Introduction

A room temperature ionic liquid (RTIL) is an organic salt that is liquid at room temperature and has specific physical properties such as noncombustibility, no vapor pressure, high heat resistance, and high ionic conductivity. These unique properties have led many researchers to use RTILs in various fields, including electronics, chemistry, analysis, and catalysis [1-6]. Kuwabata et al. have reported the successful use of an RTIL for the electronic pretreatment of biological samples, such as insects, flowers, tissues, pollen, and cells, for scanning electron microscopy (SEM). As RTILs have high electrical conductivity and no very low vapor pressure, they can maintain their liquid state even in vacuum, making them suitable for use inside an SEM sample chamber, and also facilitate clear...
Scheme 1  Chemical structure of choline-type room temperature ionic liquid used in this study.

Scheme 2  Schematic illustration of the RTIL pretreatment process for SEM observation: (a) A drop of a suspension of wet biological samples is placed on a PTFE membrane. (b) The medium is aspirated before complete drying. (c) The RTIL is dropped on the sample. (d) Allowed to sit for 1 min. (e) Excess RTIL is wiped away. (f) The sample is introduced into the SEM chamber and observed.

Scheme 3  Conductive preparation for SEM compared with the RTILs pretreatment and conventional multi-step pretreatment.
SEM images. Several RTILs are suitable for pretreating samples for SEM including imidazolium salts, pyridinium salts and ammonium-type salts, as aqueous solutions. [6-8] Some imidazolium-type RTILs have high viscosity and low hydrophilicity and biocompatibility, however, the concentration of the RTILs solution needs to be optimized for different types of samples. We designed a novel, asymmetrical choline-like hydrophilic RTIL (Scheme 1) with improved hydrophilicity and wettability, [9, 10], and investigated its use for the pretreatment of samples for SEM without dilution. By fine-tuning the length of the alkyl chain and counter anion, we successfully synthesized an RTIL that had low viscosity and high hydrophilicity and biocompatibility. In addition, we successfully observed several biological samples, including bacteria, osteoblastic cells, microbes, bacteiocellulose, and epithelial and muscle tissue using an extremely simple and rapid pretreatment process that required only a drop of the RTILs for immersion (Scheme 2). The aim of this study was to extend the usefulness of this RTILs pretreatment for SEM observation using several types of wet biological samples.

Materials and Methods

2.1 Preparation of biological samples

Streptococcus mutans was incubated in 2% heart infusion medium with 1% glucose at 37 °C for 24 h. The algae were collected at Chidoriga-ike in Tokiwa park, Asahikawa city, Hokkaido prefecture, Japan. Most of them were green alga Pleodorina sp. by optical microscopic observation. Prepared eggs of Artemia salina were incubated in 2% NaCl aqueous solution at 30 °C for 24 h. Bloods samples of several animals were purchased from Cosmo Bio Co. (Tokyo, Japan), dispersed in phosphate buffered saline (PBS; pH 7.4) washed with PBS solution, and then red blood cells were collected by centrifugation.

2.2 Preparation of RTILs

The RTILs used in this study was ethyldimethylhydroxyethylammonium methylsulfonate (Scheme 1), which was synthesized from tetraaklylammonium bromide via an anion exchange reaction. The detailed synthesis and properties of this material are described in Ref. 9, 10.

2.3 RTIL pretreatment for SEM observation

The pretreatment process is illustrated in Scheme 2. Horse red blood cells (RBCs) were dispersed in PBS (pH 7.4), following which 20 mL of the suspension was placed on a hydrophilic polytetrafluoroethylene (PTFE) membrane filter (ADVANTEC®) and aspirated through a syringe filter. The aspiration was stopped before the suspension was completely dried, and the RBCs remaining on the membrane were immersed in a drop of the RTIL (10 μL). After 1 min, any excess RTIL on the PTFE was wiped away using Kim Wipes®. This entire pretreatment process was carried out at room temperature. Following this preparation, the RBC sample on the PTFE membrane was introduced into an SEM chamber (HITACHI, S-4800) under high vacuum and observation were made using an acceleration voltage of 5 kV. The other samples were also pretreated with the RTILs following aspiration. The details are illustrated in Scheme 2.

Results

Figure 1a and 1b show typical SEM images of horse RBCs that were pretreated with the RTILs. These cells were not destroyed and had the typical round shape of RBCs. Furthermore, the RBCs in these images had a diameter of 6.2 ± 0.9 μm, compared with 3.1 ± 0.4 mm for RBCs that were treated using conventional methods (Figure 1c). The RBCs that were observed through optical microscope also had a diameter of 5.5 ± 0.5 μm (Figure 1d). This result suggests that the RTILs treatment can allow cells to be observed as “living matter,” even under high vacuum conditions. Interestingly, a very small number of echinocytes were observed (Figure 1b); however, the deformation of these cells could not have been caused by any chemical effect of the RTIL. This finding suggests that the RTIL pretreatment also enables the observation of echinocytes with their living morphology. Similar observations were made for the RBCs of other animals. As shown in Figure 2a and 2c, SEM images of the RBCs of sheep and rabbits were also successfully obtained. Here the diameter of these cells were 5.7 ± 0.5 μm and 4.1 ± 0.4 μm, respectively, which are in good agreement with their OM images (Figure 2b and 2d, respectively). The diameter of these cells were 5.4 ± 0.4 μm and 3.7 ± 0.5 μm, respectively.
Figure 1 SEM images of horse RBCs pretreated with RTILs (a) and (b), with conventional methods (c), and their OM image (d).

Figure 2 SEM images of (a) sheep RBCs, (c) rabbit RBCs pretreated with RTILs and their OM image; (b) sheep, and (d) rabbit.
Figure 3a shows a typical SEM image of the green alga *Pleodorina* sp., following conventional pretreatment using gold (Au)-sputtering. As evident, the obtained images present distorted and collapsed shapes. By contrast, algae treated with the RTILs retained their characteristic spherical shape even under high vacuum (Figure 3b). Several tubular-shaped diatoms were also observed.

The OM and typical SEM images of the nauplius of *Artemia salina* pretreated with the RTIL are shown in Figure 4a and 4b, respectively. A clear shape and good contrast were observed not only for the whole body at low magnification (Fig. 4b) but also at high magnification, where fine structures such as the antennae were clearly observed (Figure 4c and 4d). The quality of these images was the same as those obtained using conventional carbon sputtering. However, carbon coated samples exhibited some destruction of the body (Figure 4e and 4f), whereas immersion in only RTILs avoided...
Figure 4 (a) OM image of nauplius of Artemia salina, and their SEM images pretreated with RTILs (b: low magnification, c and d: high magnification) and with conventional methods (e and f). A white arrow indicates destruction of the body.
this problem and maintained the 3D structure, even under high vacuum conditions, suggesting that immersion in only one drop of the RTILs was sufficient as an electrical conductivity pretreatment.

Figure 5 shows a typical SEM image of *S. mutans*, which is a type of gram-positive facultative anaerobe that can induce dental caries. These bacteria appeared cylindrical or tubular in shape and were approximately 1 μm in length and 0.5 μm in diameter. Some of them were also tethered to each other in a head-to-tail manner.

**Discussion**

In this study, high-quality SEM images of several wet biological samples were successfully obtained using the RTILs pretreatment. Moreover, the findings suggest that only a drop of the RTILs is required to provide an appropriate conductive preparation of the surface of these samples. Conventional methods for pretreating samples for SEM observation require several steps of treatment, such as chemical fixation, staining, dehydration, drying, and conductive sputtering (as shown in Scheme 3). On the other

![SEM images of S. mutans pretreated with RTILs.](image)

**Figure 5** SEM images of S. mutans pretreated with RTILs.
hand, the RTILs pretreatment provided high-quality SEM images using only immersion, representing a rapid and simple treatment that will be a useful tool for SEM observation.

The RTILs pretreatment also provided SEM images of the samples as “living matter,” Green algae that were treated with conventional Au-sputtering appeared distorted and collapsed in the SEM images, whereas those that were pretreated with the RTILs had a swollen spherical shape. Interestingly, RBCs that were pretreated using conventional methods were shrunkken in the SEM images, whereas those that were pretreated with RTILs retained the same size as in OM observations, despite being under high-vacuum conditions. Similarly, the body of Artemia salina, was crushed or destroyed easily following conventional pretreatment techniques, whereas the RTILs pretreatment had no such effect. The exact process by which the RTIL pretreatment works is currently unknown, but it is likely to be via one of the following mechanisms: (1) this hydrophilic RTIL was exchanged with water inside the body; (2) the RTIL penetrated the body and fixed it; or (3) the RTIL coated the sample surface and fixed it. Takaku et al. previously reported the successful observation of several biological samples such as insect, plants, and bacteria as living matter using SEM by coating their surfaces with a surfactant—referred to as a “Nano-suit.” [11] They also mentioned that some of the samples were still alive following observation under high vacuum conditions. By contrast, in a previous study, we found that human RBCs that were dispersed in the RTIL did not break even after soaking for 2 h; and after a longer period of soaking, some of the RBCs gradually expanded and finally exploded. [12] The cells that were dispersed in an imidazolium-type RTIL faded away and were destroyed. This suggests that the RTIL used in this study penetrates the cells and has a completely different mechanism of interaction with RBCs than imidazolium-type RTILs. However, the exact mechanism remains unclear and so will be the subject of future work.

Conclusions
In this study, we investigated the usefulness of electroconductive preparation of wet biological samples with the RTILs for SEM observation. We found that a single drop of the RTIL immersed the samples and provide high-quality SEM images. Compared with conventional multi-step conductive pretreatments for SEM, the RTILs pretreatment is significantly rapid and simple. In addition, the RTIL pretreatment allowed wet biological samples to be observed as “living matter,” retaining their swollen morphology, and avoiding their distortion and collapse, even under high vacuum conditions. Thus, this RTIL pretreatment could be a powerful tool for SEM, particularly when observing wet biological samples.

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References

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