Submitted to JPorousMater (Manuscript)

Diatom frustules as a biomaterial: Effects of chemical treatment on organic material removal and mechanical properties in cleaned frustules from two *Coscinodiscus* species

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Acknowledgments

The Research Council of Norway is acknowledged for financial support through the SOLBIOPTA project (Contract #10358700). Egil S. Erichsen, Laboratory for Electron Microscopy, University of Bergen is acknowledged for his help in SEM/EDS analysis.

Abstract

The three-dimensional structure of silica diatom frustules offers a great potential as nanoporous material for several nanotechnological applications, but the starting point for these applications is the ability to obtain clean frustules with sufficient mechanical strength and intact structure. Here, frustules from the diatoms *Coscinodiscus centralis* Ehrenberg and *C. wailesii* Gran et Angst are characterized with respect to their structural integrity, content of residual organic biomaterial and their mechanical properties after two cleaning methods using either hydrogen peroxide as oxidizing agent or a combination of a surfactant (sodium dodecyl sulphate) and a complexing agent. Fluorescence microscopy and energy dispersive spectroscopy (SEM/EDS) analysis revealed clear differences regarding the amount of organic residual within the frustules depending on the cleaning process, with little organic material left after the oxidizing method. This method, however, induced a partial cracking of the frustules suggesting an embrittlement due to the cleaning. Nanoindentation confirmed this and showed that the oxidizing method resulted in more brittle frustules compared to the surfactant/complexing method. More efficient cleaning of organic biomaterial may result in more fragile frustules, and the choice of cleaning method must be based on the planned application.

Keywords: porous biomaterial, nanomaterial, silica frustules, cleaning protocols, oxidation, surfactant

1 Introduction

Diatoms are among the most abundant species on earth and the ability of these organisms to build small-scale complex silica-based frustule structures have attracted considerable scientific interest over the recent years [1], [2], [3], [4]. The three-dimensional nanoporous structure of diatom frustules consists mainly of silica nanoparticles, and the architecture – with layers and different sized pores – gives rise to several interesting material applications, *e.g.* nano- to micrometer scaled sieves[5] or drug carriers[6], [7] as well as vectors for drug delivery[8], protein adsorbents [9] and as photocatalysts [10]. Diatom frustules also show particular optical properties [11], [12], [13], [14], [15], [16], [17] making them interesting for several optical applications [18]. Furthermore, the biosilica can also be used as a powder to reinforce other composites [19]. In all applications the material properties depend on cleaning and removal of organic material.

An organic matrix envelopes the frustules *in vivo* [20] and the cleaning protocol must handle a variety of compounds: amino acid derivatives [21], [22], long-chain polyamines (LCPAs)[23], chitin[24] and polysaccharides [25]. Some of the organic molecules are enclosed within the biosilica in a phase separation process [26]. For material applications as well as for morphological studies of diatom frustules, controlled cleaning of the frustule is necessary to ascertain removal of the organic material. Removal of different compounds from the frustules and the cell within must take into consideration everything from easily extractable carbohydrates (which can be extracted simply using warm water) to silaffins or pleuralins embedded within the biosilica, which may not be accessible until the biosilica is dissolved using e.g HF or KOH. Controlled cleaning is therefore necessary for characterization and evaluation of the material, and for functionalization or tailoring of the surface such as in e.g. dye sensitized solar cells [27] or functionalized biomaterials [28]. The cleaning also affects the

material *per se*, and may induce brittleness, fragmentation and loss of strength. Harsh cleaning may also affect the pore structure and thereby modulate the structural properties [29]. Cleaning protocols dedicated to obtain intact frustules are based on different chemical treatments with e.g. acids, bleaching, oxidising or denaturation functions from different chemicals, and thermal treatment. There are few systematic studies with comparisons of different cleaning methods [30], [31], [32], [33], [34]. Here, a comparative study of two frustule cleaning methods is presented, using carefully described cleaning protocols based on established cleaning principles [35]. Hydrogen peroxide (H_2O_2) combined with hydrochloric acid (HCl) was used as a strong oxidizing agent in the first cleaning method, whereas the second method was a milder surfactant based cleaning method involving sodium dodecyl sulphate (SDS) combined with the complexing agent ethylene diaminetetraacetic acid (EDTA). A qualitative characterization of the structural integrity of the frustules, amounts of remaining organic material and mechanical properties of cleaned frustules was done using phase contrast optical microscopy, epi-fluorescence microscopy, scanning electron microscopy (SEM) including elemental analysis by energy dispersive spectroscopy (EDS), and nanoindentation.

2 Materials and methods

2.1 Selection of diatoms

Two different species of the diatom genus *Coscinodiscus* were chosen mainly because of their large frustule size (around 150 µm in diameter) and their promising potential as porous material for several applications. Because of the wide variety of frustule designs among diatom species it may be difficult to suggest generalized cleaning protocols. However, the large size and rather complex frustule structure of *Coscinodiscus* frustules (with loculate chambers of the areola) pose a challenge, and any cleaning protocol that is efficient here is

expected to be efficient also for other species. *Coscinodiscus centralis* Ehrenberg was collected by net haul in the Trondheim fjord (Norway), whereas *Coscinodiscus wailesii* Gran et Angst was grown in monocultures. *C. wailesii* was cultivated in monocultures using f/2 medium with Si [36] and standard laboratory conditions (100 mmol m-2 s-1 light, temperature: 20 °C) in cell cultivation flasks.

2.2 Cleaning protocols

The two different cleaning methods followed the same step-by-step progression. In the first step of the cleaning, diatom material was washed three times with Milli-Q water (0.133 g frustules·L^{-1}) to remove salt, centrifuged at 4500 rpm for 10 min between each washing and dried overnight at 60 °C. The second step was defined by the cleaning method used. For the first method, termed H (=Hard), a dispersion consisting of dried diatom material (2 mg) in hydrogen peroxide solution (10 mL 30 % H_2O_2) was stirred at 90 °C for 24 h. Thereafter, a HCl solution (37 %, 1 mL) was added, and the sample was centrifuged at 4500 rpm for 10 min. For the second method, termed S (=Soft), a dispersion consisting of dried material (2 mg) in 4 mL of sodium dodecyl sulfate (SDS)/ethylene diaminetetraacetic acid (EDTA) solution (50 g·L^{-1} SDS in 100 mM Titriplex III) at pH 5 was prepared, vortexed for 1 min, left still for 20 min, and centrifuged at 4500 rpm for 10 min. This step was repeated six times. After this method-specific step, all samples were washed and centrifuged (4500 rpm, 10 min) three times and stored in ethanol (96 %) at -20 °C. For convenience, the frustules cleaned using methods H and S are called H-frustules and S-frustules, respectively.

2.3 SEM imaging

For imaging, the *C. wailesii* frustules were collected on 1 µm pore size polycarbonate filters. SEM images were acquired using a Zeiss Ultra 55 FE-SEM, and the SEM/EDS analysis was performed using a Zeiss Supra 55VP scanning electron microscope equipped with a Thermo Electron Cooperation Noran Six X-ray microanalysis system. Light microscopy images (transmission and epi-fluorescence) were acquired using a Zeiss Axio Imager Z2 optical microscope. Transmission light microscopy pictures were taken using a dark field configuration. In epi-fluorescence mode, excitation light was 475 ± 15 nm and emitted fluorescence was observed in the 500-550 nm range.

2.4 Nanoindentation

For the nanoindentation, cleaned *C. centralis* and *C. wailesii* frustules were deposited on a glass slide and only individual valves showing their internal side (*foramen*) in the upwards direction were selected. Nano-indentation was performed using a Hysitron TI950 Triboindenter equipped with a 90° cube corner probe (radius ~ 100 nm) and set to apply a maximum force of 200 µN. For reproducibility, the indent was placed at the centre of the valve, avoiding areas in the proximity of pore edges.

3 Results and discussion

The diatom frustule can be regarded as a composite biosilica structure, with organic material both around and within the biosilica. More effective removal of organic material from the frustules (such as with acids or oxidizing agents) is expected to induce stiffness in the biomaterial, whereas the gentler SDS cleaning should render a more elastic biomaterial. On

the other hand, more effective cleaning than the one obtained using SDS may be crucial for e.g. surface functionalization. Here a strong oxidizing agent, H_2O_2, was compared to a milder surfactant/chelating based cleaning method with SDS/EDTA.

3.1 Microscopy

Different biosilica layers with varying pore sizes from *C. wailesii* valves is illustrated in Fig. 1 a. Both valves are made of two nanoporous layers separated by a honeycomb structure of vertical walls. The two valves of the frustules are joined together by girdle bands (Fig. 1 b). The images shown in Fig. 2 allow comparison of both the visual aspect of frustules and the intensity of the fluorescence (residual organic matter) after treatment with the two cleaning methods. The SEM images (Fig. 2 H1, S1) show the structural integrity of the H- and S-frustules in detail, whereas the transmission microscopy image (Fig. 2 H2, S2) and the epi-fluorescence picture (Fig. 2 H3, S3) of the same area visualize the amount of organic material present on the frustules. The results presented in Fig. 2 are representative data from one out of several cleaning experiments done with both species, all of these giving very similar results.

The *C. wailesii* H-frustules were fully disassembled, *i.e.* the valves were separated from each other and the girdle bands were detached from the valves (Fig. 2 H1). Reactive hydroxyl radicals formed from H_2O_2 is relatively non-selective, and leads to fragmentation of organic molecules. It seems to efficiently attack also the biomolecules which are involved in structural maintenance of the frustule, leading to dissembling of the frustule. Mechanical damages in some valves and girdle bands can be due to collisions between the frustules during the cleaning process as well as the forces during the centrifugation. The very weak fluorescence signal shows that cleaning method H left little fluorescent material from both protoplast and with the biosilica (Fig. 2 H3). Many organic molecules show fluorescent properties; green

auto fluorescence possibly from flavins [37] and carotenoids [38] has been observed in microalgae. Weak, red auto fluorescence has been observed from chitin exoskeletons in insects [39]. Polysaccharides such as chitin [40], [41], callose [25] and LPCAs [23] are associated with frustule construction, and some are even integrated into the biosilica structure during formation. Degradation products of various organic compounds are often determined using fluorometric analyses: Chlorophyll easily degrades to pheophytin in contact with acids or increasing temperature, and pheophytin shows red fluorescens [42]. Peroxidation of membrane lipids has been observed using fluorescence detection [43], and Dasu et al. [44] studied enzymatic polyamine catabolism using HPLC with fluorescence detection. Absence of fluorescence is therefore an indicator of efficient removal of organics.

The *C. wailesii* S-frustules, on the other hand, appear to be well preserved after the SDS/EDTA cleaning treatment. Valves without observable cracking were observed, and many S-frustules were assembled (Fig. 2 S1), i.e. the girdle bands and the valves were still joined together. The anionic surfactant SDS works on interfaces, lyzes cell membranes and unfolds proteins, and cell wall associated proteins were efficiently extracted with the complexing agent EDTA [45]. High fluorescence intensity of the S-frustules (Fig. 2 S3), however, showed that a large amount of fluorescent material remained in these frustules after cleaning. This is seen as golden brown colour (typical of diatom pigments) in the transmission light microscopy image (Fig. 2 S2). The large amount of remaining fluorescing material shows that method S is only partially removing the organic material from the cell protoplast and in/on the biosilica structure.

3.2 SEM/EDS and element analysis

Analysis by SEM/EDS of the elemental composition of *C. wailesii* frustules before and after cleaning revealed a C:Si atomic ratio of 3.4 ± 0.8 in the un-cleaned frustules, whereas the S-

and H-frustules had C:Si ratios of 1.1 and 0.1, respectively. The reduced C:Si ratio after cleaning revealed that both washing methods significantly reduced the amount of organic material in and on the surface of the frustules, and almost down to the detection limit in the case of the H-frustules. This is in accordance with the observations from light and epi-fluorescence microscopy discussed above (Fig. 2).

Furthermore, the atomic percentages of C and O detected by SEM/EDS of uncleaned frustules were 37.4 ± 4.8 and 54.7 ± 5.8, respectively. After subtraction of the oxygen signal from SiO_2 using an O:Si ratio of 2, we estimated a O:C-ratio of 1.5 ± 0.4 in the organic material associated with the frustules. The organic material could be carbohydrates [41] or lipids [46], however, the high O:C ratio excludes lipids as a likely constituent in our study. Other elements may be used to identify the sources of auto fluorescence, but here the amounts of N, P and S were close to the detection limit (0.1 %) in un-cleaned frustules, hence proteins and nucleic acids were unlikely the major constituents of the organic material associated with the frustules. It has been shown that *Thalassiosira pseudonana* frustules contained hydrocarbons, protein and lipids after a H_2O_2 treatment [35]. Furthermore, H_2O_2 treatment removed more elements from the frustules than SDS/EDTA [34], and facilitated the access to N embedded in the biosilica [33]. These observations may influence the choice of cleaning methods for frustules that are subjected to elemental analyses.

3.3 Nanoindentation

The effect of the two different cleaning protocols on the biosilica structure were analysed using nanoindentation on the central area of cleaned valves from the diatom *C. centralis* (Fig. 3 a). The force-depth curves for the H-frustules show that these frustules were much stiffer

than the S-frustules (Fig. 3 b). This is revealed by the steeper slope of the indentation curves and is consistent with the low fraction of remaining organic material in these frustules. The fact that some H-frustules appeared cracked (Fig. 2 H1) is also an indicator of their fragile structure due to the chemical treatment during the cleaning. Etching of the biosilica from H_2O_2 may have induced more brittleness in these valves. It is also clear that the variability in the stiffness of the S-frustules is larger than for the H-frustules. Because weakening of the frustules after the cleaning treatments was similar in the two species, we anticipate a similar effect for other species.

Conclusions

Two different cleaning methods for diatom frustules were compared as a basis for using clean frustules a porous biomaterial. The chemistry of the cleaning was shown to strongly affect both the amount of organic biomaterial left on the frustules and the mechanical properties of the frustules. A mild surfactant based cleaning method using sodium dodecyl sulphate (SDS) combined with ethylene diaminetetraacetic acid (EDTA) resulted in inefficient removal of organic biomaterial on the frustules, whereas the rougher cleaning based on hydrogen peroxide (H_2O_2) combined with hydrochloric acid (HCl) as a strong oxidizing agent removed most of the organic biomaterial on the frustules. The rougher cleaning treatment gave brittle frustules with a higher stiffness, and resulted in partial fracture during mechanical treatment. The softer cleaning gave more preserved frustules with a lower stiffness. Consequently, the choice of cleaning method is a trade-off between different final characteristics of the frustules and thus application dependent; More efficient removal of organic biomaterial within and on diatom frustules may result in more fragile frustules, whereas milder cleaning leaves a larger amount of organic material with more preserved frustules as a consequence.

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Figures

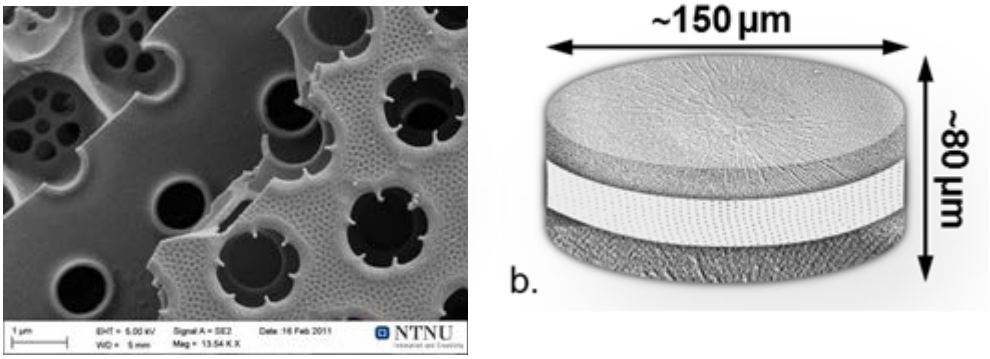

Figure 1. (a) A view through several layers in valves from *C. wailesii*, displaying different pore sizes and structural complexity; (b) a schematic representation of a whole *C. wailesii* frustule.

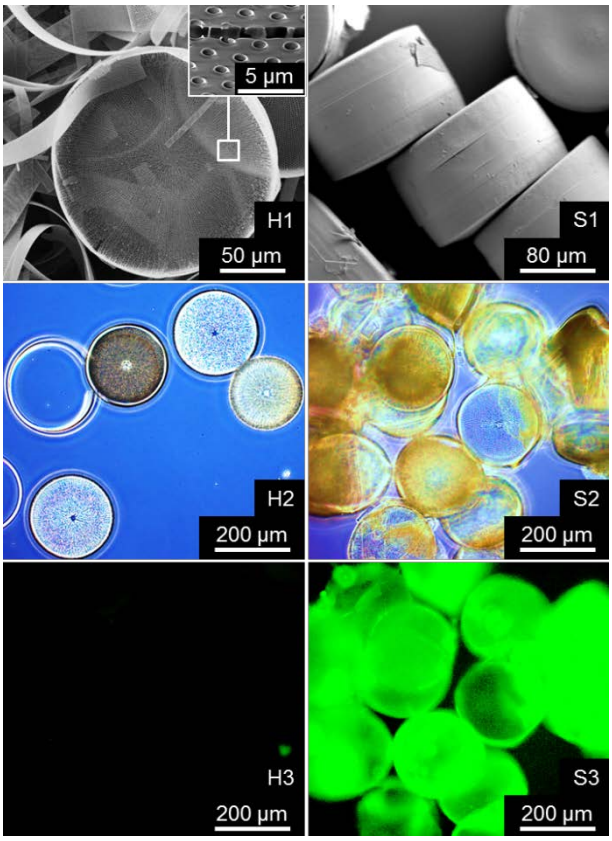

Figure 2. SEM pictures (H1, S1), transmission light microscopy pictures (H2, S2) and epi-fluorescence pictures (H3, S3) of *Coscinodiscus wailesii* diatom frustules after cleaning using the methods H (H1, H2, H3) and S (S1, S2, S3). Notice that H2/H3 and S2/S3 are the same field of view.

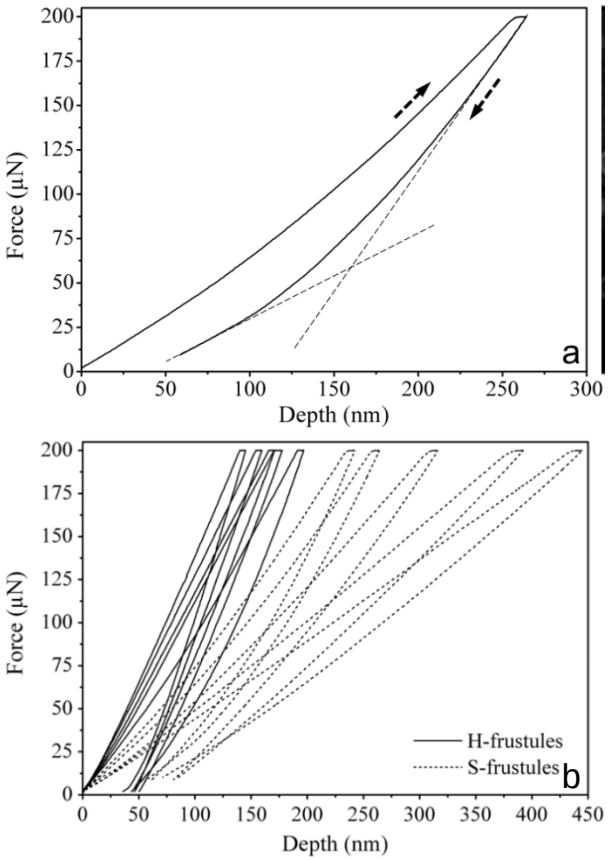

Figure 3. (a) Typical force-depth curve showing both loading and unloading parts (dashed arrows) obtained from the nanoindentation of a *Coscinodiscus centralis* S-frustule valve; (b) Typical force-depth curves (5 for each cleaning method) obtained from the nanoindentation of several H-frustule (solid lines) and S-frustule (dotted lines) valves.