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Amelogenin-assisted ex vivo remineralization of human enamel: Effects of supersaturation degree and fluoride concentration

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ABSTRACT

The formation of organized nanocrystals that resemble enamel is crucial for successful enamel remineralization. Calcium, phosphate and fluoride ions, and amelogenin are important ingredients for the formation of organized hydroxyapatite (HAP) crystals in vitro. However, the effects of these remineralization agents on the enamel crystal morphology have not been thoroughly studied. The objective of this study was to investigate the effects of fluoride ions, supersaturation degree and amelogenin on the crystal morphology and organization of ex vivo remineralized human enamel. Extracted third molars were sliced thin and acid-etched to provide the enamel surface for immersion in different remineralization solutions. The crystal morphology and mineral phase of the remineralized enamel surface were analyzed by field emission–scanning electron microscopy, attenuated total reflection–Fourier transformed infrared and X-ray diffraction. The concentration of fluoride and the supersaturation degree of hydroxyapatite had significant effects on the crystal morphology and crystal organization, which varied from plate-like loose crystals to rod-like densely packed nanocrystal arrays. Densely packed arrays of fluoridated hydroxyapatite nanorods were observed under the following conditions: $\sigma(\mathrm{HAP}) = 10.2 \pm 2.0$ with 1.5 ± 0.5 mg l^{-1} fluoride and 40 ± 10 μg ml^{-1} amelogenin, pH 6.8 ± 0.4. A phase diagram summarizes the conditions that form dense or loose hydroxyapatite nanocrystal structures. This study provides the basis for the development of novel dental materials for caries management.

1. Introduction

Dental enamel is the hardest tissue in vertebrates and is composed of more than 95% mineral by weight. Enamel is composed of highly organized nanocrystals of fluoro-substituted carbonated hydroxyapatite (HAP). This structure provides enamel with increased hardness and resistance to fracture and erosion compared to monolith hydroxyapatite. However, due to the acellular and protein-free composition of mature enamel, after trauma or decay the regeneration of enamel is not observed. Thus, the synthesis of enamel through biomimetics is of great interest to dental clinicians. In the past decades, enamel-mimicking material comprised of hydroxyapatite nanocrystal coatings have been synthesized using different in vitro methods. Biomimetic synthesis [1,2] is a highly promising approach due to the mild and biocompatible synthesis conditions in comparison to other approaches requiring harsh treatments, such as a high-temperature hydrothermal method [3] and a method using a strong acid in a calcium phosphate paste [4–6].

Dental caries originate from the demineralization of dentin, enamel or cementum by acid-producing bacteria, which alter the dynamic demineralization–remineralization process. When proper methods are used, caries can be halted at early stage and even reversed. Enamel remineralization is a well-accepted concept for controlling coronal caries. Many clinical products, including topical fluoride varnish [7], fluoride dentifrice [8] and casein phosphopeptide–amorphous calcium phosphate-containing paste [9,10] have been developed to improve enamel remineralization. However, there was an irregular morphology and organization of the crystals on eroded enamel after treatment with these remineralization agents. Previous reports on dental enamel remineralization indicated that a high content of spherical calcium fluoride [11] and loosely structured calcium phosphate [12] were commonly observed during enamel remineralization.

Amelogenin is an enamel extracellular matrix protein that controls the formation of hierarchical organized minerals [13,14]. Many in vitro studies have shown that amelogenin is a crucial promoter of mineralization and a modulator of the nanocrystalline structure of calcium phosphate [15–17]. The cooperation mechanism of protein assembly and mineralization was first demonstrated by Beniash et al., who reported the alignment of

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hydroxyapatite crystals in the presence of full-length amelogenin using homogeneous nucleation [18]. Nancollas's group recently applied constant-composition nucleation to demonstrate the role of amelogenin in the formation of elongated hydroxyapatite crystals at an initial stage of nucleation [19,20]. Fan et al. have recently reported that amelogenin and fluoride are key components for the regrowth of organized apatite nanocrystals on an enamel substratum. Nanocrystals of hydroxyapatite with a diameter of 20 nm formed bundles on an eroded enamel surface in a supersaturated remineralization solution [21,22].

Application of a remineralization microenvironment on enamel, which provides an adequate amount of amelogenin, calcium, phosphate and fluoride for enamel remineralization, can promote the formation of enamel-like structures. Previous studies revealed that the phase formation and morphology of calcium phosphate crystals may be affected by temperature, ionic strength, pH, fluoride and degree of supersaturation [23–25]. Changing the solution's supersaturation degree and the fluoride concentration in mineralization solutions were reported to affect the crystal morphology dramatically [23,26]. The self-assembly of amelogenin shows a strong dependency on pH [27]. However, the systematic effects of the above mineralization parameters on the nanocrystal morphology and organization of ex vivo enamel remineralization are not fully understood. These parameters are of great significance for the advances in enamel remineralization technology.

The aim of this study was to investigate how the crystal morphology of the ex vivo remineralized enamel is affected by pH and concentrations of calcium, phosphate, fluoride and amelogenin. The current study differs from and builds upon previous work performed with a fixed pH and supersaturation degree [21]. Our study determines the zones or ranges of pH and supersaturation degree wherein enamel remineralization with densely packed nanorods are observed. The crystal morphology and mineral phase of the remineralized enamel surface were analyzed by field emission-scanning electron microscopy (FE-SEM), attenuated total reflection-Fourier transformed infrared (ATR-FTIR) spectroscopy and X-ray diffraction (XRD). The microhardness of the remineralized enamel surface was analyzed and correlated with the optimal conditions for formation of densely bundled enamel-like crystals.

2. Materials and methods

2.1. Amelogenin preparation

Purified recombinant full-length porcine amelogenin (rP172) was prepared as described previously [13,28]. The protein was expressed in *Escherichia coli* strain BL21-codon plus (DE3-RP, Strategene), purified by ammonium sulfate precipitation and reverse-phase preparative high-performance liquid chromatography (Dionex Summit HPLC with Jupiter 250 mm × Ø21.2 mm, 10 μm C4 column, Phenominex, CA), using a mobile phase 20–60% acetonitrile/water + 0.1% trifluoroacetic acid under a flow rate of 10 ml min^{-1}. The molecular weight of purified rP172 was confirmed by a mass spectrometer (Bruker Esquire 3000) to be 19,572 ± 1 Da.

2.2. Preparation of remineralization solution

All reagents and buffers were purchased from commercial sources (Sigma or Thermo-Fisher). Supersaturated calcium phosphate solutions, Tris (*tris*(hydroxymethyl)-aminomethane) or HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered at pH 6.5–7.6 were prepared by dissolving $CaCl_2{\cdot}2H_2O$, KH_2PO_4, NaCl and Tris–HCl or Na–HEPES in distilled deionized water to achieve an ionic strength of 190 mM. In our remineralization buffer solutions, the initial molar ratio of Ca^{2+} to PO_4 ions is 1.67, and for simplicity we only report the Ca^{2+} concentration in the text. Remineralizing solutions with various pH levels, [Ca^{2+}] and [F^-] were prepared and tested as indicated in Table 1. Experiments were performed at least three times and similar results were obtained each time.

The relative supersaturation degree of octacalcium phosphate ($Ca_8H_2(PO_4)_6{\cdot}5H_2O$; OCP) HAP and fluoroapatite ($Ca_{10}(PO_4)_6F_2$; FAP) were calculated using previously described methods [24]. The definition of relative supersaturation degree (σ) of hydroxyapatite is:

$$\sigma = \left(\frac{[\alpha(Ca^{2+})]^{10}[\alpha(PO_4^{3-})]^6[\alpha(OH^-)]^2}{K_{sp}} \right)^{1/18} - 1$$

where K_{sp}(HAP) = 5.5 × 10^{-118} M^{18}, K_{sp}(FAP) = 5.0 × 10^{-123} M^{18}.α is ion activity: $\alpha_i = c_i\gamma_{\pm}$, where the ion activity coefficient (γ) was calculated from the total ion strength by the extended Debye–Huckel equation [24]. Table 1 lists the compositions of different remineralization solutions. To calculate the buffer pH at 37 °C, the temperature coefficients, −0.031 ΔpH $°C^{-1}$ for Tris–HCl buffer and −0.014 ΔpH $°C^{-1}$ for HEPES buffer [29], were used to adjust the pH from room temperature (22 ± 2 °C) to 37 °C. The relative supersaturation degrees at 37 °C were from 4.21 to 15.0 for HAP, from 0 to 56.0 for FAP and from 0.42 to 2.62 for OCP in this study.

The selection of pH is based on the physiological range of pH from 6.3 to 7.2, and in this pH range amelogenin is soluble. Amelogenin rP172 has a theoretical isoelectric point of 6.8 (by ExPASy Proteomics Server). Recombinant full-length amelogenin reportedly forms nanospheres at pH 6.5–7.4 [13,27]. The range of amelogenin concentrations was selected according to previous publications [21] and the known solubility of amelogenin of 30 μg ml^{-1} at 25 °C pH 6.5.

2.3. Mineralization of eroded enamel

Extracted human third molars (randomly selected from a tooth bank from unidentified donors at the School of Dentistry, Louisiana State University Health Science Center, approved by Intuitional Review Board) were sliced thin and acid etched to provide the ex vivo enamel surface. Teeth were cut in longitudinal slices to a thickness of 0.4 mm using a water-cooled diamond blade on a section machine (Accutom 50, Struers, Denmark). The slices were stored at 4 °C in phosphate-buffered saline. Before remineralization, the slices were etched in 5% HNO_3 for 30 s and rinsed in deionized water. The slices were then immersed in 12 of a ml freshly prepared remineralization solution containing 50 mM Tris or 20 mM HEPES buffer (pH 6.30–7.20), amelogenin rP172 (30–70 μg ml^{-1}) and fluoride (0.001–10 mg l^{-1}), and incubated at 37 °C. The samples were removed at the indicated times and rinsed with deionized water, air-dried and then analyzed as described below.

2.4. Characterization of remineralized enamel

The remineralized enamel surfaces were analyzed by FE-SEM (LEO 1530 VP). The specimens were coated with carbon using a Carbon Coater 208C (Cressington Scientific Instruments, Watford, UK). The crystal sizes in the digital SEM images were measured using Photoshop ($n > 15$).

ATR-FTIR spectra were acquired with a Nexus 670 FTIR spectrometer (Thermo-Nicolet, Madison WI) equipped with a Vision Gladi diamond ATR (Pike Technology, Madison WI). The enamel samples were pressed on the diamond crystal and testing locations were confirmed by a live video monitoring beneath the diamond crystal. The samples were scanned for 128 times at 4 cm^{-1} resolution from 4000 to 400 cm^{-1}.