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Modulated regeneration of acid-etched human tooth enamel by a functionalized dendrimer that is an analog of amelogenin

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ABSTRACT

In the bioinspired repair process of tooth enamel, it is important to simultaneously mimic the organic-matrix-induced biomineralization and increase the binding strength at the remineralization interface. In this work, a fourth-generation polyamidoamine dendrimer (PAMAM) is modified by dimethyl phosphate to obtain phosphate-terminated dendrimer (PAMAM-PO_3H_2) since it has a similar dimensional scale and peripheral functionalities to that of amelogenin, which plays important role in the natural development process of enamel. Its phosphate group has stronger affinity for calcium ion than carboxyl group and can simultaneously provide strong hydroxyapatite (HA)-binding capability. The MTT assay demonstrates the low cytotoxicity of PAMAM-PO_3H_2. Adsorption tests indicate that PAMAM-PO_3H_2 can be tightly adsorbed on the human tooth enamel. Scanning electron microscopy and X-ray diffraction are used to analyze the remineralization process. After being incubated in artificial saliva for 3 weeks, there is a newly generated HA layer of 11.23 μm thickness on the acid-etched tooth enamel treated by PAMAM-PO_3H_2, while the thickness for the carboxyl-terminated one (PAMAM-COOH) is only 6.02 μm. PAMAM-PO_3H_2 can regulate the remineralization process to form ordered new crystals oriented along the *Z*-axis and produce an enamel prism-like structure that is similar to that of natural tooth enamel. The animal experiment also demonstrates that PAMAM-PO_3H_2 can induce significant HA regeneration in the oral cavity of rats. Thus PAMAM-PO_3H_2 shows great potential as a biomimetic restorative material for human tooth enamel.

1. Introduction

In nature, there are numerous biomineralized materials which have excellent mechanical properties, such as bone and teeth. It has been revealed that these materials have a complex hierarchical organization from the nanometric to the macroscopic scale [1–4]. In the biomineralization process, inorganic minerals are generated by the regulation of biological macromolecules, such as various proteins. Tooth enamel is the hardest mineralized tissue in the human body, and it is formed under the control of organic matrices [5]. The mature enamel consists of around 96% of inorganic materials and 4% of organic materials. The inorganic composition is nanorod-like hydroxyapatite (HA) crystals, which are arranged into highly organized hierarchical microstructures (prism) [6]. It has been revealed that amelogenin secreted by ameloblasts is the major enamel protein, and constitutes of approximately 90% of all organic matrix material in developing enamel [7–10]. It plays an important role in the biomineralization process of tooth enamel, especially for the crystallites to form the well-organized prism pattern [11]. The sequence of the amelogenin molecule is characterized as bipolar, with two distinct regions. Compared to the bulk of the molecule, which has an isoelectric point (p*I*) of 8.0, the carboxyterminal telopeptide (13–15 amino acid residues) is highly charged, with a p*I* of about 4.2. This hydrophilic carboxy-terminal is probably externalized on the amelogenin nanosphere surface, creating negatively charged structures which will interact with the forming enamel apatite crystals at a very early stage of enamel formation [12,13]. Amelogenin can also affect the morphology of the crystalline, depending on its carboxy-terminal telopeptide [14]. Moreover, the in vitro experiments show that the phosphate group of amelogenin can stabilize the structures of amorphous calcium phosphate [15,16]. Further, the argument that amelogenin nanospheres adhere synthetic apatite crystals through a "polymer-bridging"

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mechanism is widely accepted [17]. Thus we can see that an analog of amelogenin should have a nanosphere structure with a modified phosphate group. Unlike other calcified tissues, mature tooth enamel has a low self-repair capability due to the shortage of living cells [18]. Thus, once enamel is damaged, whether by local cariogenic bacteria, erosive challenges or mechanical force, the body cannot regenerate it since there are no ameloblasts to secrete amelogenin. Therefore, it is of great interest to scientists in different fields to design various smart strategies to repair the defective tooth enamel.

Traditional dental restorative materials include metals, compound resins and ceramics. However, these do not fit well with natural tissues at the lesion interface because their components, structure and properties are not similar to those of the natural HA [19–21]. In recent years, the biomimetic synthesis of enamel-like HA has attracted much attention. A number of natural and synthetic materials have been developed to regenerate tooth enamel by inducing the in situ remineralization of nanorod-like HA on the defective surface [22–24]. For instance, natural amelogenin and its supramolecular assembly have been directly applied and have achieved effective regeneration of the HA layers [25–27]. Other systems, such as a self-assembled anionic peptide [28], peptide amphiphiles [29], a glycerine-enriched gelatin gel containing phosphate and fluoride ions [30], and a combination of glutamic acid and nanoapatite particles [31], have all been reported to mimic the biomineralization process and regenerate enamel-like HA. However, since it is difficult to extract/purify/store the natural protein, or there is overuse of fluoride ions and complicated multi-steps in these strategies, their further use in clinical applications is limited. Thus, it is necessary to develop a simple strategy to mimic the functions of amelogenin to induce the remineralization of the surface of defective tooth enamel.

Dendrimers and their derivatives have well-defined structures, with monodispersity and easily modified surface groups. They are termed "artificial proteins", and have been widely applied as protein analogues for various biological, medical and material applications [32–34]. As the first synthetic dendrimer, poly(amido amine) (PAMAM) dendrimer has been investigated as a biomineralized material, especially in the crystallization process of HA [34–36]. It was found that PAMAM dendrimer can regulate the size and shape of HA by the modification of different surface groups and generations, and is concentration dependent [37,38]. For example, the carboxyl-terminated PAMAM dendrimers (PAMAM-COOH) has a significant influence on the size and shape of HA nanostructures [6,39]. In our previous work, we reported that PAMAM-COOH has a similar structure and similar self-assembly behavior to amelogenin [40]. It can be immobilized within the collagen matrix and induces intrafibrillar mineralization with a hierarchical structure on human dentine, which is regulated by the amorphous precursor pathway [33,41]. We also found that alendronate-conjugated PAMAM dendrimer (ALN-PAMAM-COOH) could induce in situ remineralization of tooth enamel, which is due to the combined effect of the HA-anchored property of the ALN moiety and the remineralization capability of the –COOH moiety [42]. However, the complex synthesis of ALN-PAMAM-COOH may hinder further industrialization of the process and potential biomedical applications.

In this work, as inspired by the structure and property of amelogenin, we attempt to prepare phosphate-terminated PAMAM dendrimer (PAMAM-PO_3H_2) and investigate its application as an amelogenin analog on the remineralization process of acid-etched human tooth enamel (Scheme 1). There are two reasons for the molecular design: the phosphate group has a stronger affinity for calcium ion than the carboxyl group and it can also provide strong HA-binding capability [43–45]. Thus it can fulfill the two functions of the ALN and –COOH moieties of ALN-PAMAM-COOH at the same time. The structure and property of PAMAM-PO_3H_2 are characterized by ^{1}H nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FTIR) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Adsorption experiments are performed to examine the binding capability to HA and human tooth enamel. The acid-etched tooth enamel samples coated with PAMAM-PO_3H_2 are investigated both in vitro (artificial saliva) and in vivo (oral cavity of rats). Scanning electron microscopy (SEM) and X-ray diffraction (XRD) are performed to analyze the remineralization process.

2. Materials and methods

2.1. Materials

Trimethylbromosilane was purchased from Tokyo Chemical Industry (TCI Shanghai). Dimethyl phosphate was purchased from Damas-beta. Hydroxyapatite powder was purchased from National Research Center for Biomaterials, Sichuan University (medical grade, spherical HA powder 10 μm in diameter). Human tooth enamel samples were extracted at the Hospital of Stomatology in Sichuan University. Standard procedures for extraction were followed during the extraction. These were approved by the institution's review board. Most of the other reagents and solvents were purchased from Tianjin Bodi Chemical Holding Company. All of them were of analytical grade, except that methanol (MeOH) was of chromatographic grade. Carboxyl-terminated PAMAM dendrimer (PAMAM-COOH) was synthesized according to previously reports [33].

2.2. Synthesis of PAMAM-PO_3H_2

First, the fourth-generation PAMAM dendrimer (G-4.0) was synthesized following the classical method introduced by Tomalia et al. [35,46] for further modification. Next, G-4.0 (1.600 g, 0.232 mmol) and paraformaldehyde (0.384 g, 12.8 mmol) were dissolved in a mixture of potassium hydroxide aqueous solution (1 mol l^{-1}, 7 ml) and tetrahydrofuran (10 ml) in a 50 ml round-bottom flask. Dimethyl phosphate (1.95 ml, 21.26 mmol) was then added slowly with stirring at room temperature. The flask was placed in an oil bath at 70 °C for 14 h with vigorous stirring [47]. After removal of the solvent, the resulting mixture was dialyzed (MWCO 3500) and freeze-dried to obtain phosphate ester-terminated PAMAM (PAMAM-PO$(OCH_3)_2$) dendrimer. The phosphate ester (0.5 g, 0.034 mmol) was then dissolved in dimethylsulfoxide (DMSO, 450 ml) in a 1 l round-bottom flask. Trimethylbromosilane (2.32 ml, 17.566 mmol) was added and the flask was stirred at room temperature for 24 h [48]. Finally, excess methanol (1 ml) was added and the solution was stirred at 25 °C for another 24 h. The mixed solution was concentrated by evaporation and dialysis (MWCO 3500), and freeze-dried to obtain PAMAM-PO_3H_2. FTIR and ^{1}H NMR (400 Hz, D_2O) were used to confirm its structure.

2.3. Cytotoxicity assay

To measure the cytotoxicity of PAMAM-PO_3H_2, an MTT assay was performed on the L929 cell line. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, 100 units ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin at 37 °C, in 5% CO_2 with 95% relative humidity. The cells were seeded in a 96-well microtiter at a density of 1×10^4 cells per well and incubated in 100 μl of DMEM per well for 24 h. The culture medium was replaced with 100 μl of fresh DMEM containing serial dilutions of PAMAM-PO_3H_2. After incubation for 24 h, 10 μl of MTT solution (in phosphate buffer,

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