A self-propagating system for Ge incorporation into nanostructured silica†

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Technologically-relevant levels of Ge can be incorporated into cell wall silica of the diatom *Thalassiosira pseudonana* with no aberration in structure at low levels, whereas higher levels alter structure.

The eukaryotic algae known as diatoms populate a broad range of oceanic and freshwater environments. A characteristic feature of these single-celled organisms is a rigid, three-dimensionally (3-D) structured cell wall (the frustule) composed largely, but not entirely, of amorphous nanostructured silica (Fig. 1).[1,2] Estimates of the number of diatom species are in the tens of thousands, each having a unique silica structure.[1,3] Frustule morphology is genetically encoded and, within a given species, is reproduced with fidelity from generation to generation. Diatoms are amenable to being cultured in a laboratory setting, as they are typically photosynthetic and require minimal supplementation. The relatively rapid growth rate of diatoms allows high cell densities to be achieved in cultures, yielding large quantities of biogenic silica for experimental investigations.

Diatoms accumulate trace elements in their silica, including Ge, from the surrounding aqueous environment. Previous measurements of trace elements in diatom cell wall silica indicate a parts-per-million, and in some cases parts-per-thousand range of incorporation (Table 1); a substantial enrichment over the generally parts-per-trillion concentrations in seawater. Presumably, the enrichment is due to the fact that deposition of the trace element in an insoluble form acts as a thermodynamic sink for uptake of its soluble form into the diatom cell. Most measurements of trace element incorporation in diatom silica have been conducted on diatom-rich sediments (Table 1 and references therein). Although diatoms are the major constituents of these sediments, and rigorous techniques and convincing controls are applied to ensure removal of adsorbed elements from the surface of the diatom silica,[4–7] these sediments are not part of a controlled experimental system, and there is the possibility of repeated dissolution/polymerization of sedimentary silica, which could entrap trace elements at levels not found in the living organism. Another consideration is that the amount of trace element available for incorporation is limited by oceanic concentrations of that element. One way to thermodynamically favor increased incorporation of a trace element into diatom silica is to increase its availability in soluble form.

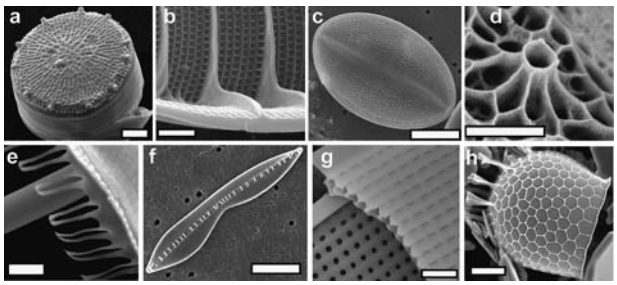

Fig. 1 Diversity of diatom silica structures. Acid-cleaned material from; (a) *Thalassiosira pseudonana*, bar = 1 μm, (b) unknown species, bar = 2 μm (c) *Cocconeis* sp., bar = 10 μm (d) rimoportula from *Thalassiosira weissflogii*, bar = 500 nm (e) corona structure of *Ditylum brightwellii*, bar = 2 μm (f) *Bacilaria paxillifer*, bar = 10 μm (g) close-up of pores in *Gyrosigma balticum*, bar = 2 μm (h) *Stephanopyxis turris*, bar = 10 μm.

We investigated whether increased availability of soluble Ge(IV) increased its incorporation into the cell wall silica of the diatom *Thalassiosira pseudonana* under controlled growth conditions. The accumulation of Ge(IV) was investigated specifically because of its technological relevance and its previously demonstrated presence in diatom silica. Hydrated forms of Ge(IV) have reasonable solubility, and are naturally transported into diatom cells. Diatom silicic acid transporters preferentially transport $Si(OH)_4$, but also $Ge(OH)_4$ with lower affinity.[9] Here we demonstrate through controlled growth experiments that incorporation of GeO_2 into the *T. pseudonana* silica cell wall can be increased far above base levels, and that maximal GeO_2

Table 1 Selected trace element concentrations in diatom silica

Element	Concn (ppm)	Comments	Ref.
Al	5840	Sediments	4
	20–16 000	Oceanic collection	8
Fe	3780	Sediments	4
	185–2800	Oceanic collection	8
Ge	925	Lab culture radiotracer (calculated)	9
	0.63	Sediments	5
Cu	2–80	Oceanic collection	8
Zn	271	Laboratory culture	10
	1–120	Oceanic collection	8
	17.2	Sediments	4
	4.6	Sediments	6
	2.17–7.34	Sediments	7

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incorporation occurs at concentrations of Ge(IV) in the growth medium that do not limit cell growth nor alter silica structure.

A systematic survey comparing the relationship between the availability of soluble Ge(IV) in the growth medium and incorporation of Ge into silica components of the diatom *Thalassiosira pseudonana* (Hustedt) Hasle et Heimdal clone CCMP1335 was performed (ESI†). Ge(IV) was added to cultures as $Ge(OH)_4$. Because $Ge(OH)_4$ and $Si(OH)_4$ are competitive inhibitors for their uptake into the cell,[9] cultures were established with a fixed amount of silicic acid (100 μmol L^{-1}) and varying amounts of germanic acid (0–5.2 μmol L^{-1}) to generate ratios of $Ge(OH)_4$: $Si(OH)_4$ of 0, 1 : 100, 1 : 60, 1 : 40, and 1 : 20. Cultures were inoculated at approximately 3×10^5 cells mL^{-1} and allowed to grow for 2 days. Cells were harvested by centrifugation and frustules were acid cleaned to remove organics. Cleaned frustules were dissolved with HF, dried under vacuum, and resuspended in 1% HNO_3. Aliquots were examined in triplicate using ICP-OES to measure Ge concentration. Silicic acid measurements were performed in triplicate on aliquots of acid cleaned frustules using the molybdate method of Strickland and Parsons,[11] but scaled down (ESI†).

Maximal Ge incorporation occurred at 1.04 μmol L^{-1}, and substantial incorporation at 1.73 and 2.6 μmol L^{-1}, dropping significantly at 5.2 μmol L^{-1} (Fig. 2a). Up to 0.42 mol% Ge relative to silica was incorporated (Fig. 2a). Addition of $Ge(OH)_4$ inhibited silica production (Fig. 2b), in close approximation to cell culture density (Fig. 2c).

Two concerns after completing this experiment were (1) whether lower concentrations of $Ge(OH)_4$ than 1.04 μmol L^{-1} would result in greater incorporation, and (2) whether any $Ge(OH)_4$ that could either be non-specifically associated with the cells or carried over as an extracellular precipitate contributed to the measurements. These concerns were addressed in a second experiment that followed a similar protocol to the first, except that 0.52 μmol L^{-1} was tested (a 1 : 200 $Ge(OH)_4$: $Si(OH)_4$ ratio), and a killed cell control for monitoring non-specific association was evaluated. The latter entailed harvesting cells from an untreated culture, and subjecting the cells to repeated freeze–thaw cycles to render them inviable, then inoculating a flask with these cells and 5.2 μmol L^{-1} $Ge(OH)_4$ and incubating for the same period of time the other cultures had been incubated. This sample was then processed using the same procedure as for the others. Results are shown in Fig. 2d, indicating that maximum $Ge(OH)_4$ incorporation still occurred at 1.04 μmol L^{-1}, and that non-specifically-associated Ge was negligible. Maximum incorporation was more than 500-fold higher than base levels, resulting in nearly 0.4 mol% Ge : Si (Fig. 2d). A recent report demonstrated a similar maximum Ge : Si incorporation ratio in a freshwater diatom.[12]

Scanning electron microscopy (SEM) was performed on *T. pseudonana* cell wall material that had been isolated and cleaned after $Ge(OH)_4$ treatment (ESI†). SEM results demonstrated that 1.04 μmol L^{-1} produced no effect on silica structure compared with controls, whereas 5.2 μmol L^{-1} had a substantial inhibiting effect on structure formation (Fig. 3). This included more pronounced rib structures, gaps in silicification between ribs, and malformation of the outer pores called rimoportulae (Fig. 3).

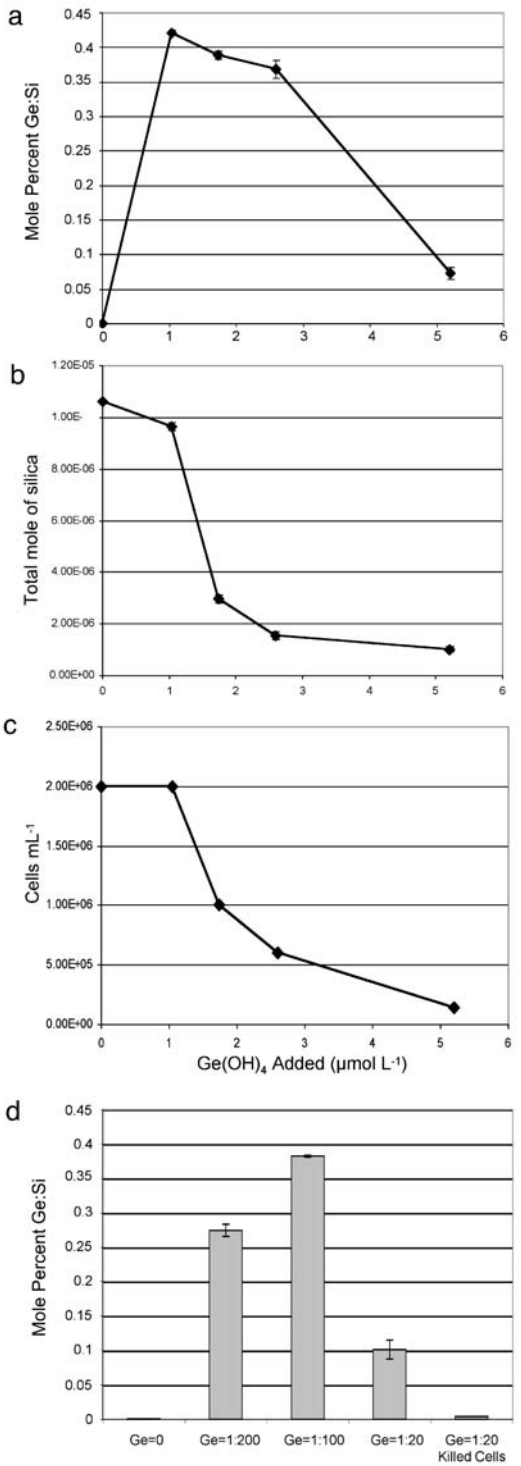

Fig. 2 (a) Mole percent Ge : Si incorporation relative to the concentration of $Ge(OH)_4$ added to the culture medium. (b) Total production of silica after two days' growth in the presence of different concentrations of $Ge(OH)_4$. (c) Cell density in the culture after two days' growth in the presence of various concentrations of $Ge(OH)_4$. (d) Mole percent Ge : Si incorporation into *T. pseudonana* silica after two days' growth. Ratio of Ge : Si is shown, along with a killed cell control sample. Samples in a, b, and d were measured in triplicate, and in c, in duplicate. Error bars are SD.

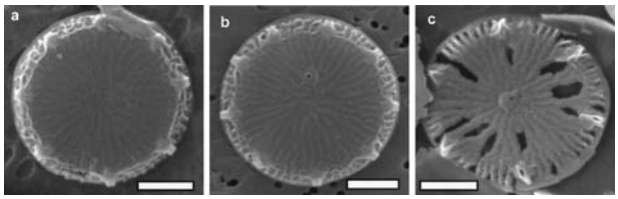

Fig. 3 SEM images of valve of *T. pseudonana*. (a) untreated control, (b) treated with 1 : 100 ratio of Ge : Si, (c) treated with 1 : 20 ratio of Ge : Si.

The mechanism of inhibition of silica incorporation by Ge is not entirely clear, and details are not documented in the literature. The data presented in Fig. 3 suggest that incorporation is directly inhibited. Around the periphery of the valve of *T. pseudonana* are large pore structures called rimoportulae, which are grossly misshapen under the highest Ge treatment (Fig. 3c), consistent with an effect on their formation process. The portulae are formed by circularization of a tube-like silica structure,[13] and weakening of that structure by inhibition of silica formation by Ge could lead to a collapse of the tube, as is seen in Fig. 3c. Similarly, the rib structures that form the body of the valve are propagated radially out from the center during formation,[13] which is consistent with the pattern of the holes in the valves observed in Fig. 3c. The holes occasionally become closed nearer to the valve rim due to later branching by the ribs. GeO_2 forms both tetrahedral and hexagonal coordinates, and the hexagonal form is predominantly precipitated from solution.[14] One possible mechanism of Ge inhibition of silica polymerization could be the accumulation of hexagonal coordinates of GeO_2, which could disrupt formation of tetrahedrally coordinated SiO_2.

The continuously propagating method performed in flasks is distinct from previous attempts to incorporate GeO_2 into diatom silica.[15,16] In these methods, cells were starved for silicic acid prior to treatments with various concentrations of $Ge(OH)_4$, and incubations were performed in bioreactors.[15,16] Despite differences in experimental setup we demonstrate maximum germanium incorporation levels on the same order as those reported previously[16] and our results agree that cells are not viable at high Ge(IV) concentrations (Fig. 2). In contrast to previous reports, our results show no silica structure aberrations in cells grown at a Ge : Si ratio that resulted in maximal germanium incorporation (Fig. 2 and 3). However, silica structure was compromised at levels of Ge that halted cell division in *T. pseudonana*. The continuous growth system described here is accomplished with a simpler experimental setup and yields comparable results.

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