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TRENDS

Advances in the analysis of biological samples using ionic liquids

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Abstract
Ionic liquids are a class of solvents and materials that hold great promise in bioanalytical chemistry. Task-specific ionic liquids have recently been designed for the selective extraction, separation, and detection of proteins, peptides, nucleic acids, and other physiologically relevant analytes from complex biological samples. To facilitate rapid bioanalysis, ionic liquids have been integrated in miniaturized and automated procedures. Bioanalytical separations have also benefited from the modification of nonspecific magnetic materials with ionic liquids or the implementation of ionic liquids with inherent magnetic properties. Furthermore, the direct detection of the extracted molecules in the analytical instrument has been demonstrated with structurally tuned ionic liquids and magnetic ionic liquids, providing a significant advantage in the analysis of low-abundance analytes. This article gives an overview of these advances that involve the application of ionic liquids and derivatives in bioanalysis.

Keywords Task-specific ionic liquid · Magnetic ionic liquid · Bioanalysis · Automation · Miniaturization · Magnetic separation

Introduction

Driven by their unique properties and tunable chemical structures, ionic liquids (ILs) have rapidly expanded into the field of bioanalytical chemistry. By definition, ILs are molten salts composed of organic/inorganic cations and anions that exhibit melting temperatures of or below 100 °C. Many of the known ILs possess low or negligible vapor pressures under ambient conditions and are derived from bulky cations, including functionalized phosphonium, imidazolium, ammonium, or pyrrolidinium, paired with weakly coordinating anions such as bis[(trifluoromethyl)sulfonyl]imide ($[NTf_2^-]$). However, the choices of cation and anion structures are virtually limitless and result in the formation of ILs with vastly different fundamental properties including viscosity, conductivity, thermal stability, and hydrophobicity.

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The customizable nature of the IL cation and anion components established the basis for what are known as "task-specific ILs," whose physicochemical properties are tailored for a given application [1]. This conceptualization of ILs is particularly useful in the field of analytical chemistry, where ILs have been designed to engage in specific intermolecular interactions to extract, purify, separate, and detect analytes [2]. For example, ILs may facilitate selective preconcentration of an analyte or analyte class by exhibiting a strong affinity toward functional group(s) in the chemical structure of the analyte. For biological macromolecules, a multitude of interactions between the IL solvent and biopolymer may exist that must be carefully considered for selective enrichment from various biological samples. In addition to the complexity of biomolecule structure, a major obstacle facing the application of ILs in bioanalytical chemistry has been the design of ILs that preserve the secondary or tertiary structure of biopolymers.

Many biologically relevant compounds are present in samples at very low concentrations and require considerable effort to preconcentrate them. IL extraction solvents that can be directly injected into analytical instrumentation without dilution are particularly desirable for detection of low-abundance analytes in biological samples. In addition to some liquid handling instruments that have been specifically designed for manipulating ILs, IL-based media have also shown promise in

this area, as demonstrated by the online implementation of these materials in chromatographic analysis.

Structural modifications to ILs may also impart magnetic susceptibility that can be exploited to reduce the analysis time and manual sample handling steps of bioanalytical methods that are notoriously difficult/expensive to automate. The covalent tethering of ILs to solid supports is a versatile modification to nonspecific magnetic materials to compensate for their limited selectivity. Magnetic ILs (MILs) also combine the structural tunability of conventional ILs with magnetic properties, but are a particle-free alternative to magnet-assisted separations that maintains extraction selectivity while avoiding the drawbacks of solid particle aggregation.

This article focuses on ILs, MILs, and IL-derived materials that have advanced the analysis of biological samples. The structural tunability of ILs, miniaturization and automation of IL-based methods, magnet-assisted platforms that capitalize on IL-derived media, and the compatibility of ILs with downstream instrumentation are topics that are emphasized herein.

Rational design of ionic liquid chemical structure for specific tasks involving biomolecules

The physical, chemical, and ultimately functional properties of an IL solvent are inextricably related to the structure of its cation and anion components. By carefully selecting and installing substituent groups in their structure, one can design ILs to engage in specific interactions with analyte molecules. Although this unique feature of ILs was articulated more than a decade ago [1], applications of task-specific ILs in bioanalytical chemistry have only recently emerged. Identifying IL solvents that are suitable for biological applications has been a formidable challenge in part because of the complexity of biopolymer structure. For proteins, enzymes, and nucleic acids, biological function is dependent not only on the primary sequence of amino acids or nucleotides but also on the three-dimensional arrangement of atoms in space. Altering this secondary or tertiary structure has many implications and can lead to aggregation, inactivation, rapid degradation, or loss of the structural features that facilitate analysis of the biomolecule. The limited stability of some biopolymers in vitro prompted the investigation of ILs as solvents to increase their solubility and long-term stability. A hydrated form of the 1-butyl-1-methylpyrrolidinium bis[(trifluoro methyl)sulfonyl]imide ($[BMPy^+][NTf_2^-]$) IL was initially discovered to preserve the secondary structure of the protein monellin at temperatures approximately 60 °C higher than its unfolding temperature in aqueous buffer [3]. Similarly, hydrated choline-based ILs were found to enhance the solubility and stability of cytochrome *c* when paired with anions that exhibit relatively low cytotoxicity such as dihydrogen phosphate [4]. These observations supported the feasibility of designing IL-based solvent systems for bioanalysis that could mitigate unwanted perturbation of higher-order structures.

The separation of intact protein mixtures is rapidly gaining interest in biopharmaceutical analysis and diagnostic applications. High-performance liquid chromatography (HPLC) is a powerful separation technique capable of fractionating a complex sample into its constituents, but can often lead to the denaturation of proteins and enzymes because of the use of organic solvent-based mobile phases. Zhou and Danielson [5] investigated the separation of proteins using HPLC with the isopropylammonium formate IL as an eluent to enhance the stability of proteins during the chromatographic process. The IL solvent was applied in a gradient elution method that permitted the retention and elution of the multisubunit enzyme lactate dehydrogenase (LDH), and was found to preserve enzymatic activity when mobile phase compositions of up to 30% (v/v) IL were used. In comparison, the use of a conventional organic eluent (methanol) resulted in the complete inactivation of LDH at just 10% (v/v). The partitioning and stability of bovine serum albumin (BSA) was investigated in aqueous biphasic systems (ABS) comprising imidazolium-, choline-, or ammonium-based ILs with various anions, including some derived from Good's buffers [6, 7]. In addition to evidence suggesting that the tertiary structure of BSA was intact, quantitative extraction of BSA into the IL-rich phase demonstrated the potential practical application of these IL-based extraction systems.

The selectivity of extraction methods for biomolecules must also be rigorously controlled so as to minimize the co-extraction of interfering agents that would otherwise inhibit downstream experiments. Tseng et al. [8] reported the selective isolation of peptides and proteins containing lysine or arginine residues by using triazolium-based ILs functionalized with crown ether moieties [8]. By increasing the cavity size of the crowned IL, they achieved selective extraction of peptides with multiple basic residues. The crowned IL solvents were also capable of quantitatively extracting proteins rich in basic side chains from aqueous solutions, but spectroscopic measurements indicated a partial loss of native protein conformation on recovery of the protein from the IL phase. The trade-off between extraction selectivity and protein stability will likely persist until new IL structures are investigated.

Precise control over the secondary and tertiary structure of nucleic acids by IL solvents has also been investigated to advance bioanalytical technologies, including selective target recognition, signal amplification, and fluorescence imaging. Choline-based ILs were found to stabilize the secondary structure of DNA via interactions with the minor groove of the double helix [9, 10]. These choline-based solvents imparted long-term stability to nucleic acids of up to 1 year at room

temperature and even in the presence of nucleases that possess strong hydrolytic activity toward DNA/RNA [11]. For some applications, such as molecular beacons (MB) or aptamer technologies, a more careful balance of stabilized/destabilized nucleic acid secondary structure is desirable. Machado et al. [12] examined the binding selectivity of a DNA molecular beacon for a complementary nucleic acid in the hydrated form of the ethylammonium nitrate IL. Within the IL medium, a partial decrease in melting temperature of the MB was accompanied by faster hybridization kinetics with the target single-stranded DNA molecule, demonstrating the potential of IL solvents to increase the speed of molecular diagnostics. Further decreasing the melting temperature of double-stranded DNA can also be highly advantageous for nucleic acid amplification. The base content of DNA has dramatic effects on the melting temperature of a given sequence, particularly for GC-rich nucleic acids that exhibit highly stable secondary structure. Shi et al. [13] investigated the influence of bicyclic imidazolium-based ILs on the polymerase chain reaction (PCR) amplification efficiency of nucleic acids with high GC content and high melting temperature (approximately 94 °C). As shown in Fig. 1, conventional methods such as treatment of the sample with dimethyl sulfoxide or betaine failed to assist target amplification, but the addition of IL to the PCR mixture resulted in successful amplification and also reduced the incidence of nonspecific amplification.

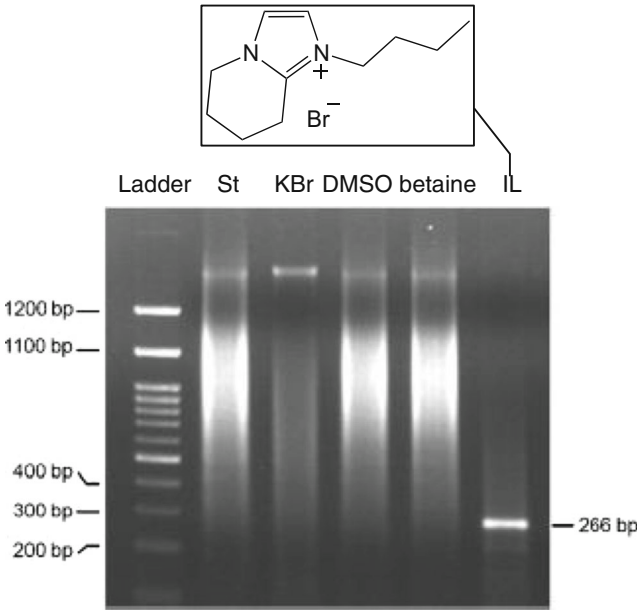

Fig. 1 Polymerase chain reaction amplification of a 266-bp DNA fragment with high GC content and high melting temperature (approximately 94 °C) using salt (St; KBr), dimethyl sulfoxide (DMSO), betaine, or ionic liquid (IL) additives. Smearing and high molecular weight bands indicate nonspecific amplification. (Reprinted with permission from [13])

The selective extraction and purification of nucleic acids is an essential step in the bioanalytical workflow that mitigates the inhibition of downstream detection methods. Initially, Wang et al. [14] demonstrated the nearly quantitative extraction of DNA from aqueous solution using the 1-butyl-3-methylimidazolium hexafluorophosphate ($[BMIM^+][PF_6^-]$) IL. Despite relatively low recoveries of DNA from the IL phase (approximately 30%) and disruption of the native B-form structure of duplex DNA, these experiments ushered in new efforts to study ILs as sorptive phases for nucleic acid applications. The extraction of RNA from biological samples was recently investigated with use of polymeric ILs (PILs) that provided a highly selective sorbent for RNA compared with traditional liquid–liquid extraction approaches [15]. Coupling the selective PIL-based sample preparation approach with reverse transcription real-time quantitative PCR demonstrated the compatibility of the IL-derived material with sensitive enzymatic assays.

Miniaturization, simplification, and automation of analytical methods using ionic liquids for the analysis of xenobiotics and biological samples

The combination of IL solvents with miniaturized and automated methods undoubtedly constitutes an important advancement toward faster and more selective analyses that align with the goals of green analytical chemistry [16]. Miniaturized and automated methods that reduce solvent consumption, analysis time, and sources of error are especially desirable in sample preparation since it is the major bottleneck of any analytical process. Furthermore, biological samples are often limited in quantity and necessitate the use of small volumes of sample and reagents.

ILs have been used as extraction solvents in newly designed extraction devices so as to simplify and miniaturize microextraction procedures. The 1-octyl-3-methylimidazolium hexafluorophosphate ($[C_8MIM^+][PF_6^-]$) IL was used as the extraction solvent for in-syringe dispersive liquid–liquid microextraction (DLLME) of danazol in drugs and mouse serum [17]. In this approach, two syringes were used for the extraction of the target analytes, while the dispersion of the IL in the sample was achieved by injection of the mixture from one syringe to another. The method developed was coupled with HPLC for the determination of danazol in mouse serum samples, providing a limit of detection of 54 $\mu g \cdot L^{-1}$, interday relative standard deviation ranging from 2.7% to 3.9%, and relative recoveries between 90.5% and 103%.

Automated and online procedures have also been designed to dramatically increase the throughput of IL-based sample preparation. Figure 2 shows a diagram of an automated dynamic DLLME procedure used for the determination of anti-inflammatory drugs in human urine [18]. The system is based on a syringe pump connected through polytetrafluoroethylene

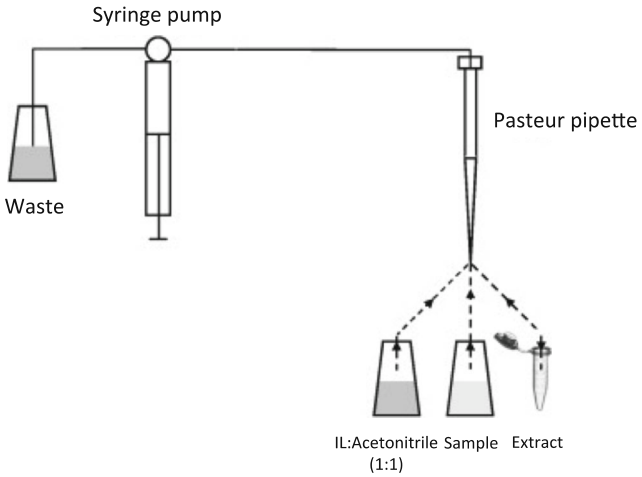

Fig. 2 The experimental setup of the dynamic liquid-phase microextraction instrument that uses ionic liquid (IL) extraction solvents. (Reprinted with permission from [18])

tubing to a Pasteur pipette that serves as the extraction unit. During the extraction, a mixture of the 1-butyl-3-methylimidazolium hexafluorophosphate ($[C_4MIM^+][PF_6^-]$) IL extraction solvent and acetonitrile (disperser solvent) fills the Pasteur pipette and the sample is continuously introduced into the system with use of the syringe pump. The IL phase is then collected and injected for subsequent detection of the analytes.

Some approaches permit the integration of sample preparation and analysis into a single system using online extraction. Suárez et al. [19] used multiple syringes connected to a multiposition valve for the extraction of ultraviolet filters in water. In this method, the syringes simultaneously propel and mix the sample, extraction solvent, and other components required for the extraction method within an extraction chamber. When the extraction ends, the system directs the analyte-enriched extraction solvent to the detection system. This method exploited the high affinity of analytes for the IL phase to achieve efficient and rapid extractions while minimizing deleterious matrix effects. IL-based sorbents have also recently been applied for the purification of analytes in online solid-phase extraction (SPE). Liu et al. [20] developed an online SPE-HPLC approach for the determination of antihypertensives in human plasma. The SPE method used a monolithic column based on a PIL prepared by the polymerization of the 1-vinyl-3-butylimidazolium chloride ($[VMIM^+][Cl^-]$) IL monomer that contributed to the elimination of the matrix effect caused by proteins in the human plasma samples.

Magnetic separations using ionic liquid-based materials and magnetic ionic liquids

Magnet-assisted procedures have increased in popularity in recent years because of the ease of handling and increased sample throughput afforded by functional magnetic substrates [21]. Magnetic separations exploit the magnetic susceptibility of an extraction phase, permitting the isolation of analytes from complex mixtures by the simple application of a magnetic field. In the last few years, ILs have been successfully applied in magnet-assisted microextraction procedures that can be divided in two groups according to the nature of the magnetic fluid used as the extraction phase: (1) magnetic/IL hybrid materials [22–25] and (2) MILs [27–29].

Magnetic/IL hybrid materials possess two components: a magnetic core based on magnetic nanoparticles (MNPs) and an IL (or derivative, including IL-based surfactants or PILs) that is usually coated/functionalized on the surface of the MNPs (MNP@IL) [22–25]. The magnetic core facilitates the magnetic separation, while the extraction performance of the material is related to the IL component. In general, MNP@IL materials are core–shell particles, but other structures such as end-grafted polymer-coated particles, fully encapsulated particles, or heterogeneous particles are also possible. The MNPs are usually based on metal or metal oxides of iron, cobalt, nickel, or a combination of metals, with magnetite (Fe_3O_4) being the most commonly used substrate. Since the magnetic core possesses extremely limited extraction selectivity, the IL component of the hybrid material is often designed to engage in specific interactions with the analytes studied.

Magnetic/IL hybrid materials have been used as sorbents in different microextraction approaches, including dual magnetic microextraction (DMME) [22] and magnet-assisted dispersive micro-SPE (μ-SPE) [23–25]. DMME is a combination of DLLME and dispersive μ-SPE where the IL is first dispersed in the sample (DLLME step) to rapidly extract analytes followed by the addition of MNPs (dispersive μ-SPE step) [22]. Weak interactions between the analyte-enriched IL and the MNPs are then established and facilitate the magnetic separation of the extraction phase from the sample. A hallmark of the aforementioned DMME procedure is the in situ generation of the magnetic/IL hybrid material during the microextraction procedure. In contrast, magnet-assisted dispersive μ-SPE applications use hybrid materials that are synthesized and fully characterized before the microextraction procedure where the extraction step and the elution step are both done with magnetic separation of the material. Higher extraction capacity, shorter extraction times, and the possibility of reusing the material are some of the advantages of magnet-assisted dispersive μ-SPE compared with conventional dispersive μ-SPE [23–25].

In contrast to magnetic particle suspensions such as magnetic hybrid materials or ferrofluids, MILs are neat liquids that have rapidly expanded into magnet-assisted microextraction procedures as an alternative to aggregation-prone solid magnetic particles [26]. MILs are a subclass of ILs that contain one or more paramagnetic components in their chemical structure. These magnetic solvents possess some of the unique

physicochemical properties of ILs and impressive solvation capabilities for both polar and nonpolar compounds. Compared with the MNP@IL materials, MILs are easily prepared and are also optically transparent solvents, rendering them useful for spectroscopic applications.

MILs have been used in magnet-assisted DLLME where the MIL is dispersed into fine microdroplets in solution and recovered with use of a magnetic field, resulting in reduced extraction times and increased extraction efficiency [27–29]. The MIL solvent may also be adapted to magnet-assisted single-drop microextraction (SDME) procedures. For SDME using MILs, the MIL solvent is suspended from a rod magnet instead of a common microsyringe and is introduced into the headspace or directly immersed in the sample during extraction [27, 28]. Because of the influence of the magnetic field on the MIL solvent droplet, a higher microdroplet volume can be suspended from the rod magnet for a prolonged sampling time.

Most applications to date have used MILs containing Fe(III)-based anions such as tetrachloroferrate(III) ($[FeCl_4^-]$) or bromotrichloroferrate(III) ($[FeBrCl_3^-]$) and have served as selective and efficient solvents for the extraction of DNA from complex matrices [27, 29]. To minimize the hydrolysis of the tetrahaloferrate(III) anions, the water solubility of the MIL was controlled with a combination of hydrophobic substituents in the cation component and the use of dicationic MIL systems that permitted the use of paramagnetic and hydrophobic heteroanions. Another strategy to increase the hydrolytic stability of MILs has been to incorporate the tetrachloromanganate(II) ($[MnCl_4^{2-}]$) anion into the MIL structure. MILs including trioctylmethylammonium tetrachloromanganate(II) ($[Aliquat^+]_2[MnCl_4^{2-}]$) and trihexyl(tetradecyl)phosphonium tetrachloromanganate(II) ($[P_{6,6,6,14}^+]_2[MnCl_4^{2-}]$) were explored as alternatives to Fe(III)-based MILs [28] since they are less susceptible to undergo hydrolysis and possess limited absorbance in the ultraviolet–visible region that is commonly used for instrumental analysis. However, the high viscosity of these tetrahalomanganate(II)-based MILs resulted in difficulties with handling and dispersion of the solvent within the sample solution. To address these issues, a new generation of low-viscosity MILs comprising anions derived from metal complexes with hexafluoroacetylacetonate have been explored as possible extraction materials for magnet-assisted procedures. Some examples include the recently described MILs trihexyl(tetradecyl)phosphonium tris(hexafluoroacetylaceto)manganate(II) ($[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$) and trihexyl(tetradecyl)phosphonium tris(hexafluoroacetylaceto)nickelate(II) ($[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$) [30], which are promising extraction materials for both biological and environmental applications. For example, the MIL $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ was recently applied as a magnetic liquid support for the sequence-specific capture of nucleic acid fragments from heterogeneous pools of genomic DNA and cell lysate [31]. The MIL-based approach exhibited superior recovery efficiency compared with use of commercial magnetic particles and provided a particle- and aggregation-free nucleic acid extraction platform suitable for interfacing with microdevices. Because of the rapid and recent advances of MIL solvents in analytical chemistry, further investigation of MILs and their extraction properties is highly anticipated.

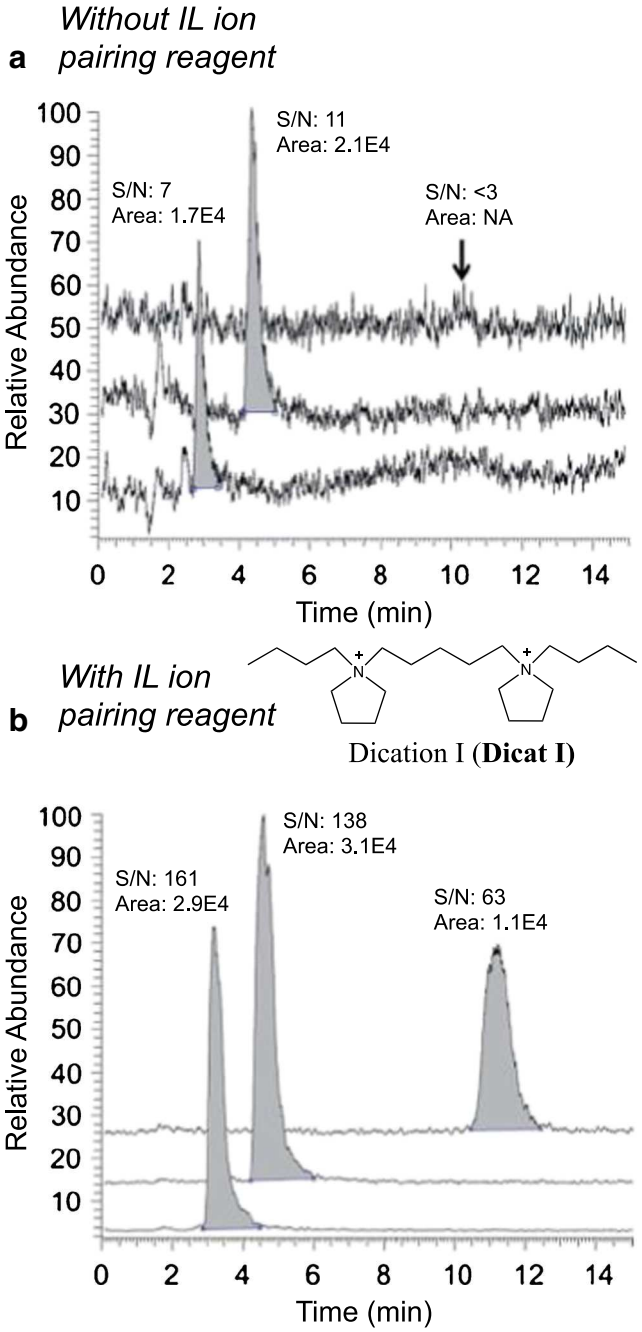

Fig. 3 Comparison of signal-to-noise ratios (S/N) and peak areas obtained for three sphingolipids analyzed by high-performance liquid chromatography–electrospray ionization mass spectrometry in positive mode **a** without an ionic liquid (IL) ion-pairing reagent and **b** with a dicationic IL ion-pairing reagent. NA not available. (Reprinted with permission from [36])

Compatibility of ionic liquids with analytical instrumentation

To capitalize on the unique solvation and physicochemical properties of ILs, the direct introduction of ILs or MILs into an analytical instrument for the measurement of dissolved analytes has become a topic of intense investigation. The detection of molecules within IL solvents is particularly useful for the coupling of extraction/purification methods with chromatographic separations. After analyte preconcentration into an IL or MIL solvent, injection of the analyte-enriched IL into the HPLC system circumvents lengthy back-extraction or recovery procedures and does not impose the need for further dilution of analytes before detection. For example, Chatzimitakos et al. [32] demonstrated the preconcentration of phenolic endocrine disrupters with Fe(III)-based MIL solvents and subsequent analysis of the analyte-enriched MIL by direct injection into an HPLC instrument. By selection of the appropriate detection wavelength (254 nm), the background signal from the MIL did not interfere with analyte quantification. Mn(II)-based MILs have also been used for extraction and direct HPLC analysis, but provided even lower background signals than Fe(III) MILs [28].

The low vapor pressure and liquid nature of ILs renders these solvents as ideal matrix substances in matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). One challenge associated with conventional crystalline MALDI matrices is the heterogeneous distribution of analytes during the co-crystallization process. The inconsistencies in analyte signal across the sample cumulatively affect signal reproducibility and quantification. To address these problems, ILs were investigated as matrix substances in MALDI-MS for the analysis of a variety of biopolymers, including proteins and peptides [33]. Improved spot homogeneity and, for some analytes, higher signal intensities were observed. IL matrix substances have also been successfully applied in matrix-enhanced secondary ion mass spectrometry (SIMS) for the analysis of lipid profiles of neuronal cells [34].

ILs have also been applied in electrospray ionization mass spectrometry (ESI-MS) to enhance the signal of acidic or anionic substances [35]. By the introduction of low concentrations of dicationic (or polycationic) IL ion-pairing reagents into the electrospray ionization stream, complexes between the IL dication and the anion analyte are formed. The complex then allows highly sensitive detection in positive ion mode because of its higher inherent sensitivity, the higher molecular weight of the complex, which avoids the chemical noise from low mass interferences, and the greater surface activity of the analyte–IL complex. Xu et al. [36] applied dicationic and tetracationic IL ion-pairing reagents for the analysis of sphingolipids in aqueous solution at picomolar and femtomolar concentrations. As shown in Fig. 3, use of structurally optimized IL ion-pairing reagents increased the signal intensities and peak areas for the sphingolipids studied by between one and three orders of magnitude.

Outlook

The unique physicochemical properties of ILs have been central to the development of task-specific solvents and materials in many areas of bioanalytical chemistry. The selective extraction and separation of biomolecules is a rapidly developing application that is expected to benefit from the design of new IL solvents that also preserve higher-order structures in the biopolymer. Simultaneously, the integration of extraction procedures using ILs with miniaturized and automated methods promises to reduce organic solvent consumption and analysis time. Magnetic separations using magnetic/IL hybrid materials have been successfully used for biological applications, and the development of new classes of MILs has expanded the possibilities for their use in many types of magnet-assisted microextraction procedures. The hallmarks of MIL-based methods, including particle-free magnetic separations and higher sample throughput, will undoubtedly be the subject of continuing investigations to advance bioanalysis techniques. To capitalize on the selectivity and speed of IL- and MIL-based approaches, it is important that these solvents be designed for direct injection/introduction into analytical instrumentation. Since MILs typically contain metal complexes that exhibit high absorption in the ultraviolet region, new MIL solvents that minimally contribute to background analytical signals are expected to have a significant impact in the separation science community. The preparation of spectroscopically pure ILs and MILs through novel synthetic methods and purification schemes will also be important for the broader application of ILs and MILs. As ILs and MILs expand into bioanalytical applications, their merits as greener alternatives compared with conventional media should also continue to be thoroughly investigated. The lower cytotoxicity and environmental risks of some classes of ILs and MILs serve as incentives for laboratories to consider these intriguing ionic media for bioanalytical applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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