

Plasma-Etching of the Organic Layer in Nacre

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Abstract

Nacre's brick and mortar structure has been motivating innovations in biomimetic materials for decades. However, there is still room to improve understanding of the structure of the organic layer in order to engineer better biomimetic composites. A plasma-etching technique that allows for the selective removal of some organic components from individual layers is developed. We conclude that this technique enables a closer examination of the organic layer such that the locations and mechanical properties of individual components can be determined. A methodology for examining nacre samples that have not been demineralized provides a more accurate substrate for understanding the structure-property relationships of the organic layer in native nacre.

Keywords

Plasma-Etching, Nacre, *Haliotis rufescens*, β -Chitin, AFM, SEM

1. Introduction

For many years nacre has been a source of biomimetic inspiration for the materials community due to its hierarchical structure and organic template-assisted self-assembly [1]. Nacre can be best visualized as a “brick and mortar” structure in which the bricks are composed of aragonite and the mortar consists of a variety of polysaccharides and protein fibers [2]. The exact structure of these two components, and the manner in which they interact to provide nacre with an array of interesting deformation mechanisms, has been the subject of scientific investigation for many years [3]-[9].

In order to simplify the discussion surrounding the interaction between the inorganic and organic layers, there has also been work focused on improving understanding of the structure of the organic layer. *Song et al.* have examined the structural and mechanical characteristics of organic matrix layers, and discussed how their find-

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ings relate to the theory of the existence of mineral bridges between adjacent inorganic layers [10]. *Sumitomo et al.* used TEM to study the deformation behavior of the organic layer under strain and found that it demonstrates a crack-bridging toughening mechanism as well as some reversible deformation [10]. *Bezares et al.* demineralized nacre samples and suggested that the organic layer might consist of intercalated silk fibroin/chitin planes that contribute to shear lag and protein connectivity [7]. There has even been biomineralization work that tests the ability of proteins and polysaccharides from nacre's organic layer to induce biomineral formation [11].

The microscopy community has also contributed work on structure-function relationships in the organic layer of nacre. Recent advances in imaging technology such as high-resolution transmission electron microscopy (HRTEM) and cryo-transmission electron microscopy (Cryo-TEM) have enabled scientists to more closely examine the organic layer than was previously possible. *Yao et al.* used HRTEM to show that many "mineral bridges" are in fact discontinuous but abutting nanoasperities [4]. *Levi-Kalishman* used cryo-TEM to investigate the interlamellar sheets and demonstrate that they consist of highly ordered and aligned β -chitin fibrils [12]. *Meyers et al.* took force measurements of both the dry and the hydrated organic layer to show that the process of drying lends the organic layer more strength and brittleness, suggesting a glass transition of the incorporated chitin [6]. Finally, AFM work by *Liu et al.* posited that the organic layer might in fact consist of multiple, mechanically-distinct organic sublayers [5]. However, nacre is structured such that these organic layers often do not cleave apart easily and therefore hinders the independent observation of different layers. The goal of this project was to investigate further the possibility of sublayers by developing a plasma-etching technique that could selectively remove organic layers.

Plasma-etching has been used before in the context of research on nacre, however, it has thus far served only to clean samples for the examination of the inorganic layer [12]-[17]. This project is therefore believed to be the first instance in which plasma-etching was used to investigate the components of the organic layer of gastropod nacre.

2. Materials and Methods

In these experiments, gastropod nacre was sampled from *Haliotis rufescens*. This type of nacre is organized in columnar form and demonstrates a lamellar structure [18]. Columnar indicates that each polygonal tablet is of similar shape and size, and that the centers of the stacked tablets roughly align [3]. Nacre is built up of alternating layers of inorganic material and organic material which can be seen in **Figure 1**.

The inorganic layers are made of calcium carbonate in the form of aragonite tablets which are 0.5 μm thick and approximately 5 μm in diameter [3]. These are indicated by the white areas of **Figure 1**. The organic layers are much thinner, only 20 nm under compression [18], and composed of a variety of proteins and polysaccharides [3] with a central core of β -chitin [1]. The organic material is indicated by the black lines in **Figure 1**. Samples were originally prepared via the methodology developed in *Wang et al.* [18]. However, one deviation from this methodology is that all samples used in this study were both stored and tested in air.

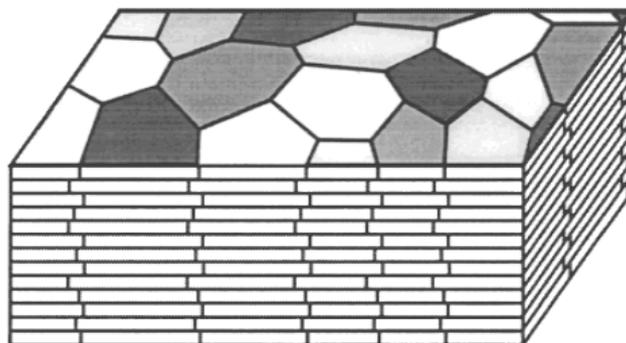


Figure 1. Illustration of the columnar structure of abalone shell. Columnar nacre is differentiated from sheet nacre by the tessellated arrangement of the intertablet boundaries that arises due to the alignment of the stacked tablet centers. (Reproduced from [16]).

Samples that were approximately 2 mm by 1 mm across and several hundred microns thick were prepared to be plasma-etched and imaged. First, a handheld vacuum pickup tool was used to place each nacre sample on a mounting adhesive attached to an AFM sample disk. Scotch tape was then pressed on the nacre surface in order to cleave the top layers off the sample and expose a fresh surface. This was repeated many times, until the sample disk was covered, in order to uncover multiple sites of interest. AFM and SEM were then used to provide preliminary images of the untreated sample surface. The AFM used was a Nanoman (Digital Instruments) and the SEM used was a FEI Quanta 200 FEG Environmental-SEM. In order to capture SEM images of an uncoated nacre sample, low vacuum examination was performed at 0.75 Torr with a 15 kV beam.

The system used was a radio frequency glow discharge plasma source which can generate a large volume of stable plasma and is illustrated in **Figure 2**.

Plasma-etching was performed in a PDC-32G Harrick Plasma Cleaner with a feed gas composed of argon (79%) and oxygen (21%). The samples were plasma-etched at low pressure in one minute increments and imaged between each etching to track changes in the organic layer. In order to reliably track changes, an area of interest was chosen in the preliminary SEM imaging session. Images were then taken of the area at successively lower magnification such that the area could be found quickly and easily after each plasma-etching.

3. Results and Discussion

In order to remove organic material from the organic layers of nacre, the technique developed here relies on the degradation reaction that results from plasma-etching. Oxygen plasma-etching rates are linearly proportional to the oxygen atomic concentration in the plasma and the process is initiated by oxygen free radicals [19]. Weight loss from degradation is dependent on the energy of the plasma and the number of oxygen functionalities and strong backbone bonds present in the polymer [19] [20]. Additionally, weight loss will be restricted to the top-most layers of the polymer [20]. This can be seen in **Figure 3** which shows the progression of the degradation of the organic layer covering a single nacre platelet with incremental plasma-etching.

Figure 3 shows that over time the plasma-etching cause small holes to develop in the organic layer of the nacre. Initially in **Figure 3(a)** the organic layer was smooth with no indications of where the holes will develop. In **Figure 3(b)** small, pale circles have begun to emerge that resemble globules. However, continued plasma-etching yields **Figure 3(c)** and **Figure 3(d)** which clearly show the holes that have been opened in the organic matrix and **Figure 3(e)** and **Figure 3(f)** show that the plasma-etching continues deeper into the organic layer to reveal more holes in the organic matrix. These holes have diameters between 10 - 50 nm and are believed to be pores in the chitin mesh.

Previous work by other groups has proven the existence of “holes” or “pores” in the organic layer. By demineralizing nacre samples, it has been shown that the inorganic component of nacre can be removed while leaving several components of the organic scaffolding intact [5] [6] [19]. *Meyers et al.* concluded that the holes result from a fibril matrix of chitin macromolecules, which form the structural component of the intertile organic layer [6]. *Blank et al.* took AFM images of demineralized multilamellar organic sheets concluded that the fibrous network of chitin contains pores of 40 - 80 nm in diameter [21]. They then incubated the demineralized organic sheets with proteinase K for two hours and found that the pore size increased slightly. However, cryo-TEM work by *Levi-Kalisman et al.* pores were found to have a diameter of approximately 5 nm in diameter and

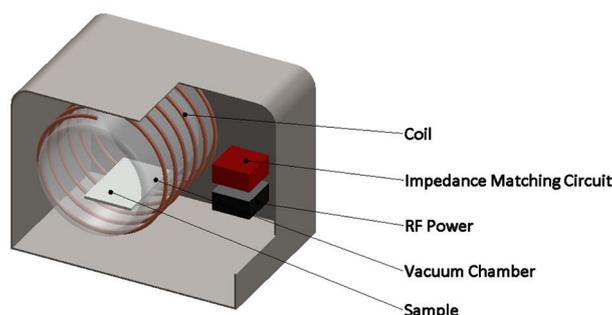


Figure 2. Schematic of the radio frequency glow discharge plasma-etching system.

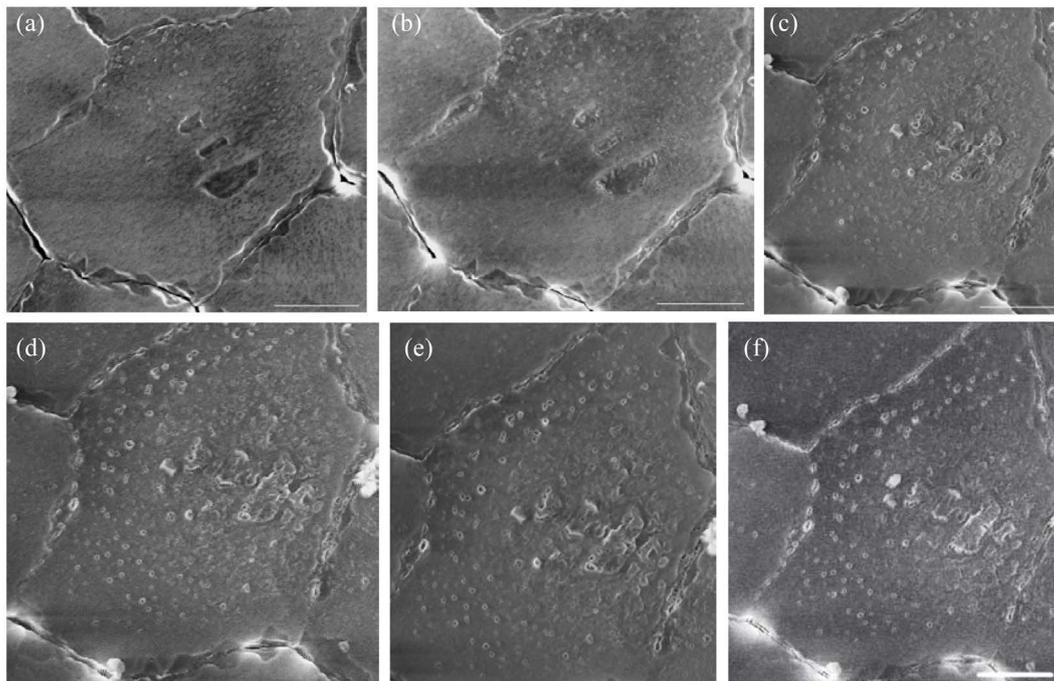


Figure 3. Samples that have been plasma-etched for (a) 1 minute; (b) 2 minutes; (c) 3 minutes; (d) 4 minutes; (e) 5 minutes; (f) 7.5 minutes. All scale bars represent 1 μm length.

they suggest that microscopy techniques necessarily involve dehydration and therefore the introduction of artifacts into the data [12].

The distance between pores was determined by *Schaffer et al.* who grew flat pearls and then fixed demineralized interlamellar organic sheets [22]. They measured a distance of 20 - 100 nm between these holes in the organic layer. Normally the chitin fibrils are contained between layers of acidic macromolecules but these have been etched away during the demineralization process. **Figure 3** shows that, like demineralization, plasma-etching is capable of revealing the chitin core of the organic layers of nacre. However, unlike demineralization, it is possible that plasma-etching can be more precise and remove only the organic components that are most susceptible to degradation.

Figure 4 offers a higher magnification of **Figure 3(d)** which better demonstrates the distribution of the pores in the organic layer across a single platelet and the nature of individual pores. The image shows the nacre sample after it has undergone four minutes of plasma-etching and the holes in the uppermost part of the chitin layer are clearly visible. *Bezares et al.* proposed that the organic layer consists of an intercalated chitin/silk fibroin core sandwiched between layers of acidic macromolecules [7]. Our work suggests that the etching process gradually removes the acidic macromolecules from the chitin mesh as the macromolecules react with the plasma, and therefore leaves behind the characteristic pore structure. Chitin and silk fibroin both have a low susceptibility to plasma-etching compared to the acidic macromolecules. This is because the acidic proteins in the organic layer are thought to have anionic side chains similar to the spacing of calcium in calcium carbonate crystals thereby giving them a role in aragonite nucleation [21].

4. Conclusion

In this study, to our knowledge for the first time, plasma-etching has been used to investigate the organic layer of gastropod nacre rather than to remove it entirely to allow for examination of the inorganic layer. By plasma-etching the organic layer of nacre samples for one minute increments, we showed that the gradual decomposition of the organic layer could be observed with time. Different components of the organic layer were shown to etch at different rates due to the nature of oxygen plasma-etching, which allowed us to conclude that the chitin fibril mesh remained after being stripped of smaller, oxygen functionality-containing molecules such as perlustrin or perlucin. Based on this work, techniques could be developed to selectively remove the various com-

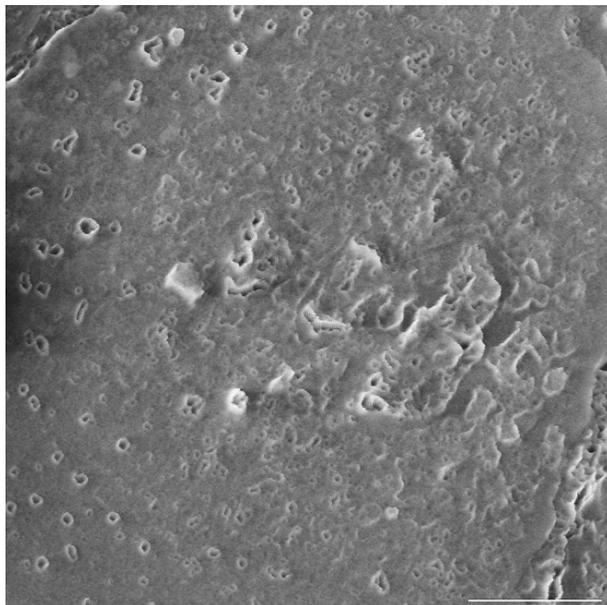


Figure 4. A higher magnification of Figure 3(d) which clearly shows the distribution of pores in the organic layer across the platelet's surface. The scale bar represents 500 nm.

ponents of the organic layer so that the organic components can be examined individually rather than the organic layer as a whole. This would lead to easier mapping of the locations of different components and also to more precise measurement of the mechanical properties.

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