Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/jden

Biomimetic mineralisation of phosphorylated dentine by CPP-ACP

Ying Cao [a,b], May L. Mei [a], Jianguang Xu [b], Edward C.M. Lo [a], Quanli Li [b,**], Chun Hung Chu [a,*]

[a] Faculty of Dentistry, The University of Hong Kong, Hong Kong, China
[b] College of Stomatology, Anhui Medical University, Hefei, PR China

ARTICLE INFO

Article history:
Received 5 March 2013
Received in revised form
18 June 2013
Accepted 19 June 2013

Keywords:
Biomimetic mineralisation
Hydroxyapatite
Dentine collagen
Intrafibrillar
CPP-ACP
STMP

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 fibres. Sodium trimetaphosphate was then used to phosphorylate the exposed collagen fibres. 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 °C 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 fibres. 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 fibres.

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.

* *Corresponding author at*: Faculty of Dentistry, The University of Hong Kong, 34 Hospital Road, Hong Kong, China. Tel.: +852 2859 0287; fax: +852 2858 7874.
** *Corresponding author at*: College of Stomatology, Anhui Medical University, Hefei, PR China. Tel.: +86 0551 5118677; fax: +86 0551 5111538.
E-mail addresses: ql-li@126.com (Q. Li), chchu@hku.hk, chchu@agd.org (C.H. Chu).
0300-5712/$ – see front matter
http://dx.doi.org/10.1016/j.jdent.2013.06.008

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.[2] This hypersensitivity is becoming a significant condition that warrants dental treatment.[3] However, the current management strategies for dentine hypersensitivity have substantial limitations.[4] Researchers are thus looking for alternative treatment strategies, such as biomimetic mineralisation,[5] 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.[6] 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%).[7] 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.[8] 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.[9]

Various approaches have been used to study the initiation of mineralisation including those using carboxylic acid-containing polyelectrolytes,[10] phosphoproteins,[11] casein phosphopeptide-amorphous calcium phosphate(CPP-ACP),[12] colloidal nano-beta-tricalcium phosphate[13] and bioactive glass particles.[14] 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.[15]

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 and Chang[16] 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.[16] 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.[17] The liquid-phase properties of the amorphous calcium phosphate (ACP) nano-precursor facilitate its passage to the mineralising zone and thus initiate mineralisation.[17] The ACP nano-precursor is transient and unstable, and CPP-ACP was used as the nano-ACP mineral precursor in this study.[12] The casein phosphopeptides (CPP) stabilise the calcium and phosphate ions through the formation of complexes.[18] CPP can also stabilise nano-ACP in a metastable solution. CPP-ACP promotes the remineralisation of dentine and enamel, in particular enamel lesions.[19] 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 and 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, IL, USA).[20] The slices were polished with a 2000-grit silicon carbide paper under running water (Buehler EcoMet 5, Lake Bluff, IL, USA). A 10× stereo microscope (Stemi DV4, Maple Grove, Minnesota, USA) was used to examine the dentine slices. Ten dentine slices without cracks or hypomineralisation 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 4 °C.

2.2. Phosphorylation of the dentine collagen matrices

Ten dentine slices were acid-etched with 37% phosphoric acid for 60 s 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 23 °C for 12 h 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, MN, 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