Effects of bioactive glass with high phosphorus content on mineralization of type I collagen fibrils

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Abstract

Purpose: To study effects of bioactive glass with high phosphorus content (10.8% P2O5, 54.2% SiO2, 35% CaO, mol%, named PSC) on mineralization of type I collagen fibrils.

Methods: (1) PSC, and PSC combining 0.1 mg/mL, 0.5 mg/mL, or 1.0 mg/mL polyacrylic acid (PAA), were used to induce the mineralization of self-assembled type I collagen fibrils. After 3 and 7 days of mineralization, collagen fibrils were observed by transmission electron microscopy (TEM) and selected area electron diffraction (SAED). (2) PSC suspension was dialyzed in simulated body fluid (SBF), or in SBF containing 0.1 mg/mL, 0.5 mg/mL, or 1.0 mg/mL PAA, to form amorphous calcium phosphate (ACP), then observed by TEM.

Results: (1) PSC and PSC combining 0.1 mg/mL or 0.5 mg/mL PAA induced mainly extrafibrillar mineralization. PSC combining 1.0 mg/mL PAA induced both extrafibrillar and intrafibrillar mineralization. (2) The ACP induced by PSC or PSC combining 0.1 mg/mL PAA partly formed lattice structure after 24 h. The particle size of the ACP induced by PSC combining 0.5 mg/mL PAA was 100-150 nm, and that induced by PSC combining 1.0 mg/mL PAA was 30-50 nm.

Conclusion: PSC induced mainly extrafibrillar mineralization, and PSC combining an appropriate concentration (1.0 mg/mL) of PAA induced both extrafibrillar and intrafibrillar mineralization.

Keywords: bioactive glass, extrafibrillar mineralization, intrafibrillar mineralization, polyacrylic acid, type I collagen fibrils

Introduction

During minimally invasive treatment of caries, some demineralized dentin is often retained. Acid etching for dentin bonding restoration will demineralize the dentin, and it is difficult for resin monomer to wrap the demineralized collagen completely, thus exposing collagen at the dentin bonding interface [1]. This exposed collagen can be hydrolyzed by the action of proteolytic enzymes [2], leading to interface aging and micro-leakage, which can affect the long-term efficacy of adhesive restorations. Therefore, it is highly desirable to achieve remineralization of the demineralized dentin.

Dentin is a mineralized tissue in which mineral crystals are oriented hierarchically both inside and outside the scaffold of type I collagen fibrils. Type I collagen fibrils consist of collagen microfibrils arranged with a dislocation periodicity of 67 nm, forming a cross-striated structure with a less dense gap zone of about 40 nm and a densely packed overlap zone of about 27 nm [3]. Studies have shown that intrafibrillar mineralization determines the mechanical properties of collagen and dentin at the nanoscale [4,5]. Therefore, some means of inducing the intrafibrillar mineralization of type I collagen to improve the mechanical properties of demineralized dentin, such as bending strength, would be desirable.

Non-collagenous proteins (NCPs) play an important regulatory role in the process of biomineralization in vivo. NCPs are composed of a large number of negatively charged acidic amino acids that allow combine with calcium and phosphorus to form amorphous calcium phosphate (ACP). They can also bind to specific sites on type I collagen fibrils where they regulate the nucleation and arrangement of hydroxyapatite [6,7]. Gower et al. have proposed a polymer-induced liquid precursor (PILP) theory, whereby calcium and phosphate ions first form clusters of nano-precursors, which further fuse to form liquid stable ACP, which then diffuses into the gap zone of collagen fibers to begin the process of mineralization. Collagen fibers act as templates for nucleation to form hydroxyapatite with an oriented arrangement, thus leading to intrafibrillar mineralization of type I collagen fibrils [8,9]. In order to simulate the process of biomineralization, biomimetic analogues (mainly polyelectrolyte macromolecules) are often used in in vitro studies to simulate the function of NCPs. Polyacrylic acid (PAA) is an anionic surfactant whose carboxyl groups can combine with calcium ions to form liquid-stable ACP and prevent the nucleation and crystallization of calcium and phosphorus ions before entering collagen fibrils [10,11]. Sodium trimetaphosphate (STMP) is rich in phosphate groups, which can combine with collagen by covalent bonding. Phosphorylated collagen fibrils can be templates for crystallization and nucleation of ACP, promoting the orderly crystallization and growth of apatite in the gap zone of collagen, and facilitating oriented intrafibrillar mineralization [12].

Bioactive glass (BG) is a calcium silicate-based bioactive material that can release mineral ions such as calcium and phosphate upon contacting with body fluids and has good mineralization performance. Previous studies have shown that when BG is applied to demineralized dentin, calcium and phosphate ions are released, leading to dentin remineralization, sealing of dentin tubules, reduction of dentin roughness, and an increase in dentin mineral content [13,14]. With regard to the mechanical properties of demineralized dentin, BG induces remineralization, which can improve the surface hardness to some extent [15]. However, Vollenweider et al. found that although BG induced hydroxyapatite formation and increased the mineral content of dentin, the Young’s modulus and bending strength recovered to only a limited degree, possibly because the newly formed hydroxyapatite in the collagen matrix lacked an orderly arrangement [16]. Another study showed that addition of fluoride-containing BG to the adhesive with biomimetic analogues led to intrafibrillar collagen mineralization in the hybrid layer to a certain extent [17]. However, because the structure of the bonding hybrid layer is complex, the possibility that collagen mineralization is attributable to residual minerals in the incompletely demineralized dentin could not be ruled out. Further studies are therefore needed to characterize the type of collagen mineralization induced by BG.

Recently, a type of bioactive glass known as PSC has been reported, which has a much higher proportion of phosphorus than traditional glass [18]. The present study was performed to explore the mineralization effects of PSC on self-assembled type I collagen fibrils, and to further clarify whether PSC could facilitate intrafibrillar mineralization when combined with STMP and PAA.
Materials and Methods

Materials

The composition of the PSC used in this study was (mol%) 10.8% P₂O₅, 54.2% SiO₂, and 35% CaO, prepared by the sol-gel method reported previously [18]. The particle size of the PSC was <10 μm.

Rat tail type I collagen solution (3.77 mg/mL, 100 mg) was purchased from Corning (Corning, NY, USA). STMP (Mw 305.89 g/mol) and PAA (Mw 2,000 g/mol) were obtained from Sigma-Aldrich (Shanghai, P. R. China). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from TIC (Shanghai, P. R. China). Milli-Q water was obtained using a Millipore filter (Millipore Corporation, Burlington, MA, USA).

Self-assembly of type I collagen

Potassium solution (0.2 mol/L KCl, 0.03 mol/L Na₂HPO₄, 0.01 mol/L KH₂PO₄) and acetic acid solution (0.1 mol/L) were prepared and filtered through a 0.22-μm filter. Rat tail type I collagen solution (3.77 mg/mL) was diluted to 0.05 mg/mL using the potassium solution and acetic acid solution with a ratio of 2:1, and after fully mixing, the pH was adjusted to 5.0 using 1.0 M NaOH. Transmission electron microscope (TEM) grids were laid upside-down on 100-μL collagen diluent droplets. Collagen fibers were allowed to self-assemble at 37°C for 48 h and then cross-linked with 0.3 M EDC/0.06 M NHS for 3 h in darkness, followed by a wash with Milli-Q water for 10 s. After air-drying at room temperature, the formation of ACP was observed using a TEM (JEM-1400PLUS, Nidec Corporation, Tokyo, Japan) at an accelerating voltage of 100 kV (Fig. 1).

Phosphorylation of type I collagen fibrils

STMP at 5 wt% was prepared in Milli-Q water, and the pH was adjusted to 12 by 1 M NaOH to open the STMP ring structure. After 5 h, the pH was further adjusted to 8.0 by 1 M HCl. The self-assembled collagen fibrils were then placed in STMP solution and phosphorylated at 37°C for 1 h.

Preparation of mineralization solution

SBF solution (136.8 mM NaCl, 4.2 mM NaHCO₃, 3.0 mM KCl, 1.0 mM K₂HPO₄·3H₂O, 1.5 mM MgCl₂·6H₂O, 2.5 mM CaCl₂, and 0.5 mM Na₂SO₄) was prepared and the pH was adjusted to 7.4 using Tris (hydroxymethyl) aminomethane, before final filtration at 0.22 μm. PAA was added to the SBF solution at concentrations of 0.1 mg/mL, 0.5 mg/mL or 1.0 mg/mL to form PAA-SBF solution. Each dialysis bag containing PSC was placed in 100 mL of each PAA-SBF solution. After magnetic stirring for 24 h, 20 μL of each PAA-SBF solution was dropped onto a separate TEM grid. After 5 h, the pH was further adjusted to 5.0 using 1.0 M NaOH. Transmission electron microscope (TEM) grids were laid upside-down on 100-μL collagen diluent droplets. Collagen fibers were allowed to self-assemble at 37°C for 48 h and then cross-linked with 0.3 M EDC/0.06 M NHS for 3 h in darkness, followed by a wash with Milli-Q water for 10 s. After air-drying at room temperature, the formation of ACP was observed using a TEM (JEM-1400PLUS, Nidec Corporation, Tokyo, Japan) at an accelerating voltage of 100 kV (Fig. 1).

Mineralization of type I collagen fibrils

The phosphorylated type I collagen fibrils were washed with Milli-Q water for 10 s, and the excess liquid was gently absorbed with chipless paper. The TEM grids with attached self-assembled collagen fibrils were floated upside-down on 5 mL PAA-PSC-SBF mineralization solution of different concentrations, and then allowed to mineralize at 37°C and 100% humidity for 3 days and 7 days. Finally, the TEM grids were washed with Milli-Q water and dried at room temperature for 24 h. The mineralized collagen fibrils were observed by TEM and selective area electron diffraction (SAED) with an accelerating voltage of 100 kV (Fig. 1).

Results

Structure and morphology of type I self-assembled collagen fibrils

After assembly of the type I collagen, thin fibrils of collagen were formed, showing a clear boundary and periodic horizontal striations comprising the less dense gap zone and the densely packed overlap zone (Fig. 2).

Mineralization of collagen fibrils induced by PSC and PSC with PAA

After 3 days in PSC solution, the observed mineralization of type I collagen fibrils was mainly extracellular, and no intrafibrillar mineralization was apparent. SAED examination of extracellular minerals demonstrated diffraction rings, indicating that minerals had crystallized and nucleated external to the collagen fibrils (Fig. 3A, B). Exposure of type I collagen fibrils to PSC with 0.1 mg/mL and 0.5 mg/mL PAA for 3 days resulted in mineral accumulation mainly on the outside of collagen fibrils, showing irregular clumping, and no obvious intrafibrillar minerals deposition. SAED demonstrated no crystal diffraction rings, indicating that the extrafibrillar minerals were amorphous (Fig. 3C-F). When collagen fibrils were exposed to PSC with 1.0 mg/mL PAA for 3 days, both extracellular and intrafibrillar mineralization was observed. The intrafibrillar minerals were deposited in an orderly manner in the collagen gap zone, forming periodic striations (Fig. 3G). The nanocrystals were confirmed to be apatite by SAED, showing a characteristic biological apatite crystal diffraction ring structure (Fig. 3H).

When type I collagen fibrils were exposed to PSC alone for 7 days,
extrafibrillar mineralization with acicular crystals was more evident, but there was no apparent intrafibrillar mineralization (Fig. 4A, B). SAED (Fig. 4B) demonstrated that extrafibrillar minerals showed more obvious crystal diffraction rings than at 3 days. Exposure of type I collagen fibrils to PSC with 0.1 mg/mL or 0.5 mg/mL PAA for 7 days resulted in no obvious intrafibrillar mineralization, and only continuous deposition of extrafibrillar minerals. SAED of extrafibrillar minerals demonstrated crystal diffraction rings (Fig. 4C-F). After 7 days of collagen mineralization induced by PSC with 1.0 mg/mL PAA, deposition of both extrafibrillar and intrafibrillar minerals was evident. Intrafibrillar minerals are deposited in an orderly manner, forming periodic striations. The white double arrows indicate a periodic structure of approximately 67 nm. SAED in the lower left corner shows the characteristic diffraction rings of apatite.

ACP formation induced by PSC with PAA

PSC in SBF was able to induce ACP, but it crystallized at an early stage and formed an apparent needle-like structure (24 h). Exposure to PSC in SBF containing 0.1 mg/mL PAA for 24 h, ACP also gradually transformed into acicular crystals. Exposure to PSC in SBF containing 0.5 mg/mL PAA for 24 h led to the formation of ACP particles. Small and large ACP particles agglomerated and fused with each other, and large ACP particles were about 100-150 nm in size. The particles of ACP formed by PSC in SBF containing 1.0 mg/mL PAA were small, measuring about 30-50 nm (Fig. 5).

Discussion

Portland cement has been commonly used as a source of mineral ions to induce collagen mineralization in previous studies. It has been reported that Portland cement with polyacrylic acid (PAA) and polyvinylphosphonic acid (PVPA) can partially facilitate intrafibrillar mineralization of demineralized dentin [10,19]. However, as a calcium silicate-based material, Portland cement lacks phosphorus, and SBF has often been used as a source of phosphorus during mineralization. In this study, PSC was synthesized using phytic acid as a precursor, which increased the phosphorus content of PSC, whose chemical content included 10.8 mol % P 2O 5, 54.2 mol % SiO 2, and 35 mol % CaO. Previous studies found that PSC showed rapid mineralization, forming hydroxyapatite in the early stage (24 h) and maintaining high contents of Si and P in SBF [18].

Hench et al. found that the mechanism of BG mineralization involved...
Ca on the BG surface, which condenses to form a silica-rich colloidal layer. Mineral crystal nucleation, leading to the formation of amorphous precur-
ser, was evident on the collagen surface; after 24 h, thus allowing ACP to achieve intrafibrillar mineralization. On increment of mineralization time was extended to 7 days, SAED showed that crystal diffraction rings and mineralization crystals were formed outside collagen fibrils induced by PSC combining 1.0 mg/mL PAA for 3 days suggested orderly deposition of minerals in the collagen, producing mineralized col-
lagen with periodic striations. STMP contains a large number of phosphate radicals, which can combine with collagen through covalent bonding. These phosphorylated type I collagen fibrils can act as a template for nucle-
ation and crystallization of calcium and phosphorus ions. STMP promotes the crystallization and growth of apatite in the collagen gap region, thus leading to orderly intrafibrillar mineralization [12]. After mineralization for 7 days, intrafibrillar mineral deposition gradually extended from the collagen gap zone to the overlap zone, and the periodic collagen striations became gradually blurred. SAED showed that crystal diffraction rings were more obvious, with characteristic apatite diffraction rings, indicating that the degree of collagen mineralization had increased. At the same time, some extrafibrillar minerals formed needle-like crystals.

It has been reported that when Portland cement was used as a miner-
alization source, intrafibrillar mineralization was achieved using 0.5 mg/
ml PAA (1800 Da) [25,26]. In the present study, the concentration of PAA used for inducing intrafibrillar mineralization was higher at 1.0 mg/mL. It had been shown that the molecular weight and concentration of PAA influ-
cenced collagen mineralization. Qi et al. used PAA at different concentrations in ultra-saturated mineralization solution to induce mineralization of type I collagen fibrils. They found that low-molecular-mass (2 kDa) PAA achieved collagen mineralization within 7 days at a low concentration of 0.01 mg/mL. When the concentration of PAA was increased (0.05 mg/mL), the ACP formed by PAA was too stable to nucleate, resulting in failure of intrafibrillar mineralization [23]. Thus, different concentrations of PAA are required for different mineralization sources. On the one hand, because PAA releases ions faster and can maintain a high P state in SBF solution, a higher concentration of PAA might be needed in order to compete for the binding of calcium ions to prevent ACP agglomeration and crystallization. In the present study, the particle size of ACP induced by PSC combining 1.0 mg/mL PAA was about 30-50 nm, and was maintained at a small size after 24 h, thus allowing ACP to achieve intrafibrillar mineralization. On the other hand, because PSC basically did not change the pH of SBF, the mineralization solution was neutral, whereas Portland cement released a large amount of OH in the process of mineralization, making the mineral-
ization solution alkaline. Some studies have shown that when the pH is 10.25, ACP can maintain maximum stability [27]. Therefore, under neutral conditions, a higher concentration of PAA might be needed to achieve a more stable ACP.

Different pH conditions affect the electric charge and collagen mineral-
ization properties of self-assembled type I collagen fibrils [28,29]. Marelli et al. found that type I collagen fibrils formed at pH 9.0 had greater electro-
negativity and induced faster collagen mineralization in SBF [28]. Under the conditions used for the present study, PSC combined with 1.0 mg/mL PAA was able to partially induce intrafibrillar mineralization, but whether or not self-assembled collagen fibrils formed under other conditions has better mineralization properties, or whether this can be further applied to the remineralization of dentin, still requires further investigation.

A number of studies have examined the dynamics of ACP entry into the collagen interstitial space. Sommerdijk et al. found that the a and c bands in the gap zone of collagen had obvious positive charge, while the mineralization precursors had negative charge. Therefore, ACP might enter collagen due to charge attraction [30]. Niu et al. postulated that the entry of ACP into the internal space of collagen might involve an internal-external balance both charge and osmotic pressure. All of these possible mechanisms still require further experimental verification [31]. Regardless of the dynamic mechanism by which ACP enters collagen, the particle size and charge of ACP might have some influence on the mineralization of collagen.

In conclusion, exposure of collagen fibrils to PSC alone led to deposition of mainly extrafibrillar minerals, while exposure to PSC combining an appropriate concentration (1.0 mg/mL) of PAA induced both intrafi-
brillar and extracellular mineralization. The present results could suggest a new strategy for promoting dentin remineralization and might further be applicable to the formulation of dentin adhesive with remineralization properties to improve the durability of dentin bonding.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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