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Silica Biotechnology

Silica Immobilization of an Enzyme through Genetic Engineering of the Diatom *Thalassiosira pseudonana***

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Organisms are adept in generating inorganic materials (biominerals) with structural and mechanical properties superior to those of abiotically formed minerals. Increasing efforts in interdisciplinary research are aimed at understanding how mineral-forming organisms achieve their outstanding control over the assembly and properties of minerals.[1–16] Emergent knowledge from biomineralization research has been exploited recently for the fabrication of functional hybrid materials under mild reaction conditions in vitro, including the formation of silica and non-silica materials by proteins from sponges and diatoms.[17–20] These methods included immobilization of enzymes and other functional proteins inside a silica matrix. Such materials are attractive for sensor technology (e.g. biosensors, microarrays, microfluidic devices), as reusable biocatalysts for organic syntheses and degradation of harmful chemicals (remediation), and for drug delivery.[21a,b] While a variety of methods are available for protein immobilization by attachment to silica/silicate surfaces or physical entrapment inside silica, such approaches rely on costly reagents and bear a high risk of denaturing the protein.[22–24] In contrast, biomineral-forming organisms have a natural ability for immobilizing proteins, since each biomineral is composed of an inorganic matrix and tightly associated proteins.[1] Of particular interest are the silica-based cell walls of diatoms (frustules), which exhibit highly porous, nanopatterned microshapes with excellent mechanical properties.[25–27] Diatom silica is, therefore, desirable for many applications including use as a support matrix for biomolecules.[28–32]

A method for the chemical attachment of DNA to diatom silica has recently been developed,[33] but attachment of functional proteins has not yet been achieved. Recent progress in analysis of the molecular mechanism of silica biogenesis in diatoms[34] has spurred us to explore a radically different approach for protein immobilization. The approach is based on genetic manipulation of the biological silica-forming machinery and enabled immobilization of the bacterial enzyme HabB in the nanoporous biosilica structures of the diatom *Thalassiosira pseudonana*. This is the first demonstration of an in vivo method for immobilization of an active protein in a biomineral. The method represents a paradigm for utilizing the unique capabilities of biomineral-forming organisms to enable the production of nanopatterned materials with tailored functionalities.

Our studies were performed with the diatom *Thalassiosira pseudonana*, because its biosilica-associated proteins are well characterized[35a] and a genetic transformation system for this organism has been established.[35b] *T. pseudonana* produces cylindrical silica structures containing a vast number of irregularly arranged, spherical nanopores with a very narrow size distribution ((18.3 ± 3.1) nm in diameter)[35c] (see the Supporting Information).

Components of the silica-forming machinery of diatoms include the silaffins, a unique family of phosphoproteins. Silaffins are tightly associated with the diatom silica and can be solubilized only by completely dissolving the silica.[35a,36] We assumed that this tight association results from immobilization of the silaffins during silica biogenesis in vivo. If so, it should be possible for silaffin fusion proteins to become immobilized in the same way, provided the natural activity of the silaffin domain remains intact. To test this hypothesis, we introduced into the *T. pseudonana* genome a fusion gene that encoded the enhanced green fluorescent protein (GFP) linked to the C terminus of the silaffin tpSil3. Expression of the tpSil3-GFP fusion gene was under the control of the constitutive promoter *Pfcp2*.[35b] The GFP fluorescence was localized on the cell surfaces rather than in the interior of the cells, and the fluorescence patterns on the valves corresponded to the porous architecture (see "cell" images Figure 1). Following extraction of the cell contents with a hot solution of sodium dodecyl sulfate (SDS), the GFP fluorescence remained associated with the biosilica (see "cell wall" images in Figure 1). These results indicate that the tpSil3-GFP fusion protein was specifically targeted to the cell wall and became incorporated throughout the diatom silica.

The above experiment did not reveal whether the GFP molecules were encapsulated within the diatom silica (in

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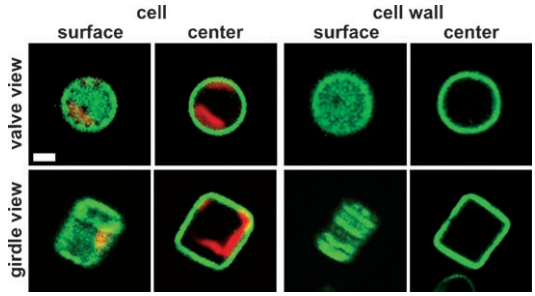

Figure 1. Images obtained by confocal fluorescence microscopy of *T. pseudonana* transformants expressing silaffin fusion protein tpSil3-GFP. The four images in the left half ("cell") were taken from live cells, the images in the right half ("cell wall") were taken from isolated biosilica. Each micrograph is an overlay of two images recorded in the "green channel" (excitation: 488 nm, emission: 505/550 nm bandpass filter) and the "red channel" (excitation: 543 nm, emission: 585 nm long-pass filter). The green color is indicative of GFP, the red color is caused by chloroplast autofluorescence. Scale bar: 2 μm (identical scale for all micrographs).

other words, completely surrounded by silica) or exposed on the silica surface. Fluorescent molecules encapsulated in a silica matrix generally have an increased stability towards photobleaching.[37,38] Therefore, fluorescence microscopy was used to determine the photostability of GFP in selected areas of live *T. pseudonana* cells. In cells expressing the tpSil3-GFP fusion protein, the half-life of GFP fluorescence was (112.4 ± 8.5) s (Figure 2, filled circles). For comparison, *T. pseudonana* cells were generated that expressed GFP without the silaffin moiety leading to accumulation of soluble GFP in the cytosol.[35b] In these cells the decay of GFP fluorescence was significantly faster, exhibiting a half-life of only (59.6 ± 12.0) s

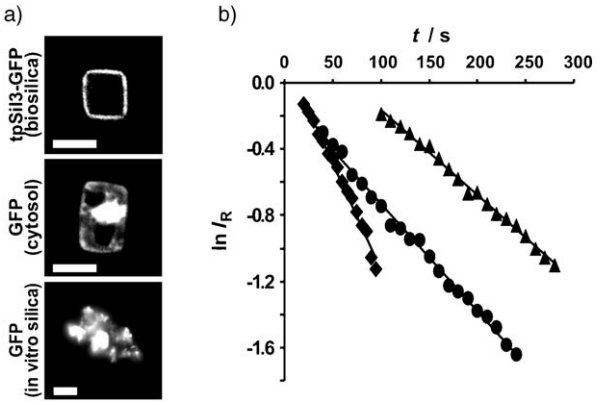

Figure 2. Kinetics of GFP photobleaching. a) Fluorescence microscopy images of *T. pseudonana* cells expressing tpSil3-GFP (top) or GFP (middle), and of GFP encapsulated in synthetic silica (bottom). Scale bars: 5 μm. b) Semi-logarithmic plot of relative fluorescence intensity I_R versus time of UV exposure under a fluorescence microscope. In each case the whole objects were exposed to UV light. Filled circles: live cells expressing tpSil3-GFP; diamonds: live cells expressing GFP in the cytosol; triangles: GFP encapsulated in synthetic silica. Each data point represents the average of at least ten independent measurements. The half-lives ($t_{1/2}$) were determined from the slopes of the corresponding trendlines assuming single-exponential decay of fluorescence photobleaching.

(Figure 2, diamonds). The half-life of GFP encapsulated in silica by an established in vitro method (using the R5 peptide[19a]) was (138.8 ± 16.8) s (Figure 2, triangles), which was significantly longer than the half-life of the diatom silica associated GFP. These results suggested that the GFP molecules associated with diatom silica are protected but not completely enclosed by the diatom silica. This interpretation was supported by protease treatment of diatom silica containing tpSil3-GFP, which resulted in the removal of (82 ± 3) % of the GFP within 4 h (see the Supporting Information).

To investigate whether it is possible to use the silaffin fusion protein approach for incorporating an active enzyme into diatom silica in vivo, we constructed a fusion gene encoding the hydroxylaminobenzene mutase (HabB) from *Pseudomonas pseudoalcaligenes*[39] linked to the C terminus of silaffin tpSil3. The product of the HabB-catalyzed reaction, 2-aminophenol, is a precursor for high-performance polymers and biologically active compounds, and the enzyme has previously been immobilized in silica using an in vitro method.[19b] The tpSil3-HabB fusion gene was placed under control of the promoter *Pfcp2* and incorporated into the *T. pseudonana* genome. Out of seven independent transformants tested, three clones (C1, C2, C3) were positive for silica-associated HabB activity (Figure 3a, squares, circles,

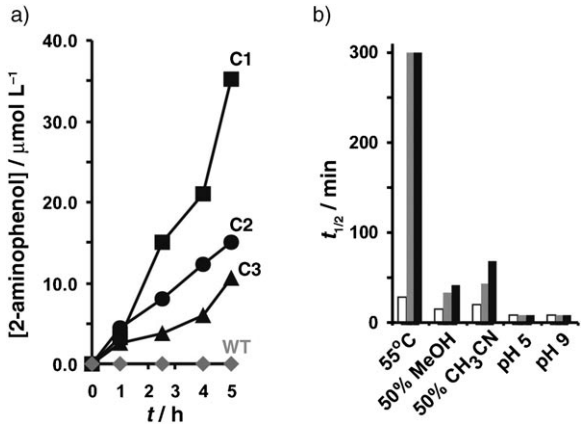

Figure 3. HabB activity in *T. pseudonana* biosilica. The activity was monitored by following the formation of 2-aminophenol from hydroxylaminobenzene.[40] a) Kinetics of 2-aminophenol formation by identical amounts of biosilica (1.2 mg) isolated from wild-type cells (WT, gray diamonds) and transformants C1 (squares), C2 (filled circles) and C3 (triangles). b) Half-life of soluble HabB (white bars), immobilized in biosilica of transformant C1 (black bars), and immobilized in silica produced by the R5 peptide in vitro (gray bars).[19] The HabB-containing samples were subjected to various treatments as indicated (note: standard assay conditions for HabB are pH 7 and 25 °C).

and triangles). No HabB activity was detected in silica from wild-type cells (Figure 3a, diamonds). The average HabB activities of the biosilica materials from the three positive clones varied significantly amounting to (130 ± 27) mU per mg of SiO_2 for C1, (64 ± 8) mU per mg of SiO_2 for C2, and (46 ± 9 mU) per mg of SiO_2 for C3 (1 mU corresponds to the formation of 1 nmol of 2-aminophenol per minute). This variation is most likely a result of different levels of HabB

gene expression, since copy number and site(s) of transgene integration into diatom genomes cannot be controlled.[35b]

To investigate whether the HabB enzyme is stabilized by the immobilization in the diatom silica, the half-life of HabB activity in the biosilica of transformant C1 was compared with that of purified HabB in solution (Figure 3). The silica-associated HabB was more stable in organic solvents (methanol: 2.8-fold higher half-life; acetonitrile: 3.5-fold higher half-life), and much more heat stable (10.3-fold higher half-life at 55 °C). The stability at high and low pH was about the same as that of the enzyme in solution (Figure 3b, compare white and black bars). The stability of diatom-silica-immobilized HabB was at least as good as, and for treatment with organic solvents even better than, the stability of HabB that was silica encapsulated in vitro using a previously described protocol[19] (Figure 3b, compare red and black bars). In long-term-storage experiments diatom-silica-immobilized HabB retained more than 80% of its activity when stored for 30 days at 4 °C or frozen (see the Supporting Information). HabB stability under such conditions was not enhanced by immobilization, because the soluble enzyme was also very stable in long-term-storage experiments.

In this report we have described the discovery that the biomineral-forming machinery of diatoms can be genetically tailored to incorporate functional proteins into diatom silica in vivo. This new strategy for protein immobilization has several striking advantages over the existing in vitro methods: 1) The protein of interest need not be purified; 2) protein immobilization proceeds under physiological conditions that are likely to be compatible with the stability requirements of a large number of proteins; 3) owing to its architecture and hierarchically nanoporous structure, diatom silica exhibits extremely high mechanical stability and excellent flow and separation properties for microfluidic devices;[27,32] 4) production of the functionalized diatom silica material is environmentally benign and sustainable, since growth of photosynthetic diatoms (like *T. pseudonana*) requires only sunlight, water, and mineral salts (including carbonate).

We expect that in addition to enzymes, a wide range of protein-based functional molecules (e.g. peptide hormones, growth factors, antibodies, receptors) may be suitable for immobilization in diatom silica using the method described here. Such silica-based materials would be highly attractive for sensing and drug delivery.[21–23,32] Apart from the technological potential, the molecular genetic methods established in this work will be powerful tools for analyzing the molecular mechanism of diatom silica formation. This knowledge may eventually enable tailoring the nanopatterned diatom silica structures. The molecular genetic manipulations reported here (expression of tpSil3-GFP and tpSil3-HabB) did not result in changes of the *T. pseudonana* silica structure as determined by scanning electron microscopy.

Experimental Section

Cells expressing silaffin-GFP fusion proteins were extracted by incubation in buffer A (1% SDS, 100 mM EDTA) for 30 min at 50 °C, and were then subjected to washing with H_2O and acetone ($3 \times H_2O$, $1 \times$ acetone, $3 \times H_2O$) to obtain colorless biosilica. Since SDS and acetone partially inactivate HabB, the non-ionic detergent octylphenyl-polyethylene glycol (Igepal CA-630) was used for biosilica isolation. The extraction and washing procedure was modified for all experiments involving HabB as described in the following: Cells were suspended in buffer B (100 mM EDTA, 20 mM sodium phosphate pH 7.0) and sonicated five times for 45 s each time. The crude biosilica fraction was collected by centrifugation (4 °C, 5 min, 13200 *g*), washed three times with buffer B, and extracted with detergent solution (1% Igepal CA-630, 20 mM sodium phosphate pH 7.0) at 4 °C, for 1 h. The extracted biosilica was collected by centrifugation (4 °C, 5 min, 13200 *g*) and washed five times with buffer B.

GFP (containing a C-terminal His_6 tag) for in vitro encapsulation was expressed in *E. coli* and purified by immobilized metal ion affinity chromatography. Gene habB was cloned as a His-tagged derivative, expressed in *E. coli*, and purified by affinity column chromatography. The details of the method will be described elsewhere. Enzyme activity of HabB was determined by monitoring the conversion of hydroxylaminobenzene into 2-aminophenol using HPLC as described previously.[40] Silica encapsulation of HabB and $GFPHis_6$ was performed using the R5 peptide as described previously.[19] The three HabB-containing preparations (each containing 0.1 U mL^{-1}) were incubated under the following conditions: 1) in 25 mM sodium phosphate pH 7.0 at 55 °C, 2) in 25 mM sodium phosphate pH 7.0 and 50% organic solvent (acetonitrile or methanol) in H_2O, 3) in 25 mM phosphate buffer pH 5.0 or pH 9.0. Samples were removed periodically and enzyme activity was determined.

For description of cloning procedures, fluorescence microscopy and photobleaching experiments, see the Supporting Information.

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