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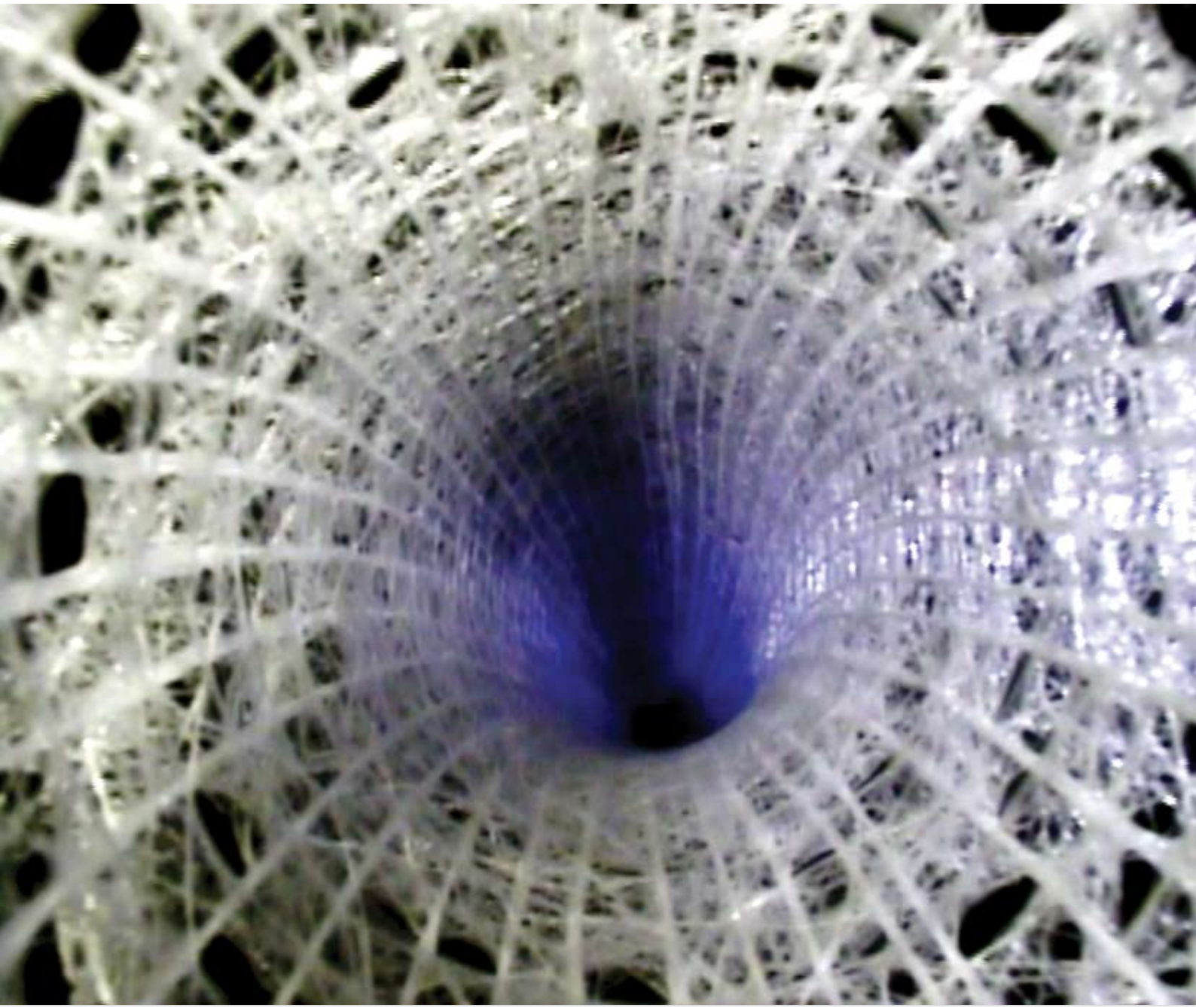

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REVIEW
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Biofabrication of biosilica-glass by living organisms

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Biosilicification is an evolutionarily old and widespread type of biomineralization both in unicellular and multicellular organisms, including sponges, diatoms, radiolarians, choanoflagellates, and higher plants. In the last few years combined efforts in molecular biology, cell biology, and inorganic and analytical chemistry have allowed the first insight into the molecular mechanisms by which these organisms form an astonishing variety of siliceous structures that cannot be achieved by chemical methods. Here we report about the present stage of knowledge on structure, biochemical composition, and mechanisms of biosilica formation, focusing our attention particularly on sponges because of the enormous (nano)biotechnological potential of the enzymes involved in this process.

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1 Introduction

Biogenic silica ("biosilica"), consisting of glassy amorphous silica, is formed in many aquatic and terrestrial organisms including sponges, diatoms, radiolarians, choanoflagellates, and higher plants.[1–4] The turnover of silicon by silica-forming organisms is enormous. Marine organisms process about 6.7 gigatonnes of silicon every year to build their silica skeletons.[5] Biosilicifying organisms including sponges (approx. 10 000 species) and diatoms (approx. 100 000 species) are able to form a huge variety of biosilica structures which are species-specific and often used as systematic characters for a given species. Biosilicification has also become an inspiration ("Nature as model") for the development of novel fabrication procedures in nanobiotechnology. Technical production of silica commonly requires high temperatures and pressures, and extremes of pH. Living organisms are, however, able to form silica under ambient conditions, at low temperature and pressure and near-neutral pH.[4] Moreover, they produce their silica skeletons with high fidelity. Understanding the mechanism(s) of biosilica formation as well as identification of the components involved in this process and the constituents of biosilica is therefore of high importance for the technological/industrial application of the unique synthetic abilities of silica-forming plants and animals.

Silica is, in principle, a mechanical fragile material. However, siliceous organisms use silica as a composite material. Several different classes of biomolecules have been found to be associated with or embedded in the biosilica, including enzyme proteins,[6–12] glycoproteins,[13] polyamines[14] and polyamine-modified peptides.[15–17]

Here we report about the present stage of knowledge on the structure, biochemical composition, and mechanisms of formation of biosilica, mainly in sponges, diatoms and higher plants. We focus our attention particularly on biosilicification in sponges because of the enormous (nano)biotechnological potential of the sponge enzymes involved in this process.

2 Silica: chemical aspects

At concentrations above 1–2 mM and neutral pH, orthosilicic acid [$Si(OH)_4$] undergoes a series of polycondensation reactions, leading to the formation of siloxane (Si–O–Si) bonds.[1,18] Such condensation reactions may involve (i) the reaction between two un-ionized silicic acid molecules or (ii) the reaction between an ionized and an un-ionized silicic acid molecule. The first process results in the release of water without a change of the pH of the system, while a hydroxyl ion which (in non-buffered media) increases the pH of the system is liberated in the latter process. The condensation reaction is based on a nucleophilic

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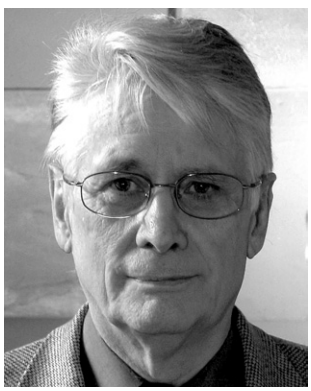

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substitution (S_N2) reaction, resulting in the intermediary formation of a pentacoordinated silicon species.[3]

The condensation process initially results in the formation of dimers which react preferentially with monomers to form trimers and higher oligomers; the latter silicic acid species easily cyclize because of the proximity of the chain ends to form rings of three to six silicon atoms. Oligomer formation renders the silicon atoms more electrophilic (increase in the density of ionized silanol groups), which then become preferential sites for the addition of further silicic acid monomers. The Ostwald ripening process then leads to the growth of larger, less soluble particles by deposition of silicic acid released from smaller, more soluble particles.[3,19] The aggregation of silica particles in solution follows a fractal growth process.[20]

As the pK_a values for the silanol groups decrease with increasing size of the particles (monomeric silicic acid is weakly acidic with a pK_a of 9.8),[18] larger silica particles are ionized and have a negative charge.[3] Therefore, the presence of positively charged molecules or ions is required to neutralize the negative, repulsive surface charge of these silica particles, so that further aggregation can occur. It is well known that the particle size, pore structure, as well as the kinetics of the process of silica formation can be modified by the presence of additives.[18] The condensation process is promoted by the presence of metal cations, which favor silica aggregation by decreasing the negative charge of the silicate particles above pH 7,[21] as well as by cationic polyelectrolytes.[22] Polyamines such as polylysine, polyarginine, and polyallylamine promote the aggregation process by adsorption of silicic acid monomers and oligomers onto the amino groups of these molecules.[23,24]

The polymeric amorphous silica formed consists of a covalently linked network of tetrahedrally coordinated and randomly arranged siloxane centers.[1] It exhibits a variable number of free silanol groups and a variable extent of hydration. Data obtained by infrared spectroscopy indicate that up to one of the four oxygens of the silica tetrahedra exist in the form of a free silanol group;[25] both fully condensed centers (Q_4 sites) and partially condensed centers with one (Q_3), two (Q_2), and three hydroxy groups (Q_1) can be distinguished. The extent of condensation of the silanol groups can be determined also by solid-state ^{29}Si NMR spectroscopy. In addition, the SiO_4 tetrahedra show variable Si–O–Si bond angles and Si–O bond distances.[3]

3 Biosilica structures

3.1 Sponge spicules

Sponges (Porifera) evolved prior to the Cambrian explosion, more than 525 Myr (million years) ago, and are the oldest Metazoa still existing that use silica as a biomineral to form their inorganic skeleton.[26–28] It should be noted that only two classes of sponges, the Demospongiae and the Hexactinellida, have a silica skeleton, while the evolutionary younger third class of sponges, the Calcarea, has spicules made of calcium carbonate.[29] The biosilica produced by sponges, as also the biosilica formed by other organisms, consists of glassy amorphous silica. Small-angle and wide-angle X-ray diffraction analyses of sponge spicules,[30] diatom cell walls[31] and higher plants[32] gave no evidence for the existence of quartz (crystalline silica) in biosilica. Sponge biosilica has a high water content (6 to 13%).[33–35] High resolution magnetic resonance microimaging studies revealed that the water in sponge spicules is largely present in a 'mobile' form.[36] Spicules contain, besides silicon and oxygen, small or trace amounts of a number of other elements (mainly Al, Ca, Cl, Cu, Fe, K, Na, S, and Zn),[35,37,38] which may influence the properties, *e.g.* the refractive index, of biosilica.[39] The silica content of sponges can amount to 75% or more of the dry mass of the animals.[6]

Sponge spicules can be subdivided into megascleres/macroscleres (*e.g.* oxeas) and (smaller) microscleres (*e.g.* spheraster) (Fig. 1).[40] The skeleton of the demosponge *Suberites domuncula* (Fig. 2A) which has been used as a model to study spicule formation (reviewed in refs. 10 and 36) is composed of only two types of megascleres, monactinal tylostyles, which form the main fraction of spicules of this siliceous sponge, and a smaller fraction of diactinal oxeas. The tylostyles have one pointed end and one swollen knob (Fig. 1A), whereas the oxeas have two pointed ends. The spicules of *S. domuncula* can reach lengths of up to 450 μm and diameters of 5 to 7 μm (Fig. 2B). *S. domuncula* is currently the best-studied sponge on a genetic level (reviewed in ref. 10).

In the center of siliceous spicules is an axial canal 0.3 to 1.6 μm wide (Fig. 2C) which harbors an organic (proteinaceous) axial filament (Fig. 2D). The silica is deposited around this axial filament to form concentric layers ~0.3 to 1 μm thick.[38,41] These lamellar silica layers become visible after partial etching of broken spicules (Fig. 2C) and are particularly prominent in spicules from hexactinellid sponges, *e.g. Hyalonema sieboldi* (Fig. 2E,F). Analyses by high-magnification scanning electron microscopy (SEM) and atomic force microscopy (AFM) revealed that the biosilica in spicules has a nanoparticle substructure with a diameter from about 70 nm[42,43] to 100–200 nm.[44]

The formation of sponge spicules is a fairly fast process, *e.g.* in the freshwater sponge *Ephydatia fluviatilis* (spicule length

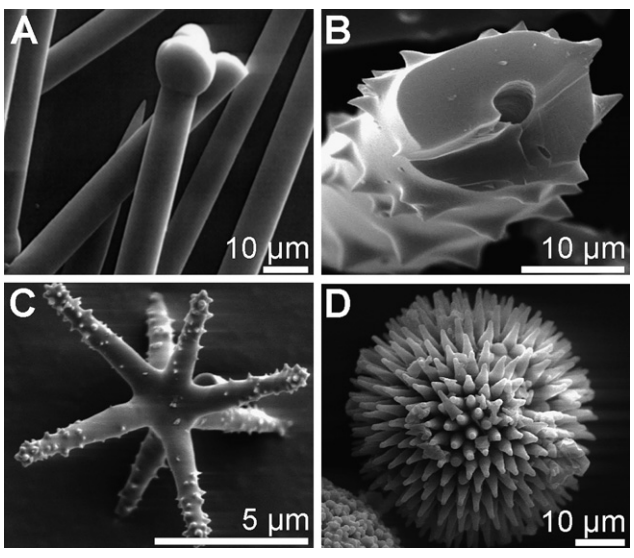


Fig. 1 SEM analysis of (A) spicules from the marine demosponge *Suberites domuncula*, (B) a spicule from the freshwater demosponge *Lubomirskia baicalensis*, (C) spheraster and (D) sterraster from the marine demosponge *Geodia cydonium*.

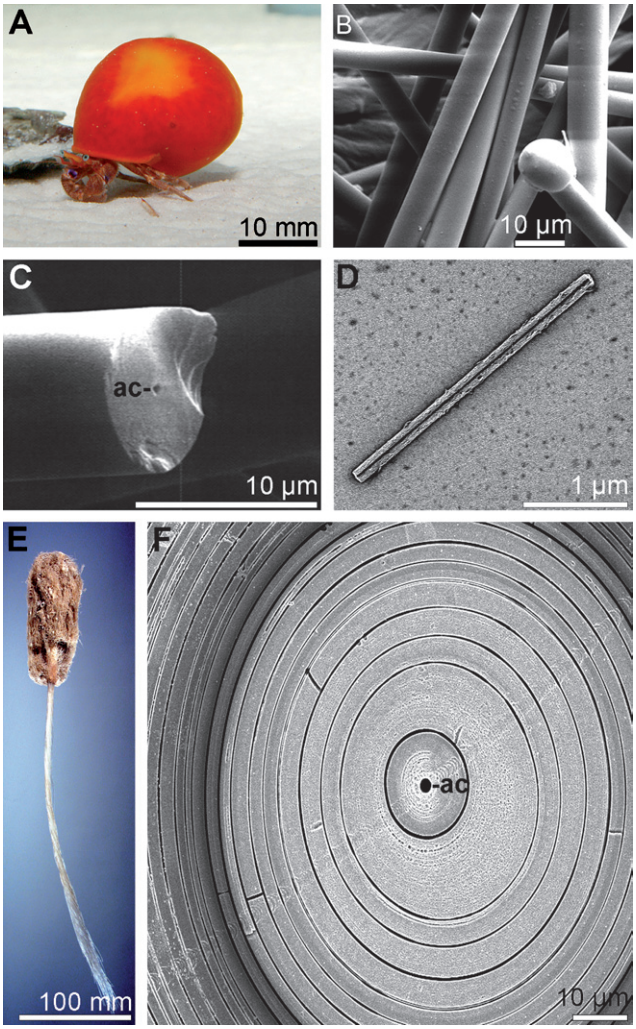


Fig. 2 (A) The demosponge *Suberites domuncula*. SEM analysis of (B) spicules and (C) a broken spicule from *S. domuncula* showing the axial canal (ac). (D) Axial filament. (E) The hexactinellid sponge *Hyalonema sieboldi*. (F) Cross-section (polished) of *H. sieboldi*.

100–300 µm), they are formed within 40 h.[45,46] The 3D-primmorph culture system of sponges is a very suitable model to study spicule formation.[9] Primmorphs are cell aggregates formed from sponge single cells in the presence of Ca^{2+} ions that can be obtained from marine and freshwater demosponges (examples: *S. domuncula*[47,48] and *Lubomirskia baicalensis*[49]). Primmorphs contain totipotent archaeocytes which can differentiate to the spicule-forming cells, the sclerocytes, in the presence of exogenous silicate.[50] A number of genes involved in biosilica formation and spiculogenesis are upregulated in primmorphs in the presence of silicon, such as those encoding silicatein,[8] silicase,[51] galectin,[12] collagen,[8,52] and noggin, a morphogenetic protein.[53] The optimal concentration of silicon for spicule formation in *S. domuncula* primmorphs is about 60 µM.[8]

3.2 Diatom frustules

Diatoms are unicellular algae living in marine and freshwater habitats; they are divided into two classes, the radially symmetric centrics, and the pennates with an axis of symmetry. Diatoms

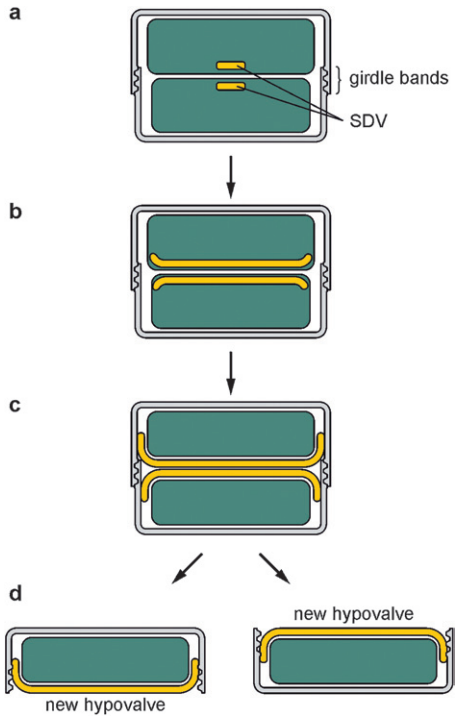


Fig. 3 The diatom reproductive cycle. The two daughter cells are formed inside the parent cell (a). The new hypovalve is formed within the SDV of each daughter cell (b), which is then released by exocytosis (c) before the cells separate (d).

arose later in evolution than sponges (~260 Myr ago). It is assumed that their occurrence resulted in a strong reduction of the silicon levels of the oceans.

The nanostructured silica patterns of the diatom cell walls (frustules) are species-specific and genetically determined. The frustules are built by silica nanoparticles (50–100 nm) that are surrounded by carbohydrate- and protein-containing organic matrices. They consist of two parts, the epitheca (upper half) and the hypotheca (lower half), which are composed of a valve and several girdle bands (silica strips) (Fig. 3).[54] The valves are usually perforated with pores that show a species-specific pattern. The epitheca and the hypotheca overlap in the girdle band region. Both thecae are produced in a specialized vesicle, the silica deposition vesicle (SDV). The SDVs form a weakly acidic compartment (pH 5).[55] Each daughter cell formed during the diatom reproductive cycle has one original frustule and one new valve (Fig. 3). The formation of a new valve is very fast, as demonstrated in studies with synchronized cells of the diatom *Navicula salinarum*, using fluorescent stains (PDMPO and rhodamine 123) which are entrapped in the newly formed silica.[56] The architecture and the biosilica composite material of the frustule exhibit remarkable mechanical strength and may provide mechanical protection to the diatom cell.[57] In addition, diatom silica has been proposed to exhibit proton-buffering activity.[58]

3.3 Biosilica in higher plants

Less is known about biosilica in higher plants than about biosilica in sponges and diatoms. Silicon accumulation and silica deposition vary considerably among different plant species.[59]

The silicon content of the shoot dry weight may range from 0.1% to 10% in silicon-accumulating plants such as rice (*Oryza sativa*).[60–63] Silica is deposited on the cell wall of epidermal cells of leaves, stems, and hulls[64,65] where it provides structural support and increases the resistance of plants to diseases, drought and other biotic and abiotic stresses.[60–62,66–69] The silica–cuticle double layer may act as a physical barrier against the penetration of fungi and other pathogens into the epidermal cells.[68] Silicon is furthermore able to induce some enzymatic stress response mechanisms, *e.g.* the activity of peroxidases, polyphenol oxidases and chitinases.[68,70] Induction of systemic acquired resistance (SAR) in cucumber (*Cucumis sativa*) has been shown to involve the expression of a gene encoding a strongly cationic proline-rich protein (PRP1) with a high amount of lysine and arginine residues; a synthetic peptide derived from this sequence was able to precipitate silica.[71]

3.4 Vertebrates/humans

Silicon is also an essential element in human and many other vertebrates. It is the third most abundant trace element in the human body. The highest silicon concentrations are found in connective tissues such as in bone, skin and blood vessels.[72] Dietary silicon has a critical role in the development of the extracellular matrix (collagen) and bone (hydroxy-apatite) formation.[73–76] Silicon stimulates the formation of type I collagen in human osteoblasts and osteoblast-like cells;[74] it may be involved in the radical-dependent prolyl-hydroxylase pathway during type I collagen formation.[77] Silicon has been identified as a constituent of certain glycosaminoglycans, [78,79] and is absorbed in the gastrointestinal tract in the form of orthosilicic acid.[80]

4 Biosilica formation

4.1 Sponges: silicatein

The major breakthrough in understanding the molecular mechanism of spicule formation was the discovery of the principle enzyme involved in this process. The axial canal of the spicules harbors an organic filament, the axial filament. This axial filament consists predominantly of a cathepsin L-related enzyme, which was first described by the group of Morse[6,7] and termed silicatein. Their studies (reviewed in ref. 43) and later studies using biocatalytically active recombinant silicatein[8,11,12] (reviewed in ref. 81) demonstrated that silica formation in sponges is an enzymatic process. Hence, the mechanism of biosilicification in sponges is distinct from the mechanism of silica formation in diatoms, which does not involve any enzyme and is mediated by polyamines[16] and/or polycationic peptides (silaffins).[15,82] Native silicatein isolated from axial filaments as well as the recombinant protein are capable of forming silica from soluble silicon alkoxide precursors in solution.[6–8,81]

4.1.1 Silicatein genes/cDNAs. The first silicatein cDNA has been cloned from the marine demosponge *Tethya aurantium*; two isoforms of silicateins (silicatein-α and silicatein-β) have been characterized.[7] Shortly afterwards, the genes/cDNAs encoding silicatein-α and silicatein-β were cloned from *S. domuncula*[8,10,53,83–85] (Fig. 4), and then from other marine sponges (*Petrosia ficiformis*),[86] as well as from freshwater sponges.[87–89] Besides the silicatein cDNA from the ubiquitous freshwater sponge *E. fluviatilis*,[87] the genes/cDNAs coding for four isoforms of silicatein-α (silicatein a1 to a4) have been identified and characterized in the endemic Baikalian freshwater sponge *L. baicalensis*.[88,89]

The silicateins are related to the cathepsin family of proteases; the greatest similarity is shared with cathepsin L (Fig. 4).[6,8,85] The most striking difference between the two groups of enzymes is the presence of a serine residue in the catalytic center of silicateins[6,8] instead of a cysteine residue present in the cathepsins[90] (Fig. 4). The serine residue in silicatein is thought to be essential for the catalytic mechanism of the enzyme (see below). Besides the three amino acids Ser, His and Asn, which form the catalytic center (catalytic triad) of the silicatein, a cluster of hydroxy amino acids (serine cluster) is present (Fig. 4),[6,8] which is located

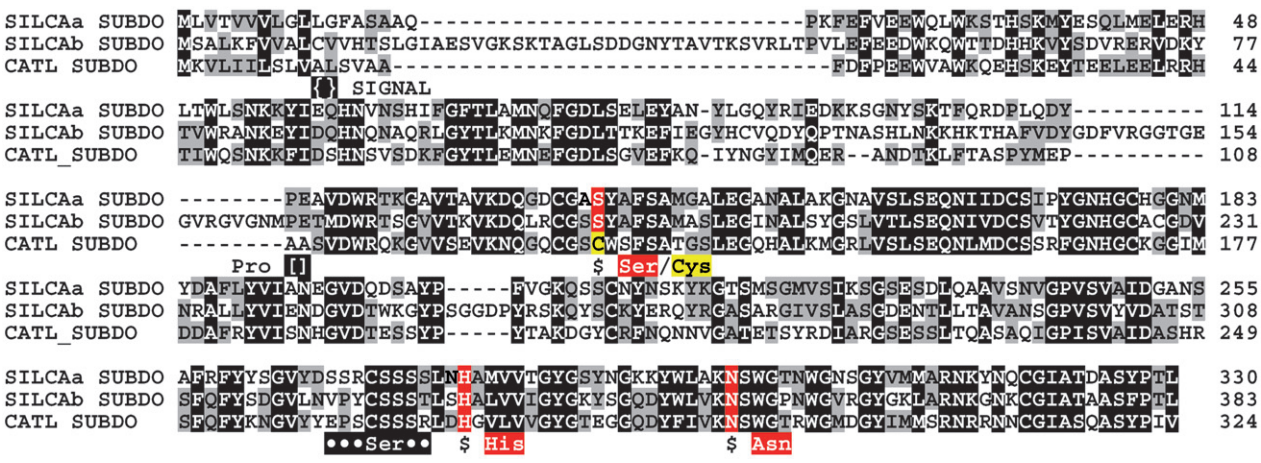


Fig. 4 Sponge silicateins. Alignment of the deduced polypeptide sequences of silicatein-α and silicatein-β from the sponge *S. domuncula* (SILICAa_SUBDO and SILICAb_SUBDO) with cathepsin L from *S. domuncula* (CATL_SUBDO). Residues conserved (similar or related with respect to their physico-chemical properties) in all sequences are shown in white-on-black and those in at least two sequences in black-on-gray. The characteristic sites in the sequences are marked; the catalytic triad amino acids, Ser (**$**; red) in silicateins [Cys (**$**; yellow) in cathepsins] and His (**$**; red) and Asn (**$**; red), and the processing sites for the conversion of the proenzyme to the mature enzyme (Pro [ ]), the signal peptide (SIGNAL { }), and the serine cluster (•Ser•).

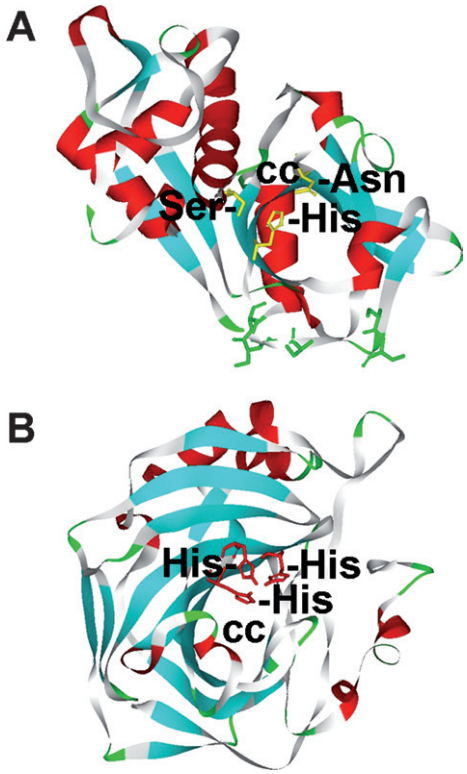


Fig. 5 Modeling of the 3-D structure of silicatein-α (A) and silicase (B). The published X-ray structures of human procathepsin S and carbonic anhydrase were used as a reference for modeling the silicatein-α and silicase structure, respectively.[12,109] The catalytic center (cc) of silicatein, comprising the amino acids Ser, His and Asn (yellow) and the localization of the Ser residues (green) of the serine cluster are marked. The silicase model shows the three histidine residues (red), which bind the zinc ion, in the catalytic center of the enzyme. Secondary structure elements are marked in red (α-helices) or blue (β-strands).

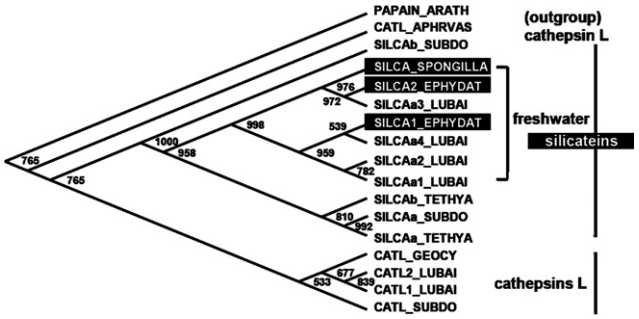


Fig. 6 Phylogenetic relationship of sponge silicateins. Four deduced silicatein sequences of the isoform silicatein-α (α-1, α-2, α-3 and α-4) from *L. baicalensis* (SILICAa1_LUBAI; SILICAa2_LUBAI; SILICAa3_LUBAI; SILICAa4_LUBAI) and the two cathepsin L sequences (CATL1_LUBAI; CATL2_LUBAI) were aligned with silicatein-α from *S. domuncula* (SILICAa_SUBDO) and *T. aurantium* (SILICAa_TETHYA), and with the β-isoenzymes from *S. domuncula* (SILICAb_SUBDO) and *T. aurantium* (SILICAb_TETYHA), as well as with the cathepsin L sequences from *S. domuncula* (CATL_SUBDO), *G. cydonium* (CATL_GEOCY) and *Aphrocallistes vastus* (CATL_APHRVAS) and the related papain-like cysteine peptidase XBCP3 from *Arabidopsis thaliana* (PAPAIN_ARATH) [outgroup]. In addition, the deduced silicateins from the cosmopolitan freshwater sponges *E. fluviatilis* (SILCA1_EPHYDAT and SILCA2_EPHYDAT) and *S. lacustris* (SILCA_SPONGILLA) were included in this analysis. The numbers at the nodes are an indication of the level of confidence for the branches as determined by bootstrap analysis (1000 bootstrap replicates). According to ref. 95 with modifications.

on the surface of the molecule (see Fig. 5A) and may act as template for silica deposition.

The alignment of the deduced amino acid sequence of silicatein-α with the silicatein-β sequence and the cathepsin L sequence from *S. domuncula* is shown in Fig. 4. Prominent sites within the silicatein sequence are the catalytic triad (silicatein-α: Ser aa_{138}, His aa_{277}, and Asn aa_{297}) and the hydroxy amino acid (serine) cluster (aa_{267} to aa_{274}).[6,8] Cathepsins are known to be processed at the C-terminal end of the molecules. The probable processing sites of the primary translation product of silicatein, comprising the signal peptide (aa_1 to aa_{17}), the propeptide region (aa_{18} to aa_{112}) and the mature chain (aa_{113} to aa_{330}) to the final mature enzyme (aa_{113} to aa_{290}), can be predicted based on the closely related human cathepsin L sequence.[91]

Analyses of the silicatein-α and β genes from *S. domuncula* revealed that they consist of six exons.[92,93] The silicatein-β gene is flanked by an ankyrin repeat gene at one side and by a tumor necrosis factor receptor-associated factor and a protein kinase gene at the other side.[93] All genes of this gene cluster are expressed synchronously in primmorphs following exposure to silicic acid.

In the presumptive 3D structure of silicatein-α obtained by computer modeling using the human cathepsin S as a reference (Swissprot approach), the three amino acids of the catalytic triad, Ser aa_{138}, His aa_{277}, and Asn aa_{297} are present in the active site pocket of the enzyme, while the serine residues of the hydroxyl amino acid cluster are located at the surface of the molecule (Fig. 5A).

The phylogenetic analysis of the silicatein and cathepsin L sequences from marine and freshwater sponges revealed that the silicatein sequences derive from a common ancestor of the cathepsin L sequences (Fig. 6).[11] In addition, the results indicate that the silicateins of the Baikalian endemic sponge *L. baicalensis* evolved from the silicateins of the cosmopolitan species *Spongilla lacustris* and *E. fluviatilis* (see also refs. 94 and 95). The four silicateins of *L. baicalensis* are phylogenetically closely related, suggesting their emergence by gene duplication.[89]

4.1.2 Catalytic mechanism. Silicatein catalyzes the formation of silica from monomeric silica precursors in solution, such as synthetic tetraethoxysilane (TEOS), the most commonly used substrate of the enzyme.[7] TEOS is relatively stable when mixed with water at neutral pH, and is also used in the industrially applied Stöber process.[96]

Cha *et al.*[7] proposed a two-step mechanism for silicatein-mediated silica formation. Step 1 is the (rate-limiting) hydrolysis of the alkoxide substrate (Fig. 7) and step 2 is the subsequent (poly)condensation reaction of the resulting silanol compounds.

The interaction of the hydroxyl group of the side chain of the serine residue with the imidazole group of the histidine residue in the active site of the enzyme is essential for the catalytic mechanism of the enzyme, as revealed by site-directed mutagenesis experiments.[97] The nucleophilicity of the serine hydroxyl group

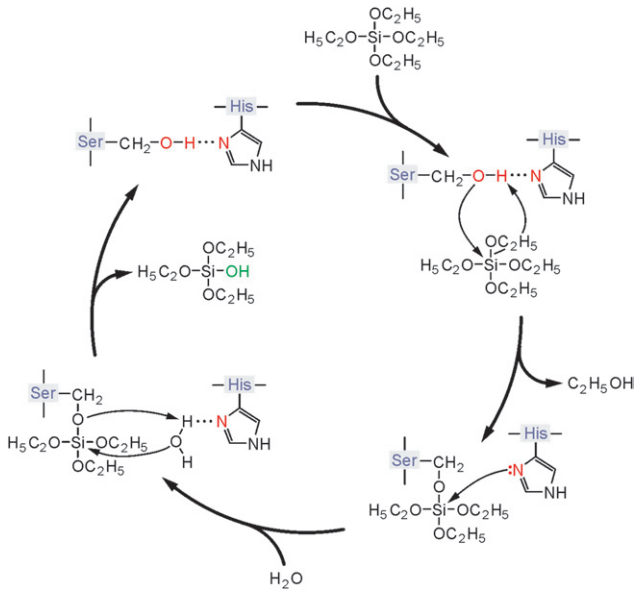


Fig. 7 Proposed mechanism of silicatein reaction. The hydrolysis of the alkoxide substrate (rate-limiting step) is shown. According to ref. 7 with modifications.

is thought to be increased by formation of a hydrogen bond to the imidazole nitrogen, thus facilitating the nucleophilic attack (S_N2 type) of the hydroxyl group on the silicon of the alkoxide substrate; a pentavalent intermediate is formed (Fig. 7).[7,97] The transitory covalent linkage between the enzyme and the substrate is then hydrolyzed by water. The reactive silanol molecules generated by hydrolysis subsequently undergo condensation reactions.

It should be noted that silicon alkoxides which are used in *in vitro* assays of silicatein activity are no natural precursor molecules of biosilica formation in sponges. The natural substrate of the enzyme is not yet known. It is known that silicic acid forms complexes with catechols, certain sugars or polyols,[98–103] and silicatein has been shown to be closely associated with (sugar-binding) lectins.[12,89] Therefore, silicic acid–sugar complexes might be possible substrates for silicatein. It has been proposed that such complexes are involved in biosilicification in diatoms.[104] It should be noted that silicatein exhibits besides a silica-polymerizing activity, a proteolytic (cathepsin-like) activity, which might also be involved in spicule formation.[92]

The mechanism of the condensation reaction (step 2) of the silanol compounds formed in step 1 of silicatein reaction is not known (enzyme/silicatein-mediated *vs.* purely chemical reaction). Molecular simulation experiments indicated that two orthosilicic acid units can be brought together by the rotation of the serine and histidine residues in the catalytic site of the silicatein molecule, through the formation of hydrogen bonds, favoring the condensation reaction.[105] Model studies of silica formation have been performed using recombinant silicatein and a silicon–catecholate complex (see Scheme 3 in Section 7) that liberates silicic acid in aqueous solution at neutral pH.[106] The results of these studies indicate that silicatein may also control the kinetics of silica polymerization (step 2).

It has also been proposed that the hydroxyl groups of the serine residues are involved in silica deposition by orienting the silicic acid monomers on the protein template.[7] However, the hydroxyl groups of the serine clusters at the surface of the silicatein molecule cannot be responsible *per se* for the silica deposition at the surface of axial filaments. Silica condensation and deposition have not been observed for cellulose or silk fibroin, although these fibers also have high numbers of hydroxyl groups at their surface.[7]

Silicatein has also been reported to catalyze in *in vitro* systems the formation of organically modified polysiloxanes (silicones) from monomeric precursors.[7] This might be of biotechnological interest (silicon industry).

4.1.3 Maturation of the protein. Silicateins are synthesized as proenzymes that undergo subsequent processing steps to form the mature, active enzymes as demonstrated in protein chemical studies and analyses of the silicatein genes/cDNAs.[9,92] In the first processing step the signal peptide is cleaved from the primary translation product (36.3 kDa) to generate a 34.7 kDa polypeptide; a terminal propeptide is then cleaved from the proenzyme to form the mature 23-kDa enzyme.[9] Axial filaments contain only the mature silicatein (23 kDa) which lacks the signal peptide and the propeptide, whereas the proenzyme (34.7 kDa) is the dominant form in the extra-spicule space.[9] Silicatein in the extra-spicule (extracellular) space has been shown to bind to galectin strings, forming hollow cylinders along the axis of the growing spicules (see below).[9,10,107] At present it is not known if silicatein is cleaved when associated with spicules.

It is likely that the silicatein proenzyme is enzymatically inactive, like the related cathepsin proenzyme.[108] Computer modeling studies revealed that the catalytic pocket in the silicatein proenzyme is blocked by the terminal propeptide sequence.[109] Only after cleavage of the propeptide sequence does the catalytic center become accessible to the substrate. This cleavage also occurs during formation of mature (active) cathepsin L from its inactive precursor; it may be mediated by either a second protease or autolysis.[110] The predicted cleavage site of the *S. domuncula* silicatein proenzyme is at aa_{112}/aa_{113} (Gln↓Asp; see Fig. 4).

4.1.4 Post-translational modification. The peptides/proteins involved in silica deposition are extensively modified by post-translational processes both in diatoms and sponges, and these modifications are essential for the function of these molecules. Two-dimensional gel electrophoretic analysis of sponge axial filament protein revealed that silicatein undergoes multiple phosphorylations; five phospho-isoforms with pI values of 5.5, 4.8, 4.6, 4.5, and 4.3 have been identified.[9] Each of these five silicatein phospho-isoforms can be resolved as two or three spots in 2D gels.[11] Electrospray ionization mass spectrometry analysis of these spots revealed that silicatein also undergoes post-translational modification by dehydroxylation of tyrosine; the resulting phenylalanine residue is most likely localized on the surface of the molecule as shown by computer modeling of silicatein-α with the human cathepsin S as a reference.[11] The post-translational modifications of the silicatein molecule differ from those of cathepsins, which do not form filaments and are only modified by glycosylation and formation of disulfide bonds.[108]

4.1.5 Self-assembly of silicatein filaments. Electron microscopy, small-angle X-ray diffraction (SAXS), protein chemical

and computer modelling studies allowed the first insights into the mechanism of self-assembly of silicatein monomers to oligomers and long protein filaments. These studies involved the analysis of both isolated axial filaments and soluble, native or recombinant silicatein protein. The first SAXS analyses of axial protein filaments revealed evidence for a regular, repeating structure of silicatein units with a periodicity of 17.2 nm.[6] However, the filaments used in these studies had been extracted from spicules using a rather harsh procedure (HF treatment), which may cause structural changes of the protein. In a later, more detailed study,[105] the structural organization of spicules from both demosponges and Hexactinellida was investigated. The results from SAXS experiments revealed a very high degree of organization (hexagonal) of the protein units forming the axial filament.[105,111]

Murr and Morse[112] proposed a mechanism for the self-assembly of silicatein monomers to filamentous structures, which proceeds through intermediary formation of a fractal silicatein network. This model assumes that silicatein monomers associate into oligomers by hydrophobic interactions. Silicatein dimers/oligomers can be seen in 1D and 2D gels of axial filament protein, but their occurrence strongly depends on the method used for isolation of the filament.[9,11] The silicatein oligomers are thought to be stabilized by intermolecular disulfide bonds.[112] These oligomers then assemble into fractal intermediates that subsequently form a filament. There is, however, no evidence that in native silicatein sulfhydryl groups are exposed towards the surface of the protein.

It must, however, be taken into account that the method used for isolation of axial filaments may have affected the results of studies on silicatein filament formation. HF is known to cause cleavage of O-glycosidic and phosphate ester bonds in proteins.[113] This treatment may therefore result in a loss/change of function of proteins that undergo post-translational modification, as reported for silaffins.[15,17] The availability of *soluble* native or recombinant silicatein protein is absolutely crucial for such studies, aiming to elucidate the mechanism of the self-assembly process of the protein.[9,11]

4.1.6 Localisation of silicatein. Silicatein is present not only in the axial filament but also on the surface of the spicules, as shown in immunofluorescence studies and immunogold labeling experiments using anti-silicatein antibodies.[10] The results support the view that growth of spicules occurs through apposition of lamellar silica layers.[9] Detailed electron microscopy analyses highlighted the presence of concentric rings where silica deposition occurs.[10] These rings are arranged at a distance of 0.2 to 0.5 μm from each other and fuse at a later stage of spicule formation.

4.1.7 Silicatein-associated proteins. Although silicateins are the predominant proteins of the axial filament, a number of further "silicatein-associated" proteins have been identified by analysis of native axial filament protein isolated by a milder extraction procedure than the harsh HF treatment.[12] These proteins include a 35 kDa protein (galectin-2) and collagen.[12] Furthermore, some minor protein bands are found in SDS-PAGE of spicule extracts, among them a 14 kDa protein (selenoprotein M).[114]

Incubation of primmorphs of *S. domuncula* without or with 60 μM Na-silicate and analysis of the differentially expressed RNA allowed the identification of the cDNA coding for a lectin, galectin-2.[12] Galectin-2 is a galactose-binding lectin, which forms aggregates in the presence of Ca^{2+} ions. It was demonstrated that silicatein-α molecules bind to these aggregates.[12] In competition experiments the interaction between galectin-2 and silicatein-α was abolished in the presence of a synthetic oligopeptide corresponding to a highly hydrophobic stretch which is present at the C-terminus of galectin-2.[12] Electron microscopy and immunogold labeling experiments provided evidence that, together with collagen, galectin-2 serves as a structural matrix for the assembly of silicatein molecules during spicule formation (see Section 8.1).[12]

The expression of two proteins was found to be upregulated after exposure of primmorphs from *S. domuncula* to selenium: selenoprotein M and silicatein-associated protein.[114] The sponge selenoprotein M (14 kDa) is related to other metazoan selenoproteins, whereas the silicatein-associated protein represents a new, sponge-specific protein.[114] The function of the selenium-dependent spicule-associated proteins is not yet known. Immunofluorescence studies revealed that they are present both at the axial filament and the surface of the spicules.[114]

4.2 Diatoms: silaffins and polyamines

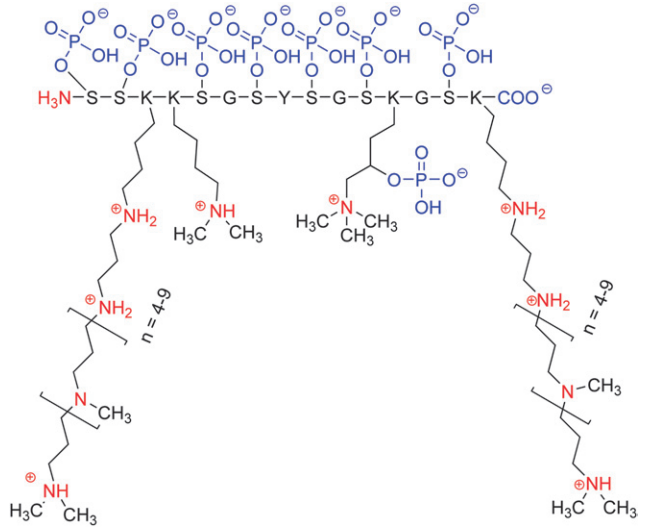
4.2.1 Silaffins. Three groups of proteins have been identified in the cell wall of the marine diatom *Cylindrotheca fusiformis*: (i) the frustulins, (ii) the pleuralins, and (iii) the silaffins (for a review, see refs. 115 and 116).

The EDTA-extractable frustulins are a family calcium-binding glycoproteins with molecular masses of 75–200 kDa which form the protective outer coat of the diatom cell wall.[13,117,118]

The pleuralins, or HF-extractable proteins (HEPs), are highly acidic molecules with molecular masses of 75–200 kDa.[119] They comprise a proline-rich region and several repeats of a PSCD domain. The pleuralins do not induce silica formation. The 200 kDa HF-extractable protein HEP200 from *C. fusiformis* has been characterized.[119] HEP200 is located within the girdle band region of the diatom cell wall where the epitheca overlaps the hypotheca (Fig. 3).[119]

The silaffins have low molecular masses of 4–17 kDa and can also be extracted from the diatom cell wall using anhydrous HF.[17] Three polypeptides, silaffin-1A, silaffin-1B and silaffin-2, have been identified in *C. fusiformis*.[15] Silaffin-1A comprises two peptides, silaffin-$1A_1$ and silaffin-$1A_2$, which are very similar and, like silaffin-1B, contain lysine groups which are modified (ε-*N*,*N*-dimethyllysine and δ-hydroxy-ε-*N*,*N*,*N*-trimethyllysine).[120] A silaffin gene has been cloned and sequenced.[15,82] The deduced polypeptide comprises seven lysine and arginine clusters containing repeat units (R1–R7). Silaffin-1B derives from R1, while silaffin-$1A_1$ and silaffin-$1A_2$ originate from R2–R7.

Using a milder extraction procedure (NH_4F/HF), native silaffins (termed natSil) could be isolated from *C. fusiformis*.[17] In the native silaffins both lysine and serine residues were found to be modified. The ε-amino groups of the lysine residues in natSil-$1A_1$ (molecular mass 6.5 kDa) are modified by forming ε-*N*-dimethyllysine or ε-*N*,*N*,*N*-trimethyl-δ-hydroxylysine residues or they carry a long-chain polyamine consisting of about 5 to 10 *N*-methylpropylamine units.[15,17,82] The serine residues and the hydroxyl group on the modified lysine are phosphorylated.[17]



Scheme 1 Schematic structure of silaffin 1A (natSil-1A) from *C. fusiformis*. The structures of the side chains of the modified lysine (K) residues and the phosphorylated serine (S) residues are shown. According to ref. 17 with modifications.

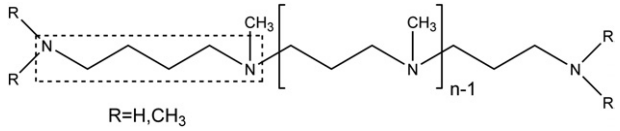
Thus natSil-1A$_1$ carries a high number of both positive and negative charges. The structure of natSil-1A$_1$ is shown in Scheme 1.

Mixtures of silaffins 1A, 1B and 2 from *C. fusiformis* are able to cause silica precipitation from a solution of silicic acid, forming nanoparticles with diameters of less than 50 nm.[15] Larger particles of 500–700 nm in diameter are obtained in the presence of purified silaffin-1A$_1$ and silaffin-1A$_2$.[82] The polypeptides are occluded in the precipitated silica. The size of the silica nanospheres formed *in vitro* by the purified proteins is larger than the size in diatoms where silica particles of 10 to 100 nm in diameter are found. Silaffins isolated by the harsh HF procedure require addition of phosphate to the incubation mixture for silica precipitation.[15,16,82] Native, phosphorylated silaffins (natSil-1A) are also able to precipitate silica in the absence of additional phosphate.[17] It is assumed that a template for silica precipitation is formed through self-assembly of the zwitterionic silaffin molecules.[17]

In addition to the proteins described above, a homologue of ubiquitin (8.5 kDa) that might be involved in silica biomineralization has been identified by screening of polypeptides with a high affinity to silica from the diatom *Navicula pelliculosa*.[121]

The R5 peptide, a synthetic polypeptide derived from silaffin-1A,[122,123] as well as synthetic propylamines derived from silaffins,[124] have been used to synthesize bioinspired organic–inorganic nanocomposites. The results indicate that even the nonphosphorylated protein is able to promote silica deposition. The R5 peptide has also been used in the form of a chimeric protein with the self-assembling domain of the MaSp1 protein of spider dragline silk for the synthesis of nanocomposites.[125]

4.2.2 Polyamines. Another group of molecules involved in biosilicification in diatoms are linear long-chain polyamines (molecular mass < 3.5 kDa), which have been identified in several diatom species.[16] These long-chain polyamines consist of up to 20 repeats of *N*-methylaminopropyl or aminopropyl units (Scheme 2). They seem to be species-specific and represent



Scheme 2 Chemical structure of polyamines.

the main organic constituent of the cell walls of diatoms, and occur either alone or combined with silaffins.

The studies of Sumper and Lehmann[126] revealed a great structural variety of the long-chain polyamines involved in silica precipitation in diatoms. The mechanism of silica precipitation from orthosilicic acid solutions induced by polyamines is not completely understood. Mizutani *et al.*[23] proposed that polycondensation of silicic acid by synthetic polyamines (polyallylamine and poly-L-lysine) follows a catalytic mechanism. It is hypothesized that, at pH 8.5, the polyamine acts as an acid–base catalyst, facilitating the condensation reaction.

A catalytic mechanism for the polyamine-mediated hydrolysis and condensation of organosilicates, which resembles the mechanism proposed for silicatein, has also been proposed by Delak and Sahai.[127] It is assumed that the conjugate base of the amine performs a nucleophilic attack on the silicon to form a pentacoordinate reactive intermediate. The second amine group then forms a hydrogen bond to a water molecule, facilitating hydrolysis of the intermediate. The condensation step is thought to occur by an acid-catalyzed S_N2 mechanism.

Phosphate ions can induce the self-assembly of polyamines.[128–130] Polyamines exhibit amphiphilic properties. The aggregation and phase separation of polyamines in solution might be explained by the electrostatic interactions between the positively charged polyamine molecules and the negatively charged phosphate ions.[129] In addition, formation of hydrogen bonds may occur. The aggregates (microemulsions) formed in aqueous solution have positively charged surfaces. Multivalent anions can promote the formation of higher-order assemblies of the polyamine droplets. Silicic acid present in the aqueous phase could adsorb to the surface of the droplets, resulting in coacervate formation and finally silica formation. Based on these properties, Sumper[131] proposed a model for silica nanosphere formation and nanopatterning in diatoms, which is based on multiple phase-separation processes. The characteristic "honeycomb"-like larger structures which are initially formed during silica formation are step-wise transformed into smaller structures, mediated by subsequent phase-separation processes. An alternative "upscaling" model based on the results of *in situ* time-resolved ultrasmall-angle X-ray scattering analyses has been proposed by Vrieling *et al.*[132]

4.3 Higher plant proteins

Early analysis of plant biosilica in *Phalaris canariensis* (canary grass) and *Equisetum telmateia* revealed low levels of entrapped proteins but also monosaccharides.[133] Model studies of silica precipitation using silicon catecholate complex as the source of soluble silicic acid showed that protein-containing biosilica extracts from *Equisetum telmateia* influence the kinetics of the early stages of the silica oligomerisation.[134] These proteins present in extracts from higher plants have not yet been well characterized.

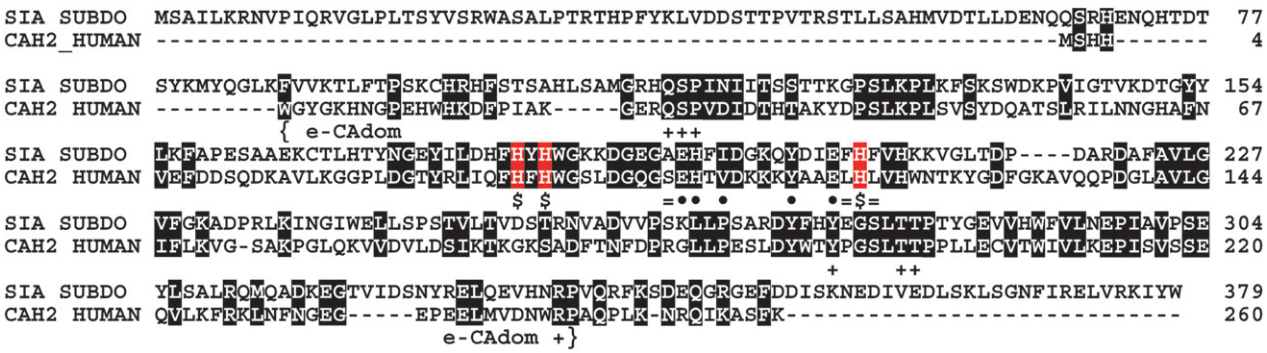


Fig. 8 Sponge silicase. Alignment of the silicase from the sponge *S. domuncula* (SIA_SUBDO) with the human carbonic anhydrase II (CAH2_HUMAN). The carbonic anhydrase domain is framed ({e-CAdom}). Similar amino acid residues in both sequences are shown in white-on-black. The three zinc-binding histidine residues (**$**; red) and the characteristic amino acids forming the eukaryotic-type carbonic anhydrase signature (●, found in both sequences; =, present only in the carbonic anhydrases but not in the silicase) are indicated; residues marked + are those forming the active-site hydrogen network.

5 Biosilica dissolution

5.1 Sponges: silicase

Sponges contain also an enzyme which is able to etch or dissolve silica. Dissolution of intact spicules is comparatively slow, but seems to be faster at the ends of spicules which have been mechanically broken.[135] Sponges may also be able to digest diatom frustules, as has been observed in vacuoles of mobile cells of the freshwater sponge *E. fluviatilis*.[136]

The cDNA encoding the silica-catabolic enzyme, termed silicase, has been isolated from the marine sponge *S. domuncula*. This enzyme is able to dissolve amorphous silica, releasing free silicic acid.[51] The expression of the silicase gene is strongly upregulated in the presence of exogenous silicate (60 μM), like the expression of silicatein.[8] The deduced polypeptide (43 kDa) is closely related to the carbonic anhydrases; the characteristic eukaryotic-type carbonic anhydrase signature is also present in the sponge enzyme (Fig. 8).[51] Carbonic anhydrases are a family of zinc metal enzymes.[137] The three conserved histidine residues which bind the zinc ion are present in the deduced amino acid sequence of silicase at aa_{181}, aa_{183} and aa_{206} (Fig. 8; and structure obtained by computer modeling, Fig. 5B).

The proposed mechanism of silicase reaction follows the mechanism of other zinc-dependent enzymes involved in ester hydrolysis (Fig. 9).[51] The reaction is initiated by a hydroxide ion which is bound to the zinc ion (a Lewis acid) and formed by splitting of the water molecule (a Lewis base). The hydroxide ion performs a nucleophilic attack at one of the silicon atoms of the polymeric silicate. Thereby the zinc-complex binds to the silicon, and the oxygen bond in the polymeric silicate is cleaved. The transiently formed zinc-bound silicate is then hydrolyzed by water, resulting in the release of silicic acid and regeneration of the zinc-bound hydroxide.

5.2 Diatoms

A silicase, like the sponge enzyme, has not been identified in diatoms. A ferredoxin-NADP reductase (a zinc-binding protein) has been detected among the proteins from *N. pelliculosa*, that show high affinity for solid silica.[138] Although the deduced

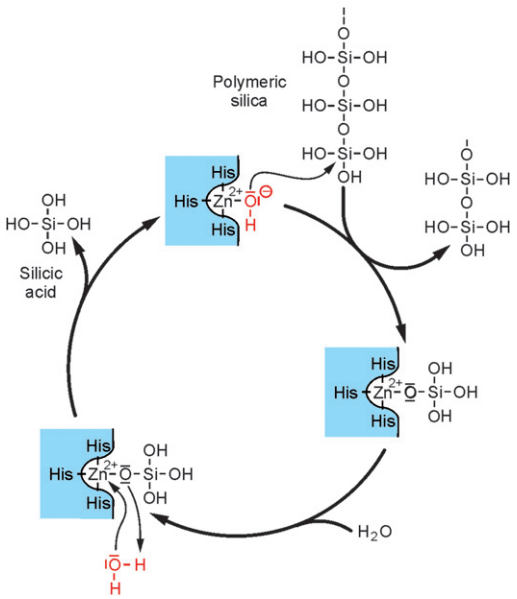


Fig. 9 Proposed mechanism of silicase reaction. According to ref. 51 with modifications.

amino acid sequence of ferredoxin-NADP reductase from *Thalassiosira pseudonana* was found to be homologous with carbonic anhydrase and sponge silicase, no evidence has been found for an involvement of this enzyme in silica dissolution.[138] However, it has been demonstrated that diatoms when encapsulated into artificial silica sol–gel matrices are able to dissolve the surrounding silica.[139]

6 Silicic acid transport

Aqueous organisms live in an environment which is undersaturated with respect to silica. The average concentrations of dissolved silicon in seawater, which is mostly present as undissociated orthosilicic acid, $Si(OH)_4$, is about 70 μM.[5] In surface waters this concentration is even lower (<3 μM) due to the biological consumption of $Si(OH)_4$.[5,140] On the other hand, the intracellular concentrations of silicon, *e.g.* in diatoms, can reach

>100 mM,[141] thus exceeding the extracellular silicon concentration by >1000-fold. This can be seen as an indication that these organisms have developed efficient mechanisms for the active uptake of silicic acid from the surrounding water to form their silica skeleton. The uptake of silicon into sponge cells[84] and diatoms is linked to the transport of sodium.[142,143]

6.1 Sponge silicic acid transporter

The uptake of silicic acid into sponge cells is an energy-consuming process.[144] The technique of differential display of mRNA from primmorphs (*S. domuncula*) that had been incubated in the absence and presence of exogenous silicate (60 μM) was used to isolate the cDNA encoding the putative silicic acid transporter.[84] The sponge transporter displays high sequence similarity to the Na^+/HCO_3^- (NBC) cotransporter; both transporters are inhibited by the stilbene compound DIDS (Fig. 10A).[84] The expression of the sponge transporter ($Na^+/HCO_3^-[Si(OH)_4]$ or NBCSA cotransporter) is strongly up-regulated in response to increased concentrations of silicic acid (60 μM) in those sponge cells that are located adjacent to the spicules.[84] The $Na^+/HCO_3^-[Si(OH)_4]$ cotransporter in sponges is not identical with the silicon transporter in diatoms[143]

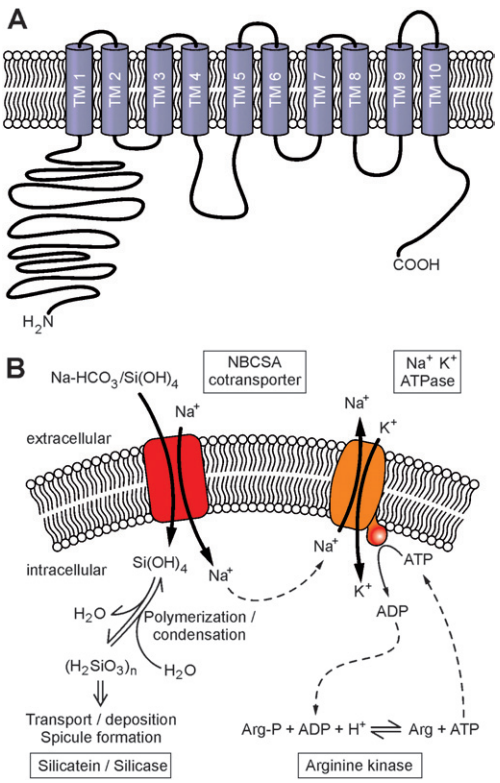


Fig. 10 (A) Predicted structure of the sponge silicic acid transporter (*S. domuncula*). The cylinders indicate ten transmembrane segments TM1-TM10. The larger intracellular loop is located between TM4 and TM5. (B) Schematic presentation of silicic acid transport in sponge cells mediated by the Na^+-driven $Na^+/HCO_3^-[Si(OH)_4]$ cotransporter (NBCSA cotransporter). The orthosilicic acid in the cytoplasm undergoes polymerization/condensation during spicule formation. The sodium gradient is maintained by Na^+ K^+ ATPase. ATP is supplied by arginine kinase reaction, which functions as a phosphagen kinase in sponge cells.

(reviewed in ref. 116). The activities of both transporters seem, however, to depend on the presence of sodium ions (sodium/silicic acid symport).

The Na^+-driven transport of silicic acid depends on the supply of ATP required for Na^+ K^+ ATPase activity to maintain the sodium gradient. ATP is most likely supplied by arginine phosphate mediated by the sponge arginine kinase activity.[144] Fig. 10B shows a schematic presentation of the concerted action of the $Na^+/HCO_3^-[Si(OH)_4]$ cotransporter and arginine kinase. The expression and activity of this kinase in sponge primmorphs are increased in the presence of silicic acid.[144]

Silicon transport and spicule formation in sponges are inhibited by germanium at a Ge/Si molar ratio of 1.0.[145–147] At lower Ge/Si ratios, the growth of the spicules in length but not in width is inhibited, resulting in shorter spicules with normal morphology.[148]

6.2 Diatom silicic acid transporter

The silicon transporter from the marine pennate diatom *C. fusiformis*, consisting of a family of five silicic acid transporters (SIT1–5), has been identified and characterized by Hildebrand *et al.*[149,150] The amino acid sequences deduced from the SIT genes contain up to 12 putative transmembrane segments, a signature amino acid sequence for sodium symporters and a long hydrophilic carboxy-terminus (Fig. 11A).[149,151]

The less conserved carboxy-terminal segment of the SITs has a predictable coiled-coil structure. It is assumed that this segment of the molecule interacts with other proteins or other SITs, and may be involved in the control of the activity of the protein.[150] The transmembrane domain contains nine conserved cysteine residues, which may explain the sensitivity of silicon transport to sulfhydryl blocking agents.[142] The silicon transporter in diatoms acts as a co-transporter with sodium ions with a 1 : 1 ratio of $Si(OH)_4$ to Na^+.[143] The expression patterns of the five SIT genes change during the cell cycle and are correlated with the silica deposition.[150,152] The diatom silicon transporter shares no similarity with the sponge silicon transporter, which is related to the Na^+/HCO_3^- (NBC) cotransporter, and the rice silicon transporter, which is an aquaporin homolog (see Section 6.3). SIT homologs have also not been found in sponges.

The activity of the diatom silicon transporter was determined in *Xenopus laevis* oocytes that had been microinjected with SIT1 RNA.[149] The uptake of ^{68}Ge, which was used as an isotopic tracer for silicon, into the oocytes was abolished by silicic acid, as revealed in competition experiments, and was sodium-dependent.[149] It was inhibited by sulfhydryl blockers like *N*-ethylmalemide.

Two models have been proposed for SIT-mediated silicon transport. The first model is based on the identification of a conserved sequence motif, CMLD, in the deduced amino acid sequences of the silicic acid transport proteins from four diatom species.[153] Grachev *et al.*[154] proposed that the functional groups in the side chains of the C, M and D residues of the conserved CMLD motif act as a potential binding site for a Zn^{2+} ion. Zinc has been shown to affect the uptake of silicic acid by diatoms.[155] It is hypothesized that the bound Zn^{2+} acts as a Lewis acid and is involved in binding and transport of silicic acid by facilitating the nucleophilic attack of a functional group

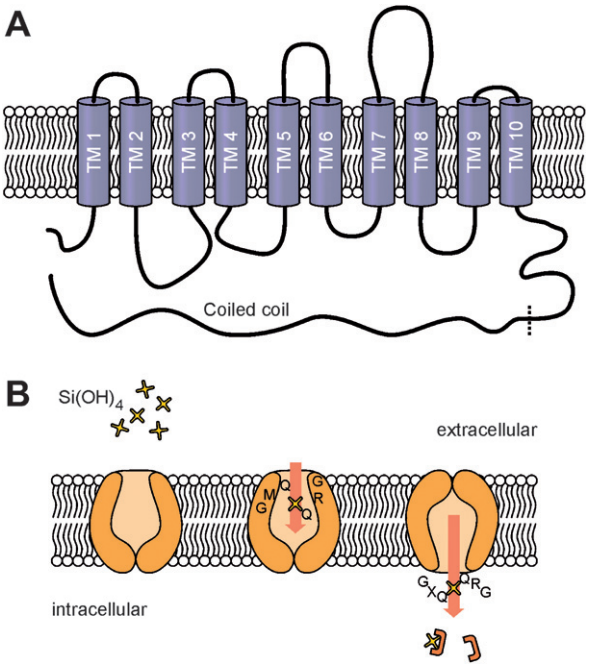


Fig. 11 (A) Predicted structure of the diatom silicic acid transporter (*C. fusiformis*) comprising ten transmembrane domains (TM1–TM10). The position of the coiled coil region is indicated. According to ref. 225 with modifications. (B) Model of SIT-mediated silicon transport. The sequential conformational changes of the silicon transporter occurring during silicic acid transport are shown. (i) Binding of extracellular silicic acid through hydrogen bonding by two conserved glutamine residues located in transmembrane segments TM7 and TM8. (ii) Induction of a conformational change of the transporter allowing binding of silicic acid to two additional conserved glutamine residues located in the loop between TM2 and TM3. (iii) Release of silicic acid into the cell and binding to an unknown cellular component. According to ref. 156 with modifications.

of the transporter (or of another silicic acid molecule) at the silicon atom. This mechanism is similar to that proposed for the sponge silicase.[51]

However, it should be noted that the conserved cysteine residue is not found in all SIT species.[156] The authors assume that SIT species that carry a substitution of cysteine by alanine are inactive and not involved in silicic acid transport.[154]

Another model for silicon transport proposed by the group of Hildebrand is based on sequence comparison of SIT genes from eight diatom species (centrics and pennates); a conserved motif, GXQ (X = Q, G, R, or M) has been identified in four distinct regions of the deduced proteins.[156] Two of these motifs are located in predicted transmembrane sequences TM7 and TM8 (Fig. 11B). It is assumed that the (two) carbonyl groups in the side chains of the glutamine residues of the two conserved GXQ motifs in TM7 and TM8 (either alone or together with the amine groups) form hydrogen bonds to the hydroxyl groups of the silicic acid molecule.[156] A similar complex can be formed with the two glutamines of the GXQ motifs near the transmembrane sequences TM2 and TM3. This assumption also takes into account that silicic acid is co-transported with sodium;[143] the sodium gradient acts as a driving force for the uptake of silicic acid. Binding of silicic acid to the conserved glutamine residues of the GXQ motifs in TM7 and TM8 and sodium (bound to an unknown binding site) would induce a conformational change of the transporter from an outward-facing conformation to the inward-facing conformation. This conformational change results in the release of silicic acid *via* the conserved glutamine residues near TM2 and TM3 into the intracellular space (Fig. 11B). Intracellularly, silicic acid may be bound to an unknown factor preventing the polymerization of oversaturated solutions of the molecule.[151] This model requires an (energy-dependent) mechanism to pump the sodium out of the cell (as in sponges). Alternatively, a coupling of the conformational change of the transporter with silica deposition might be possible.[156]

6.3 Silicic acid transport in chrysophycean algae

Recently a gene responsible for silicic acid transport in chrysophycean algae has been identified.[157] These algae originate from the Late Precambrian (about 600 Myr ago),[158] when siliceous sponges were already present;[159] diatoms are evolutionarily younger (originating about 240 Myr ago).[160] The data revealed that the mechanism of silicon transport in chrysophycean algae is mediated by a SIT protein, as in diatoms.[157] This protein contains the conserved CML(I)D motif that is proposed to form the active center of the silicon transporter protein. This motif is present in the predicted polypeptide sequence of most but not all SITs studied (see above).[161] The C, M, and D residues of this motif has been proposed to bind a zinc ion that may be involved in binding and transport of silicic acid (see above).[153,154]

6.4 Silicic acid transporter of higher plants

The concentrations of silicic acid in groundwater solutions are typically below the solubility of quartz (6–12 ppm SiO_2). Therefore, plants must concentrate silicic acid to exceed the solubility of amorphous silica (about 120 ppm SiO_2). The concentration of soluble silicic acid in soil in equilibrium with solid SiO_2 (such as quartz, cristobalite or amorphous silica) can reach ~0.1–2.0 mM.[162] Silicon uptake by the roots occurs in the form of uncharged silicic acid.[163] The uptake of silicic acid by rice roots is an energy-dependent process which is inhibited by metabolic inhibitors (*e.g.*, NaCN, dichlorophenoxy acetate and 2,4-dinitrophenol) and low temperature.[164–166] The silicic acid is then translocated – *via* the xylem – to the shoot, also in the form of monomeric silicic acid,[167,168] and finally to the leaf where it precipitates beneath the cuticle layer to form a silica–cuticle double layer, as well as a silica–cellulose double layer.[61,63] The concentration of monomeric silicic acid in the xylem sap of rice exceeds the concentration at which silicic acid normally polymerizes, $[Si(OH)_4]$ >2 mM.[168] The concentration of silicic acid increases further in the shoot through transpiration of water, resulting in the polymerisation of silicic acid and silica gel formation.[61]

The different ability of higher plant species to accumulate silicon has been explained by differences in the silicon uptake mechanisms of their roots.[61] The ability of plant species to accumulate silicon varies over a huge range. A comparative study of three plant species with low (tomato), medium (cucumber) and high (rice) levels of silicon accumulation revealed that the K_m

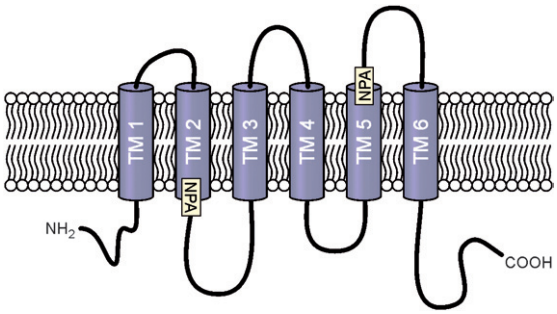


Fig. 12 Predicted structure of the plant (rice) silicic acid transporter comprising six transmembrane domains (TM1–TM6). The location of the two NPA motifs is shown. According to ref. 63 with modifications.

value (but not V_{max}) of silicon uptake is similar in all three species and amounts to 0.15 mM; the V_{max} values increased from tomato to cucumber and rice.[166] These results suggest that all three plant species have a similar silicon transporter but that the densities of this transporter are different.

Recently, the gene encoding a silicon transporter has been isolated from rice.[169] This gene (*Lsi1*), which has been identified using a rice mutant defective in silicon uptake,[165] comprises five exons and four introns.[170] The silicon transporter in higher plants (rice) is not a homolog of the silicon transporter in diatoms and sponges. The protein encoded by *Lsi1* belongs to the aquaporin family of proteins,[169] which form water channels in cellular membranes. These proteins have also been identified and functionally characterized in freshwater sponges (*L. baicalensis*), but no evidence has been obtained that aquaporins are involved in uptake of silicic acid in sponge cells.[171] The deduced amino acid sequence of the rice silicon transporter comprises six transmembrane domains and, like aquaporins, two NPA motifs (Fig. 12).[169] The transporter is expressed in the plasma membrane of the exodermis and endodermis cells of the roots, next to the Casparian strips, which without a transporter would not allow a free passage of silicic acid for further translocation to the shoot and leaf.

The ability of Lsi1 to act as a silicon transporter was confirmed in microinjection experiments of Lsi1 RNA using frog (*X. laevis*) oocytes.[169] The transporter seems to be specific for silicic acid as revealed by competition experiments with glycerol.

The affinity for silicic acid of the transporter is relatively low (K_m = 0.15–0.3 mM). The uptake of silicic acid in plant (rice) cells is inhibited by phloretin, a nonspecific inhibitor of aquaporin-mediated transport of water and glycerol,[172] but not DIDS,[173] an inhibitor of the sponge silicon transporter and other NBC transporters.

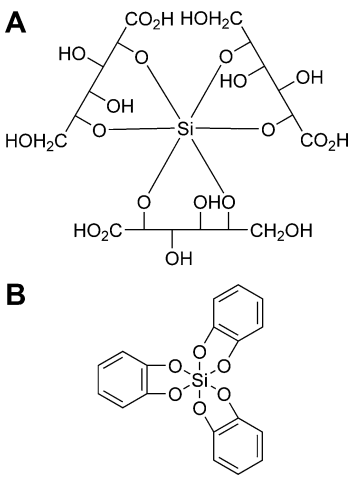
6.5 Silicic acid/silica uptake in vertebrates

A silicic acid transporter has not been identified in vertebrate cells although silicon has been shown to be an essential element in animals and humans (see Section 3.4). However, vertebrate cells are able to take up crystalline silica particles. Silica uptake in vertebrate cells appears to be mediated by surface-bound scavenger receptors. The primary receptor interacting with silica has recently been identified in alveolar macrophages from C57BL/6 mice. This receptor (macrophage receptor with collagenous structure, MARCO) is thought to be involved in silica cytotoxicity.[174]

7 Formation of organosilicon complexes: polyols

Siliceous organisms such as diatoms may contain high concentrations of soluble silicic acid, besides the deposited silica. The estimated concentration of soluble silicon of diatom cells is in the range 19–340 mM (given as orthosilicic acid).[151] The solubility limit for orthosilicic acid is about 2 mM. Also, sponges (silicasomes; see Section 8.1) might contain high concentrations of soluble silicic acid. Quantitative ^{29}Si MAS NMR experiments on diatoms revealed the existence of intracellular pools of less condensed silica; the Q^4:Q^3 ratios for complete cells are significantly smaller (1.8–1.9) than those for cell walls (2.5–2.8).[175] The mechanism by which diatoms can maintain such high concentrations of silicon in solution is not yet known. Chemically, this could be realized by the generation of molecules containing hypervalent silicon through complex formation. Stable six-coordinate silicon complexes can be formed between $H_3SiO_4^-$ and catechol (1,2-dihydroxybenzene), tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one) or 2-hydroxypyridine *N*-oxide. Formation of complexes ("chelates") of silica with catechol is favored at high pH, whereas the reaction with tropolone is favored at low pH. Formation of a complex, bis[citrato(3−)-O^1,O^3,O^6]silicate, with hexa-coordinate silicon and two tridentate dioato(2−)olato(1−) ligands has been reported.[176] The formation of silica from tris(catecholato)silicate complexes, $(M^+)_2[Si(C_6H_4O_2)_3]$ (Scheme 3A) has been extensively studied by the group of C. C. Perry.[99,177]

Silicic acid has been shown by the group of Kinrade also to form (water-stable) complexes with certain sugars or polyols.[102] Such molecules could indeed be identified inside cells of the diatom *Navicula pelliculosa* by ^{29}Si NMR spectroscopy.[104] Polyalcohols with at least four adjacent OH groups and a *threo* configuration of the two middle OH groups (such as threitol, mannitol, sorbitol, and xylitol) are able to form stable five- and six-coordinated organosilicon complexes with silicate under



Scheme 3 Structure of (A) the hexa-coordinated tris(catecholato)silicate and (B) the tri[η^2-2,5-(+)-gluconato]silicate complex.

aqueous alkaline solutions.[102] Such complexes are also formed by certain aliphatic acid carbohydrates (such as gluconic acid, saccharic acid and glucoheptonic acid);[178] the structure of the hexa-coordinate tri[η^2-2,5-(+)-gluconato]silicate complex is shown in Scheme 3B. The formation of hexa-coordinate species over both penta- and tetra-coordinate species is favoured at increasing pH or decreasing temperature. The affinity of complex-forming alcohols increases with increasing number of hydroxy groups.[103] The existence of stable alkoxy-substituted silicate anions formed with these ligands in aqueous alkaline silicate solutions has been proved by ^{29}Si NMR.[103] NMR studies even provided evidence for the existence of pentaoxo organosilicon complexes in dilute neutral aqueous silicate solutions.[179] Complexation of $H_3SiO_4^-$ ions with polyols enhances the solubility of silica; under these conditions, stable silica solutions containing up to 3 mol l^{-1} silica can be prepared without gelation.[102] Formation of soluble 2 : 1 complexes of certain sugars with basic silicic acid in aqueous solution has also been reported by other groups.[180] The sugar silicates mainly consist of pentacoordinate silicate.

Carbohydrates may also play a potential role in silicate transport.[104,178]

8 Special aspects of sponge spicule formation

8.1 Demosponges

Primmorphs from the sponge *S. domuncula* were used to study spicule formation. Electron microscopy studies revealed that the initial steps of spicule formation and the formation of the first silica layer around the (short) axial filament occur in vesicles of sclerocytes.[9] Small spicules (up to 10 μm long) are then extruded from the cells,[9] and in the extracellular space, the spicules grow through apposition of lamellar silica layers up to a length of 450 μm and a diameter of 5 μm.[9,85] In the initial stage, the axial canal is primarily filled with the axial filament and additional string- and net-like structures which very likely represent clumped galectin.[12] In the final stage, it is almost completely filled with the axial filament, which displays the characteristic triangular form (Fig. 2D). Immunofluorescence studies revealed that silicatein exists both in the axial canal and on the surface of the spicules.[9,10,107]

Immunogold electron microscopy analysis showed that silicatein is present also in the extracellular space and arranged along galectin-containing strings, which are organized parallel to the surfaces of the spicules.[9,12] In the presence of Ca^{2+}, silicatein binds to the galectin-containing strings, allowing the appositional growth of the spicules. The silicatein–galectin-2 complexes are closely associated with the inorganic silica phase, both at the inner and the outer surface. The galectin-containing strings are organized by collagen fibers to net-like structures. These collagen fibers form an ordered network[12,181] and thereby control the spatial arrangement of the silicatein–galectin-2 complexes, *i.e.* the formation of concentric rings around the axis of a growing spicule (Fig. 13B) and the spicule shape.[9,10] The new silica lamellae are produced in hollow cylinders which have a diameter of 0.2 to 0.4 μm and are bordered by an outer net-like galectin layer with associated silicatein molecules and the surface of the spicule.[44] These tube-like cylinders are stabilized by collagen fibers.[181] The 1–3 μm thick lamellae are formed by silica nanoparticles with a diameter of about 100 to 200 nm, which are particularly visible in SEM images of partially etched spicules.[44] The assembly and packaging of the initially formed silica granules to form larger silica layers and the process (and the factors) that determine the species-specific shape of the spicules are not yet completely understood.

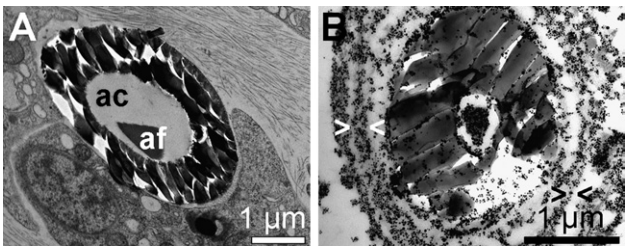


Fig. 13 (A) Cross-section through a spicule within a primmorph (*S. domumcula*) showing the axial canal (ac) with the triangular axial filament (af). The spicules are closely, but never intimately, associated with cells (sclerocytes). Electrodense granules (silicasomes) are visible within the cell. (B) Immunogold electron microscopy of a cross-section through a growing spicule in primmorphs using polyclonal anti-silicatein antibodies. The immune complexes formed were visualized with nanogold anti-rabbit IgG. Concentric rings (> <) are seen that surround the first layer of silica at the growing spicule. The silicatein antibodies react with the axial filament and the silica-forming silicatein–galectin rings.

Further studies revealed that the spicules in the mesohyl are surrounded by sclerocytes,[12,44,181] that, however, never come into close contact with the silica surface (Fig. 13A).[12,181] They are filled with electron-dense vesicles. Energy dispersive X-ray analysis showed that these vesicles have a high silicon content; they were termed silicasomes.[44] The silicasomes are released by the sclerocytes into the extracellular space and transported into the space around the spicules.

Based on these studies it has been thought that the silica lamellae are produced from silica released by silicasomes into the hollow cylinders around the growing spicules.[44] These silicasomes are produced in sclerocytes which accumulate silica within these vesicles through their $Na^+/HCO_3^-[Si(OH)_4]$ cotransporter.

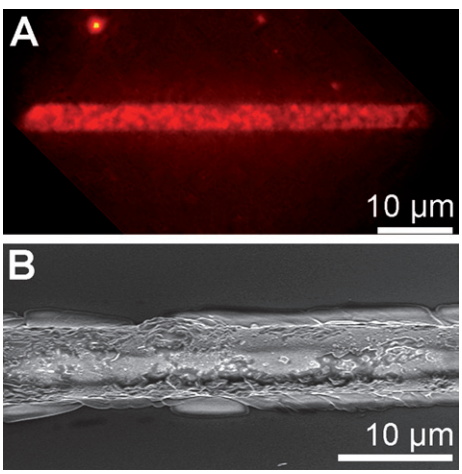


Fig. 14 (A) Immunostained silicatein immobilized on a spicule of *S. domuncula*. (B) SEM image of the biosilica layer formed by silicatein immobilized on a *S. domuncula* spicule.

This model was also corroborated in biomimetic studies in which recombinant silicatein was immobilized on the surface of spicules freed of organic material, after functionalization with aminopropyltriethoxysilane (APS) and binding of a reactive ester polymer [poly(acetoxime methacrylate)] to the primary amine created on the surface by APS treatment; this matrix was then used for immobilisation of His-tagged silicatein through Ni(II) complexation (Fig. 14A). Fourier transform infrared spectroscopy (FTIR) and electron microscopy (Fig. 14B) analysis revealed that new biosilica layers/lamellae deposited on the surface of the spicules are produced by the matrix-bound enzyme after incubation with substrate.[44]

8.2 Lithistids

Some siliceous sponge species, the lithistids, show an articulated skeleton. These sponges have hypersilicified spicules, so-called desmes, forming arms which interlock with each other.[182] The silica of the desmes of lithistid sponges consists of silica nanospheres which may fuse to form larger spheres.[182] It should be noted that hypersilicification of spicules can also occur in the presence of high concentrations of silicon. Maldonado *et al.*[183] presented experimental evidence that the concentration of silicon in seawater modulates the phenotypic expression of the various spicule types in a sponge species.

A special group of demosponges are keratose sponges, which do not form their own spicules. These sponges may use foreign silica (sand) particles as inorganic skeletal material to stiffen their spongin fiber network.[184] In the keratose sponge *Dysidea etheria*, active transport of silica particles to areas where skeletal fibers are formed has been observed.[185]

8.3 Hexactinellida

Hexactinellid sponges are characterized by a syncytial organization – in contrast to demosponges that have a cellular organization – and are the oldest sponge taxon as revealed by molecular biology[186] and geology data.[187] They evolved between two global glaciation ("snowball Earth")[188] periods 720–585 Myr ago (Neoproterozoic). The hexactinellid sponges form the largest biosilica structures known on Earth. For example, the giant spicules (basalia) of the deep sea hexactinellid *Monorhaphis chuni* have a length of up to 3 m and a maximum diameter of 8.5 mm.[189] Located in the center of these spicules is a relatively small axial canal (diameter ~1 μm), which harbors the axial filament (see Fig. 2F). The cross-section of the axial filament in hexactinellid sponges is quadrangular, whereas the axial filaments in demosponges are triangular or hexagonal. The axial canal is surrounded by an electron-dense, 100–150 μm thick axial cylinder composed of silica. This – homogeneous – silica core structure is surrounded by up to 500 highly regularly arranged concentric silica lamellae.[189,190]

The siliceous skeleton of the hexactinellid sponge *Euplectella aspergillum* shows a hierarchical ordered structure.[191,192] At least six hierarchical levels in the length scale from nanometres to centimetres can be identified in the skeleton of *E. aspergillum*.[192,193] The mechanical properties of the spicules of *E. aspergillum* are influenced by the thin organic interlayers (5–10 nm) between the thicker concentric silica layers of the spicules.[194]

Recent studies indicate that the radial growth process of the hexactinellid spicules most likely occurs as in demosponges. Collagen is the predominant protein species of these spicules on the outer surface, which has an important influence on the physicochemical/mechanical properties of this composite material.[190,195] Some physicochemical properties of these spicules may also be influenced by their sodium and potassium contents, which show regional variations as revealed by electron microprobe analysis.[190]

8.4 Optical fibers

Spicules from siliceous sponges, in particular spicules from the hexactinellid sponges *Rosella racovitzae*,[196] *E. aspergillum*[39] and *H. sieboldi*,[197] turned out to be excellent light transmitters, exhibiting some advantages over technical optical fibers.

The stalk spicules of *H. sieboldi* are composed of approximately 40 siliceous layers around the central axial filament and can reach 30 cm in length and a diameter of 300 μm (Fig. 2E). These spicules have been demonstrated to act as sharp high- and low-pass filters. Only light with wavelengths between 615 nm and 1310 nm can pass through the spicule fiber, while wavelengths <615 nm and >1310 nm are filtered out.[197]

Investigations of the optical properties of the basalia spicules (up to 15 cm long, and up to 70 μm diameter) of the hexactinellid sponge *E. aspergillum*[39,198] revealed a high refractive index in the core region, a low refractive index in the surrounding cylindrical tube, and a progressively increasing refractive index in the outer portion of the spicules. The differences in the refractive index could be explained by different organic material content and/or different degrees of hydration of the silica in the spicule core, the cylindrical tube and silica layers. In addition, the high refractive index in the core region may be caused by the enhanced concentration of sodium in this region.

The presence of lens-like structures at the end of the pentactinal spicules from the Antarctic hexactinellid sponge *R. racovitzae* has been shown to improve the light-collecting efficiency of the spicule optical fibers for ambient light;[196] this may allow delivery of sunlight to endosymbiotic algae.

Sponge spicules exhibit advantages over technical optical fibers: the composite structure of the sponge biosilica and the lamellar architecture of the spicule fibers, resulting in enhanced fracture toughness, the low-temperature synthesis, and the presence dopants (sodium) raising the refractive index of the fibers.

9 Model systems of biosilicification

A number of model systems have been used to study formation of biogenic silica nanostructures.[199] In principle, two different strategies can be applied;[200] (i) investigation of the effect on silica formation of biomolecules extracted from silica forming organisms, and (ii) study of the effect of model molecules that exhibit similar structural/functional properties to the naturally occurring molecules (biomimetic approach). The first strategy has been used in most studies described above. Model molecules that have been used to mimic silica formation following the second strategy include amine-containing compounds (polyamines) such as spermine, spermidine, and putrescine homologues,[201,202] polyallylamine hydrochloride, polylysine and polyarginine,[23,203,204]

bifunctional molecules (*e.g.* cysteamine),[205] block copolypeptides (*e.g.* cysteine–lysine block copolypeptides),[206] and model biomolecules such as the R5 peptide (see Section 4.2.1).[122,123,207] Block copolypeptides contain covalently linked domains of water-soluble and water-insoluble polypeptides. These amphiphilic molecules are able to self-assemble into structured aggregates that display both hydrolytic and structure-directing activity, resulting in the formation of ordered silica structures from alkoxide substrates (TEOS). In addition, phage display libraries have been used as a further strategy to identify peptides showing silica-forming activity.[208]

10 Biosilica and nanobiotechnology

The enzymes involved in silica metabolism, in particular silicatein[209] and silicase,[210] have attracted increasing attention because of their potential applications in the field of nanobiotechnology and biomedicine. Silica-based materials are used in many high-tech products including microelectronics, optoelectronics, and catalysts. Biocatalysis of silica formation from water-soluble precursors, in particular silicatein-mediated biosilica production, occurs under mild physiological conditions and is advantageous compared to technical (chemical) production methods that require high temperatures, pressures or extremes of pH. In addition, biological organisms such as sponges and diatoms are able to fabricate their skeletons with high fidelity and in large copy number. These properties are of extreme importance for many applications in nano(bio)technology.

Silicatein remains functionally active even after immobilization of the protein onto metal or metal oxide surfaces.[109,211] Recombinant silicatein immobilized on a gold surface was able to catalyze the formation of interconnected silica nanospheres with a diameter of about 70–300 nm.[81] The gold surface had been functionalized with the chelator nitrilotriacetic acid (NTA) alkanethiol, which bound the recombinant His-tagged protein through Ni^{2+} complexation.[81] Binding of silicatein to metal or metal oxide surfaces was also achieved applying the method of self-assembled monolayers (SAM) formation.[212]

The range of potential applications of silicatein is increased by the fact that this enzyme is also able to catalyze – besides silica deposition – the (non-physiological) formation of other metal oxides like titania (TiO_2),[212–214] zirconia (ZrO_2),[212,215] and GaOOH/spinel gallium oxide[216] from the water-stable precursors at room temperature and neutral pH. These metal oxides are known to exhibit semiconductor, piezoelectric, dielectric and/or electro-optic properties.

Based on these findings, new strategies towards the application of the silica-forming enzymes have been designed. Biocatalytically (silicatein) formed silica may be used as coating of metal implants used in surgery (increase in biocompatibility), for the encapsulation of bioactive compounds (control of drug delivery) and in lithography (fabrication of microelectronics). For example, cultivation of bone-forming cells on silicatein (biosilica)-modified culture plates has been shown to result in an enhanced mineralization (formation of calcium phosphate) of the cells.[217] Thus biocatalytically formed silica may be applied in the production of bone-repairing materials and in dentistry. Silicatein has also been shown to catalyze the (ring-opening) polymerization of (cyclic) L-lactide to the biocompatible and biodegradable polymer poly(L-lactide), which is used as a scaffold in tissue engineering.[218]

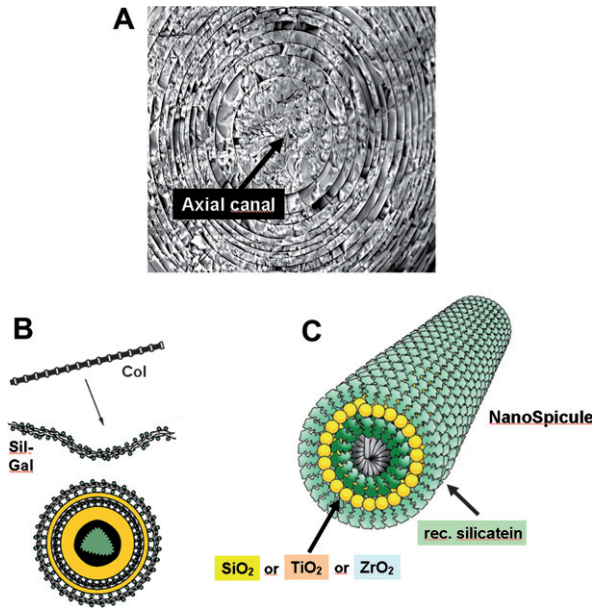


Fig. 15 (A) Tree ring structure of sponge spicules. (B) Nature as model: appositional growth of spicule around the axial (silicatein [green]) filament by formation of alternating organic (silicatein–galectin [Sil-Gal]) layers (organized by extra-spicular collagen [Col] fibrils) and inorganic (silica [yellow]) layers catalyzed by silicatein [green]. (C) Scheme highlighting the structure formation of amphiphile micelles (silicatein [green] + hydrophobic chain [grey]) that could be used for fabrication of artificial sponge spicules (silica [yellow]).

The biosilica products formed by siliceous bioorganisms, *e.g.* the porous structures of diatom frustules, may also be used, *e.g.*, for the construction of nanostructured membranes for particle separations.[219] Alternatively, functional analogues of the silica-forming enzymes (silicatein) could be used to synthesize, *e.g.*, micro- and mesoporous molecular sieves at room temperature and neutral pH.[220]

Silicatein also exhibits a reductive activity, which might be of technological interest; it catalyzes the reductive formation of colloidal gold nanoparticles, which further aggregate to form gold nanocrystals, from tetrachloroaurate anions ($AuCl_4^-$) in solution.[211] His-tagged silicatein immobilized on TiO_2 nanowires has been used to produce gold nanoparticles at the surface of the nanowires;[211] a multifunctional polymeric ligand containing dopamine/catechol (attachment of the polymer to the metal oxide surface) and NTA residues (binding of the His-tagged silicatein) has been applied. Following a similar approach, the fabrication of sponge spicule-like core–shell materials of alternating metal and metal oxide layers with complex properties was found to be feasible (Fig. 15).[109]

11 Conclusion

Biosilicifying organisms are capable of producing a huge variety of siliceous skeletal structures from the nano-scale to the macro-scale. In the past few years, the first insights have been obtained into the mechanisms underlying biosilica formation and the

molecules involved. New groups of enzymes (sponge silicateins) and proteins/polyamines (diatom silaffins) acting at the interface of inorganic chemistry and biochemistry have been discovered. The nanocomposite materials formed in particular by the sponge enzymes, silicatein and silicase (protected by patents; silicatein,[209] and silicase),[210] are of extreme interest for application in nano(bio)technology;[221] thus nature is used as a model to produce new nano-scale devices. The unique feature of biocatalytic (silicatein-mediated) silica formation is the possibility to produce silica glass under mild conditions (low temperature, low pressure, and near-neutral pH); current methods require the application of high temperatures and pressures, and the use of caustic chemicals. This innovative technology will allow the production of metal oxide (silica and other metal oxides) coatings on organic (bio)material surfaces and the development of biomimetic fabrication procedures for new nanostructured materials and devices in opto- and microelectronics industry.[209,210,222,223] The enzymes involved in silica formation and degradation might also be of interest for the organosilicon chemistry, in particular drug design (synthesis of new drug analogs by replacement of a specific carbon atom with a silicon atom; see ref. 224).

12 Acknowledgements

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