Acta Biomaterialia 9 (2013) 7289–7297

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

An amelogenin–chitosan matrix promotes assembly of an enamel-like layer with a dense interface

Qichao Ruan [a], Yuzheng Zhang [b], Xiudong Yang [a], Steven Nutt [b], Janet Moradian-Oldak [a,*]

[a] Center for Craniofacial Molecular Biology, Herman Ostrow School of Dentistry, University of Southern California, Los Angeles, CA 90033, USA
[b] Mork Family Department of Chemical Engineering and Materials Science, University of Southern California, Los Angeles, CA 90089, USA

ARTICLE INFO

Article history:
Received 18 January 2013
Received in revised form 4 March 2013
Accepted 1 April 2013
Available online 6 April 2013

Keywords:
Enamel
Amelogenin
Chitosan hydrogel
Apatite
Biomimetic

ABSTRACT

Biomimetic reconstruction of tooth enamel is a significant topic of study in materials science and dentistry as a novel approach to the prevention, restoration, and treatment of defective enamel. We have developed a new amelogenin-containing chitosan hydrogel for enamel reconstruction that works through amelogenin supramolecular assembly, stabilizing Ca-P clusters and guiding their arrangement into linear chains. These amelogenin Ca-P composite chains further fuse with enamel crystals and eventually evolve into enamel-like co-aligned crystals, anchored to the natural enamel substrate through a cluster growth process. A dense interface between the newly grown layer and natural enamel was formed and the enamel-like layer improved the hardness and elastic modulus compared with etched enamel. We anticipate that this chitosan hydrogel will provide effective protection against secondary caries because of its pH-responsive and antimicrobial properties. Our studies introduce an amelogenin-containing chitosan hydrogel as a promising biomaterial for enamel repair and demonstrate the potential of applying protein-directed assembly to biomimetic reconstruction of complex biomaterials.

1. Introduction

Enamel is the exterior layer of the mammalian tooth and a hard biomaterial with significant resilience that protects the tooth from external physical and chemical damage [1]. The remarkable mechanical properties of enamel are associated with its hierarchical levels of structure from the nanoscale to the macroscale [2]. The building blocks of enamel, the enamel rods, are densely packed arrays of elongated apatite crystals organized into an intricate interwoven structure [2]. Cellular activity and the protein-controlled process of mineralization are key to achieving such precisely organized structures [1]. The proteins that mediate the mineralization of apatite crystals are gradually degraded and eventually removed during enamel maturation [1,3,4]. Mature enamel is non-living and cannot regenerate itself after substantial mineral loss, which often occurs as dental caries or erosion. Currently the conventional treatments for carious lesions include refilling with amorphous materials like amalgam, ceramics, or composite resin [5]. However, even after those treatments secondary caries often arises at the interface between the original enamel and the filling materials due to weakening adhesion over time [6]. There is therefore a need for alternative restorative materials with improved adhesion to the tooth surface. One such alternative is a synthetic enamel-like material that can be prepared by biomimetic regrowth on the enamel surface.

Various biomimetic systems have been developed to repair enamel defects, including liquids and pastes that contain nano-apatite or different organic additives, for the remineralization of early, sub-micrometer sized enamel lesions. A glycerine-enriched gelatin system has been used to form dense fluorapatite layers on human enamel [7,8]. Growth in small cavities of enamel-like nanocrystals from a paste containing fluoride-substituted hydroxyapatite has been achieved in vitro [9], and a compacted fluorapatite film with a prism-like structure was synthesized on metal plates using a hydrothermal technique [10]. Formation of enamel-like structures under ambient conditions was also performed in vitro using a liquid and pastes with different organic additives [11–15]. Recently an electrospun hydrogel mat of amorphous calcium phosphate (ACP)/poly(vinylpyrrolidone) nanofibers was developed for the in vitro remineralization of dental enamel [16]. These investigations constitute significant progress in the study of enamel-like structures. Overall, however, biomimetic strategies still face an ongoing challenge in the fields of dentistry and material science.

In natural enamel the formation of apatite crystals occurs in an amelogenin-rich matrix that plays a critical role in controlling the oriented and elongated growth of apatite crystals [4,17–20]. Accordingly, we have used several strategies to prepare enamel-like materials that contain nano- and microstructures using amelogenin to control the crystallization of biomimetic

* Corresponding author. Tel.: +1 323 442 1759; fax: +1 323 442 2981.
E-mail address: joldak@usc.edu (J. Moradian-Oldak).

1742-7061/$ - see front matter © 2013 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2013.04.004

calcium and phosphate [20–23]. The results have opened up the promising possibility of remodeling complex enamel minerals in an amelogenin-containing system.

Here we report development of a new amelogenin-containing chitosan (CS-AMEL) hydrogel to synthesize an organized, enamel-like mineralized layer on an acid-etched enamel surface used as an early caries model. Compared with a previously developed amelogenin-containing system, CS-AMEL is easier to handle under clinical conditions. It is biocompatible, biodegradable, and has unique antimicrobial and adhesion properties that are practical for dental applications [24–26]. Chitosan has been observed to have antimicrobial activity against fungi, viruses, and some bacteria, including streptococci and lactobacilli, which are known as the principal etiological factors of dental caries [27–29]. Therefore, we expect that the "synthetic enamel" formed in the CS-AMEL hydrogel will have antimicrobial properties that can prevent bacterial infection and subsequent demineralization. In addition, chitosan is mucoadhesive to both hard and soft surfaces [30]. Importantly, the newly formed crystals in the CS-AMEL hydrogel grow directly on the original enamel, achieving complete adhesion of the repaired layer to the natural enamel with a dense interface. The robust attachment of the newly grown layer demonstrated in the present work can potentially improve the durability of restorations and avoid the formation of new caries at the margin of the restoration.

2. Materials and methods

2.1. Amelogenin preparation

Recombinant full-length porcine amelogenin rP172 was expressed in *Escherichia coli* and purified as previously described. The rP172 protein has 172 amino acids and is an analog of full-length native porcine P173, but lacking the N-terminal methionine as well as a phosphate group on Ser16 [20–23].

2.2. Tooth slice preparation

Human third molars (extracted following the standard procedures for extraction at the Ostrow School of Dentistry of the University of Southern California and handled with the approval of the Institutional Review Board) without any restored caries were selected. Slices 0.1–0.2 cm thick (Fig. 1a) were cut longitudinally using a water-cooled low speed diamond saw. To simulate early caries lesions tooth slices were acid etched with 30% phosphoric acid for 30 s and rinsed with deionized water.

2.3. Etched enamel repaired by the amelogenin-containing chitosan hydrogel

The amelogenin-containing chitosan hydrogel was prepared by mixing chitosan (medium molecular weight, 75–85% deacetylated, Sigma–Aldrich) solution (960 μl, 1% m/v), Na_2HPO_4 (15 μl, 0.1 M), $CaCl_2$ (25 μl, 0.1 M) and amelogenin rP172 (200 μg), followed by stirring at room temperature overnight. The pH value was adjusted to 6.5 with 1 M NaOH. 20 μl of chitosan-based hydrogel was carefully applied to the enamel surface and dried in air at room temperature. The tooth slices were then immersed in 30 ml of artificial saliva (AS) solution (0.2 mM $MgCl_2$, 1 mM $CaCl_2{\cdot}H_2O$, 20 mM HEPES buffer, 4 mM KH_2PO_4, 16 mM KCl, 4.5 mM NH_4Cl, 300 p.p.m. NaF, pH 7.0, adjusted with 1 M NaOH) [16] at 37 °C for 7 days. After the allotted time the tooth slice was removed from the solution, rinsed with running deionized water for 50 s and air dried.

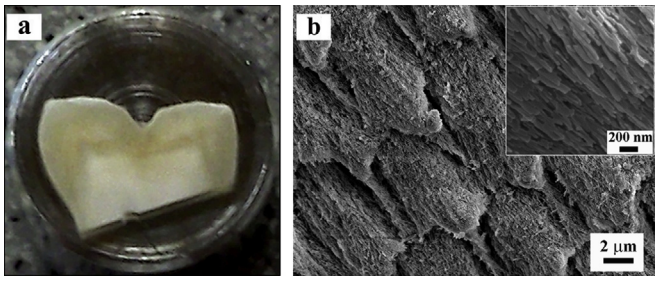

Fig. 1. (a) Optical micrograph of a tooth slice used in this work. (b) SEM image of an acid etched enamel surface.

2.4. Characterization

Scanning electron microscopy (SEM) imaging was performed in a field emission scanning electron microscope (JEOL JSM-7001F), operating at an accelerating voltage of 10 keV. X-ray diffraction (XRD) patterns were recorded in a Rigaku diffractometer with Cu K_α radiation (λ = 1.542 Å) operating at 70 kV and 50 mA with a step size of 0.02°, at a scanning rate of 0.1°s^{-1} in the 2θ range 10–60°. Thin sections (~100 nm) between enamel and the newly grown layer for the TEM observations were prepared in a SEM/FIB (JEOL JIB-4500) with an ion accelerating voltage of 30 kV. The device was also equipped with an in situ lift-out system (Omniprobe Autoprobe 200), which had a tungsten needle attached to a micromanipulator inside the FIB vacuum chamber. High resolution transmission electron microscopy (HR-TEM) images were obtained on a JEOL JEM-2100 microscope using an accelerating voltage of 200 keV. The hardness and elastic modulus were measured at 20 test points in each sample (n = 3) using a nano-indenter (Agilent-MTS XP) with a Berkovich tip. Circular dichroism (CD) spectropolarimetry was performed using a J-815 spectropolarimeter (JASCO, Easton, MD). The spectra were recorded between 190 and 260 nm with a step size of 0.5 nm and a scan rate of 50 nm min^{-1}. Fluorescence spectroscopy was performed using a PTI QuantaMaster QM-4SE spectrofluorometer (PTI, Birmingham, NJ). The amelogenin solutions were excited at 290 nm. The emission spectra were monitored between 300 and 400 nm with a step size of 1 nm.

2.5. Antimicrobial evaluation

Human saliva was collected as described in the literature [31] for the antimicrobial experimentation. Healthy adults were chosen as the subjects for saliva collection. Subjects were asked to refrain from eating, drinking, and oral hygiene procedures for at least 1 h prior to collection. Subjects were given distilled drinking water and asked to rinse their mouths out with it for 1 min. Five min after this oral rinse the subjects were asked to spit into a 50 ml sterile tube, which was placed on ice while more saliva was collected. The subjects were instructed to tilt their head forward and let the saliva run naturally to the front of the mouth; Upon collection of approximately 5 ml of saliva from a subject the saliva sample was immediately taken to the laboratory for processing. Twenty μl of saliva were added to tubes with 1 ml of lysogeny broth (LB) medium containing chitosan–amelogenin hydrogel or amelogenin, and then incubated at 37 °C overnight. The OD_{600} of the overnight cultures was measured using a Beckman DU-640 spectrophotometer.

2.6. Statistical analysis

Enamel remineralization experiments were repeated three times. The mechanical tests and antimicrobial experiments were conducted in triplicate and data were expressed as means ± standard deviations. For mechanical testing Student's t-test was applied to identify differences in the hardness and elastic modulus between etched and repaired enamel (n = 3). For the antimicrobial experiments (n = 3) the OD values were compared between control and