Nanoorganized biocomposites: from biomimetic potential to development of new biomaterials

by

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Content

• Silica-aragonite-chitin biocomposite
• Silica-calcite biocomposites
• Phenomenon of multiphase biomineralization
• Extreme Biomimetics

Light microscopy view: 3D skeleton of *Sarostegia oculata* glass sponge
Motivation

Our discoveries of:

Silica-chitin composites in cell walls of diatoms (2010)
Silica-chitin composites in spicules and skeletal frameworks of glass sponges (2007 - 2010)
Silica-collagen composites in glass sponge spicules (2005-2010)

Deep-sea *Aspidoscopulia sp.* glass sponge (up to 2 m high and 70 cm wide). It skeleton is made of silica-chitin composite.
Sponges (Porifera)

Glass sponges (Hexactinellida)

Horn sponges (Demospongia)

Calcereous sponges (Calcarea)

Silica or CaCO₃

Silica

CaCO₃

Biominerals
First evidence of the presence of chitin in sponges

Staining indicating the presence of chitin, as shown in a 3D-reconstruction of a multichannel confocal LSM image stack (a) as well as in wide-field fluorescence images revealing marked difference between unstained (b) and stained (c) alkali-insoluble sponge fibers (bars: 200 µm)


What is chitin?

Chitin, or poly-(beta-[1→4]-2-acetamido-2-deoxy-D-glucopyranose), is crystalline in its native state.
\begin{align*}
\textbf{\(\alpha\)-chitin} & \\
\text{Orthorhombic} & \text{Two chain/unit cell} \\
\text{Antiparallel} & \\
ap = 4.74 \text{Å} \\
b = 18.86 \text{Å} \\
c = 10.32 \text{Å} \\
\textbf{\(\beta\)-chitin} & \\
\text{Monoclinic} & \text{One chain/unit cell} \\
\text{Parallel} & \\
ap = 4.85 \text{Å} \\
b = 9.26 \text{Å} \\
c = 10.38 \text{Å}
\end{align*}

Scheme 1. Crystalline structures of chitin.

Chitin-based Sponges: Verongida (Demospongia)

*Aplysina archeri*  
*Aplysina sp.*
Fibrous skeleton of *Verongula gigantea*

**A**, *Verongula gigantea* from Trindade Island, 10 cm tall.

**B**, Skeletal fibers of *V. gigantea* showing pith (dark coloured) and bark (yellow and dark-brown coloured).

**C**, reticulated fiber skeleton of *V. gigantea* seen in SEM.
Fibrous skeleton of *V. gigantea*

**A**, cross-section of a skeletal fibre showing multilayered structure, resembling silica spicules as described for hexactinellids and demosponges

**B**, STEM image of cross-section of inner layer of the fibre showing the presence of electron-dense inclusions (dark)
V. gigantea (a) show no presence of foreign particles. However, skeletal spongin-based fiber of *Dysidea avara* (b) shows typical accumulation of sediment particles including debris of sponge spicules. Heating of these fibers at 500°C during 5 h leads to more definitive visualization of the fiber contents after thermal damage of spongin (c). Image (d) represents results of the 1M HCl action on the particle agglomerates observed in image (c).
Identification of silica and CaCO₃

A. Photoemission spectra of natural sponge fibres showing the presence of silicon oxide bound to an organic matrix.

B. X-ray absorption spectra showing that calcium carbonate is the second mineral component present within three Verongida species of different geographical origin.

Silica and calcium carbonate were additionally identified using FTIR and Raman spectroscopy.
TEM images (b and c) of the FIB cut of the both fiber regions represented in (a) show that silica-based layers are located in the outermost region (b), however calcium carbonate is grown on the chitin nanofibers (c, d) which are localized near the axial channel.
Both micro- and nanoparticles (arrows) of the mineral are tightly bound on and into organic matrix that additionally rules out any kind of foreign mineral particles.
HR-TEM and electron diffraction analysis

**A,** high resolution TEM image of the crystalline phase within this agglomerate, displaying spacings of 9.19 Å, 4.45 Å and 2.10 Å corresponding to (002), (120) and (143)/(136) lattice planes typical for \textit{alpha-chitin} (B).

**C,** HR-TEM image showing the presence of crystalline aragonite within the same mineral agglomerate.

**D,** Fast Fourier transform of C displaying orthorhombic structure of \textit{aragonite} with denoted spacings of 3.96 Å, 3.00 Å, 2.78 Å and 2.11 Å corresponding to (020), (002), (121) and (220) reflections.
Selective Demineralization

HCl

Sponge skeleton

NaOH

Silica layers

Chitin
HCl-based demineralization of the sponge skeleton

A, SEM image of skeletal fibre after treatment with 3 M HCl at 37 °C for 3 weeks, showing perforated layers that cover the pith region.

B, light microscopy micrograph of isolated layers showing their perforated structure.

C, SEM image showing three dimensional organisation of the layers. D, nano- and microscale apertures visible using SEM within these formations corresponding to the mineral compounds that were dissolved in HCl.
Identification of nanofibrillar chitin within silica layers

**A**, SEM image of the same fragment prepared at higher magnification show the presence of nanofibrillar network.

**B**, Nanofibers in the silica matrix samples containing a nano-crystallite with diameter 2 nm (arrows), typical for α-chitin crystallites.
Overview: The Model of Silica-Chitin-Aragonite Unit

From: Ehrlich et al., Chemistry of Materials (2010) 22(4): 1462-1471
Examples of multiphase biomineralization


**silica**-chitin-*willemite* composite in copepoda teeth

Williams, A., Lüter, C. & Cusack, M., 2001:

**silica**-chitin-*apatite* composite in Brachiopoda

Sone, E. D., Weiner, S. & Addadi, L., 2007:

**silica**-chitin-*goethite* composite in limpet teeth

TEM image: Section of the tooth of *Calanus pacificus* made of silica, chitin and crystalline $[\text{Zn}_2\text{H}_4\text{SiO}_4]/[\text{H}^+]_4$ (scale bar=2.6 µm)

From Miller et al, 1990.
Sponges 
(Porifera)

- Glass sponges (Hexactinellida)
- Horn sponges (Demospongia)
- Calcareous sponges (Calcarea)

Silica or CaCO₃

Biominerals

Silica

CaCO₃
Mushroom-like deep-sea glass sponge *Caulophacus sp.* (image courtesy NOAA).

The Eiffel tower – an example of man-made hierarchical construct (photos courtesy Vasily V. Bazhenov).
Caulophacus sp. glass sponge

The stalks of Caulophacus sp. possess complex network of glassy spicules
There are several similarities between structural motives in Eiffel tower (a,c,e) and within skeletal framework of *Caulophacus* sp. stalk (b,d,f) observed using light microscopy.
It is possible to destroy structural integrity of the stalk using pincers (left). After this procedure club-formed structures were observed (right).
Club-formed structures and their role

Club morphology of the spicules could be also observed using light microscopy. The stalks were incubated in alkali solution at 37°C during two weeks. After partial dissolution of silica-based articulations club-formed spicule (arrows) are well seen within articulation (right).
SEM studies on club-formed structures

The same phenomenon could be confirmed using SEM
The material of articulation is corroded in alkali, however the spines of the club-formed spicule are resistant to this kind of chemical treatment.
Caulophacus sp. spicule articulations

SEM image (right) show that club-formed structures are distributed within spicules. The model view of these articulations is represented on the left image.
SEM image (right) shows with strong evidence that club-formed structures are responsible for mechanical coupling also within one spicule. Corresponding model of this coupling is represented on the left image.
Mother Nature vs. Human: how to connect?

SEM image: club-formed spicule released from articulations after their particular dissolution using 2.5 M NaOH at 37°C on the 10th day of insertion

Nature - made articulation

Articulation: Made in Germany
SEM studies on natural club-formed spicules

Mechanically disrupted club-formed spicule:
the spines of club-formed structures possess **seed-like** formation which is covered with silica layers
Calcium identification within club-formed structures

For EDX analysis air-dried samples were embedded in Epoxy resin without additional staining and cut on a Leica EM UCT ultramicrotome to obtain a flat block face. Samples were coated with carbon and analysed in an ESEM XL 30 Philips. EDX-analysis and elemental mapping was done with an EDAX detecting unit and EDAX software.

![Image of calcium identification within club-formed structures]

- **Ca**: Calcium peak
- **Si**: Silicon peak
- **O**: Oxygen peak

Graph showing the elemental composition with peaks for calcium (Ca), silicon (Si), and oxygen (O).
Silica and CaCO$_3$ identification

Left: Photoemission spectra of natural *Caulophacus* sp. spicules showing the presence of silicon oxide bound to an organic matrix.

Right: X-ray absorption spectra showing that calcium carbonate is the second mineral component present within the same spicules.
TEM and electron diffraction analysis

A, TEM image of the natural *Caulophacus* sp. spicule with a fragment of club-formed structure (B).

TEM diffraction analysis of the spicule shows the presence of two phases: amorphous (C) and crystalline (D).

Electron diffraction pattern corresponding to the spines shown as a TEM image in (B) corresponds to the (0001) zone axis of **calcite** with d spacings:

100 *(1010)* 4.32 Å  
010 *(0110)* 4.32 Å  
and  
210 *(2110)* 2.49 Å
Results of alkali-based demineralization

Silica

Calcite

Collagen?  Chitin?  Silicatein?

Light microscopy image

SEM image
Content

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# Temperature diapason of biosilicification

<table>
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<th>Temperature, °C</th>
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<th>Temperature, °C</th>
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<tbody>
<tr>
<td>Bacteria, Cyanobacteria</td>
<td>45-90</td>
<td>Sponges (Porifera):</td>
<td>-1.5 -10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hexactinellida Demospongiae</td>
<td></td>
</tr>
<tr>
<td>Protista: Silica plankton</td>
<td>0-10</td>
<td>Plants: bamboo</td>
<td>10-18</td>
</tr>
<tr>
<td>Diatoms (Algae)</td>
<td>-1.5 - 80</td>
<td>Brachiopoda</td>
<td>2-10</td>
</tr>
</tbody>
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Adapted from: Ehrlich H., *Biological materials of marine origin*. Springer. 2010
Biosilicification at -1.9°C

*Scolymastra joubini* glass sponge:
- Up to 2 m tall
- Up to 1.4 m in diameter
- Up to 600 kg wet weight
- Up to 50 kg biosilica
Deep sea glass sponges and „cold biomineralization“

Glass sponges are the most ancient multicellular organisms: the oldest hexactinellid spicules date from Edicarian (630 to 542 Myr)
Does *Hyalonema sieboldi* spicules contain collagen?
Unique flexibility of anchoring spicules of *H. sieboldi*
Amino acid sequencing of *H. sieboldi* collagen

List of peptides:

G A Q G (P) L G P
G G F G L 4Hyp G R
G (V) D G N 4Hyp G I X G A T G S
G S V G 3Hyp 4Hyp G N 4Hyp G V Q G V S G 3Hyp
I G P D E P L K G K I
G I 4Hyp G P Q G F T G A I G V T G S 4Hyp G E I G A 4Hyp G
V G D 4Hyp G L V G D L G A Q G P Q G S Q G L V G
G A T G 3Hyp 4Hyp G I S G 3Hyp 4Hyp G P Q G Q 4Hyp G T 4Hyp G I
I G P A G P Q G Q 4Hyp G 3Hyp 4Hyp G P G G P X G 3Hyp 4Hyp
G G S G A 4Hyp G L 4Hyp G A I G N Q G A 4Hyp
Possible role of **Gly-3-Hyp-4-Hyp** in Silicification

![Chemical structure of Gly-3-Hyp-4-Hyp and silicate ions](image)

trans-3-hydroxyproline  trans-4-hydroxyproline
Unique feature of *H. sieboldi* collagen

Some doubts about stability?
HR-TEM images of silicification on *H. sieboldii* collagen. Silicification is apparent as nano particles after exposure of nanofibrillar *H. sieboldii* spicular collagen to a solution of sodium methasilicate solution for 30 minutes (a). However after protection of 3- and 4-hydroxyproline residues by ketal groups there is no visible silica deposition (b). Cleavage of the ketal protecting groups from collagen leads to a functional recovery with respect to silicification (c). The layer of silica nanoparticles is formed around the nanofibril of native spicular collagen (d, e) during the first 30 minutes of silicification as seen in the native collagen fibre The results are in good agreement with measurements of activity (f) of nonprotected collagen (▲), which is lost following protection (◆) and partially restored when this protection is removed (●).
“Collagen, isolated from meter-long silica spicules of a primitive glass sponge, contains an unusual [Gly-3Hyp-4Hyp] motif which is shown to structure the spicule and provide a site for silica deposition. This is central to understanding the role of this unusual collagen as a novel and specific template for biosilicification in nature.”


Mineralization of the meter-long silica structures of glass sponges is templated on hydroxylated collagen

Biosilicification in Glass Sponges (Hexactinellida)


Creatures from the deep freeze: ice fish

Principles of calcification at minus 1.9 °C are unknown.

What are the nature and origin of skeleton, teeth and otoliths (otoconia) of ice fish species?
Chionodraco hamatus, one of the Antarctic's ice fish, can withstand temperatures (-1.9°C) that freeze the blood of all other types of fish.
Through the replacement of bone by connective tissue and decreased mineralization of the skeleton as a whole, many Antarctic fish species have evolved reduced bone density. This adaptation increases their buoyancy in water, a characteristic that enables them to move easily in the water column for feeding. This adaptive trait clearly mimics the detrimental human condition osteopenia, a reduction in bone mineral density.

Open question: if the skeleton of this fish is really non-mineralized, what is about their teeth and otoliths?
Ice fish have long snouts, wide mouths, and large teeth (!)
Open question: What is the mineral composition and nanostructure of these teeth?
Extreme Biomimetics: life between 50°C and 400 °C

Deep-sea images of hydrothermal vent (a) as well as of vestimentiferan fauna (b, c, d), which is well adapted to these extreme environment. (Images from the IMAX film “Volcanoes of the Deep Sea”, courtesy Rutgers University and The Stephen Law Company).
Examples of inspiration for “extreme biomimetics”: (a) silica microparticles of geyserites from Kamchatka surrounding by organic matter; (c and d) different kind of silicified microorganisms observed using SEM within these formations (images courtesy Gennady Karpov); (b) Thermus and Hydrogenobacter are predominant components among the indigenous microbial community in huge siliceous deposits formed within the pipes and equipment of Japanese geothermal power plants.
Thermotolerant Diatom

Light microscopy images (a, b) of *Amphora veneta* isolated from hot spring (80°C) in Kamchatka region (images courtesy Philipp Sapozhnikov).
Chitin Scaffold as Integral Part of the Diatom Cell Wall

SEM images of SDS/EDTA treated *T. pseudonana* samples harvested using a flow centrifuge (left). The right image shows an organic scaffold extracted by NH$_4$F treatment. Scale bar: 2 μm.

TEM imagery: Chitinous scaffold of *T. pseudonana*

- Chitin fibril $\varnothing$ 20 nm
- Chitin fibril $\varnothing$ 85 nm
phytoplankton – bacillariophyceae

Thalassiosira rotula

abundance: permanent abundant
life-form: in chains
diameter: 30 - 60μm

LM (North Sea, German Bight)

LM (coastal station Heiligendamm)

REM (North Sea, SWWBA)
**Thalassiosira rotula**: alkali-based desilification

SEM images: lyophilized cells (left) and cells after treatment using 2.5 M NaOH (16 h at 37°C) (right)

Samples courtesy: Karen Wiltshire & Alex Kraberg, AWI Helgoland
Thalassiosira rotula: alkali-based desilicification

SEM images: lyophilized cells (left) and cells after treatment using 2.5 M NaOH (16 h at 37°C) (right)
Thalassiosira rotula: alkali-based desilicification

SEM images: lyophilized cells (left) and cells after treatment using 2.5 M NaOH (16 h at 37°C) (right)
**Thalassiosira rotula**: Nanoimagery of alkali-based desilicification

SEM images: residual silica nanoparticles (arrows) on the partially demineralized chitinous scaffold
Nanostructural organization of the naturally occurring silica-chitin composite unit

From:
Ehrlich et al.,
J. Nanomat., 2008
Possible silica-chitin interactions

Schematic view shows a possible distribution of silica between chitin nanolamellae within silica-chitin-based outermost layer of *S. hawaiicus* spicule.

We suggest that silicate ions and silica oligomers preferentially interact with glycopyranose rings exposed at the chitin surface, presumably by polar and H-bonding interactions.
Vauxia gracilenta a 505 Myr old Chitin-based Sponge

Exceptional preservation because of low grade metamorphism (260°C).
Sample courtesy Ontario Royal Museum, Canada
A Stereo microscopy view of the Vauxia (Verongida: Porifera) sponge skeleton fragment

B Light microscopy image of the skeletal fibre isolated from Vauxia skeleton

C and D: fluorescence microscopy images of the same fibre show autofluorescence typical for chitin and observed in studies on recent Verongida sponges
The representative thermogravimetric curve of the crab chitin, Verongida sponge chitin and Verongida chitinous sponge skeleton (courtesy Dawid Stawski).
Light microscopy images of ZrO$_2$ crystalline phase (right) obtained within sponge chitinous matrix (left).
Precursor: Ammonium Zirconium (IV) Carbonate
Temperature: 150 °C
Reaction time: 24 h
Extreme Biomimetics *in vitro*

SEM images of \( \text{ZrO}_2 \) nanocrystals obtained on the surface of sponge chitinous matrix. Precursor: Ammonium Zirconium (IV) Carbonate

Temperature: 150 °C

Reaction time: 24 h
Extreme Biomimetics: TEM and X-ray pattern of ZrO2 - chitin

\[ ZrO_2: \ 3.73 \text{ Å (01-1)} \ 2.88 \text{ Å (111)}, \ 3.17 \text{ Å (11-1)} \ ZrO_2 \ 1.83 \text{ Å (21-2)} \ \nu (02-2) \ \nu (220) \]
Conclusions

• Nanoorganized silica-aragonite-chitin biocomposite in demosponges as well as silica-calcite biocomposite in glass sponges are the additional evidence regarding to the presence of more complex multiphase biomineralization in nature.

• These findings motivate us to develope novel strategies to design nanostructured hybride biocomposites \textit{in vitro}.

• \textit{Extreme Biomimetics} is proposed as a challenging strategy to develope of new generation of biomaterials.
Unique hierarchically structured skeletons of up to 2 m high and 70 cm wide *Aspidoscopulia* sp. deep-sea glass sponges are present in our collection.

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Thank you for your attention!