

Original article

Effects of bioactive glass with high phosphorus content on mineralization of type I collagen fibrilsQiuju Li^{1,2,3,4}), Junhe Shi⁵), Sainan Wang^{1,2,3,4}), and Yanmei Dong^{1,2,3,4})¹) Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology, Beijing, P. R. China²) National Clinical Research Center for Oral Diseases, Peking University School and Hospital of Stomatology, Beijing, P. R. China³) National Engineering Laboratory for Digital and Material Technology of Stomatology, Peking University School and Hospital of Stomatology, Beijing, P. R. China⁴) Beijing Key Laboratory of Digital Stomatology, Peking University School and Hospital of Stomatology, Beijing, P. R. China⁵) State Key Laboratory of Polymer Physics and Chemistry, Institute of Chemistry, Chinese Academy of Sciences, Beijing, P. R. China

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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 microleakage, 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 fibers [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.

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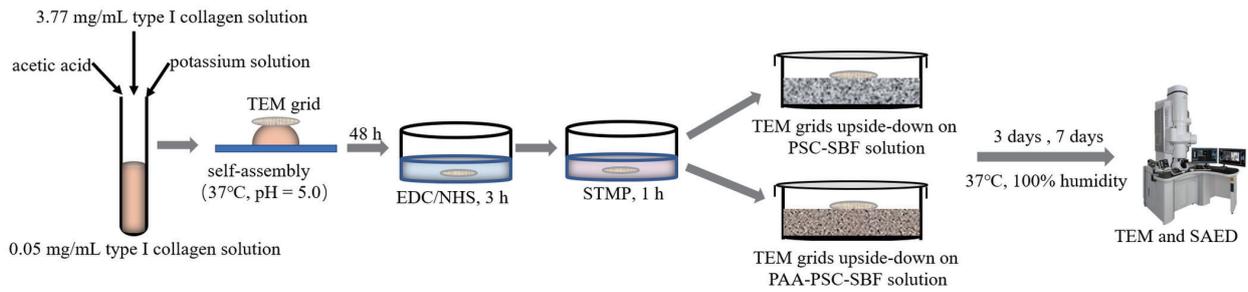


Fig. 1 Schematic diagram showing mineralization of self-assembled type I collagen fibrils by PSC.

Materials and Methods

Materials

The composition of the PSC used in this study was (mol%) 10.8% P_2O_5 , 54.2% SiO_2 , and 35% CaO , prepared by the sol-gel method reported previously [18]. The particle size of the PSC was $<10 \mu m$.

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 Na_2HPO_4 , 0.01 mol/L KH_2PO_4) 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 morphology of the self-assembled type I collagen fibrils 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 with 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 $NaHCO_3$, 3.0 mM KCl, 1.0 mM $K_2HPO_4 \cdot 3H_2O$, 1.5 mM $MgCl_2 \cdot 6H_2O$, 2.5 mM $CaCl_2$, and 0.5 mM Na_2SO_4) 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 0.1 mg/mL, 0.5 mg/mL or 1.0 mg/mL to form different concentrations of PAA-SBF solution. The PAA-PSC-SBF mineralization solution was formed by dispersing PSC at 0.05 g/mL in the above PAA-SBF solution.

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).

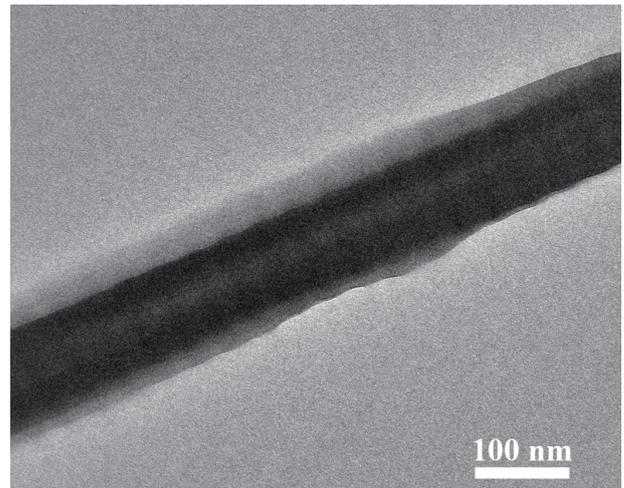


Fig. 2 Morphology of type I collagen fibrils after self-assembly demonstrated by TEM. Self-assembled collagen showed periodic alternating light and dark horizontal striations.

Formation of amorphous calcium phosphate

Five grams of PSC was dispersed in 2 mL of SBF and then placed in a dialysis bag with a pore size of 3,500 Da. 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 air-drying at room temperature, the formation of ACP was observed using the TEM.

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 extrafibrillar, and no intrafibrillar mineralization was apparent. SAED examination of extrafibrillar 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 extrafibrillar 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,

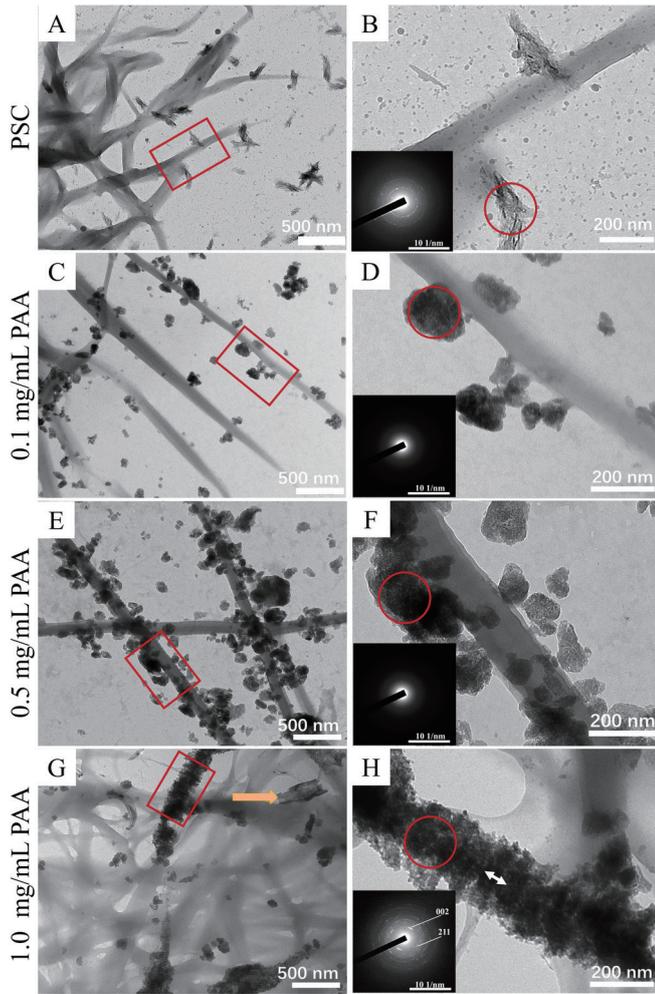


Fig. 3 TEM morphology and SAED pattern of type I collagen fibril mineralization induced by PSC combining PAA after 3 days. (A) Type I collagen showed no obvious intrafibrillar mineralization, but mainly extrafibrillar mineralization. (B) Shows the area of the red box in (A). Extrafibrillar minerals are indicated by red circles, and SAED in the lower left corner shows crystal diffraction rings. (C) and (E) show type I collagen fibril mineralization induced by PSC combining 0.1 mg/mL or 0.5 mg/mL PAA for 3 days, with no obvious intrafibrillar mineralization. (D) and (F) are enlarged views of the red boxes in (C) and (E), respectively. The red circles indicate irregular extrafibrillar minerals, and SAED shows diffraction rings in the lower left corner. (G) Type I collagen fibril mineralization induced by PSC with 1.0 mg/mL PAA for 3 days. Both extrafibrillar (yellow arrow) and intrafibrillar (red box) mineralization are evident. (H) Shows an enlarged view of the red box in (G). 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.

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 nanocrystals gradually extended from the collagen gap zone to the overlap zone, and the periodic striations in the collagen fibrils gradually became blurred. SAED showed that characteristic crystal diffraction ring structure of biologic apatite was more obvious. Simultaneously, collagen extrafibrillar minerals also formed needle-like crystals (Fig. 4G, H).

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

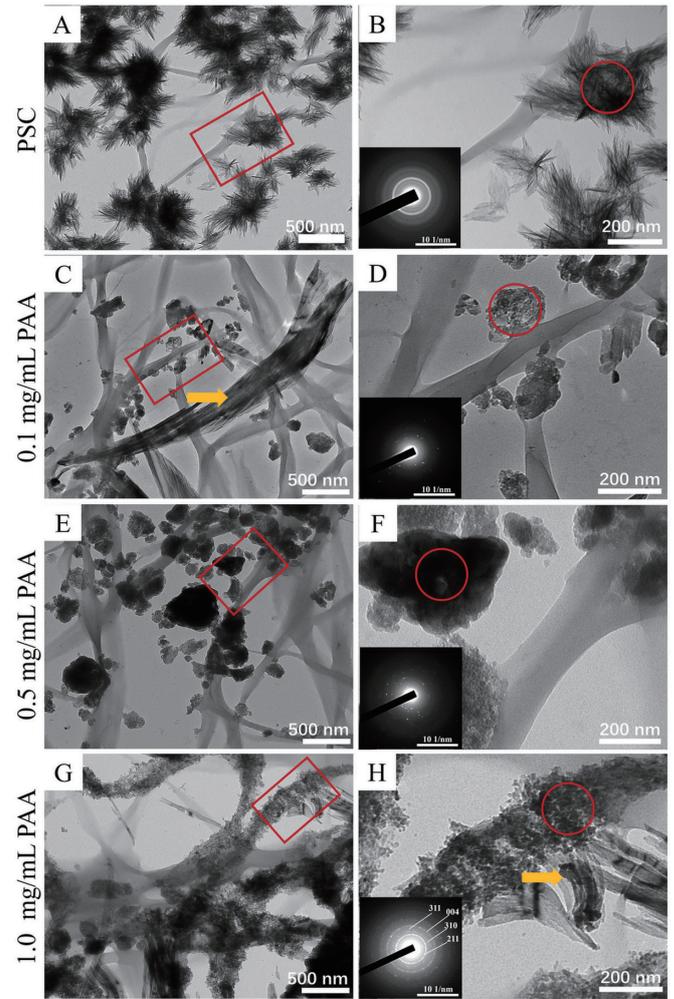


Fig. 4 TEM morphology and SAED pattern of type I collagen fibril mineralization induced by PSC with PAA for 7 days. (A) Type I collagen fibril mineralization induced by PSC for 7 days shows no obvious intrafibrillar mineralization. (B) Shows the area of the red box in (A). Extrafibrillar minerals are indicated by red circles, and in the lower left corner SAED shows crystal diffraction rings. (C) and (E) show type I collagen fibril mineralization induced by PSC with 0.1 mg/mL or 0.5 mg/mL PAA for 7 days, respectively, with no obvious intrafibrillar mineralization and mainly extrafibrillar mineralization. (D) and (F) show enlarged views of the red boxes in (C) and (E), respectively. The red circles show irregular extrafibrillar minerals, and in the lower left corner SAED demonstrates diffraction rings. (G, H) Type I collagen fibril mineralization induced by PSC with 1.0 mg/mL PAA for 7 days. Both extrafibrillar (yellow arrow) and intrafibrillar (red box) mineralization are evident. (H) Intrafibrillar minerals gradually extend from the collagen gap zone to the overlap zone, and the periodic collagen striations become gradually blurred. SAED shows the characteristic diffraction rings of apatite.

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

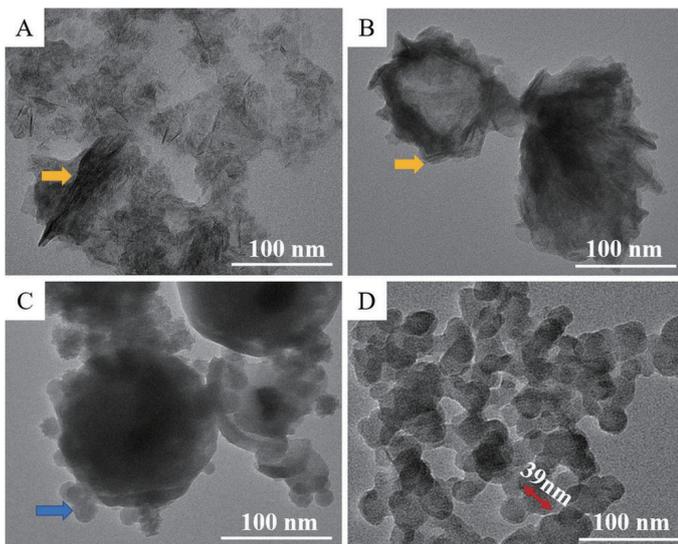


Fig. 5 TEM morphology of ACP induced by PSC with different concentrations of PAA for 24 h. (A) ACP induced by PSC alone in SBF showed development of acicular crystals (yellow arrow) after 24 h. (B) ACP induced by PSC with 0.1 mg/mL PAA in SBF showed nucleation and crystallization from an amorphous state, with formation of acicular crystals (yellow arrow). (C) ACP induced by PSC with 0.5 mg/mL PAA in SBF with a large particle size of about 100-150 nm, and some ACPs with a small particle size are also evident (blue arrow). (D) The morphology of ACP induced by PSC with 1.0 mg/mL PAA in SBF for 24 h. The ACP particles formed were small, about 30-50 nm (red double arrow indicates a small ACP particle of 39 nm), without any obvious acicular crystal structure.

ion exchange. When BG comes in contact with body fluid, Ca^{2+} and Na^{+} in the BG are rapidly exchanged with H^{+} and H_3O^{+} in solution. OH^{-} breaks the Si-O-Si chemical bond of BG and SiO_3^{2-} is released, followed by the further release of Ca^{2+} and Na^{+} . At the same time, more Si-OH is formed on the BG surface, which condenses to form a silica-rich colloidal layer. Ca^{2+} and PO_4^{3-} in solution migrate to the BG surface and react with the silica gel layer to form an amorphous mineralized layer, which gradually crystallizes and transforms into hydroxyapatite [20]. In this study, when PSC alone was used to induce mineralization of type I collagen fibrils over a 3-day period, minerals were evident on the collagen surface; after 7 days of mineralization, these minerals were continuously deposited and more apatite was formed. Previous studies have shown that PSC is able to maintain a high Si and high P state in solution [18], and that Si-OH can bind and cooperate with the carbonyl group of collagen and $-\text{C}=\text{N}-$ in the side chain, by which can achieve the combination of newborn minerals and collagen [21]. The Si-OH released by PSC might bind to collagen and form a silica gel layer, which then binds with calcium and phosphorus ions, leading to the gradual formation of hydroxyapatite on the surface of the collagen fibrils.

In the process of biomineralization, calcium and phosphorus can form clusters of nano-precursors, which fuse further to form liquid-stable ACP, thereafter diffusing into the collagen gap space to undergo mineralization using collagen as a template. The presence of liquid-stable ACP is prerequisite for the mineralization of collagen [8]. As a polyanion electrolyte, PAA can partially simulate the function of non-collagen proteins that inhibit and delay the crystallization of apatite, and combine with calcium and phosphorus in solution to form ACP, which penetrates into nanoscale gap regions of collagen fibers [22]. ACP formed by low-molecular-weight PAA is more stable [23]. For the present study, PAA with a molecular weight of 2 kDa was selected for collagen fiber mineralization. When PSC combining 0.1 mg/mL or 0.5 mg/mL PAA was used to induce type I collagen fibril mineralization for 3 days, amorphous extrafibrillar minerals were formed. However, mineralization induced by PSC alone for 3 days led to formation of minerals that crystallized outside the collagen. Therefore, PAA at a concentration of 0.1 mg/mL or 0.5 mg/mL appeared to partially inhibit mineral crystal nucleation, leading to the formation of amorphous precursors, but induction of intrafibrillar mineralization was difficult. When the mineralization time was extended to 7 days, SAED showed that crystal diffraction rings and mineralization crystals were formed outside collagen fibers at PAA concentrations of 0.1 mg/mL and 0.5 mg/mL. This might have been due to continuous aggregation of ACP at low concentrations of

PAA, leading to formation of large and irregular amorphous mineralization precursors. Along with the constant release of Si, Ca and P from PSC, these mineral ions nucleated and crystallized. Collagen fibers have a molecular elimination mechanism whereby molecules smaller than 6 kDa can freely enter the collagen internal space, while molecules larger than 40 kDa can barely do so [24]. Therefore, the poor degree of intrafibrillar mineralization was presumably due to the large size of the mineralized precursors.

The extrafibrillar and intrafibrillar mineralization of type I collagen fibrils induced by PSC combining 1.0 mg/mL PAA for 3 days suggested orderly deposition of minerals in the collagen, producing mineralized collagen 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 nucleation 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 mineralization 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 influence 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 PSC 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 mineralization 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 mineralization 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 intra-

brillar and extrafibrillar 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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