Dual functions of methacryloyloxydecyl dihydrogen phosphate monomer with de- and re-mineralizing ability

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Research Article

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Abstract

Background

Methacryloyloxydecyl dihydrogen phosphate (MDP) is one of the most commonly used acidic functional monomers in dentin adhesives. MDP has been speculated to induce mineralization, but there has been no convincing evidence of its ability to induce intrafibrillar mineralization. Polymers play a critical role in biomimetic mineralization as the stabilizers/inducers of amorphous precursors. Hence, MDP-induced biomimetic mineralization without the addition of polymers has not been fully verified or elucidated.

Results

MDP-collagen fibrils were heavily mineralized after 48 h. Amphiphilic MDP can not only demineralize dentin (0.5-1 μm) but also induce remineralization of demineralized dentin at 28 days without polymer additives. By combining three-dimensional stochastic optical reconstruction microscopy (3D-STORM), surface zeta potentials, contact angle measurements, inductively coupled plasma-optical emission spectroscopy, transmission electron microscopy, atomic force microscopy, and Fourier transform infrared spectroscopy with circular dichroism, we show that amphiphilic MDP can not only demineralize dentin by releasing protons but also infiltrate collagen fibrils, including dentin collagen, break hydrogen bonds between polypeptides, unwind the triple helical structure and finally immobilize collagen by the formation of chemical bonds. MDP-bound collagen functions as huge collagenous phosphoprotein (HCPP) unlike chemical phosphorylation modifications. HCPP can induce biomimetic mineralization itself without polymer additives by alternatively attracting calcium and phosphate through electrostatic attraction.

Conclusions

We herein propose the dual functions of amphiphilic MDP monomer with de- and re-mineralizing ability. MDP in the free state can demineralize dentin substrates by releasing protons, whereas MDP in the collagen-bound state as HCPP can induce intrafibrillar mineralization. The dual functions of MDP monomer with de- and re-mineralization properties might create new epochs in adhesive dentistry and preventive dentistry.

Background

Dentin and bone are biomineralized hard tissues in humans [1]. Their main components are similar, consisting of type I collagen and hydroxyapatite (HAP) [1]. HAP nucleation and growth inside collagen fibrils have been widely studied [2]; however, the precise mechanism of intrafibrillar mineralization remains controversial [3]. Intrafibrillar mineralization has been found to play an important role in the mechanical properties of bone and dentin [4, 5]. Thus, understanding the mechanisms of collagen mineralization is critical for the development of new materials for bone and dentin repair [6]. Type I

collagen, as a template, plays a key role in the biomineralization of dentin and bone that has been well established; however, noncollagenous proteins (NCPs) that mediate the transformation of amorphous calcium phosphate (ACP) nanoparticles into HAP in the collagen matrix should not be neglected [7, 8]. NCPs play crucial roles in the formation and maintenance of the mineralized matrix [9]. The phosphate and carboxyl groups on the surface of organic templates can attract calcium ions via electrostatic forces as nucleation sites for crystallization [10–12]. Previous studies have revealed that synthetic polymers such as polyacrylic acid (PAA) and polyaspartic acid (PAsp) alone or in conjunction with other molecules such as polyvinylphosphonic acid (PVPA), sodium trimetaphosphate (STMP) and sodium tripolyphosphate (TPP) can be used to mimic the functions of NCPs to bioinspire apatite formation in collagen [9].

The demineralization of dentin is the initial stage in the development of caries and the main cause of dentin hypersensitivity. However, the remineralization of demineralized dentin is an intractable challenge for scientists and dentists [13]. Biomimetic mineralization provides a promising application for tooth hard tissue repair, such as dentin decay [14, 15]. Self-etching adhesives containing phosphoric acid ester as a polymerizable acidic monomer, such as the amphiphilic molecule methacryloyloxydecyl dihydrogen phosphate (MDP), have been widely used in dentistry for decades and have achieved excellent dentin bond durability in laboratory and clinical investigations [16, 17]. Amphiphilic MDP containing two P-OH groups could dissociate H^+ in aqueous solutions to demineralize the dentin substrate, and subsequently, the P-O- groups could react with calcium ions [18]. Nurrohman et al. [19] reported that the immobilization of MDP on collagen could induce biomimetic mineralization. Certain physicochemical characteristics were used to support the formation of apatite within the collagen matrix in their study [19], but there was no convincing evidence of intrafibrillar apatite deposition within the collagen fibrils [9]. Certain NCPs, such as dentin matrix protein (DMP1) and dentin phosphophoryn (DPP), are believed to contribute to the regulation of collagen fibril biomineralization in human dentin [20]. These macromolecules are phosphoproteins that contain highly phosphorylated serine and threonine residues, in which the phosphate groups can capture calcium ions for biomimetic mineralization [20]. In this study, MDP immobilized in collagen fibrils was surmised to form huge collagenous phosphoprotein (HCPP), which could induce itself biomimetic mineralization without NCPs or polymer additives. However, whether MDP could induce the intrafibrillar mineralization of reconstituted type I collagen and demineralized dentin without ACP polymeric stabilizers has not been fully verified. The detailed mechanism by which MDP promotes intrafibrillar mineralization without polymer additives has not been fully elucidated.

Herein, we proposed the dual functions of amphiphilic MDP monomer with de- and re-mineralizing ability. This means that MDP in the free state could demineralize dentin substrates, while MDP in the collagen-bound state could form HCPP to induce itself biomimetic mineralization. The dual functions of MDP with de- and re-mineralizing ability might provide a novel strategy in adhesive dentistry and preventive dentistry. Whether other amphiphilic phosphate monomer possess this same function needs to be further studied.

Materials And Methods

Chemicals

All chemical reagents used in this study were of analytical chemical grade, purchased commercially and used as received. All solvents were used without further purification. These reagents and solvents included $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, K_2HPO_4 , 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Mw: 238 Da), KCl, NaOH, NaN_3 , dimethyl sulfoxide (DMSO; Mw: 78 Da), glycine (Mw: 75 Da) and BODIPY (Mw: 248 Da) from Sigma–Aldrich (MO, USA); (3-aminopropyl) triethoxysilane (APTES), tert-butyldimethylsilyl chloride (TBSCl; Mw: 151 Da), tetra-n-butylammonium fluoride (TBAF; Mw: 262 Da), dibenzyl chlorophosphate (Mw: 297 Da), decane-1,10-diol (Mw: 174 Da), anhydrous pyridine (Mw: 79 Da) and calcein (Mw: 623 Da) from Aladdin Reagent Co., Ltd. (Shanghai, China); 10-methacryloyloxydecyl dihydrogen phosphate (MDP; Mw: 323 Da) was purchased from Watson International Co., Ltd. (Jiangsu, China); and blocking buffer solution and washing buffer solution were from Beyotime Co., Ltd. (Shanghai, China). Pure type I collagen solution derived from rat tails (3 mg mL^{-1}) was purchased from Gibco-Invitrogen (CA, USA). Collagen-I rabbit anti-mouse antibody (ab34710) and goat anti-rabbit IgG (H + L) superclonal secondary antibodies were purchased from Abcam Co., Ltd. (Cambridge, UK) and Thermo Fisher (CA, USA), respectively. Distilled water was used in the study, and all solutions were filtered through 0.22 μm Millipore film prior to use.

Mineralization of collagen fibrils

A single-layer reconstructed collagen model was employed in this study and prepared as described in our previous studies [21, 22]. This study comprised two groups: a Control group (Bare collagen): collagen-coated TEM grids without MDP pretreatment were immersed in distilled water for 10 min, and an Experimental group (MDP-collagen): collagen-coated TEM grids were pretreated with 10 wt % MDP solution for 10 min, then, the collagen-coated TEM grids were rinsed with distilled water for 10 min. Next, MDP-collagen and bare collagen were incubated in artificial saliva (AS) solution (1.5 mM CaCl_2 , 0.9 mM KH_2PO_4 , 20 mM HEPES, 260 mM KCl, 2 mM NaN_3 , pH 7.0) at 37°C for 6 h, 12 h, 24 h and 48 h. Two TEM grids were floated on 60 μL of AS solution. The AS solution was changed every day. Four TEM grids from each group were removed at each predetermined time and washed, in sequence, with distilled water and 50% and 100% ethanol. All samples were analyzed using TEM (JEM-1230, JEOL, Tokyo, Japan) at 110 kV. SAED patterns were recorded using a HRTEM (JEM-2100F, JEOL, Tokyo, Japan). ImageJ software (National Institute of Health, Bethesda, MD) was used to obtain the diameters of the collagen fibrils. For each analysis, at least five TEM images were examined to obtain the mean value and standard deviation.

Dentin remineralization

Freshly extracted third molars without caries were collected from patients, as approved by the Ethics Committee of Zhejiang University School of Stomatology (#2018017). The teeth were thoroughly cleaned and stored as previously described [23]. One-millimeter-thick mid-coronal dentin disks were prepared using a slow-speed diamond saw (Isomet 1000, Buehler Ltd., IL, USA) and then polished using 360-grit

silicon carbide paper under running water. The polished dentin disks were acid-etched with 37% phosphoric acid for 10 s and rinsed with distilled water for 20 s to produce a 5- to 7- μ m thick layer of mineral-free collagen matrix.

A total of 30 demineralized dentin disks were prepared and randomly divided into 2 groups ($n = 15$). Briefly, for the control group, demineralized dentin disks without MDP pretreatment were rinsed with distilled water for 10 min. For the experimental group, demineralized dentin disks were pretreated with 10 wt % MDP solution for 10 min and then rinsed with distilled water for 10 min. Twenty samples were each incubated in 10 mL of AS solution (changed every day) in a 100% humidity chamber at 37°C for 4, 7, 14 and 28 days. Three samples from each group were retrieved at predetermined time and dehydrated in an ascending ethanol series (50–100%) and acetone before being immersed in propylene oxide and embedded in epoxy resin. Ultrathin sections (approximately 70 ~ 90 nm) were prepared for TEM analysis with SAED (JEM-2100F, JEOL, Tokyo, Japan).

Fourier transform infrared (FTIR) spectroscopy analysis of collagen

An FTIR spectrophotometer (Spectrum 400, PerkinElmer, MA, USA) was used to collect infrared spectra of commercial MDP and the collagen gels with or without MDP solution pretreatment. The collagen gels employed in this study were prepared as described in our previous studies [24]. Commercial MDP was prepared by the potassium bromide (KBr) pellet pressing method. The tablets contained MDP and KBr at a ratio of 1:200. Infrared spectra were collected between 4,000–400 cm^{-1} at a resolution of 4 cm^{-1} using 32 scans.

Circular dichroic (CD) spectroscopy analysis of the collagen fibrils

CD measurements were carried out to detect the changes in the triple helical structure of collagen (0.2 mg/mL) in the presence of 0.3 mM ethanol and 0.3 mM MDP solution ($n = 3$). A quartz cuvette with a 1-mm path length was used. The CD spectra were measured in the far-ultraviolet region (260–190 nm) using a J-1500 spectropolarimeter (Jasco; Easton, MD, USA) with a scan speed of 100 nm/min at 25°C. Baseline correction was performed by background subtraction of the solution spectrum. Each spectrum represents an average of 3 scans.

Collagen solid surface zeta potential

Collagen gel samples were prepared as mentioned above. The collagen gels were pretreated with or without MDP solution ($n = 3$) and then rinsed with distilled water for 10 min. After that, the collagen gels were dried in an electro-thermostatic blast oven (DHG-9070A, Shanghai Jinghong Experimental Equipment Co., Ltd., Shanghai, China) at 37°C overnight. The zeta potentials of the collagen gels were determined with an electrokinetic analyzer for solid surface analysis (SurPASS 3, Anton Paar, Steiermark, Australia). This experiment was performed in triplicate.

Collagen contact angle measurements

Collagen gels pretreated with or without MDP solution ($n = 5$) were prepared as described above. A 4- μ L drop of artificial saliva solution was dropped onto the surface of the gel, and the contact angle was analyzed by the sessile drop technique (OCA15plus, DataPhysics Instruments GmbH, Germany). The contact angle of each drop was recorded at equilibrium. The right and left contact angles were averaged to obtain a mean θ value.

Absorption of calcium ions by collagen pretreated with MDP solution

The chelation capacities of MDP from the collagen gels were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES; 720ES, Agilent, California, USA). The collagen gels were pretreated with or without 10 wt % MDP solution ($n = 18$). Each gel was added to 10 mL of 1.5 mM CaCl_2 solution and left uninterrupted for 1, 12, 24, 48, 96 and 168 h. Then, 226 μ L of solution was withdrawn at each predetermined time and diluted with distilled water to 10 mL. The Ca^{2+} concentration in each solution was measured. The experiment was conducted in triplicate.

Three-dimensional stochastic optical reconstruction microscopy (3D-STORM) image acquisition of collagen fibrils

Synthesis and characterization of MDP-BODIPY

MDP-BODIPY was synthesized through chemical reactions from a series of intermediate compounds. All of the intermediate products were characterized by proton and carbon nuclear magnetic resonance spectroscopy (JNM-ECZR, JEOL, Tokyo, Japan). The ^1H NMR data were reported in the following order: chemical shift (δ) in ppm; multiplicities indicated as s (singlet), d (doublet), t (triplet), dd (doublet of doublets), or m (multiplet); and coupling constants (J) in Hertz (Hz). ^{13}C NMR data were reported in terms of chemical shift (δ , ppm). MDP-BODIPY was characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry (Bruker MALDI-TOF Ultraflex, Munchen, Germany) for mass analysis.

In general, the fluorescent probe (Compound 6: MDP-BODIPY), as a mimic of MDP, was synthesized from commercially available reagents (Compound 2), as shown in Fig. 1. The synthesis process and characterization of the compounds are described in detail in the supporting information (SI). Characterization of Compounds 1–6 is provided in the supporting information (Fig. S1–5).

Sample preparation and 3D-STORM image acquisition of the collagen fibrils

Single-layer reconstructed type I collagen fibrils were assembled on APTES-modified LCCD glass substrates and labelled with fluorescent reagents as described in previous studies [24–26]. Afterward, MDP-BODIPY and BODIPY were used for fluorescence imaging. Specifically, 200 μL of 10 wt % MDP-BODIPY and a BODIPY ethanol-aqueous solution were added to the LCCD glass substrate at 37°C for 10 min and gently rinsed with 5 mL of PBS buffer solution ten times. The samples were soaked in imaging buffer before STORM image acquisition. STORM imaging experiments were performed as previously described [24, 27]. STORM data acquisition of the collagen fibrils was performed on a Nikon Ti-E inverted optical microscope equipped with a 100 $\times$ oil immersion objective (CFI Apo 100 $\times$ oil NA 1.49 TIRF WD 0.12) and an Andor camera (iXon 897 back illuminated EMCCD). The STORM image data were analyzed by Nikon NIS-Elements AR 4.51 software.

3D-STORM image acquisition of the collagen fibrils after incubation in CaCl_2 solution

3D-STORM was employed to locate calcium ions in MDP-pretreated collagen. The collagen fibrils were labelled with a fluorescent reagent by immunofluorescent staining and STORM images were acquired as described above. Control group: type I collagen fibrils were added to 200 μL of 1.5 mM CaCl_2 solution and incubated for 48 h followed by rinsing with 5 mL of PBS ten times, and then labelled with 10 mM calcein for 10 minutes. Experimental group: FITC-labelled type I collagen fibrils pretreated with MDP were incubated in calcium solution for 48 h, rinsed with 5 mL of PBS ten times and labelled with 10 mM calcein for 10 min.

Atomic force microscopy of the collagen fibrils

The surface morphology and average diameter of the collagen fibrils were determined by atomic force microscopy (AFM). Single layers of rat tail type I collagen were assembled on freshly cleaved APTES-modified muscovite mica (Grade V1, Ted Pella Inc., CA, USA). The experiment was carried out with an IconScan atomic force microscope (Bruker Corporation, USA) in ambient air. Identical AFM tips (Bruker SNL-10) were utilized only once for each sample. AFM was performed in contact mode with a scan rate of 1.0 Hz. The surfaces of the collagen fibrils were immersed in 2 mL of solution before AFM observation, and a closed fluid cell was employed.

The bare collagen fibrils were treated with distilled water, 50% ethanol-aqueous solution, and 10 wt % MDP solution for 5 and 10 min followed by rinsing with distilled water for 10 min before they were immersed in AS for 1, 2 and 3 h. Images (field size: 5 $\times$ 5 μm) of the same collagen fibrils were collected by AFM. The acquired images were analyzed using NanoScope Analysis version 1.90 to obtain the diameters and heights of the collagen fibrils. Each collagen fibril was measured three times, and the mean value was used as the diameter of the fibril.

Statistical analysis

After the zeta potential and contact angle data followed the normality and homoscedasticity assumptions, one-way ANOVA was used to analyze these data between the MDP-collagen and bare

collagen samples. One-way ANOVA followed by Tukey's multiple comparisons test was utilized to detect significant differences between the diameter data from the TEM images among different groups. The differences in fibril diameters detected by AFM were examined using one-factor repeated measures analysis. Post hoc multiple comparisons were conducted using the Holm-Sidak statistic. For statistical analyses, a significance level was set at 0.05.

Results And Discussion

This study showed that MDP immobilized in collagen fibrils could induce collagen intrafibrillar mineralization after incubation in an artificial saliva (AS) solution without an amorphous CaP precursor stabilizer. However, bare collagen as the template could not.

Mineralization of collagen fibrils

A single-layer collagen model was used to verify the efficacy of MDP to direct intrafibrillar mineralization in the study. Representative TEM images and changes in the diameters of the collagen fibrils are shown in Fig. 2. The bare collagen fibrils displayed the typical 67 nm D-period with a fibril diameter of approximately 66.64 ± 9.62 nm ($n = 30$) (Fig. 2a **and b**). The bare collagen fibrils were not mineralized after incubation in AS for 48 h (Fig. 2c-j). This was confirmed by the SAED patterns (inset in Fig. 2j). There was no significant change in the diameters of the bare collagen fibrils after they were incubated in AS (Fig. 2k). However, the MDP-pretreated collagen fibrils did not display D-periodicity (Fig. 2A **and B**), and their diameter increased 2-fold ($P < 0.05$) (Fig. 2k). After incubation in AS for 6 h, the MDP-collagen fibrils became partially swollen as a result of the infiltration of mineral ions or amorphous CaP precursors into the internal water compartments of the collagen (Fig. 2C **and D**). Spheroidal CaP precursors, which were confirmed by the SAED pattern (insets in Fig. 2D), formed on the MDP-collagen fibrils. As the incubation duration extended to 12 h, the diameter of the whole MDP-collagen fibril increased by 3-fold (Fig. 2E **and F, k**). The SAED pattern (insets in Fig. 2F) indicates that the minerals are amorphous. Partial intrafibrillar mineralization could be observed in MDP-collagen fibrils after 24 h of incubation (Fig. 2G **and H**), and heavy mineralization occurred with slight shrinkage of the fibrillary diameter after incubation for 48 h (Fig. 2I **and J**). The SAED patterns (inset in Fig. 2H, J) indicate the crystalline minerals inside the MDP-collagen fibrils. At the early stage of intrafibrillar mineralization, infiltration of amorphous CaP precursors results in an increase in the diameter of the collagen fibril. However, the loss of water caused by the crystallization of amorphous CaP precursors results in a reduction of intrafibrillar volume [28].

Dentin remineralization

Remineralization of demineralized dentin is a convincing strategy to improve dentin bonding durability and inhibit primary and secondary caries [14]. Evidence of the mineralization potential of MDP was further acquired using a 3-D model of natural tissue, including demineralized human dentin (Fig. 5). A thin demineralization layer approximately 5–7 μ m thick was created in this study by etching with 37% phosphoric acid (Fig. S6, SI). MDP is one of the most commonly-used acidic functional monomers in

dentin adhesives. Its phosphate groups can dissociate to release protons in aqueous solutions, which contribute to demineralization when it is applied to the enamel or dentin surface [29]. In the experimental group, pretreatment with MDP solution resulted in an extra partially demineralized layer approximately 0.5–1 μm thick in addition to the fully demineralized layer (Fig. S7, SI). The TEM images show that the demineralized dentin in the control group was not remineralized even after incubation in AS for 28 days (Fig. S8, SI). In the experimental group (MDP-pretreated dentin), remineralization of the demineralized dentin could be identified after incubation in AS for 4 days (Fig. 3a-c). The remineralization of the demineralized dentin increased over time from weak crystallinity after 7 days of incubation (Fig. 3d-f) to good crystallinity with partial remineralization after 14 days of incubation (Fig. 3g-i). Complete remineralization could be observed (Fig. 3j-l) with diffraction rings of 002, 211 and 004 after incubation in AS for 28 days (inset in Fig. 3l). The microstructures of the remineralized dentin zone are similar to those of the underlying intact dentin. The findings in this study demonstrated, for the first time, that the remineralization of demineralized dentin could be gradually and completely induced via MDP-bound collagen as HCPP without polymer additives. MDP-bound collagen (HCPP) with remineralizing potential might increase dentin bond durability [19, 30].

Fourier transform infrared (FTIR) spectroscopy analysis of collagen

FTIR was used to analyze the interactions between MDP and collagen. Figure 4a displays the typical FTIR spectra of type I collagen with and without MDP pretreatment. After MDP pretreatment, new peaks (carbonyl groups ($\sim 1720\text{ cm}^{-1}$), C-O-C stretching ($\sim 1169\text{ cm}^{-1}$), O = P ($\sim 1300\text{ cm}^{-1}$), P-O-C ($\sim 1019\text{ cm}^{-1}$) and P-OH ($\sim 2860\text{ cm}^{-1}$) (marked as asterisk)) derived from MDP were detected (Fig. 4a). Compared with bare collagen, MDP-collagen possessed an intense amide I peak (C = O stretch, $\sim 1640\text{ cm}^{-1}$) that overlapped with the peaks of MDP. A slight redshift was found in the absorbance peak of amide B (CH_2 asymmetrical stretch), shifting from 2945 cm^{-1} to 2927 cm^{-1} , and amide III, shifting from 1240 cm^{-1} to 1235 cm^{-1} , which are indicative of a change in collagen structure [31, 32]. According to a previous study, the amide III band, which is assigned to C-N stretching and N-H in-plane deformation vibration [33, 34, 35], has been shown to be very sensitive to changes in the collagen triple helical structure, and the CH_2 stretching frequency was proposed by Bykov et al. [36] as another conformational marker. This result might hint that the triple helical structure could be affected by MDP.

Circular dichroism (CD) analysis of the collagen fibrils

To determine the conformational changes of collagen, circular dichroism (CD) should be used to study the effects of MDP on the triple helical structure. CD analysis (Fig. 4b) revealed that natural collagen has a type-II polyproline structure with optical activity and shows a maximum positive absorption peak at 220 nm and a minimum negative absorption peak at 197 nm, while the unordered structure of collagen is characterized by a weak negative band at 197 nm and the absence of a positive band at 222 nm [37]. Figure 4b clearly shows a change to the triple helical structure of the collagen molecules in the MDP-

collagen solutions with a reduction in intensity of both peaks, suggesting the occurrence of triple helix unfolding. In this study, the Rpn (ratio of the absolute values of the positive (222 nm) and negative (197 nm) peak intensities) can be utilized to analyze the quality of the collagen solution, in which the Rpn value of native collagen with a triple helical structure is approximately 0.12 [38]. It should be noted that the Rpn ratio of MDP-collagen (0.0911 ± 0.002) was lower than that of bare collagen (0.1200 ± 0.0013) and ethanol-collagen (0.1198 ± 0.0013), indicating that MDP significantly unfolds the triple helical structure of collagen, while ethanol does not.

MDP is an amphiphilic molecule interspersing a decyl moiety between a hydrophilic phosphate and a hydrophobic methacrylate. It has a great ability to form hydrogen bonds for interactions with the amino acids of collagen. Previous computational modeling has shown that the functionalization of collagen with MDP could be achieved via hydrogen bonding between the amino and carbonyl groups of MDP and the carbonyl groups of collagen and P-OH groups of MDP, respectively [39]. Therefore, it could be assumed that MDP favors intra- and inter-molecular hydrogen bonding between C = O and N-H groups in the peptide backbone, thereby disrupting the interstrand hydrogen bonding between the C = O and N-H groups in the triple helical structure of collagen, inducing a partial nonnative helical structure in solution. Aside from hydrogen bonds, intermolecular forces such as hydrophobic interactions and electrostatic interactions also make significant contributions to the conformation of collagen [40, 41, 42]. Therefore, the hydrophobic methacrylate group of MDP may interfere with the hydrophobic interactions between the collagen fibrils and thereby destabilize the native collagen structure [19]. A previous study showed that long-range electrostatic interactions could affect the conformational stability of proteins [43, 44], in which the majority of the long-range ion pairs destabilize the proteins. Hence, it is assumed that the conformational changes in collagen could be caused by long-range electrostatic interactions between the negatively charged phosphate groups of MDP and the positively charged collagen residues [19]. Furthermore, the affinity of certain functional groups binding to calcium ions follows the order of phosphate > carboxyl > amide > hydroxyl [45]. The P-OH group, P = O and P-O-C of MDP could act as a spatial cage for the nucleation and growth of HAP [46].

Collagen solid surface zeta potential and contact angle measurements

According to the classic biomineralization principle—‘interfacial molecular recognition’ mechanism—when MDP is immobilized in collagen, its phosphate groups can form strong ionic bonds with Ca^{2+} ions via electrostatic interactions, and then these calcium ions can accumulate at specific sites on the surface, further attracting sufficient numbers of phosphate groups. The repeated Ca-P attraction cycles finally lead to nucleation of calcium phosphate minerals [47]. On the surface of phosphorylated type I collagen, phosphate groups trapping calcium ions as preferential nucleation sites could lower the surface energy of the intended crystal phase to overcome the activation energy and form stable nuclei [48].

Electrostatic interactions have been proposed to be the driving force for attracting mineral ions/amorphous CaP precursors to the collagen fibril surface. Figure 4c shows that after collagen fibrils

were pretreated with MDP, MDP-collagen had a more negative zeta potential (-35.62 ± 0.68 mV, $P < 0.05$) than bare collagen (-11.29 ± 0.82 mV), indicating that MDP could affect the collagen surface potential. Thus, MDP-collagen may have the potential to attract more calcium ions or amorphous CaP precursors from the mineralization solution to promote nucleation. The contact angle of the bare collagen was significantly greater ($101.43 \pm 1.51^\circ$) than that of MDP-collagen ($60.55 \pm 1.85^\circ$, $P < 0.0001$) (Fig. 4d). The decrease in the contact angle indicates that the collagen surface is more hydrophilic due to MDP pretreatment, which may decrease the surface energy to promote collagen nucleation [30].

Adsorption of calcium ions by collagen pretreated with MDP

DMP1 and DPP are phosphoproteins containing highly phosphorylated serine and threonine residues, and these phosphate groups can capture calcium ions to form nuclei [20]. The phosphate and carboxyl groups on the surface of organic templates could attract Ca^{2+} ions via electrostatic forces as nucleation sites for crystallization [10–12]. The grafting of MDP molecules into collagen as nucleation sites to trigger initial nucleation mimics the natural mechanism, and crystallization is therefore possible [48]. The ICP-OES results showed that compared with bare collagen, MDP-collagen possessed a higher binding affinity for Ca^{2+} ions when the collagen was immersed in AS solution (Fig. 4e). These results showed that MDP pretreatment resulted in the MDP-collagen fibrils showing bioactive behaviors, while bare collagen did not.

Three-dimensional stochastic optical reconstruction microscopy (3D-STORM) image acquisition of the collagen fibrils

MDP, a common acidic functional monomer used in dentin adhesives, has a dihydrogen phosphate group and a decyl chain. Its molecular weight is approximately 320 Da, and the mass analysis in our study showed a single peak at 321.15 m/z (Fig. S9a, SI), indicating that MDP is monodisperse in solution. Size exclusion has been proposed as a potential transportation mechanism for the migration of amorphous CaP precursors into the internal water compartments of collagen fibrils [49]. As the MDP used in this study is a small molecule, it could potentially diffuse into collagen fibrils [50]. The STORM technique has been utilized to confirm the spatial distribution of MDP relative to type I collagen fibrils. MDP labelled with BODIPY (MDP-BODIPY; MW: 557.27 Da, Fig. S9b, SI) as a fluorescent tag was synthesized to detect whether MDP could penetrate into the collagen fibrils. We provided direct experimental evidence to validate that MDP was present within the collagen fibrils (Fig. 4). The 3D-STORM images revealed that MDP-BODIPY could be detected inside the collagen fibrils (Fig. 4f-h and Movie S1, SI), while only a small amount of fluorescein (BODIPY, 248 Da) could be found when only BODIPY without MDP was applied (Fig. 4i-k and Movie S2, SI). These observations might be attributable to the high steric hindrance of the aromatic BODIPY monomer with a weak affinity for collagen, since the low steric hindrance of the aliphatic MDP monomer with a strong affinity for collagen brings BODIPY monomer into the intrafibrillar space [19]. These findings indicated that the infiltration of molecules into the water compartments within

the collagen fibril could be affected by molecular weight, a flexible molecular structure, molecular affinity and electronegativity [50, 51, 52]. Figure 4l **and m** show that collagen pretreated with MDP could absorb calcium ions while the bare collagen could not (Fig. 4n **and o**), which are consistency with the elemental analysis of the bare and MDP-collagen fibrils (Fig. S10, SI).

Amorphous CaP precursors are easily formed in calcium phosphate solutions regardless of the use of polymers as additives [2, 53]. Generally, amorphous CaP precursors without polymeric additives (process-directing agents) cannot induce intrafibrillar mineralization [2, 53]. This study directly demonstrated that MDP monomer without polymeric additives could induce the biomimetic mineralization of collagen. Hence, we concluded that the MDP monomer could diffuse into the intrafibrillar water compartments and be immobilized in the collagen fibrils, thereby attracting calcium ions or amorphous CaP precursors into the MDP-collagen fibrils to induce collagen intrafibrillar mineralization.

Atomic force microscopy of the collagen fibrils

Atomic force microscopy (AFM) imaging of the same collagen fibril in different solutions was used to detect the value of MDP on collagen and the initial intrafibrillar mineralization process. Representative AFM images and collagen fibril diameter changes under different conditions are shown in Fig. 5. As seen from Fig. 5, the characteristic morphology of bare collagen fibrils is a D-periodicity pattern of ~ 67 nm in distilled water as well as in 50% ethanol-aqueous (v/v) solution, and their diameters are approximately 184.07 ± 5.86 and 187.50 ± 0.43 nm, respectively (Fig. 5a **and b, i**). After pretreatment with MDP, the D-periodicity pattern disappeared, and the diameter of the fibril increased (Fig. 5c **and d, i**). As the immersion time increased, the diameters of the collagen fibrils gradually increased by approximately two fold (327.67 ± 15.82 nm at 5 min and 389.67 ± 8.74 nm at 10 min, $P > 0.05$). After the MDP-collagen fibril was washed with distilled water for 10 min, the collagen fibril shrunk slightly (333 ± 6.08 nm, $P > 0.05$), suggesting that some of the unconjugated MDP was washed off, but most of the MDP remained in the collagen (Fig. 5e, i). Following incubation in AS for 1 h, the diameter of the collagen fibrils increased (409.44 ± 20.50 nm, $P > 0.05$) (Fig. 5f, i), indicating that mineral ions or amorphous CaP precursors were attracted to the MDP-collagen fibrils. After further incubation in AS for 2 h, a beaded substance, such as amorphous CaP precursors, was attached on the surface of collagen fibril followed by gradual fusion and then penetrate into the collagen, thereby increasing the diameter of collagen fibril (Fig. 5g **and h, i**) (455.99 ± 21.86 nm to 512.56 ± 12.42 nm, $P > 0.05$). The AFM image results are completely consistent with the TEM results. It has been previously reported that the diameters of collagen fibrils by TEM are smaller than those measured by AFM, which might result from the difference in hydrophobicity and charge between the surfaces of a TEM grid and a mica disc and the moisture content between the samples [54].

The D-periodicity structure with a length of 60–70 nm results from alternating overlap and gap zones, which is produced by the rearrangement of collagen molecules due to intermolecular forces [55, 56]. The inconsistent presence of the D-periodicity pattern suggests a compromised supramolecular collagen structure [57]. In this study, the disappearance of the periodic pattern indicates that the inter- and intra-strand hydrogen bonding and water bridges of the collagen molecules could be disrupted by MDP due to

its small size, strong hydrogen bonding capacity, and hydrophobic end. This finally induces conformational changes in the triple helical structure of collagen. Generally, polar solvents with low hydrogen bonding ability cannot expand collagen fibrils, while solvents with higher hydrogen bonding capacities can [54]. It is believed that water bridges also contribute to maintaining the conformation of the native collagen molecule by bridging amide and carbonyl groups [58]. This might be explained by the fact that MDP can replace the intrafibrillar water molecules, which may disrupt the water-mediated bridges and further break the hydrogen bonding forces between the collagen polypeptides. This produces a pronounced expansion of the collagen fibril with no D-periodicity. In addition, expansion of the collagen structure could also result from the infiltration of mineral ions/amorphous CaP precursors into the intrafibrillar space [59]. The sizes of the collagen fibrils continuously increase, which finally initiates collagen mineralization (Scheme 1).

Generally, chemical phosphorylation of collagen without polymer additives can induce extrafibrillar mineralization but not intrafibrillar mineralization [60, 61]. This can be explained by the fact that collagen phosphorylated by STMP and TPP can induce mineralization on collagen surfaces by increasing the number of mineralization nucleation sites and facilitate Ca^{2+} aggregation at these sites to form stable nuclei [48, 61, 62]. Large amorphous Ca/P precursors cannot infiltrate into the intrafibrillar space without polymer additives as sequestration analogs, and they instead undergo extrafibrillar mineralization [60, 61]. Furthermore, numerous studies have revealed that the chemical phosphorylation of collagen in combination with polymer additives could induce intrafibrillar mineralization [62, 63, 64].

Taken together, this study, for the first time, demonstrated that MDP-bound collagen fibrils functioning as HCPP could induce the biomimetic mineralization of reconstituted type I collagen fibrils and demineralized dentin without polymer additives. In detail, MDP in the free state can demineralize dentin by releasing protons. Additionally, MDP in the collagen-bound state can induce biomimetic mineralization because MDP immobilized in collagen can break the hydrogen bonds between the collagen polypeptides, replace the intrafibrillar water molecules, and expand the intrafibrillar space, and the MDP-bound collagen, such as HCPP, can easily alternatively trap Ca^{2+} ions and phosphate groups or amorphous Ca/P precursors by electrostatic attraction due to the expanded intrafibrillar space. The dual functions of amphiphilic MDP with de- and re-mineralizing ability could provide insight into biomimetic mineralization for preventive dentistry and adhesive dentistry. Whether other amphiphilic phosphate ester monomer possess the same functions needs to be further studied in the future.

Conclusion

In summary, the MDP monomer in the free state demineralizes the dentin surface due to release of protons in aqueous solutions. However, MDP in the collagen-bound state, by means of the chemical bonds between MDP and collagen, lowers the surface electrostatic potential and increases the affinity of the collagen fibrils. Finally, the electronegative MDP-bound collagen fibrils functioning as HCPP induce itself biomimetic mineralization by alternatively trapping Ca^{2+} ions followed by binding phosphate groups via electrostatic attraction without any polymer additives. Hence, we

propose that the amphiphilic MDP monomer possesses dual functions with de- and re-mineralizing ability.

Declarations

Supplementary Information

The online version contains supplementary material available at...

The following files are available free of charge.

Information on the synthetic procedure and characterization of compound MDP-BODIPY (Fig. S1-S5); mineralization of collagen (Fig. S6-S8), characterization of MDP solution (Fig. S9), STEM images with elemental mapping analysis of collagen (Fig. S10).

Movie S1 and S2 showing the 3D representation of MDP-collagen fibril and bare collagen fibril, with the viewing angle rotated to show different perspectives.

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Author's contribution

Haiyan Zheng: Methodology, Validation, Formal analysis, Roles, Writing - original draft. Writing - original draft. Ying Shi, Ling Bi: Methodology, Validation, Formal analysis, Roles, Writing - review & editing. Zhixin Zhang, Zihuai Zhou: Writing - review & editing. Changyu Shao: Resources. Dezhi Cui, Xiamin Cheng: Methodology, Validation, Formal analysis. Ruikang Tang, Haihua Pan: Methodology, Validation, Data curation, Formal analysis. Zhifang Wu: Conceptualization, Writing - review & editing, Project administration, Supervision, Funding acquisition. Baiping Fu: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition. ‡These authors contributed equally: Haiyan Zheng, Ying Shi and Ling Bi.

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Availability of data and materials

All relevant data supporting the findings of this study are either included within the article and its Supplementary Information files or available upon request from the corresponding author.

Declarations

Ethics approval and consent to participate

The study (Reporting in Dentin Remineralization Experiments) was approved by the Ethics Committee of Zhejiang University School of Stomatology (#2018017).

Consent for publication

All authors agree to be published.

Competing interests

The authors declare that they have no competing interests.

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References

1. Goldberg MA, Kulkarni B, Young M, Boskey A. Dentin. Structure, Composition and Mineralization. *Front Biosci Elite Ed.* 2011;3(2):711–35.
2. Nudelman F, Bomans PHH, George A, de With G, Sommerdijk NAJM. The role of the amorphous phase on the biomimetic mineralization of collagen. *Faraday Discuss.* 2012;159:357–70.
3. Wang QQ, Miao LY, Zhang HS, Wang Q, Li QL, Sun WB. A novel amphiphilic oligopeptide induced the intrafibrillar mineralisation via interacting with collagen and minerals. *J Mater Chem B.* 2020;8(11):2350–62.
4. Cointry GR, Capozza RF, Chiappe MA, Feldman S, Meta MD, Daniele SM, Fracalossi NM, Reina P, Ferretti JL. Novel experimental effects on bone material properties and the pre- and postyield behavior of bones may be independent of bone mineralization. *J Bone Miner Metab.* 2005;23:30–5.
5. Kinney JH, Habelitz S, Marshall SJ, Marshall GW. The importance of intrafibrillar mineralization of collagen on the mechanical properties of dentin. *J Dent Res.* 2003;8(12):957–61.
6. Deshpande AS, Beniash E. Bio-inspired Synthesis of Mineralized Collagen Fibrils. *Cryst Growth Des.* 2008;8(8):3084–90.
7. Palmer LC, Newcomb CJ, Kaltz SR, Spoerke ED, Stupp SI. Biomimetic Systems for Hydroxyapatite Mineralization Inspired by Bone and Enamel. *Chem Rev.* 2008;108(11):4754–83.
8. Hoang QQ, Sicheri F, Howard AJ, Yang DS. Bone recognition mechanism of porcine osteocalcin from crystal structure. *Nature.* 2003;425(6961):977–80.
9. Jiao K, Niu LN, Ma CF, Huang XQ, Pei DD, Luo T, Huang Q, Chen JH, Tay F. Complementarity and Uncertainty in Intrafibrillar Mineralization of Collagen. *Adv Funct Mater.* 2016;26(38):6858–75.
10. Kikuchi M, Itoh S, Ichinose S, Shinomiya K, Tanaka J. Self-organization mechanism in a bone-like hydroxyapatite/collagen nanocomposite synthesized in vitro and its biological reaction in vivo. *Biomaterials.* 2001;22(13):1705–11.
11. Hartgerink JD, Beniash E, Stupp SI. Self-assembly and mineralization of peptide-amphiphile nanofibers. *Science.* 2001;294(5547):1684–88.
12. Zhang RY, Ma PX. Porous poly (L-lactic acid) /apatite composites created by biomimetic process. *J Biomed Mater Res.* 1999;45(4):285–93.

13. Chen Z, Cao SS, Wang HR, Li YQ, Kishen A, Deng XL, Yang XP, Wang YH, Cong CH, Wang HJ. Biomimetic Remineralization of Demineralized Dentine Using Scaffold of CMC/ACP Nanocomplexes in an in Vitro Tooth Model of Deep Caries. *Plos one*. 2015;10(1):e0116553.
14. Niu LN, Zhang W, Pashley DH, Breschi L, Mao J, Chen JH, Tay F. Biomimetic remineralization of dentin. *Dent Mater*. 2014;30(1):77–96.
15. Dhivya S, Saravanan S, Sastry TP, Selvamurugan N. Nanohydroxyapatite-reinforced chitosan composite hydrogel for bone tissue repair in vitro and in vivo. *J Nanobiotechnology*. 2015;13:40.
16. Van Meerbeek B, Peumans M, Poitevin A, Mine A, Van Ende A, Neves A, De Munck J. Relationship between bond-strength tests and clinical outcomes. *Dent Mater*. 2010;26(2):e100-12.
17. Giannini M, Makishi P, Ayres APA, Vermelho PM, Fronza BM, Nikaido T, Tagami J. Self-etch adhesive systems: A literature review. *Braz Dent J*. 2015;26(1):3–10.
18. Fukegawa D, Hayakawa S, Yoshida Y, Suzuki K, Osaka A, Van Meerbeek B. Chemical interaction of phosphoric acid ester with hydroxyapatite. *J Dent Res*. 2006;85(10):941–44.
19. Nurrohman H, Nakashima S, Takagaki T, Sadr A, Nikaido T, Asakawa Y, Uo M, Marshall SJ, Tagami J. Immobilization of phosphate monomers on collagen induces biomimetic mineralization. *Bio-med Mater Eng*. 2015;25(1):89–99.
20. George A, Veis A. Phosphorylated proteins and control over apatite nucleation. crystal growth, and inhibition. *Chem Rev*. 2008;108(11):4670–93.
21. Wang Z, Ouyang Y, Wu ZF, Zhang LQ, Shao CY, Fan JY, Zhang L, Shi Y, Zhou ZH, Pan HH, Tang RK, Fu BP. A novel fluorescent adhesive-assisted biomimetic mineralization. *Nanoscale*. 2018;10(40):18980–87.
22. Wu ZF, Wang XK, Wang Z, Shao CY, Jin XT, Zhang LQ, Pan HH, Tang RK, Fu BP. Self-Etch Adhesive as a Carrier for ACP Nanoprecursors to Deliver Biomimetic Remineralization. *ACS Appl Mater Interface*. 2017;9(21):17710–17.
23. Shi Y, Shen DN, Zheng HY, Wu ZF, Shao CY, Zhang LQ, Pan HH, Tang RK, Fu BP. Therapeutic Management of Demineralized Dentin Surfaces Using a Mineralizing Adhesive To Seal and Mineralize Dentin, Dentinal Tubules, and Odontoblast Processes. *ACS Biomater Sci Eng*. 2019;5(10):5481–88.
24. Zhou ZH, Zhang LQ, Li JC, Shi Y, Wu ZF, Zheng HY, Wang Z, Zhao WJ, Wang Q, Jin XG, Pan HH, Tang RK, Fu BP. Polyelectrolyte-calcium complexes as a pre-precursor induce biomimetic mineralization of collagen. *Nanoscale*. 2020;13(2):953–63.
25. Verne E, Vitale-Brovarone C, Bui E, Bianchi CL, Boccaccini AR. Surface functionalization of bioactive glasses. *J Biomed Mater Res Part A*. 2009;90(4):981–92.
26. Qian L, Gao Q, Song Y, Li Z, Yang X. Layer-by-layer assembled multilayer films of redox polymers for electrocatalytic oxidation of ascorbic acid. *Sens Actuators B*. 2005;107(1):303–10.
27. Bates M, Jones SA, Zhuang XW. Stochastic optical reconstruction microscopy (STORM): a method for superresolution fluorescence imaging. *Cold Spring Harb Protoc*. 2013; 2013 (6): 498–520.

28. Chesnick IE, Mason JT, Giuseppett AA, Eidelman N, Potter K. Magnetic resonance microscopy of collagen mineralization. *Biophys J.* 2008;95(4):2017–26.
29. Hiraishi N, Tochio N, Kigawa T, Otsuki M, Tagami J. Monomer-Collagen Interactions Studied by Saturation Transfer Difference NMR. *J Dent Res.* 2013;92(3):284–88.
30. Tian FC, Wang XY, Huang Q, Niu LN, Mitchell J, Zhang ZY, Pranatik C, Zhang L, Chen JH, Breshi L. Effect of nanolayering of calcium salts of phosphoric acid ester monomers on the durability of resin-dentin bonds. *Acta Biomater.* 2016;38:190–200.
31. Riaz T, Zeeshan R, Zarif F, Ilyas K, Muhammad N, Safi SZ, Rahim A, Rizvi SAA, Rehman IU. Ftir analysis of natural and synthetic collagen. *Appl Spectrosc Rev.* 2018;53(9):703–46.
32. Muyonga JH, Cole CGB, Doudu KG. Fourier transform infrared (ftir) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (*Lates niloticus*). *Food Chem.* 2004;86(3):325–32.
33. Gordon PL, Huang C, Lord RC, Yannas IV. The far-infrared spectrum of collagen. *Macromolecules.* 1974;7(6):954–56.
34. Chen XF, Zhou LL, Xu HZ, Yamamoto M, Shinoda M, Tada I, Minami S, Urayama K, Yamane H. The structure and properties of natural sheep casing and artificial films prepared from natural collagen with various crosslinking treatments. *Int J Biol Macromol.* 2019;135:959–68.
35. Stani C, Vaccari L, Mitri E, Birarda G. Ftir investigation of the secondary structure of type I collagen: new insight into the amide III band. *Spectrochim Acta A.* 2020;229:118006.
36. Bykov SV, Myshakina NS, Asher NS. Dependence of Glycine CH₂ stretching frequencies on conformation, ionization state, and hydrogen bonding. *J Phys Chem B.* 2008;112(18):5803–12.
37. Mu C, Li D, Wei L, Ding Y, Zhang G. Temperature induced denaturation of collagen in acidic solution. *Biopolymers.* 2007;86(4):282–87.
38. Feng YB, Melacini G, Taulane JP, Goodman M. Acetyl-terminated and template-assembled collagen-based polypeptides composed of Gly-Pro-Hyp sequences. 2. Synthesis and conformational analysis by circular dichroism, ultraviolet absorbance, and optical rotation. *J Am Chem Soc.* 1996;118(43):10351–58.
39. Vaidyanathan J, Vaidyanathan TK, Kerrigan JE. Evaluation of intermolecular interactions of self-etch dentin adhesive primer molecules with type I collagen: Computer modeling and in vitro binding analysis. *Acta Biomater.* 2007;3(5):705–14.
40. Zhu C, Yan S, Wang Y, Luo Y, Fan D. The preparation and characterization of novel human-like collagen metal chelates. *Mater Sci Eng C Mater Biol Appl.* 2013;33(5):2611–19.
41. Usha R, Maheshwari R, Dhathathreyan A, Ramasami T. Structural influence of mono and polyhydric alcohols on the stabilization of collagen. *Colloids Surf B.* 2006;48(2):101–5.
42. Buerck J, Heissler S, Geckle U, Ardakani MF, Schneider R, Ulrich AS, Kazanci M. Resemblance of electrospun collagen nanofibers to their native structure. *Langmuir.* 2013;29(5):1562–72.

43. Freudenberg U, Behrens SH, Welzel PB, Müller M, Grimmer M, Salchert K, Taeger T, Schmidt K, Pompe W, Werner C. Electrostatic interactions modulate the conformation of collagen I. *Biophys J*. 2007;92(6):2108–19.
44. Kumar S, Nussinov R. Close-range electrostatic interactions in proteins. *Chem Bio Chem*. 2002;3(7):604–17.
45. Wang XH, Ma JB, Wang YN, He BL. Bone repair in radii and tibias of rabbits with phosphorylated chitosan reinforced calcium phosphate cements. *Biomaterials*. 2002;23(21):4167–76.
46. Hayakawa T, Yoshinari M, Sakae T, Nemoto K. Calcium phosphate formation on the phosphorylated dental bonding agent in electrolyte solution. *J Oral Rehabil*. 2004;31(1):67–73.
47. Wang S, Yang YD, Wang RH, Kong XD, Wang XM. Mineralization of calcium phosphate controlled by biomimetic self-assembled peptide monolayers via surface electrostatic potentials. *Bioact Mater*. 2020;5(2):387–97.
48. Zhang X, Neoh KG, Kishen AA. Biomimetic strategy to form calcium phosphate crystals on type I collagen substrate. *Mat Sci Eng C Mater Biol Appl*. 2010;30(6):822–26.
49. Price PA, Toroian D, Lim JE. Mineralization by Inhibitor Exclusion the Calcification of Collagen with Fetuin. *J Biol Chem*. 2009;284(25):17092–101.
50. Toroian D, Lim JE, Price PAD. The size exclusion characteristics of type I collagen-Implications for the role of noncollagenous bone constituents in mineralization. *J Biol Chem*. 2007;282(31):22437–447.
51. Chen L, Jacquet R, Lowder E, Landis WJ. Refinement of collagen-mineral interaction: A possible role for osteocalcin in apatite crystal nucleation, growth and development. *bone* 2015; 71: 7–16.
52. Beniash E. Biomaterials-hierarchical nanocomposites: the example of bone. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*. 2011;3(1):47–69.
53. Nudelman F, Pieterse K, George A, Bomans PH, Friedrich H, Brylka LJ, Hilbers PA, de With G, Sommerdijk NAJM. The role of collagen in bone apatite formation in the presence of hydroxyapatite nucleation inhibitors. *Nat Mater*. 2010;9(12):1004–9.
54. Niu LN, Jee SE, Jiao K, Tonggu L, Li M, Wang L, Yang YD, Bian JH, Breschi L, Jang SS. Collagen intrafibrillar mineralization as a result of the balance between osmotic equilibrium and electroneutrality. *Nat Mater*. 2017;16(3):370–78.
55. Gautieri A, Vesentini S, Redaelli A, Buehler MJ. Hierarchical structure and nanomechanics of collagen microfibrils from the atomistic scale up. *Nano Lett*. 2011;11(2):757–66.
56. Gutsman T, Fantner GE, Kindt JH, Venturoni M, Danielsen S, Hansma PK. Force spectroscopy of collagen fibers to investigate their mechanical properties and structural organization. *Biophys J*. 2004;86(5):3186–93.
57. Zeugolis DI, Khew ST, Yew E, Ekaputra AK, Tong YW, Yung LYL, Hutmacher DW, Sheppard C, Raghunath M. Electro-spinning of pure collagen nano-fibres - just an expensive way to make gelatin? *Biomaterials*. 2008;29(15):2293–305.

58. Bella J, Brodsky B, Berman HM. Hydration structure of a collagen peptide. *Structure*. 1995;3(9):893–906.
59. Shao CY, Zhao RB, Jiang SQ, Yao SS, Wu ZF, Jin B, Yang YL, Pan HH, Tang RK. Citrate improves collagen mineralization via interface wetting: a physicochemical understanding of biomineralization control. *AdvMater*. 2018;30(8):1704876.
60. Gu L, Kim YK, Liu Y, Ryou H, Wimmer CE, Dai L, Arola DD, Looney SW, Pashley DH, Tay F. Biomimetic Analogs for Collagen Biomineralization. *J Dent Res*. 2011;90(1):82–7.
61. Du TM, Niu XF, Hou S, Li ZW, Li P, Fan YB. Apatite minerals derived from collagen phosphorylation modification induce the hierarchical intrafibrillar mineralization of collagen fibers. *J Biomed Mater Res A*. 2019;107(11):2403–13.
62. Liu Y, Li N, Qi YP, Dai L, Bryan TE, Mao J, Pashley DH, Tay F. Intrafibrillar Collagen Mineralization Produced by Biomimetic Hierarchical Nanoapatite Assembly. *Adv Mater*. 2011;23(8):975–80.
63. Gu LS, Kim J, Kim YK, Yan L, Dickens SH, Pashley DH, Ling JQ, Tay FA. Chemical phosphorylation-inspired design for type I collagen biomimetic remineralization. *Dent Mater*. 2010;26(11):1077–89.
64. Kim YK, Gu LS, Bryan TE, Kim JR, Chen L, Liu Y, Toon JC, Breschi L, Pashley DH, Tay F. Mineralisation of reconstituted collagen using polyvinylphosphonic acid/polyacrylic acid templating matrix protein analogues in the presence of calcium, phosphate and hydroxyl ions. *Biomaterials*. 2010;31(25):6618–27.

Figures

a **b**

Figure 1

Synthetic procedure to generate MDP-BODIPY. (a) The synthetic route of MDP-BODIPY. (b) The molecular structure of BODIPY.

Figure 2

TEM images of bare collagen (a-j) and MDP-collagen (A-J). Panels b, d, f, h, j, B, D, F, H and J are higher magnifications of the red circled areas in Panels a, c, e, g, i, A, C, E, G and I, respectively. (a-b) Uranyl acetate-stained TEM images showing bare collagen fibrils displayed D-periodicity patterns of ~67 nm. (c-j) Unstained TEM images showing that no mineralization was observed in bare collagen after incubation in AS for 6 h (c-d), 12 h (e-f), 24 h (g-h) and 48 h (i-j), as confirmed by the SAED pattern (insets in h and j).

(A-B) Uranyl acetate-stained TEM images showing MDP-collagen fibrils did not display D-periodicity patterns. (C-D) Unstained TEM images of MDP-collagen show swelling of part of the collagen fibril without mineralization after 6 h of incubation in AS. (E-F) With further incubation, MDP-collagen continued to swell. However, no mineralization in MDP-collagen was observed. The SAED pattern (insets in D, F) indicates that the mineral deposited in the collagen fibrils are amorphous phase. (G-H) Moderate mineralization was observed after 24 h. (I-J) MDP-collagen fibrils were heavily mineralized after 48 h. The SAED patterns (inset in H, J) reveal the following characteristic diffraction rings: 002, 004 and 211. (k) Changes in the diameters of the bare collagen fibrils and MDP-collagen fibrils after immersion in AS for different lengths of time using conventional TEM. Groups labeled with the same lowercase letters are not significantly different ($P > 0.05$).

Figure 3

TEM images of demineralized dentin pretreated with MDP after incubation in AS for 4 days (a-c), 7 days (d-f), 14 days (g-i) and 28 days (j-l). Panels b and c, e and f, h and i, k and l are higher magnifications of Panels a, d, g and j, respectively. The dotted lines represent the boundary between the remineralized dentin (RD) and intact natural dentin (ID). The thickness of the remineralized layer increased over time, and the demineralized dentin was completely remineralized after incubation for 28 d. The SAED patterns

(insets in Panels f, i and l) of the minerals within the collagen matched those of HAP from the poorly crystalline phase (Panel f) to the well-ordered crystalline phase (Panels i and l). RD: remineralized dentin. DD: demineralized dentin. ID: intact dentin. T: dentinal tubule. E: epoxy resin.

Figure 4

Characterization of bare collagen and MDP-collagen. (a) FTIR spectra of bare collagen, MDP-collagen and MDP. The new peaks (*) at $\sim 1019\text{ cm}^{-1}$, $\sim 1169\text{ cm}^{-1}$, $\sim 1300\text{ cm}^{-1}$, $\sim 1720\text{ cm}^{-1}$ and $\sim 2860\text{ cm}^{-1}$ were assigned to P-O-C, C-O-C, O=P, C=O, and P-OH stretching bands, respectively. (b) The CD spectra of bare collagen and ethanol-collagen show a positive band at 222 nm followed by a strong negative band at 197 nm, while the intensities of both peaks were significantly reduced in the CD spectrum of MDP-collagen. (c) Solid surface zeta potential of bare collagen ($-11.29 \pm 0.82\text{ mV}$) and MDP-collagen ($-35.62 \pm 0.68\text{ mV}$). (d) The contact angles of bare collagen ($101.43 \pm 1.51^\circ$) and MDP-collagen ($60.55 \pm 1.85^\circ$). (e) The residual concentration of Ca^{2+} in the AS solution after an immersion period of 168 h. (f, g) The xy projections (f) and 3D STORM image (g) of MDP-collagen. The collagen fibrils were labeled with secondary antibodies, which emitted red fluorescence. MDP labeled with BODIPY emitted green fluorescence. (h) The z-slice of the STORM image of MDP-collagen. The yellow of the MDP-collagen fibril indicate the MDP was inside collagen fibrils due to the merged image of the red and the green. (i-k) The xy projections (i) and 3D STORM image (j) and of z-slice images (k) of bare collagen. A small amount of fluorescein was observed inside the bare collagen fibril. Z=0, 45, 90, 135, 175 and 210 nm (Image from left to right). (l-o) STORM images of MDP-collagen (l-m) and bare collagen (n-o) immersed in CaCl_2

solution and then labeled with calcein. (l-m) The merged image indicates that calcium was inside the MDP-collagen fibrils. (n-o) The merged image indicates that calcium was not inside the bare collagen fibrils. Z= 0, 70, 140 and 210 nm (images from left to right).

Figure 5

Representative AFM amplitude and height images of the collagen fibrils immersed in different solutions. (a) Distilled water. (b) 50% ethanol-aqueous solution. As shown in (a) and (b), the fibril exhibits a

characteristic D-periodicity pattern. (c-d) A swollen collagen fibril with no D-periodicity pattern after treatment with MDP for 5 min (c) and 10 min (d). (e) Following washing with distilled water for 10 min, the collagen shrunk slightly. (f) Further immersion in AS resulted in reexpansion of the collagen fibril. (g) After immersion in AS for 2 h, large, amorphous CaP precursors (red circled area) were absorbed on the surface of the collagen. (h) The amorphous CaP precursors were gradually fused into the collagen fibril after immersion in AS for 3 h, causing the fibril to thicken. (i) Changes in diameter of the collagen fibril. Groups labelled with different lowercase letters depict significantly difference ($P < 0.05$).

Supplementary Files

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- [S2.mp4](#)
- [SupportingInformation.docx](#)
- [GraphicalAbstract1.tif](#)