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Nanoscale architecture

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2006

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Hazelaar, S. (2006). *Nanoscale architecture: The role of proteins in diatom silicon biomineralization*. s.n.

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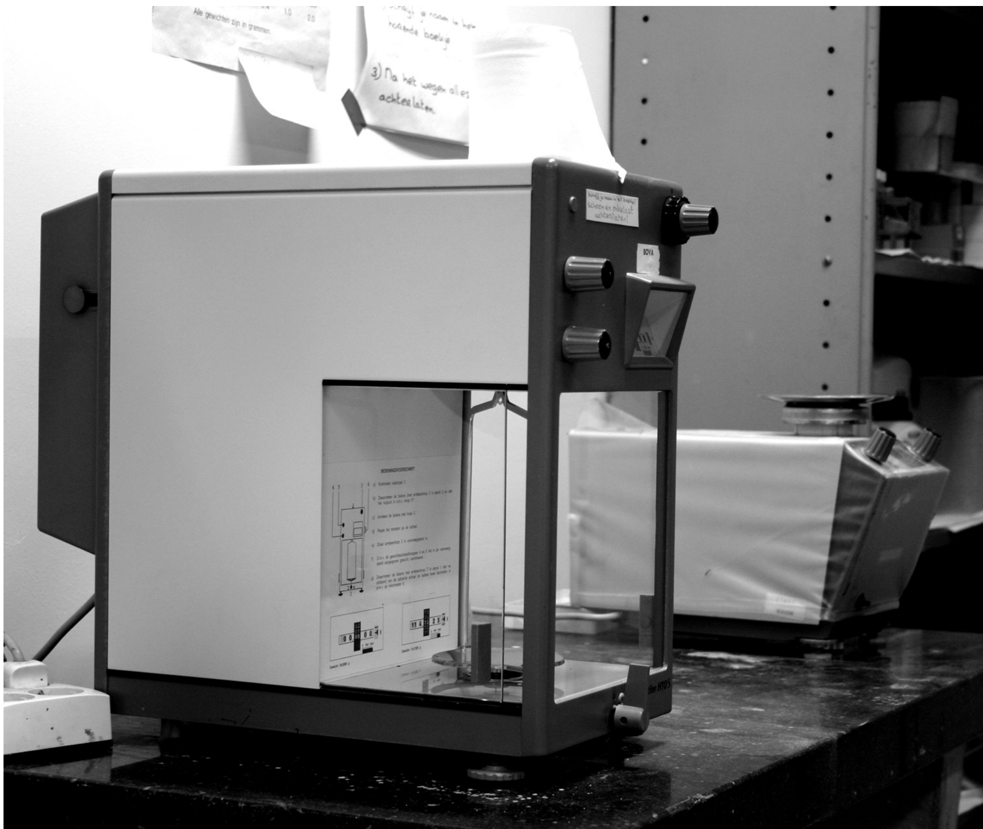
Summary



'Diatoms', in the scientific literature known as the heterokontophyte class of Bacillariophyceae, are either 'centric' (discoid or cylindrical cells with a radial symmetry of the cell wall) or 'pennate' (exhibiting a bilateral symmetry). There are more than 250 genera of living diatoms, with more than 10,000 different species, which all have a species-specific cell wall structure and cell shape and size. The diatom cell wall, the 'frustule', is composed of two overlapping valves that fit together like a petri-dish. Wide and small angle X-ray scattering studies have recently confirmed the suspicion that the rigid siliceous part of the cell wall consists of amorphous silica. This silica is enclosed in an organic casing composed of polysaccharides, lipids, and proteins that protect the siliceous structures against dissolution in both fresh and salty marine water environments.

During cell division every cell divides in its own frustule, whereafter each of the two daughter cells a new hypovalve develops that starts to grow in a cell organelle called the silica deposition vesicle (SDV). In this SDV morphogenesis of the frustule architecture is accomplished, a process during which polypeptides such as silaffins and long-chain polyamines (LCPAs) are believed to play a role in silica precipitation, and possibly in the subsequent structure-direction of the cell wall.

A constant "rain" of dead diatoms to the bottoms of seas, oceans and lakes has resulted over millions of years in thick geological deposits known as 'diatomaceous earth'. It has been mined from quarries where these deposits have reached the surface in several regions on land. This silica source has been used in many applications, but mainly as filler or abrasive; the most spectacular application, however, is seen in the stabilization of the very explosive nitroglycerine in the production of dynamite by Alfred Nobel. Because of aging and impurities due to contamination with unwanted catalytic elements, the use of diatomaceous earth has remained rather limited. Artificial silicas therefore have been produced on an industrial scale to obtain specific porous structures and surface properties that make them useful for applications such as filling materials, filter agents, catalyst support, ion-exchange materials, whiteners in for instance washing powders, tooth pastes, paints, pharmaceuticals, cosmetics, and even in rubber industries (mainly in tires). In general artificial silicas are produced at high temperatures and pressures, and often under reaction conditions where very strong acids are used. In contrast, silicon biomineralization in *diatoms* proceeds under ambient conditions. The Research and Development divisions of silica industries are therefore, and not surprisingly, interested in understanding Nature's ways of mineralization and in particular to mimic these processes to obtain materials that resemble the striking natural features such as the meso- to macroporous patterns in diatom frustules. So far, little was known of the molecular and physico-chemical aspects of diatom biomineralization. Experimental work with model compounds has suggested that organic molecules are essential as inducers of silica precipitation or as structure-directors in the growth of artificial micro- and mesoporous materials and zeolites.

Recently, several proteins have been identified that seem to play key roles in diatom silica precipitation. The mechanistic principles of the protein-silica interactions, however, remain elusive, but are considered of crucial importance for understanding the natural processes that give rise to the complicated frustule architecture in diatoms.

The aim of this thesis was to identify novel proteins that are involved in silica cell wall formation in diatoms, and to determine their role in silica structure formation at the nano- to micrometer scale.

In order to focus on proteins involved in silicon metabolism of diatom cells, affinity for solid silica was chosen as the selection criterion using the species *Navicula pelliculosa* (Brébisson et Kützing) Hilse as model organism. One protein isolated in this way appeared to be a homologue of ubiquitin based on its N-terminal amino acid sequence. Ubiquitin is a highly conserved 8.6 kDa protein involved in protein degradation. Immunocytochemical localization of ubiquitin in *N. pelliculosa* indicated that it is not only present intracellularly. It is located along the cell walls and inside pores; in particular during certain stages of valve formation in dividing cells. This finding agreed with results of a previous study in our group on the role of peptides and polymers involved in phase separation processes, which had led to an upscaling model to explain micromorphogenesis in diatoms. This model implies that during silica formation protein-rich and silica-rich phases separate, a process that depends on organic molecules present inside the SDV and where the smaller molecules (silaffins and LCPAs) induce rapid silica precipitation, while larger molecules induce phase separation and subsequently become enclosed as protein-rich phases when the silica-rich phases solidify and collide to the solid silica. This model seemed attractive but so far the natural inducers of phase separation were unknown; they were expected to be involved in pore formation since protein-rich phases do remain present in the final cell walls. How the protein-rich phases are removed, finally leaving cavities (the pores) in the silica, during valve formation in diatoms has not been determined yet. With the identification of ubiquitin and its apparent specific localization along the cell walls in dividing cells a hypothesis has now been formulated: the protein degradation machinery depending on the ubiquitination pathway is involved in the removal of the protein-rich phases (chapter 2).

The next step (chapter 3) was to determine at what stage the ubiquitination machinery is activated during cell wall formation. Therefore, expression levels of ubiquitin, present as poly-ubiquitins or ubiquitinated proteins, were assessed in cells of *N. pelliculosa* over the period of valve formation by analyzing cell-free extracts containing soluble proteins and in two fractions that contained cell wall-associated proteins (EDTA- and SDS-extracts). It was demonstrated that 7 soluble and 6 SDS-extractable proteins ((poly)ubiquitin and ubiquitinated proteins)

identified by Western blotting fitted significantly to positive second order polynomial correlations, indicating an increased amount of ubiquitin in the first 120 min of valve formation followed by a steady decrease in the remaining period. For EDTA-extractable proteins no clear correlations were found. After analyzing the contribution of large (> 50 kDa) and small (< 50 kDa) ubiquitinated proteins – on the basis of the ratios of their abundance between cell free-, EDTA-, and SDS-extracts – a clear difference in expression patterns of ubiquitinated proteins was observed between early stages of valve formation (< 90 min) and the later stages. The obtained results suggest that ubiquitination of proteins associated with the diatom cell wall during cell wall formation is an important process where the timing of the process depends on the phase of cell wall formation. The ubiquitination machinery could therefore have a functional role in diatom valve formation.

Using the fluorescent probe 2-(4-pyridyl)-5-((4-(2-dimethylamino-ethylamino-carbamoyl) methoxy)phenyl)oxazole (PDMPO) the timing of the early formation of the new valve was visualized (chapter 4), confirming that 2D expansion of the valve is a rapid process that is completed in less than 15 minutes in *Navicula salinarum* (Grunow) Husted. The 3-D completion of the valve appeared to proceed slower, lasting most of the time valve formation takes. The results are clearly relevant in understanding the timing of molecular processes involved in valve formation in relation to micromorphogenesis (chapters 2 and 3). Also, PDMPO proved to be excellent for studying distinct developmental stages for further detailed analyses of diatom biosilica formation and it was suggested that with the aid of fluorescent probing new strategies for enrichment of SDV factions could be developed.

Other proteins that were identified in *N. pelliculosa* using the silica affinity approach were actin, oxygen-evolving enhancer protein, and ferredoxin-NADP reductase (FNR). The latter was studied in more detail because it appeared to be a homologue of carbonic anhydrase and silicase after alignment of their amino acid sequences. Silicase has been first identified in sponges where it was proposed to be involved in silica restructuring and possibly maintenance. The proposed mechanism of action is the ability of silicase to bind a zinc ion which functions as a so-called Lewis acid that acts as a catalyst in cleaving the ester bond between silicon and oxygen. Because of the homology and ability of FNR to bind zinc, it was hypothesized that this enzyme identified in *N. pelliculosa* could function in a similar way in diatom biosilica restructuring or maintenance.

In order to test this hypothesis the genome of *Thalassiosira pseudonana* Hasle and Heimdal was mined for homologues of *N. pelliculosa* FNR. Three FNR genes with homologous nucleotide sequences were found to be present in the *T. pseudonana* genome (newV2.0.genewise.31.175.1, newV2.0.genewise.39.343.1, and newV 2.0.genewise.91.65.1) for which the expression was quantified by Quantitative Polymerase Chain Reaction (Q-PCR) for cells grown at three defined conditions;

namely, during synchronized cell division, during silicon limitation, and during a light-dark-light regime (which dictates photosynthesis activity). The mRNA expression patterns of the 3 FNR genes did not correlate with the observed stages of valve and girdle band formation (that was determined also by PDMPO probing). FNR is known to play a role in photosynthesis and as such it was difficult to assign a clear role of FNR in the silicification events in *T. pseudonana* cells. FNR's potential role in silica restructuring was also tested *in vitro* by incubating diatom biosilica with a commercially available homologue (spinach FNR) and monitoring silicic acid release. In addition, the occurrence of nanostructural changes of defined artificial silicas and diatom biosilicas was examined by X-ray scattering analysis. It was shown that spinach FNR did not enhance release of silicic acid *in vitro* and that also the nanostructural characteristics of the tested silicas did not change. Although FNR is homologous to silicase (and also carbonic anhydrase), has a relatively high affinity for solid silica, and is known to bind a catalytic zinc ion, no evidence was found for a role of FNR in restructuring silica *in vitro*. Therefore, it was not considered likely that FNR plays a direct role in silica restructuring or silicon resorption in diatoms. It is not excluded, however, that restructuring of silica occurs in diatoms, since other proteins (e.g. carbonic anhydrase) may well be involved in this process. Restructuring processes have never been assessed in diatom silicon biomineralization research, but this notion provides perspectives for new studies that eventually may become useful for designing biomimetic approaches resulting in industrial or nanotechnological applications.

Concluding remarks

Our research has been performed from a biological point of view, whereas our partners in the project from Eindhoven Technical University complemented this work by adding the silica chemistry. This dual approach provided the project with a significant added value. At the start of our joint project ´biomolecules´ had never been used to artificially produce porous silica. Instead, polymers such as polyethyleneglycol (PEG) and derivatives thereof were used as cheap substitutes for natural compounds in experiments to reveal the action of organic molecules on silica formation. Using PEG of different chain lengths and at various PEG/waterglass ratios it appeared to be possible to create silicas containing mesopores with diameters between 2 and 20 nm; the obtained silicas did not resemble morphologies of diatom frustules. Organic polymers, however, certainly can be considered as attractive substitutes for biomolecules such as the proteins, enzymes and carbohydrates in silica production. In view of the studies on phase separation processes, our partners at the Eindhoven Technical University were further able to obtain laminarily ordered hollow silica spheres, using block-copolymers as

surfactants. These hollow spheres have potential for use in drug-delivery applications. Summarizing: nature's ways of silicon biomineralization have inspired us to create artificial structures with physico-chemical properties that quite well resembled the biological ones.

Already in 1960 it was suggested that diatom valve formation is a very fast process. Forty-five years later we have been able to visualize the intermediate stages of diatom valve formation, producing clear evidence that this is a very rapid process indeed. It already is confirmed for different species such as *Navicula pelliculosa, N. salinarum* and *Thalassiosira pseudonana*. These visualizations are a first step to tune in on specific developmental stages for further detailed analysis of diatom biosilica formation. Fluorescent probing is a good basis that may well lead to sensitive strategies for enrichment and purification of SDV fractions, enabling a precise characterization of the proteins and reaction conditions inside the SDV.

The notion that the activity of the ubiquitination machinery correlates to valve formation in diatoms is in line with the hypothesis developed in our group that pore formation in frustules during silicon biomineralization implies the removal of protein-rich phases to leave pores in the formed silica. By identifying the cell wall associated proteins that become targeted for degradation and unravel their specific role prior to ubiquitination a better insight can be gained in how diatoms make their fabulous siliceous cell wall structures. The collaboration with the Eindhoven Technical University should be maintained in order to functionally characterize the role of these proteins in silica syntheses using biophysical methods.