Remineralization of Natural Tooth Enamel in Artificial Saliva Environment

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This paper investigates in situ remineralization of the acid-etched natural enamel surface, by incubation in a phosphate solution with neutral pH, at 37 °C for 4-10 days, without using any enamel matrix derivative (EMD). We investigated the morphology, crystallinity, chemical composition and structure of the newly grown layer onto the natural enamel surface stored in contact with artificial saliva (AS) having a composition similar to natural oral environment. The crystalline phases, crystallite size and orientation, as well as the chemical and phase composition of the remineralized dental enamel samples were studied by scanning electron microscopy coupled with energy dispersive X-ray spectrometry, Fourier transform infrared spectroscopy and X-ray diffraction. The experimental results showed that the enamel crystals grown on the demineralized enamel surface are mainly Ca-deficient apatite hexagonal structure. Fluoridated hydroxyapatite and sylvite-type KCl crystallites, which amount considerable increased in the case of sample stored in AS for 10 days, were also identified.

Keywords: artificial saliva environment, natural dental enamel, hydroxyapatite, crystalline structure

Tooth enamel is a highly mineralized extracellular matrix [1] and the hardest tissue in the human body [2]. Enamel is composed of 92-94% inorganic material, 2-3% water, 2% carbonate, 1% trace elements (sodium, magnesium, potassium, chloride, zinc), less than 1% lipids and about 0.01-0.05% fluoride [3-4]. The main inorganic material is crystalline hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$) [2] that is contained in enamel rods, which are the basic structural unit of enamel [5]. As the outer layer of teeth, enamel has to withstand a range of physical and chemical challenges like compressive forces, abrasion, attrition and importantly acidic challenges from plaque and diet [6-7]. Enamel is relatively stable in the healthy oral environment, where saliva and plaque fluid promote the balance between dissolution and deposition of minerals [1]. The rate and amount of dissolution depends not only on pH, but also on the concentration of calcium and phosphate ions in solution, according to the reaction described bellow. The enamel mineralization is relatively stable due to the natural processes that take place at the tooth/saliva interface, approximated with the following equation [8, 9]:

$$5\,Ca^{2+} + 3\,PO_4^{3-} + OH^- \rightleftharpoons Ca_5(PO_4)_3OH \quad (1)$$

In general, saliva is a neutral (*p*H≈7) body fluid, but acidic agents can be introduced into the mouth by food and drinks [10]. For example, *p*H values of acidic drinks can range from 1 to 6 [5]. At *p*H<5.5, HAP crystals can dissolve in the process known as demineralization which ocurrs on the surface of enamel [11]. Erosive substance loss of enamel is a dynamic process with demineralization and remineralization. Remineralization is the process of restoring mineral ions into the hydroxyapatite latticework structure. In a healthy oral environment, at *p*H 7.0–7.4, the remineralization of the upper layers of tooth enamel occurs naturally, ensuring the maintenance of the natural composition of enamel, which is an acellular tissue.

Saliva is essential for tooth remineralization because it supplies calcium and phosphate ions to build HAP blocks [12] into crystal voids of the demineralized enamel. It can act as a natural buffer to neutralise acid and restrict the dissolution process. At *p*H>5.5 and high concentration of calcium and phosphate ions, the equilibrium can be shifted to the re-precipitation of calcium phosphate with the surface remineralization of the demineralised enamel [13]. The remineralization of the acid-eroded enamel in saliva medium is now considered a very convenient method to restore tooth superficial lessions. Most studies focused on the contribution of the remineralization treatments of the enamel caries lesion [5]. It has been revealed that the surface of enamel softened by acidic beverages could be rehardened by following exposure to saliva and artificial saliva [14-15]. Zheng et al. succeeded to remineralize *in vitro* acid-eroded human tooth enamel by immersing in artificial saliva for 12 h [5].

This paper investigates *in situ* remineralization of the acid-etched dental enamel surface, by incubation in a phosphate solution, at 37°C for 4-10 days, without using any enamel matrix derivative (EMD). We have investigated the morphology, crystallinity, chemical composition and structure of the newly layer grown in enamel surface in contact with artificial saliva with composition similar to natural oral environment.

Experimental part

Selected human molars without caries or restored caries were collected from local dental clinics ('Ovidius' University of Constanta, Faculty of Dentistry) according to the ethic protocol and the patients were informed and consented to the use of teeth. The samples were treated with sodium hypochlorite solution (3 wt%) and phosphate buffer saline to remove bacteria and stored in distilled water at 4°C until use. The molars were sectioned longitudinally (2 mm thick slices) with a diamond blade under water cooling. All

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samples were individually sonnicated in demineralized water for 5 min to remove the residual abrasives. Enamel surfaces were demineralized by acid-etching using 36% phosphoric acid for 60s, followed by cleaning under ultrasounds for 2 min and rinsed with deionized water 3 times (denoted as S0 sample). The demineralized surfaces were immersed in artificial saliva (AS) at 37 °C for 4, 7 and 10 days (S1-S3 samples), to investigate to what extent saliva in neutral environment, can promote the nucleation and growth of apatite crystals. The AS was prepared according to the recipe proposed by Fletcher et al [16]: 0.2 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM Na_2HPO_4, 16 mM KCl, 4.5 mM NH_4Cl and 20 mM HEPES (4-(2-hydroxyethyl) piperazine-1-ethane-sulfonic acid) buffer. The *p*H of the resulted solution was adjusted to 7.0 with 1 M NaOH and stored at 4 °C. Before using the solution, sodium fluoride (300 ppm) was added. The morphology and elemental chemical composition on the surface of the investigated samples were examined by scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM/*EDX*) using a FEI Q 200 microscope, in low vacuum conditions. Before examination, the samples were coated with a 4-5 nm thick conducting layer of Au using a SPI-Module™ sputter coater system. Fourier transform infrared spectroscopy (FTIR, Nicolet 6700) was used for investigation of chemical structure and composition of the obtained samples. The FTIR spectra of samples were obtained with 4.0 cm^{-1} resolution. Each spectrum was obtained with 80 scans in the range of 4000 to 400 cm^{-1}. The XRD patterns were collected using a Rigaku diffractometer type Ultima IV equipped with thin film attachment for grazing incidence X-ray measurements, at an incidence angle ω=0.3°. The source of the X-rays was a Cu tube (λ = 0.15418 nm) operating at 40 kV and 30 mA. The XRD data were recorded in the range of 10-80°. Rigaku's PDXL software package, connected to the ICDD database was used for the phase identification and crystallite size calculation. The XRD pattern was fitted using Whole Pattern Powder Fitting (WPPF) method. Crystallite size (D) has been calculated using Scherrer's formula:

$$D = k \cdot \lambda / (FWHM) \cdot \cos(\theta) \quad (2)$$

where: k is a shape factor (0.94), FWHM is the full width at half maximum of the intensity vs. 2θ profile, λ is the wave length of the Cu Kα radiation (1.54056 Å) and θ is the Bragg's diffraction angle.

Results and discussions

Morphological and elemental chemical composition

Figure 1 shows the SEM top-view images of the acid-etched natural enamel sample (S0) together with the samples obtained after immersion into artificial saliva (AS) for 4 (S1), 7 (S2) and 10 (S3) days. According to SEM images, even the specific HAP honeycomb-like structure generated by assembling nanorod crystals can be clearly seen, the surface tooth enamel crystals of the acid etched sample become discontinuous and broken (S0 in fig 1). As shown in SEM images in figure 1, newly grown layers were formed on the surface of the remineralized tooth enamel samples (S1-S3) after being soaked by AS between 4 and 10 days. Newly-growth crystals formed after 4 days (S1) of immersion into AS are different from the original HAP crystals of acid-demineralized enamel (S0). The honeycomb-like profile of acid-etched tooth enamel is still clearly visible after storing in AS for 4 days, although the original surface is almost covered by the new grown crystals (S1). For the sample stored in AS for 7 days (S2)

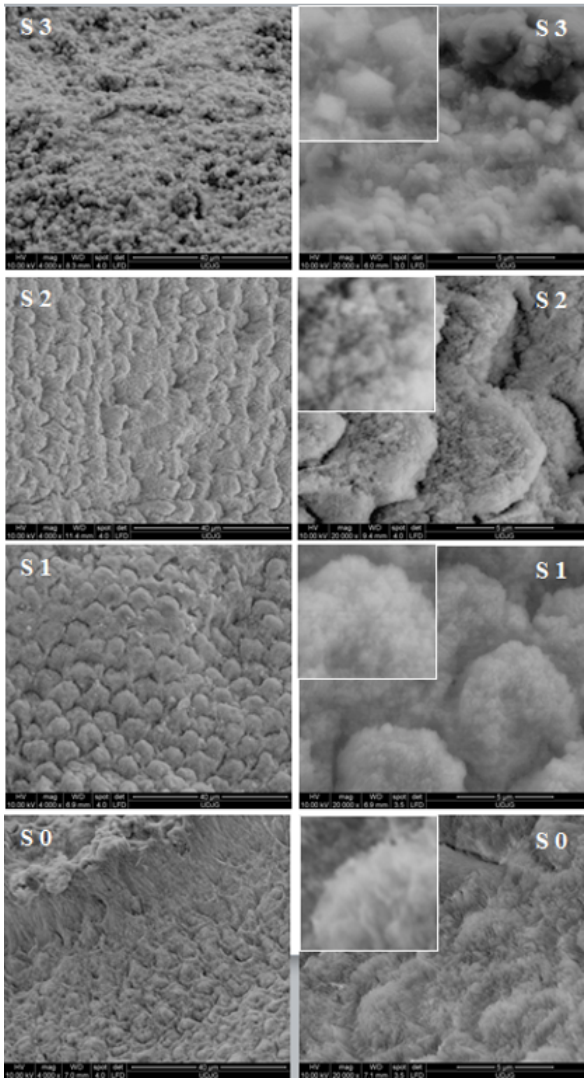

Fig. 1 SEM images of acid-etched enamel (S0) and remineralized acid-etched enamel samples stored in artificial saliva for 4 (S1), 7 (S2) and 10 days (S3); 40μm (left) and 5μm (right) magnifications

one can be seen nanorod-like crystals with small aspect ratio (length/diameter), formed due to a certain capability of exposed surface of natural enamel crystals to attract calcium and phosphate ions from soaking saliva to form patterned nanorod-like HAP crystals [17]. However, this remineralizated layer shows not very good structural control, due to lack the structural regulation by organic matrix. The length of HAP nanorod crystals observed in the SEM images in figure 1 ranges from 0.36μm (S0) to 1 μm (S1) and 1.27μm (S2). In the case of sample stored in AS for 10 days (S3), the surface is almost totally covered by individual and agglomerated crystals with a totally different geometry. In the case of the sample S3, the average size of the crystals grown on the top of the remineralized layer, observed from SEM images in figure 1, ranges from 450 nm for smaller crystals to about 1μm for the largest crystals.

The EDX spectra of the investigated samples, recorded on 50 x 40 mm areas (fig. 2) revealed that the main elements from the composition of the remineralized layer were calcium, phosphate and oxygen for samples. It was found that Ca/P average molar ratio was increased from 1.26 for acid-etched natural enamel (S0) to 1.49 for the sample immersed in AS for 7 days (S2), but decreased to 1.40 for the sample immersed in AS for 10 days (S3), together with an important increase of fluoride, chloride and potassium amounts. In the last case, the increase of F, Cl, K and Mg atomic concentration suggests that several

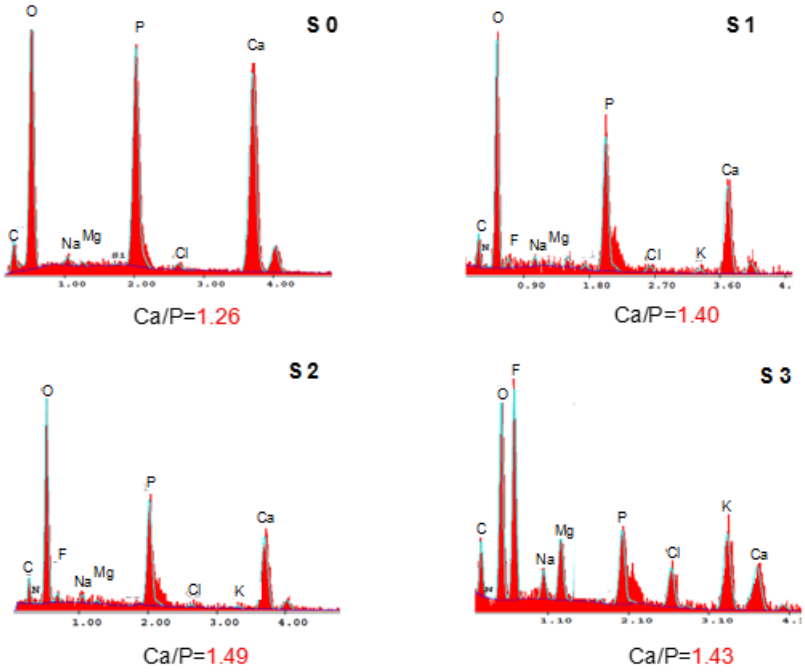

Fig. 2 EDX spectra of acid-etched enamel (S 0) and remineralized acid-etched enamel stored in artificial saliva for 4 days (S1), 7 days (S2) and 10 days (S3)

different types of calcium phosphate and other phases, like fluoroapatite (FAP), carbonatoapatite or fluoride and carbonates may be precipitated during the remineralization process [9]. The above mentioned values of Ca/P atomic ratio below 1.67, specific to stoichiometric hydroxyapatite crystal with chemical formula $Ca_{10}(PO_4)_6(OH)_2$ [2], can suggest that the new grown layers contain fluoroapatite calcium-deficient hydroxyapatite.

Figure 3 shows EDX spectra of samples S1 and S3, recorded on selected punctual area, in order to obtain more information about the elemental composition of specific areas. In the case of sample S3, EDX spectrum confirms that the big increase of F, Cl and K contents comes from the individual or agglomerated polyhedral crystals grown onto the surface of enamel (S3, fig. 1). SEM-EDX mapping of chemical elements on the surface of the acid-etched natural enamel (S0) and the samples immersed in artificial saliva (AS) for 4 (S1), 7 (S2) and 10 (S3) days (fig 4), confirms the relatively uniform distribution of Ca, P and O elements within the remineralized layer grown onto the natural enamel surface. An increased quantity of fluoride atoms is clearly visible for sample stored in AS for 10 days (S3), at the same time with a decrease of elemental concentarion of Ca and P.

Chemical and crystalline structure of remineralized samples

For the qualitative and semi-quantitative analysis, the FTIR absorption bands considered for this study were the superposition of the ν_1 and ν_3 vibration modes of phosphate (1300-900 cm^{-1}), amide I (1680-1600 cm^{-1}), amide II (1580-1480 cm^{-1}), amide III (1200-1300 cm^{-1}), the ν_2 vibration mode of carbonate (around 870 cm^{-1}) and the superposition of the stretching μ_3 and bending μ_4 vibration mode of carbonate (between 1600-1300 cm^{-1}) [18].

Figure 5 shows the FTIR spectra for the acid-etched enamel sample (S 0) and samples S1-S 3 stored in artificial saliva for 4 - 10 days. The characteristic bands of PO_4^{3-} group appears in FTIR spectra as absorption bands at 900-1140 cm^{-1} (ν_1 and ν_3); 615-623 cm^{-1} (ν_4) and 589-575(ν_4) and at 448-474 cm^{-1}(ν_2). CO_3^{2-} group forms weak peaks between 900 and 780 cm^{-1} (ν_2). Similar bands for PO_4^{3-} group and CO_3^{2-} group were recorded by others authors in bones and hydroxyapatite spectra [18-22]. Regarding the bands for PO_4^{3-} group in infrared spectra of hydroxyapatite depending on preparation route their position can be shifted to higher or smaller wavenumbers. Acid-etched enamel sample has similar FTIR spectra, characteristic to carbonated hydroxyapatite. In the case of samples S1 to S2, one can observe an increase of bands intensities for

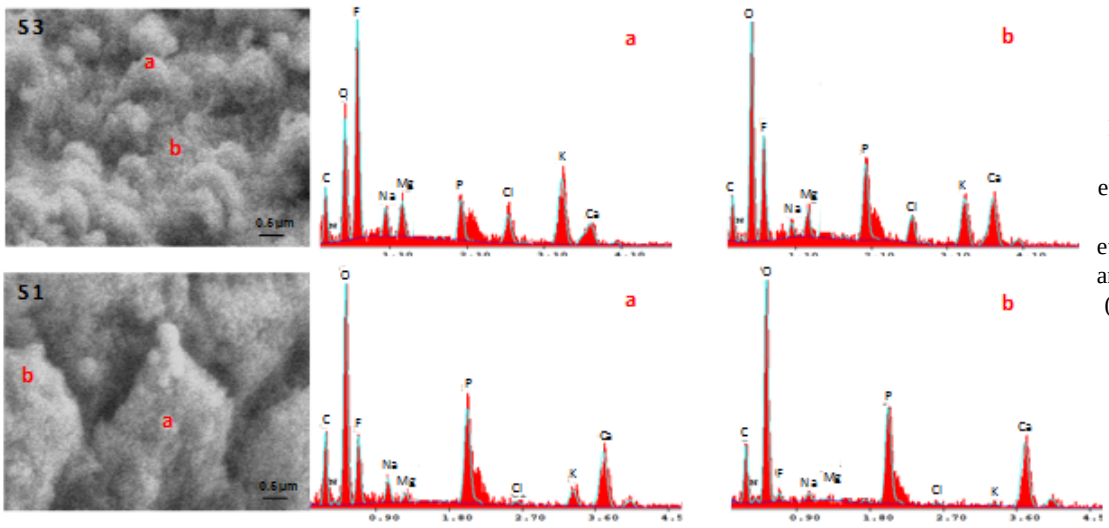

Fig. 3 SEM images and EDX spectra of acid-etched enamel (S 0) and remineralized acid-etched enamel stored in artificial saliva for 4 days (S1), 7 days (S2) and 10 days (S3)

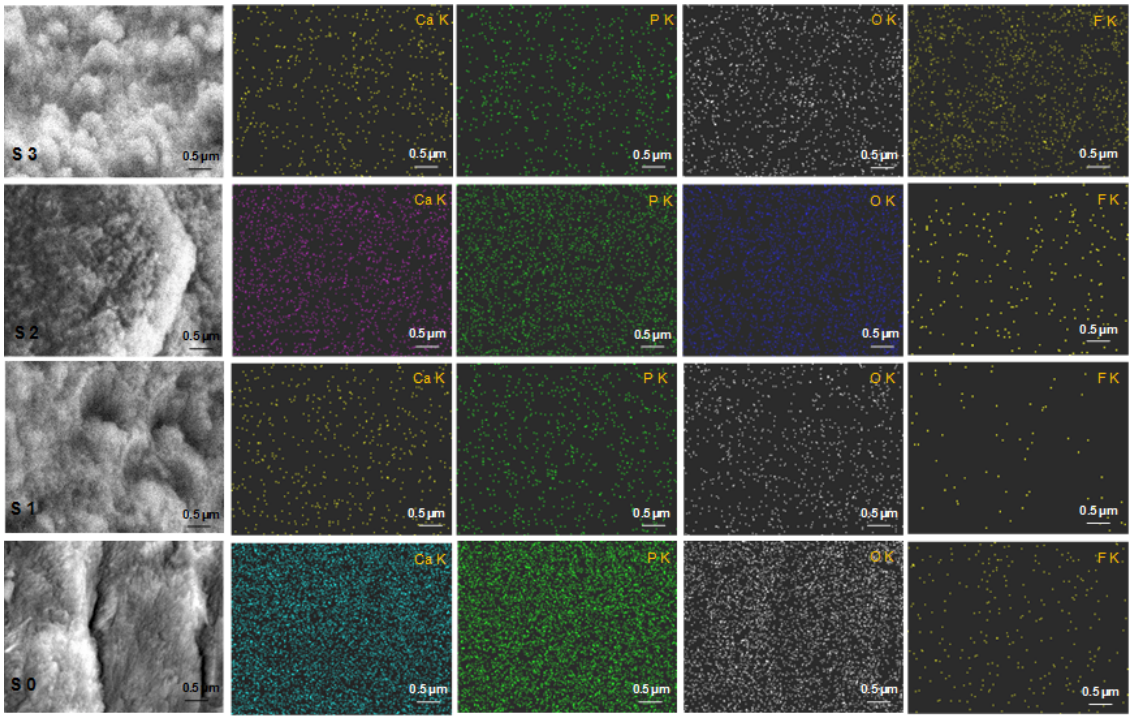

Fig.4 SEM-EDX elemental mapping for Ca, P, O and F elements on the acid-etched enamel (S0) and remineralized acid-etched enamel stored in artificial saliva for 4 days (S1), 7 days (S2) and 10 days (S3)

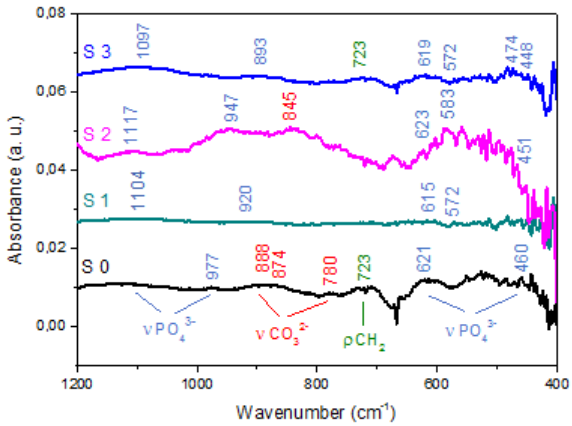

Fig. 5. FTIR spectra of acid-etched enamel (S0) and remineralized acid-etched enamel stored in artificial saliva for 4 days (S1), 7 days (S2) and 10 days (S3).

hydroxyapatite (HAP) demonstrating an increasing of the cristallinity HAP crystals The spectrum of S3 sample shows less intense bands corresponding to the decrease of quantity of HAP crystallites. Also the bands of carbonate disappear from the spectrum of sample S3.

Figure 6 shows the diffraction patterns of the investigates samples. On can clearly see that in the case of acid-etched (S0) and remineralized samples for 4 and 7 days (S1 and S2), the main peaks can be assigned to the hexagonal HAP (JCPDS card No. 09-0432). More specifically, the diffraction peaks at 2θ of 25.7°, 31.8° and 32.9° are consistent with (002), (211) and (300) reflections of hexagonal HAP, respectively. Further XRD examinations showed that the deposits on the remineralized surface mainly consist of HAP crystals, which is the major inorganic component of natural enamel, with preferential c-axes orientation growth corresponding to (002) as the most intense peak. The crystals on the original surface of enamel show specific preferred orientation demonstrated by the sharp and well defined peak corresponding to the (002) crystal plane of HAP. Well defined peak at 2θ=25.9° was observed for the remineralized surface. The diffraction patterns of samples S1 and S2 also show small diffraction peaks that can be assigned to KCl crystals and even to small KF (2θ of 44.7°) crystals grown on the top of the

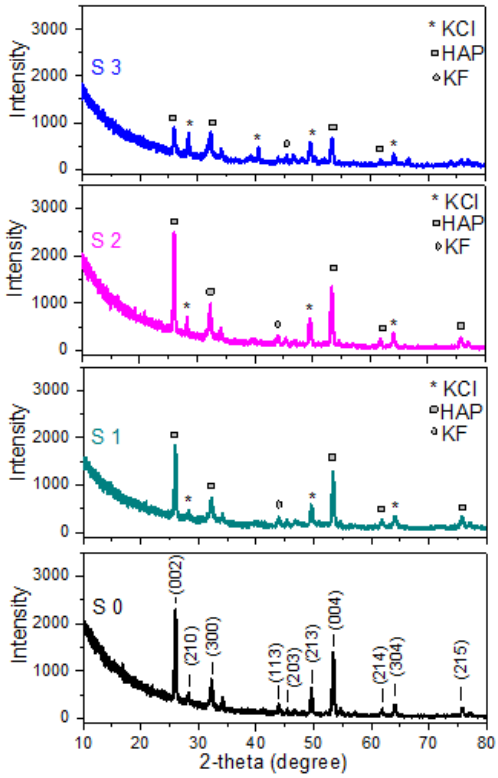

Fig. 6. XRD patterns of demineralized enamel (S 0) and remineralized enamel stored in artificial saliva for 4 days (S1), 7 days (S2) and 10 days (S3)

remineralized layers. The intensities of the peaks assigned to KCl sylvite phase increase in the case of sample stored in AS for 10 days (S3) together with the decrease of the intensity of HAP peaks, in agreement with the EDX spectra and mapping results mentioned before.

In conclusion, the crystallinity on the remineralized HAP layers is lower than that of the original enamel; it increased with increasing duration of incubation in the AS from 4 to 7 days and decreased for samples stored in AS for 10 days. The structural parameters of crystalline phases, calculated using Scherrer's equation and XRD (002) peak fitting, identified into the remineralized layer newly deposited on

No. crt.	Sample cod	Crystalline phase	Lattice parameters		Crystallite size (nm)	Ca/P ratio [at%]
			a (A)	c (A)		
1.	S0	Hydroxylapatite, syn	9.2908	6.8869	20.7	1.26
2.	S1	Hydroxylapatite, syn	9.3478	6.8848	19.4	1.40
3.	S2	Hydroxylapatite, syn	9.3662	6.8887	18.8	1.49
4.	S3	Hydroxylapatite, syn	9.4142	6.9015	10.4	1.43
		Sylvite, syn	6.2983	6.2983	23.7	-

Table 1
STRUCTURAL PARAMETERS OF CRYSTALLINE PHASES OF THE REMINERALIZED NATURAL ENAMEL SAMPLES

the surface of the acid-etched natural enamel samples by incubation into artificial saliva, are presented in table 1.

From table 1, one can observe an increase of the lattice parameter values, when comparing to the natural enamel (S0), but also with increasing the incubation time, from 4 to 10 days. At the same time, a decrease of crystallite size was observed, from 20.7 nm for the reference sample (S0) to 19.4, 18.8 and 10.4 nm for samples stored for during 4, 7 and 10 days (S1-S3).

Sylvite phase of synthetic KCl crystallites of 50nm size with 6.2983 Å lattice parameter values was identified by XRD data processing.

Our results demonstrated that artificial saliva could induce *in situ* remineralization of HAP onto the surface of acid-etched natural enamel. The new generated HAP crystals after 4 and 7 days of storage into AS are nanorod-like with almost uniform size and shape and crystalline structure, similar to natural tooth enamel. The lattice parameter values of the newly grown HAP phase increase and the crystallite size decreases, when comparing to the natural enamel. This behavior continues with increasing the storage time in AS from 4 to 10 days. Sylvite-type KCl crystallites, which amount considerable increased in the case of sample stored for 10 days in AS, were also identified onto the surface of the remineralized samples.

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References

1. CHUNG, H.Y., LI, C.C, HSU, C.C., J. Mech. Behav. Biomed. Mat., **6**, 2012, p. 74-79.
2.MANN, A., DICKINSON, M., Nanomechanics, Chemistry and Structure at the Enamel Surface (Chapter 5) in: The Teeth and Their Environment. Monographs in Oral Science, vol. 19, DUCKWORTH, R.M., Editor, Karger, Basel, 2006, p. 105-131.
3. HICKS, J., GARCIA-GODOY, F., FLAITZ, C., J. Clin. Pediatr. Dent., 2**8**, no. 2, 2005, p. 119-124.
4. FEATHERSTONE, J.D.B., Oral Health. Prev. Dent., **2**, no. 3, 2004, p.259-264.
5. ZHENG, L., ZHENG, J., WENG, L.Q., QIAN, L.M., ZHOU, Z.R., Wear, **271**, 2011, p. 2297-2304.
6. JONES, F.H., Surf. Sci., **42**, 2001, p. 75-205.
7. WEST, N.X., JOINER, A., J. Dent., **42**, suppl. 1, 2014, p. S2-S11.
8. DOROZHKIN, S.V., J. Colloid Interf. Sci., 191, 1997, p. 489-97.
9. WILLIAMS, D. R., WU, L., Chem. Spec. Bioavailab., 5, no. 3, 1993, p. 107-117.
10. LEWIS, R., DWYER-JOUCE, R. S., J. Eng. Tribol., 219, 2005, p. 1-18.
11. LeGEROS, R.Z., Calcium Phosphates in Oral Biology and Medicine. Monographs in Oral Science, vol. 15, Karger, Basel, 1991, p. 1-201.
12. ZERO, D.T., LUSSI, A., Int. Dent. J., **55**, 2005, p. 285-290.
13. LI, X., WANG, J., JOINER, A., CHANG, J., J. Dent., **42,** suppl. 1, 2014, p. S12-S20.
14. AMAECHI, B.T., HIGHAM, S.M., J. Dent., **33**, 2005, p. 243-252.
15.DEVLIN, H., BASSIOUNY, M. A., BOSTON, D., J. Oral Rehabil., **33**, 2006, p. 26-30.
16. FLETCHER, J., WALSH, D., FOWLER, C.E., MANN, S., CrystEngComm., **13**, 2011, p. 3692–3697.
17. CHEN, H., BANASZAK HOLL, M., ORR, B.G., MAJOROS, I., CLARKSON, B.H., J. Dent. Res., **82**, 2003, p. 443-448.
18. ZEZELL, D.M., BENETTI, C., VELOSO, M.N., CASTRO, P.A.A., ANA, P.A., J. Braz. Chem. Soc., **26**, no. 12, 2015, p. 2571-2582.
19. VRÂNCEANU, M.D., ANTONIAC, I., ªABAN, R., ALBU, M., MICULESCU, F., UPB. Sci. Bull., Series B, **74**, no. 3, 2012, p. 145-156.
20. QUERIDO, W., ROSSI, A.L., CAMPOS, A.P. C., ROSSI, A.M., FARINA, M., Mater. Res., **16**, no. 5, 2013, p. 970-974.
21. SLOSARCZYK, A., PASZKIEWICZ, Z., PALUSZKIEWICZ, C., J. Molec. Struct. **744–747**, 2005, p. 657–661.
22. BERZINA-CIMDINA, L., BORODAJENKO, N., Chapter 6, Research of Calcium Phosphates Using Fourier Transform Infrared Spectroscopy, in: Infrared Spectroscopy – Materials Science, Engineering and Technology (THEOPHANIDES, T., Editor, InTech Europe, Rjeka, 2012, p. 127.

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