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Short Communication

Probing the Enamel Topography After Acid Erosion by Scanning Electrochemical Microscopy

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In this work, we present an investigation on the thickness of the eroded enamel layer in tooth samples after exposure to citric and hydrochloric acid by using Scanning Electrochemical Microscopy (SECM). Approaching curves with typical negative feedback behavior were obtained in enamel samples for evaluation of topographic changes. In a control experiment, SECM images showed no significant difference in the current monitored during the scan, implying that enamel demineralization did not occur in mineral water medium. Topographic SECM images obtained after contact with citric and hydrochloric acid for different periods of time showed a significant increase in the current relative to a previously protected surface, indicating the structural loss of enamel. The thickness of the enamel layer eroded after contact with hydrochloric acid was significantly higher when compared to the one obtained with citric acid. Hence, our results showed that the enamel acid erosion is a relatively fast process, which is strongly dependent on parameters such as pH, time, acid strength and acid concentration.

Keywords: Scanning Electrochemical Microscopy (SECM), acid erosion, demineralization tooth, microelectrodes

1. INTRODUCTION

The Scanning Electrochemical Microscopy (SECM) has been widely employed in investigations of interfacial processes [1-3]. The possibility of obtaining quantitative information with high spatial resolution allows using SECM in several areas, as shown in some review papers published in the literature [4-7]. Microelectrodes are used as mobile probe in SECM because of some unique

properties, such as appropriate size, stability along the time scale of the experiment, low detection limits, selectivity and high signal / noise ratio [8]. Since the inception of SECM in 1989, different modes of operation have been developed to investigate the chemical properties of interfaces [9, 10]. The feedback mode is an amperometric mode of operation, widely employed in SECM to provide highly localized information on the concentration of electroactive species on a substrate [11], flow of electroactive species in membranes [12-14] as well as for obtaining information on surface topography [15]. The current detected at the tip is directly related to the composition of the solution, nature of substrate and separation (d) between the tip and substrate [16]. Approaching curves are obtained when the tip is driven from an infinite distance to the surface of the substrate and the plot relates the monitored current as a function of tip-substrate distance. The information obtained can be used to evaluate the geometrical characteristics of the tip [17], to determine the reactivity of a surface [18], to observe the profile concentration of a given species [19] and to obtain with considerable accuracy tip-substrate separation if the radius of the tip is known [9, 17].

The SECM has been often used in studies involving tooth as substrate. Important information about the flow of electroactive species that permeate through the dentin tubules [20] and the efficiency of precipitating agents [21] to restrain the transport have been obtained and they contributed significantly to investigations on tooth hypersensitivity. SECM was also used for the quantitative study of localized acid-induced dissolution in bovine enamel. The dissolution was observed as a fast process and the high rate of mass transport allowed to evaluate the kinetics of surface dissolution [22].

Dental hypersensitivity is largely studied in the dental community because it affects much of the population [23]. This problem occurs when the dentin is exposed to the oral environment and it becomes susceptible to variations in thermal, chemical, mechanical and evaporative stimuli on dental tubules [24]. The exposure of dentin may occur due to gingival recession, friction between the most external part of the tooth (enamel) or dental erosion caused by daily contact with acidic substances [25].

Dental erosion is a relatively new risk factor for dental health, caused by a lifestyle which includes the consumption of foods and drinks with high acidity [26, 27]. One of the highest mineralized tissues in the human body, tooth enamel is a composite material, organized into prisms, which is permeable and sensitive to ion exchange in the oral cavity. Thus, dental erosion occurs by dissolution of mineralized tissues without the involvement of bacteria. The protective hard coating, calcium hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, is demineralized with the resulting loss of tooth structure, in a process that takes place in accordance with equation 1 [28, 29].

$$Ca_{10}(PO_4)_6(OH)_2 + 14\ H^+ \rightleftharpoons 10\ Ca^{2+} + 6\ H_2PO_4^- + 2\ H_2O \qquad (1)$$

Dental erosion is a serious problem that can be caused by intrinsic and extrinsic factors. The dental erosion is associated with the intrinsic action of endogenous acids, i.e., acids produced by the body such as HCl in gastric juice. The extrinsic factors are due to exogenous acids, such as those present in soft drinks, sport drinks, juices, foods and drugs. The structural loss of enamel causes health hazards, such as pain, poor appearance and also hypersensitivity if the dentin becomes exposed.

Accordingly, studying this chemical process is important, since the acid erosion of enamel has often been discussed in dental offices.

The enamel loss after acid erosion has been investigated by techniques such as Electrochemical Impedance Microscopy (EIS) [30], Confocal Laser Scanning microscopy (CLSM) [31], Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM), Nanoindentation [26], Ultrasonic Pulse Echo [32] and Profilometry [33]. In this work, SECM was used as a new tool to examine the tooth enamel wear by acidic environments. Significant differences in the topography of bovine enamel were observed after a forced exposure to an erosive challenge using citric acid and hydrochloric acid.

2. EXPERIMENTAL

2.1. Reagents and Solutions

All chemicals employed were of analytical-reagent grade and used as received. Potassium ferrocyanide was obtained from Baker. Potassium chloride and citric and hydrochloric acid were obtained from Merck. All reagents were dissolved or diluted in water purified via a NanoPure Infinity System (Barnstead, Dubuque, 18 MΩ cm^{-1}). The mineral water (Crystal, Coca-Cola, Brazil) was obtained in a local market.

2.2. Instrumentation

Microelectrodes were fabricated using a Flaming/Brown Micropipette Puller model P-97 equipment and borosilicate glass capillaries (Sutter Instrument Company®). The capillary had the following dimensions: outside diameter 1.5 mm, internal diameter 0.86 mm and length 15 cm. Sealing of the microelectrodes was performed with the aid of a vacuum pump model NOF-650 (New Pump). An NI 150 High Intensity Illuminator (Nikon Instruments Inc.) optical light microscope was used to inspect the surface of the microelectrodes. The polishing of the platinum microdisc and the lateral sides of the glass capillary was done employing the BV-10 Micropipette Beveler (Sutter Instrument Company®) equipment. SECM experiments were performed on a Sensolytics GmbH Bochum (Germany) instrument using a conventional three electrodes system: a working electrode (Pt disc microelectrode), a $Ag/AgCl/KCl_{(sat)}$ reference electrode and a platinum wire as auxiliary electrode. Enamel samples were eroded in acid solutions under gentle agitation on an automatic shaker (Fisatom 752). The pH of the solutions was measured using a Metrohm 713 pH meter.

2.3. Fabrication of microelectrodes

Fig. 1 shows a scheme of the procedure used for the fabrication of microelectrodes using a Micropipette Puller. Firstly, a glass capillary containing a platinum microfiber (nominal radius of 10 μm and length 1.5 cm) is placed between two mechanical arms. An electrical resistance heats the capillary and leaves it soft and susceptible to stretching (step 1). Then, a second heating temperature is

employed for the sealing of the microfiber (step 2). At this stage, vacuum is applied to minimize subsequent problems arising from the possible presence of air bubbles. The cut of the whole microfiber/capillary is performed to expose the fiber and one capillary should produce 2 identical microelectrodes (step 3). The last part involves the polishing procedure performed and the electrical contact, which is done using indium wire (step 4). The main advantage of using this equipment is to allow a more accurate control of heating during manufacture, which is applied to only a fraction of the whole microfiber/capillary. Hence, very thin devices can be fabricated [34].

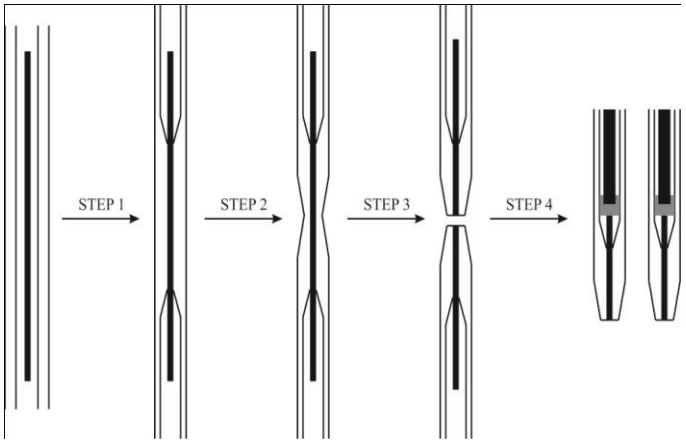

Figure 1. Schematic representation of the manufacturing process of disc microelectrodes using a Micropipette Puller.

2.4. Samples Preparation

Bovine mandibular incisor teeth were extracted without visible fractures and the soft tissue was removed using an appropriate instrument [35]. The samples were subjected to prophylaxis with pumice and water, and then stored in sanitizing solution of 0.5% chloramine T at 4 °C for a period of one month [36]. Subsequently, the teeth were fixed in a cutting machine (Extec ® Labcut 1010) with water cooling to obtain a flat surface. Enamel pieces were embedded in epoxy resin (Araldite®) for the mold manufacture. The polishing of the enamel surface was performed using water sandpaper (# 600 and 1200) followed by the use of felt alumina 1μm (Alfa Aesar). Samples were cleaned by ultrasonication in ethanol before and after polishing and stopped in deionized water after preparation.

2.5. Acid erosion of the enamel samples

Two different aqueous solutions, 20 mmol L^{-1} citric acid and 80 mmol L^{-1} hydrochloric acid, were employed for erosion experiments. The pH values of the solutions were measured with a pH-meter and adjusted using NaOH solution to give pH 2.2 and 1.2 for the citric acid and hydrochloric acid, respectively. The choice of pH and acid employed in the experiments was based on oral cavity exposure to gastric acids due to abnormalities in the gastrointestinal tract and unusual or abusive consumption of demineralizing acidic foods and beverages [37]. Mineral water of pH 7.1 was used in a

control experiment. Fig. 2 shows a sketch of the sample preparation for SECM studies. Fig. 2A shows the polished enamel, whose half area was covered with a protective resin layer to leave an exposed window of enamel (Fig. 2B). The covered surface was unaffected by erosion and was used as a control surface. The samples were then treated with 30 mL of each acid solution at room temperature. To prevent a local increased concentration of dissolved Ca^{+2} and PO_4^{-3} ions at the sample surface during the erosion process, the solutions were stirred with an automatic shaker at 50 rpm. At this experimental condition, the dissolution process takes place continuously as the layer of solution adjacent to the tooth is constantly replaced and the local saturation is not reached.

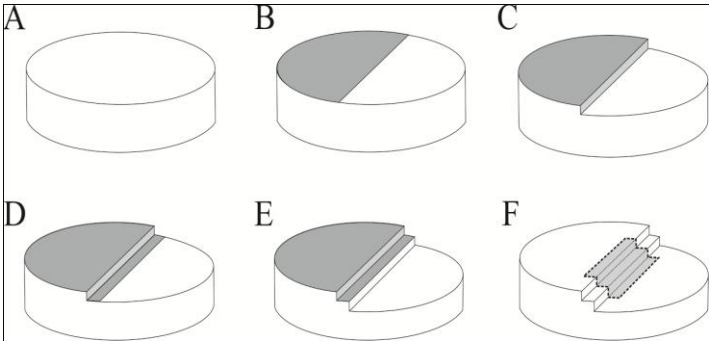

Figure 2. Sketch of the preparation of the samples for SECM studies.

After erosion time 1 (Table 1), the sample (Fig. 2C) was removed from the solution, rinsed with deionized water for 30 s and subsequently dried with compressed air. Then, the enamel surface was covered again with a new protective resin layer, as shown in Fig. 2D (width around 300 μm). After drying, the teeth samples were placed again in the acid solution (erosion time 2). Table 1 shows the parameters used in this experiment [31, 38].

Table 1. Solutions, pH and erosion time.

Solution	Concentration (mmol L^{-1})	pH	Erosion time 1	Erosion time 2
Mineral water	-	7.1	30	30
Citric acid	20	2.2	25	25
Hydrochloric acid	80	1.2	10	10

After erosion time 2 (Fig. 2E), the protective resin layer was removed by using acetone and a cotton swab. The sample surfaces were examined in an optical light microscope and any remaining resin was carefully removed. Topographic SECM images were recorded over a specific hatched area, as shown in Fig. 2F.

3. RESULTS AND DISCUSSION

Acids present in some fruits, drinks and even gastric juice demineralize the inorganic matrix of the tooth structure and leads to a softening step followed by a collapse of the enamel. The stability of the material comprising the enamel depends on the concentration of calcium, phosphate and fluoride ions (which were not investigated in this study), pH, acid strength, as well as the exposure time. To investigate the erosion process in vitro and to create a reference area, the enamel surface was covered by a protective resin layer. The enamel loss, based on an in vitro erosion process, results in a gap between the eroded enamel surface and the protected area (Fig. 2 C and 2E). The gap represents the thickness of the enamel layer consumed in the chemical reaction with acid substances.

Hence, approaching curves and topographic SECM images were obtained in the enamel samples for evaluation of topographic changes. Firstly, approaching curves were obtained over the reference area (polished enamel surfaces) using $K_3[Fe(CN)_6]$ as a probe. A Pt microdisc (r = 10 μm and RG = 10) was biased at -100 mV *vs*. $Ag/AgCl/KCl_{(sat)}$, a potential value that corresponds to the diffusion limiting current for the reduction of the electroactive species. The approaching curve obtained in the experiment (Fig. 3) confirms the insulator nature of the enamel, as the tip current tended towards zero as *d* (distance between tip-enamel) approached zero [16].

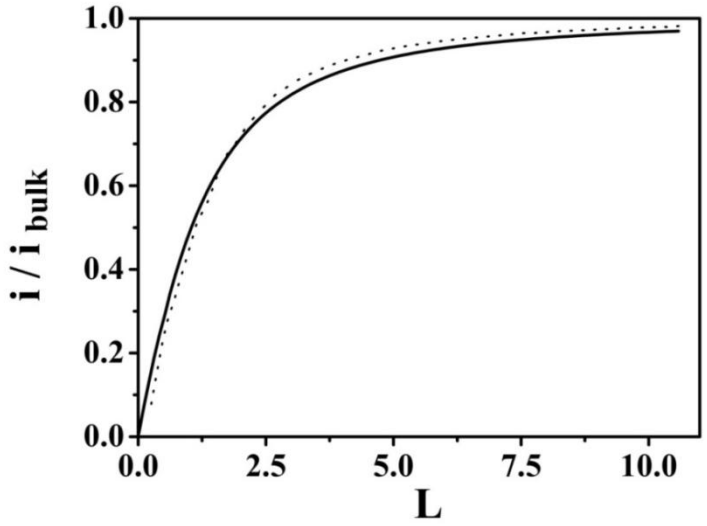

Figure 3. Approaching curve obtained with a Pt SECM tip (r = 10 μm) above a reference area (polished enamel surface) in 15 mmol L^{-1} $K_3[Fe(CN)_6]$ + 0.1 mol L^{-1} KCl solution (---). The solid line (—) represents the theoretical approaching curve for a tip with RG =10. E_{tip} = - 100 mV, ν = 1 μm s^{-1}. L= ratio between tip-substrate distance and microelectrode radius.

Fig. 4 displays topographic SECM images of dental enamel after exposure to mineral water (A), citric acid (B) and hydrochloric acid (C). No significant current changes were observed during the scan in the experiment carried out with mineral water (control), i.e., i/i_{bulk} = 0.19 (mean of all points). By taking into account this value and the approaching curve shown in Fig. 3, the distance between the

tip and substrate was found to be at around 4 μm, as L = 0.42 and the microelectrode radius was 10 μm.

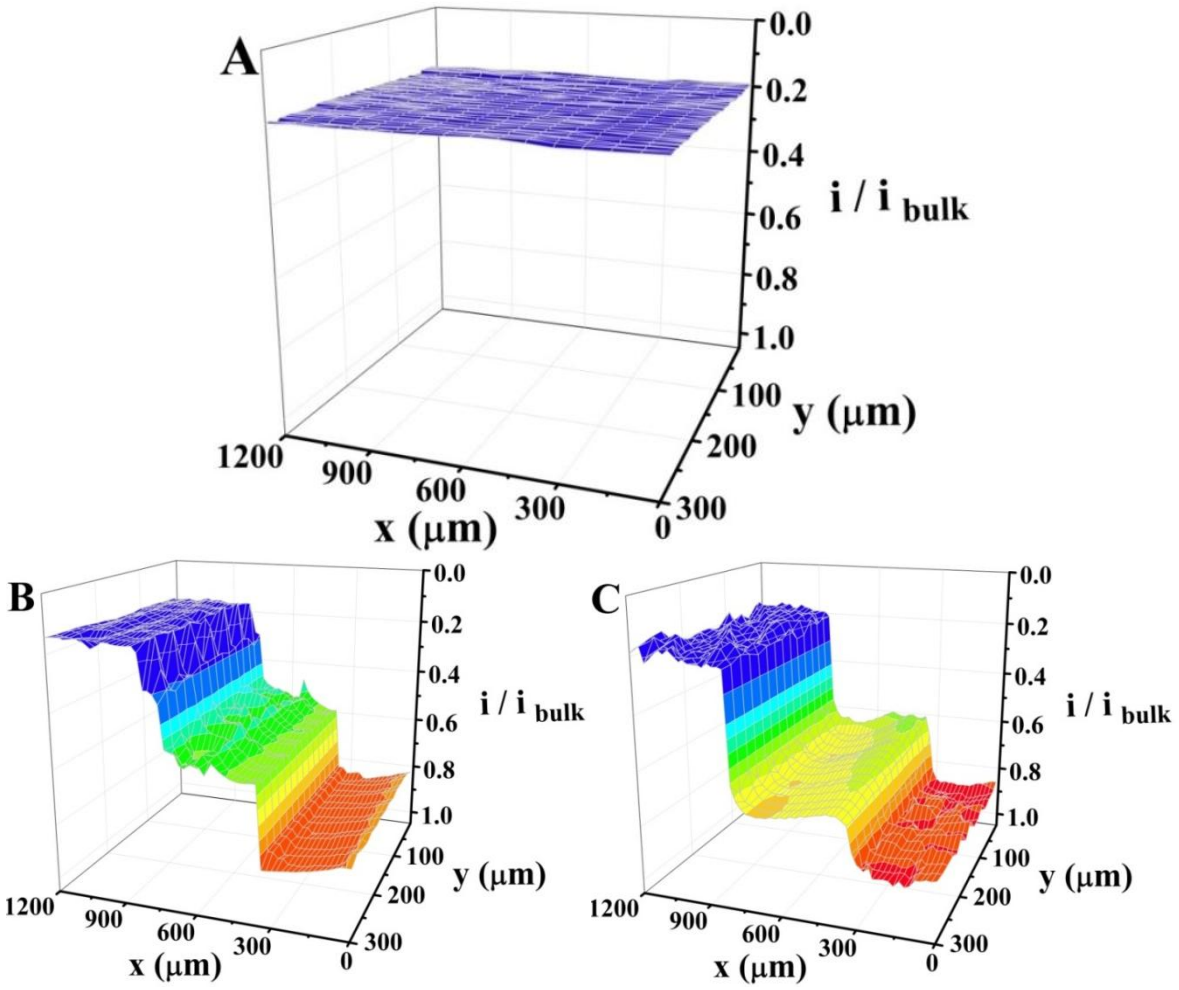

Figure 4. Topographic SECM images of the enamel surface after etching in mineral water (control experiment) (A), 20 mmol L^{-1} citric acid solution pH 2.2 (B) and 80 mmol L^{-1} HCl solution pH 1.2 (C). Experiments carried out with a Pt tip (r = 10 μm and RG = 10) in 15 mmol L^{-1} $K_3[Fe(CN)_6]$ + 0.1 mol L^{-1} KCl solution. E_{tip} = - 100 mV, and ν = 20 μm s^{-1}.

After the erosion process in citric acid and hydrochloric acid, enamel surfaces were examined in an optical microscope. The macroscopic appearance of the surfaces exposed to both acids with respect to the reference area became whitish, cretaceous and opaque, indicating that both solutions are potentially erosive. Fig. 4B and 4C shows topographic SECM images obtained after exposure to citric acid and hydrochloric acid, respectively. The current variation is a consequence of the increase in the tip-substrate separation, caused by the enamel demineralization after its contact with both acid solutions [29, 39].The thickness of the eroded enamel layer can be estimated using the relationship between i/i_{bulk} and L when the tip is close to the substrate [40, 41] and the results are shown in Table 2.

Table 2. Thickness of the enamel layer eroded in citric acid and hydrochloric acid.

	Thickness of the eroded layer in citric acid (μm)	Thickness of the eroded layer in hydrochloric acid (μm)
Erosion time 1	7	12
Erosion time 2	21	26

The amount of mineral dissolved from enamel is a function of the solution acidity, the buffering effect, the exposure time and the concentration of Ca^{2+}, PO_4^{3-} and F^-. By analyzing equation 1, it can be observed that the solubility of hydroxyapatite increases dramatically with decreasing pH. Accordingly, data shown in Table 2 confirms that the thickness of the enamel layer eroded after contact with hydrochloric acid was significantly higher when compared to the one obtained with a weak acid (pK_a for citric acid is 3.1), even though the exposure time was much lower. With respect to citric acid, this is also an important erosion agent because of its chelating action on calcium. Hence, the enamel erosion process may continue even after the pH rising at regions adjacent to the tooth surface.

Other methodologies have already been used to determine the thickness of eroded enamel after contact with citric acid. For instance, by using profilometric measurements Schlueter *et al* [33] found that the thickness of the eroded layer of a tooth sample immersed in 50 mmol L^{-1} citric acid (pH 2.3) for 20 and 60 min was (10.3 ± 6.5 μm) and (17.6 ± 6.1 μm), respectively. These values are consistent with those obtained in this work. It is worth noting that the structural loss is slower in the oral environment due to the remineralization effect of saliva, but at long times the erosion may be significant.

In vivo and in vitro ultrasonic measurements of thickness of eroded enamel have also been reported, but problems associated with reproducibility were noticed [32]. Confocal Laser Scanning Microscopy (CLSM) has the advantage of high resolution and fast recording of the surface topography, but the laser may penetrate the translucent enamel leading to background noise and artifacts due to water loss [31]. The in vitro enamel dissolution has been studied by AFM and SEM [26], but the use of SEM to evaluate the effects in enamel surface morphology requires special conditions for imaging, as low pressure ambient and coating with a conducting material, which change the natural conditions of the specimen structure. Besides, SEM imaging does not provide three-dimensional quantitative measurements of the surface morphology [42]. AFM allows one to obtain images with high spatial resolution and complete information about surface morphology, requiring minimal sample preparation. However, the cantilever tip may damage the enamel surface because after the erosion process it becomes fragile and liable to suffer scratches. Hence, the use of SECM to measure the thickness of the enamel layer dissolved by acid erosion may be considered a non-invasive technique that does not introduce artifacts due to water loss.

4. CONCLUSIONS

In this study, SECM has shown to be a powerful tool for investigating the topographical changes of bovine enamel after erosion in citric acid and hydrochloric acid. This approach can be used to measure the amount of enamel loss by directly determining the current monitored with the SECM tip positioned close to the tooth sample. Our results demonstrated the influence of exposure time, pH, acid strength and acid concentration on the thickness of the enamel layer eroded. In summary, SECM can be considered a facile and non-invasive technique, as it requires no special sample preparation steps and minimizes artifacts due to water loss.

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