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BIOMINERALIZATION FOR LITHOGRAPHY AND MICROELECTRONICS

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Thematic Priority: NMP Priority

Final Activity Report

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Johannes Gutenberg Universität Mainz

Contents

Part I: Project Execution

Introduction

The impact of biomineralization processes on lithographic and microelectronic production processes has not yet been explored. As opposed to conventional industrial manufacturing, the biological synthesis of silica occurs under mild physiological conditions of low temperatures and pressures, with clear advantages in terms of cost-effectiveness, parallel production, and impact on the environment. The integration of nature-mimic biomineralization processes with micro- and nanofabrication provide a unique route to make them usable in the medium-long term for industrial application and production. In particular, some peculiar proteins of sponges (silicateins) catalyze the reaction of silica polymerization to give ordered structures. Besides this catalytic activity, when the proteins are assembled into mesoscopic filaments, they serve as scaffolds that spatially direct the synthesis of polysiloxanes over the surface of the protein filaments. Hence, these biomolecules present the combined characteristics of: (i) chemical action (catalysis) for the formation of silica, and (ii) patterning action, by driving the silica on the surface of the filaments. In this project, we exploited this unique combination within a novel technology, whose demonstrator was the realization of patterned, aligned assembly of silica fibers, and their employment as insulating layers for prototype transistor devices. Two parallel strategies were pursued for the production of silicatein: (i) expression of the recombinant proteins, and (ii) development of *in vitro* primmorph cultures. Soft lithography techniques were used for the controlled patterned deposition of molecules. Specific approaches were designed and implemented, for the hierarchical assembly of silicatein fibers into functional networks.

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The state of the art

To date, the nanolithography approach applied to inorganic crystals (semiconductors), has been very successful in the electronic industry. Even more successful, although scarcely under human control, are nanometer-scale growth bioprocesses that have reached an unparalleled level of complexity. The objective of this project was to explore in depth this new and exciting field of science, enabling full exploitation of the properties of growth bioprocesses by the realization of opto- and microelectronic devices, including nanopatterned silica arrays, optical waveguides, and semiconductor field effect transistors, with the controlled linking of silicatein molecules to surfaces by micro- and nanopatterning.

Within the past several years, the capability of producing patterns of biological molecules with micrometer-level resolution has attracted increasing interest with respect due to their possible use in a wide number of applications. These applications include microarray technologies for genomics and proteomics, medical diagnostics, biological assays and sensors, molecular electronics, control of cellular adhesion, growth and functionality, and bacterial detection. To fabricate biomicropatterns, one can use photografting, inkjet printing, printing robots, and soft lithography. However, soft lithography is particularly suitable due to its experimental simplicity, low cost, and extreme flexibility in terms of the employable substrates, solvents, and deliverable molecules. Imprinting processes, microfluidic networks, and microcontact printing (μCP) have succeeded in achieving chemical contrast among different biomolecular monolayers. In particular, μCP, which was originally proposed to fabricate self-assembled monolayers (SAMs) of alkanethiolates onto gold, was soon applied to the synthesis of patterned proteins on surfaces.

Many uni- and multicellular organisms like diatoms and sponges, have an inorganic skeleton consisting of amorphous silica (biosilica). Siliceous sponges are unique in their ability to synthesize their silica skeleton enzymatically. The responsible enzymes, the silicateins which have been isolated from demosponges, have been described to polymerize alkoxide substrates to silica. The cDNAs and/or the genes encoding these enzymes, which belong the cathepsin subfamily of proteases, have been cloned from the marine sponges *Tethia aurantium*, *Suberites domuncula* and *Petrosia ficiformis*, and the freshwater sponge *Lubomirskia baicalensis*. The recombinant silicateins, silicatein-α and -β, are able to catalyze biosilica synthesis using tetraethoxysilane (TEOS) as substrate.

Other members of the cathepsin subfamily of proteases including human cathepsins do not precipate silica. In the sponge silicatein sequence, the cysteine residue of the catalytic triad of these cysteine proteases (consisting of the three amino acids Cys, His and Asn) is replaced by serine which is thought to be essential for the catalytic mechanism of the enzyme. In addition, a hydroxy amino acid (serine) cluster is present in the *T. aurantium* and *S. domuncula* molecules.

In this project, nature was used as a model for the structure-directed synthesis of amorphous silica (biosilica). Usually the synthesis of glass-like silica structures requires high temperature or high pressure processes limiting the possibility to built up functional biomolecule-silica composite structures. The new enzymes (silicateins patented by the consortium) allow the controlled formation of silica patterns on surfaces and the construction of specifically designed nanostructures.

Work performed and results achieved

1. Model: Nature (Biosilicification)

1.1. Sponge collection and purification of axial filament

Several specimen of marine and freshwater sponges were collected for purification of axial filaments and silicatein. To approach the question about a possible role of silicatein as a form-guiding molecule for the spicules **P1** selected the marine demosponge *Geodia cydonium*. This sponge species is built of two types of spicules, the larger megascleres (oxeas and triaenes) and the smaller microscleres (sterrasters and oxyasters); Fig. 1.

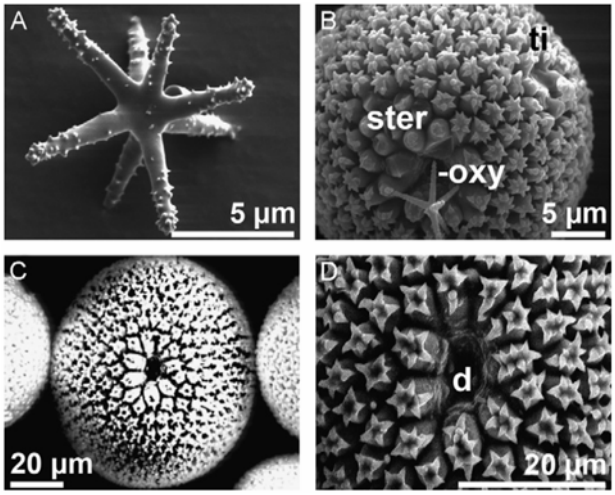


Fig. 1. SEM analyses of the two types of microscleres (*G. cydonium*). **(A)** The small oxyaster displays 8 rays around which silica is appositionally deposited. **(B)** One sterraster (ster) which is associated with an oxyaster (oxy). **(C)** One sterraster, showing that the tips of the silicified rays are arranged around a central dent. **(D)** At a higher magnification the central dent (d) is visible which is surrounded by mature tips.

P1 separated the microscleres from the megascleres and determined the molecular composition of the axial filaments. To determine if also in the axial canals of the microscleres axial filaments are present, a step-wise dissolution of the silica material of the sterrasters was performed (Fig. 2A-C). The light microscopical data could be confirmed by SEM analysis (Fig. 2D-F).

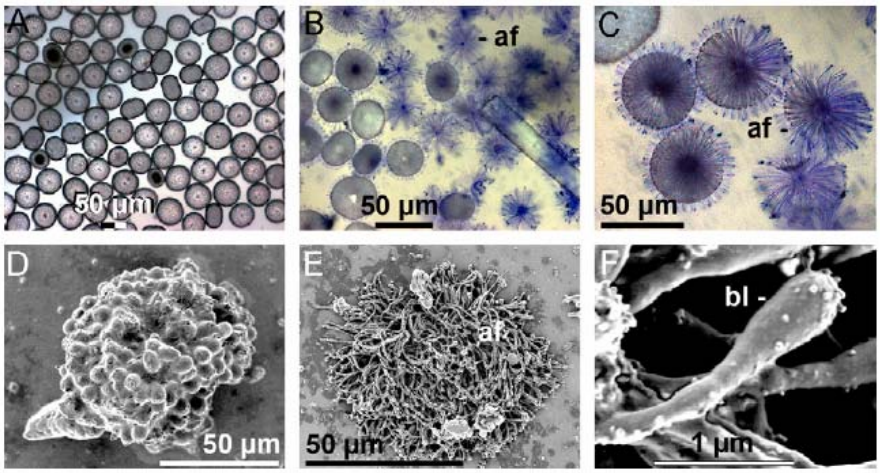


Fig. 2. Dissolution of silica around the microscleres with hydrofluoric acid in a time lapse period. (**A** to **C**) The light optical micrographs were taken progressively after 1 min (A), 5 min (B) or 15 min (C). Simultaneously with the liberation of the axial filaments, these fibers became stained blue, using Coomassie brilliant blue. (**D** to **F**) Electron microscopical images (SEM). The spicules were treated with hydrofluoric acid for 3 min (D) or 30 min (E and F). At a higher magnification swollen tips/blebs (bl) of the axial filaments are seen.

P1 and **P7** isolated spicules and axial filaments from the Baikalian sponge *Lubomirskia baicalensis*. The skeleton of *L. baicalensis* is composed of fusiform amphioxea, which have an average length of 180 µm and a diameter of 10 µm (Fig. 3A and B); the surfaces of the spicules are spined (Fig. 3A). Broken spicules show a 2-4 µm wide axial canal (Fig. 3B), which harbors the axial filaments. The axial filaments were isolated by treatment of the spicules with hydrofluoric acid (Fig. 3C-a).

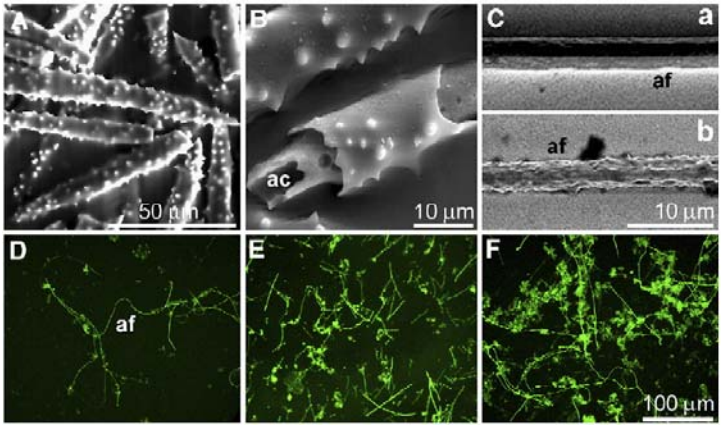


Fig. 3. Spicules and axial filament/silicatein from *L. baicalensis*. **(A)** SEM photograph of amphioxea. **(B)** Broken spicules show the axial canal (ac); SEM analysis. **(C)** Axial filaments which are composed of silicatein (SEM). One axial filament is shown immediately after isolation from a spicule **(C-a)**, or after incubation with TEOS for 180 min **(C-b)**. The time-dependent formation of biosilica from TEOS and mediated by silicatein is shown after staining with Rhodamine 123 **(D–F)**. The silica production was analyzed with the dye Rhodamine 123 at time zero **(D)**, or after the incubation period of 30 min **(E)** or 180 min **(F)** with TEOS. The reaction products are visualized by immunofluorescence microscopy.

The filaments from *L. baicalensis* were incubated with TEOS under the standard incubation conditions. After a reaction period of 180 min the filaments were washed and analyzed by SEM. The images revealed that the smooth surfaces from the axial filaments (Fig. 3C-a) changed after incubation with TEOS and were decorated with silica lumps (Fig. 3C-b). In a second approach to demonstrate that the axial filaments catalyze the formation of biosilica from TEOS, the filament samples were stained with Rhodamine 123 and inspected by fluorescence microscopy (Fig. 3D-F).

1.2. MS analysis of the axial filament proteins

Axial filaments from *P. ficiformis* spicules were extensively analyzed by mass spectrometry. Several post-translational modifications were detected in our experiments. Three phosphorylation sites Tyr 97, Ser 213 and Ser 66 were found. An extensively oxidized histidine was found at H15. The thionyl group of Cys 157 is modified to cysteic acid. The results of the tandem mass analysis are summarized in Fig. 4.

meLPETVDWRTGGAVToxH^{15}VKDQLRCGCSYAFAAVGALEGAAALARGRTASLSEQ
NVV^{54}DCSVPYGNHGCpS^{66}CEDVNNAFMYVIDNGGLDTTSSYPYVSRQYpY^{97}CKFKSS
GVGATATGIVTISSGDESSLESALATAGPVAVYIDASHSSFQFYKYGVLNVPNcaC^{157}S
RSKLSHAMILIGYGTTSSKKYWLLKNSWGPNWGISGYIKMSRGMSNQCGIATYApS^{213}
^{3}FPTL

Fig. 4. Results of tandem mass analysis of the effectively expressed form of silicatein: detected post-translational modifications and the correction to the genetic sequence determined by tandem mass analysis are underlined. The position of the modification is indicated as superscript. Abbreviations used: me, methylation; ox, oxidation; p, phosphorylation; ca, cysteic acid.

1.3. Purification of the silicatein proteins from axial filaments

P1 showed that in contrast to the published data, silicateins composing the axial filament, exist in the native state as monomers or as non-covalently linked dimers. In our studies, a new extraction procedure was introduced, which allowed the preparation of native silicateins [extraction with Tris/glycerol buffer].

P1 described the composition of the protein components of the axial filament from small spicules, the microscleres, using the demosponge *G. cydonium* that possesses besides megascleres also microscleres. Unexpectedly the protein composition of the microscleres was less divers as the one from megascleres. While three isoforms of silicatein could be identified in megascleres (silicatein-α, -β and -γ), the axial filaments of the microscleres contain only one form of silicatein, termed silicatein-α/β, with a size of 25 kDa. Silicatein-α/β undergoes three phosphorylation steps. The gene encoding silicatein-α/β was identified and found to comprise the same characteristic sites, described previously for silicateins-α of -β.

1.4. Characterization of the purified proteins

The purified proteins were analyzed by 1D and 2D electrophoresis (Fig. 5). **P1** could demonstrate that silicatein occurs in the axial filament in several isoforms. In the axial filament silicatein undergoes stepwise phosphorylation (Fig. 5B). Five phospho-isoforms with pI values of 5.5, 4.8, 4.6, 4.5 and of 4.3 have been identified. The sizes of these phosphorylated proteins are around 25 kDa, compatible with the predicted mature form of the silicatein. The silicatein proteins (around 25 kDa) appeared as two or three spots.

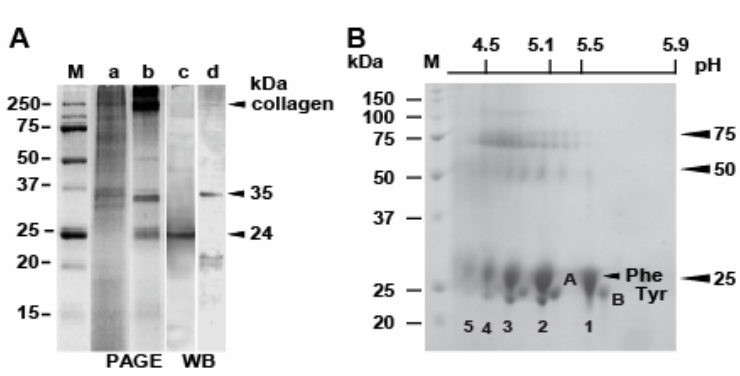


Fig. 5. SDS-PAGE analysis of silicatein in the axial filaments from *S. domuncula*. (**A**) Isolation of proteins under mild conditions and not with HF. Spicules were prepared, pulverized and extracted with urea. Lane b: proteins from spicule extracts were separated and the gels stained with Coomassie brilliant blue. The proteins were blot transferred and reacted with polyclonal antibodies against silicatein-α (lane c; 24 kDa band) or galectin-2 (lane d; 35 kDa band); the immunocomplexes were identified with labeled secondary antibodies. Lane a: protein pattern after SDS-PAGE of primmorphs extracted with the urea-lysis buffer. The region around which collagen had been identified is marked with an arrow. (**B**) Analysis of the proteins in the axial filament by 1D gel electrophoresis. The arrows mark the position of the monomers (25-28 kDa), dimers (55 kDa) and trimers of silicatein (75 kDa). In addition, the different phosphorylated isoforms of silicatein (#1 to #5), as well as the two spots (#A and #B) of the 25-28 kDa silicatein which had been analyzed by ESI-MS are highlighted. Spot B comprises the unmodified tyrosine at position aa_{261}, while in spot A this amino acid is replaced by phenylalanine.

To clarify whether they represent three different post-translational modifications, the spots were subjected to electrospray ionization (ESI)-mass spectrometry (MS) (**P1**). The results revealed a posttranslational modification, a de-hydroxylation from tyrosine to phenylalanine. A computer modeling of silicatein-α with the human cathepsin S as a reference using the Swissprot approach revealed that this modified amino acid (Tyr/Phe) is localized on the surface of the silicatein molecule (Fig. 6A). In a further approach to model the silicatein dimer. The results revealed that the cleft of the active centers of the two molecules is directed to the surrounding milieu (Fig. 6B).

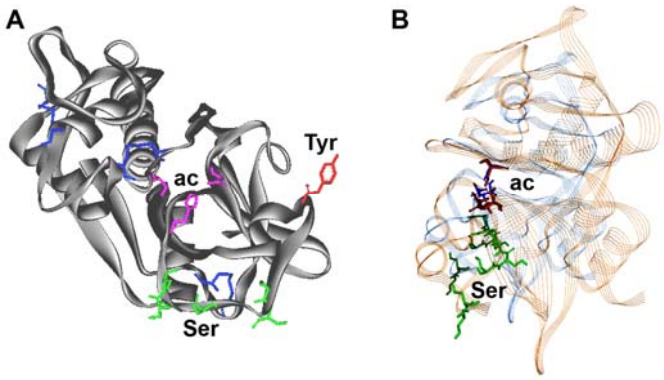


Fig. 6. Computer modeling of silicatein-α. (**A**) The silicatein-α had been subjected to 3D modeling using the Swissprot approach and the cathepsin S sequence as a reference. The active center of the enzyme (ac), comprising Ser, His and Asn, the disulfide bridges (in blue) and the localization of the Ser-stretches are marked in green. In addition, the tyrosine residue which undergoes de-hyroxylation to phenylalanine is marked (Tyr). (**B**) To explain the dimer formation, two silicatein-α molecules (colored in blue [back] and brown [front]) have been docked together, showing the cleft of the active centers of the two molecules in one row. Again the Ser-stretches are marked.

Experiments on *P. ficiformis* silicatein revealed that silicatein is eluted as a sharp peak at 27.5 minutes (see Fig. 7, panel A) and appears in the mass spectrum as a multiply charged pattern of three molecules, very close in mass, at 46865, 47010 and 47125 Da respectively.

These masses correspond to dimeric forms of three molecules of 23432, 23505 and 23562Da respectively (see Fig. 7, panels C and D). This inclination of silicatein to form dimeric aggregates is in good agreement with literature data on gel electrophoresis. Since no direct evidence for covalent peptide linking was collected in bottom up experiments with trypsin and chimotrypsin, these silicatein dimers should form in a non-covalent way.

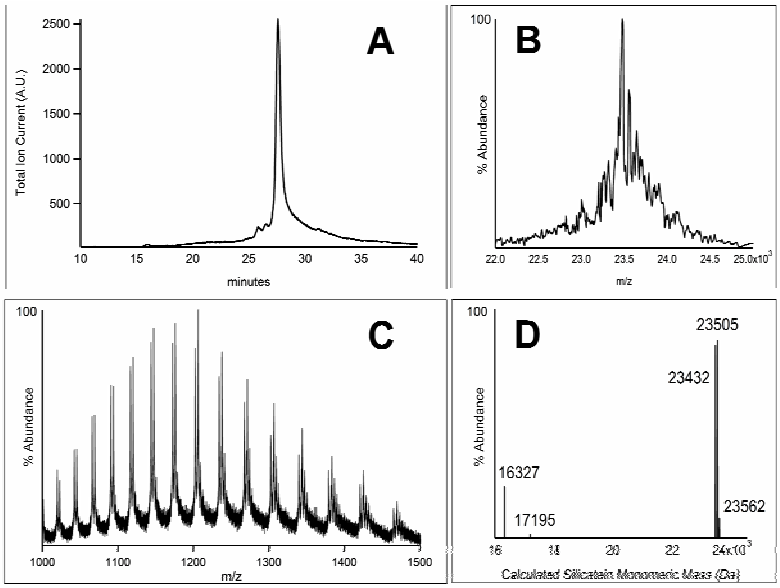


Fig. 7. MS analysis of intact silicatein: Panel A shows the Total Ion Chromatogram (Arbitrary Units) of the NanoLC-MS analysis of intact Silicatein extracted from spicules by HF dissolution. Panel C shows the recorded mass spectrum of peak at 27.5 minutes. Panel D summarizes the calculated molecular masses of the monomeric forms of silicatein (detected in the LC run as dimers). Panel B show the MALDI mass spectrum of the same sample.

1.5. Identification of silicatein genes/cDNAs from further sponge species

In order to identify the gene encoding the silicatein-α/β from *G. cydonium*, the corresponding cDNA was isolated by PCR technique (**P1**). The cDNA was obtained using a degenerate primer set designed against conserved regions in silicatein-α cDNAs. In addition, silicatein-α and cathepsin L cDNA have been cloned from *L. baicalensis* (**P1** and **P7**).

P1 also succeeded for the first time to identify and to characterize a silicatein cDNA from the second class of siliceous sponges, the Hexactinellida. The major protein in the spicules of the hexactinellid sponge *Crateromorpha meyeri* is a 24-kDa protein that strongly reacts with anti-silicatein antibodies in Western blots. Its cDNA has been successfully cloned; the deduced hexactinellid silicatein comprises, in addition to the characteristic catalytic triad amino acids (Ser, His, Asn) and the conventional Ser cluster, a further Ser cluster (Fig. 8).

1.6. 3D sponge cell culture for the study of spicule morphogenesis

A 3D sponge cell model of *P. ficiformis* has been established (Fig. 9 left). The model will be used to study spicules formation in different environmental conditions and in the presence of different silica substrates. Preliminary results demonstrate active spicologenesis in the standard cultural conditions (Fig. 9 right)

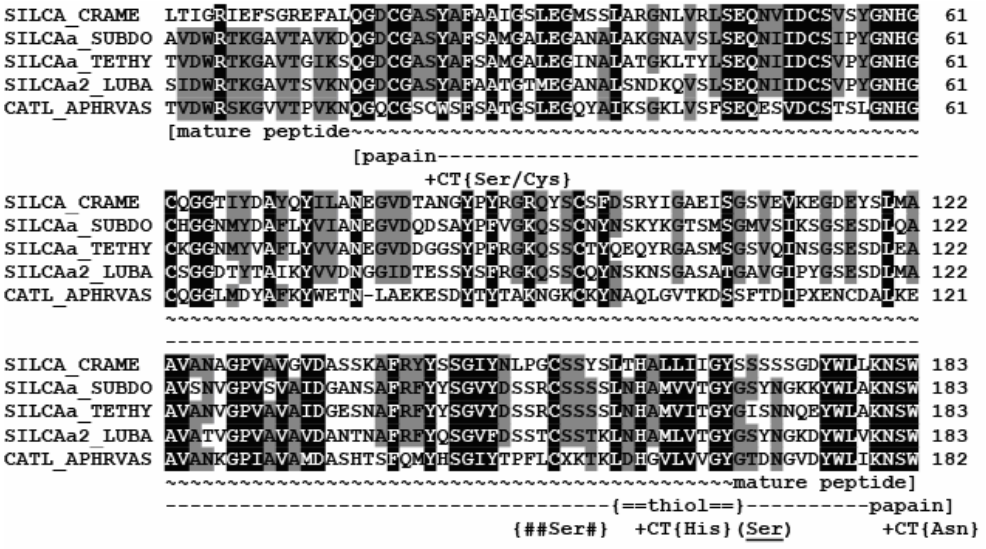


Fig. 8. Silicatein protein deduced from *C. meyeri* cDNA. From the cDNA segment of this sequence (CMSILCA), the protein (SILCA_CRAME) was deduced and aligned with silicatein-α from *T. aurantium* (*Tethya aurantia*; SILCAa_TETYA) and silicatein-α from *S. domuncula* (SILCAa_SUBDO) and from one isoform of silicatein-α from *L. baicalensis* [α-2] (SILCAa2_LUBAI5) and the cathepsin L sequence from hexactinellid *A. vastus* (CATL_APHRVAS). 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 four sequences in black on gray. The characteristic sites in the sequences are marked; the catalytic triad (CT) amino acids, Ser in silicateins and Cys in cathepsin, and His and Asn. The borders within the mature silicatein (mature peptide) of the following domains are given: the peptidase-C1 papain family cysteine protease domain (papain) and the thiol-protease-His signature (=thiol=). The "conventional" Ser cluster (##Ser#), and the additional Ser cluster (underlined Ser) are marked.

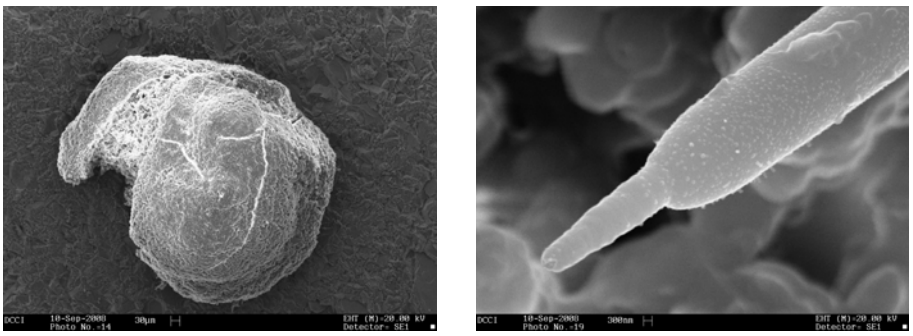


Fig. 9. SEM picture of a primmorph from *P. ficiformis* (left) and magnification of newly forming spicule inside a primmorph (right).

1.7. Understanding the biocontrol mechanisms involved in the assembly of silicateins into filaments and the patterning of fibers into functional networks

As a functional assay for the different preparations (HF- and Tris/glycerol-extracts) polymerization studies *in vitro* were performed.

SDS-PAGE studies (in the presence of β-mecaptoethanol and after pre-heating of the sample) revealed that the silicatein-α form is more abundant than the β-form; the ratio was determined to be 4:1. On this basis, a 4:1 ratio between silicatein-α to -β, a computer model for the proposed arrangement between the two isoforms of silicatein was outlined.

Three directions of orientation of the active centers can be proposed (Fig. 10A-C). Under the assumption that the Ser-clusters are crucial segments in the silicateins, the con-axial orientation of all silicateins-α is most likely, since only in this arrangement the Ser-

clusters of the silicateins are ordered and direct towards the center of the tetrameric silicateins (see: Fig. 10C). It can be proposed that in this center the tetrameric silicateins-α could interact with one silicatein-β molecule, as proposed in Fig. 10D. At present the con-axial orientation of all silicateins-α appears to be most likely since such an arrangement allows also an understanding of the directional growth of the filaments.

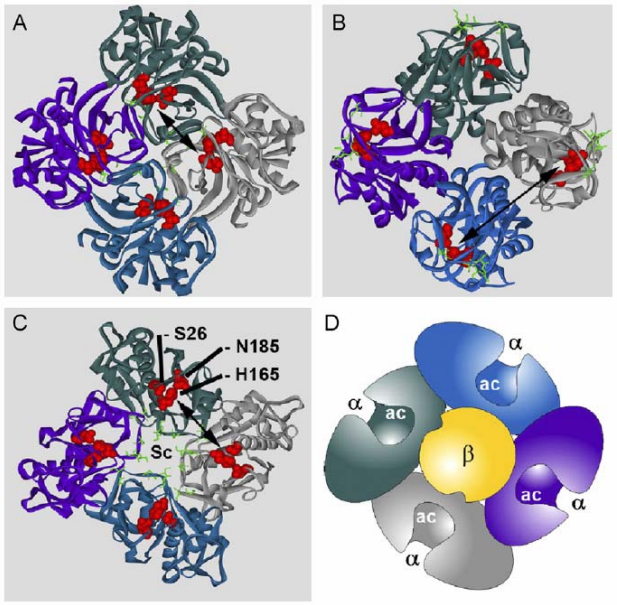


Fig. 10. Silicatein-α tetramer. The amino acids involved in the active centers of the silicateins are marked; serine (S26), histidine (H165) and asparagine (N185). (**A**) The silicatein molecules are oriented with their active centers towards the center of the axis (syn-axial), or (**B**) in the opposite direction of the axis (anti-axial), or, (**C**) the entrances of the con-active centers are located in the plane of the tetramer (con-axial). Only in the axial-orientation the Ser-rich clusters (Sc) are directed towards the center of tetramer. (**D**) Graphical model of the silicatein-α tetramer (green-grey-blue-pink) with the silicatein-β (yellow) in the center. The active centers in the silicatein-β molecules are marked (ac).

The assembly process of the silicatein samples was followed by electron microscopy. These studies revealed that after an already 60 min incubation period of the silicatein mono-/dimers first filaments are seen (Fig. 11).

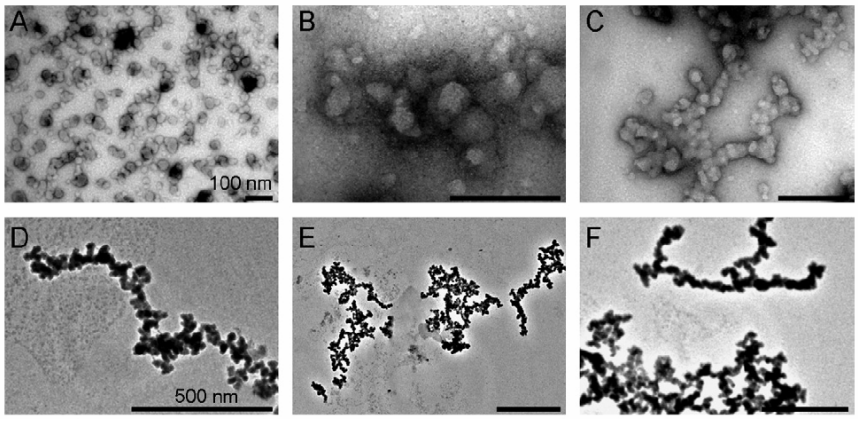


Fig. 11. TEM analysis of polymerized silicatein from axial filaments. (**A-F**) The proteins were transferred into re-assembly buffer. (A) Self assembly period: 0 min, (B) 15 min, (C) 30 min, (D) 60 min, (E) 120 min, or (F) 180 min. Magnification: Bars A-C: 100 µm; D-F: 500 µm.

2. System I: Recombinant silicateins and primmorphs

2.1. Expression of silicatein cDNAs using prokaryotic and eukaryotic expression systems

The preparation of recombinant silicatein-α and -β was performed in *E. coli*. The purification of the recombinant protein using the histidine-tag, which is present in the protein, was performed on a Ni-NTA-matrix. In addition, the yeast *Pichia pastori* eukaryotic expression system was used for the expression of silicatein-α from *S. domuncula*.

Polyclonal antibodies (PoAb-aSILIC) raised against purified, recombinant silicateins-α and -β from *S. domuncula* in female rabbits were used for the immunodetection of the recombinant proteins (Fig. 12).

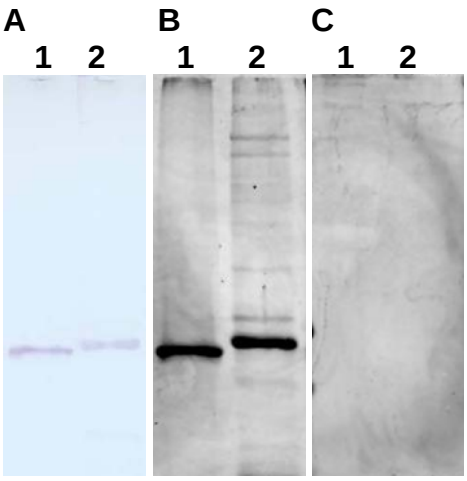


Fig. 12. Immunodetection of silicatein. Axial filament protein from *Suberites domuncula* (lane 1) and recombinant silicatein-α (*S. domuncula*; lane 2) were subjected to 10% SDS-PAGE. (**A**) Gel was stained with Coomassie blue. (**B**) Western blot analysis using PoAb-aSilic. (**C**) In controls adsorbed antibodies incubated with recombinant silicatein were used.

2.2. Preparation of biosilica using the purified recombinant silicateins

In a biomimetic approach, using "Nature as model", **P1** used the biosynthetic abilities of silicatein to synthesize materials which imitate the lamellar architecture found in sponge spicules with their unique physicochemical properties. This approach was indeed found to be feasible. Using recombinant silicatein immobilized on the surface of spicules freed of organic material, fabrication of spicule-like core-shell materials of alternating metal and metal oxide layers with complex architectures and properties turned out to be possible.

Purified recombinant silicatein was linked onto spicules after amino-group-functionalization of the silica surface with aminopropyltriethoxysilane (Fig. 13A). The NH_2-functionalized spicules were reacted with a reactive ester polymer which allowed binding of the recombinant protein, with its His-tag, via Ni(II). We demonstrated that the immobilized enzyme synthesized a new silica lamella using TEOS as substrate. SEM analysis revealed that the spicules that were covered with silicatein have a wrinkled surface caused by the proteinaceous layer (Fig. 13B). Backscattered SEM images showed a 200–500 nm thick additional surface layer at the surface of spicules; EDX analysis of this layer showed a distinct silicon peak which was absent in reference samples. The presence of silicatein molecules at the surface of the spicules could also be visualized using the antibodies against this protein, as revealed by fluorescence microscopical analysis (Fig. 13C). Functionalized spicules which had been treated with the polymer but not with silicatein (controls) showed a completely smooth surface (Fig. 13D).

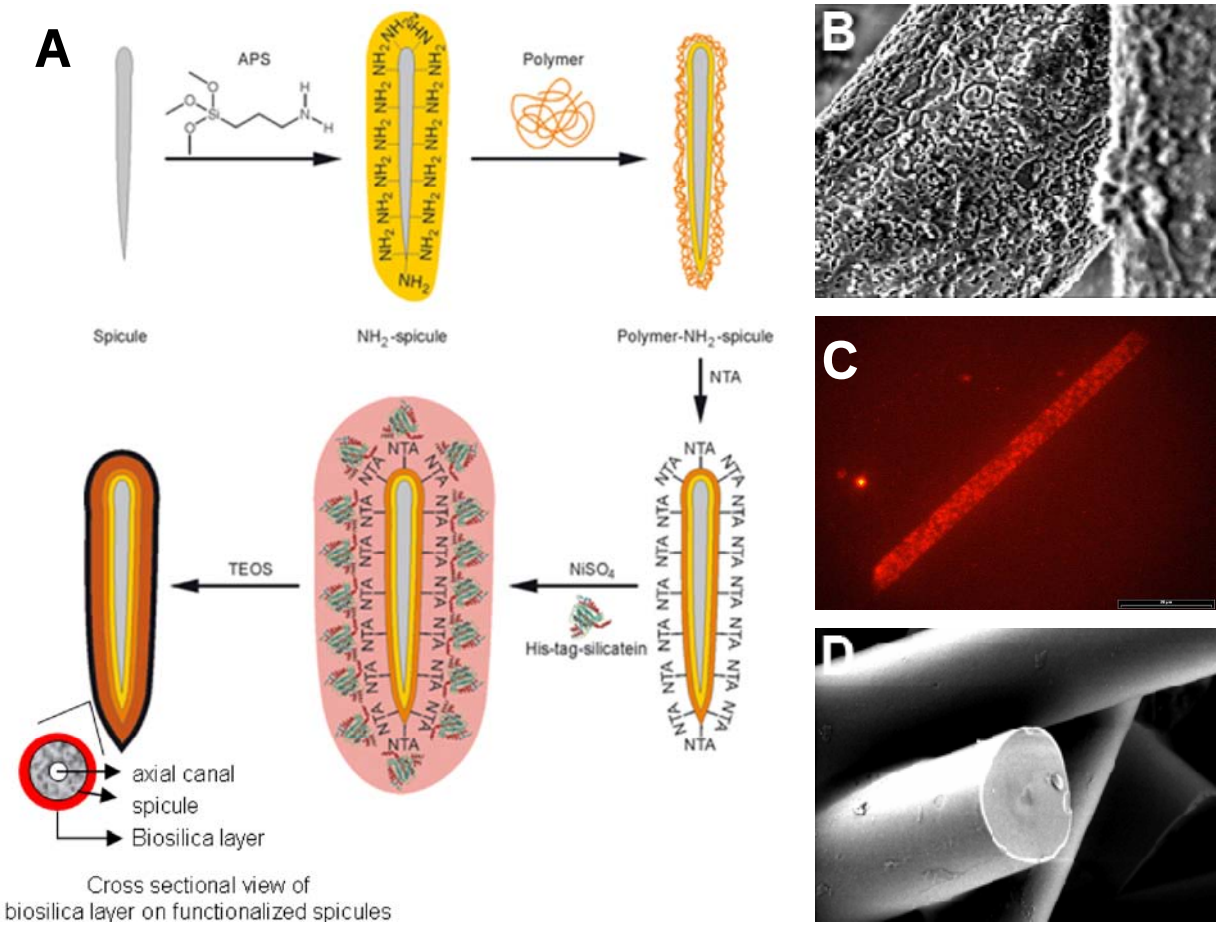


Fig. 13. (A) Schematic representation: Functionalization of spicule surface and formation of biosilica mediated by immobilized recombinant silicatein. The NH_2-functionalized spicules are reacted with the polymer and then with the His-tagged silicatein. **(B)** SEM analysis of spicules of *S. domuncula* with biosilica layer. **(C)** Immunostaining of spicule of *S. domuncula* with immobilized silicatein. **(D)** Non-functionalized spicules.

2.3. Analysis and comparison of the enzymatic activities of the recombinant and native silicateins and identification of the most suitable substrates

P1 showed for the first time that silicatein comprises, in addition to its silica-polymerase activity, also a silica-esterase function. The linkage between silicate or tetrahedral silica units in poly(silicate) can be considered as an ester-like bonding. In order to test if silicatein also functions as an silica-esterase, **P1** studied its hydrolytic function on bis(*p*-aminophenoxy)-dimethylsilane [BAPDsilane]. This compound comprises two ester-like bonds between silicon and *p*-aminophenol and two methyl silane linkages (Fig. 14). It is proposed that hydrogen-bonding between the imidazole nitrogen of the conserved His and the hydroxyl of the active-site Ser increases the nucleophilicity of the Ser oxygen, facilitating the attack of silicatein on the silicon atom of the substrate. This reaction can be monitored spectroscopically on the basis of the release of *p*-aminophenol..

The enzymatic parameters of the silica-esterase activity were determined. The K_m value is 22.7 µmol/l. The turnover number (molecules of converted substrate per one enzyme molecule per sec) is 5.2. The temperature optimum of the silica-esterase activity is in the range of 20°C to 25°C; the temperature coefficient (Q_{10}) above 25°C decreases by 2.5-fold and increases below 25°C by 2.9-fold.

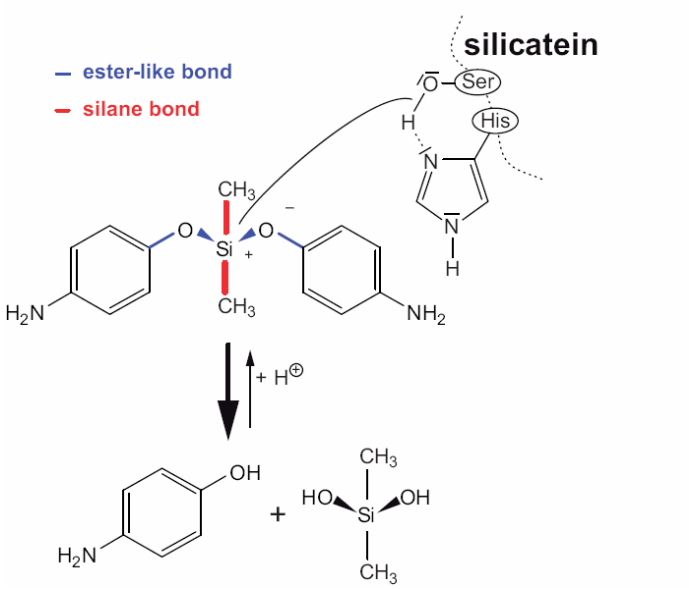


Fig. 14. Proposed silicatein-α mediated reaction mechanism of hydrolysis of bis(*p*-aminophenoxy)-dimethylsilane, comprising two silicic ester-like (blue) and two silane bonds (red). In the catalytic center of silicatein, the serine oxygen starts a nucleophilic attack on the silicon resulting in a displacement of *p*-aminophenol and the formation of a (alkoxyl)-monosilane. This reaction is facilitated by the hydrogen-bonding between the imidazole nitrogen of the conserved histidine (His) and the hydroxyl of the serine (Ser).

In addition, **P1** demonstrated, also for the first time, that silicatein, with its silica-polymerase activity, causes polymerization/condensation via a successive addition of monomeric silica units. The synthesis of polymerized derivates of silicic acid, poly(silicate), in terms of siloxanes, was performed with silicatein and dimethyl-dimethoxy-silane as substrate.

The optimal conditions for stabilization of different silicateins have been worked out. To find out the optimal buffer conditions to measure silicatein activity in solution, the effect of various buffer solutions of different pH on the activity of the enzyme was determined.

2.4. Establishment of the primmorph system to synthesize nanostructured biosilica material

Primmorphs have been prepared from the marine sponge *S. domuncula* (**P1**) and the freshwater sponges *L. baicalensis* and *B. bacilifera* (**P7**). The primmorphs were used for the production of biosilica structures. Formation of spicules was monitored by electron microscopy. **P7** optimized the growth conditions and searched for further factors involved in biosilica synthesis in primmorphs. Addition of iron salts to the solution was found to stimulate spicule formation. Primmorphs from the marine sponge *P. ficiformis* were prepared and in the presence of dissolved silica they actively produce new spicules (**P2**).

2.5. Investigation of the specific, potentially distinct function of the various isoforms of silicatein in endemic freshwater demosponges

The branches of the Baikalian sponge, *L. baicalensis* are composed of modules, arranged along the apical–basal axis. **P1** and **P7** studied the regional expression level of silicatein along the branches (modules) and in the crust zone. The relative steady-state expression levels of silicatein-α1 were determined by Northern blotting. The house-keeping gene α-tubulin was used as a quantity control for the loading of RNA. The results showed that the expression of the relative expression of silicatein five-fold higher in the apices compared to the bases of the modules.

In parallel to the Northern blotting experiments, RT-PCR amplification studies were performed. The expression of silicatein-α is higher at the apices, in contrast to the bases of the individual modules.

To confirm the Northern blot and the RT-PCR amplification studies indicating a higher expression of the silicatein gene at the apex region than at the base of a module within a branch, *in situ* hybridization studies were performed. Sections were prepared and hybridized with a DIG-labeled silicatein-α1 (LBSILICAa1) probe from this sponge species (Fig. 15).

If sections were reacted with the sense probe, no strong reaction could be visualized with anti-DIG-Fab-fragments conjugated to alkaline phosphatise (Fig. 15A). However, if the antisense probe was applied a strong staining could be seen at the apex of the module and also of the rim regions, while the basis of the module showed a much weaker reaction (Fig. 15B). In order to demonstrate that the amounts of cells in these regions are similar, higher magnifications of the sections are given (Fig. 15C-F). The slices were reacted with the antisense probe and additionally stained with DAPI. The apex/rim region of the module shows again a strong signal with the antisense probe (Fig. 15C) and the blue DAPI-stained nuclei (Fig. 15D). If the images were taken from the center of the module a significant lower reaction with the antisense probe is seen (Fig. 15E), while the DAPI signal (Fig. 15F) is highly similar to that seen at the apex/rim.

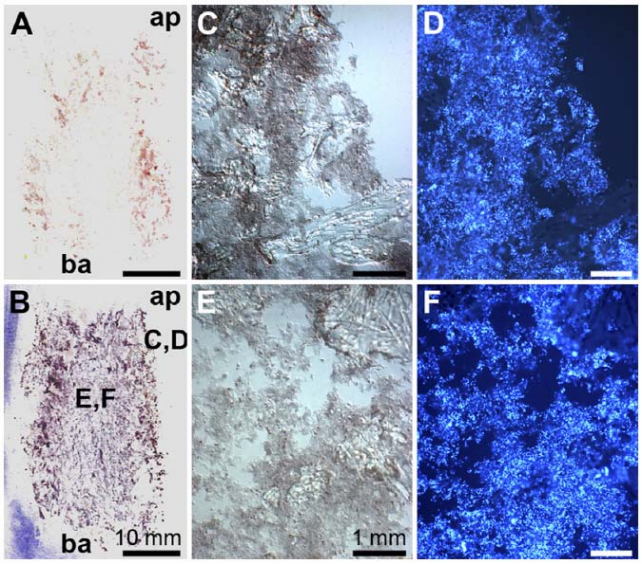


Fig. 15. *In situ* hybridization of one module of a branch with a DIG-labeled probe of silicatein (LBSILICAa1). The sections were hybridized either with a sense probe (**A**) or an antisense probe (**B**); in the latter experiment strong hybridization signals were seen around the apex region (ap) and also at the rim of the module, while the staining was lower at the basis (ba). Bars: 10 mm. Higher magnification of the regions of the apex/rim (**C** and **D**) and the center of the module (**E** and **F**). The regions from which the images were taken are marked in (**B**). The images **C** and **E** show the signals of the hybridization with the antisense probe, while **D** and **F** (the same region) show the DAPI-stained nuclei in the corresponding area. All bars: 1 mm.

3. System II: Biological and biomimetic tools for building higher ordered structures of silicatein/biosilica

3.1. Identification of adhesive proteins/molecules suitable for attachment of silicatein/ biosilica

To attach silicatein and silica nanoparticles to surfaces, **P1** isolated an adhesive protein from the sea cucumber *Holothuria forskali*. In addition, **P1** demonstrated that a fusion protein comprising a silicatein sequence and a silk fibroin sequence can be used to synthesize

biosilica (Patent application US60/839,601). Silk fibroin is used as an adhesive protein ("underwater glue") to attach silicatein and silica nanoparticles formed by silicatein to surfaces.

3.2. Development of a procedure for preparation of DOPA-containing polypeptides

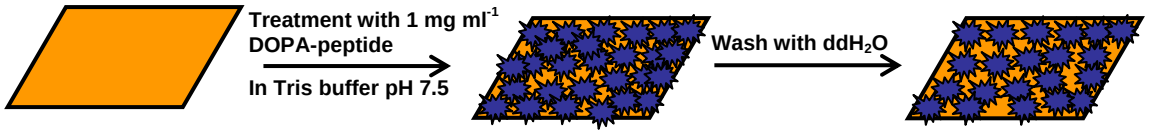
P1 has shown that *S. domuncula* expresses a tyrosinase which synthesizes diphenols from monophenolic compounds. Applying the technique of PCR cloning, the complete cDNA encoding the sponge tyrosinase-related protein has been obtained.

P4 at NTU has synthesized chemically DOPA containing peptide of sequence H-Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-DOPA-Lys-NH2. In this work, a DOPA containing peptide was used as a linker between the substrate's surface and silicatein to maintain the activity of the adsorbed protein. A DOPA-containing peptide, H-Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-DOPA-Lys-NH2, derived from repeats of a mussel adhesive protein was synthesised in-house using a solid-phase microwave synthesis of the peptide by the 9-fluorenylmethoxycarbonyl (Fmoc) strategy. The synthesized and purified DOPA-containing peptide were used for coatings of gold surfaces and further adhesion of silicatein.

3.3. Development of a procedure for binding silicatein/biosilica building blocks on surfaces using DOPA-containing polypeptides

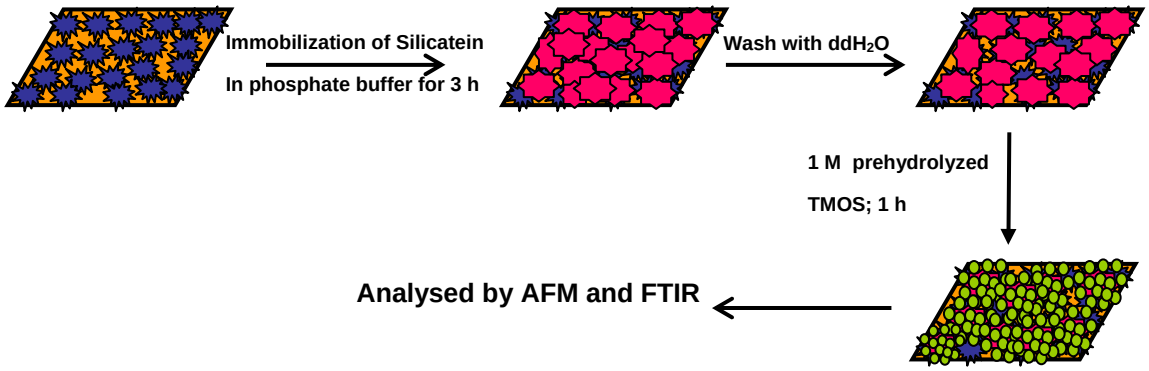
P4 has developed the protocol for binding of DOPA containing peptide on the gold surface.

Schematic diagram of protocol



P4 has developed a procedure to uniform deposition of silicatein on DOPA-containing peptide coated gold surfaces.

AFM and FTIR analysis were performed after every stage of coating to characterize the formation of different chemical layers on the gold surfaces.



3.4. Generating patterned silicatein/biosilica structures on surfaces using glue protein/ substances

DOPA containing peptide was also used to functionalize the gold surface for the uniform immobilization of silicatein. DOPA-containing peptide acts a glue or linker to adsorb silicatein protein with maintenance of its activity by increasing the distance been the surface and the protein. Homogeneous deposition of DOPA-containing peptide with a thickness of 8 nm is observed by AFM (Fig. 16, Au-DOPA-pep). Silicatein adsorbed uniformly on the DOPA-containing peptide coated surface with the thickness of 20 nm. Silicatein is like a globular protein and has a molecular weight of 24kDa with a diameter of ca. 3.2 nm (Fig. 16, Au-DOPA-pep-Silicatein). This result indicated that silicatein adsorbed in multilayers as also suggested in the protein quantification data, which showed multisteps adsorption of silicatein. AFM image showed the uniform silica films, which was formed by the adsorbed silicatein (Fig. 16, Au-DOPA-pep-Silicatein-Si). The line profile analysis shows that the uniform silica films with thickness of 45 nm were formed on the surface, indicating an increment of thickness of films after every stage of coatings.

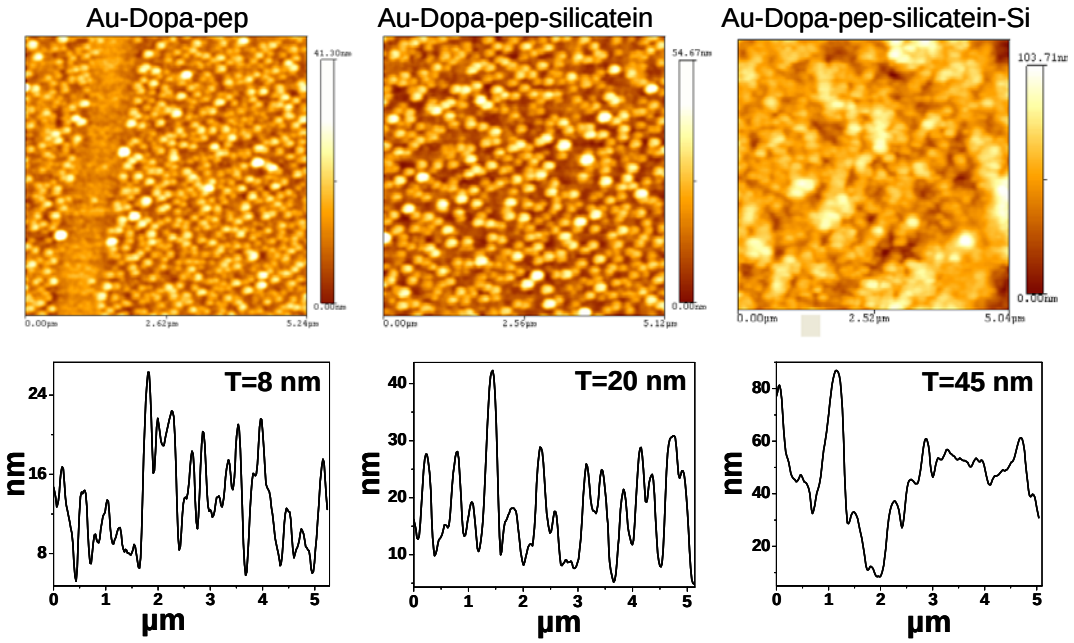


Fig. 16. AFM image of DOPA-containing peptide coated gold surface, silicatein-DOPA-containing peptide surface (left and middle) and the silica coated surface (right).

4. System III: Materials chemistry

4.1. Precursor selection

P4 used aqueous based silica precursors such as tetraethoxy silane (TEOS), tetramethoxy silane (TMOS) and sodium silicates often termed as water glass for the controlled synthesis of biosilica using silicatein protein under ambient conditions of temperature, pressure and pH. Model silica precipitation experiments have been performed in order to understand the effects of concentration of silica precursors and silicatein protein.

4.2. Studies of the activity of different amount of silicatein on silica production in solution with the aim of optimising reaction conditions

The condensation and precipitation of silica particles with increasing time were monitored using turbidity measurement (at 350 nm) and dynamic light scattering (DLS).

Spherical silica particles with few aggregated structures were observed in SEM images (Fig. 17).

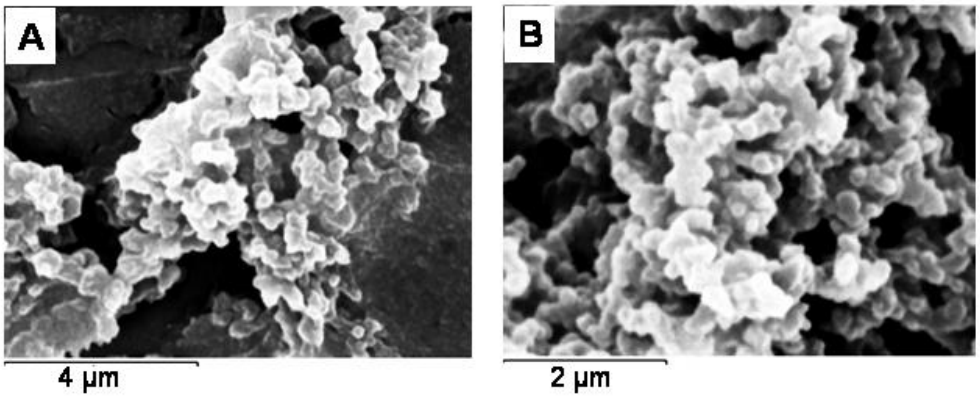


Fig. 17. SEM images of silica particles synthesized from 200 mM TMOS using 50 ng/mL **(A)** and 200 ng/mL **(B)** silicatein protein.

4.3. Studies of the activity of silicatein attached to surfaces in silica precipitation

Different amounts of silicatein protein were immobilized on cystamine and cysteamine-glutardialdehyde coated gold surfaces. FTIR analysis showed a poor amide-I band for the 50 ng/mL and a strong amide-I band for the 200 ng/mL silicatein deposited surfaces, indicating large amount of adsorption of silicatein (Fig. 18) and therefore this surface (200 ng/mL) is probably more suitable for the precipitation and deposition of silica particles. These silicatein bound gold surfaces were treated with prehydrolyzed TMOS (200 mM) solution for 2 h. Silica particles were deposited on the uncoated areas, leading to fabrication of uniform silica films on the surface after 2 h (Fig. 18). This result also indicates an even coating of silicatein protein on cystamine and cysteamine-glutardialdehyde bound surfaces.

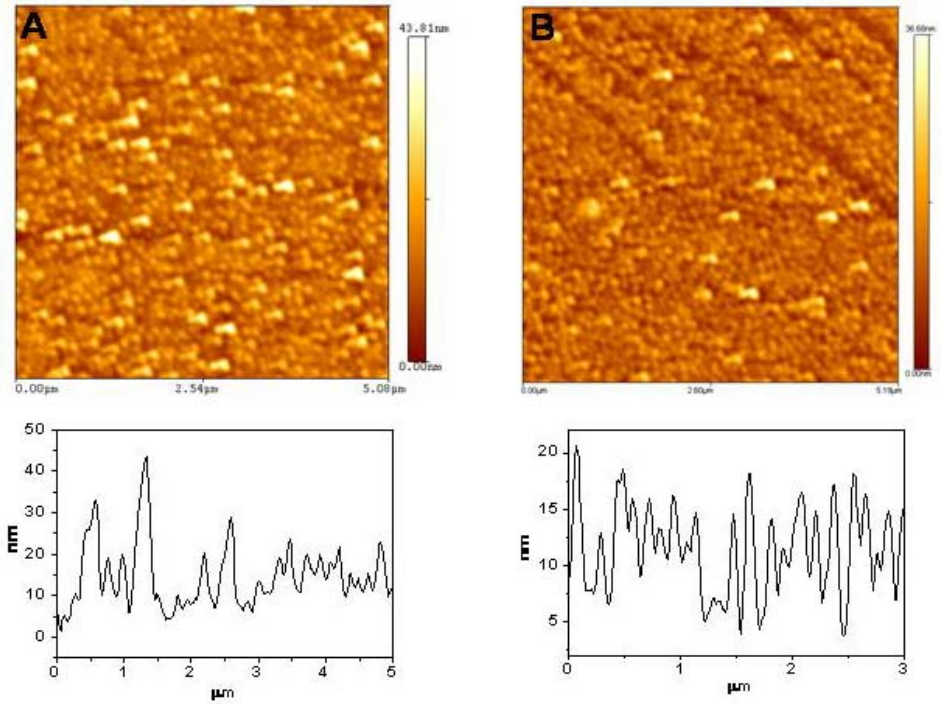


Fig. 18. AFM images of silica deposited on cystamine-glutardialdehyde-silicatein **(A)** and cysteamine-glutardialdehyde-silicatein **(B)** coated gold surface. Line profile analysis of the corresponding AFM images is shown below each image.

The specific activity of immobilized silicatein on cystamine-GDA-Au and cysteamine-GDA-Au surfaces was calculated and estimated to be 90 ± 2% as compared to free silicatein with the reduction most likely being due to the dense packing and unavailability of a few silicatein protein molecules to the silica precursors. AFM images clearly show interconnected structures of silica particles of size from 20 to 40 nm on both surfaces (Fig. 19). The total thickness of the films after silica deposition on the Au-cystamine-GDA-silicatein surface was estimated to be 105 nm and 48 nm on the Au-cysteamine-silicatein bound surface in line with the differences in bound silicatein. These results confirm that the activity of silicatein was maintained after immobilization on the amine-GDA modified surfaces.

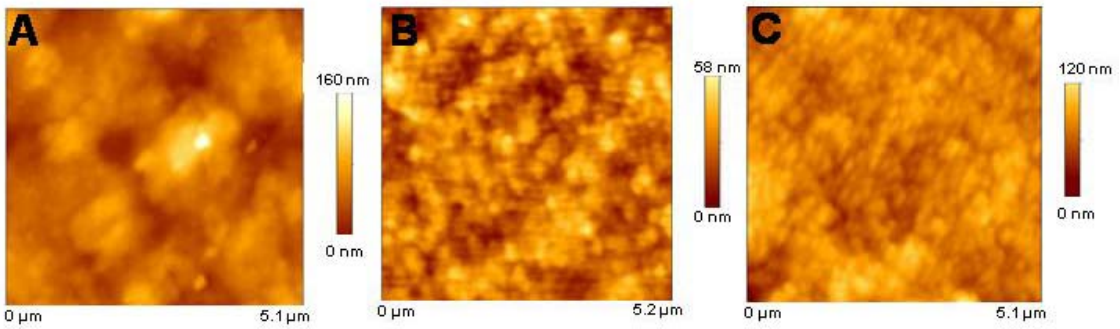


Fig. 19. Representative AFM images of silica films deposited on silicatein-Au (A), silicatein-GDA-cysteamine-Au (B) and silicatein-GDA-cystamine-Au surfaces.

FTIR and EDXA analysis were used to confirm the presence of silica on the surfaces. No signature of silica was observed on a bare gold surface nor GDA modified amine treated surfaces after treatment with hydrolyzed TMOS for 2 hr (Curve 1, 2, 3, Fig. 20A). Peaks arising from vibrations of Si-O containing species were identified at ~ 1040 cm^{-1}, 950 cm^{-1} and 800 cm^{-1}, corresponding to Si-O-Si (asym), Si-OH and Si-O-Si (sym) bonds respectively after the silica films were formed on the Au-amine-GDA-silicatein surfaces, (Curves 4 and 5 in Fig. 20A). Furthermore, energy dispersive X-ray analysis (EDXA) showed (Fig. 20B) a strong silicon signal at 1.75 keV only for the Au-amine-GDA-silicatein-silica coated surfaces (curves 1 and 2).

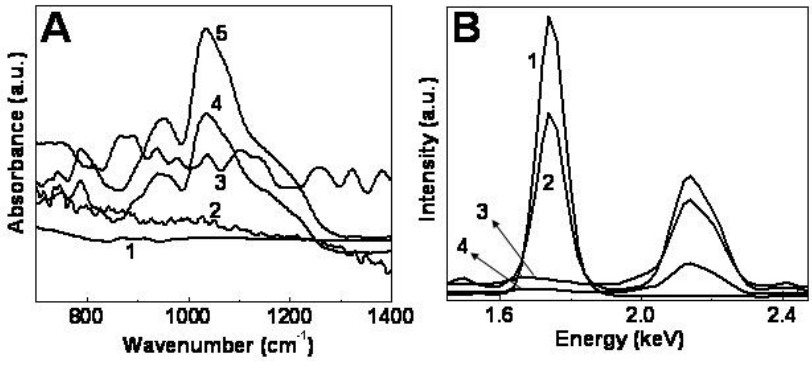


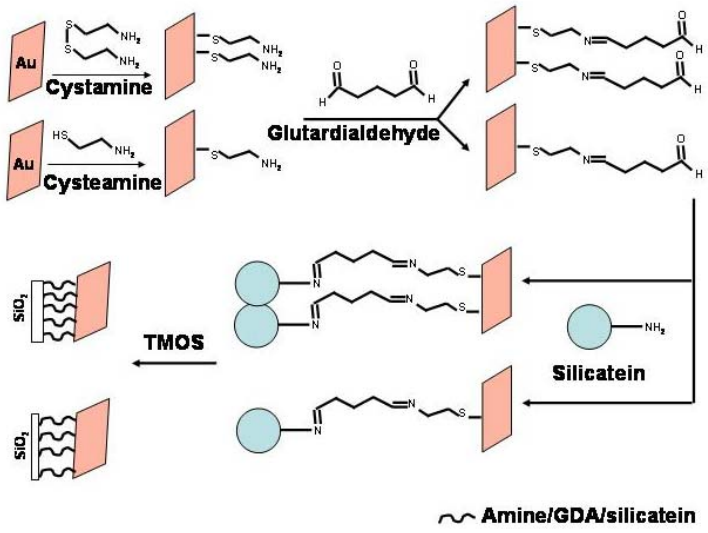
Fig. 20. A. FTIR spectra of a bare gold surface (curve 1), silica films deposited on Au-Cystamine-GDA (curve 2), Au-cysteamine-GDA (curve 3), Au-cysteamine-GDA-silicatein (curve 4) Au-cystamine-GDA-silicatein (curve 5). **B.** EDXA analysis of Au-cystamine-GDA-silicatein (curve 1) and Au-cysteamine-GDA-silicatein (curve 2) as well as after silica deposition on Au-cysteamine-GDA-silicatein (curve 3) and Au-cystamine-GDA-silicatein (curve 4).

4.4. Studies of the attachment of silicatein and model polymers to surfaces

P4 has developed a reproducible protocol for uniform immobilization of silicatein on glutardiladehyde (GDA)-cystamine-Au and GDA-cysteamine-Au surfaces. Florescamine

assay was standarize to quantify the adsorbed amount of silicatein in nanogram amount on both surfaces.

Schematic diagram shows stepwise coatings of gold surfaces using various chemicals



5. Immobilization: Protocols for the controlled deposition

5.1. Chemical Methods of immobilization by modifying surfaces and/or modifying the surface of the protein itself by addition of a 'chemical tag'

Silicatein protein was immobilized on GDA-cystamine-Au and cysteamine-Au surfaces. Silicatein immobilized uniformly on both surfaces with maintenance of its activity. The activity of immobilized and free silicatein was estimated using molybdenum blue assay which indicate that the immobilized silicatein was 90% active as compared to the free silicatein. The detail description of immobilization protocol and results were discussed above (section 4: System III: Materials chemistry).

5.2. Physical Methods of immobilization

Analysis of surface bound silicatein was performed in conjunction with WP3 on materials chemistry to also assess activity of the silicatein. UV-vis spectroscopy, Grazing angle Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), energy-dispersive X-ray analysis (EDXA), atomic force microscopy (AFM) and dynamic light scattering (DLS) have been used to characterize the adsorption of silicatein and coatings of silica on glass coated with gold.

6. Lithography/control: Production of nanostructures

6.1. Realization of master templates

Electron-beam lithography (EBL), photolithography and reactive ion etching have been developed for master production. In addition to the preliminary silanisation and physisorption methods, functionalisation techniques based on polyelectrolyte coatings have been applied and tested for printing silicatein molecules. Master templates for soft lithography were fabricated by photolithography on Si/SiO_2 (SiO_2 thickness 100 nm) substrates and EBL/reactive ion etching on Silicon.

During the first stage of the project, **P3** focused on the production of master templates with feature resolution up to 1 µm by photolithography. Next, high-resolution, monolithic master patterns were fabricated by EBL on Silicon substrates. **P3** realized one-dimensional gratings on silicon substrates with periods ranging from 1 µm to 200 nm, and a lateral resolution of the features in the range 90-570 nm.

P3 tested different immobilization protocols on various substrates to obtain the immobilization of silicatein on planar surfaces. The critical steps of cleaning and pre-treating substrates were optimized. Fig. 21 shows an AFM picture of the silicatein patterned surface.

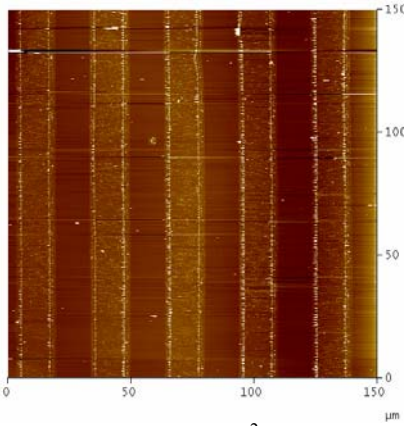


Fig. 21. Tapping AFM micrograph (scan size 250×250 µm^2, vertical data scale 100 nm) of µCP of 50 µg/ml recombinant silicatein on Si/SiO_2 substrates.

6.2. Soft lithography

Replica Molding (REM) and Microcontact Printing (µCP) have been implemented and optimised in order to produce well defined microstructures by silicatein. Different immobilization protocols, including chemisorptions and physisorption approaches, have been combined to µCP.

Poly(dimethylsiloxane) (PDMS) elastomer building blocks were fabricated starting from photolithographic masters (with features in the range 15-100 µm), by mixing the two polymer components (base and curing agent) in a ratio of 4:1 by weight, and then casting the blend on the surface of the wafers. After heating (75°C, 15 min), the PDMS was peeled off (scheme: Fig. 22) and temporarily activated by oxygen plasma under various processing conditions to increase hydrophilicity for the subsequent adsorption of silicatein molecules.

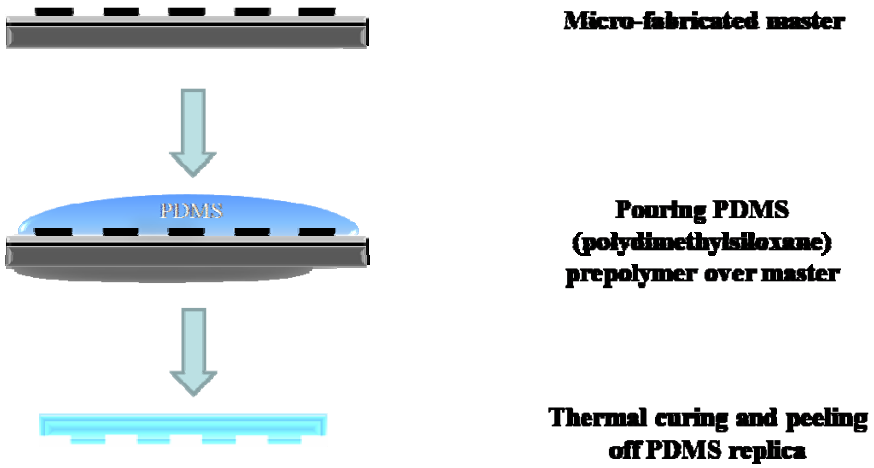


Fig. 22. Schematic diagram of the implemented Replica Molding (REM).

6.3. Microfluidics on silicateins

Microfluidics has been also applied with very good patterning results to favour the alignment of silicatein filaments onto a target substrate (scheme in Fig. 23). For microfluidic experiments, PDMS elastomeric stamps having relief and recessed features on their patterned side were placed on the substrate. The microflow behaviour (in terms of penetration rate) of silicatein proteins into microchannel environment was investigated.

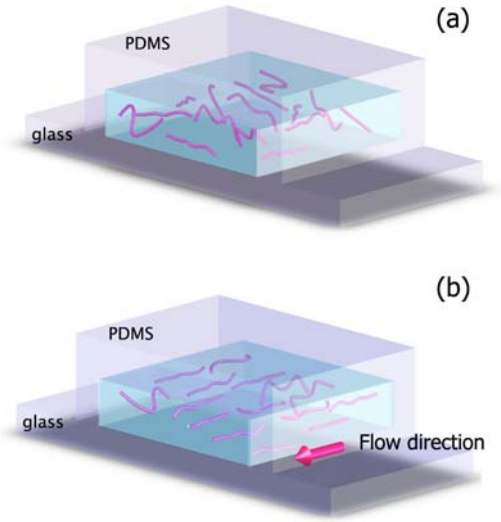


Fig. 23. Schematic representation of the procedure of the silicatein fiber alignment: Solution of fibers with random orientation in the liquid volume (a) and with enhanced alignment induced by pressure-driven flow (b).

7. Devices: Microelectronic and optical components relying on biosilica

7.1. Morphological characterisation

P3 patterned soluble silicatein on target surfaces using a master with typical parallel features of lateral size ranging from 30 μm to several mm. Silicatein patterns were realized and immobilized by soft lithography (REM plus μCP) technologies on surfaces. After soft lithography, the silicon substrates with patterned silicatein were incubated in a precursor solution (TEOS). The growth of the biosilica layer was followed microscopically. Fluorescence methods by fluorescein isothiocyanate and rhodamine B isothiocyanate, allowed easy imaging of the patterned areas (Fig. 24). Similar experiments were carried out on silica generated by silicateins deposited by microfluidics.

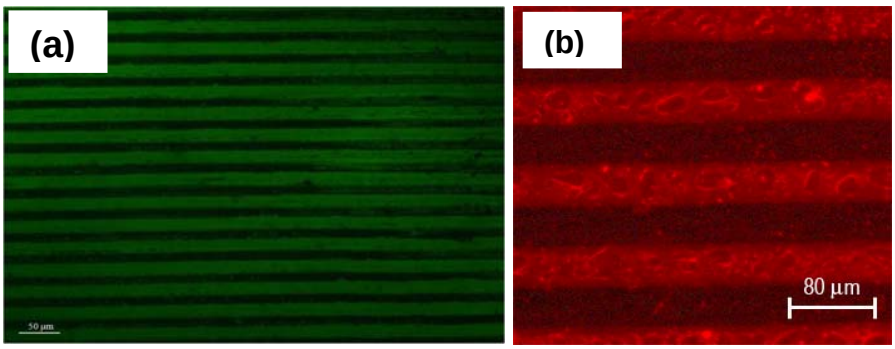


Fig. 24. Fluorescence microscopy of silicatein patterns. (a) Protein labelling by fluorescein (peak excitation wavelength, λ_{ex} = 492 nm, peak emission wavelength λ_{em} = 518 nm). (b) Protein labelling by rhodamine (peak excitation wavelength, λ_{ex} = 555 nm, peak emission wavelength λ_{em} = 588 nm). Excitation provided by a 100 W mercury lamp.

7.2. Waveguiding optical devices

In order to assess the performances of the biogenic optical waveguides in terms of light losses, a system for the characterization of the waveguide properties was realized. A He-Ne laser is used as light source. The light transmitted by the waveguide is detected by a Si photodiode. The transmitted light power is also measured by the Si photodiode. The transmitted light is imaged by a CCD camera, allowing measurement of the transmitted mode profile. The biosilica refractive index and its wavelength dispersion were measured by confocal imaging of the light back-reflected from the interfaces between borosilicate glass and recombinant silicatein and biosilica, respectively, and found to be very close to typical values and dispersion of amorphous quartz (Fig. 25). The coupling of the laser light into the waveguides was optimised, by measuring and maximizing the optical power transmitted by both free-standing fibers and fibers deposited on the employed substrates, fabricated by pressure-driven microfluidics. The light transmitted by the fibers and collected in a plane perpendicular to the waveguide axis was found to formed an almost circular spot.

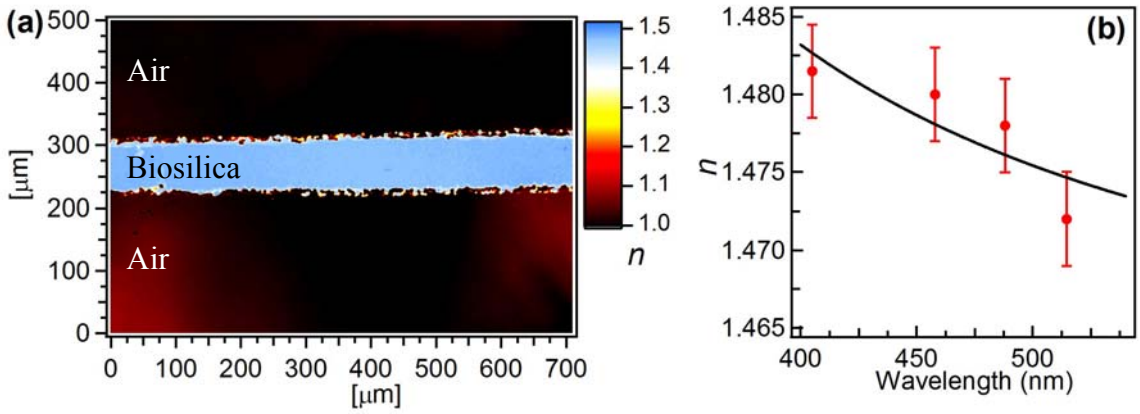


Fig. 25. (a) Refractive index map of a biosilica waveguide. (b) Biosilica refractive index wavelength dispersion. The continuous line is a fit to the data by a Cauchy model (see text).

7.3. Bio-grown silica based electrical devices

By using soft lithography techniques, biosilica layers were grown by biosilicification and tested as a possible dielectric layers for electronic or optoelectronic devices, such as the gate insulator in an organic thin film transistor (OTFT). Several measurements performed on the same layer produced similar electrical performances, thus showing the reproducibility of the measurement and the stability of the device under test. Next, the electrical characterization was further developed also on 3-terminal devices. In order to investigate the resulting electrical properties, conductivity measurements were carried out on both native silicatein films and on biomineralised layers after incubation in TEOS solution for different time intervals.

Fig. 26 displays a scheme for the performed 2-terminal measurements of the insulating properties of the gate dielectric (Fig. 26a), and for the 3-terminal measurement of the field effect behaviour (Fig. 26b).

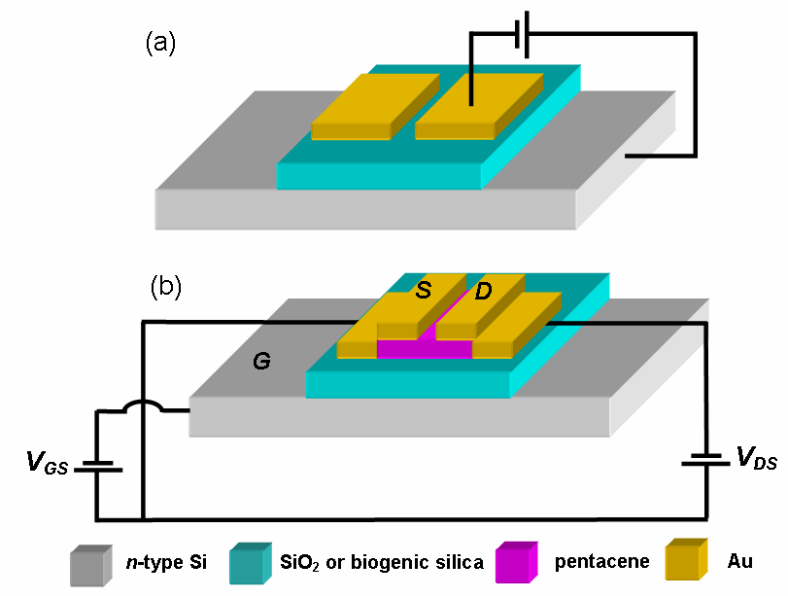


Fig. 26. Scheme of the 2-terminal (a) and 3-terminal (b) measurements for the assessment of the insulating properties of the silicatein-induced biosilica layer and for the performances of the OFETs, respectively.

8. Industrial perspectives

The project exploits the unique ability of siliceous sponges to synthesize their skeleton enzymatically. The described bionic approaches are of enormous commercial importance in view of their application in nanobiotechnology. The realized FET devices could be competitive candidates for existing and novel applications that require large area coverage, structural flexibility, and especially low temperature processing and low fabrication costs. In the framework of large-scale manufacturing, there is currently a strong demand for inexpensive deposition and patterning processes that can be easily incorporated within existing device concepts.

Part II: Dissemination and use

Exploitable knowledge and its Use

The following overview table presents exploitable results achieved in BIO-LITHO project, which have a potential for industrial or commercial application.

Overview table

Exploitable Knowledge	**Exploitable product(s) or measure(s)**	**Sector(s)** of application	Timetable for commercial use	Patents or other IPR protection	**Owner** & Other Partner(s) involved
Silicatein-mediated synthesis of biosilica and siloxanes and use thereof	Silicatein and applications	Nanobio-technology: lithography, Micro-electronics; Nanobio-medicine	≥2009	**European Patent No. EP1320624; US Patent No. 7169589; National phases in different countries**	**P1**; **P8** (owner) Poss. licensing to other companies
Silicase-meadiated degradation of silica	Silicase and applications	Nanobio-technology: lithography, Micro-electronics; Nano-medicine	>2010	**German Patent No. DE10246186; European Patent No. EP1546319; National phases in different countries**	**P1**; **P8** (owner) Poss. licensing to other companies
Enzyme- and template-directed synthesis of silica	Application to non-organic silicon compounds	Nanobio-technology: lithography, Micro-electronics	>2009/2010	**Patent applications in Germany, Europe and USA**	**P1**; **P8** (owner) Poss. licensing to other companies
Controlled fabrication of silver and gold nanoparticles	Application of silicatein; nanowires / nanotubes	Nanobio-technology: lithography, Micro-electronics	>2010	**Patent application in Germany**	**P1**; **P8** (owner) Poss. licensing to other companies
Enzymatic method for producing bioactive surfaces	Application of silicatein	Nano-medicine	>2009/2010	**Patent applications in Germany, Europe and USA**	**P1**; **P8** (owner) Poss. licensing to other companies
Enzymatic synthesis, modification and degradation of	Application of silicatein and silicase	Nanobio-technology: lithography, Micro-electronics	>2009/2010	**Patent applications in Germany, Europe and USA**	**P1**; **P8** (owner) Poss. licensing to other companies

Exploitable Knowledge	**Exploitable product(s) or measure(s)**	**Sector(s)** of application	Timetable for commercial use	Patents or other IPR protection	**Owner** & Other Partner(s) involved
silicon(IV) and other metal(IV) compounds					
Biosilica-adhesive protein nanocomposite materials	Application of silicatein and adhesive proteins	Nanobio-technology: lithography, Micro-electronics; Nanobio-medicine	>2010	**Patent application in Europe and USA**	**P1**; **P8** (owner) Poss. licensing to other companies
Application of silicatein in lithography	Fabrication of micro-electronics	Nanobio-technology: lithography, Micro-electronics	>2011	**Joint patent application EP09159065.3**	**3,8**, Poss. licensing to kit manuf.
Silicatein fusion proteins – adhesive proteins	Coating of surfaces	Nanobio-technology: lithography, Micro-electronics; Nanobio-medicine	>2011	**Patent application in preparation**	**P1; P8** Poss. licensing to kit manuf.
Production of specifically designed biosilica structures in primmorphs	Fabrication of specifically designed biosilica structures	Nanobio-technology	>2011	Patent planned for 2010	**P1; P2; P8** Poss. licensing to kit manuf.
Antibodies against silicatein, silicase and other proteins	Antibodies	Mainly for research	≥2007	No patenting	**P1**

Patents/patent applications

Patents (selection)

Silicatein:

- **German Patent No. DE10037270.** Silicatein-vermittelte Synthese von amorphen Silikaten und Siloxanen und ihre Verwendung. Inventors: Müller WEG, Lorenz A, Krasko A, Schröder HC

- **European Patent No. EP1320624.** Silicatein-mediated synthesis of amorphous silicates and siloxanes and use thereof. Inventors: Müller WEG, Lorenz A, Krasko A, Schröder HC

- **United States Patent No. US7169589.** Silicatein-mediated synthesis of amorphous silicates and siloxanes and use thereof. Inventors: Müller WEG, Lorenz A, Krasko A, Schröder HC

- **Chinese Patent No. CN1460110.** Silicatein-mediated synthesis of amorphous silicates and siloxanes and use thereof. Inventors: Müller WEG, Lorenz A, Krasko A, Schröder HC

- **New Zealand Patent No. NZ523474.** Silicatein-mediated synthesis of amorphous silicates and siloxanes and use thereof. Inventors: Müller WEG, Lorenz A, Krasko A, Schröder HC

- **Australia Patent No. AU2001289713.** Silicatein-mediated synthesis of amorphous silicates and siloxanes and their uses. Inventors: Müller WEG, Lorenz A, Krasko A, Schröder HC

Silicase:

- **German Patent No. DE10246186.** In vitro and in vivo degradation or synthesis of silicon dioxide and silicones, useful e.g. for treating silicosis or to prepare prosthetic materials, using a new silicase enzyme. Inventors: Müller WEG, Krasko A, Schröder HC

- **European Patent No. EP1546319.** Decomposition and modification of silicate and silicone by silicase and use of the reversible enzyme. Inventors: Müller WEG, Krasko A, Schröder HC

Patent applications (selection)

- **EP1740707.** Enzym- und Template-gesteuerte Synthese von Silica aus nicht-organischen Siliciumverbindungen sowie Aminosilanen und Silazanen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

- **US11579019.** Enzym- und Template-gesteuerte Synthese von Silica aus nicht-organischen Siliciumverbindungen sowie Aminosilanen und Silazanen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

- **JP2007535319.** Enzym- und Template-gesteuerte Synthese von Silica aus nicht-organischen Siliciumverbindungen sowie Aminosilanen und Silazanen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

- **DE102006001759.5.** Kontrollierte Herstellung von Silber- und Gold-Nanopartikeln und Nanokristallen definierter Größe und Form durch chirale Induktion mittels Silicatein. Inventors: Tremel W, Tahir MN, Müller WEG. Schröder HC

- **EP05012162.3.** Selenium-enriched liquid media for the cultivation of siliceous sponges and for biogenic silica production. Inventors: Müller WEG, Schröder HC, Osinga R, Schwertner H

- **EP1740706.** Enzymatisches Verfahren zur Herstellung bioaktiver, Osteoblasten-stimulierender Oberflächen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

- **JP2007535320.** Enzymatic method for producing bioactive, osteoblast-stimulating surfaces and use thereof. Inventors: Müller WEG, Schwertner H, Schröder HC

- **US2008213851.** Enzymatic method for producing bioactive, osteoblast-stimulating surfaces and use thereof. Inventors: Müller WEG, Schwertner H, Schröder HC

- **US2006029939.** Decomposition and modification of silicate and silicone by silicase and use of the reversible enzyme. Inventors: Müller WEG, Krasko A, Schröder HC

- **JP2006501832.** Decomposition and modification of silicate and silicone by silicase and use of the reversible enzyme. Inventors: Müller WEG, Krasko A, Schröder HC

- **CA2501208.** Decomposition and modification of silicate and silicone by silicase and use of the reversible enzyme. Inventors: Müller WEG, Krasko A, Schröder HC

- **AU2003276044.** Decomposition and modification of silicate and silicone by silicase and use of the reversible enzyme. Inventors: Müller WEG, Krasko A, Schröder HC

- **DE10352433.** Enzymatische Synthese, Modifikation und Abbau von Silicium(IV)- und anderer Metall(IV)-Verbindungen. Inventors: Müller WEG, Schwertner H, Schröder HC

- **EP1682658.** Enzymatische Synthese, Modifikation und Abbau von Silicium(IV)- und anderer Metall(IV)-Verbindungen. Inventors: Müller WEG, Schwertner H, Schröder HC

- **US2007280921.** Enzymatic synthesis, modification and degradation of silicon(IV)- and other metal(IV)-compounds. Inventors: Müller WEG, Schwertner H, Schröder HC

- **CN1922315.** Enzymatic synthesis, modification and degradation of silicon(IV)- and other metal(IV)-compounds. Inventors: Müller WEG, Schwertner H, Schröder HC

- **EP20064329.** Biosilica-adhesive protein nanocomposite materials: synthesis and application in dentistry. Inventors: Müller WEG, Schröder HC,Geurtsen WK

- **US2010047224.** Biosilica-adhesive protein nanocomposite materials: synthesis and application in dentistry. Inventors: Geurtsen WK, Müller WEG, Schröder HC

- **EP09159065.3.** Silicon derivate layers/films produced by silicatein-mediated templating and process for making the same. Inventors: Pisignano D, Biasco A, Camposeo A, Pagliara S, Polini A, Müller WEG.

Further patent applications are planned.

Exploitable results will be used by the SME partners in the project. The plans for use and exploitation are briefly outlined below.

NanotecMARIN GmbH is patent owner of the (granted) patents on silicatein and its technical use (DE10037270, EP1320624, US7169589, CN1460110, NZ523474, AU2001289713) as well as patent owner of the (granted) patents on silicase and its (nano)technological applications (DE10246186, EP1546319). This company will exploit these enzymes for their future commercial use. In addition, this company will also exploit the technology described in the related patent applications EP1740707, US11579019, JP2007535319, DE102006001759.5, EP1740706, JP2007535320, US2008213851, US2006029939, JP2006501832, CA2501208, AU2003276044, DE10352433, EP1682658, US2007280921, CN1922315, EP20064329, and US2010047224. **NanotecMARIN GmbH** will further elaborate the technology in cooperation with partners from big industry. **BIOTECmarin GmbH** will be involved in the further development (scale-up) of the enzymes in frame of the project.

National Nanotechnology Laboratory of INFM-CNR and **NanotecMARIN GmbH** are owners of the patent application EP09159065.3 ("Silicon derivate layers/films produced by silicatein-mediated templating and process for making the same") and will exploit the new technology in soft lithography and for the fabrication of microelectronics.

Dissemination of knowledge

The following overview table summarizes the dissemination activities of BIO-LITHO project in the past and future.

Overview table

Planned/ actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
During the project period and thereafter	Publications	Research	All	Large	All
During the project period and thereafter	Conferences/Oral Presentations/Posters	Research	All	Medium	All
3/3	Project web-site	Research/General public	All	Large	**P1**; **P5**
During the project period and thereafter	Press release	General public	EU	Large	**P1** and the partners in the respective countries
During the project period and thereafter	Contribution to TV	General public	EU	Large	**P1** and the partners in the respective countries
21/20	Summer school	Research	EU	Small / Medium	**P1**, some partners
33/33	Summer school	Research	EU	Small / Medium	**P1**, all
36/36	Final project meeting	General public	EU	Medium	**P1**, all

Publications

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4. S.V. Patwardhan, K. Shiba, H.C. Schröder, W.E.G. Müller, S.J. Clarson and C.C. Perry: The interaction of silicon with proteins: Part 2. The role of bioinspired peptide and recombinant proteins in silica polymerisation. In: Science and Technology of Silicones and Silicone-Modified Materials (S.J. Clarson, J.J. Fitzgerald, M.J. Owen, S.D. Smith and M.E. Van Dyke, eds.). ACS Symposium Series **964**, 328-347 (2007).

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Biosilica in Evolution, Morphogenesis, and Nanobiotechnology: Case Study Lake Baikal.
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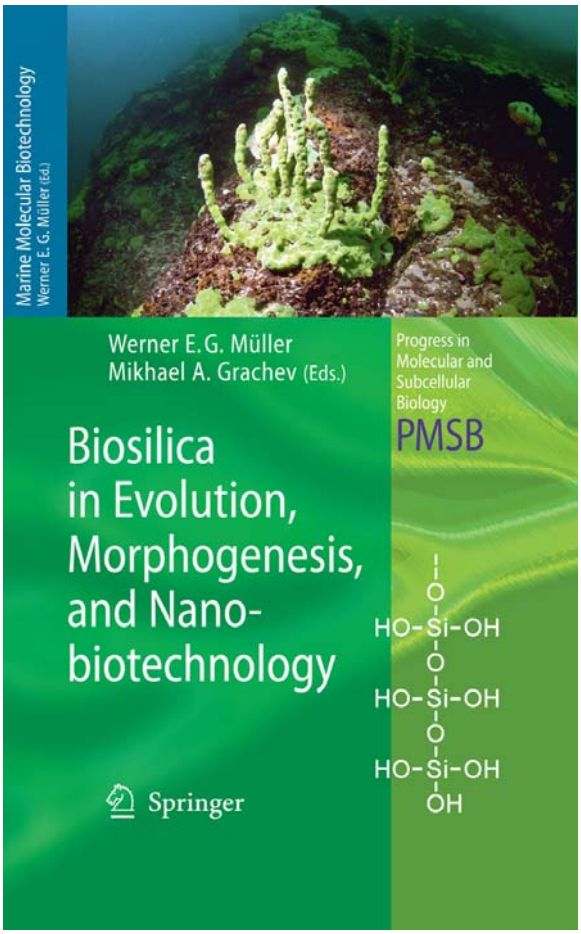

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Web site

A web site has been created. The web site contains a short introduction describing the BIO-LITHO project and links to the different work packages and activities.

Website address: www.bio-litho.de

Summer school

The Summer school of BIO-LITHO project was annually held in Rovinj, Croatia (Center for Marine Research, Ruder Boskovic Institute). This Summer school was organized together with the German Center of Excellence "BIOTECmarin" and the Marie-Curie Research Training Network "BIOCAPITAL". About 35-50 scientists (PhD students, postdocs, senior researchers) participated in the summer school. In the morning sessions, the participants presented the results of their work. In the afternoon, experimental courses on specific topics were organized, as well as catching/dredging excursions with the Institute boat to the islands around Rovinj and to the Limski Canal.

Dissemination to the public

A number of measures has been undertaken to disseminate the results of the project to the public. These activities did not only include contributions to conferences or workshops but also contributions to exhibitions/fairs or the distribution of information of material to potential customers by the industrial partners involved in this project. In 2007, **P1** and **P5** made two major contributions in the German TV channels "WDR" and "3sat" about the topic "Silicatein and biosilica". Further TV contributions in 2008 were in the "ARD-Morgenmagazin" and in "Die große Show der Naturwunder" with Frank Elstner and Ranga Yogeshwar (4 million viewers).