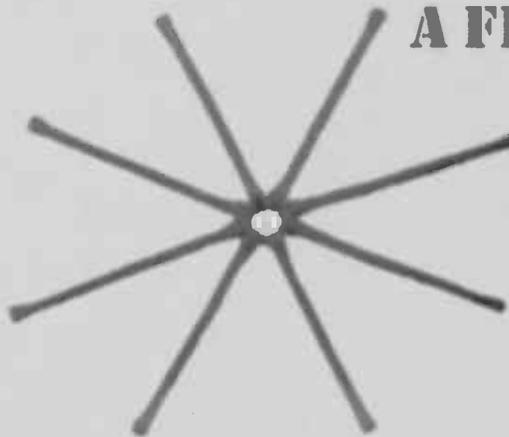


THE ART OF BUILDING A FRUSTULE

By Johanneke Oosten
Supervised by Dr. W.W.C. Gieskes

Werkgroep Mariene Biologie
Rijksuniversiteit Groningen
Biologisch Centrum
Postbus 14
9750 AA Haren

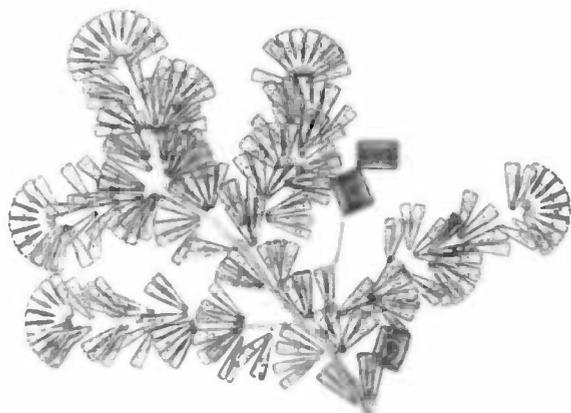


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Diatoms from the genus *Licmophora*. They are epiphytes that can grow on algae or rock. The individuals grow like fans from a common stalk.

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DIATOMS & NANO- TECHNOLOGY

Scientists have been fascinated by diatom shells ever since the first one was discovered under a microscope. Until recently, this fascination was the main motive for studying this unicellular creature. Then some scientists suggested that biomimicking the genesis of the creature's shell, in lingo referred to as the 'frustule', could have many useful applications.

Many artificial products, such as filter agents, washing powders and even car tires, require a component of silicon structures. These structures need to be ordered at a nanometre scale. The current industrial production methods have many disadvantages. They require high temperatures, increased pressure, and extremely acid conditions. Three dimensional objects have to be built up plane by two-dimensional plane. Designing the desired patterning is difficult and expensive^{30,49}.

Diatom shells consist of silica. Their shapes vary greatly. So does their patterning, which is more detailed than anything the nano-industry can manufacture. Both shape and patterning are species-specific. These characteristics form the basis for diatom systematics. Considering that there are at least twelve thousand diatom species, there is a huge variety in morphologies (*figure 1*). The frustules' many potentials gave diatom research a huge boost, and much of this research was directed at how the creature builds its frustule. Can this be imitated?

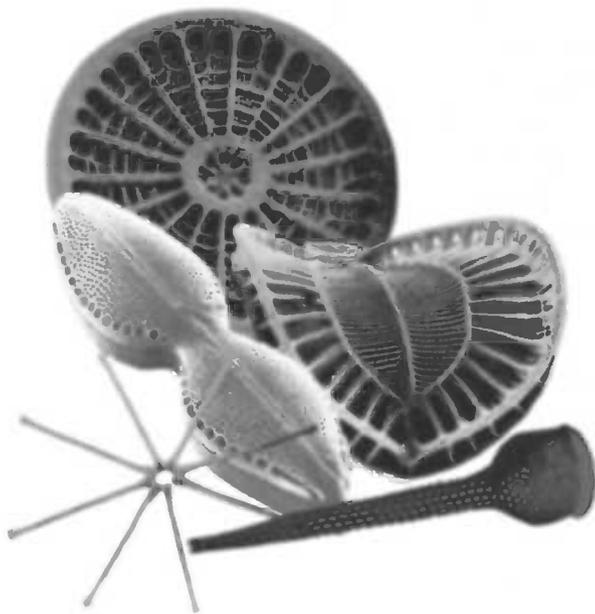


Figure 1: Some frustules:

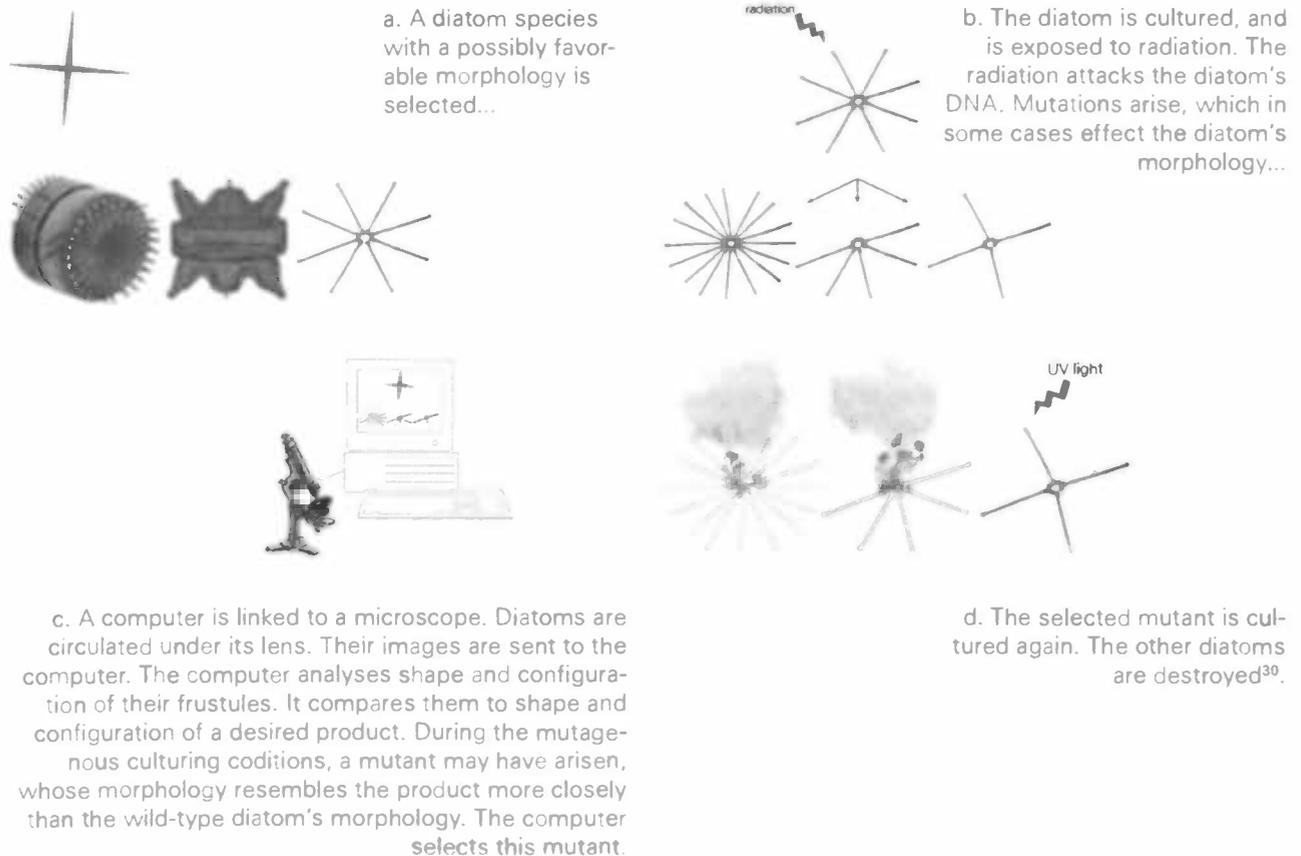
- | | |
|------------------------|--------------------------|
| 1: <i>Pyxilla</i> | 4: <i>Campylodiscus</i> |
| 2: <i>Asterionella</i> | 5: <i>Arachnoidiscus</i> |
| 3: <i>Dictyoneis</i> | |

In the past, scientists have explored potential uses of *diatomaceous earth*. This 'earth' consists of dead diatoms' shells sunken to the ocean's bottom. Millions of years passed before these geological deposits reached substantial amounts. Over such time scales most of the shells' attractive structure and patterning gets lost, the silica is no longer pure and it is easier and cheaper to produce silicon structures artificially⁴⁹. Of course, fresh diatom frustules are another option. Their owners make them at moderate temperatures and physiological pH. If scientists could manipu-

late the frustule morphology under these mild conditions, this would make silica production far more flexible. Biomimicking the process of diatom silica mineralization could be the key to industrial success!

To adjust a diatom shell's shape and patterning to a product's requirements, one could use a *compustat* (figure 2). A compustat is a computer capable of comparing a shell's shape to a desired shape. If the similarity is not satisfactory, the diatom is destroyed³⁰.

Figure 2: compustat

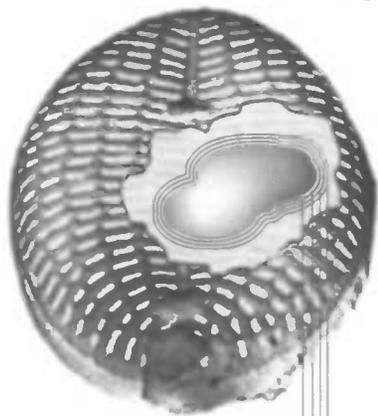


THE RISE OF THE DIATOMS

The oldest diatom fossils were dated back 120 million years³⁵. These fossils were centric forms. The pennate form first appeared 70 million years ago⁴⁷. This would mean that the pennates descend from a centric form. Phylogenetic data agrees with this^{12,27}. Phylogenetic data even suggest that diatoms are 250 million years old²⁷. Why are there no fossils of the first 130 million years? Silicified shells are usually well preserved. Other genera, like certain sponges, also contain silicified parts. Some of these fossils date back 580 million years ago!²⁵. Perhaps diatom fossils became lost due to some unknown environmental cause. But a more likely explanation is that diatoms have been silicified for only half the time of their exist-

ence³⁵. What then made diatoms incorporate silica into their walls? Some data suggests that 120 million years ago, the ocean was supersaturated with Si(OH)_3^1 . Under these circumstances, silica polymerizes of its own accord. The diatom would not need a system to concentrate the silica before incorporating it into the cell wall³⁸. It has also been suggested that a silica wall is energetically cheaper than a carbon one³⁶. The present-days's sea is definitely not supersaturated with silica⁴⁵, but over the centuries diatoms have developed expert techniques to collect it from their environment.

Diatoms came into existence by an endosymbiotic event between a red eukaryotic alga



Plasma membranes of:
 prokaryotic endosymbiont
 prokaryotic host
 (1st endosymbiotic event)
 red alga
 flagellate
 (2nd endosymbiotic event)

figure 3: four membranes

and a heterotrophic flagellate²⁷. They have four membranes around their plastids. The inner two originate from 'The primary endosymbiotic event' between two prokaryotic organisms, of which one became the chloroplast of the other. The third membrane is what remains of the red alga's plasma membrane and the fourth was built by the hosting flagellate: it is continuous with the diatom's endoplasmic reticulum (figure 3)⁵⁴.

The genome of *Thalassiosira pseudonana* was recently sequenced. It was compared to the genomes of three organisms: a mouse, a red alga and a green plant. Half of *T.pseudonana*'s protein repertoire resembled proteins of these three organisms: 806 matched only the mouse's proteins, 182 matched only the red alga's proteins, and 865 matched only the green plant's proteins. The other half was unlike any protein these three organisms possessed. These findings confirm that the divergence of the diatom's ancestor and the ancestors of the mouse, red alga and plant is ancient. Also, the diatom is as much or as little an animal as it is an alga.

CREATING A VALVE

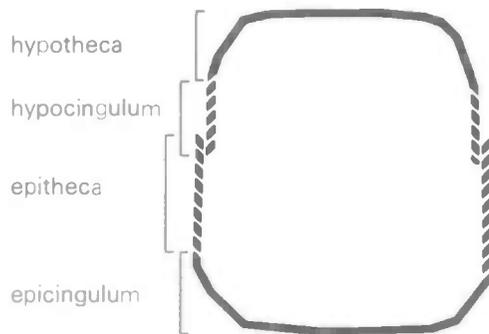


figure 4

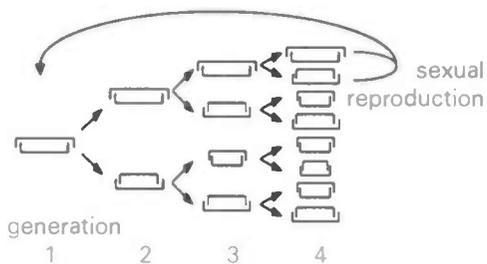


figure 5³⁰

The name *Diatom* is derived from the Greek words: *duo* and *atomos*, which mean 'two' and 'indivisible' respectively. The name refers to the two valves, which together constitute the diatom's silicified wall. One valve is usually smaller. This one is called the *hypotheca*; the other, larger one is called the *epitheca*. Attached to the thecas' edges are the *hypocingulum* and *epicingulum*, also referred to as the *girdle bands*. The two sets of girdle bands are fused together. Epitheca, hypotheca and girdle bands together form the *frustule*³² (figure 4).

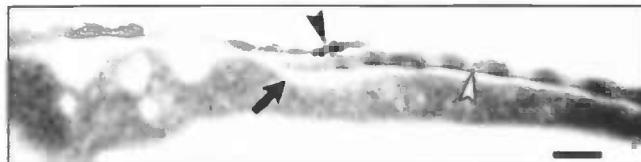
When a diatom divides, one daughter cell inherits its mother's epitheca and produces a new hypotheca. The other daughter cell inherits the hypotheca. This hypotheca will become the daughter cell's epitheca and she will complement it by creating her own hypotheca. This means that one daughter cell is the same size as the mother; one daughter cell is smaller than the mother. In other words: the average cell size in a diatom population decreases every generation! To make up for this size loss, diatoms reproduce sexually: sexual offspring is larger³⁷ (figure 5).

figure 6³⁷: schematic representation of a dividing diatom.

The diatom grows by adding girdle band to its hypotheca

The cytoplasm divides

Inside the mother's walls the daughter cells make new valves



- ▶ epicingulum
- ▷ hypocingulum
- plasma membrane



figure 7¹⁹: Electron Microscopy image of *C.fusiformis*' pleural bands. The red dots are gold-labeled pleuralins.

Before making new wall elements, the cytoplasm divides. The DNA is duplicated, a membrane cuts the cell in half, the mother's organelles are redistributed to both compartments. What was once one individual are now two, in every aspect, except for the cell wall. The daughter cells share their mother's walls. They build their hypovalve inside these wall, close to where the mother's cytoplasm divided (figure 6).

There are two important differences between the hypotheca and the epitheca.

- A mother cell's hypotheca can differentiate into a daughter cell's epitheca. An epitheca however, can never turn back into an hypotheca.
- An hypotheca grows by the continuous addition of newly formed girdle bands. Once the hypotheca becomes an epitheca, the addition of girdle bands stops and never continues³⁷.

Little is known about the transition of hypotheca into epitheca. Cationic proteins called *pleuralins*, seem involved. These proteins are present only in the *pleural bands* of the epitheca¹⁹. Pleural bands are the parts where the epitheca overlaps the hypotheca (figure 7).

During the transformation of hypotheca into epitheca, pleuralins appear in the pleural bands! Perhaps the conversion of hypo- into epitheca attracts pleuralins, or perhaps the pleuralins induce the differentiation. For the time being, this remains a mystery¹⁹.

The pleuralins are bound tightly to the silica of the pleural bands. The bindings can only be broken by dissolving the silica. The binding's nature is unknown. Possibly the cationic pleuralins bind non-covalently to the anionic silica particles. Also, they may bind covalently to organic material incorporated in the silica¹⁹.

Checking for silica

To complement their frustules, the daughter cells first need to collect silica from their watery environment. Diatoms do this very efficiently: if their silica supply is limited, they can reduce the silica concentration of their surroundings to mere micromolars⁴⁵. The diatom checks for silica availability in the environment at two stages of its cell cycle. If the environmental silica concentration is too low, the cycle will halt and continue only when the situation becomes more "to the diatom's liking". The first stage is the G1/S boundary. The second stage is the G2/M boundary. A diatom, silica-starved at the G1/S boundary, reacts differently to newly provided silica, than a diatom starved at the G2/M boundary: it needs less time to recover from its

starvation and will pick up its life cycle sooner⁴⁸. During the G1/S stage, the diatom begins duplicating its DNA. Possibly, the diatom's DNA synthesis machinery is silica dependent. A silica deficiency would stop DNA-duplication and thus the reproduction cycle. During the G2/M stage new valves are built: without silica, this building can not continue (*figure 8*)¹⁰.

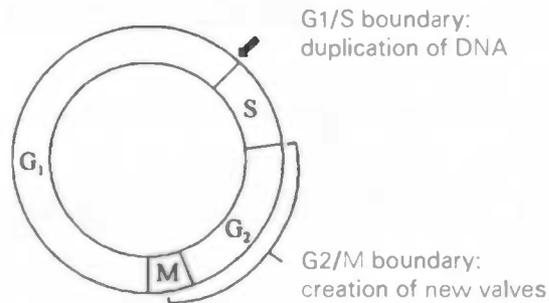
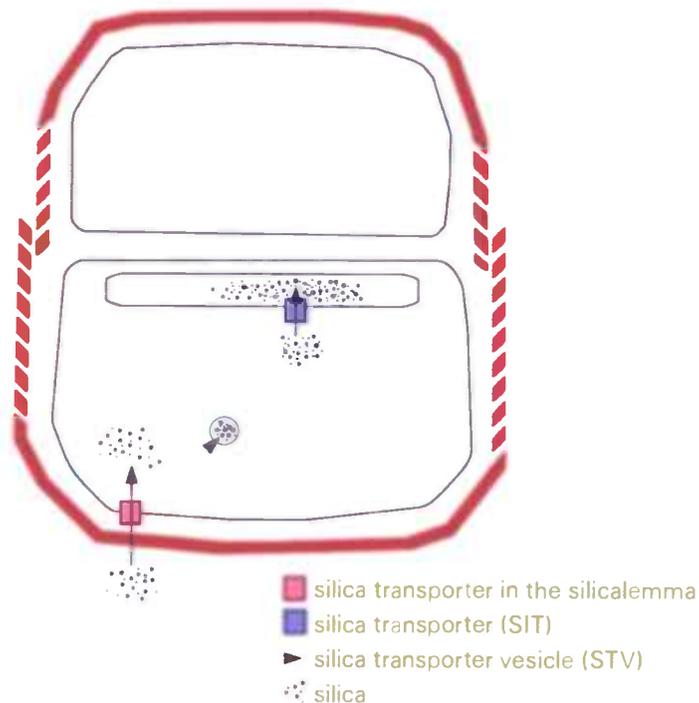


figure 8

Silica transport

Diatoms can make their walls in just an hour. The silica in sea water is mainly present in the form of $\text{Si}(\text{OH})_4$ and partly in the form of $\text{Si}(\text{OH})_3^-$. Diatoms can work with both²⁶. To transport silica from the environment across the plasma membrane, diatoms are said to use five specialized transporters, encoded by five genes called *Silicon Transporter genes* or *SITs*. All five are sodium/silicic acid symporters². The transporters are thought to be built just before maximum silica uptake. The cellular concentrations of the five transporter species differ and change over time. This suggests that the five have different roles in the silica import process. It seems silica import is no simple matter, but a process carefully regulated by the diatom at all times¹⁴. Of what happens once the silica is inside the cell, little is known. It needs to be directed to specialized compartments called the *Silicon deposition Vesicles* (SDVs). In these compartments elements of the diatom's wall are formed. The SDV is surrounded by a membrane called the silicalemma. In theory, only transporters in the silicalemma are needed to get the silica inside the SDV. These silicalemma transporters are definitely not *SITs*⁵⁴.

figure 9: schematic representation of silica uptake and internal transport of a diatom.



Another option is that specialized vesicles, *Silica Transportation Vesicles* (STVs), transport the silica through the cell and fuse with the SDV, depositing their contents inside³⁹. But evidence for this is lacking (figure 9). In *Nitzschia alba* some intracellular silica transport occurs with the help of ionophores (which cause a leakage in the membrane so that ions, like silica, can pass). Until now, this mechanism has only been found in *N. alba*¹.

Silica pools

Diatoms possess pools of solubilized silica inside their cells³. The location of these pools varies, but every organelle may have one^{43,7,3}. The silica concentration of the pools is supersaturated and one would think that the silica polymerizes, but it does not. In the vacuole the pH is elevated. A high pH prevents silica polymerization. How the diatom prevents polymerization in other organelles is unknown. Most species take up silica from the environment only when they need it: it is transported quickly to the forming valve, and internal pools are small^{7,42}. Some species do take up silica some time in advance. The storage pools of these species are far larger. When it is time to build a new valve, these diatoms use their stored resources^{7,3,6}.

Each element of the diatom cell wall (epivalve, hypovalve, first girdle band, second girdle band, etc.) is built in a separate SDV. During the formation of a new wall element, the SDV's pH is kept low by ion pumps in the silicalemma. An acidic environment stimulates silica polymerization and prevents silica dissolution^{5,16,28}. At first a daughter cell builds its new hypotheca in two dimensions only. The SDV expands, until the valve reaches its mature proportions. Only then does the valve grow into the third dimension^{32,46}. After it is finished, the diatom starts building two girdle bands, each in their own SDV. The diatom adds a third girdle band just before the entire frustule is exocytosed. Finally, the daughter cells separate⁴¹.

Organic casing

The diatom's cell wall is surrounded by an organic casing. This casing has been studied in only a few diatom species. It seems to be secreted and replenished through pores in the valves and girdle bands¹⁵. The casing's function is under debate. According to one theory, it protects the polymerized silica from the sea's caprices⁸. After all, the frustule is built in a protective environment: the SDV. The SDV's silica concentration and pH favour silica polymerization. The surrounding water is undersaturated in silica and has a slightly basic pH, which damages silica aggregates. Without protection, the diatom's wall would slowly dissolve. A mother cell could only pass on a partly dissolved epitheca to its daughter cells, if it could pass on anything at all. Experiments showed that cell walls without casing sooner dissolved, than cell walls with casing^{3,17,24,49}.

In pennate diatoms special proteins, called *frustulins*, are distributed uniformly through the organic casing⁴⁶. They are inter-connected by non-covalent Ca^{2+} bridges. They are not related to any other known protein but form a separate family. To be called *frustulin*, a protein needs at least three of five structural elements:

- Targeting signals for both the endoplasmatic reticulum and the cell wall.
- Acidic cysteine-rich domains (*ACR domains*).
- A proline-rich domain.
- A polyglycine domain.
- A tryptophan-rich domain²⁰.



DIATOM'S

DE- TAILS

In the SDV, the silica is deposited in the form of small spheres, about 40 nm in diameter⁹. The diameters vary per species and sometimes even per frustule element, but in a single element diameters are the same (*table 1*). When deposited, the spheres' stacking is loose. As more and more enter the SDV, holes in the stacking are filled up and the silica becomes a solid structure⁹.

The size of the spheres may be influenced by the environmental salinity: it was determined that a lower salinity leads to a higher silica content per diatom cell. Possibly the silica sphere size decreases at lower salinities. This would result in a more efficiently packed silica structure^{15, 52}. The diatoms frustule contains many, regularly arranged pores and slits. This makes diatoms especially interesting for nanotechnology: it is difficult to manufacture silica structures that contain the same orderly detail as a diatom's wall (*figure 10*). The pore and slit sizes are at least as small as 3.0 nm and can be as large as 1000 nm⁵². The architecture of the pores is similar in all species and this indicates that they are all formed in a similar manner³.

Table 1: Sphere diameters with their variance of two diatom species⁹.

	Valve sphere size	Girdle band sphere size
<i>P. viridis</i>	44.8 ± 0.7 nm	40.3 ± 0.8 nm
<i>H. amphyoaxis</i>	37.1 ± 1.4 nm	38.1 ± 0.5

Moulding the SDV

How does the diatom create such a symmetrical and complex shape? And how does this shape obtain its often intricate patterning of pores and slits? The cytoskeleton, some membranes and organelles 'mould' the SDV's shape and the silicella's surface. This building strategy is called *macromorphogenesis*. It can influence the overall wall shape and perhaps even more detailed aspects⁴⁰.

Many of the wall's characteristics can definitely not be explained by macromorphogenesis. E.g. the diatom *Pinnularia viridis* perforates its frustule with tiny pores, called *puncti*. Observation with a microscope showed that *P.viridis* forms these *puncti* from the inside to the outside.

Figure 10: The variation in diatom frustules and their patterns is huge. The regularity is impressive. As is the tiny scale on which all these patterns are formed.

*Diffusion Limited
Aggregation*



figure 11

A model designed by Parkinson, Brechet & Gordon³¹ postulates that the formation of the diatom's frustule is comparable to the formation of a snowflake (figure 11). Just like diatom cell walls, snow flakes have symmetrical, crystal-like forms that can be quite beautiful. The formation of snow flakes is thought to be an 'automatic' process, guided by the characteristics of the flake's building material and by its environment. This automatic process can be simulated with a specially designed algorithm: the *DLA-mode*^{29,28}. DLA stands for: *Diffusion Limited Aggregation*. In contrast to snow flakes, frustule growth can not be accurately simulated by the DLA model. But when the model is modified, that is when two additional processes are incorporated, simulation accuracy becomes much larger³¹.



figure 12

The first process involves the interaction between a silica aggregate and a loose silica particle. The aggregate's surface consists mainly of silanol groups. A silica particle can 'slide' along this surface and place itself into an energetically favourable position. This sliding and repositioning is called *sintering*. Sintering results in a smooth surface. The amount of sintering depends on pH and salt concentrations¹¹.

The second process involves the predetermined location of silica release in the SDV. If the silica is not released in specific places only, the DLA model can not produce aggregates with an orderliness comparable to diatom frustules (figure 12). The model's designers attribute these specific release sites to microtubuli. The microtubuli originate from a centrosome located near the centre

A diatom frustule
and
a DLA simulation without microtubuli³¹
and
a DLA simulation with microtubuli³¹

of the SDV. From there they form a radial array over the silicalemma and transport silica inside STVs (figure 13: SDV with centrosome). They release the STVs at specific locations. There the STVs fuse with the silicalemma and release their content into the SDV³¹. Though scientists have found microtubuli near the SDV, it is unclear if these transport STVs¹¹. Then again, very little is known about the SDV's surroundings.

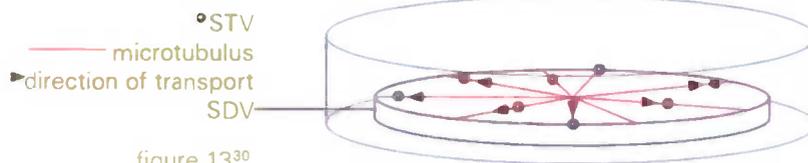


figure 13³⁰

In the DLA model, each silica release site gives rise to a rib in the simulated aggregate, resembling diatoms' costae. The number of costae in diatoms differs per species and ranges from twenty to fifty, so the model places twenty to fifty release sites. These give the simulated aggregate an orderly structure (figure 13)³¹. Some diatom species have frustules that are not orderly at all. Perhaps in these species the silica release sites move during frustule formation³¹. Temperature influences the roundedness of the simulated aggregate. Lower temperatures lead to more faceted aggregates: they reflect the underlying network of release sites. Higher temperatures make the aggregates more rounded (figure 14). Temperature and amount of sintering together influence

- thickness of the costae.
- presence of a large central mass. From this mass 'costae' originate (figure 14)³¹.

The amount of sintering is pH and salinity dependent¹¹. Thus the model predicts that the SDV's pH and salinity influence the aggregate's morphology.

At low surface tensions, holes appear inside the simulated aggregate (figure 14). This process might resemble pore formation in frustules: perhaps molecular agents inside the SDV reduce surface tension and thus prevent silica particles from sintering. The particles cannot slide into the energetically favourable holes and the holes turn into pores³¹.

The model shows that with few parameters, many aspects of diatom frustules can be simulated. It shows that the regularity of diatom frustules may be caused by microtubuli. The model predicts that the SDV's internal environment influences the morphology of the silica aggregate. Experiments with *T.weisflogii* and *N.salinarium* support this. Lower temperatures increase silicification in diatoms¹⁶. A low pH facilitates the aggregation of silica particles⁵¹. And lower salinities lead to a higher silica content per cell⁵².

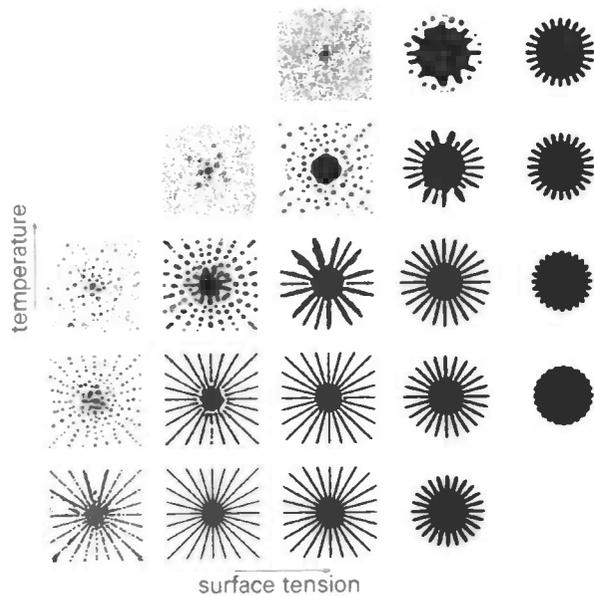
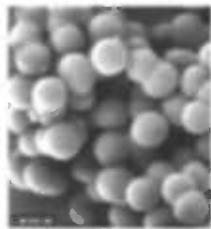


figure 14³¹

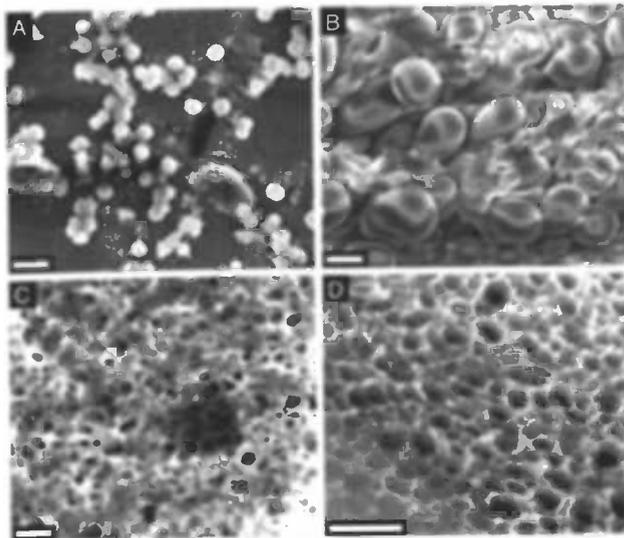
But, like all models, the DLA model has its limitations. Its simulations are two-dimensional structures³¹, while diatom frustules are three-dimensional. The appearance of holes at specific surface tensions is interesting³¹. But these holes hardly possess the diversity, regularity and architectural complexities of natural diatom pores. The model can not produce pennate or more complex centric forms³¹.

Every diatom generation faithfully reproduces the shape and patterning of the frustule of its ancestors. So it seems that the formation of the frustule greatly depends on the genetic material³². Two protein families are tightly connected to the silica of diatoms' frustules: *Long Chain PolyAmines (LCPA)* and *silaffins*. Both families are crucial for silica polymerization. They have been studied extensively in *C.fusiformis* and *T.pseudonana*^{21, 22, 23, 33, 34}. LCPA are polycationic molecules. Researchers sought and found them in five diatom species. Amongst those species were both pennate and centric forms. In a solution that contains both silicic acid and anionic molecules, LCPA polymerize silica. Under these conditions, LCPA forms large silica spheres (diameters between 50 and 900 nm)²² (figure 15). The required anionic groups may also be provided by silaffins. They possess negatively charged phosphate and carbohydrate groups. But silaffins do more than providing LCPA with anionic groups. They regulate the rate of silica polymerization and influence the morphology of the formed silica aggregates^{23, 33, 34}. The silaffin protein family can be divided into two groups: silaffins that can not polymerize silica without LCPA, and silaffins that can. The first group contains *C.fusiformis*' natSil-2 and all silaffins of *T.pseudonana*'s. The second group contains *C.fusiformis*' natSil-1A.

figure 15²²

Silica structures
polymerized by
Navicula angularis'
LCPA *in vitro*.

Figure 16²³
Silica structures polymerized by *Cylindrotheca fusiformis*' natSil-1A *in vitro*.



The interactions between LCPA and silaffins are complex. The silaffin's effect on silica polymerization differs per silaffin species. It also depends on the silaffin/LCPA concentration ratio. Two examples:

C.fusiformis' natSil-1A in a silicic acid solution polymerizes silica into spheres (400-700 nm in diameter)²³ (figure 16). Low natSil-2/natSil-1 concentration ratios form similar, but larger silica spheres (100-1000 nm in diameter)³⁴ (figure 17A). High natSil-2/natSil-1 concentration ratios form interconnected pear-shapes that differ only little from the spheres³⁴ (figure 17B). But at intermediate natSil-2/natSil-1 concentration ratios silica blocks with numerous irregularly arranged pores (100-1000 nm in diameter) are formed³⁴ (figure 17C & D). The amount of silica polymerization also depends on the natSil-2/natSil-1A concentration ratio^{22,23,34} (figure 18).

figure 17³⁴

Silica polymerized by natSil-1A and natSil-2 *in vitro*.

A natSil-2 at 0.5 units/ml, natSil-1A at 0.3 mM.

B natSil-2 at 5.0 units/ml, natSil-1A at 0.3 mM.

C natSil-2 at 2.0 units/ml, natSil-1A at 0.3 mM.

D natSil-2 at 1.6 units/ml, natSil-1A at 0.2 mM.

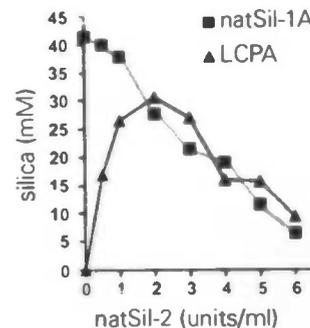


figure 18³⁴

NatSil-2 inhibits polymerization by natSil-1A.

NatSil-2 concentrations up to 2.4 stimulate polymerization by LCPA.

Higher natSil-2 concentrations inhibit LCPA polymerization.

T.pseudonana has five silaffins: tpSil1L, tpSil2L, tpSil1H, tpSil2H and tpSil3. In a monosilicic acid solution that contains LCPA and tpSil1L or tpSil2L, a higher silaffin concentration leads to more polymerized silica (figure 19). In a solution that contains LCPA and low concentrations of tpSil1H, tpSil2H or tpSil3, increasing the silaffin concentration leads to more polymerized silica. But at a boundary concentration, the silaffins

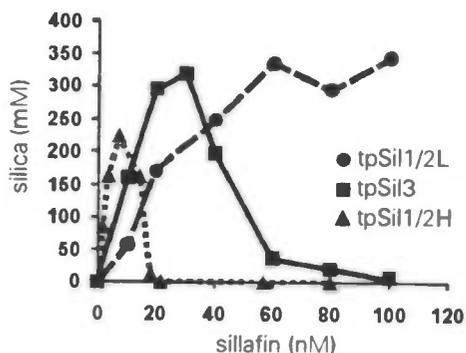


figure 19³³

Silica polymerization by silaffins of *T.pseudonana*. All measurements were done *in vitro* in the presence of 0.75 mg/ml LCPA

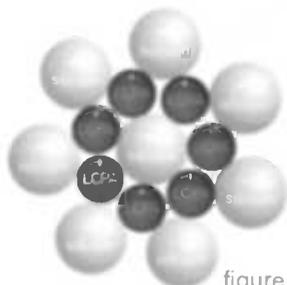


figure 21

start to inhibit polymerization, and the amount of polymerized silica decreases (figure 19).

Silica polymerized by LCPA and tpSil3 has two morphologies. The first morphology consists of large spheres (900 nm to 4.2 micrometer in diameter). This form becomes increasingly dominant at lower silaffin/LCPA concentration ratios (figure 20A). The second morphology consists of densely packed plates, made up of extremely small silica particles. This form becomes increasingly dominant at higher silaffin/LCPA concentration ratios³³ (figure 20B).

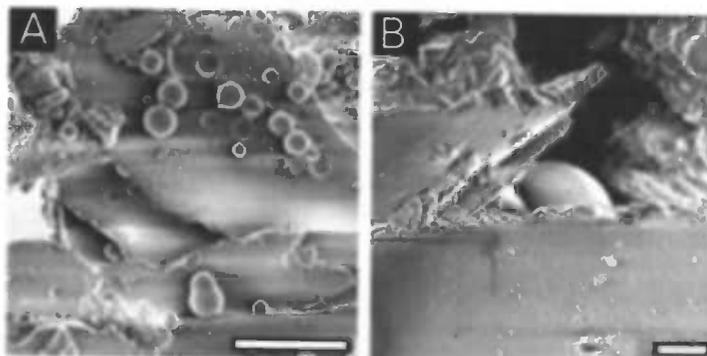


figure 20³³

Silica polymerized by *in vitro* by 0.75 $\mu\text{g}/\mu\text{l}$ *T.pseudonana* LCPA and

- A 20 μM tpSil1/2L
- B 3.5 μM tpSil1/2H

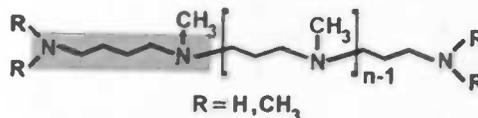


figure 22²²

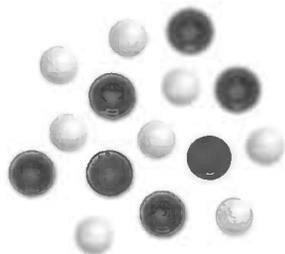
Molecular structure of LCPA. *C.fusiformis*' LCPA have up to 20 repeated units of N-methyl-propylamine, *T.pseudonana*'s vary from 6 to 9.

How do LCPA and silaffins direct silica polymerization?

LCPA are polycationic molecules, while silica particles are negatively charged. LCPA electrostatically interconnect the silica particles (*figure 21*)²². *C.fusiformis*' natSil-1A contains many

posttranslational modifications: eleven of its fifteen residues are modified by ionized groups. These groups are both positively and negatively charged: thus natSil-1A is a *zwitterionic* molecule. Negative groups consist of phosphate residues (*figure 23*). Positive groups of a peculiar modification of the lysine residues: LCPA are attached to them. Without these LCPA, the molecule could not polymerize silica below pH 7. The SDV is acidic so natSil-1A needs the LCPA to precipitate silica inside the SDV. The positive and negative groups together make natSil-1A a neutral molecule^{21,23}.

figure 24
The cationic LCPA are shielded by the anionic natSil-2. The LCPA can not reach the silica particles.



Structure of the ingredients: natSil-2

NatSil-2 can not polymerize silica, but greatly effects the polymerization activity of LCPA and natSil-1A. NatSil-2's structure resembles natSil-1A's and contains LCPA-modified lysines. But contrary to natSil-1A, natSil-2 contains many glycolysated and sulphated groups. These groups shield NatSil-2's cationic LCPA. This shielding is called "electrostatic shielding". The result is that natSil-2 can not polymerize silica³⁴ (*figure 24*).

T.pseudonana's silaffins have no sequence homology to natSil-2. Their lysine residues contain no LCPA, but shorter uncharacterized modifications. They do have similar posttranslational modifications and a similar amino acid composition³³.

Facts about LCPAs

- LCPA are not a single molecular species, but consist of a collection of conserved molecules that differ only in the degree of methylation and in mass. Masses vary from 600 to 1500 kDa.
- Every diatom species' LCPA collection is unique.
- The LCPA molecular structure consists of repeated units of N-methyl-propylamine, attached to methylation isoforms of putrescine (*figure 22*).
- *C.fusiformis*' LCPAs have up to 20 repeated units.
- *T.pseudonana*'s LCPAs contains from 6 to 9 repeated units²².

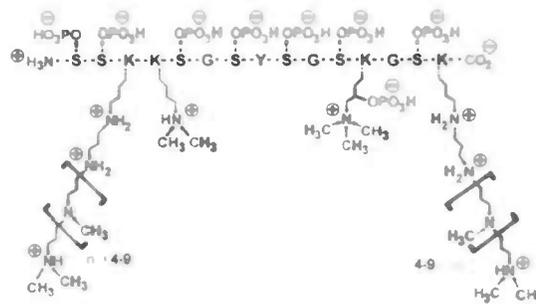


figure 23²³
Molecular structure of *C.fusiformis*' natSil-1A. The protein's backbone is negatively charged. Connected to the lysine residues are positively charged LCPA. The exact charge distribution is pH-dependent. In this schematic the pH is around pH 5²³.

Formation of a template

How could silica particles, silaffins and LCPA together form the patterning of diatom shells? A model designed by Vrieling and co-workers suggests that the process resembles the "cire perdue" method of bronze statue casting⁵⁵. In the favourable circumstances of the SDV (slightly acidic pH, a continuous import of silica particles) LCPA and silaffins polymerize silica. Both LCPA and silaffin particles become encapsulated within the formed silica aggregates. In the SDV a second aggregate is present, which is composed of interconnected LCPA and silaffins: the cationic LCPA form covalent bonds with the anionic groups of *C.fusiformis'* natSil-2 or *T.pseudonana's* silaffins. The size and composition of these LCPA-silaffin aggregates depends on the concentrations of both building materials. At some parts of the aggregates the LCPA is exposed. Here the LCPA can polymerize silica or bind silica aggregates. Other parts are shielded by natSil-2. These parts can only bind other LCPA.

If the silaffin/LCPA ratio is too high, the cationic LCPA all become shielded by silaffins: LCPA can no longer reach or polymerize silica. The silaffins inhibit polymerization. At low silaffin/LCPA concentration ratios the silaffins merely function to interconnect the LCPA and hardly regulate the morphology of the silica aggregate: large spheres are formed (*figure 20A*). These spheres are not found *in vivo*. At carefully regulated intermediate ratios, the silaffins and LCPA form large aggregates. Many LCPA polymerize silica, but there are many silaffins as well. Their interaction with the LCPA and the silica is unclear, but their effect on morphology is large³³. As silica polymerization progresses, the silica aggregates and the LCPA-silaffin aggregates fill up the SDV. In the final stages of valve formation, the LCPA-silaffin aggregates are removed and become pores^{13, 55}.

After silica polymerization has finished, the frustule's pores are filled with the silaffin-LCPA aggregates. How do diatoms clean out their pores? Perhaps they use a ubiquitin homologue¹³. Ubiquitin is a conserved protein found in all eukaryotes. Its function often lies in the degradation of short-lived proteins. Scientists discovered a homologue in *Navicula pelliculosa* that had a high affinity for silica. It was located in newly formed and mature valves, sometimes inside the silica or even inside the pores. But to know the homologue's function for certain, more research is necessary¹³.

A phase separation model by Manfred Sumper⁴⁴

Coscinodiscus is a large, centric diatom genera. Some of its members are: *C.asteromphalus*, *C.granii*, *C.radiatus* and *C.wailesii* (figure 25). These diatoms have LCPA, but no silaffins. The patterning of their frustules is honeycomb-like: it contains an inner layer and an outer layer, connected by hexagonally arranged walls: the *areolae*.

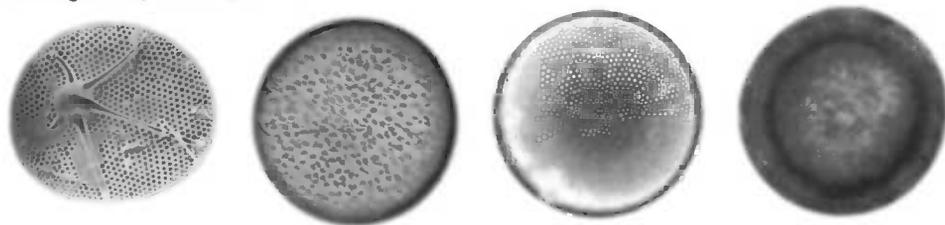


figure 25

C.asteromphalus

C.granii

C.radiatus

C.wailesii

The outer layer has a peculiar structure: it contains a set of hexagonally arranged pores: the *cribrum*. Each of these pores contains yet another set of hexagonally arranged pores: the *cribellum* (figure 26). A simple model explains how the patterning of the outer wall is formed.

LCPA are *amphiphilic*: they contain both water-soluble and water-insoluble groups. In a watery solution they form micelles, just like soap which is also an amphiphilic molecule. At the contact sites between micelles and solution, LCPA polymerizes silica. This polymerization extracts some of the LCPA from the micelles. Also, the polymerized silica exposes many negative groups. These groups 'pull' at the LCPA inside the micelles. Both processes result in breaking of the micelles into many smaller micelles (figure 27). The contact sites of these smaller micelles again polymerize silica. Again the polymerization extracts LCPA. Again the micelles break apart. This self-similar pattern formation stops when all LCPA is used up. Imperfections along the areolae walls originate from fusion between two or more micelles.

In this model only one parameter determines the species-specific patterning. This parameter is the wall-to-wall distance of the areolae. It defines the diameter of the micelles, formed at the very start.

Some notes

The model assumes a two-dimensional, static space, which the SDV is not. The model can explain the complex cribrilla formation but not other aspects of frustule formation, like the transition of base layer formation to areolae formation. The high symmetry of the frustule might reflect its relatively simple building blocks: silica and LCPA. Creation of more complex and asymmetric frustule shapes probably requires silaffins.

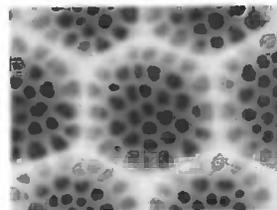


Figure 26⁴⁴
Cribrillum of
C.asteromphalus.

Figure 27⁴⁴



DISCUSSION AND OUTLOOK

over the years, diatomists have obtained large amounts of knowledge. Using this knowledge they recently designed models to explain the formation of diatom's silica shells, the so-called 'frustules'. The models describe only the simplest of diatom structures. More complex frustules, the twisted or asymmetrical ones, are still beyond the modeller's reach.

The Diffusion Limited Aggregation model assumes that frustule formation is a physical process, shaped by the environment: no silica-shaping proteins are required. The discovery of silaffins makes this assumption dubious. But some of the DLA-model's predictions could prove valuable, such as the importance of microtubuli and STVs for the frustule's regularity, or the influence of pH, salinity and temperature on silica polymerization. It is likely that diatoms carefully regulate these, and other characteristics of the SDV. So far, no diatomist has been able to prove the existence of STVs.

We distinguish between diatom species by the shape and patterning of their frustules. These frustules are faithfully reproduced every generation. Thus it is argued that frustule shape and patterning are genetic traits. But they don't have to be. The DLA-model suggests that at least part of frustules' shape depends on environmental factors. Daughters may reproduce their mother's walls, not because they have the same genes, but because they live in the same environment. In this case our system of diatom systematics may not be accurate.

The discovery and analysis of the Long Chain Polyamides and silaffins is a big step forward.

These molecules give insight into how the diatom could create the complex patterning of its shell. LCPA seem to polymerize silica particles into one form only: large round spheres. Silaffins can influence their polymerization activity and induce the formation of far more intricate shapes. Thus silaffins, and not LCPA, seem responsible for species-specific patterning. Silaffins of two species only have been studied: those of *Cylindrotheca fusiformis* and those of *Thalassiosira pseudonana*. They share no sequence homology. One diatom family, *Coscinodiscus*, has no silaffins at all⁴⁴. Perhaps identification and description of additional species' silaffins will shed light on how these molecules influence frustule patterning. How do newly discovered silaffins differ from the silaffins already known? Is there a correlation between the silaffins' chemical structure and the frustule's shape? The silaffins' influence on silica polymerization probably depends on electrostatic interactions with LCPA and silica particles. Which silaffin groups interact with which LCPA groups? What do these interactions look like?

On the other hand, perhaps little or no silaffin homologues will be found in other species. Even if the proposed model of silaffin-LCPA template formation is correct for *C.fusiformis* and *T.pseudonana*, it might not be a universal system. Silaffins and LCPA explain how the diatom may achieve patterning. But how does the diatom direct the location and form of a template? How is pore shape decided? What parts of the frustule become perforated and what parts do not? These are questions that can not be answered with our current knowledge.

The discovered ubiquitin homologue may remove the template molecules when frustule forma-

tion is nearing completion, leaving pores in the silica as in "cire perdue". Further research should determine the exact location of the homologue during frustule formation. It has been found inside the pores which might be the standard situation. This would support both the function of the homologue, and the diatom's use of template molecules. After all, if the diatom does use template molecules, it should have a mechanism to remove them after they've fulfilled their task. The genetic code of *T.pseudonana* was unravelled in 2003¹. This feat gives diatomists many new research angles. For example: one could knock out the genes encoding pleuralins. Perhaps in this way their function in theca differentiation can be unraveled.

Naturally far more is known about what happens outside of the diatom, than what happens within. *SITs* carry silica across the plasma membrane, but intracellular silica transport is largely unknown territory. No known signal sequence directs a molecule to the SDV. Silaffins and LCPA polymerize silica. Together these molecules can create a rich diversity of silica structures. But this was seen only *in vitro*. The molecules not be studied *in vivo* as long as the SDV can not be isolated. Until then, we do not know how removal from their natural environment affects their functioning. This is nicely illustrated by a problem that Kröger's team ran into. Originally they extracted *C.fusiformis'* natSil-1A with Hydrogen Fluoride (HF). HF removes many of a molecule's post-translational modifications. The team characterized the molecule's structure. They found that it could polymerize silica with the help of attached cationic LCPA. Some time later they used a more

gentle extraction method. This method left all modifications intact. It turned out that natSil-1A possessed many phosphorylated groups. Without these groups the molecule could not polymerize silica. How had the originally unphosphorylated natSil-1A been able to polymerize silica? In the team's earlier experiments, the silicic acid solution had been buffered by phosphate! The silaffin extracted the anions it needed from the buffer! If Kröger's team hadn't revised their experiments, they would probably not have found that natSil-1A was a zwitteronic molecule, with cationic LCPA and anionic phosphate groups. Its zwitteronic structure is essential for template formation and silica polymerization.

Researchers obtained the silaffins and LCPA by dissolving the diatom's cell wall. Next they searched for organic material in the solvent. With this method only proteins that are incorporated into the wall are discovered. Enzymes and molecular agents involved in the polymerization process, but not built into the wall, remain unknown. Hazelaar's team looked at a diatom's entire protein repertoire. They selected molecules that had affinity for silica. The team found an ubiquitin homologue. Perhaps this method will prove useful for finding other molecules involved in polymerization as well.

Even with all our knowledge we understand only little of the mechanisms of frustule formation. Building a frustule is a complex and fascinating art. And there is still much to discover.

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