Project no. 031541

Project acronym: BIO-LITHO

Project title:

BIOMINERALIZATION FOR LITHOGRAPHY AND MICROELECTRONICS

Instrument: STREP

Thematic Priority: NMP Priority

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Project coordinator:	Prof. Dr.Dr. Heinz C. Schröder
Project coordinator organisation:	Johannes Gutenberg Universität

Project public website: www.bio-litho.de.

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Publishable executive summary

Project objectives

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 will be 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. We plan to exploit this unique combination within a novel technology, whose demonstrator will be the realization of patterned, aligned assembly of silica fibers, and their employment as insulating layers for prototype transistor devices. Two parallel strategies will be pursued for the production of large amounts of silicatein: (i) expression of the recombinant proteins, and (ii) development of in vitro primmorph cultures. Soft lithography techniques will be used for the controlled patterned deposition of molecules. Specific approaches will be designed and implemented, for the hierarchical assembly of silicatein fibers into functional networks. The multidisciplinary team involved in the proposed project has the specific know-how in biosilicification/lithography and the intellectual property rights in enzymatic silica formation.

Work performed and results achieved

Partner **P1** demonstrated that silicatein, which has been isolated from axial filaments of the demosponge *Suberites domuncula* using a new extraction procedure, is able to polymerize under formation of long filaments. The filament formation from disassembled silicatein could be visualized by both light and transmission electron microscopic analyses. This self assembly process, which is accompanied by an increase in catalytic activity of the protein, occurs in the absence of glycerol, while silicateins present in glycerol-containing extraction buffer are monomers/dimers. Polymerization of silication proceeds through formation of fractal-like intermediates, which subsequently form the filaments. These self-assembly features were not observed with silicatein from axial filaments isolated using HF extraction procedure; these axial filaments had to be disassembled by urea to obtain monomeric silicatein. In *S. domuncula* silicatein-α is 4-times more abundant in the axial filament than silicatein-β. Based on computer modeling studies **P1** concluded that first tetramers consisting of four silicatein-α molecules are formed, which then bind one silicatein-β molecule, reflecting the 4:1 stoichiometry of silicatein in axial filaments.

P1 demonstrated that silicatein comprises dual enzymatic activities (silica-polymerase and silica-esterase). The polymerizing growth of the silica chains, mediated by the silica-polymerase activity of silicatein, involves a step-wise addition of single silica monomeric units. In addition, **P1** demonstrated that recombinant silicatein remains active when bound to surfaces. The recombinant protein was linked to the surface of silica spicules; after incubation with TEOS biosilica was deposited onto the spicules.

Silicatein was also isolated from the freshwater sponge *Lubomirskia baicalensis* where it occurs in isoforms with sizes of 23 kDa, 24 kDa and 26 kDa (**P1** and **P7**). The silicatein

isoforms are post-translationally modified by phosphorylation; at least four isoforms exist with pI's of 5.4, of 5.2, of 4.9 and of 4.7. Surprisingly silicatein does not only mediate polymerization of silicate, but also displays proteolytic activity which is specific for cathepsin L enzymes, thus underscoring the high relationship of the silicateins to cathepsin L.

The work of **P2** focused on the characterization of the natural model of biosilica structures in sponges. In particular, the work of this group addressed the biochemical characterization of native silicatein, the mass spectrometry analysis of the primary structure and its comparison with the putative sequence obtained with cDNA.

Several specimens of *Petrosia ficiformis* (600 grams-dry weight) were collected in the Ligurian Sea. The sponges were air dried and stored at room temperature. They were used for spicules extraction.

Dry sponges were cut in small pieces and the organic material was removed by means of treatment with acid nitric/acid sulphuric. The cleaned spicules were then washed several times with distilled water and dried under vacuum after a final washing with ethanol and acetone. All the procedures were done under a clean hood and with protecting clothes and gloves, in order to avoid dust and keratin contamination. Each preparation gave 15 g of cleaned spicules. The original amount of dried sponge used was 60 g.

The silicatein preparation was performed to deliver the proteins to the other partners. Cleaned spicules were dissolved with ammonium fluoride procedure and the unsoluble protein was recovered after dialysis. The axial filament was then sent to **P3** and **P5**. Each preparation gave 10 mg of protein from 10 g of cleaned spicules. The amount of protein was determined by means of Bradford protein assay method performed on some aliquots of silicatein suspension previously dissolved with 8 M urea.

Samples were analyzed as follows: intact proteins were mixed with sinapic acid and analyzed by MALDI-MS. For the bottom-up analysis, peptides were prepared from excised 1D electrophoresis bands and directly from HF spicules preparation, by means of proteolytic digestion with trypsin and chymotrypsin. Peptides chromatography and sequencing were performed on a high resolution LS/MS/MS system. Data interpretation was achieved with web-based algorithms and with the extensive use of *de novo* sequencing softwares.

The full length cDNA of silicatein-β was amplified from freshly processed tissues of *P. ficiformis* using standard procedures. The PCR product was then inserted inside a pCR 2.1 vector and expanded in *E. coli*. The plasmid was then extracted and sequenced. 50 μg of the plasmid was sent to **P5**.

The work of **P3** was focused on extensive experiments on patterning of silicateins by soft lithography and injecting silicatein filaments into microfluidic elastomeric circuits. Notwithstanding the difficulties due to the withdrawning of **P6** from the BIO-LITHO consortium, our aim for the first year of the project has been accomplishing the realization of silicatein micrometer-scale structures on different substrates, with resolution in the range between 10-100 μm. The soft lithography techniques implemented and developed for the controlled patterned deposition of silicatein molecules include micromolding in lithographically defined capillaries (MIMIC) and imprinting technologies, with particular attention paid to the Microcontact Printing (μCP). In addition, we have used replica molding (REM), which consists in the fabrication of elastomeric elements with patterned relief microstructures on their surface, to enable both the soft lithography techniques mentioned above.

Poly(dimethylsiloxane) (PDMS) elastomer building blocks were fabricated starting from previously realized 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 to 75°C for 15 minutes, the PDMS was peeled off (procedure schematised in Fig. 1) and temporarily activated by oxygen plasma under various processing conditions to increase hydrophilicity for the subsequent

adsorption of silicatein molecules. The best patterning results were obtained by a plasma power of 50 W, applied for 5 seconds.

The patterned surfaces were characterized by optical and atomic force microscopy. Silicatein in both filament and recombinant soluble form were patterned by MIMIC and μCP, and extensive experiments were carried out to identify the most performing approach and system allowing one to realize a controlled pattern deposition for the subsequent device realization. In particular, we found that the most suitable processing for filaments of silicatein seems to be the MIMIC technique, since allowing the introduction of whole filaments in a fitting microfluidic channel without breakes, thus potentially realizing a natural catalytic template for biomineralization reaction. Instead, the μCP technique has given very good results for patterning recombinant silicatein molecules, which provide homogeneous protein layers very suitable for being transferred to the target substrated via the conformal contact performed during printing.

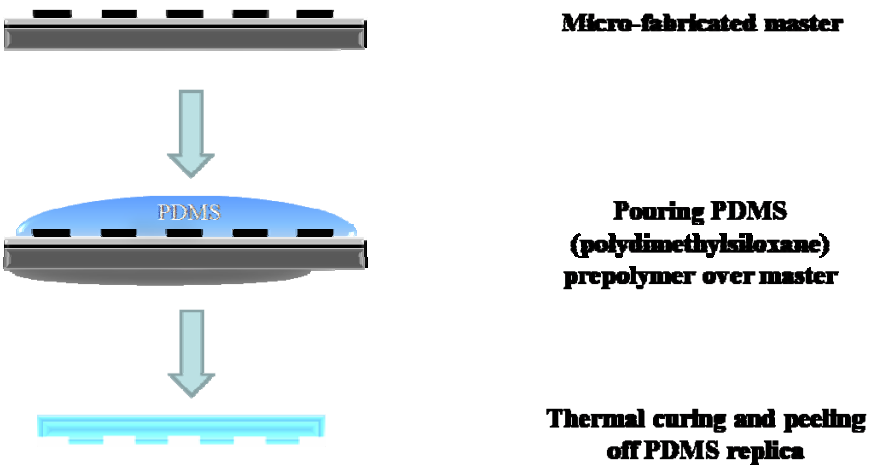

Fig. 1. Schematic diagram of the implemented Replica Molding (REM).

Gold and glass surfaces have been coated with different polymers, for instance, poly(allylamine hydrochloride) (PAH), poly(ethyleneimine) (PEI) and octadecyl amine (ODA), followed by immobilization of BSA and lysozyme at pH 7.2 (**P4**). These proteins were chosen for preliminary studies as they provided for variations in intrinsic pI, charge and size. Gold and glass surfaces were coated with amine containing molecules to achieve high loading with retention of the functionality of the adsorbed proteins. UV-vis spectroscopy, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) with curve-fitting analysis of amide-I bands has been used to assess protein conformation, scanning electron microscopy (SEM), energy-dispersive X-ray analysis (EDXA), and atomic force microscopy (AFM) have been used to characterize the adsorption of different proteins and coatings of silica on glass coated with gold and glass surfaces alone. From the data obtained, lysozyme coated on PAH or ODA bound surfaces and BSA coated on PAH bound surfaces are ideal for the preparation of homogeneous coatings of silica. The technology is being transferred to p and n doped Si(100) samples provided by **P3** and will be used for further studies involving the silicatein protein.

Expected end results

It is expected that the achievement of the goals of the project (development of novel techniques for lithography and microelectronics based on principles of biomineralization /

biosilicification) and the new knowledge based on “using nature as a model” will result in a long-term innovation in the rapidly growing field of nano(bio)technology, in particular lithography and microelectronics.

Intentions for use

A strong focus of the BIO-LITHO programme is to spread excellence by promoting the exploitation of results generated within this project. This will include the following innovation-related activities: (a) protection of intellectual property, (b) developing a plan for the use and dissemination of the knowledge, and (c) assessing the socio-economic impact of the knowledge and technologies generated within the project.

The industrial participation in this project will guarantee that the technologies developed in the course of the project will have a real chance to be introduced to the market. The availability of methods for using silicatein to induce controlled biosilicification under ambient conditions will also be of benefit for other high-tech industries in the European Union.

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.

Impact

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 availability of techniques to utilize the capability of sponges to form silica under ambient conditions will strengthen the competitiveness of the European Union.