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# 1-Dodecyl-3-Methylimidazolium Chloride-Assisted Sample Preparation Method for Efficient Integral Membrane Proteome Analysis

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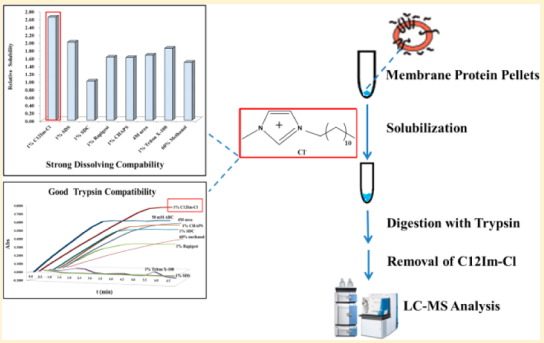
Supporting Information

**ABSTRACT:** Due to their extremely hydrophobic nature, the analysis of integral membrane proteins (IMPs) is of great challenge. Although various additives have been applied to improve the solubility of IMPs, they still suffer from low solubilization efficiency, incompatibility with trypsin digestion, or interference with MS detection. Herein, the systematic study on the effect of ionic liquid structure on membrane protein solubilization and trypsin biocompatibility was performed, based on which 1-dodecyl-3-methylimidazolium chloride (C12Im-Cl) was selected for the sample preparation of IMPs. Compared with other commonly used additives, such as sodium dodecyl sulfate (SDS), Rapigest, and methanol, C12Im-Cl showed the best performance. In addition, with a strong cation exchange trap column, it could be easily removed after trypsin digestion, which not only was beneficial to avoid protein precipitation during digestion but also had no adverse effect on LC-MS-based separation and detection. Such a C12Im-Cl-assisted sample preparation method was further applied to the membrane proteome analysis of rat brain. Compared with the SDS-assisted method, 1.4 and 3.5 times improvement on the identified IMP and hydrophobic peptide number were achieved (251 vs 178, and 982 vs 279). All these results demonstrated that the C12Im-Cl-assisted sample preparation method is of great promise to promote the large-scale membrane proteome profiling.




Cellular membranes function as a natural barrier and a communication interface between intracellular compartments, cells, and their environments, within which integral membrane proteins (IMPs) act as critical components and play unique roles in cell−cell communication, vesicle trafficking, signal transduction, ion transport, and drug targets.[1−3] However, the analysis of IMPs is challenging because of their hydrophobic nature. To solve this problem, several types of additives, such as chaotropes,[4−6] detergents,[7−12] organic solvents,[13,14] and organic acids,[15−17] have been used to solubilize membrane proteins; however, the use of these additives still has critical disadvantages. Although urea can be easily removed by desalting before liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis, its solubilization efficiency for IMPs is limited.[18] Detergents could efficiently solubilize IMPs but must be diluted during trypsin digestion to retain enzymatic activity, which may lead to IMP aggregation and poor digestion efficiency. Organic solvents, especially 60% (v/v) methanol, are also powerful for extracting membrane proteins but would reduce trypsin activity. In addition, 90% (v/v) formic acid is a strong solubilizing agent for membrane proteins; however, the harsh acidic condition restricts the use of most enzymes.

In the past 10 years, over 6000 papers have been published regarding ionic liquids (ILs).[19] The desirable features of low vaporization pressure, high thermal stability, and structure tunability have attracted more and more researchers.[20−27] In our previous study, 1% (v/v) IL (1-butyl-3-methyl imidazolium tetrafluoroborate) was successfully applied to membrane proteome analysis[28−30] and showed excellent solubilizing ability and trypsin activity compatibility, compared with urea, Rapigest SF, sodium dodecyl sulfate (SDS), and methanol. Unfortunately, a systematic study on the effects of IL structure on membrane protein solubilization has not been performed. Therefore, it is important and imperative to reveal the key factors that affect the solubilization capacity of ILs for IMPs, which could facilitate the selection or design of ideal ILs for membrane proteome analysis.

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In this study, we systematically studied the effects of IL structure on membrane protein solubilization by evaluating the capability of various ILs to dissolve a membrane protein, bacteriorhodopsin. With the further consideration on the biocompatibility with trypsin activity, a membrane protein preparation method using 1-dodecyl-3-methylimidazolium chloride (C12Im-Cl) was developed and successfully applied to large-scale membrane proteome analysis, in which C12Im-Cl proved superior to other additives, such as SDS.

## EXPERIMENTAL SECTION

**Reagents and Materials.** Bacteriorhodopsin, bovine serum albumin (BSA), immunoglobulin G (IgG) from human serum, protease inhibitor cocktail, Nα-benzoyl-L-arginine ethyl ester hydrochloride (BAEE), dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). SDS was purchased from Biomol (Hamburg, Germany), and Rapigest SF was obtained from Waters (Milford, MA, USA). Trypsin was ordered from Promega (Madison, WI, USA). Formic acid (FA) and urea were purchased from Fluka (Buches, Germany). Acetonitrile (ACN, HPLC grade) and methanol were ordered from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q system (Millipore, Milford, MA, USA). Otherwise, the chemicals were of analytical grade.

Strong-cation-exchange (SCX) particles (10 μm, 1 000 Å pore) were obtained from TOSOH (Tokyo, Japan), and Luna C18 particles (5 μm, 100 Å pore) were obtained from Phenomenex (Torrance, CA, USA). Fused-silica capillaries (75 μm i.d./365 μm o.d.) were acquired from Sino Sumtech (Handan, China), and the rat cerebellums were ordered from the Experimental Animal Center of the Dalian Medical University (Dalian, China).

**Membrane Protein Isolation.** Five male Sprague−Dawley rats (180−200 g) were killed by decapitation, and the cerebellums were dissected quickly and freshly frozen in liquid nitrogen followed by storage at −80 °C. The procedure for IMP extraction was performed according to the previously described method with minor modifications.[31] Briefly, five rat cerebellums (approximately 1.4 g) were washed 3 times with cold phosphate-buffered saline (PBS) and further homogenized in 10 mL of high salt buffer (2 M NaCl, 1× PBS, pH 7.4, and 1% (v/v) protease inhibitor cocktail) using Tissue Tearor from Biospec Products (Bartlesville, OK, USA) at approximately 20 000 rpm for 3 min, followed by ultrasonication (Cole-Parmer, Vernon Hills, IL, USA) for 3 min on ice at 100% pulse power to break the cells and extract proteins. The resulting solution was centrifuged at 1500*g* for 10 min at 4 °C to remove unbroken cells and debris. The supernatant was collected and centrifuged at 38 000*g* for 1 h at 4 °C to collect the membrane protein pellet. The pellet was re-extracted in 20 mL of a high pH buffer (0.1 M $Na_2CO_3$ and 1% (v/v) protease inhibitor cocktail at pH 11.3) and incubated on ice for 1 h to remove loosely associated peripheral membrane proteins, followed by centrifugation under the same conditions. Subsequently, the pellet was washed with 20 mL of urea buffer (4 M urea and 1× PBS at pH 7.4) to further enrich the membrane proteins. After a 10 min incubation on ice, the supernatant was discarded under the same centrifugation conditions. The pellet was suspended in 1.5 mL of cold water and homogenized by Tissue Tearor, followed by protein quantification with a Bradford assay kit (Bio-Rad, Hercules, CA, USA) using BSA as a standard, which was further divided equally into several aliquots for analysis. Then, each aliquot of cell lysates was precipitated with methanol/chloroform to remove the lipids. Finally, the pellet was lyophilized and stored at −80 °C before use.

**Solubility Measurement for Bacteriorhodopsin.** Equal aliquots of bacteriorhodopsin (25 μg) were solubilized in 30 μL of 0.05 M ILs, including 1-butyl-3-methylimidazolium tetrafluoroborate (C4Im-$BF_4$), 1-butyl-3-methylimidazolium acetate (C4Im-Ac), 1-butyl-3-methylimidazolium trifluoromethanesulfonate (C4Im-$CF_3SO_3$), 1-butyl-3-methylimidazolium bromide (C4Im-Br), 1-butyl-3-methylimidazolium chloride (C4Im-Cl), 1-ethyl-3-methylimidazolium chloride (C2Im-Cl), 1-octyl-3-methylimidazolium chloride (C8Im-Cl), 1-dodecyl-3-methylimidazolium chloride (C12Im-Cl), 1-allyl-3-methylimidazolium chloride (C=C2Im-Cl), 1-aminopropyl-3-methylimidazolium tetrafluoroborate ($NH_2$C3Im-$BF_4$), 1-hydroxyethyl-3-methylimidazolium tetrafluoroborate (HOC2Im-$BF_4$), *N*-butylpyridinium tetrafluoroborate (C4Py-$BF_4$), and *N*-butylpyridinium chloride (C4Py-Cl) in 50 mM ammonium bicarbonate (ABC) buffer. The samples were mixed using a vortex mixer for 1 min and sonicated for 15 min in a water bath at room temperature. Afterward, the samples were centrifuged to remove insoluble materials, and the supernatants were collected. The samples were then quantified by the BCA method at 562 nm with BSA as the standard protein. All the measurements were repeated three times in parallel. For comparison, 1% (m/v) SDS, 1% (m/v) sodium deoxycholate (SDC), 1% (m/v) CHAPS, 1% (m/v) Triton X-100, 1% (m/v) Rapigest, 60% (v/v) methanol, and 4 M urea were chosen to evaluate their ability to dissolve membrane proteins.

**Trypsin Activity Assay.** With BAEE as the trypsin substrate, the trypsin activity was measured by monitoring the hydrolytic rate of BAEE into a UV-active product, Nα-benzoyl-L-arginine (BA), using a UV spectrophotometer (Cary 60, Agilent) at 253 nm, according to the method previously reported.[32] Briefly, the trypsin activity assays were performed in buffer solutions containing 50 mM ABC, 0.05 M C12Im-Cl, 4 M urea, 1% (m/v) CHAPS, 1% (m/v) SDC, 60% (v/v) methanol, 1% (m/v) Triton X-100, 1% (m/v) Rapigest, and 1% (m/v) SDS. For each assay, 30 μg of trypsin was added to BAEE (2.4 mM) dissolved in 50 mM ABC (pH 8.0). The UV absorbance of the reaction solutions was recorded every 0.5 min for 6.5 min. The slope of the reaction kinetics curve defined the trypsin activity. The equilibrium stage of the curve indicated the termination of enzymolysis. The changes in trypsin activity caused by the addition of the different additives were compared using 50 mM ABC (no additive added) as the control.

**Transmission Electron Microscopy (TEM).** Immunoglobulin G (IgG, 5 mg/mL), respectively, solubilized in water and 10% (m/v) C12Im-Cl was pretreated in the negative staining mode and mixed with phosphotungstic acid before being added to the carbon grid. The images were collected with a Tecnai G2 Spirit microscope operated at 120 kV (Tecnai G2, FEI, Eindhoven, Netherlands) at a magnification of 18 500 times.

**C12Im-Cl Removal and Desalting.** After BSA was digested in 4% (m/v) C12Im-Cl, the solution was adjusted to pH 9.5 with 5 mM Tris-HCl. The C12Im-Cl was removed by SCX chromatography using a Shimadzu LC-20AD HPLC system with a homemade SCX trap column (4.6 mm × 10 mm). The mobile phase A used in the SCX chromatography consisted of 1 mM Tris-HCl and 5% ACN (pH 9.2), and


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mobile phase B consisted of 1000 mM NaCl added to mobile phase A. After the sample was loaded onto the column with mobile phase A, C12Im-Cl was captured by the stationary phase, and the eluted peptides were collected. Finally, C12Im-Cl was eluted from the SCX trap column with 100% mobile phase B.

The samples were desalted by reverse-phase liquid chromatography (RPLC) using a Shimadzu LC-20AD HPLC system with a homemade C18 trap column (4.6 mm × 10 mm). After the sample was loaded, the column was flushed with 98% mobile phase A (0.1% TFA/$H_2O$) for 10 min to remove salts. Then, 80% mobile phase B (0.1% TFA/ACN) was used to elute peptides. Finally, the collected peptides were dried with a SpeedVac and reconstituted in 0.1% FA aqueous solution.

**Protein Solubilization and Digestion.** C12Im-Cl- and SDS[14]-assisted solubilization and proteolysis of proteins were performed. In brief, lyophilized protein pellets (100 μg) were resuspended in 1%, 2% C12Im-Cl (m/v, in 50 mM ABC, pH 8.0) and 1% SDS (m/v, in 50 mM ABC, pH 8.0), respectively. The suspension was sonicated for 40 min in a water bath at room temperature and maintained at 90 °C for 20 min for thermal denaturation. Subsequently, the samples were cooled, followed by reduction in 10 mM DTT at 56 °C for 2 h. Afterward, the cysteines were alkylated in darkness in 25 mM IAA for 40 min at room temperature.

For the C12Im-Cl-assisted method, the tryptic digestion was directly performed with a trypsin/protein ratio (m/m) of 1:25 at 37 °C for 20 h. After the digest solution was adjusted to pH 9.5, the IL was removed with an SCX trap column, followed by desalting with a RP trap column.

For the SDS-assisted method, the sample was diluted ten times to decrease the SDS concentration to 0.1%. Then, 4 μg of trypsin (trypsin/protein, 1:25) was added, and the digestion was performed at 37 °C for 20 h. Subsequently, the solution was acidified with FA (final concentration, 1%) to stop proteolysis. Finally, SDS was removed by an SCX trap column, followed by desalting with a RP trap column.

**LC-ESI-MS/MS Analysis.** The buffers used for the nanoflow RPLC-electrospray ionization tandem mass spectrometry (nano-RPLC-ESI-MS/MS) analysis were $H_2O$ with 2% ACN and 0.1% FA (A), ACN with 2% $H_2O$ and 0.1% FA (B), and 1000 mM $CH_3COONH_4$ at pH 3 (C).

The protein digests were analyzed by nano-RPLC-ESI-MS/MS with an LTQ-Orbitrap Velos mass spectrometer equipped with a quaternary Surveyor pump and an ESI probe Ion Max Source with a nanospray kit. The spectrometer was controlled by Xcalibur software version 2.1.0 (Thermo Fisher, Waltham, MA, USA). The ion transfer tube temperature was held at 200 °C, and the mass spectrometer was operated in the positive ion mode. Full MS scans were acquired in the Orbitrap analyzer over the 300−1800 $m/z$ range with a resolution of 60 000 ($m/z$ 400). The 15 most intense ions with a charge state ≥2 in each full MS scan were selected for sequencing and fragmented in the data-dependent CID mode with a normalized collision energy of 35%, activation $Q$ of 0.25, activation time of 10 ms, and one microscan.

The nano-RPLC separation was performed using a C18 capillary column (15 cm, 75 μm i.d./375 μm o.d.) packed with C18 silica particles (5 μm, 100 Å). The gradient of the mobile phase was set as follows: 10% to 35% B in 110 min, then 80% B in 5 min, and maintenance at 80% B for 10 min. The flow rate was 300 nL/min.

The two-dimensional (2D)-SCX-nano-RPLC separation was performed with a 9-step salt elution, consisting of 20, 40, 60, 80, 100, 150, 200, 300, and 1000 mM $CH_3COONH_4$ to elute the peptides from the SCX trap column (3.5 cm, 200 μm i.d./375 μm o.d.) onto a C18 capillary column (15 cm, 75 μm i.d./375 μm o.d.). The nano-RPLC gradient was set as follows: 2% to 10% B in 5 min, then 35% B in 100 min, 80% B in 10 min, and maintenance at 80% B for 10 min.

**Database Searching.** All of the nano-LC-MS/MS raw files were converted to *.mgf files by pXtract v1.036 and searched with the MASCOT search engine v2.3.2 against the database IPI. RAT. v3.75 (39 713 protein entries) for membrane proteins extracted from rat cerebellums. Peptides were searched using fully tryptic cleavage constraints, and up to two internal cleavage sites were allowed for tryptic digestion. Cysteine carbamidomethylation was set as a static modification, and methionine (Met) oxidation was set as a variable modification. The mass tolerances were 10 ppm for parent ions and 0.5 Da for fragment ions. After database searching, the results were filtered by pBuild[33] to control the false discovery rate (FDR) of the peptide level at less than 1% and reduce the apparent redundancy in protein identification.

**Bioinformatics Analysis.** The grand average of the hydropathicity (GRAVY) values of the identified peptides was calculated using the ProtParam program (http://tw.expasy.org/tools/protparam.html). The peptides with positive and negative GRAVY values were named as hydrophobic and hydrophilic peptides, respectively.[34] The TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) algorithm was used to predict the transmembrane domains (TMDs) of identified proteins. The proteins with at least one predicted TMD were considered IMPs.[35] The cellular components and molecular functions based on the Gene Ontology (GO) Consortium were assigned with GoMiner.

## RESULTS AND DISCUSSION

**Effect of IL Structure on Solubilizing IMPs.** To study the effect of the anion of imidazolium-based ILs on the dissolving capability for IMPs, five types of ILs with the same cation but different anions were chosen, including C4Im-$BF_4$, C4Im-Ac, C4Im-$CF_3SO_3$, C4Im-Br, and C4Im-Cl, and a commercially available IMP (bacteriorhodopsin) with a 7-transmembrane helix was used as the protein sample. As shown in Figure 1A, at a solubilizing ability for C4Im-$BF_4$ of 1, the abilities of the other ILs were less than 1. In addition, the pyridinium-based ILs that were examined, namely, C4Py-BF4 and C4Py-Cl, with another type of body cation, showed relative solubilities of 0.64 and 6.14, respectively. These results indicate that the electrostatic interaction of the anion and body cation affects the dissolving capability of ILs. However, the trypsin activity was completely suppressed with the pyridinium-based ILs, whereas good activity was maintained using the imidazolium-based ILs. Further investigation revealed that the inactivation of trypsin was due to the pyridinium body itself (Supporting Information, Figure S1), in accordance with a previous study.[36] Therefore, the following discussion is focused on the imidazolium-based ILs.

Because of the higher hydrophilicity of ILs with $Cl^-$ than those with $BF_4^-$, four ILs with $Cl^-$ and longer alkyl chains were selected to investigate the hydrophobic interaction between ILs and IMPs. As shown in Figure 1B, the capacity to dissolve bacteriorhodopsin increased with longer alkyl chain length, especially for C8Im-Cl and C12Im-Cl, demonstrating that the


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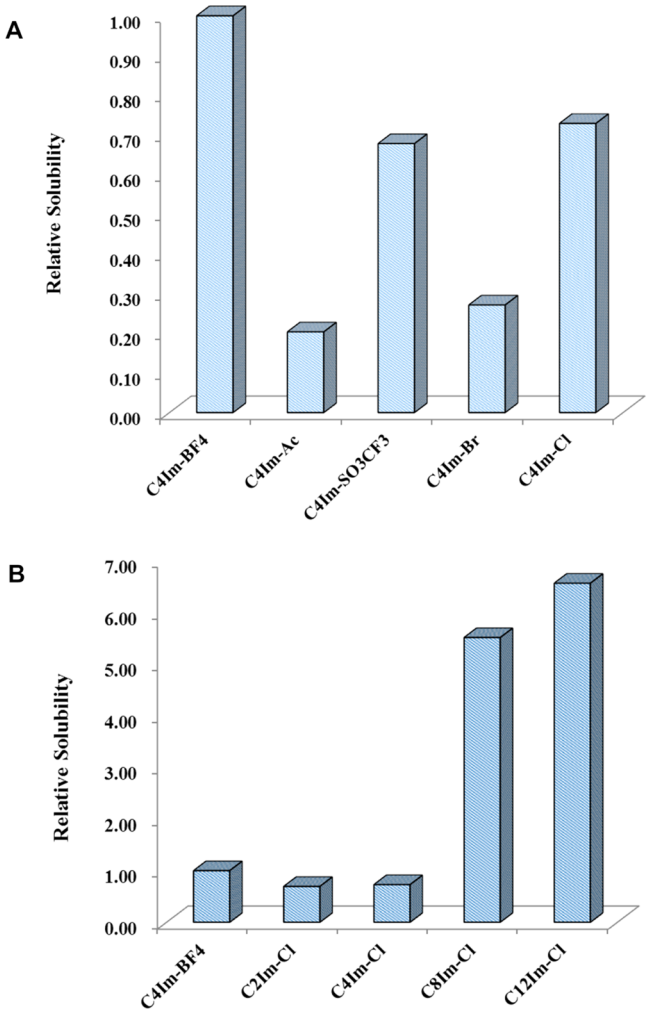


**Figure 1.** Comparison of the relative solubility of bacteriorhodopsin in various ILs with (A) different anions and (B) increasing length of alkyl chains.

hydrophobic interaction of alkyl substituents played a significant role in IMP solubilization. This result may have been possible because of two factors: (1) Because ILs are amphipathic molecules containing both hydrophobic and hydrophilic domains (e.g., C12Im-Cl in Supporting Information, Figure S2A), their long, flexible alkyl chains, which are hydrophobic, could interact with the hydrophobic region of membrane proteins; such interaction is beneficial in that it fosters good dispersal of membrane proteins in ILs, resulting in an efficient protein extraction and a favorable solubilization state. (2) Water molecules could tend to isolate from each other as they interact with the anions of ILs via hydrogen bonding; meanwhile, the anions of ILs attract the cations with a weaker electrostatic interaction. Therefore, ILs could further self-associate and form ionic cluster.[19,37−39] The negatively charged anions with the positively charged cations of ILs could disrupt the protein−protein interactions and prevent protein aggregation, which was demonstrated by the TEM images; the IgG tended to aggregate together in water (Supporting Information, Figure S2B) but was more dispersed in the C12Im-Cl solution (Supporting Information, Figure S2C).

The effects of $\pi-\pi$ conjugate interactions and hydrogen bond interactions between ILs and IMPs were also studied (Supporting Information, Figure S3). The hydrogen bond interaction of the cations with $-NH_2$ and $-OH$ substituents contributed little to the ILs for membrane protein solubilization compared with C12Im-Cl and C4Im-BF4, whereas the $\pi-\pi$ conjugate interactions of cations with the unsaturated alkyl substituent (C═C2Im-Cl) had obvious effects on the solubility.

Therefore, among all the aforementioned interactions between imidazolium-based ILs and IMPs, the hydrophobic interactions of alkyl substituents played a dominant role in solubilizing the IMPs, followed by the $\pi-\pi$ conjugate interactions of unsaturated alkyl substituents and the electrostatic interaction of anion groups; however, the hydrogen bonding interaction of the cation groups had little influence. Thus, ILs with long chain alkyl substituents (e.g., C12Im-Cl) should be excellent solubilizers for IMPs analysis.

**Comparison of C12Im-Cl with Other Additives.** As shown in Figure 2, compared with other additives, in 1%

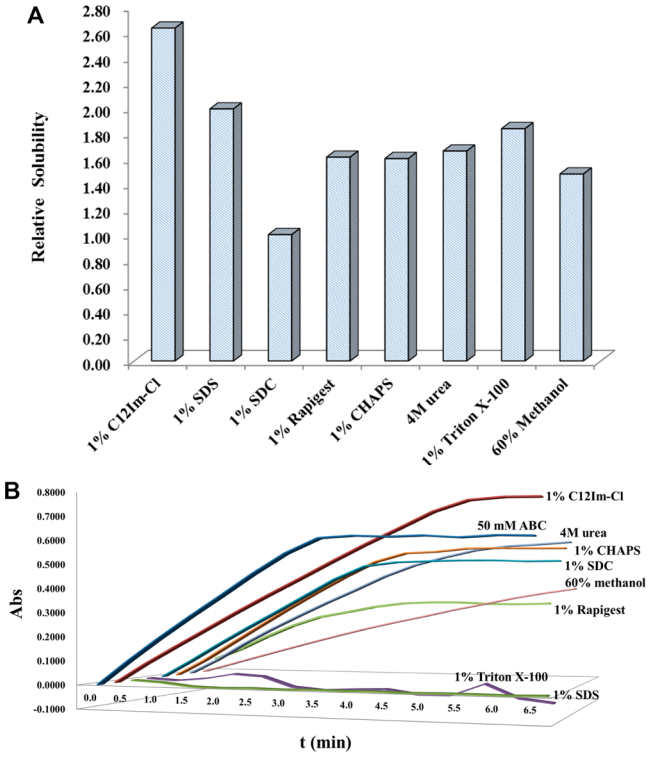


**Figure 2.** (A) Relative solubility of membrane proteins and (B) trypsin activity with different additives added.

C12Im-Cl, both the dissolving capacity for IMP and the trypsin activity were the highest, further demonstrating the superiority of such an IL for membrane protein analysis. Note that a significant difference existed between SDS and C12Im-Cl regarding their compatibility with trypsin activity, even if both of them contain dodecyl substituent. The reason might be contributed by ILs in the liquid phase existing as hydrogen-bonded polymeric supramolecules[21] and the existence of spare space in the binding site of the ILs with the membrane proteins that may facilitate the accessibility of trypsin to the cleavage site.

**Removal of C12Im-Cl before Mass Spectrometry Analysis.** Considering the positive charge of imidazolium, whereas most peptides have a negative charge, at pH 9.5, we employed SCX materials at pH 9.5 to remove C12Im-Cl after IMP digestion, followed by MALDI-TOF MS analysis. For


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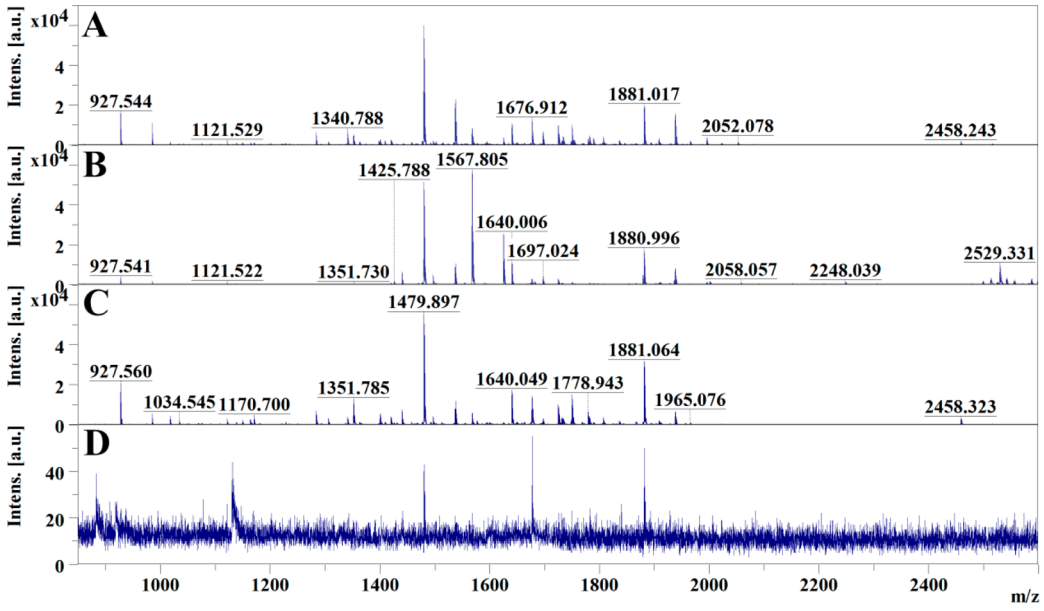


**Figure 3.** MALDI-TOF MS spectra of the tryptic digests of BSA (A) without C12Im-Cl, (B) after removal of 4% C12Im-Cl, and with (C) 0.00001% and (D) 0.01% C12Im-Cl added.

comparison, BSA digests without C12Im-Cl and with 0.01% and 0.00001% (m/v) C12Im-Cl added were also analyzed.

As shown in Figure 3, quite good MS signal was obtained after the removal of 4% C12Im-Cl, with the identified sequence coverage of BSA as 50%, comparative to that without and with 0.00001% (m/v) IL added (49%) and much better than that with 0.01% C12Im-Cl (undetected) added. These results illustrated that the removal of C12Im-Cl from the digests by SCX materials was of high efficiency and that interference of residual ILs during MS detection was negligible.

In addition, the recovery of the whole sample preparation with the C12Im-Cl method including protein digestion and IL removal was evaluated using 10 $\mu$g of BSA as the sample. The produced tryptic digests were quantified by UV detection (214 nm) according to previous work.[17] As a result, 8.24 ± 0.28 $\mu$g of tryptic digests was obtained, with the recovery as 82 ± 3% ($n$ = 3).

**Analysis of IMPs Extracted from Rat Brain.** To evaluate the applicability of C12Im-Cl for the preparation of real membrane proteome samples, the C12Im-Cl (1%, m/v)-assisted method was applied to the analysis of IMPs extracted from rat brains. Followed by nano-RPLC-ESI-MS/MS analysis, 251 IMPs and 982 hydrophobic peptides (GRAVY > 0) were identified, improved by 1.4 and 3.5 times compared to those obtained by the SDS-assisted method (178 IMPs and 279 hydrophobic peptides). The outstanding performance of C12Im-Cl should be attributed to its strong solubility for IMPs and good biocompatibility with tryptic digestion. In contrast, SDS needed to be diluted during digestion to avoid the decreased tryptic acitivity, which could reduce the solubility of some hydrophobic proteins and lead to the loss of some hydrophobic peptides.

Furthermore, the distribution of the TMDs of the identified IMPs was analyzed. As illustrated in Figure 4A, the IMPs identified by the C12Im-Cl-assisted method with 1 to 6 and 12 TMDs were more than those obtained by the SDS-assisted method. Besides, the identified peptides were compared with respect to their GRAVY values (Figure 4B). Due to the better solubilization of IMPs during digestion in C12Im-Cl than in

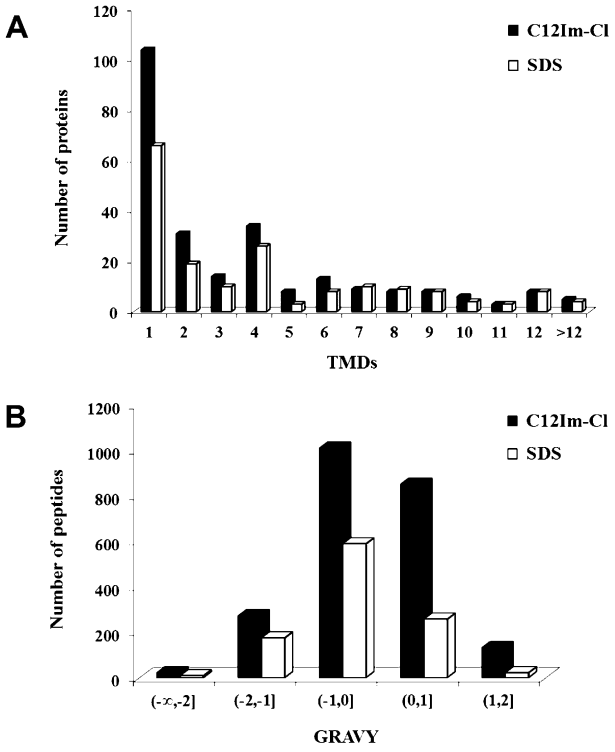


**Figure 4.** Comparison of distribution of (A) TMDs and (B) peptide GRAVY values of IMPs extracted from rat cerebellum by the C12Im-Cl-assisted method and the SDS-assisted method, both followed by nano-RPLC-ESI-MS/MS analysis.

SDS, more peptides could be identified with the C12Im-Cl-assisted method. It was worth noting that the identified peptides with GRAVY values >0 increased significantly using the C12Im-Cl-assisted method compared with the SDS-assisted method, which further indicated the superiority of C12Im-Cl over SDS for the identification of peptides with high hydrophobicity. Then, the missed cleavage sites of the


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identified peptides with the C12Im-Cl-assisted method were investigated and compared with those of the SDS-assisted method. As a result, the percentage of identified peptides without missed cleavage sites in the C12Im-Cl-assisted method was 84%, much higher than that identified by the SDS-assisted method (61%), while the percentages of identified peptides with 1 and 2 missed cleavage sites were 14% and 2% in the C12Im-Cl-assisted method, respectively, much lower than those identified by the SDS-assisted method (32% and 7%). The results further demonstrated higher digestion efficiency could be achieved with the C12Im-Cl-assisted method than that with the SDS-assisted method.

To achieve the global IMP analysis of rat brains, a 2D-nano LC-ESI-MS/MS analysis was performed on the digests of 15 $\mu$g (pretreated) using the C12Im-Cl method. After database searching, in triplicate runs, 2876 proteins and 12 208 peptides were identified from rat brains, of which 1025 proteins contained at least one predicted TMD, and 4031 hydrophobic peptides were identified. Furthermore, 128 peptides containing partial or whole TMDs were identified and recognized as transmembrane peptides (Supporting Information, Table S1), which was important and challenging for IMP analysis. In comparison, Chen et al.[11] reported that 2645−3291 proteins were identified from 300 $\mu$g of rat brain homogenate using a combination of mixed organic and aqueous solvents with three MS-compatible detergents, respectively, of which 423−556 proteins were predicted to be transmembrane proteins and 23−88 transmembrane peptides were identified. It is clear that, with our method, more transmembrane proteins could be identified with less starting materials, demonstrating the superiority of our proposed method for the large scale membrane proteome analysis.

In addition, we further analyzed the distribution of the identified IMPs with respect to the number of TMDs (Supporting Information, Figure S4), which showed that the IMPs identified were distributed from 1 TMD to 19 TMDs, demonstrating that our method could efficiently identify IMPs with various TMDs without any bias.

## CONCLUSIONS

A systematic study of the effects of ILs on the solubilization and analysis of IMPs was performed. The hydrophobic interactions of the alkyl substituents of imidazolium-based ILs were proven to play a dominant role in IMP solubilization, followed by the $\pi-\pi$ conjugate interactions of unsaturated alkyl substituents. With the further consideration of biocompatibility with trypsin digestion, a C12Im-Cl-assisted method was proposed for analysis of the membrane proteome and shown to be superior to an SDS-assisted method. The results demonstrated the great promise of our proposed IL-based sample preparation method for the large-scale membrane proteome profiling.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org/.

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### Notes

The authors declare no competing financial interest.

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## REFERENCES

(1) Whitelegge, J. P. *Anal. Chem.* **2013**, *85*, 2558−2568.
(2) Speers, A. E.; Wu, C. C. *Chem. Rev.* **2007**, *107*, 3687−3714.
(3) Vuckovic, D.; Dagley, L. F.; Purcell, A. W.; Emili, A. *Proteomics* **2013**, *13*, 404−423.
(4) Ye, X.; Li, L. *Anal. Chem.* **2012**, *84*, 6181−6191.
(5) Wu, C. C.; MacCoss, M. J.; Howell, K. E.; Yates, J. R., 3rd. *Nat. Biotechnol.* **2003**, *21*, 532−538.
(6) Melo-Braga, M. N.; Schulz, M.; Liu, Q.; Swistowski, A.; Palmisano, G.; Engholm-Keller, K.; Jakobsen, L.; Zeng, X.; Larsen, M. R. *Mol. Cell. Proteomics* **2014**, *13*, 311−328.
(7) Catherman, A. D.; Durbin, K. R.; Ahlf, D. R.; Early, B. P.; Fellers, R. T.; Tran, J. C.; Thomas, P. M.; Kelleher, N. L. *Mol. Cell. Proteomics* **2013**, *12*, 3465−3473.
(8) Srivastava, V.; Malm, E.; Sundqvist, G.; Bulone, V. *Mol. Cell. Proteomics* **2013**, *12*, 3874−3885.
(9) Yu, Y.; Xie, L.; Gunawardena, H. P.; Khatun, J.; Maier, C.; Spitzer, W.; Leerkes, M.; Giddings, M. C.; Chen, X. *Anal. Chem.* **2012**, *84*, 9008−9014.
(10) Masuda, T.; Tomita, M.; Ishihama, Y. *J. Proteome Res.* **2008**, *7*, 731−740.
(11) Chen, E. I.; McClatchy, D.; Park, S. K.; Yates, J. R., 3rd. *Anal. Chem.* **2008**, *80*, 8694−8701.
(12) Takahashi, D.; Kawamura, Y.; Uemura, M. *J. Proteome Res.* **2013**, *12*, 4998−5011.
(13) Blonder, J.; Chan, K. C.; Issaq, H. J.; Veenstra, T. D. *Nat. Protoc.* **2006**, *1*, 2784−2790.
(14) Zhang, N.; Chen, R.; Young, N.; Wishart, D.; Winter, P.; Weiner, J. H.; Li, L. *Proteomics* **2007**, *7*, 484−493.
(15) Ma, J.; Hou, C.; Sun, L.; Tao, D.; Zhang, Y.; Shan, Y.; Liang, Z.; Zhang, L.; Yang, L.; Zhang, Y. *Anal. Chem.* **2010**, *82*, 9622−9625.
(16) Rietschel, B.; Bornemann, S.; Arrey, T. N.; Baeumlisberger, D.; Karas, M.; Meyer, B. *Proteomics* **2009**, *9*, 5553−5557.
(17) Zhao, Q.; Liang, Y.; Yuan, H.; Sui, Z.; Wu, Q.; Liang, Z.; Zhang, L.; Zhang, Y. *Anal. Chem.* **2013**, *85*, 8507−8512.
(18) Wei, J.; Sun, J.; Yu, W.; Jones, A.; Oeller, P.; Keller, M.; Woodnutt, G.; Short, J. M. *J. Proteome Res.* **2005**, *4*, 801−808.
(19) Hallett, J. P.; Welton, T. *Chem. Rev.* **2011**, *111*, 3508−3576.
(20) van Rantwijk, F.; Sheldon, R. A. *Chem. Rev.* **2007**, *107*, 2757−2785.
(21) Dupont, J. *J. Braz. Chem. Soc.* **2004**, *15*, 341−350.
(22) Tseng, M. C.; Tseng, M. J.; Chu, Y. H. *Chem. Commun.* **2009**, 7503−7505.
(23) Li, Z.; Pei, Y.; Wang, H.; Fan, J.; Wang, J. *TrAC, Trends Anal. Chem.* **2010**, *29*, 1336−1346.
(24) Huang, J. L.; Noss, M. E.; Schmidt, K. M.; Murray, L.; Bunagan, M. R. *Chem. Commun.* **2011**, *47*, 8007−8009.
(25) Ho, T. D.; Zhang, C.; Hantao, L. W.; Anderson, J. L. *Anal. Chem.* **2013**, *86*, 262−285.
(26) Sun, P.; Armstrong, D. W. *Anal. Chim. Acta* **2010**, *661*, 1−16.
(27) Dong, K.; Zhang, S. *Chemistry* **2012**, *18*, 2748−2761.
(28) Sun, L.; Tao, D.; Han, B.; Ma, J.; Zhu, G.; Liang, Z.; Shan, Y.; Zhang, L.; Zhang, Y. *Anal. Bioanal. Chem.* **2011**, *399*, 3387−3397.
(29) Tao, D.; Qiao, X.; Sun, L.; Hou, C.; Gao, L.; Zhang, L.; Shan, Y.; Liang, Z.; Zhang, Y. *J. Proteome Res.* **2011**, *10*, 732−738.
(30) Qiao, X.; Tao, D.; Qu, Y.; Sun, L.; Gao, L.; Zhang, X.; Liang, Z.; Zhang, L.; Zhang, Y. *Proteomics* **2011**, *11*, 4274−4278.

(31) Zhao, Q.; Sun, L.; Liang, Y.; Wu, Q.; Yuan, H.; Liang, Z.; Zhang, L.; Zhang, Y. *Talanta* **2012**, *88*, 567−572.
(32) Lin, Y.; Zhou, J.; Bi, D.; Chen, P.; Wang, X.; Liang, S. *Anal. Biochem.* **2008**, *377*, 259−266.
(33) Li, D.; Fu, Y.; Sun, R.; Ling, C. X.; Wei, Y.; Zhou, H.; Zeng, R.; Yang, Q.; He, S.; Gao, W. *Bioinformatics* **2005**, *21*, 3049−3050.
(34) Blonder, J.; Goshe, M. B.; Moore, R. J.; Pasa-Tolic, L.; Masselon, C. D.; Lipton, M. S.; Smith, R. D. *J. Proteome Res.* **2002**, *1*, 351−360.
(35) Zhang, H.; Lin, Q.; Ponnusamy, S.; Kothandaraman, N.; Lim, T. K.; Zhao, C.; Kit, H. S.; Arijit, B.; Rauff, M.; Hew, C. L.; Chung, M. C.; Joshi, S. B.; Choolani, M. *Proteomics* **2007**, *7*, 1654−1663.
(36) Luis, P.; Ortiz, I.; Aldaco, R.; Irabien, A. *Ecotoxicol. Environ. Saf.* **2007**, *67*, 423−429.
(37) Chen, S.; Zhang, S.; Liu, X.; Wang, J.; Wang, J.; Dong, K.; Sun, J.; Xu, B. *Phys. Chem. Chem. Phys.* **2014**, *16*, 5893−5906.
(38) Lopez-Pastor, M.; Ayora-Canada, M. J.; Valcarcel, M.; Lendl, B. *J. Phys. Chem. B* **2006**, *110*, 10896−10902.
(39) Dominguez-Vidal, A.; Kaun, N.; Ayora-Canada, M. J.; Lendl, B. *J. Phys. Chem. B* **2007**, *111*, 4446−4452.