Full Paper

Application of Some Room Temperature Ionic Liquids in the Development of Biosensors at Carbon Film Electrodes

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Abstract
Two room temperature ionic liquids, 1-butyl-3-methylimidazolium bistriflimide and 1-butyl-3-methylimidazolium nitrate, were employed for enzyme immobilization in a new sol-gel matrix and, for the first time, were successfully applied as electrolyte carriers in a biosensing system. The new sol-gel matrix, based on 3-aminopropyltrimethoxysilane and 1-butyl-3-methylimidazolium bistriflimide mixtures, did not crack even after several weeks when kept dry, and exhibited similar analytical properties to aqueous sol-gel based glucose biosensors. The linear range was up to 1.1 mM of glucose, sensitivity was 62 nA mM^{-1} and the limit of detection was 28.8 μM. The optimum ionic liquid electrolyte carrier was found to be 1-butyl-3-methylimidazolium nitrate, where the biosensor was made by electrodeposition of the redox mediator, poly(neutral red), and the enzyme was immobilized by cross-linking with glutaraldehyde. The results showed that application of room temperature ionic liquids to biosensors is very promising and can be further exploited.

Keywords: Room temperature ionic liquids, Electrochemical enzyme biosensors, Sol-gel, Glutaraldehyde cross-linking, Poly(neutral red), Glucose

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1. Introduction

Recently, a particular class of organic solvents, room temperature ionic liquids (RTILs), has been explored to identify their advantages in biocatalysis [1, 2]. RTILs are usually organic or mixed organic-inorganic salts that have melting points less than 100 °C and are frequently liquid at room temperature [1, 3]. Because of their lack of vapor pressure and resulting ease of containment, RTILs are considered to be green solvents. Moreover, these solvents do not deactivate enzymes like other nonaqueous solvents [4], and their ability to dissolve a wide range of dissimilar substrates facilitates reaction of polar substrates such as sugars [1].

RTILs have been used in fundamental biochemical investigations including: hemoglobin [5, 6], peroxidase [7, 8], hemin, and cytochrome c [7], amino acid esters [9], papain [10], and in the analysis of ethanol [11] and dopamine [12]. Also, the development of enzyme-based biosensors for hydrogen peroxide (horseradish peroxidase, [13]), glucose (glucose oxidase was immobilized using polymerized ionic liquids [14, 15]), and paraoxon (acetylcholinesterase inhibition, [16]) have been reported. Polymerized ionic liquid, poly(propylene glycol)-block-(ethylene glycol)-block-(propyleneglycol)-bis(2-aminopropyl ether), as electrolyte was reported only in a sensing system for ethanol determination [11], but in other cases polymerized ionic liquids were used as a matrix for enzyme immobilization [14] or for modification of the electrode surface [15].

In recent years, carbon film electrodes fabricated from carbon film electrical resistors have been introduced in electroanalytical chemistry. These electrodes have been evaluated [17, 18] and successfully used in electroanalytical [19–22] and bioelectroanalytical chemistry [23–26]. Also, these electrodes have similar electrochemical properties to glassy carbon, especially after electrochemical preconditioning; other advantages include their physical robustness and ease of preparation.

This study reports the application of imidazolium-based RTILs in biosensing systems. The first methodology investigated is the immobilization of enzymes in sol-gel matrices incorporating RTIL. The second approach investigated is the use of a RTIL as the carrier electrolyte for electrochemical biosensors. In contrast to previous work, non-polymeric RTILs were used here.

2. Experimental

2.1. Chemicals and Solutions

The two room temperature ionic liquids used for electrochemical studies were 1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide (denoted in the text as bistri-

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flimide) ($BmimNTF_2$) and 1-butyl-3-methylimidazolium nitrate ($BmimNO_3$) which were synthesized as previously described [27]. The structure of these compounds is shown elsewhere [27].

Neutral red (NR) monomer (N^8,N^8 – 3-trimethylphenazine-2,8,-diamine [30]) was obtained from Aldrich (Germany). The three oxysilanes used for enzyme encapsulation were 3-glycidoxypropyltrimethoxysilane (GOPMOS), methyl-trimethoxysilane (MTMOS; Aldrich, Germany), and 3-aminopropyltriethoxysilane (APTOS; Fluka, Switzerland). Glucose oxidase (GOx) from *Aspergillus niger*, anhydrous α-D-(+)-glucose, bovine serum albumin (BSA), and glutaraldehyde (GA) were obtained from Sigma (Germany).

Solutions were prepared either in RTILs, or in Milli-Q nanopure deionized water (≥ 18 Ω cm). Experiments were carried out at room temperature (25 ± 1 °C).

2.2. Methods and Instruments

The three-electrode electrochemical cell (of 300 μL volume when RTIL was used as electrolyte, or of 10 mL volume in the case aqueous electrolyte) contained the modified carbon film working electrode and a platinum wire counter electrode. In RTIL media, a silver wire (coated with solid-state AgCl, i.e., $Ag/AgCl_{ss}$) served as pseudo-reference electrode; whereas, in aqueous media, a saturated calomel electrode (SCE), was used as reference. Measurements were performed using a computer-controlled μ-Autolab Type II potentiostat/galvanostat with GPES 4.9 software (Eco Chemie, Netherlands).

2.3. Electrode Preparation

Electrodes were made from carbon film electrical resistors (2.0 ± 0.1 Ω resistance) as described elsewhere [17, 18]. The final exposed geometric area of the electrode was ca. 0.20 cm^2.

Neutral red was polymerized electrochemically from an aqueous solution containing 1 mM NR monomer, 0.05 M phosphate buffer (pH 5.5) and 0.1 M KNO_3, by cycling the applied potential 15 – 20 times between -1.0 and $+1.0$ V vs. SCE at a scan rate of 50 mV s^{-1}.

Glucose oxidase was either immobilized using glutaraldehyde cross-linking or was encapsulated in a sol-gel matrix. For glutaraldehyde immobilization, a mixture comprising 10 μL of 10% GOx solution (in 0.1 M phosphate buffer saline (PBS) pH 7.0); 10 μL of 10% BSA solution (in 0.1 M PBS pH 7.0); 1 μL of $BmimNTF_2$; 1 μL of 23% GA solution (in water), was used. All the components were carefully mixed, and 5 μL of the mixture was applied on the surface of the PNR-modified carbon film electrode. The coated electrode was left in air for 1 hour to dry out.

For the sol-gel approach, solutions were prepared by mixing the sol-gel precursor with the RTIL and water (in the proportion 150:200:450 μL), followed by the addition of 2 μL of 6 M HCl. This mixture was intensively mixed for two minutes in a vortex mixer (UniEquip, Germany) and then sonicated for 15 min in order to accelerate hydrolysis of the precursors. The alcohol formed during hydrolysis was removed by heating at ca. 70 °C for up to 30 min. until the solution lost 40% of its volume. The solution was then allowed to cool and was neutralized to pH 7 with either 0.1 M NaOH or HCl solution. 50 μL of the sol-gel mixture was mixed with 15 μL of 10% GOx solution (0.1 M PBS/pH 7.0), and left for several hours for gelation to start. When gelation began, PNR-coated carbon film electrodes were immersed in the sol-gel-enzyme solution for 5 min. After removal, the electrodes were stored at 4 °C for 2 – 3 days allowing sol-gel formation. All biosensors were stored dry at 4 °C when not in use.

2.4. Biosensing Procedure in RTIL

RTIL was used as electrolyte directly, i.e., without the addition of any pH buffering capacity. A volume of 300 μL of ionic liquid was placed into the electrochemical cell and aliquots of the standard glucose solution, prepared in the same RTIL, were added. The current was measured at a fixed applied potential of -350 mV vs. $Ag/AgCl_{ss}$. The enzyme was cross-linked with glutaraldehyde for these experiments.

3. Results and Discussion

3.1. RTIL Sol Gel Matrix for Enzyme Immobilization

Sol-gel enzyme encapsulation is often a better enzyme immobilization method than cross-linking because it does not deactivate the enzyme and increases its stability [24, 28, 29]. Nevertheless, sol-gel matrices are prone to cracking during storage under dry conditions, and enzyme leaching can occur due to porosity of the matrix [24, 29]. It has already been demonstrated that addition of 1-butyl-3-methylimidazolium tetrafluoroborate prevents sol-gel matrices, based on tetraethoxysilane, from cracking [13].

The sol-gel formation protocol, used here for producing RTIL-containing sol-gels, is a variant of the optimized procedure established for aqueous sol-gel solutions [29], in which RTIL is simply added to the standard trioxysilane-water mixture. The results obtained showed that neither RTIL examined was compatible with MTMOS. In both cases, precipitation appeared either during hydrolysis or during removal of alcohol from the mixture. Similarly, for the combination of GOPMOS and $BmimNO_3$, a silicate precipitate was formed after 15 hours of gelation. However, the other three combinations (i.e., GOPMOS/$BmimNTF_2$, APTOS/$BmimNTF_2$ and APTOS/$BmimNO_3$), formed homogeneous mixtures. It was also observed that the GOPMOS/$BmimNTF_2$ and APTOS/$BmimNTF_2$ mixtures formed dry gel within 38 and 48 hours, respectively; while the APTOS/$BmimNO_3$ mixture required at least 7 days for

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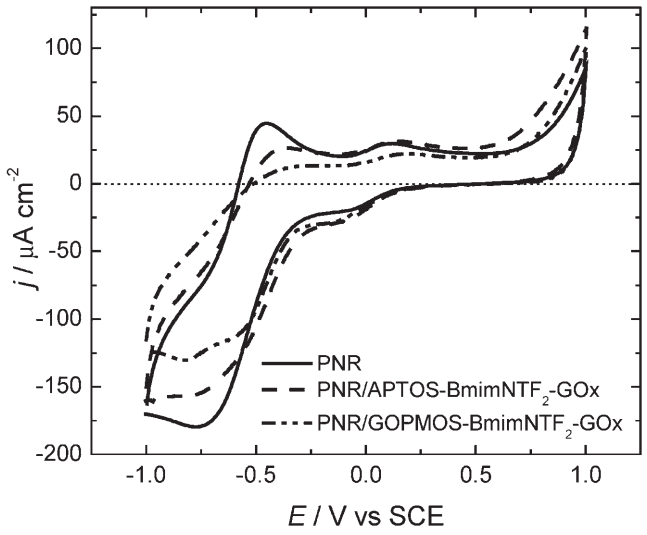

Fig. 1. Cyclic voltammograms at carbon film electrode modified with PNR, PNR with APTOS-$BmimNTF_2$-GOx layer, and PNR with GOPMOS-$BmimNTF_2$-GOx layer in 0.1 M PBS, pH 7.0. Potential scan rate 50 mV s^{-1}.

gelation. Thus, only the GOPMOS-$BmimNTF_2$ and APTOS-$BmimNTF_2$ combinations were used for subsequent the sol-gel immobilization of glucose oxidase on PNR-modified electrodes.

Because of its well-known enzymatic properties in aqueous and sol-gel media, glucose oxidase was used here to probe the behavior of the active protein within the RTIL-containing sol-gel matrices. The responses of these electrodes to analyte were examined after 3 days of gelation, i.e., when the gel-enzyme layer was completely dry. Biosensors were kept dry as described in Experimental.

Cyclic voltammograms at both types of sol-gel biosensor, as well as at PNR-modified carbon film electrodes, are presented in Figure 1. In these voltammograms, it can be seen that the sol-gel layer coating the PNR film creates a diffusion barrier to charge-compensating counter-ions, a behavior which is evident from the lower peak currents relative to the uncovered PNR modified electrode [30]. Also, the sol-gel-RTIL matrices based on GOPMOS (Fig. 1) exhibited similar voltammetric behavior to those formed from purely aqueous sol-gels [24, 29]. Significantly, in the case of APTOS-RTIL, the peak currents are enhanced relative to aqueous systems, thus potentially giving better electrode performances. These observations indicate that the introduction of RTIL into the sol-gel matrix is not detrimental to the electrochemical performance of the biosensors, as outlined in detail below.

The biosensors' performances were examined using chronoamperometry in 0.1 M PBS, pH 7.0 at −250 mV vs. SCE, as previously reported for aqueous sol-gel matrices [24, 29]. The calibration data are presented in Table 1, along with the corresponding data for aqueous based sol gels for comparison. The response at the GOPMOS-$BmimNTF_2$ based biosensor exhibited a linear range from 0.05 to 0.40 mM with a sensitivity of 101.5 nA mM^{-1}, and a limit of detection of 13.8 μM. Although the linear range for this sensor was identical to the corresponding aqueous-based sol-gel system [24], the sensitivity was almost a factor 2 higher, and the limit of detection was one third lower. For the APTOS-$BmimNTF_2$ biosensor, the linear range extended from 0.05 to 1.1 mM, and exhibited a sensitivity of 62.0 nA mM^{-1} and a limit of detection of 28.8 μM. Obviously, the linear range, sensitivity and LOD are improvements relative to the corresponding aqueous sol-gel system [24].

The stability of the biosensors based on the $BmimNTF_2$ RTIL sol-gel matrices was very different from that with aqueous sol-gel matrix. For the GOPMOS-RTIL based biosensor, 80% of its activity was lost after one day although the aqueous GOPMOS sol-gel matrix ensured much better stability than the other sol-gel precursors used by Pauliukaite et al. [29]. The reason for the loss of response is uncertain but could be due to leaching of the enzyme or its deactivation or, most likely, mechanical instability of the xerogel membrane.

The APTOS based sol-gel-RTIL matrix exhibited better stability than the aqueous equivalent; the signal dropped by only 20% after 1 day, and was relatively stable thereafter, with the biosensor exhibiting 70% of its initial activity after 15 days. However, over the same time period, the linear range decreased to 0.05 – 0.70 mM of glucose. Significantly, no visible cracking of the sol gel-RTIL matrices was observed even after 3 weeks storage under dry conditions at 4 °C. In contrast, aqueous sol-gel matrices crack after one week and, therefore, have to be stored in buffer solution while not in use [24]. The enhanced stability of this biosensor is probably due to the nonvolatile nature of the RTIL that prevents the xerogel formed from cracking as opposed to the situation observed with aqueous sol-gel matrix.

Table 1. Calibration data for glucose at the biosensors based on sol-gel-RTIL-GOx and sol-gel-aqueous matrix (Pauliukaite et al., 2006). Applied potential −0.25 V vs. SCE, supporting electrolyte 0.1 M PBS, pH 7.0.

Biosensor	Linear range (mM)	Sensitivity (nA mM^{-1})	Correlation coefficient (R^2)	*LOD* (μM)	K_M (mM)
Sol-gel-RTIL matrix					
GOPMOS-$BmimNTF_2$-GOx	0.05 – 0.40	101.5	0.997	13.8	1.6
APTOS-$BmimNTF_2$-GOx	0.05 – 1.10	62.0	0.997	28.8	2.7
Sol-gel-aqueous matrix (from Pauliukaite et al., 2006)					
GOPMOS- GOx	0.05 – 0.40	57.9	0.998	37.8	1.0
APTOS- GOx	0.05 – 0.60	53.1	0.997	38.7	1.0

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3.2. RTILs as Electrolytes in Biosensing Systems

The application of RTILs as the carrier electrolytes in the biosensing systems was performed for the first time using a 300 μL microcell. The working electrodes were electrodeposited PNR modified with GOx, via glutaraldehyde cross-linking. A 0.1 M glucose stock solution was prepared in RTILs and left for one day for anomeric equilibration to occur. Both RTILs studied, $BmimNTF_2$ and $BmimNO_3$, were used as electrolytes, and for glucose stock solution preparation. The RTILs were not deoxygenated since oxygen is necessary for the enzymatic reaction converting glucose to gluconolactone. Since solution stirring was not possible in the microcell, analyte sample was injected as close as possible to the working electrode in order to avoid a long response time due to slow diffusion within the RTILs.

Initially, cyclic voltammograms were recorded for the biosensors in both RTILs without glucose (Fig. 2a dotted line). The well known neutral red/leuco-neutral red redox peaks were barely evident in either ionic liquid. However, after addition of glucose to the RTIL carrier electrolytes, the expected redox peaks appeared between −0.20 and −0.15 V vs. $Ag/AgCl_{ss}$ in both $BmimNTF_2$ and $BmimNO_3$, at potential sweep rates of ≤ 20 mV s^{-1} (Fig. 2, solid and dashed lines, respectively).

Also, in $BmimNO_3$, an additional oxidation peak was observed at 0.15 V, which was not present at the same kind of biosensor in aqueous PBS solution (see Sec. 3.1 and [27]). This oxidation peak (at 0.15 V) increased with increasing glucose concentration up to 4 mM, and then remained constant; the origin of this redox process is not clear and will require further investigation. However, the neutral red/leuco-neutral red redox peaks were observed to decrease with increasing glucose concentration, as expected. The cathodic current decreased because oxygen was consumed during the enzymatic reaction and at the same time the hydrogen peroxide produced caused an increase of the anodic current at 0.17 V.

Although it possesses a common cation, voltammetry in $BmimNTF_2$ was different from that in $BmimNO_3$. Firstly, although the neutral red/leuco-neutral red redox peaks appeared with the addition of glucose, no clearly visible changes to the voltammetry were obtained with glucose addition from 2 to 6 mM (Fig. 2b). Secondly, no additional oxidation peak for glucose was evident.

Both $BmimNO_3$ and $BmimNTF_2$ were applied as electrolytes for glucose determination by chronoamperometry at the electrochemical biosensor described above. Since RTILs extend the negative potential limit of the biosensor, a more negative potential (−0.35 V vs. $Ag/AgCl_{ss}$) was used in chronoamperometric measurements as compared to aqueous solutions (−0.25 V vs. SCE). Moreover, at this

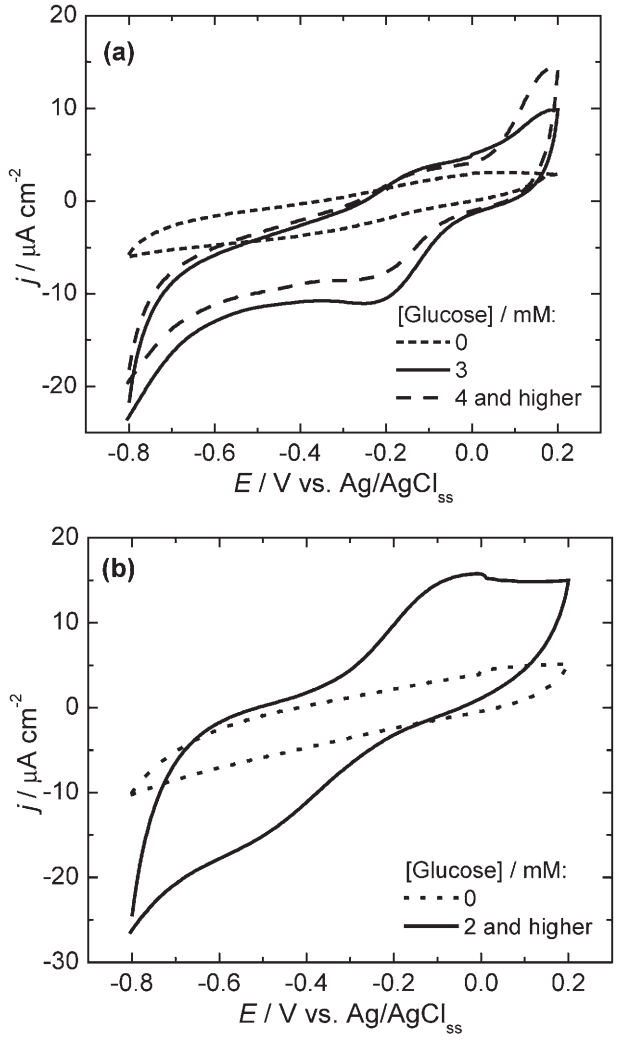

Fig. 2. Cyclic voltammograms at PNR/GOx-BSA electrode in a) $BmimNO_3$ and b) $BmimNTF_2$with specified additions of glucose. Scan rate 20 mV s^{-1}.

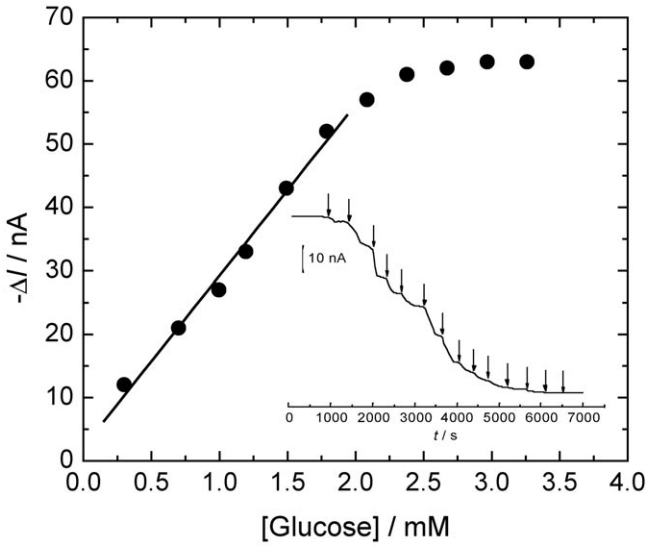

Fig. 3. Calibration curve at a PNR/GOx-BSA electrode in $BmimNO_3$ from chronoamperometric determination of glucose. Applied potential −0.35 V vs. $Ag/AgCl_{ss}$. The insert shows the recorded current–time trace; the arrows indicate injections of a standard glucose solution in $BmimNO_3$: the first is of 50 μM, and the others are of 250 μM.

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potential the background current is constant and does not interfere with the measurements. Unsurprisingly, the chronoamperometric results obtained were a reflection of their respective voltammetric response, i.e., good reduction responses were observed in $BmimNO_3$ (Fig. 3) while no clear responses were seen in $BmimNTF_2$ (not shown). This can probably be related to the fact that NTF_2-containing RTILs are quite hydrophobic whereas NO_3^- is a hydrophilic anion; as such, $BmimNO_3$ possesses significantly more water than hydrophobic ionic liquid [27]. Since most of enzymes, especially those that are water soluble, require an aqueous environment to maintain their activity [31, 32], the relative performance of the RTILs is probably a reflection of their respective water-content. Similar behavior has been reported previously where a sol/RTIL/Nafion/enzyme electrode assembly exhibited the reversibility of the enzyme redox centre [33] where the Nafion provided an aqueous microenvironment for the enzyme. The observations reported here support the hypothesis that some water in the RTIL is necessary to retain enzyme activity [31, 32].

The current responses for the PNR-GOx electrode obtained in $BmimNO_3$ exhibit typical Michaelis-Menten-type kinetics, yielding an apparent Michaelis-Menten constant (K_M, calculated according to Lineweaver – Burk linearization) of 2.2 mM. The first significant observation that can be made regarding biosensor response within RTIL media is the significant response time (τ_{95}) of 300 s. The slowness of response is due to the slow mass-transfer within the viscous RTIL medium. Also, because electrochemical sensors measure the amount of species reacting at the electrode surface (i.e., $I = dQ/dt$), the slow mass transfer within the RTIL also dictates that the magnitude of the current signal will be much smaller than in less viscous media (e.g., aqueous) and, consequently sensor performances (linear range, sensitivity and *LOD*) may be less favorable.

In the $BmimNO_3$ medium investigated here, the biosensor exhibited a linear range extending from 0.3 to 1.7 mM, which compares reasonably well with the aqueous electrolyte system where the linear range was from 0.09 mM to 1.8 mM [24]. Also, the sensitivity and limit of detection of the biosensor operated in $BmimNO_3$ were found to be 27.0 ± 1.7 nA mM^{-1} ($n = 3$) and 49.3 μM, respectively, values which compare somewhat less favorable with the corresponding values in aqueous electrolyte i.e., 58 nA mM^{-1} ($n = 3$) and 22 μM. Overall, these analytical performances are acceptable for glucose detection, and quantification, and could be improved with appropriate cell design, and electrode geometry. The main difference in chronoamperometric response between aqueous solution and ionic liquid was in the current response at the same potential at PNR/GOx electrode: an anodic response was observed in aqueous solutions and a cathodic response in RTIL. This leads to the conclusion that ionic liquid blocks the direct electron transfer mediation of PNR with the FAD co-factor in glucose oxidase, observed in aqueous solutions [30]. The reason is probably the diminished charge compensation ability within the film due to large electrolyte ions.

It is also worth noting that $BmimNO_3$ may be a useful carrier electrolyte for biosensors using enzymes that are unstable in aqueous solutions, or for substrates that are insoluble in water, such as lipids. Further investigations of biosensor construction, electrochemical cell design and RTIL composition will be performed to improve response times and sensitivities.

4. Conclusions

Two imidazolium-based room temperature ionic liquids have been used in the preparation of redox-mediated biosensors, $BmimNTF_2$ and $BmimNO_3$. Of these, $BmimNTF_2$ was the more compatible with the GOPMOS and APTOS sol-gel precursors for preparation of the enzyme layers, and prevented sol-gel films from cracking for several weeks. Glucose oxidase enzyme immobilized in the sol-gel-$BmimNTF_2$ matrix had approximately the same activity as aqueous gel prepared from the same precursors, but the stability of the APTOS-based biosensor was increased. Further optimization of the sol-gel-RTIL matrix composition will be carried out in order to improve the analytical properties of such biosensors with different enzymes.

Using PNR deposited from aqueous solution as redox mediator and with enzyme cross-linked with glutaraldehyde, $BmimNO_3$ was the better RTIL as electrolyte carrier in the biosensing system. Nevertheless, the performance of the biosensor in $BmimNO_3$ was not as good as in aqueous phosphate buffer saline solution due to slower diffusion caused by the higher RTIL viscosity. Further improvement of microcell construction will be performed in order to reduce the effects of the slow diffusion, and enzymes that are not stable in aqueous solutions will be used in this system.

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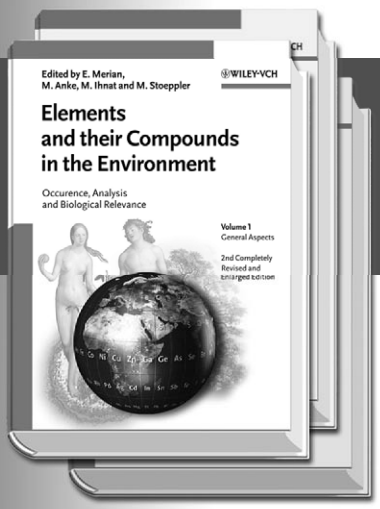

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