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ORIGINAL PAPER

Diatom Cells Grown and Baked on a Functionalized Mica Surface

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Abstract We demonstrate the cultivation of diatom cells on a functionalized mica surface and the preparation of frustules on a mica surface by baking. Diatom cells were successfully grown on a mica surface treated with 3-aminopropyltriethoxysilane. After baking at 400˚C for 2 h, frustule structures without the organic components of the diatom cells were successfully observed by scanning electron microscopy and atomic force microscopy. Furthermore, the frustules deformed and became slender when a sample was baked at 800˚C for 2 h. Our method is effective for the direct characterization of frustule structures and physical properties without changing the configuration of the diatom cells grown on the mica surface.

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1 Introduction

Diatoms are a major group of photosynthetic plankton, which produce 25% of oxygen on the earth [1–4]. Diatoms are widely found in seas, rivers, lakes, and sometimes in hot springs. One of the unique behaviors of diatoms is the formation of nanoporous biosilica inside their body [5–7]. Diatoms collect Si from water and form several micrometers to several hundreds of micrometers of biosilica, and they contain several nanometers to several hundreds of nanometers of nanopores.

Nanoporous silica, referred to as frustules, has been widely used as filters, carriers, supports for chromatography, and building materials [8–11]. Recently, several research groups have focused on combining frustules and nanotechnology [12–19]. A typical example of diatom nanotechnology is the preparation of replicas of the nanoporous structures of frustules. For example, Bao et al. have demonstrated that a single frustule can be used as a NO*x* sensor [18]. The nanoporous structures of a frustule are useful for obtaining the high sensitivity of the sensor. Diatom frustules have become one of the most attractive nanoporous materials in nanotechnology.

Frustules are usually purified from living diatom cells by chemical treatments such as boiling in concentrated sulfuric acid. By such chemical treatments, organic components such as proteins and DNA are removed completely, thereby yielding pure frustules [20, 21]. However, although these are established methods, frustules are divided into several components after the treatment.

In our previous study, we established a method for culturing diatom cells on a functionalized glass surface in contrast to the usual method of culturing diatoms in a bottle or flask [22]. As a result, diatom cells were grown on modified glass surfaces, especially on a surface treated with 3-aminopropyltriethoxysilane (APS). In this study, we demonstrate the cultivation of diatom cells on an APS-treated mica surface instead of a glass surface. Because of the high melting point of a mica substrate, living diatom cells were able to be directly baked on the mica surface after cultivation. By this baking treatment, it is possible to prepare undivided frustules on a mica surface. Observations of the directly baked diatom cells by scanning electron microscopy (SEM) and atomic force microscopy (AFM) elucidate the effect of baking diatom cells. We suggest that this method can be applied to the study of nanoscopic structures and the physical properties of frustules.

2 Materials and Methods

2.1 Functionalization of the mica surface

3-Aminopropyltriethoxysilane (APS) was bought from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). For functionalizing the mica surface, a cleaved mica substrate (1 × 1 cm, artificial mica for electron microscopy, Oken Shoji Co., Tokyo, Japan) was immersed in 1% APS ethanol solution for 3 h at room temperature [20, 21]. The mica surface was rinsed with ethanol thrice after incubation. Then the substrate was annealed at 90˚C for 1 h. Finally, the mica substrate was rinsed with ethanol.

2.2 Preparation of Diatom Samples

The marine diatom *Navicula* sp. was cultured in the Daigo IMK culture medium (Nihon Pharmaceutical Co., Ltd., Osaka, Japan) using old sea water [22]. One millimolar of Na_2SiO_3 (Wako, Japan) was added to the culture medium as the Si source. The sea water containing the culture medium was filtrated with a 0.2 μm Millipore filter and sterilized by autoclave before cultivation. The functionalized mica surface was immersed overnight in a diatom suspension, followed by rinsing thrice with the culture medium. Then, the mica substrate was incubated in the culture medium for more than 1 month. All the facilities for the culture were sterilized before use. After cultivation, the samples were rinsed with pure water, followed by annealing in an electric furnace (Super 100, Shirota Denki Co., Tokyo, Japan) at 400 or 800˚C for 2 h. For characterization of the un-baked cells by microscopy, the samples were fixed with 10% glutaraldehyde for 1 h at room temperature and were gradually dehydrated with 10, 30, 50, 70, and 100% methanol.

2.3 Microscopy

A scanning electron microscope (S-4100, Hitachi Co., Tokyo, Japan) was employed for observing the sample surfaces. Prior to observation, the samples were coated with platinum–palladium. For AFM imaging, Nanoscope IIIa (Veeco Inc., Santa Barbara, CA, USA) was used with the tapping mode in air.

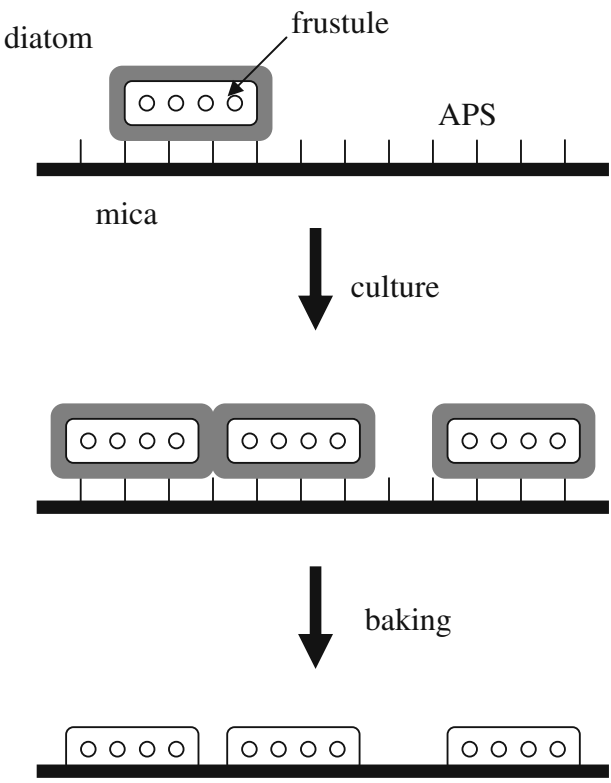

Fig. 1 A schematic view of sample preparation procedure. First, the living diatom cells were adhered onto the AP-mica surface. After the removal of the floating cells, the sample was cultured in the culture medium. After the cultivation, the sample was baked at 400 and 800˚C. This procedure is expected to remove the organic components of the diatom cell, resulting in the presence of only the pure frustules on the mica surface without changing the configuration of the cells

3 Results and Discussion

Figure 1 shows a schematic view of the diatom (*Navicula* sp.) cultivation on the functionalized mica surface. An amino-terminated mica (AP-mica) surface was prepared as described in the previous section. Then, the mica substrate was immersed overnight in the diatom suspension for adhesion of the diatom cells onto the mica surface. On the second day, floating cells were removed by rinsing the mica surface with the culture medium, followed by culturing for more than a month. Then, the samples were baked at 400 or 800˚C after rinsing them with pure water. Usually, diatom frustules are purified in a bottle by chemical treatments such as etching with concentrated sulfuric acid. By our method, it is expected that the purified frustules can be observed on the mica surface without any change in the configuration after cultivation.

Figure 2 shows a typical example of diatom cells baked on the AP-mica surface observed by SEM. Because only a few cells existed at the beginning of the cultivation (data not shown), it is clear that the diatom cells were increased on the mica surface. A uniform distribution of frustules was observed over the entire area of the sample. In our previous study, we reported the culturing of diatom cells on a glass surface that was functionalized with APS. Although a glass substrate is suitable for observing living cells by an optical microscope, we selected a mica surface for the direct baking of the samples. The manner of growth of the diatom cells on AP-mica was almost the same as that on a glass surface. We can conclude that diatom cells could be grown on the AP-mica surface. Although we did not measure growth speed exactly, it took several weeks to 1 month for diatom growth. Acceleration of growth speed is necessary for commercial use of this method.

Figure 3 shows typical SEM images of the frustules on the mica surface. After cultivation, samples were baked at 400 or 800˚C for 2 h. In the case of baking at 400˚C, surface patterns of a diatom frustule were clearly observed in most frustules (Fig. 3a and b). Because the pattern was observed as holes, the organic components were successfully removed from the diatom frustules by baking. In order to confirm the results, the baked diatom surface was observed by AFM (Fig. 4). It is clear that the pattern is an array of actual

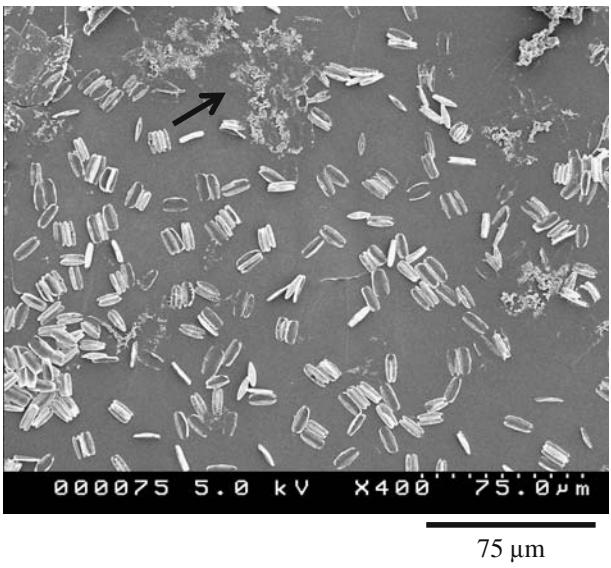

Fig. 2 Typical SEM image of diatom cells baked on the mica surface at 400˚C for 2 h. The *scale bar* is 75 µm

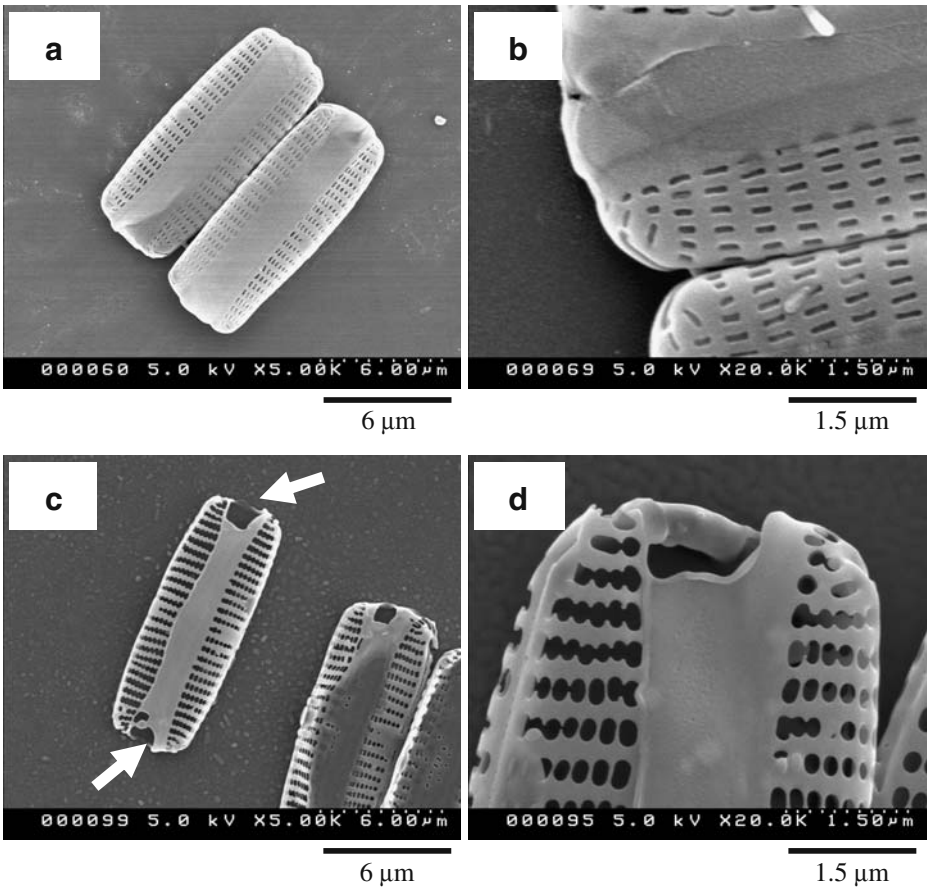

Fig. 3 Typical SEM images of diatom cells baked on a mica surface at 400˚C (**a** and **b**) and 800˚C (**c** and **d**) for 2 h. The *scale bars* in (**a**)/(**c**) and (**b**)/(**d**) are 6 and 1.5 µm, respectively. The *arrows* in (**c**) indicate the holes at the end of the baked diatom cell

three-dimensional holes from the cross-section of the AFM image (Fig. 4b). The depth of each hole was almost 50 to 60 nm in the cross-section. Further experiments are necessary for quantitative analysis of baked frustule structures. On the other hand, some unknown materials (see arrow in Fig. 2) remained around the frustules. Although each frustule was well purified by baking, organic components still remained on the surface at this condition. Further experiments are necessary to understand the mechanism of the baking treatment.

The ordering of frustules on the surface was almost amorphous, although some adjacent cells were lined up. In order to prepare well-arranged, large nanopatterns with some frequency, further breakthroughs are necessary. On the other hand, most frustules showed the lateral side of their body. Vertical piling of the components was not observed. In our previous work using a glass surface, attached diatom cells on APS did not move during cultivation [22]. Although we did not observe movement of living diatom cells on the mica surface because of lack of transparency of mica, diatom cells probably immobilized on the APS-treated mica surface. For this reason, we conclude that diatom cells lay sideways during growth.

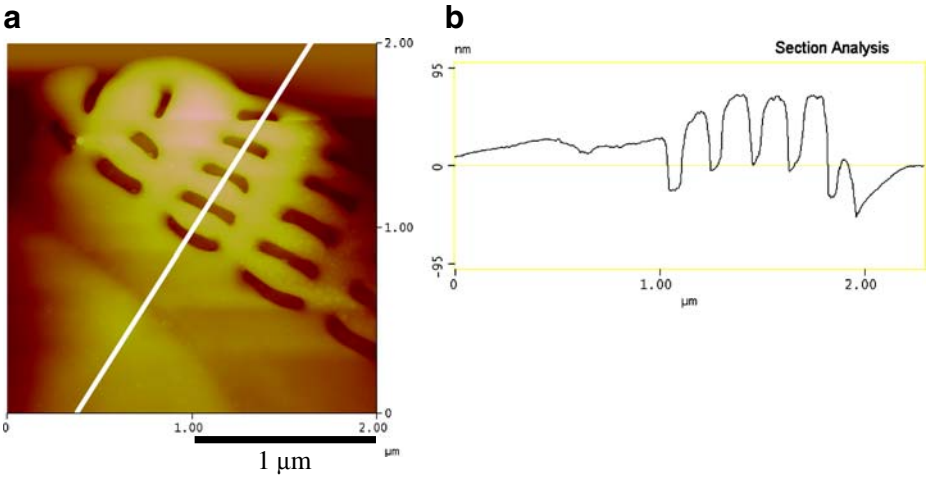

Fig. 4 Typical AFM image of diatom cell surface baked at 400˚C. **a** Topograph. The image size is 1 µm. **b** Cross-section of (**a**)

When diatom cells were baked at 800˚C for 2 h, the size of the nanopores on the frustules increased significantly. Some of the adjacent pores were connected to each other. This suggests that the frustules probably melted and evaporated at 800˚C. However, there was no significant deformation of the overall shape of the frustules. Furthermore, the central area of the frustules, termed bands, was not deformed even at 800˚C. On the other hand, large holes appeared at the end of many frustules only at 800˚C (see arrows in Fig. 3c). Such holes were not observed in the case of baking at 400˚C. These results suggest that tolerance against baking is dependent on the components of the frustules. It is known that the main

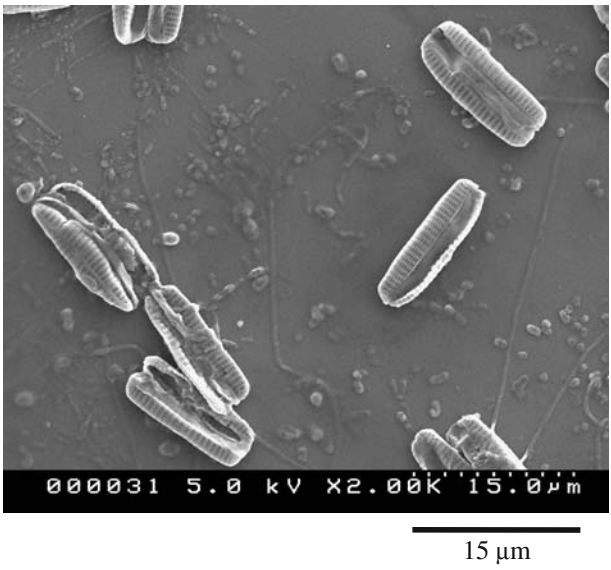

Fig. 5 SEM image of un-baked diatom cells. The sample was fixed with 10% glutaraldehyde and then dehydrated. The *scale bar* is 15 µm

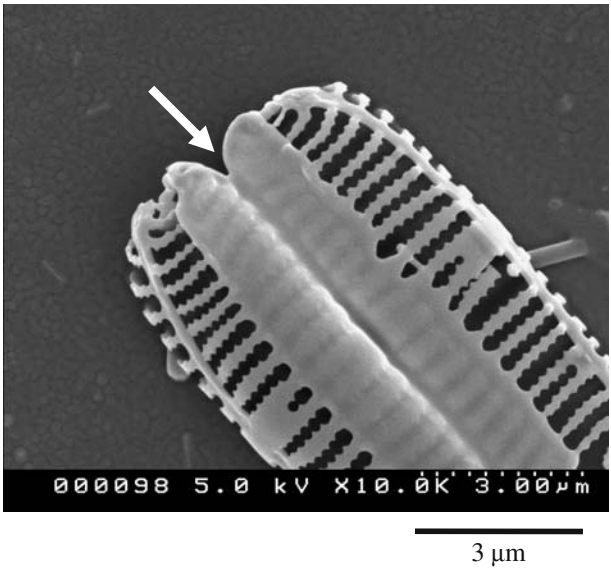

Fig. 6 SEM image of diatom cell baked at 800˚C for 2 h. The *arrow* indicates a groove on a diatom cell undergoing cell division. The *scale bar* is 3 µm

components of frustules are silica. However, it is difficult to assess the uniformity of the structures and physical/chemical properties by macroscopic measurements such as X-ray spectra. On the other hand, although AFM and SEM are good instruments for nanoscopic observation, physical/chemical properties are not easily deduced from micrographs. We suggest that observation of baked diatom cells provides valuable information in nanoscopic characterization of frustules.

Figure 5 shows an SEM image of un-baked diatom cells grown on a mica surface. The sample was fixed with 10% glutaraldehyde and dehydrated with methanol. Although some of the cells kept a square shape, other cells were collapsed. Even in the case of cells with square shape, we cannot determine whether this is a native shape or not. Because *Navicula* sp. is a marine diatom, fixation of the living cells is a tricky subject. Direct comparison of the un-baked and baked cells under the same conditions is not easy. In this sense, direct baking is a convenient way for SEM observation without complicated pre-treatment.

Figure 5 shows an SEM image of the specific structures of the diatom baked at 800˚C. A groove in the central area suggests that this cell was in the process of cell division [23]. It is known that a frustule of *Navicula* sp. is composed of two parts, the epitheca and the hypotheca. The epitheca and hypotheca are connected by a band (central area of the frustule in Fig. 6). In the cell division process, the epitheca and hypotheca dissociate into two cells.

Although the central area was covered with bands even after baking, backbone-like structures were observed inside the bands. Because the diatom cell was directly baked on the mica surface after cultivation, the detailed effect of the baking on the frustule structures was characterized easily.

4 Conclusion

In this paper, we have described a method for the direct characterization of diatom frustules on mica surface without changing the configuration of diatom cells after cultivation. Our

data shows that diatom frustules melt and evaporate at 800˚C. We anticipate that our method will be useful for the nanoscopic characterization of frustules in diatom nanotechnology.

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