Biomaterials 34 (2013) 5036–5047

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Hydroxyapatite-anchored dendrimer for in situ remineralization of human tooth enamel

Duo Wu [a], Jiaojiao Yang [a], Jiyao Li [b,c], Liang Chen [b,c], Bei Tang [b,c], Xingyu Chen [a], Wei Wu [a], Jianshu Li [a,*]

[a] College of Polymer Science and Engineering, Sichuan University, Yi Huan Road, South Section One, No. 24, Chengdu 610065, China
[b] State Key Laboratory of Oral Diseases, Sichuan University, Chengdu 610065, China
[c] West China School of Stomatology, Sichuan University, Chengdu 610065, China

ARTICLE INFO

Article history:
Received 7 January 2013
Accepted 15 March 2013
Available online 8 April 2013

Keywords:
Dendrimer
Specific adsorption
Hydroxyapatite
In situ biomineralization
Dental restorative material

ABSTRACT

In situ remineralization of hydroxyapatite (HA) on human tooth enamel surface induced by organic matrices is of great interest in the fields of material science and stomatology. In order to mimic the organic matrices induced biomineralization process in developing enamel and enhance the binding strength at the remineralization interface, carboxyl-terminated poly(amido amine) (PAMAM–COOH)–alendronate (ALN) conjugate (ALN–PAMAM–COOH) was synthesized and characterized. PAMAM–COOH has a highly ordered architecture and is capable of promoting the HA crystallization process. ALN is conjugated on PAMAM–COOH due to its specific adsorption on HA (the main component of tooth enamel), resulting in increased binding strength which is tight enough to resist phosphate buffered saline (PBS) rinsing as compared with that of PAMAM–COOH alone. While incubated in artificial saliva, ALN–PAMAM–COOH could induce in situ remineralization of HA on acid-etched enamel, and the re-generated HA has the nanorod-like crystal structure similar to that of human tooth enamel. The hardness of acid-etched enamel samples treated by ALN–PAMAM–COOH can recover up to 95.5% of the original value with strong adhesion force. *In vivo* experiment also demonstrates that ALN–PAMAM–COOH is effective in repairing acid-etched enamel in the oral cavity. Overall, these results suggest that ALN–PAMAM–COOH is highly promising as a restorative biomaterial for in situ remineralization of human tooth enamel.

1. Introduction

In human tooth enamel, nanorod-like hydroxyapatite (HA) crystals are arranged into highly organized prism to form the main unit. In the oral cavity, the tooth enamel can be damaged by the local cariogenic bacteria in plaque (caries), non-bacterially derived erosive challenges (such as acidic beverages) or mechanical force. Traditional dental restorative materials involve metal, compound resin and ceramics. However, since their structures, components and properties are different from the natural material, they do not fit well with natural tissues in the lesions interface. Many attempts, including inorganic paste approaches [1–3], hydrothermal method [4–6], self-assembly or agents-mediated methods [7–10], have been taken to repair acid-etched enamel or to obtain enamel-like structure. However, most of the above methods need conditions such as extreme acidic pH, high temperature, high pressure, or in the presence of a concentrated solution of surfactant. Therefore, the in situ regeneration or remineralization of HA under physiological conditions as an alternative restorative material is very desirable in stomatology.

The biomineralization process in nature, such as the construction of tooth enamel, is controlled by organic matrices (including various proteins such as amelogenin for tooth enamel). By now, quite a few natural or synthetic materials have been developed to mimic the organic matrices to regenerate dental tissues, i.e., restoring the injured enamel by inducing HA remineralization on dental surface. For example, amelogenin and its supramolecular assembly have been directly used to obtain mineral layers containing organized needle-like fluoridated HA crystals on the surface of etched enamel [11–13]. Kirkham et al. utilized a self-assembled anionic peptide to form scaffolds on caries-like lesions in dental enamel under simulated intra-oral conditions, leading to a significant net HA remineralization [14]. Peptide amphiphiles are also used as artificial matrices for biological synthesis of tooth enamel

* Corresponding author. Tel.: +86 28 85466755; fax: +86 28 85405402.
E-mail addresses: jianshu_li@scu.edu.cn, fredca2005@163.com (J. Li).

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.03.053

[15]. In addition, a glycerine-enriched gelatin gel containing phosphate and fluoride ions [16], and a combination of glumatic acid and nano apatite particles [17] have both been reported to be capable of inducing enamel-like structure of reconstructed mineral on dental surface. However, most of the above strategies still have limitations, such as the difficulty in preparation of proteins/peptides, the overuse of fluoride ions and the complicated multi-steps in clinic applications. Thus, it is needed to develop a simple strategy to mimic the functions of organic matrices to induce biomineralization on the surface of tooth enamel.

It is widely known that dendrimer is a class of mono-dispersed polymeric nanomaterials with plenty of branches radiating from one central core and highly ordered architecture. It has been referred as 'artificial protein' due to its biomimetic properties and well-defined/easily tailored structure, i.e., its functional group, generation and spatial structure are controllable [18]. Several kinds of dendrimers or their derivatives have been applied in biomineralization field [19–22]. Specifically, poly(amido amine)-type (PAMAM) dendrimer has been widely investigated in the crystallization process of HA in recent years. The size and shape of HA could be regulated by PAMAM dendrimer with different surface groups, generations and concentrations [23,24]. Recently, an amphiphilic PAMAM dendron has been synthesized with aspartic acids on the periphery and an aliphatic chain at the focal point, which exhibited a self-assembly behavior similar to that of amelogenin in the oriented growth of HA *in vitro*, i.e., initially aggregating to nanospheres and further translating to linear chains [25]. However, because the free dendrimer or its derivatives in aqueous solution could not contribute to the crystal growth on a specific substrate, it is necessary to increase the binding capability between PAMAM and the substrate of the human hard tissues (mainly HA) to achieve in situ regeneration or remineralization.

It has been proved that alendronate (ALN) could easily adsorb on the HA crystals. Palazzo et al. investigated the adsorption and desorption kinetics of ALN towards synthetic HA nanocrystalline materials. They found that the interaction between ALN and HA surface takes place by ligand exchange in which the two phosphate groups of the ALN molecule replace two surface phosphate groups of HA [26]. This adsorption affinity is further proved to be a two-site model, i.e., with two different binding sites, by using isothermal titration calorimetry method [27]. Therefore, ALN has been conjugated with different drug carriers to provide them with the HA binding specificity as bone- or tooth-targeted drug delivery systems [28–33]. In this work, we synthesize an HA-anchored ALN–PAMAM–COOH dendrimer and then evaluate its in situ biomineralization behavior on the surface of acid-etched human tooth enamel (both *in vitro* and *in vivo*). It may mimic the role of natural organic matrices in biomineralization. Anionic PAMAM dendrimer with carboxyl groups on the periphery is linked with ALN to obtain ALN–PAMAM–COOH conjugate. The ALN–PAMAM–COOH conjugate should adsorb on the surface of tooth enamel due to the HA binding specificity of ALN, and then induce in situ HA regeneration in artificial saliva (Scheme 1). Several instrumental methods have been used to characterize the HA binding capability of ALN–PAMAM–COOH, the morphology and the mechanical properties of newly generated HA on the surface of acid-etched enamel. Ultimately, we hope to investigate the ALN–PAMAM–COOH as a restorative material for in situ remineralization of tooth enamel.

2. Materials and methods

2.1. Materials

Dicyclohexyl carbodiimide (DCC), ethyl (dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), alendronate (ALN), fluorescein isothiocyanate (FITC) were purchased from Aldrich. Hydroxyapatite powder was purchased from National Engineering Research Center for Biomaterials, Sichuan University (medical grade, spherical HA powder of 10 μm in diameter). Human tooth samples were extracted following standard procedures for extraction at the Hospital of Stomatology in Sichuan University, and handled with permission of Institutional Review Board. All other reagents and solvents if not specified were purchased from Tianjin Bodi Chemical Holding Company and were all of analytical grade except for chromatographic grade methanol (MeOH).

2.2. Synthesis of carboxyl-terminated PAMAM dendrimer (PAMAM–COOH)

The divergent synthesis of PAMAM dendrimer includes two-step iterative sequence to produce amine terminated structures. It involves alkylation with methyl acrylate (MA) and followed by amidation with excessive 1, 2-ethylenediamine (EDA). The alkylation step produces ester terminated dendrimer that is referred as 'half-generations'. The second step involves amidation of the ester terminated intermediates with a large excess of EDA to produce amine terminated dendrimer, which is named as 'full-generations'. PAMAM dendrimer was synthesized step by step following the classical method reported by Tomalia [34,35]. G3.5 PAMAM dendrimer was synthesized for further modification: ^{1}H NMR (400 Hz, $CDCl_3$) δ (ppm) = 2.41–2.49 (−CH_2−NCH_2−), 2.28–2.38 (−CH_2CONH−), 3.27–3.28 (−$NHCH_2$), 2.75 (−CH_2COOCH_3), 3.66 (−$COOCH_3$), 7.27 (−NH−).

G3.5 PAMAM (1.681 g, 0.280 mmol) was dissolved in MeOH (10 mL) in a reflux condenser, NaOH (0.430 g, 10.752 mmol) was added to the dendrimer solution and then reacted at 60 °C for 8 h. MeOH was evaporated and then the cold MeOH was used to dissolve dendrimer and simultaneously precipitate unreacted NaOH. After filtrating, the filtrate was evaporated again to obtain the alkaline hydrolyzed dendrimer G3.5–COONa, and then dissolved in water. Then pH value of the solution was adjusted to 3 by adding 0.1 M HCl, followed by dialysis against water (molecular weight cut-off: 3500) to remove NaCl and then lyophilized. Yield: 85%. ^{1}H NMR (400 Hz, D_2O) δ (ppm) = 2.81–2.99 (−CH_2−NCH_2−), 2.62–2.68 (−CH_2CONH−), 3.42–3.60 (−$NHCH_2$−), 3.26–3.29 (−CH_2COOH).

2.3. Synthesis of ALN–PAMAM–COOH dendrimer

The carboxyl groups of PAMAM–COOH were activated by NHS (0.097 g, 0.840 mmol) in dimethyl sulfoxide (DMSO) for 0.5 h. After that, DCC (0.173 g, 0.840 mmol) was added under stirring and the reaction was continued for overnight at 25 °C. The reaction system was filtrated to remove the by-product 1, 3-dicyclohexylurea (DCU) and the filtrate containing NHS–PAMAM–COOH was collected. The filtrate was then added into ALN (0.379 g, 1.400 mmol) aqueous solution in three batches, 4 h apart. After the last batch the reaction was continued for another 36 h at room temperature. The solution was then filtrated again and dialyzed against water (molecular weight cut-off: 3500), followed by lyophilization to obtain the final product ALN–PAMAM–COOH. Yield: 52%. ^{1}H NMR (400 Hz, D_2O) δ (ppm) = 2.81–2.88 (−CH_2−NCH_2−), 2.69 (−CH_2CONH−), 3.44–3.56 (−$NHCH_2$−), 3.27–3.29 (−CH_2COOH), 1.31–1.36 (−CH_2CH_2−).

2.4. Cytotoxicity assay

MTT assays were performed to measure the cytotoxicity of the synthesized ALN–PAMAM–COOH. HepG2 cells were cultured in Dulbecco's modified eagle medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL of streptomycin at 37 °C, with 5% CO_2 and 95% relative humidity. The cells were seeded in a 96-well microtiter plate (Nunc Co., Wiesbaden, Germany) at a density of 10^4 cells/well and incubated in DMEM/well (100 μL) for 24 h. The culture media was replaced with fresh culture media containing serial dilutions of ALN–PAMAM–COOH, and the cells were incubated for 24 h. Then, 10 μL of sterile-filtered MTT stock solution in 5 mg/mL PBS was added to give a final MTT concentration of 0.5 mg/mL. The unreacted dye was removed by aspiration after 5 h. The formed formazan crystals were solubilized in DMSO (100 μL/well). The absorbance was measured using a microplate reader (Spectra Plus, Tecan, Zurich, Switzerland) at a wavelength of 570 nm. The cell viability (%) was calculated from $100 \times ([A]_{test} - [A]_{PEI})/[A]_{control}$, where $[A]_{test}$, $[A]_{PEI}$ and $[A]_{control}$ represent the absorbance values of the wells with ALN–PAMAM–COOH, with PEI (positive control) and without ALN–PAMAM–COOH (negative control), respectively. The absorbance was the average value measured from six wells in parallel for each sample.

2.5. Preparation of tooth enamel samples

Sound human third molars were obtained from and approved by the Hospital of Stomatology in Sichuan University. After extraction, the organic contaminants on the teeth were removed with a scalpel blade. Then, the teeth were further cleaned with 3% sodium hypochlorite (to remove adhered bacteria) and rinsed with PBS. The teeth were stored at 4 °C in water containing 0.05% thymol before use. A diamond-coated band saw was used to separate root from crowns and to cut sections longitudinally, resulting in an approximately 5 × 5 mm^2 square plate. They were ground flat and polished with water-cooled carborundum discs. All the surfaces of sections were protected with acrylic resin except the polished enamel surface. The sample