Biomimetic mineralisation of phosphorylated dentine by CPP-ACP

Abstract

Objectives: Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has the potential to induce the biomimetic mineralisation of dentine collagen fibrils. This study aimed to demonstrate *in vitro* the ability of CPP-ACP to form apatite crystals on phosphorylated dentine collagen fibrils.

Methods: Dentine slices with a 2-mm thickness were prepared from sound human third molars. The slices were etched with phosphoric acid to expose the collagen fibrils. Sodium trimetaphosphate was then used to phosphorylate the exposed collagen fibrils. CPP-ACP paste was topically applied to the surface of the phosphorylated slices, which were then immersed in a metastable calcium phosphate remineralising solution and incubated at 37℃ for 10 days. The CPP-ACP paste and the remineralising solution were replaced every two days. Phosphorylated dentine slices without a CPP-ACP application and non-phosphorylated dentine slices with a CPP-ACP application were prepared and used for comparison. The slices were examined using scanning electron microscope (SEM), diffuse reflectance-Fourier transform infrared spectroscopy (DR-FTIR) and X-ray diffraction (XRD).

Results: The SEM results revealed the presence of intrafibrillar and interfibrillar crystal nucleation and growth along the phosphorylated dentine collagen fibrils. The DR-FTIR and XRD confirmed that the crystals were hydroxyapatite. No apatite crystal nucleation and growth were observed in either the slices that had no non-phosphorylation or those without CPP-ACP application.

Conclusions: CPP-ACP can induce the biomimetic mineralisation of dentine through apatite formation along and between the phosphorylated dentine collagen fibrils.

Clinical Significance: The *in vitro* study imitated the application of CPP-ACP to exposed dentine tooth surfaces in the mouth. This could lead to the development of a new therapeutic technique for the treatment of tooth hypersensitivity.

1. Introduction

Dentine can be lost or exposed due to trauma, tooth fracture, dental caries or by the effects of non-caries related tooth loss such as abrasion or erosion.[1] Tooth hypersensitivity often develops when the exposed dentine is subjected to stimuli. This hypersensitivity is becoming a significant condition that warrants dental treatment.[2] However, the current management strategies for dentine hypersensitivity have substantial limitations. Researchers are thus looking for alternative treatment strategies, such as biomimetic mineralisation,[3] for the management of hypersensitivity.

Histologically, dentine is a mineralised collagenous and living tissue containing by weight approximately 70% inorganic minerals, 20% organic substances and 10% water.[4] The inorganic minerals are primarily composed of hydroxyapatite crystals, and the organic substances are mainly Type I collagen (about 90 wt%) and non-collagenous proteins (about 10 wt%).[5] Although the collagen itself cannot initialise hydroxyapatite nucleation and growth in the absence of apatite seed crystallite, non-collagenous proteins might be involved in the regulation of mineralisation.[6] The negatively charged non-collagenous proteins contain highly phosphorylated serine and threonine residues; these residues can trap calcium ions and form nuclei via their phosphate groups. This attribute of non-collagenous proteins is crucial for the initiation of biomimetic mineralisation.[7]

Various approaches have been used to study the initiation of mineralisation including those using carboxylic acid-containing polyelectrolytes,[8] phosphoproteins,[9] casein phosphopeptide-amorphous calcium phosphate(CPP-ACP),[10] colloidal nano-beta-tricalcium phosphate[11] and bioactive glass particles[12]. These studies were relatively successful in controlling the dimensions of calcium phosphate, but achieved limited success in reproducing the structural hierarchy of apatite deposition within the collagen matrices.[13]

Incorporating a phosphate group of biomimetic molecules into the collagen fibrils that make up dentine creates a phosphorylated and negatively charged dentine surface. This

phosphorylated surface attracts calcium ions through electrostatic interaction, leading to the nucleation and growth of hydroxyapatite. This is a plausible biomimetic method for simulating the role of non-collagenous proteins in biomimetic mineralisation. In addition, Li et al.[14] showed that the interfibrillar deposition of large spherical hydroxyapatite will occur around STMP-phosphorylated bovine collagen matrices in a metastable calcium solution. Sodium trimetaphosphate (STMP, $Na_3P_3O_9$) forms chemical bonds with the hydroxyl groups of proteins. This reaction introduces the phosphate functional group to the protein molecules.[14] However, the deposition of calcium phosphate minerals on a collagen surface alone does not produce a highly mineralised collagen matrix, and therefore this process cannot be regarded as true mineralisation. The key issues in the mineralisation and regeneration of a dentine microstructure are replicating the hierarchical structure of the demineralised dentine collagen fibrils and inducing intrafibrillar mineralisation.

Olszta et al. suggested that an amorphous, liquid-phase precursor could facilitate the formation of calcium-based biominerals.[15] The liquid-phase properties of the amorphous calcium phosphate (ACP) nano-precursor facilitate its passage to the mineralising zone and thus initiate mineralisation.[15] The ACP nano-precursor is transient and unstable, and CPP-ACP was used as the nano-ACP mineral precursor in this study.[10] The casein phosphopeptides (CPP) stabilise the calcium and phosphate ions through the formation of complexes.[16] CPP can also stabilise nano-ACP in a metastable solution. CPP-ACP promotes the remineralisation of dentine and enamel, in particular enamel lesions.[17] It is commercially available as a paste (Tooth Mousse, GC International, Itabashi-ku, Tokyo, Japan) that restores minerals lost from a demineralised tooth surface. At present there are no published reports on the biomimetic mineralisation ability of CPP-ACP within dentine collagen fibrils; in particular, there are no studies of its potential to induce intrafibrillar apatite formation. This study examined *in vitro* the ability of CPP-ACP to induce the biomimetic mineralisation of phosphorylated dentine collagen fibrils.

2. Materials & Methods

2.1. Preparing the dentine slices

This study was approved by The University of Hong Kong/Hospital Authority Hong Kong West Cluster Institutional Review Board (IRB UW10-210). Extracted sound human third molars were collected and the soft tissue attached to the teeth was removed. The teeth were disinfected with 3% sodium hypochlorite and rinsed with phosphate-buffered saline. Two-millimetre thick dentine slices were prepared in the following manner. Each tooth was sliced perpendicular to its longitudinal axis using a diamond saw (IsoMet Low Speed Saw, Buehler, Lake Bluff, Illinois, USA). The slices were polished with a 2000-grit silicon carbide paper under running water (Buehler EcoMet 5, Lake Bluff, Illinois, USA). A 10x stereo microscope (Stemi DV4, Maple Grove, Minnesota, USA) was used to examine the dentine slices. Ten dentine slices without cracks or hypo-mineralisation were selected for use in this study. They were cleaned ultrasonically with detergent, followed sequentially by acetone, ethanol, and deionised water and stored in a polyethylene tube in a refrigerator at 4ºC.

2.2. Phosphorylation of the dentine collagen matrices

Ten dentine slices were acid-etched with 37% phosphoric acid for 60s to demineralise the hydroxyapatite and expose the dentine collagen. After being rinsed with deionised water, the etched dentine slices were immersed in 0.2 M STMP solution (Sigma-Aldrich, St. Louis, MO, USA) at 23ºC for 12 hours to incorporate the phosphate ions into the demineralised dentine collagen matrices. The phosphorylated dentine slices were then rinsed with copious amounts of deionised water. X-ray Photoelectron spectroscopy (XPS) (Thermo ESCALAB 250Xi, Maple Plain, Minnesota, USA) was used to analyse the surface chemistry of the two phosphorylated dentine slices and two non-phosphorylated dentine slices. Diffuse reflectance-Fourier transform infrared spectroscopy (DR-FTIR) **(**Nicolet 8700 Research FT-IR Spectrometer, Thermo Scientific Instrument Co., Friars Drive Hudson, New Hampshire, USA) was used to collect the IR spectra from aforementioned dentine slices.

2.3. Biomimetic remineralisation of the dentine collagen matrices

The remaining four phosphorylated and two non-phosphorylated slices were used to study the biomimetic remineralisation of the dentine collagen matrices. They were divided into three groups. In group 1, a commercially available CPP-ACP paste (Tooth Mousse, GC Corp., Tokyo, Japan) was applied to the surface of two phosphorylated dentine slices. Two phosphorylated dentine slices with no CPP-ACP application were assigned to group 2 for comparison. In group 3, CPP-ACP was applied to the two dentine slices with no STMP-phosphorylation. The specimens of the three groups were then separately put into sealed polyethylene tubes filled with 10 ml of freshly prepared metastable calcium phosphate remineralising solution (2.58 mM Ca^{2+} and 1.55 mM PO_4^{3-}, buffered by 50 mM Tris buffer to pH 7.6) and incubated at 37°C for 10 days. The CPP-ACP paste and the metastable calcium phosphate remineralising solution were replaced every two days. After 10 days of biomimetic mineralisation, the dentine slices were dehydrated with ethanol and then dried in the critical evaporator so that they could be assessed using scanning electron microscopy (SEM) (Sirion 200, FEI Company, Hillsboro, Oregon, USA), X-ray Diffraction (XRD) (X'Pert PRO, Philips, Almelo, Netherlands) and FTIR.

3. Results

3.1. Phosphorylation of the dentine collagen matrices

The XPS spectrum of the P2p scan on the dentine collagen surface showed that the peak of the characteristic binding energy was at 132-134eV. The intensity of the P2p peak on the phosphorylated dentine collagen surface was higher than on the demineralised dentine collagen surface (Fig. 1). This observation corroborated the increase of phosphorus on the surface of the demineralised dentine collagen matrices in the slices that had been immersed in the 0.2 M STMP solution for 12 hours.

Fig. 2 shows the DR-FTIR spectra of the dentine collagen matrices before and after phosphorylation. The PO_4 ν_3 band (1033 cm^{-1}) and the PO_4 ν_4 band (600 and 554 cm^{-1}) indicated the presence of phosphorus on the surface of the dentine collagen matrices.[18] Compared to non-

phosphorylated dentine collagen matrices (spectrum in black), the relative intensity of the peak was enhanced on the phosphorylated one (spectrum in red), which suggested that more phosphorus were introduced to the dentine collagen matrices after phosphorylation.

3.2. Precipitation on the surface or within the dentine collagen matrices

The SEM demonstrated the precipitation of nano-particles along the surface of the fibrils (Fig. 3a, b) and within the collagen matrices (Fig. 3c, d) in the experimental group. However, no precipitation of nano-particles was observed along the surface of the fibrils in the two control groups (Fig. 4). The precipitated nano-particles on the surface (Fig. 3a, b) of the collagen matrices were larger than those precipitated within the collagen matrices (Fig. 3c, d).

The transverse section micrographs showed that the nano-particles precipitated along the collagen fibrils of the peritubular (Fig. 3c, Arrow) and intertubular dentine (Fig. 3c, Triangle), which suggested the mineral precursors had penetrated into the collagen matrix.

SEM observation made at a high magnification (x50,000) revealed that numerous nano-crystals distributed regularly and homogenously along the collagen fibres like a string of bead, which was similar to natural calcified collagen fibres, and suggested that the collagen fibres was recalcified. We speculated that crystallites were deposited intrafibrillarly (Fig. 3d, Arrow). In some areas, the recalcified collagen fibres connected together exhibiting a “corn-on-the-cob” appearance, which resulted from the substantial mineralisation of the calcified collage fibres (Fig. 3, Rectangle). The spaces between the collagen fibres were occupied by nano-crystals, thereby embedding the collagen fibres and making them difficult to identify (Fig. 3d, Oval), and which may imply interfibrillar mineralisation.

In the control samples without STMP-phosphorylation, sporadic precipitated particles were found on the collagen surfaces, but no crystals formation was detected inside the collagen matrices (Fig. 4a). For the control samples without a CPP-ACP application, plate-like hydroxyapatite crystals were observed to have precipitated in the dentine tubules (Fig. 4b), but no collagen fibrils were remineralised. There was no evidence of biomimetic mineralisation on

the dentine collagen fibrils found in the two control groups. Unequivocally, there was no intrafibrillar or interfibrillar crystal formation in the collagen matrices of either the dentine slices with phosphorylated dentine collagen and no CPP-ACP application or the slices with non-phosphorylated dentine collagen and a CPP-ACP application.

3.3. Structure and composition of the precipitation

The surface and subsurface structure of the dentine slices in all of the samples were characterised using XRD. The XRD spectra of all of the groups are shown in Fig. 5. The peaks at 2θ^{o} were 25.968^{o}, 31.699^{o}, 32.079^{o} and 32.782^{o} corresponded to the expected peaks for hydroxyapatite at 002, 211, 112 and 300 planes, respectively (JCPDS No. 09-0432). [19] This demonstrated that the precipitates were hydroxyapatite crystals, but their broad breadths suggested low crystallinity. In all of the control groups, the HAP peaks came from the subsurface of non-demineralised dentine, which is the natural HAP. However, the relative intensities of the peaks were enhanced on the dentine surfaces of group 1 (spectrum 2) compared to group 2 (spectrum 3), group 3 (spectrum 4) and demineralised dentine surface (spectrum 5), which suggested that more HAP precipitated on the dentine surface in group 1. In addition, the peaks of 211, 112 and 300 were more clearly separated in group 1 (spectrum 2) than those in group 2 (spectrum 3) and group 3 (spectrum 4), suggesting that the precipitated HAP on the dentine surface had similar crystallinity as the natural dentine HAP (spectrum 1).

The DR-FTIR spectra of the experimental and controls samples are shown in Fig. 6. The HPO_4 v_1 band (997-1124 cm^{-1}) and the HPO_4 v_4 band (600 and 554 cm^{-1}) indicated the presence of hydroxyapatite crystals in the experimental group (Spectrum 1 in black) and in the control group without CPP-ACP (Spectrum 2 in red). [18] The relative intensities of these peaks were enhanced on the dentine surface of group 1 (spectrum 1) compared to group 2 (spectrum 2) and group 3 (spectrum 3), which suggested that more HAP precipitated onto the dentine surface. The Reflection Absorption bands at 1650 cm^{-1}and 1453 cm^{-1} corresponded to the collagen protein amide I and II bands which cannot find on sound dentine surface (spectrum 4).

4. Discussion

A calcified collagen matrix is the basic microstructure of dentine, and the apatite phase of mineralised dentine is classified as intrafibrillar crystallites. These crystallites are found within the hole zones and pore spaces of the collagen fibrils. They are oriented along and occupy the interstitial spaces that separate the collagen fibrils.[20] Our study demonstrated that both intrafibrillar (along the collagen fibres and within the collagen fibres) and interfibrillar (between the collagen fibres) remineralisation can be replicated in the phosphorylated dentine collagen scaffold of human dentine in the presence of amorphous calcium phosphate nano-precursors (i.e. a CPP-ACP complex).

Type I collagen accounts for about 90% of the organic matrix of dentine. The remaining organic substances are mainly non-collagenous proteins (NCPs). NCPs are poly-anionic protein molecules. It is suggested that NCPs can bind to the collagen substrate which possesses high calcium binding capacity.[21] The phosphate groups of STMP anions can function as a biomimetic analogue of NCPs; and they adsorb to dentine collagen surface and form covalent bonds with the collagen under an alkaline pH.[22,23] The results of the XPS and FTIR showed that STMP could phosphorylate dentine collagen matrices. The relative intensity of the peak of P2p scan was enhanced after phosphorylation. DR-FTIR spectra demonstrated that phosphorus increased on the surface of the demineralised dentine collagen matrices after phosphorylation. This suggested that a phosphate group was incorporated into the dentine collagen fibrils after the slices had been immersed in an STMP solution. STMP is considered a phosphorylation chemical for bio-organic compounds and a coupling agent for the oligomerisation of nucleosides and amino acids.[24] In an alkaline environment, it can generate covalent bonds between the phosphate group and the hydroxyl or amino groups on proteins; it is plausible that this is a mechanism through which phosphate functional groups are built into dentine collagen.[25] The phosphate groups of the STMP became attached to the collagen fibrils and contributed to the binding sites for calcium ions, thereby facilitating the apatite nucleation onto the collagen. The collagen phosphorylation simulated the function of the NCPs (such as the dentine matrix protein-1 or the dentine phosphophoryn) [23], binding the collagen fibrils and

initiating the biomimetic mineralisation of human dentine.[26, 27]

This study found that the precipitated particles distributed regularly and homogenously along the collagen fibres like a string of bead structure, not only on dentin surface, but also in the peritubular and intertubular dentine. This might indicate that the mineral precursors diffuse into the cross-linking dentine collagen matrices and mineralise the collagen fibrils. Although the SEM results could not provide the confirmatory evidence that nano-crystals have penetrated into the collagen fibres resulting in intrafibrillar mineralisation, previous studies have found crystallites deposited interfibrillarly using TEM [8]. It is very important to remineralise dentine collagen fibrils, especially intrafibrillar mineralisation, for recovering the mechanical property of dentine. On the other hand, there was substantial mineralisation on the collagen fibrils, which exhibited a "corn-on-the-cob" appearance due to the connections between the remineralised collagen fibres. This pattern suggested interfibrillar mineralisation. The intrafibrillar mineralisation might act as apatite seed crystallites to facilitate the growth of nano-crystals along the collagen, and resulting in crystallites epitaxial growth. The hydroxyapatite nano-crystals grow and become connected to the mineralised intrafibrillar collagen fibrils.[8] Furthermore, we found that nano-particles precipitated both on the surface and within the collagen matrices. The precipitated particles on the surface of the collagen matrices were larger than those within the collagen matrices. This might suggest a faster precipitation rate on the surface than within the collage matrices. Thus, our results indirectly led to the conclusion that intrafibrillar mineralisation and interfibrillar mineralisation can occur on phosphorylated dentine collagen surface in the presence of CPP-ACP.

The phosphorylated dentine collagen surface attracts calcium ions to the collagen scaffold by electrostatic force and then acts as a nucleation site to trigger the remineralisation needed for the growth of hydroxyapatite crystals.[8] The samples in groups 2 and 3 demonstrated that collagen did not attract nano-precursors from CPP-ACP into the dentine collagen scaffold without STMP phosphorylation, and could not bring about the remineralisation of dentine collagen fibrils. In addition, the phosphorylated dentine collagen could not trigger the intrafibrillar or interfibrillar remineralisation of dentine collagen in the absence of CPP-ACP;

therefore, only large mineral spheres were deposited in the dentinal tubules.

Collagen mineralisation is a bottom-up mineralisation approach based on the non-classical theory of crystallisation.[28] In such cases, crystallisation often proceeds via a sequential transient-precursor phases transformation, wherein fluidic nano-precursors, stabilised by polymer molecules, are transformed into mesocrystalline intermediates that eventually fuse to become single microscopic crystals.[28, 29] In this study, CCP-ACP was used as the nano-ACP mineral precursor.[10] Due to the sequestering mechanism, CCP prevents ACP nano-precursors from aggregating or precipitating, giving CCP-ACP a liquid-like property. It also renders them small enough to diffuse into the collagen matrix scaffolds and to coalesce in the collagen fibrils.[30] Driven by kinetic force, the ACP then transforms into nano-apatite crystals. A schematic diagram of the biomimetic mineralisation of collagen fibril as induced by CCP-ACP is illustrated in Fig. 7.

In this *in vitro* study, we applied a commercially available CCP-ACP paste to a phosphorylated dentine surface to induce the biomimetic mineralisation of dentine in the presence of a metastable calcium phosphate solution. This design aimed to simulate the clinical scenario of applying CPP-ACP (Tooth Mousse) paste to a tooth surface in the mouth. Like a metastable calcium phosphate solution, saliva promotes the release of calcium ions from CCP-ACP. A previous study demonstrated that 5 min was sufficient for STMP to phosphorylate the dentine collagen.[22,23] Hence, this study design is a promising idea and could help in the development of a new therapeutic technique for the treatment of tooth hypersensitivity.

5. Conclusions

This study demonstrated that CCP-ACP can induce biomimetic mineralisation by forming intrafibrillar apatite and interfibrillar apatite on phosphorylated dentine collagen fibrils. The *in vitro* design imitated applying CPP-ACP to an exposed dentine tooth surface in the mouth and the results point towards a novel treatment of tooth hypersensitivity.

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