Human Dentine Remineralization Under Non-colagen Materials Action

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The purpose of this work is to induce biomimetic remineralization of acid etched coronal human dentine in artificial saliva (AS) under agarose (A) hydrogel or chitosan (CS)-A hydrogel action. The investigations focused on the morphology, chemical composition and crystalline structure of the new remineralized layers grown onto the etched dentinal surface (R) using scanning electron microscopy coupled with energy dispersive X-ray spectrometry and micro-Raman spectroscopy. Experimental results showed that remineralized layers grown in the presence of A or CS-A hydrogels consist in B-type Ca-deficient hydroxyapatite (HAP). After 7 days treatment into artificial saliva under agarose hydrogel, nanorod-like extrafibrilar HAP crystals randomly self-assembled in a discontinuous layer were formed, while in presence of chitosan-agarose hydrogel a continuous compact CS-HAP composite layer was obtained. The new biomimetic layer (A-CS4) formed after 4 days on dentine surface under A-CS hydrogel has higher crystallinity. Longer exposed (7 days) dentine in the presence of agarose hydrogel shows a higher mineral-to-collagen ratio (A7). Since dentine mineralization increases, the collagen quality factor decreases in succession A-CS4>R>A7. Results show a benefic effect of chitosan on remineralization of etched dentine.

Keywords: artificial saliva, agarose hydrogel, chitosan, human dentine, hydroxyapatite

Dentine is a complex tissue [1], which contains 50-70 vol.% calcium-deficient and carbonated hydroxyapatite (HAP, Ca₁₀(PO₄)₆(OH)₂) and 30 vol.% of organic components [2]. The organics are represented by 90% type I collagen with a minimal amounts of types III and V collagen, and 10% water containing non-collagenous proteins. A percentage of 20% of the dentine volume is made of free and bound water [3]. Dentine is covered by enamel in the crown and cementum in the root and completely surrounds the pulp [4]. Dentine consists of dentinal tubules which are microscopic channels radiating from pulp to the cementum or enamel of tooth. Dentine tissue surrounding the tubules (peritubular dentine) is relatively densely mineralized, while the remaining dentine tissue between the tubules (intertubular dentine) is hypomineralized [5].

Dentine exposure resulting from loss of enamel and gingival recession is considered a key predisposing factor leading to dentine hypersensitivity [6]. Pulpo-dentine complex is one structure due to pulp cell knobs and nerve fibers placed within dentinal tubules. Dentine sensitivity to stimuli depends on the pulp and pulp reactions depend on changes in dentine. The interdependence of the two tissues is observed in inflammatory processes of the pulp [7-10]. Pulp damaging factors include attrition, erosion, abrasion, tooth decay, recession, as well as mechanical, chemical and thermal factors [11]. Acid-producing bacteria feed on fermentable carbohydrates and produce organic acids as byproducts [12]. These acids dissolve hydroxyapatite (HAP), forming a caries lesion [13]. If this process continues, the cavitation occurs. Prior to cavitation, a subsurface lesion with partial demineralization is present, and this lesion can be reversed and remineralized under appropriate conditions. Saliva is essential for tooth remineralization because it supplies calcium and phosphate ions to build HAP blocks [14]. In natural oral environment, the remineralization of dental tissues is the natural repair process that takes place at the tooth/saliva interface [15]. However, natural remineralization can only overcome a certain level of demineralization and initial caries. When bacterial acid challenge is severe, natural remineralization is insufficient to halt or reverse the caries process [16]. Remineralization of demineralized dentine is the process of restoring minerals through the formation of inorganic mineral-like materials [17].

Biomimetic remineralization of dentine has been investigated using different non-collagen materials, from solution containing Ca^{2+} , SiO_4^{-4} , F or PO_4^{-3} ions [18] or ions leached from ultrafine bioactive glass particles [19]. Gandolfi et al. developed bioactive calcium-releasing lightcurable hydrophilic composites with tailored remineralizing properties to be used as restorative base-liner materials in sandwich restorations [20]. Among many non-collagen remineralization materials reported in literature [21], agarose (A) polysaccharide has been less used for teeth remineralization [22]. Ning et al. designed a biomimetic remineralization system of dentine with agarose gel loaded with calcium phosphate which can aid in the development of a new method to treat dentine hypersensitivity and dental caries [22]. In the absence of residual crystals, demineralised dentine could not induce spontaneous nucleation of minerals inside of the organic matrix and consequently can not facilitate deposition of calciumphosphate minerals [21]. Agarose hydrogel not only provides the microenvironment to mimic mineral formation, but also can be used as template to control dentine remineralization due to it molecular structure that can chemically interact with the collageneous proteins from dentine. Thus, agarose agarose, which is a polyanionic polysaccharide containing repetitive anionic -OH groups,

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may bond to positive charged groups of collagen molecules and then induce hydroxyapatite crystals to nucleate and grow [22]. To improve the quality and stability of adhesive restoration layers, some studies have evaluated the use of biopolymers, such as chitosan (CS) [23-24] that is a natural biocompatible, biodegradable and non-toxic polysaccharide [25], to increase the cross-linking between the collagen fibers and agarose molecules [26].

The purpose of this work is to induce biomimetic remineralization of acid etched coronal human dentine in artificial saliva under agarose and chitosan-agarose hydrogels action. The investigations focused on the morphology, chemical composition and crystalline structure of the new remineralized layers grown onto the etched dentinal surface (R) by using scanning electron microscopy coupled with energy dispersive X-ray spectrometry and micro Raman spectroscopy.

Experimental part

Selected human molars without caries or restored caries were collected from local dental clinics (OvidiusUniversity of Constanta, Faculty of Dentistry) according to the ethic protocol and the patients were informed and consented to the use of teeth. 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. Flat slices of about 1.5 mm in thickness were cut using a diamond blade under water cooling. The sliced samples were individually sonnicated in demineralized water for 5 min to remove residual abrasives. Dentine surfaces were demineralized by acid-etching using 36% phosphoric acid for 60 s, followed by cleaning under ultrasounds for 2 min and rinsed 3 times with deionized water (denoted as control sample R).

The agarose based hydrogel used for remineralization of the dentine samples was obtained by adding 0.15 mL of agarose solution (1wt %) over 960 µL of 1% (v/v) acetic acid solution and mixing until the solution was clear. To this solution, 25 μ L of 0.1 M CaCl, and 15 μ L of 0.1 M Na, HPO_4 solutions were added and the pH of the agarose solution was adjusted to 6.5 by adding 1 M NaOH solution. The chitosan-agarose hydrogel used for remineralization of dentine was obtained by mixing the agarose hydrogel prepared as mentioned before with 960 µL 1% chitosan solution, using a vortex, until the solution was clear. The chitosan solution was prepared by dissolving 1% (w/v) chitosan in a 1% (v/v) acetic acid solution followed by stirring at 80 °C. After cooling to room temperature, the solution was filtered using a 0.8 µm filter. Artificial saliva (AS) was prepared according to the recipe proposed by Fletcher et al. [27]: 0.2 mM MgCl., 1 mM CaCl., 4 mM Na, HPO, 16 mM KCl, 4.5 mM NH, Cl 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.

For the remineralization of the acid-etched dentine samples, 20 μ L agarose hydrogel or chitosan-agarose hydrogel was applied to cover the acid-etched dentine surface and dried at room temperature. The dried samples were immersed into artificial saliva at 37 °C for 4 or 7 days, without replacing the hydrogels. At the end of the immersion period, tooth slices were removed and air-dried.

The morphology and elemental chemical composition of the investigated sample surface 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, samples were coated with a 4-5 nm thick conducting layer of Au using a SPI-Module[™] sputter coater system.

Micro Raman spectra of two remineralized molar dentine samples were collected by using a LabRam HR800 spectrometer calibrated with silicon wafer as reference. Samples were excited with a HeNe laser of 633 nm wavelength through an x50LWD/0.55 NA air objective assuring a laser spot size of ~1.4 μ m. Raman signal was collected using a 600-line/mm grating. Tooth specimens were mounted on a computer-controlled, high-precision x-y stage with a spatial resolution of 0.05 mm. Depth profile spectra were collected with 1 mm step within 400-1400 cm⁻¹ range for the A7 sample. Spectra were cubic baseline corrected and fitted by using Igor software [28].

Results and discussion

Morphological and chemical compositions

Figure 1 shows the SEM top-view images of the acidetched natural dentine sample (control sample R) together with the samples obtained after biomimetic mineralization in the presence of agarose gel with immersion into AS for seven days (sample A7) or in chitosan-loaded agarose gel with immersion into AS for four days (sample A-ČS4). One can observe from figure 1 the formation of nanorod-like extrafibrilar HAP crystals randomly self-assembled in a discontinuous layer on the dentine sample surface stored in AS for 7 days under agarose gel (sample A7), when compared with the control etched dentine sample (R). As mentioned before [21], the acid-etched demineralized surface of the dentine consisting of only type I collagen matrix can not promote the mineral crystal deposition because the absence of noncollagenous proteins and/or lack of seed mineral crystals [29]. The presence of agarose gel has facilitated the formation of nanorod-like HAP crystals during immersion into AS solution by attaching to the collagen matrix and inducing the formation of transient amorphous phosphates [21]. These new grown crystals are randomly self-assembled onto the etched dentine surface. Some HAP crystals deposited around the dentinal tubules and an initiation of the dentinal tubules occlusion can be observed under agarose hydrogel remineralization (sample A7) when comparing with the aspect of dentinal tubules from control acid-etched dentine sample (R). Also, a homogeneous surface of a continuous compact layer can be observed for sample mineralized under agarose gel loaded with chitosan (A-CS4, fig. 1). In this case, chitosan molecules acting as a chelating agent [30] served as a scaffold for the formation of a complex composite calcium phosphate based layer onto the surface of the demineralized dentine. Comparing to the peritubular dentine area of control demineralized sample (R), the surface of this A-CS4 remineralized dentine sample shows an shrinking of tubule diameter promoted by the development of the new composite layer grown under CSagarose hydrogel in AS saliva.

The EDX spectra and elemental analysis of the investigated samples (fig. 2) revealed that the main elements composing the remineralized layers were carbon, oxygen, calcium, phosphorus and nitrogen. Higher concentration of C and O and decreased concentration of Ca and P show high organic and low mineral contents on dentine type tissues. EDX elemental analysis shows an increase in the Ca and P and a decrease in C and O contents when mineralization time increases from 4 days (sample A-CS4) to 7 days (sample A7), demonstrating an increased mineralization of A-CS4 sample. In the same time, the new grown layer of A-CS4 sample mineralized under



Fig.1. SEM images of control acid-etched dentine (R), remineralized dentine under agarose gel (A7) , and remineralized dentine under chitosanagarose gel (A-CS4)

Fig. 2 SEM-EDX spectra of acid-etched dentine (R), remineralized dentine with agarose gel (A 7), and remineralized dentine with agarose gel loaded with chitosan (A-CS 4)

chitosan-agarose hydrogel contains an increased amount of nitrogen, thus confirming the presence of chitosan. The value of Ca/P ratio for sample mineralized seven days under agarose hydrogel (fig.2) is 1.44, very closed to that of the control sample (R), but significantly decreased to 1.32 in the case of sample mineralized for four days under chitosanagarose hydrogel, suggesting a modification in mineral hydroxyapatite phase composition.



Fig. 3. EDX-elemental mapping for C, O, Ca and P elements on the remineralized dentine with agarose gel (A 7) and remineralized dentine with agarose gel loaded with chitosan (A-CS 4)

EDX mapping (fig. 3) showed a homogeneous distribution of calcium and phosphorus within the biomimetic dentin layers remineralized both in agarose and in chitosan-agarose hydrogels. The Ca, P and N maps confirmed the higher mineralization of sample A7 treated under agarose hydrogel for seven days and higher content of nitrogen in the layer formed under chitosan-agarose hydrogel (A-CS4). EDX map highlights lower C content for sample A7 in the area where self-assembled crystals are present (circled area on SEM image), confirming the mineral apatite phase of the crystals.

Molecular and crystalline structure

Raman spectroscopy is a useful quantitative technique in investigating molecular and crystalline structure of preand post reparatory treated human dentine and enamel.

All Raman spectra of the A7, A-CS4 and reference R illustrated in figure 4 show the presence of major hydroxyapatite phase with its main band at about 960 cm⁻¹ (ν_1 (PO₄³⁻)) and minor phases of carbonate and hydroxyl ions (ν_1 (CO₃²⁻) at 1070 cm⁻¹ and ν (HO) at 3574 cm⁻¹), respectively [31-32]. Both remineralized layers grown in the presence of agarose (Sample A7) and chitosan-agarose hydrogels (sample A-CS4) consist of B-type Ca-deficient hydroxyapatite (fig. 4b). In contrast to





Fig. 4. Raman spectra of: (a) etched R sample; (b) R, A-CS4 and A7 dentines (baseline corrected) within 660-1780 cm⁻¹ range; (c) A7 dentine (depth profile)

the enamel spectrum of the R sample in figure 4a, existence of collagen is depicted by its main bands located at 1673 (backbone amide I), 1455 (CH₂), 1274 (amide III) and 1242 cm⁻¹ (amide III) [31-32]. Relative mineral concentration of R, defined as area ratio of 960 and 1070 cm⁻¹ bands, RRM_p of 1.83 in table 1, indicates demineralized dentine for etched R. This is consistent with presence of bands at 920 and 940 cm⁻¹ as reported in case of materials with Ca/P ratio lower than hydroxyapatite [33]. Also small value of RRM_c (0.62) or area ratio for bands at 960 and 1455 cm⁻¹ was recorded for R sample.

Closed MMR_c values for R and A⁻CS4 in table 1 indicate slightly different mineral-to-collagen matrix ratio. Conversely, longer exposed A7 dentine (7 days) in the presence of agarose shows a higher mineral-to-collagen ratio of 0.91, confirming the SEM-EDX results. Also it is confirmed the formation of an amorphous apatite layer, e.g. shifting of the v_1 (PO₄³⁻) band towards lower wave numbers along with a FWHM of 15.59 cm⁻¹. Since dentine mineralization increases, collagen quality factor (band at about 1660 cm⁻¹) decreases in succession A-CS4 > R > A7 (table 1), as a consequence of collagen damage and/or fibril removal [34].

Although there is a similar spectral feature for both R and A-CS⁴ dentine samples (fig. 4b), the new layer formed on the surface of the A-CS4 dentin has a higher crystallinity, i.e. FWHM for both 13.67 cm⁻¹ for A-CS4 comparison with 16.81 cm⁻¹ in case of R dentine. Moreover, higher MMR_p ratio (5.37) is obtained for new layer of the A-CS4 dentine. Slightly different mineral-to-collagen matrix ratio, MMR_c, is recorded for R (0.62) and A-CS4 (0.5). Conversely, longer exposed A7 dentine (7 days) in the presence of agarose shows a higher MMR_c of 0.91, confirming the SEM-EDX results and formation of an amorphous apatite layer, e.g. shifting of the $\mu_1(PO_4^{3})$ band towards lower wave numbers along with a FWHM of 15.59 cm⁻¹. Since dentine mineralization increases, collagen quality factor (band at about 1660 cm⁻¹) decreases in succession A-CS4>R>A7 (table 1) as a consequence of collagen damage and/or

Sample	Peak (cm ⁻¹)	HWHM(cm ⁻¹)	Area (cm ⁻²)	MMR₽	MMRc**
R	960	16.81	23.899	1.83	
	1070	33.61	13.054		
	1455	43.32	38.62		0.62
A-CS4	962	13.67	19.964	5.37	
	1071	19.73	3.7174		
	1456	50.58	40.317		0.50
A7	958	15.59	17.928	2.57	
	1072	26.26	6.9589		
	1456	62.38	19.639		0.91

Table 1DECONVOLUTION RESULTS (PEAKPOSITION, FULL WIDTH AT HALFMAXIMUM, AND AREA) OF THE R,A-CS4 AND A7 CORONAL DENTINESAMPLES

*Area ratio of 960 and 1070 cm⁻¹peaks ** Area ratio of 960 and 1455 cm⁻¹ peaks

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fibril removal [34]. This behaviour is consistent with superficial formation of a new enamel-like layer.

To emphasize structural differences between newly formed layer and starting coronal dentine of A7, depth profile measurements were carried out (fig. 4c). Thus, spectrum collected for 1 mm inside dentine area contains a tiny band at about 1005 cm⁻¹ assignable to the phenyl groups in organic content of natural dentine substrate [32]. This is a consequence of the thin layer formed superficially.

Conclusions

Our results showed that agarose and chitosan-agarose hydrogels successfully induced biomimetic remineralization of acid etched coronal human dentine in artificial saliva. Both remineralized layers grown in the presence of agarose or chitosan-agarose hydrogels consist of B-type Ca-deficient hydroxyapatite. After 7 days storage into artificial saliva under agarose hydrogel, nanorod-like HAP crystals self-assembled into a discontinuous layer were formed, while in the presence of chitosan-agarose hydrogel a continuous compact chitosan-hydroxyapatite composite layer was obtained. The new biomimeticlayer formed after 4 days on dentine surface under agarosechitosan hydrogel has higher crystallinity. Longer exposed dentine (7 days) in the presence of agarose hydrogel (A7) shows higher mineralization. Since dentine mineralization increases, collagen quality factor decreases in succession A-CS4>R>A7. Results show a benefic effect of chitosan on remineralization of etched dentine. Longer remineralization tests are in progress.

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