IONIC LIQUIDS AS A SOLVENT FOR BIOELECTROCATALYSIS USING ELECTRODE-IMMOLIZED CYTOCHROME C

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ABSTRACT

Immobilized Cytochrome c on mercaptothiol self-assembled monolayers exhibit a characteristic Fe(III)/Fe(II) redox signal that is lost when exposed to ionic liquids composed of a butylimidazolium cation combined with either hexafluorophosphate or bis(trifluoromethylsulfonylimide) anion. In this study it was shown that exposure to the aqueous solubilized ionic liquid components, butyl-, hexyl-, and octyl-imidazolium cations and bis(trifluoromethylsulfonylimide) anion, resulted in partial loss of the electrochemical signal. Spectroscopic measurements, including both absorbance and fluorescence, showed that signal loss due to the cationic liquid component followed a different mechanism than that of the anionic component. Although a portion of the signal was recoverable, irreversible signal loss also occurred in each case. The source of the irreversible component is suggested to be the loss of protein secondary structure through complexation between the ionic liquid components and the protein surface residues. The reversible electrochemical signal loss is likely due to interfacial interactions imposed between the electrode and the cytochrome heme group. Microperoxidase-11 was also used as a simple model protein to explore the influence of the amount of exposed surface residues.

INTRODUCTION

Biocatalytic systems in non-aqueous environments have garnered much attention over the past decade due to their unique synthetic opportunities.[1] For example, hydrolytic enzymes (e.g. lipases and esterases) in non-polar, organic solvents with low water content have been shown to carry out reversed hydrolytic and transferase-type reactions. Ionic liquids (ILs) have recently emerged as an alternative media to non-polar, hydrophobic solvents for supporting biocatalytic processes.[2-6] A myriad of organic cation and inorganic or organic anion combinations have allowed for these room temperature molten salts to be structured with convenient physical and chemical properties. ILs possess negligible vapor pressure, nonflammability, chemical and thermal stability, high ionic conductivity, and excellent electrochemical stability.[7, 8] It should be noted that the pharmacological and toxicological effects of most ILs have yet to be
delineated. With thoughtful selection of cationic and anionic components, ILs represent a neoteric, non-aqueous, polar class of solvents suitable for the development of ‘green’ biocatalytic processes.[9]

Intrinsic conductivity and electrochemical stability of ILs make them an especially attractive non-aqueous media in which to study bioelectrocatalytic systems.[8, 10] Relatively little data exists on the enzymatic activity of oxidoreductases (e.g. desaturases, oxidases, and peroxidases) in ILs.[11-13] Recent studies have shown that the classic redox-active hemoprotein, cytochrome c (cyt-c), and analogues of its active site, microperoxidase-11 (MP-11) and protoporphyrin(IX) iron(III) chloride (hemin), conditionally retain peroxidase activity in the ILs, 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([bmim][tf2N]) and the hexafluorophosphate salts of 1-butyl- and 1-octyl-3-methylimidazolium ([bmim][PF6] and [omim][PF6], respectively).[14] Cyt-c and its analogue MP-11 must be coordinated with a multidentate ligand (i.e. 2-(hydroxymethyl)-18-crown-6 ether) to retain peroxidase activity in the ILs. The lariat ether is necessary to solubilize the protein and is presumed to help maintain the non-supported enzyme’s active conformation in polar, non-aqueous solvents.[15]

To further investigate the potential use of redox active proteins and enzymes for bioelectrocatalytic synthesis in ILs. Cyt-c modified electrodes can be used to directly examine the protein’s redox activity in ILs. Initial studies determined that the Fe(II)/Fe(III) redox couple of hemin-modified electrodes was stable in [alkylimidazolium][PF6] salts without the need for additional electrolyte.[16] Similar studies were performed on cyt-c immobilized to mixed self-assembled monolayers (SAMs) of 11-mercaptio-1-undecanol (11-MU) and 11-mercaptoundecanoic acid (11-MUA) supported on gold electrodes.[17] The redox activity of the immobilized cyt-c was lost upon prolonged exposure to neat [bmim][tf2N] and [bmim][PF6]. The cyt-c redox activity was reconstituted, however, when the electrodes were subsequently exposed to aqueous buffer. Polar organic solvents generally result in the loss of enzyme function, presumably due to the stripping of essential water from the enzyme.[18] This rationalization, however, is insufficient as water-saturated [bmim][tf2N] (1.4% w/w) also did not support the redox activity of the immobilized cyt-c.[17] Thus, other confounding effects must contribute to the deleterious impact ILs have on the redox activity of the immobilized cyt-c.

The present work employs electrochemical, absorbance, and fluorescence methods to examine the specific effects of the aqueous solubilized IL components, [bmim]⁺, 1-hexyl-3-methylimidazolium [hmim]⁺, [omim]⁺, [tf2N]⁻, [PF6]⁻, [BF4]⁻, and [PO4]³⁻ on the electron transfer response of cyt-c covalently bound to 1:1 mixed 11-MU/11-MUA SAMs supported on gold electrodes.

EXPERIMENTAL

Materials

Horse heart cyt-c prepared without trichloroacetic acid was purchased from Sigma-Aldrich and stored at -4 °C. Cyt-c purified via ion-exchange chromatography[19]
was found to have identical behavior compared to the unpurified protein. Therefore, cyt-c was used as received and without further purification for all experiments in this study. Microperoxidase-11 (MP-11), 11-mercaptop-1-undecanol (11-MU), 11-mercaptoundecanoic acid (11-MUA), 1-[3(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-mercaptopropanoic acid (3-MPA), 3-mercaptop-1-propanol (3-MP) and reagent grade ethanol were also obtained from Sigma-Aldrich and used as received. ILs were obtained from ACROS Organics and stored in a dry nitrogen atmosphere chamber prior to use. Potassium phosphate buffer solutions (Pb) were prepared by using a mixture of potassium dihydrogen phosphate and potassium hydrogen phosphate with a combined total phosphate concentration of 10 mM. A pH of 7.0 was achieved by using a ratio between the two salts and confirmed by electronic pH measurement. Sodium chloride, ammonium chloride, and potassium nitrate were obtained from Fisher Scientific. Deionized water for this study was obtained using a Barnstead NANOpure Diamond model D11911 water purification system.

Electrode preparation. High purity gold wire (>99.99% pure) with a diameter of 0.5 mm was formed into a coil with a surface area of between 2.0 and 2.4 cm² either by Bioanalytical Systems (BAS) of West Lafayette, IN, or in house. Cleaning of electrodes was accomplished before each modification scheme using chemical and electrochemical polishing. Chemical polishing was composed of swirling the electrode in piranha solution (a 3:1 mixture between concentrated H₂SO₄ and 30% H₂O₂) for 60 seconds followed by a 15 minute ultrasonic rinse in D.I. H₂O. After three repetitions of chemical polishing, the electrode was subjected to an electrochemical polish step. Electrochemical polishing was accomplished by using the gold coil electrode as the working electrode (WE) in a three electrode cell in combination with a platinum wire counter electrode (CE) and a Ag/AgCl/NaCl(3M) reference electrode (RE, E vs. SHE = 206 mV). The working electrode was subjected to a 50 cycle potential sweep between 1.50 V and -0.30 V in 1.0 M H₂SO₄, after which the characteristic cyclic voltammogram for this system was obtained.[20] The electrode surface roughness was determined by integrating the charge under the gold oxide reduction peak in the sulfuric acid voltammograms. A value of 482 μC cm⁻² for monolayer coverage of oxygen on a gold electrode was equated to a surface roughness of 1.0.[21]

After polishing, the electrodes were immediately coated with a mixed SAM of 11-MU and 11-MUA. Coating was accomplished by incubating the electrode for 24 h in a solution composed of both 5 mM 11-MU and 5 mM 11-MUA dissolved in ethanol. Electrodes were rinsed with ethanol and a solution of 10% acetic acid in ethanol after removal from the SAM bath. This step was included to remove electrostatically bound 11-MU and 11-MUA from the electrode surface.[22]

Covalent attachment of cyt-c was accomplished by first activating the SAM surface by placing the modified electrode into an aqueous solution of 200 mM EDC and 50 mM NHS for 10 min. The activated electrode was then rinsed with buffer and placed into a 50 μM cyt-c solution for 3 h. Electrostatically adsorbed cyt-c was removed by rinsing the electrode with aqueous 10% acetic acid, followed by D.I. H₂O immediately prior to use.
Measurements

Cyclic voltammetry (CV). Voltammograms were recorded using a BAS CV-50W voltammetric analyzer in combination with a BAS C3 cell stand. A standard three electrode configuration was used for all experiments. This configuration utilized a platinum wire CE, a Ag/AgCl/NaCl(3M) RE, and the modified gold coil as the WE. Oxygen was purged from the system by bubbling dry nitrogen through the cell solution before experiments and a nitrogen atmosphere was maintained over the solution during measurement of voltammograms. CV data were recorded with a scan rate of 0.050 V/s. The pH 7.0 reduction potential (E°') was calculated as the midpoint between the oxidation and reduction peaks and listed as relative to the RE potential (E = 206 mV vs. SHE) unless otherwise noted.

Peroxidase Assay. Electrode-immobilized MP-11 was examined for peroxidase activity by following the oxidation of 1.0 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate (ABTS) optically at 416 nm with a Shimadzu 1240 spectrophotometer equipped with a kinetics module. A cuvette (1 x 1 cm) was charged with 2.5 mL of 10 mM Pi, pH 7.0, containing 1.0 mM ABTS and 1.0 mM H2O2. Following immobilization on the SAM (11-MU:11-MUA) modified surface, the electrode was rinsed extensively with water to remove adhering solution phase MP-11. The electrode was then transferred to the cuvette, agitated by hand, and then briefly withdrawn at 60 s intervals.

Fluorescence measurements. Fluorescence measurements were obtained on a Varian Cary-Eclipse fluorometer where the samples were temperature-controlled in a four-position Peltier cuvette holder. Temperature was maintained at 23 ± 0.03 °C. Emission spectra were measured with an excitation of 295 nm and slits (excitation/emission) at 10 nm each. Cyt-c was added at 10 μM to [bmim][Cl] or Li[tf2N] at various concentrations. Due to the high native fluorescence of [bmim][Cl] overwhelming the signal of all other components, however, it was not possible to obtain useful information from measurement of solutions containing the salt. The [bmim][Cl] fluorescence results are, therefore, not included. Reported spectra were the result of averaging the individual spectra of three or four samples, each sample having been scanned three to six times to ensure spectral stability.

RESULTS AND DISCUSSION

Immobilization of cyt-c. When CV was performed on cyt-c modified electrodes in Pi buffer, a reversible current signal was observed for a redox process with a E°' of -42 ± 7 mV and a peak separation of 34 ± 3 mV (n=10 electrodes). This signal is consistent with the Fe(II)/Fe(III) redox process of cyt-c covalently immobilized on a MU:MUA electrode as reported by Lisdat[23] as well as previously measured by Compton and Laszlo.[17] This signal was stable for more than ten successive cycles and for greater than 120 minutes in pH 7.0 Pi buffer. Through integration of the background subtracted reduction peak and the assumption of a one electron process, the cyt-c surface coverage was calculated to be 4.0 ± 0.5 pmol/cm². This value is consistent with the surface coverage previously measured by Compton and Laszlo[17] and approximately 50% of the coverage reported by Lisdat using a similar electrode modification scheme.[23]
To verify covalent immobilization of cyt-c to the MU:MUA modified gold, the electrodes were exposed to high ionic strength aqueous solutions. The cyt-c redox signal was observed in up to 1.0 M NaCl, LiCl, KNO$_3$, and NH$_4$Cl aqueous solutions without the loss of current signal. Electrostatically bound cyt-c typically desorbs from the MU:MUA surface at these molar salt concentrations resulting in the loss of redox signal.[24] The lack of observed desorption indicates that the covalent immobilization of cyt-c was successful.

A positive shift in the cyt-c reduction potential was observed with increasing ionic strength and is consistent with previous findings by Margalit and Schejter of the effects of ionic strength on buffer-solubilized cyt-c.[25] A similar positive shift of the cyt-c redox potential was observed upon exposure to the IL components (Fig. 1). The deleterious effects that the IL components have on the cyt-c current signal discussed below, however, cannot be attributed to simple ionic strength effects. Furthermore, these adverse effects on the cyt-c current cannot be attributed to the influence of the IL component counter ions (Li$^+$, Na$^+$, K$^+$, NH$_4^+$, and Cl$^-$) or the desorption of the cyt-c from the electrode surface.

Ionic liquid cation exposure. A shift in the cyt-c E' to more positive potentials was observed in the presence of [bmim]Cl (Fig. 2). This effect can be explained by the increase in ionic strength of the solution, similar to the potential shift seen in NaCl, LiCl, KNO$_3$, and NH$_4$Cl solutions discussed above. Although the IL cation component is organic in nature, the redox potential of the cyt-c does not become more negative as observed when the protein is exposed to other water-miscible organic solvents such as dimethyl sulfoxide, dimethylformamide, and acetonitrile. These organic solvents lower the dielectric of the aqueous solution, resulting in alkaline-like misligated states of the cyt-c.[26] The IL components studied herein, however, dissociate as ions in water, effectively raising the dielectric of the media. Thus, the positive shift of the cyt-c redox potential would be expected.

![Figure 1. Ionic liquid cationic and anionic components.](image)
Contrary to the unchanged current response observed with increasing concentration of NaCl, increasing concentrations of [bmim]Cl resulted in a progressive decrease in the cyt-c current signal (Fig. 2). The loss of the cyt-c current signal was independent of the scan rate from 0.050 to 0.500 V/s. At 1.0 M [bmim]Cl, the electrochemical signal was not entirely suppressed as it was in neat [bmim][PF₆] and [bmim][tf₂N].[16] After a series of [bmim]Cl solution treatments (up to 1.0 M), ~65% of the cyt-c redox signal returned when the electrode was rinsed well and placed into fresh Pi buffer. This recovered redox signal occurred at an E°' value that was negatively shifted ~15 mV in comparison to the unexposed cyt-c signal. Recovery of the redox signal confirms that cyt-c is not removed from the electrode surface upon exposure to [bmim]Cl. The lesser current and negative shift in E°' in the recovered signal indicates that an irreversible process is occurring in addition to a reversible signal loss.

Loss of the cyt-c current signal in [bmim]Cl solution may be caused by one or more different effects such as a structural change within the heme ligation state that suppresses the Fe(II)/Fe(III) redox couple, an interruption of the electron transfer pathway between the electrode and the cyt-c heme, or an increase in electrode to heme distance.
The loss of the cyt-c signal is not likely caused by a direct interaction of the [bmim]$^+$ with the heme iron, resulting in a change of the heme ligation state. The dialkyl imidazolium cation (Fig. 1) lacks a free nitrogen electron lone pair found in such bases as N-methyl imidazol and pyridine. These nitrogenous coordinating bases shift the redox potential of cyt-c heme center more negative.[16] Exposure of immobilized cyt-c to [bmim]$^+$ results in a positive shift in its redox potential (Fig. 2). The loss of the cyt-c redox signal when exposed to [bmim]$^+$ would more likely be due to the cation's influence on the protein's secondary structure. Changes in the extended protein structure have been shown to have a strong impact on the thermodynamics of redox couples.[27] If the redox thermodynamics shift to unfavorable conditions through unfolding or denaturation of the protein then loss of the cyt-c current signal within the potential window of our experiments would be observed. The possible influence of [bmim]$^+$ on the cyt-c secondary structure is explored using absorbance and fluorescence spectroscopy, discussed below.

As [bmim]$^+$ contains a significant non-polar organic region (Fig. 1) one must consider the possibility that the cation's tail may likely partition into the hydrophobic environment of the SAM. This accumulation of the [bmim]$^+$ in the SAM layer could possibly lessen the cyt-c redox signal by increasing the organic density between the electrode and the protein, which would most likely lessen the electron's ability to tunnel to the protein. Two additional IL cations were used with longer hydrophobic chain lengths, [hmim]Cl and [omim]Cl, where the butyl alkyl chain is replaced by a hexyl and octyl group respectively to examine this possibility. The increased hydrophobic nature of the [hmim]$^+$ and [omim]$^+$ as compared to [bmim]$^+$ would be expected to increase the level of partitioning into the highly hydrophobic inner regions of the SAM layer. A greater signal loss at lower concentrations would then be expected with the longer chain length species as they more significantly partition into the SAM, insulating against the electrode-protein electron-transfer.

Both of the longer chain length cations were found to result in a greater loss of the cyt-c current signal as shown in Figure 3. Because the initial coverage of cyt-c varied somewhat between electrodes, the experiments were normalized by reporting the redox active surface coverage as the percentage of the initial redox active coverage remaining after exposure to an increasing concentration of IL. The [omim]$^+$ ion might be expected to partition more greatly than [hmim]$^+$ into the SAM due to its greater hydrophobic surface area, yet [omim]$^+$ resulted in less of a deleterious effect on the cyt c redox signal (Fig. 3). A possible explanation for the lesser effect of [omim]$^+$ is from steric hinderance arising from a non-uniform density of the SAM layer. Similar to the straws in the head of a broom where the point of attachment is denser than the unattached outer edge, the density of the SAM molecules may be more diffuse at the solution-SAM boundary than at the electrode interface. This increasing density at greater SAM depth could sterically hinder molecules partitioning into the layer after a maximum chain length is reached. Overall, both the longer chain length IL cations caused greater loss of the cyt-c redox current than the [bmim]$^+$. These data support the possibility that the IL cations may intercalate into the SAM, and that the intercalation is dependent on the IL cation alkyl chain length.
Figure 3. Influence of [bmim]Cl, [hmim]Cl, and [omim]Cl on immobilized cyt-c redox current. Error bars represent one standard deviation (n=4).

If any intercalation of the IL cations into the SAM occurs, it appears to be reversible, as a significant portion of the cyt-c signal returns after rinsing the electrode with 10 mM P1 buffer. As complete reversibility is not achieved, the question of the nature of the unrestored portion of the CV signal naturally arises. To examine the effect of the IL cations on the SAM, electrodes were coated with 11-MU:11-MUA and then incubated for 30 minutes in 200 mM solutions of [bmim]Cl, [hmim]Cl, or [omim]Cl. The IL-treated electrodes were then rinsed with 10% acetic acid and D.I. H2O to remove any electrostatically adsorbed species before covalent attachment of cyt-c was performed. No difference in cyt-c signal compared to untreated electrodes was observed, indicating that the exposure of the SAM to the IL cations did not result in irreversible alteration of the alkylthiol monolayer. Electrochemical impedance spectroscopy measurements of the 11-MU:11-MUA SAM layer before and after exposure to the three IL cationic components confirmed these findings. The impedance of the SAM did not change after exposure to 1.0 M solutions of the three cations for 72 h followed by rinsing and immersion in P1 buffer. Thus, the unrecoverable loss of the immobilized cyt-c redox signal upon exposure to [bmim]Cl cannot be attributed to an irreversible interaction of the [bmim]+ with the 11-MU:11-MUA SAM. The possibility that the partial, unrecoverable loss of redox signal results from some irreversible change in cyt-c structure resulting from interactions with the IL cation is examined further in the spectroscopy section (vide infra).
Increase in electron donor-receptor distance is a third possibility for the loss of the cyt-c redox signal when exposed to the IL cations. An increase in electrode-protein distance has generally been described to result in an exponential decrease in the electron transfer rate.\[28\] The loss of the redox signal of cyt-c due to increased distance between the heme and the electrode would imply that the distance has increased to where the electron transfer becomes less favorable to the extent that it is not observed at the scan rates used in this study.

Many lysine residues exist on the protein surface that may be required for maintaining a functional orientation and electrode-heme distance through electrostatic interactions, although cyt-c is covalently anchored to the electrode surface through one or more lysine residues for the present study, including most likely Lys13.\[29\] Niki et al [30] have shown that the electron transfer rate can decrease by over an order of magnitude for proteins on a carboxyl terminated alkylthiol SAM with each additional CH\(_2\) group beyond a 10 carbon chain length. Disruption of the lysine-surface interactions, outside of the covalent anchor, by the [bmmim]\(^+\) cation may cause reorientation of the protein that could increase the heme-electrode distance.

Due to the complexity of the possible effects caused by the cationic IL components, absorption spectroscopy, as well as comparison to MP-11, were used to better elucidate the mechanism of the cyt-c redox signal loss.

**Ionic liquid anion exposure.** In addition to cationic IL components, the anionic component [tf\(_2\)N]\(^-\) was examined by using the lithium salt, Li[tf\(_2\)N]. Like the cationic IL components, Li[tf\(_2\)N] produced a loss of cyt-c signal (Fig. 4), which was not observed in the presence of the simpler Cl\(^-\) anion. This loss occurred to a degree similar to that of the [bmmim]\(^+\) and [omim]\(^+\) and more than that of [bmmim]\(^+\). As [tf\(_2\)N]\(^-\) is a much more polar molecule than the [imidazolium] cations, incorporation into the SAM layer would not be expected to occur to an appreciable extent.

The influence of two other common IL anions on the cyt-c redox signal was examined. The ammonium salts of [PF\(_6\)]\(^-\) and [BF\(_4\)]\(^-\) produced a loss of cyt-c signal with increasing concentration (Fig. 4). The [PF\(_6\)]\(^-\) was more deleterious to the cyt-c redox signal than [BF\(_4\)]\(^-\). The loss of cyt-c signal in the presence of the poorly coordinating [tf\(_2\)N]\(^-\) anion was greater than that due to the [BF\(_4\)]\(^-\), and less than the loss due to the [PF\(_6\)]\(^-\).

Resulting in the complete loss of signal at a concentration of 27 mM (data not shown), [PO\(_4\)]\(^3-\) proved to be the harshest anion on the cyt-c redox signal. The pH of the 27 mM [PO\(_4\)]\(^3-\) solutions were >10, thus the alkaline environment is presumed to have caused the conformational rearrangement to a mixture of misligated states. These conformational changes most likely render the immobilized cyt-c redox inactive.

The pH of the [tf\(_2\)N]\(^-\), [BF\(_4\)]\(^-\), [PF\(_6\)]\(^-\) solutions (>500 mM) was found to be ~7, therefore, the cyt-c was not subjected to the same alkali-induced conformational changes in these solutions. These complex anions, however, must interact with the immobilized protein in some way which renders it redox inactive. These anion-protein interactions were investigated using absorbance and emission spectroscopy.
Figure 4. Influence of Li[tf₂N], [NH₄][BF₄], and [NH₄][PF₆] on immobilized cyt-c redox current. Error bars represent one standard deviation (n=4).

Absorbance spectroscopy. Loss of the cyt-c 695 nm visible absorption band at room temperature indicates a disruption of the coordination of the native Met80 heme ligand, such as that seen with the acid-unfolded pH 2.7 form of the protein.[31] The position and intensity of the Soret band is also indicative of the integrity of the heme pocket. P₁ buffer (pH 7.0) solutions of cyt-c containing up to 1.0 M NaCl showed no change in the Soret band position compared to cyt-c freshly reconstituted in P₁ buffer from the lyophilized state, but did show a modest increase in the intensity at 695 nm (Table 1). The increased intensity of the 695-nm charge-transfer band may result from closer heme-Met80 interactions. In contrast, [bmim]Cl added above 0.2 M led to an increasing loss of the 695-nm band, with about 52% retention of Met80 ligation at 1.0 M. There was no significant change in the position of the Soret absorption maximum with NaCl or [bmim]Cl, indicating that the heme environment remained otherwise intact with either salt. The progressive loss of the Met80 ligation at higher [bmim]⁺ concentrations corresponds to the progressive loss of the immobilized cyt-c redox signal. If the initial loss of the cyt-c redox current observed below 200 mM [bmim]⁺ (Fig. 3) results from a protein-[bmim]⁺ interaction, this interaction does not perturb the heme pocket. Perturbation of the heme pocket was only observed at higher [bmim]⁺ concentrations (>500 mM, Table 1).
Table 1. Changes in cyt-c absorbance spectrum due to [bmim]Cl and Li[tf2N]

| Salt conditions | 695 nm absorption intensity b | Soret band position c / nm |
|----------------|-------------------------------|---------------------------|
| 10 mM buffer   | 100                           | 409                       |
| 0.2 M NaCl     | 121                           | 409                       |
| 0.5 M NaCl     | 121                           | 409                       |
| 1.0 M NaCl     | 124                           | 409                       |
| 0.2 M [bmim]Cl | 103                           | 408                       |
| 0.5 M [bmim]Cl | 86                            | 408                       |
| 1.0 M [bmim]Cl | 52                            | 408                       |
| 0.2 M Li[tf2N] | 5                             | 405                       |
| 18 mM HCl      | 0                             | 394                       |

a All solutions, except 18 mM HCl, contained 10 mM phosphate buffer, pH 7.0
b Relative absorption of 250 μM cyt-c in the indicated salt solution, less the absorption of acid unfolded cyt-c:
(Salt695 nm - HCl695 nm)/(Phosphate695 nm - HCl695 nm)
c Cyt-c concentration 5μM

[Bmim]Cl treatment of immobilized cyt-c results in a loss of redox activity that is only partially recovered with removal of the IL. As discussed above, this irretrievable portion of the cyt-c redox activity could not be attributed to changes in the SAM layer. As an alternative possibility, permanent changes in the cyt-c structure may have been induced by the IL. This possibility was examined by treating cyt-c with either 1.0 M NaCl or 1.0 M [bmim]Cl, followed by exhaustive dialysis (5 d) of the protein against Pi buffer. The NaCl-treated cyt-c fully retained its enhanced 695-nm absorbance (120% compared to buffer-alone solubilized cyt-c) after dialysis. This suggests that 1.0 M NaCl induces a permanent change in the heme cavity, i.e., greater heme-Met80 interaction. Dialysis of [bmim]Cl-treated cyt-c dramatically reversed the loss of the charge-transfer interactions in the heme cavity to the extent that the 695-nm band intensity increased to 137% of that of the control, untreated cyt-c. Thus [bmim]Cl-induced heme ligation changes appear to be fully reversible, resulting in a state comparable to that induced by NaCl treatment. Recalling that 1.0 M NaCl did not alter the redox signal intensity of the immobilized cyt-c, the [bmim]Cl-induced irretrievable cyt-c redox activity is not attributable to changes in heme ligation, nor any heme cavity change detectable by absorption spectroscopy. Differences between the NaCl- and [bmim]Cl-treated, dialyzed, cyt-c observed by fluorescence spectroscopy are described below.

The effect of [tf2N]⁺ on the cyt-c absorbance spectrum was much more pronounced than that of [bmim]⁺. Li[tf2N] (0.2 M) effectively eliminated Met80 ligation in the heme pocket and was accompanied by a 3 nm blue shift in the Soret absorption band. Concentrations of 0.5 M Li[tf2N] and above resulted in protein aggregation and precipitation. Thus, [tf2N]⁺ is assumed to be far more disruptive than [bmim]⁺ on the structure of cyt-c in aqueous media. A far more disruptive influence of [tf2N]⁺ compared to [bmim]⁺ is not observed in the electrochemical measurements. Instead, the loss of the cyt-c redox signal observed in the presence of 0.2 M [tf2N]⁺ is only ~ 5% greater than in
the presence of 0.2 M [bmim]+. It is likely that the immobilization of the cyt-c on the alkylthiol SAM stabilizes the protein, reducing the [tf₂N]⁻ ability to disrupt the protein’s secondary and tertiary structure.

**Fluorescence spectroscopy.** Fluorescence emission has been used to follow structural changes induced by the aqueous environment of cyt-c.[32-34] Emission from the tryptophan of cyt-c is seen at 348 nm with excitation at 295 nm. Within the folded native cyt-c environment the tryptophan fluorescence is strongly quenched by the heme-group as well as nearby amino acids. When protein unfolding occurs, the distance between the tryptophan and the heme increases. This increased distance reduces the level of quenching and results in greatly increased tryptophan fluorescence, which can be monitored directly as an indication of the loss of secondary structure in cyt-c.[35] Figure 5 shows the fluorescence spectra of 10 μM cyt-c in its native (pH 7.4) and acid denatured (pH 2.7) states.

![Fluorescence spectra of cyt-c in pH 7.4 and pH 2.7 solutions.](image)

Figure 5. Emission spectra of cyt-c in (—) pH 7.4 and (--) pH 2.7 solutions.

The emission spectrum of cyt-c in the presence of Li[tf₂N] showed that the [tf₂N]⁻ had only a moderate effect on the cyt-c tryptophan fluorescence at concentrations of 30 to 273 mM (Fig. 6). The slightly less quenched tryptophan peak (348 nm) at 273 mM Li[tf₂N] shows that the protein structure is only slightly changed. This is counter to what was observed in the absorbance assay where the cyt-c experiences complete loss of the Met80 ligation to the heme iron (Table 1). Therefore, [tf₂N]⁻ must more directly affect the cyt-c heme pocket than the protein tertiary structure at moderate concentrations. This, however, results in only a slightly increased loss of cyt-c redox current when
Figure 6. Emission spectra of cyt-c in (—) 0, (••) 30, (—•—) 59, (••••) 143, (— —) 273, and (•••) 500 mM Li[tf₂N] concentration. Signal strength increases with increasing concentration of Li[tf₂N].

compared to the influence of the [bmim]⁺ at similar concentrations (273 mM). At higher [tf₂N]⁺ concentrations (500 mM) the emission spectrum indicates that the cyt-c is completely denatured. This is consistent with the nearly complete loss of cyt-c redox signal observed at similar [tf₂N]⁺ concentrations (Fig. 4).

Microperoxidase-11. In order to examine a simplified system, MP-11/SAM modified electrodes were also constructed. MP-11 consists of an iron-containing heme moiety in conjunction with an eleven amino acid secondary structure. MP-11 therefore acts as a model of the heme pocket with a minimum of additional protein scaffolding. This simplification allows for further elucidation of IL component interactions with the cyt-c protein scaffolding.

CV did not show a redox process within a -400 — +600 mV potential window for MP-11 on 11-MU:11-MUA electrodes, but the presence and continued enzyme activity of MP-11 on the electrode was confirmed through a colorometric microperoxidase activity assay (Fig. 7). A catalytic wave was also detected using the MP-11 modified electrode in the presence of 30 mM H₂O₂. The bare SAM surface did not display a catalytic wave CV when exposed to H₂O₂. Catalytic current signals can be observed in
CV when both an enzyme and an appropriate substrate for that enzyme are present. The presence of a catalytic wave when attempted immobilization of MP-11 had been performed, along with the absence of the wave from bare SAM electrodes, is additional evidence that MP-11 was successfully immobilized on the electrode surface. Although the MP-11 was successfully immobilized on the 11-MU:11-MUA SAM and exhibited catalytic activity, the Fe(II)/Fe(III) redox couple was not observed by CV.

The lack of a CV signal from the MP-11/11-MU:11-MUA/Au electrodes may be due to the more flexible tether of the MP-11 as compared with cyt-c. This greater flexibility would be expected to cause a greater average distance between the heme center and the electrode with MP-11. Therefore, a modified electrode consisting of MP-11 covalently attached to a 1:1 mixture of 3-MP and 3-MPA on gold was prepared to reduce the electrode to heme distance. Under this modification scheme, a semi-reversible redox couple ($E^{o'} = 121 \text{ mV}, \Delta E = 107 \text{ mV}$) was observed and attributed to MP-11.

When the MP-11/3-MPA:3-MP/Au electrode was exposed to increasing concentrations of [bmim]Cl or Li[(t$_2$)N], the peak current was found to shift to more positive potential values and to decrease in current signal, similar to the response of the cyt-c modified electrodes (data not shown). Returning the electrode to buffer solution after thorough rinsing recovered nearly all of the original current signal. The peak potential was found to be shifted to a more negative potential than the original buffer-only results, again similar to the cyt-c electrode results. One difference in the results was

![Figure 7](image-url)
that the potential shift upon return of the electrode to a buffer-only solution was approximately half of that seen with the cyt-c modified electrodes, 7 mV vs. 15 mV. The lesser shift of MP-11 compared with that obtained from cyt-c electrodes indicates that the amount of available surface residues is a factor in the level of irreversibility in current change. Additionally, the degree of signal loss was much greater with the MP-11 modified electrode, with the complete loss of redox active MP-11 signal in solutions of less than 500 mM [bmim]Cl. Exposure of the MP-11/3-MPA:3-MP/Au electrode to Li[tf$_2$N] resulted in a moderate loss of the current signal, requiring higher concentrations to reach a similar level of loss as the cyt-c modified electrodes, possibly due to the lesser amount of disruptive secondary structure in MP-11 as compared to cyt-c.

CONCLUSIONS

The loss of Fe(II)/Fe(III) redox activity of cyt-c covalently attached to 11-MU/11-MUA modified electrodes in the presence of the full ionic liquid [bmim][tf$_2$N] was found to also occur when the electrodes were exposed to the cationic and anionic components separately. The mechanism by which the redox activity is lost differs, however, in the two cases. From the use of different chain length alkylimidazolium salts it is shown that the cation may intercalate into the SAM layer causing a disruption of the attachment site leading to non-ideal distance and orientation of the heme center. Absorbance spectroscopy shows that the local heme environment of solutionary cyt-c, as evidenced by the Met80-heme ligation, is only affected at concentrations of [bmim]$^+$ over 0.5 M and that the heme cavity structure, as monitored by the Soret band, is unchanged even at 1.0 M [bmim]$^+$ (Table 1). This evidence is consistent with the continued integrity of the protein scaffold during exposure to alkylimidazolium cations, with an additional non-destructive interaction. When the cyt-c modified electrode is exposed to the longer chain substituant alkylimidazoliums, the loss of cyt-c redox signal, as measured by CV, occurs at a lower concentration of salt (Fig. 3). This indicates that partitioning of the cation into the monolayer may be occurring and that the increased hydrophobicity of the longer chain IL cation leads to greater partitioning, and therefore a greater extent of redox signal loss.

The partial irreversibility of the redox signal change even upon rinsing and return of the modified electrode to pure buffer (Fig. 2) indicates that either a cyt-c/IL cation interaction is not completely dissociated by return to buffer or that with a protein covered SAM, diffusion of the IL cation out of the SAM is hindered. Experiments with exposure of the SAM to [bmim]$^+$ before incubation in the cyt-c bath show that without the protein layer [bmim]$^+$ is not retained inside the SAM.

In contrast to the [alkylimidazolium]$^+$ components, [tf$_2$N]$^-$ was found to directly cause protein unfolding. Absorbance spectra indicated that Met80 ligation is lost at concentrations of [tf$_2$N]$^-$/below 200 mM, as evidenced by the loss of the 695 nm band (Table 1) although the lack of a large shift in the Soret band (409 nm vs. 408 nm in the native cyt-c) shows that only partial unfolding of the protein secondary structure has occurred at this concentration. Emission results also support an interpretation of a partial loss of structural integrity as the spectrum of the [tf$_2$N]$^-$ exposed cyt-c shows only minor changes in tryptophan intensity up to 200 mM [tf$_2$N]$^-$ concentration (Fig. 6), although
complete loss of secondary structure is indicated by the large change in the emission spectrum at concentrations at or above 500 mM.

Electrode immobilization of the protein appeared to impart an enhanced stability, as seen from comparison of the loss of protein integrity in solution to the loss of redox active cyt-c signal from the modified electrode surface. This increased stability may be due to interactions with neighboring cyt-c molecules as well as through reduced surface area for interaction between the protein and solution components. Experiments with MP-11 seem to support this hypothesis. Similar to the effect of reducing the amount of exposed cyt-c surface area through tightly packed immobilization, MP-11 with fewer overall residues, resulted in less irreversible electrochemical signal change than cyt-c (7 mV vs. 15 mV negative shift in E°).

Future experiments will focus on several areas to prevent loss of the redox signal in ILs. The addition of protecting groups to the protein surface can be explored to determine if the secondary structure of the protein can be protected from solutionary component interaction. Several varieties of surface modified cyt-c have been produced and are commercially available. The addition of a protecting layer over the existing cyt-c modified electrode surface is a second option. Under this scheme, the secondary layer selected should be permeable to substrate, but exclude potentially damaging IL from reaching the enzyme or SAM. Thirdly, with the large number of existent and emerging IL cations and anions, and the ability to tune characteristics of these solvents through structural modifications, use of different IL cation or anion components may allow for selection of specific IL solvents that are less damaging to the enzyme structure.

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† Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

Electrochemical Society Proceedings Volume 2004-24 599