Constructed amphiphilic gel as natural auxiliary medium to mimick remineralized dental hydroxyapatite

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Abstract: For obtain enamel-like hydroxyapatite, we are trying to induce the growth of crystal with modified chitosan which can simulate the organic template like extracellular matrix--Amelogenin to induce the deposition of miner ions. Phosphorous acid modification was applying into natural polycation polysaccharide chitosan to synthesize polyanion derivate phosphorylated chitosan. Amphiphilic hydrogels combined with phosphorylated chitosan (+cation) and gelatin (-anion) were built through peptide binding reaction which using Genipin as crosslinker. The gels self assembled on the inert surface of tooth which stimulated by ultraviolet radiation. $CaCl_2$ and Na_3PO_4-$12H_2O$ solutions provided mineralized ion then the hydroxyapatite assembled and grown in situ of the tooth. All of the samples detected by FTIR, SEM and XRD to confirm the character of neonatal crystal. The amphiphilic gel can enhance the proportion of organic elements on the surface of dentin which confirmed by FTIR. After mineralized reaction, we observed bunch and column (15-25nm) crystal arranged on the dentin base. XRD result showed the neonatal crystal is hydroxyapatite and scratch tester testified the coalescent force between 22-25N. The amphiphilic gel can be induced the enamel-like crystal grown under demineralizing tooth basement.

Introduction

Enamel is the exterior layer of human tooth and a highly mineralized biomaterial with significant resilience that protects the human tooth from physical and chemical damages [1]. The basic component of mature enamel is hydroxyaptite (HAP) and Fluor-hydroxyapatite (FHAp), it is highly ordered structural organization which fundamental units are rod and inter-rod woven by parallel crystals boundless[2]. As adult tooth enamel is not living tissue, it can hardly remineralize after the substantial mineral loss. Thus, the synthesis of enamel through biomimetics is of great interest to dental clinicians. The key of remineralization or regeneration of tooth hard tissue is finding an appropriate organic template and inducing the process of mineralization. The frequently used agents are fluoride [3], salivary proteins [4], casein phosphopeptide amorphous calcium phosphate [5], or organic polymers [6]. However, these approaches either call for too severe conditions to apply in clinics, or reconstruct structures unlike natural enamel.

The formation of Calcium matrix proteins in bones (mainly collagen type I) and teeth (amelogenin) are associated with the nucleation and growth of hydroxyapatite crystals [7]. For mimicked the physiological course to obtain neonatal hydroxyapatite we chose phosphonic chitosan as the crystal growth monolayer.

Chitin, a naturally abundant mucopolysaccharide, and the supporting material of crustaceans, insects, etc., is well known to consist of 2-acetamido-2-deoxy-b-D-glucose through a β (1,4) linkage [8]. Chitosan, obtained by alkaline N-deacetylation of chitin, (Fig. 1) is one kind of the most widely used natural cationic polysaccharides. Although it is insoluble at physiological pH, but still shows particularly high biocompatibility and fairly low cytotoxicity [9].

Reports reveal that apatite-chitosan composite would be a candidate for a bioactive material. When a functional group e.g., carboxyl group that is effective for apatite nucleation, is present on the surfaces of carboxymethylated chitin and gelatin gum gels, then apatite spontaneously forms on these surfaces [10] [11]. Chemical modification of chitosan including acylation, carboxylation, hydroxylation, etherification, Schiff base reaction, N-alkylation, esterification and hydrolysis, oxidation, halogenation and other graft modification. Researchers attaches great importance to phosphate esterification reaction for phosphate group were induced which have special affinity with calcium ions.

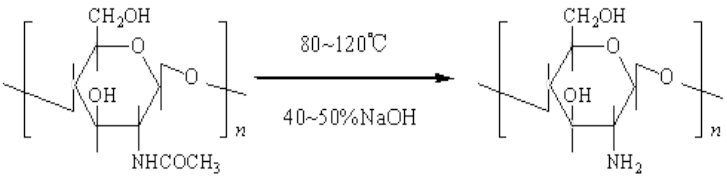


Figure 1. Structure of Chitin and Chitosan

Based on the basic theory, we use phosphonicchitosan molecules model to induce the crystallization of hydroxyapatite to synthesized tooth-like calcium phosphate/hydroxyapatite with 3D-structure in a controllable way in vitro.

Materials and methods

Phosphonized chitosan

One equivalent of phosphorous acid in water was added dropwise to one equivalent (w/w) of 2% chitosan solution in acetic acid with continuous stirring for 1 h. Raised the temperature to 70 ºC and one part of 36.5%(w/w) formaldehyde was added drop-wise over 1 h with reflux and heating for 6 h. After dialyzed against demineralized water for 48 h, frozen and freeze-dried finally [12]. Figure 2 showed the reaction of phosphonized chitosan. The variation elements on the surface of sample were investigated by Fourier transformed infrared spectroscopy (FTIR, EQUINOX 55, Bruker Optics, Switzerland).

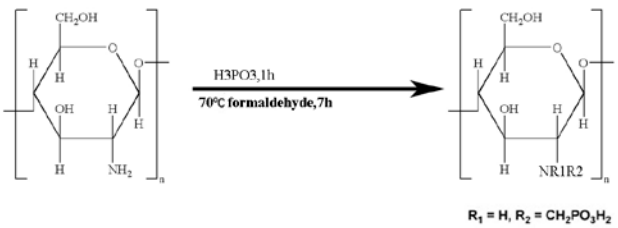


Figure 2. Synthesis of N-methylene phosphonic chitosan

Sample preparation

Human molars were sliced into 1mm thickness disks and etched with 0.5N EDTA to reveal the different orientations of the dentinal tubule. The disks were immersed in 0.02M Tris-HCL buffer (pH7.2,) after cleaned by ultrasonic. The samples were studied by scanning electron microscopy (SEM) (S-2460N, Hitachi, Tokyo, Japan).

Phosphorylation of tooth surface

The molars disks were daubed with phosphate ions dental adhesive agent equably (Prime& BondNT, DentsplyDetreyGmbh). The solidification process induced by curing light is 10 s at least. N-methylene phosphonic chitosan solution(test group) and chitosan solution(contrast group) were daubed to disks of molar after phosphorylation.

Mineralization in vitro

Biomimetic calcification solution with 2.58 mM calcium ($CaCl_2 \cdot 2H_2O$, >74.4% $CaCl_2$) and 1.55 mM phosphates (KH_2PO_4, >99%) at 37°C, buffered by 50 mM pH=7.6 trihydroxymethylaminomethane (Tris) – hydrochloric acid and 180 mM NaCl. The calcification solution pH was adjusted by 1 M HCl to 7.60 using a Metrohm 718 pH-STAT. [13]. All the samples were studied by X-ray diffraction (XRD, X'Pert Pro MPD, Philips, Holland), SEM.

Determination of coalescent force

The coalescent force between tooth surface and neonatal crystal Scratching Method(WS-92 scratch tester). The loading range between0.01N-30N, then automatic continuous loading in the following way: trial is conducted initially in precision 0.1N，scratch speed 2mm/min，loading rate for 10N/min,loading pressure head is cone Angle 120 ° of the diamond, and the tip radius is 0.2mm.

Results and discussion

Characterization of CS and NMCS

FTIR spectrogram showed organic compounds were grafted to the surface of tooth section. Figure 3 showed the FTIR spectrum of chitosan(CS) and N-methylene phosphonic chitosan(NMCS). 894 cm^{-1} and 1154 cm^{-1} absoprtion band were the characteristic band of glycosyl in chitosan. Compared with CS, the NMCS spectrogram showed weak band of $-NH_2$ in 1600 cm^{-1} wave area and sturdy band of acidamideI in 1635 $cm^{-1}$and acidamide II in 1524 cm^{-1}. The stretching vibration peak of alcohol hydroxyl lower in1000~100 cm^{-1}, and new vibration peak of PO_4 were observed in 1048 cm^{-1}. There were broad absoprtion band between 2000～3500 cm^{-1} that suggest dissociative H^+in $-NH_2$ were insteaded by $-CH_2$-PO_3.

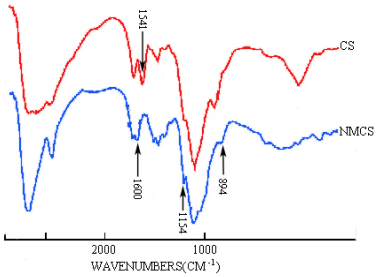


Figure 3. FTIR spectrum of CS and NMCCS

Calcium Phosphate Formation on tooth

After aging in SCS for four hours, SEM micrographs showed that the enamel rods were blocked by neonatal hydroxyapatite layer. Fig 4(A) showed the structure of enamel rod after decalcificated of EDTA 1h. Type II etching pattern, where the prism boundary is preferentially eroded. Continuous hydroxyapatite layer were formed within 24 hours, and a crystal shape were observed on test groups which NMCS covered the surface of enamel(Fig 4(B)). The mean diameter of the rod was 15±6 nm. Type I pattern with the prism core preferentially eroded were showed in Fig 4(C). In this section the neonatal hydroxyapatite were mutual parallel arrangement in the interspace after eroded(Fig 4(D)). The habit and size of the crystals were similar to those crystals obtained in prism boundary eroded section.

In contrast group which without chitosan crosslink to enamel after daubed with phosphate ions dental adhesive, only sheet and irregular neonatal apatite could be observed. There were about 2μm columned precipitation of mineral crystals formed on the surface within 24 hours (Fig2(E)). On the surface of tooth, hollow tubules of enamel were only filled in by shot prismatical crystal dispersedly and irregularly(Fig 2(F)). Furthermore, both test group and contrast group have a continuous structure of columns crystal with size of 10-20nm.

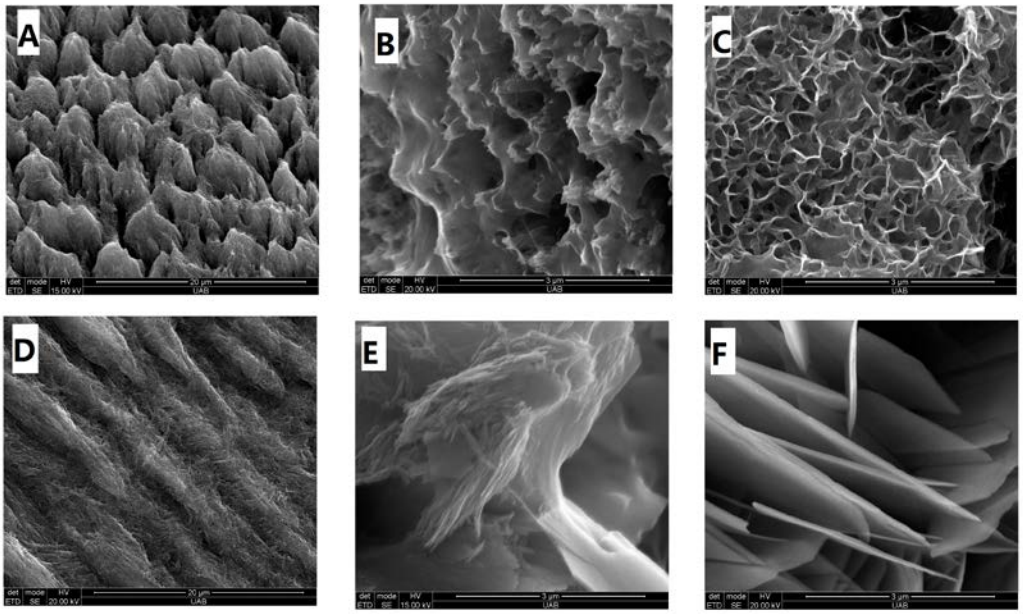


Figure 4. SEM images of neonatal crystal

A.normal enamel rod after decalcificated 24 h(type II). B.the form of crystal after mineralization in test group 48 h(type II). C.normal enamel rod after decalcificated 24 h(type I). D.the form of crystal after mineralization in test group 48 h(type I). E.the form of crystal after mineralization in contrast group 48 h(typeII). F. the form of crystal after mineralization in contrast group 48 h(type I).

XRD spectrum

XRD spectrum of the neonatal crystal (Figure 5) suggested that several diffraction peaks around 2θ=32˚ which are corresponding to the expected Bragg peaks for hydroxyapatite, implying that HA crystals were formed. In test group, the peaks around 25-26˚ were sharper than contrast group, and peaks around 42-47˚ were smoother than others(Figure 5). This may came from the plentiful organic molecules on the surface of experiment groups which induced the growth of hydroxyapatite and didn't hydrolyze on time.

Test group shows sharper characteristic peak than contrast group in 2θ = 26°,29°,32°,39°which corresponding to $Ca_{10}(PO_4)_3(CO_3)_3(OH)_2$, $Ca_{10}(PO_4)_5CO_3(OH)F$, $Ca_{10}(PO_4)_6(OH)_2$ and $Ca_{10}(PO_4)_5CO_3(OH)F$.

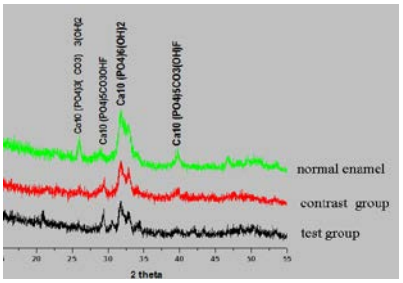
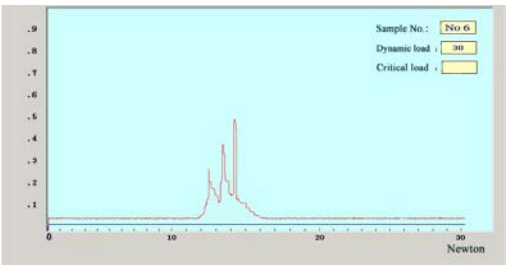


Figure 5. XRD of precipitation formed on tooth surface

Determination of coalescent force

Figure 6 and 7 showed the coalescent force between tooth surface and neonatal crystal in contrast group and test group are 13~15N and 22~25N.



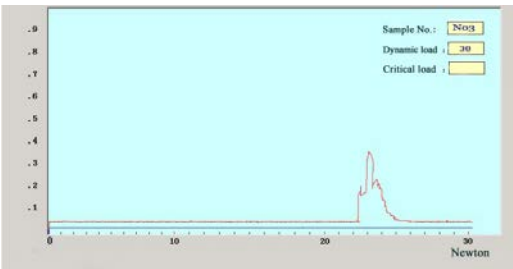


Figure 6. Coalescent force between neonatal crystal and tooth surface in contrast group(CS).
Figure 7. Coalescent force between neonatal crystal and tooth surface in test group(NMCS).

Discussion

Although dentin and bone are similarly composed of about 70% hydroxyapatite and 30% organic material [14], histological analyses demonstrate distinct cellular and morphological differences between these tissues. That's why we chose N-methylene phosphonic chitosan instead collagen to induce the synthesis of Hydroxyapatite which full of phosphate group has special affinity with calcium ions. It should be pointed out that we have tried to deposit the PO_4^{3+} onto different substrates, only the iron-plate substrateis able to produce the enamel-like films. The others may support the growth and orientation of the HA crystals but do not produce the closely aligned prismlike structure. The reason for this is still under investigation.

There is an interesting phenomenon that the eroded pattern would decide the direction of the neonatal crystal(Fig 4 (B)(D)). Acid attack initiated at the rod sheath space then penetrated into the rod core before extending into neighbouring rods through the rod tail [15]. So in the further study the time of eroded and the variety of acid etching agent such as EDTA and 37% phosphate etc would be observed.

Phosphate ions dental adhesive agent is polyanion organic molecules which not only inducted the demineralization on surface of tooth, but also adsorbed on enamel through calcium phosphate chemical bond. The phosphate groups and halogenated phosphoric acid would bonding to Ca^{2+} on tooth surface by coordinate bond or formed hydrogen bonds and intermolecular forces with collagen [16]. So dentin adhesive agent can intake calcium and phosphorus from the SCS, then generation apatite is induced.

The successful growth of enamel-like HA under the guide of NMCS not only shows that they are biocompatible, but also shows that under the control of the appropriate signaling molecules and cell-culture conditions it might be possible to induce dentin and pulp formation on the synthetic enamel layer in vitro. The key to specificity in controlled crystallization is the "molecular recognition" effect at the interface between functional groups on the organic macromolecules and ions in the surface of a crystal nucleus [17-18]. The organic model dental collagen-biologic N-methylene phosphonic chitosan connected by peptide bond and the $–COO^-$ headgroup on the peptide can accumulate Ca^{2+} and PO_4^{3-},OH^- ions and build the beneficial lattice configuration for the plane of hydroxyapatite [19]. Overall, the amphiphilic gel combined with the implementation of Ca^{2+} in the solution which provide three dimensional sites for directed self-assembly arrangement of biomolecule template in the tissue of tooth surface. All of these sites will regulate the hydroxyapatite crystals arrange in parallel shape which the length of neonatal crystal is more close to beam crystal like nature enamel column. These studies provides reliable basic data for regeneration of enamel crystals.

Conclusion

In summary, the present work shows we are able to modifie the model of mineralization by phosphonized reaction which partly mimiced the physiological process of mineralization. Both of these neonatal structures are very similar in chemical composition and in structural dimensions to natural tooth enamel. The specific organic molecule model can be used as a potential effective crystal growth modifier.

Acknowledgement

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