Chitin in Biosilica

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Chitin-Based Organic Networks: An Integral Part of Cell Wall Biosilica in the Diatom *Thalassiosira pseudonana***

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Diatom[1] cell walls are outstanding examples of natural hybrid materials and exhibit extraordinarily interesting mechanical and optical properties.[2] Their structure and composition continue to inspire a variety of biomimetic synthesis approaches.[3] Diatoms are preferred model organisms in silica biomineralization studies.[4] Their hierarchically structured cell walls contain amorphous silica as well as special biomolecules. Over the past decade, three different classes of such biomolecules have been identified: 1) the silaffins, highly post-translationally modified peptides/proteins;[5] 2) long-chain polyamines (LCPAs);[6] and 3) the highly acidic silacidins.[7] The zwitterionic silaffins self-assemble into supramolecular aggregates. The same was observed for LCPAs[8] provided a properly chosen counterion such as orthophosphate or pyrophosphate, or a negatively charged peptide such as silacidin is present. Both the aggregated silaffins as well as the long-chain polyamines induce rapid silica precipitation in vitro from silicic acid containing solutions. To identify these molecules, biosilica was dissolved in HF or NH_4F. Silaffins, LCPAs, and silacidins were then found to be dissolved in the extraction solutions.

The diatom species *Thalassiosira pseudonana* is an established model organism in this area;[4] its genome has been completely sequenced.[9] Recent ion-abrasion scanning electron microscopic[10] as well as atomic force microscopic studies[11] on cell wall formation in *T. pseudonana* revealed the presence of filamentous nano- and microscale structures within the growing cell wall which apparently contain central templating organic structures ("linear proteins").[10] Chitin (poly-*N*-acetyl-D-glucosamine) occurs in numerous calcium-based biominerals.[12] It is assumed to form insoluble scaffolds or compartments, wherein chitin-associated biomolecules control calcium biomineralization events. So far, however, chitin has not been identified in biosilica formation in diatom cell walls. On the other hand, several diatom species such as *Thalassiosira* sp. synthesize external fibers from highly crystalline β-chitin.[13] Interestingly, recent gene expression studies of *T. pseudonana* indicate a possible role of chitin in cell wall biosynthesis.[14] Furthermore, signals characteristic for polysaccharides such as chitin were observed in solid-state NMR spectroscopic analyses of *T. pseudonana* cell walls.[15] The aim of the present work is the elucidation of the possible role of chitin in *T. pseudonana* cell walls, in particular with respect to the presence of "internal" chitin embedded in or tightly bound to the biosilica.

SEM images of *T. pseudonana* cell walls are shown in Figure 1. The cell walls were extracted using the established method based on treatment with sodium dodecylsulfate (SDS) and ethylenediamine tetraacetic acid (EDTA) (see the Experimental Section). Cultures were grown under identical conditions but harvested either with the use of a flow centrifuge or a filter (see Experimental Section). The filtered samples contain large amounts of the well-known external chitin fibers (Figure 1, top). This observation is confirmed by ^{13}C solid-state NMR spectroscopy (Figure 2). The spectrum of the filtered sample is dominated by intense, narrow resonances at chemical shifts characteristic of crystalline β-chitin[16]. An intense fluorescence of the material can be observed after staining with Calcofluor White, a fluorescence dye that preferentially binds to β-1,4-bound polysaccharides. This confirms the presence of high amounts of external, dye-accessible chitin (see the Supporting Information).

In contrast, external chitin is removed from samples harvested with a flow centrifuge (Figure 1, middle). This is confirmed by the complete absence of fluorescence for centrifuge-harvested Calcofluor White stained cell walls (see the Supporting Information). The ^{13}C solid-state NMR spectrum of centrifuge-harvested samples is a superimposition of various resonances. However, a well-resolved signal at $\delta = 104$ ppm is observed which is characteristic for the C1 position of polysaccharides like poly-*N*-acetyl-D-glucosamine.[17] In monomers such as *N*-acetyl-D-glucosamine this signal would occur at approximately $\delta = 93$ ppm.[17] In other words, a significant amount of polysaccharide is indeed integrated in the biosilica. The linewidth (full width at half maximum height, FWHM) of the C1 signal is approximately 240 Hz. In contrast, for the external chitin fibers the linewidth of this signal amounts to only 110 Hz (see Figure 2A). This observation indicates that the polysaccharide bound in the biosilica is more disordered than that in the crystalline external chitin fibers.

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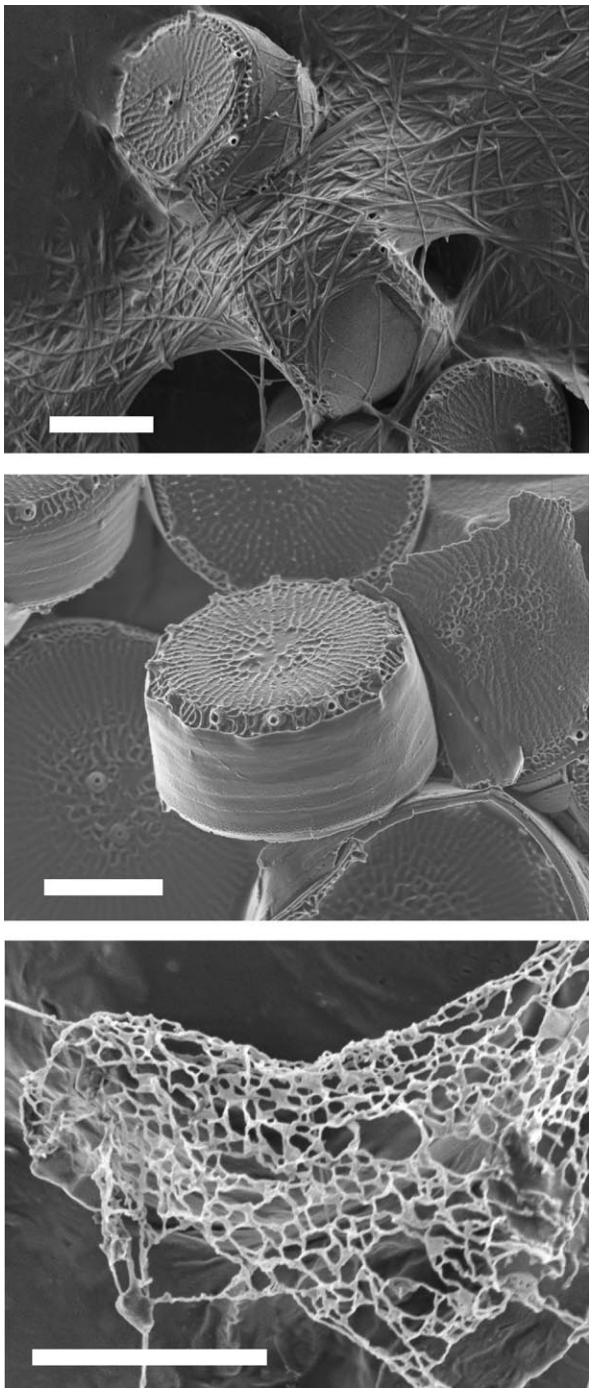

Figure 1. SEM images of SDS/EDTA-treated *T. pseudonana* samples harvested using a filter (top) and a flow centrifuge (middle). The majority of the siliceous cell walls withstands both the of harvesting procedures as well as the subsequent SDS/EDTA treatment. The bottom image shows an organic scaffold extracted by NH_4F treatment. Scale bar: 2 μm.

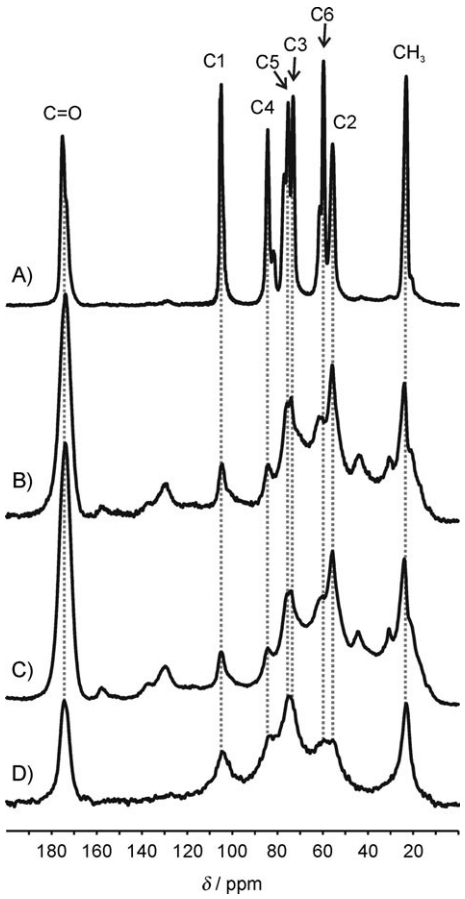

Figure 2. ^{13}C solid-state NMR spectra of SDS/EDTA-treated *T. pseudonana* samples harvested using a filter (A) and a flow centrifuge (B). Spectrum C was obtained after NH_4F treatment (desilicification) of the material shown in (B) and represents the organic scaffold shown in Figure 1 (bottom). The bottom spectrum (D) was recorded after NaOH treatment of the scaffolds.

To obtain SEM images of the intact silica-embedded organic matrix, diatom cell walls were placed on a sample holder and desilicified using NH_4F without any mechanical treatment such as centrifugation (see the Experimental Section). After this treatment, NH_4F-resistant organic scaffolds remained (see Figure 1, bottom).

These networklike scaffolds resemble the size and shape of the diatom cell walls and consist of crosslinked fibers with an average diameter of approximately 25 nm. The diameter of the fibers varies between approximately 5 and 50 nm. The ^{13}C solid-state NMR spectrum of this material is shown in Figure 2C. It is almost identical to the spectrum of the whole, centrifuge-harvested cell walls shown in Figure 2B although the linewidth of the former is somewhat greater. For example, the FWHM of C1 amounts to 260 Hz after NH_4F treatment. The organic scaffolds remaining after NH_4F treatment correspond to approximately 8—10 wt% of the SDS/EDTA-cleaned cell walls. GC–MS and HPLC (Dionex, see the Supporting Information) analyses confirmed glucosamine as a major constituent of this material. Since the solid-state NMR investigations revealed significant amounts of a polysaccharide, these observations indicate the existence of internal chitin or chitosan within the cell wall biosilica.

However, the ^{13}C solid-state NMR spectra show the presence of further organic compounds in addition to chitin/chitosan in the scaffolds. Chitin–protein composites as well as chitin crosslinked by other organic compounds are well

known from various organisms.[18] It is, furthermore, interesting to note that a chitin-binding protein was already identified in the girdle band region of *T. pseudonana*.[19] In order to remove this organic material, the samples were subsequently treated with 2.5 M NaOH;[20,21] chitin is known to resist this treatment. The ^{13}C solid-state NMR spectrum of the remaining material is shown in Figure 2D. Comparison with Figure 2C reveals that the NaOH treatment results, for example, in the disappearance of signals for aromatic carbon atoms at $\delta = 120$–140 ppm and for aliphatic carbon atoms at $\delta = 30$–50 ppm, which probably correspond to amino acids with aromatic and aliphatic side chains, respectively. The amide groups in proteins/peptides contribute to the C=O signal; their removal by basic hydrolysis necessarily results in a decreased intensity of the C=O signal (Figure 2C,D).

The spectrum of the remaining material exhibits strongly broadened resonances at positions characteristic for chitin. The polysaccharide found in the internal organic scaffolds from *T. pseudonana* biosilica is acetylated, as can be seen from the signals due to C=O and CH_3. This means, it can clearly be identified as poly-*N*-acetyl-D-glucosamine (chitin), as was also confirmed by Raman spectroscopy (see the Supporting Information). Gravimetric analyses show that the chitin obtained after NH_4F and NaOH treatment makes up about 2—3 wt% of the SDS/EDTA-cleaned cell wall material, which corresponds to about 25–40% of the NH_4F-extracted organic scaffolds. The aforementioned C1 signal exhibits an FWHM of 500 Hz after NaOH treatment. Removal of the other organic material from the scaffolds apparently results in an even more disordered poly-*N*-acetyl-D-glucosamine. It is important to note that β-chitin extracted from squid pen as well as α-chitin extracted from marine sponges also exhibit broadened ^{13}C solid-state NMR signals.[16,20a] This broadening was explained by the presence of high amounts of surface-exposed poly-*N*-acetyl-D-glucosamine molecules in these types of chitin. Electron diffraction experiments with the extracted scaffolds shown in Figure 1 (bottom) did not exhibit the defined reflections typical for crystalline chitin phases (see the Supporting Information). This behavior confirms the highly disordered nature of the extracted scaffold materials and is in agreement with the severe broadening of the ^{13}C solid-state NMR signals. Similar conclusions were made, for example, based on the X-ray diffraction patterns of chitin-based fibrils isolated from squid pen[16] and the alga *Poteriochromonas stipitata*[22] as well as the chitin-based scaffolds from the marine sponge *I. basta*.[20a]

In summary, we have shown for the first time that the cell walls of the diatom species *T. pseudonana* contain a network-like chitin-based scaffold that resembles the size and shape of the biosilica. These scaffolds consist of interconnected fibers with an average diameter of about 25 nm that contain other yet unknown biomolecules apart from chitin. It is tempting to speculate that the chitin-based networks provide the scaffold structure for silica deposition while other biomolecules—maybe silaffins—actively deposit silica on these superstructures in analogy to calcium carbonate biomineralization processes (see above). It is also possible that the chitin-based networks are necessary to mechanically stabilize the cell walls.

Further work is in progress in order to further analyze this scaffold material and its function, and to analyze the cell walls of other diatom species with respect to the presence of similar scaffolds. In any case, the involvement of the polysaccharide poly-*N*-acetyl-D-glucosamine (chitin) in the formation of diatom biosilica could be shown within the present work.

Experimental Section

T. pseudonana (clone CCMP1335) was cultured in a 20 L plastic vessel in axenic culture medium prepared according to the recipe from the North East Pacific Culture Collection.[23] In order to obtain a sufficient signal-to-noise ratio in ^{13}C solid-state NMR spectroscopy, the diatoms were ^{13}C-labeled by adding $NaH^{13}CO_3$ to the culture medium.

Harvesting procedures: Filtered cell walls: Whole cells were sampled by consecutive filtration of the culture medium on a 1 μm nylon sieve cloth (Stockhausen Sieb- und Filtererzeugnisse) and a 0.2 μm ZAPCAP-CR nylon filter (Whatman). Centrifuged cell walls: Cells were centrifuged in a Westfalia separator at maximum speed.

Isolation of chitin-based scaffolds: Step 1: Harvested cells were boiled twice in a buffer containing 0.1 M EDTA and 2% SDS. The suspension was centrifuged and washed in distilled water until the supernatant remained colorless and was then freeze-dried over night. Step 2: Diatom silica was dissolved under relatively mild conditions using an acidified ammonium fluoride solution (8 M NH_4F/2 M HF at RT, pH 4–5, 20 min). Afterwards, the samples were centrifuged, rinsed four times with distilled water, and freeze-dried overnight. Step 3: The samples were treated with 2.5 M NaOH at 37 °C for 2 h. Afterwards the samples were centrifuged, rinsed four times with distilled water, and freeze-dried overnight. Gravimetric analyses were made on an analytical balance (Kern).

SEM: To avoid mechanical destruction of the scaffolds, experiments were conducted directly on SEM sample holders (Plano GMBH). Diatom cell walls were treated with NH_4F and NaOH as described above.

NMR spectroscopy: Solid-state ^{13}C NMR experiments were performed on a Bruker Avance 300 spectrometer operating at 75.47 MHz for ^{13}C using a commercial double-resonance 2.5 mm MAS NMR probe. Ramped ^{1}H–^{13}C cross-polarization (CP[24]) was used (contact time: 4 ms). Spectra were acquired with a sample spinning rate of 14 kHz and with SPINAL ^{1}H decoupling[25] during signal acquisition.

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[1] a) F. Round, R. Crawford, D. Mann, *The diatoms.* Cambridge University Press, Cambridge, **1990**; b) E. V. Armbrust, *Nature* **2009**, *459*, 185–192.
[2] a) C. E. Hamm, R. Merkel, O. Springer, P. Jurkojc, C. Maier, K. Prechtel, V. Smetacek, *Nature* **2003**, *421*, 841–843; b) T. Fuhrmann, S. Landwehr, M. El Rharbi-Kucki, M. Sumper, *Appl. Phys. B* **2004**, *78*, 257–260.
[3] a) M. Sumper, E. Brunner, *Adv. Funct. Mater.* **2006**, *16*, 17–26; b) R. Gordon, D. Losic, M. A. Tiffany, S. S. Nagy, F. A. S. Sterrenburg, *Trends Biotechnol.* **2009**, *27*, 116–127.
[4] a) M. Sumper, E. Brunner, *ChemBioChem* **2008**, *9*, 1187–1194; b) M. Hildebrand, *Chem. Rev.* **2008**, *108*, 4855–4874; c) N. Kröger, N. Poulsen, *Annu. Rev. Genet.* **2008**, *42*, 83–107.

[5] a) N. Kröger, R. Deutzmann, M. Sumper, *Science* **1999**, *286*, 1129 – 1132; b) N. Kröger, S. Lorenz, E. Brunner, M. Sumper, *Science* **2002**, *298*, 584 – 586; c) M. Sumper, R. Hett, G. Lehmann, S. Wenzl, *Angew. Chem.* **2007**, *119*, 8557 – 8560; *Angew. Chem. Int. Ed.* **2007**, *46*, 8405 – 8408.

[6] a) N. Kröger, R. Deutzmann, C. Bergsdorf, M. Sumper, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 14133 – 14138; b) M. Sumper, E. Brunner, G. Lehmann, *FEBS Lett.* **2005**, *579*, 3765 – 3769.

[7] S. Wenzl, R. Hett, P. Richthammer, M. Sumper, *Angew. Chem.* **2008**, *120*, 1753 – 1756; *Angew. Chem. Int. Ed.* **2008**, *47*, 1729 – 1732.

[8] M. Sumper, S. Lorenz, E. Brunner, *Angew. Chem.* **2003**, *115*, 5350 – 5353; *Angew. Chem. Int. Ed.* **2003**, *42*, 5192 – 5195.

[9] E. V. Armbrust, J. A. Berges, C. Bowler, B. R. Green, D. Martinez, N. H. Putnam, S. Zhou, A. E. Allen, K. E. Apt, M. Bechner, M. A. Brzezinski, B. K. Chaal, A. Chiovitti, A. K. Davis, M. S. Demarest, J. C. Detter, T. Glavina, D. Goodstein, M. Z. Hadi, U. Hellsten, M. Hildebrand, B. D. Jenkins, J. Jurka, V. V. Kapitonov, N. Kröger, W. W. Lau, T. W. Lane, F. W. Larimer, J. C. Lippmeier, S. Lucas, M. Medina, A. Montsant, M. Obornik, M. S. Parker, B. Palenik, G. J. Pazour, P. M. Richardson, T. A. Rynearson, M. A. Saito, D. C. Schwartz, K. Thamatrakoln, K. Valentin, A. Vardi, F. P. Wilkerson, D. S. Rokhsar, *Science* **2004**, *306*, 79 – 86.

[10] M. Hildebrand, S. Kim, D. Shi, K. Scott, S. Subramaniam, *J. Struct. Biol.* **2009**, *166*, 316 – 328.

[11] M. Hildebrand, G. Holton, D. C. Joy, M. J. Doktycz, D. P. Allison, *J. Microsc.* **2009**, *235*, 172 – 187.

[12] a) J. L. Arias, M. S. Fernández, *Chem. Rev.* **2008**, *108*, 4475 – 4482 and references therein; b) I. M. Weiss, V. Schönitzer, *J. Struct. Biol.* **2006**, *153*, 264 – 277.

[13] a) J. McLachlan, A. G. McInnes, M. Falk, *Can. J. Botany* **1965**, *43*, 707 – 713; b) M. Falk, D. G. Smith, J. McLachlan, A. G. McInnes, *Can. J. Chem.* **1966**, *44*, 2269 – 2281; c) J. Blackwell, K. D. Parker, K. M. Rudall, *J. Mol. Biol.* **1967**, *28*, 383 – 385; d) W. Herth, W. Barthlott, *J. Ultrastruct. Res.* **1979**, *68*, 6 – 15.

[14] C. A. Durkin, T. Mock, E. V. Armbrust, *Eukaryotic Cell* **2009**, *8*, 1038 – 1050.

[15] a) B. Tesson, S. Masse, G. Laurent, J. Maquet, J. Livage, V. Martin-Jézéquel, T. Coradin, *Anal. Bioanal. Chem.* **2008**, *390*, 1889 – 1898; b) E. Brunner, K. Lutz in *Handbook of Biomineralisation* (Eds: P. Behrens, E. Baeuerlein), Wiley-VCH, Weinheim, **2007**, pp. 19 – 38.

[16] S. F. Tanner, H. Chanzy, M. Vincendon, J. C. Roux, F. Gaill, *Macromolecules* **1990**, *23*, 3576 – 3583.

[17] A. Webster, P. O. Osifo, H. W. J. P. Neomagus, D. M. Grant, *Solid State Nucl. Magn. Reson.* **2006**, *30*, 150 – 161.

[18] a) K. M. Rudall, W. Kenchington, *Biol. Rev.* **1973**, *49*, 597 – 636; b) J. Schaefer, K. J. Kramer, J. R. Garbow, G. S. Jacob, E. O. Stejskal, T. L. Hopkins, R. D. Speirs, *Science* **1987**, *235*, 1200 – 1204; c) J. F. V. Vincent, U. G. K. Wegst, *Arthropod Struct. Dev.* **2004**, *33*, 187 – 199; V. A. Iconomidou, J. H. Willis, S. J. Hamodrakas, *Insect Biochem. Mol. Biol.* **2005**, *35*, 553 – 560; I. M. Weiss, S. Kaufmann, B. Heiland, M. Tanaka, *J. Struct. Biol.* **2009**, *167*, 68 – 75.

[19] L. G. Frigeri, T. R. Radabaugh, P. A. Haynes, M. Hildebrand, *Mol. Cell. Proteomics* **2006**, *5*, 182 – 193.

[20] a) E. Brunner, H. Ehrlich, P. Schupp, R. Hedrich, S. Hunoldt, M. Kammer, S. Machill, S. Paasch, V. V. Bazhenov, D. V. Kurek, T. Arnold, S. Brockmann, M. Ruhnow, R. Born, *J. Struct. Biol.* **2009**, *168*, 539 – 547; b) H. Ehrlich, M. Krautter. , T. Hanke, P. Simon, C. Knieb, S. Heinemann, H. Worch, *J. Exp. Zool. Part B* **2007**, *308*, 473 – 483.

[21] L. Heux, J. Brugnerotto, J. Desbières, M.-F. Versali, M. Rinaudo, *Biomacromolecules* **2000**, *1*, 746 – 751.

[22] W. Herth, A. Kuppel, E. Schnepf, *J. Cell Biol.* **1977**, *73*, 311 – 321.

[23] P. J. Harrison, R. E. Waters, F. J. R. Taylor, *J. Phycol.* **1980**, *16*, 28 – 35. (http://www3.botany.ubc.ca/cccm/NEPCC/esaw.html).

[24] a) A. Pines, M. G. Gibby, J. S. Waugh, *J. Chem. Phys.* **1973**, *59*, 569 – 590; b) G. Metz, X. Wu, S. O. Smith, *J. Magn. Reson. Ser. A* **1994**, *110*, 219 – 227.

[25] B. M. Fung, A. K. Khitrin, K. Ermolaev, *J. Magn. Reson.* **2000**, *142*, 97 – 101.

