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# The unique skeleton of siliceous sponges (Porifera; Hexactinellida and Demospongiae) that evolved first from the Urmetazoa during the Proterozoic: a review

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## Abstract

Sponges (phylum Porifera) had been considered as an enigmatic phylum, prior to the analysis of their genetic repertoire/tool kit. Already with the isolation of the first adhesion molecule, galectin, it became clear that the sequences of the sponge cell surface receptors and those of the molecules forming the intracellular signal transduction pathways, triggered by them, share high similarity to those identified in other metazoan phyla. These studies demonstrated that all metazoan phyla, including the Porifera, originate from one common ancestor, the Urmetazoa. The sponges evolved during a time prior to the Ediacaran-Cambrian boundary (542 million years ago (myr)). They appeared during two major “snowball earth events”, the Sturtian glaciation (710 to 680 myr) and the Varanger-Marinoan ice ages (605 to 585 myr). During this period the aqueous milieu was silica rich due to the silicate weathering. The oldest sponge fossils (Hexactinellida) have been described from Australia, China and Mongolia and were assessed to have existed coeval with the diverse Ediacara fauna. Only little younger are the fossils discovered in the Sansha section in Hunan (Early Cambrian; China). It has been proposed that only the sponges had the genetic repertoire to cope with the adverse conditions, e.g. temperature-protection molecules or proteins protecting them against ultraviolet radiation. The skeletal elements of the Hexactinellida (model organisms *Monorhaphis chuni* and *Monorhaphis intermedia* or *Hyalonema sieboldi*) and Demospongiae (models *Suberites domuncula* and *Geodia cydonium*), the spicules, are formed enzymatically by the anabolic enzyme silicatein and the catabolic enzyme silicase. Both, the spicules of Hexactinellida and of Demospongiae, comprise a central axial canal and an axial filament which harbors the silicatein. After intracellular formation of the first lamella around the channel and the subsequent extracellular apposition of further lamellae the spicules are completed in a net formed of collagen fibers.

The data summarized here substantiate that with the finding of silicatein a new aera in the field of bio/inorganic chemistry started. For the first time strategies could be formulated and experimentally proven that allow the formation/synthesis of inorganic

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structures by organic molecules. These findings are not only of importance for the further understanding of basic pathways in the body plan formation of sponges but also of eminent importance for applied/commercial processes in a sustainable use of biomolecules for novel bio/inorganic materials.

## 5 1 Introduction

The origin of the first ancestor of all metazoan phyla remained enigmatic until the first sequences coding for informative proteins from a sponge (phylum Porifera) had been identified by application of molecular biological techniques (Pfeifer et al., 1993). Before, it had been speculated that the sponges are metazoans that are composed of individually reacting and acting cells (see: Pechenik, 2000). With the identification of the first sponge sequence, a galectin, it became overt that these animals have the genetic toolkit to allow their cells to differentiate from omnipotent via pluripotent to finally determined somatic cells (reviewed in: Pilcher, 2005; Müller, 2006); most of the functional analyses were performed with the sponges *Suberites domuncula* and *Geodia cydonium* (see: Müller et al., 2004). With the first cell-matrix adhesion molecule, an integrin (Pancer et al., 1997), it could be substantiated that the sponges represent organisms that are composed of cells expressing cell surface molecules allowing their cross talks and in turn also divisions/restrictions of their physiological functions (Müller and Müller, 2003). The individuality of a sponge specimen and the morphogenetic arrangement of its cells according to a defined body plan was underscored by the discovery of apoptotic as well as organizer-specific axis-forming molecules in *S. domuncula* (reviewed in: Wiens et al., 2004; Müller, 2005; Wiens and Müller, 2006). The next challenge was to identify those factors which allowed the evolution of the phylogenetically oldest animals, the sponges. One of the key elements, allowing the emergence of these animals was silicon, which comprises both morphogenetic and also structural properties.

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## 2 Role of silicon and silicate

Most skeletal structures in Metazoa are composed of calcium-salts, e.g. calcite or apatite. However, as identified in vertebrates, silica deposition is seen prior to the ossification process forming apatite bones (Carlisle, 1986). The morphogenetic role of silicon is not restricted to this crown taxon, but has also been identified already in sponges (Krasko et al., 2000). In *S. domuncula* this element causes the expression of those genes which are required for the formation of the skeletal elements. Silicon/silicate has been used in the two sponge classes, Hexactinellida and Demospongiae, as a starting substrate for the enzymatic synthesis of their silica-based spicules which are the key structures, allowing the formation/arrangement of the differentiated cells within an individual according to a body plan.

Silicon/silicate is in general precipitated passively onto inorganic matrices, e.g. Roman glasses (Fig. 1A), or on organic matrices like in diatoms (Fig. 1B). In contrast to those processes, the formation of “polymerized”/condensated silicate is guided in siliceous sponges by an enzyme, termed silicatein (Cha et al., 1999). This finding is already amazing and surprising; however, almost not to anticipate was the fact that the silicate-based spicules are the structural basis for the formation of two meters large and highly elaborated sponges, e.g. the hexactinellidan sponges *Monorhaphis chuni* (Fig. 1C) or other *Monorhaphis* species (Fig. 1D) and also Demospongiae, e.g. *Lubomirskia baicalensis* (Fig. 1E; Carlisle, 1986). The question arises when and in which environment did the sponges appear/evolve.

## 3 Evolution during the Proterozoic: evolution of sponges in the silicon ocean

Macroevolution reflects the mechanisms during which evolutionary novelties are brought about. This process might be an unbroken continuation of microevolution or could be independent of microevolutionary processes (see Mayer, 2001). Nevertheless, through macroevolutionary changes new anatomical, developmental innovations

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were introduced which surely reflect genetic innovations. One striking example for the evolution of a new biotic kingdom in a drastically changing environment, with all its dependent physical and chemical living conditions, is the appearance of Metazoa. They emerged from their hypothetical ancestor, the Urmetazoa (Müller et al., 2001).

5 It is surprising that 542 million years ago (myr), near the Ediacaran-Cambrian boundary, a rapid appearance of different animal types occurred; this event was called “Cambrian Explosion”. Exciting examples of these emergences were the so-called Ediacaran biota (Knoll and Carroll, 1999). The development and divergence of the major animal clades were surely driven by the genetic tool kits available at that time. The major, or perhaps even only, phylum existing at the border from Late Neoproterozoic to Cambrian (approximately 543 MA) which did not become extinct are the sponges. Consequently, sponges were also termed “living fossils” (Müller, 1998); they represent the evolutionary oldest, still extant taxon which testifies the developmental level of the animals that lived in the Neo-Proterozoic eon (1000 to 520 MA); Figs. 2 and 3. 10 This must be especially mentioned since two major “snowball earth events” occurred, the Sturtian glaciation (710 to 680 myr) and the Varanger-Marinoan ice ages (605 to 585 myr), which very likely caused the covering of the earth by a continuous ice layer. It was proposed that as consequence of these ice ages most organisms went extinct (Hoffman et al., 1998); perhaps more than 85% (Mayr, 2001).

20 The primordial earth surface comprised initially insoluble silicates and carbonates as well as, to a small extent, phosphates. During the silicate weathering-carbonate precipitation cycle, prior or simultaneously with the glaciations, a dissolution of these surface rocks composed of insoluble silicates ( $\text{CaSiO}_3$ ) resulted in formation of soluble calcium carbonate ( $\text{CaCO}_3$ ) and soluble silica ( $\text{SiO}_2$ ), under consumption of atmospheric  $\text{CO}_2$  25 (Walker, 2003). The resulting soluble minerals leached into the waters of the rivers, lakes and oceans. There, they were again re-precipitated into new composites, as part of the sedimentary rocks. Such processes depend upon temperature, pH and atmospheric carbon dioxide. Passively, the minerals are transformed diagenetically to secondary structures.

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In contrast to passive re-precipitation, biogenic deposition of minerals by metazoans is first seen in sponges. The oldest sponge fossils (Hexactinellida) have been described from Australia, China and Mongolia (from more than 540 MA) (Gehling and Rigby 1996; Li et al., 1998; Brasier et al., 1997). Hence, the Hexactinellida are the 5 oldest group of sponges as documented there and later in fossil records of the Sansha section in Hunan (Early Cambrian, China; Steiner et al., 1993). There, in both lower and upper levels of the Niutitang Formation, more or less completely preserved sponge fossils, e.g. *Solactiniella plumata* (Figs. 4A-a to 4A-c), have been discovered (Steiner et al., 1993). The occurrence of almost intact sponge fossils also in the basal part of 10 this formation is real (Rigby and Guang, 1996) and is stratigraphically equivalent to the Chengjiang assemblage in Yunnan (China). So far the base of the Niutitang Formation has been correlated with the Tommotian black shales, “Badaowan” Member. However, the transgression that deposited the black shale of the Niutitang Formation and the “Badaowan” Member was a diachronous event, which first became evident in 15 the basin environment, Hunan/SE – Guizhon/S-Anhui, and progressed across the platform, Yunnan/Sichuan (Steiner, 1994). Therefore, the base of the Niutitang Formation, which contains basinal phosphate concretions and cherts, may be correlated with the Tommotian or even the Nemakit-Daldynian. The important point is that the sponges at the Sansha section definitely occur at lower levels than the famous, diverse fauna at 20 Chengjiang. Except for one questionable demosponge, all the sponges described from the Sansha section can be attributed to the Hexactinellida.

Very likely the oldest isolated spicules were also found in China, observed in thin sectioned material from the Dengying Formation, “Shibantan” Member, Hubei Province (Steiner et al., 1993). These spicules are mainly monaxial spicules, but some are also rather definite “crosses”, the evidence of triaxones from hexactinellids. The 25 “Shibantan” Member in Hubei is of Late Proterozoic age and stratigraphically equivalent with the Ediacara (South Australia), which is famous because of its exceptionally preserved Vendian fossils. The Ediacara fauna has normally been considered to begin at about 600 MA, although new stratigraphic data place the base of the Ediacara

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fauna at  $\approx 565$  MA, and suggest that it ranges up to the Precambrian/Cambrian boundary (Grotzinger et al., 1996). Still, the Ediacara-type Vendian fossils are the oldest megafossils, which may, at least partly, be interpreted as metazoans, although this interpretation is still controversial (Seilacker, 1989; Morris, 1993, 1994; Retallack, 1994).  
5 It is justified to call the Porifera the oldest organismic group in the Earth history, which can now be proven as definitely belonging to the animal kingdom, and which furthermore has survived successfully until present times. In contrast, to the marine Hexactinellida and Demospongiae, the freshwater demosponges are much younger; one of the first fossil freshwater sponges, *Spongilla gutenbergiana*, had been described  
10 from the Middle Eocene (Lutetian); (Müller et al., 1982; Figs. 4B-a to 4B-c).

The earliest evidence of the Demospongiae and the Calcarea is the presence of isolated spicules in thin sections of rocks from Early Cambrian (Atdabatian) Archaeocyath mounds of the Flinders Ranges (South Australia). The Calcarea documented from the Flinders Ranges, Archaeocyath mounds, are both isolated spicules and also more or  
15 less complete rigid skeletons of small calcaronean sponges sitting on Archaeocyaths. The spicules are of the triaene, equal-angular type characteristic of the modern Calcarea, but quite different from those typical of the Paleozoic Heteractinellida. Very similar, perhaps identical, spicules from the Early Cambrian of Sardinia were described as *Sardospongia triradiata* and attributed to the Heteractinellida (Mostler, 1985). The complete calcarean sponge *Gravestockia pharetronensis* (Reitner, 1992) from the Flinders Ranges possessed a rigid calcitic skeleton with affinity to the modern Pharetronida. As  
20 one of the earliest families of the Demosponges the Geodiidae have been described on the basis of their sterrasters (Reitner and Mehl, 1995).

Those taxa that survived the “snowball earth” episodes must have had the genetic  
25 repertoire to cope with the adverse conditions, meaning: (i) proteins to protect against adverse temperature, (ii) possibility to survive food restriction and (iii) – not to forget – also the protection machinery against ultraviolet radiation. During the pre-Sturtian period atmospheric CO<sub>2</sub> had been consumed and removed from the atmosphere resulting in extreme temperature amplitudes. In sponges, *cryoprotective proteins* exist,

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e.g. galactose-specific lectins (Pfeifer et al., 1993; Wiens et al., 2003) which display cryoprotective protein-membrane activity, or, interestingly, also  $\beta$ -crystallins, proteins which contain only low amounts of water and hence are resistant to adverse protein  
5 folding (Krasko et al., 1997). *Food restriction* was probably compensated by the establishment of a symbiotic relationship with microorganisms (Breter et al., 2004), such as gram-positive (Thakur et al., 2005) and gram-negative bacteria (Wiens et al., 2005) as well as fungi (Perovic-Ottstadt et al., 2004a). This eukaryotic-prokaryotic “labor-division” allowed the sponges a flexible and rapid adaptation to the changing environment. It is furthermore amazing that sponges have an unexpected variety of protection  
10 systems against *mutagens*, including also ultraviolet *radiation*. Several protection systems against radiation have been described; e.g. the (6-4) photolyase system (Krasko et al., 2003b) or the SOS-response-like mechanism (Krasko et al., 1998), and there is an extensive literature on protection systems against environmental stress (e.g. Efremova et al., 2002). Perhaps the greatest fortune for the sponges was their ability  
15 to utilize silicic acid as substrate for their skeleton. When sponges emerged, the insoluble silicate minerals were converted to monomeric, soluble silicate providing the sponges with an advantageous basis for survival and diversification, with respect to the number of the species and their abundance. Early assumptions postulated that those taxa which survived mass extinction, e.g. the Foraminifera, became ecological  
20 and morphologically generalized species (Cifelli, 1969). Furthermore, the number of species, the diversity of the biota, increased rapidly after each period of extinction (Futuyama, 1986). Hence, the sponges (survivor taxon) became the beneficiaries of the glaciation crises and received the chance to colonize those habitats which had been de-populated.

25 The urmetazoans/sponges had already the basic genetic toolkit for all deriving metazoans which emerged during “Cambrian Explosion” (Figs. 2 and 3). This statement implies that the genetic repertoire of the sponges, which survived the glaciations, gives the frame, potentials/potentialities but also the limits of the body plan construction, seen in higher “crown” groups and which exist in the present day animal phyla. The

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“crown” taxa utilized the pre-existing molecules and pathways for their diversification of patterning and for an increase in the genetic network complexity. It can be postulated that during the progress of evolution the degree of entropy decreased, whereas the complexity increased. This progressive “perfection” might be detrimental to the stability and survival of most of the species which are evolutionary younger.

The oxygen level in the atmosphere and the water during early Proterozoic was lower than at present (Hayes, 1994). It had been postulated that with the rise of oxygen the synthesis of collagen became possible (Towe, 1981); sponges contain and express collagen genes (Garrone, 1998). The ability to form these extracellular fibrils had been considered to be a crucial prerequisite for the origin of multicellular animals and the establishment of a metazoan body plan (Towe, 1981). Until now, no experimental evidence for an existence of a primordial, blood oxygen-transporting system in sponges has been found. Therefore, we assume that most of the oxygen required by these animals to allow intermediary metabolism is provided by diffusion. Perhaps oxygen is partly transported/generated via the tyrosinases (Breter et al., 2004). However, this apparent disadvantage was surely of benefit for the sponges to survive the oxygen deficiency in waters because of an ice cover during the glaciations.

#### 4 Unique formation and degradation of biomaterial (biosilica) in sponges: silicatein and silicase

Sponges are sessile filter-feeding organisms; their body is composed of an epithelial layer which surrounds a mesohyl compartment. This is reticulated in a highly organized manner by a canal system. The main structural and functional novelties, which evolved during the major evolutionary transitions to the Porifera and Cnidaria, are summarized in Fig. 3. The characteristic trait of the sponges are the spicules (the sclerocytes) which stabilize the sponge bodies and provide the platform on which the body plan can develop (reviewed in: Müller et al., 2004). In the center of the spicules is a hollow canal of varying diameter around which the silica is deposited under formation of concentric

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layers (Uriz et al., 2000). This axial canal harbors an organic filament called axial filament; this filament is composed of silicatein (Fig. 5). Interestingly enough, the spicules with the axial canal can be identified in fossil sponges with all their characteristic features (Fig. 4A-c). The inorganic silica phase of the siliceous spicules contains 6–13% water, yielding an approximate formula of  $(\text{SiO}_2)_{2-5} \cdot \text{H}_2\text{O}$  (reviewed in: Sanford, 2003). High resolution magnetic resonance microimaging studies revealed that this water is largely present in a “mobile” form at least in certain freshwater sponges (Müller et al., 2006b). In addition, spicules contain trace amounts of other elements, mainly S, Al, K, and Ca, but also Cl, Fe, Na, Zn, and Cu (reviewed in: Uriz et al., 2003a, b; Sanford, 2003).

One major breakthrough in the understanding of spicule formation came after the discovery of the key enzyme involved in spiculogenesis. The group of Morse discovered that the organic filament in the central canal of spicules is composed of a cathepsin L-related enzyme, which they termed silicatein (Shimizu et al., 1998; Cha et al., 1999); Fig. 5. They cloned two of the proposed three isoforms of silicateins, the  $\alpha$ - and  $\beta$ -forms, from the marine demosponge *Tethya aurantium* (Cha et al., 1999). Then these molecules were cloned also from other sponges, among them the marine sponge *S. domuncula* and the freshwater sponge *L. baicalensis* (Schröder et al., 2004b; Wiens et al., 2006).

The *S. domuncula* cDNA encoding silicatein was isolated and characterized; the predicted translation product of 330 amino acids has a calculated size of  $M_r$  36 306. As outlined before, silicatein is a new member of the cathepsin subfamily (Cha et al., 1999; Müller et al., 2003, 2005). The three amino acids Cys, His and Asn, which form the catalytic triad of cysteine proteases, are present in the sponge cathepsin at the characteristic sites: Cys<sub>125</sub>, His<sub>164</sub> and Asn<sub>184</sub>. Silicatein contains a Ser residue instead of Cys. Furthermore, the silicatein comprises one cluster of characteristic hydroxy amino acids (serine). We could demonstrate that silicatein occurs in the axial filament in several isoforms (Schröder et al., 2004b). By two-dimensional gel electrophoretic analysis of the axial filament (silicatein) we could demonstrate that in this compartment silicatein

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undergoes stepwise phosphorylation.

A further enzyme of potential interest for application in nanobiotechnology is the silicase. This enzyme is able to depolymerize amorphous silica (Schröder et al., 2003a). The cDNA encoding the silicase has been identified in primmorphs from *S. domuncula*,  
5 applying the technique of differential display of transcripts. The expression of the silicase gene is strongly upregulated in response to higher silicon concentrations in the incubation assays (Schröder et al., 2003b), like the expression of silicatein. The deduced polypeptide has a  $M_r$  of 43 131. Silicase is a member of the family of carbonic anhydrases. An alignment of the sponge silicase sequence with the human  
10 carbonic anhydrase II shows that most of the amino acids which are characteristic for the eukaryotic-type carbonic anhydrase signature are also present in the sponge silicase (Schröder et al., 2003b). The recombinant silicase was found to display besides carbonic anhydrase activity the ability to dissolve amorphous silica under the formation of free silicic acid (Schröder et al., 2003b).

15 Carbonic anhydrases are a family of zinc metal enzymes (Sly and Hu, 1995). In the *S. domuncula* silicase the three conserved His residues which bind the zinc ion are found in the deduced protein at aa<sub>181</sub>, aa<sub>183</sub> and aa<sub>206</sub>. The proposed mode of action of the silicase to depolymerize amorphous silica follows the reaction mechanism known for other zinc-dependent enzymes involved in ester hydrolysis. The zinc ion is a Lewis  
20 acid that interacts with water, a Lewis base. The zinc-bound hydroxide ion formed by splitting the water molecule mediates a nucleophilic attack at one of the silicon atoms linked by oxygen bond(s). In the next step the zinc-complex binds, within the polymeric silicate, to one silicon atom under cleavage of the oxygen linkage. Through consumption of H<sub>2</sub>O, the silicic acid product is released and the zinc-bound hydroxide  
25 is regenerated allowing the start of the next catalytic cycle. The enzyme has been expressed in *E. coli* using the glutathione S-transferase (GST) fusion system (Schröder et al., 2003b).

Recent studies have shown that during spicule formation in primmorphs of *S. domuncula*, the expression of both, silicatein and silicase, is seen at those sites within the

3-D-cell aggregates where the growth/building of these siliceous structures starts (Eckert et al., 2006). The role of the silicateins as synthesizing enzymes also in vivo was elucidated by in situ hybridization studies (Schröder et al., 2004a). In a recent study immunofluorescence analyses have been applied to demonstrate that in primmorphs  
5 silicatein is associated with the fibrous structures in primmorphs that represent the primordial spicules. In parallel with the application of anti-silicatein it could be shown that also the silica catabolic enzyme (silicase) is localized at the same structures within the primmorphs. This result had been supported also by immunofluorescence studies of sections through tissue samples. Like silicatein(s) also silicase belongs to a protein  
10 family known from triploblasts, the carbonic anhydrases (Schröder et al., 2003a).

## 5 Hexactinellida: first approaches to understand spicule formation

The monophyletic group of Hexactinellida is, according to the form and organization of its spicules, divided into two subclasses, Hexasterophora and Amphidiscophora. In the latter subclass amphidiscs are the (main) microscleres that never fuse. One  
15 member of this subclass is the family Monorhaphididae which includes also the three species – *Monorhaphis chuni*, *M. dives* and *M. intermedia*. Since the discovery of these sponges during the first German Deep Sea Expedition (RV “Valdivia”) and the first descriptions of these animals by Chun (1900) and Schulze (1904), only little additional information has been added about the form and construction of their skeletal systems.  
20 Nothing has been published about the synthesis of their giant spicules. Worldwide *M. chuni* has been documented only in few sampling sites, suggesting a distribution in the deep sea in the Indian Ocean and in the West Pacific. One outstanding feature of *Monorhaphis* is the anchoring spicule which can reach lengths of up to 3 m with a maximum diameter of 8.5 mm (Schulze, 1904). *M. chuni* thus produces the largest  
25 biosilica structure known on Earth. Polished cross sections confirm the existence of up to 500 highly regular concentric rings which are arranged around a 150  $\mu$ m thick, less structured core (Schulze, 1904). In the middle of this core, a square axial channel with

an approx. 2  $\mu\text{m}$  thick proteinaceous filament is visible, which runs through the entire spicule from one end to the other (Figs. 6A, C and D). The silica layers are composed of smaller granules and spheres within a network of (perhaps) proteinaceous material (Fig. 5B).

5 An additional hexactinellid *Hyalonema sieboldi* (Müller et al., 2006c) likewise possesses long stalk spicules that attach them to the substratum; the animals live in a depth of more than 600 fathoms (1000 m); (Wyville Thomson, 1874). The long stalk spicules are composed of distinct siliceous layers which are also superposed in a stratified pattern around a central axial filament (Schulze, 1904). It could be demonstrated  
10 that these giant spicules from the root-tuft of *H. sieboldi* transmit light with high efficiency. Surprisingly, however, the blue light with a wavelength between 400 and 600 nm is filtered out. Data elaborated in our group suggest that the spicules from *H. sieboldi* act as optical absorbent in a novel photoreception system (Müller et al., 2006c).

Very recently, Aizenberg et al. (2005) published a detailed structural analysis of the spicule formation in the hexactinellid *Euplectella*. They demonstrated structural hierarchies of the spicule synthesis starting from the nanometer-sized particles of silica to the final mature spicules. The compositions of the proteins which are associated with and found in the spicules have not been described. Ehrlich et al. (2005) dissolved the basal spicules of the hexactinellid *H. sieboldi* in alkaline solution for 14 days and showed that  
15 the abundant structural protein, associated with the spicules might be collagen.

Recently, we performed microscopic analyses of the spicules from *M. chuni* with major emphasis of the large-sized giant spicules (giant basal spicules or basalia; size of 1 m) and also the large comitalia (size of around 60 mm). The focus of the study was put on the organic components of these spicules, including the collagen fibrils  
20 which surround these spicules. Furthermore, electron microprobe analysis data had been given which show a regionally different composition of sodium and potassium within the spicules. With respect to the organic components, it should be highlighted that after dissolution of the spicules several proteins could be visualized; (i) one of them cross-reacted with antibodies raised against silicatein, while (ii) others displayed

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proteolytic activity (to be published).

After having substantiated, by application of molecular biological techniques, that also Hexactinellida have at least one gene coding for collagen (the cDNA was isolated from the hexactinellid *Aphrocallistes vastus*) a schematic outline of the spicule growth  
5 both in longitudinal and axial direction could be given (Fig. 7). In the center of the spicules the axial canal is filled with an axial filament which comprises a protein composition highly similar to the one described for demosponges. After formation of a first silica layer, assumed to be the result of silicatein or of a silicatein-related protein, the next layer is formed through centrifugal and centripetal synthesis of a silicatein(-related)  
10 protein. In analogy to Demospongiae (Schröder et al., 2006) it is adopted that the silicatein(-related) protein is associated with a lectin which form a tube-like cage around the existing silica lamella. After deposition of newly formed silica clusters followed by a solid silica lamella the existing proteins are hydrolyzed by protease(s). In continuation a third lamella of silica is formed. The model also proposes that collagen guides the  
15 silicatein(-related) protein/lectin associates concentrically along the spicules.

## 6 Demospongiae: silica deposition during spicule formation

The morphology of sponge spicules had been analyzed recently using the marine demosponge *S. domuncula* (reviewed in: Müller et al., 2006a). From this species primmorphs had been prepared and applied for the analysis of spicule formation. Primmorphs, a special type of 3-D-cell aggregates, containing proliferating and differentiating cells, allow during incubation in medium supplemented with silicic acid to study the differentiation of archaeocytes to sclerocytes. The spicules were analyzed by electron microscopical procedures, e.g. scanning electron microscopy (SEM) analysis of spicules, transmission microscopical analysis (TEM) and electron immunogold labeling.  
20 The skeleton of *S. domuncula* is composed of only two types of megascleres, monactinal tylostyles and a smaller fraction of diactinal oxeas. The spicules reach up to 450  $\mu\text{m}$  in length and diameters of 5 to 7  $\mu\text{m}$  (Figs. 8A to C); they grow through

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apposition of lamellar silica layers. While the two ends of the oxeas are pointed, the tylostyles have one pointed end and one swollen knob. All spicules have an axial canal of 0.3 to 1.6  $\mu\text{m}$  in width in the center. As shown in SEM images (Figs. 8A to C) the central canal is surrounded by lamellated layers approximately 0.3 to 1  $\mu\text{m}$  thick. To  
5 monitor the location and distribution of silicatein in and around the spicules, polyclonal antibodies were raised against purified filaments from spicules in rabbits (Müller et al., 2005). Immunofluorescence studies with cryosections through tissue revealed that the surfaces of the spicules in the tissue (Fig. 8D) and of isolated spicules (Figs. 6E and F) are surrounded by silicatein. From these studies we conclude that silicatein is not only  
10 present in the axial filament of the spicules, but is also located on their surface.

Based on our studies, it had been deduced that the process of spicule formation should be divided into the following phases; the initial intracellular steps and the extracellular final and shaping phase (Müller et al., 2006a; Eckert et al., 2006).

(i) *The intracellular phase in the sclerocytes:* Silica is taken up actively by a  
15  $\text{Na}^+/\text{HCO}_3^-[\text{Si}(\text{OH})_4]$  cotransporter. Very likely during the transport through the endoplasmic reticulum and the Golgi complex, silicatein undergoes phosphorylation and is transported into vesicles where it forms rods, the axial filaments (Fig. 8A). After assembly to filaments the first layer(s) of silica is (are) formed. Silica deposition occurs in two directions; first from the axial canal to the surface (centrifugal orientation) and second from the mesohyl to the surface of the spicule (centripetal). Finally the spicules  
20 are released into the extracellular space where they grow in length and diameter by appositional growth (Fig. 8B).

(ii) *Extracellular phase (appositional growth):* Silicatein is present also in the extracellular space. It came surprising that also there the silicatein molecules are organized  
25 to larger entities. The immunogold electron microscopical analysis showed that the silicatein molecules are arranged along strings, which are organized in parallel to the surfaces of the spicules (Schrüder et al., 2006). In the presence of  $\text{Ca}^{2+}$ , silicatein associates with galectin and allows the appositional growth of the spicules (Fig. 8B). Since the surface of a new siliceous spicule is also covered with silicatein, the appo-

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sitional growth/thickening of a spicule hence proceeds from two directions (centrifugal and centripetal).

(iii) *Extracellular phase (shaping):* In the next step, the galectin-containing strings are organized by collagen fibers to net-like structures. It is very likely that collagen, which  
5 is released by the specialized cells, the collencytes, provides the organized platform for the morphogenesis of the spicules (Fig. 8B). The longitudinal growth of the spicules can be explained by the assumption that at the tips of the spicules, the galectin/silicatein complexes are incorporated into deposited biosilica under formation and elongation of the axial canal.

## 10 **7 Change in a paradigm**

In 1828 Wöhler succeeded to copy nature by producing an organic compound from inorganic reactants. He synthesized urea from the inorganic ammonium cyanate, establishing the first rules in organic chemistry. However, to copy organic (bio)synthetic reactions comprehensively, the existence of enzymes had to be discovered, an important step which dates back to Pasteur (1857). He discovered that lactic acid is  
15 a fermentation product, and thus proved the basis for the discipline biochemistry. The causal-analytical understanding of organic reactions in biological systems became possible after the deciphering of the genetic code and the subsequent elucidation and application of molecular biological, recombinant techniques. However, it took until recently when first strategies had been formulated and experimentally proven, to outline  
20 the biosynthesis of inorganic structures formed in uni- and multicellular organisms. At present, in a self-accelerating progress, the matrices (templates), e.g. collagen, and the organic catalysts (enzymes), e.g. silicatein, required for the synthesis of those inorganic structures and skeletal elements have been illuminated with the help of inorganic/organic chemists, biochemists, molecular biologists and material scientists, to  
25 establish the discipline "bio-inorganic material science". The first opportunities have been touched in biomedicine and electronics providing us with a first indication about

400



the power and potential of this new technology (Wang and Wang, 2006).

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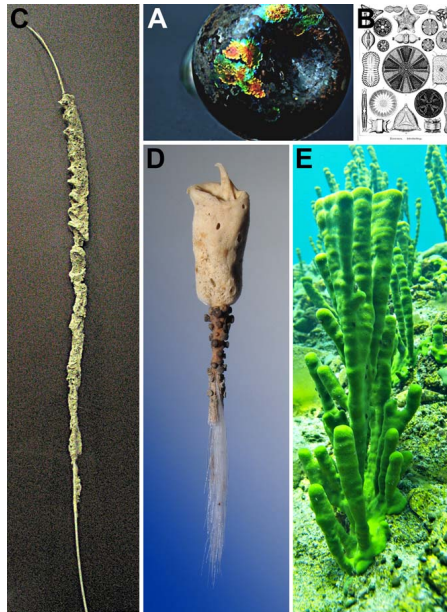
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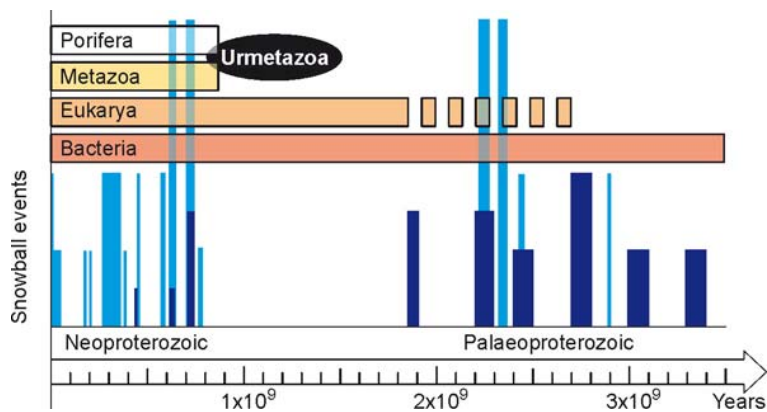
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**Fig. 1.** Non-enzymatic precipitation of silica (A and B) and enzymatic formation of biosilica by sponges (C to E). **(A)** Romanic vase (150 years AC) showing opalescence [silicon-based inorganic films] on the surface of the glass (size: 7 cm). **(B)** Lithography from Haeckel (1899) showing the diverse forms of diatoms (non-enzymatic silica formation). **(C)** A giant spicule from *Monorhaphis chuni* (height: 1.3 m). **(D)** A *Hyalonema* species (Hexactinellida), collected by Doflein (1906) (height: 12 cm). **(E)** *Lubomirskia baicalensis* (Demospongiae), an endemic freshwater sponge from Lake Baikal (height: 40 cm).

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**Fig. 2.** Frequency of occurrence of major glacial periods (blue). The appearance of the different organismic groups (Bacteria; Eukarya; Metazoa and Porifera) are indicated. The Urmetazoa evolved during the inter-ice period between 600 MA and approximately 800 MA (modified after Hoffmann and Schrag, 2002).

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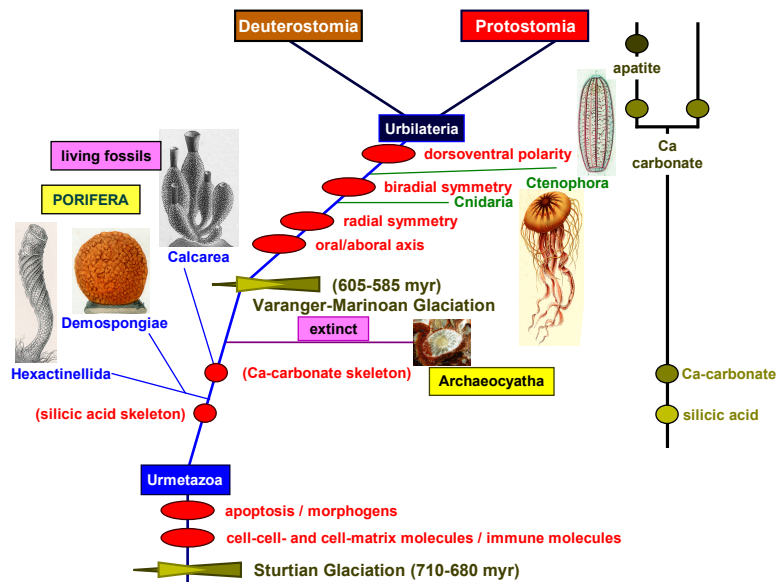
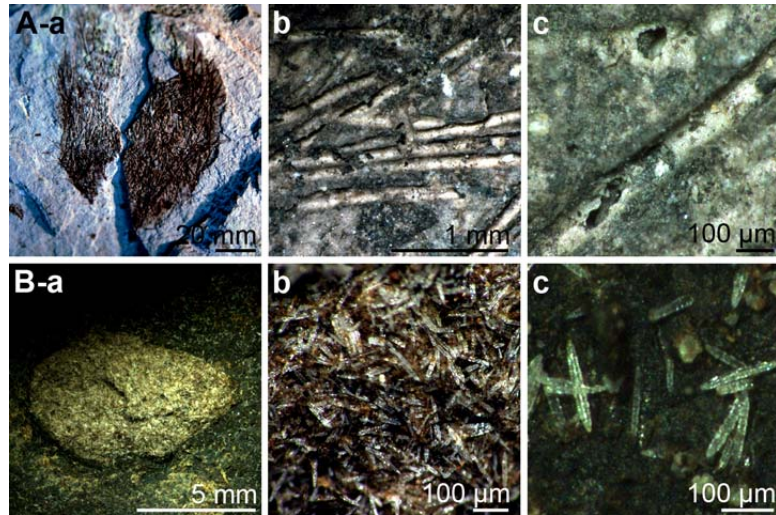


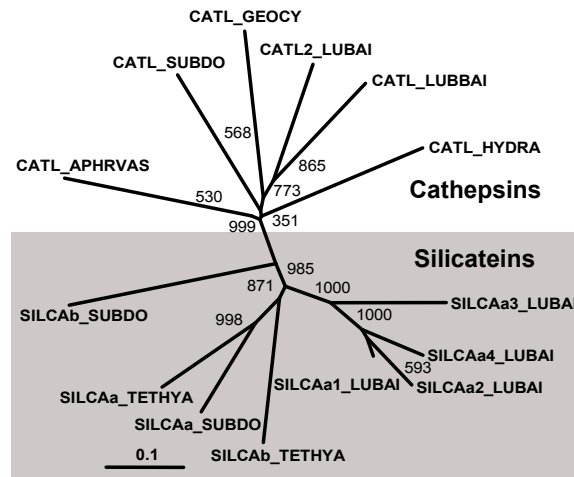
Fig. 3.

**Fig. 3.** Phylogenetic position of the Porifera between the Urmatazoa and the Urbilateria. The major evolutionary novelties which have to be attributed to the Urmatazoa are those molecules which mediate apoptosis and control morphogenesis, the immune molecules and primarily the cell adhesion molecules. The siliceous sponges with the two classes Hexactinellida and Demospongiae emerged first and finally the Calcarea, which possess a calcareous skeleton, appeared. These three classes of Porifera are living fossils that provide a reservoir for molecular biological studies. The Archaeocyatha, sponge related animals with a calcareous skeleton, became extinct. The Calcarea are very likely a sister group of the Cnidaria. From the latter phylum the Ctenophora evolved which comprise not only an oral/aboral polarity but also a biradial symmetry. Finally the Urbilateria emerged from which the Protostomia and the Deuterostomia originated. Very likely the Urmatazoa emerged between the two major “snowball earth events”, the Sturtian glaciation (710 to 680 myr) and the Varanger-Marinoan ice ages (605 to 585 myr). In the two poriferan classes Hexactinellida and Demospongiae the skeleton is composed of amorphous and hydrated silica, while the spicules of Calcarea are composed of Ca-carbonate. The latter biomineral is also prevalent in Protostomia and Deuterostomia. In vertebrates the bones are composed of Ca-phosphate (apatite).



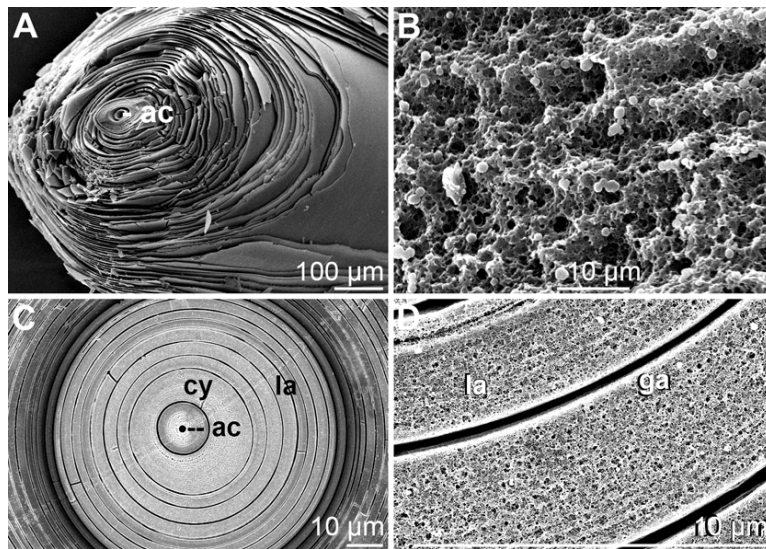
**Fig. 4.** Fossil marine and freshwater sponge. **(A)** One of the oldest fossil marine sponge in body preservation are known from the Lowermost Cambrian Sansha section (Hunan, China); *Solactiniella plumata* (Hexactinellida) **(A-a)**. This fossil is composed of highly intact spicules **(A-b)**. **(A-c)** Some spicules are broken and expose the internal axial canals. **(B)** One of the oldest fossil freshwater sponge; *Spongilla gutenbergiana* from the Middle Eocene (Lutetian) near Messel (Darmstadt, Germany). **(B-a)** Spicule assembly, reminiscent of a complete animal. **(B-b)** Oxeas in these nests. **(B-c)** Oxeas with the flashing centers, representing the axial canals. Size bars are given.

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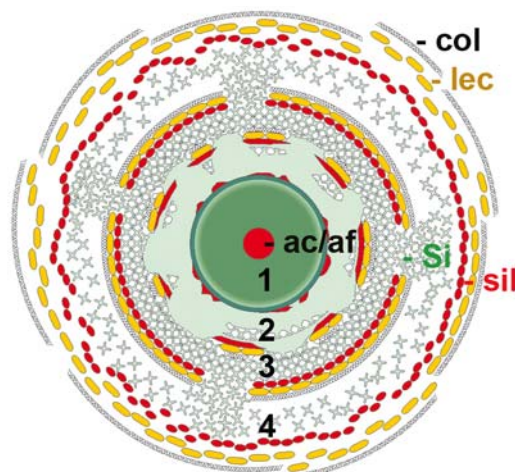
**Fig. 5.** Phylogenetic relationship of the silicateins, the enzyme which catalyzes the polymerization process of biosilica in the sponge spicules. Four deduced silicatein sequences of the isoform silicatein- $\alpha$  ( $\alpha$ -1,  $\alpha$ -2,  $\alpha$ -3 and  $\alpha$ -4) from *Lubomirskia baicalensis* (SILICAa1.LUBAI; SILICAa2.LUBAI; SILICAa3.LUBAI; SILICAa4.LUBAI) and the two cathepsin L sequences (CATL.LUBAI; CATL2.LUBAI) were aligned with silicatein- $\alpha$  from *S. domuncula* (SILICAa.SUBDO) from *Tethya aurantium* [*T. aurantium*] (SILICAa.TETYA) and with the  $\beta$ -isoenzymes from *S. domuncula* (SILICAb.SUBDO) and *Tethya aurantium* (SILICAb.TETYA), as well as with the cathepsin L sequences from sponges *Suberites domuncula* (CATL.SUBDO), *Geodia cydonium* (CATL.GEOCY) and *Aphrocallistes vastus* (CATL.APHRVAS) as well as from the cnidarian *Hydra vulgaris* (CATL.HYDRA). The radial phylogenetic tree was constructed after the alignment of these sequences. The numbers at the nodes are an indication of the level of confidence for the branches as determined by bootstrap analysis (1000 bootstrap replicates).

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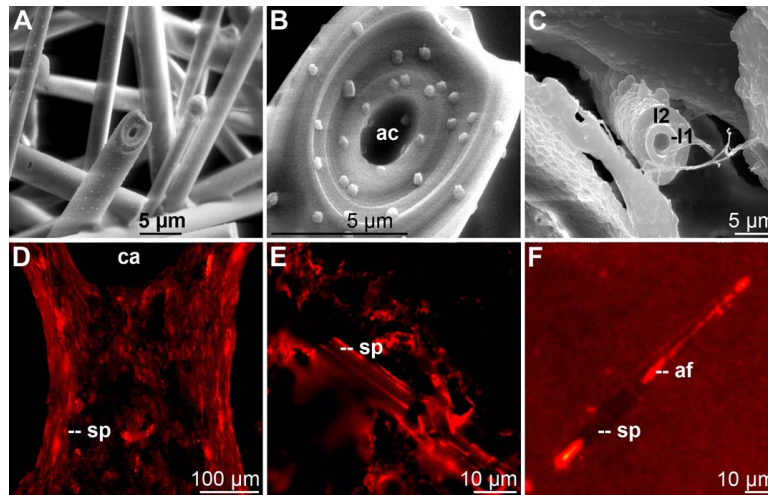
**Fig. 6.** Morphology of hexactinellid spicules. Concentric organization of the silica layers of giant spicules and giant basal spicules from *Monorhaphis intermedia* (A to D). **(A)** The diagonal scanning electron micrograph (SEM) shows the layered surface of the spicules. The layers are separated by small gaps, which harbored the proteinaceous material, synthesizing the silica layers. **(B)** Granules and spheres of silica which form the lamellae of the spicules. **(C and D)** Giant basal spicules (basalia) from *M. intermedia*. The polished cross sections can be divided in three parts: (i) The axial canal (ac) in which the axial filament is located. (ii) An axial cylinder (cy), formed by dense homogeneous silica and finally (iii) the main part of the spicule with the regularly arranged concentric silica lamellae (la). **(D)** Higher magnification of the lamellar region is shown. Between the lamellae (la) a gap (ga) exists.

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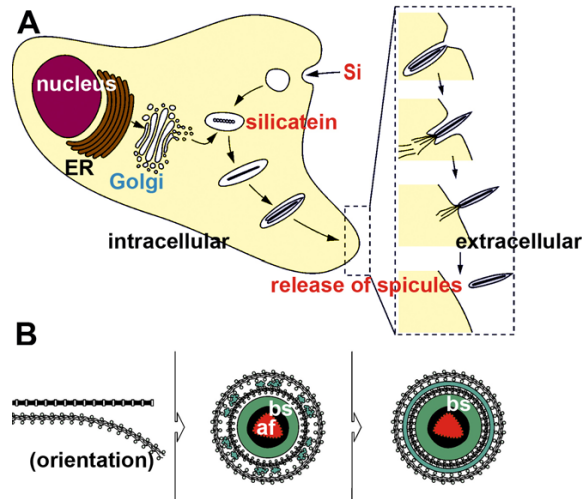
**Fig. 7.** Proposed schematic illustration of the spicule formation in the hexactinellid *M. chuni* and *M. intermedia*. The center of the spicule comprises the axial canal (ac) which is filled with the axial filament (af; in red). Around the axial filament a first lamella has been formed (1). The formation of the next silica lamella is supposed to be mediated by silicatein(-related) proteins (red ellipsoid balls) which are arranged both on the surface of the first lamella and a proteinaceous tube/cage which is stabilized in its outer layer by lectin molecules (yellow balls). The final orientation of the tube is provided by the collagen mat. Within the cage a solid silica lamella is formed through an association of the silica clusters. The concentric arrangement of the silicatein(-related) proteins/lectin associates is proposed to be stabilized by collagen (col; grey fibers).

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**Fig. 8.** Morphology of demospongian spicules showing the zonation of the silica shell of the spicules from *Suberites domuncula*. (A to C) SEM analysis of broken spicules in primmorphs, after an incubation period of 20 days. Around the axial canal (ac) two layers of lamellae (l1 and l2) can be distinguished. (C) and (D) Immunostaining of spicules with antibodies, raised against silicatein (PoAb-aSILIC). (D) Immunostaining of cryosections through tissue of *S. domuncula*. 8- $\mu$ m thick frozen sections were reacted with polyclonal silicatein antibodies. Spicules (sp) and one canal of the aquiferous system (ca) are marked. (E) and (F) Reaction of antibodies against silicatein with partially digested (hydrofluoric acid) spicules. The preparations were placed onto glass slides and treated with the antibodies (F). The immuno-stained specimen showed that both the surface of the spicules (sp) and also the axial filaments (af) were brightly stained.

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**Fig. 9.** Schematic outline of spicule formation in *S. domuncula*. (A) The initial steps of spicule synthesis occur intracellularly where the silicatein is processed to the mature form. During this processing the silicatein undergoes phosphorylation. Very likely with the help of other proteins the silicatein molecules assemble to a rod, the axial filaments. Around this filament the first deposition of silica proceeds. (B) Process of the appositional growth of the spicules in the extracellular space (mesohyl). In the mesohyl, galectin molecules associate in the presence of  $\text{Ca}^{2+}$  to strings (nets) that allow binding of silicatein molecules. Collagen fibers orient the silicatein-galectin strings concentrically round the growing spicules. In the last step, biosilica deposition is mediated in two directions, originating both from the silicatein-galectin strings and from the surface of the spicules (centripetal and centrifugal). Finally an additional further biosilica lamella (bs) is formed which is layered onto the previous two lamellae. The initial biosilica layer is formed around the silicatein rod, existing in the axial canal (ac) of the spicules.

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