Journal of Structural Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Reconstituting the formation of hierarchically porous silica patterns using diatom biomolecules

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ARTICLE INFO

Keywords:
Biomineralization
Biosilica
Morphogenesis
Long-chain polyamines
Organic matrix
Self-assembly

ABSTRACT

The genetically-controlled formation of complex-shaped inorganic materials by living organisms is an intriguing phenomenon. It illustrates our incomplete understanding of biological morphogenesis and demonstrates the feasibility of ecologically benign routes for materials technology. Amorphous SiO_2 (silica) is taxonomically the most widespread biomineral, with diatoms, a large group of single-celled microalgae, being the most prolific producers. Silica is the main component of diatom cell walls, which exhibit species-specific patterns of pores that are hierarchically arranged and endow the material with advantageous properties. Despite recent advances in characterizing diatom biomolecules involved in biosilica morphogenesis, the mechanism of this process has remained controversial. Here we describe the *in vitro* synthesis of diatom-like, porous silica patterns using organic components that were isolated from biosilica of the diatom *Cyclotella cryptica*. The synthesis relies on the synergism of soluble biomolecules (long-chain polyamines and proteins) with an insoluble nanopatterned organic matrix. Biochemical dissection of the process revealed that the long-chain polyamines rather than the proteins are essential for efficient *in vitro* synthesis of the hierarchically porous silica patterns. Our results support the organic matrix hypothesis for morphogenesis of diatom biosilica and introduce organic matrices from diatoms as a new tool for the synthesis of meso- to microporous inorganic materials.

1. Introduction

Diatoms are single-celled, eukaryotic algae that produce silica-based cell walls with species-specific morphologies. A hallmark of diatom biosilica is the presence of hierarchically arranged patterns of pores that have diameters in the range of ~10 nm up to ~1000 nm. The porous architecture endows the material with interesting optical properties including light confinement and selective optical transmission (De Tommasi et al., 2017). Despite the high porosity, diatom silica exhibits a remarkably high mechanical stability (Hamm, 2003; Aitken et al., 2016; Dimas and Buehler, 2012). Morphogenesis of diatom biosilica is therefore regarded as a paradigm for the synthesis of multifunctional low-density, high-strength materials.

Previous research on the cell biology, molecular genetics, and biochemistry of diatom cell wall biogenesis has provided insight into the intracellular organization and molecular composition of the machinery of biosilica formation. This process takes place inside the cell within lipid bilayer bound compartments called silica deposition vesicles (SDVs). The two types of biosilica building blocks (plate- or dome-shaped valves and ring-shaped girdle bands) are produced in different SDVs during different stages of the cell cycle. Valves are produced during cell division and girdle bands during interphase when the cell expands. The deposition of solid silica and its shaping and patterning is accomplished entirely within the valve and girdle band SDVs. When morphogenesis is completed, the SDVs undergo exocytosis and the newly formed valve or girdle band is incorporated into the cell wall. Diatom biosilica is a hybrid material with amorphous SiO_2 as the main component (~90% by weight) and the remainder being organic components, which include unique proteins (e.g., silaffins, silacidins, cingulins, silicanins, SiMat proteins, SAPs), long-chain polyamines (LCPA), and polysaccharides (5). When the silica is dissolved using ammonium fluoride, silaffins, silacidins, and LCPA become solubilized, whereas

Abbreviations: AFSC, ammonium fluoride soluble components; *DAP*, 1,3-diaminopropane; *DLA*, diffusion-limited aggregation; *LCPA*, long-chain polyamines; *NPI*, N-methylated propyleneimine; *PAGE*, polyacrylamide gel electrophoresis; *PI*, propyleneimine; *SDS*, sodium dodecyl sulfate; *SDV*, silica deposition vesicle; *EDTA*, ethylenediaminetetraacetic acid; TMOS, tetramethyl-orthosilicate

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https://doi.org/10.1016/j.jsb.2018.07.005
Received 11 May 2018; Received in revised form 6 July 2018; Accepted 7 July 2018

cingulins, silicanins and SiMat proteins are part of insoluble organic matrices which exhibit mineral-free nanopatterns that match structural features of the silica (Hildebrand et al., 2018).

Previously, several hypotheses have been put forward regarding the mechanism for biosilica morphogenesis. These can be grouped into two main categories: (i) template-independent mechanisms, which rely on diffusion limited aggregation (DLA) (Gordon and Drum, 1994; Parkinson et al., 1999) or a reaction-diffusion system (Willis et al., 2010) and (ii) template-dependent mechanisms (Robinson and Sullivan, 1987; Schmid, 1994; Sumper, 2002; Lenoci and Camp, 2008; Kröger, 2007). In the following paragraph, these hypotheses are briefly summarized.

(i) Template-independent morphogenesis: The DLA process assumes that 1–10 nm-sized silica nanoparticles are imported into the leading edge of the growing SDV lumen, subsequent diffusion throughout the SDV, and nucleation of silica deposition in the SDV center. Computer simulations of this process demonstrated the formation of radial, branched ribs of silica that resemble the spoke-like silica patterns of diatoms (Gordon and Drum, 1994; Parkinson et al. 1999). However, the process is unable to explain the formation of cross-connections between the ribs and the pore patterns in the silica plates. In contrast, computer simulations based on the Turing equation (reaction-diffusion system) have been shown to generate patterns of pores within pre-existing large pores (Willis et al., 2010). It is yet unknown whether reaction-diffusion systems would be able to explain morphogenesis of the basic diatom biosilica architecture that is made of cross-connected ribs. *(ii) Template-dependent morphogenesis* mechanisms rely on biomolecular clusters that self-assemble into patterned organic scaffolds with long-range order. The assembly of the scaffold may occur within the SDV lumen (i.e. internal patterning) (Sumper, 2002; Lenoci and Camp, 2008; Kröger, 2007) or on the cytoplasmic surface of the SDV (i.e. external patterning) (Robinson and Sullivan, 1987; Schmid, 1994). External patterning is thought to involve the cytoskeleton and other intracellular structures (mitochondria, spacer vesicles). Microtubules, actin, mitochondria and spacer vesicles are believed to mold the SDV membrane into the species-specific shapes including indentation patterns that template the pores (Hildebrand et al., 2018; Schmid, 1994). Cytoskeleton filaments have been hypothesized to mediate the positioning of transmembrane proteins that are able to nucleate silica formation in the SDV lumen (Robinson and Sullivan, 1987). In contrast, internal patterning mechanisms are believed to be governed by some of the previously identified biosilica associated biomolecules (see above). *In vitro* experiments have demonstrated that mixtures of silaffins and LCPA undergo phase separation generating biomolecule-rich liquid droplets within the bulk aqueous solution (Poulsen et al., 2003; Poulsen and Kröger, 2004; Sumper and Brunner, 2008). It has been proposed that the formation of hierarchical pores in the diatom genus *Coscinodiscus* is the result of an iterative process of liquid-liquid phase separation in hexagonal arrays of LCPA-rich droplets inside the SDV (Sumper, 2002). Computer simulations of the phase separation process were able to generate diatom-like porous patterns albeit no hierarchical pores were obtained (Lenoci and Camp, 2008). In an alternative model, it was proposed that the porous basal layer of biosilica is templated by a silaffin-LCPA matrix that spans the entire lumen of the SDV. The porous biosilica patterns are believed to reflect the distribution of activating and inhibiting silaffin-LCPA clusters (Poulsen and Kröger, 2004; Kröger and Sandhage, 2010) that define areas of silica deposition and silica free sites, respectively, within the matrix (Kröger, 2007).

The presence of insoluble organic matrices with nanopatterns that match structural features of the silica in diatoms seems to support the model of template-directed silica morphogenesis within the SDV lumen (Reimann et al., 1966; Brunner, 2009; Scheffel et al., 2011; Tesson and Hildebrand, 2013; Kotzsch et al., 2016). However, it has been argued that the insoluble organic matrices may be identical to the diatotepum, which is an organic layer that is added onto the biosilica after SDV exocytosis and thus cannot be involved in silica morphogenesis (Tesson and Hildebrand, 2013). On the other hand, circumstantial experimental evidence has been presented that the insoluble organic matrices from the diatom *Thalassiosira pseudonana* may be largely covered by silica (Kotzsch et al., 2016; Kotzsch, 2017), which would only be possible if the organic matrices are present during silica formation inside the SDV. When exposed to a metastable solution of silicic acid, silica was rapidly deposited on the surfaces of *T. pseudonana* organic microrings (i.e., organic matrices derived from girdle band biosilica), yet no porous silica patterns were observed (Scheffel et al., 2011). It has previously been hypothesized that the soluble biosilica associated components (e.g., silaffins, LCPA, silacidins) need to bind to the insoluble organic matrices to template the formation of porous biosilica patterns (Scheffel et al., 2011). Here we have investigated this hypothesis using the valve-derived organic matrix and the soluble biosilica-associated components from the diatom *Cyclotella cryptica*.

2. Materials and methods

2.1. Chemicals

Tetramethyl-orthosilicate (TMOS), Tris base, cyanoborohydride, formaldehyde, and ammonium formate, chitinase (from *Streptomyces griseus*) and proteinase K (from *Tritirachium album*) were purchased from Sigma-Aldrich. NH_4F, 37% HCl, ethylenediamine tetraacetic acid (EDTA), 85% glycerol, acrylamide, ammonium acetate, β-mercaptoethanol, formic acid (98–100%), acetic acid (p.a.), acetonitrile (Prepsolv®) and sodium dodecyl sulfate (SDS) were purchased from Merck. Tricin base and chloroform were purchased from Roth. Tetramethylethylendiamin and ammoniumpersulfate were purchased from Bio-Rad. Pronase (from *Streptomyces griseus*) was purchased from Roche and methanol from VWR. MilliQ-purified H_2O (resistivity: 18.2 MΩ cm) was used throughout this study except for mass spectrometry analysis for which LC-MS grade H_2O (LiChrosolv®) from Merck was used.

2.2. Culture conditions

C. cryptica strain CCMP332 was grown in an enriched artificial seawater medium according to the Canadian Center for the Culture of Microorganisms at 18 °C under constant light at 5000–10,000 lx for 20–26 days.

2.3. Isolation of ammonium fluoride soluble components (AFSC)

C. cryptica biosilica was isolated according to the same method as described previously for *T. pseudonana* (Poulsen and Kröger, 2004) and incubated with 10 M NH_4F (adjusted to pH 4.5 with 6 M HCl) for 1 h at room temperature to dissolve the silica. After centrifugation (30 min, 3200*g*) the supernatant was dialyzed against 100 mM ammonium acetate (MWCO 6–8 kDa, Spectra/Pore RC) and concentrated using an Amicon Ultra-15 centrifugal filter unit (MWCO 3 kDa; Merck). The concentrated solution contained the AFSC. The proteins in the AFSC were quantified via their phosphate content according to a previously described method (Buss and Stull, 1983).

2.4. Preparation of LCPA and LCPA-free AFSC proteins

To isolate LCPA and prepare LCPA-free AFSC proteins, the isolated AFSC was separated into LCPA and proteins using the following protocol. The lyophilized AFSC was resuspended in 2 M NaCl, centrifuged (4 °C, 30 min, 3200*g*), filtered through a polyethersulfone syringe filter (pore size 0.2 µm; Carl Roth) and subjected to gel filtration chromatography on a Superose 12 10/300 GL column (GE Healthcare) equilibrated with 200 mM ammonium formate (flow rate: 0.4 ml/min). Mixtures of AFSC proteins and LCPA eluted between 17.5 and 43.5 min (fraction F1), and pure LCPA eluted between 45 and 48.5 min (fraction