ORIGINAL ARTICLE

Simple and Rapid Conductive Preparation of Wet Biological Samples for SEM Observation: Use of an Asymmetrical Choline-like Room Temperature Ionic Liquid as a Visualizing Agent

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Synopsis
In this study, we prepared a hydrophilic, choline-like room temperature ionic liquid (RTIL), and investigated its use as an electroconductive pretreatment for scanning electron microscopy (SEM) investigations of wet biological samples. Wet biological samples generally require pretreatment before SEM observation because of their properties. Conventional pretreatment methods consist of multiple tedious steps that take from several hours, to a day or more. In contrast, our pretreatment only requires the samples to be immersed in an RTIL. This gives the sample suitable electroconductivity for SEM analysis, which can then be carried outperformed rapidly. In addition, samples pretreated with RTILs can remain wet even in the vacuum chamber of SEM. This property allows morphological observation of wet biological samples in "a life-like manner" because our method avoids the chemical fixation, dehydrogation and drying processes required by conventional pretreatment. Moreover, some samples can be successfully visualized after pretreatment using RTILs without dilution. These results suggest that this method can allow simple and rapid conductive pretreatment of wet biological and insulating samples without optimizing their concentrations.

Key words*: room temperature ionic liquis, SEM visualization, simple and rapid conductive preparation*

Introduction

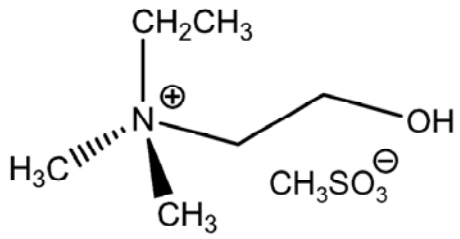
A room temperature ionic liquid (RTIL) is a molten organic salt that is very different from water and oil. Because RTILs have unique and specific physical properties, such as non-combustibility, no vapor pressure, high heat resistance, and high ionic conductivity, they have great potential for further development [1-4]. They have recently been used for electron microscopy observation because of their useful properties, such as fluidity, conductivity and no vapor pressure [5]. Because RTILs have no or very low vapor pressure, they can be maintained in a liquid state even under vacuum, such as in a

scanning electron microscopy (SEM) sample chamber. Their electrical conductivity can allow to observation of their SEM images as liquid state. It suggests that they can use for SEM visualizing agent when they coat on sample surface. Then they are suitable for pretreating samples for SEM. Recently, Kuwabata *et al*. reported that they succeeded in observation of SEM images of biological specimens, including insects, flowers, tissues, pollen, and cells [6-8]. In these cases, they used several ionic liquids, such as imidazolium salts, pyridinium salts, and ammonium-type salts, as aqueous solutions. We also prepared an imidazolium-type RTIL ethanol solution at a low concentration for pretreatment of several nanocarbon materials for SEM. We succeeded in visualization of these materials with a high resolution [9]. These results suggest that homogeneous coating on the surface is a key factor for high quality SEM observation. For improvement of high fluidity and hydrophilicity, we designed asymmetrical quaternary ammonium-type RTILs [10]. The chemical structures were close to a typical bioactive material, choline. The higher fluidity and hydrophilicity were expected for excellent affinity to biological wet samples compared with RTILs used previously. In this study, we investigated the usefulness of hydrophilic choline-like RTILs for conductive pretreatment of several insulating biological samples for SEM observation.

Materials and Methods

RTIL

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



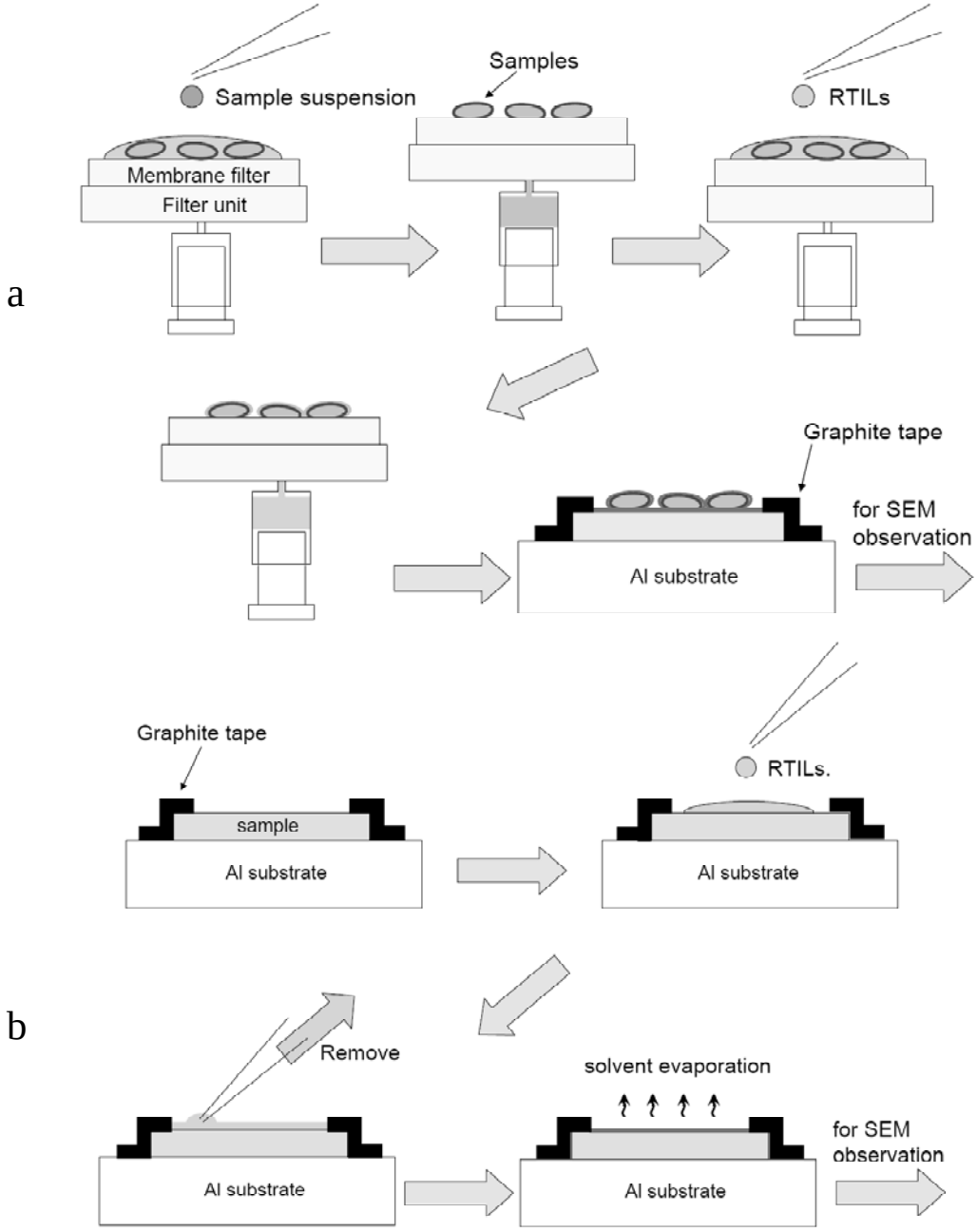
Scheme 1 Chemical structures of asymmetrical choline-like RTILs.

Preparation of biological samples

Mouse osteoblastic cells (MC3T3-E1) were seeded on a culture dish at a density of 2×10^4 cells/well and incubated for 48 h at 37 ˚C in a humidified atmosphere of 5% CO_2. The adherent cells were fixed on the dish with glutaraldehyde solution and then washed with phosphate-buffered saline (PBS). The details are described in Ref. [11]. Fresh red blood cells (RBCs) collected from a healthy donor were dispersed in PBS at pH 7.4 and then purified by centrifugation. *Acetobacter xylinum* dispersion was added to 20 mL of HS medium and incubated for 2 weeks at 30°C. After 2 weeks of incubation, a white cellulose gel was produced at the air–water interface of the medium. To lyse the werebacteria, the gel was immersed in 1% aqueous NaOH for a day, washed with distilled water, and freeze–dried. Other bacteria (Bacillus subtilis var. natto and Lactococcus family's bacteria) were derived from food samples (natto and yogurt, respectively) and purified by centrifugation.

SEM observation

We selected different methods of electroconductive pretreatment, depending on the nature of the samples. When the sample was suspended in solution, the suspension was placed on a hydrophilic polytetrafluoroethylene (PTFE) membrane filter (ADVANTEC, Tokyo, Japan) and aspirated through the filter with a syringe. The aspiration was halted before the suspension was completely dried, and the samples left on the membrane were immersed in a drop of the RTILs (tens of ⌈L) which was used without dilution. A minute later, any excess RTIL on the PTFE was wiped away with Kim Wipes (Kimberly Clark and Everett, WA, USA). The procedure is illustrated in Scheme 2(a). In contrast, when the samples were on a substrate, an aqueous solution of the RTIL was poured on the sample surface and the solution was quickly removed by a pipette as shown in Scheme 2(b). Any excess RTIL that remained on the surface was absorbed by careful patting with Kim Wipes. After the pretreatment described above, the edges and backside of the samples were affixed with electrically conductive tape (Nisshin EM, Tokyo, Japan) to an aluminum sample stub for SEM. Sample



Scheme 2 Schematic illustration of RTILs pre-treatment. a) for suspended samples, b) for samples on substrate.

morphology was analyzed with a scanning electron microscope (S–4800, Hitachi, Japan). The accelerating voltage was adjusted to 5 kV.

Results

Figure 1 shows a typical SEM image of MC3T3-E1 osteoblastic cells after pretreatment with a 10 % (v/v) aqueous RTIL solution. The cells were spindle-shaped and elongated in one direction. They also showed elongation of several filopodia that had an ultrafine structure (Fig. 1(b)). When the sample was pretreated with an RTIL solution at a lower concentration, the SEM image showed considerable noise, such as many

scanning lines. Figure 2 shows a typical SEM image of human RBCs. The cells observed are not destroyed and show the disk-like shape typical of RBCs. The RBCs in these images had diameter of approximately 8 μm. We also applied this method to some bacteria. Figure 3(a) shows a typical SEM image of *A. xylinum* on cellulose. *A. xylinum* is an acetic acid bacterium that can produce cellulose. An RTIL solution adjusted to 10% (v/v) was used. These bacteria appeared cylindrical or tubular in shape and approximately 2 μm in length and 0.5 μm in diameter. Some of them were tethered to each other in a head-to-tail manner (Fig. 3(a)). Figure 3(b) is an SEM image of *B. subtilis var. natto*, which was obtained from natto. *B. natto* is a gram-positive, catalase-positive bacterium. These bacteria appeared rod-shaped and were approximately 2–3 μm in length and 0.5 μm in diameter. They were tethered to each other in a

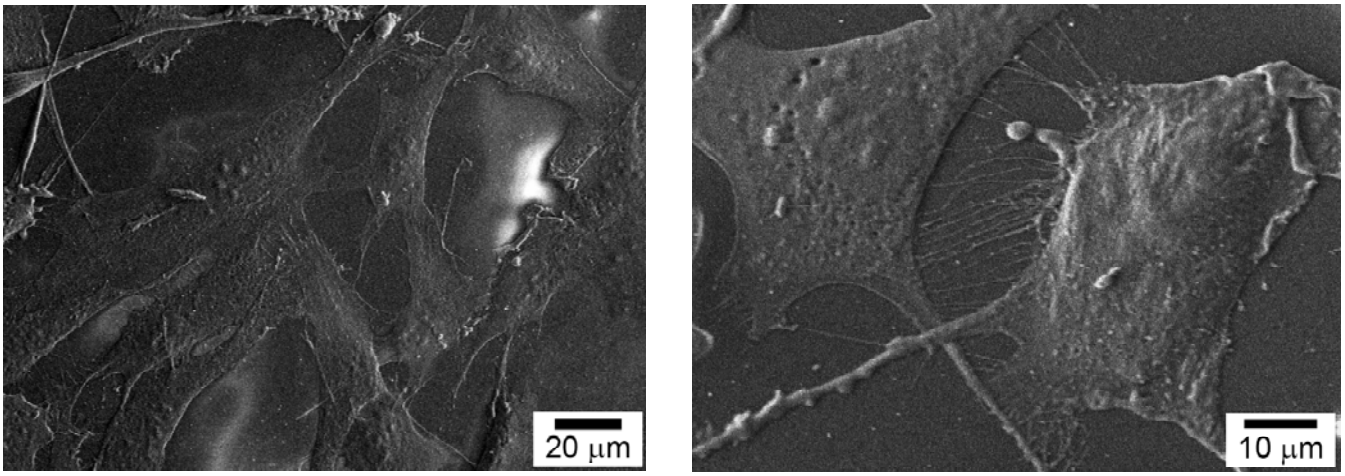


Figure 1 SEM images of mouse MC3T3-E1 osteoblastic cells pretreated with 10% aqueous RTIL.

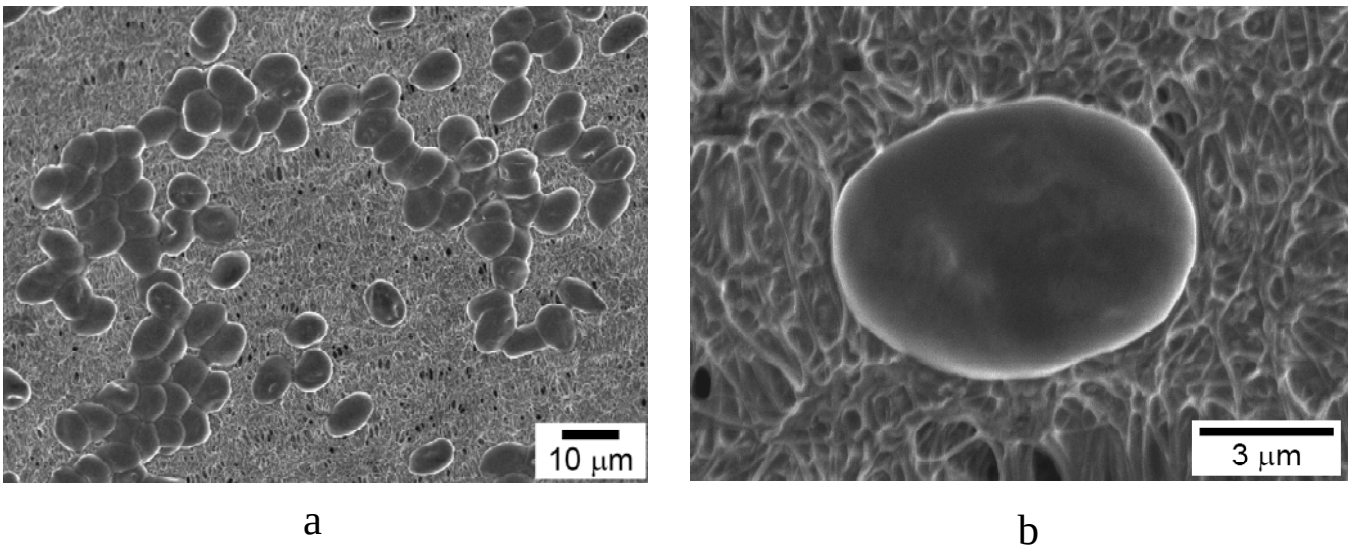


Figure 2 SEM images of RBCs pretreated with RTILs.

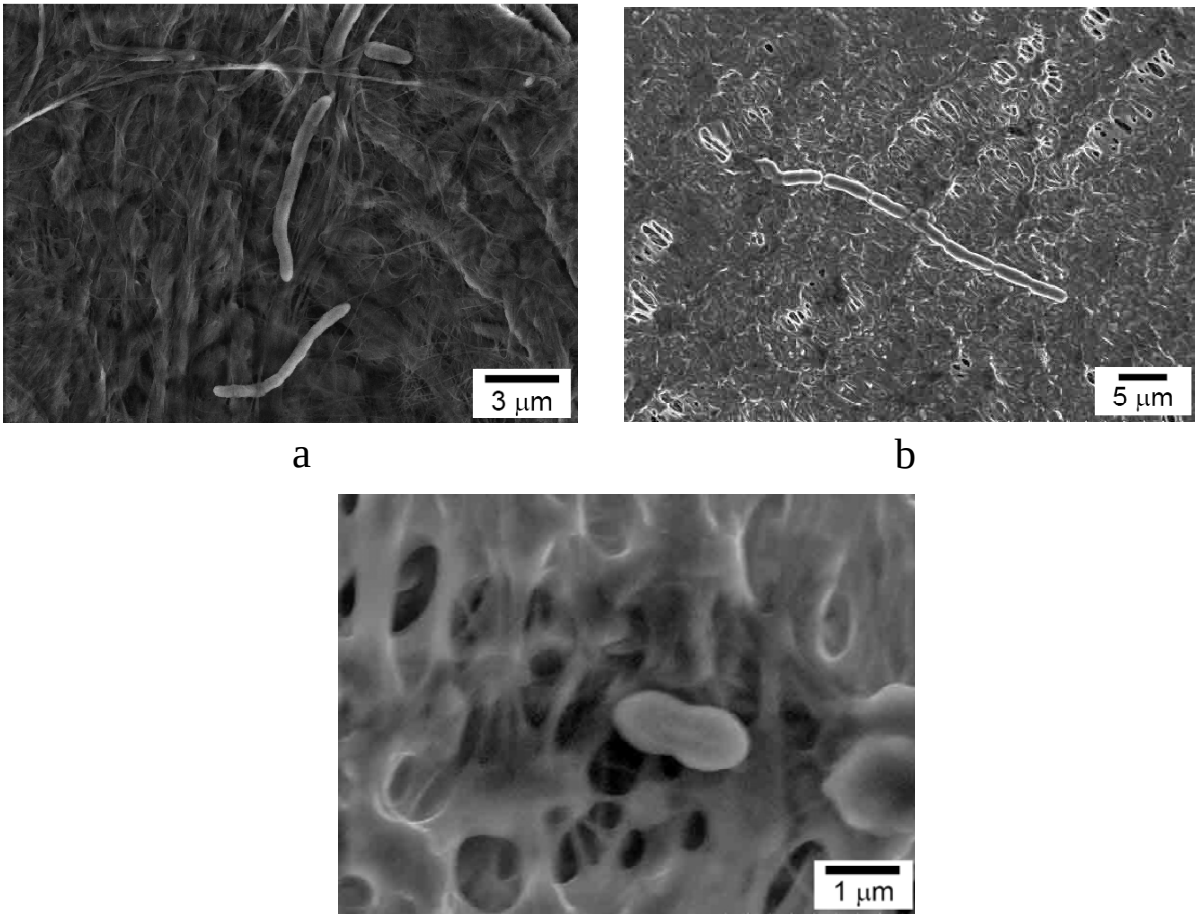


Figure 3 SEM images of (a) *A. xylinum*, (b) *B. natto*, and (c) *Lactococcus* family''s bacteria pretreated with RTILs.(a: 10% aqueous RTIL, b, c: RTILs without dilution).

head-to-tail manner, and then the pairs were also tethered in a head-to-tail manner (Fig. 3(b)). We also obtained *Lactococcus* family's bacteria, such as *Lactococcus lactis*, from yogurt. Their SEM images were also observed after IL pretreatment (Fig. 3(c)). These bacteria appeared ellipse-shaped and were approximately 0.5 μm in size. They were also tethered to each other in a head-to-tail manner.

Discussion

Figure 1 shows a typical SEM image of MC3T3-E1 osteoblastic cells after RTIL pretreatment. The concentration of the aqueous RTIL solution was adjusted to 10% (v/v). The cells were spindle-shaped and elongated in one direction (Fig. 1(a)). They also showed elongation of several filopodia that had an ultrafine structure (Fig. 1(b)). When the sample was pretreated with an RTIL solution with a lower concentration (0.1% or 1%), the SEM image showed considerable noise, such as many scanning lines. These lines resulted from the surface possessing insufficient electrical conductivity under these pretreatment conditions. To obtain clear SEM images using this pretreatment method, the amount of RTIL on the sample surface is a key factor. For efficient pretreatment, it is important to optimize the concentration, and amount of the dropped solution, and the immersion time.

As shown in Fig. 2, RBCs pretreated with RTILs had a diameter of approximately 8 μm. When RBCs were observed with a conventional optical microscope, their diameter was close to that observed for cells pretreated with RTILs. RBCs pretreated using conventional methods usually show a smaller diameter (approximately

5–6 mm) [12]. This indicates that the cells are shrunken by conventional pretreatment through multiple tedious steps. This result suggests that our pretreatment method can avoid sample shrinkage during conductive treatment of insulating biological samples.

The RTILs used in this study has high hydrophilicity and fluidity compared with conventional RTILs. In addition, its chemical structure is close to that of choline, which is a typical bioactive material. These properties enable it to be an excellent visualizing agent for the observation of insulating biological wet samples by SEM. Its physical and chemical properties can be tuned by modification of the structures of the cationic and anionic portions of the compound. Although we have not yet adequately addressed the mechanism of this electroconductive pretreatment, this method can serve as a powerful tool during electron microscopy of biological samples.

Conclusion

In this study, we investigated a simple and rapid conductive pretreatment method for biological wet sample. Using a hydrophilic choline-like RTIL as a visualizing agent, we obtained SEM visualizations of insulating biological samples under wet conditions. This method allows the rapid electroconductive pretreatment of samples. Because the method avoids dehydrogenation and drying processes, the sample morphology can be maintained in a life-like manner.

Acknowledgments

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