Repair of enamel by using hydroxyapatite nanoparticles as the building blocks

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The application of calcium phosphates and their nanoparticles have been received great attention. However, hydroxyapatite (HAP) is not suggested in dental therapy to repair the damaged enamel directly although this compound has a similar chemical composition to enamel. We note that the size-effects of HAP are not taken into account in the previous studies as these artificial particles frequently have sizes of hundreds of nanometres. It has recently been revealed that the basic building blocks of enamel are 20–40 nm HAP nanoparticles. We suggest that the repair effect of HAP can be greatly improved if its dimensions can be reduced to the scale of the natural building blocks. Compared with conventional HAP and nano amorphous calcium phosphate (ACP), our in vitro experimental results demonstrate the advantages of 20 nm HAP in enamel repairs. The results of scanning electron microscopy, confocal laser scanning microscopy, quantitative measurement of the adsorption, dissolution kinetics, and nanoindentation, show the strong affinity, excellent biocompatibility, mechanical improvement, and the enhancement of erosion-free by using 20 nm particles as the repairing agent. However, these excellent in vitro repair effects cannot be observed when conventional HAP and ACP are applied. Clearly, nano HAP with a size of 20 nm shares similar characteristics to the natural building blocks of enamel so that it may be used as an effective repair material and anticaries agent. Our current study highlights the analogues of nano building blocks of biominerals during biomedical applications, which provide a novel pathway for biomimetic repair.

Introduction

Dental caries is a prevalent chronic and world-wide oral disease.1 At the initial stage of caries lesions, bacteria cause the damage of enamel, which is the exterior coating of teeth with remarkable hardness and resistance.2 As the most highly mineralized structure in vertebrate bodies, enamel is composed of numerous needle-like apatite crystals, which are bundled in parallel ordered prisms to ensure the unique mechanical strength and biological protection.3 The principal proteins such as amelogenins, ameloblastins, and protinases are involved in the hierarchical construction of enamel apatite.4–7 However, the proteins that induce/control the crystallization of apatite are almost completely degraded or removed during enamel maturation.8–10 As a non-living tissue, the main composition (97 wt%) of mature enamel is inorganic apatite so enamel is scarcely self-repaired by living organisms after substantial mineral loss.11,11 Filling with artificial materials is a conventional treatment to repair damaged enamel. Currently, enamel defects are frequently refilled with unstructured substitutes such as amalgam, metal alloys, ceramics, or composite resins.12,13 But secondary caries frequently arises at the interfaces between the tooth and foreign materials.14,15

Hydroxyapatite [Ca10(PO4)6(OH)2, HAP] is always considered as a model compound of enamel due to the chemical similarity.3,16 Therefore, the remineralization of enamel minerals by using synthetic apatite or metastable calcium phosphate is always suggested in dental research.17–19 Unfortunately, these chemically analogous compounds of enamel are not widely applied in clinic practices. The native structure of enamel is too complex to be remodelled and the synthesized apatite crystallites often have different dimensions, morphologies, and orientations from the natural ones, which result in poor adhesion and mechanical strength during the restoration.20–22 Despite the complicated hierarchical structures, it is revealed that the basic building blocks of enamel are generally 20–40 nm particles of HAP.23,24 Tens to hundreds of these nanoparticles, in the collagen matrix, combine into self-assembled apatite during enamel formation.4,21,23–26 Although some dental nano-composites with high mechanical strength and low abrasion have been introduced, their dimensions are still away from the natural building blocks.27 Recent advances in biomineralization also highlight that features of smaller HAP nanoparticles may more closely approximate to features of biological apatite than features of the larger HAP particles that are conventionally used.28,29 Our previous study has also demonstrated that HAP nanoparticles can be self-assemble to form enamel-like structures in aqueous solution.30

Herein, a biomimetic technique is suggested in which the localized repair of enamel surface can be improved by using the analogue of the basic building blocks of enamel. Different from the other frequently used calcium phosphates in mineralization such as conventional HAP (cHAP, rod-like with lengths of hundreds of nanometres) and amorphous calcium phosphate (ACP), the biocompatibility between 20 nm artificial HAP and enamel can be greatly improved. Our in vitro experiments show that the nano HAP can adsorb onto enamel surfaces strongly.

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The formed nano HAP layer can even prevent the demineralization of hard tissue. It is also important that the mechanical strength of the restored enamel surface is maintained after the treatment. These in vitro results imply that 20 nm sized HAP is a better candidate than any restorative material used to date and a perfect repair of enamel is approached.

Materials and methods

The human molars were provided by the First Affiliated Hospital of Zhejiang University College of Medicine under an agreement with the patients. The use of human tissue specimens followed a protocol that was approved by the ethical committee of the Hospital, and was also agreed by the patients. The teeth were stored in a thymol solution and were sectioned perpendicular to the dental crown using a diamond saw. The samples were polished on silicon carbide paper under the running water, and were demineralized by 37% phosphoric acid for 10 s. The dimensions of a typical enamel window are 5 × 5 × 0.5 mm³.

The 20 nm HAP particles (nano HAP) were synthesized in our laboratory and the details of these materials can be found in our previous paper. Conventional HAP crystals were prepared by the method suggested by Mohan and Nancollas. The ACP was synthesized at room temperature (20 °C). 100 ml Na₂HPO₄ (0.004 M, pH = 9.5 ± 0.1) solution was added to 100 ml CaCl₂ (0.006 M, pH = 9.5 ± 0.1) solution quickly and reacted for 1 min. The precipitate was separated by centrifugation (12 000 rpm) and washed using acetone three times. The morphologies and the particle size of these materials were examined by transmission electron microscopy (TEM, JEM-200CX, Japan). All solids were dried under vacuum at room temperature and their 0.1 wt% aqueous slurries were prepared.

3 μl of a slurry of calcium phosphate solid was carefully dropped onto the enamel surface and dried in air at room temperature. 10 ml ethanol was used to wash the enamel surface to remove the unabsorbed materials. In order to estimate the adsorption ability of the samples onto the enamel, the repaired windows with different materials were put into distilled water and the ultra sonications (45 KHz, 120 W) was applied for 30s to remove the weakly adsorbed materials. Then, the windows were washed with 10 ml ethanol again and were dried in air. The details of the sample treatment were also illustrated in Scheme 1.

The dried enamel surfaces were examined directly under a SEM (S-4800, Hitachi, Japan). The quantitative examination of the adsorbed particles on the enamel surfaces was performed using a quartz crystal microbalance (QCM-D300, Q-sense, Sweden).

Some enamel samples in the absence and presence of the coated nano HAP layer were put in 50 ml of 1.0 × 10⁻⁴ M Rhodamine B aqueous solution for 2 h and they were washed with distilled water. CLSM observation was carried by an Axiovert 200M (Carl Zeiss, Germany). In order to examine the dissolutions, the samples were put in 500 ml of 0.15 M NaCl solution with a pH of 4.50 (adjusted using diluted HCl) at room temperature. The enamel surfaces were exposed to the solution and the other surfaces were covered by epoxy glue (protection against dissolution). The dimensions of the samples were measured before the demineralization experiment and their mass was examined every day so that the rate of mass loss could be estimated. At least four parallel experiments were taken simultaneously to confirm the reproducibility of data.

Nanoindentation was performed by a multiple scanning probe microscope (SPM, Nanoscope IVa, Veeco, USA) with a diamond-tipped probe (k = 300 N m⁻¹, Veeco, USA). The tip was calibrated with a fused-silica sample prior to examination. During the indentation, the applied load forces, F, and the depth of penetration into the sample were continuously monitored leading to highly reproducible force–displacement curves. Hardness, H, and elastic modulus, E, were determined from the unloading portion of force–displacement curves as eqn 1 and 2:

$$H = \frac{F_{\text{max}}}{A}$$  \hspace{1cm} (1)

$$E = \frac{S\left(\frac{h}{A}\right)^{1/2}}{2\left(\frac{h}{A}\right)^{1/2}}$$  \hspace{1cm} (2)

where $F_{\text{max}}$ was the maximum applied load, S, the max stiffness of the system, and $A$, the calibrated tip area. At least five profiles were taken on each sample surface.

Results and discussion

TEMs of the different artificial calcium phosphate materials are shown in Fig. 1. These confirmed that the nano HAP particles are grain-like with diameters of approximately 20 nm (Fig. 1a). The particles of cHAP have the typical rod-like morphology and their lengths are hundreds of nanometres (Fig. 1b). The ~20 nm ACP particles are sphere-like (Fig. 1c). The inserts are their corresponding selected area electron diffraction (SAED) patterns. The diffraction dots or rings reflect the crystallinity of samples. The SAED results of nano HAP and cHAP are in good agreement with the lattice structure of hydroxyapatite and exhibit excellent crystallinity. However, there is no obvious diffraction pattern for ACP, which indicates that its main composition is amorphous phase.

In the in vitro repair, the enamel window is obtained from human molars and is treated by 37% phosphoric acid for 10 s to obtain an experimental model for the enamel surface. Under a scanning electron microscope (SEM), the characteristic fish-scale “keyhole” structure of the native enamel is observed.
Numerous needle-like apatite crystallites are oriented to form the organized structure (Fig. 2b) on the enamel surface. Previously, various attempts at in situ remineralization have been undertaken using supersaturated calcium phosphate solution and ACP to mimic the biological formation of enamel. However, the enamel-structured HAP cannot be induced by these methods. It is worse that the adhesion of the newly formed calcium phosphates on the enamel surfaces is relatively weak. A similar conclusion is achieved from our control experiments. cHAP aqueous slurry (0.1 wt%) is dropped carefully onto the enamel surface. After drying in air, the sample is washed using ethanol to remove the unabsorbed particles and residuals. It seems that the adsorption of cHAP is easy and that the enamel surface can be fully covered by crystals (Fig. 2c). Compared with the natural apatite crystallites (Fig. 2b), the synthesized cHAP crystallites have larger dimensions, despite this they are also rod-like. However, these artificial HAP crystals randomly aggregate on the enamel surface and they cannot form any enamel-like structures by the oriented assembling. However, the ordered orientation is a key factor to ensure the physical and biological functions of enamel. Clearly, these disorderly adsorbed cHAP crystallites cannot ensure the repair of enamel. It is noted that the attachment of cHAP onto the enamel substrate is actually poor. Almost the adsorbed cHAP crystallites can be detached from the enamel readily under ultrasonic conditions (25Hz, 120W, 30 s). Only a few cHAP crystallites are still present on the surface after the treatment (Fig. 2d). Actually, even the HAP layer obtained by in situ growth on the enamel surface can be destroyed readily by the ultrasonic treatment. These phenomena imply that cHAP is not a suitable agent for enamel repair.

ACP is often encountered as a transient phase during enamel formation. It is suggested that the adsorption and dissociation of this metastable phase can provide the calcium and phosphate sources for production of new biological apatite by the body. However, the attachment of ACP onto enamel surface is even weaker than that of cHAP. Only a few ACP particles (arrows, Fig. 2e) can adsorb onto the enamel and almost all of them are washed out by ethanol (the solubility of ACP in ethanol is extremely low and ethanol is always used as a solvent to protect the amorphous phase). It indicates that the affinity of ACP to enamel is extremely low. Moreover, the attached ACP disappears completely from the enamel surface after the ultrasonic treatment (Fig. 2f). This phenomenon may be explained by
the different chemical composition and structure between enamel apatite and ACP so that they cannot recognize and match with each other. Besides, in situ phase transformation of the adsorbed ACP are not observed during the experiments.

It is not surprising that the repair effects of cHAP and ACP are not satisfactory. Although enamel is not a living part and it has a close chemical structure to HAP, this hard tissue is biologically constructed by living organisms with well-organized structures. The chemical similarity of HAP is not enough to ensure the restoration, and better biomedical materials require a perfect biocompatibility to reduce the interface between implanted materials and natural materials. Since it is too complicated to remodel the functions of living organism to induce new enamel apatite on a non-living surface, HAP nanoparticles which approximate to the basic building blocks of enamel may be a candidate since the in vitro biomimetic construction of enamel-like apatite by using nano calcium phosphate has already been achieved. It is suggested that 20 nm sized HAP is analogous to the subunits of biological apatite. As we expected, the repair of enamel surfaces can be improved dramatically by reducing the size of HAP. The typical “fish scale like” structure remained after the surface adsorption of numerous 20 nm HAP particles, which tend to array along the natural rod structures (Fig. 2g and inset). The individual nanoparticles can be well identified under SEM. These particles are not observed on the original enamel surface. Thus, they can only be attributed to the added nano HAP. Different from that of cHAP and ACP, the detachment of these HAP nanoparticles from the enamel surface is not obvious under the ultrasonic conditions (Fig. 2h). Most of the adsorbed nano HAP remains on the enamel surface after the vigorous treatment.

The quantitative measurement of mass change (the adsorption of artificial calcium phosphates) during the restoration is summarised in Fig. 3. It indicates the strong affinity between enamel and the nanoparticles. Although the initial adsorbed amount of cHAP is greater, its poor affinity results in the easy loss of the crystallites from the enamel surface. A number of studies have demonstrated that the size- and crystallinity-effects of calcium phosphates play essential roles in the formation of hard tissues. It is suggested that the relatively high HAP–solution interfacial energy can drive the aggregation and even the fusion of the nanoparticles in the presence of water. In this paper, we reveal that these effects are also important in adhesion since nano HAP exhibits better attachment onto enamel than the large ones and the amorphous one. In this adsorption process, the interfacial energy may still play an key role.

It is surprising that a layer of HAP nanoparticles can inhibit the future mineral loss from the enamel surface significantly. Under an acidic solution, the enamel dissolution is frequently initiated at the prism–prism sheath interfaces and develops anisotropically along the long axes of apatite, leading to caries formations. This demineralization stage can be retarded by the 20 nm HAP layer. The protection of the prism–prism sheath interface by the nanoparticles is confirmed using a confocal laser scanning microscopy (CLSM). Rhodamine B can fluorescently dye the interface due to the selective adsorption and high fluorescence yield. There are many defects at the interfaces on the original damaged enamel so that Rhodamine B can infiltrate together with the solution. Thus, the interfaces are dyed red and display luminescence under CLSM (Fig. 4a). These red sites

Fig. 3 Mass change, \( \Delta m \) (the enamel mass after treatment – the initial enamel mass), during the repair. 1: Initial adsorption state before the ultrasonic treatment. 2: After the ultrasonic treatment.

Fig. 4 CLSM images of an enamel surface dyed by Rhodamine B: (a) original enamel surface; (b) enamel surface restored by nano HAP. SEM images of the enamel surface after the acidic treatment: (c) erosion of natural enamel; (d) dissolution of the enamel surface is not initiated, the layer of nano HAP stays unchanged in the acidic solution. (e) Dissolution curves (mass loss vs. time).
(bright area in Fig. 4a) can provide active dissolution sites during the early stage of caries formation. However, the fluorescent intensity decreases significantly after the surface treatment when using the nano HAP (Fig. 4b). The CLSM result indicates that the penetration of the solution (Rhodamine B) into the prism-shape sheath is blocked when the surface is covered by a layer of HAP nanoparticles. Thus, the demineralization of enamel apatite cannot be triggered so readily according to the enamel dissolution model. It also implies that secondary caries formation is inhibited accordingly by the restoration. It is emphasized that 20 nm HAP particles themselves can be resistant to dissolution in the biological milieu too, which can be understood by a nano dissolution model. Briefly, the model suggests that the active dissolution pits cannot be produced on nanoparticles. The nanostructured materials can be kinetically protected on account of their sizes and can remain relatively stable under undersaturated conditions.

Therefore, the prevention of enamel erosion is enhanced by the nano HAP, which is also confirmed experimentally. Without any treatment, the demineralization of the natural enamel surface is remarkable in an acidic solution (pH = 4.5 ± 0.1, experimental time period of 2 day) and the corrupted sites can be observed (Fig. 4c). In contrast, the layer of nano HAP on the treated enamel surface is almost unchanged (Fig. 4d) in the acidic solution. The mass loss of the enamel windows with time in the acidic solutions are also examined with the extended experimental periods (Fig. 4e). It is determined that the averaged mass loss rate of the natural enamel surface is about 0.12 ± 0.04 mg mm⁻² day⁻¹ and that of the nano HAP-coated enamel approaches zero (<0.02 mg mm⁻² day⁻¹), which is beyond the sensitivity of the method. Since the new nano layer is insensitive to dissolution, the underlying enamel surface is well protected under the acidic condition.

As the hardest biological part, the mechanical strength of the restored enamel surface is a key parameter, which can be examined using nanoindentation. The 20 nm HAP-coated (the thickness of the layer is about 40–50 nm) enamel surface has a hardness of 4.6 ± 0.4 GPa and an elastic modulus of 95.6 ± 8.4 GPa. These results are very similar to those of the natural ones, which are 4.2 ± 0.2 and 94.1 ± 5.4 GPa, respectively. We mention that this measurement is performed at the early stages of the adsorption and the particles are not partially fused. Previously, it has been reported that the nanometre scale of crystals in many biological hard tissues such as enamel or vertebral bone is chosen by nature to ensure optimal strength and maximum tolerance of flaws (robustness).

This feature confers the 20 nm HAP, or the basic building blocks of enamel, the improved mechanical strength at the earlier stage. And a similar mechanism is suggested in the restoration by using 20 nm HAP. It is important that the featured hardness of enamel remains after the artificial repair.

Conclusions

In summary, the similarity of the 20 nm HAP and the building blocks of enamel apatite result in the effective adsorption of the artificial materials to the natural tissue. The enamel structure is even reinforced by the nano HAP since the secondary caries formation is suppressed and the hardness is almost remained. This strategy may have prospective applications in dentistry as it offers an easy but effective method to reconstruct tooth enamel that is suffering from mineral loss. Generally, the study also suggests that the analogues of the nano building blocks of biominerals shall be highlighted in biomimeralization rather than despite the complicated morphology and structures of the natural materials.

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