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Use of Ionic Liquid for Scanning Electron Microscopy of Protists

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***Abstract*— We investigated the use of ionic liquids to observe the cell surface of protists rapidly and conveniently. By using ionic liquid, SEM observation is possible without complicated pretreatment such as drying of sample or metal coating. Therefore, it has been shown so far that it is particularly useful for observation of biological samples. It has been shown that good results can be obtained for some biological samples with hard surface structures such as foraminifera and pollen. However, for other unicellular organisms this method has not been validated. In this paper, it is shown that by adding ionic liquid directly after fixation, good results are obtained with diatoms and dinoflagellates without metal coating. In unicellular organisms such as ciliates with soft cells, however, significant shrinkage of the samples was observed.**

***Index Terms*— Ionic liquid, *Paramecium*, Protist, Scanning Electron Microscopy.**

I. Introduction

It is known that an ionic liquid has a melting point at a temperature lower than room temperature and is extremely stable even in vacuum and hardly generates vapor pressure [1]. So far ionic liquids have been used for SEM observation due to their low vapor pressure and high ionic conductivity. For example, Kuwabata et al. observed various biological samples using ionic liquids and reported the results of good observation [2-5]. They stated that the advantage of using ionic liquids is that we can observe wet samples close to their natural conditions. They have succeeded in observing samples in physiological condition by applying ionic liquids to water-containing seaweeds and foraminifers. Furthermore, they used ionic liquids to observe water-containing cells such as cultured fibroblasts in a water-containing state.

Many preprocessing such as pre-fixation, post-fixation, dehydration, freeze-drying, and metal coating are necessary for general sample preparation for scanning electron microscopy. These treatments lead to artifacts such as cell deformation and morphological change of the surface structure such as dropping of microstructure. By using an ionic liquid, SEM observation becomes possible without dehydration, drying and metal coating treatment, so it is possible to exclude artifacts resulting from these treatments. In addition, it is also a great advantage that the sample can be quickly observed due to reduced work.

In this study, we investigated the use of ionic liquids to observe protists rapidly using ionic liquids. Various species of protists exist, but diatoms, dinoflagellates, and ciliates were used as observation materials in this report. For the identification of diatoms, the form of striations and longitudinal grooves (raphe) present on the surface of cells is important, and detailed observation of the surface structure by SEM is often required for reliable identification. Dinoflagellates are a very diverse group of organisms such as *Peridinium* which has hard thecal plates on the cell surface and those which do not have thecal plates such as *Gymnodinium*. There are many small species, and identification of species often requires SEM observation. Ciliates have a large number of cilia on the cell surface. SEM observation is useful, as biological species are often identified by cilia array status, cell surface pattern, and the like. However, in the observation by SEM, since the cilia are of very fine structure, they may be damaged during the pretreatment work and careful handling is required.

This study is the first report that high quality scanning electron microscopic images could be obtained by a simple method of immersing chemically fixed cells in an ionic liquid and then removing excess ionic liquid with a membrane filter.

II. Materials and Methods

A. Protist samples

Samples observed in this study were diatoms and dinoflagellates collected using a plankton net from Nakaumi, a brackish water lake, and the ciliate *Paramecium caudatum* cultured in the laboratory.

B. Ionic Liquid

Ionic liquid BMI-BF_4 (1-butyl-3-methylimidazolium tetrafluoroborate) was used in this study. Since BMI-BF_4 is a water-soluble, it was dissolved in distilled water and adjusted to a final concentration of 2% before use.

C. Sample preparation

Three sample preparation methods were used for observation of *Paramecium*. For the samples to be observed after fixation and air-drying, the cells were centrifuged and fixed with 1.5% paraformaldehyde and 1.5% glutaraldehyde in 40 mM HEPES buffer (pH 7.0), washed with distilled water, and filtered through a membrane filter (Fuji Film, Japan, pore size 0.8 μm), then dropped on a membrane filter and air-dried. The sample subjected to ionic liquid treatment was fixed with 1.5% paraformaldehyde and 1.5%

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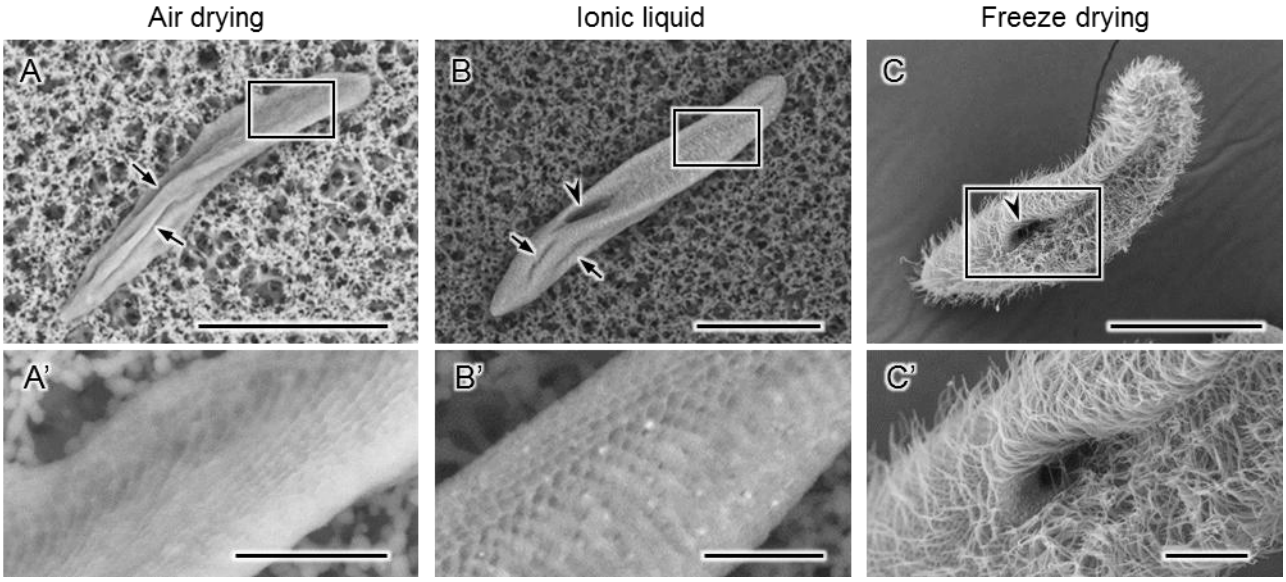

Fig. 1. *Paramecium caudatum* as observed by three sample preparation methods. A and A': specimen was air dried after chemical fixation and observed without metal coating. Due to air drying, the cells shrank greatly and wrinkles (arrows) were formed. Most of the cilia are detached from the surface of the cells. Since it is not metal-coated, the contrast of the cell surface is low and the fine structures of the cell surface are not distinguished. B and B': Samples were treated with ionic liquid after chemical fixation and were observed without metal coating. Cells were not shrunk as much as samples made by air drying, but were wrinkled on the surface as indicated by arrows. Most cilia fall off from the cell surface, but the undulation pattern of the cell surface can be observed well. Arrowhead indicates cell pharynx. C and C': After chemical fixation, the cells were dehydrated with ethanol, freeze-dried using t-butyl alcohol and observed after metal coating. Cell contraction is not observed and cilia are not detached. Because the cilia are present, the undulation pattern of the cell surface cannot be observed. Arrows indicate wrinkles on the cell surface. Arrowhead indicates cell pharynx. A', B', and C' are enlarged micrographs of the areas indicated by rectangles in A, B, C, respectively. Scale bars in A, B, and C indicate 50 µm. Scale bar in A', B', and C' are 10 µm.

glutaraldehyde, in 40 mM HEPES buffer solution (pH 7.0), and then washed with 40 mM HEPES buffer. The washed samples were mixed with the ionic liquid in a microtube, allowed to stand still for 15 minutes, and then dropped onto a membrane filter, and thereafter the membrane filter was adhered to a sample stage to which a conductive carbon tape was attached. For the sample to be freeze-dried, the pre-fixed specimens were washed and post-fixed with 1% osmium tetroxide in 40 mM HEPES buffer. This sample was washed with a buffer solution, dehydrated in ethanol series, and infiltrated with t-butyl alcohol. Thereafter, it was dried in a freeze dryer (Vacuum Device VDF-21S, Tokyo), placed on a sample stage to which a conductive carbon tape was attached and Pt-Pd was coated by an ion sputter coater (Hitachi High Technologies E-102, Japan).

Samples collected in the field were fixed with 2% paraformaldehyde immediately after sampling and stored until use. Three types of sample preparation methods were also used for outdoor samples as in *Paramecium*.

D. Scanning electron microscopy

Most of the samples were observed with a low vacuum SEM (Hitachi High-Technologies TM-3000). *Dinophysis* subjected to freeze-drying were observed with a high vacuum SEM (Hitachi High-Technologies S-4800).

III. Results

Paramecium caudatum was observed by three different sample preparation methods. In chemically fixed, air-dried, samples without metal coating, the cells shrank greatly and wrinkles were formed (Fig. 1A). Most of the cilia were removed, and the fine pellicle structure of the cell surface was almost not retained (Fig. 1A'). Also, the cell pharynx was not clearly observed. Cells fixed chemically, treated with 2% ionic liquid, and observed without metal coating are shown in Fig. 1B and B'. The shrinkage of the cells was less than that of the sample prepared by air - drying. On the surface layer, wrinkles were observed, but it was less than air-dried cells (Fig.1B). From the surface of the cell, many cilia were dropped as in the case of air-drying, but the microstructure of the cell surface was retained better. The structure of the cell pharynx was also clearly observed (Fig.1B'). When chemically fixed and ethanol dehydrated, freeze-dried with t-butyl alcohol and coated with metal, cell shrinkage was scarcely observed and wrinkles on the cell surface were not observed (Fig.1C). In addition, since the cilia remained without dropping out, the microstructure of the cell surface layer was rather difficult to observe (Fig. 1C'). The cellular pharyngeal structure was clearly observed.

Next, samples taken from the field were observed. The diatom *Thalassiosira* sp. was well preserved in samples which were chemically fixed and air-dried, and the honeycomb structure on the shell surface was clearly observed (Fig 2A). However, surface charging occurred in protrusions around the cell and part of the honeycomb structure, which hindered observation (arrows and arrowheads in Fig. 2A). For samples that were fixed

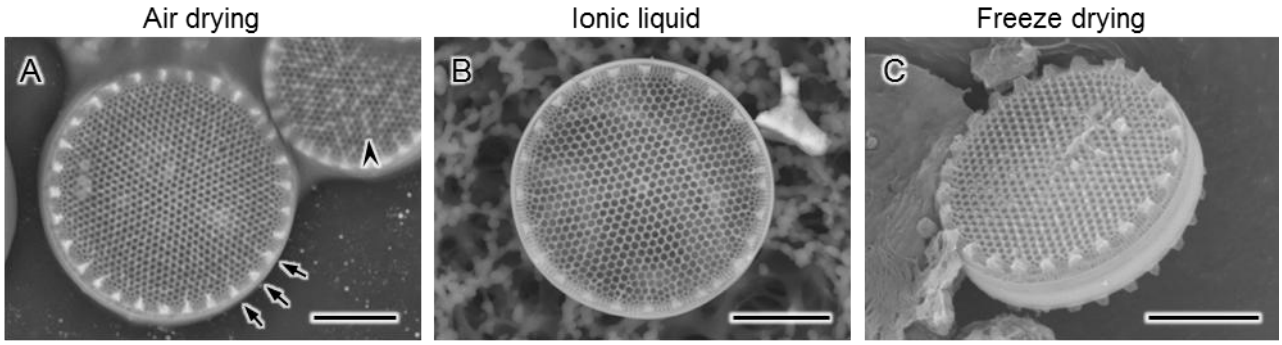

Fig. 2. *Thalassiosira* sp. as observed by three sample preparation methods. A: The specimen was air dried after chemical fixation and observed without metal coating. B : The sample was treated with 2% ionic liquid after chemical fixation and was observed without metal coating. C: After chemical fixation, the cell was dehydrated with ethanol, freeze-dried using t-butyl alcohol, and observed after metal coating. No significant difference was observed in the surface structure of the cells observed by any method. However, in the air-dried sample (A), surface charging was observed on small protrusions (strutted processes) at the periphery of the cell (indicated by arrows). In addition, charging artefacts were also present in parts of the honeycomb structure on the surface of the cell (arrowhead). When an ionic liquid was used (B), good image quality was obtained as in the case of freeze-drying (C). Scale bars: 10 µm.

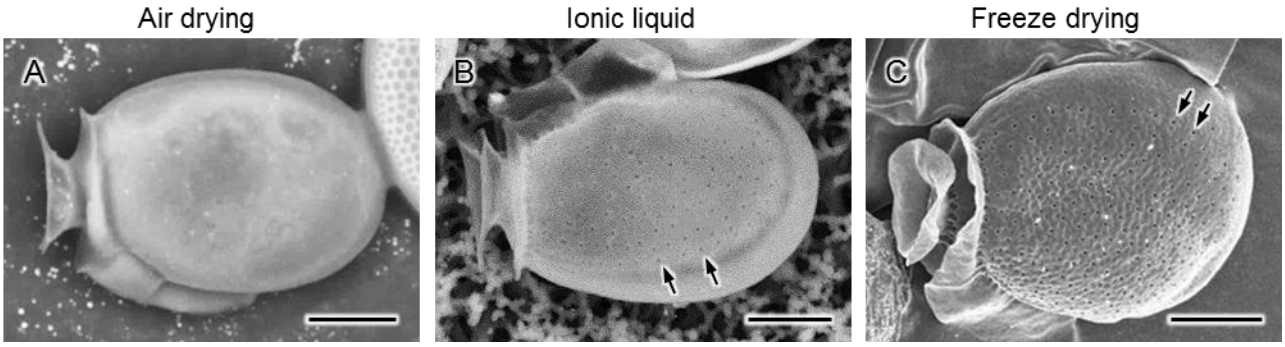

Fig. 3. *Dinophysis* sp. as observed by three sample preparation methods as described in Fig. 2. As in the case of *Thalassiosira*, there was no significant difference in the external shape of the cells regardless of the sample preparation method. However, in the air-dried sample (A), due to charging of the cell surface, it is difficult to distinguish fine holes on the cell surface. In the case of using ionic liquid (B), although the image contrast was lower than the metal-coated sample after freeze-drying (C), small pores on the cell surface were clearly observed (arrows). Scale bars: 10 µm.

chemically, treated with ionic liquid, and not coated with metal, the ultrastructure of the cell surface layer was preserved to fine details (Fig. 2B). In the cells treated with the ionic liquid, surface charging as observed in untreated cells was small, and good images comparable to the samples coated with metal after freeze-drying (Fig. 2C) were obtained.

In the samples collected from the field, the dinoflagellate *Dinophysis* sp. showed little cell deformation even after chemical fixation and air drying. However, small holes on the thecal plates were difficult to be observed, and a part of the cell showed surface charging (Fig 3A). In the cells treated with ionic liquid after chemical fixation, although the contrast was slightly lower, pores on the thecal plate could be observed well, and surface charging was scarcely detected (Fig 3B). For cells that were chemically fixed, ethanol dehydrated, freeze-dried with t-butyl alcohol and coated with metal, the contrast of the image was high and a good image was obtained (Fig 3C). The pores of the armor plate could be observed well, and the surface charging was small.

In the outdoor samples, the presence of various types of ciliates was also confirmed, but it was difficult to obtain a good image due to insufficient retention of cellular morphology. In addition, it was observed that some components of the washing buffer were partly crystallized, which sometimes hindered the observation. When the concentration of the ionic liquid was high, the ionic liquid remained excessive and became a state of submergence, and when the concentration was low, surface charging was likely to occur. Therefore, the concentration range of the observable ionic liquid was narrow, and the optimum concentration was 2-3%. When the sample was submerged by ionic liquid treatment, excessive ionic liquid could be effectively removed by using a membrane filter.

IV. Discussion

There are several merits to using ionic liquids in SEM observation. Since deformation often occurs when the sample is dried, it is common to prevent deformation during drying by using a freeze-drying apparatus or a critical point dryer. Since this process requires a considerable amount of work and time, it is one of the merits of being able to prevent deformation of cells to some extent by using ionic liquid. Although detailed data was not shown here, it was found that deformation of the cell can be prevented to some extent by selecting an appropriate ionic liquid concentration. Also, when trying to observe microscopic samples such as protists, it is more likely that cells will be lost as a result of frequent fluid exchanges in work such as dehydration. Although it is possible to lengthen the centrifugation time for recovering the cells, there is also a disadvantage that the cells deform accordingly. It is also a merit of ionic liquids that we can

reduce working procedures. Another merit is that conductivity can be given up to a fine structure. Complex protrusions on the surface of protists may sometimes cause surface charging due to insufficient metal reaching the interior even if metal coating is performed. When ionic liquid was treated, conductive ionic liquid was uniformly coated on the cell surface, and it was possible to observe finely the structure of the cell well.

Depending on the type of cells, the suitability for SEM observation using ionic liquid was different. The diatom was covered with a very hard shell and was sufficiently resistant to air drying treatment, so an ionic liquid was not necessary to retain the morphology of the cell. However, in observation with a low vacuum SEM without imparting conductivity on the surface of the sample, protrusions on the surface tended to cause surface charging. By applying ionic liquid to diatom, surface charging on the surface was greatly suppressed, and images close to the sample by freeze drying were obtained. Many dinoflagellate algae have hard shell but some do not. In case of having a hard shell, there is little deformation during sample preparation, but for those without shell, deformation is suppressed to some extent by applying ionic liquid and good images close to the freeze-drying method were obtained. Of dinoflagellates, those with a cell length of 20 µm or less are difficult to identify by optical microscopy and observation by SEM is often required. On the other hand, when taking samples from the field, fixing them, dehydrating them, applying freeze-drying and metal coating, it takes much time and labor, so when you need to observe a large number of samples or need data quickly, these efforts are often obstacles. However, if an ionic liquid is used, it is possible to quickly obtain images necessary and sufficient for species identification.

Ciliates are softer than diatoms and dinoflagellates and are very easy to deform. When cells are fixed and air-dried, the cells collapse and often do not retain the original shape. In addition, the shape of cilia on the cell surface was not adequately retained, and often dropped off. In this respect, ciliates were considered not to be suitable for observation using ionic liquids. However, from the observation of *Paramecium* in this study, it was revealed that the deformation of the cell was suppressed to some extent by using the ionic liquid, and the structure of the cell surface remained firmly. Cilia are often lost by ionic liquid treatment, but it seems that it may be more suitable for observation of the cell surface layer by dropping cilia.

Komai et al. observed pollen using ionic liquids and obtained good images [5]. When compared with their condition (1% ionic liquid), it was found that a slightly higher concentration is better for obtaining a good image of protists. This is a major factor in that the type of ionic liquid used is different (choline lactate), and when comparing pollen and protists, the hardness of the surface also differs. Also, it seems to reflect whether or not to preprocess with sucrose. It is considered necessary to study whether Komai's method can be applied to protists. Tsuda et al. tried various ionic liquids and reported ionic liquids suitable for cell observation and their concentrations and treatment times [7, 8]. In the future, it is desirable to search for ionic liquids that can be used to observe samples taken outdoors together, and to decide the optimum concentration and treatment method.

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