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Nanofibrous Bio-inorganic Hybrid Structures Formed Through Self-Assembly and Oriented Mineralization of Genetically Engineered Phage Nanofibers

Tao He, Gopal Abbineni, Binrui Cao, and Chuanbin Mao, Prof.*

Department of Chemistry & Biochemistry, University of Oklahoma, Norman, OK 73019, USA

Nanofibrous organic–inorganic hybrid structures, where inorganic nanocomponents are formed or assembled within the aligned organic nanofibrous matrix, are important materials that can find applications in electronics, photonics, catalysis, and tissue engineering.^[1–11] They not only provide a means for supporting and ordering the functional inorganic materials such as nanoparticles,^[4–6,8] but also can serve as building blocks to further self-assemble into higher-order structures.^[5,11,12] The common approaches to the synthesis of the nanofibrous organic–inorganic hybrid structures include electrospinning,^[2,4,10] polymer-templating,^[3] biotemplating,^[5–7,11] and directional freezing.^[1] As a matter of fact, the nanofibrous organic–inorganic hybrid structures are important building blocks in natural mineralized biomaterials. One of the best examples is the mineralized collagen fibrils constituting the extracellular matrix (ECM) of bone.^[13] Bone is made of cells embedded in ECM, which is hierarchically organized from proteins, including type I collagen and non-collagenous proteins (NCPs) such as bone sialoprotein (BSP), and calcium hydroxylapatite (HAP, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). The collagen molecules (~1.5 nm wide and 300 nm long) are self-assembled into wider (up to 200 nm wide) and longer (several μm long) fibrils in a side-to-side and head-to-tail format, which are further hierarchically self-assembled to form much wider and longer collagen fibers (up to several tens μm wide and long).^[14,15] HAP is found within the gaps and grooves of the collagen fibers with its c-axis preferentially along the collagen fibers.

Here we demonstrate a new biomimetic strategy to synthesize nanofibrous bio-inorganic hybrid structures by taking advantage of the genetic engineering and self-assembly of M13 phage, which is a virus that specifically infects bacteria and is non-toxic to human beings,^[5,16] and using HAP as a model inorganic material component. This strategy includes the following steps (Figure 1). First, the M13 phage nanofiber (Figure 1a) is genetically modified to display negatively charged peptides on the side wall to become anionic (Figure 1b). Second, the free cationic precursors (e.g., Ca^{2+}) for the inorganic component (e.g., HAP) initiate the self-assembly of the anionic nanofiber into a bundle-like fiber through electrostatic interaction in the presence of the anionic precursors (e.g., PO_4^{3-}) for the same inorganic component (Figure 1c). Finally, the cationic and anionic precursors are accumulated within the bundle, making the local environment supersaturated with respect to the inorganic material component, which results in the nucleation of the inorganic component within the bundle and the formation of mineralized bundle (i.e., nanofibrous bio-inorganic hybrid structure, Figure 1d).

M13 phage (Figure 1a) is a nanofiber-like virus (~880 nm long, ~7 nm wide) that can be mass-produced by specifically infecting bacteria.^[5,16] Filamentous phage has found some biomedical applications. For instance, phage can carry therapeutic drugs on its coat, be infused into nose, enter the brain and then deliver the drugs to brain to treat brain-related diseases without inducing adverse events in animals.^[17,18] It can be circulated in the body to identify a peptide that recognizes a specific tissue or organ (e.g., brain and kidney).^[19,20] Phage with foreign peptides displayed on the coat can be injected into the human body to bind to tumors without inducing obvious toxicity and immune response.^[21,22]

Both collagen and non-collagenous proteins (NCPs) constitute the organic matrix in bone. Among the NCPs in bone, BSP has been found to be a potent HAP nucleator, and its negatively charged domains with a contiguous sequence of 8 Glu (E) residues, that is, the peptide motifs with a sequence of E₈, are responsible for HAP nucleation.^[23,24] Therefore, at the molecular level (Figure 1b), we genetically fuse the peptide E₈ to the major coat protein (pVIII) of phage to make the side wall of M13 phage anionic. ~2700 copies of the pVIII self-assemble into a highly ordered array along the length of M13 phage (Figure 1a,b), providing a high density of sites for electrostatically interacting with cations in the solution. We chose M13 phage as a model bioorganic nanofiber in this work for three reasons: (1) it can be genetically modified^[16] to display any short peptides such as bone protein-derived peptides to promote mineralization; (2) it can self-align^[5,16] in a side-by-side and head to tail format to form higher-order structures by increasing its concentration; and (3) the fiber-like shape may allow it to co-self-assemble with other filamentous biomolecules such as collagen molecules. We chose HAP as a model inorganic material because of the following considerations. First, it is still controversial whether collagen or NCPs in bone initiate HAP nucleation.^[25,26] Thus the protein-mediated HAP nucleation on phage displaying bone protein-derived peptides such as the peptide E₈ derived from BSP may shed some light into this issue. Second, developing bone biomaterials mimicking the hierarchical architecture and chemical composition of the bone ECM is highly demanded in bone regeneration medicine.^[13,27,28] Our strategy can duplicate some aspects of bone biomineralization and result in mineralized bundles mimicking some structural features of mineralized collagen fibers, which may be further assembled into a bone-like biomaterial. Biomimetic mineralization has been proposed to be one of the promising strategies in the synthesis of bone-like biomaterials.^[27,29]

We first verified that the E₈-displayed phage can be self-assembled into a nanofibrous structure under the electrostatic mediation of free Ca²⁺ ions. Figure S1a shows that the phage is assembled into a bundle when 200 μ L phage (with a concentration of 3.0×10^{12} PFU/mL) is added to a 200 μ L CaCl₂ solution (4 mM) and incubated at 37 °C for 24 h, indicating the self-assembly of phage into nanofibrous structure in the presence of Ca²⁺ ions. Moreover, the effective diameter of individual phage particles determined by dynamic light scattering (DLS) is 141.6 nm while that of phage particles in the presence of CaCl₂ (4 mM) was increased to 180.9 nm, indicating the formation of phage bundles due to the presence of Ca²⁺ (Figure S6). In order to further confirm the formation of phage bundles was due to the electrostatic interaction between anionic phage and cationic calcium ions, zeta potential measurement was conducted. The zeta potential of individual phage nanofibers in water was -20.25 mV, while the value for phage nanofibers in the presence of 4 mM CaCl₂ was increased to -6.39 mV, indicating that calcium ions tended to neutralize the negative surface charges of the phage nanofibers displaying E₈ peptide when the complexes of phage-Ca²⁺ are electrostatically assembled into a bundle. However, when the same amount of phage is added to a 4 mM Na₃PO₄ solution, only individual phage nanofibers were found and they could only be visualized after being stained with uranyl acetate (Figure S1b). It should be noted that single phage nanofibers are not visible under TEM unless stained by heavy metal ions or staining agents such as uranyl acetate. In

addition, we found that when the wild-type phage and phage displaying positively charged peptides (K_8) were incubated in the presence of free Ca^{2+} ions, no bundles were formed and only individual phage nanofibers were found under TEM after staining. These results indicate that the electrostatic interaction between anionic phage (due to the display of the negatively charged peptide E_8 on the side wall) and free cations can induce the assembly of phage into a nanofibrous structure where anionic phage nanofibers are “crosslinked” by cations (Figure 1c). Our finding is consistent with the recent report that positively charged gold nanoparticles can bridge the anionic phage nanofibers to form networks.^[30]

We then tested our idea that mineralized nanofibrous structures can be formed after E_8 -displayed phage is mixed with a HAP precursor solution where both Ca^{2+} and PO_4^{3-} ions are present with a molar ratio (Ca^{2+}/PO_4^{3-} molar ratio = 1.67) same as that in HAP. In order to make the solution gradually become supersaturated with respect to HAP, we allowed a mixture of a phage suspension and a supersaturated HAP precursor solution to slowly evaporate in a closed humidified incubator. First, the aqueous suspension of the E_8 -displayed phage was mixed with a supersaturated HAP solution ($[Ca^{2+}] = 4$ mM, Ca^{2+}/PO_4^{3-} molar ratio = 1.67), which we consistently used in our previous publications,^[31–34] to form an unsaturated HAP solution ($[Ca^{2+}] = 2$ mM). The mixture was then aged to vaporize the solvent water slowly in the closed humidified incubator. With solvent evaporation, the concentration of both phage and HAP precursor ions gradually increased. DLS measurements were used to monitor the growth of the phage bundles and the formation of the minerals within the phage bundles. We found that the size of phage-HAP composites determined by DLS was increased with the prolonged incubation (Figure S6), indicating the formation of mineralized phage bundles. During the early incubation period ($t \leq 40$ h), individual non-mineralized phage bundles were found, which is verified by TEM (Figure 2a). The electron diffraction (ED) study of the structure shown in Figure 2a shows no diffraction spots or rings (data not shown), confirming the absence of crystalline phase. We believe that such bundles were formed due to the self-assembly of anionic phage nanofibers in the presence of Ca^{2+} ions (Figure S1a).

At $t = 52$ h, the concentration of Ca^{2+} ions of the solution phase reached $[Ca^{2+}] = 4$ mM according to the induction coupling plasma (ICP) chemical analysis of the mixture, and correspondingly, the bundle was mineralized with an amorphous calcium phosphate (ACP) phase (Figure S2), which at $t = 69$ h was further converted to a weakly crystalline HAP phase with c-axis preferentially oriented along the bundle (Figure S2). The formation of HAP phase was also confirmed from its Fourier transform infrared spectroscopy (FTIR) spectrum where peaks corresponding to PO_4^{3-} , OH, NH_2 , CH, CH_2 , and CH_3 groups could be found (Figure S7). The initial deposition of ACP followed by its conversion to oriented HAP is consistent with the most recent theory of bone biomineralization.^[35] With prolonged incubation, many nanofibrous structures with crystalline HAP are formed (Figure 2b,c and S3). The ED pattern (Figure 2c) of the bundle shown in Figure 2b shows arcs corresponding to (00l) planes of HAP, indicating that HAP in the bundle is oriented with its c-axis preferentially parallel to the fiber. Without phage templates, HAP crystals nucleated from the solution under the similar condition were large particles with flower-like shape (Figure S8). The ED pattern showed typical diffraction rings corresponding to HAP crystal without any preferred orientation (Figure S8). To verify that the nanofibrous structures are assembled from phages, we dissolved the basic HAP phase out of the bundles by using an acetic acid solution. The remained material after acid treatment shows parallel phage nanofibers after negative staining (Figure 2d), further confirming that phages self-assembled into a bundle before mineralization (Figure 1c,d and S1a). It should be noted that phage is chemically stable in acetic acid,^[36] and we did not find that adding acetic acid to phage destroyed phage structure by TEM observation. These results show that the anionic phage can self-assemble and induce oriented HAP nucleation.

The HAP nucleation ability of collagen has been very controversial. Some groups found that it could initiate the nucleation of HAP while others did not.^[25,26,37] It is recently found that type I collagen cannot initiate HAP nucleation although it is the major protein component of the ECM.^[25,26] In natural bone, the ECM is co-assembled from both collagen and NCPs and HAP is nucleated in the presence of both, making whether collagen or NCPs initiate HAP nucleation a long-standing controversial issue.^[25,26] Inspired from the co-assembled status of the ECM, we studied the co-self-assembly of the phage displaying peptide (E₈) derived from one of NCPs (BSP) and collagen molecules, and found that the preferred orientation of nucleated HAP is enhanced and the mineralized structure is more compact by the co-assembly.

We conducted two mineralization experiments starting with type I collagen molecules in the HAP precursor solution in the absence (Exp-1) and presence (Exp-2) of the E₈-displayed phage. In Exp-1, the material formed was made of nonmineralized collagen fibers surrounded by a HAP phase, the EDS spectra of the collagen fibers did not show the signals of Ca, and the ED patterns of collagen fibers show the absence of crystals (Figure S4). These results indicate that the collagen molecules did not initiate HAP nucleation within the collagen fibers in Exp-1. However, the nanofibrous structures, that is, mineralized phage-collagen complex fibers, were formed in Exp-2 (Figure 3a). Compared with the loosely packed structure of the mineralized phage fibers (Figure 2b), the mineralized phage-collagen fibers are more compact (Figure 3a). ED gives shorter and stronger arcs corresponding to (001) planes of HAP (Figure 3b), indicating a higher degree of c-axis preferred orientation of HAP in the phage-collagen fibers than the phage fibers (Figure 2c). We also found that the fibers formed in Exp-2 were still intact after a strong ultrasonic agitation (Figure S5), probably indicating the mechanical stability of the mineralized phage-collagen fibers. To visualize the organic phase of the mineralized phage-collagen fibers, an acetic acid solution was used to remove the basic HAP phase. TEM image of the resultant fibers (Figure 3c) shows that the phage-collagen fibers have a 36 nm periodic transversal band structure that is different from the periodic band (67 nm) of collagen fibrils (Figure S4),^[14] indicating that collagen fibril assembly together with E₈-displayed phage results in an altered structure than collagen and phage alone. Therefore, the better mineralization of the phage-collagen fibers in terms of stronger c-axis preferred orientation and more compact mineralized structures than the collagen fibers alone is attributed to the existence of HAP-nucleating phage.

Inter-chain hydrogen bonding promotes the self-assembly of collagen molecules into fibers.^[14] It is possible that the peptide E₈ displayed on the side wall of phage forms hydrogen bonding with the collagen molecules, leading to their hierarchical co-assembly into phage-collagen fibers. The collagen was selectively removed from the phage-collagen fibers by using an acetic acid solution.^[38] TEM image of the resultant fibers (Figure 3d) shows a structure similar to phage bundles (Figure 2a), confirming the assembly of phages in the phage-collagen fibers. Furthermore, Alexa Fluor 488 (green) and Alexa Fluor 546 (red) dye, were conjugated to M13 phage and collagen molecules through the anti-M13 phage pIII antibody (specifically binding to the pIII tip of phage shown in Figure 1a) and anti-collagen antibody as two separate linkers, respectively. Then mineralization same as Exp-2 was conducted using the dye-labeled phage and collagen molecules. The fluorescence images of the fibers formed after co-assembly further confirm the co-assembly of the phage and collagen molecules (Figure 3e–g). Both TEM and confocal microscopy study confirm the co-assembly of phage and collagen in one bundle during the biomimetic mineralization process.

In conclusion, we have demonstrated a novel biomimetic strategy for the synthesis of nanofibrous bio-inorganic hybrid materials by using phage as a model biological nanofiber and HAP as a model inorganic material. In this strategy, the anionic biological nanofibers

are induced to self-assemble into nanofibrous phage-cations complex structures through the electrostatic interaction between anionic phage nanofibers and the free precursor cations and the inorganic materials are formed in situ within the nanofibrous structures through the reaction of the precursor cations and anions for the inorganic materials. The oriented mineralization and co-self-assembly of phage nanofibers and other filamentous biomolecules such as collagen molecules are also successfully demonstrated, which may lead to a novel building block for hierarchically assembling bone ECM-like materials. In the co-assembled materials, collagen promotes the formation of hierarchical architecture similar to the framework of the ECM while E₈-displayed phage enhances the mineralization of the matrix. The improved control over the assembly and orientation by the co-assembly is attractive because the organization and orientation of both proteins and HAP determine the superior mechanical properties of bone.^[39] Our results indicate that the co-self-assembly of collagen and NCPs are important in forming bone and suggest that controlled mineralization within a protein matrix co-assembled from collagen and HAP-nucleating protein fibers is a promising strategy for developing bone-like biomaterials. Such biomaterials are desired scaffolds for bone tissue engineering as cells seeded on them will be in an environment similar to bone and deposit new ECM to form new bone tissue.

Experimental Section

Display of E₈ peptide on phage

We used standard phage display technology to fuse foreign peptide (Glu₈, i.e., E₈), to the N-terminus of each copy of the major coat protein of M13 bacteriophage. The molecular cloning experiments were performed as described in the literature.^[40] We used *E. coli* XL1 blue bacteria and phagemid for peptide display. The oligonucleotide encoding E₈ was amplified by PCR using replicative form of DNA (RF DNA) as a template and the following primers synthesized by Invitrogen:

5'ATCCATGGCG

GAAGAAGAAGAAGAAGAAGAA

GAAGATCCCGCAAAAGCG3'

(*Nco*I restriction site is underlined)

5'GCAAGCTTTTATCAGCTTGCTTTCGAG3'

(*Hind*III restriction site is underlined).

The PCR products were purified using QIAGEN PCR product purification kit according to the protocols provided with the kit. The purified DNA was digested with both *Nco*I and *Hind*III restriction enzymes. The digestion reactions were carried out in a sterile microcentrifuge tube. After digestion, the DNA fragments were loaded into 1% agarose gel and isolated by electrophoresis in 0.5×TBE buffer. The digested fragment was extracted from the agarose gel using QIAGEN gel DNA extraction kit. The ligation reaction was carried out at 25 °C for 2 h using T4 ligase. The DNA was then transfected into competent *E. Coli* TG1 cells by CaCl₂ method. 100 μL of transfected bacteria was spread onto SOB plate containing suitable antibiotics. The vector fragments without ligation were used as a control. A well separated clone from the plate was inoculated into 3 mL 2×YT broth and incubated at 250 rpm at 37 °C. The recombinant phagemid DNA was isolated using QIAGEN miniprep plasmid extraction kit. The DNA was eluted with sterile ddH₂O. To analyze the recombinant phagemid DNA, the phagemid DNA was digested with both *Nco*I and *Hind*III restriction enzymes. The digested products were analyzed by electrophoresis in agarose gel and observed under UV light. The correctly inserted fragments were verified by DNA sequencing. To realize display of E₈ on the major coat of M13 phage, 0.5 μL recombinant

phagemid DNA was transfected into competent host strain XL1 blue *E. Coli* by CaCl_2 method. The transfected bacteria were spread on a SOB plate containing $20 \mu\text{g mL}^{-1}$ tetracycline and $35 \mu\text{g mL}^{-1}$ chloramphenicol. Then a well-spread clone from the plate was inoculated into 3 mL 2xYT medium containing the appropriate antibiotics. The culture was incubated in the tube for 4–6 h at 37°C with moderate shaking. 10^{10} pfu M13KO7 virions were added to the tube and the tube continued to be incubated for 30 min at 37°C . The mixture in the tube was transferred to 150 mL 2xYT medium containing appropriate antibiotics. Kanamycin was added to reach a final concentration of $70 \mu\text{g/mL}$ and IPTG was added to a final concentration of 0.1 mM. The mixture was incubated overnight at 37°C with vigorous shaking.

Engineered Phage Propagation and Purification

E. Coli XL1 blue strain bacterial cultures were grown in Luria-Bertani (LB) medium (20 g L^{-1} LB and pH adjusted to 7.0 with 1N NaOH) at 37°C . The phage displaying E₈ on the major coat (i.e., engineered phage) was amplified by incubating phage suspension with *E. Coli* XL1 blue strain. Two tubes of 5 mL bacterial culture was incubated with the phage in a 1 liter LB medium on a shaking incubator at 37°C overnight. Antibiotics including chloramphenicol ($35 \mu\text{g/mL}$), tetracycline ($35 \mu\text{g mL}^{-1}$), and kanamycin ($70 \mu\text{g/mL}^{-1}$) were used for the selection of respective plasmid and strains. The overnight culture was first centrifuged at 2400 g for 10 min at 4°C using Beckman Coulter™ High performance centrifuge, and the supernatant was collected and re-centrifuged at 6700 g for 10 min at 4°C . Then PEG/NaCl solution was added and the suspension was stored in a refrigerator overnight. Phage precipitates were collected at 10100 g for 60 min at 4°C , dissolved in TBS and centrifuged at 10100 g for 10 min until clear.

Self-assembly and mineralization of E8-displayed phage bundles

(1) *Preparation of supersaturated hydroxylapatite (HAP) solution*: The HAP supersaturated solution was prepared by following the protocols in our previous publications.^[31–34] Briefly, a stock solution was prepared by dissolving HAP (calcium/phosphate ratio of 1.67, sigma) powder in a solution containing 100 mM of hydrochloric acid and had a final concentration of 50 mM of calcium. 40 mL stock solution was pipetted into a clean polythene container, and the volume was increased to 450 mL by adding with distilled water. The pH value was adjusted to 7.01 with 0.05 M potassium hydroxide. Sodium chloride was added to the solution to reach a final concentration of 200 mM, and then the final volume was adjusted to 500 mL with distilled water. The concentration of the resultant HAP-supersaturated solution is $[\text{Ca}^{2+}] = 4 \text{ mM}$. (2) *Mineralization*: 200 μL solution of phage (with a concentration of 3.0×10^{12} PFU/mL, pH ~7.5) and 200 μL supersaturated HAP solution (pH ~7.01, $[\text{Ca}^{2+}] = 4 \text{ mM}$) were mixed together in a glass tube (diameter ~1.5 cm and length ~4.5 cm) to form a clear solution. And then the solution was aged in an incubator at 36.7°C to vaporize the solvent water slowly. After different times, the mixture was centrifuged. The solid materials obtained were re-suspended in water, transferred onto a TEM grid, rinsed to remove soluble salts, and characterized by transmission electron microscopy (TEM). 200 μL water and 200 μL supersaturated HAP solution were also mixed to conduct a control experiment. The $[\text{Ca}^{2+}]$ of the solution phase during the mineralization was monitored using inductively coupled plasma (ICP) analysis.

Fourier transform infrared spectroscopy (FTIR) and Dynamic light scattering (DLS) characterization

HAP-phage complex after 72 h incubation was freeze dried, mixed with KBr and characterized by FTIR. DLS was used to characterize the effective size and zeta potential of the aqueous phage solution in the absence and presence of 4 mM CaCl_2 as well as the HAP-phage complex formed after 24 h, 36 h, and 72 h incubation.

Co-self-assembly and mineralization of E₈-displayed phage and collagen molecules

(1) *Exp-2 (in the presence of both phage and collagen molecules)*: 100 μ L type 1 collagen solution (8.40 mg mL⁻¹, purchased from BD Bioscience as a liquid in 0.02 N acetic acid, from rat tail tendon) and 200 μ L E₈-displayed phage were mixed in a glass tube (diameter ~1.5 cm and length ~4.5 cm) and then vortexed for 10 seconds until a homogenous and transparent solution was formed. 200 μ L supersaturated HAP solution was added to the solution, which was vortexed for another 10 s. Then, the glass tube was kept in a 36.7 °C incubator. After different aging times, the mixture was centrifuged. The solid materials obtained were re-suspended in water, transferred onto a TEM grid, rinsed to remove soluble salts, and characterized by TEM. (2) *Control experiment (Exp-1) in the presence of only collagen molecules*: the 200 μ L E₈-displayed phage solution in Exp-2 was replaced with 200 μ L TBS buffer solution. Thus, the only difference between Exp-1 and 2 was the existence of E₈-displayed phage in the latter. The rest experimental procedure is same as Exp-2.

Selective removal of HAP or collagen fibrils from the mineralized fibers

(1) Selective removal HAP from mineralized phage bundles and staining: Acetic acid solution (pH = 3.5) was dropped on the surface of the TEM copper grid, which held the mineralized phage bundles. After 2 minutes the acid solution containing dissolved HAP was sucked off by using a small piece of tissue paper. The TEM grid was then rinsed with water. When the TEM grid was dried, a drop of an acidic uranyl acetate (UA) solution (1.0 wt%, pH = 3.5) was dropped on the surface of the TEM grid. The UA solution was sucked off after 10 seconds. When the TEM grid was dried entirely, another drop of UA solution was dropped. The staining process was repeated for 3 times before TEM characterization. (2) Selective removal collagen fibrils from the mineralized fibers: Acetic acid was used to selectively remove collagen fibrils.^[38] The TEM copper grid holding the mineralized fibers was first immersed in 0.1 mol L⁻¹ acetic acid for 1 h to selectively dissolve out collagen molecules and HAP. And then the TEM grid was stained with uranyl acetate after it was taken out and dried in air at room temperature for 10 min.

Confocal fluorescence microscopy characterization of mineralized co-assembly of dye-labeled phage and collagen

Before the nucleation experiments, the dye *Alexa Fluor 546* (from Invitrogen) was conjugated to Anti-collagen α 1 Type I antibody (Goat, form SANTA CRUZ BIOTECHNOLOGY, INC.) at 4 °C overnight, and then 5 μ L of the conjugated dye was mixed with 100 μ L of type 1 collagen (*catalog # 354249*, from BD Biosciences) antibody at 4 °C overnight; the dye *Alexa Fluor 488* (from Invitrogen) was conjugated to Anti-M13 pIII Monoclonal Antibody (from MoBiTec, Inc.) and then 2 μ L of the conjugated dye was mixed with 200 μ L surface engineered M13 phage 4 °C over night. It should be noted that the Anti-M13 pIII monoclonal antibody can specifically bind to the tip (pIII) of the phage (Figure 1a), thus the dye-labeling will not affect the assembly and mineralization behavior of the side-wall-displayed M13 phage. The detailed procedure for conjugation of dye and antibody and purification of dye conjugated antibody from free dye can be obtained from *Invitrogen Alexa Fluor 546 and 488 Monoclonal antibody labeling Kits* (cat# A-20183 and A-20181). The dye *alexafleur 488* was excited at 488 nm to give the green image (550 nm) and the dye *alexafleur 546* was excited at 546 nm to give the red image (573 nm). Then the mineralization experiment same as Exp-2 was conducted.

Materials Characterization

The morphology, phase structure as well as crystal orientation of the mineralized products were characterized by using TEM (Zeiss 10, 80 kV and JEOL 2000, 200 kV). The samples

without mineralization or with mineral removed were stained using uranyl acetate before observation under TEM unless otherwise stated.

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Figure 1.

a) Structure of wild-type M13 phage. b) Genetic fusion of E₈ peptide to major coat protein (pVIII) on the side wall of phage. c) Self-assembly of anionic phage into a nanofibrous structure (bundle) in the presence of Ca²⁺ ions. d) Mineralization of the phage bundle to form a mineralized fiber.

Figure 2.

a) TEM image of a stained phage bundle at $t = 40$ h before mineralization. b) TEM image of a mineralized phage bundle formed at $t = 90$ h. c) ED pattern of the bundle in (b). d) TEM image of the mineralized phage bundle after removal of HAP and uranyl acetate staining.

Figure 3.

a) TEM image of a mineralized phage-collagen fiber. b) ED pattern of the same fiber shown in (a). c) TEM image of the mineralized phage-collagen fiber after removal of mineral phase and uranyl acetate staining. d) TEM image of the mineralized phage-collagen fiber after removal of both mineral phase and collagen fibrils by acid-treatments and uranyl acetate staining. e,f) Confocal fluorescence image of a mineralized, dye-labeled phage-collagen bundle excited at 488 nm and 546 nm to give green (e) and red (f) image, respectively. g) is a merged image of (e) and (f).