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A biomimetic model for mineralization of type-I collagen fibrils

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Abstract

Bone and dentin mainly consist of type-I collagen fibrils mineralized by hydroxyapatite (HAP) nanocrystals. *In-vitro* biomimetic models based on self-assembled collagen fibrils have been widely used in studying the mineralization mechanism of type-I collagen. In this chapter, the protocol we used to build a biomimetic model for the mechanistic study of type-I collagen mineralization is described. Type-I collagen extracted from rat tail tendon or horse tendon is self-assembled into fibrils and mineralized by HAP *in vitro*. The mineralization process is monitored by cryoTEM in combination with two-dimension (2D) and three-dimension (3D) stochastic optical reconstruction microscopy (STORM), which enables *in-situ* and high resolution visualization of the process.

Key words: Bone, Type-I collagen, Calcium Phosphate, cryoTEM, STORM

1. Introduction

Type-I collagen is the main organic constitution of the hard tissues of vertebrates, e.g., dentin and bone.^{1, 2, 3} The type-I collagen fibrils are assembled by ~300 nm long triple helix molecules which are ~1.5 nm in diameter.⁴ Recent X-Ray study indicate that in type-I collagen fibrils, subunit called ‘microfibrils’ are formed by five 1d-staggered and twisted collagen molecules.⁵ These microfibrils

pack into superstructures with triclinic symmetry ($P1$, $a \approx 40.0 \text{ \AA}$, $b \approx 27.0 \text{ \AA}$, $c \approx 678 \text{ \AA}$, $\alpha \approx 89.2^\circ$, $\beta \approx 94.6^\circ$, $\gamma \approx 105.6^\circ$) and form larger collagen fibrils which display a $\sim 67 \text{ nm}$ periodical structure. Each $\sim 67 \text{ nm}$ unit contains a less dense gap region and an overlap region, which are respectively $\sim 37 \text{ nm}$ and $\sim 30 \text{ nm}$ long,⁵ while the values varies slightly depending on the sources of the collagen.⁶ The type-I collagen fibrils in bone are embedded by $\sim 4 \text{ nm}$ thick carbonated hydroxyapatite (HAP) nanoplatelets with their c -axis oriented along the fibril axis,^{7, 8} which significantly improves the mechanical performances of the fibrils.^{9, 10} The formation mechanism of the intrafibrillar HAP crystals has long been an attractive research topic. Due to the difficulty of *in-vivo* study of bone formation process, the majority of the studies on collagen mineralization was performed *in-vitro* based on biomimetic models, using demineralized collagen from bone or dentin,^{11, 12} collagen sponge,^{8, 13} but most commonly self-assembled collagen fibrils.^{14, 15, 16, 17} Type-I collagen molecules could self-assemble *in-vitro* at $\text{pH} = 7$ to 10 at temperatures from 20 to 37 degrees with presence of electrolytes (e.g., KCl),^{18, 19} forming fibrils with $\sim 67 \text{ nm}$ periodical structures similar to those found *in-vivo*. It has been shown that collagen could be intrafibrillarly mineralized *in-vitro* by HAP platelets oriented in c -axis,^{8, 15} with electron diffraction pattern indistinguishable to mineralized collagen fibrils found in bones. Charged macromolecules mimicking the acidic non-collagenous proteins (NCP)²⁰ in bone or dentin such as polyaspartic acid (pAsp),^{8, 15} polyacrylic acid (pAA),¹⁷ fetuin^{15, 21} and poly(allylamine) hydrochloride (pAH)¹⁶ were found to be essential for the *in-vitro* mineralization of the type-I collagen fibrils. This is probably related to their ability to stabilize amorphous calcium phosphate (ACP) precursor and inhibit the formation of HAP. Recently we have found that charged small biomolecules, such as citrate, could further promote the type-I collagen mineralization by reducing the interfacial energy between collagen and the ACP precursor.¹⁴ In summary, the *in-vitro* biomimetic models have been shown to be a powerful tool which provide profound insight into the collagen mineralization process.²²

However, these models were often based on different collagen sources and different methods to obtain and mineralize the collagen fibrils, which brings uncertainty for interpreting and comparing experimental results.

In this chapter we describe the protocol we used for assembling and mineralizing type-I collagen fibrils.^{14,15} In a typical procedure, a type-I collagen (from rat tail tendon or horse tendon) stock solution was mixed with an assembling buffer solution and then dripped onto transmission electron microscopy (TEM) grids to assemble the collagen fibrils on the grids. The grids were then exposed to the mineralization solution to induce the HAP mineralization. After mineralization, the products were visualized using conventional an cryogenic TEM (cryoTEM) in combination with 2D/3D STORM images in order to examine the intrafibrillar and extrafibrillar HAP mineralization of type-I collagen fibrils *in-situ* with nano-level accuracy. We hope our method will contribute for establishing a standard experimental model, which will benefit all the researchers in this field.

2. Materials

Type-I collagen stock solution (3 mg ml⁻¹) from rat tail tendon was purchased from Gibco-Invitrogen (USA). Type I collagen extract from horse tendon was kindly provided by Dr. Giuseppe Falini (Department of Chemistry, University of Bologna, Italy). (3-aminopropyl)triethoxysilane (APTES) was purchased from Aladdin Reagent (Shanghai, China), ethanol, polyaspartic acid (pAsp, Mw = 27 kDa or 2~11 kDa), glycine, sodium hydrate (NaOH), glutaraldehyde, ammonia, potassium chloride (KCl), sodium chloride (NaCl), calcium chloride (CaCl₂), disodium hydrogen phosphate (Na₂HPO₄), dipotassium hydrogen phosphate (K₂HPO₄), glucose oxidase (G2133), catalase (C40), cysteamine (300070), glucose and β-mercaptoethanol (63689) were supplied by Sigma-Aldrich. The blocking buffer (Product Code: P0023B) and the washing buffer (Product Code: P0023C6) were purchased from Beyotime (Product Code, P0023B, China). The type-I

collagen rabbit anti-mouse antibody (1:50) and mouse anti-rabbit Cy3B-conjugated secondary antibodies (1:100) were supplied by Proteintech (USA). All chemicals used in the experiments were of analytical grade and used directly without further purification. Deionized water and all the solutions were filtered through 0.22- μ m Millipore films prior to use.

3. Methods

3.1 Conventional TEM experiments

3.1.1 Self-assembly of type-I collagen fibrils

0.3754 g glycine and 1.481 g KCl were dissolved in 100 ml of deionized water and ultrasonicated for 20 min at room temperature to obtain an assembling buffer solution containing 50 mM glycine and 200 mM KCl. The pH value was adjusted to 9.2 with 1M NaOH solution. The assembling buffer solution was kept at 4 °C prior to use.

In order to assemble the collagen on TEM grids, 8.33 μ l of type-I collagen stock solution from rat tail tendon was dripped into 0.5 ml of assembling buffer solution and incubated at 37 °C for 20 minutes with stirring (see **Note 1**). 200-mesh gold TEM grids (see **Note 2**) coated by carbon films were glow discharged for 40 s using a Cressington 208 carbon coater and then transferred into a glass petri-dish. 3 μ l of incubated type-I collagen solution were gently dripped onto the carbon-film face of the TEM grids (see **Note 3**).

Sealed TEM grids were incubated at 37 °C for 12 h (see **Note 4**) and then gently rinsed with deionized water. Then the type-I collagen fibrils were further cross-linked with 0.05% glutaraldehyde for 2 h and gently rinsed with deionized water and air dried. To increase the contrast of the periodical structure within the type-I collagen fibrils, 2% (w/v) uranyl acetate staining

solution was dripped onto the TEM grids loaded with type-I collagen fibrils and then washed after 15 s with deionized water and air dried.

3.1.2 Collagen mineralization

To prepare the mineralization solution, 2 ml of 3.34 mM CaCl₂ was dripped into a 10 ml Petri-dish, then 0 to 240 ml of 10 mg ml⁻¹ pAsp (27 kDa) stock solutions were added into the calcium solution. 2 ml phosphate solution containing 19 mM Na₂HPO₄ and 300 mM NaCl was then mixed with the above solution to obtain the mineralization solution. 5% (w/v) NaN₃ (8 µl) was added to the mineralization solution to retard bacterial growth. The solutions should be mixed quickly and the as-prepared mineralization solution should be colorless and transparent. Slow mixing would lead to a light-blue colored, cloudy dispersion (**Figure 1**). TEM grids loaded with type-I collagen fibrils were floated upside-down on the mineralization solution at 37 °C for designated times. After mineralization, the TEM grids were taken out and washed in sequence by deionized water, 25% ethanol, 50% ethanol and 100% ethanol.

3.1.3 TEM imaging and mineralization degree calculation

The assembled (**Figure 2**), stained (**Figure 3**) and mineralized (**Figure 4 and 5**) type-I collagen fibrils were visualized by TEM operating at 100 kV (HT-7700, Hitachi, Japan) or 200 kV (JEM-2100F, JEOL, Japan). The mineralization degree increased with increasing the pAsp concentration from 0 to 240 µg ml⁻¹ (**Figure 4**), and increasing the mineralization time from 6 h to 24 h (**Figure 5**). The mineralization degree (*m.d.*) was calculated by first splitting the pixel intensity histogram of the TEM images into three Gaussian distributions (**Figure 6**), which correspond to the

background, collagen fibrils, and mineralized collagen fibrils, respectively. The thresholds (T) between the three areas are determined by $T = \bar{I} - 2\sigma$,²³ where \bar{I} is the mean pixel intensity of each Gaussian distribution, and σ is the standard. The areas corresponding to all of the collagen fibrils (S1) and the mineralized part of the collagen fibrils (S2) could then be segmented from the image, and *m.d.* could then be given by comparing S1 and S2

$$m.d. = \frac{S1}{S2}$$

For each analysis, at least five samples were examined to obtain the mean value and standard deviation of *m.d.*

3.2 CryoTEM experiments

3.2.1 Self-assembly of type-I collagen extracted from rat tail tendon

Type-I collagen stock solution from rat tail (3 mg/l) was mixed with the buffer solution containing 10 mM Hepes (pH=7.4, see **Note 5**), 200 mM NaCl and 30 mM KH₂PO₄ in a 1:29 ratio. 300 μ l of as-prepared incubation solution was applied on a parafilm. Gold Quantifoil grids were floated upside-down on the incubation solution droplet (**Figure 7**). The parafilm was then covered by a glass petri dish and incubated for 200 min. After incubation, the grids were transferred to the Vitrobot (FEI, Mark III) in 100% humidity (see **Note 6**), blotted for 5 s by filter papers, and then vitrified in liquid ethane to visualize the collagen fibrils; or transferred to the mineralization solution (see 3.2.3)

3.2.2 Self-assembly of type-I collagen extracted from horse tendon

1 g of type-I collagen extracted from horse tendon was mixed overnight with 10 ml of aqueous acetic acid (50 mM, pH 2.5), centrifuged at 5000 rpm for 10 minutes and the supernatant was collected and stored at 4 °C. CryoTEM grids were laid on a 15 µl drop of collagen solution for 10 seconds. The excess of collagen solution was manually blotted and the grids were transferred to a 15 µl drop of Hepes buffer (10 mM, pH 7.4) containing NaCl (150 mM), for 30 minutes. This procedure was performed inside a glove box, where temperature and humidity are controlled at 22 °C and 100 % relative humidity. The grids were then vitrified as described in 3.2.1, or transferred to the mineralization solution (see 3.2.3).

3.2.3 Collagen mineralization

The following two solutions were mixed 1:1 to prepare the mineralization solution in a glass petri dish: (1) 5.4 mM CaCl₂, 10 mM Hepes, pH = 7.4; (2) 2.7 mM K₂HPO₄, 10 mM Hepes, 20 mg/l pAsp (mw=2000~11000). The gold Quantifoil grids were rinsed in two 300 µl water droplets applied on parafilm for 1 min each immediately after collagen assembly to remove the residual salts, and then transferred into the petri dish containing the mineralization solution. The petri dish was then sealed by parafilm and heated at 37 °C for 72 h for mineralization. After mineralization, the grids were transferred to the Vitrobot (FEI, Mark III) in 100% humidity, blotted for 5 s by filter papers, and then vitrified in liquid ethane.

3.2.3 CryoTEM imaging

CryoTEM imaging of the assembled (**Figure 8** and see **Note 7**) and then mineralized (**Figure 9**) collagen was performed under ~3 µm defocus on a FEI Titan cryoTEM equipped with a field emission gun (FEG) and operating at 300 kV, or a FEI Tecnai 20 (Type Sphera) TEM equipped

with a LaB₆ filament operating at 200 kV and a Gatan cryo holder operating at -170 °C. Images were recorded in FEI Titan using a 2k x 2k Gatan CCD camera equipped with a post column Gatan Energy Filter (GIF), or in FEI Tecnai using a 1k x 1k Gatan CCD camera. The electron dose used is 10 e/Å² per image (see **Note 8**).

3.3 STORM experiments

3.3.1 Modification of the laser confocal culture dish (LCCD) and self-assembly of type-I collagen fibrils

Type-I collagen fibrils (rat tail) were immobilized on the amino-silanized LCCD. In the experiments, 200 µl APTES was dripped onto a LCCD substrate avoiding the light. The APTES solution was then removed and dried in an oven at 100 °C prior to use (see **Note 9**).

100 µl of 50 µg ml⁻¹ type-I collagen (rat tail) solution was dripped on the modified LCCD substrate and incubated at 37 °C for 12 h, then gently rinsed with deionized water. After that, the type-I collagen fibrils were further cross-linked with 0.05 wt% glutaraldehyde for 2 h and then gently rinsed with deionized water (see **Note 10**).

3.3.2 Staining and mineralization of the type-I collagen fibrils

The immunofluorescent staining was performed according to the following steps. First, the type-I collagen (rat tail) fibrils were incubated with blocking buffer for 1 h and then incubated with the type-I collagen rabbit anti-mouse antibody at 37 °C for another 2 h. Second, the samples were washed by the washing buffer for 3 times (10 min each) and incubated with mouse anti-rabbit Cy3B-conjugated secondary antibodies for 1 h at 37 °C. At last, the samples were washed by 3 extra times in the washing buffer (10 min each) and mineralized with the mineralization solution. The mineralization process was as follows: 2 ml of 3.34 mM CaCl₂ solution and 48 µl of 10 mg

ml⁻¹ pAsp solution were dropped into a LCCD which was loaded with immunofluorescence stained collagen. Then, 2 ml of solution containing 19 mM Na₂HPO₄ and 300 mM NaCl was mixed into the solution. The samples were mineralized in the as-prepared mineralization solution at 37 °C for 24 h, and rinsed with deionized water for three times. Afterwards, the mineralized type-I collagen fibrils were labeled by 2 ml of 10 μM calcein which specifically bind with HAP for 20 min at room temperature. Residual calcein was removed by washing using deionized water.

3.3.3 STORM imaging

The type-I collagen (rat tail) fibrils were immersed in the imaging buffer before STORM imaging. The imaging buffer contained 1.6 mg ml⁻¹ glucose oxidase, 50 mM Tris, 10 mM NaCl, 50 mM cysteamine, 10% (w/v) glucose, 32 μg ml⁻¹ catalase and 1% (v/v) β-mercaptoethanol. The pH of the imaging buffer was tuned to 8 by 1 M NaOH and HCl solution. 2D STORM imaging experiments were performed on a Nikon Ti-E inverted optical microscope. For z-stack images of the mineralized type-I collagen fibrils, the images were recorded, processed, and analyzed using the Imaris software (Bitplan AG, Zurich, Switzerland). 3D STORM imaging experiments were performed on a Nikon N-STORM microscope equipped with a 100 × oil immersion objective and an Andor camera.²⁴ To identify the zone of interest, a low magnification fluorescence image was acquired prior to STORM imaging. After switching to higher magnification (100× objective), conventional fluorescence images were first acquired. STORM data acquisition was then started using imaging cycles at one frame of activation laser illumination (405 nm laser) followed by five frames of imaging laser illumination (561 nm laser). The integration time of the camera was set to the 1 frame mode with an EM gain of 305,000-10,000 cycles per channel, which was used for the reconstruction of each 3D super-resolution image using Nikon NIS Elements 4.30 software. The STORM images confirm the intrafibrillar mineralization of type-I collagen fibrils. In the 2D images,

the type-I collagen fibrils were labeled before mineralization, which emitted red fluorescence. After mineralization, the HAP was labeled with calcein, which emitted fluorescence with the wavelength of green light. The data showed that the ACP infiltrated into the type-I collagen fibrils and transformed into HAP inside the type-I collagen fibrils (**Figure 10**). The z-slice of the 3D STORM images of the mineralized type-I collagen fibrils indicated the mineralization of HAP inside and outside the type-I collagen fibrils. Besides, the HAP was homogeneously dispersed in the type-I collagen fibrils (**Figure 11**). The movie and images were analyzed by Nikon NIS-Elements AR software.

4. Notes

1. Incubation time less than 20 min will lead to an inhomogeneous dispersion of the type-I collagen fibrils on the TEM grid (**Figure 12**).
2. The mineralization experiment could not work on copper grids because of copper leaching in the solution which inhibits the crystallization of HAP.²⁵
3. 3 μ l is the optimal volume which neither dries very quickly (less than 4h) nor leads to overflow of the TEM grids.
4. The mineralization solution was replaced each 6 h to keep the supersaturation constant.
5. This buffer solution induce a faster assembly of rat tail type-I collagen, which benefits the cryoTEM study.
6. The solution left between the tweezers has to be carefully removed by filter paper before the grids were transferred into Vitrobot, in order to prevent the tweezers from being frozen during the vitrification process.

7. No ~67 nm periodical structure could be visualized without staining for the assembled rat tail collagen fibrils, while the horse tendon collagen fibrils clearly show the periodical structure. This is due to the fact that these rat tail collagen fibrils (60~80 nm in diameter) are much thinner than the horse tendon collagen fibrils (~300 nm in diameter).

8. Collagen fibril is very sensitive to the electron beam and beam damage could be clearly observed after the fibril was exposed to $200 \text{ e}/\text{\AA}^2$ (**Figure 13**).

9. The APTES solution was obtained by dissolving 0.5 ml APTES into 9.5 ml anhydrous ethanol. The LCCD must be fresh and clean prior to use. After removal of the APTES solution, the modified LCCD was ultrasonicated with anhydrous ethanol and deionized water for 20 min, respectively, to remove the physical absorbed chemicals. $50 \mu\text{g ml}^{-1}$ of type-I collagen solution was obtained by mixing 5 μl of type-I collagen stock solution with 295 μl of assembling solution and then incubating the mixed solution at room temperature for 20 min. After 12 h, the LCCD was gently rinsed with deionized water for at least three times to remove un-assembled type-I collagen.

10. Incubation times of anti-bodies less than 2 h or calcein labelling time less than 20 min would lead to inhomogeneous stained type-I collagen samples (**Figure 14**). The labelling process should avoid the light.

5. References

1. Weiner S, Traub W, Wagner HD. Lamellar bone: Structure-function relations. *J Struct Biol* **126**, 241-255 (1999).
2. Beniash E, Traub W, Veis A, Weiner S. A transmission electron microscope study using vitrified ice sections of predentin: Structural changes in the dentin collagenous matrix prior to mineralization. *J Struct Biol* **132**, 212-225 (2000).

3. Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. *Frontiers in bioscience (Elite edition)* **3**, 711-735 (2011).
4. Hodge AJ. Recent studies with the electron microscope on ordered aggregates of the tropocollagen macromolecule. *Aspects of protein structure*, 289-300 (1963).
5. Orgel JPRO, Irving TC, Miller A, Wess TJ. Microfibrillar structure of type I collagen in situ. *Proc Natl Acad Sci U S A* **103**, 9001-9005 (2006).
6. Quan BD, Sone ED. Cryo-TEM analysis of collagen fibrillar structure. In: *Methods in enzymology*. Elsevier (2013).
7. Weiner S, Traub W. Organization of hydroxyapatite crystals within collagen fibrils. *FEBS Lett* **206**, 262-266 (1986).
8. Olszta MJ, *et al.* Bone structure and formation: A new perspective. *Mater Sci & Eng R* **58**, 77-116 (2007).
9. Gao HJ, Ji BH, Jager IL, Arzt E, Fratzl P. Materials become insensitive to flaws at nanoscale: Lessons from nature. *Proc Natl Acad Sci U S A* **100**, 5597-5600 (2003).
10. Nair AK, Gautieri A, Chang S-W, Buehler MJ. Molecular mechanics of mineralized collagen fibrils in bone. *Nat Commun* **4**, 1724 (2013).
11. Chen J, Burger C, Krishnan CV, Chu B, Hsiao BS, Glimcher MJ. In vitro mineralization of collagen in demineralized fish bone. *Macromol Chem Phys* **206**, 43-51 (2005).
12. Wang J, *et al.* Remineralization of dentin collagen by meta-stabilized amorphous calcium phosphate. *CrystEngComm* **15**, 6151-6158 (2013).
13. Olszta M, Douglas E, Gower L. Scanning electron microscopic analysis of the mineralization of type I collagen via a polymer-induced liquid-precursor (PILP) process. *Calcif Tissue Int* **72**, 583-591 (2003).
14. Shao C, *et al.* Citrate improves collagen mineralization via interface wetting: a physicochemical understanding of biomineralization control. *Adv Mater* **30**, (2018).
15. Nudelman F, *et al.* The role of collagen in bone apatite formation in the presence of hydroxyapatite nucleation inhibitors. *Nat Mater* **9**, 1004-1009 (2010).
16. Niu LN, *et al.* Collagen intrafibrillar mineralization as a result of the balance between osmotic equilibrium and electroneutrality. *Nat Mater* **16**, 370-378 (2017).

17. Wang Y, *et al.* The predominant role of collagen in the nucleation, growth, structure and orientation of bone apatite. *Nat Mater* **11**, 724 (2012).
18. Jiang F, Hörber H, Howard J, Müller DJ. Assembly of collagen into microribbons: effects of pH and electrolytes. *J Struct Biol* **148**, 268-278 (2004).
19. Williams BR, Gelman RA, Poppke DC, Piez KA. Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results. *J Biol Chem* **253**, 6578-6585 (1978).
20. George A, Veis A. Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition. *Chem Rev* **108**, 4670-4693 (2008).
21. Price PA, Toroian D, Lim JE. Mineralization by inhibitor exclusion the calcification of collagen with fetuin. *J Biol Chem* **284**, 17092-17101 (2009).
22. Nudelman F, Lausch AJ, Sommerdijk NAJM, Sone ED. In vitro models of collagen biomineralization. *J Struct Biol* **183**, 258-269 (2013).
23. Smeets PJM, *et al.* A classical view on nonclassical nucleation. *Proc Natl Acad Sci U S A* **114**, E7882-E7890 (2017).
24. Bates M, Jones SA, Zhuang X. Stochastic optical reconstruction microscopy (STORM): a method for superresolution fluorescence imaging. *Cold Spring Harb Protoc* **2013**, pdb. top075143 (2013).
25. Nudelman F, Bomans PH, George A, Sommerdijk NA. The role of the amorphous phase on the biomimetic mineralization of collagen. *Faraday Discuss* **159**, 357-370 (2012).

Figure Captions

Figure 1. Photos showing the effect of mixing speed on preparing the mineralization solution. (a) The colorless and transparent mineralization solution obtained by fast mixing. (b) The light-blue colored, cloudy dispersion obtained by slow mixing.

Figure 2. Conventional TEM image of the assembled type-I collagen fibril.

Figure 3. Conventional TEM image of the assembled type-I collagen fibril after uranyl acetate positive staining, displaying the ~67 nm periodical structures together with fine band structures. Reproduced with permission.¹⁴ Copyright 2018, Wiley-VCH.

Figure 4. Conventional TEM images of the mineralized collagen-I fibrils in the presence of 0 (a), 10 (b), 15 (c), 50 (d), 120 (e), 240 (f) $\mu\text{g ml}^{-1}$ pAsp. Insets: Selected area electron diffraction (SAED) patterns of the samples, which match with the HAP diffractions and indicate the oriented crystallization. Reproduced with permission.¹⁴ Copyright 2018, Wiley-VCH.

Figure 5. Conventional TEM images of the collagen-I fibrils mineralized at 6 h (a) and 24 h (b). Insets: SAED patterns indicating HAP mineralization. Reproduced with permission.¹⁴ Copyright 2018, Wiley-VCH.

Figure 6. Mineralization degree calculation. (a) Segmentation thresholds determined by analyzing the pixel intensity histogram of the TEM image and splitting the histogram into 3 Gaussian distributions. (b–d) Determination of the regions of the mineralized collagen-I fibrils (b), unmineralized collagen-I fibrils (c), the background (d). Reproduced with permission.¹⁴ Copyright 2018, Wiley-VCH.

Figure 7. Photo of the gold Quantifoil TEM grids floating on a droplet of buffer solution applied on a parafilm.

Figure 8. Figure 8. CryoTEM image of assembled type-I collagen fibrils. (a) Rat tail type-I collagen fibrils, which are 60~80 nm thick. (b) Zoom-in image of the fibrils, showing no periodical structure. (c) A horse tendon type-I collagen fibril, which is ~300 nm thick and clearly shows the ~67 nm periodical structure. The 10 nm particles highlighted by yellow circle are gold fiducial markers used for tomography.

Figure 9. CryoTEM image of mineralized type-I collagen fibril. (a) Image showing a mineralized rat tail type-I collagen fibril, displaying stacks of HAP platelets. (b) SAED pattern of the mineralized rat tail collagen fibril, which shows a narrow but very weak HAP (002) diffraction arc in the collagen fibril axis direction as highlighted by the yellow arrow. (c) Image showing a mineralized horse tendon type-I collagen fibril. The fibril is completely impregnated with HAP platelets and therefore the ~ 67 nm periodical structures are not distinguishable. (d) SAED pattern of the mineralized horse tendon type-I collagen fibril, which clearly shows the HAP (002) diffraction arc in the collagen axis as highlighted by the yellow arrow. The collagen fibril axis directions in (c) and (d) are highlighted by the blue arrows.

Figure 10. The xy projects of the 2D STORM images of the mineralized collagen-I fibrils, with collagen labeled by cy3B and showing red fluorescence. The HAP was labeled with calcein, which emitted green fluorescence. Reproduced with permission.¹⁴ Copyright 2018, Wiley-VCH.

Figure 11. The z-slices of the STORM image of the mineralized collagen-I fibrils, indicating that the mineralization of HAP inside and on the surface of the collagen-I fibrils. Reproduced with permission.¹⁴ Copyright 2018, Wiley-VCH.

Figure 12. Conventional TEM image of the TEM grid inhomogeneously covered by collagen-I fibrils.

Figure 13. CryoTEM image of collagen fibrils after exposure of $200 \text{ e}/\text{\AA}^2$, showing the beam damage (white spots).

Figure 14. 2D STORM image of the non-homogenous stained collagen-I fibrils.