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Biomimic Enamel Remineralization by Hybridization Calcium- and Phosphate-Loaded Liposomes with Amelogenin-Inspired Peptide

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Abstract. We here report a novel biomimetic mineralization strategy for enamel remineralization by intergration of calcium phosphate loaded and thermally triggered liposomes and a self-assembly amelogenin-inspired peptide. Firstly, calcium and phosphate loaded temperature sensitive liposomes were synthesized by Interdigitation-fusion method with 1,2-bis(palmitoyl)-sn-glycero-3-phosphor-choline (DPPC) and 1,2-bis(myristoyl)-sn-glycero- 3-phosphocholine (DMPC) at mass ratio of 9:1. The liposomes were stable at room temperature, but slowly released calcium and phosphate ions if heated to 37 °C. Secondly, a novel polyanion amelogenin-inspired oligopeptide $(Gln\text{-}Pro\text{-}Ala)_4$-Thr-Lys-Arg-Glu-Glu-Val-Asp) was synthesized by standard solid-phase. Finally, the mixture of peptide and liposomes solution was exposed to enamel surface at 37 °C. The results showed oriented enamel-like hydroxylapatite evenly deposited on enamel surface.

Introduction

Matured enamel is composed of 95 wt% nano rod-like hydroxyapatite (HA). The nano HA crystals have a cross-section of 25-100 nm and an undetermined length of 100 nm to 1000 nm or longer along the c-axis, and self-assemble into prism microstructure [1]. Biomineralization process is self-assembly and an organic-matrix-mediated biomineralization, where some types of organic macromolecules self-assemble and may be used as templates for controlling the nucleation and growth of mineral crystals to form hierarchical hybrid materials [2]. Recently, with the development of understanding the mechanisms of biomineralization, and nanomaterial fabrication technique, biomimetic mineralization strategy has been used to regenerate the hierarchical microstructure of the dental tissue [3-5].

During enamel formation, amelogenin, the major enamel protein constituting approximately 90% of all organic matrix material, spontaneously self-assembles into nanospherical supermolecular structures. This supermolecular structure plays a vital role in HA nucleation and growth, and furtherly directs the HA crystals to form a well-organized prism pattern [6]. The molecular mechanism of amelogenin self-assembly is that its primary structure preserves a bipolar nature, especially proline-rich sequences that contain a repetitive sequence of (Gln-Pro-X)n forms with a β spiral secondary structure showing a unique globular monomers, and the hydrophilic flexible C-terminal (-Thr-Lys-Arg-Glu-Glu-Val-Asp) ‘‘tail’’ which interacts with calcium and initializes HA nucleation exposes on the surface of the globular monomers [7]. Thus, we designed a novel amelogenin-like oligopeptide ((Gln-Pro-X)n- Thr-Lys-Arg-Glu-Glu-Val-Asp) to control enamel remineralization.

On the other hand, a biological strategy during mineralized tissue formation is the use of matrix vesicle (lipid vesicle compartments) to sequester calcium and phosphate ions, control ion transport, and to control mineral particles deposited within developing tissues[8, 9]. Messersmith have

developed a temperature-sensitive liposomes system to mimetic matrix vesicle to control mineralization study [10, 11]. The liposomes loaded calcium and phosphate salts, and were stable at room temperature, but slowly released calcium and phosphate ions when heated to 37 °C.

In the present study, We intergrated the calcium phosphate loaded liposomes and the self-assembly amelogenin-inspired peptide to build a novel biomimetic mineralization strategy for enamel remineralization.

Experimental

Calcium- and phosphate-loaded liposomes preparation Calcium- and phosphate-loaded liposomes were prepared using the interdigitation-fusion (IF) method as reported by Messersmith with some modification [10,11]. Briefly, 1,2-bis(palmitoyl)-sn-glycero-3-phosphocholine (DPPC > 99%) and 1,2-bis(myristoyl)-sn- glycero-3-phosphocholine (DMPC> 99%) (Sigma-Aldrich Trading Co., Ltd, American)were prepared at the mass ratio of 9:1, and hydrated with aqueous $CaCl_2$ or Na_2HPO_4. Then, the resultant suspensions were probe-sonicated and centrifuged. Absolute ethanol was added to the liposome suspensions. The samples were sealed and incubated for 15 min at room temperature followed by incubated for 15 min at 50 °C. Then allow ethanol evaporation resulting in Ca^{2+} and PO_4^{3-}-loaded vesicles. Vesicle suspensions were then combined with an equal volume of buffered NaCl solution, vortexed, and centrifuged at 16,000 g for 5 min. This process was repeated until Ca^{2+} (or PO_4^{3-}) could not be detected in the supernatant using a colorimetric test with AIII dye. The liposomes were characterized with SEM,TEM and Dynamic light scattering(DLS).

Prepared of the amelogenin-like oligopeptide The amelogenin-like oligopeptide of $(Gln\text{-}Pro\text{-}Ala)_4$-Thr-Lys-Arg-Glu-Glu-Val-Asp was synthesized by standard solid-phase peptide synthesis. The HPLC-purified peptide was checked by analytical HPLC and mass spectrometry.

Biomimetic remineralization of etched enamel surface 1 mm thick slabs of human enamel were etched with 37% phosphoric acid. Ca^{2+} and PO_4^{3-}-loaded liposomes were diluted with 50 mM HEPES at the ratio of about 1.7:1. PA solution were prepared and dissolved in deionized water at a concentration 20% by weight. The solution was adjusted to a pH of 9.0 using 1 M NaOH. Lastly, the mixture of peptide and liposomes solution was exposed to enamel surface at 37 °C in oven for two weeks. The enamel surface were characterized with SEM, DR-FTIR and film-XRD.

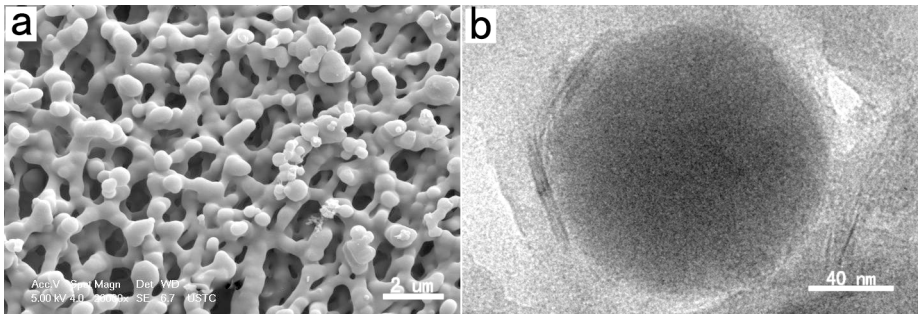

Fig. 1 a) SEM images of calcium -loaded temperature-sensitive liposomes; b) images of the liposomes of TEM

Results and Discussion

Liposomal synthesis and thermally triggered Ca^{2+} and PO_4^{3-} release We prepared the temperature-sensitive liposomes by the interdigitation-fusion method (IF) as reported by Messersmith with some modification. The important factor to induce interdigitatio is the concentrations of ethanol. This method formed large unilamellar liposomes with a high encapsulation efficiency [12]. The encapsulation efficiency of Ca-loaded vesicles was 70~85%, whereas the encapsulation efficiency of PO_4^{3-}-loaded vesicle was 60~80%. It is found that liposomes morphology was a uniform global particles with the diameters about 161nm (Fig. 1). The size of the Ca^{2+} and PO_4^{3-} -loaded liposomes of

size similar to matrix vesicles [13,14]. If the liposomal was exposed to 37 °C enviroment, Ca^{2+} and PO_4^{3-} could slowly released from the liposomes tested with AⅢ dye. Release of entrapped ions at this temperature was due to increased permeability of phospholipid bilayers at the lipid chain melting temperature. Messersmith *et al* have proved the diffusion of Ca^{2+} and PO_4^{3-} out of liposomes and their reaction to form crystalline calcium phosphate minerals, which were deposited on dentin and enamel surfaces or formation of mineral/collagen composite [11,12]. In the present study, we integrated the liposomes and the self-assembly amelogenin-inspired peptide to for enamel remineralization.

Biomimetic mineralization of etched enamel It is found that the enamel surface only treated with liposomes suspension had a few amount of HA crystal deposition, and the precipitate distributed randomly. In contrast, the experimental groups treated with the mixture solution of liposomes and polyanion peptide showed that the rob-like crystals almost fully covered the etched enamel surface, and distributed evenly to make the surface smoothly (Fig. 2). The deposited crystals assembled along its c-axis.

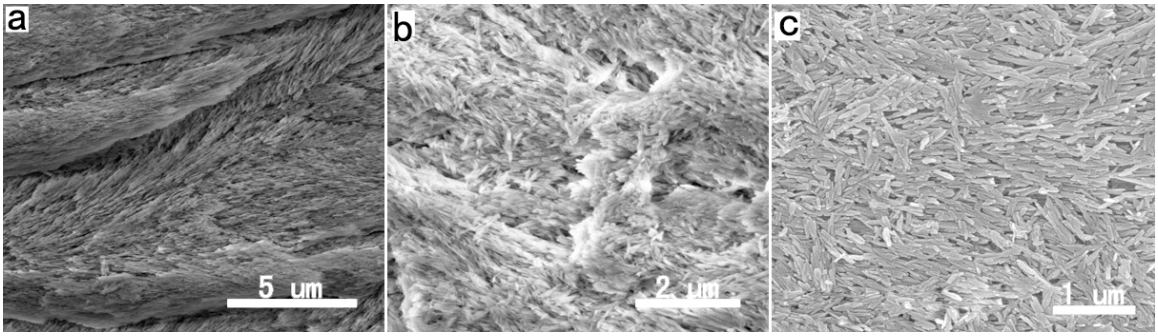

Fig. 2 SEM images of mineral crystals deposited on etched enamel surface. a) enamel etched with 37% phosphoric acid only; b) etched enamel surface treated with liposomes suspension; c) etched enamel surface treated with with liposome and polyanion peptide

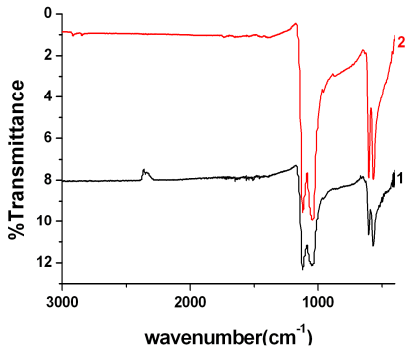

Fig. 3 DR-FTIR spectra of the precipitate, (1) control group (mineralization with liposome only); (2) experimental group(mineralization with liposome and polyanion peptide)

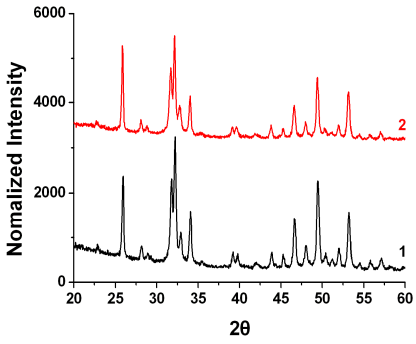

Fig. 4 X-ray diffraction pattern of the precipitate, (1) control group (mineralization with liposome only); (2) experimental group (mineralization with liposome and polyanion peptide)

In the FTIR spectrum of the treated enamel surface (Fig. 3), the peak at approximately 1100 cm^{-1} , 1040 cm^{-1} ,650 cm^{-1} , 565 cm^{-1} represent PO_4^{3-}, respectively, but the intensity of the experiment groups was much more stronger than the control groups. All the XRD patterns of the control and experimental groups showed the characteristic peaks of hydroxyapatite. All the 002 lattice plane ($2\theta=26^{\circ}$) were enhanced, which suggested the HA crystal arranged along its c-axis. The orientation in experimental groups came from the coating HA, while the control groups came from the substrate enamel primes.

From the above results, we found that the amelogenin-inspired oligopeptide polyanion peptide could induce enamel remineralization with high effectively, and could induce HA crystals to self-assemble into enamel like HA orientation. Thus, the novel amelogenin-inspired oligopeptide polyanion peptide may be a useful tool to be used in biomimetic mineralization study for enamel regeneration.

Conclusions

Temperature sensitive Ca^{2+} and PO_4^{3-} loaded liposomes combining with self-assembly amelogenin-inspired oligopeptide polyanion peptide can effectively induce enamel remineralization. This biomimetic mineralization strategy provides a potential method mimicking the processes that occur during natural mineralized tissue formation.

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