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In situ synthesis carbonated hydroxyapatite layers on enamel slices with acidic amino acids by a novel two-step method

Xiaoguang Wu [a], Xu Zhao [b], Yi Li [a,*], Tao Yang [c], Xiujuan Yan [a], Ke Wang [a]

[a] Department of Pediatric Dentistry, The Hospital of Stomatology, Jilin University, Changchun 130021, China
[b] College of Chemistry, Jilin University, Changchun 130021, China
[c] Department of Stomatology, Children's Hospital of Changchun, 130051, China

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ABSTRACT

In situ fabrication of carbonated hydroxyapatite (CHA) remineralization layer on an enamel slice was completed in a novel, biomimetic two-step method. First, a $CaCO_3$ layer was synthesized on the surface of demineralized enamel using an acidic amino acid (aspartic acid or glutamate acid) as a soft template. Second, at the same concentration of the acidic amino acid, rod-like carbonated hydroxyapatite was produced with the $CaCO_3$ layer as a sacrificial template and a reactant. The morphology, crystallinity and other physicochemical properties of the crystals were characterized using field emission scanning electron microscopy (FESEM), Fourier transform infrared spectrometry (FTIR), X-ray diffraction (XRD) and energy-dispersive X-ray analysis (EDAX), respectively. Acidic amino acid could promote the uniform deposition of hydroxyapatite with rod-like crystals via absorption of phosphate and carbonate ions from the reaction solution. Moreover, compared with hydroxyapatite crystals coated on the enamel when synthesized by a one-step method, the $CaCO_3$ coating that was synthesized in the first step acted as an active bridge layer and sacrificial template. It played a vital role in orienting the artificial coating layer through the template effect. The results show that the rod-like carbonated hydroxyapatite crystals grow into bundles, which are similar in size and appearance to prisms in human enamel, when using the two-step method with either aspartic acid or acidic glutamate (20.00 mmol/L).

1. Introduction

Tooth enamel is a highly mineralized extracellular matrix and the hardest tissue in the human body. The main component of enamel is 95–97 wt.% of nanorod-like hydroxyapatite (HA) crystals [1]. The ordered architecture of enamel plays an important role in controlling the mechanical strength of the teeth and acts as a buffer to protect them [2,3]. Mechanical forces can damage tooth enamel, while acidic beverages and cariogenic bacteria can corrode it, causing the formation of caries [4]. Besides, the mature enamel is a nonliving tissue and the damaged part is scarcely self-repairable. Dentistry uses metals, compound resins and ceramics as traditional restorative materials; however, these materials exhibit poor adhesion and weak mechanical strength. Their chemical composition and physical properties are different from those of the body's natural material [5]. To restore the eroded enamel, a favorable and effective treatment is to synthesize an enamel-like material comprised of HA crystals. Fluorides have been used widely in clinic because of the low solubility of fluoride-substituted hydroxyapatite [6,7]. However, the excessive fluoride ions can impel serious dental fluorosis [8].

Biomimetic mineralization is a methodology by which minerals are produced under the control of organic matrices (including various proteins, such as amelogenin, in tooth enamel). Organic matrices can regulate the crystal nucleation, morphology, and crystal structure of the mineral [9,10]. It has been reported that organic matrices could allow dental tissues to regenerate and thus restore the injured enamel via HA remineralization on dental surface. For example, organic matrices in saliva have been shown to bond to the enamel surface, which induces inorganic mineral deposits and reduces enamel demineralization [11–14].

Studies have found during enamel mineralization that apatite crystals assembled into parallel arrays under strict biological controls by amelogenin [11,15], enamel matrix proteins (EMPs) [16,17] and some other non-collagens. Unfortunately, EMPs only exist in the growth period of enamel and degrade in the mature stage. Note that most non-collagenous proteins in the matrix of biological hard tissue (such as bone, dentin, and enamel) are acidic macromolecules. Dentin phosphoprotein (DPP), composed of collagen and parts of non-collagenous proteins, is a powerful initiator and regulator for hydroxyapatite crystal formation and growth [18]. Most of the proteins mentioned above contain highly repetitive peptide sequences (aspartic acid (Asp), glutamate (Glu), and serine), forming a three-dimensional structure. Researchers [19,20] developed octuplet repeating units of aspartic–serine–serine

* Corresponding author.
E-mail address: lyi99@jlu.edu.cn (Y. Li).

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polypeptides to promote the nucleation of calcium phosphate carbonate from the free ions. This research successfully promoted mineral deposition onto human enamel and improved the surface properties of demineralized enamel. Therefore, it is reasonable to speculate that hydroxyapatite nucleation might depend on individual repeating units of proteins.

Asp or Glu was used as water-soluble additives, which have been known to strongly react with Ca^{2+} [21,22]. In fact, the interaction of HA with biological macromolecules, such as amino acids, is a promising method to control the surface charge of HA and thus influence the morphology of synthesized HA powder [23–26]. However, major aspects of the formation mechanism of enamel-like HA crystal synthesized on enamel are still unclear, e.g., HA nucleation, crystal growth and crystal orientation.

Based on the properties of biological hydroxyapatite on natural human enamel, a novel, two-step method was proposed for in situ synthesis of carbonated hydroxyapatite layers by which $CaCO_3$ was used as an intermediate active bridge layer. The aim of this work was to functionalize HA particles with different amino acids to obtain an enamel regeneration material. In addition, we explain the mechanism by which carbonated HA (CHA) crystals grow on the surface of enamel prisms in situ with Asp and Glu when using the two-step method.

2. Materials and methods

2.1. Materials

All the chemical reagents used in this experiment were analytical grade, and the solvent was distilled water. Anhydrous calcium chloride ($CaCl_2$), ammonium bicarbonate (NH_4HCO_3), twelve crystallization water disodium hydrogen phosphate with molecules ($Na_2HPO_4 \cdot 12H_2O$) and sodium hydroxide (NaOH) were provided by Beijing Chemical Reagent Factory. Asp and Glu were purchased from J&K Technology Company Limited.

2.2. Preparation of enamel slices

We collected healthy premolar teeth, recently removed because of orthodontic reasons, and sliced them into 0.5 cm × 0.5 cm × 0.2 cm pieces with a low speed diamond saw. The slices were treated with 3% sodium hypochlorite to remove bacteria and polished them sequentially with 800 grit to 2000 grit silicon carbide (SiC) sandpaper. These enamel pieces were rinsed to remove the residual abrasives. Enamel surfaces were demineralized with 37% phosphoric acid for 30 s, rinsed thoroughly and dried at room temperature. Half of every slice was covered with a nail polish as the control.

2.3. Synthesis CHA by the two-step method

2.3.1. Preparation of active $CaCO_3$ layer (the first step)

$CaCO_3$ particles were synthesized as described previously [27,28]. The procedure was carried out in a closed desiccator at 80 °C for 1 h. Calcium carbonate crystals were precipitated into a three-necked flask containing 0.10 mol/L $CaCl_2$ solutions. In addition, different concentrations of either Asp or Glu (1.00 mmol/L, 5.00 mmol/L, 10.00 mmol/L, and 20.00 mmol/L) were respectively added to $CaCl_2$ solutions. Each demineralized enamel surface was exposed to one of the above-mentioned mixed solutions. Excessive amounts of NH_4HCO_3 were hung above the solution in a small beaker to produce a carbon source CO_2 vapor by volatilization.

2.3.2. Preparation of HA particle layer (the second step)

As described by Ying Liu [29], aqueous solutions of 0.03 mol/L $Na_2HPO_4 \cdot 12H_2O$ containing the same concentrations of amino acid (Asp or Glu: 1.00 mmol/L, 5.00 mmol/L, 10.00 mmol/L, and 20.00 mmol/L) were respectively added dropwise to the above-mentioned suspensions. The Ca/P molar ratio was adjusted to 1.67 in this system. The pH was controlled to be about 10 with a 20 wt.% NaOH solution. The mixed solutions were stirred for 2 h at 60 °C with a magnetic bar. Finally, the enamel surfaces were gently washed and dried for further analysis. The powder was rinsed three times with distilled water and then dried in a desiccator at 80 °C for 24 h.

2.4. Synthesis HA by the one-step method

The enamel pieces were placed into a three-necked flask containing 0.10 mol/L $CaCl_2$ solution. Then, 0.03 mol/L $Na_2HPO_4 \cdot 12H_2O$ was added dropwise to the suspension. Subsequent procedures were carried out following the steps in Section 2.3.2.

2.5. Structural and compositional analyses

The morphological growth of the $CaCO_3$ and HA particles on the enamel was studied under an FEI XL30 ESEM-FEG Field Emission

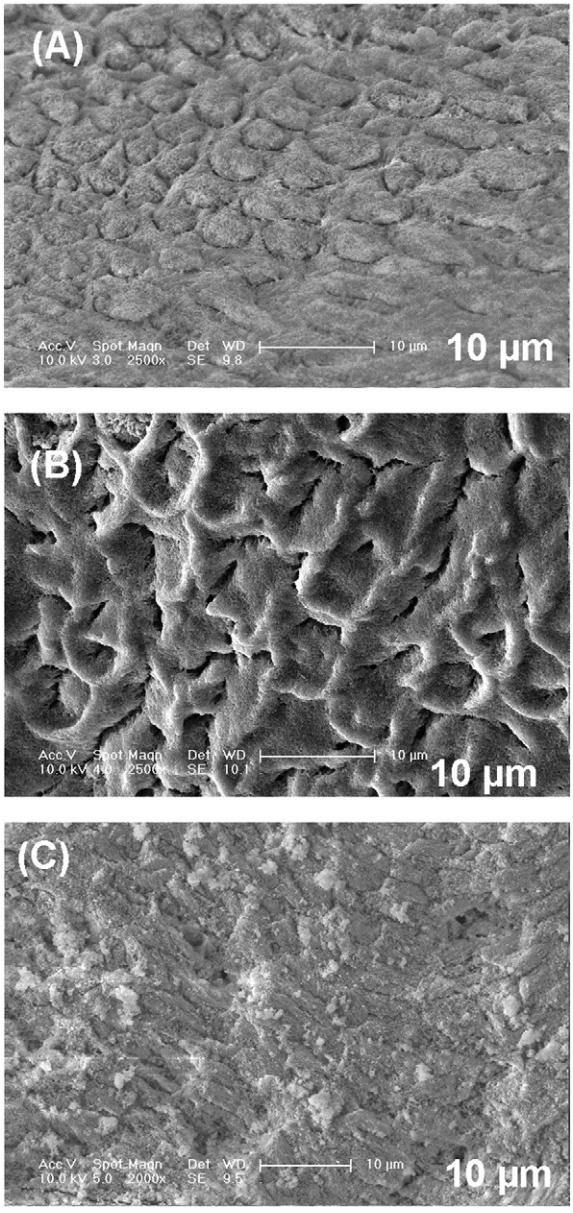

Fig. 1. SEM images of HA film prepared on the enamel pieces. (A) Etched enamel surface, (B) $CaCO_3$-con, (C) HA-con.