by nanoindentation measurements.

1. Introduction

Dental erosion has been defined as the dissolution of dental hard tissue without involvement of bacteria [1]. It is well known that acid containing food and beverages may lead to erosive damage of dental enamel [2,3]. The acids in soft drinks are the major aetiological factor of dental erosion [4]. Moreover, a wide range of extrinsic and intrinsic factors are involved in the process of dental erosion. A combination of these factors, e.g. high soft drink consumption, or eating disorders with acid regurgitation, leads to greatly enhanced tooth mineral loss [5–7].

The investigation of dental erosion has been recently of increasing interest for the dental materials science community [8–10]. Since the mid-1990s studies investigated the intensity of enamel loss and surface softening caused by the consumption of acidic soft drinks. Citric and phosphoric acid containing soft drinks were frequently investigated with respect to their erosive effect of dissolution and softening of human enamel [1,11–13]. The early stages of enamel erosion were characterized by demineralization of the enamel surface leading to softening and nanostructural changes. The subsequent stages of enamel erosion were characterized by material loss and the structural collapse of dental enamel [14,15].

To reduce or avoid erosive effects caused by acids in soft drinks, different strategies have been investigated. One strategy is to change the degree of saturation of the erosive agent with respect to hydroxyapatite (HA). Adding Ca^{2+} and PO_4^{3-} ions to the citric acid solution (CAS) changes the chemical equilibrium of these ions between the surface and the erosive agent. Therefore, a reduced enamel softening during the acid exposure was possible [16,17].

Furthermore, other compounds have been tested for their capability to reduce enamel erosion. Some beverage-modifying agents, such as citrate [18] and fluoride [19,20], have been successfully added to acidic solutions to reduce the dissolution of human enamel. The effect of proteins in the saliva which bind to the enamel surface has been investigated with respect to the reduction of erosive effects [21–23]. Moreover, the interaction of milk-proteins with human enamel has been tested [24–26]. In both cases, a reduced erosive enamel damage has been demonstrated.

Only a few studies have shown that polymers can affect the dissolution of the HA surface [27–29]. Polymers have been investigated with respect to their potential to prevent or reduce the dissolution of Ca^{2+} and PO_4^{3-} ions from the HA prisms [28,30]. Such polymers may be useful to reduce erosion or to study enamel remineralization mechanisms [31]. Barbour et al. [28] have shown on artificial HA discs that some food-approved polymers, such as xanthan gum and carboxymethylcellulose reduce the dissolution of Ca^{2+} and PO_4^{3-} ions caused by CAS. Two studies have shown in situ that xanthan gum [32] and the combination of xanthan gum with calcium or polyphosphate reduce the softening of the enamel [27,29].

To the authors' best knowledge, a potential effect of food-approved polymers on reducing the erosion of human enamel measured by nanoindentation has not been reported so far. Highly esterified pectin (HP), propylene glycol alginate (PGA) and gum arabic (GA), the polymers used in this current in vitro study, are normally used in sweets, jams, bakery products, ice cream and soft drinks. In these food systems, the polymers form gels to stabilize the food.

Therefore, the first aim of this in vitro study was to test the hypothesis that the above mentioned food-approved polymers, added to citric acid solutions (CAS), decrease the erosion of human dental enamel compared to CAS without such polymers, as measured by nanoindentation. The second aim was to test the hypothesis that these polymers, added to CAS, form a polymer layer on the enamel surface.

2. Materials and methods

2.1. Sample preparation

For this in vitro study non-erupted human third molars ($n = 87$) were used and prepared as described previously [33]. In brief, teeth were extracted and disinfected, and the roots were carefully removed. Pieces (approximately 2 mm × 3 mm) were cut from all over the tooth with a low-speed saw (Isomet, Buehler GmbH, Düsseldorf, Germany) using a water-cooled diamond blade (Buehler GmbH, Düsseldorf, Germany), and embedded in a resin (Stycast 1266, Emerson & Cuming ICI, Westerlo, Belgium). The samples were then ground with SiC paper (grit 1200–4000; Buehler GmbH, Düsseldorf, Germany) and polished with diamond powder dispersions (particle size ranging from 6 to 1 μm) to obtain smooth surfaces. Samples were stored in deionized water at room temperature until use. Immediately before treatment with the test solutions, one half of the surface of each enamel sample was covered with PVC tape (Tesa AG, Hamburg, Germany) to obtain an untreated area (UT) which was protected against solution exposure.

2.2. Test solutions

The solutions were prepared by dissolving the following polymers (1%, w/w) in deionized water: highly esterified pectin (HP), propylene glycol alginate (PGA) and gum arabic (GA). Polysaccharides were supplied by WILD GmbH & Co. KG (Eppelheim, Germany). All specifications known are given in Table 1 and their use or purity degree is regulated by the European Union directive (200/84/EG). To obtain the polymer-modified citric acid solutions (PMCAS), 50% (w/w) cit-

Table 1 – Specifications of the polymers used in this in vitro study. All polymers were supplied by WILD GmbH & Co. KG (Eppelheim, Germany) and their use and purity is regulated by the European Union directive (200/84/EG).

	Highly esterified pectin (HP)	Propylene glycol alginate (PGA)	Gum arabic (GA)
Source	Apple pectin	–	Acacia senegal
E-number	E 440	E 405	E 414
Molecular weight	Not known	10,000–600,000 g/mol	350,000 g/mol
Level of esterification	70–76%	≥75%	–
Viscosity	55 ± 5 mPa s, 2.5% solution	50–175 mPa s, 2% solution	≥60 mPa s, 25% solution
Others	Protein < 1%	–	–

ric acid was added to adjust the pH to three different values (2.30 ± 0.01, 3.30 ± 0.01 and 4.00 ± 0.01) and controlled with a pH meter (Knick pH meter 765 Calimatic, Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany). This addition of citric acid may lead to minute changes in the polymer concentration. We estimate the deviation to be small because of adding only few droplets of citric acid (i.e. final polymer concentration 1%, w/w, ±0.01%). No other substances that could influence the erosive effect of enamel were added to the solutions. Control CAS was prepared similar but without addition of polymers.

2.3. *Erosive treatment of the enamel samples*

The enamel samples were treated at room temperature with PMCAS or CAS for 30, 60, and 120 s, respectively, to simulate the normal intake times of soft drinks. For the erosion investigations three samples ($n = 3$) were used for every PMCAS or CAS. For every sample 30 ml of PMCAS or CAS was used and the solution was stirred with a magnetic stirrer. After treatment, the samples were immediately rinsed with deionized water for 30 s and dried with compressed air. The PVC tape or residues of glue from the tape were removed carefully from the untreated areas of the enamel samples with ethanol soaked cotton swab. Samples were stored in air before investigation by nanoindentation.

2.4. *Nanoindentation*

An atomic force microscope (Digital Instrument Dimension 3100, Veeco Instruments, Santa Barbara, CA, USA) equipped with a Hysitron TriboScope® nanoindenter (Hysitron Inc., Minneapolis, MN, USA) was used to measure the nanohardness and the reduced elastic modulus of the enamel samples [14,34,35]. For the indentations, the standard Berkovich diamond indenter with equilateral pyramidal area was calibrated with fused silica, and a standard tip area function was used. The indentations were made in air at room temperature in three steps: linear loading up to 5 mN from 0 to 15 s, holding this load for 5 s, and linear unloading to 0 mN within 15 s. An image of the surface was recorded before every indent to ensure that it was flat, clean, and free of damages. Indentations were made five times in both the treated area and the untreated area on every sample. The distance between the indents was kept to at least 20 μm to avoid interferences between them. Nanohardness and reduced elastic modulus were calculated with the TriboScope® software (version 3.5).

2.5. *SEM*

Scanning electron microscopy (SEM) was performed with a LEO 440i SEM Scanning Electron Microscope (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany) operated at 15 kV. For SEM all samples were gold sputter-coated (approx. 10 nm) in vacuum.

2.6. *Statistical analysis*

A multiway ANOVA and the Scheffe *t*-test (95% confidence interval, $p < 0.05$) analysis were performed with StatGraphics Centurion XV (StatPoint Technologies Inc.; Warrenton, VA, USA) to test the statistical significance of the nanohardness loss and the reduced elastic modulus of the human enamel samples. The factors investigated for significance were erosion time ($t = 30$, 60, 120 s), polymer substance (HP, PGA, GA or CAS), and pH value (pH 2.3, 3.3, 4.0). For the measurements the samples were randomly divided to the different test groups.

3. Results

Fig. 1A–C shows the nanohardnesses of enamel samples treated with different PMCAS or CAS compared to the untreated areas (UT) of enamel for pH 2.3, 3.3 and 4.0 as a function of exposure time. There is a general trend of a reduced enamel nanohardness with decreasing pH of the PMCAS and CAS, except the enamel nanohardness at pH 3.3 for $t = 30$ s. Additionally, there is also a general trend of decreasing enamel nanohardness with increasing exposure time, with the exception of the enamel nanohardness values at pH 4.0 and $t = 60$ s.

For the enamel samples treated with PMCAS containing GA, there is a statistically significantly ($p < 0.05$) higher nanohardness than for enamel samples treated with CAS for all exposure times at pH 2.3 (Fig. 1A). For pH 3.3 and a treatment time of 60 s (Fig. 1B) the enamel samples treated with PMCAS containing PGA or HP showed a statistically significantly higher nanohardness than samples treated with CAS. For pH 4.0 the PMCAS containing PGA showed a statistically significantly higher nanohardnesses for 30 and 60 s than the samples treated with CAS, and for PMCAS containing GA for 60 s, respectively (Fig. 1C).

In comparison, there were no statistically significant differences ($p > 0.05$) for the reduced elastic modulus (Fig. 2A–C) between enamel samples treated with PMCAS or CAS for all treatment times (30, 60 and 120 s) and pH values (2.3, 3.3 and 4.0).

The enamel sample surfaces were investigated by SEM (Fig. 3A–D) after treatment ($t = 120$ s) with PMCAS or CAS (both

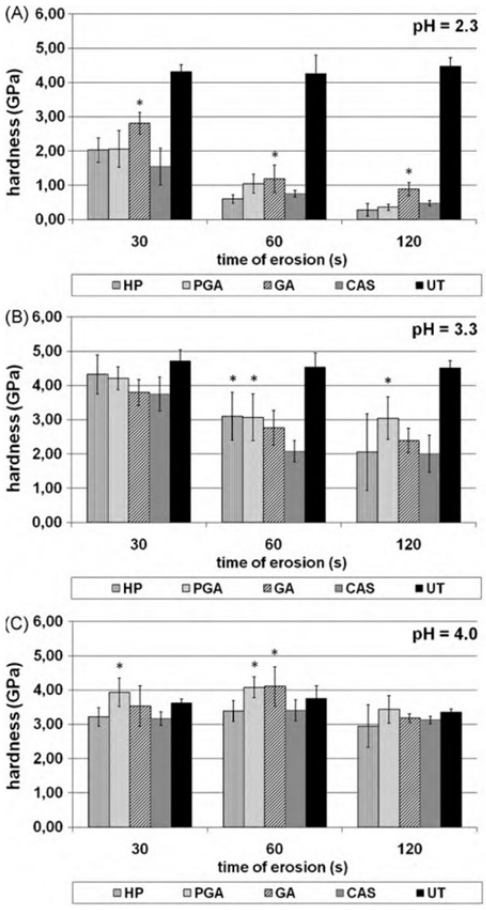

Fig. 1 – (A–C) Nanohardness of enamel. Samples were treated with different PMCAS dependent on the pH [2.3 (A); 3.3 (B) and 4.0 (C)] and the polymers [highly esterified pectin (HP), propylene glycol alginate (PGA), gum arabic (GA)]. Black bars show the nanohardness of the untreated areas (UT); grey bars show treatment with CAS without a polymer; *Denotes a statistically significant higher nanohardness of the PMCAS treated samples than the samples treated with CAS.

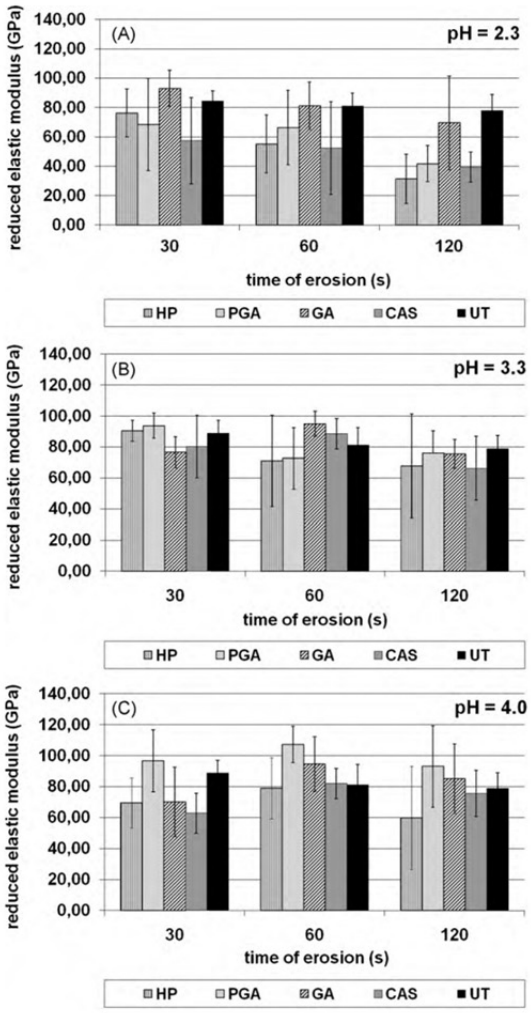

Fig. 2 – (A–C) Reduced elastic modulus of enamel treated with different PMCAS dependent on pH [2.3 (A); 3.3 (B) and 4.0 (C)] and the addition of polymers [highly esterified pectin (HP), propylene glycol alginate (PGA), gum arabic (GA)]. Black bars show the reduced elastic modulus of the untreated area (UT) of the enamel; grey bars show samples treated with CAS.

solutions pH 3.3). On all images, the left, dark area is the untreated area (UT) whereas the right, bright area shows the enamel surface treated with CAS (Fig. 3A) or PMCAS (Fig. 3B–D). Only for the samples treated with CAS (Fig. 3A) the typical eroded HA prism structure of enamel is visible on the right side of the micrograph. The PMCAS treated enamel surfaces shown in Fig. 3B–D are relatively smooth compared to the enamel surface treated with CAS. The etch patterns typical for CAS treatment on the enamel surface are not detectable after treatment with PMCAS.

4. Discussion

Dental erosion caused by acidic food, such as soft drinks has become one of the major factors of tooth mineral loss. Especially for children an increase of the clinical cases of dental erosion during the last decades has been observed [36]. To reduce the erosive effects soft drinks have on dental enamel, different strategies have been employed so far. These include the change of the degree of saturation with respect to hydroxyapatite [16,17], beverage-modifying agents, such as

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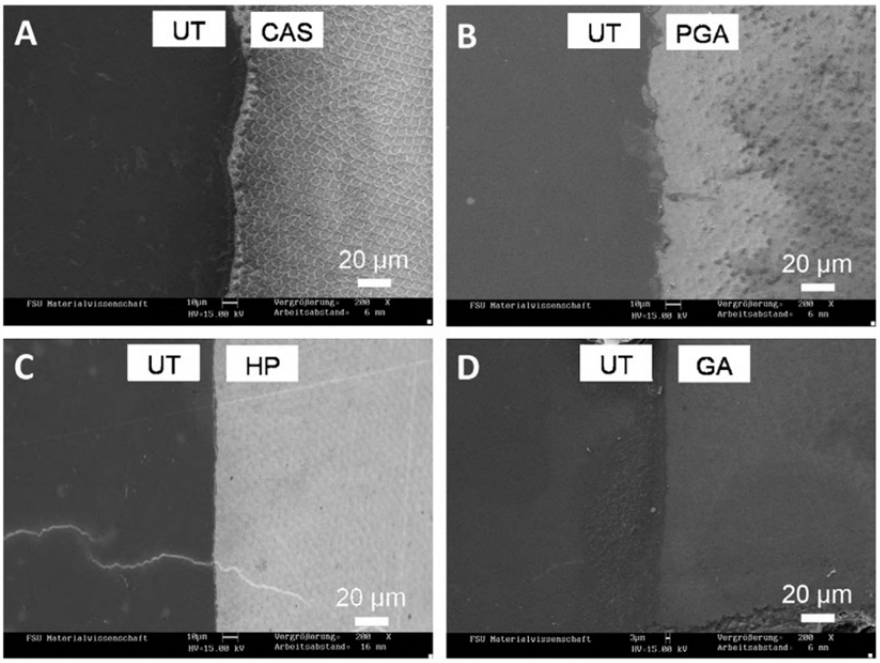

Fig. 3 – (A–D) SEM images of human enamel surfaces treated (120 s, pH 3.3) with CAS (A) and PMCAS containing PGA (B), HP (C), GA (D); UT: untreated area.

citrate [18] and fluoride [19,20], saliva proteins [21–23] and milk-proteins [24–26].

As described previously, only a few studies have investigated the effect of polymers in citric acid solutions against erosion so far [27–29,32]. These studies used profilometry [27,29] to measure the material loss of enamel caused by polymer-modified citric acid solutions. The results of these studies show reduced erosion effects of the PMCAS for the different polymers (xanthan gum, carboxymethylcellulose) used compared to unmodified CAS. Nevertheless, the experimental parameters of these previous studies, such as long exposure times up to 30 min [27–29], profilometry measurement [27,29] and artificial HA discs [28] differ from the parameters used in the current study.

To the best knowledge of the authors, there are no studies reported so far using nanoindentation to quantify the effect of PMCAS on erosion with in vitro enamel samples. This method is suitable to detect the early softening of human enamel caused by erosion [2]. Therefore, the current in vitro study determines firstly the effect of polymers (PGA, GA and HP) in PMCAS on erosion using short-time treatment (up to 120 s). To our best knowledge, the present study is the first using highly esterified pectin (HP) and propylene glycol alginate (PGA), as erosion inhibitors.

The nanomechanical properties of eroded human enamel are often characterized by nanohardness and reduced elastic modulus [14,34]. For PMCAS containing PGA or GA, tested in this current study, generally a higher nanohardness of human enamel samples was observed compared to samples treated with CAS (Fig. 1A–C). For GA, our results differ in some respect from the results reported by Barbour et al. [28]. These authors demonstrated that there is no effect on the dissolution of artificial HA discs when exposed to PMCAS containing GA (pH 3.2; 0.02% polymer). In the present study, with shorter treatment times, a statistically significantly lower nanohardness loss was observed for enamel treated with PMCAS containing GA compared to samples treated with CAS. Our nanohardness data indicate that using food-approved polymers GA and PGA in PMCAS leads generally to a higher nanohardness of enamel compared to enamel treated with CAS (Fig. 1A and B). The variation of the mean nanohardness might be based on the biological variance of human third molars.

The PMCASs investigated in the current study show different capacities to reduce the citric acid induced enamel erosion depending on pH and exposure time. Generally, it is accepted that longer erosion times and lower pH values lead to more pronounced softening effects on enamel [14,37] which has been confirmed in the current study. For a pH of 4.0, there are no statistically significant differences of the enamel nanohardness between the PMCAS treated areas and the untreated areas. This means that no erosive effects, i.e. enamel softening, were detectable under this condition. For a common pH value of soft drinks (pH 3.3), after 60 and 120 s treatment with PMCAS containing PGA a lower nanohardness loss was observed than for samples treated with CAS. In comparison, for pH 2.3 PMCAS containing GA showed a lower nanohardness loss than PMCAS containing PGA.

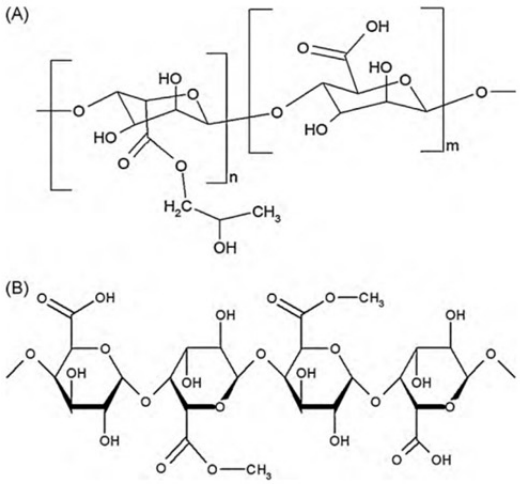

Fig. 4 – (A and B) Chemical structure (drawn with ISIS Draw 2.3) of propylene glycol alginate (A) and esterified pectin (B).

The observed pH dependent protection effect against erosion of PMCAS containing PGA can be explained by neutralization of charged functional groups (carboxyl groups) in PGA (Fig. 4A) with the changing pH value. This can be explained as follows. It is known that neutral and anionic polymers adsorb on HA surfaces and prevent the dissolution process of artificial HA [38,39]. The polymers used in the current study may show similar properties since they contain anionic functional groups as well. For PGA (Fig. 4A: copolymer with homopolymeric blocks of (1,4)-linked-β-D-mannuronate and α-L-guluronate) and HP (Fig. 4B: mostly α-1,4-linked-D-galacturonic acid) it seems to be possible that their anionic groups (carboxyl groups) are able to interact with the Ca^{2+} cations at the enamel surface [40] and form ionic bonds with them. In the presence of bivalent positively charged ions, e.g. Ca^{2+}, PGA and HP polymers start to gelatinize and a chelate complex is formed [41]. During this interaction, the polymer builds a gel-like matrix. At a low pH value (pH 2.3), the structure of the PGA polymer is more influenced by the H^+-ions in citric acid, and its carboxyl groups are not negatively charged any longer. The interaction between Ca^{2+} ions and the polymer for gel formation stops under these conditions. The gelatinizing effect seems, therefore, to be not possible or at least disturbed. Therefore, a stronger softening of enamel is expected for pH 2.3 than for pH 3.3 using PMCAS containing PGA. This is what we observed in this current nanoindentation study. Contrary to the positive erosion reducing effects of PGA, the shielding effect of HP is only detectable for short treatment times of 30 s but with a similar chelate building mechanism [40]. Compared to pH 2.3 the neutralization is not predominant for pH 3.3. We hypothesize that near the enamel surface the concentration of positively charged free Ca^{2+} ions increases during erosive treatments due to the dissolution process [17,42]. Therefore, based on these positively charged Ca^{2+} ions, the gel-forming process for pH 3.3 may start at the enamel surface immediately when the enamel is exposed to PMCAS containing PGA or HP. The proposed interactions of such polymers with the enamel surface and between polymer molecules are graphically depicted in Fig. 5.

GA consists of four different structural units (L-arabinose, D-galactose, L-rhamnose und D-glucuronic acid). To our best knowledge, such a gel-building process is not known for GA polymers. From literature it is known that GA adsorb on HA nanoparticles [43]. The interaction of this polymer with the HA nanoparticles is described by electrostatic and hydrogen-bond interactions with positively charged surface sites [44]. We hypothesize that a similar binding effect of GA exists depending on positively charged ions, e.g. Ca^{2+} present at the

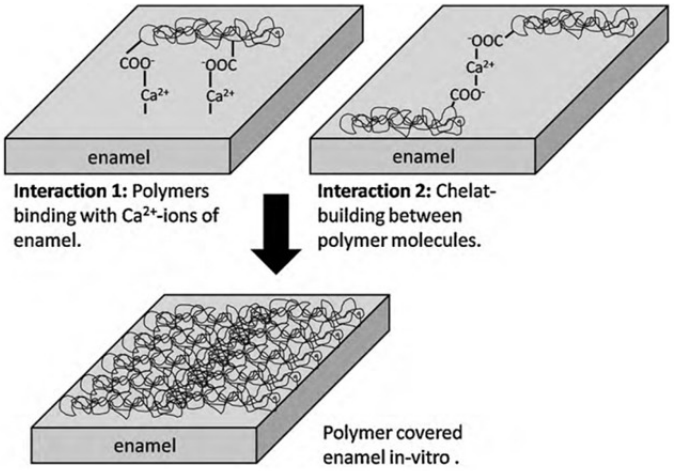

Fig. 5 – Model of erosion inhibiting polymers in citric acid solutions (PMCAS). Two interactions are hypothesized: interaction (1) between negatively charged carboxyl groups of polymers and Ca^{2+}-ions of the enamel surface. Interaction (2) between negatively charged carboxyl groups of different polymer molecules in presence of positively charged Ca^{2+}-ions by forming a chelate complex.

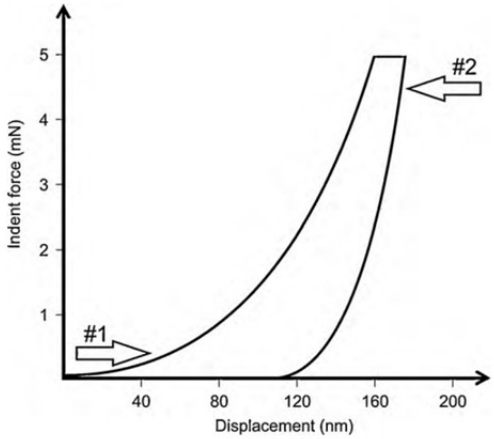

Fig. 6 – Force–displacement-curve of a typical indent with a Berkovich indenter.

enamel surface. Furthermore, the hydrogen-bond interaction between polymer molecules leads to a multilayer formation with a potential stronger effect to reduce enamel erosion (Fig. 1). A higher nanohardness was observed for enamel samples treated with PMCAS containing GA than for samples treated with CAS. Perhaps the effect of reducing the enamel nanohardness loss of PMCAS containing GA is caused also by a viscosity effect of the polymer solutions, which is discussed below, rather than by the formation of a protective layer. There may also be a combination of mechanisms, layer formation and increasing viscosity of the solution.

To measure the nanohardness of the enamel and not the nanohardness of a soft polymer layer adsorbed on the enamel, two indentation forces of 3 and 5 mN were used in this study. Using these forces it is assured that the Berkovich indenter easily penetrates the thin polymer layer on the enamel. Identical results were obtained for both indentation forces. Moreover, a polymer layer would potentially affect the initial part of the force–displacement–curve shown in Fig. 6 (arrow #1) when lower indentation forces were used [45,46]. The nanohardness of the enamel, however, is determined from the initial part of the force–displacement–curve when the indenter tip retracts from the sample Fig. 6 (arrow #2). We are convinced that our measurements represent the nanohardness of the enamel and are not affected significantly by the presence of thin polymer layers on the enamel surface.

The SEM micrograph shown in Fig. 3A shows the typical prism patterns of enamel eroded with CAS. This typical prism pattern is observed when enamel is exposed to citric acid [47,48]. After treatment with PMCAS (Fig. 3B–D), the typical eroded structure (prism patterns) of the enamel (Fig. 3A) is not visible for samples treated with PGA, HP and GA containing PMCAS at pH 3.3 for 120 s treatment time. All samples were rinsed with deionized water after treatment, however, the polymer layers are visible on the surfaces. Nevertheless, it is clear from Fig. 1A–C that for samples treated with PMCAS enamel erosion (softening) occurs. Polymer layers adsorbed on the enamel surface may explain the absence of the typical prism pattern on the PMCAS treated samples. In fact, in Fig. 3B a weak prism pattern can be seen on an PGA containing PMCAS treated sample. This pattern appears weakened potentially by an overlaying adsorbed polymer layer. For samples treated with GA or HP containing PMCAS the adsorbed polymer layer seems to cover the prism pattern completely. A similar effect of adsorbed xanthan gum on enamel was demonstrated previously using tapping mode AFM investigations [30]. These polymer films may reduce enamel erosion probably by a shielding effect as evident from a higher nanohardness of most of the PMCAS treated samples compared to samples treated with CAS.

An alternative explanation for the absence of the prism pattern for PMCAS treated samples could be the reduced erosion potential of these polymer containing solutions. The onset of the prism pattern formation may be delayed or perhaps reduced in this case. Therefore, no evidence of the prism pattern would be present which is what we observed in SEM pictures. Future studies should clarify if the absence of this prism pattern of PMCAS treated samples is caused by the adsorbed polymer layer or just an effect of the reduced erosion potential of polymers.

Another mechanism to explain the effect of polymers to reduce erosion is the increase of viscosity of PMCAS compared to CAS. A higher viscosity of a solution leads to a reduced ion mobility in the solution and slower dissolution kinetics. It is known that the polymers used in this current study increase the viscosity of the test solutions [40,49]. The increase in viscosity of the PMCAS compared to CAS may lead to slower dissolution kinetic of ions out of the enamel surface and therefore less softening. This effect has been shown previously on HA discs with GA modified PMCAS [28]. It is logical to hypothesize that a similar mechanism applies also for PGA and HP modified PMCAS which were used in the current study for the first time as erosion inhibitors.

The erosion reduction mechanism proposed in this study can be described as a self-formation of a gel-like polymer layer on enamel surfaces. This mechanism could potentially be used as a new way to protect human enamel against erosion caused by citric acid after short-time treatment. The mechanism to form an erosion protective layer of PMCAS containing PGA, HP and GA directly on the enamel surface has not been reported up to now.

In summary, we demonstrated that highly esterified pectin, propylene glycol alginate and gum arabic modified citric acid solutions show a reduced enamel erosion compared to pure citric acid solutions with similar pHs. For the first time this current study shows the reduction of erosion of CAS by adding HP, PGA and GA as protective substances, as measured with AFM-based nanoindentation after short-time treatment. A polymer-triggered “shielding effect” by forming a protective layer on enamel gives the opportunity to develop new food applications against human dental erosion. Moreover, our results identify different suitable polymers for reduced enamel erosion dependent on the given pH value of soft drinks or other food. The reduced enamel erosion may be caused by polymer layers. In future a potential formation of polymer layers on enamel to reduce erosion needs to be studied in in situ experiments and using native and unpolished enamel.

5. Conclusion

This study shows that PGA, GA and in one case HP can significantly reduce nanohardness reduction of human enamel caused by citric acid. It was shown for the first time, measured with AFM-based nanoindentation, that PGA, HP and GA polymers protect the human enamel against citric acid solutions. The protection is mainly based on a "shielding" effect of the polymers, which is self-formed directly on the human enamel surface during treatment with PMCAS. Possibly, the modification of acidic soft drinks with such polymers may reduce the erosive effect significantly. Further research is necessary to characterize the polymer layer formed on the enamel surface.

Acknowledgments

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MANUSCRIPT III (IN PREPARATION)

Morphology and structure of polymer layers protecting dental enamel against erosion

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Morphology and structure of polymer layers protecting dental enamel against erosion

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Abstract

Objectives Human dental erosion caused by acids is one major factor for tooth decay. Adding polymers to acidic soft drinks is one of the most important approaches to reduce human dental erosion caused by acids. The aim of this study was to investigate the thickness and the structure of polymer layers adsorbed in vitro on human dental enamel from polymer modified citric acid solutions.

Methods The polymers propylene glycol alginate (PGA), highly-esterified pectin (HP) and gum arabic (GA) were used to prepare polymer modified citric acids solutions (PMCAS, pH 3.3). With these PMCAS enamel samples were treated for 30, 60 and 120 s to obtain polymer layers on the enamel surface. Profilometer scratches on the enamel surface were used to estimate the thickness of the polymer layers via atomic force microscopy (AFM). In addition the polymer-enamel interaction was investigated with zeta-potential measurements and scanning electron microscopy (SEM).

Results It could be shown, that the profilometer scratch depth on the enamel with deposited polymers showed a depth in the range of 10 nm (30 s treatment time) up to 25 nm (120 s treatment time). Contrary to this the scratch depth of the CAS treated surface showed a range of nearly 30 nm (30 s treatment time) up to 60 nm (120 s treatment time). Based on scanning electron microscopy (SEM) micrographs, zeta-potential and XPS measurements a model was hypothesised. The deposited layer is composed of an opposing gradients of polymer molecules and HA particles.

Significance A layer of two opposing gradients of polymers and HA particles released from the enamel surface developed. The polymers seem to interact with the dissolved HA particles and build up the polymer-HA particle layer that protects the underlying enamel surface against the citric acid caused dental enamel erosion.

Introduction

Dental enamel is one of the hardest materials of the human body [1]. It is well known that this tissue can be destroyed by acids such as present in certain food. This process without the involvement of bacteria is known as erosion [2]. Erosion leads to the dissolution of the human dental enamel triggered by acid containing food especially soft drinks. The process of dental erosion is influenced by a number of extrinsic and intrinsic factors, like properties of the soft drinks, biological factors or socio-economic status of the consumer [3]. An important approach to reduce erosion is to modify the erosive food and thus to reduce the erosive effects against human dental enamel.

The process of enamel dissolution caused by acids was investigated in several studies. The surface softening and the enamel loss are most often caused by the consumption of acidic soft drinks containing citric and/or phosphoric acid [2,4-6]. The enamel surfaces can be characterised by profilometry [7,8] to measure the loss of dental enamel dependent on e.g. pH value, acid concentration and time. A relatively new advanced method is to use atomic force microscopy based nanoindentation [9,10] to characterise the nano mechanical properties of the enamel surface. Using this method it is possible to determine the erosive effects of acids against human dental enamel after short time (early stage) erosion [9-13].

There are some mechanisms known which can influence and decrease the dissolving process of human dental enamel caused by the acids. The degree of saturation with respect to hydroxyapatite (HA) of the erosive solution can be changed, the main component of the dental hard tissue. Adding Ca^{2+} and PO_4^{3-} ions to the citric acid solution changes the chemical equilibrium of these ions between the enamel surface and the erosive agent and leads to a reduced enamel softening [13,14]. Also the addition of different compounds such as citrate [15] and fluoride [16,17] as additives to citric acid solutions has been successfully tested for their capability to reduce dental enamel erosion.

Another complementary approach to reduce dental erosion is to modify the soft drinks with food-approved polymers. To date only few studies investigated the erosion reducing effect of polymers added to acidic solutions [18-20]. In our previous study we showed, that the treatment of human dental enamel samples with polymer modified citric acid solutions led to a reduced softening of the enamel surfaces compared to samples treated with unmodified citric acid solutions [21]. In addition, we presented a qualitative evidence that the polymers may built up a layer on the enamel surfaces. The investigated polymers propylene glycol alginate (PGA), highly-esterified pectin (HP) and gum arabic (GA) were highly useful to protect the dental enamel against citric acid induced dental erosion. Nevertheless, until now it is not completely known how the morphology of the polymer layers looks like and in which way these polymers interact with the HA from the enamel surface. Therefore, the aims of the current study were to build up polymer layers on human dental enamel depending on pH value and treatment time and to characterise the morphology and structure of these polymer layers on the enamel surfaces. This study shows, what happens on the enamel surface during the consumption of polymer modified citric acid solutions and a model of the thickness and structure of the protective polymer layers is given.

Materials and Methods

Sample preparation

For this in vitro study non-erupted human third molars (n = 12) were used and prepared as described previously [8]. The teeth were extracted and disinfected, and the roots were carefully removed. Pieces (approx. 2 mm×3 mm) were cut from all over the tooth with a low-speed saw (Isomet, Buehler GmbH, Düsseldorf, Germany) using a water-cooled diamond blade (Buehler GmbH, Düsseldorf, Germany), and embedded in a resin (Stycast 1266, Emerson & Cuming ICI, Westerlo, Belgium). The samples were then ground with SiC paper (grit 1200–4000; Buehler GmbH, Düsseldorf, Germany) and polished with diamond powder dispersions (particle size ranging from 6 to 1 µm) to obtain smooth surfaces. Immediately before treatment with the test solutions, one

half of the surface of each enamel sample was covered with PVC tape (Tesa AG, Hamburg, Germany) to obtain an untreated control area (UT), which was protected against acid solution exposure.

Test solutions and layer building

The erosive solutions were prepared as described previously [21]. Three different polymer solutions were prepared containing propylene glycol alginate (PGA), highly-esterified pectin (HP) and gum arabic (GA) (1% wt/wt), respectively. To obtain the polymer modified citric acid solutions (PMCAS) the pH of the polymer solutions was adjusted with citric acid (Carl Roth GmbH & Co KG, Karlsruhe, Germany) to 3.3 using a pH meter (Knick pH meter 765 Calimatic, Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany). As control a non-polymer modified citric acid solution (CAS) with the same pH was used. For erosive treatment the enamel samples were placed in a glass beaker with approx. 50 mL of each acid solution under constant stirring. For each solution 3 samples were used and treated for 30, 60 and 120 s, respectively. The erosive treatment was stopped by rinsing the enamel samples with deionized water for 20 s. Then the samples were dried with compressed air and the PVC-tape was removed carefully.

Profilometry and atomic force microscopy (AFM)

Directly after the PMCAS or CAS treatment of the enamel samples a profilometer (Taylor Hobson FormTalysurf Series 2, Taylor Hobson, Leicester, England) was used to make scratches in the polymer layers on the enamel and subsequently the scratch depths were measured with AFM. The scratches (line scans) with a length of approximately 4 mm were created in direction from the reference surface area to the eroded surface area. The cone shaped diamond tip had a tip radius of 2.00 µm. The testing force applied to the enamel surface was 0.87mN [7].

Topographic images (picture size 25 x 25 µm) of the profilometer scratches were recorded with an atomic force microscope (AFM, Digital Instruments Dimension 3100, Veeco Instruments, Santa Barbara, CA, USA) operating in tapping mode and using silicon cantilevers (OMCL AC160TS-W, Olympus, Tokyo, Japan). The spring constant of the cantilevers ranged between 20 M/m and 100 M/m and their length was 125 µm with a resonance frequency of 200–300 kHz. The AFM was used to measure the depth of the surface scratches caused by the profilometer [7]. It was necessary to capture the AFM images in tapping mode to avoid damaging the softened enamel or the polymer layer. Raw AFM images were subjected to first order flatten prior to further analysis. Image processing was performed with Gwyddion 2.21 free SPM data analysis software (http://gwyddion.net/). With the software (negative U-profile over the scratch) 9 line profiles every 2.5 µm were used to determine the scratch depth.

Zeta-potential measurements

To characterise the surface charge (zeta-potential) of the polymer molecules and the HA particles (Merck KGaK, Darmstadt, Germany) a Zetasizer Nano-ZS (Malvern Instruments GmbH, Herrenberg, Germany) was used. The zeta-potential was measured in a pH range from 2 to 11.

Scanning electron microscopy (SEM)

SEM was performed with a LEO 440i SEM Scanning Electron Microscope (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany) operated at 15 kV, working distance 6-8 mm. For these investigations the polymer solutions were prepared with 1 % (wt/wt) polymer and 1 % (wt/wt) HA particles (Merck KGaK, Darmstadt, Germany; also used for SEM measurements) in deionized water. The pH was adjusted with citric acid to pH 3.3 and the solutions were freeze dried for 48 h. For SEM imaging samples were gold sputter-coated with approximately 10 nm (Edwards High Vacuum International, Crawley, West Sussex, UK) in vacuum.

X-ray photoelectron spectroscopy (XPS)

To investigate the atomic composition of the layer deposited on the enamel surfaces the energy of the binding electrons was determined by angle dependent X-ray photoelectron spectroscopy with a Quantum 2000 (PHI Co., Chanhassen, MN, USA) and a focused monochromatic Al Kα source (1486.7 eV) for excitation. The pass energy was 23.5 eV and the reference was the C1s peak of the C–C bond. The signal at 346.2-349.7 eV represents the Ca electrons of the 2p orbital. With increasing the angle of the focused X-ray beam from 0° (horizontal to the enamel sample surface) to 90° (perpendicular to the enamel sample surface) the penetration depth of the X-ray beam increases. This gave the opportunity to measure the atomic composition of the polymer layers depending on the penetration depth of the focused X-ray beam.

Results

It is known that profilometer measurements can cause scratches on eroded enamel surfaces [7]. A typical light microscopy micrograph of a profilometer-caused scratch on an eroded polymer-coated dental enamel surface obtained in our study is shown in **Figure 1**. All samples showed scratches caused by the profilometer. The scratches were investigated with AFM measurements (**Figure 2**) to determine the scratch depth. Enamel samples treated with PMCAS showed smooth and less eroded surfaces (**Figure 2**) compared to samples treated with pure CAS without polymer addition where strong erosion effects were recorded with AFM (**Figure 2**).

The profilometer scratches on each samples imaged with AFM were analysed with 9 line profiles and a mean scratch depth was calculated (**Figure 3**). With increasing erosion time the scratch depth increased for all samples. There were no statistically significant differences ($p > 0.05$) between the profilometer scratches depths of all PMCAS treated samples after 30 s treatment time. Generally, samples treated with GA containing PMCAS showed the lowest scratch depths depending on erosion treatment times (7.7 ± 2.7 nm, 10.2 ± 2.4 nm, 12.8 ± 1.3 nm). For samples treated with PGA

containing PMCAS the scratch depth increased for 120 s treatment time (21.0 ± 3.1 nm) compared to 30 s (8.4 ± 2.2 nm) and 60 s (11.1 ± 3.4 nm). The scratch depth of the samples treated with the unmodified CAS were almost the highest and increased with increasing treatment time (28.9 ± 9.7 nm, 38.9 ± 7.1 nm, 60.2 ± 9.8 nm). In addition, the roughness (Ra and Rms) of the surfaces was calculated and is given in **Table 1**.

To characterise the charge of the polymer molecules in citric acid solutions the zeta-potential was measured (**Figure 4**) in a pH range from 2 to 11. With increasing pH-value the zeta-potential of all PMCAS decreased. For HP the zeta-potential was the most negative and decreased from pH 2 (-10 mV) to pH 5 (-35 mV), after that it remained stable. For GA the zeta-potential was less negative and decreased from pH 2 (-2 mV) to pH 7 (-23 mV). Until pH 11 no further decrease occurred. The zeta-potential of PGA decreased over the whole measured range from pH 2 (-2 mV) to pH 11 (-25 mV). In the range of 5 to 8 it showed a stable zeta potential of around -11 mV. For the HA particles the zeta-potential also decreased over the whole pH range from pH 2 (+1 mV) to pH 11 (-20 mV). At pH 3.3 the polymer molecules of HP (-25 mV) and GA (-15 mV) were more negatively charged than the HA particles, whereas the polymer molecules of PGA and the HA particles were similar charged (-6 mV).

Freeze dried polymer solutions were characterised using SEM (**Figure 5**). The micrographs of the polymers without HA particles showed for all samples smooth polymer matrixes with holes, which apparently appeared during the freeze drying process. When the polymer solutions were modified with the HA particles the morphology of the freeze dried polymer solutions changed. In the PGA+HA sample the HA particles seemed to be incorporated in the polymer matrix. In the HP+HA sample some HA particles were also incorporated in the polymer matrix but most of them seemed to be only attached to the surface. Whereas in the GA+HA polymer solution most of the HA particles were not incorporated in the polymer matrix. Only particles in nanometre scale were found within the polymer matrix.

XPS data will be included here

Discussion

Since a few years it is known that polymers can act as protective agents against dental erosion [18-20]. In a previous study we have shown that polymers added to citric acid solutions lead to less enamel softening compared to citric acid solutions without polymer addition. A polymer layer deposits on the dental enamel surface and protects it against erosion induced enamel softening [21]. Until now it remains unclear in which way the interaction between the polymers and the enamel surface is caused. It may be forced by a different charge between the enamel surface and the polymer molecules or by the formation of a chelate complex of the polymer molecules. In the current study we investigated the thickness and structure of the polymer layers, when they adsorb from polymer modified acid solutions (PMCAS) to in vitro enamel surfaces.

With AFM images (**Figure 2**) of the PMCAS treated enamel samples it could be shown that the surfaces are very smooth and less rough (**Table 1**) compared to the CAS treated samples. This may indicates that a polymer layer was deposited on the in vitro dental enamel. This finding were consistent to our previous study, where we showed with SEM images that due to the overlaying polymer layer the etch morphology was not visible and that the enamel surfaces were very smooth [21], whereas CAS treated enamel surfaces showed strong eroded and very rough surfaces (**Figure 2**). The typical etch prism morphology known from literature [22,23] occurred in the current study only for CAS treated enamel surfaces. In comparison, samples treated with PMCAS showed less eroded surfaces with smoother surfaces.

Using the profilometer a scratch was made into the surface of the treated samples as known from literature [7]. Using the AFM image analysis software the scratch depth was calculated for the PMCAS- and CAS-treated samples (**Figure 3**). The scratches of PMCAS treated enamel samples were less deep compared to that of the CAS treated enamel samples. Thus, the addition of polymers to citric acid solutions caused less

erosion due to less deep scratches (approximately 10 to 20 nm) than the non-modified citric acid solutions with deeper scratches (approximately 30 to 60 nm). The polymers may build up a protective layer onto the dental enamel surface, which led to less enamel softening and thus to a less deep scratch. The scratch depth not represent the polymer layer thickness, rather it seems to represent the thickness of the deposited polymer and the softened and destroyed enamel together. It has to be considered that the profilometer tip scratches over the soft surface of the treated enamel samples. This surface consists of polymer molecules on the one hand and softened enamel and dissolved enamel particles, e.g. HA crystals [24], on the other hand. When material will be removed by the profilometer tip there should be raised material right and left-hand of the scratch. But, the enamel seems to be compressed and not removed by the tip, as the AFM micrographs showed no raised material next to the scratches (**Figure 2**). At the bottom of the scratches the prism edge was visible. The prism plain was eroded and thus collapsed when the profilometer force of the tip was applied. Therefore the measured scratch depth did not only represent the polymer thickness, rather it includes both, the eroded enamel surface and the polymer layer. Despite the additional polymer layer on the PMCAS-treated samples the scratch depth of these samples was less deep than that of the CAS-treated samples.

A model was developed about the morphology and structure of the enamel/polymer layer (**Figure 4**) based on previous findings of Hannig and Hannig (Referenz). They observed in their study, that during the process of erosion HA particles were dissolved out of the enamel surface, not only Ca^{2+} ions as stated in previous studies. We hypothesize, that the soft upper layers of the PMCAS-treated enamel surfaces consists of three parts. The lowest part is an HA rich region, whereas the upper part is rich in polymer molecules. An intermediate region connects the lower and the upper part. This region consists of both, polymer molecules and HA particles. These three regions are composed as two opposing gradients of polymer molecules and HA particles. From the underlying enamel surface to the outer parts the amount of polymers increase,

whereas the amount of HA particles decrease. Because of this gradient structure no clear separation of the polymer layer and the enamel surface is possible.

With increasing treatment time, thus with an increasing erosion, more HA particles are released from the enamel surface and diffuse into the polymer layer, whereas the polymer molecules can penetrate into the more rough and eroded enamel surface. According to our model two opposing gradients of HA particles and polymer molecules occur.

XPS data will be included here.

To find out, if the citric acid dissolved HA particles interact with the polymer matrix the zeta potential of the PMCAS (charge of the polymer molecules) and of the HA particles (charge of the HA particles) was measured depending on the pH value of the solutions (**Figure 5**). Our results indicated that the interaction seemed not to be caused by a different charge between the polymer molecules and the HA surface. For the pH value relevant for our study (pH 3.3) all investigated polymer solutions as well as the HA particles showed more or less negative zeta potentials (-5 mV to -25 mV). Therefore we assume that a charge caused interaction between the HA surface and the polymer molecules is at most minimal. Thus the adsorption of the polymers to the enamel surface seems to be caused by the formation of a chelate complex as assumed before [21].

Our suggestion of the interaction between the polymers and the HA particles and the enamel surface, respectively, was also supported by SEM images (**Figure 6**). For HP and PGA it was clearly shown that the HA particles are incorporated in the polymer matrix. There seemed to be a strong chemical interaction between the HA particles (Ca^{2+} ions) and the carboxyl groups of the HP and PGA polymer molecules. In case of the GA polymer solution only very small HA particles seemed to be incorporated in the polymer matrix. These results are supported by our previous study, where HP and PGA

showed strong erosion inhibiting effects, whereas GA had only little erosion inhibiting effects at pH 3.3 [21]. We assume that the interaction between the HA particles and the polymer solutions as shown by the SEM micrographs can be also expected in a similar way for the interaction between the PMCAS and the in vitro enamel surface. HA particles are dissolved from the enamel surface by the citric acid [24]. The HA particles dissolved from the enamel surface behave similar to the HA particles added to the polymer solutions. The developed layer consisting of the polymers and the dissolved HA particles (**Figure 4**) act as a protective barrier against the erosive effect of the citric acid solution. Thus the process of erosion can be reduced by adding polymers to the acid solution what was shown before [21].

Summary

The different methods used in our study to describe and characterize the polymer layers developed onto the dental enamel surfaces suggest that there is no clear separation between the polymer layer and the enamel surface. Moreover, according to our model two opposing gradients of polymer molecules and HA particle released from the enamel surface during erosion occur. This polymer-HA particle layer act as a protective layer against the acids and thus reduce the dental enamel erosion.

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Table 1: Roughness values (Ra = average roughness and Rms = Root mean square) of the PMCAS and CAS treated enamel surfaces.

	Treatment time	**CAS**	**PGA**	**HP**	**GA**
Ra	30 s	6.6 (0.5)	1.9 (0.1)	2.1 (0.0)	1.7 (0.1)
	60 s	7.6 (0.8)	1.4 (0.1)	1.9 (0.0)	1.9 (0.1)
	120 s	52.1 (4.2)	1.9 (0.1)	2.0 (0.3)	2.3 (0.2)
Rms	30 s	9.0 (0.8)	2.8 (0.2)	3.5 (0.0)	2.8 (0.6)
	60 s	11.4 (0.8)	2.1 (0.1)	3.7 (0.1)	2.6 (0.2)
	120 s	68.4 (6.1)	3.2 (0.8)	2.7 (0.4)	3.1 (0.2)

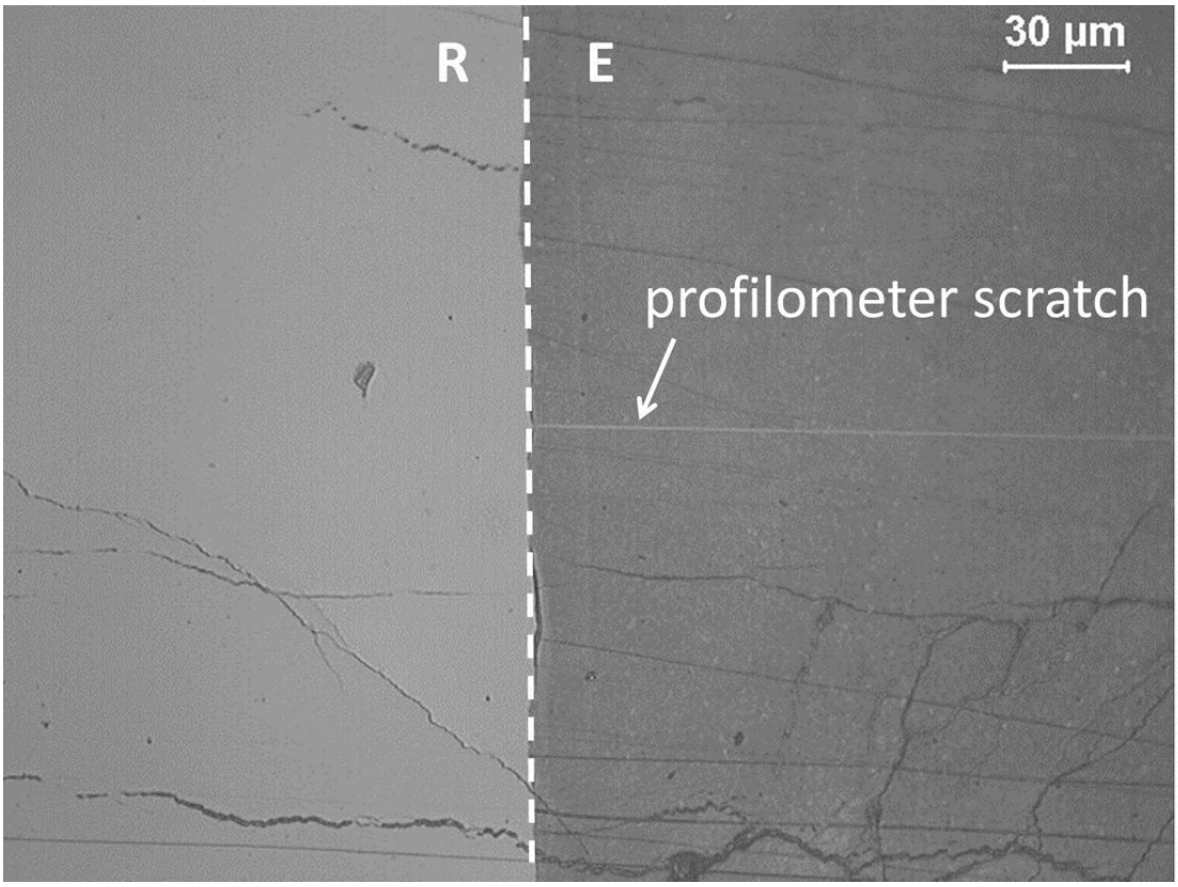

Figure 1: Light microscopy image of a typical profilometer scratch (R = reference, untreated area; E = eroded, treated area).

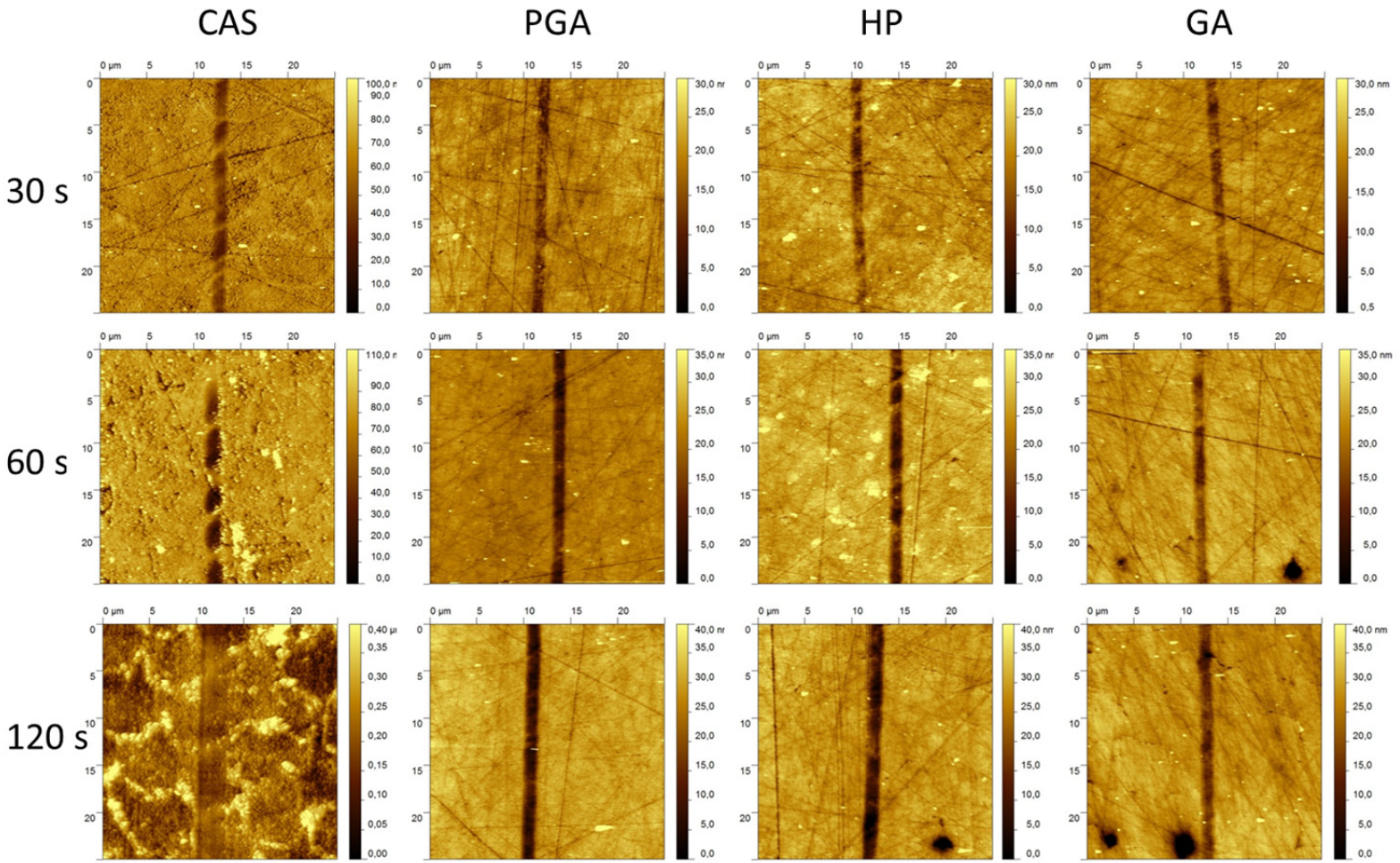

Figure 2: AFM height images (tapping mode, 25×25 µm) of the profilometer scratches on enamel samples dependent on the treatment time /30,60 and 120 sec) and the erosive treatment with citric acid solution (CAS) or the polymer modified citric acid solutions PMCAS containing PGA, HP or GA.

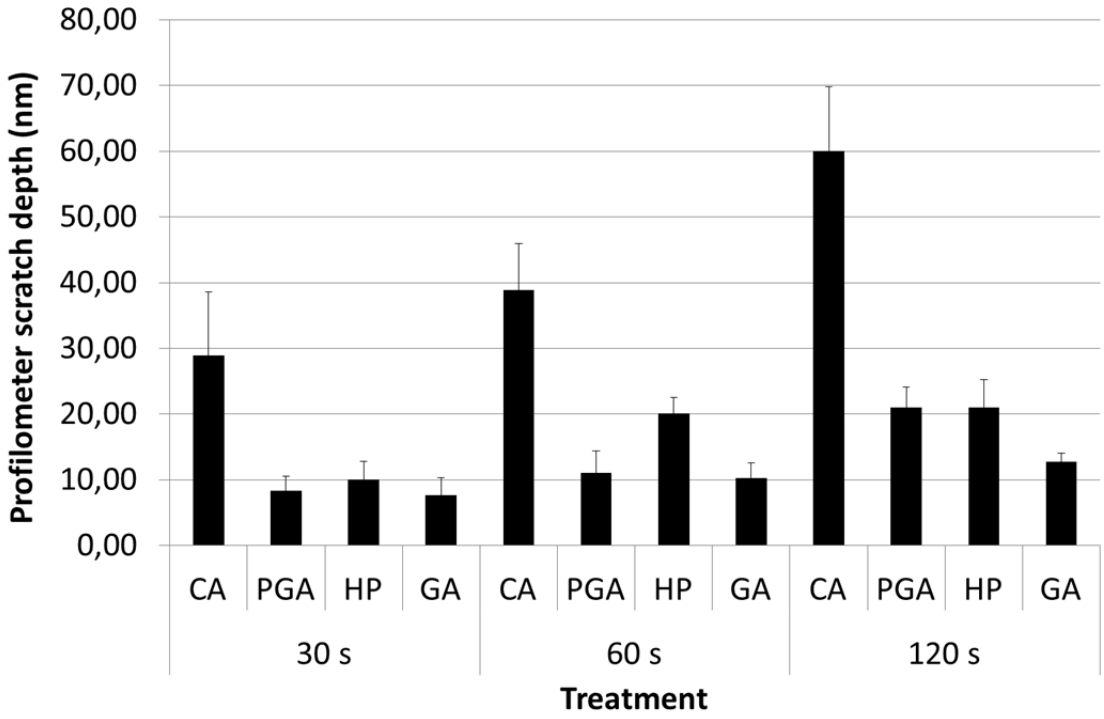

Figure 3: Depth (Δz) of profilometer-caused scratches measured with AFM of the CAS treated and PMCAS containing PGA, HP or GA treated samples dependent on treatment time.

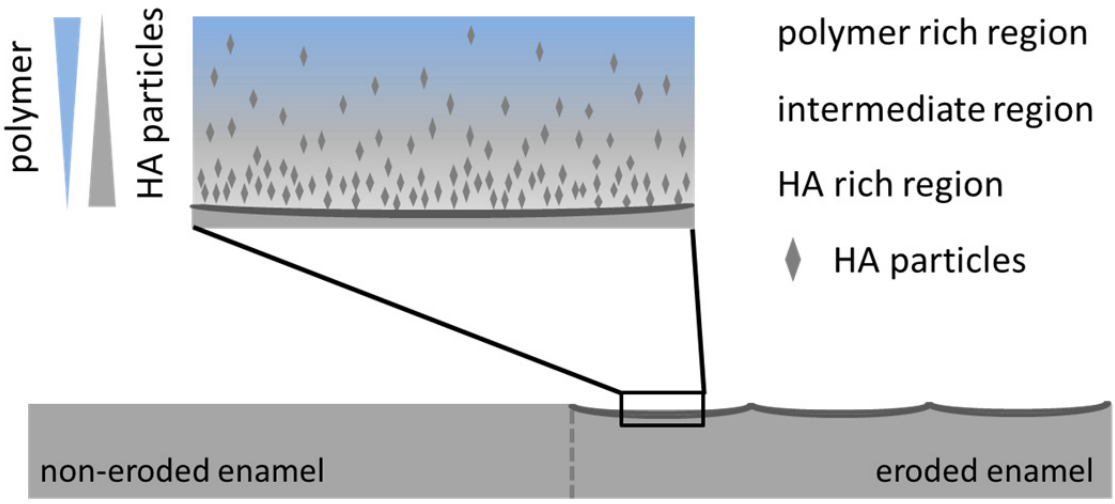

Figure 4: Modell of the polymer layer morphology. The citric acid induced dissolving of HA particles lead to the inclusion of these particles in the polymer matrix. The polymer/enamel layer therefore composed as a gradient of three regions: an upper polymer rich region, a lowest HA particle rich region and the intermediate region.

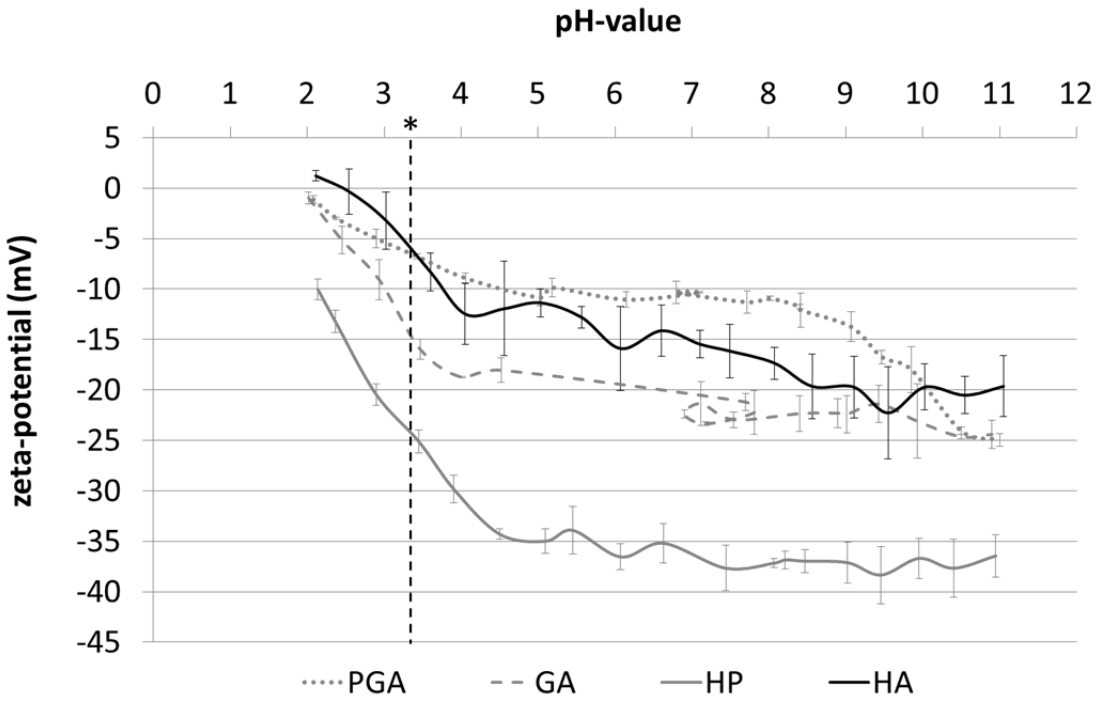

Figure 5: Zeta-potential measurements of the three polymer solutions (PGA = propylene glycol alginate; GA = Gum arabic; HP = highly-esterified pectin) and of the hydroxyapatite particles (HA). * ≙ the pH used for CAS and PMCAS of the current study.

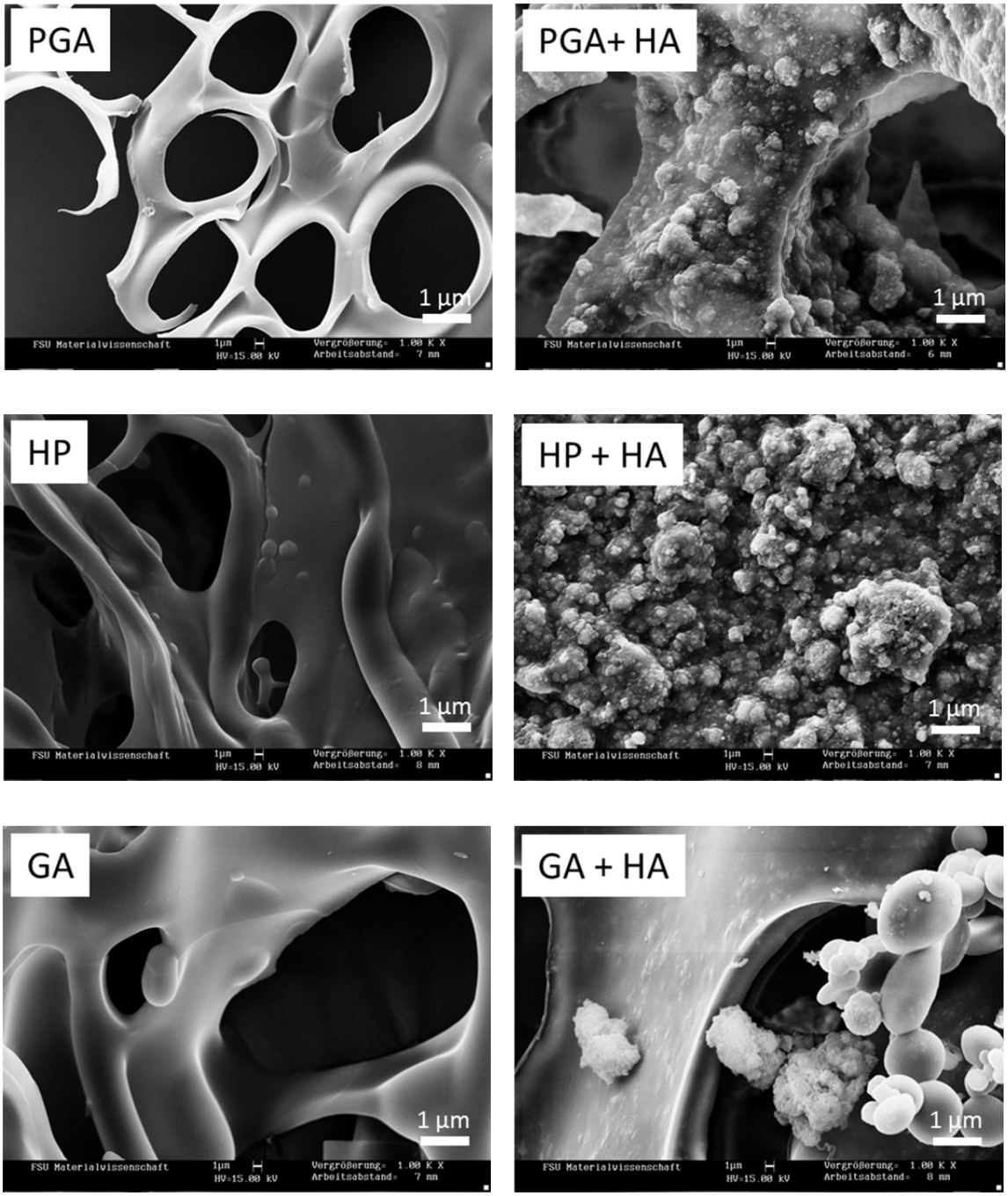

Figure 6: SEM micrographs of freeze dried polymer solutions (left) of propylene glycol alginate (PGA); highly-esterified pectin (HP) and Gum arabic(GA) and of similar polymer solutions with hydroxyapatite particles (right).

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Acids with an equivalent taste lead to different erosion of human dental enamel

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Acids with an equivalent taste lead to different erosion of human dental enamel

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ABSTRACT

Objectives. The consumption of acidic soft drinks may lead to demineralization and softening of human dental enamel, known as dental erosion. The aims of this in vitro study were to determine: (i) if different acids with a similar sensorial acidic taste lead to different hardness loss of enamel and (ii) if the fruit acids tartaric, malic, lactic or ascorbic acid lead to less hardness loss of enamel than citric or phosphoric acid when their concentration in solution is based on an equivalent sensorial acidic taste.

Methods. Enamel samples of non-erupted human third molars were treated with acidic solutions of tartaric (TA), malic (MA), lactic (LA), ascorbic (AA), phosphoric (PA) and citric (CA) acids with a concentration that gave an equivalent sensorial acidic taste. The acidic solutions were characterized by pH value and titratable acidity. Atomic force microscopy (AFM) based nanoindentation was used to study the nano mechanical properties and scanning electron microscopy (SEM) was used to study the morphology of the treated enamel samples and the untreated control areas, respectively.

Results. The investigated acids fell into two groups. The nano hardnesses of MA, TA and CA treated enamel samples (group I) were statistically significantly greater ($p < 0.05$) than the nano hardnesses of PA, AA and LA treated enamel samples (group II). Within each group the nano hardness was not statistically significantly different ($p > 0.05$). The SEM micrographs showed different etch prism morphologies depending on the acid used.

Significance. In vitro, the acids investigated led to different erosion effects on human dental enamel, despite their equivalent sensorial acidic taste. This has not been reported previously.

1. Introduction

Dental health is influenced by various factors [1]. One of them is dental erosion. This is defined as the dissolution of dental hard tissue without involvement of bacteria [2]. Acids present in food and beverages are one major etiological factor responsible for the erosive lesions of dental enamel [3,4]. Since the mid-1990s the effects of surface softening and enamel loss caused by the consumption of acidic soft drinks have been investigated. Soft drinks containing citric acid were investigated with respect to their erosive effects,

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specifically dissolution and softening of human enamel [2,5–8].

Different parameters determine the erosive effects of soft drinks on enamel. Important parameters to characterize the erosive potential of acidic solutions are the pH value and the titratable acidity [9,10]. The primary dissolution of human enamel depends on the pH value and the corresponding pKa value of acids [11]. With lower acid pH the erosive softening of human enamel increases dramatically [8]. Hannig et al. tested different acids with the same pH value for their calcium and phosphate dissolution from bovine enamel [12]. It was shown that ascorbic acid caused the highest erosion effect and malic acid the lowest. In addition, the titratable acidity parameter best described the erosive potential of acid solutions. The titratable acidity is the amount of sodium hydroxide solution (NaOH), which is required to reach a neutral pH [1]. It was shown that the higher the value of titratable acidity the greater the erosion effects [9,11].

Different compositional aspects are important for the use of different acids in soft drinks. From the perspective of the manufacturer, the concentration of acid in soft drinks is limited and the acid used should be able to reach a specific pH, which is necessary for the application. A further aspect for consumers is that, besides a generally preferred acidic taste of the soft drink the pH-values, concentration or type of acids or titratable acidity are not important for their choice of soft drinks. Rather, for consumers, the sensorial taste of a beverage is important [13]. There is a wide range of fruit acids that can be used in soft drinks or other food, e.g. malic acid, tartaric acid, ascorbic acid or lactic acid [14,15].

It is understood how acids interact with hydroxyapatite (HA) surfaces [16]. It has been shown that mono-, di- and tricarboxylic acids chemically adsorb onto the enamel surface and dissolve Ca^{2+} ions out of the HA surface [17]. The carboxylic acids: MA, TA and LA showed increased dissolution of Ca^{2+} and PO_4^{3-} ions out of a bovine HA surface compared to other acids tested [12].

A newer approach for using acids in soft drinks is to establish an adjusted concentration of the different acids that results in an equivalent sensorial acidic taste. From sensorial tests it is known that different acids can have an equivalent sensorial acidic taste by using the concentrations shown in Table 1 [18,19]. The useful and sensitive sensory method [20–22] can be used to adjust the acidic concentration so that it becomes sensorially equal for soft drinks. The previous sensorial tests were conducted by a trained panel. This panel consisted of various people who individually assessed the taste of the acid solutions. The panel identified the concentrations that give an equivalent acidic taste. The erosive effects of such sensorially adjusted acidic solutions on human dental enamel have not been investigated previously. Therefore, the aims of this in vitro study were to determine: (i) if different acids with a similar sensorial acidic taste lead to different hardness loss of enamel and (ii) if the fruit acids tartaric, malic, lactic or ascorbic acid lead to less hardness loss of enamel than citric or phosphoric acid when their concentration in solution is based on the equivalent sensorial acidic taste.

2. Materials and methods

2.1. Sample preparation

For this in vitro study non-erupted human third molars ($n = 53$) were used and prepared as described previously [23]. In brief, teeth were disinfected and the roots were carefully removed. Enamel pieces (approximately 2 mm × 3 mm) were cut from the tooth with a low speed saw (Isomet; Buehler GmbH, Düsseldorf, Germany) using a water cooled diamond blade (Buehler GmbH, Düsseldorf, Germany) and embedded in a resin (Stycast 1266; Emerson & Cuming, ICI, Wasterlo, Belgium). To obtain smooth surfaces, the samples were finally ground with SiC paper (grit 1200–4000; Buehler GmbH, Düsseldorf, Germany) and polished with aluminum oxide powder dispersions (particle size ranging from 6 μm to 1 μm). Prior to use, the samples were stored in deionized water at room temperature. Immediately before treatment with the test solutions, one half of the surface of each enamel sample was covered with PVC tape (Tesa AG, Hamburg, Germany) to obtain a reference area that was protected against acid solution exposure.

2.2. Acid solutions

The acid solutions were prepared in deionized water according to the acid concentrations given in Table 1 [18,19]. The acids: tartaric acid (TA), malic acid (MA), lactic acid (LA), ascorbic acid (AA), citric acid (CA) and phosphoric acid (PA) were supplied by Rudolf Wild GmbH & Co. KG (Eppelheim, Germany). To quantify the erosive potential of the different acid solutions the pH value was measured with a pH-meter (Knick pH-meter 765 Calimatic, Berlin, Germany). In addition, the titratable acidity was calculated by measuring the amount of NaOH (0.1 mol/L) which was necessary to reach a neutral pH of 7.00.

2.3. Erosive treatment of the enamel samples

Four samples chosen at random ($n = 4$) were used to investigate the erosive effect of each of the six acid solutions (overall $n = 24$). Each enamel sample was placed in a beaker with the acid solutions (approx. 40 mL) for 60 s under constant stirring. This treatment time relates to the neutralization properties of saliva [24] and short term pellicle building time of saliva [25]. Immediately after treatment, the samples were rinsed with deionized water for 30 s and dried with compressed air. The PVC tapes were removed from the untreated areas (UT) of the enamel samples. Residues of glue from the tape were removed carefully with ethanol soaked cotton swabs.

2.4. Nanoindentation

An atomic force microscope (Digital Instrument Dimension 3100; Veeco Instruments, Santa Barbara, CA, USA) equipped with a Hysitron TriboScope® nanoindenter (Hysitron Inc., Minneapolis, MN, USA) was used to measure the nano hardness and the reduced elastic modulus [26,27] of the enamel samples. For the indentations, a standard Berkovich diamond indenter with an equilateral pyramidal area was calibrated

Table 1 – Concentration of acids (g/L and mmol/L) required to give an equivalent sensorial taste [18,19], measured pH values, pKa values, degree of dissociation of the acids (%), titratable acidity (amount of mmol NaOH/L) and hardness loss (%) compared to the nano hardness of the untreated enamel areas for the acids used.

	Acid	c_{acid} g/L (mmol/L)	pH	pKa	Degree of dissociation (%)	Titratable acidity c_{NaOH} (mmol/L)	Hardness loss (%)
Group I	Malic acid (MA)	1.12 (8.35)	2.64	$pKa_1 = 3.46$	15.14	0.80	48.6
	Tartaric acid (TA)	1.00 (6.66)	2.56	$pKa_1 = 2.98$	38.02	0.42	47.5
	Citric acid (CA)	1.22 (6.35)	2.62	$pKa_1 = 3.13$	30.90	0.72	48.7
Group II	Lactic acid (LA)	1.36 (15.1)	2.63	$pKa_1 = 3.90$	5.37	0.68	66.4
	Ascorbic acid (AA)	3.00 (17.03)	2.87	$pKa_1 = 4.20$	4.68	0.67	59.6
	Phosphoric acid (PA)	0.85 (8.67)	2.15	$pKa_1 = 2.16$	97.72	0.53	64.0

with fused silica and a standard tip area function was used. The enamel indentations were made in air at room temperature in three steps: linear loading up to 5 mN from 0 s to 15 s, holding this load for 5 s and linear unloading to 0 mN within 15 s. Before every indentation an image of the surface was recorded with the Berkovich tip to ensure that it was flat, clean, and free of damage. Five indentations were made in both the reference area and the treated surface area on every sample. The distance between the indents was kept to at least 20 μm to avoid interferences between them. Nano hardness and reduced elastic modulus were calculated with the TriboScope® software (version 3.5).

2.5. *SEM measurements*

Scanning electron microscopy (SEM) was performed with a LEO 440i SEM Scanning Electron Microscope (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany) operated at 15 kV and with a working distance of 6 mm. For SEM images all samples were gold sputter coated (approx. 10 nm) with an Edwards sputter coater S150B (Edwards High Vacuum International, Crawley, West Sussex, UK).

2.6. *Statistical analysis*

ANOVA analysis and a Scheffe *t*-test (95% confidence interval, $p < 0.05$) were performed with StatGraphics Centurion XV (StatPoint Inc., Warrenton, USA) to test the statistical significance of the nano hardness loss and the reduced elastic modulus of the human enamel samples. The factor investigated for significance was the acid type.

3. Results

All acidic solutions of the current study were characterized for pH values and the titratable acidity (Table 1). The pH of the acids TA, CA, LA and MA differed slightly with values in a range between 2.56 and 2.64. PA showed the lowest pH value (2.15) whereas AA had the highest pH value (2.87). The measured pH and the pKa values were used to calculate the degree of dissociation following the equation:

$$pH = pKa - lg\left(\frac{HA}{A^-}\right).$$

The quotient HA/A^- (HA = undissociated acid; A^- = dissociated acid) represents the degree of dissociation of the acid (Table 1). The titratable acidity values differed between the acids. The lowest quantity of alkali to neutralize the acid was found for TA (0.42 mmol/L NaOH) and the highest for MA (0.80 mmol/L NaOH). LA (0.68 mmol/L NaOH) and AA (0.67 mmol/L NaOH) had similar values, whereas PA was lower and CA slightly higher than LA and AA.

The nano hardness data of the enamel samples treated with the six different acid solutions are shown in Fig. 1. All acid solutions led to decreased enamel nano hardness during erosive treatment. The measured nano hardness data can be divided into two groups. Group I (samples treated with MA, TA and CA) with an average nano hardness of the enamel samples of 1.86 ± 0.06 GPa and group II (samples treated with PA, AA and LA) of 1.27 ± 0.10 GPa, respectively. Samples treated with group II acids showed a statistically significantly lower nano hardness ($p < 0.05$) than samples treated with group I acids. Within each group there were no statistically significant differences ($p > 0.05$) among the measured nano hardness. Enamel samples treated with AA showed a slightly less reduced elastic modulus than the moduli of the enamel samples treated with the other acids (Fig. 2). Nevertheless, there was no statistically significant difference between the reduced elastic moduli of all acid treated enamel samples. The etch morphology of the enamel samples treated with the different acid solutions of the enamel surfaces is shown by SEM micrographs (Fig. 3). For every treated sample shown, surface defects such as very rough surfaces or voids are visible. For group II (PA, AA and

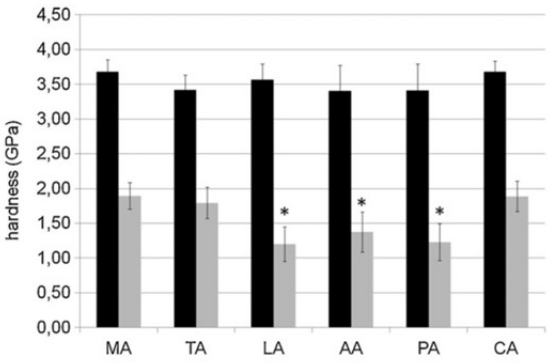

Fig. 1 – Nano hardness of enamel samples which were treated with different acids: malic acid (MA), tartaric acid (TA), lactic acid (LA), ascorbic acid (AA), phosphoric acid (PA) and citric acid (CA). Black bars show the control nano hardness (untreated area); * denotes a statistically significantly lower hardness than enamel treated with CA.

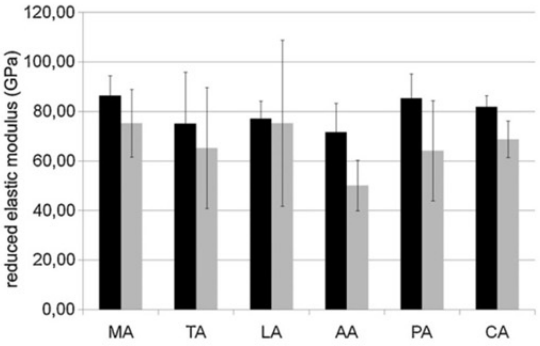

Fig. 2 – Reduced elastic modulus of enamel samples which were treated with different acids: malic acid (MA), tartaric acid (TA), lactic acid (LA), ascorbic acid (AA), phosphoric acid (PA) and citric acid (CA). Black bars show the control reduced elastic modulus (untreated area).

LA) all SEM images show rougher and more eroded surfaces compared to the images of group I (MA, TA and CA) where the surfaces seem to be much smoother with very flat areas (white arrows). Especially for AA and PA the enamel surfaces appear strongly eroded with voids and loss of material over the whole surface. For MA, TA and CA non-eroded areas are visible.

4. Discussion

Acids in soft drinks are the major extrinsic factor for dissolution and softening of human dental hard tissue [7]. Different studies have reported strategies to reduce the softening of enamel by modifying the soft drinks. The erosion process can be reduced by changing the degree of saturation [28], by adding enamel protecting polymers [6,29], by changing the pH value of the acids [30] or by the use of different acids with identical pH [12]. From the consumer's view the parameters of these studies are of minor interest, as they are mainly interested in a similar acidic taste [13]. The approach of this current study was to investigate the erosive behavior of acids in vitro when they have concentrations adjusted to give an equivalent acidic taste [18,19]. Besides their taste, the acidic solutions can be characterized by pH and titratable acidity. The pH values of the acidic solutions in our study (Table 1) were very similar for all acid solutions and were in the range of many acidic soft drinks [31]. With the exception of PA and AA all acid solutions had the same pH value of about 2.61 ± 0.04. For PA the pH was lower (pH = 2.15) and for AA it was higher (pH = 2.87) than the pH of the other acid solutions. In this current study, the pH seems not to be the dominant parameter that influences erosion. There was no significant difference in hardness loss caused by PA and AA despite the greatest deviance in the pH (Fig. 2). This indicates that other parameters are necessary to determine the effects of acids on human dental enamel during the process of erosion.

The higher the values of the titratable acidity for the acids the more the erosion on human dental hard tissue occurs [9,11]. Therefore, this parameter seems to be more critical than the pH value in determining the likely erosive effects expected in vivo from different acids with equivalent sensorial acidic taste. Table 1 shows the amounts of a 0.1 M NaOH solution that are needed to reach a neutral pH of 7.00 for the acidic solutions. The titratable acidity, however, does not correspond well to the enamel nano hardness loss found in this current in vitro study. The higher the amount of added NaOH solution to reach a neutral pH value, the stronger the erosive effect is expected. The strongest erosion effects were, therefore, hypothesized for MA and CA, and the lowest for TA. Surprisingly, the measured nano hardness showed the opposite behavior. MA and CA caused a similar hardness loss of enamel, but this hardness loss was not the strongest as expected from titratable acidity.

In Table 2 we arranged the acid solutions by an anticipated in vivo erosion potential (EP) dependent on the measured parameter values. The influence of the parameters with respect to human dental erosion is reported in previous literature: acid concentration (the higher the concentration, the higher the erosion effect) [30], pH value (the lower the value, the higher the erosion effects) [8,30], and titratable acidity (the higher the value, the higher the erosion effects) [9,11]. For the used acid concentrations, the EP was hypothesized as: PA lowest (0.85 g/L) and AA highest (3.00 g/L). Measuring the pH value, the EP was hypothesized as: AA lowest (pH 2.87) and PA highest (pH 2.15). For the titratable acidity the following order was hypothesized: TA lowest (0.42 mmol/L) and MA highest (0.80 mmol/L). The measured hardness loss (%) of the enamel samples gave this EP: TA lowest (47.5%) and LA highest (66.4%). It has to be considered that this ordering is based on the literature that investigated the acids by itself and is not completely usable for the parameters investigated in this current study. With the EP we demonstrate that the commonly used parameters are not suitable, when different strong acids were used based on equivalent taste.

Table 2 – Expected in vivo erosion potential (EP) of the acids on human dental enamel depending on the measured parameters (ordered numerically) compared to the measured in vitro hardness loss of enamel samples of the current study. Acids from group I are shown with this character (*).

Investigated parameter	Expected in vivo erosion potential depending upon the measured parameter value[a]
Concentration of acid (g/L)	$PA < TA^* < MA^* < CA^* < LA \ll AA$
pH value	$AA < MA^* \cong LA \cong CA^* < TA^* \ll PA$
Titratable acidity (mmol NaOH/L)	$TA^* < PA < AA \cong LA < CA^* < MA^*$
Nano hardness loss (% compared to untreated area)	$TA^* \cong MA^* \cong CA^* < AA \cong PA \cong LA$

[a] The terms on the left hand side (PA, AA, TA*, TA*) show lower expected EP and on the right hand side (AA, PA, MA*, LA) higher expected EP.

Nevertheless, only the nano hardness loss directly represents the softening effect of human dental enamel when acids interact with these surfaces, whereas acid concentration, pH and titratable acidity can only indirectly indicate the erosion behavior.

The pH value alone, for example, does not represent the erosive potential of the acids on human dental enamel, as AA with the highest pH value did not show the lowest nano hardness loss. Moreover, a high acid concentration alone (e.g. AA with 3.00 g/L) was not responsible for the highest hardness loss. A high amount of titratable acidity, such as for MA (0.80 mmol/L) and CA (0.72 mmol/L), does not represent the highest erosive effects because MA and CA led to the lowest hardness loss for enamel samples in this current study (Table 1). The pH and titratable acidity values did not fall into the same two groups found by nanoindentation measurements. From the current study, the commonly used parameters to classify or appraise erosion effects seem to be unsuitable when different strong acids were used (Table 1). The nano hardness loss of the enamel samples seems to be related either in a complex manner – or not at all – to the other chemical parameters.

All acids of this in vitro study had an equivalent acidic taste but different erosion effects on human dental enamel. Several parameters have been previously identified as influencing the erosion process of acids against human dental enamel [1]. From the literature it is known that mono-, di- and tricarboxylic acids interact with hydroxyapatite (HA) and chemically adsorb onto the enamel surface [17]. This adsorption leads to a dissolution of Ca^{2+} ions out of the HA surface.

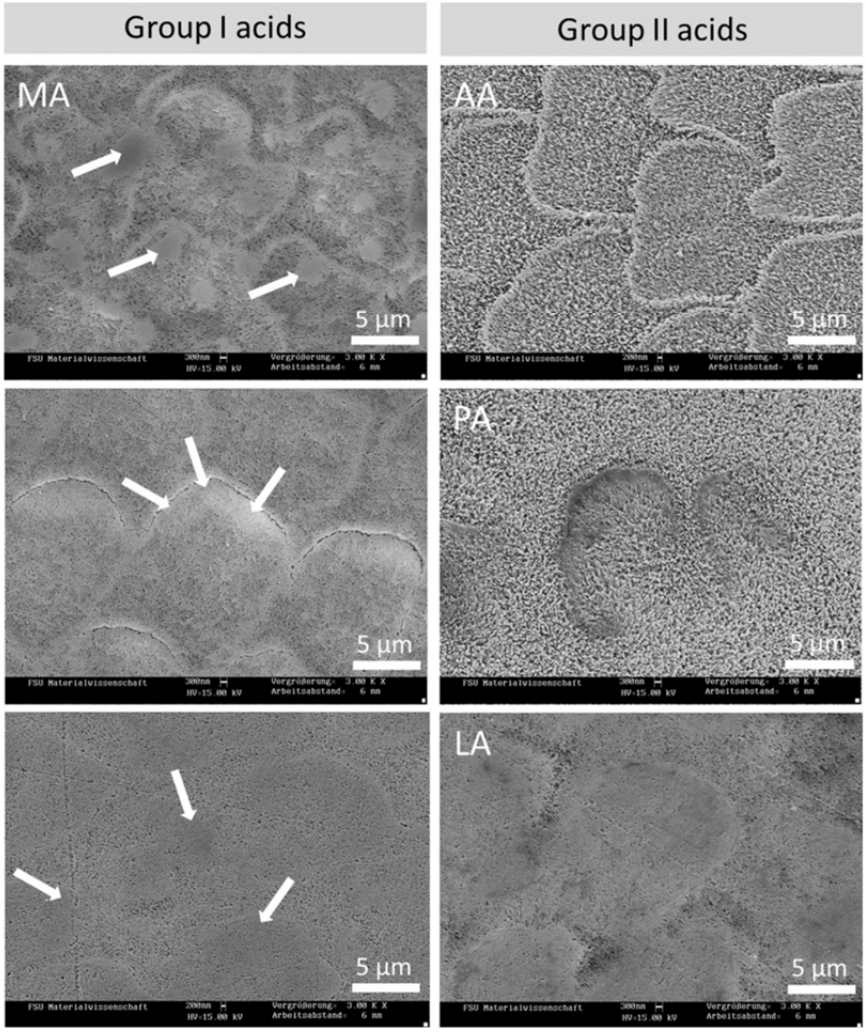

Fig. 3 – SEM images of the enamel surfaces treated with group I acids [left: malic acid (MA); tartaric acid (TA); citric acid (CA)] and enamel samples treated with group II acids [right: ascorbic acid (AA); phosphoric acid (PA); lactic acid (LA)]. The white arrows represent smooth and less eroded areas of the acids from group I.

This chemical binding to the HA surfaces is not influenced by the change of pH of the acids. MA, TA, LA and CA interact with the enamel surface and lead to dissolution processes [12]. However, these acids show different capacities to reduce enamel nano hardness as shown in this current study. Acid anions can build up nearly insoluble salts, e.g. calcium tartrate, calcium malate or calcium citrate with the Ca^{2+} ions [17]. In addition, the dissociated acids directly bind with the Ca^{2+} ions of HA surface and build up chelate complexes [32]. In the current study, the monocarboxylic acid (LA) and the non-carboxylic acids (AA and PA) showed the strongest erosion effects in vitro on human dental enamel. This could imply steric interactions between the acid molecular structure and the enamel structure. For the acids of group II (LA, AA and PA) the acidic group is strongly localized within the molecular structure. These localized acidic groups may possibly lead to a faster and closer arrangement between acid molecules and the HA surface. Thus, we hypothesize that acids from group II better adapt spatially to the Ca^{2+} ions of the hexagonal crystal structure of enamel and may lead to faster dissolution.

The effect of acids against human enamel depends on nanochemical and nanophysical interactions between the acids and the human enamel [16]. Thus, we assume that these interactions lead to the loss of hydroxyapatite crystals at the surface and an ensuing erosion process. To illustrate this effect of acids against human dental enamel, the etched enamel prism surfaces after erosive treatment are shown in SEM micrographs (Fig. 3). From the literature it is known that the different acids lead to different etch morphologies on bovine enamel surfaces [12]. The SEM micrographs showed different etch morphologies that support the nano hardness data and interpretations of the current study. The greater the hardness loss that was measured by nanoindentation, the rougher the surface appears. For PA and AA the surface was strongly eroded which corresponds to the measured nano hardness loss. For MA and TA the surface was less affected. Thus, the roughness of the surface corresponds to the nano hardness loss. As shown in Fig. 3 the surfaces of LA, PA and especially AA showed the highest erosion represented by very rough surfaces. In contrast to the SEM micrographs of LA, PA and AA treated samples, the SEM micrographs of MA, TA and CA treated samples showed smooth and less eroded areas (white arrows).

The present in vitro study shows that the erosive effects of acids with equivalent acidic taste lead to different erosion effects on human dental enamel for the first time. For the given parameters, we hypothesize that CA can be exchanged by MA or TA without higher erosion effects on human dental enamel. However, when using LA, PA or AA, the erosion effect was increased. It has to be considered that the results of the current study based on simple acidic solutions does not fully represent the soft drink recipes of the manufacturers. In further studies the findings should be investigated in vivo and concerning complex and modern soft drink recipes under in vivo circumstances. Despite an equivalent sensorial acidic taste, it is therefore possible to influence significantly the erosive effect of soft drinks against human dental enamel by an appropriate selection of the acid.

5. Conclusion

This study shows that acids with an equivalent sensorial taste lead to different erosion effects on in vitro enamel surfaces. CA, MA and TA lead to a significantly lower nano hardness loss than LA, AA and PA. Acids with equivalent adjusted sensorial acidic taste show different erosion effects on enamel samples that may provide new approaches for the formulations of acids in soft drinks.

Acknowledgments

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4 FINAL DISCUSSION

Dental erosion is one of the main causes for dental tooth diseases and is influenced by several factors [35,49,67,77,108]. This disease becomes more and more important [36,37] and it is necessary to develop and investigate strategies to reduce erosion of human dental enamel [109]. One of the major scientific approaches is to modify acidic drinks that cause dental erosion [78]. One of the first ideas was to reduce dental erosion by adjusting the degree of saturation. Ca^{2+} and PO_4^{3-} ions were added to citric acid solutions in a range, that erosion was inhibited [79,80,106,110,111]. Another very promising idea was the addition of protective polymers to acidic solutions to reduce the erosion effects caused by the acids in the soft drinks [94,95,98,112,113]. But until now these effects were investigated only for long time acidic treatments. Therefore, the aim of this study was to investigate the erosion effects caused by acids on *in vitro* human dental enamel after short time treatments in the range of seconds depending on different modifications of acidic solutions. First, citric acid solutions were modified with food-approved polymers to reduce erosion and to develop polymer based strategies against human dental erosion induced by the citric acid. In addition, also the erosive effects of different acidic solutions, but with an identical sensorial taste, were investigated. Parts of this work were supported and accompanied by an industrial partner and their specifications.

4.1 Measurement methods used to characterise human dental erosion

There is a wide range of methods that are useful to characterise and investigate the erosion process on dental enamel surfaces [99,114,115]. In our studies we characterised the erosive effects of acidic acid solutions on human dental enamel quantitatively with profilometry, confocal laser scanning microscopy (CLSM) and AFM-based nanoindentation (NI). For qualitative investigations of the enamel surfaces scanning electron microscopy (SEM) was used. Profilometry measurements are one of

the mainly used and very simple methods to investigate long time erosion on enamel within the range of minutes [100-102], hours [48] and days [83]. Nevertheless, profilometry measurements causes scratches in softened and eroded dental enamel [47]. To exclude unfavourable effects of the profilometry scratches on the measured results, they were compared to the results of the non-tactile and non-destructive CLSM method. Comparing the measured material loss we could show that the tactile profilometry measurements show reliable values of the erosive enamel material loss compared to the non-tactile confocal laser scanning microscopy (CLSM). There were no statistically significant differences of the measured material loss between both measurement systems. Although profilometry is a destructive method, the deviation of the results was very low, so profilometry provides comparable and reliable results compared to non-tactile methods (CLSM).

AFM-based nanoindentation (NI) is a suitable [45] and commonly used method to investigate erosive effects of acids on human dental enamel after short time treatments [25,80,105,114,116]. According to these previous studies AFM-based NI was used in our studies to characterise the erosive softening of enamel surfaces after 30 s up to 120 s treatment times. In addition, AFM-based NI seems to be also a suitable tool to investigate the effectiveness of polymers added to citric acid solutions to reduce dental enamel erosion after short time treatments. First the method was verified by measuring water treated enamel samples (**Figure 4-1**) compared to citric acid treated enamel samples to ensure that AFM-based NI is an useful and sensitive method. It could be shown clearly, that Volvic® mineral water (VM), as a control not led to any changes in the hardness of enamel samples compared to the untreated enamel samples. Moreover, the treatment with citric acid solutions (CAS) decreased the hardness of enamel samples already after 30 s dependent on the pH value.

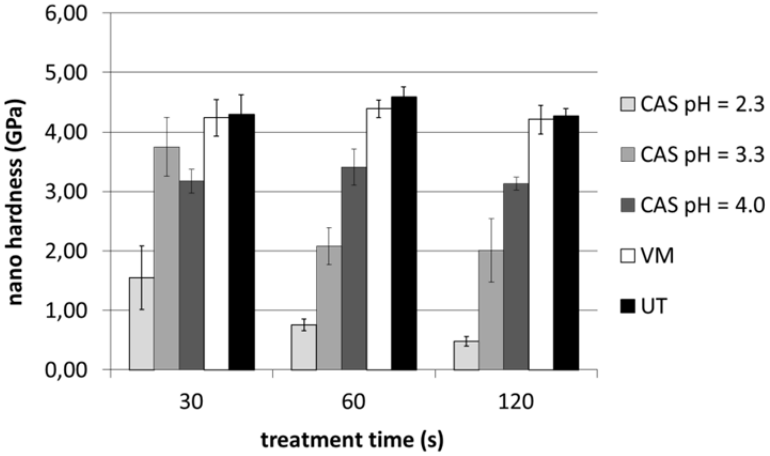


Figure 4-1: Nano hardness of *in vitro* enamel samples treated with CAS (pH 2.3, 3.3 or 4.0) or Volvic® mineral water for 30, 60 and 120 s. The black bars represent the nano hardness of the untreated reference area (UT).

AFM-based NI is a very sensitive method to measure very small changes in the nano hardness after short time treatments. It is therefore one of the best methods to characterize the enamel hardness and the protective effects polymers have as additives in citric acid solutions on the hardness of enamel samples. In our study we could show for the first time that AFM-based NI is a powerful technique to investigate the protective effects of polymers in acidic solutions after short time enamel treatment.

Scanning electron microscopy (SEM) was used to investigate the eroded enamel surfaces and the interaction between the surfaces and the polymers [112,114,117,118]. It was shown, that SEM micrographs represent the enamel surface adequately to characterise the prism morphology when acidic [114,117,119] or alkaline agents [31] eroded the enamel surface. According to these previous studies SEM micrographs were used to compare the morphology of acid treated human dental enamels samples to each other and to the untreated enamel surface.

Using profilometry, AFM-based NI and SEM in the current study it was possible to characterise with a good reliability and comparability the erosive effects of modified

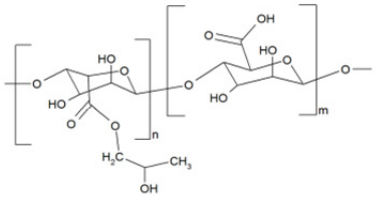
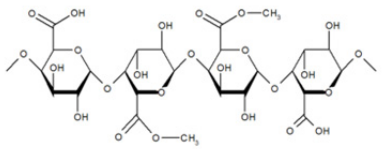
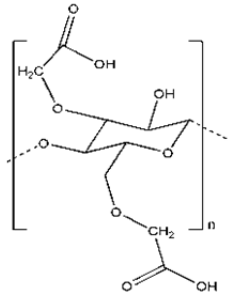
and un-modified citric acid solutions on human dental enamel and thus get also new insights into and deepen our knowledge about the process of dental erosion.

4.2 The protective effect of polymers added to citric acid solutions against the process of human dental erosion

There is a wide range of polymers that can be used as additives in citric acid solutions [93-95]. These polymers are normally widely found in food products and drinks as thickening, gelling and stabilizing agents. In the European Union (EU) legislation there are more than 40 E-numbered ingredients with stabilising properties for food use listed (E400-445). Although not more than 10 of these were regularly and widely used in soft drink formulations: alginates (E401-405), carrageens (E407), pectin (E440, 440ii), acacia (E414), guar (E412), tragacanth (E413), xanthan (E415) and carboxymethyl cellulose (E466) [120]. **Table 4-1** lists all polymers that were tested in our studies for its enamel protective properties, even if they have not shown any positive effects against erosion.

Our investigations have shown that three of the listed polymers (**Table 4-1**) showed promising effects against dental erosion caused by citric acid, when they were added to the citric acid solutions (PMCAS): propylene glycol alginate (PGA), highly esterified pectin (HP) and gum arabic (GA). These three polymers showed significant erosion reducing effects when they were added 1.0 % (wt/wt) to citric acid solutions [121]. Thus we could show for the first time that polymers added to citric acid solutions reduce erosion on human dental enamel also after short time treatments. In previous studies prolonged treatment times (in a range of minutes) [93,94] not comparable to a normal soft drink intake (in the range of seconds), and artificial HA discs [94], that are inappropriate compared to *in vitro* enamel, were used.

Table 4-1: Names, E-numbers, properties and the chemical structure of all polymers used in the current study, even if they do not show any positive effects against erosion. The use and purity of these food ingredients are regulated by the European Union directive (200/84/EG) and the 33rd Session of the Codex Alimentarius Commission (2010) of the FAO/WHO Food Standards (INS = International Numbering System; E-number = European classification).

Polymer	INS (E-number)	Properties	Chemical structure
propylene glycol alginate (PGA)	405	bulking agent, carrier, emulsifier, glazing agent, stabilizer and thickener	 1,4-linked β-D-mannuronate and α-L-guluronate
highly esterified pectin (HP) / low-esterified pectin (LP)	440	emulsifier, gelling agent, stabilizer and thickener in the food industry level of esterification 70-76 % (HP) level of esterification 5-50 % (LP)	 α-1,4-linked-d-galacturonic acid
gum arabic (GA)	414	as stabilizer for food industry (e.g. soft drinks, candies)	structure consist of statistically ordered L-arabinose, D-galactose, L-rhamnose und D-glucuronic acid
Na-carboxymethyl cellulose (Na-CMC)	466	viscosity modifier and thickener stabilizer for emulsions wide use for non-foods (e.g. toothpaste, paints)	 glucopyranose backbone with carboxyl groups bounded to the hydroxyl groups

As mentioned above the number of available polymers as additives to soft drinks is very large. The application and suitability for the industrial producer is sometimes limited by EU regulations and restrictions (200/84/EG). Thus the concentration dependency of the erosion reducing effect of two of the promising polymers (PGA and GA) was investigated in citric acid solutions (pH = 3.3) to find minimal effective concentrations of the polymers against enamel softening. Next to a concentration of 1 % additional concentrations of PGA (0.03 %) and GA (0.3 %, 0.6 % and 2.0 %) were tested. In **Figure 4-2** (PGA) and **Figure 4-3** (GA) the results are given.

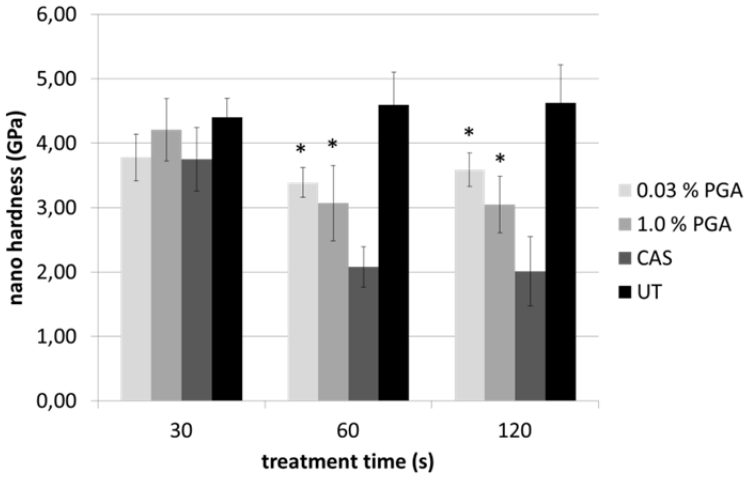


Figure 4-2: Nano hardness values of enamel samples treated with PMCAS depending on the concentration of PGA, compared to the nano hardness values of enamel samples treated with pure CAS and the untreated reference (UT) area. *denotes a statistically significantly higher nano hardness than CAS treated enamel samples.

Both concentrations of PGA showed statistically significant erosion reducing effects compared to the citric acid solution without PGA addition. After 30 s treatment time no significant effects were observed for the PGA polymer addition. However, there are no enhanced or reduced erosion inhibiting effects found for PMCAS containing 0.03 % of PGA compared to 1.0 % PGA within the standard deviation. A similar result can be seen for the different GA concentration in PMCAS. For treatment times of 60 s PGA concentrations of 0.3 %, 0.6 % and 2.0 % GA showed statistically significant erosion reducing effects.

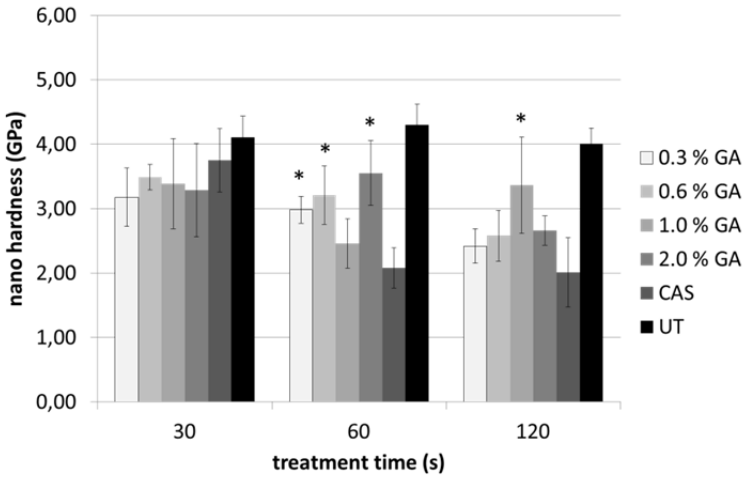


Figure 4-3: Nano hardness values of enamel samples treated with PMCAS depending on the concentration of GA compared to the nano hardness values of enamel samples treated with pure CAS and the untreated reference (UT) area. *denotes a statistically significantly higher nano hardness than CAS treated enamel samples.

For longer erosion times (120 s), only the concentration of 1.0 % GA showed statistically significant erosion reducing effects compared to the pure CAS. These promising results indicate that the use of the tested polymers is possible over a large range of concentrations. So for the industrial application of such polymers, like PGA or GA, as additives in soft drinks it is possible to use relatively low concentrations of the polymers, which not unfavourable influence the sensorial properties of the drinks, but significantly reduce their erosive properties.

Since both polymers PGA and GA showed high erosion reducing effects, it was also tested, if there was a synergistic effect of the two polymers when they are added both together to a citric acid solution. The results indicate that the combination of 0.03 % PGA and 0.6 % or 1.0 % GA showed no enhanced effect against enamel erosion compared to PMCAS with only one of the two polymers [121]. Moreover, the combination of these two polymers showed no statistically significant erosion reducing effects at all (**Figure 4-4**).

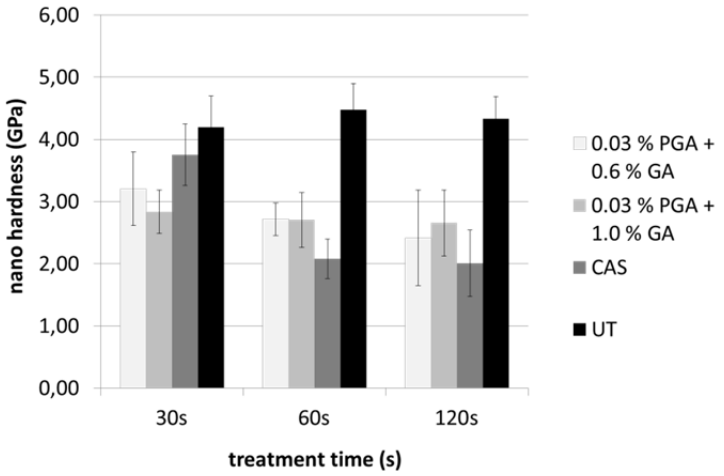


Figure 4-4: Nano hardness values of enamel samples treated with a combination of two different polymers (PGA and GA) depending on time compared to the untreated reference (UT) area.

Until now it is not clear why the synergistic use of PGA and GA polymers did not show any significant erosion inhibiting effects compared to the pure CAS. Previous studies showed that xanthan gum [98] reduces erosion. Moreover, it could be shown, that there is a synergistic effect of xanthan gum and calcium or polyphosphates [93,95]. From the current results it has to be considered, that only the addition of a single polymer showed significant less enamel softening compared to CAS treated enamel samples. Maybe the interaction of the polymers with the enamel surface is influenced and disturbed. This could be due to negatively effects of the polymers to each other, e.g. a binding of the polymers. At the end the protecting effect they showed when they were added to the CAS alone was repealed.

For the consumers the sensorial properties like refreshing properties [122], flavour [123] or the viscosity of the soft drinks are important. As mentioned above, polymers are used as thickener agents in soft drinks and food. They increase the viscosity of water solutions and of course of soft drinks. Thus for the use of a polymer as additive to soft drinks it is of high interest, in which way it changes the sensory properties, especially the viscosity of the drink. Therefore, the changes in viscosity were measured (shear rate 200 s^{-1}) depending on the concentration of the polymers in PMCAS and the

pH of the PMCAS (pH 2.3 and 3.3). The data are shown in **Figure 4-5** compared to the viscosity of water (0.64 mPa s, dashed line).

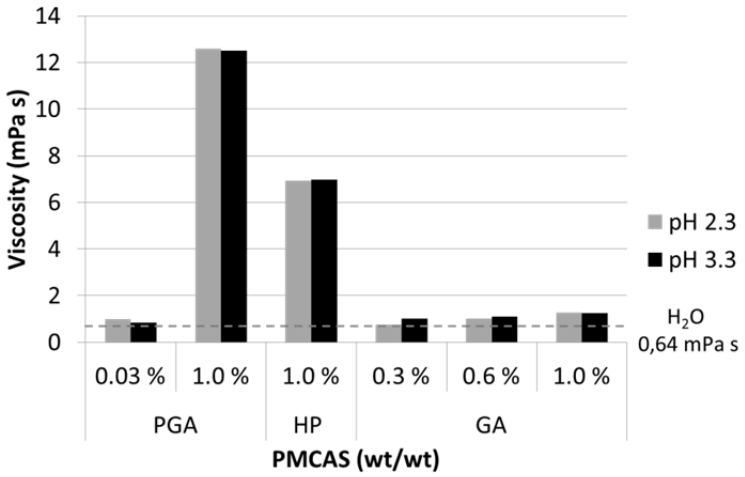


Figure 4-5: Viscosity of the PMCAS depending on the concentration of polymers used (HP = highly esterified pectin; GA = gum Arabic; PGA = propylene glycol alginate) for pH 2.3 (grey bars) and pH 3.3 (black bars). The dashed line represents the viscosity of pure water.

PMCAS containing 1.0 % PGA and 1.0 % HP, respectively, showed a clearly increased viscosity compared to the viscosity of water. Despite the addition of the polymers for all other PMCAS the viscosity was not changed markedly compared to that of water and remained in a similar range like the viscosity of other common soft drinks [124]. This indicates that GA can be used in concentrations of 0.3 up to 1.0 % in PMCAS and PGA with 0.03 % without negative changes in the viscosity and thus without sensorial unfavourable consequences for the consumers.

Referring the aim of the suitability of polymers as protective additives to citric acid solutions to reduce erosion it could be shown, that the polymers PGA, HP and GA have properties that statistically significant reduce erosion on human dental enamel [121]. These polymers protect the enamel surface against the influence of citric acid and are therefore promising and useful additives to soft drinks to reduce their erosive potential. Concerning the viscosity results it has to be considered that two of the

polymers (PGA and GA) can be used in soft drinks in a wide range without appreciable sensorial changes for the consumer.

4.3 Insights into the polymer-enamel interaction during the process of erosion

Next to the investigation of the erosion-inhibiting effect of polymers added to citric acid solutions, one main aim of the current *in vitro* study was to characterise and investigate in more detail the specific interaction between the polymers and the enamel surface as well as the structure and morphology of the polymer layers deposited on the enamel. First impressions were given by SEM micrographs, which showed that PMCAS treated enamel surfaces showed not such enamel prism rods that would be expected, when acids affect the enamel surface [121]. The prisms seem to be covered by the polymers. We therefore assume that the polymers were deposited on the enamel surface and may protect this surface against the erosive influence of the citric acid. Thus two models were hypothesised, which describe the polymer-enamel interaction:

I Ca^{2+} ions triggered chelate bonds between the polymer molecules themselves and between the polymer molecules and the enamel surface (**Figure 4-6**)

II A protecting polymer-HA particles barrier is formed by the dissolved HA particles and the surrounding polymer matrix (**Figure 4-7**)

We assume that both mechanisms in different proportions determine the build-up and structure of a polymer layer on the enamel surface. A protecting barrier develops, that comprise both polymer-polymer interactions and polymer-HA interactions.

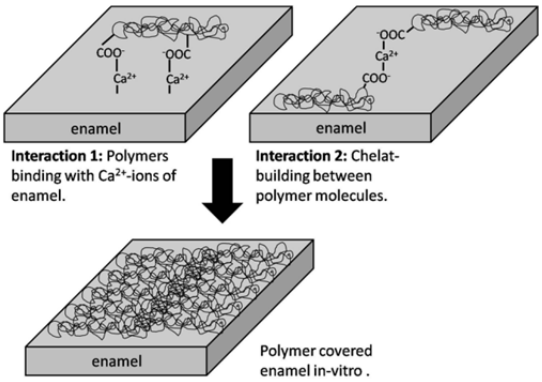


Figure 4-6: Ca^{2+} ions trigger chelate bonds between the polymer molecules themselves and between the polymer molecules and the enamel surface [121].

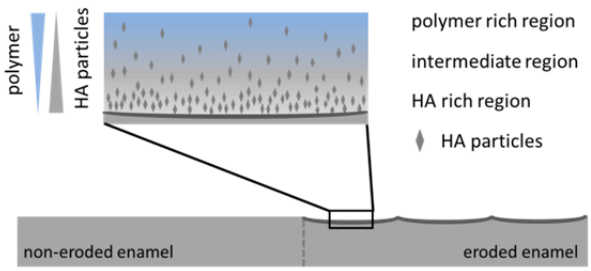


Figure 4-7: Polymer-enamel barrier formed by opposing gradients of dissolved HA particles and the surrounding polymer matrix (Chapter 3.3).

In cooperation with the Carl Zeiss NTS GmbH (Oberkochen, Germany) we imaged the plan view of the PMCAS treated enamel surfaces (**Figure 4-8, left**) and the same area was then imaged to visualize the material contrast between carbon and calcium (**Figure 4-8, right**). Using different ion beam energies and detecting the back scattered electrons an element contrast between the carbon of the polymers and the calcium of the hydroxyapatite appears. From reference materials and measurement experiences the material contrast of calcium was assumed by Carl Zeiss NTS GmbH of about 10 Å or less below the plan view of the surface. This finding fits well to the model described in **Figure 4-7,** where only a very thin upper layer of the pure polymers exists. Moreover the structure of the protective layer seems to be dominated by two opposing gradients of polymers and HA particles. This assumption is based on the previous results of Hannig & Hannig, who found that during the process of dental erosion mainly HA particles are dissolved from the enamel surface [46]. Thereby the HA particles and the underlying enamel give the material contrast shown in the SEM micrographs on the right side of **Figure 4-8**. The measured thicknesses of the pure polymer layer are qualitatively values that are appraised in comparisons of reference materials from Carl Zeiss NTS GmbH.

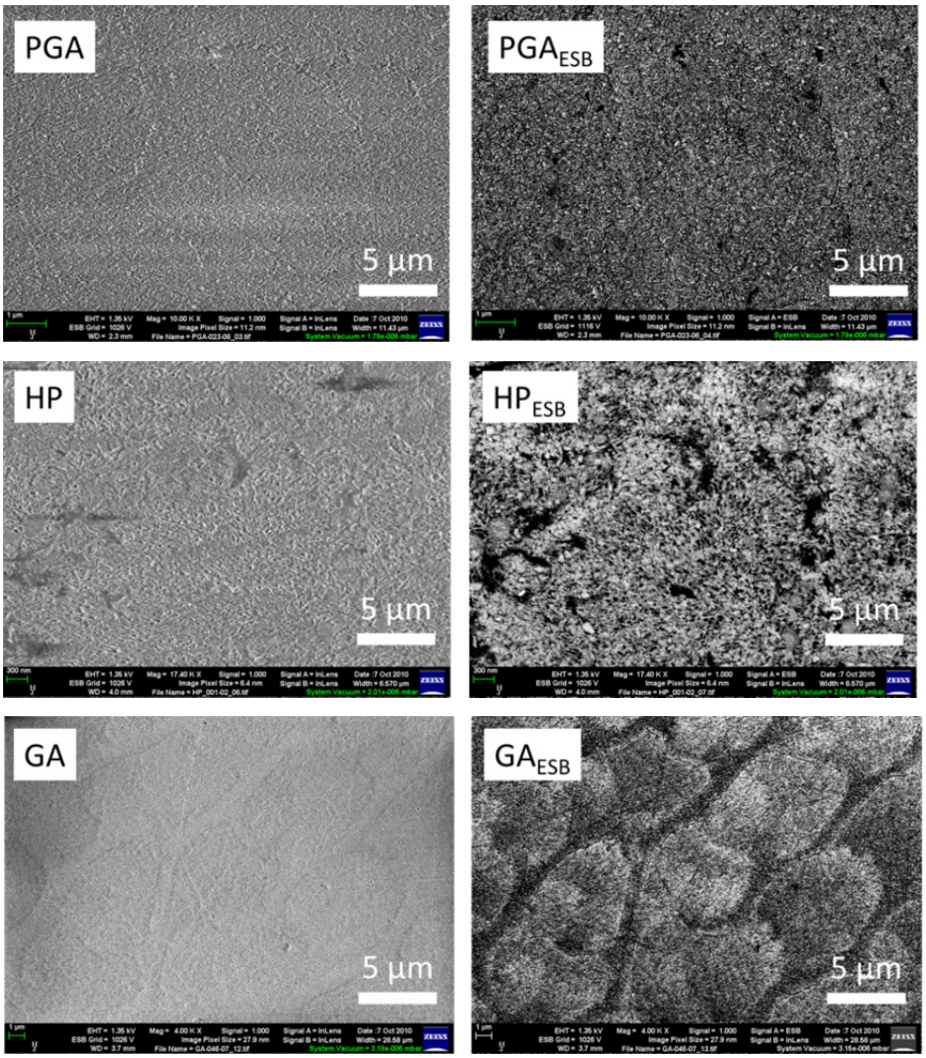


Figure 4-8: SEM micrographs (Carl Zeiss NTS GmbH, Oberkochen, Germany) of polymer coated enamel surfaces. On the left side the topography of the PMCAS treated surfaces (PGA; HP and GA) is shown; on the right side the underlying enamel surface is shown by the use of an Energy Selected Backscattered (ESB) detector with very low accelerating voltage that visualizes the material contrast.

The SEM pictures in **Figure 4-8** clearly show that the pure polymer layer was very thin and estimated about 10 Å. To support these findings and the hypothesised opposing gradients structure the chemical composition of the layer was measured with angle dependent X-ray photoelectron spectroscopy (XPS) [125]. With this method it is possible to vary the angle of the focused X-ray beam to the sample surface from 90° (perpendicular to the surface) to 0° (horizontally to the surface). This gives the opportunity to measure the atomic concentration of the surface dependent on the penetration depth of the focused X-ray beam. Using angle dependent XPS we could

show, in preliminary results, that the amount of calcium increased with increasing angle, which means increasing penetration depth of the X-ray beam (**Figure 4-9**). The intensity of the 2p orbital electrons of calcium (346.2-349.7 eV) [126] increases with increasing the angle of the X-ray beam (15-75°). The XPS measurements on the PMCAS treated enamel surfaces are still in progress and the final results will be added to the manuscript in Chapter 4. Nevertheless, these preliminary XPS results support the assumed gradient of calcium within the polymer layer deposited from PMCAS on human enamel (**Figure 4-7**).

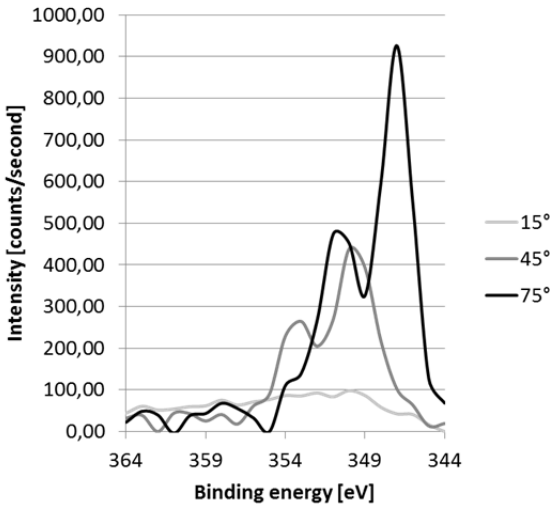


Figure 4-9: Angle dependent XPS measurements (15-75°) of an enamel surface with a deposited polymer layer from a PMCAS treatment. The increasing calcium peak (atomic concentration, 346.2-349.7 eV) is shown depending on the penetration depth of a focused X-ray beam. The intensity peak of calcium at 15° is very weak compared to the strongly increased calcium peak at 75°.

SEM (**Figure 4-8**) and XPS (**Figure 4-9**) results indicate that there is a layer composed of polymer molecules and incorporated HA particles that were dissolved out of the enamel surface due to the erosion process and form a gradient within this layer.

To further clarify the morphology of the layer coating the enamel surface, the thickness was estimated through profilometry scratches on the PMCAS treated enamel surfaces (Chapter 3.3). Despite the fact that the profilometry scratch also affects the softened enamel [47], this method gives an opportunity to roughly estimate the layer

thickness. Exemplary the enamel surface of two samples is shown in **Figure 4-10**. The samples were treated for 120 s with PMCAS (left side) and CAS without polymer addition (right side), respectively.

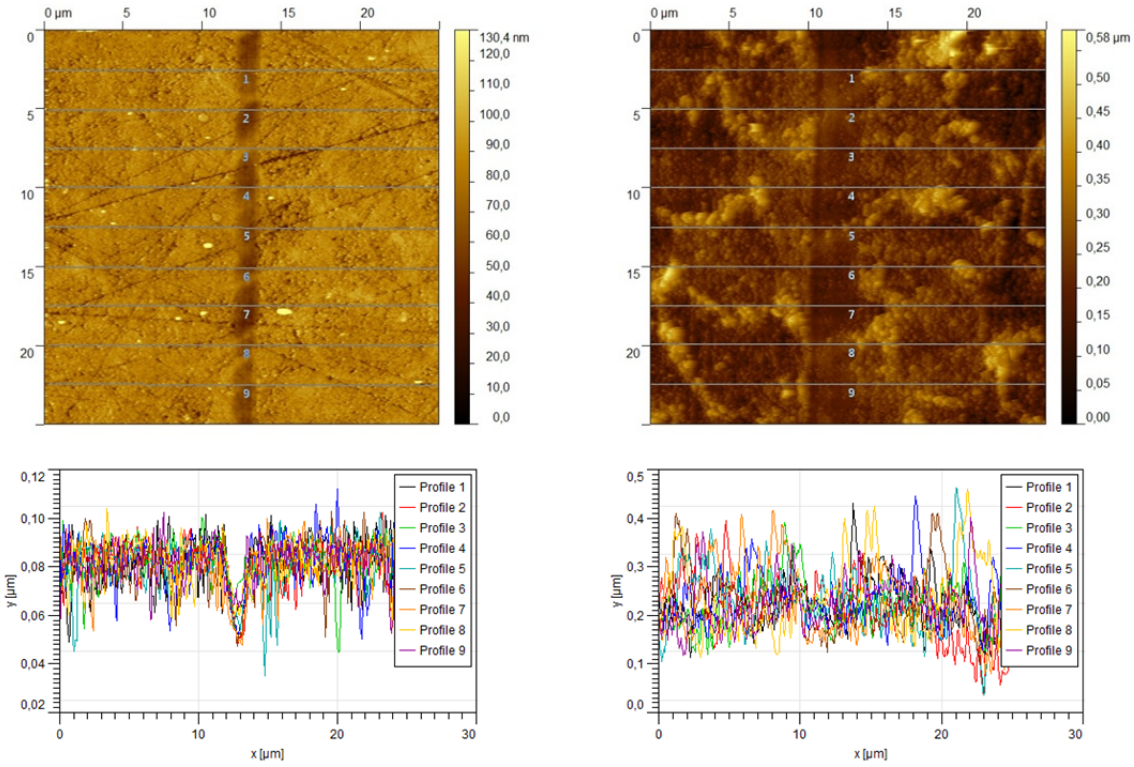


Figure 4-10: Enamel samples treated for 120 s with a PMCAS (left) and CAS (right) solutions (pH = 3.3) and the corresponding depth profiles of the profilometry scratch measured with AFM.

It could be shown, that the scratch on the enamel with deposited polymers showed a depth in the range of 10 nm (30 s treatment time) up to 25 nm (120 s treatment time). Contrary to this the scratch depth of the CAS treated surface showed a range of nearly 30 nm (30 s treatment time) up to 60 nm (120 s treatment time). These results indicate that with the scratch depth profiles it was not exactly possible to estimate the real polymer layer thickness. Rather the scratch depth seems to represent the thickness of the deposited polymer and the softened and destroyed enamel together, which corresponds also to our hypothesised model of the mixed polymer HA particle layer. Nevertheless the thickness of this layer on the PMCAS treated samples must be 25 nm

or less after 120 s, while the scratch depth on the CAS treated sample represents the depth of the erosion (60 nm after 120 s).

With SEM it could be shown, that the polymer coated PMCAS treated enamel surfaces appear smoother compared to that of the CAS treated samples [121]. The AFM micrographs shown in **Figure 4-10** support this observation. Thus the surface morphology of the PMCAS eroded samples seems to be noticeably different compared to the surfaces of the CAS treated samples and also to acid treated enamel surfaces as they are described in literature [117,119]. This finding clearly supports our assumption that the polymers in the PMCAS were deposited on the enamel surface in form of a protective layer, which overlaid the eroded surface and led to less softening of the enamel surface [121].

To support our assumption that the polymers adsorb onto the enamel surfaces and build a protective layer this interaction was investigated also via fluorescence measurements during a student`s project. The polymer layers were deposited from PMCAS on *in vitro* enamel samples and were than stained with a fluorescence dye (fluoresceinisothiocyanat, FITC; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The most intensive fluorescence was observed for enamel samples treated with PGA- and GA containing PMCAS (**Figure 4-11**).

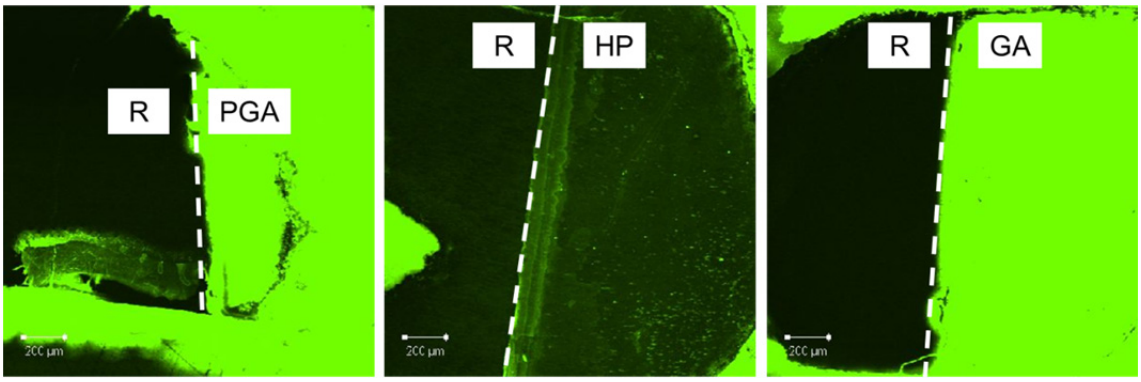


Figure 4-11: Fluorescence micrographs of PMCAS treated *in vitro* enamel samples. The dashed lines represent the interfaces between the untreated reference areas (R) and the eroded surface areas treated with PMCAS containing PGA, HP or GA. The scale bar is 200 µm.

This observation might be due to a higher amount of polymer molecules deposited on the enamel surface where therefore more FITC molecules could bind. For HP coated surfaces less fluorescence was observed that may indicate that fewer polymer molecules were deposited on the enamel surface. Another possibility would be that FITC showed a different binding behaviour to the different polymer molecules. However, this question could not be answered during this student`s project and should be further investigated. What could be shown with the fluorescence images was that the polymers adsorbed to the enamel surface during the treatment of the samples with the PMCAS. The polymers built up a nearly closed layer on the surface, which than according to our previous results protected the enamel surface against erosion.

Our results clearly show that the polymers in the PMCAS were deposited on the enamel surface in form of a protective layer. Our first presented model hypothesise that the polymers bind to the enamel surface dependent on electrostatic and chelate bonds. Our second model refines and clarifies the first one in that way that the protective layer on the enamel surface is hypothesised as two opposing gradients of polymer molecules and from the enamel dissolved HA particles. This polymer HA particle layer had a smooth surface and covered completely the enamel surface. Depending on the PMCAS treatment times the layer thickness was between 10 nm and 25 nm.

4.4 Human dental erosion caused by different acids with an identical sensorial taste

Besides the addition of polymers to acidic solutions to reduce dental erosion a completely different approach is to simply use different acids to reduce dental erosion, since different acids lead to different erosion effects [43,127,128]. For industrial application it is easily possible to exchange one acid against another in the soft drinks [78]. The influence of different acids on the extent of the erosion was investigated with

respect to the pH value, the acid concentration and time dependent [78,127,129]. So our approach was to investigate the influence of these well characterised and most often used acids (tartaric, malic, lactic, ascorbic, phosphoric and citric acid) on the process of erosion dependent on an identical sensorial acidic taste. This approach provides the opportunity to influence the erosive effects of soft drinks with a proper choose of the used acid. Studies about the interests of consumers could clearly show that their choice is mostly based on added functional ingredients (vitamins, stimulants), the type of packaging and the price [123]. Anyhow, besides these extrinsic factors, also intrinsic factors, e.g. the carbonation level, the flavour and the taste are important for the consumers preference [123,130]. The most important parameters to characterize the erosive potential of acidic solutions are the pH value and the titratable acidity [131,132]. To investigate the enamel softening AFM-based NI [80,121] was used.

Our results could clearly show that different acidic solutions with an identical acidic taste led to a different extent of erosion. For the investigated acids we found, that there were two groups of acids, the first one caused stronger enamel softening effects (phosphoric acid, ascorbic acid and lactic acid) compared to the other one, which caused lower enamel softening (citric acid, malic acid, tartaric acid). This finding is of high importance due to the fact that for consumers the acidic taste is one main important factor for their choice of soft drinks [123,130]. All acids led to dental erosion, but our results showed that some acids led to less softening of the dental enamel than others, but have an identical acidic taste. Thus, with a proper choice of the used acid the erosion caused damages of the dental enamel surfaces by the soft drinks can be regulated. The erosive effects of soft drinks or other acidic food can be reduced without any changes in taste for the consumers.

4.5 Conclusions

The acid induced process of dental erosion cause softening and after prolonged treatment times loss of the enamel material. We could demonstrate that the tactile profilometry measurements showed reliable values of material loss after long time erosion treatments compared to the non-tactile reference methods (CLSM). Concerning the short time erosion treatments we present that AFM-based NI is a useful method to measure and compare the softening of enamel caused by PMCAS and CAS. Samples treated with PMCAS showed significantly decreased enamel softening compared to samples treated with unmodified CAS. Thus, the addition of polymers to citric acid solutions is a promising and suitable tool to significantly reduce the erosive damage of the enamel surfaces by the acids. To summarize the main results of the current work the influence of CAS and PMCAS during the process of erosion of human dental enamel is shown in **Figure 4-12**.

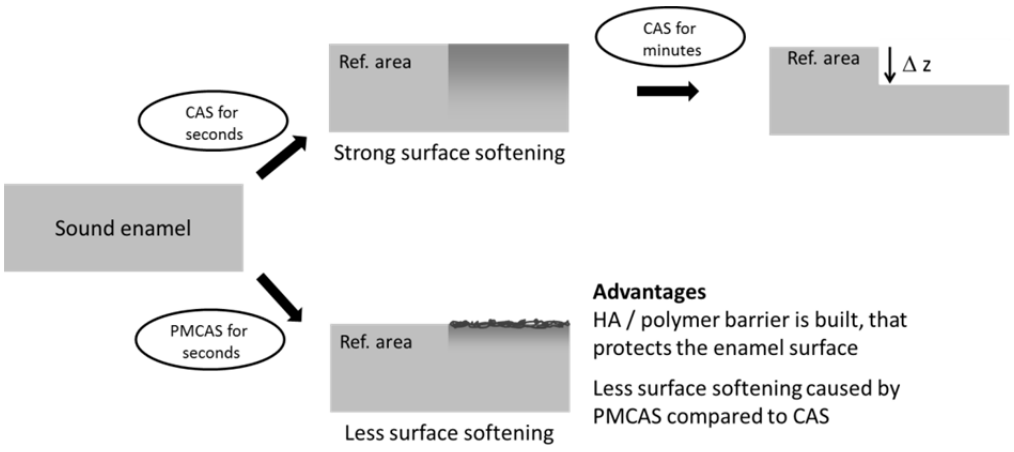


Figure 4-12: Erosion process of the enamel surface treated with CAS and PMCAS. The shadowed surfaces represent softened enamel after short time treatments with acidic solutions. Delta z (Δ z) represents material loss that occurs after long time erosion processes.

The used food-approved polymers (propylene glycol alginate, highly-esterified pectin and gum arabic) added to CAS showed binding interactions to the hydroxyapatite surface of human dental enamel. The presented results lead to the conclusion, that these polymers (PGA, HP and GA) directly interact with the HA surface of human

dental enamel. Within this contact a polymer-HA particle layer is built that acts as a protective barrier for the underlying enamel surface against the influence of the citric acid solutions after short time treatments. The interactions between the polymers and the enamel surface are triggered by chemically and electrostatically bonds and are presented in two complementary models. Besides the modification of acidic solutions with protective polymers there are other promising approaches to reduce the process of erosion caused by acids. It could also be shown that with a proper selection of the acid used in soft drinks the softening and erosion process can be influenced. Overall there are different promising approaches to reduce dental enamel erosion by modifications of soft drinks or food. Nevertheless, there is still research needed to deepen our knowledge about the process of dental erosion and to effectively and comprehensively reduce dental erosion on human enamel. Further strategies are required to lessen the erosive behaviour of acid containing food and drinks and to raise awareness among the consumers.

5 SUMMARY

Background Dental erosion is a dental disease that becomes more and more important in humans and is an increasing problem especially for children. The surface of the tooth, the enamel, is already been softened by the acids after short treatment times. A prolonged acid exposure to human dental enamel leads to irreversible material loss. The acids that are responsible for the erosion process are found in acidic food, especially in soft drinks. There are different approaches known that showed promising results to reduce dental erosion. One of them is the modification of the soft drinks in that way that they cause less erosion on human dental enamel.

Objectives Therefore, softening and material loss of enamel caused by acidic solutions comparable to soft drinks were investigated. Moreover, typical citric acid solutions were modified with food-approved polymers to investigate their erosion-reducing effect on human dental enamel. New polymer based strategies against dental erosion induced by citric acid were developed. In addition, the current study should give new insights into the interaction of polymers with enamel surfaces during the process of erosion. A second approach of this study was to investigate the effects equivalent tasting acids have on the erosion process of human dental enamel. Parts of this work were supported and accompanied by an industrial partner and industrial specifications.

Methods The material loss caused by citric acid solutions was measured with profilometry dependent on time (10, 20 and 40 min) the pH value (2.3 and 3.3) and compared to a non-tactile optical method (CLSM). AFM-based nanoindentation was used to investigate the softening of human dental enamel caused by citric acid solutions or other acidic solutions. Citric acid solutions were modified with three different polymers: propylene glycol alginate (PGA), highly esterified pectin (HP) and gum arabic (GA). The effects of such polymer modified citric acid solutions (PMCAS) on the nano hardness of human dental enamel samples were investigated dependent on time (30, 60 and 120 s) and pH value (2.3, 3.3 and 4.0) and were compared to the nano

hardness of enamel samples treated with citric acid solutions without polymer addition (CAS). To observe and compare the etch morphology of human dental enamel surfaces scanning electron microscopy (SEM) was used.

Results The profilometer measurements showed that both the tactile method (profilometer) and the non-tactile optical method (CLSM) showed similar material loss of human dental enamel. The profilometry led to scratches of 57.6 nm to 607.6 nm depth on the softened and eroded enamel dependent on the pH value and the treatment time. The profilometer tip caused scratches on the surface of the softened enamel. However, the scratch did not statistically significant increase the measured material loss.

The enamel softening caused by PMCAS was investigated depending on pH value, treatment time and used polymer. PMCAS treated enamel surfaces showed softening as well as the CAS treated enamel surfaces. However, the hardness loss (percentage) was increased over time (30 s to 120 s). Enamel samples treated with PMCAS (pH 3.3) showed, depending on the time, a lower hardness loss (PGA 7.7 % to 35.2%; HP 15.6 % to 53.1 % and GA 9.9 % to 49.8 %) than the loss of hardness of samples treated with CAS (19.9 % to 52.8 %).

SEM micrographs as well as AFM images showed that PMCAS treated enamel surfaces were less eroded and the typical enamel prism morphology, as described in literature for acid treated enamel surfaces, was not visible. The SEM micrographs indicated that the polymers were deposited on the enamel surfaces. In addition, XPS measurements indicated that the layer consists of polymer molecules and incorporated HA particles. On these layers profilometer scratches were made to estimate the layer thickness. The scratch depth of the PMCAS treated surfaces depended on treatment time (30 to 120 s) and the used polymer (PGA 8.4 nm up to 21.0 nm, HP 10.1 nm up to 21.07 nm and GA 7.7 nm up to 12.8 nm).

Different acids that were adjusted to an equivalent acidic taste caused different enamel softening. Two groups were found, whereas acid group one (MA, TA, CA) showed significant lower enamel softening then the acid group two (PA, AA, LA).

Conclusion Profilometry and AFM-based nanoindentation were useful methods to investigate the long time and the short time erosion process on human dental enamel. The investigated polymers were suitable as additives to citric acid solutions to statistically significantly reduce the hardness loss of enamel compared to unmodified CAS. The interaction of the polymers and the enamel surface led to the development of a layer consisting of polymer molecules and HA particles that protect the underlying enamel against erosion caused by citric acid. In addition, with a proper choice of the acid used for soft drinks, the enamel softening can be significantly influenced.

6 ZUSAMMENFASSUNG

Hintergrund Zahnerosion ist eine Erkrankung, die in letzter Zeit mehr und mehr an Bedeutung gewonnen hat und ein zunehmendes Problem besonders für Kinder darstellt. Die Oberfläche des Zahns, der Zahnschmelz, wird durch Säuren bereits nach kurzen Einwirkzeiten erweicht. Eine anhaltende Säureexposition führt zu einem massiven Materialverlust an Zahnschmelz. Die Säuren, die für den Erosionsprozess verantwortlich sind, findet man häufig in sauren Lebensmitteln, vor allem in alkoholfreien Getränken (Soft Drinks). Es sind verschiedene viel versprechende Ansätze bekannt, die gute Ergebnisse bei der Verringerung von Erosion zeigen. Einer davon ist die Modifikation der Soft Drinks auf die Weise, dass sie weniger Erosion des menschlichen Zahnschmelzes verursachen.

Zielsetzung Ziel war es die Erweichung und den Materialverlust des Zahnschmelzes ausgelöst durch saure Modelllösungen vergleichbar mit Soft Drinks zu untersuchen. Darüber hinaus wurden Zitronensäurelösungen mit für Lebensmittel zugelassenen Polymeren modifiziert, um deren erosionsreduzierende Wirkung auf den Zahnschmelz zu untersuchen. Neue Polymer-basierte Strategien gegen Säure-bedingte Zahnerosion sollten entwickelt werden. Weiterhin sollten neue Einblicke in die Wechselwirkung von Polymeren mit Zahnschmelzoberflächen während des Prozesses der Erosion gewonnen werden. Ein zweiter Ansatz der Studie war es, die Auswirkungen gleich sauer schmeckender Säuren auf den Erosionsprozess von Zahnschmelz zu untersuchen. Teile dieser Arbeit wurden durch einen industriellen Partner unterstützt und durch deren Vorgaben begleitet.

Methoden Der Zahnschmelzverlust verursacht durch Zitronensäurelösungen wurde mittels Profilometrie abhängig von der Zeit (10, 20 und 40 min) und dem pH-Wert (2,3 und 3,3) gemessen und mit den Ergebnissen einer nicht-taktilen optischen Methode (CLSM) verglichen. AFM-basierte Nanoindentation wurde verwendet, um die Erweichung des menschlichen Zahnschmelzes durch Zitronensäurelösungen oder andere saure Lösungen zu untersuchen. Zitronensäurelösungen wurden mit drei

verschiedenen Polymeren modifiziert: Propylenglycolalginat (PGA), hochverestertes Pektin (HP) und Gummi arabicum (GA). Die Auswirkungen solcher polymermodifizierter Zitronensäurelösungen (PMCAS) auf Erweichung des Zahnschmelzes wurde abhängig von der Zeit (30, 60 und 120 s) und dem pH-Wert (2,3, 3,3 und 4,0) untersucht, und mit der Erweichung verursacht durch Zitronensäurelösungen ohne Polymerzusatz (CAS) verglichen. Um die Ätzmorphologie des Zahnschmelzes hervorgerufen durch PMCAS, CAS oder anderen Säurelösungen zu vergleichen, wurden die erodierten Oberflächen mittels Rasterelektronenmikroskopie (SEM) untersucht.

Ergebnisse Profilometermessungen zeigten, dass sowohl mit der taktilen Methode als auch mit der nicht-taktilen optischen Methode (CLSM) ein ähnlicher Materialverlust an menschlichem Zahnschmelz gemessen wurde. Die Profilometrie erzeugt jedoch abhängig vom pH-Wert und der Behandlungszeit Kratzer zwischen 57,6 nm und 607,6 nm Tiefe auf den erweichten und erodierten Zahnschmelzoberflächen. Die entstandenen Kratzer erhöhen aber nicht statistisch signifikant den gemessenen Materialverlust.

Die Zahnschmelzerweichung durch PMCAS wurde abhängig vom pH-Wert, der Behandlungszeit und dem verwendeten Polymer untersucht. PMCAS als auch CAS behandelte Zahnschmelzoberflächen zeigten eine Erweichung der Schmelzoberflächen. Der Härteverlust (in Prozent) nahm mit zunehmender Behandlungszeit (30 s bis 120 s) zu. Zahnschmelzproben die mit PMCAS (pH 3,3) behandelt wurden, zeigten in Abhängigkeit der Zeit einen geringeren Härteverlust (PGA 7,7 % bis 35,2 %; HP 15,6 % bis 53,1 % und GA 9,9 % bis 49,8%) als mit CAS behandelte Proben (19,9 % bis 52,8 %).

Rasterelektronenmikroskopische (REM) Aufnahmen sowie AFM-Aufnahmen zeigten, dass PMCAS behandelt Schmelzoberflächen weniger erodiert erschienen und die typische erodierte Schmelzprismenmorphologie, wie sie in der Literatur für Säure-behandelte Schmelzoberflächen beschrieben wurde, nicht sichtbar war. Die REM-

Aufnahmen lassen zudem vermuten, dass die Polymere auf den Zahnschmelzoberflächen abgelagert wurden. XPS Messungen lassen vermuten, dass die Schicht aus Polymer-Molekülen und eingebundenen HA Partikeln besteht. Profilometerkratzer auf diesen Schmelzoberflächen wurden genutzt, um die Schichtdicke dieser Schichten abzuschätzen. Die Kratzertiefe hing dabei von der Behandlungszeit (30 bis 120 s) und dem verwendeten Polymer ab (PGA 8,4 nm bis 21,0 nm; HP 10,1 nm bis 21,1 nm; GA 7,7 nm bis 12,8 nm).

Säuren mit gleichem Säuregeschmack verursachten eine unterschiedlich starke Erweichung der Zahnschmelzoberfläche. Es konnten zwei Gruppen von Säuren gefunden werden, wobei die Säuren der Gruppe I (MA, TA, CA) statistisch signifikant weniger Zahnschmelzerweichung verursachten, als die Säuren der Gruppe II (PA, AA, LA).

Schlussfolgerung Profilometrie und AFM-basierte Nanoindentation sind geeignete Methoden, um die Langzeit- und Kurzzeiterosionsprozesse am menschlichen Zahnschmelzes zu untersuchen. Die untersuchten Polymere (PGA; HP; GA), die als Additive den Zitronensäurelösungen zugesetzt wurden, reduzierten den Härteverlust des Zahnschmelzes statistisch signifikant im Vergleich zu unmodifizierten Zitronensäurelösungen. Die Wechselwirkung der Polymere mit der Schmelzoberfläche führte zur Bildung einer Schicht aus Polymer-Molekülen und HA-Partikeln, welche den darunterliegenden Zahnschmelz vor Erosion durch Zitronensäure schütze. Darüber hinaus kann mit einer richtigen Wahl der Säure für die Verwendung in Soft Drinks die Zahnschmelzerweichung signifikant beeinflusst werden.

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SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass ich die Arbeit selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Die vorliegende Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt.

Jena, den 30.11.2011 _____

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LIST OF PUBLICATIONS, POSTERS AND ORAL PRESENTATIONS

PUBLICATIONS

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