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Controlled Hierarchical Biomaterial Utilizing Diatom Nanostructure

Benjamin Lazarus

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Controlled Hierarchical Biomaterial Utilizing Diatom Nanostructure

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in the Applied Science Department from The College of William and Mary

by

Benjamin Seth Lazarus

Accepted for Honors (Honors, High Honors, Highest Honors)

Professor Hannes Schniepp
Professor Walker Smith
Professor Jonathan Frey

Williamsburg, VA
April 23, 2019
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Abstract

Materials with hierarchical structure often exhibit impressive material properties and functionalities; however, modern manufacturing techniques cannot produce centimeter-scale components with geometries on the scale of nanometers. Biofabrication has the potential to overcome this trade-off by taking advantage of naturally occurring micro- and nanoscale architectures. This paper explores the biofabrication of large-scale hierarchical geometries using the intricate nanostructure of live diatoms as a microscale building block. Diatom growth patterns through hydrogels produced via 3D printing simulations are studied. Diatom manipulation with light and nutrient access during the incubation process of these gels is also explored. Thermal processing experiments are conducted on diatom xerogels and cryogels to fuse the diatoms’ silica frustules together and form a bonded material. Two different species of diatoms, *Navicula trivialis* and *Thalassiosira pseudonana*, are utilized in this project and the different growth patterns and post-processing results of each are analyzed.
Introduction

Hierarchical structures in nature have captured scientists’ imaginations for decades due to its ability to improve a vast swath of material properties. Hierarchically structured materials are substances that have geometries at multiple different length scales [i.e., has nanostructure (structures at a scale of $10^{-9}$ meters), microstructure (structures at a scale of $10^{-6}$ meters), and macrostructure (structures down to about $10^{-3}$ meters)]. Over thousands of iterations, evolution has found a way to perfect intricate and complex structures on a number of length scales that imbue biological materials with properties that humans have struggled to replicate [1]. A prime example of nature’s success with hierarchical structures is nacre, the iridescent inner shell layer of some mollusks that is 95% aragonite. This material bears geometries at five length scales, ranging from centimeters to tens of nanometers, and has mechanical properties that easily outstrip bulk aragonite. Nacre has a strength of 170 MPa compared to bulk aragonite’s 30 MPa and a fracture toughness of 2.9 MPa m$^{1/2}$, which is 3000 times higher than standard aragonite [2]. Furthermore, nacre has been shown to be extremely good at arresting cracks from external sources and has a very high work of fracture [3][4].

Attempts to replicate these nano- and microscale structures for large scale components have fallen short. While additive manufacturing processes are ever-improving, it is challenging to get a resolution in the regime of nanometers [2][3]. Meanwhile, subtractive manufacturing processes have successfully manipulated individual atoms; however, the time requirements to do so is too demanding to make the production of any large-scale product feasible [5]. Thus, there is an inherent trade-off between resolution and part size. To engineer large scale objects with traditional manufacturing techniques, the detail on multiple length scales that makes natural hierarchical structures so impressive must be sacrificed.
Biofabrication has the potential to bridge this gap between resolution and part size. By growing and manipulating living organisms with naturally occurring nano- and microstructures and then 3D printing the macrostructure, large scale objects can be produced without foregoing the properties that come with finely detailed multi-scale structural geometries.

One choice for this building-block organism is microscopic silica-encased diatom. Diatoms are unicellular, photosynthetic eukaryotes that produce silica frustules through a biomineralization process [6][7][8]. These frustules have incredibly intricate geometries on the scale of micrometers as shown in (Figure 1), which give rise to very impressive mechanical properties. The specific strength of the diatom Coscinodiscus sp. was reported to be 1,702 kN m kg⁻¹, one of the highest specific strengths ever reported for a biological material [4]. There are over 100,000 different species of diatoms, each bearing uniquely shaped frustules. This variety would allow for tunability within a biomaterial composed of these microscopic organisms, as differently shaped frustules can be used to achieve different functionalities. Interest in these singular organisms has grown rapidly in the fields of genomics, materials science, nanotechnology, and biofuels over the past decade. As a result, significant research has gone into studying diatom growth rates and responses to environmental conditions, paving the way for research into the many exciting applications of these microorganisms [9].

Diatom Selection

For this project, we chose to explore two different species of diatoms, Navicula trivialis and Thalassiosira pseudonana. Navicula trivialis is a freshwater, benthic, motile, pennate diatom that is approximately 30 microns long and 8 microns wide (Figure 1 and 2C). These diatoms live and grow attached to a surface (i.e., rocks on the bottom of a lake) rather than in suspension.
These diatoms are capable of independent movement and can maneuver to better access nutrients and light [10]. *N. trivialis* also produce a polymer, carbohydrate based Extracellular Matrix (ECM) that allows it to adhere to underwater surfaces [11][12]. *Thalassiosira pseudonana* on the other hand is centric and marine and is approximately 4 microns in diameter; it lives in suspension, can form chains, and is non-motile. *T. pseudonana* is noted for being the first diatom species to have its entire genome sequenced, and more importantly for this project, is hardy and capable of surviving in numerous environments. These diatoms rely on physical processes to find nutrients and other *T. pseudonana* cells for sexual reproduction [13][14]. Both *T. pseudonana* and *N. trivialis* diatoms reproduce sexually and asexually [15][16], although sexual reproduction is relatively uncommon. These differences in motility and environment gave rise to different growth and harvesting techniques.

Methods and Materials

Process Overview

A four-step process was utilized to create a 3D printed hierarchical structure out of diatoms. First, a small number of diatoms were harvested from an existing culture (Figure 2) and mixed into a biocompatible gel to create a printable ink. This ink was then printed into a desired macroscopic shape and cured via a crosslinking reaction, producing a free-standing hydrogel. Several shapes were printed to show that customization is possible and to explore different growth patterns within complex geometries. These diatom infused structures were then incubated in nutrient enriched growth medium, allowing the diatoms to grow and spread throughout the
printed shape. Finally, the hydrogel was either dried to be imaged or heat treated to burn off all organic material and sinter the remaining frustules together.

We focus on the exploration of diatom growth through the hydrogel based on different variables. We first showed that diatoms could survive a printing environment by extruding a diatom inoculated ink through a syringe to study survivability and controlled growth through a printed gel. We also studied how diatom development could be manipulated using gels with complex shapes. Finally, we used post processing techniques to create a contiguous macroscopic material out of the diatom frustules.

**Diatom Cultivation**

A custom built photobioreactors (Figure 3) were used to grow *N. trivialis* and *T. pseudonana*. *N. trivialis* diatoms were purchased from the University of Texas (Utex) Algal Culture Collection along with the recommended growth medium, CR1-S. *T. pseudonana* was purchased from Bigelow Laboratory for Ocean Sciences along with its recommended growth medium, L-1. The photobioreactor consisted of five sealable chambers that were exposed to four strips of cool white LED lights for twelve hours per day and constant air cooling by fans to maintain constant temperature. *N. trivialis* was grown in one-liter glass jars equipped with an air pump and a three-piece plastic airlock that ensured aeration of the growth medium without allowing unfiltered air into the chamber. The growth of *N. trivialis* began to decrease after 14 days, so cultures were continuously replaced every two weeks. To create a new culture, all components that came into contact with the diatoms were cleaned with surfactant and deionized water before being sterilized in an autoclave or with isopropyl alcohol. Then, 300 mL of CR1-S was added to the one-liter chambers along with 10 ml of starter *N. trivialis* culture or 10 ml of
the previous culture. *T. pseudonana* was grown in 250 ml Erlenmeyer flasks equipped with a fitted cork, an air filter, and an air pump to aerate the medium. *T. pseudonana* cultures were replaced every 8 days to avoid growth reductions using the same process as with *N. trivialis*.

**Hydrogel Production**

Hydrogel inks were used to create the macroscopic shape for the diatoms to grow through because of their proven biocompatibility and applicability in bioprinting [17][18]. Lode et al. (2015) and Krujatz et al. (2015) showed that diatoms could be grown throughout a hydrogel matrix [19][20]. Not only did these studies find that diatoms could survive within a hydrogel, but they also showed that diatoms could photosynthesize while in the gel and increase in number. Two different types of hydrogels were produced to be bioprinted. The hydrogels were based on processing procedures for Direct Ink Writing (DIW) 3D printing. DIW is an extrusion method for low viscosity materials that creates a predetermined structure by depositing material one layer at a time [21][22]. One type of hydrogel was produced using a 2% weight by volume sodium alginate solution. These gels were produced by slowly adding sodium alginate to deionized water and mixing at 400 RPM and 37°C for 4 hours. To create neat gels, this sodium alginate solution was then cured in a 0.1M CaCl₂ solution and stored in a refrigerator. This curing process replaces the sodium in the gels with calcium and allows the polymer chains to crosslink. The result was a jello-like material that held its shape at room temperature. Typically, 25 ml of uncured sodium alginate solution was produced at each time. To print this low viscosity solution, the sodium alginate ink was printed into a supportive bath to hold the gel in place before it is cured.
The other hydrogel was a 0.02 weight by volume sodium alginate gel with 0.06 weight by volume methylcellulose added. The methylcellulose acted as a thickening agent, allowing the gel to support its own when printing [22]. Production of this material followed the same initial steps as the sodium alginate hydrogels. The sodium alginate solution was then brought to 55°C and the 0.06 weight/volume methylcellulose was slowly stirred in. The mixture was then placed in an ice water bath keeping the methylcellulose in suspension and allowing it to dissolve with time. The mixture was then refrigerated for storage.

To print *T. pseudonana* hydrogels, 32 ml of *T. pseudonana* were harvested from a two-week-old culture using a pipette. Since these diatoms are non-benthic and live in an evenly dispersed suspension (Figure 4a), cells were considered to be randomly dispersed throughout the culture. This sample was then centrifuged for 10 minutes at 8000 RPM to separate the diatoms from the medium (Figure 4b). The precipitated diatoms were then harvested and manually mixed into the uncured Sodium Alginate gel. The sample could then be centrifuged again with the same settings to extract even more diatoms. Typically, 32 ml of medium yielded 4-5 ml of concentrated diatoms.

Since *N. trivialis* is benthic and lives in freshwater, their growth medium has a significant amount of silt and soil particulate that settles on the bottom of the chamber. This debris can be difficult to remove with post-processing techniques, so this separation was done during the harvesting stage. Microscope slides were placed vertically in the cultures and diatoms were allowed to grow upon the sides of the slide (Figure 2a). These slides were then removed from the culture and the diatoms scraped off and mixed in with the uncured Sodium Alginate gel.

*3D Printing Simulation and Molds*
The goal of the printing simulation was to verify that diatoms could survive being printed within a hydrogel and explore how they would spread through a gel on the centimeter scale. Multiple techniques were used to produce complex macroscopic gels.

The first method was to simply open-air print the sodium alginate methylcellulose ink in a thin bead geometry. *N. trivialis* ink was loaded into a 5 ml syringe, then extruded through a 0.4 mm in diameter nozzle into the CaCl$_2$ bath which would cross link the sodium alginate chains immediately on contact. This allowed the gels to maintain their bead structure. These gels were then left in the curing bath for 10 minutes to ensure crosslinking occurred throughout the gel. The cured gels were then transferred into 200 ml of fresh deionized water three times for 2 minutes to wash off any residual salts from the curing process.

A similar process was used to produced spherical gels that were approximately 2-3 mm in diameter. These spheres were made from the sodium alginate ink without methylcellulose and were loaded with diatoms prior to printing. This diatom-infused ink was then dripped out of the nozzle into the CaCl$_2$ bath. These spheres were also cured for 10 minutes and cleaned in the same way as the beads.

The third technique was to manually extrude uncured sodium alginate into a 3D printed PLA mold (Figure 5) to explore how design impacted diatom growth. Multiple different molds were used to create complex geometries and explore how diatoms grew with differently shaped gels. These molds were then cured in a CaCl$_2$ bath, which would cross link the sodium alginate chains. Small 1 ml puck-shaped molds were cured for one hour to allow the gels to cure completely. Larger molds were cured overnight. The gels were then removed from the PLA mold and washed in deionized water. Some of the gel scaffolds made from the molds (Figure 5) were prepared without a diatom inoculation and *N. trivialis* was grown on the sides of the material.
This was done to test how the geometries affected the growth of diatoms on the surface of the gels. Other gels were prepared with a diatom inoculation, and the growth of diatoms within the material was observed.

**Incubation**

To provide the necessary nutrients and irradiance for the diatoms to grow on and through the gels, cured gels were then placed into the photobioreactor and exposed to the same conditions as the original cultures. Gels were typically incubated for two weeks or longer. Figure 6B shows a sphere prior to incubation and Figure 6C shows that same sphere after 3 weeks of growth. Figure 6D shows the sphere once it has been freeze-dried and Figure 6E is an SEM image of the *N. trivialis’* growth on the surface of the gel. During incubation the gels darken as the diatoms accumulate. For *N. trivialis*, this darkening is largely a result of the ECM that they produce and the pigmentation of the diatoms themselves. For *T. pseudonana*, colonies of diatoms usually appear as the dark clusters that are composed of single diatoms.

**Cryogels and Xerogels**

Wet hydrogels are nearly impossible to image optically or with an electron microscope. Therefore, to prepare samples xerogels were produced by simply leaving uncovered hydrogels to air dry. This allowed the water to evaporate leaving behind the gel’s polymer matrix and the grown diatom structures. However, since hydrogels are over 90% water, this drying process resulted in significant shrinkage of the gel [23]. This distorted the macroscopic shape of the material and disrupted the microstructure of the diatoms. Cryogels, on the other hand, only experience a 15-30% shrinkage by volume reducing the macro and microstructural distortion.
[24]. These were produced by freeze drying the hydrogels. To do so, gels were frozen in liquid nitrogen and then placed in a vacuum where the water sublimated from the gel. This process reduces the surface tension that causes the gels to shrink when they are dried under ambient conditions [25][26].

_Sintering and Post-Processing_

Once the gels have incubated and the diatoms have spread throughout the structure, the material must be post-processed to remove the water, the sodium alginate, methylcellulose polymer, and other organic material produced by the diatoms. The growth medium also has various amounts of inorganic debris that needs to be removed. The _N. trivialis_ CR1-S growth medium contained organic matter and soil that was essential for its growth. These particulates would settle on top of the gel or get mixed with ink when the diatoms were harvested. For the _T. pseudonana_ diatoms’ L-1 growth medium, this included salt crystals that would appear on the gels during incubation. The post-processing techniques also needed to bond the individual diatoms together to create a contiguous material.

The goal of this section is to explore how thermal post-processing effects the density of the diatoms, the dimensional stability of the material at the macro- and nanoscale, and bonding within and between diatoms. Sintering was attempted with cryogels; however, the densities of diatoms were not large enough to create intimate contact between the silica frustules. When the ECM and other organic materials were incinerated, there was significant shrinkage and little inter-diatom bonding. This problem was solved by increasing the diatom density by adding a bonding material that could fill in gaps between the diatoms. Alternatively, diatomite (diatomaceous earth) could be used to increase the frustule density, but this sacrifices uniformity
in frustule shape and quality since many frustules in diatomite are fractured and of a variety of species.

To avoid this problem, sintering focuses primarily on xerogels where the macrostructure had already experienced significant shrinkage and diatoms were in close proximity to each other. Figure 7A shows a bead xerogel that has a surface layer of *N. trivialis* on it. This xerogel was then processed in a furnace at 600°C for 6 hours to remove organic materials (Figure 7B). Most of the debris and organic materials have been removed and the remaining diatoms are densely packed. Some salt crystals and silica were still observed on the surface of the material. These were likely deposited on the surface during the incubation stage. Changes to the photobioreactor design and reduction of debris in the medium could help remove these impurities. Other post processing steps, like allowing the gel to sit in water prior to thermal treatment, could also help dissolve some of these particles, but increased handling risks removing diatoms and decreasing frustule density on the surface of the material. The final step was to bond the frustules together by firing the gel for 2 hours at 640°C to remove any remaining polymer, and then at 1000°C for 4 hours to induce bonding between the silica frustules. These values were chosen to allow the silica to flow and create bonds between the frustules, while keeping the nanostructure intact [27].

Processing the *T. pseudonana* xerogels under the same conditions at 800°C resulted in a fine white powder that neither bonded nor maintained the intended microstructure. We suspected that this was a result of the residual material that remained after the calcination of the polymer, coating the diatoms and obstructing the intimate contact necessary for bonding. An alternative approach was to process freeze dried, centrifuged diatoms that were harvested directly from the culture excluding the gel entirely. These samples were fired between room temperature and 1000°C and characterized with Thermogravimetric Analysis (TGA).


_Growth on Complex Gels_

Early tests with simple shapes showed that diatoms seemed to grow better on certain parts of the gel. This gave rise to the question: How does part design effect diatom growth? Furthermore, can diatoms grow uniformly through a gel that is more complex than the simple shapes (spheres, pucks, and beads) described above? What can be done to increase the density so that diatoms can be more easily sintered?

The design for our complex gels consisted of a rectangular block with four square channels through its middle, introducing a macroscale level of structure. This design was chosen because vascular networks and channels are a solution to nutrient depletion in bioprinting [28]. Neat sodium alginate was poured into the white mold shown in the center (Figure 5) and the mold was placed into the calcium chloride bath so that the top of the mold was submerged in the curing solution. The gels were cured overnight.

A previous experiment involving _T. pseudonana_ indicated that replacing the growth medium in the culture during incubation led to a marked increase in biomass accumulation. Figure 8 shows images of an incubating gel after 8 days, 13 days, 17 days, and 21 days. The growth medium was replaced after day 8 and 17. Without replacing the medium, growth would typically remain steady through 14 days and then decrease. This experiment generated gels that became noticeably darker after the medium was replaced and resulted in the largest accumulation within a gel. As diatoms are consuming nutrients in the medium (therefore decreasing the concentration of these nutrients that are able to diffuse through the gel), replacing nutrients enhanced growth and accumulation. This replacement seems especially important later in the
incubation process when there are more diatoms present, resulting in quicker consumption of nutrients. This theory was further tested with experiments concerning complex geometries.

A neat complex gel was produced using the aforementioned technique and placed into a live *N. trivialis* culture. After six weeks the gel was removed and imaged. A significant number of diatoms had grown on the front surface of the gel that faced the light source. The PBR is designed so that irradiance is only projected from one side and hence more diatoms would grow where exposed to the greatest irradiance. Diatoms had also grown on the surface of the square channels that faced the light source. This growth pattern could be seen in images of the hydrogel (Figure 9A) and the images of the freeze dried cryogel (Figure 10A). Diatoms and their extracellular matrix appear as a brown slime on the hydrogels but turn green once freeze dried. Clumps of diatoms could be seen growing on the other sides of the gel that were not facing the light source (Figure 9B and 10B). The diatoms grew densely in a thin sheet along the surface (Figure 9D and 9E) of the gel and, as before, could easily be peeled off along with their extracellular matrix once the gel was freeze dried. The brittle, fractured diatom surface can be seen bowing out from the rest of the gel (Figure 10B). These fractures occurred naturally during the freeze-drying process, likely a result of internal stresses from the shrinkage that occurs when the water sublimates off of the frozen hydrogel. This thin layer could be peeled off, leaving the clean cryogel surface (Figure 9F). The interface between the ECM laden with diatoms and the clean cryogel surface was imaged optically in Figure 9C and with SEM in Figure 10C.

Diatom densities from the interior channel wall facing the light source and the front wall facing the light source were determined by manually counting the number of diatoms in an SEM image and then dividing by the area shown in the image. Seven images of each surface, taken of different subsections, were analyzed and averaged to determine cellular density on both faces of
We found the diatom density on the front wall to be 21.3% higher than the density of interior channel wall. We hypothesized that this was caused by the decrease in irradiance from the front wall to the first interior wall as light passed through a layer of diatoms and gel before it reached the interior channel.

These preliminary results led to several more questions concerning diatom growth on complex structures. Could diatom growth be manipulated by varying their access to light? How does the orientation of the macroscale geometry during the incubation period effect the growth of the diatoms? How does replacing the culture during the incubation process effect the growth of the diatoms?

To probe these questions, neat gels were placed into three different chambers in the photobioreactor with 300 ml of CR1-S growth medium. 10 ml of the *N. trivialis* were collected from a culture and added to these chambers. After two weeks of incubation, one gel was rotated 90 degrees and had its culture and medium changed. Another gel was left unrotated but had its culture and medium changed. The third gel was left unrotated with the same culture and medium. After two more weeks the gels were harvested, imaged, and the densities of the diatoms analyzed.

**Imaging**

Images were taken using a Phenom ProX Tabletop SEM and an Olympus Inverted Optical Microscope. Aerogels and Xerogels were sputter coated with gold palladium for 2 minutes prior to imaging on the SEM. Hydrogels, Aerogels, and Xerogels were imaged microscopically. For *N. trivialis*, these images were used to ascertain algal densities. For *T. pseudonana*, the hydrogels were dissolved in sodium carbonate before being loaded into a
hemocytometer and imaged. Algal densities were then calculated using on the measured volume of the hydrogel and the average density of diatoms.

Results and Discussion

Incubation

Microscopy showed that large numbers of diatoms grew through the gel (Figure 6A and 6C). SEM images of freeze dried spheres with *N. trivialis* mixed into the pre-printed ink are shown in Figure 6E. An optical image of the sphere is shown in Figure 6D. We found that *N. trivialis* diatoms primarily grow on the outer surface of the gel. This can also be seen in the optical image shown in Figure 6A, where diatoms are evidently growing all along the bead’s exterior, but very few diatoms appear to be growing within the bead itself. The thick exterior layer of diatoms and extracellular matrix could easily be peeled off, reinforcing the notion that the diatoms grew more densely on the surface than within the gel. Diatoms were also seen moving along the surface of the gel. Figures 6F-H show the movement of a single diatom over 20 seconds. This movement was observed at multiple different depths of focus, indicating that diatoms survived and retained their motility throughout the gel. However, it should be noted that the speed and regularity of diatoms moving within the gel was lower than those of the diatoms on the surface of the gel. Movement within the gel is likely limited by the constrictive nature of the cured polymer matrix.

SEM imaging showed that *N. trivialis* successfully propagated along the surface of the gels during incubation. Figure 11A shows a cryogel prior to incubation and Figure 11B shows a gel after 4 weeks of incubation. We suspect that the large amounts of growth of *N. trivialis* along
the surface of the gels was a result of the diatoms maneuvering to find better access to light and nutrients. Figure 12 shows the diffusion of food dye through spherical hydrogels over time. The hydrogels were immersed in a 10:1 by volume solution of red food dye for 0.5, 2, 6, 10, and 20 minutes. Over 20 minutes, the spheres continued to be more saturated with food dye, suggesting that while hydrogels do allow nutrients in the surrounding fluid to penetrate the gel, diffusion is not instantaneous. This provided further evidence that diatoms in the ink migrated to the outside of the gel during the incubation process to better access nutrients.

During the incubation stage, incubating gels were placed in the photobioreactor in sterilized chambers filled with sterilized growth medium. However, _T. pseudonana_ and _N. trivialis_ were often observed growing freely in the chambers when their respective gels were incubating. This suggests that diatoms often escape the gel itself and continue to grow wherever conditions are favorable. Therefore, inoculation of the diatom gels might not be necessary if the geometry of the gels allow the diatoms to easily move to areas where their nanostructure is desired.

While the diatoms survived the printing process and were successfully grown on and within the gels, it should be noted that often there was a significant amount of variance in diatom density within a batch of gels placed in the same culture, between trials with similar conditions, or even within a single sample. This could result from a variety of causes. One reason was diatom loading density. When _T. pseudonana_ were centrifuged after being harvested and loaded, effectively increasing their loading density, far more successful diatoms gels were produced. This increased density in the gels increased the number of surviving diatoms and increased the rate at which the diatoms procreated within the gel. Other possible reasons for the variable
growth could be differing amounts of nutrient access within a gel or variance in the viability of the culture that was used to inoculate the gel ink.

**Benefits of Xerogels**

While cryogels reduce the structural distortion and shrinkage of the gels they also had several disadvantages that became clear over the course of the project. When *N. trivialis* diatoms were grown on the surface of a gel and then freeze dried, the surface layer of ECM and diatoms became very brittle. When xerogels were produced from the same samples, the ECM and diatom layer conformed to the shape of the xerogel and clung tightly to the polymer matrix. Cryogels also take on a tan color as can be seen in Figure 10B. This makes it difficult to optically image the gels at different depths. Xerogels on the other hand remain clear allowing gels to be more easily optically imaged.

This project worked primarily with cryogels since we were more interested in the structure that was occurring on the unprocessed hydrogels. However, we noticed several other interesting aspects relating to diatoms on xerogels. Figure 10B shows an SEM image of a bead xerogel and the diatoms on the surface seem to have more alignment than diatoms on images of cryogels do. We believe this a result of the shear forces on the diatoms during the drying process. While this was not investigated further, orienting the diatoms would provide another level of tunability and control to the design. Xerogels also tend to experience the most shrinkage along the axis (the z-axis) that is perpendicular to the substrate that it is dried on, while retaining its dimensions in the other directions. The result is a thin film with the same shape in the x and y directions as the original hydrogel. Diatoms that are growing within the gel or on top of each
other on the surface get pressed together as the xerogel shrinks increasing the visible diatom density.

*Variation by Species*

One of the most exciting aspects of this project was the differences seen between the two species of diatoms. Not only does each species have different microscopic shapes and nanostructures, but they grow differently as well. As a motile, benthic diatom, *N. trivialis* migrated to the interfaces of gels. Their maneuverability allows their growth to be manipulated to a degree with light and access to nutrients. However, it makes it very difficult to grow these diatoms within a gel. As benthic creatures, they like growing attached to surfaces rather than suspended in a gel and use their motility to move to the surface of a part where access to nutrients and light are greater. While this motility can present certain design challenges, it is beneficial in that the diatoms tended to grow evenly over a surface (Figure 11B). Their motility allows them spread out to find nutrients and other diatoms to procreate with on their own.

Planktonic *T. pseudonana* diatoms on the other hand grow naturally in suspension. As a result, these creatures grew well within a gel but often were absent in the area 1 mm from the edge of the gel. We suspected this was a result of the diatoms getting sucked out of the gel at the edges since they did not have any way to cling to the material like *N. trivialis* diatoms did. Furthermore, the non-motile *T. pseudonana* diatoms have no way to find nutrients for themselves and so nutrient delivery must be taken into consideration when designing a macroscopic gel shape. Large cubic gels inoculated with *T. Pseudonana* were produced using the yellow mold shown in Figure 5. In these gels, diatoms grew in small clusters along the edge of the gel, but no clusters were seen more than about 1 cm into the gels surface. We suspected that this was because
nutrients were not able to penetrate deep enough, in high enough concentrations to support the
diatom colonies. Unlike the *N. trivialis* diatoms, the *T. pseudonana* diatoms did not grow evenly
spaced, instead preferring to grow in clusters (Figure 13B and 13C). These images were taken on
the optical microscope of the gel (Figure 13A). These clusters can also be seen in the images of
puck shaped gels (Figure 13D).

There are a number of reasons why the diatoms might grow in these similarly sized
clusters, despite being evenly mixed before the crosslinking stage when the gel is just an ink.
One is that the crosslinked calcium alginate polymer matrix restricts the diatoms from
dividing. Colonies form around small groups of diatoms and without any motility, the diatoms have no
way to maneuver and spread through the gel. As the colonies grow in size, the restrictive
pressure on these colonies from the polymer matrix builds up and prevents the colonies from
spreading further. Another reason may be that there is limited access to nutrients within the gel.
Certain areas of the gel may allow for better diffusion and thus better nutrient access. Diatoms in
these areas are able to divide, creating a colony around them, while diatoms in lower nutrient
areas do not form colonies. These successful colonies may then reach a critical mass where the
colony is consuming more nutrients than it has access to and so growth slowly stagnates. This
colony formation may restrict overall *T. Pseudonana* biomass accumulation, since the closely
packed diatoms compete for nutrients and may obscure each other’s access to light.

*Diatom Growth Manipulation on Complex Gels*

The second set of experiments involving growth on complex gels (i.e., rotating one gel
and changing its culture, changing one gel’s culture but leaving it unrotated, and neither rotating
the last gel or changing its culture) yielded noticeably less growth than the previous experiment
involving *N. trivialis* growing on the sides of a neat gel. This is probably because the seed culture was weaker after having been unchanged for six weeks during the previous experiment. However, the comparisons between the growth on each gel still gave insights into how diatoms prefer to grow on macrogeometries that are more complicated than spheres, pucks, and beads.

After four weeks the gels were removed from the culture and SEM images were taken of the top and bottom portions of the front wall and top and bottom portions of the second interior column wall. The algal densities of these images were then analyzed (Figure 14). Error bars were determined based on the size of the images that were taken. It was assumed that ± three diatoms could have been miscounted in a 328 x 328 micron image. It should be noted that these measurements are not meant to be an extensive count of all of the diatoms that grew on the surface of the material. Rather, these counts are merely a count of the visible diatoms on the surface after processing. Handling such fragile surface layers meant that bits of the ECM and its diatoms were disrupted during harvesting, freeze drying, and sample preparation for imaging. Furthermore, there were often several layers of diatoms buried in the ECM and under particles that built up on the surface of the material. This measuring technique did not take into account the depth of the surface layer, so these diatoms were not included in the counts. However, since each sample was exposed to the same conditions, these measurements are a simple way to draw comparisons between the algal densities of different parts of the gel.

One of the most notable findings of this experiment was the large increase in diatoms on the top portions of the rotated gel. We expected to find much higher diatom densities at the base of all of the gels, since this is where diatom propagation onto the gel would begin. However, the rotated gels had significantly higher densities at the tops of the gel (Figure 15B). Large networks of ECM were found at the bottom of the gels with very few diatoms present (Figure 15A). This
indicated that the diatoms had migrated through the lower the parts of the gel towards the top. Furthermore, the tops of the rotated gels had markedly denser growth than either the unrotated gels or the unchanged gels. This reinforces the theory that the diatoms had moved away from the bottom of the gels once the gels were rotated. There may exist a slight irradiance gradient that naturally existed in the cultures when they are changed. This gradient occurs because particulate matter is suspended into the water when the cultures are changed, and the gels are moved. This debris could take days to settle, and over time more particulates could gather lower in the water, attenuating the light. Also, the PBR was set up so that there were five rows of horizontally oriented LED lights that illuminated the incubation chambers (Figure 3). The tops of these complex gels barely extended above the first row of lights, so that most of the light came from above. When the gels were rotated, the side that the diatoms once grew on no longer receives the optimal irradiance, so the diatoms moved. Since there is a slight irradiance gradient along the vertical axis, the diatoms moved towards a higher photon flux density. While it may be more advantageous to move around an edge to the side of the gel or to make its way towards the gel front, it is impossible for diatoms to detect the larger structure of the material. Thus, we found that the diatoms move towards a local maximum of light rather than towards the area that had the most light. In this case, that meant moving towards the top of the gel. This makes a strong case for being able to manipulate the diatom growth with light; however, diatoms may move away from areas where they once grew if light is no longer present.

The unchanged gels which were neither rotated or had their culture changed had higher growth than the unrotated gels that had their cultures changed (except on the front wall, which we suspect may be an anomaly resulting from excessive handling since very little ECM or diatoms were present). Unlike the *T. pseudonana*, which were locked into the gels, *N. trivialis*
clinging loosely to the side of the gels, so changing the cultures may have been another handling step that disrupted diatoms and decreased their density on surface. Also, changing the culture entails removing most of the diatoms that are in the chamber and replacing them with new nutrients and a smaller seed colony. The replenished nutrients stimulated *T. pseudonana* growth without removing any of the diatoms that were already in the chamber (since they were all inside the gel itself). For *N. trivialis* the nutrient change meant that the colony size decreased significantly, reducing the number of new diatoms that could climb onto the gel.

**Bonding and Production of a Contiguous Material**

Once the diatoms have been grown on the gel, the silica frustules need to be fused together to form a single material. The results of the thermal treatment on the *N. trivialis* xerogels is shown in Figure 10 C. While the nanostructure of the frustules survived the processing procedure, the material was extremely brittle and fell apart upon handling. This was due to insufficient silica bonding between the diatoms and impurities that caused defects in the silica structure. Increasing the diatom densities would help cure this problem. Sintering of diatomite or diatomaceous earth has shown some promise as the diatoms are closely packed and have few impurities [29]. However, diatomite is composed of many different species of diatoms and the frustules are often fractured, reducing control in the design process. These struggles were a large part of the motivation for increasing diatom density and exploring the impact that macroscale geometries on diatom growth.

Gels inoculated with *T. pseudonana* were also thermally treated. Freeze dried gels containing *T. pseudonana* diatoms were fired at 640°C to burn off organic materials and 800°C to bond the silica structure. Three distinct transitions occurred in the first phase that
corresponded to the degradation of the polymer. Figure 7C-7F show the impact of the firing on the remaining frustules. While the silica has bonded, the nano- and microstructure of the diatoms has disappeared entirely. We believe the reason that *T. pseudonana* melt and bond at such a low temperature compared to the *N. trivialis*, which showed only marginal bonding after being fired at 1000°C, is a result of the salts (sodium, potassium, etc.) that are present in the L-1 growth medium (which is essentially nutrient-enhanced, seawater). These salts lower the effective melting point of the silica frustules. It is unclear if these salts are present both within the diatoms themselves and on the surface of the incubated gels, so to adjust this melting point and the resultant post-processing steps, modifications would need to be made to the incubation technique.

These results illustrate how the selection of a specific diatoms species restricts the designs that can be made from them. The high density loading and alignment of the *N. trivialis* on xerogels lend themselves to applications that require thin films and better control over the microstructure. Meanwhile, *T. pseudonana* have the potential to form solids that are bonded together and form a specific printed shape. Either way, as far as thermal processing is concerned, there seems to be a tradeoff between how well the structure is bonded and how much of the frustules’ nanostructure remains intact.

**Conclusion**

This project explored the basis of biofabricating hierarchical structures from live diatom frustules. Printing simulations revealed that diatoms can grow successfully within and on a gel. Experiments also showed that these diatoms can be manipulated with light to reach higher densities. The different growth patterns of diatoms species underscored the importance of part
design in the development of this type of biomaterial. While thermal processing techniques were unsuccessful bonding the silica frustules together while maintaining the nano- and microstructure of the material, it did successfully remove organics and showed that the macroscopic integrity of the material could be maintained. Extensive work with cryogels and xerogels revealed certain characteristics, such as dimensional stability, brittleness, and imageability, that will be important for future work on this topic.

_Future work_

This project has laid the groundwork for inquiries into this exciting, multidisciplinary topic. Future work will involve true 3D printing using a modified or custom-built bioprinter to print low viscosity diatom inoculated inks into a supportive bath. These inks could then be cured and incubated to create far more complex gel scaffolds that incorporate diatom nanostructures. Further work needs to be done on the post-processing of the diatom material to create a contiguous usable material, with dimensional stability, that has firm inter-diatom bonds while also maintaining the nanostructure of the frustules. This will require modifications to the harvesting and incubation processes to remove unwanted debris from the system prior to firing. This project could also benefit from the use of CRISPR to genetically manipulate diatom density and colonization. Diatom orientation may also be controlled by inducing a flow during the incubation steps or by using different post-processing techniques to induce a shear force once the gels have been incubated (as discussed in the Benefits of Xerogels section). Finally, experimenting with different diatom species would introduce exciting new growth and bonding properties to the project that might solve some of the current challenges.
Appendices

**Figure 1:** High resolution image of *N*. *trivialis* nanostructure.

**Figure 2:** Images of *N*. *trivialis* harvesting process.
Figure 3: Image of the Photobioreactor used to grow diatoms and incubate hydrogels.

Figure 4: Images of T. pseudonana harvesting process.
Figure 5: Image of molds used to create various hydrogel shapes.
Figure 6: Images A-C show N. trivialis growth through bead and sphere geometries. Figure E shows an SEM image of the cryogel pictured in Figure D. Figures F-H show the motility of an N. trivialis diatom over time.
Figure 7: Figure A shows an N. trivialis bead gel after being dried into a xerogel and Figure B shows this xerogel after it has been fired at 600°C for 6 hours. Figures C, E, and F show a T. pseudoana cryogel after firing. Figure D shows the TGA analysis of this process.
Figure 8: This figure shows T. pseudonana growth over the course of three weeks. Growth medium was changed after day 8 and day 17. Images were taken of the surge in growth that ensued.
Figure 9: Image A shows a neat hydrogel after six weeks in a N. trivialis culture. Diatoms can be seen growing densely on the sides of the gel that faced the light. Image B and C are optical images of the edge of the gel. Images D and E are SEM images of the diatom growth on the front of the gel and image F is the gel appearance after the surface layer of ECM was pulled away.

Figure 10: Image A and B show a cryogel of the hydrogel pictured in Figure 9. Image C shows the interface between the diatoms and ECM shown in Figure 9D and 9E and the bare gel shown in Figure 9F.
Figure 11: This figure shows the growth of *N. trivialis* on the surface of a hydrogel.

Figure 12: This figure shows the diffusion of food dye through spherical hydrogels over time. From left to right, the gels were soaked in food dye for 0.5, 2, 6, 10, and 20 minutes.
Figure 13: These images show the growth patterns of *T. pseudonana* within a hydrogel. Unlike *N. trivialis*, these diatoms grow in clusters that appear to be nucleated colonies.
Figure 14: This graph shows the growth densities of the *N. trivialis* on complex gels under different conditions. Rotated gels (yellow) were rotated after 2 weeks and had their culture changed. Unrotated gels (green) had their cultures changed but were left with the same side facing the light. Unchanged gels (blue) were left in the same culture, unmoved.

Figure 15: Image A is an SEM image of the bottom of a rotated gel. Image B shows a higher diatom density towards the top of the same gel.
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