RESEARCH ARTICLE

MATERIALS SCIENCE

Diatom-inspired multiscale mineralization of patterned protein-polysaccharide complex structures

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ABSTRACT

Marine diatoms construct their hierarchically ordered, three-dimensional (3D) external structures called frustules through precise biomineralization processes.
Recapitulating the remarkable architectures and functions of diatom frustules in artificial materials is a major challenge that has important technological implications for hierarchically ordered composites. Here, we report the construction of highly ordered, mineralized composites based on fabrication of complex self-supporting porous structures—made of genetically engineered amyloid fusion proteins and the natural polysaccharide chitin—and performing in situ multiscale protein-mediated mineralization with diverse inorganic materials, including \( \text{SiO}_2 \), \( \text{TiO}_2 \), and \( \text{Ga}_2\text{O}_3 \). Subsequently, using sugar cubes as templates, we demonstrate that 3D fabricated porous structures can become colonized by engineered bacteria and can be functionalized with highly photoreactive minerals, thereby enabling co-localization of the photocatalytic units with a bacteria-based hydrogenase reaction for a successful semi-solid artificial photosynthesis system for hydrogen evolution. Our study thus highlights the power of coupling genetically engineered proteins and polysaccharides with biofabrication techniques to generate hierarchically organized mineralized porous structures inspired by nature.

**Keywords:** biomimetic mineralization, patterned porous structure, genetic engineering, amyloid protein, artificial photosynthesis

**INTRODUCTION**

Biological organisms such as diatoms, sponges, and radiolarians are able to make hierarchically organized, mineralized structures across multiple length scales [1-3]. One particularly interesting example is diatoms—unicellular, eukaryotic algae—which are well known to produce a wide variety of hierarchically ordered porous silica structures in a genetically controlled manner [1-4]. The diatom frustule (i.e., the hard and porous external layer of diatoms) is multifunctional: it serves as a protective barrier between the cytoplasm and the exterior environment, and it also provides mechanical strength to predation [3, 4]. Furthermore, the micro-scale,
periodic structure of the frustule possesses interesting light-directing properties, which diatoms are known to exploit to enhance their photosynthetic capacity [5-7]. Accordingly, diatoms have long served as a fruitful source of inspiration for the fabrication of novel bio-inspired hierarchically porous materials.

Over the last several decades, numerous strategies have been used for the \textit{in vitro} generation of nanostructured silica-based material structures, including for example the use of organic molecules (such as surfactants, polymers, and organo-gelators) to guide silica mineral formation [8-13]. Most organic templates used in these efforts have been based on long-chain cationic polymers, mainly polyamines, in order to mimic the positively charged sequences of the silaffin proteins known to orchestrate diatom frustule biogenesis [14-19]. These organic templates can control the size and morphology of the silica particles that form; however to date these strategies have yielded relatively simple shapes like rods, spheres, or hexagonal platelets [14, 20-23]. Other research efforts have focused on the application of various three-dimensional assemblies of organic matrices (cellulose, collagen, and virus) for the structured aggregation of silica particles to form higher-order silica structures [14, 15, 24-30]. These \textit{in vitro} scaffolds have led to the controlled deposition and organization of silica and some metal oxide minerals [31-33]. Additionally, diatom frustule-inspired scaffolds such as DNA origami architectures and micro-patterned functional silk structures have been explored to produce spatially ordered, nano- or micro-sized mineralized composites [34, 35]. Despite important advances in biomimetic mineralization research, the development of mineralized structures recapitulating the hierarchically ordered porous structures of diatoms across multiple length scales remains elusive.

Here, aiming to recapitulate the hierarchically porous mineral structures and functional properties of diatom frustules (Fig. 1A), we developed a biomimetic mineralization strategy in which complex porous structures made of genetically engineered multifunctional amyloid fusion proteins and the polysaccharide chitin are used as scaffolds for \textit{in situ} mineralization of both SiO\textsubscript{2} and metal oxides across multiple length scales. Our design was inspired by the biogenesis of mineralized
frustule structures. First, a three-domain fusion protein was engineered to comprise the CsgA protein from the well-known curli amyloid system of \textit{Escherichia coli} (E. coli) \cite{36, 37}, a chitin binding domain (CBD) from \textit{Bacillus circulans} \cite{38}, and the R5 silica-nucleation peptide from \textit{Clavulinopsis fusiformis} \cite{24, 39-41}. This design was aimed to mimic the molecular interactions intrinsic to natural diatom systems in which chitin-based networks serve as a framework for silica mineralization during frustule formation, while soluble biomolecules such as silaffins are then used as silica-nucleating proteins to promote mineralization within the chitin matrix \cite{39-42}. Second, two types of porous structures were produced in order to morphologically resemble the porous features of the diatom frustule, including a highly ordered 2D porous sheet fabricated via replica molding and a 3D irregular porous cube created using sugar cube as a template (Fig. 1B and C).

Using a biomimetic mineralization strategy, the 2D patterned and 3D irregular porous self-supporting protein/chitin composite structures can further template the formation of complex mineral architectures across multiple length scales (from nm to cm). Extending the application of these multiscale mineralized structures, we demonstrate a hybrid artificial photosynthesis system based on a sugar-cube templated, 3D, biocompatible structure mineralized with photoreactive TiO$_2$ and colonized with hydrogenase-expressing bacteria for successful hydrogen production.

RESULTS

Bio-inspired construction of silicified self-supporting porous R5CsgA$_{CBD}$/chitin sheets.

To mimic the multiscale mineralization of diatom frustules, we started by rationally designing self-assembling nanofiber networks capable of both promoting local silica nucleation and mineralization at the molecular level and recapturing protein-polysaccharide interactions that occur in diatom frustules. Specifically, we initially constructed and tested mineralization-promoting CsgA fusion proteins by
genetically appending a SiO$_2$-nucleation peptide (R5) at the N-terminus of CsgA (R$_5$CsgA), a bacterial structural amyloid protein of *E. coli* biofilms that is amenable for genetic modification to endow functional properties without disrupting its nanofiber self-assembly capability [37, 43]. We confirmed that the R$_5$CsgA fusion proteins could self-assemble into amyloid nanofibers (Supplementary Fig. 1) and found that they could indeed serve as a template for promoting silica formation (Supplementary Fig. 2). To engineer protein-polysaccharide molecular interactions, we designed CsgA fusion proteins containing CBD at C-terminus (CsgA$_\text{CBD}$). For context, the CsgA$_\text{CBD}$ fusion proteins had previously been demonstrated by our research group to enable strong and specific protein-chitin molecular interactions, resulting in networked chitin-amyloid nanofiber soft hybrid materials [37].

Our final functional protein module was therefore settled on engineered multidomain amyloid fusion proteins (R$_5$CsgA$_\text{CBD}$) in which the R5 peptide and CBD were genetically fused at the N- and C-terminus of CsgA, respectively (Supplementary Figs. 3 and 4). Morphological characterization by atomic force microscopy (AFM) and transmission electronic microscopy (TEM) revealed that the R$_5$CsgA$_\text{CBD}$ fusion proteins in solution could self-assemble into nanofibers, with an average fiber diameter of 1.7 ± 0.4 nm and an average length of 1,097.5 ± 181.0 nm (Fig. 2A, B and Supplementary Fig. 5). Further, structural characterization by X-ray fiber diffraction revealed that the nanofibers displayed a typical β diffraction pattern, with a meridional reflection (denoted as d$_2$ in the diffraction pattern) at 4.8 Å, reflecting the spacing between β-strands within each layer of β-sheets and an equatorial reflection (denoted as d$_1$ in the diffraction patterns) at 9.5Å, corresponding to inter-sheet packing distances (Fig. 2C) [43].

We then tested the self-assembling nanofibers composed of R$_5$CsgA$_\text{CBD}$ fusion proteins as organic templates for silica formation. After 10-min incubation in a precursor solution of tetramethoxysilane (TEOS) at room temperature, spherical SiO$_2$ particles were found to appear around the nanofibers, clearly suggesting that the nucleation process of SiO$_2$ was guided by the nanofibers (Fig. 2D, left). In contrast, other CsgA fusion proteins that do not contain R5 domain seemed not to promote the
deposition of SiO$_2$ nanoparticles under the same silicification conditions (Supplementary Fig. 6). In addition, a series of chemical mapping analyses using energy dispersive spectrometer (EDS) coupled with electron microscopy imaging confirmed the silica mineralization of the amyloid fibers comprising the R$_5$CsgA$_{CBD}$ fusion monomers (Fig. 2D, right).

We next designed self-supporting porous structures consisting of R$_5$CsgA$_{CBD}$/chitin complex components for multiscale silica mineralization, largely inspired from the porous structure of the diatom frustule. Using a simple microtransfer molding process, we fabricated complex self-supporting structures made of R$_5$CsgA proteins and the polysaccharide chitin (Fig. 2E and G) [44]. Through a methanol-assisted in situ curing process, CsgA fusion proteins in the fabricated structures would regain their amyloid β-sheet structures with fibrous morphology [37]. Briefly, using an hexafluoroisopropanol (HFIP) solution containing both the engineered R$_5$CsgA$_{CBD}$ protein monomers and dissolved squid pen β-chitin molecules, we printed and cured structures that we term patterned porous sheets (PPS) (Fig. 2E). Upon methanol vapor exposure the protein monomers in the fabricated complex structures indeed reassembled into their characteristic nanofiber structures (Fig. 2G, and I). In addition, the PPS composed of chitin and R$_5$CsgA$_{CBD}$ fusion proteins (length: ~1cm; width: ~1cm; thickness: 412 ± 15 nm and pore diameter: ~10 μm) exhibited impressive durability and stability after exposure to aqueous solution, in sharp contrast to the deterioration we observed for incomplete PPS structures built solely of R$_5$CsgA$_{CBD}$ fusion proteins (i.e., lacking the polysaccharide chitin) (Supplementary Fig. 7). When these patterned porous sheets were exposed to a mineralization precursor solution of tetramethoxysilane for in situ mineralization (Fig. 2F), silica spheres appeared to form on the PPS surface, as revealed by AFM and scanning electron microscopy (SEM) (Fig. 2H, and J). In contrast, deposition of silica spheres was not clearly found on the pure chitin PPS sheets, highlighting the role of fusion proteins in promoting silica formation (Supplementary Fig. 8). In addition, silica deposition was found to occur preferably at neutral pH (7.5), rather than at basic (10.5) or acidic (4.5) pH value (Supplementary Fig. 9), in agreement with phenomena
reported in previously published works [24, 45]. Examination of the mineralized structures by X-ray photoelectron spectroscopy (XPS) confirmed successful PPS silicification: these mineralized PPS exhibited characteristic binding energies of 102 eV and 153 eV for electrons found in the 2s and 2p3 electron shells of the silicon atom, respectively (Fig. 2K). Further characterization by Fourier Transform Infrared Spectroscopy (FTIR) revealed peak wavenumbers at 1,095 cm$^{-1}$, indicating that Si–O–Si antisymmetric stretching was occurring in the sheets (Fig. 2L). Highlighting the impressive mechanical properties of PPS after mineralization, we also used peak force quantitative nanomechanical (PK-QNM) AFM to measure the Young's modulus of non-mineralized and mineralized sheets and found that mineralization substantially increased the Young’s modulus of the material by around 200% (from 4.84 ± 0.46 GPa to 14.16 ± 0.58 GPa) (Fig. 2M).

Collectively, by integrating rationally designed mineralization-promoting amyloid proteins with a microtransfer molding process, we precisely fabricated ordered porous structures that can be mineralized in situ across multiple scales, thereby resulting in hierarchically ordered mineralized structures resembling the exquisite frustules of diatoms [44]. These results implied that the $\text{R}_5\text{CsgA}_{\text{CBD}}$ fusion proteins can serve as self-assembling nanofibers that promote local silica mineralization. The SiO$_2$ mineral substantially enhances the mechanical properties of the free-standing PPS structures, thus potentially broadening a wide range of applications including serving as protective shelters for biomolecules and mechanical strengthening building materials [34]. For example, immobilization of enzymes in mechanically stable and chemically inert silica matrices would provide the embedded enzymes with better environmental tolerance and extended life-time storage, likely extending application of these biomolecules even under non-physiological conditions [25, 46].
**Biomimetic mineralization of R5CsgA\textsubscript{CBD}/chitin materials with TiO\textsubscript{2}**

Recalling the known ability of the R5 peptide to mediate the mineralization of TiO\textsubscript{2} [31], we next investigated whether the engineered R5CsgA\textsubscript{CBD} amyloid nanofibers could nucleate metal oxide to generate mineralized nanostructures. The use of biomimetic mineralization approaches to produce porous metal oxide-based composites at room temperature is particularly attractive, given the fact that metal oxide-based materials are known to be useful for a wide variety of applications in photocatalysis and photovoltaics [32].

We started to assess whether the engineered R5CsgA\textsubscript{CBD} amyloid nanofibers could template the nucleation and growth of TiO\textsubscript{2}. To such ends, we added nanofibers to buffered aqueous solutions containing Ti (IV) bis-(ammonium lactato)-dihydroxide (TiBALDH) to trigger TiO\textsubscript{2} mineralization. We found that the solutions became turbid when the R5CsgA\textsubscript{CBD} nanofibers were added, implying their potential as self-assembling scaffolds to promote TiO\textsubscript{2} mineralization. We confirmed the successful mineralization of the R5-interacting TiO\textsubscript{2} by using multiple analyses including EDS, selected area electron diffraction (SAED), lattice fringe high-resolution transmission electron microscope (HR-TEM). TEM and corresponding EDS analysis indicated the presence of Ti on the surface of the R5CsgA\textsubscript{CBD} nanofibers exposed to the mineralization precursor solution and the presence of N (from the proteinaceous components) (Fig. 3A and B). Further, the SAED pattern obtained from the coatings close to the nanofiber surface revealed rings with spots having d-spacing of 3.5, 2.4, and 2.3 Å, corresponding to the lattice plane (101), (004), and (103), respectively (anatase phase, JCPDS 84-1286) (Fig. 3C). The relative intensities of the diffraction patterns matched the three most intense values for nanocrystalline TiO\textsubscript{2} (anatase phase, JCPDS 84-1286). Further, the lattice fringe spacings were consistent with the (101) plane spacing of anatase (anatase phase, JCPDS 84-1286) (Fig. 3D).

Having demonstrated successful mineralization at fibril level, we next assessed TiO\textsubscript{2} mineralization using the self-supporting porous sheets (Fig. 3E). After
mineralization, we used nanoscale-infrared spectroscopy (nano-IR) at a wavenumber of 750 cm$^{-1}$ to reveal the presence of TiO$_2$ on the mineralized sample, and indeed an overlay of the reconstituted topography image (3D) with a color code representing the absorption intensity at 750 cm$^{-1}$ clearly indicated the homogeneous distribution of TiO$_2$ on the sheets (Fig. 3F and G). Moreover, corresponding chemical mapping analyses based on EDS furthered indicated the successful TiO$_2$ mineralization (Supplementary Fig. 10). In addition, XPS spectral analysis revealed that the mineralized sheets had distinct signals for oxygen (O 1s and 2s), carbon (C 1s), nitrogen (N 1s) and titanium (Ti 2p and 3p) (Fig. 3H), again supporting successful TiO$_2$ mineralization of the PPS. We next measured the Young’s modulus to assess the effects of TiO$_2$ mineralization on the mechanical properties of PPS based on PK-QNM AFM methodology: the mineral indeed increased the elasticity of the PPS (from 4.84 ± 0.46 GPa to 7.21 ± 0.51 GPa) (Supplementary Fig. 11). Notably, using a similar biomimetic mineralization approach, Ga$_2$O$_3$, an ultra-wide bandgap oxide semiconductor, could also be mineralized on both nanofibers and porous $\text{R}_5\text{CsgA}_{CBD}$/chitin sheets (Supplementary Figs. 12, 13, and 14). Collectively, these results establish that porous $\text{R}_5\text{CsgA}_{CBD}$/chitin sheets can serve as scaffolds for templating the in situ growth of metal oxides to produce mineralized porous sheets over multiple length scales.

Fabrication of self-supporting and high surface area mineralized porous structures

The hierarchical porous structures of diatoms endow them with adaptive functions [5-7], providing inspiration for reconstructing artificial hierarchical porous materials for numerous applications. Previous studies have demonstrated that hierarchically structured porous bioceramic-silk composites could enhance cell attachment, proliferation and gene expression, and porous silica-based structures could facilitate enzyme immobilization, while porous organic polymers serve as a promising platform for designing heterogeneous catalysts [47-49]. Motivated by the applications of such
diatom-inspired structures, we turned to construct porous mineralized \( R_5 \text{CsgA}_{CBD} \)/chitin composites with high-surface areas and photo-reactivity for proof-of-concept demonstration of a solar-driven hydrogen evolution system.

To such ends, we started by preparing a 3D porous \( R_5 \text{CsgA}_{CBD} \)-chitin complex scaffold for TiO\(_2\) mineralization. We first applied HFIP ink containing both \( R_5 \text{CsgA}_{CBD} \) monomers and chitin, along with a porous sugar cube as a bulk template, to fabricate 3D complex self-supporting structures (Fig. 4A). After 12h immersion in HFIP ink, the sugar cube was cured under methanol vapor to trigger the reassembly of amyloid proteins into nanofibers throughout the whole matrix of the cube. After dissolving the sugar cube template in aqueous solution, a self-supporting 3D amyloid-chitin complex structure was obtained. The successful replication of the sugar cube shape as a sponge-like structure was evident in normal photographs (Fig. 4B), and the porous surfaces of the scaffold were found to comprise large amount of nanofiber structures under SEM (Fig. 4C and D). Notably, the air-dried 3D porous structures could spontaneously restore their shapes in their hydration state after immersion in different solutions (e.g., distilled water and Congo red solution) (Fig. 4E-H and Supplemental Fig. 15), and rapidly reached equilibrium swelling ratio in solution within 25 seconds (Fig. 4I). Additionally, these structures exhibited reversible hydration/dehydration behaviors with nearly identical swelling/de-swelling ratios even after multiple cycles (Fig. 4J), thus allowing for recyclable loading and release of specific reagents in a controlled manner.

Owing to its rapid swelling feature and reversible swelling behavior, we hypothesize that the fabricated porous cubes would first serve as sponge-like materials to homogeneously absorb a mineralization precursor solution for TiO\(_2\) mineralization, and then harbor a mixed reaction solution containing bacterial cells for artificial photosynthesis. Ideally, in the final constructed artificial photosynthesis system, mineralized TiO\(_2\) nanoparticles act as the light-antennae component converting photons into electrons, and an engineered \textit{E. coli} strain harboring a hydrogenase gene cluster, upon receiving electrons transported by methyl violet (MV), can catalyze continuous hydrogen evolution when the system is illuminated.
We next probed if the fabricated porous 3D cubes could promote local TiO$_2$ mineralization and if the mineralized porous cubes could further absorb and harbor bacterial from aqueous solution (Fig. 5A and B). After 2h incubation in a TiBALDH mineralization precursor solution, the 3D cube structures could still maintain its original shape (Fig. 5C). SEM and EDS analysis revealed that mineralized TiO$_2$ nanoparticles could directly form on the fibers comprising the cube (Fig. 5D and Supplemental Fig. 16). The UV-Vis absorption spectra showing a sharp absorption edge at ~385 nm representative of TiO$_2$ band gap excitation further confirmed the successful mineralization of TiO$_2$ in the porous cube (Supplementary Fig. 17).

Nanoindentation measurements showed that the Young’s modulus of the TiO$_2$-mineralized cube was obviously higher than the unmineralized cube (Supplementary Fig. 18), with a value of 8.58 ± 1.63 GPa and 33.14 ± 6.58 GPa for unmineralized and mineralized cube structures, respectively. In addition, after incubation of the mineralized cube in an aqueous solution containing the engineered E. coli strain, SEM analysis clearly indicated that the bacterial could distribute homogeneously inside the porous structure of the mineralized cube (Fig. 5E and F). These results therefore confirm that the 3D structures can serve as 3D scaffolds for biomimetic mineralization and for trapping bacterial cells.

**Artificial photosynthesis of the photoreactive mineralized porous structures**

Biogenic hydrogen production coupling microbial with protective shelters has been a well-known technique in the field. In particular, cyanobacteria (e.g., Synechocystis sp., Microcystis aeruginosa, Phormidium valderianum.) has been utilized for hydrogen production via direct photolysis [51, 52]. When encapsulated in a 1.5% agar matrix, the cyanobacteria Oscillatoria sp. has shown that the rate and longevity of hydrogen production increased significantly compared to the free cells [52]. The cyanobacteria Synechocystis sp. PCC 6803 in silica matrices showed that the hydrogen production of encapsulated cells was higher than the cells in suspension [53, 54]. Despite these
advances, hydrogen production efficiency using biological organisms has been limited by its low volumetric productivity. As such semi-artificial photosynthesis coupling inorganic semiconductor catalysts with engineered cells has gained great interests for their high performance in terms of broad-spectrum absorption and light harvesting efficiency [55].

We next explored the use of mineralized TiO$_2$ cubes in an artificial photosynthesis system for solar-driven hydrogen production. To such ends, we first measured the transient photocurrent of a mineralized amyloid cube by placing it onto a piece of FTO-conductive glass and dried the sample at 37°C overnight (Supplementary Fig. 19). The glass coated with the dried sample was then successfully used as an electrode: the transient photocurrent of 12.7 ± 1.1 μAcm$^{-2}$ was detected when it was exposed to light and the value dropped to almost zero when illumination was terminated, thus highlighting its pronounced property of photoresponse (Fig. 5G). Moreover, we tested the voltammetry responses of a bare FTO substrate and of TiO$_2$-mineralized cube deposited on the FTO electrode in 0.50 M aqueous Na$_2$SO$_4$ (pH = 6.3). No redox peak was observed for both mineralized TiO$_2$ and bare FTO glass over a wide voltage range of -0.1~1.4 V vs RHE, and the curves of their cyclic voltammetry are identical, implying the electrochemical stability of the TiO$_2$-mineralized cube under aqueous conditions (Supplementary Fig. 20).

With this photocatalytic mineralized cube reaction system established, we next carried out H$_2$ evolution in the aforementioned semi-solid artificial photosynthetic system comprising a bacterial strain that expresses a hydrogenase (and its required maturases), TEOA as the sacrificial agent, and methyl violet (MV) as the mediator. UV spectroscopy revealed peaks between 300-400 nm and 500-700 nm, confirming the MV$^{+•}$ formation upon illumination (but not under dark conditions) (Supplementary Fig. 21) [56]. We first assessed how the reaction system performed in terms of hydrogen production under different conditions. As revealed, a final H$_2$ concentration of 0.21 μmol/μL was obtained after 120 h reaction based on the mineralized cube reaction system (navy blue curve), which was substantially higher than the system
utilizing the excessive amount of free TiO$_2$ nanoparticles (0.014 μmol/μL) (pink curve). In contrast, almost no H$_2$ evolution was found for the cubic system lacking engineered strain (green curve), and for the cubic system containing the engineered strain but lacking mineralized TiO$_2$ (indigo curve). Meanwhile, H$_2$ production was not detected in the SiO$_2$-mineralized scaffolds containing engineered strain (light green curve) (Fig. 5H). Based on the results, we concluded that the optimized conditions for our semi-artificial cubic system: the initial cell density applied is around 1×10$^{9}$ colony forming units (cfu) and solution pH = 8. (Supplementary Fig. 22). Conceivably, the 3D porous cube provides an ideal confined space in which mineralized TiO$_2$ and engineered bacterial are in good contact with each other, ensuring high-efficiency solar-driven hydrogen evolution. In addition, the results indicated that the hydrogen production efficiency of a biological enzymatic system could be substantially enhanced when combined with mineralized TiO$_2$. We envision that our 3D porous mineralized material systems coupled with colonized engineered bacterial may serve as a viable alternative route for conducting various artificial photosynthetic reactions.

CONCLUSION

Diatom frustules represent exquisite masterpiece examples of natural hierarchical composite materials, generating keen interest for exploring various diatom-inspired scaffolds for biomimetic mineralization. Despite important advances, it is still challenging to fully recapitulate the multiscale mineralization features and functions of natural diatom structures in current state-of-the-art biomimetic composites. In our view, the difficulties of biomimetic mineralization research to reconstruct the spectacular morphologies and their remarkable material properties of natural composite materials arise at least in part from the inability to precisely order the molecular recognition and interactions at the organic-inorganic interfaces (for example, closely mimicking the polysaccharide-protein-mineral interactions) across
multiple scales with existing bio-derived or bio-inspired scaffolds. Our self-supporting PPS and porous sponge-like architectures, made of genetically programmable amyloid proteins and polysaccharide chitin, for biomimetic multiscale mineralization have thus brought us a step closer to nature. As demonstrated, the produced 3D architectures can template the formation of diverse minerals across multiple length scales, and the resultant mineralized porous composites can be further utilized for performing solar-driven hydrogen evolution reactions, opening the door for exploiting porous mineralized structures for various artificial photosynthesis systems. Additionally, our studies will spur new interests in designing sophisticated 3D scaffolds for multiscale mineralization by rationally incorporating the hidden molecular recognitions among protein-polysaccharide-mineral interfaces of natural minerals through genetic engineering.

Moving forward, given the genetically engineerable aspect of our mineralization scaffolds, it should be quite straightforward to develop additional biomimetic mineralization fusion proteins by swapping the silica-nucleating R5 peptide for other peptides that are known to mediate the mineralization of inorganic nanomaterials, thereby substantially expanding the versatility and applications of this general biomimetic mineralization design strategy. For example, porous CoPt catalytic materials should be easily generated based on a cobalt-binding peptide Co1-P10 that can control the nucleation of CoPt nanoparticles, while porous hydroxyapatite (HA) composites can be fabricated by applying fusion proteins containing HA-promoting protein domains found in bone.

**STATISTICS**

Data analysis was performed on Origin 2019b software and presented as mean ± s.d. (standard deviation), which were calculated based on at least three replicates. Statistical comparisons between two groups were based on Student’s t test with two tailed distribution, and P-values less than 0.05 was considered statistically significant.

Supplementary data are available at NSR online.
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AUTHOR CONTRIBUTIONS

C.Z. conceived the concept and directed the research. C.Z., K.L. and Y.L. conceived the technical details, designed the experiments, and analyzed the data. M.C. assisted in performing the mechanical tests. B.A. participated in the construction of the plasmid. X.W. and H.P. participated experiments in building up the artificial photosynthesis system. J.L., B.Z. and G.M. assisted in measuring the H₂ production by gas chromatography.

CONFLICT OF INTEREST STATEMENT

The authors have applied for a provisional patent based on this work with the China Intellectual Property Office (PCT/CN2018/085988).
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Figure1. Bio-inspired construction of self-supporting porous structures by using engineered multifunction amyloid fusion proteins as scaffolds for in situ mineralization. (A) Schematic of a typical diatom ordered porous structure comprising a chitin scaffold, silica-associated proteins, and biosilica. (B) Schematic of the components of self-supporting patterned porous sheets (PPS) obtained by engineering strong amyloid/chitin interactions within the nanofiber matrix. (C) Schematic illustration of an artificial photosynthesis system for H₂ evolution based on 3D mineralized R₅CsgA_CBD/chitin cubes.
Figure 2. Morphologies, structural characterization, and mechanical properties of rationally designed self-assembling R5CsgA_{CBD} nanofibers, self-supporting PPS, and their corresponding mineralized architectures. (A-C) Morphological and structural characterization of the native amyloid nanofibers which self-assemble from the R5CsgA_{CBD} fusion proteins: (A) AFM image, (B) TEM images, and (C) X-ray fiber
diffraction pattern. (D) TEM images and STEM-EDS of the silicified \( R_5 \text{CsgA}_{CBD} \) nanofibers. (E) Schematic showing the bio-inspired construction of self-supporting PPS by coupling microtransfer moulding with HFIP ink composed of dissolved \( R_5 \text{CsgA}_{CBD} \) monomers and squid pen polysaccharide β-chitin. (F) Schematic showing biomimetic mineralization of PPS in the presence of TEOS solution to produce silicified PPS. (G-H) AFM images of non-mineralized (G) and mineralized (H) complex PPS structures. (I-J) SEM images of non-mineralized (I) and mineralized (J) complex PPS structures. (K) XPS analysis of the silicified sheets on Al foil at the characteristic binding energies of 102 eV and 153 eV for electrons found in the 2s and 2p3 electron shells of the silicon atom, respectively. (L) ATR-FTIR spectroscopy of the silicified PPS; numbers indicate the peak wavenumbers (in 1095 cm\(^{-1}\)) of the Si–O–Si antisymmetric stretching modes. (M) Young’s modulus of \( R_5 \text{CsgA}_{CBD}/\)chitin PPS before and after silicification. \(* P < 0.05, \) Student’s \( t\)-test. Note: The data was obtained by statistics from 256 \( \times \) 256 spots in a 4 μm\(^2\) square per sample.
Figure 3. Biomimetic mineralization of TiO$_2$ with self-assembling $R_5$CsgA$_{CBD}$ nanofiber networks and self-supporting PPS. (A-C) Morphological, elemental, and structural analysis of the mineralized $R_5$CsgA$_{CBD}$ nanofibers: (A) TEM image, (B) TEM-EDS images and (C) Diffraction pattern. Note: the diffraction ring representative of a typical anatase phase for TiO$_2$ mineral. (D) HR-TEM analysis of a microsphere fragment showing the (101) lattice fringes of the crystal. (E-H) Morphological and structural analysis of the mineralized PPS: (E) SEM image, (F) AFM topography (bottom) and IR absorption (top) images showing where mineralized TiO$_2$ (using Ti-O stretch absorption at 750 cm$^{-1}$) were localized on the sheets. (G) The 3D overlaid image combining topography and IR absorption showing the homogeneous and topographic distribution of TiO$_2$ on the patterned sheets. Note: red and blue color stands for strong and weak Ti-O stretch absorption, respectively. (H) XPS survey spectra of TiO$_2$ mineralized $R_5$CsgA$_{CBD}$ PPS showing the chemical identity.
Figure 4. Morphological characterization and reversible dehydration/hydration behaviors of the fabricated porous $\text{R}_5\text{CsgA}_{\text{CBD}}$/chitin cube. (A-B) Digital photographs of the commercial sugar cube (A) and the fabricated porous $\text{R}_5\text{CsgA}_{\text{CBD}}$/chitin cube in initial hydration state (B). (C) SEM image showing the internal porous structures of the fabricated $\text{R}_5\text{CsgA}_{\text{CBD}}$/chitin cube. (D) SEM image of a zoomed-in area from (C), revealing the cube are composed of nanofibers. (E-H) Digital camera snapshots of the same porous $\text{R}_5\text{CsgA}_{\text{CBD}}$/chitin cube switching between dehydration (dry) state and hydration state in different solutions. (E-F): a dry sample (E) swells and floats at the surface of aqueous solution, resulting in swollen sponge (F). (G-H) the dehydrated sample (G) from (H) re-swells into original sponge-like shape in Congo red solution. (I) Weight changes of the porous $\text{R}_5\text{CsgA}_{\text{CBD}}$/chitin cube as a function of swelling time in aqueous solution. Data are presented as Mean ± SD (n = 3 repeats). (J) Weights of the same cube in hydration and dehydration state after multiple cycles of hydration/dehydration, illustrating the reversible hydration/dehydration behavior of
the porous R5CsgA_{CBD}/chitin cube. Note: To obtain completely dry samples, filter papers and constant N₂ blowing were applied to remove the trapped water in the hydrated samples.
Figure 5. Bio-inspired construction of an artificial photosynthesis system for hydrogen evolution by coupling high surface area, self-supporting, 3-D porous mineralized cubes with colonized engineered *E. coli* cells. (A) Schematic illustration
of an artificial photosynthesis system for H₂ evolution based on the mineralized \text{R}_5\text{CsgA}_{CBD}/chitin cubes, in which mineralized TiO₂ nanoparticles act as light-antennae for converting photons into electrons and engineered \textit{E. coli} cells harboring a hydrogenase gene cluster are in close proximity to the TiO₂ structures, enabling constant hydrogen evolution upon receiving electrons delivered by methyl violet (MV). (B) Photograph image of hydrogen evolution equipment for the artificial photosynthesis systems based on mineralized porous cube structures. (C-D) Morphologies of non-mineralized and mineralized porous \text{R}_5\text{CsgA}_{CBD}/chitin cubes before loading with engineered \textit{E. coli} cells; (C) photograph images of mineralized porous \text{R}_5\text{CsgA}_{CBD}/chitin cubes; and (D) SEM images of mineralized porous \text{R}_5\text{CsgA}_{CBD}/chitin cubes. (E-F) Morphologies of the mineralized porous \text{R}_5\text{CsgA}_{CBD}/chitin cubes after incubation with engineered \textit{E. coli} cells: (E) photographic and (F) SEM images. (G) Transient photocurrents generated by mineralized TiO₂ \text{R}_5\text{CsgA}_{CBD}/chitin porous cubes. The green curve represents the photocurrent of the mineralized \text{R}_5\text{CsgA}_{CBD}/chitin cube structure deposited on FTO glass, when illuminated (on) or shielded (off) from visible light. The black curve represents the photocurrent of non-mineralized \text{R}_5\text{CsgA}_{CBD}-chitin materials on FTO glass. (H) H₂ evolution over time catalyzed with different hybrid systems: the mineralized cube reaction system (navy blue curve), the system utilizing excessive amount of free TiO₂ nanoparticles (pink curve), the cubic system lacking engineered strain (green curve), the cubic system containing the engineered strain but lacking mineralized TiO₂ (indigo curve), and the SiO₂ mineralized scaffolds containing engineered strain (light green curve). Note: The light source used here was a 300 W Xenon lamp (CEL-HXF300, CEAULIGHT) with the illumination intensity of 20.07 mW/cm². A three-electrode system was used for photocurrent testing. A platinum wire and an Ag/AgCl were used as counter and reference electrodes, respectively. The applied potential was 0 V, and the electrolyte solution was 0.5 M Na₂SO₄. The solution was purged with continuous N₂ bubbles for 30 min to get rid of oxygen in the electrolyte cell. The experiments in G and H were replicated at least 3 times with identical results.