

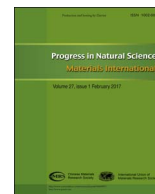
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Original Research

Characterization and analysis of Coscinodiscus genus frustule based on FIB-SEM

Yuan Xing^a, Lihua Yu^{b,*}, Xueli Wang^a, Jiaqi Jia^a, Yingying Liu^a, Jianying He^c, Zhihong Jia^a^a College of Material Science and Engineering, Chongqing University, Chongqing 400044, China^b College of Life Science, Chongqing University, Chongqing 400044, China^c Department of Structural Engineering, Norwegian University of Science and Technology, Trondheim 7491, Norway

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ABSTRACT

Coscinodiscus genus, a type of diatoms with complex frustule, has been widely studied and reported because of their delicate nanostructures. In this paper, a dual beam system focused ion beam and scanning electron microscope (FIB-SEM) were applied to precisely investigate the microstructure of diatom cell walls, in particular, aiming to reveal the hierarchical pores of the valve. The microstructures of the valve, valve mantle and girdle bands were illustrated in details through a series of high resolution images after performing the cross section milling of the specific region. The 3D morphology of frustule valve was reconstructed based on the 2D image series, which demonstrated the presence of cribellum in valve, the external foramen, hexagonal cavity and the internal foramen. The four dense porous membranes and girdle bands were characterized clearly based on FIB-SEM dual beam system. An integrated model of Coscinodiscus frustule could be simulated based on the 2D and 3D results. This study provided a systematic approach to measure the morphological features of diatoms at a nanoscale, which could be applied to other nanoporous structure in three dimensions.

1. Introduction

Diatoms, a kind of unicellular and photosynthetic eukaryotes in both fresh water and seawater, were first observed in the 18th century with a simple microscope [1,2]. Its frustules were composed of a unique biosilica with hierarchical pores [3,4]. Along with the progress and development of microscopic imaging technology, further researches on diatoms were more in-depth and detailed, which focused on not only the characterization of the diatom morphology, but also the internal microstructures [2,5], especially the frustule. Diatoms have always been a kind of important environmental monitoring indicator species and used in water quality research. With the discovery of the nano-patterned surfaces of diatoms, especially the Coscinodiscus genus, more and more potential applications have been exploited, such as the fabrication of frustules morphologies by replica molding [6,7], photonic applications of diatom valves [8], drug delivery [9] etc. The specific nanoporous structure of cytoskeleton has excellent mechanical, filterable and carrying properties.

The Coscinodiscus sp. was established by Ehrenberg in 1839 based on the morphology and size of frustule [2,10]. The frustule was composed of two thecae: an epitheca and a hypotheca. They consisted of valve and several copulae (cingulum), and the cingula were formed

by griddle bands [2,5]. The multi-layer pores of valve was formed by orthosilicic acid in silica deposition vesicle [1]. The natural nanoporous of diatom frustule have exhibited good mechanical properties, the carriers, antireflection and so on [8,9,11], so the studies of structure simulation of diatom frustules seem very meaningful. As a novel nano-structure, it was still a challenge to simulate the veritable structure of Coscinodiscus frustule by artificial work, though there were a lot of researchers dedicating to the nano-scale fabrication and simulation on Coscinodiscus genus [12,13]. It was generally accepted that the pore size was the most important factor for the properties of the frustules. Gnanamoorthy et al. characterized the morphological features of frustules and made a statistics for different pores of Coscinodiscus sp. by FESEM [14]. Zhang used the hydrofluoric acid to enlarge the diatom frustules pores for improving the size of each pores [15]. In order to investigate the actual morphology of the Coscinodiscus frustules, different techniques, such as SEM, TEM, AFM, have been applied [5,16,17]. The ultrafine structure of frustules could be restored based on the 2D-images. But it was still not persuasive to suspect the actual morphology of its cell wall based on the information gained by a series of 2D-image of frustules fragment [10,18]. Then the resin embedding and cutting section method were used to analyze the ultrafine structure of sectioned diatom frustules by SEM. Through this

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* Corresponding author.

E-mail address: lihuayu@cqu.edu.cn (L. Yu).<http://dx.doi.org/10.1016/j.pnsc.2017.04.019>

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method, the ultrastructural valve features of diatom frustule were observed, which were generally not easy to obtain using TEM. Moreover, using this method to prepare SEM sample, the background caused by the internal morphology of scallops could be removed [17]. The 2D morphology could provide a lot of information of the *Coscinodiscus* frustule structures, but sometimes it might also lead to the erroneous information. Friedrichs used the data from SEM coupled with confocal laser scanning microscopy to determine the exact three-dimensional models of diatom frustules and proposed a nice finite element analysis model [19]. Combining confocal microscopy and an image analysis system (NIS Elements AR software, Nikon), Roselli obtained the shape and biovolume of *Coscinodiscus cf. granii* specimens by means of a 3D reconstruction [12]. A 3D imaging of the marine diatom *Thalassiosira pseudonana* was reconstructed by ion-abrasion scanning electron microscopy [20]. Catalina et al. approached a new method for the 3D reconstruction based on the digital image correlation of several SEM images [21].

However, the 3D models obtained by Friedrichs, Roselli and Hildebrand [12,19,20] et al. could not provide an intricate micro-structure of the *Coscinodiscus* frustule. Focus Ion Beam combined with Scanning Electronic microscope has recently been developed to produce 3D images by contrast mechanisms of the SEM [22], but it was rarely applied to the research of diatom frustules. In this article, the FIB-SEM technique was used to analyze the *Coscinodiscus* frustules systematically combining with several 2D-images and 3D models of the fragment.

2. Material and methods

2.1. Preparation and embedding of diatom frustule

Coscinodiscus sp. provided by Marine Biological Culture Collection Centre (collected from the seaside of Qingdao, Shandong province) was used in this study. The *Coscinodiscus* frustule had to be free of organism in order to obtain a high-quality SEM imaging. The diatom cells were soaked in the mixture of Tetra-n-octylammonium bromide (TOAB) and ethyl alcohol for 3 h. The frustule powders were collected by centrifugation, and then cleaned by ethanol with ultrasonic cleaning machine. The cleaning process was repeated five times, and the *Coscinodiscus* frustule powder were subsequently staved at 80 °C in the oven for 12 h [23].

Diatom frustule belonging to porous materials was used in a powdered form after cleaning procedure. In order to get a series of SEM images for the section of diatom valves, the cavities of the diatom were filled with resin. The polymerization resin, Epon-812, was the mixture with SPI-PON 812, DDSA, NMA, DMP-30; and Table 1 showed the solution ratio of each solvent [24]. The diatom powder was bathed in embedded 812 resins with an ultrasonic vibration, and the polymerization of the resin was accelerated in the vacuum drying oven at 60 °C for 48 h.

2.2. Imaging and processing based on FIB-SEM system

For FIB studies, cleaned frustule and embedded block were coated with a thin gold layer for dissipating charge to get good SEM image contrast. SEM imaging and FIB processing were conducted on Zeiss

Auriga FIB dual-beam system. The high-resolution 2D-images were obtained by the in-lens detector to present the morphology of *Coscinodiscus* sp. frustule. To analyze the hierarchical pore, the feature of the cross-section and 3D-reconstruction model was obtained by employing a 30 kV Ga ion beam with different-level current to cut the frustule. A schematic diagram for micro/nano fabrication was illustrated in Fig. 1. Since there was an 54° angle between the FIB and the SEM column, the stage was always tilted to accordingly to make sure that the sample surface was oriented perpendicular to the ion beam during milling, and the Energy Selective Backscattered electron (EsB) images were taken with a calibration of projection angle. Fig. 1a shows the model of an intact frustule with partial milling. In this process, an ion beam with 30 kV/16 nA was selected to remove most of the materials and got the morphology of the cross-section, followed by a fine etching with 30 kV/1 nA.

Fig. 1b shows the principle of 3D-imaging by FIB-SEM dual beam system [22]. The embedded block of frustule was fixed on the sample holder by carbon tape to put in the sample chamber, and it was tilted to a 54° angle as well as placed at a working distance about 5 mm. A layer of platinum was deposited upon the interested area to prevent Ga ion irradiation and curtaining effects from etching by ion beam. To gain a cube-like apex, we used an ion beam of 30 kV/20 nA to avoid re-depositing and to exclude the effect of shielding the detector while imaging the face of the section. The “slice and view” mode was adopted to acquire slicing and imaging sequence (Fig. 2). As the size of the hierarchical pores varied from 50 nm to 2 μm, two intervals (20 nm and 100 nm) between each slice for reconstruction were taken to characterize multi-size pores by employing 30 kV/1 nA and 30 kV/600 pA, respectively. A series of images were collected and aligned. The 3D-reconstruction from these images in sequence were generated by a software named Image J.

3. Results and discussion

Fig. 3 shows the frustule panorama of *Coscinodiscus* sp. with complete structure. The frustule appeared roughly to be cylindrical varying from 10 μm to 90 μm in diametrical dimension, and was composed of epitheca and hypotheca. The valve of frustule tended to swing outward and there was an arc chamfering at the edge of the valve. In the frontal view, the areolae, a honeycomb of hexagonal chambers, was found in the valve. A little gap (marked with a white arrow in Fig. 3a) and several round holes (marked with the white rectangle in Fig. 3a) were demonstrated from the side view.

To understand the internal structure of *Coscinodiscus* sp. frustule, the intact frustule milled by FIB and imaged by the SEM was shown in Fig. 4. It was demonstrated that the diatom cytoskeleton comprised of valve, valve mantle, griddle bands, and pleural bands. Obviously, it was more persuasive and intuitive to study the diatom frustule in this way. There was an irregular band on the inner surface of pleural bands marked with white arrow in Fig. 4a and white ring-like embossment array on the internal surface of valve marked by the black arrow in Fig. 4a. Fig. 4b shows a high-resolution map of the cross-section, which was stitched by 9 pictures, and the morphology with a two-tier valve was formed at the position of pleural bands of the *Coscinodiscus* sp. frustule in division. Each layer of the two-tier valve was similar to the epivalve without cribellum.

Fig. 5a and b are the partial enlarged view of Fig. 4a and the multi-level pores of the valve are illustrated more clearly. The valve was an areola consisting of four dense porous membranes, cribellum, the sieve membrane (outer), loculate wall structure and the pore membrane (inner). As shown in Fig. 5a, the cribellum consisted of pores with different size (the outermost was around 100 nm and the inner was about 50 nm). The external foramina ranged from 1.2 μm to 2 μm, the diagonal of the hexagonal cavity was approximately 2.6 μm, and the internal foramina fluctuated from 0.8 μm to 1 μm (Fig. 5c-f). It is known that the hexagonal cavity is the largest pore and the cribellum is

Table 1
Composition of Epon-812 used for embedding.

Reagent	Dosage (mL)
SPI-EPON 812	9.8
DDSA	3.3
NMA	6.9
DMP-30	0.3–0.4

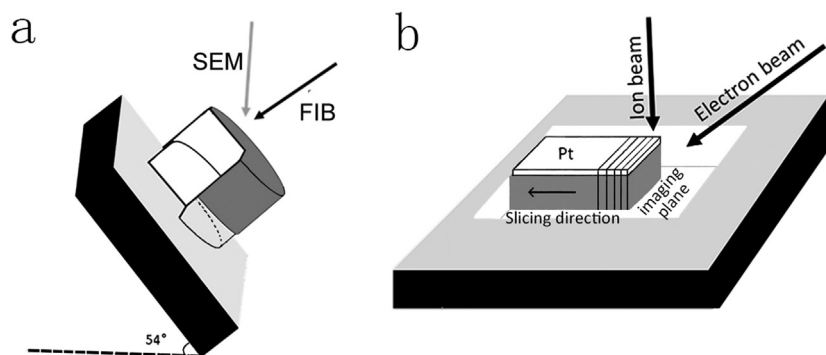


Fig. 1. Schematic illustration of the FIB-SEM method. (a) the imaging and fabrication of the diatom cross-section with complete structure, (b) the sample geometry for the 3D imaging process.

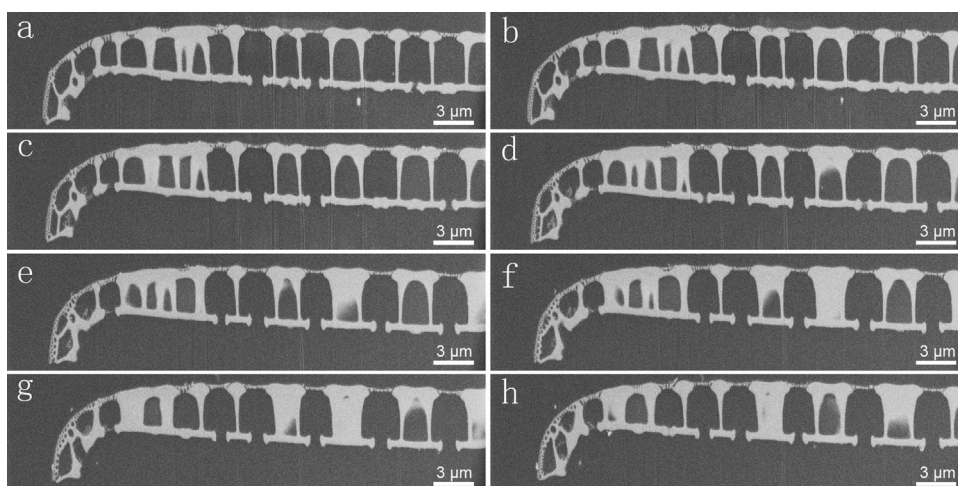


Fig. 2. A series of 2D SEM images.

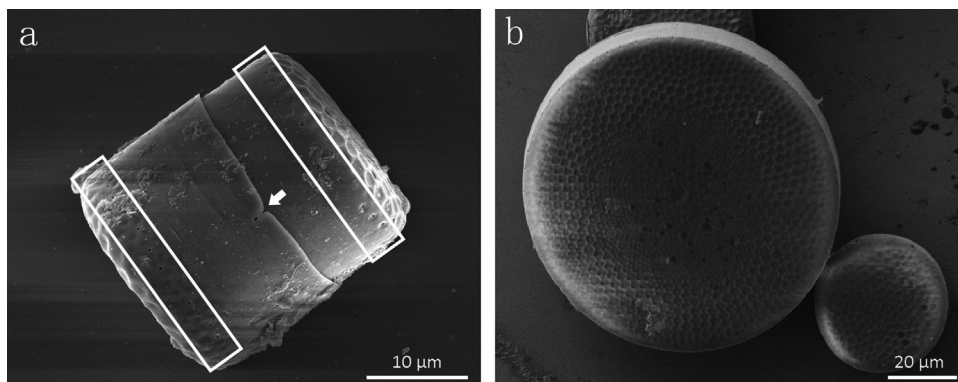


Fig. 3. SEM images of diatom frustule with complete structure. (a) side view (b) frontal view.

the smallest in the four-level pores. Fig. 5d shows that the external foramen was larger than the internal one. In the valve mantle, the hexagonal cavity was replaced by the irregular cavity to guarantee the structure stability.

The 3D structure of the frustule fragments was obtained by reconstruction of 123 series images with a thickness of 20 nm for each slice, and four snapshots are shown in Fig. 6. The 3D structure has a volume of 15 μm×7 μm×2.4 μm. The subtle structure of the frustule could be displayed distinctly. It was verified that the hierarchical pores of valve mantle marked in Fig. 6 were different from that of the valve. The cribellum and the external foramen were replaced by high-density pores, and the size was around 50 nm. The irregular cavity of valve mantle could be observed by the 2D image (Fig. 5a), but it was found that irregular cavity fluctuated from 0.7 μm to 1 μm in size and

distributed randomly in the three-dimensional space (Fig. 6a, b). However, the reconstructed result shown in Fig. 6 was too diminutive to characterize the valve of diatom and it was questionable in the periodic arrangement of frustule valve. Therefore, a typical stack of 72 images taken from the slices, where the thickness of each slice was 100 nm, was used to reconstruct the 3D structure with a large volume of 31 μm×7 μm×8 μm, shown in Fig. 7. The valve structure was revealed in this way. Finally, an intact 3D structure of the *Coscinodiscus* frustule was simulated by software named Unigraphics (Fig. 8) based on the experimental data in this work.

4. Conclusions

The *Coscinodiscus* frustule in two and three dimensions has been

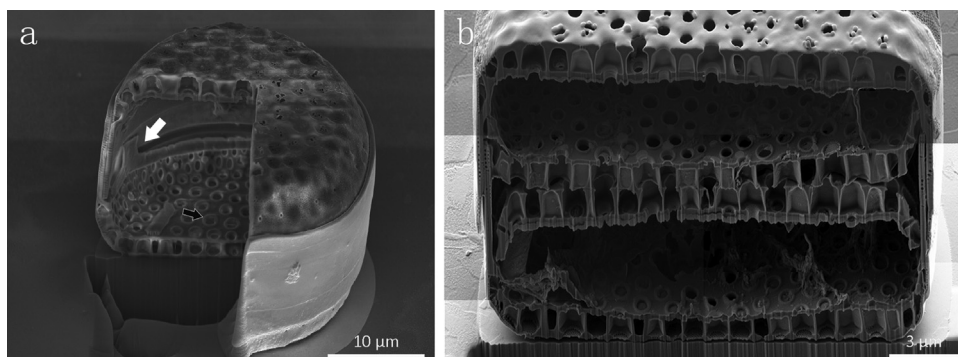


Fig. 4. Fabrication and imaging for one selected frustule by FIB-SEM. (a) image of the frustule with a quarter removed by ion beam, (b) Cross-section of a cell in division at the valve centre of *Coscinodiscus* genus.

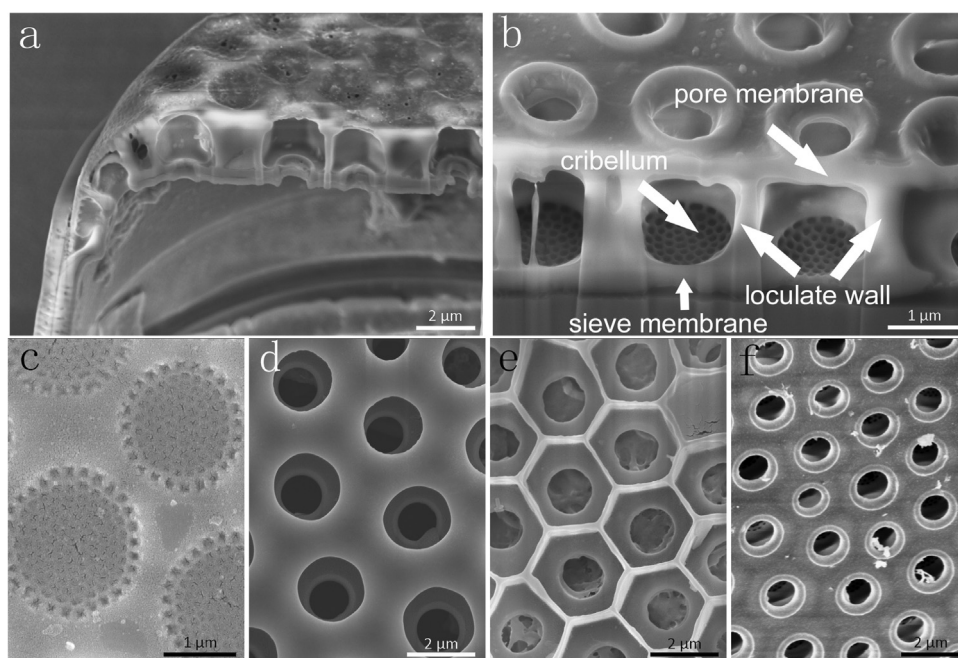


Fig. 5. The hierarchical pores of hypovalve: (a) the cross-section of the valve mantle, (b) The image of the valve with an intact areolae, (c) The cribellum, (d) the sieve membrane (outer), (e) loculate wall structure, (f) the pore membrane(inner).

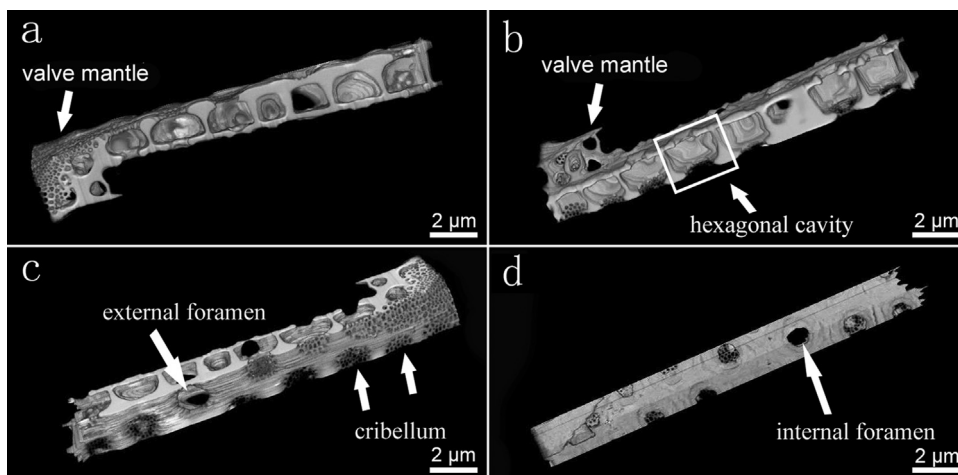


Fig. 6. Four snapshots taken from different angles of the 3D structure of the partial frustule, which was reconstructed by 123 images with each slice thickness of 20 nm.

systematically characterized and analyzed by means of FIB-SEM and 3D reconstruction and simulation techniques. The valve was an areola consisting of four dense porous membranes: cribellum, external foramen, hexagonal cavity and the internal foramen. The valve mantle was

comprised of cribellum, irregular cavity and an internal foramen. An irregular band was found in the frustule at the position of pleural bands and a two-tier valve was observed in the divisive frustule. The partial and whole structures of *Coscinodiscus* frustule were clearly revealed in

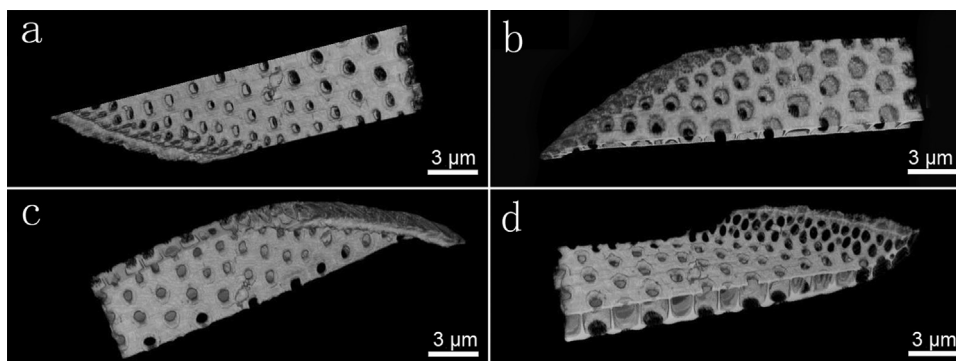


Fig. 7. Four snapshots taken from different angles of the 3D structure of the partial frustule, which was reconstructed by 72 images with each slice thickness of 100 nm.

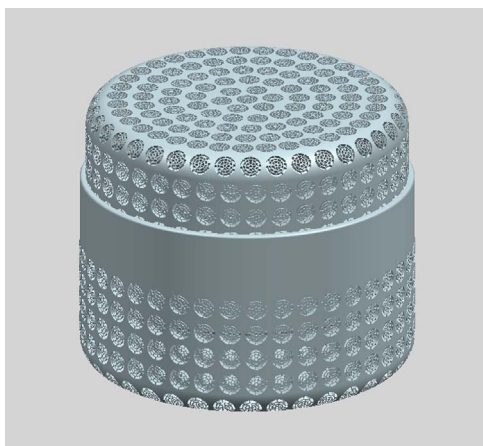


Fig. 8. A 3D model made based on the information from FIB-SEM characterization.

a 2D and/or 3D manner. The FIB-SEM is an effective and powerful tool in studying the microstructure of *Coscinodiscus* genus. The 3D model of *Coscinodiscus* frustule is useful for exploring new ideas for the applications in photonic diatom valves and drug delivery etc.

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