Electron Microscopy Imaging Applications of Room Temperature Ionic Liquids in the Biological Field – *A Review*

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Biographical Sketches:

Liza-Anastasia DiCecco is a Ph.D. candidate in Materials Science and Engineering at McMaster University where she holds a prestigious Vanier Canada Graduate Scholarship, a top doctoral distinction in Canada. She utilizes and develops advanced microscopy techniques to characterize mineralization processes and bone structure. Notably, her research interests center on liquid-phase imaging methods including liquid-phase transmission electron microscopy and exploring the use of ionic liquids to observe cells, biofilms, bone, and other biological samples in electron microscopy systems.

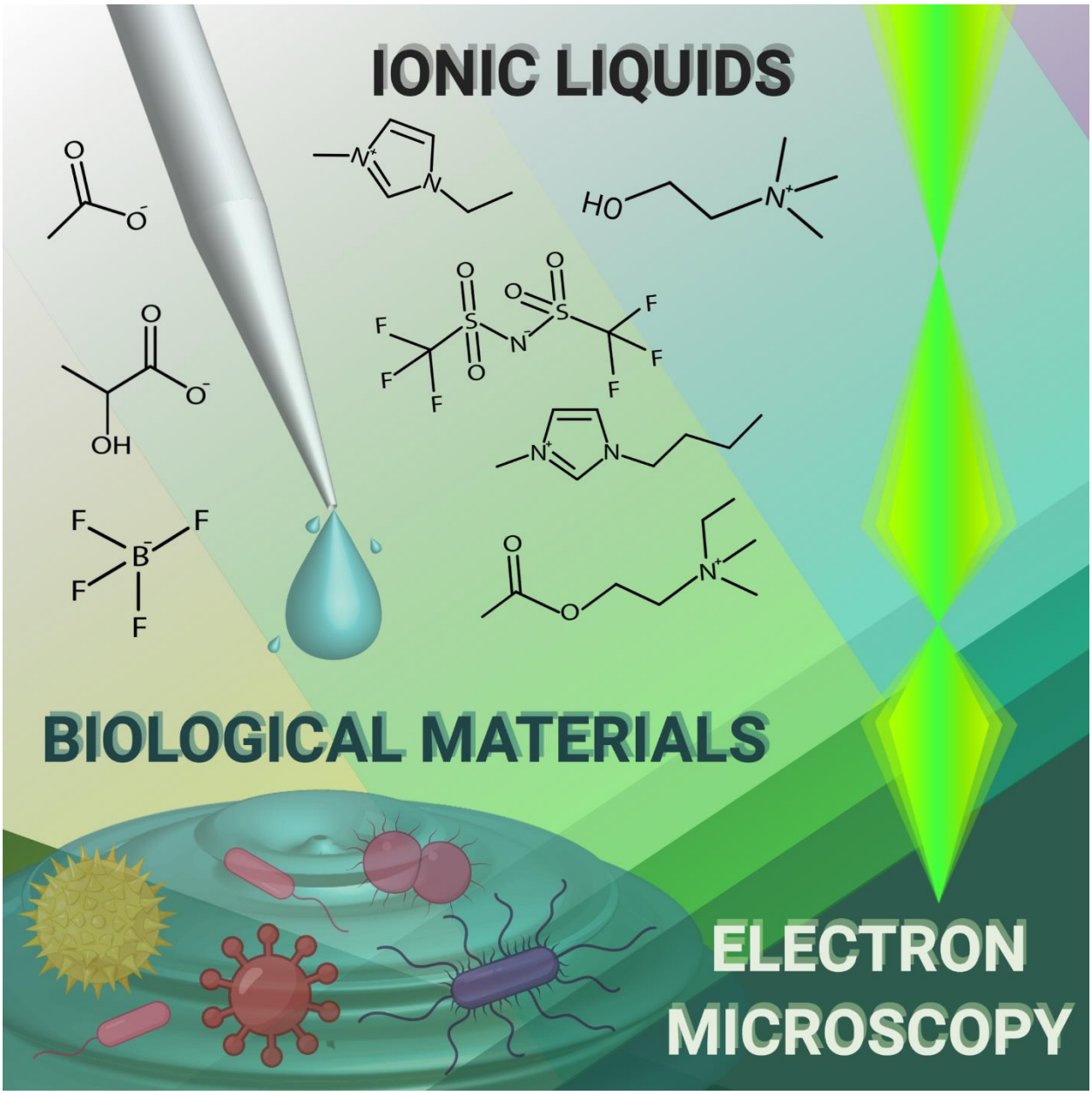
Kathryn Grandfield received her Ph.D. in Engineering Sciences in 2012 from Uppsala University prior to completing postdoctoral training at the University of California San Francisco. Currently, she is an Associate Professor in Materials Science and Engineering at McMaster University and Canada Research Chair in Microscopy of Biomaterials and Biointerfaces. Her group focuses on the development of biomaterials and multi-scale characterization techniques for biomineralization, osseointegration and live-cell imaging.

Abstract:

For biological imaging using electron microscopy (EM), the use of room-temperature ionic liquids (RTILs) has been proposed as an alternative to traditional lengthy preparation methods. With their low vapour pressures and conductivity, RTILs can be applied onto hard-to-image soft and/or wet samples without dehydration – allowing for a more representative, hydrated state of material and opening the possibility for visualization of *in situ* physiological processes using conventional EM systems. However, RTILs have yet to be utilized to their full potential by microscopists and microbiologists alike. To this end, this review aims to provide a comprehensive summary of biological applications of RTILs for EM to bridge the RTIL, *in situ* microscopy, and biological communities. We outline future research avenues for the use of RTILs for the EM observation of biological samples, notably i) RTIL selection and optimization, ii) applications for live cell processes and iii) electron beam and ionic liquid interaction studies.

Keywords:

Ionic Liquids, Electron Microscopy, Biology, Life Science, *in situ* Electron Microscopy, Imaging



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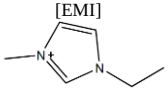
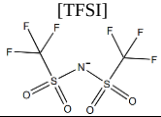
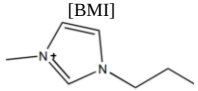
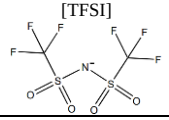
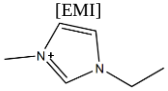
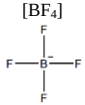
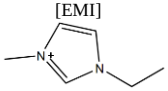
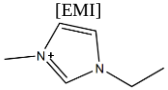
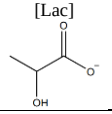
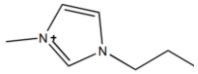
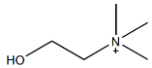
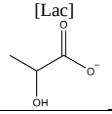
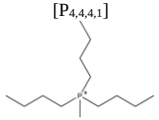
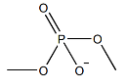
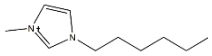
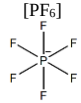
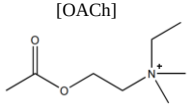
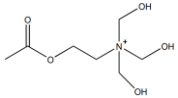
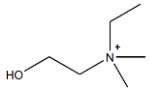
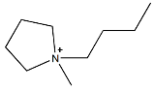
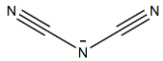
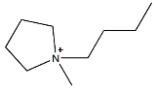
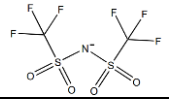
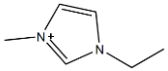
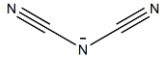
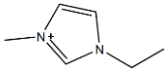
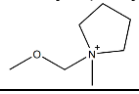
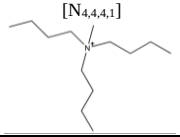
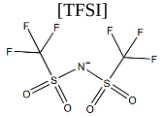
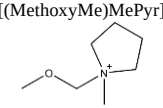
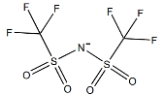
1. Introduction

Room temperature ionic liquids (RTILs), also referred to as molten or fused salts, are composed of ions and short-lived ionic pairs that are liquid at room temperature. They are known for their attractive properties such as low vapour pressure, high ionic conductivity, non-combustibility, and capacity to dissolve many kinds of substances.[1–4] In the last forty years, water-stable RTILs have been studied in a wide range of applications within chemical fields,[1,5,6] such as for biocatalysis and biotransformations,[7–9] electrolytes for electrochemical applications,[10] specifically for batteries[11–13] and electrodeposition,[14] micro-CT imaging,[15] and lubrication,[16] among others. However, only recently has the potential of RTILs as a preparation method for biological sample imaging in electron microscopy (EM) been explored.[17–21] Applications of RTILs in the place of conductive coatings,[10,22,23] provide comparable resolution and contrast to that of conventional preparation methods for biological EM imaging.[24] Notably, their low vapour pressures enable samples wetted with ionic liquid solutions to be imaged under high vacuum conditions.[18] Combined, the unique properties of RTILs allow them to play the role of a solvent, a discharging layer for electron charge, and a vacuum-stable solution that enables hydrated EM imaging at high vacuum. While conventional methods of preparing biological samples for EM constitute dehydration and fixation protocols that can significantly alter the structure of these materials,[25–27] preparation methods with RTILs involve simpler and shorter steps.[17–21] The unique properties of RTILs have provided new avenues for EM applications involving soft and/or hydrated biological materials as well as novel mediums for *in situ* EM. However, RTILs in EM have yet to be explored to their full potential by researchers including microscopists and microbiologists.

This review paper aims to offer a fresh perspective on the capabilities, challenges, and outlook of using RTILs in the EM study of biological materials. Here, we present the use of RTILs in common preparation schemes for biological samples and highlight their use in a diverse range of applications, summarized in Tables 1 & 2. We intend this review paper to inspire others working with biological samples to explore the use of RTILs to investigate new avenues for hydrated sample preparation.

Table 1. Chemical and physical properties of RTILs mentioned in this review

RTIL Index	Chemical Name	RTIL [Cation][Anion]		Molecular Weight [g/mol]	Viscosity [cP]	Density [g/cm^3]	Hydrophilicity	Ref.(s)
1	1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[EMI] 	[TFSI] 	391.3	56 (293K)	1.54 (293K)	Hydrophobic	[28,29]
2	1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[BMI] 	[TFSI] 	419.4	52 (293 K)	1.44 (293 K)	Hydrophobic	[23,29,30]
3	1-ethyl-3-methylimidazolium tetrafluoroborate	[EMI] 	[BF_4] 	198.0	37 (298 K)	1.28 (298 K)	Hydrophilic	[21], [31,32]
4	1-ethyl-3-methylimidazolium acetate	[EMI] 	[AcO] 	170.2	144 (298 K)	1.10 (298 K)	Hydrophobic	[31]
5	1-ethyl-3-methylimidazolium lactate	[EMI] 	[Lac] 	200.2	184 (303 K)	1.14 (298 K)	Hydrophilic	[34]
6	1-butyl-3-methylimidazolium tetrafluoroborate	[BMI] 	[BF_4] 	226.0	99 (298 K)	1.20 (298 K)	Hydrophilic	[31,32]
7	2-hydroxy-N,N,N-trimethyl-Ethanaminium 2-Hydroxypropionate Methylglycolate (Choline lactate)	[Ch] 	[Lac] 	193.2	895 (303 K)	1.14 (298 K)	Hydrophilic	[34]
8	tri-n-butylmethylphosphonium dimethylphosphate	[$P_{4,4,4,1}$] 	[DMP] 	342.4	439 (298 K)	1.03 (303 K)	Hydrophilic	[35]
9	1-hexyl-3-methylimidazolium hexafluorophosphate	[HMI] 	[PF_6] 	312.2	497 (298 K)	1.29 (298 K)	Hydrophobic	[30]
10	2-carboxylethylethyldimethylammonium methylsulfonate	[OACh] 	[MS] 	255.3	-	1.31 (298 K)	Hydrophilic	[36]

11	2-carboxylethyl-tris(2-hydroxylethyl)ammonium methylsulfonate	[XXCh] 	[MS] 	289.3	-	1.37 (298 K)	Hydrophilic	[36]
12	Hitachi Ionic Liquid HILEM© IL1000 (2-hydroxyethyl-ethyl-dimethyl-ammonium methylsulfonate)	[MECh] 	[MS] 	213.3	-	-	Hydrophilic	[36], [37], [38]
13	1- butyl-1- methylpyrrolidinium dicyanamide	[BMP] 	[DCA] 	208.3	50 (293K)	1.02 (293K)	Hydrophilic	[28]
14	1- butyl-1- methylpyrrolidinium bis(trifluoromethylsulfony)imide	[BMP] 	[TFSI] 	422.4	307 (293K)	1.46 (293K)	Hydrophobic	[28]
15	1-ethyl-3-methylimidazolium dicyanamide	[EMI] 	[DCA] 	177.2	21 (293K)	1.11 (293K)	Hydrophilic	[28]
16	1-MEethyl-3-methylimidazolium methylphosphonate	[EMI] 	[MP] 	316.3	107 (298K)	1.21 (293K)	Hydrophilic	[39]
17	1-methoxymethyl-1-methylpyrrolidinium tetrafluoroborate	[(MethoxyMe)MePyr] 	[BF_4] 	217.0	95 (298K)	-	Hydrophilic	[40]
18	Tributylmethylammonium bis(trifluoromethylsulfonyl)amide	[$N_{4,4,4,1}$] 	[TFSI] 	480.5	551 (298K)	1.27 (293K)	Hydrophobic	[40]
19	1-methoxymethyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)amide	[(MethoxyMe)MePyr] 	[TFSI] 	243.2	40 (298K)	1.46 (293K)	Hydrophobic	[40]

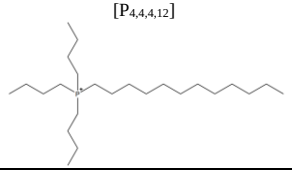
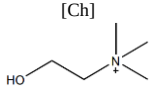
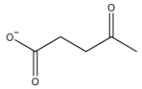
20	tri-n-butyldodecylphosphonium tetrafluoroborate	$[P_{4,4,4,12}]$ 	$[BF_4]$ 	458.7	1310	-	Hydrophobic	[20]
21	2-hydroxy-N,N,N-trimethyl-Ethanaminium 4-oxopentanoate (Choline Levulinate)	[Ch] 	[Lev] 	219.3	-	-	Hydrophilic	[41]

Table 2. Select RTIL treatment for biological material applications (RTIL: room temperature ionic liquid; SE: secondary electron; BSE: backscatter electron; SEM: scanning electron microscopy; S1: RTIL addition method (Fig. 1B); S2: RTIL immersion method (Fig. 1B); Var.: variable)

Biological Material Sample Observed	RTIL Used (Index # from Table 1)	Initial Sample Condition	Treatment Method and Time	v/v% RTIL solution (optimal in parenthesis if applicable)	EM Observation Parameters (optimal in parenthesis if applicable)	Authors, Reference, & Year
Microbial Studies						
Grains of star sand (*foraminifera* shells)	1	-	S2	100%	20kV SE-SEM	Kuwabata et al.[22] 2006
Basidiospores (*Laccaria, Lactarius lignyotus*, & *Russula integra*)	3	Fixed, hydrated	S2: 1 minute	Var. 1-100% (5-10%)	15kV SE-SEM	Yanaga et al.[19] 2012
Aspergillus fumigatus, mixed culture yeast (*Saccharomyces cerevisiae*) and bacteria (bac)	12	-	-	Var. (10%)	10kV SE-SEM, 2-3kV FESEM, 15kV for VP-SEM	Joubert and McDonald[37] 2016
Protists (*Paramecium caudatum, Thalassiosira*, & *Dinophysis*)	6	Fixed, hydrated	S2: 15 minutes	2%	BSE-SEM, low vacuum	Ishida et al.[42] 2016
Staphylococcus aureus	6, 12	Unfixed, hydrated	S2: 5 minutes	50%	15kV SEM	Kawai et al.[43] 2013
Leptospira biflexa, *Salmonella* Senftenberg, vaccinia, and Ebola virus	6	Unfixed, hydrated	S1: 60 seconds	2.5%	4kV SE-SEM	Golding et al.[21] 2016
Streptococcus mutans biofilm	2, 4, 7, 20	Unfixed and fixed, hydrated	S1: 10 minutes	Var. 1-20% (RTILs 7 & 4 – 10%)	Var. 1-20kV SE-SEM (5kV), 200kV TEM	Asahi et al.[20] 2015
Herpes simplex virus 1	3-7, 21	Unfixed, hydrated	S1: 3 minutes	2%	TEM	Tsuda et al.[41] 2018
Cellular Biology						
A549 (adenocarcinomic human alveolar basal epithelial cells) cells on wrapping film & Panc-1 (pancreatic carcinoma)	2, 3, 6, 14, 17,18,19	Fixed and/or treated with 50X diluted colloidal gold labeled IgG for improved contrast	S1: 60 second	Var. 0-75% (RTILs 3 & 6 – 10-25%)	Var. kV SE-SEM (0.9-1.2kV)	Ishigaki et al.[40,44,45] 2011
Mouse-derived fibroblast L929 cells	5, 7	Unfixed, hydrated	S2**:** RTIL 5 for 60 seconds; RTIL 7 for 10 seconds	0.5%	SE-SEM	Tsuda, et al.[18] 2011
Chinese Hamster Ovary (CHO) cells	12	-	7 -	10%	10kV SE-SE4M, 2-3kV FESEM, 15kV for VP-SEM	Joubert and McDonald[37] 2016
Indian muntjac spread chromosomes and polyamine (PA) isolated human chromosomes from HeLa cell line	3, 5, 6	Fixed, hydrated	S1: 1 minute	Var. 0.1-3% (RTIL 6 – 0.1-1%)	Var. 1.5-10kV SE-SEM (1.5kV), high and low vacuum	Dwiranti et al.[46] 2012
Forelimb buds of embryonic mice	12	Fixed, hydrated	S2: 60 seconds	-	SE-SEM	Iezaki et al.[47] 2018
Human osteoblast-like Saos-2 cells on untreated and laser-modified Ti surfaces	3	Live, unfixed, hydrated	S2: 5 minutes	5%	BSE-SEM, low vacuum	Lee et al.[48] 2020
Plant Structures						
Seaweed leaf	2, 6	Dried, re-hydrated	S2: 2 hours	100% (RTIL 6)	SE-SEM	Arimoto et al.[23] 2008
Seaweed leaf	2, 6, 10,11,12,	Dried, re-hydrated	S2: 2 hours	100%	15kV SE-SEM	Kawai et al.[36] 2011
Pollen from a stamen of *Camellia sasanqua (C. sasanqua)*	6, 12	Unfixed, hydrated	S2: 30 minutes	0.5g	15kV SEM	Kawai et al.[43] 2013

Pollen from *Lillium* "Casa Blanca"	2	Unfixed, hydrated	S2: 30 seconds	5%	10kV SE-SEM	Tsuda, et al.[18] 2011
Pollen from *Primula juliae, Anemone coronaria, Leucoglossum paludosum, and Lathyrus odoratus*	6	Unfixed, hydrated	S2: 600 second	10%	5kV SE-SEM	Tsuda, et al.[18] 2011
Mature pollen grains of *Lilium* cultivar	7	Unfixed, hydrated	S2: 10 seconds	Var. 1-100% (1 & 5%)	5kV, SE-SEM	Komai et. al.[49] 2014
Petal and stamen of Asteraceae flower	2	Unfixed, hydrated	S2: 30 seconds	5%	10kV SE-SEM	Tsuda, et al.[18] 2011
Pelargonium leaf, Pollen grains	12	-	-	Var. (10%)	10kV SE-SEM, 2-3kV FESEM, 15kV for VP-SEM	Joubert and McDonald[37] 2016
Dried wood *(Cryptomeria japonica)*	4	-	S2: 60 seconds	5%	5kV SE-SEM	Tsuda et al.[17] 2012
Modern wood (*Cryptomeria Japonica, Larix gmelinii*, Zelkova serrata Makino, *Fagus crenata Blume*) & archeological wood (*Chamaecyparis Obtuse)*	16	Originally dried and then boiled in water	S2: 10 minutes	Var. 1-30% (10%)	10kV SE-SEM (10kV)	Yamashita et al.[39] 2018
Modern wood, archeological wood (violin c. 1750, 18th-century spruce wood, Chinese zither)	1, 13, 14, 15	-	S2: 30 seconds	Var. 5-100% (RTIL 14 – 7.5%)	5kV SE-SEM	Lu et al.[28] 2019
Arthropods						
Scaled butterfly wing	1	Unfixed, hydrated	S2: 60 seconds	5%	10kV SE-SEM	Tsuda et al.[17] 2012
Small Crustaceans (*Gammaridea, Tanaidacea*, and *Myodocopida*)	12	-	S2: 1-3 hours	10%	5kV SE-SEM	Shiono et al.[38] 2014
Yellow jacket (*Vespula*)	2	Unfixed, hydrated	S2: 60 seconds	5%	10kV SE-SEM	Tsuda et al.[18] 2011
Live Tick (*Haemaphysalis flava*)	-	Unfixed, live	-	-	SEM	Ishigaki et al.[50] 2012
Other Biological and Related Specimens						
Bacterial cellulose gel (produced by *acetobacter xylinum*), red king crab shell (tsubugai), Neptune Whelk shell (tsubugai), scallop shell (hotate)	7	Freeze dried	S1: <30 seconds	Var. 0.1-10% (10%)	5kV SE-SEM	Abe et al.[51] 2012
Villi of mouse small intestine	3	Unfixed, hydrated	S2: 600 seconds	5%	5kV SE-SEM	Tsuda et al.[18] 2011
Epithelial and muscular chicken tissue	6, 12	Unfixed, hydrated	S2: 30 minutes	100%	15kV SE-SEM	Kawai et al.[36] 2011
Liposomes	5, 7	Hydrated	S1	Var. 10-30% (10%)	TEM	Hayakawa et al.[52] 2013
Carbon nanotubes (CNTs), fullerene C_{60} nanocrystals and nanowhiskers	9	-	S1: <30 seconds	Var. 0.01-0.1% (0.02%)	5kV SE-SEM	Abe et al.[53] 2013
Hydrous superabsorbent polymer (SAP)	3-8	Dry and hydrous SAP particles (pretreated with water overnight)	S1: 5 minutes	Var. 10-100% (RTILs 4 & 8 – 100%)	5kV SE-SEM	Tsuda et al.[34] 2014

2. Brief Historical Review

Early work produced by Hurley et al. in 1951 highlighted initial research in the field of ionic liquids with the exploration of fused quaternary ammonium salts used for electrodeposition of metals.[54] However, they found the ionic liquid solution was highly sensitive to moisture and unstable at room temperature,[54] which remained a limiting factor in their application for years to come. Further development of RTILs was pioneered by Wilkes et al. starting with their key 1982 published work,[5] though it was the 1992 article produced by Wilkes et al. that revolutionized the field by presenting the first water-stable RTIL, 1-ethyl-3-methylimidazolium tetrafluoroborate or [EMI][BF_4].[1] In parallel to Wilkes, Cooper and O'Sullivan prepared 1-ethyl-3-methylimidazolium with triflate or [EMI][CF_3SO_3], a novel water-stable RTIL.[6]

Previously limited by chemical instability in the presence of moisture, the new generation of water-stable RTILs have received wide acclaim for their synthetic and electrochemical applications, with an electrochemical window of larger than three Volts.[4] Since then a variety of commercially available water-stable ionic liquids have been developed featuring various cationic and anionic combinations, each comprising a unique subset of physicochemical properties. Also referred to as "designer solvents,"[55] ionic liquids can be defined as a material in the liquid state at or below 373 K (100 ºC), composed entirely of cations and anions that do not contain a molecular solvent.[4] Those that remain liquid at or below room temperature (298 K) are more specifically referred to as RTILs.[56] Table 1 summarizes select RTILs commonly used in EM applications, their chemical structures, and other properties, such as molecular weight and hydrophobicity.

The first application of RTILs in EM was not shown until 2006 by Kuwabata et al. in their fundamental work which describes the first published observation of RTILs in scanning EM (SEM).[22] They featured the use of [EMI][TFSI] applied to the star-shape shells of *Foraminifera*, that post-RTIL treatment exhibited a significant reduction of charging effects in SEM compared to untreated shells.[22] A simple immersion method was used where a sample is directly dipped into an RTIL and excess liquid is removed, which allowed the shell's features to be imaged efficiently and clearly.[22] In electron microscopy, RTILs lend similar electrical conductivity to those of metal or carbon coatings commonly used in EM sample preparation.[10,22,23] Electron ejection from RTILs has been shown to be effective due to the solvation nature of the condensed ions which compose this solution,[44,57] leading to improved electron signal detection in EM.

Further to this, Kuwabata et al. postulated if the morphology of samples is not influenced by replacing water constituents of biological samples with RTILs, they could be used to image hydrated samples in normal high-vacuum EM modes.[22] Arimoto et al.'s subsequent work using both [BMI][TFSI] and [BMI][BF_4] for imaging rehydrated seaweed demonstrated RTIL treatment could facilitate imaging hydrated specimens through water replacement with RTIL.[23] Arguably, these works[22,23] paved the way for the consideration of RTILs as treatment methods for biological samples in EM studies, with several works since exploring the application of RTILs in EM in the biological field, as discussed in the following subsections. A summary of biological applications of RTILs in EM is presented in Table 2.

3. Challenges of Biological Sample Imaging in Electron Microscopy and Advantages to RTIL Application

Prior to introducing applications in which RTILs have been used for imaging biological samples, it is important to discuss the current protocols and challenges with biological sample preparation, particularly of soft and/or wet samples for EM. EM generally must be performed under high-vacuum, low moisture conditions on conductive, dry specimens with negligible vapour pressure. Moreover, to probe specimens with high resolution, electron charging effects must be mitigated.

Traditional EM preparation methods for biological materials, such as cells and soft tissue, require hours of work prior to observation. An example of a typical workflow for a traditional cell treatment methodology for SEM is shown in Fig. 1A, where a biomaterial disc is considered after being incubated with cells. First, a fixation media such as glutaraldehyde can be applied which creates crosslinks between proteins and fixes membranes for shape preservation. However, through fixation, the soft fluid-like behaviour of the membrane is compromised, proteins lose their function, and cells quickly die.[58] Moreover, the high-vacuum EM necessitates biological samples to be dehydrated to maintain the vacuum, prevent damage to the instrument, and mitigate sample evaporation. This may be accomplished via dehydration in increasing concentrations of ethanol followed by critical-point or freeze-drying. Fixation and

subsequent dehydration are known to influence the structure of biological samples, leading to significant morphological changes such as shrinkage or wrinkling and potential imaging artifacts in EM.[58,59] This poses a greater limitation of the observations made with EM methods, being that biological structures observed in EM may not be representative of the naturally hydrated state. In consideration of this, users can alternatively employ more involved critical point drying processes which offer improved preservation of the morphological structures of samples but with the trade off of increased complexity in preparation.

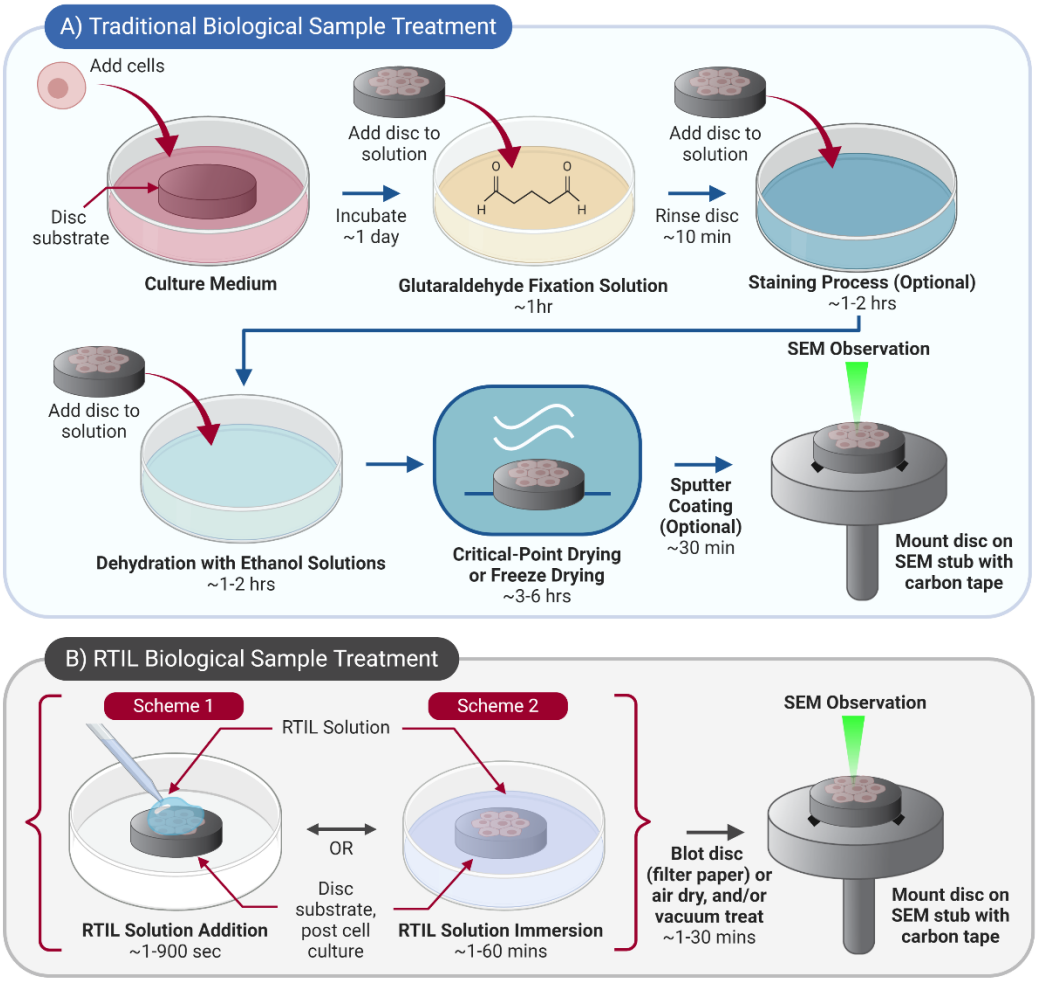


Figure 1. A) Illustration of the traditional biological sample preparation treatment procedure for SEM observation of cells. B) Illustration of RTIL biological sample treatment procedure, highlighting RTIL solution via addition (Scheme 1) or immersion (Scheme 2) techniques to prepare cells for SEM observation. Note the decrease in preparation time from longer than six hours to minutes. Image created with BioRender.com.

Biological specimens are most commonly insulators, soft, and composed of low atomic number elements, which render samples susceptible to several chemical and physical electron beam damage phenomena. Relevant damage examples include electrostatic charging effects, burn artifacts, carbon deposition from sample preparation itself or storage, and sample degradation,[60] which can of course be exacerbated further by poor vacuum conditions. To prevent artifacts related to conductivity, samples are often sputter-coated with a metal or carbon to improve surface conductivity. Sputter coating may result in non-uniform thickness over the surface of the substrate, a particular challenge for samples with distinct geometries, such as biological entities. Alternatively, methods that employ heavy metal stains, such as uranyl acetate, lead citrate and osmium tetroxide, can be used with the disadvantages of toxicity, and potential for altering sample morphology.[25,27,61]

Environmental-SEM (ESEM) and Cryogenic-EM (Cryo-EM) have been used as alternative methods to observe biological materials, suspended solutions, and liquid-solid interactions.[62–66] However, shortcomings and limitations

to these techniques for hard-to-image soft and/or wet samples[62,67–69] have resulted in the exploration of new methods to facilitate EM observation of biological specimens. In contrast to these established techniques, RTILs leverage properties that, in theory, are better equipped to handle the unique demands of biological specimens. By maintaining the hydrated nature of these specimens, RTIL treatments may be able to preserve delicate morphological structures often damaged through dehydration, providing a means to image samples in a representative state of their true size and morphology.[21] Golding et al. suggest RTILs can provide superior capabilities of conserving the size of microbes while minimizing the effects of cracking and wrinkling.[21] Figure 2 featured from their work provides a visual on how RTIL treatment for imaging various microbes compare to conventional preparation methods, notably sputter coating for SEM and negative staining for TEM imaging.[21] They posit that RTIL treatment can produce images of similar quality to traditional sputter coating in a fraction of the time,[21] without the need of specialized equipment required for ESEM or Cryo-EM.

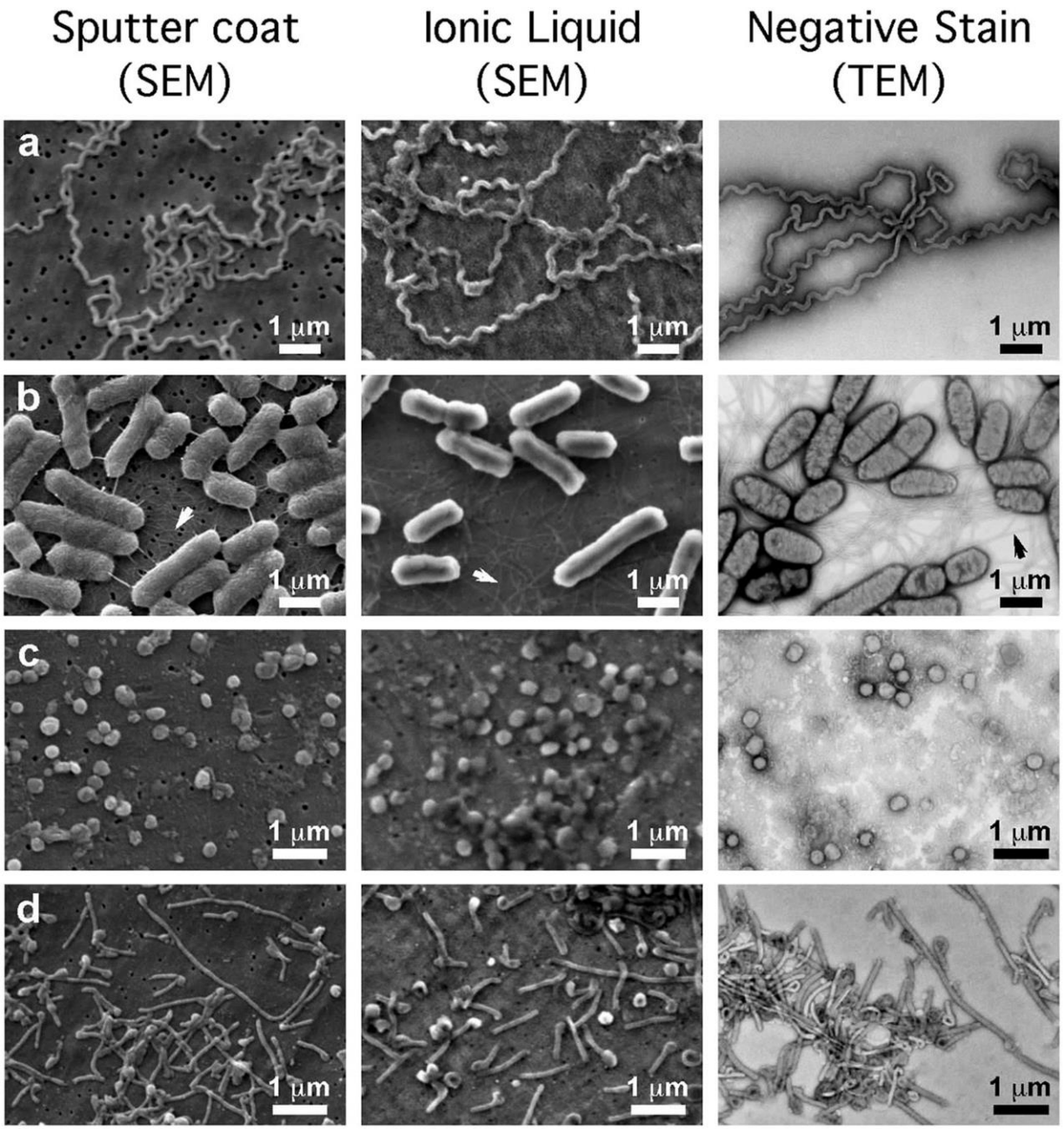


Figure 2. Comparison of conventional sputter coating SEM sample preparation methods (left column) with [BMI][BF_4] RTIL treatment (middle column) and conventional TEM (right column) for the observation of microbes:

(a) *Leptospira biflexa*, (b) *Salmonella* Senftenberg, (c) vaccinia, and (d) Ebola virus. Sputter coating was done with gold on plain uncoated filters, RTIL treatment was on pre-coated aluminum filters, and TEM utilized methylamine tungstate negative staining. Image reproduced under the Creative Commons CC BY 4.0 License from Christine G. Golding et al.[21]

4. Applications of Ionic Liquids in Biological Imaging

RTIL treatment can facilitate EM imaging of biological samples by offering an alternative to coatings for conductivity-improvement and the ability to view samples in a hydrated state. With the elimination of dehydration and/or fixation steps, RTIL-based protocols can drastically reduce preparation time.[17,18] Research in this area suggests that RTILs produce a thin, uniform conductive layer on samples for their imaging,[10,22,23] thus their capacity to spread evenly on a wet surface is key in this type of work.

Table 1 summarizes relevant material properties of the RTILs used in EM studies and shows the anions and cations which form these solutions. Table 2 displays a variety of biological samples where RTILs have been used to observe in the literature while also showing the various sample conditions that have been applied to commonly fixed or unfixed and dehydrated or hydrated samples. Two direct and facile RTIL sample preparation protocols for EM are summarized through the illustration in Fig. 1B. These include: 1) the addition of an RTIL solution directly to the sample of interest through directly pipetting solution to the sample for a given exposure period (Fig. 1B – Scheme 1); and 2) the RTIL solution immersion treatment, an infiltration method where samples are immersed in solution for a given amount of time for sample integration (Fig. 1B – Scheme 2),

Most works using RTILs for EM preparation fall within these two schemes, with some variation in protocols and preliminary sample preparation. Typically, the Scheme 1 (Fig. 1B) addition method is applied for smaller samples or for quick surface treatment of drier samples while the Scheme 2 (Fig. 1B) immersion method may be required with large, hydrous samples that are being impregnated by the RTIL solution.[70] The treatment time associated with solution addition or immersion is variable in literature, though can be found to normally last under 5 minutes (Table 2). With this minimal exposure time in treatment, an unfixed, hydrated sample may be prepared for EM imaging in under an hour. Heating the solution prior to treatment is recommended, reducing the viscosity of the solution to spread more evenly across a surface and mitigating liquid accumulation on the surface referred to as pooling.[18,23] If high-purity RTILs cannot be obtained, purification methods of RTILs may be considered.[71,72] Post-treatment, samples are typically blotted using filter paper or other means to remove excess liquid, often followed by vacuum treatment in the range of 1-30 mins before SEM observation to evaporate excess liquid from the sample surface. After being appropriately prepared and mounted onto an SEM stub, RTIL-treated biological samples are typically observed using acceleration voltages within the range of 5-10kV in SEM (Table 2). Similarly, Fig. 3 shows how the Scheme 1 (Fig. 1B) addition method can be adapted for use in transmission electron microscopy (TEM).

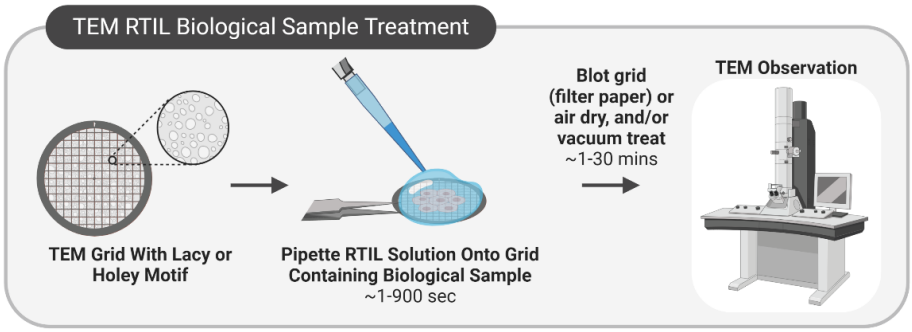


Figure 3. Illustration of sample addition RTIL method for TEM biological sample observation. Image created with BioRender.com.

4.1. Microbial Studies

Since Kuwabata et al.'s first-time observation of star sand grains, *Foraminifera* shells, using RTILs,[22] many other microbial-based applications have been explored, outlined within this section. Golding et al. proposed that the RTIL treatment may better conserve microbe size, making it a representative preparation model for studying microbes.[21]

Similarities between morphological measurements of the Ebola virus prepared for EM using [BMI][BF_4][21] are noted to Cryo-EM observations,[73] suggesting that the RTIL preparation is a closer representation of the natural hydrated state.[21] This is attributed to the low vapour pressure of [BMI][BF_4], which avoided artifacts in samples such as wrinkling, shrinkage and cracking, as well as the preserved hydrated state of the sample.[21] Traditionally prepared samples were reported to have flattening and collapse of samples due to dehydration.[21]

Microbial samples with pointed or sharp features are often hard to image in SEM due to a phenomenon known as the "edge effect". For samples that have sharp edge-like features, higher amounts of escaping surface electrons cause charge accumulation in these areas, which can be difficult to mitigate using conventional sample preparation methods apart from using lower electron-beam energies.[74] Yanaga et al. used varying concentrations of [EMI][BF_4] in pure water to study the ultrastructure of various basidiospores, which are highly topographical with pointed features.[19] Notably, at an optimal concentration between 5-10%, imaging artifacts such as electron charging at point tips were mitigated using the RTIL treatment, showing that RTILs present a method to improve imaging of sharp edge-like features.[19] Similarly, Ishida et al. found an optimal treatment of 2-3% [BMI][BF_4] resolved fine structural details of highly textured protists with minimal amounts of charging.[42] Both works further note that low concentrations outside of these optimal ranges resulted in surface charging while higher concentrations resulted in liquid pooling on the surface.[19,42]

Other applications of RTILs can be found in literature reporting quick and efficient observation of microbes in SEM. In the study of biofilms, Asahi et al. were able to use RTILs to study the native structure of *Streptococcus mutans*, in hydrated fixed and unfixed states.[20] Biofilms are hydrous and otherwise severely impacted by dehydration and harsh fixation steps used in conventional EM preparation.[75,76] Hydrophilic RTILs resulted in better imaging quality for biofilms while highly concentrated RTIL solutions led to liquid pooling on regions of interest.[20] Typical features of conventionally prepared biofilms such as sample cracking and fibrous features of extracellular matrix constituents were not observed in RTIL treatment.[20]

Recently, works produced by Tsuda et al. explored the use of RTILs in TEM imaging applications for microbial research.[41] Following a pipette-based IL treatment protocol (similar to Fig. 3), RTILs successfully facilitated the visualization of herpes simplex virus I (HSVI) in TEM.[41] Kamlet-Taft parameters for polarity factors were consulted in evaluating RTILs for TEM imaging, where RTILs such as [EMI][BF_4] and [Ch][Lac] with a high hydrogen bond accepting ability (denoted by parameter β) performed optimally and caused no noticeable damage to specimen morphology.[41] This is one of the few studies to effectively report the successful use of RTILs in TEM, while most available studies use RTIL treatment in SEM.

4.2. Cellular Biology

Key works produced in 2011 were the first to collectively explore fundamental concepts of RTIL usage for cellular samples, notably the feasibility of imaging cells with various RTILs at different concentrations, the influence of hydrophilicity, and its application for different cellular samples.[18,40,44,45] Since then, RTIL solutions have been applied to a variety of cellular samples and proposed as a potential medium for live-cell imaging.

Tsuda et al. were the first to describe RTILs as a "facile" treatment in studying a wide range of biological samples, from insects and plants to single-cell observation applications.[18] For cellular studies, Tsuda et al. suggested using biocompatible RTIL candidates that may better preserve cellular structures, where [EMI][Lac] & [Ch][Lac] were identified as the most suitable for imaging mouse-derived L929 cells after evaluating their viability using flow cytometry at different RTIL and cell medium concentrations.[18] While the cells could be quickly imaged using this method, finer cellular features were lost due to low secondary electron contrast and cells appeared flattened, a potential artifact from the drying and/or vacuum conditions.[18] Fixed hydrated samples were found to provide stable and improved imaging quality in their work in comparison to unfixed samples.[18] Joubert and McDonald further note that fixation may allow for better sample preservation when using diluted 10% Hitachi Ionic Liquid HILEM© IL1000 solution to perform single-cell SEM observation of Chinese Hamster Ovarian (CHO) cells.[37]

The influence of RTIL concentration and selection for cellular samples have been explored in great depth for fixed A549 cells, treated with and without gold-conjugated antibodies.[40,44] It was highlighted that RTILs can efficiently facilitate cellular imaging without SEM charge build-up, resolve fine cellular features such as microvilli, and perform optimally at low SEM accelerating voltages of 0.9-1.2 kV (Fig. 4).[40,44] Hydrated A549 cell images of exceptional

quality are shown using a reported 1-minute [EMI][BF_4] RTIL treatment protocol in Fig. 4, which was further improved upon using gold-conjugated antibodies treatment prior to RTIL treatment.[44] In exploring the influence of concentration, similarly to previously presented microbial studies,[19,20,42] it was found that higher concentrated RTILs resulted in solution surface pooling due to their highly viscous nature while low concentration solutions resulted in charge buildup.[40,44] Moreover, the study of these cells helped show that the hydrophobicity of the RTILs greatly affects imaging, with hydrophilic RTILs diluted in aqueous solution giving far superior imaging quality in comparison to hydrophobic RTILs diluted in acetone-based solutions.[40]

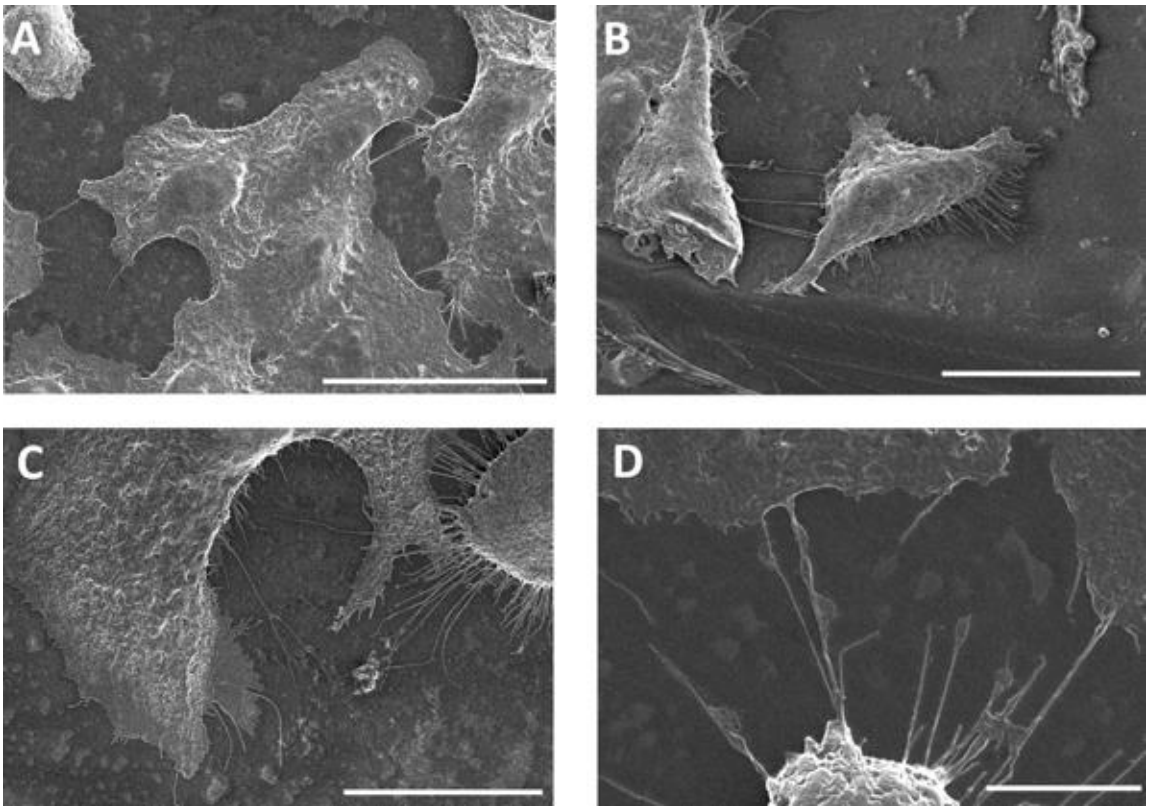


Figure 4. Fixed, hydrated A549 cells observed with [EMI][BF_4], diluted to concentrations within 10-40 v/v%. Fine topographical features and microvilli were resolved with this treatment. Scalebars are 50 μm in panel A, 30 μm in B and C, and 10 μm in D. Image reproduced with permission from Yasuhito Ishigaki et al.[44] (Copyright © 2010 Wiley-Liss, Inc.)

The potential of RTILs to image different types of cells and cellular processes was highlighted by Ishigaki et al., who studied the epithelial-mesenchymal transition of A549 as well as Panc-1 (pancreatic carcinoma) cells in the presence of TGF-β1, a transforming growth factor.[45] Fine features such as cell-to-cell bridging as well as filopodia in cells could be observed using a 25% [EMI][BF_4]-aqueous solution in SEM, features that were also observed at lower resolution in their EVOS microscope.[45] Moreover, the authors distinguished key morphological differences between cells with and without TGF-β1 treatment, noting that the number of filopodia reduced in size and number significantly with exposure to TGF-β1, one of the first demonstrations of this novel effect in SEM.[45]

A novel application of RTILs in imaging of cellular structures by Dwiranti et al. used combinations of platinum blue (Pt-blue) staining and one of EMI-BF_4 or BMI-BF_4 to image Indian Muntjac and human chromosomes.[46] The optimal concentration of these RTILs for imaging was found to be within the range of 0.1-1%.[46] Notably, phase contrast microscopy of these chromosomes confirmed that the treatment had no detrimental effects on chromosome morphology, indicating that RTILs can be used to image DNA without significantly altering its structure.[46] The results and discussion of this study suggested simply trading traditional methods out for RTILs is insufficient.[46] The use of an ionic liquid comes with its own unique set of parameters to achieve the best results, ranging from sample preparation technique to optimal instrument operating conditions. However, the best practices and operating

parameters for SEMs, such as optimal spot size and accelerating voltage, have yet to be fully characterized for the use of ionic liquids.

While RTIL treatment for cells in these works shows promising results due to their comparable imaging quality and resolution to conventional preparation techniques,[18,37,40,44,45] several aspects must still be understood and further studied in this field. Differences in morphological features in RTIL treated samples and conventionally prepared samples have been noted in the literature and should be further explored. Smooth surfaces obtained in platinum-coated cells versus ragged surfaces of those treated with RTIL have been observed as well as different lengths of microvilli of cells.[44] It is unclear as to whether these morphological features are preserved natural structures or artifacts from the treatment itself.

A new avenue explored by a limited number of researchers is the potential of RTILs for studying "live" cell imaging and surface interactions. Recently, Lee et al. featured a new treatment scheme that utilized a 5% [EMI][BF_4] solution in McCoy's 5A modified medium to image mammalian cells adhered to nanotopographic laser-modified titanium substrates (Fig. 5).[48] In Fig. 5, low-vacuum backscattered SEM imaging mode is used with the RTIL treatment, where cells appear dark in contrast to the bright titanium substrate. The work compared the influence of untreated titanium substrates (Fig. 5A/B) and laser-modified titanium (Fig. 5 C/D), where sub-micron features induced from the laser-modification appear as thin lines most clearly represented in Fig. 5D.[48] Prior to imaging, an optimal percentage of ionic liquid that would cause limited influence on cell viability while maintaining improved imaging in SEM was determined. Human osteoblast-like Saos-2 cells were imaged using SEM and subsequently evaluated using biochemical assays as well as fluorescence microscopy to confirm cell viability.[48] Cells from this specimen were viable after treatment with the RTIL-media solution and after imaging; however, directly irradiated cells were killed due to electron beam damage.[48] This demonstrates treatment applicability to facilitate live imaging of cells in near-native environments while warning that cells are still highly vulnerable to the extreme environment required for EM.

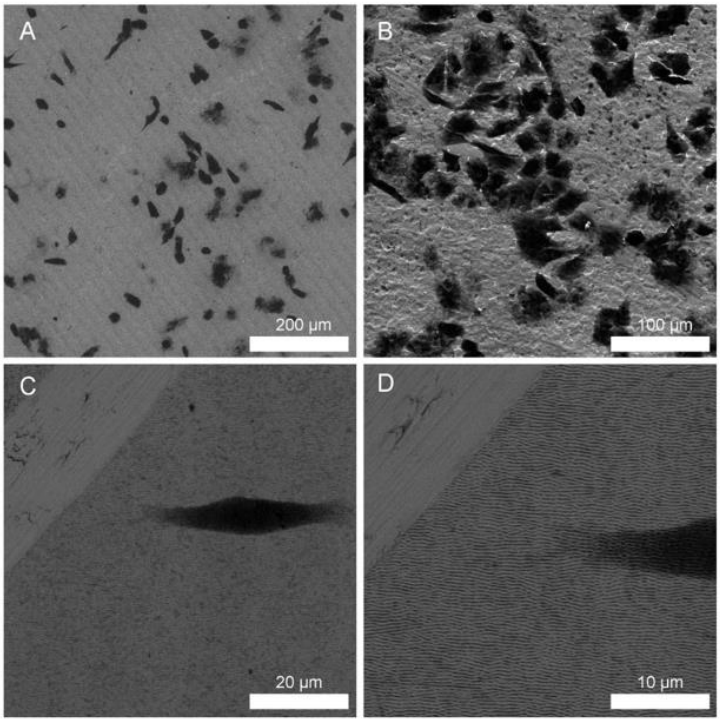


Figure 5. Cells adhered to titanium substrates after 5 minutes of a 5 v/v% aqueous [EMI][BF_4] RTIL treatment. Cells can be observed across the entire substrate of the unmodified sample (A/B) and in detail with respect to sub-micron features from the laser-modification treatment, which appear as fine lines on the metallic substrate (C/D). Image reproduced with permission from Bryan E. Lee et al.[48] (Copyright © 2020 Wiley-VCH GmbH)

In separate works, the viability of yeast cells in RTIL solutions was explored using hydrophilic [Ch][Lac] and [EMI][AcO] diluted with cell culture medium.[77] Observation characteristic bands following treatment revealed that

yeast cells were able to maintain their bioactivity in the presence of RTILs,[77] further highlighting RTILs as a potential medium for live studies. Evidence of blueshift in the Raman spectrum corresponding to [EMI][AcO], however, was reflective of protein denaturation.[77] These results warn that only select RTILs such as [Ch][Lac] may be suitable for the investigation of cellular events that are contingent upon proper protein functioning, an incipient advantage of RTIL-based EM. A choline-like [EHACh][MO] RTIL used in the observation of red blood cells in SEM by Hyono et al. was noted to have minimal effect on the shape and size of cells observed with minimum charging.[78] When observation was attempted using imidazolium-based ionic liquids, no cells could be visualized, speculated due to induced cell lysis with high osmotic pressure on the cell membrane.[78] This suggests that choline-based RTILs could be used to accurately study blood cells without causing significant damage to their structure. Additionally, [Ch][Lac] has been demonstrated to be an effective medium for the analysis of lipid vesicle fusion in TEM.[52] It was posited that ionic liquids induce this process via localization at the water-lipid interface between the vesicles and cytosol, which separates the water layer and promotes fusion of the membrane with the vesicle.[52] With lactate anions, comparison with vesicle fusion induced by [EMI] cations showed that cholinium cations yield smaller vesicles.[52] The observation of these sub-cellular processes *in vitro* and *in vacuo* provides further support for the potential to view live cellular processes in EM through sample preparation methods using choline-based RTILs.

4.3. Plant Structures

Arimoto et al. were the first to study plant structures in SEM using pure [BMIM][TFSI] and [BMIM][BF_4] to observe water-swelled seaweed through an immersion treatment method.[23] In later works, Kawai et al. further explored seaweed in SEM using bioinspired choline-like RTILs, noting they were more easily integrated through the immersion protocol.[36] Moreover, choline-like solutions better conserved the morphology of the seaweed with higher impregnation rates and size retention of samples.[23] Subsequently, several others have used RTILs to observe plant structures, such as delicate flower petals and stems,[18] leaf structures,[37] pollen,[18,37,43] and wood.[17,28,39].

Pollen specimens have been a key target for RTIL treatment due to their inherently fragile and highly topographical, nonconductive structures. Tsuda et al. featured a variety of pollen specimens (*Primula juliae, Anemone coronaria, Leucoglossum paludosum, and Lathyrus odoratus*) with sharp features that were easily imaged using a 10% [BMI][BF_4]-water solution, shown in Fig. 6.[18] Similarly, Joubert and McDonald used a 10% Hitachi Ionic Liquid HILEM© IL1000 solution to observe pollen grains.[37] While fine structures could be observed, excess solution remained on the sample, highlighting the need to further optimize the treatment method.[37] When RTILs are used in pure condition states, the application of choline-type RTILs to study pollen samples has been shown to lead to improved imaging results in comparison to non-choline-like [BMI][BF_4].[43] However, Komai et. al. noted that highly concentrated RTILs can effectively hide surface features from observation and found that low concentrations of 1% and 5% [Ch][Lac]-ethanolic solutions provided improved resolution in imaging hydrated, unfixed mature pollen grains of *Lilium* cultivar in SEM.[49]

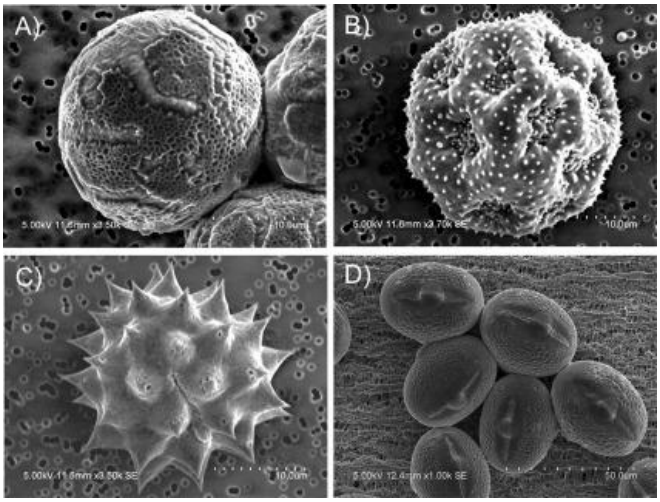


Figure 6. SEM images featuring pollen pretreated using an RTIL immersion-based method using a 10 v/v% [BMI][BF_4] aqueous solution for 600 seconds. Pollen specimens shown are: A) *Primula juliae*, B) *Anemone*

coronaria, C) *Leucoglossum paludosum*, and D) *Lathyrus odoratus*. Image reproduced with permission from Tetsuya Tsuda et al.[18] (Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim)

The study of wood and its hierarchical structure is another area that researchers have explored the use of RTILs for imaging treatment. Wooden specimens can be difficult to image using EM methods due to their insulating nature, requiring conductive coating for SEM.[79] Often, wood-based structures have complex topographical features due to their hierarchical structure, and sample features such as fractured surfaces can be even more difficult to coat, leading to potential uneven coverage. Tsuda et al. first studied wood (*Cryptomeria japonica*) using [EMI][AcO]. Uniquely, the RTIL was used as a solvent to better understand the mechanism behind wood liquification used for the depolymerization of cellulose[17] as high β value RTILs can easily dissolve cellulose.[80] SEM imaging of samples before and after liquification below 393K was done to observe morphology changes in samples, exposing them to an immersion-based treatment of [EMI][AcO].[17]

More recently researchers have used RTILs to study a wide variety of modern and unique archaeological woods. Yamashita et al. used varying concentrations of [EMI][MP] ethanolic solutions to study soft, hard, and archeological wooden specimens.[39] The study found a 10% ethanolic RTIL solution to be ideal for imaging and avoiding surface charging without deforming the fine wooden structures being observed.[39] Lu et al. further explored several RTILs as a means to effectively observe archaeological and spruce wood hierarchical structures, using a "facile" immersion-based treatment to quickly prepare samples and obtain high-quality SEM images (Fig. 7).[28] It was noted that hydrophilic RTILs in this study performed better in imaging than hydrophobic solutions and that water content, viscosity, and density of the RTILs were also key parameters that influenced imaging quality.[28] The study found [BMP][DCA] at a 7.5% concentration in an ethanol solution gave the best imaging quality results, which outperformed conventional coatings in this work.[28] Delicate features such as torus/margo of wood pit membranes were imaged at varying temperatures without specimen damage using this scheme.[28] Given proper optimization procedures, RTIL preparation can give high-quality imaging with reduced time and higher throughput in sample preparation, particularly useful for studying precious wooden samples where only small pieces are available for study.

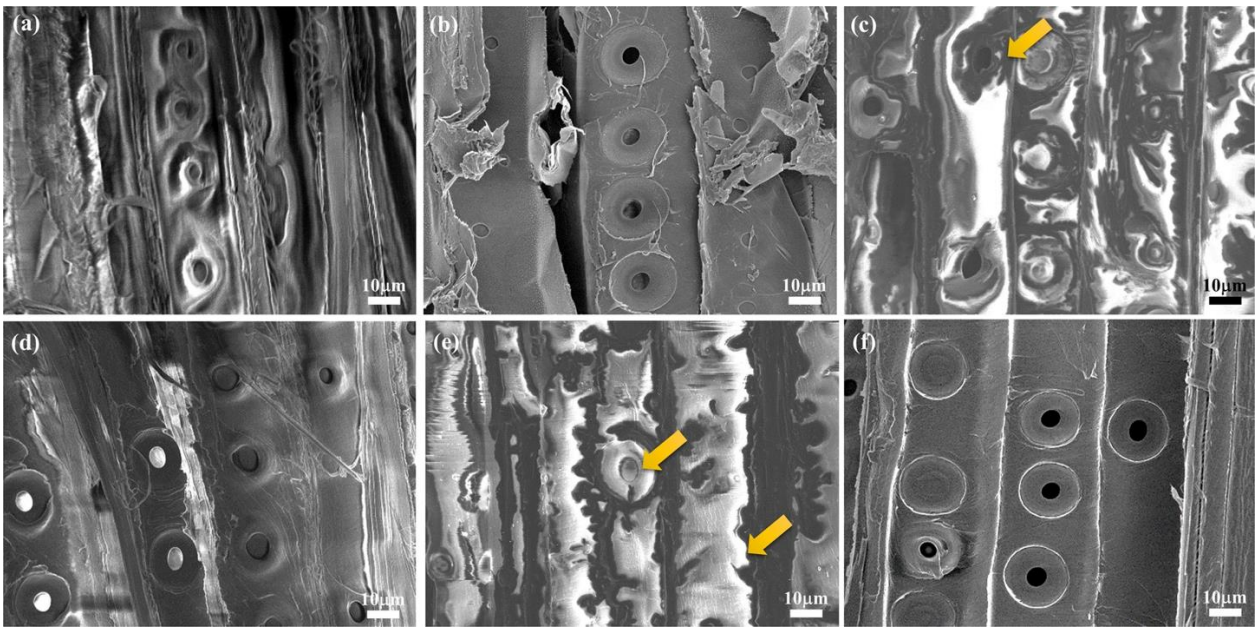


Figure 7. SEM images of modern spruce pretreated with (a) no treatment, (b) platinum sputtering, (c) 5% [EMI][TFSI], (d) 5% [EMI][DCA] (e) 10% [BMP][TFSI], and (f) 7.5% [BMP][DCA]/ethanol solutions. The acceleration voltage is 5 kV. Arrows highlight regions of excess RTIL accumulation. Image reproduced under the Creative Commons CC BY 4.0 License from Bing-Jyun Lu et al.[28]

4.4. Arthropods

On the mesoscale, in between the macro and micro levels, RTILs have been applied to arthropods. Arthropods can be challenging samples to coat due to their inherent complex structures as well as the occasional presence of exoskeletons

which can make it difficult and slow to impregnate with many conventional SEM preparation solutions. While acidified 2,2-dimethorypropane (DMP) has been proposed as a quick alternative to traditional fixation and dehydration of insects with hard-to-penetrate cuticles, this still requires that conductive coatings be applied post-application.[81] These harsh treatments can also be destructive for fragile and soft insect tissues. Moreover, traditional treatments do not facilitate the observation of wet or soft samples, such as soft-shelled eggs, winged structures, and larvae which, due to high-vacuum SEM conditions, can alter their morphology and appearance, leading to crumpling and wrinkling.

To address these difficulties, researchers have turned towards alternative preparation techniques using RTILs. Tsuda et al. describe the versatility of RTIL treatment for biological applications and the use of [BMI][TFSI] in a 5% ethanolic solution to efficiently image yellow jacket (*Vespula*) specimens using a 60-second immersion method.[18] Fine features of the yellow jacket, such as its antennal fossae, were able to be imaged as shown in Fig. 8.[18] Tsuda et al. show a similar immersion-based technique using [EMI][TFSI] to successfully observe delicate, unfixed scaled butterfly wings.[17] A comparison between the morphological observations of the RTIL treated sample to a glutaraldehyde fixed and gold sputter-coated specimen showed differences in size, shape, and SEM quality.[17] Due to the complex scaled butterfly wing structure and likely uneven coating, charging was observed when gold sputter coating technique was used, and imaging artifacts noted.[17] The avoidance of the fixation step in this work saved several hours of laborious sample preparation and enabled quick observation of these samples.[17] At the macro scale, Shiono et al. use the Hitachi Ionic Liquid HILEM© IL1000 to observe small crustaceans, *Gammaridea*, *Tanaidacea*, and *Myodocopida*, in SEM using an immersion-based RTIL treatment protocol.[38] Notably, the soft and delicate parts of the crustaceans, including the specimen's eggs, could be imaged using this solution without evaporating in the microscope.[38]

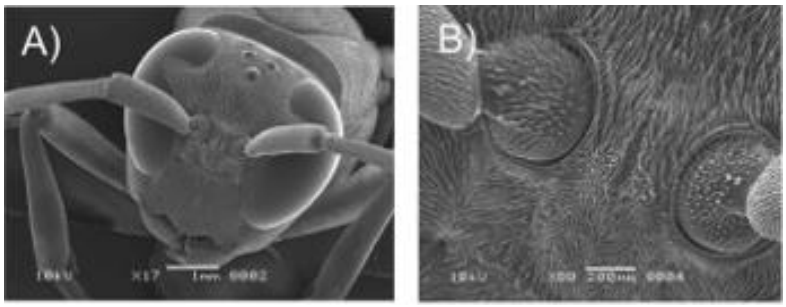


Figure 8. SEM images highlighting the A) head of a yellow jacket and B) antennal fossae of a yellow jacket. Both were treated with a 5 v/v% ethanolic [BMI][TFSI] RTIL solution for 60 seconds. Image reproduced with permission from Tetsuya Tsuda et al.[18] (Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim)

Both hydrophobic and hydrophilic ionic liquids have been applied to larger macro-scale observations done in the works of Ishigaki et al. for the observation of live ticks.[50] To reduce the potential of death, ticks were observed in the study at low vacuum conditions of 1.5×10^{-3} Pa and lower acceleration voltages in the 2-5 kV range.[50] However, in this application, it was noted that the RTIL treatment techniques did not show significant improvement in imaging and that some RTILs were toxic to the live specimens being observed.[50]This highlights the need for understanding how RTILs may interact with specimens being observed, particularly toxicity effects in live imaging, as well as the need to study dilute solutions to optimize imaging parameters and decrease toxicity.

4.5. Other Biological and Related Specimens

Other works can be highlighted which look at the use of RTILs relevant to unique biological applications. One avenue which has not been discussed is the imaging of soft and mineralized tissue using RTILs.

Kawai et al. showed that immersion-based treatment methods can successfully be applied to soft tissue samples, notably the epithelial and muscular tissue of chicken, by using an immersion-based technique with choline-like RTILs.[36] Contrary to imidazolium-based hydrophilic RTIL leaving hydrated samples relatively stiff, specimens treated with choline-based RTILs remained flexible.[36] The sub-standard performance of imidazolium-based ionic liquids in this study is attributed to their relative lipophilicity compared to choline-based ionic liquid, and their inherent structural differences that inhibit cell-mediated movement of the liquid across the membrane.[36] These findings suggest that choline-based ionic liquid treatments may be suitable for the investigation of other soft tissue samples.

Using a similar technique, Tsuda et al. were able to image the villi of the small intestine of a mouse, revealing fine details such as microvillus from epithelial cells of the small intestine villi.[18] For successful imaging of soft tissue, longer immersion times can be noted in sample preparation using RTILs compared to other biological materials in Table 2, ranging from 5-10 minutes, allowing the RTIL to better impregnate the soft, wetted tissues considered.

Soft and hard tissue has been successfully imaged in SEM using [Ch][Lac] by Abe et al. to study mineralized shellfish and a soft bacterial cellulose gel.[51] The work achieved similar imaging quality to conventional Pt/Pd sputter coating techniques using [Ch][Lac] at a 10 v/v% concentration in ethanol instead. Energy-dispersed fluorescence X-ray spectroscopy (EDS) and elemental distribution mapping could also be done on RTIL-treated mineralized samples.[51] This suggests that similar preparation techniques could be effectively applied for imaging and analytical analysis of other tissues quickly and efficiently in SEM, a presentable area of growth for RTIL applications.

Research in non-biological sample centred RTIL imaging literature also has implications towards the imaging of biological samples. Carbon nanotubes (CNTs) are highly studied for their unique properties and characteristics. Notably in biomedical studies, CNT structures are being considered for their nanotextured features which may play an important role in protein adsorption and cellular adhesion.[53] To study potential hydrated unfixed samples on CNTs, Abe et al. showed that fine nano-scale features could be resolved in RTIL treatment using [HMI][PF_6], obtaining a resolution below 30 nm in their work and comparable quality to that of conventional Pt/Pd sputtering coating treatment.[53] This has implications not only for imaging CNTs in biological fields at high resolution but also for the potential to study other nanostructured surfaces and their interactions with biological specimens.

Tsuda et al. present a study that uses a variety of RTILs and studies their effectiveness in observing hydrous superabsorbent polymer (SAP) particles through an addition-based treatment method.[34] While not a typical biological material, SAP particles are used for a variety of applications in hygienic products and information from this RTIL study can be used, for instance, to infer the behaviour of RTILs on similarly structured polymers used in biological material scaffolds. The application of several RTILs found [$P_{4,4,4,1}$][DMP] to be the most effective in imaging these SAP samples while commonly used [EMI][BF_4] and [BMI][BF_4] were found to damage particles and lead to a reduction in the size of samples.[34] Potential degradation of [EMI][BF_4] and [BMI][BF_4] through hydrolysis of the [BF_4]$^-$ anion can occur with exposure to water and temperature increase, which could result in the formation of HF by-products.[82,83] Other RTILs studied by Tsuda et al. did not sufficiently integrate into structures and led to a reduction of size in SAP particles.[34] Overall, as exemplified by this work, as well as other applications mentioned, users need to consider how samples interact with the RTIL solutions, and how that may influence their structure and interpretation of images.

Related to the tissue applications mentioned, and the potential of RTILs to study non-conductive polymer-based materials such as SAP particles,[34] the use of RTILs in the application for visualizing substrates such as nanostructured surfaces, scaffolded materials, and polymeric based materials like hydrogels, is a natural point of progression for their application in biological imaging. Particularly, given the number of applications observing hydrated cells and microbes highlighted in Table 2, the use of RTILs for imaging interactions between complex substrates and biological samples holds great potential. In past work, Lee et al. eluded to this application, showing the success of visualizing SAOS-2 osteoblast-like cells imaged in their hydrated state on nanostructured, laser-modified titanium substrates.[48] The titanium substrates were of interest for orthopedic applications and using the RTIL technique, cellular interactions between the submicron features and cells could be elicited.[48] While limited works have been produced in this area, we believe this could be a key area of development for future growth of RTIL biological imaging.

5. Outlook

5.1. Acclaim of the Scientific Community

While the previous sections presented several examples of RTIL applications facilitating EM imaging for biological samples, the use of these RTILs is limited in comparison to the wide application of conventional EM techniques applied within the biological field. Typically, studies that use RTILs explore the application of RTILs themselves, rather than using RTILs to facilitate the investigation of other research topics. EM imaging presented by Iezaki et al. is one of few studies where RTILs are mentioned solely in the method section of the work and not as a focus of the research itself.[84] This is in part due to a lack of repetition studies as well as the limited availability of standardized treatment protocols for new RTIL users, despite the facile nature of this treatment. Moreover, limited studies exist where RTIL treatments are thoroughly compared to various control preparation methods, which could help identify

whether observations are of natural structures or artifacts from the treatment itself. One suggested approach to improve confidence in these techniques would be to explore correlative imaging methods combined with different preparation methods to gain better insight into these methods. The works of Golding et al. do this quite well, where their RTIL treatment was compared to conventional TEM and SEM preparation and structural measurements of samples were correlated to past published Cryo-EM research for technique validation.[21] The expansion of casual RTIL use in biological studies as well as the exploration of these methods against various imaging controls may further encourage the application by others in the field and help establish it as an equivalent method to conventional protocols.

Another challenge to its adaptation, emphasized by Torimoto et al., is the reluctance of microscopists and scientists alike for putting liquid, wetted, and/or hydrated samples in vacuum-based microscopes.[16] This apprehension persists despite several works highlighting the possibility of observing samples hydrated with RTILs and has slowed the adoption and development of this technique, leaving many avenues of research unexplored and the true potential of RTILs insufficiently characterized.

Undoubtedly, using a technique such as this raises concerns of possible water and other contamination to the vacuum system should users not be careful in how they treat samples. To mitigate these risks, we suggest researchers use a minimal amount of RTIL solution where possible to treat samples. Moreover, blotting away excess solution with filter paper and using an intermediate vacuum treatment before EM observation is suggested to minimize the amount of solution being effectively introduced to the EM system. A vacuum station or intermediate vacuum stage that can be monitored is ideal where users can survey the stability of the vacuum and wait for it to stabilize to mitigate EM system risks. Lastly, it is suggested that for users considering samples treated with greater amounts of RTIL solution, observations should be conducted first in low vacuum mode to evaluate the risk of high vacuum operation by visually surveying to note if excess solution has accumulated on the sample surface.

Until RTILs are more widely accepted, traditional biological sample preparation methods are likely to continue and with it the limitations previously discussed. While this has persisted as a challenge, with increasing research in fields of liquid-phase electron microscopy, we expect that researchers will move towards accepting these methods and this reluctance will be alleviated.

5.2. RTIL Selection and Protocol Optimization

With limited works available exploring these techniques, strategies need to be optimized to provide scientists guidelines on imaging techniques. Literature has focused more on the synthesis and application of these solutions, but little work has been done to comprehensively summarize and make recommendations on their utilization for biological samples for EM. Typical questions include: What are the optimal concentrations for imaging particular sample types and which RTILs are most suitable? How should the time of ionic liquid immersion change with sample and RTIL types in accordance with osmolarity and RTIL infiltration rate?

Table 1 and Table 2 may act as a good starting point towards identifying these trends; however, significantly more work and meta-analysis must be accomplished in this area. RTILs can vary greatly with respect to their interactions with different samples. Interactions include, but are not limited to, the extent of infiltration, water substitution, changes to sample structure, and changes to sample function in the case of live specimens. Thus, research must be done to establish interaction trends between sample type and the RTILs so that protocols can be designed accordingly. Expansion of statistical analysis of EM-relevant RTILs' Kamlet-Taft parameters[85] and their corresponding performance in EM may help answer part of this question and guide RTIL selection.[41]

Another important consideration relates to the customization of RTILs to yield properties that will work best for each biological sample type. Ganske and Bornscheuer used anion type, a determinant of water-miscibility, to inform their choice of RTIL paired with considerations of bacterial viability.[86] It was found that certain bacteria, such as *E. coli*, are intolerant to ionic liquids while others, such as *Bacillus cereus* and *Pichia pastoris*, experience only mildly toxic effects.[86] These findings suggest RTIL selection may be species-specific, and thus requires extensive investigation to characterize the applicability of each ionic liquid for a variety of specimens.

The suitability of certain RTILs for biological observation is owed in part to their structure, notably their hydrophobicity—with hydrophilic RTILs often performing more optimally for hydrated biological samples.[23,40] The phospholipid bilayer of cells in biological specimens are particularly sensitive to RTILs. Cations with long alkyl side

chains are largely hydrophobic and can insert themselves in between phospholipids of the cell membrane.[70] This disturbance in the cell membrane can create artifacts in the morphology of the cell surface.[70] On the method of using RTILs in imaging, the mechanism by which ionic liquids diffuse and substitute water in hydrated samples must also be further characterized. Although the replacement of water by RTILs has been performed in theory,[23] the extent, efficiency, and precision to which RTILs can substitute water is not yet demonstrated experimentally. This is an important consideration for future works, as failure to properly integrate RTILs into hydrated tissues and sufficiently displace water molecules can impact imaging and affect sensitive EM instruments.

5.3. RTIL Sample Longevity and Purity

The impermanence of RTIL treatment hinders the long-term viability of samples and is another challenge facing future adopters.[45] The impermanence of the RTIL treatment is generally due to its wetted state, which can accumulate contaminants overtime even with careful storage and lead to reduced imaging quality. Thus, samples pretreated with RTILs should be imaged within the first 6-7 days following treatment, after which samples exhibit charging effects and must be treated again to re-establish conductivity.[44] Careful storage to mitigate dust or contamination could extend hydrophilic RTIL coated samples up to one month.[40] However, little is known about how successive treatments with ionic liquids affect image quality or sample integrity and is an area for further exploration.

In addition, the importance of RTIL purity in yielding high-quality EM images has been noted in various works.[18,46] The purity of an RTIL constitutes various measures, including water, chloride ion, and oxygen contamination.[56] Increasing ionic liquid purity can have profound effects on the clarity of images,[18] with sufficiently high purity (>99%) proven to eliminate the need for additional chemical treatments in some cases.[46] Should highly pure RTILs be unavailable, purification methods are available for RTILs,[71,72] however, these procedures require specialized equipment and related chemistry training. Purity has implications on the viability of RTILs for potential live-cell microscopy avenues. Dilute water-based RTIL solutions may interact with the electron beam to form acidic solutions at high accelerating voltages.[87] If not destroyed by radiation itself, sufficiently high H^+ concentrations from electron beam water interactions may significantly damage cellular structures.[87]

5.4. Application of RTIL to Live Microscopy

Despite preliminary success shown by Lee et al. in maintaining cellular activity in RTIL solutions and placing living cells under low-vacuum conditions in EM,[48] radiation of the electron beam can ultimately swiftly induce cellular and microbial death, a challenge in the investigation of live cellular processes.[88] De Jonge and Pecky stress that live-cell EM is likely impossible due to the difficulties of the method.[88] Principally, they highlight the electron dosage required to yield quality images is much higher than the lethal dose.[88] They argue that certain reports of live cell studies feature questionable interpretations of live/dead assay results.[88] Kennedy et al. presented a strong case refuting these conclusions, with proof showing that *E. coli* could survive up to 90 mins of beam exposure in low electron dose imaging (20e-/nm^2/frame) in TEM.[89,90] While live imaging may be possible in EM, its methodologies are difficult and our understanding of assessing "life" at the micro length and time scale is limited. The superposition of these works demonstrates two core challenges in live EM: mitigating the effects of electron dosage and the scientific challenge of the quantification of live cells post EM observation.

A potential solution with respect to creating a more biologically-friendly environment for live cell EM may reside in choline-based RTILs. As mentioned earlier, Kawai et al. demonstrated the ability for choline-based ionic liquids to not only provide adequate conductivity to the surface but also preserve the size and structure of samples by traversing the cell membrane.[36] This conclusion was supported by Hyono et al. in their research on red blood cells and seaweed samples using another choline-like ionic liquid.[78] In possessing a structure similar to precursors of naturally occurring biosynthetic pathways, choline-like RTILs can make use of cellular channels and machinery to better infiltrate the tissues while still preserving the natural structure and flexibility of samples.[36] Although the mechanism of choline-like RTIL impregnation is not yet understood, its resemblance to choline derivatives may permit their movement across the cell membrane via membrane transport mechanisms.[36] This speculation has, in part, been addressed by the work of Sung and Johnstone, who demonstrate that despite a lack of active transport mechanisms, cells can stockpile choline derivatives in accordance with their metabolic needs.[91] These findings and the success of choline-based RTILs in imaging suggest that they may be better suited for creating a less hostile environment for biological sample observation, though more research is required in this field to address this.

In parallel, we must further consider how these materials combined with RTIL treatment behave in EM regarding fundamental electron beam interactions, such as the interaction volume, backscattered electron signals, the potential formation of radiolysis products in these media, and the generation of characteristic X-rays, which rely on deeper sample interactions for their signals. Answers to these questions will help develop an understanding of how instrument parameters, such as accelerating voltage, beam current, and electron dose should be adjusted for different RTIL treatments and samples under investigation, especially considering the assessment of live cells.

6. Summary

In this review, key works outlining the use of RTILs in biological EM have been identified and reviewed. These studies ranging from applications on microbes, arthropods, cells, and plant structures, highlight the potential advantages of RTILs as EM imaging agents for a wide variety of hard-to-image soft and/or wet samples. RTIL preparation protocols can drastically reduce the time spent preparing samples for EM, enabling users to image unfixed, hydrated samples while providing an equivalent, or even superior, image quality to that of traditional preparation methods.

Currently, RTIL treatment in the literature is highly sample-specific. Limited studies dedicated to protocol optimization are available, and these studies are largely restricted to the comparison of relative hydrophilicity, dilution and viscosity. Broadly, several challenges still exist in the use of RTILs in EM concerning the complexity of the interactions between RTILs, the sample to which they are being applied, and the electron beam they are later subject to.

While the use of RTIL treatment in EM is still in its infancy, it holds promise as a new conventional preparation method in the biological field. Although preliminary trends and conclusions can be drawn for certain subsets of RTILs and samples, overarching principles and best practices for optimal results remain difficult to identify. Standardized protocols for various biological sample types must be developed and shared with biological imaging communities for their consideration. As with any methodology, there exists a positive feedback relationship between knowledge of a method and its acceptance for general use, so it is expected that RTILs will gradually become better characterized as the apprehension over introducing wet samples into high-vacuum EM environments recedes. At this stage, it is paramount that not only successful results and methodologies be documented, but also that key points for failure, every refinement, and every observation leading to these results be identified, documented, and characterized. In doing so, we may eventually identify more definitive trends that will inform and shape the use of RTILs as a next-generation imaging treatment in future work.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figure Captions

Figure 1. A) Illustration of the traditional biological sample preparation treatment procedure for SEM observation of cells. B) Illustration of RTIL biological sample treatment procedure, highlighting RTIL solution via addition (Scheme 1) or immersion (Scheme 2) techniques to prepare cells for SEM observation. Note the decrease in preparation time from longer than six hours to minutes. Image created with BioRender.com.

Figure 2. Comparison of conventional sputter coating SEM sample preparation methods (left column) with [BMI][BF_4] RTIL treatment (middle column) and conventional TEM (right column) for the observation of microbes: (a) *Leptospira biflexa,* (b) *Salmonella* Senftenberg, (c) vaccinia, and (d) Ebola virus. Sputter coating was done with gold on plain uncoated filters, RTIL treatment was on pre-coated aluminum filters, and TEM utilized methylamine tungstate negative staining. Image reproduced under the Creative Commons CC BY 4.0 License from Christine G. Golding et al.[21]

Figure 3. Illustration of sample addition RTIL method for TEM biological sample observation. Image created with BioRender.com.

Figure 4. Fixed, hydrated A549 cells observed with [EMI][BF_4], diluted to concentrations within 10-40 v/v%. Fine topographical features and microvilli were resolved with this treatment. Scalebars are 50 μm in panel A, 30 μm in B and C, and 10 μm in D. Image reproduced with permission from Yasuhito Ishigaki et al.[44] (Copyright © 2010 Wiley-Liss, Inc.)

Figure 5. Cells adhered to titanium substrates after 5 minutes of a 5 v/v% aqueous [EMI][BF_4] RTIL treatment. Cells can be observed across the entire substrate of the unmodified sample (A/B) and in detail with respect to sub-micron features from the laser-modification treatment, which appear as fine lines on the metallic substrate (C/D). Image reproduced with permission from Bryan E. Lee et al.[48] (Copyright © 2020 Wiley-VCH GmbH)

Figure 6. SEM images featuring pollen pretreated using an RTIL immersion-based method using a 10 v/v% [BMI][BF_4] aqueous solution for 600 seconds. Pollen specimens shown are: A) *Primula juliae*, B) *Anemone coronaria*, C) *Leucoglossum paludosum*, and D) *Lathyrus odoratus*. Image reproduced with permission from Tetsuya Tsuda et al.[18] (Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim)

Figure 7. SEM images of modern spruce pretreated with (a) no treatment, (b) platinum sputtering, (c) 5% [EMI][TFSI], (d) 5% [EMI][DCA] (e) 10% [BMP][TFSI], and (f) 7.5% [BMP][DCA]/ethanol solutions. The acceleration voltage is 5 kV. Arrows highlight regions of excess RTIL accumulation. Image reproduced under the Creative Commons CC BY 4.0 License from Bing-Jyun Lu et al.[28]

Figure 8. SEM images highlighting the A) head of a yellow jacket and B) antennal fossae of a yellow jacket. Both were treated with a 5 v/v% ethanolic [BMI][TFSI] RTIL solution for 60 seconds. Image reproduced with permission from Tetsuya Tsuda et al.[18] (Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim)

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