NEW BIOCATALYSTS: MYOGLOBIN IN PHOSPHONIUM IONIC LIQUIDS

by

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ABSTRACT

New biocatalysts based on myoglobin in phosphonium ionic liquids were developed. This involved four general areas of research: (1) preparation of biocompatible phosphonium ionic liquids; (2) characterisation of myoglobin incorporated into ionic liquids; (3) biocatalysis involving myoglobin in phosphonium ionic liquids; and (4) prevention of protein denaturation by covalent modification of the protein.

Three synthetic routes for preparation of new, biocompatible ionic liquids were investigated: sodium-salt metathesis, silver-salt metathesis, and acid-base neutralisation. Silver-salt metathesis was determined to be the most effective method for complete anion exchange. Spectroscopic analyses of myoglobin in ionic liquids demonstrated varying levels of denaturation depending on the concentration of ionic liquid. Nevertheless, myoglobin was shown to exhibit peroxidase-like activity in aqueous ionic liquid solutions. Unusual activity was observed for dibenzothiophene oxidation mediated by myoglobin in one ionic liquid. Covalent modification of myoglobin by poly(ethylene oxide) demonstrated improved stability of the protein in ionic liquids.

Keywords: Ionic liquid; Biocatalysis; Myoglobin; Covalent protein modification; Denaturation

For my parents.

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d	Doublet (NMR spectroscopy)
DBT	Dibenzothiophene
DBTO	Dibenzothiophene sulfoxide
DBTO2	Dibenzothiophene sulfone
DCM	Dichloromethane
dd	Doublet of doublets (NMR spectroscopy)
DMPO	5,5-dimethylpyrroline-N-oxide
dq	Doublet of quartets (NMR spectroscopy)
dt	Doublet of triplets (NMR spectroscopy)
ENDOR	Electron-nuclear double resonance
EPR	Electron paramagnetic resonance
EtOAc	Ethyl acetate
Gu-HCl	Guanidine hydrochloride
HPLC	High performance liquid chromatography
IL	Ionic Liquid
IL 101	Tetradecyl(trihexyl)phosphonium chloride
IL 105	Tetradecyl(trihexyl)phosphonium dicyanamide
IL 106	Methyl(triisobutyl)phosphonium p-toluene sulfonate
IL 109	Tetradecyl(trihexyl)phosphonium bistriflamide

IL 169	Ethyl(tributyl)phosphonium diethylphosphate
IL-H2PO4	Tetradecyl(trihexyl)phosphonium dihydrogen phosphate
IL-NO3	Tetradecyl(trihexyl)phosphonium nitrate
IL-PO4	Tetradecyl(trihexyl)phosphonium phosphate
I.S.	Internal standard
m	Multiplet (NMR spectroscopy)
Mb	Myoglobin
NMR	Nuclear magnetic resonance
PEO	Poly(ethylene oxide)
quin	Quintet (NMR spectroscopy)
s	Singlet (NMR spectroscopy)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
t	Triplet (NMR spectroscopy)
TMS	Tetramethyl silane
δ	Chemical shift (NMR spectroscopy)

1 INTRODUCTION

This thesis seeks to demonstrate the viability of phosphonium ionic liquids for use as solvent media for biocatalysis. To show that this class of ionic liquids is in fact capable of supporting proteins and, more specifically, capable of sustaining the protein in such a way that it is catalytically active, the research was broken into four aspects. First, the design and synthesis of new, biocompatible phosphonium ionic liquids was sought through various anion exchange reactions. The second aspect of the research was to devise a method for introduction of the protein into the ionic liquid and to probe the general structure of the protein using several analytical techniques. Third, a model reaction was chosen to investigate whether the protein, now incorporated into the phosphonium ionic liquid, was still active. Finally, covalent modification of the protein was used in an attempt to prevent denaturation.

1.1 Ionic Liquids

Room-temperature ionic liquids are salts that are liquid at room temperature. They are unique as solvents because they are purely ionic, consisting of solely cations and anions. The melting point of a salt is a reflection of the electrostatic potential between cations and anions. This potential can be expressed as the lattice energy, E, which is given by:

$$\mathrm{E} = k\frac{Q_1 Q_2}{d}$$

The lattice energy describes the strength of the interaction between two charges Q_1 and Q_2 (multiplied by the Madelung constant, k) in relation to the interionic distance, d. Common salts, such as sodium chloride, have a high lattice energy since the ions are small and the distance between the charges is short. Ionic liquids have asymmetric and often bulky ions that cannot pack into tight lattices rendering them liquids. Efficient ion packing is also impeded by mismatched ion sizes or shapes, which results in diminished lattice energies and reduced melting point temperatures.[1] Therefore, ionic liquids are often composed of ions that are size-mismatched.

Ionic liquids possess inherent properties that deem them ideal for use as reaction media. For example, many reaction systems require multiple solvents to dissolve both polar and non-polar materials, however several ionic liquids are capable of dissolving both polar and non-polar substances without use of a second solvent.[2] Ionic liquids are a relatively benign class of solvents with low volatility and in most cases are non-flammable.[3,4] Unlike conventional reaction media, ionic liquids allow for facile catalyst re-use and solvent recycling. Furthermore, ionic liquids have high thermal stability, often reaching temperatures in excess of 300 ^{o}C before decomposition. Most importantly, the physico-chemical properties of ionic liquids are modifiable by variation of the cation and anion.[5] Tunable properties include polarity, hydrophilicity, and solvent miscibility behaviour.[6,7] Because of the range of properties available in ionic liquids as solvents, new and unusual chemical reactivity is being discovered.[3,4,8] There are four general types of ionic liquids most commonly referred to in the literature; these are based on the cations given in Figure 1.1. The first three cations are nitrogen-based cations ammonium, imidazolium and pyridinium; the fourth is the phosphorus-based phosphonium cation.

The nitrogen-based ionic liquids have been more extensively studied than their phosphorus counterparts.

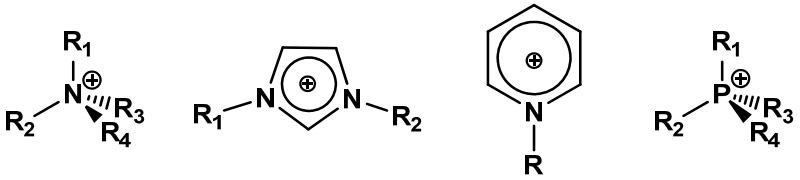


Figure 1.1 Common ionic liquid cations: ammonium, imidazolium, pyridinium, and phosphonium.

1.1.1 History of Ionic Liquids

The first room-temperature molten salt was observed by Walden in 1914 when he described the product, ethylammonium nitrate, formed by the reaction of ethylamine and concentrated nitric acid.[9] Thereafter, publications regarding molten salts or ionic liquids were infrequent until the 1970s when the groups of Osteryoung[10] and Wilkes[11] reignited research interest in the area. The first report of organic synthesis in room temperature molten salts was from Wilkes a few years later with the demonstration of Friedel-Crafts reactions in binary mixtures of 1-methyl-3-ethylimidazolium chloride and aluminium chloride.[12] Wilkes *et al.*[13] advanced the field further in 1992 when they reported the first synthesis of air- and water-stable imidazolium based salts that are liquid under ambient conditions. Since then, ionic liquids have been shown to be useful solvents in many areas, for example, organic reactions, transition metal-mediated catalysis, and biocatalysis.[6,14-16]

1.1.2 Phosphonium Ionic Liquids as Reaction Media

Phosphonium ionic liquids have been much less investigated than imidazolium or ammonium ionic liquids due in part to their comparatively recent discovery. Though phosphonium salts have been reported previously as phase-transfer catalysts and

precursors to ylides[4] their use as room-temperature ionic liquids only arose in the late 1990s.[17,18] Preparation of phosphonium ionic liquids has been primarily reported by researchers at Cytec Industries.[19,20]

The first literature account of the use of a phosphonium ionic liquid as a reaction medium occurred in 1996 with the report of palladium-catalysed Heck coupling in tributyl(hexadecyl) phosphonium bromide.[17] Interesting work in the area of reaction chemistry in phosphonium ionic liquids has been reported by McNulty[21,22] and Clyburne[3,8,23] who have demonstrated the potential for phosphonium ionic liquids to be used for improved synthetic chemistry. Synthetic reactions that have been performed in phosphonium ionic liquids include the Heck,[17,23,24] Suzuki,[21] Diels-Alder,[25] and Grignard[8] reactions as well as hydroformylations.[4,26] Furthermore, we have previously shown that electron transfer reactions can be supported in these media.[8] These ionic liquids can promote reactivity and reaction pathways that are different from nitrogen-based ionic liquids or conventional organic solvents.[4,8] Phosphonium ionic liquids have been shown to be superior to the much-used imidazolium ionic liquids in that they are more thermally stable,[20] and phosphonium cations have been demonstrated to be more robust toward bases and reducing agents.[3] We have chosen to utilise phosphonium ionic liquids in our studies because of the potential for new reactivity in these solvents as well as the lack of literature reports of enzyme-catalysed reactions in phosphonium ionic liquids. Very recently, one report of biocatalysis by lipase in phosphonium ionic liquids was published.[27]

1.2 Protein Folding and Denaturation

Protein folding is a complex process that is not yet fully understood. Proteins consist of a chain of amino acid residues linked by peptide bonds to make a polypeptide chain. The polypeptide chain folds into a three-dimensional conformation determined by the sequence of the amino acid residues. Protein folding is a reversible, thermodynamically-driven process that is influenced by environmental conditions and is frequently controlled in vivo by chaperone proteins.[28]

A spontaneous chemical process has a negative change in free energy (ΔG), which is related to the change in enthalpy (ΔH) and entropy (ΔS) by:

$$\Delta G = \Delta H - T\Delta S$$

Protein folding is a result of the cooperation of several interactions or forces. Folding results in an increase in the order of the system, or a decrease in the conformational entropy (negative ΔS). Therefore, compensation must be obtained from the energy gained by redistribution of intramolecular interactions between the protein groups and the environment, resulting in an increase in ΔS or a large, negative ΔH.

The intramolecular forces that act to cause the folding of a polypeptide chain into the native, folded state are: van der Waals interactions, interactions between charged groups (salt interactions), interactions between polar groups (hydrogen bonds), and interactions between nonpolar groups (hydrophobic interactions).[29] van der Waals interactions are induced by the close proximity of groups in a folded protein and are enthalpic. Charge-charge interactions are also enthalpic in nature and result from the interaction of charged amino acid residues at neutral pH, forming ionic bonds. Hydrogen bonds are weak interactions individually, however, proteins bear many hydrogen bond

donating and accepting groups and cooperatively this enthalpic force contributes considerably to protein stability. Hydrophobic substances in aqueous solutions cause an ordering of the water molecules to form clathrate structures around them. This ordering of the water results in a decrease in the randomness of the system and a decrease in the total system entropy. Folding the hydrophobic groups into the core of the protein allows for an increase in the entropy of the system as the water molecules gain freedom of motion. Hence, it is entropically more favourable to fold the hydrophobic amino acid residues into the core of the protein away from solvent water molecules. A small enthalpic contribution results from the van der Waals interactions between the buried hydrophobic residues. The hydrophobic effect is thought to be the largest contributor to the free energy of folding for proteins.[29,30]

1.2.1 Determination of Protein Conformation

Protein conformation can be probed spectroscopically using techniques such as UV-Visible absorption, fluorescence spectroscopy, optical rotary dispersion, circular dichroism, and electron paramagnetic resonance (EPR) spectroscopy. This thesis utilises UV-Visible absorption and fluorescence as well as EPR spectroscopies to determine whether myoglobin remains in its native conformation when incorporated into phosphonium ionic liquids. UV-Visible absorption spectroscopy can provide information about the coordination environment of the heme prosthetic group of myoglobin, specifically the spin state and coordination of the iron atom. Likewise, EPR spectroscopy (described in Appendix A) can be used to probe the local environment of the iron ion since iron(III) is paramagnetic. Fluorescence spectroscopy can been used to determine the

extent of unfolding of the protein, due to quenching of the fluorescence emission for the native, folded myoglobin.

The purpose of this thesis is not to determine the mechanism of denaturation for ionic liquids nor to provide further insight into the problem of protein folding, but to establish whether myoglobin is in its native, active conformation when solvated in phosphonium ionic liquids.

1.3 Biocatalysis in Non-Aqueous Media

Enzymes are found to be in their native, folded state when in aqueous environments such as buffer, serum, or within cells. Traditionally it was believed that when placed in non-aqueous environments enzymes would denature. Intact secondary and tertiary structures are required for an enzyme to remain active. Therefore, completely denatured enzymes are not active; however, reduced activity may be observed for partially denatured enzymes. In 1985, Zaks and Klibanov[31] demonstrated that enzymes can be prepared in certain organic solvents so that they are still are capable of catalysing reactions.

There are several motivations for using non-aqueous media for biocatalysis. Enzymatic reactions in aqueous media are limited by: (1) insolubility of compounds, (2) unwanted side reactions, (3) degradation of common organic reagents, and (4) difficult product recovery.[32] Each of these limitations can potentially be circumvented by using non-aqueous solvents as reaction media.

1.3.1 Enzymes in Organic Solvents

Zaks and Klibanov[33] proved that enzymes could be active in anhydrous organic solvents when they showed that subtilisin and α-chymotrypsin were able to catalyse transesterification reactions very effectively in dry organic solvents. Furthermore, they hypothesized that the variability of catalysis with solvent choice was related to the stripping of essential water from the protein by more hydrophilic solvents. For this reason, hydrophobic media are more tolerated than hydrophilic ones by enzymes in organic solvents. The unusual stability of enzymes in dry organic solvents is due to the macromolecule's rigidity in these environments. Hydrogen bonding with water allows the protein to be flexible and unfold when a sufficient amount is present in solution. However, in neat organic solvents reduced hydrogen bonding as compared to water means that the protein does not have the requisite mobility to unfold, even though the tendency to unfold is enormous.[32]

New and useful properties of enzymes have been discovered by performing reactions in organic solvents. For example, lipases and esterases commonly catalyse hydrolysis reactions in the presence of water but in organic media the hydrolysis reaction is suppressed and transesterification is dominant.[32] Not only can enzymatic behaviour be altered by changing from aqueous to organic media but shifts in reaction selectivity can be obtained by varying the organic solvent used. Types of enzyme selectivity that may be influenced by solvent choice are substrate, enantiomeric, prochiral, regio- and chemoselectivities.[34,35] Commercial processes such as those used by the food, pharmaceutical, and polymer industries benefit from performing biocatalytic reactions in organic media through: high product concentrations, the lack of undesired by-products as well as elimination of expensive cofactors in most cases.[36] Additionally, improved

selectivity eliminates the need for costly and time-consuming protecting and deprotecting steps in chemical synthesis.

Studies of enzymes in aqueous solutions of organic solvents have shown that enzymes are more denatured in aqueous organic solvents than anhydrous solvents.[37] Specifically, Klibanov and Griebenow[37] found that an inverse bell-shaped relationship exists between the amount of organic solvent and protein denaturation (Figure 1.2). Proteins are less denatured and have more native-like secondary structure in pure organic solvents (and pure water) than most organic solvent-water mixtures. It follows that enzymes remain more catalytically active in neat solvents than aqueous mixtures. The greatest amount of denaturation was determined to be at 60% (v/v) organic solvent for two unrelated enzymes, lysozyme and subtilisin, in three different organic solvents (acetonitrile, tetrahydrofuran, and 1-propanol). These results demonstrate that, in general, enzymes are more denatured in aqueous-organic solvent mixtures than pure organic solvents. Klibanov[32,33,38] reported on several occasions that the catalytic activity of an enzyme could generally be restored upon dilution of the aqueous organic solvent with water, indicating that the denaturation of the enzyme is often reversible. As previously mentioned, the rationalisation for the denaturation of proteins in aqueous mixtures of organic solvents relates to the conformational mobility of proteins in aqueous solutions.

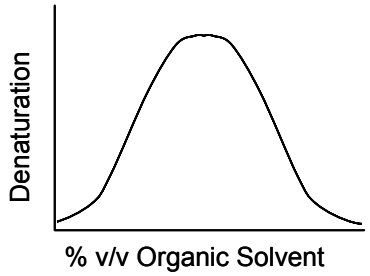


Figure 1.2 Relationship between protein denaturation and volume fraction of organic solvent, after Klibanov and Griebenow.[37]

It is important to note that although enzymes perform reactions in organic media, the observed activity is typically lower than in water. The diminished activity observed for enzymes in organic media may be related to lyophilisation, a method frequently used to prepare enzyme samples, since it was shown to cause denaturation.[39,40] Most proteins do not dissolve in organic media but remain as a suspension of lyophilised protein, creating a two-phase (solid/liquid) system. Another reason for the lower activity observed may be the formation of such a two-phase system. Alternatively, decreased enzymatic activity may be related to the structural rigidity of enzymes in organic solvents which prevents the conformational mobility required for optimal catalysis.[32] Strategies such as using surfactants,[39] adding small amounts of water,[33] and lyophilising from optimal pH[31,33] with lyoprotectants[38,41] have been developed to overcome these issues, and aqueous-like activity for enzymes in nearly anhydrous organic solvents has been reported.

1.3.2 Enzymes in Ionic Liquids

Enzyme-catalysed transformations in ionic liquids were first reported in 2000.[42-44] One favourable characteristic of ionic liquids as reaction media is their potential as alternatives to volatile organic solvents, which are frequently used in industrial synthetic procedures.[36] Particularly important to this work are the unique and tunable solvent properties that make ionic liquids attractive solvents for biocatalysis. Variability in water-miscibilities of ionic liquids has allowed for a wide variety of studies of enzymes in both aqueous and anhydrous ionic liquids.[6]

The first report of the examination of an enzyme in an aqueous mixture of an ionic liquid involved the oldest known ionic liquid, ethylammonium nitrate.[45] Increased

activity and stability relative to buffer solutions of alkaline phosphatase was found for concentrations of the ionic liquid up to 10% (v/v) or 1.1 M. Biocatalysis in aqueous-ionic liquid mixtures has been used successfully to circumvent the issue of solubility of hydrophobic reactants or products.[6] Because the mixtures are aqueous, Hofmeister effects have been used to explain the stability of the enzymes in such situations.

The effect on protein stability of various ions in aqueous solution was initially described by Hofmeister.[46] The Hofmeister series is a set of cations and anions arranged according to their effect on protein stability. Loosely correlated with the series is the kosmotropic or chaotropic character of ions. Kosmotropicity and chaotropicity describes the interaction of ions with water and therefore the effect on the structure of water, namely the ability of water to form hydrogen bonds with itself. Kosmotropic (structure making) ions tend to have high charge densities and interact strongly with water, causing an increase in hydrogen bonding between water molecules. For chaotropic (structure breaking) ions, the opposite is true; they interact weakly with water and cause breaking of water-water hydrogen bonds.[6,47] It has been found that enzymes are stabilised by kosmotropic anions and chaotropic cations. A full understanding of the Hofmeister effects has not yet been achieved. For example, whether the stabilisation effects seen with enzymes are due to the direct interaction of the ion with the enzyme or an interaction with the surrounding water causing a change in the water structure has not yet been determined.

It has been noted that caution should be used in discussing the cations of ionic liquids on a kosmotropic-chaotropic scale as ionic liquid cations in general do not fit into the pattern that Hofmeister described.[6] More recent literature has loosely fit ionic liquids

into the kosmotropic-chaotropic scale based on the position of the anion in the Hofmeister series.[48-52] Examples of enzymes being denatured in ionic liquids with kosmotropic anions have also been reported,[53] while ionic liquids containing the chaotropic tetrafluoroborate anion have been found to enhance the catalytic activity and thermostability of the enzyme compared to buffer solution.[53] Enzymes that have been studied in aqueous solutions of ionic liquids include: chloroperoxidase,[49,54] formate dehydrogenase,[55] subtilisin,[48] horseradish peroxidase,[53,56,57] papain,[53] and β-galactosidase.[55,58] There is conflict in the literature as to whether the position of the anion in the Hofmeister series actually dictates enzyme stability in ionic liquids.[6] It appears that an ionic liquid that stabilises one enzyme may destabilise another. Therefore kosmotropicity of an anion may be a starting point for the choice of a biocompatible ionic liquid, but accurate predictions cannot be made based solely on the position of the anion in the Hofmeister series. It has been suggested that a theory based on the idea that proteins lose stability in the presence of ions or solvents that interact more strongly with the unfolded, denatured enzyme than the native form could predict whether an enzyme would be compatible with an aqueous ionic liquid.[6,59]

Erbeldinger *et al.*[43] reported the first successful biocatalytic reaction in a nearly anhydrous ionic liquid in the year 2000. The reaction involved the synthesis of Z-aspartame catalysed by thermolysin in a hydrophobic imidazolium ionic liquid containing 5% water, which gave 40% of the turnover observed in ethyl acetate. Since the first reported biocatalytic reaction, most literature reports for enzymatic reactions in ionic liquids have involved lipases, though some other enzymes have been studied.

Lipases are highly stable enzymes that have been used frequently for biocatalysis in both organic solvents and ionic liquids. Several lipases were shown to be catalytically active in imidazolium and pyridinium ionic liquids possessing the anions tetrafluoroborate, hexafluorophosphate, and bis(trifluoromethanesulfonyl)imide.[6,7,16] Indeed, the majority of biocatalytic reactions reported in ionic liquids involve hydrophobic ionic liquids containing the previously mentioned anions.[6,60] The efficiency of lipase-catalysed reactions in ionic liquids was found to be comparable to commonly used organic solvents such as t-butanol,[44] dioxane,[61] and toluene.[62] In fact, one type of lipase was 10-times more active in imidazolium and pyridinium ionic liquids than diisopropyl ether.[63] Improved enantiomeric selectivity for lipases has also been established in ionic liquids.[63-65] Early reports of lipase-catalysed reactions in ionic liquids were inconsistent reportedly due to impurities present in the ionic liquids, hence a purification procedure for the use of ionic liquids in biocatalytic reactions is required.[60,62] The tolerance of lipases for ionic liquids is not universal. Lipases previously shown to be active in hydrophobic ionic liquids produced no reaction in water-miscible ionic liquids possessing the same cations as the hydrophobic ionic liquids.[16,63] The results also varied between types of lipases. Where an ionic liquid gave the best results for one lipase, another lipase was inactivated.[63]

Other enzymes have been studied in nearly anhydrous ionic liquids in addition to lipases, although to a much lesser extent. For example, α-chymotrypsin, which has also been applied in organic solvents for biocatalytic reactions,[33,34,66,67] was studied in low-water (< 2%) containing ionic liquids[68-70] as well as in biphasic reaction systems with supercritical carbon dioxide.[68] Esterases,[71] epoxide hydrolases,[2] and heme proteins, such

as microperoxidase-11,[72] cytochorome c,[72] horseradish peroxidase,[73] and hemin chloride[72] have been demonstrated to be active in imidazolium and ammonium ionic liquids.

The following report describes the use of phosphonium ionic liquids as reaction media for biocatalytic reactions. Initially discussed is the preparation of biocompatible phosphonium ionic liquids with anions that are based on the anions of common buffer salts. Then, myoglobin incorporated into commercial phosphonium ionic liquids was characterised by various spectroscopic methods, and the protein was probed for new reactivity. Finally, spectroscopic characterisation of covalently modified myoglobin was performed in order to determine whether denaturation of the protein in ionic liquids and by common denaturants could be prevented.

2 PHOSPHONIUM IONIC LIQUIDS

2.1 Introduction

The motivation for the synthesis of new phosphonium ionic liquids, in addition to those available commercially, was to develop new biocompatible systems. It was hypothesised that this could be achieved through use of anions commonly used as buffer salts for solvation of proteins in aqueous solution. Three synthetic routes to new ionic liquids were employed and are described in this chapter: sodium-salt metathesis, silver-salt metathesis, and acid-base neutralisation. This chapter compares the effectiveness of the methods as determined by the success of each reaction for anion exchange. Furthermore, the purification and important properties of several commercially available phosphonium ionic liquids are described.

2.1.1 Synthesis of Ionic Liquids

There are three basic methods for preparation of ionic liquids:

(1) Quaternization reactions, which are reactions in which a quaternary species is produced. Quaternization of a phosphine involves the combination of a tertiary phosphine and a haloalkane:[20]

$$PR_3 + R'X \rightarrow [R'PR_3]^+X^-$$

(2) Metathesis reactions, in which the halide ionic liquid can be converted by metathesis with an alkali or silver salt of the desired anion (A) to a new ionic liquid:[20]

$$[R'PR_3]^+X^- + MA \rightarrow [R'PR_3]^+A^- + MX$$

(3) Acid-base neutralisation reactions, by which a halide-containing ionic liquid can be exchanged with a hydroxide anion and subsequently reacted with the acid form of the desired anion to give the preferred ionic liquid:[20]

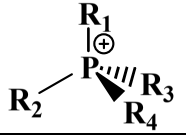
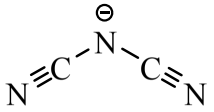
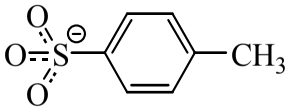
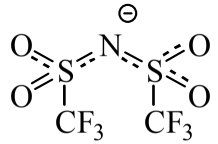
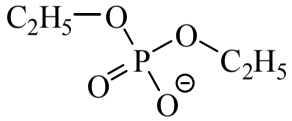
$$[R'PR_3]^+X^- + MOH \rightarrow [R'PR_3]^+OH^- + MX$$

$$[R'PR_3]^+OH^- + HA \rightarrow [R'PR_3]^+A^- + H_2O$$

Cytec Industries Inc., currently the only commercial supplier of phosphonium ionic liquids, reports the synthesis and related impurities for each of their ionic liquids (listed in Table 2.1) in their product literature.[74-78] Ionic liquids typically contain impurities in one or more of four common categories.[79] The first of these are halide ions, commonly left over from the synthesis of ionic liquids. The second impurity is water, resulting from either preparation of the ionic liquid or absorption from the atmosphere due to the hygroscopic nature of many ionic liquids. Unreacted organic starting materials are the third common impurity; while the fourth group of impurities includes inorganic salts left after incomplete metathesis reactions between organic halides and alkali salts.

The commercially available ionic liquids used in these studies include IL 101, IL 105, IL 106, IL 109, and IL 169. Tetradecyl(trihexyl)phosphonium chloride, known as Cyphos IL 101, is prepared through the quaternization reaction of trihexylphosphine with one equivalent of 1-chlorotetradecane.[20,80] Literature reports of the impurities include volatile components such as hexane, tetradecene isomers, excess 1-chlorotetradecane, trihexylphosphonium hydrochloride, hydrochloric acid, and dialkyl phosphine.[20,80] Cytec Industries reports that when saturated, IL 101 will contain 14.0% water, as determined by Karl-Fischer titration.[74]

Table 2.1 **Commercial phosphonium ionic liquids: component cations and anions, synthetic methods and impurities.**

Ionic Liquid (Commercial Name)	Cation 	Anion	Synthesis Method	Impurities
IL 101 (Cyphos IL 101)	$R_1 = C_{14}H_{29}$, $R_2=R_3=R_4= C_6H_{13}$	$Cl^{\ominus}$	Quaternization (trihexylphosphine and 1-chlorotetradecane)	Hexane, tetradecene isomers, 1-chlorotetradecane, hydrochloric acid, trihexylphosphonium hydrochloride, dialkyl phosphine[20,80]
IL 105 (Cyphos IL 105)	$R_1 = C_{14}H_{29}$, $R_2=R_3=R_4= C_6H_{13}$		Metathesis (IL 101 with sodium dicyanamide)	Same as IL 101, chloride (0.5%) [2,75]
IL 106 (Cyphos IL 106)	$R_1 = CH_3$, $R_2=R_3=R_4= (CH_3)_2CHCH_2$		Quaternization (triisobutylphosphine and methyl *p*-toluene sulfonate)	Triisobutylphosphine oxide, methyl *p*-toluene sulfonate[76]
IL 109 (Cyphos IL 109)	$R_1 = C_{14}H_{29}$, $R_2=R_3=R_4= C_6H_{13}$		Metathesis (IL 101 with lithium bistriflamide)	Same as IL 101, chloride (0.1%) [2,77]
IL 169 (Cyphos IL 169)	$R_1 = C_2H_5$, $R_2=R_3=R_4= C_4H_9$		Quaternization (tributylphosphine and triethylphosphate)	Ethyl(sec-butyl) dibutylphosphonium cation, tributylphosphine oxide[78]

Tetradecyl(trihexyl)phosphonium dicyanamide, known as Cyphos IL 105, and tetradecyl(trihexyl)phosphonium bistriflamide (Cyphos IL 109) are prepared via metathesis routes from the chloride salt, IL 101.[80] Chloride is present as an impurity due to the preparation of the ionic liquids from the chloride salt. Cytec reports that their products contain chloride (less than 0.5%, determined by titration with silver nitrate) and water as impurities.[75,77] When fully saturated, IL 105 can dissolve up to 3.1% water[75] and IL 109 only 0.7%.[77] There are no other impurities reported by the supplier although, inevitably, the impurities from the initial preparation of IL 101, as listed above, also should be present in these ionic liquids.

The preparation of IL 106 and IL 169 are entirely halide-free as they are prepared directly from non-halide containing starting materials. Methyl(triisobutyl)phosphonium *p*-toluene sulfonate (or tosylate), known as Cyphos IL 106, is prepared by quaternization of triisobutylphosphine and methyl tosylate.[20] Cytec reports the possible impurities to be triisobutylphosphine oxide and methyl tosylate.[76] Likewise, ethyl(tributyl)phosphonium diethylphosphate, Cyphos IL 169, is prepared directly from tributylphosphine and triethylphosphate.[20] Minor impurities reported by Cytec are tributylphosphine oxide, and the ethyl(sec-butyl)dibutylphosphonium cation, produced from a 3% impurity of sec-butyl(dibutyl)phosphine in tributylphosphine.[78]

This thesis outlines the purification methods used and developed with consideration of the properties of each ionic liquid, namely miscibility with water and organic solvents. Previous literature methods were used for the purification of the water-immiscible hydrophobic ionic liquids, IL 101, IL 105, and IL 109.[81] New procedures were developed for water-miscible ionic liquids IL 106 and IL 169.

2.2 Experimental

^{1}H NMR spectra were collected using a Varian Inova 500 MHz NMR spectrometer, operational frequency of 499.77 MHz. A 600 MHz Bruker AVANCE II spectrometer was used for $^{31}P\{^{1}H\}$, and $^{13}C\{^{1}H\}$ measurements by Mr. Colin Zhang and Dr. Andrew Lewis. Operational frequencies for the 600 MHz instrument were: ^{31}P, 243.94 MHz, ^{13}C, 150.95 MHz. All ^{1}H and $^{13}C\{^{1}H\}$ spectra are referenced to the solvent peak, chloroform-d_1. $^{31}P\{^{1}H\}$ spectra are referenced to an external standard of phosphoric acid. Operational parameters for NMR experiments are given in Appendix B.

2.2.1 Commercial Phosphonium Ionic Liquids

The ionic liquids obtained from Cytec Industries Inc. varied in their miscibility with water. Hydrophobic ionic liquids, IL 101, IL 105, and IL 109 were purified according to literature methods using both water and hexanes to extract impurities.[81] The hydrophilic ionic liquids, IL 106 and IL 169, were unable to be extracted with water since both are water-miscible, therefore new methods were developed for their purification.

Purification of Hydrophobic Ionic Liquids, IL 101, IL 105, and IL 109

The hydrophobic ionic liquids were purified according to literature methods.[81] To ionic liquid (50 mL) was added saturated aqueous $NaHCO_3$ solution (25 mL). This caused bubbling (due to the release of CO_2) and the mixture was gently stirred for 20 minutes after which the bubbling subsided. Care must be taken not to form an emulsion between the aqueous layer and the ionic liquid by stirring too vigorously. The solution was transferred to a separatory funnel and washed with distilled water (2 × 150 mL). The ionic liquid was subsequently extracted with water (4 × 25 mL) and hexanes (4 × 25 mL)

alternately to yield three layers. The ionic liquid layer was transferred to a side-arm flask to which toluene (5 mL) was added to azeotropically remove the water and the flask was evacuated with stirring on a dual manifold vacuum line for 24 hours at ambient temperature. The flask was then placed in a sand bath and heated to 50 ^{o}C under vacuum for 48 hours to further dry the ionic liquid. ^{1}H NMR was used to determine whether the ionic liquid was free of hexanes, toluene and water. **IL 101: ^{1}H NMR** (22 ^{o}C, $CDCl_3$, Varian Inova 500 MHz) δ (ppm) 2.45 (m, 8H), 1.50 (m, 16H), 1.27 (m, 32H), 0.89 (t, 9H, $^{3}J_{H,H}$ = 6.5 Hz), 0.86 (t, 3H, $^{3}J_{H,H}$ = 7.0 Hz); **^{13}C{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 31.81, 31.00, 30.76, 30.67, 30.44, 30.34, 29.54 (m), 29.41, 29.22 (m), 28.89, 22.58, 22.26, 21.836 (m), 19.34, 19.03, 14.03, 13.84; **^{31}P{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 33.33; **IL 105: ^{1}H NMR** (22 ^{o}C, $CDCl_3$, Varian Inova 500 MHz) δ (ppm) 2.20 (m, 8H), 1.52 (m, 16H), 1.28 (m, 32H), 0.90 (t, 9H, $^{3}J_{H,H}$ = 7.0 Hz), 0.87 (t, 3H, $^{3}J_{H,H}$ = 7.5 Hz); **^{13}C{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 119.81, 31.72, 30.75, 30.58, 30.48, 30.24, 30.14, 29.48 (m), 29.32, 29.16, 29.10, 28.77, 22.49, 22.12, 21.43 (m), 18.89, 18.58, 13.93, 13.74; **^{31}P{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 33.27; **IL 109: ^{1}H NMR** (22 ^{o}C, $CDCl_3$, Varian Inova 500 MHz) δ (ppm) 2.10 (m, 8H), 1.48 (m, 16H), 1.27 (m, 32H), 0.90 (t, 9H, $^{3}J_{H,H}$ = 7.0 Hz), 0.87 (t, 3H, $^{3}J_{H,H}$ = 7.0 Hz); **^{13}C{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 119.85 (q, $J_{C,F}$ = 321.8 Hz), 31.86, 30.80, 30.52, 30.42, 30.18, 30.08, 29.59 (m), 29.42, 29.29, 29.19, 28.72, 22.62, 22.21, 21.41 (m), 18.73, 18.42, 14.04, 13.77; **^{31}P{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 33.25.

Purification of Hydrophilic Ionic Liquid, IL 106

Distilled water (10 mL) was added to IL 106 (45 mL) and the solution was extracted with a 1:1 mixture of hexanes and ethyl acetate (7 × 50 mL). The ionic liquid was dried azeotropically under vacuum as previously described for the hydrophobic ionic liquids IL 101, IL 105 and IL 109. ^{1}H NMR was used to determine whether the ionic liquid was free of hexanes, toluene and water. **^{1}H NMR** (22 ^{o}C, $CDCl_3$, Varian Inova 500 MHz) δ (ppm) 7.67 (d, 2H, $^{3}J_{H,H}$ = 8.0 Hz), 7.05 (d, 2H, $^{3}J_{H,H}$ = 8.0 Hz), 2.26 (s, 3H), 2.13 (dd, 6H, $^{2}J_{H,P}$ = 13.0 Hz, $^{3}J_{H,H}$ = 6.5 Hz), 1.95 (m, 3H), 1.88 (d, 3H, $^{3}J_{H,P}$ = 13.0 Hz), 0.97 (dd, 18H, $^{3}J_{H,H}$ = 6.5 Hz, $^{4}J_{H,P}$ = 1.0 Hz); **^{13}C{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 143.58 (s), 138.92 (s), 128.26 (s), 125.85 (s), 29.89 (d, $J_{C,P}$ = 46.1 Hz), 24.26 (d, $J_{C,P}$ = 8.9 Hz), 23.44 (m), 21.05 (s), 6.92 (d, $J_{C,P}$ = 50.1 Hz); **^{31}P{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 29.00.

Purification of Hydrophilic Ionic Liquid, IL 169

IL 169 (25 mL) was extracted with hexanes (5 × 25 mL). Toluene was added to the ionic liquid which was dried azeotropically under vacuum as for the hydrophobic ionic liquids. ^{1}H NMR was used to determine whether the ionic liquid was hexanes-, toluene-, and water- free. **^{1}H NMR** (22 ^{o}C, $CDCl_3$, Varian Inova 500 MHz) δ (ppm) 3.88 (quin, 4H, $^{3}J_{H,H}$ = 7.0 Hz), 2.46 (dq, 2H, $^{2}J_{H,P}$ = 13.0 Hz), 2.30 (m, 6H), 1.50 (m, 12H), 1.24 (dt, 3H, $^{3}J_{H,H}$ = 7.5 Hz, $^{3}J_{H,P}$ = 18.5 Hz), 1.20 (t, 6H, $^{3}J_{H,H}$ = 7.0 Hz), 0.93 (t, 9H, $^{3}J_{H,H}$ = 7.0 Hz); **^{13}C{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 60.33 (d, $J_{C,P}$ = 5.4 Hz), 23.82 (d, $J_{C,P}$ = 15.3 Hz), 23.63 (d, $J_{C,P}$ = 4.7 Hz), 18.12 (d, $J_{C,P}$ = 47.4 Hz), 16.53 (d, $J_{C,P}$ = 7.6 Hz), 13.31 (s), 12.45 (d, $J_{C,P}$ = 48.8 Hz), 5.97(d, $J_{C,P}$ = 5.6 Hz); **^{31}P{^{1}H} NMR** (22 ^{o}C, $CDCl_3$, Bruker AVANCE II 600 MHz) δ (ppm) 35.04, 0.10.

2.2.2 Sodium-Salt Metathesis

Sodium-salt metathesis reactions were carried out according to literature procedure.[20] To pre-purified (see Section 2.2.1) IL 101 (50 mL, 0.079 mol) was added an excess of the sodium salt of the desired anion (Table 2.2), followed by the addition of distilled water (4.5 mL). The solution was heated to 50 °C with stirring for 5 hours. It was then transferred to a separatory funnel and washed with water (5 × 125 mL). The upper ionic liquid layer was transferred to a side-arm flask to which toluene (10 mL) was added to allow azeotropic removal of the water which was achieved by evacuation with stirring for 24 hours at ambient temperature. The flask was placed in a sand bath and heated to 50 °C under vacuum for 48 hours to remove residual toluene from the ionic liquid. ^{1}H NMR was used to determine whether the ionic liquid was free of both water and toluene.

Table 2.2 Amounts and salts used for sodium-salt metathesis reactions.

Anion	Sodium Salt	Mass (g)	Moles (mol)
Phosphate	Na_3PO_4	15.05	0.092
Dihydrogen phosphate	$NaH_2PO_4 \cdot H_2O$	12.46	0.090
Citrate	$C_6H_8O_7Na_3 \cdot 2H_2O$	25.24	0.086
Acetate	$CH_3COONa \cdot 3H_2O$	13.00	0.096
Glycinate	$C_2H_4NO_2$ (+ NaOH)	7.67	0.102
Formate	HCOONa	6.60	0.097

2.2.3 Silver-Salt Metathesis

IL 101 (16.41 g, 0.032 mol) was dissolved in acetonitrile (75 mL). Separately, silver nitrate (16.22 g, 0.095 mol) in three-times molar excess compared to IL 101 was dissolved in acetonitrile (40 mL). Each solution was stirred for 15 minutes to allow complete dissolution. On combination of the two solutions a white precipitate was formed immediately. The combined solution was stirred for 90 minutes. The precipitated silver chloride was removed by filtration to give the tetradecyl(trihexyl)phosphonium

nitrate (IL-NO_3) salt dissolved in acetonitrile. The solvent was removed under vacuum to yield an orange-brown viscous liquid.

The ionic liquid, IL-NO_3, was washed by dissolution in chloroform (120 mL), to give a cloudy orange-brown solution, followed by extraction with water (7 × 100 mL) until no free Ag^+ was present in the water layer. Presence of Ag^+ was tested by drop-wise addition of aqueous 2 M potassium chromate to an aliquot of the water layer. The yellow solution becomes red when free Ag^+ is present due to formation of Ag_2CrO_4. Chloroform was removed from the ionic liquid solution under vacuum to yield IL-NO_3 as a viscous pale-orange liquid. The ionic liquid was tested for complete replacement of the chloride by titration with 0.05 M $AgNO_3$ solution.

2.2.4 Acid-Base Neutralisation

The acid-base neutralisation reactions were based on literature reports for the preparation of other types of ionic liquids.[82] Phosphonium ionic liquids are inherently viscous liquids, therefore, toluene was added to reduce the viscosity of the solution for ease of filtration during the preparation. Two different amounts of organic solvent were used (minimal, 1 mL and excess, 10 mL). Minimal organic solvent was used only to reduce viscosity with the solution remaining mostly ionic liquid. Excess organic solvent was used as the primary reaction medium in the second reaction.

Minimal Organic Solvent Used

Silver nitrate (4.0883 g, 0.0236 mol) was dissolved in water (20 mL). After stirring for 5 minutes sodium hydroxide was added (0.9637 g, 0.0241 mol) to form a brown precipitate. The solution was stirred for 15 minutes. Brown silver hydroxide

(AgOH) was removed by filtration. To IL 101 (8.087 g, 0.0156 mol) was added the freshly prepared AgOH. Toluene (1 mL) was added to reduce the viscosity of the solution, which was stirred for 1 hour. Precipitated AgCl was removed by filtration. Phosphoric acid (85%, 0.017 mol) was added to the filtrate, the brown solution turned grey/white in colour after stirring for 15 minutes. This viscous solution was dissolved in acetone (10 mL). Sodium carbonate (0.50 g, 0.00472 mol) was added to neutralise the acid as per literature methods.[82] The solution was filtered to remove a precipitate, which resembled AgH_2PO_4, most likely formed due to the reaction of dihydrogen phosphate with excess Ag^+ in solution. Acetone was evaporated to leave a somewhat viscous faintly yellow coloured ionic liquid, IL-H_2PO_4.

Excess Organic Solvent Used

Silver hydroxide was prepared by dissolving silver nitrate (3.8350 g, 0.0226 mol) in water (20 mL) and adding sodium hydroxide (0.9247 g, 0.0231 mol). The brown precipitate was collected by filtration and rinsed with water (120 mL). IL 101 (6.0395 g, 0.0231 mol) was dissolved in toluene (10 mL). Silver hydroxide was stirred into the dissolved ionic liquid solution to yield a white precipitate. The solution was placed in a warm water bath (35 ^oC) and stirred for 4 hours. It was then filtered to remove precipitated silver chloride. Phosphoric acid (85%, 0.79 mL, 0.0116 mol) was added to the filtrate, which was stirred for 1 hour. The cloudy white solution was filtered to give the final ionic liquid dissolved in toluene. The solvent was removed under vacuum to give a pale yellow ionic liquid, IL-H_2PO_4.

2.3 Results

2.3.1 Properties of Commercial Phosphonium Ionic Liquids

The physicochemical properties of ionic liquids, such as viscosity, density, and melting point can vary depending on the impurities present.[79,83] Commercially available ionic liquids are known to contain impurities resulting from synthesis (Section 2.1). Cytec Industries Inc., the supplier of the phosphonium ionic liquids used in these studies, details the impurities found in each ionic liquid (refer to Table 2.1), which typically include the starting materials.

An impurity found in each ionic liquid is water since most ionic liquids, even water-immiscible ones, are hygroscopic and can readily absorb water from the atmosphere.[6] Literature purification methods,[81] previously demonstrated to successfully remove impurities, were used for the hydrophobic ionic liquids IL 101, IL 105, and IL 109. Acidic impurities, such as hydrochloric acid, were neutralised through washing the ionic liquid with a saturated aqueous sodium bicarbonate solution. The products of the neutralisation as well as any water-soluble impurities were removed by repeatedly washing with distilled water. Organic impurities were removed by extracting with hexanes. Dry ionic liquids are miscible with hexanes, however, when saturated with water, the water-ionic liquid-hexanes system is triphasic. Location of the ionic-liquid layer is dependent on the density of the ionic liquid but it is generally located in the middle. Water and volatile impurities were removed from the ionic liquids under vacuum. New procedures were established for the hydrophilic ionic liquids, IL 106 and IL 169, eliminating the water-washing step. Neutralisation of acidic species in the hydrophilic

ionic liquids was not necessary since IL 106 and IL 169 are chloride-free ionic liquids. ^{1}H and ^{31}P NMR of the purified ionic liquids indicated insignificant amounts of impurities.

After purification of the Cytec ionic liquids the density was measured and the concentration of anions and cations in the ionic liquid was calculated. Table 2.3 gives the results of the calculations.

Table 2.3 Physicochemical properties of Cytec phosphonium ionic liquids.

Ionic Liquid	Molar Mass (g/mol)	Density (g/mL)	Ion Concentration (mol/L)
IL 101	519.32	0.84	1.6
IL 105	549.91	0.88	1.6
IL 106	388.55	1.03	2.7
IL 109	764.14	1.02	1.3
IL 169	384.48	0.97	2.5

Solvent miscibility is not easily predicted for ionic liquids. For the Cytec ionic liquids several organic solvents were tested for miscibility with the ionic liquids (Table 2.4).

Table 2.4 Solvent miscibilities of commercial phosphonium ionic liquids.

Ionic Liquid	Water†	Hexanes‡	Hexanes/ Water	Methanol	Methanol/ Water	DCM*	DCM*/ Water	EtOAc*	EtOAc*/ Hexanes
IL 101	Very slightly miscible	Fully miscible	3 layers, IL in middle	Miscible	2 layers, IL on top	Miscible	2 layers, IL miscible with DCM	Miscible	Miscible
IL 105	Slightly miscible	Partially miscible	3 layers, IL in middle	Miscible	2 layers, IL on bottom	Miscible	2 layers, IL miscible with DCM	Miscible	Miscible
IL 106	Miscible	Immiscible	2 layers, hexanes on top, IL/water bottom	Miscible	Miscible	Miscible	2 layers, IL miscible with DCM	Miscible	2 layers, IL on bottom
IL 109	Immiscible	Fully miscible	3 layers, IL in middle	Miscible	2 layers, IL on top	Miscible	2 layers, IL miscible with DCM	Miscible	Miscible
IL 169	Miscible	Partially miscible	2 layers, hexanes on top, IL/water bottom	Miscible	Miscible	Miscible	2 layers, IL/water on top, DCM on bottom	Miscible	Miscible

†Miscibility with water: IL 169 ≈ IL 106 > IL 105 > IL 101 > IL 109
‡ Miscibility with hexanes: IL 101 ≈ IL 109 > IL 105 > IL 169 > IL 106
* DCM = Dichloromethane; EtOAc = Ethyl Acetate

2.3.2 Sodium-Salt Metathesis

Sodium-salt metathesis reactions were performed according to literature methods[20] for the preparation of various new phosphonium ionic liquids containing biocompatible anions such as phosphate, dihydrogen phosphate, citrate, acetate, glycinate, and formate. The reactions were all carried out by reacting IL 101, tetradecyl(trihexyl)phosphonium chloride, with the sodium salt of the desired anion. In this way, a series of ionic liquids containing the same cation could be prepared and their biocompatibility compared. Success of the anion exchange reactions was determined by measuring the remaining concentration of free chloride via silver nitrate titrations; the technique used commonly in literature reports.[79] When the replacement anion contained ^{31}P, as for phosphate and dihydrogen phosphate, $^{31}P\{^{1}H\}$ NMR studies were used to confirm the presence of the anion in the anion-exchanged ionic liquid.

When silver nitrate titrations were used to determine the chloride content of the new ionic liquids, IL 101 was titrated under the same conditions for comparison. Titrations of IL 101 gave a chloride concentration of 1.28 ± 0.03 M, as determined from three independent measurements. Unless otherwise noted, all reported amounts of chloride exchanged were determined from three separate titrations.

The phosphate ionic liquid (IL-PO_4) was titrated with silver nitrate to determine the amount of chloride present following attempted exchange as described in Section 2.2.2. These results indicate approximately 17% (± 3%) exchange of chloride for phosphate as determined by comparison of the concentration of chloride versus the original concentration of chloride in IL 101 ($[Cl^-]_{IL\text{-}PO4}$ = 1.06 M vs. $[Cl^-]_{IL\ 101}$ = 1.28 M). $^{31}P\{^{1}H\}$ NMR was used to directly establish whether phosphate was exchanged for

chloride. The ^{31}P NMR spectrum of purified IL 101 shows a single peak at 33.28 ppm arising from the tetradecyl(trihexyl)phosphonium cation (literature δ 33.39 ppm).[20] The presence of phosphate exchanged for chloride was expected to give a second resonance in the chemical shift region of 0 to 6 ppm as predicted by reports of phosphate in deuterium oxide.[84-87] The large range in chemical shift for phosphate in deuterium oxide is due to its dependence on pH. Comparison to the phosphate chemical shift of sodium phosphate in deuterated chloroform was not possible since the salt is not soluble in this solvent. Given the level of exchange indicated by the silver nitrate titrations it was anticipated that the phosphate would be readily observable. However, surprisingly no such peak could be observed, even when a wide spectral window was used. A possible reason for this absence may be insolubility of phosphate in the solvent used, chloroform-d_1. Whatever the reason for this absence, it is not significant to the conclusions of this thesis since the titration experiments clearly demonstrate that a large majority of chloride remains and thus the attempt to make pure tetradecyl(trihexyl)phosphonium phosphate was not successful.

The results of the silver nitrate titration of the attempted preparation of dihydrogen phosphate ionic liquid by anion exchange (Section 2.2.2) indicate that 1% (± 2%) of the chloride was exchanged for dihydrogen phosphate as determined by comparing the concentration of chloride in the prepared ionic liquid versus the chloride ionic liquid, IL 101. These results indicate that no exchange occurred within experimental error. Correspondingly, $^{31}P\{^{1}H\}$ NMR gives only one peak at 33.20 ppm, attributed to the tetradecyl(trihexyl)phosphonium cation. It was expected that dihydrogen phosphate would give a resonance between 0 and 1 ppm (reported in deuterium oxide[88]) in the

^{31}P NMR spectrum, its absence supports the titration results that the chloride was not replaced by dihydrogen phosphate to form tetradecyl(trihexyl)phosphonium dihydrogen phosphate as a new ionic liquid.

Titration with silver nitrate of the citrate ionic liquid (preparation described in Section 2.2.2) to determine the amount of chloride gave 1% (± 6%) exchange indicating that within experimental error no chloride was exchanged for citrate as determined by comparison to the concentration of chloride in pure IL 101. Likewise, the results of titrations of the anticipated tetradecyl(trihexyl)phosphonium acetate ionic liquid demonstrated that approximately 22% (± 1%) exchange of chloride for acetate occurred. The glycinate ionic liquid was titrated with silver nitrate and the results indicate that 16% (± 7%) of the chloride was exchanged for glycinate. Finally, titration of the formate ionic liquid for determination of the total chloride content gave 40% (± 11%) exchange of chloride for formate as determined by comparison with the concentration of chloride in IL 101 (as described above). NMR studies to determine the exact concentration of anion exchange were not performed for these ionic liquids since the only NMR-active nuclei in significant quantities is ^{1}H, and the numerous resonances of tetradecyl(trihexyl)phosphonium protons were anticipated to overlap with those of the anions. The titration experiments demonstrate that significant quantities of chloride remain for each anion exchange reaction, indicating that preparation of pure, halide-free tetradecyl(trihexyl)phosphonium ionic liquids was not successful.

2.3.3 Silver-Salt Metathesis

Preparation of a new phosphonium ionic liquid, tetradecyl(trihexyl)phosphonium nitrate (IL-NO_3) was achieved by silver-salt metathesis using the chloride salt, IL 101

(Section 2.2.3). As described in the previous section, silver nitrate titrations were used to determine the amount of chloride remaining in the ionic liquid solution. The results indicate that there is essentially no chloride in solution (less than 1%) within experimental error since the first drop of silver nitrate produced a red precipitate indicating nearly complete anion exchange of the nitrate anion for chloride. By comparison to the concentration of chloride in IL 101 there was at least 98.4% (± 0.4%) exchange of chloride for nitrate. The lower limit for these titrations is one drop, an approximate volume of 0.02 mL, or a concentration of 1.0×10^{-4} M chloride. This is the first report of tetradecyl(trihexyl)phosphonium nitrate.

2.3.4 Acid-Base Neutralisation

Tetradecyl(trihexyl)phosphonium dihydrogen phosphate (IL-H_2PO_4) was prepared by acid-base neutralisation from IL 101 (Section 2.2.4). Silver nitrate titrations were performed to determine the amount of chloride exchanged for dihydrogen phosphate as described for the sodium-salt metathesis reactions in Section 2.3.2. Initially, a small amount of toluene (1 mL) was added to reduce the viscosity of the ionic liquid solution since it is known that the physical properties of ionic liquids can vary significantly with even a small amount of organic solvent.[79] The results of the titration indicate that 41% (± 6%) chloride was exchanged for dihydrogen phosphate. Presence of dihydrogen phosphate in the anion-exchanged ionic liquid, IL-H_2PO_4, was confirmed using $^{31}P\{^1H\}$ NMR. Two peaks were present in the ^{31}P NMR: 33.24 ppm and 2.29 ppm. The peak at 33.24 ppm is attributed to tetradecyl(trihexyl)phosphonium as can be observed in the ^{31}P NMR of IL 101.[20] The new peak at 2.29 ppm is attributed to $H_2PO_4^-$.

The solution containing 1 mL toluene (as described in Section 2.2.4) yielded only a small amount of anion exchange. In addition, the solution was difficult to filter because of its high viscosity. A second attempt at synthesizing the new ionic liquid was made using a larger amount of toluene (10 mL) to aid in filtration by significantly reducing the viscosity to nearly that of toluene itself. The results of the silver nitrate titration indicate that 29% (± 1%) chloride was exchanged for dihydrogen phosphate, determined from two independent measurements, as compared to the concentration of chloride in IL 101 (described in Section 2.3.2). Analysis by ^{31}P NMR gave two peaks, 33.13 ppm and 1.51 ppm. The peak at 33.13 ppm is assigned as the tetradecyl(trihexyl)phosphonium cation as was observed in the ^{31}P NMR of IL 101.[20] It follows that the new peak at 1.51 ppm is $H_2PO_4^-$.

2.4 Discussion

2.4.1 Commercial Phosphonium Ionic Liquids

Literature reports of ionic liquids rarely describe important characteristics such as the concentration of ions in ionic liquids. This information is important for considering the effect of ionic strength on a reaction or for comparison of the concentration of species in solution. The concentrations of ions for the commercially available ionic liquids IL 101, IL 105, IL 106, IL 109, and IL 169 range from 1.3 M to 2.7 M (Table 2.3). Comparison of these concentrations with the concentration of ions in a saturated aqueous solution of sodium chloride, 8 M, demonstrates that the concentration of ions in these ionic liquids is actually not particularly high. The concentration of ions in ionic liquids becomes very important when considering dilution with organic solvent. A simple calculation demonstrates the importance of this concept, a 15% (v/v) solution of toluene

(density 0.86 g/mL, molar mass 92.14 g/mol)[89] in IL 101 (density 0.84 g/mL, molar mass 519.34 g/mol) gives a 50:50 molar ratio of toluene to IL 101.

2.4.2 Anion Exchange Reactions

Metathesis reactions for these studies were focused on the chloride salt of the tetradecyl(trihexyl)phosphonium cation since it is readily available in large quantities. However, metathesis with any halide salt is feasible. The only manufacturer of phosphonium ionic liquids, Cytec Industries Inc., reports that the chloride anion of tetradecyl(trihexyl)phosphonium chloride (IL 101) can be readily exchanged by almost any other anion provided that the free acid or alkali metal salt is available. Despite these claims, silver nitrate titrations following the sodium-salt metathesis experiments demonstrate that this is commonly not the case. The chloride concentration determined in the ionic liquids following exchange was typically comparable to the amount of chloride in the starting material, IL 101, indicating very little anion exchange. Furthermore, in the case of the phosphorus containing anions, ^{31}P NMR demonstrated no new phosphorus peak. These results indicate that the affinity of the sodium ion for the desired anions is much greater than that of the phosphonium cation.

Anions with different ionic charges were used for the sodium-salt metathesis reactions. For example, phosphate and citrate are both trianionic ions. It is fitting that the anion exchange of chloride for either of these two anions was unsuccessful since conservation of an overall neutral charge of the ionic liquid would yield three cations per anion. Tetradecyl(trihexyl)phosphonium is a bulky cation hence steric constraints would prevent three cations from surrounding one anion. Nevertheless, phosphate-containing

ionic liquids involving polyammonium or imidazolium cations have been reported in the literature.[90]

Sodium-salt metathesis is one of the most common methods for preparation of new ionic liquids via anion exchange.[91] Some examples of ionic liquids that have been prepared via this method include phosphonium ionic liquids tetradecyl(trihexyl)phosphonium tetrafluoroborate,[20] tetradecyl(trihexyl)phosphonium dicyanamide,[80] and tetradecyl(trihexyl)phosphonium trifluoromethanesulfonate[80] as well as a plethora of other types of ionic liquids containing anions such as hexafluorophosphate.[92] The results of this thesis demonstrate some of the limitations in using the sodium-salt metathesis method for anion exchange. First, there exist only a limited number of anions that can be successfully exchanged from the sodium salt in high yield; and second, the conditions of the reaction such as solvent choice, temperature, and reaction time are crucial and need to be optimised for specific ionic liquids.

Metathesis reactions in general are driven by one of three driving forces: (1) formation of a precipitate, which relates to the lattice energy of the solid formed; (2) neutralisation to form a salt and water; or (3) formation of a gas. Since the metathesis reactions with ionic liquids involve exchange of a sodium salt to form sodium chloride, the driving force of the sodium-salt metathesis reactions containing water cannot be formation of a precipitate since sodium chloride is soluble in water. It is possible that selection of another solvent, one in which sodium chloride would precipitate, would drive the metathesis reaction to occur more effectively.

The silver-salt metathesis reaction for the preparation of IL-NO_3 was shown to be successful as demonstrated by complete exchange of the chloride anion, which was

determined by silver nitrate titrations. The reaction involved the precipitation of insoluble silver chloride, which has very high lattice energy and acts as the driving force for the anion exchange. Furthermore, the solubility of the starting silver salt simplified the reaction procedure. Examples of other ionic liquids in the literature that have been prepared using soluble silver salts include imidazolium salts containing the anions acetate, tetrafluoroborate, sulfate, and nitrite.[13]

Acid-base neutralisation was employed to prepare IL-H_2PO_4 with partial success as demonstrated by silver nitrate titrations and ^{31}P NMR. There are two steps in this procedure (Scheme 2.1) and each is driven by a different force.

(A) $[R'PR_3]^+Cl^- + AgOH \rightarrow [R'PR_3]^+OH^- + AgCl$

(B) $[R'PR_3]^+OH^- + H_3PO_4 \rightarrow [R'PR_3]^+H_2PO_4^- + H_2O$

Scheme 2.1 Two-step preparation of phosphonium dihydrogen phosphate ionic liquid by acid-base neutralisation reaction.

Reaction (A) of Scheme 2.1 is driven by the precipitation of insoluble silver chloride, AgCl, to give the phosphonium hydroxide salt. The second reaction (B) is driven by the formation of water and the dihydrogen phosphate ionic liquid. Since the titrations with silver nitrate indicate chloride was still present in IL-H_2PO_4, the formation of the hydroxide-containing ionic liquid was not complete under the given reaction conditions. Longer reaction times or increased temperatures may result in increased anion exchange.

Binary ionic liquids such as halogenoaluminate ionic liquids, which are composed of a mixture of a pyridinium or imidazolium halide and an aluminium (III) halide (usually $AlCl_3$), are well known in the literature.[10,11] The success of these ionic liquids for various

applications[14] leads to the question of the possible benefits of ionic liquids with a mixture of anions. An ionic liquid containing a mixture of anions, especially those that are known to stabilise proteins, may improve the biocompatibility of an ionic liquid as compared to an ionic liquid containing, for example, only chloride anions. IL-H_2PO_4 was shown to contain both chloride and dihydrogen phosphate anions. The presence of dihydrogen phosphate, commonly used in buffers, may provide for enhanced stability of proteins. Further studies regarding ionic liquids with a mixture of anions are required to determine the effect of multiple anions however it is beyond the scope of this research since the goal was to prepare essentially chloride-free phosphonium ionic liquids.

Based on the results in this thesis, the silver-salt metathesis reaction was most successful in exchanging the chloride ion for another anion, in this case nitrate. Unfortunately, the nitrate anion has been demonstrated to be a poor choice for biocatalytic reactions.[16,93] The titrations demonstrated that the acid-base neutralisation reactions, though not providing complete chloride exchange, were partially successful in exchanging the chloride. Finally, the sodium-salt metathesis method for preparation of new phosphonium ionic liquids containing the anions phosphate, dihydrogen phosphate, citrate, acetate, glycinate, or formate proved ineffective for these anions under the reaction conditions used. This is particularly significant since sodium-salt metathesis is commonly applied in the literature for preparation of ionic liquids.

3 MYOGLOBIN IN PHOSPHONIUM IONIC LIQUIDS

3.1 Introduction

This chapter describes the first report of a biocatalytic reaction mediated by myoglobin in ionic liquids. It is only the second report of biocatalysis in phosphonium ionic liquids.[27] The motivation for the study of myoglobin-catalysed reactions in phosphonium ionic liquids was the expectation that phosphonium ionic liquids would promote new or unusual chemistry when used as reaction media.[8] Furthermore, myoglobin, the first protein for which the crystal structure was determined,[94] is a well-characterised protein, with the ability to perform chemical transformations under appropriate conditions.[95-97]

Myoglobin in ionic liquids IL 101, IL 105, IL 106, IL 109, and IL 169 was characterised by UV-Visible, fluorescence, and EPR spectroscopies. Analyses of myoglobin in aqueous solutions of hydrophilic ionic liquids IL 106 and IL 169 were also carried out. Finally, the biocatalytic oxidation of dibenzothiophene mediated by myoglobin in the presence of hydrogen peroxide was shown to proceed in aqueous solutions of IL 106 and IL 169. Ionic liquids IL 106 and IL 169 were the focus of the biocatalytic reactions since they are miscible with water and the protein could be dissolved in the reaction solvent mixture. Lyophilised myoglobin is not soluble in the hydrophobic ionic liquids, therefore only the water-miscible ionic liquids were studied for biocatalysis.

3.1.1 Myoglobin

Myoglobin, an oxygen storage protein, consists of 8 α-helices and a heme prosthetic group, which is the active site of the protein (Figure 3.1). The heme, iron protoporphyrin IX, contains a planar porphyrin macrocycle with an iron ion bound through four nitrogen atoms. In myoglobin, the heme is held in the protein by coordination of a histidine residue to the iron in the proximal position (Figure 3.1 (B)), non-covalent van der Waals interactions in the hydrophobic pocket of the protein, and hydrogen bonding between the propionate groups of the protoporphyrin and the protein. In aqueous solutions of the oxidised form of the protein, met-myoglobin, the sixth ligand of iron is a single water molecule; however, the water can be exchanged for other axial ligands. Oxygen binding by myoglobin occurs at this sixth position.

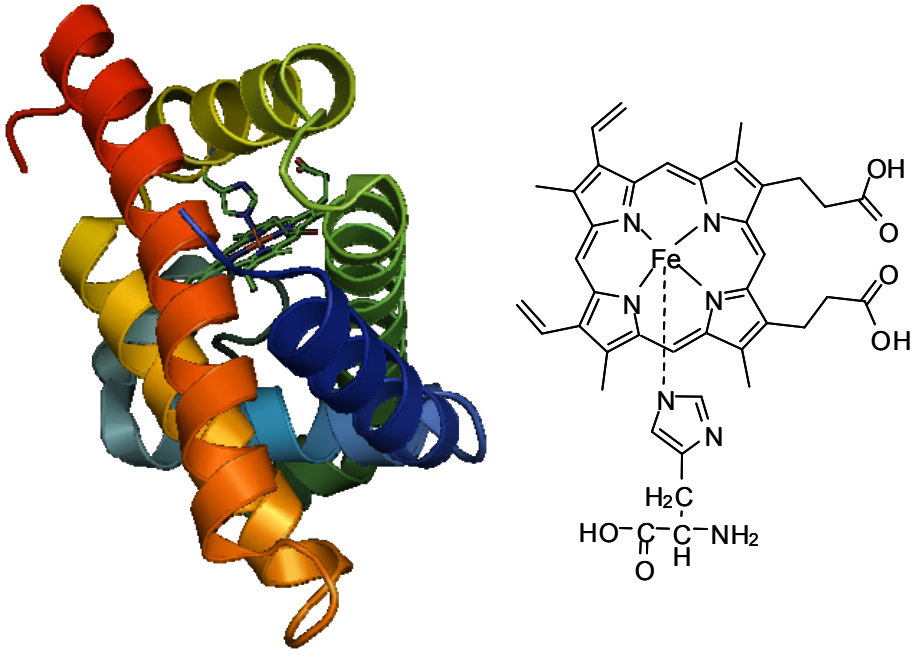


Figure 3.1 **Myoglobin. (A) Structure of myoglobin showing α-helices and heme active site in the hydrophobic pocket; (B) Heme: iron protoporphyin IX coordinated to a histidine residue at the proximal position.**

3.1.2 Protein Denaturation by Guanidine-Hydrochloride and Methanol

Denaturation of proteins by denaturing agents such as urea, guanidine-hydrochloride, and alcohols has been extensively studied for determination of protein stability. Similar to the understanding of protein folding, the exact nature of the interactions between denaturants and proteins is not completely understood. Guanidine-hydrochloride and urea are commonly used to study the stability of proteins, however a full understanding of the mechanism by which these chemicals cause a protein to unfold has not yet been reached.[98] Early investigators of the issue proposed a direct interaction model in which guanidine-hydrochloride was presumed to interact directly with polar side-chains and the peptide backbone by electrostatic interactions and hydrogen bonding.[99] Recently, molecular dynamics calculations have shown that unfolding by altering charge-charge interactions either through solvation of charged residues or by hydrogen bonding was the most probable mechanism for guanidine-hydrochloride and urea denaturation.[98] Denaturation of proteins by alcohols is also not completely understood. It is assumed that alcohols denature proteins through the "preferential solvation model" in which alcohols interact more strongly with hydrophobic amino acid residues causing the residues to be transferred into solution rather than remain folded into the core of the protein.[100] Alcohols are known to stabilise helical conformations of proteins. Therefore alcohol-denatured proteins often assume more helical structures than their native conformation.[100-104]

3.1.3 Peroxidase-Like Activity of Myoglobin

Peroxidases are proteins that catalytically oxidise substrates in the presence of peroxides, generally hydrogen peroxide. Oxidation by peroxidase enzymes with

hydrogen peroxide is a two-step process that involves the one-electron oxidation of two substrate (R) molecules:[105]

$$2RH + H_2O_2 \rightarrow 2R^{\bullet} + 2H_2O$$

Heme peroxidases contain the same iron-protoporphyrin IX active site as myoglobin. It follows that myoglobin, possessing the same active centre, could also exhibit peroxidase-like activity. Keilin and Hartree[95] were the first to report the peroxidative activity of myoglobin in the presence of peroxide. Since that discovery, few reports of native myoglobin-catalysed peroxidase reactions have been published. Myoglobin was recently shown to mediate the nitration of phenolic compounds by nitrite and hydrogen peroxide.[96] Klyachko and Klibanov[97] reported that myoglobin, and other non-enzymic heme proteins, could catalyse the oxidation of dibenzothiophene in the presence of a peroxide in mixtures of buffer and organic solvent.

The oxidation of dibenzothiophene is important because it is a model compound for sulfur impurities in coal and crude oil.[97] Oxidation of organic sulfur compounds allows for their subsequent removal thus reducing the sulfur emissions produced from combustion of these fuels. Dibenzothiophene (Figure 3.2) is only sparingly soluble in water, and this was the motivation for Klyachko and Klibanov[97] demonstrating that dibenzothiophene dissolved in organic solvent-buffer solutions could be oxidised by myoglobin. The biocatalytic oxidation of dibenzothiophene was chosen as a model reaction to demonstrate the activity of myoglobin in ionic liquids in order to compare with this earlier research and because it was shown to be successful in aqueous mixtures of organic solvents.

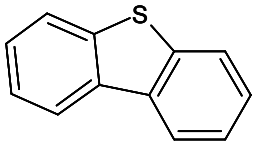


Figure 3.2 Structure of dibenzothiophene, the compound used for myoglobin biocatalysis studies.

3.2 Experimental

UV-Visible absorption spectra were collected on a Hewlett-Packard HP 8453 spectrometer. The spectra were recorded in 4 mL acrylic cuvettes (Sarstedt, Montréal, QC) with a path length of 1 cm. Spectral resolution was 2 nm.

Steady-state fluorescence measurements of myoglobin in ionic liquid and aqueous solution were conducted using a PTI fluorimeter (Photon Technology International, London ON). Emission spectra were obtained by excitation at 280 nm and monitoring the emission between 290 nm and 500 nm with 1 nm resolution. The path length was 1cm.

EPR data were recorded using either a Bruker ECS-106 X-band or a Bruker EMX Plus X-band spectrometer. 3 mm outer diameter quartz EPR tubes (Wilmad, Buena, NJ) were used in all measurements. Samples measured at liquid nitrogen temperatures (77 K) were immersed in liquid nitrogen in a finger dewar, which was placed directly into the cavity of the instrument. Helium gas was bubbled through the upper portion of the liquid nitrogen to reduce bubbling of liquid nitrogen and improve spectral stability. Operating frequencies were generally between 9.4 and 9.6 GHz; frequencies for each experiment are reported in the text.

NMR spectroscopy was performed on a 500 MHz Varian Inova spectrometer or by Dr. Andrew Lewis and Mr. Colin Zhang on a 600 MHz Bruker AVANCE II spectrometer, as described in Section 2.2. Operational frequencies for the 600 MHz

instrument were: ^{1}H, 600.13 MHz, $^{31}P\{^{1}H\}$, 242.94 MHz. All 600 MHz ^{1}H spectra are referenced to the solvent peak, benzene-d_6. $^{31}P\{^{1}H\}$ spectra are referenced to an external standard of phosphoric acid.

High performance liquid chromatography (HPLC) measurements were performed using a Hewlett Packard Series 1050 HPLC equipped a variable wavelength detector and an Agilent Zorbax Eclipse XDB-C18 column (4.6 × 75 mm, 3.5 μM). Dibenzothiophene and its oxidation products were monitored at 230 nm. Separation of dibenzothiophene (DBT), dibenzothiophene sulfoxide (DBTO) and dibenzothiophene sulfone ($DBTO_2$) was achieved using a mobile phase consisting of acetonitrile and water in the following regime: a linear gradient of acetonitrile:water (50:50 to 80:20) over 7 minutes, at a flow rate of 0.6 mL/min; maintaining an 80:20 acetonitrile:water mixture the flow rate was reduced to 0.2 mL/min and held for 3.5 minutes after which the flow rate was increased to 1.5 mL/min and the acetonitrile content increased to 95% (over 1 minute) to obtain the DBT peak.

3.2.1 UV-Visible and Fluorescence of met-Myoglobin

Aqueous Solutions of IL 106 and IL 169

Solutions of varying amounts of ionic liquid (IL 106 or IL 169) and buffer were prepared according to Table 3.1. The general procedure is described below:

Ionic liquid was measured using a disposable 5 mL pipet (Fisher Scientific, Ottawa, ON) into a centrifuge tube, followed by the corresponding amount of buffer (phosphate, pH 7.0). The mixture was then vortexed until a homogeneous solution was obtained and placed on a rocking platform to mix for 15 minutes. After mixing, the samples were allowed to stand at room temperature overnight to equilibrate.

The myoglobin solution was prepared by dissolving horse heart myoglobin (0.0063 g, 3.7×10^{-7} mol) in 6.0 mL buffer (phosphate, pH 7.0). The solution was gently mixed on a rocking platform. The met-myoglobin (myoglobin in which the iron is in the 3+ oxidation state) solution (10 mL) was added to the previously prepared ionic liquid-buffer solutions. The tubes were placed on a rocking platform to mix for 20 minutes prior to being allowed to equilibrate in a 25 °C (± 0.2 °C) water bath for 2 hours before fluorescence and UV-Vis measurements.

Table 3.1 Volumes for preparation of ionic-liquid buffer solutions.

Percent (v/v) Ionic Liquid	Volume Ionic Liquid (μL)	Volume Myoglobin Solution (μL)	Volume Buffer (μL)
90%	4500	500	0
75%	3750	500	750
50%	2500	500	2000
25%	1250	500	3250
10%	500	500	4000

Sample blanks were prepared for subtraction of fluorescence and UV-Visible absorption backgrounds. In place of the myoglobin solution, buffer (500μL) was added to the ionic liquid-buffer solution.

Two sets of all solutions were prepared to ensure reproducibility and precision.

Solutions of Myoglobin in Neat Ionic Liquids

A stock myoglobin solution was prepared by dissolving myoglobin (0.0046 g, 2.71×10^{-7} mol) in 3 mL buffer (phosphate, pH 7.0). An aliquot (250 μL) of the myoglobin solution was added to ionic liquid (5 mL). The solution was gently stirred for 1 hour to allow complete mixing of the solution. Water was removed under vacuum for 18 hours to give met-myoglobin in neat ionic liquid.

The amount of residual water in the solutions of myoglobin in neat ionic liquid was determined by ^{1}H NMR (22 ^{o}C, C_6D_6, Bruker AVANCE II 600 MHz) after two months in ambient conditions. An aliquot of the ionic liquid solution (50 μL) was dissolved in benzene-d_6 (550 μL) containing 0.05% (v/v) TMS (Cambridge Isotope Laboratories, Inc. Andover, MA) to give a final amount of 0.046% TMS. The NMR solvent was dried using 3 Å molecular sieves. Percent by volume of water was calculated by comparing the integrated water peak for each solution against the TMS peak. The amount of water in solution was corrected for water in the solvent. Reported integrals are an average of 10 integral calculations for each sample. Residual water content was not able to be determined for IL 109 since the water peak overlapped with the ionic liquid peaks. Calibration of this experiment was performed by spiking samples of ionic liquids with known amounts of water (1% (v/v), 5% (v/v)) and comparing the calculated water content from NMR measurements with the known amounts of water added.

Table 3.2 Water content determination in neat ionic liquids by ^{1}H NMR.

Sample	δ (ppm)	Integral	Moles Water	% (v/v) Water in Ionic Liquid
IL 101	4.04	10.71	1.28×10^{-4}	4.62
IL 101 with Mb	4.04	11.34	1.36×10^{-4}	4.89
IL 105	2.51	2.64	3.00×10^{-5}	1.08
IL 105 with Mb	2.54	2.84	3.25×10^{-5}	1.17
IL 106	6.79	9.76	1.17×10^{-4}	4.20
IL 106 with Mb	6.74	10.15	1.21×10^{-4}	4.37
IL 169	4.74	20.98	2.53×10^{-4}	9.12
IL 169 with Mb	4.74	18.69	2.25×10^{-4}	8.11

3.2.2 EPR Experiments in Phosphonium Ionic Liquids

Myoglobin-azide in Phosphonium Ionic Liquids

Myoglobin (0.3621 g, 2.1×10^{-5} mol) was dissolved in buffer (5.0 mL, 20 mM phosphate, pH 7.0) with vortexing to mix. Excess potassium ferricyanide ($K_3Fe(CN)_6$)

was added to oxidise the protein to met-myoglobin. The oxidant was removed using a Sephadex PD-10 desalting column (GE Healthcare). Two aliquots of the myoglobin solution were eluted with buffer (3.5 mL each), to give a total volume of 7.0 mL. Sodium azide (0.3884 g, 6.0×10^{-3} mol) was dissolved in buffer (750 μL). The colour of the myoglobin solution changed from dark brown to deep red on addition of the azide solution. The concentration of myoglobin in solution was 2.7 mM.

Ionic liquid (1.5 mL) was gently stirred with an aliquot of the myoglobin-azide solution (750 μL) for 5 minutes. Water was removed from the samples under vacuum. The final myoglobin concentration was 1.4 mM. An aliquot of the solution was transferred into a quartz EPR tube. The samples were then stored in liquid nitrogen.

Hemin Chloride and Hemin Chloride-Azide in Phosphonium Ionic Liquids

Hemin chloride (0.0028 g, 4.3×10^{-6} mol) was dissolved in IL 169 (2 mL) with stirring for 3 hours. The concentration of hemin chloride in solution was 2.2 mM. An aliquot of the solution was added to an EPR tube and stored in liquid nitrogen.

Hemin chloride (0.0027 g, 4.1×10^{-6} mol) was dissolved with stirring in IL 169 (1 mL). Separately, sodium azide (0.0412 g, 6.3×10^{-4} mol) was dissolved in IL 169 (1 mL). After stirring overnight, the two solutions were combined and stirred for 2 hours. The hemin chloride solution turned from dark green to dark brown upon addition of the azide solution. An aliquot was added to an EPR tube and stored in liquid nitrogen.

Hemin chloride was dissolved in the other ionic liquids as described above. Sodium azide was not soluble in any ionic liquid except IL 169.

DMPO Spin Trapping in IL 169

To an aliquot of IL 169 (4.0 mL) was added an excess of 5,5-dimethylpyrroline-*N*-oxide (DMPO) (0.025 mL, 2.2×10^{-4} mol). The solution was stirred for 25 minutes under ambient conditions. A small aliquot was removed and added to a quartz EPR tube. Final concentration of DMPO in solution was 56 mM.

A portion of the DMPO-IL 169 sample (2 mL) was removed. Sodium azide (0.0950 g, 1.4×10^{-3} mol) was added to the DMPO-IL 169 solution and was stirred overnight under ambient conditions. An aliquot was removed and added to a quartz EPR tube. The final azide concentration was 0.7 M.

To the remaining DMPO-IL 169 solution was added myoglobin-azide in buffer (1 mL, 3.0 mM). The solution was gently stirred and the water removed under vacuum overnight. The final myoglobin-azide solution concentration was approximately 3.0 mM. The concentration of azide in the protein solution was 0.5 M. An aliquot was removed and added to a quartz EPR tube.

Anaerobic Studies of Hemin Chloride in IL 169

IL 169 was degassed by cycling between vacuum and nitrogen gas on a vacuum line (3 cycles). To degassed IL 169 (2 mL) was added sodium azide (0.1047 g, 1.61×10^{-3} mol). The solution was stirred overnight to allow dissolution. To a second aliquot of IL 169 (2 mL) was added hemin chloride (0.0086 g, 1.32×10^{-5} mol), which was also stirred overnight. Under a positive pressure of nitrogen gas the azide solution was added to the hemin chloride solution. The combined solution was stirred for 20 minutes under nitrogen before being cycled between nitrogen gas and vacuum to degas

the solution. The solution was stirred under nitrogen for 6 hours. An aliquot (0.5 mL) was removed and placed in a purged EPR tube and was immediately frozen in liquid nitrogen.

The following procedures were performed in an inert atmosphere glovebox. Hemin chloride powder was degassed under vacuum for 15 minutes. IL 169 was degassed using the freeze-pump-thaw method (4 cycles). To degassed hemin chloride (0.0094 g, 1.44×10^{-5} mol) was added degassed IL 169 (2.0 mL). Separately, to sodium azide (0.1063 g, 1.63×10^{-3} mol) was added degassed IL 169 (2.0 mL). The solutions were stirred overnight, then combined and stirred. A sample was removed and placed into an EPR tube and sealed with a septum. The sample was frozen in liquid nitrogen immediately after removal from the glovebox.

3.2.3 Oxidation of Dibenzothiophene by Myoglobin

Catalytic Oxidation of Dibenzothiophene in IL 169

Solutions of dibenzothiophene (DBT) in IL 169 (amounts according to Table 3.3) were prepared with stirring. One-half of the dibenzothiophene-ionic liquid solution was removed. To the ionic liquid solution was added buffer (10 mM acetate-phosphate, pH 5.2; amounts according to Table 3.3) bringing the total volume of the solutions to 4.75 mL. Hydrogen peroxide (5.7 μL, 10 mM) was added to the solution which was then stirred for 5 minutes to ensure adequate mixing. Separately, myoglobin (0.0168 g, 9.88×10^{-7} mol) was dissolved in 2.0 mL buffer (pH 5.2). An aliquot of the myoglobin solution (250 μL) was added to the ionic liquid-dibenzothiophene solution. Final volume was 5.0 mL, the final myoglobin concentration was 24.7 μM, and the final concentration of dibenzothiophene was 543 μM. The solutions were stirred in 3-dram vials in a 34 ^{o}C sand bath for 24 hours.

Table 3.3 Solution preparation for the oxidation of dibenzothiophene by myoglobin in IL 169.

% (v/v) Ionic Liquid	Volume IL 169 (mL)	Volume Buffer (mL)	Mass DBT (g)
10	1.0	8.5	0.0010
25	2.5	7.0	0.0010
50	5.0	4.5	0.0010
75	7.5	2.0	0.0010
90	9.0	0.5	0.0010

Extraction of the reactants and products was accomplished using a two-phase mixture in which the ionic liquid was mixed with distilled water (for amounts see Table 3.4) then extracted with dichloromethane (3 × 1 mL). The extracts were combined and the solvent removed under vacuum. For 10% to 50% IL 169, the residue was dissolved in ethyl acetate and purified through a silica gel column (1 cm × 8 cm) using ethyl acetate as the eluent to remove residual ionic liquid. Fractions that did not contain ionic liquid were combined. The residual ionic liquid from 75% (v/v) was removed using two columns. Extraction and column chromatography of the 90% IL 169 solution was unsuccessful because of inadequate separation of the ionic liquid from the desired compounds. The solvent was removed under vacuum and the solids were dissolved in 10 mL acetonitrile:water (1:1). Internal standard (9,10-dihydrophenanthrene, 40 μM) was added for HPLC measurements.

Table 3.4 Amounts of water for extraction of IL 169 reaction solutions.

% IL 169	Volume water (mL)
10	2.0
25	2.0
50	4.0
75	5.0
90	6.0

Control reactions were performed using the second-half of the prepared dibenzothiophene-ionic liquid solution. Reaction conditions were the same as previously described but in the absence of hydrogen peroxide.

Catalytic Oxidation of Dibenzothiophene by Myoglobin in IL 106

Solutions of dibenzothiophene (Table 3.5) with IL 106 and buffer (10 mM acetate-phosphate, pH 5.2) were prepared with stirring and warming of the solution to 34 ^{o}C. One-half of the dibenzothiophene-ionic liquid-buffer solution was removed. The reaction was performed as described above for IL 169. The final myoglobin concentration was 24.8 μM, and the final concentration of dibenzothiophene in each case was 597 μM.

Table 3.5 Solution preparation for the oxidation of dibenzothiophene by myoglobin in IL 106.

% (v/v) Ionic Liquid	Volume IL 106 (mL)	Volume Buffer (mL)	Mass DBT (g)
10	1.0	8.5	0.0011
25	2.5	7.0	0.0011
50	5.0	4.5	0.0011
75	7.5	2.0	0.0011
90	9.0	0.5	0.0011

To the reaction mixture was added distilled water (Table 3.6), which was then shaken to obtain a homogeneous solution. The aqueous reaction solution was extracted with a 1:1 mixture of ethyl acetate and hexanes (5 × 2 mL). The solvent was removed under vacuum and the residue was dissolved in 10 mL acetonitrile:water (1:1). 9,10-dihydrophenanthrene was added as an internal standard (40 μM) for HPLC measurements.

Table 3.6 Amounts of water for extraction of dibenzothiophene oxidation in solutions of IL 106.

% IL 106	Volume water added (mL)
10	0.0
25	0.0
50	2.0
75	5.0
90	6.0

Control reactions were performed in the absence of hydrogen peroxide using the second-half of the solutions used for the reactions.

HPLC Calibration Curves for Oxidation of Dibenzothiophene by Myoglobin

Reverse-phase HPLC was used to determine the extent of dibenzothiophene oxidation as described in Section 3.2. Typical retention times for dibenzothiophene sulfoxide (DBTO), dibenzothiophene sulfone ($DBTO_2$), and dibenzothiophene (DBT) were 2.43 min, 3.87 min, and 11.00 min respectively. 9,10-dihydrophenanthrene was selected as an internal standard because it is structurally similar to dibenzothiophene. The calibration lines for standard solutions of dibenzothiophene (DBT) (Figure 3.3), dibenzothiophene sulfoxide (DBTO) (Figure 3.4), and dibenzothiophene sulfone ($DBTO_2$) (Figure 3.5) in the concentration range of 0.40 μM to 200 μM were constructed using a 40 μM internal standard solution. The ratios plotted were calculated from either the height or area count of each standard versus that of the internal standard peak.

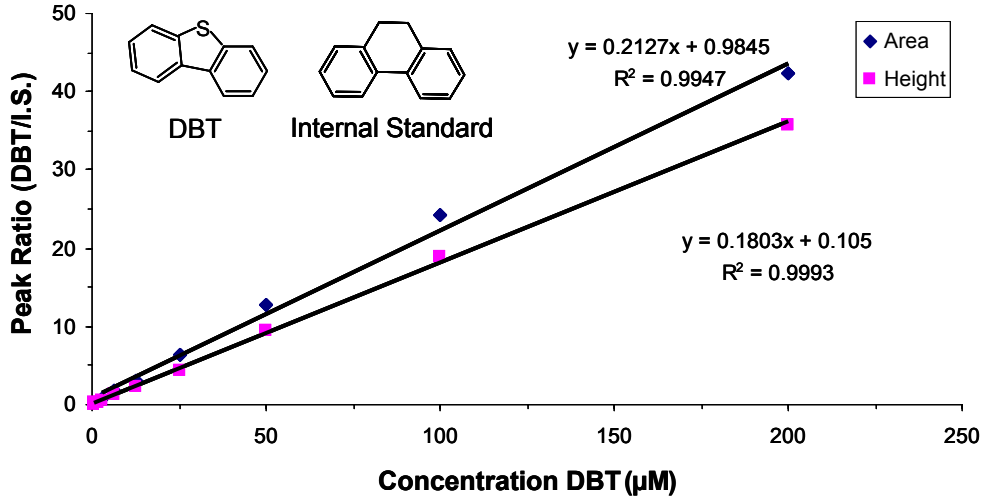


Figure 3.3 **Calibration line for HPLC of 0.40 µM to 200 µM dibenzothiophene (DBT) standard solutions with 40 µM 9,10-dihydrophenanthrene as internal standard.**

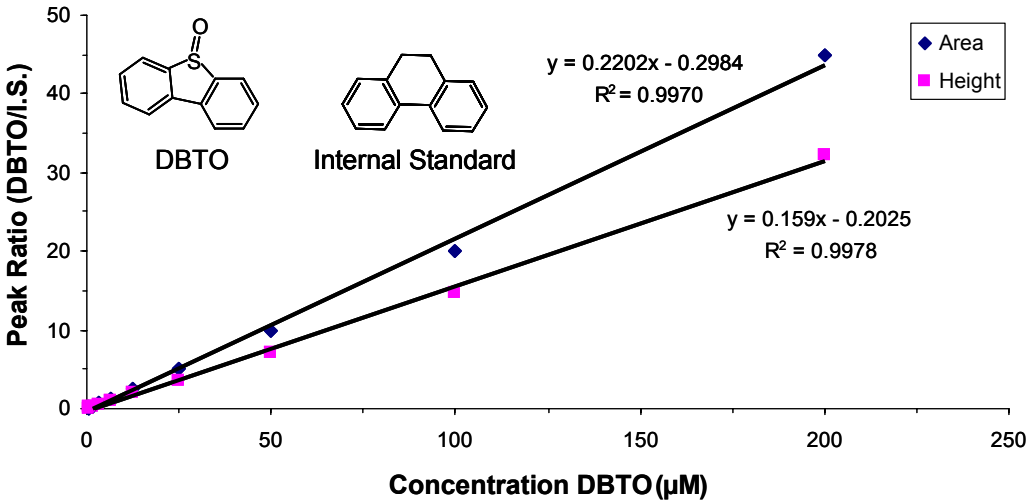


Figure 3.4 **Calibration line for HPLC of 0.40 µM to 200 µM dibenzothiophene sulfoxide (DBTO) standard solutions with 40 µM 9,10-dihydrophenanthrene as internal standard.**

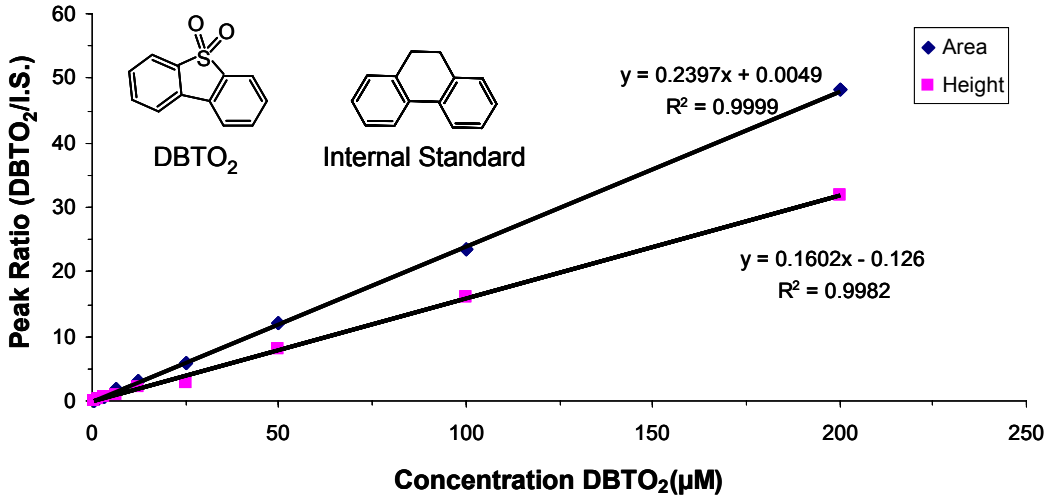


Figure 3.5 Calibration line for HPLC of 0.40 µM to 200 µM dibenzothiophene sulfone ($DBTO_2$) standard solutions with 40 µM 9,10-dihydrophenanthrene as internal standard.

In each case a linear relationship was found for the concentrations plotted for both height ratios and area ratios. Only the area ratios were considered in the subsequent experiments. A linear fit applied to the calibration line for dibenzothiophene gave the equation: $y = 0.2127x + 0.9845$ with an R^2 value of 0.9947. The calibrations of dibenzothiophene sulfoxide gave the equation: $y = 0.2202x – 0.2964$ with an R^2 value of 0.9970. Dibenzothiophene sulfone calibrations gave a linear fit of the equation: $y = 0.2397x + 0.0049$ and 0.9999 for R^2. These fits were used to calculate the amount of each compound in solution and subsequently the extent of dibenzothiophene conversion. The calibration line was used to more accurately determine the initial concentration of dibenzothiophene since the amount used was small and lead to uncertainties in the measured mass. In addition, extraction from the ionic liquids with organic solvent may affect the amount of dibenzothiophene detected in the HPLC. The initial amount of dibenzothiophene was calculated as the sum of each compound in solution: $[DBT]_o = [DBT] + [DBTO] + [DBTO_2]$. Conversion to dibenzothiophene sulfoxide

(DBTO) was calculated from the ratio $[DBTO]/[DBT]_o$. Total conversion represents the total amount of sulfoxide and sulfone produced compared to the initial amount of dibenzothiophene.

3.3 Results

3.3.1 UV-Visible and Fluorescence Studies of met-Myoglobin

One of the most useful techniques for analysing myoglobin in ionic liquids is UV-Visible absorption spectroscopy since it provides information about the environment and coordination of the heme. The spectrum of high-spin ferric myoglobin with a histidine coordinated in the proximal position and water in the sixth, distal position exhibits a sharp Soret band at 409 nm, a Q-band at 502 nm and a charge-transfer band at 630 nm in buffer solution.[106] The Soret band arises from allowed heme $\pi \rightarrow \pi^*$ transitions and is of much greater intensity than the forbidden $\pi \rightarrow \pi^*$ transition of the Q-band. The charge-transfer band is also of lower intensity and arises from the ligand-to-metal charge transfer. For the studies of myoglobin in ionic liquids, only the Soret band was used as it has the greatest intensity and, therefore, is most sensitive to changes in the coordination environment of the heme.

Myoglobin in Aqueous IL 169

The UV-Visible absorption spectra of 3.9 μM myoglobin in buffer (pH 7.0) show a marked change in the absorption in the Soret region (409 nm) with varying amounts of IL 169 (Figure 3.6). Native myoglobin in buffer with 0% (v/v) IL 169 exhibits a Soret band at 409 nm consistent with literature reports.[106] Addition of 10% (0.25 M) IL 169 resulted in almost complete loss of the Soret band absorption (shifted to 399 nm, Figure

3.6) coupled with the occurrence of a weak band at about 370 nm, however, the weakness of this band makes it difficult to determine whether it was initially present under the Soret band or whether it is a new band. At 25% (0.62 M) IL 169, the absorption of the Soret band is greater than with any other volume fraction of IL 169 and is slightly less blue shifted than 10% IL 169 with a maximum wavelength, λ_{max}, at 401 nm, but still within the uncertainty of the instrument. Intermediate between the strongest and weakest absorptions are 50% (1.25 M), 75% (1.88 M), and 90% (2.25 M) IL 169. Both 50% and 90% exhibit similar maximum absorptions at 402 nm and shoulders at approximately 370 nm, while the absorption for 75% appears to be less than 50% and 90% but greater than 10% (v/v) IL 169. The considerable loss of the Soret absorption and presence of the band near 370 nm upon addition of the ionic liquid are consistent with a loss of the heme. from the protein matrix.[107-109] Alternatively, it has been proposed that myoglobin may be unfolded but the heme remains coordinated to the proximal histidine residue.[110] Both cases describe incidences in which the protein was denatured, which is consistent with

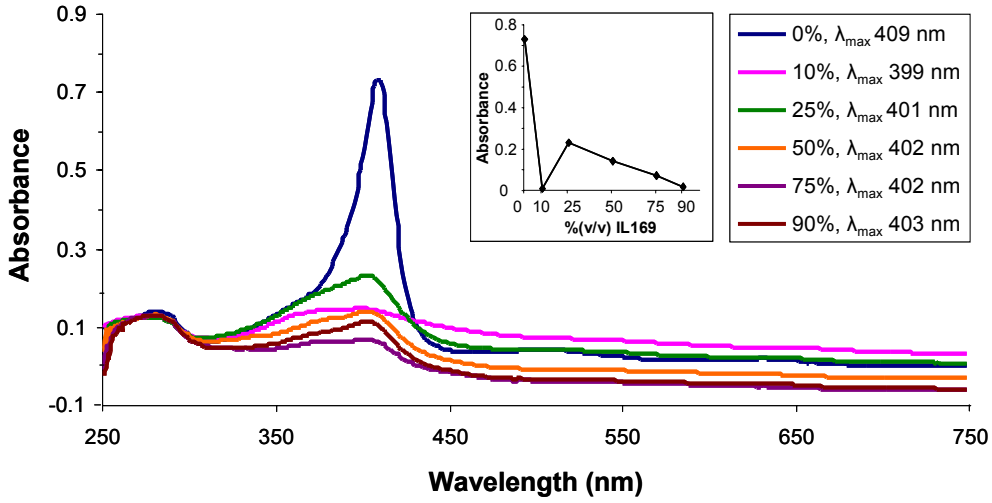


Figure 3.6 **UV-Vis spectra of 3.9 µM myoglobin in aqueous solutions of IL 169 (% v/v). (Inset) Absorbance versus concentration for peaks ~ 409 nm.**

the data presented here. Only for the case of 10% (v/v) IL 169 is the shoulder near 370 nm larger than the Soret band, indicating that at 10% IL 169, the greatest amount of heme is released from the protein

Myoglobin in Aqueous IL 106

Buffered solutions of myoglobin (4.3 μM) containing varying amounts of IL 106 (Figure 3.7) show different UV-Vis absorption spectra than for IL 169. The solutions containing 10% (0.27 M) IL 106 were cloudy, with the blank solution (buffer and IL 106) being cloudier than the myoglobin solution thus causing a negative reading in the measurement. With 25% (0.68 M) IL 106 the spectrum exhibits a broad absorption with the Soret maximum being blue shifted from that of myoglobin in buffer to a λ_{max} of 402 nm. The broadness of the absorption band indicates the presence of two species, native myoglobin and what appears to be free heme (from comparison to the λ_{max} of hemin chloride). The λ_{max} of the Soret bands for 50% (1.35 M), 75% (2.03 M), and 90% (2.43 M) are identical, 402 nm, 403 nm, and 403 nm respectively, and are exactly the same wavelength as observed for hemin chloride dissolved in IL 106 thus indicating that the Soret band at 403 nm is probably that of free heme. Increasing the amount of IL 106 resulted in greater heme loss as is apparent from the increase of the free heme Soret absorption with increasing ionic liquid concentration.

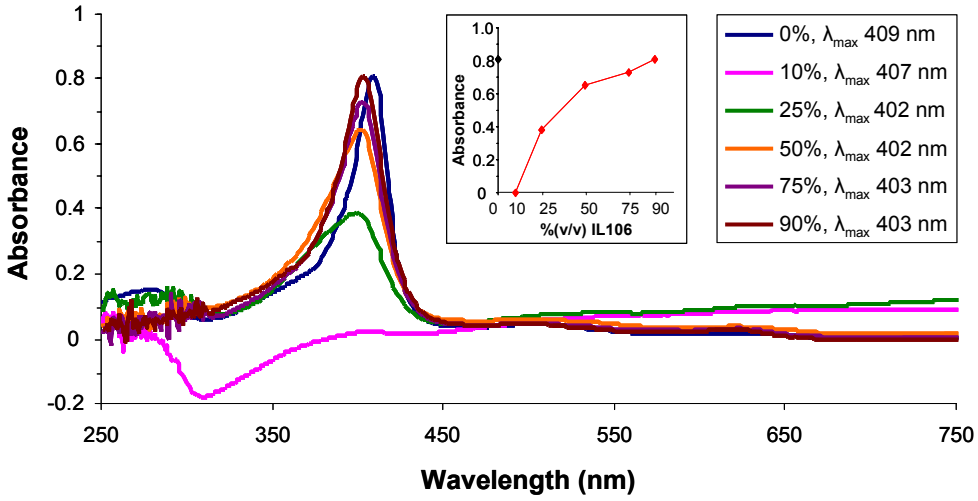


Figure 3.7 **UV-Vis spectra of 4.3 µM myoglobin in aqueous solutions of IL 106 (% v/v). (Inset) Absorbance versus concentration, black point: peak ~ 409 nm; red points: blue shifted peaks.**

Denaturation of Myoglobin in Solutions of Guanidine-Hydrochloride or Methanol

To provide a frame of reference, denaturing experiments were performed using guanidine-hydrochloride and methanol. Guanidine-hydrochloride (Gu-HCl) is known to cause protein denaturing by unfolding proteins when its concentration is greater than 2.0 M.[111] UV-Vis absorption spectra (Figure 3.8) show the effect of different concentrations of Gu-HCl on myoglobin in buffer (4.8 µM) consistent with literature reports.[111,112] Concentrations up to 1.0 M exhibit very little effect on the Soret band (λ_{max} 409 nm) of the protein. However at a concentration of 2.0 M Gu-HCl, complete loss of the protein Soret band is observed. These data indicate that for concentrations of 2.0 M and above, the protein is unfolded causing the heme to be released into solution where it is known to aggregate.[109] Aggregation of the heme causes its precipitation and consequently a decrease in the absorption of the Soret band, as can be seen in the UV-Vis spectrum (Figure 3.8).

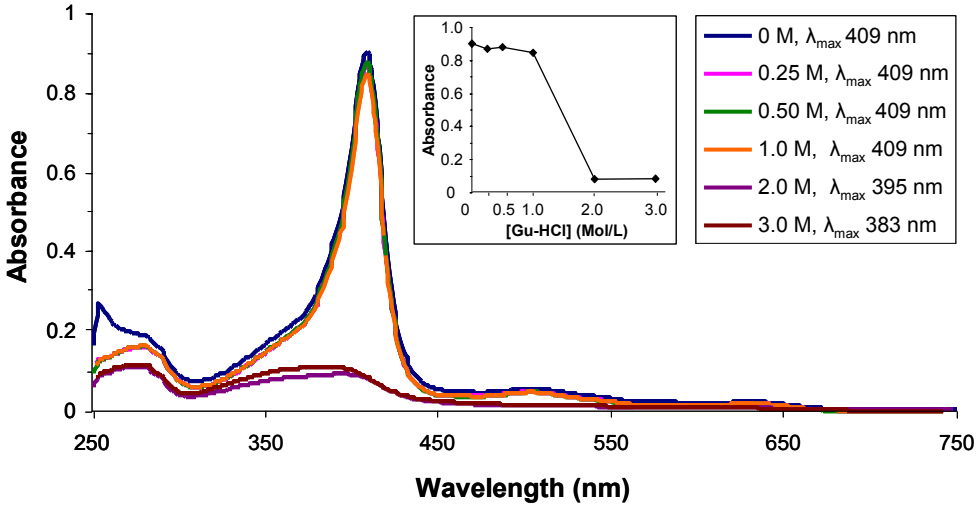


Figure 3.8 UV-Vis spectra of 4.8 µM myoglobin in aqueous solutions of Gu-HCl. (Inset) Absorbance versus concentration for peaks ~ 409 nm.

Alcohols are also known to denature proteins with concentration-dependent structural changes, although by a different mechanism from Gu-HCl. Differences in the UV-Vis spectra demonstrate the dissimilar effect each denaturant has on the protein. Figure 3.9 shows the effect of methanol on a 4.1 µM solution of myoglobin, which is consistent with literature reports.[107,108,113,114] As with Gu-HCl, lower concentrations of methanol cause no significant change in the myoglobin Soret band absorption. However at 50% (12.5 M) methanol a decrease in the Soret absorption (λ_{max} 409 nm, Figure 3.9) is observed indicating a decrease in the interaction between the heme and the protein.[107] With 75% and 90% methanol a decrease in the absorption is accompanied by a larger blue shift of the λ_{max} to 399 nm. Free hemin chloride exhibits a Soret band at 398 nm in methanol, indicating that the species observed for concentrations of methanol greater than 75% is free heme. Also evident in the spectra of solutions containing 50% to 90% methanol is a shoulder near 360 nm, a characteristic band observed for denatured heme proteins,[107-109] which is not present in the Gu-HCl denatured protein spectra.

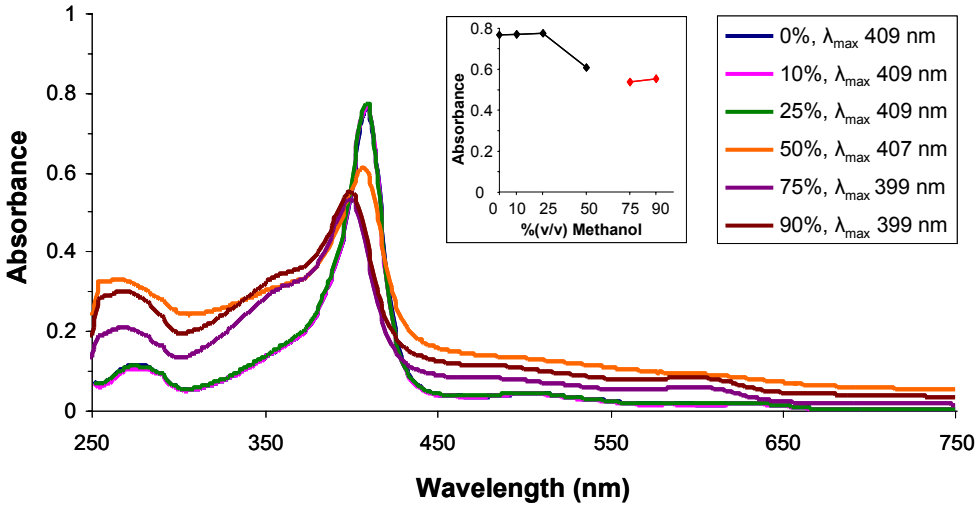


Figure 3.9 **UV-Vis spectra of 4.1 µM myoglobin in aqueous solutions of methanol (% v/v). (Inset) Absorbance versus concentration, black points: peak ~ 409 nm; red points: blue shifted peaks.**

Myoglobin in Neat Ionic Liquids

The UV-Visible spectra of myoglobin (2.7 µM) in neat ionic liquids show the distinct behaviour of the protein in each ionic liquid (Figure 3.10). The blue trace in each spectrum is from the buffer solution of myoglobin with the same concentration as in the ionic liquids after removal of the water from the ionic liquid-myoglobin solution. Different ionic liquids cause diverse shifts of the λ_{max} for the Soret band. For instance, the solution of myoglobin in IL 101 has a λ_{max} at 386 nm and a shoulder at approximately the same wavelength as the Soret band for the native protein in buffer solution. IL 105, on the other hand, caused a red shift in the Soret band to 419 nm. This is the only ionic liquid that caused the maximum to shift to a higher wavelength. There is also a significant decrease in the absorption of the λ_{max} possibly indicating decreased interaction between the heme and the protein. The spectrum for IL 106, (orange line, Figure 3.10) shows a large negative signal below 350 nm probably due to background subtraction

caused by cloudy solutions and a large Soret band at 404 nm; as previously mentioned, this band is consistent with free heme in IL 106. Myoglobin in IL 109 produced the least

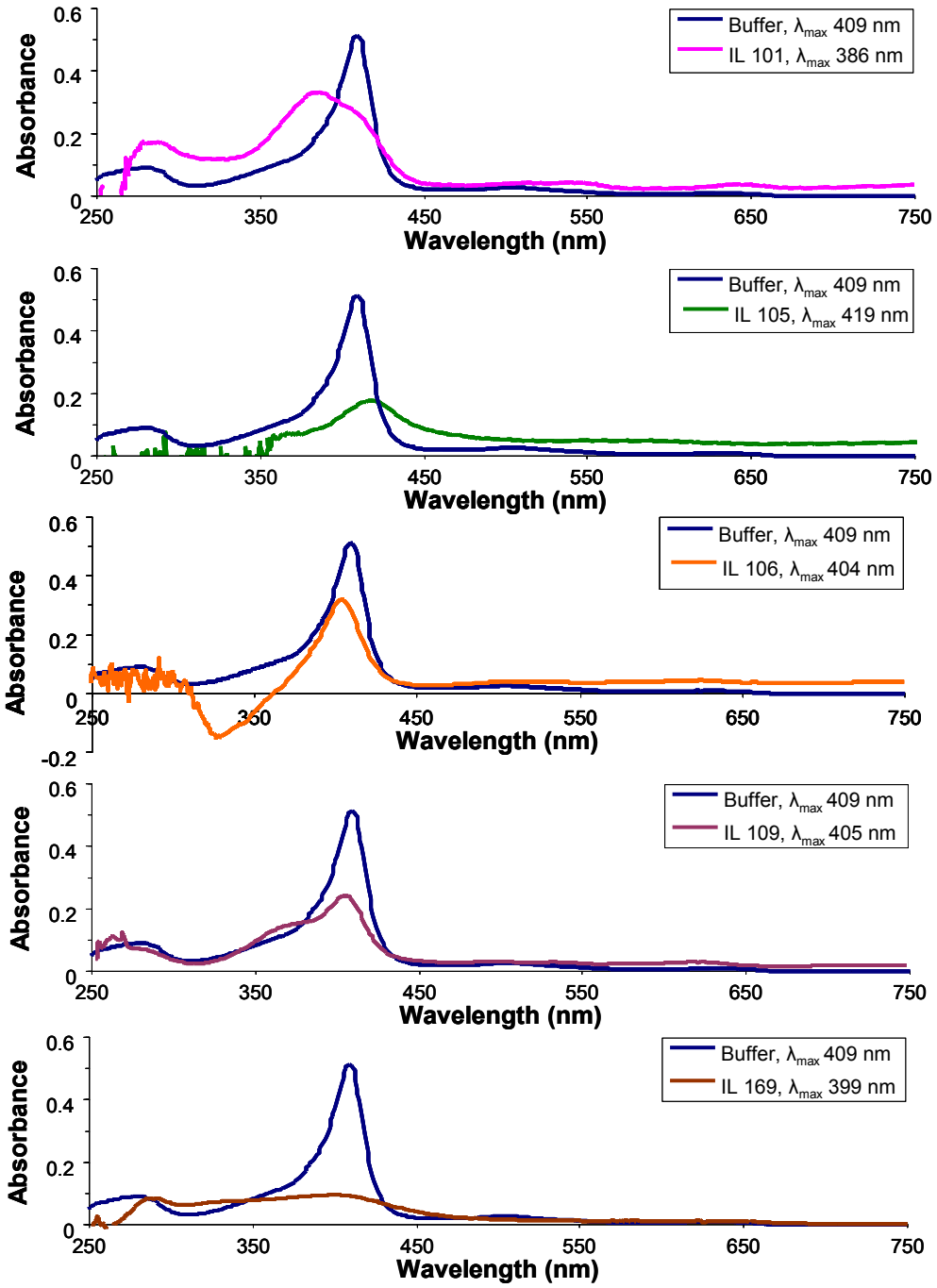


Figure 3.10 UV-Vis spectra of 2.7 µM myoglobin in neat phosphonium ionic liquids: IL 101, IL 105, IL 106, IL 109 and IL 169.

blue shifted Soret band at 405 nm and exhibits a strong second absorption visible as a shoulder at about 370 nm, caused by loss of the heme. Demonstrating the most significant suppression of the Soret band is IL 169 in which the absorption is very small and is shifted to 399 nm in neat ionic liquid.

The UV-Vis spectra also give information on the usefulness of the ionic liquids as media for absorption spectroscopy. A good solvent for UV-Visible measurements should not absorb in the region that the measurement is to take place. The spectrum containing IL 101 shows that it has a cut-off at about 270 nm, whereas IL 105 cannot be usefully measured below approximately 360 nm. IL 106 has a cut-off near 310 nm while IL 109 and IL 169 appear to have the largest windows for UV-Vis measurements.

Fluorescence Studies of Myoglobin

Myoglobin contains two tryptophan residues which are folded into the hydrophobic pocket of the protein and are not available for interaction with solvent molecules in the native state. Native myoglobin in buffer solution demonstrates only a very weak fluorescence emission (λ_{max} 326 nm). The low intensity of this peak is due to the quenching of the tryptophan fluorescence by the near-by porphyrin ring. Upon unfolding of the protein the tryptophan residues move away from the porphyrin ring yielding a stronger fluorescence emission as well as a red shift in the maximum fluorescence intensity due to exposure of the tryptophans to polar solvent molecules.[111] Fluorescence measurements were conducted on the same samples as those used for UV-Vis analyses. Optimal instrumental conditions were determined for each experiment. Tryptophan fluoresence at 25 ^{o}C (± 0.2 ^{o}C) was measured for each set of solutions. Uncertainties in the measurements were within ± 1 nm. Native myoglobin in buffer was

used for comparison in each set of measurements since the concentration of the protein varied between sets of solutions.

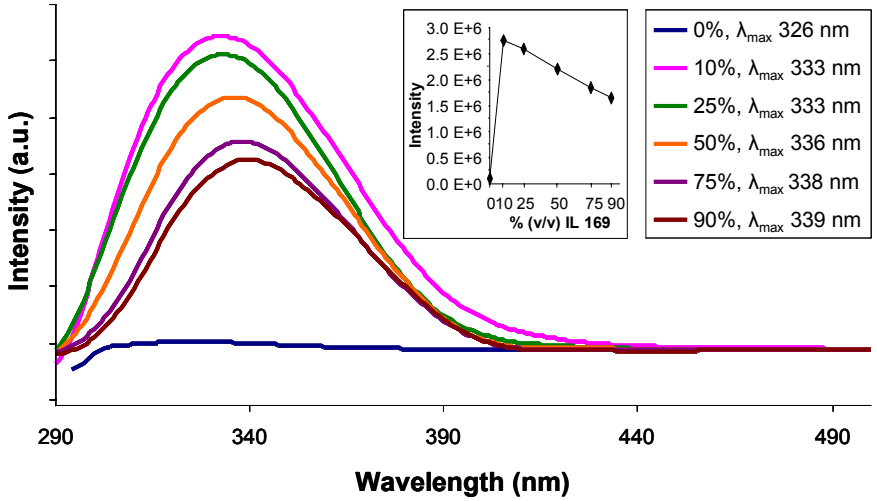


Figure 3.11 Fluorescence emission spectra of 3.9 µM myoglobin in aqueous solutions of IL 169 (% v/v). (Inset) Intensity versus concentration.

The fluorescence spectra of myoglobin with IL 169 indicate that this ionic liquid causes significant unfolding of the protein (Figure 3.11). At 10% (v/v) IL 169, the fluorescence intensity is the greatest and a red shift of 7 nm from the native myoglobin maximum is observed (λ_{max} 333 nm). The largest emission intensity indicates that 10% IL 169 causes the most unfolding of myoglobin. Increasing the amount of ionic liquid in solution to 25% produced a decrease in the fluorescence intensity but no significant shift to λ_{max} (333 nm). With 50% of the solution volume being IL 169, the intensity decreased further and a shift in λ_{max} to 336 nm was observed. The fluorescence intensity was even less for 75% (λ_{max} 338 nm) and 90% (λ_{max} 339 nm) IL 169 together with an additional red shift of λ_{max}.

Fluorescence measurements were performed for the solutions containing varying amounts of IL 106, however it was determined that IL 106 is not a useful medium for

fluorescence measurements. The anion of IL 106 contains an aromatic ring which is believed to cause quenching of the tryptophan fluorescence by energy transfer.[115] This conclusion is based on the negative fluorescence peaks observed when the background solution spectrum is subtracted from the protein solution spectrum. All fluorescence intensities were less than native myoglobin fluorescence.

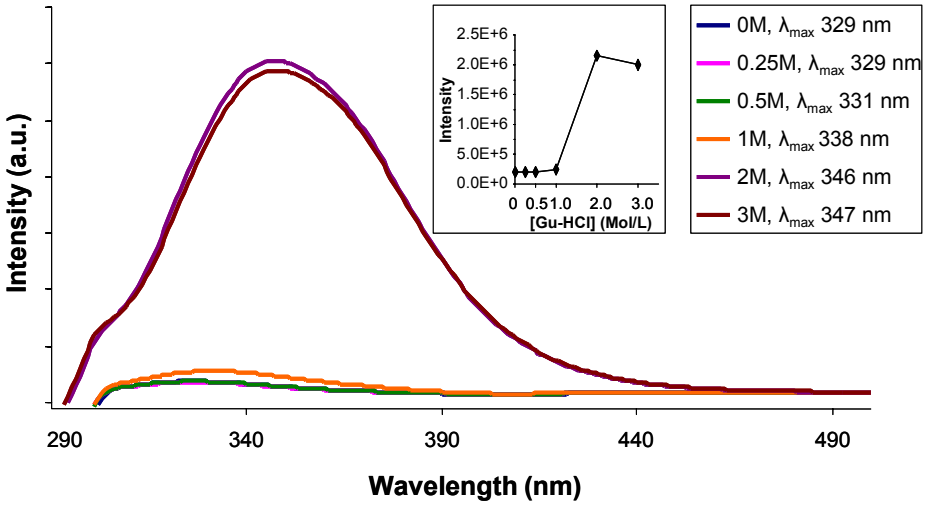


Figure 3.12 Fluorescence emission spectra of 4.8 µM myoglobin in aqueous solutions of Gu-HCl. (Inset) Intensity versus concentration.

The fluorescence of solutions of guanidine-hydrochloride (Gu-HCl) with myoglobin were also measured (Figure 3.12) and are consistent with literature reports.[111] Concentrations of up to 0.50 M Gu-HCl caused no increase in the fluorescence intensity and the spectra overlap almost exactly with the native myoglobin spectrum demonstrating no significant unfolding of myoglobin. Addition of 1.0 M Gu-HCl to a solution of myoglobin caused a very small increase in the intensity as well as a red shift of the emission peak by 5 nm (λ_{max} 331 nm). At higher concentrations, 2.0 M and 3.0 M, the protein appears to be significantly unfolded as the intensity of the fluorescence is considerably greater and the λ_{max} for both solutions is 348 nm, a red shift of 12 nm from

that of native myoglobin (λ_{max} 327 nm). These data clearly indicate that for concentrations of Gu-HCl greater than 2.0 M the protein is completely unfolded.

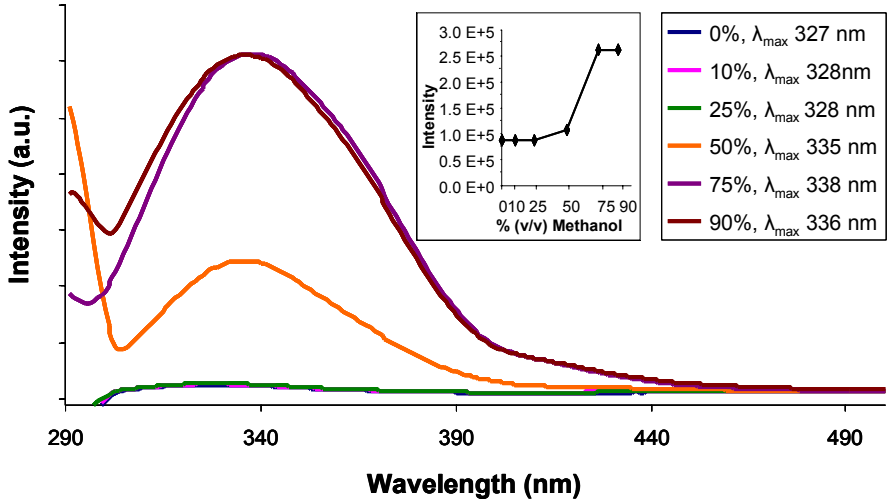


Figure 3.13 Fluorescence emission spectra of 4.1 µM myoglobin in aqueous solutions of methanol (% v/v). (Inset) Intensity versus concentration.

The fluorescence measurements of myoglobin with varying amounts of methanol (Figure 3.13) indicate that up to 25% (v/v) methanol is tolerated without significant structural change of the protein, consistent with literature reports.[107,108] The intensities and λ_{max} for both 10% and 25% are equivalent to that of native myoglobin in buffer. At 50% (v/v) methanol, the protein appears to be partially unfolded, with a shift in λ_{max} to 335 nm. Both 75% and 90% methanol produced similar fluorescence intensities and λ_{max} values of 338 nm and 336 nm respectively.

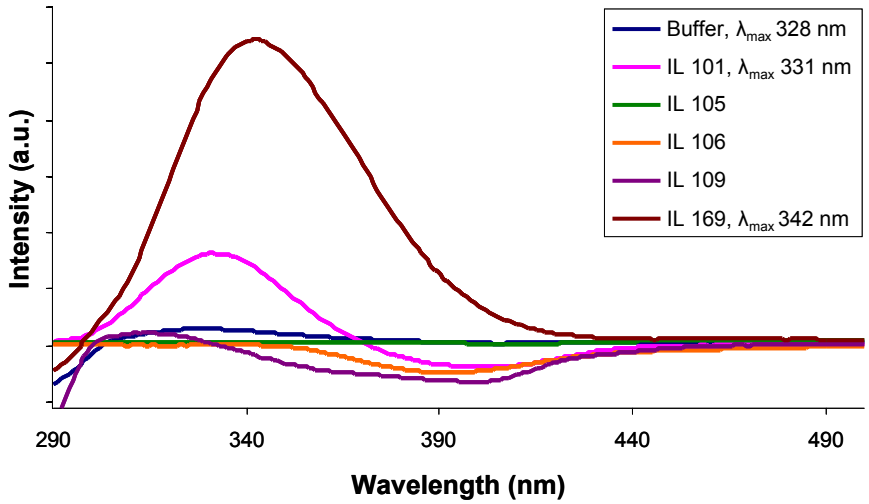


Figure 3.14 Fluorescence emission spectra of 2.7 μM myoglobin in neat phosphonium ionic liquids: IL 101, IL 105, IL 106, IL 109 and IL 169.

Tryptophan fluorescence was measured for myoglobin in neat ionic liquids (Figure 3.14). The blue line is the reference solution of native myoglobin in buffer. Unlike in the previously described fluorescence measurements, where the effect of different amounts of ionic liquid in aqueous solution were compared, Figure 3.14 is a comparison of the effect of different ionic liquids on the tryptophan fluorescence of myoglobin. The steady-state fluorescence of myoglobin in IL 169 exhibits the greatest fluorescence intensity as well as the largest red shift of the λ_{max} to 342 nm. Myoglobin in IL 101 produced the next largest fluorescence emission, but a much smaller shift in λ_{max} (to 331 nm) than IL 169. Fluorescence measurements of solutions of IL 105, IL 106 and IL 109 produced spectra in which the emission was less than the native protein in buffer. Furthermore, λ_{max} could not be determined for myoglobin in these ionic liquids. Just as IL 106 in aqueous solution may be quenching the tryptophan fluorescence, these three ionic liquids prove to be poor media for fluorescence measurements, with the anions possibly also quenching the tryptophan fluorescence.[116]

3.3.2 EPR Experiments in Phosphonium Ionic Liquids

EPR of Myoglobin-azide in Phosphonium Ionic Liquids

The top spectrum in Figure 3.15 shows the EPR of a solution of myoglobin-azide (MbN_3) in phosphate buffer (pH 7.0), with 20% glycerol as a glassing agent, at 77 K. A glassing agent is used to prevent the formation of long-range crystalline order in the sample which contributes to line broadening through increased relaxation rates. The spectrum of MbN_3 is characterised by a rhombic spectrum typical for low-spin Fe(III) with measured g-values g_1, g_2, and g_3 being 2.8, 2.2, and 1.7 (literature values 2.80, 2.22, 1.72).[117] Several portions of the original MbN_3 solution in buffer were stirred into phosphonium ionic liquids IL 101, IL 105, IL 106, IL 109 and IL 169 and the water was removed under vacuum. The spectrum of MbN_3 changes to give not only the low-spin azide species but also a second, axial high-spin Fe(III) species with $g_\perp = 5.8$, in varying amounts in the phosphonium ionic liquids. The $g_\parallel$ for high-spin Fe(III) species is typically at approximately g = 2.0. However, in this case it is of lesser intensity[118] and is obscured by the low-spin spectrum. IL 101, IL 105 and IL 109 appear to have similar amounts of the high-spin and low-spin species. In contrast, IL 106 exhibits only a high-spin signal while IL 169 has both high- and low-spin species as well as a significant amount of a new species near g = 2 not present in any other ionic liquid. Since measurements were made with the more sensitive EMX-plus spectrometer with a high-sensitivity resonator (Bruker ER 4102ST), the solution of MbN_3 in IL 101 was of a lower concentration. The sharp signal at g = 2 in many of the spectra (marked with *) is due to a background radical species in the finger dewar.

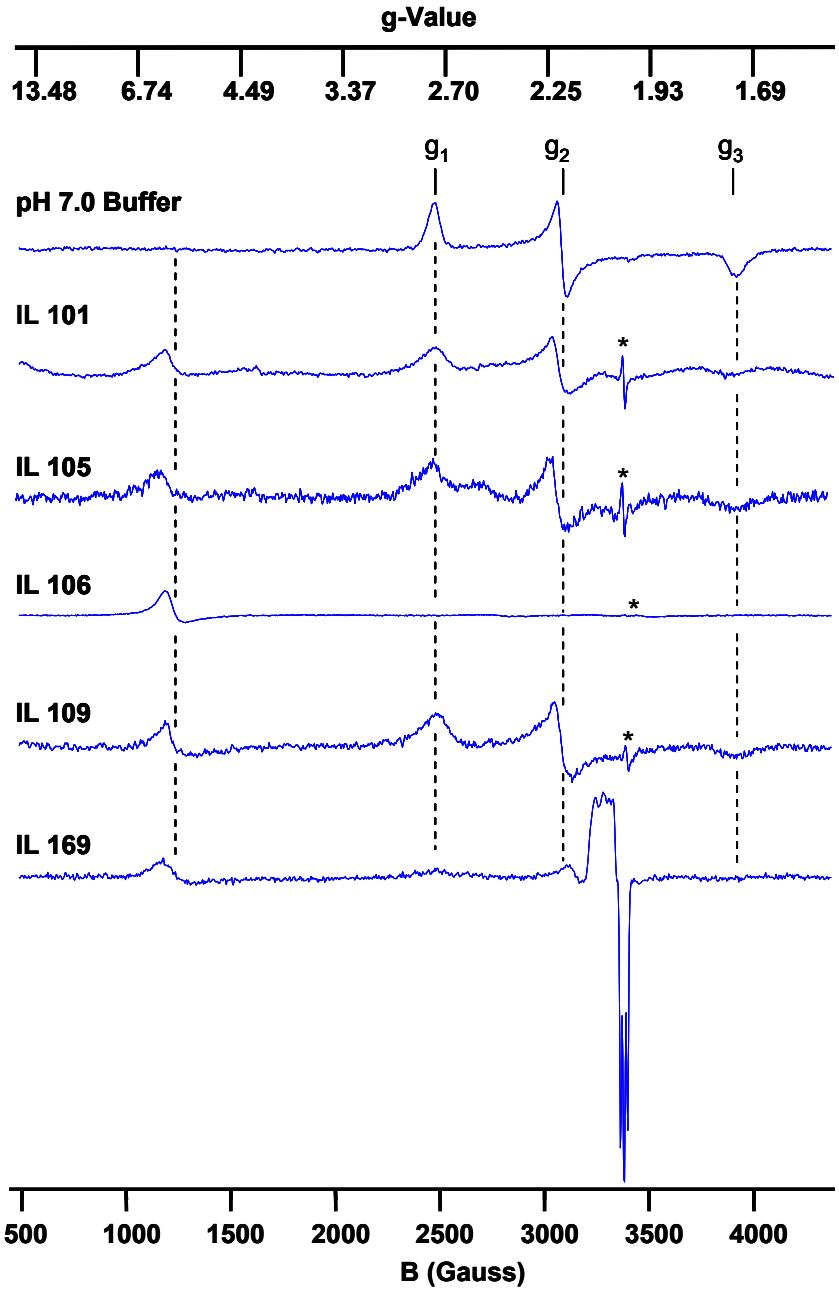


Figure 3.15 X-Band EPR Spectra of 1.4 mM MbN_3 (1.1 mM MbN_3 in IL 101) in buffer and phosphonium ionic liquids at 77 K, all spectra are scaled to the high-spin component. (*Experimental parameters*) Microwave frequency = 9.57 GHz, modulation amplitude = 8 G, microwave power = 0.64 mW, receiver gain = 2×10^4, time constant = 20.48 (IL105, IL 106) 40.96 ms, scan time = 40.96 s (buffer), 83.89 s (IL 101, IL 105, IL 106, IL 109, IL 169), average of 1 scan (6 scans, IL 105).

Formation of Nitric Oxide Species in IL 169

Unexpectedly, during the course of these experiments MbN_3 in IL 169 was found to produce a significant amount of a new species near g = 2. Although this is not directly related to the initial experiments of studying the denaturation of myoglobin in ionic liquids, it is an interesting example of myoglobin reactivity in phosphonium ionic liquids.

The EPR spectrum of MbN_3 in IL 169 exhibits three paramagnetic species (Figure 3.16). A high-spin species at g = 5.8 is present as well as a small amount of the low-spin azide species with g-values in agreement with those in both the buffer solution as well as the other ionic liquids. The inset focuses on the third species present at around g = 2. The distinct three-line pattern is evidence of coupling to a single $I = 1$ nucleus, such as one nitrogen (^{14}N, $I = 1$, 99.64% abundance) nucleus. Surprisingly, this spectrum is indicative of a 5-coordinate NO-myoglobin (MbNO) species with a nitrogen hyperfine coupling constant a_N (^{14}N) = 16.8 G and is very similar to 5-coordinate MbNO[119] and hemoglobin-NO[120] EPR spectra reported in the literature.

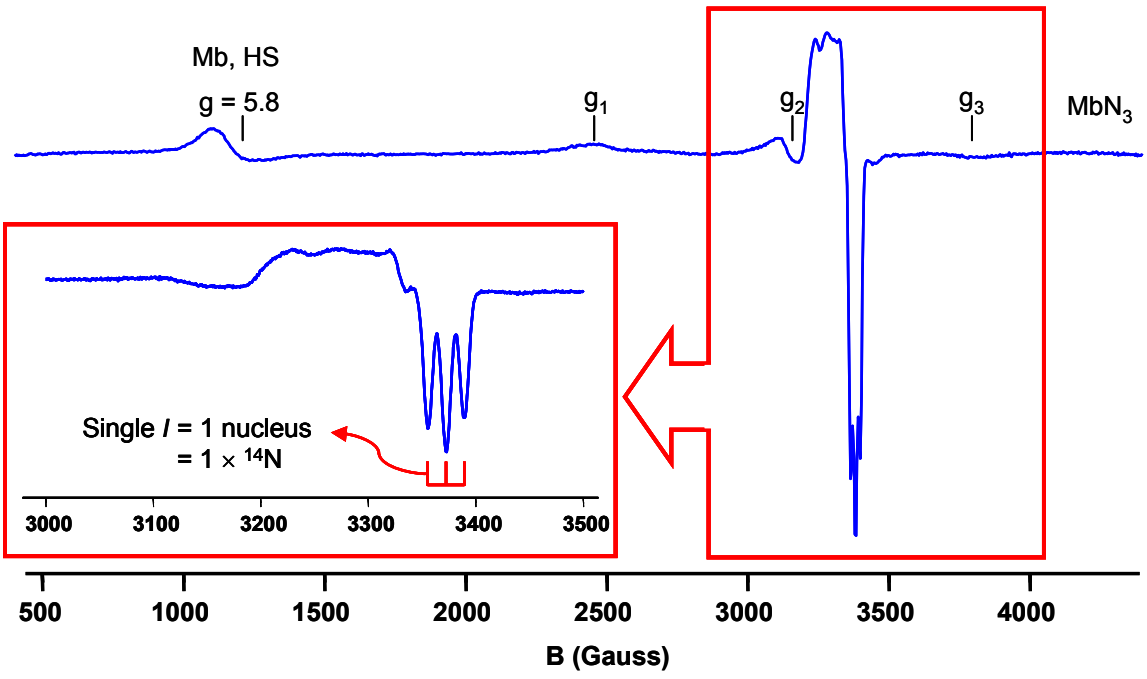


Figure 3.16 **X-Band EPR Spectrum of 1.4 mM MbN_3 in IL 169 at 77 K showing three species; (Inset) Expanded g = 2 region showing new species with three-line coupling pattern. (*Experimental parameters*) Microwave frequency = 9.56 GHz, modulation amplitude = 12 G, microwave power = 0.64 mW, receiver gain = 2×10^4, time constant = 81.92 ms, scan time = 83.89 s, average of 5 scans. (*Inset*): Microwave frequency = 9.56 GHz, modulation amplitude = 8 G, microwave power = 0.64 mW, receiver gain = 2×10^4, time constant = 81.92 ms, scan time = 83.89 s, average of 6 scans.**

To investigate whether the species observed in IL 169 was indeed MbNO, the species was generated directly in buffer by reacting met-myoglobin with nitric oxide gas. When nitric oxide gas was bubbled through a solution of met-myoglobin in buffer (0.1 M phosphate, pH 6.5), spectrum (A) in Figure 3.17 was produced. The three g-values (2.098, 2.070, 2.009) and nitrogen hyperfine coupling constant (a_N = 16.6 G) are consistent with literature values for heme protein - nitric oxide complexes.[119,120] Addition of the solution containing MbNO to IL 169 and removal of the water produced spectrum (C) of Figure 3.17. Comparing spectrum (C) of MbNO in IL 169 with spectrum (B), MbN_3 in IL 169, the g-values, hyperfine coupling constant, and overall shape of the EPR

spectrum are almost indistinguishable. Therefore the third species in solution can be assigned definitively as MbNO generated from MbN_3.

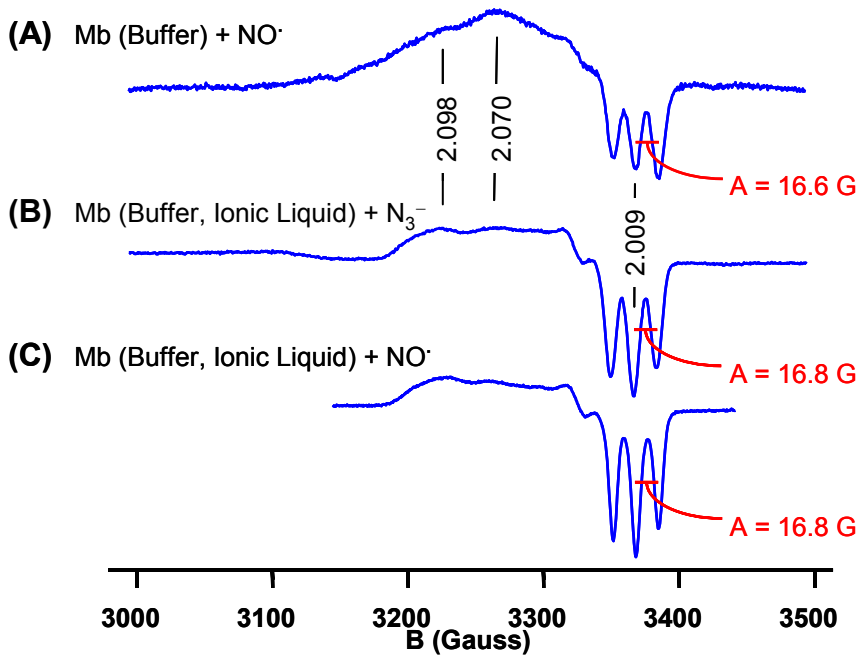
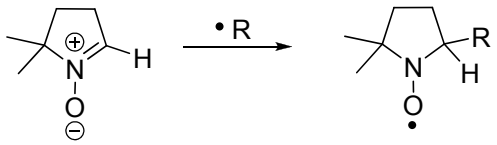


Figure 3.17 **X-Band EPR spectra comparing MbNO with MbN_3-generated species at 77 K. (A) MbNO in buffer generated directly by bubbling with NO gas; (B) MbN_3 originally prepared in buffer, added to IL 169, with water removed under vacuum; (C) MbNO in IL 169, MbNO in buffer added to IL 169, water was removed under vacuum. (*Experimental parameters*) (A) Microwave frequency = 9.57 GHz, modulation amplitude = 8 G, microwave power = 0.64 mW, receiver gain = 2×10^4, time constant = 81.92 ms, scan time = 83.89 s, average of 11 scans. (B) Microwave frequency = 9.56 GHz, modulation amplitude = 8 G, microwave power = 0.64 mW, receiver gain = 2×10^4, time constant = 81.92 ms, scan time = 83.89 s, average of 6 scans. (C) Microwave frequency = 9.58 GHz, modulation amplitude = 4 G, microwave power = 0.64 mW, receiver gain = 2×10^4, time constant = 40.96 ms, scan time = 83.89 s, average of 1 scan.**

DMPO Spin Trapping in IL169

Spin trapping involves the addition of a transient radical species to a diamagnetic compound to form a more stable paramagnetic species, which can then be characterised by EPR spectroscopy. The spin trap, usually a nitrone, can give information about the nature of a radical species via hyperfine interactions with adjacent nuclei. In the case of

5,5-dimethylpyrroline-N-oxide (DMPO), a radical (·R) adds to the unsaturated carbon to form a stable nitroxyl radical (Scheme 3.1).



Scheme 3.1 DMPO spin trapping of radical (·R) to form a stable spin-adduct.

The unpaired electron is delocalised over the nitrogen and oxygen atoms of the nitroxyl radical leading to significant hyperfine interactions with the nitrogen centre (^{14}N, I = 1) as well as the β hydrogen. In cases where the radical (·R) contains spin-bearing nuclei (*e.g.* ^{14}N, I = 1; ^{1}H, I = 1/2), hyperfine splitting can be seen from interactions between the unpaired electron and these nuclei. Hyperfine coupling constants for many known spin-adducts have been tabulated in literature[121] as well as an online database[122] and are readily available for comparison to establish the identity of the trapped radical.

After an extensive review of the literature, it is apparent that DMPO spin trapping experiments, especially for oxygen containing radicals, have several limitations that have led to conflicting reports.[121-125] There are many claims of the same hyperfine coupling constants for different radical adducts[121,122] making the assignment of these adducts ambiguous, especially when comparing adducts under different experimental conditions. Several DMPO spin-adducts have been shown to be unstable and undergo subsequent reactions after the initial trapping has occurred. Finkelstein *et al.*[123] have demonstrated that the reaction between DMPO and superoxide ($O_2\cdot^-$) results not only in the superoxide adduct but also subsequent decomposition to produce the hydroxyl adduct. It has also been proposed that peroxyl radical adducts of DMPO are highly unstable and rapidly

decompose at room temperature to give alkoxyl radical adducts in aqueous media.[124] The recent work of Mason and coworkers has shown through ^{17}O-enrichment[126] and mass spectrometry[127] experiments that the spin-adducts previously assigned to be alkyl peroxyl radical adducts were in fact alkoxyl radical adducts. Additionally, an adduct may form with a non-radical species which can undergo a successive reaction to form the stable nitroxyl radical, such as the reaction of DMPO, methanol, and a ferric salt to produce the DMPO-OH radical adduct.[128] These limitations often make the definite assignment of a trapped radical species difficult. The potential for ionic liquids to stabilise some unstable species also complicates the identification of a DMPO spin-adduct since it has been shown that phosphonium ionic liquids can mediate different reaction pathways, as compared to organic solvents, which may lead to stabilisation of usually unstable spin-adducts.[8]

Figure 3.18 shows the room-temperature EPR spectra of spin trapping experiments in IL 169 using DMPO. A paramagnetic species was produced when a spin-adduct was formed with DMPO, generating a 6-line spectrum. In the absence of the spin trap no EPR spectrum was observed (not shown). Spectrum (A) in Figure 3.18 was obtained after incubating a high concentration of the DMPO spin trap (56 mM) in neat IL 169. Incubation of DMPO in IL 169 produced a very weak paramagnetic DMPO spin-adduct spectrum in the g = 2 region. When sodium azide (0.7 M) was added to an aliquot of the IL 169-DMPO solution a more intense spectrum was produced (spectrum (B)). The increase in signal intensity observed for sodium azide in IL 169 with DMPO indicates a higher concentration of spin-adduct and consequently a higher concentration of radical species in solution. Addition of aqueous myoglobin-azide (final concentration 3.0 mM)

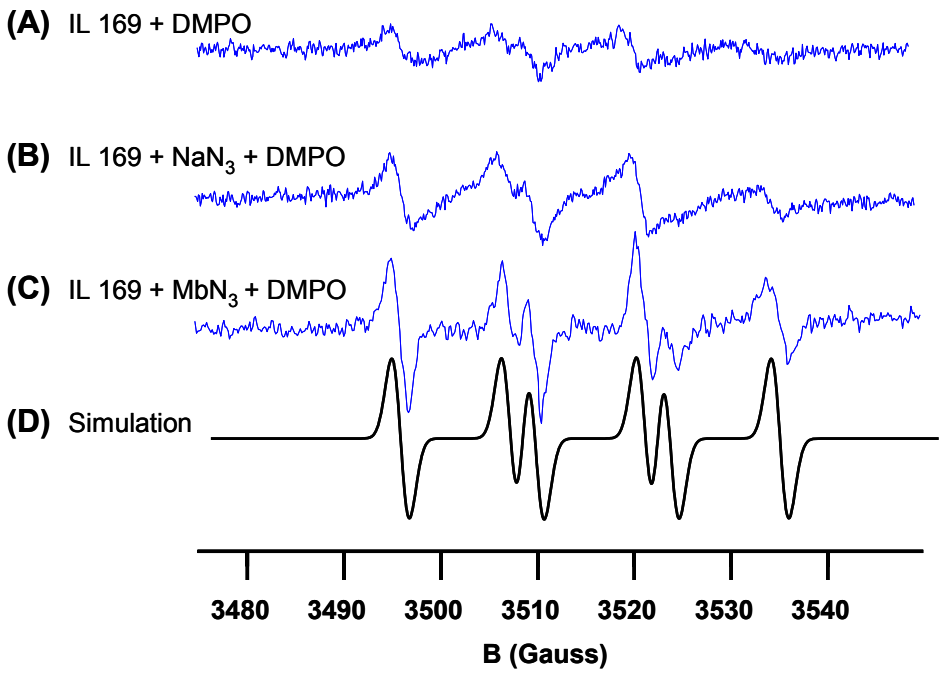


Figure 3.18 **X-Band EPR spectra of DMPO spin trapping experiments in IL 169 at room temperature. (A) IL 169 with excess DMPO (56 mM); (B) Solution from (A) with (0.7 M) NaN_3 added; (C) Solution from (A) with aqueous (3.0 mM) MbN_3 added, water removed under vacuum; (D) Computer simulation of DMPO spin-adducts. (*Experimental parameters*) (A) and (B) Microwave frequency = 9.869 GHz, modulation amplitude = 2 G, microwave power = 2.0 mW, receiver gain = 1×10^6, time constant = 327.68 ms, scan time = 368.64 s, average of 10 scans. (C) Microwave frequency = 9.869 GHz, modulation amplitude = 2 G, microwave power = 2.0 mW, receiver gain = 1×10^6, time constant = 163.84 ms, scan time = 184.32, average of 10 scans. (*Simulation parameters*) a_H = 11.3 G, a_N = 13.9 G, linewidth = 1.82 G.**

to the IL 169-DMPO solution resulted in spectrum (C). The spectrum of the myoglobin-azide solution in IL 169 appears to exhibit a greater concentration of the radical species than either the neat ionic liquid or sodium azide in IL 169. It is evident from the spectra that a similar paramagnetic species is present in all three ionic liquid solutions and the concentration of the species increased in the presence of sodium azide or myoglobin-azide. The experimental spectra all display some asymmetry due to the relatively high viscosity of the ionic liquid.[129-131] The concentration of the spin trap remained constant in each experiment. Myoglobin-azide was introduced into the ionic liquid in aqueous

solution; however, the sample was evacuated for the same period of time as previously described to ensure complete removal of water, producing the same concentration of DMPO as the initial ionic liquid solution.

The experimental spectra were simulated using the parameters a_H = 11.3 G, a_N = 13.9 G and a linewidth of 1.82 G (spectrum (D), Figure 3.18). The overall shape of the simulated spectrum does not exactly reflect the shape of the experimental spectra because of solvent viscosity effects from the ionic liquid, which caused asymmetry in the spectra, as mentioned above.[129-131] From the simulation parameters the trapped radical species can be assigned to a peroxyl radical, ·OOX, or a superoxide radical, $\cdot O_2^-$. The measured hyperfine coupling constants are consistent with literature constants for the hydroperoxyl radical adduct (DMPO-OOH) (a_H = 11.7 G, a_N = 14.1 G in water;[132] and a_H = 11.7 G, a_N = 14.1 G in ethanol[133]), the ethylperoxyl radical adduct (DMPO-OOC_2H_5) (a_H = 11.0 G, a_N = 14.6 G in water[134]) and the superoxide adduct (DMPO-OO) (a_H = 11.3 G, a_N = 13.8 G;[135] a_H = 11.4 G, a_N = 14.1 G in water;[136,137] and a_H = 10.9 G, a_N = 13.6 G in ethylene glycol[137]). Hyperfine splitting can be affected by the solvent in which the spin-adduct is formed. For example, DMPO-OOH has been reported to produce hyperfine coupling constants ranging from a_H = 11.7 G, a_N = 14.3 G in aqueous media to a_H = 9.8 G, a_N = 12.9 G in heptane.[121] Therefore the coupling constants measured in ionic liquid may not exactly coincide with literature values. The identity of the substituent (X) on the peroxyl radical cannot be ascertained from the spin trapping experiments since hyperfine interactions with nuclei adjacent to the peroxyl oxygens are too small to observe.[129-131]

Hemin Chloride Studies

The EPR spectrum of hemin chloride in IL 169 displays a strong axial high-spin Fe(III) spectrum at $g_{\perp} = 5.9$ (Figure 3.19). Addition of azide to hemin chloride in IL 169 produces three paramagnetic species, much the same as MbN_3 in IL 169. A high-spin species at $g_{\perp} = 5.8$ is present as well as the low-spin azide species with g-values (2.8, 2.2, 1.8) essentially equivalent to those of MbN_3 in both the buffer solution as well as the other ionic liquids (2.8, 2.2, 1.7). The inset in Figure 3.19 focuses on the hemin-NO species present at around g = 2 with a nitrogen (^{14}N) hyperfine coupling constant equal to 16.7 G. These results demonstrate that the MbNO and hemin-NO spectra cannot be distinguished under these experimental conditions. Therefore, it appears that the nitric oxide spectra are consistent with a heme-like nitric oxide (heme-NO) species. The relative amounts of the species in solution are different for hemin chloride-azide than for myoglobin-azide. Hemin chloride dissolved in the other ionic liquids exhibited identical axial high-spin Fe(III) spectra (not shown) to that in IL 169. Sodium azide was not soluble in IL 101, IL 105, IL 106, or IL 109; hence a spectrum of hemin chloride-azide was not obtained for these ionic liquids. In addition, hemin chloride was not soluble in buffer solution therefore comparison of hemin chloride-azide in IL 169 to that in buffer was not possible.

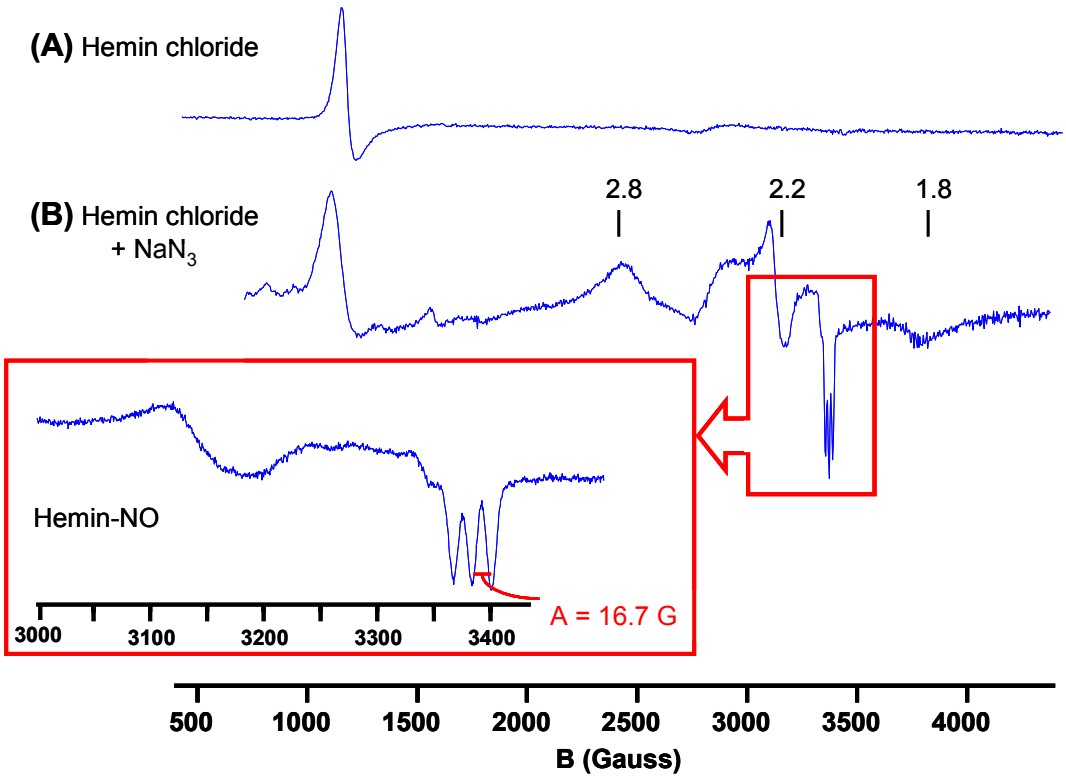
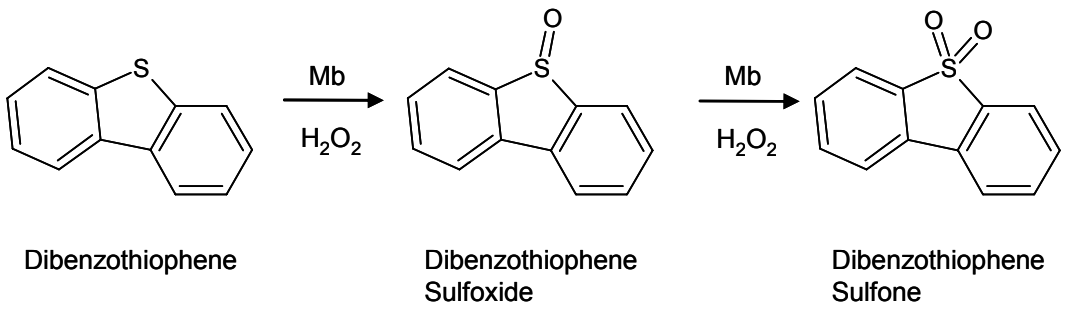


Figure 3.19 **X-band EPR of hemin chloride and hemin chloride with sodium azide in IL 169 at 77 K. (A) Hemin chloride in IL 169; (B) Hemin chloride with sodium azide in IL 169; (Inset) g = 2 region of hemin chloride with sodium azide spectrum showing hemin-NO. (*Experimental parameters*) (A) Microwave frequency = 9.60 GHz, modulation amplitude = 8 G, microwave power= 0.64 mW, receiver gain = 2 × 10^4, time constant = 20.48 ms, scan time = 41.94 s, 1 scan. (B) Microwave frequency = 9.57 GHz, modulation amplitude = 8 G, microwave power = 20.2 mW, receiver gain = 2 × 10^4, time constant = 20.48 ms, scan time = 41.94 s, 1 scan. (Inset) Microwave frequency = 9.59 GHz, modulation amplitude = 8 G, microwave power = 0.64 mW, receiver gain = 2× 10^4, time constant = 20.48 ms, scan time = 41.94 s, average of 5 scans.**

3.3.3 Oxidation of Dibenzothiophene by Myoglobin

The catalytic oxidation of dibenzothiophene by myoglobin (Scheme 3.2) was investigated in aqueous solutions of IL 106 or IL 169 using a procedure similar to Klyachko and Klibanov in organic molecular solvent.[97]



Scheme 3.2 Oxidation of dibenzothiophene to dibenzothiophene sulfoxide (single oxidation) and dibenzothiophene sulfone (double oxidation).

Oxidation of dibenzothiophene by myoglobin in aqueous solutions of hydrophilic ionic liquids IL 106 and IL 169 were performed. In addition, reactions in 25% aqueous 1-propanol were carried out and the reactivity compared.

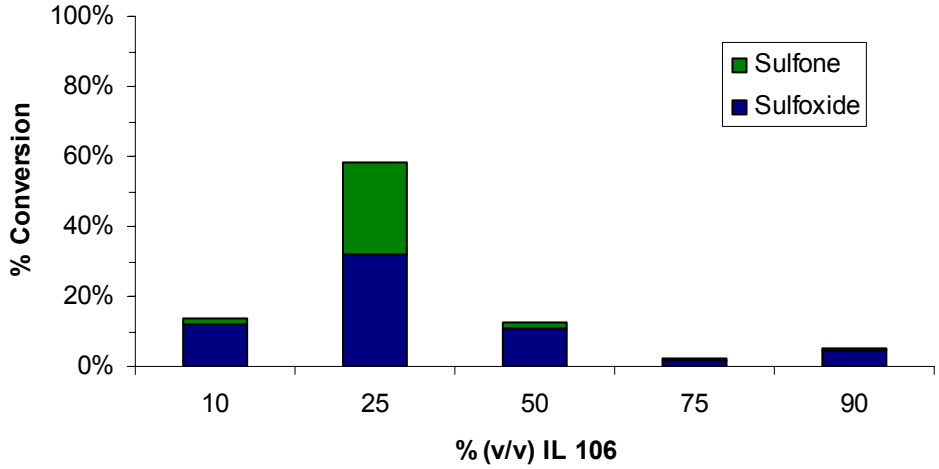


Figure 3.20 Oxidation of dibenzothiophene catalysed by myoglobin in aqueous solutions of IL 106.

The oxidation of dibenzothiophene (DBT) in IL 106 produced varying amounts of oxidation depending on the concentration of ionic liquid (Figure 3.20). At 10% (v/v) IL 106 the total amount of conversion of DBT was 14%, 12% being dibenzothiophene sulfoxide, DBTO, and the remaining 2% further oxidised to dibenzothiophene sulfone, $DBTO_2$. Maximum conversion was found for the reaction in 25% IL 106, with oxidation

of 58% of DBT. Interestingly, the conversion yielded nearly equal amounts of the oxidation products: DBTO, 32% and $DBTO_2$, 26%. A reaction solution containing 50% IL 106 produced similar results to the 10% solution, total conversion was 12%, with 11% being DBTO, a very small amount was further oxidised to $DBTO_2$ (1%). Negligible conversion was observed for the solution with 75% (v/v) IL 106, with total conversion 2%. Finally, myoglobin in 90% IL 106 produced slightly more conversion than in 75% IL 106 (5% total) and no further oxidation to the sulfone. For this reaction it was determined that 25% IL 106 is the optimal concentration.

Control reactions containing myoglobin and dibenzothiophene confirmed that negligible oxidation occurred in the absence of hydrogen peroxide, small amounts (< 3%) of DBT and $DBTO_2$ were found in the starting material and were subtracted from the final concentrations of oxidation products. A control reaction using hemin chloride demonstrated that the results obtained for the oxidation reactions involving myoglobin are not a result of the heme in solution. Hemin chloride was only sparingly soluble in IL 106, so a saturated solution of hemin chloride was used for the reaction. The same conditions were used for the hemin chloride control reaction as the myoglobin-catalysed reaction. Less than 1% conversion of DBT was observed. The absence of the double oxidation product in most cases indicates that the oxidation of DBTO to $DBTO_2$ does not occur without myoglobin even in the presence of excess hydrogen peroxide.

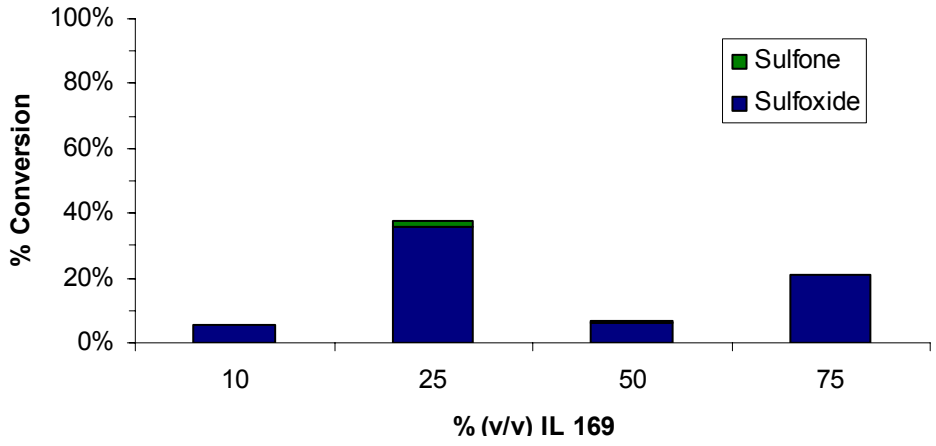


Figure 3.21 Oxidation of dibenzothiophene catalysed by myoglobin in aqueous solutions of IL 169.

In general, the oxidation reaction in solutions of myoglobin in IL 169 resulted in less oxidation of DBT than observed for myoglobin in the optimal concentration of IL 106. A 10% (v/v) solution of IL 169 and buffer produced a total conversion of 6%, the only oxidation product being DBTO (Figure 3.21). The optimal concentration of IL 169 was found to be 25% IL 169, which resulted in 38% total oxidation of DBT, the major product being DBTO (36%). A solution of 50% IL 169 produced minimal oxidation of DBT with a total oxidation of 6% and complete suppression of the double oxidation product $DBTO_2$. At 75% IL 169 only 24% of the DBT was oxidised, with DBTO as the major product (21%). Solutions containing 90% IL 169 could not be analyzed by HPLC since extraction of the dibenzothiophene compounds from the ionic liquid was not possible. Thin-layer chromatography showed that some oxidation of dibenzothiophene did occur. Control reactions containing myoglobin and dibenzothiophene confirmed that negligible oxidation occurred in the absence of oxidant, a small amount (< 6%) of DBT and $DBTO_2$ were found in the starting material and was subtracted from the final concentrations of oxidation products.

For comparison, the oxidation reaction was also performed in 25% 1-propanol under identical conditions to those used for the ionic liquids. Total conversion of dibenzothiophene was determined to be 83% ± 5% as determined from three independent experiments. The conversion to dibenzothiophene sulfoxide was 80% ± 4% while further conversion of the sulfoxide to the sulfone was 3% ± 1%. Literature reports[97] of the oxidation reaction reported a best total conversion of 95%. The observation that the oxidation in 1-propanol generally ceased after single oxidation of dibenzothiophene is consistent with the literature.

3.4 Discussion

3.4.1 Spectroscopic Studies of Myoglobin Denaturation

Myoglobin in Aqueous Solutions of IL 169 and IL 106

Enzymes in aqueous mixtures of organic solvents were demonstrated by Klibanov to be more denatured than in neat organic solvents.[37] Furthermore, it was shown that the extent of denaturation varies with the amount of organic solvent. An inverse bell-shaped relationship was found for the correlation of the extent of denaturation of enzyme in organic solvent with the amount of water (see Figure 1.2). At low and high concentrations of organic solvent the structure of the enzyme is in a near-native conformation. In this thesis UV-Vis and fluorescence spectroscopic studies were carried out on myoglobin in aqueous solutions of IL 169 and IL 106 to determine the effect of different concentrations of these water-miscible ionic liquids on the protein.

The effect of different concentrations of IL 169 on myoglobin was determined by monitoring the Soret absorbance at 409 nm by UV-Vis spectroscopy. It appears that a direct correlation between the concentration of ionic liquid and myoglobin denaturation

cannot be made. In fact, the lowest concentration of IL 169, 10% (v/v) caused the greatest amount of denaturation. This effect has been observed previously in which lesser amounts of ionic liquid have caused the greatest amount of denaturation of proteins.[49] The relative order of denaturation observed by UV-Vis for myoglobin in IL 169 follows the order (least to most denatured): 0% < 25% < 50% ≈ 90% < 75% < 10% IL 169. The optimal concentration of IL 169 was determined to be 25% (v/v) which demonstrated the least amount of denaturation in comparison to the other amounts of ionic liquid, though still significant in comparison to the original buffer solution. The fluorescence data indicate that significant unfolding of myoglobin occurs at all concentrations of IL 169 although specific trends relating unfolding to ionic liquid concentration could not be determined, as will be discussed later in this chapter.

The solutions of myoglobin in IL 106 exhibit similar behaviour to IL 169 in that the protein appears to be significantly denatured by the ionic liquid. Solutions containing 10% (v/v) IL 106 showed precipitation and possibly significant denaturation of the protein; the presence of the precipitate precluded analyses by UV-Vis. The relative order of denaturation of myoglobin in IL 106 follows the order (least to most denatured): 0% < 25% < 50% < 75% < 90% IL 106, suggesting that less IL 106 is best.

It is not immediately clear from the UV-Vis data whether the shifted Soret maxima (λ_{max} 403 nm) observed in the spectra of myoglobin in IL 106 arise from the dissociated iron-heme[107] (λ_{max} 398 nm in methanol) or from 5-coordinate high-spin myoglobin with loss of the distal water ligand (λ_{max} 394 nm in aqueous solution)[138-140] since changing the solvent can cause shifts in λ_{max} making deductions based on literature reports difficult. Changes in the local environment of the active site have previously been

shown to cause loss of the distal water ligand resulting in a 5-coordinate high-spin myoglobin species.[138,139] The nature of the iron coordination environment, including the presence of the distal water ligand, can be determined through Raman spectroscopy, magnetic circular dichroism, and x-ray absorption spectroscopy as has been shown previously by Ikeda-Saito *et al.*[139] in studies of myoglobin mutants. In order to determine whether the species observed in IL 106 is dissociated heme the UV-Vis spectrum of hemin chloride dissolved in IL 106 was measured (spectrum not shown). The Soret band observed for hemin chloride in IL 106 is located at the same λ_{max} as myoglobin in IL 106 (403 nm) however the broadness and intensity of the hemin chloride spectrum do not resemble that of myoglobin in the ionic liquid. The broadening of the peak may be caused by differences in solubility between hemin chloride and free heme in IL 106. However, the discrepancy does leave open the possibility that the species may be the 5-coordinate myoglobin species since the difference in λ_{max} between the heme and 5-coordinate myoglobin is small. Further studies, such as Raman spectroscopy, could be performed to convincingly determine the exact nature of the high-spin species. Based on the reactivity studies discussed later in this chapter it appears that the Soret band at 403 nm does in fact arise from free heme.

Literature reports of enzyme-catalysed reactions in aqueous-ionic liquid mixtures demonstrate not only that some water-miscible ionic liquids can enhance the activity of enzymes[53,141] but also that the activity of enzymes in aqueous-ionic liquid solutions is unpredictable.[6] The finding that an intermediate concentration of ionic liquid is the optimal concentration for a given enzyme has been reported previously. Other accounts of optimal concentrations of ionic liquids have been reported in which increasing or

decreasing the amount of ionic liquid from that optimal concentration results in a decrease of enzymatic activity.[49,55,58] For example, chloroperoxidase, a heme enzyme responsible for halogenation of organic substrates as well as other oxidation reactions, was demonstrated to exhibit maximum activity at 50% dimethylimidazolium dimethylphosphate in aqueous buffer whereas other ionic liquids were much less tolerated.[49] No attempts have been made in literature to explain the observed optimal concentrations of ionic liquids.

Myoglobin in Neat Solutions of Ionic Liquid

Denaturation of myoglobin was studied in neat ionic liquids using UV-Vis, fluorescence, and EPR spectroscopies. The spectral data for myoglobin in neat solutions of ionic liquid display the diversity of the behaviour of myoglobin in different ionic liquids. As previously mentioned, IL 169 and IL 106 significantly denature the protein with loss of the heme from the protein in 100% ionic liquid. The hydrophobic ionic liquids IL 101, IL 105, and IL 109 exhibit less denaturation than the hydrophilic ionic liquids.

Comparison of IL 101, IL 105, and IL 109 is indicative of the effect of different anions on the protein since all contain the tetradecyl(trihexyl)phosphonium cation. Myoglobin in IL 101 produced similar concentrations of free heme and myoglobin as evidenced by the UV-Vis spectrum. Despite the presence of an absorption band corresponding to free heme in the IL 101 spectrum, it appears that the chloride anion caused the least amount of denaturation based on the magnitude of the protein Soret band absorption. Machado *et al*.[57] reported successful biocatalysis in aqueous solutions of a chloride-containing ionic liquid by a heme protein however many literature accounts of

the detrimental effect of halide ions on proteins have been also reported.[6,53,142] The finding that the ionic liquid containing the dicyanamide anion (IL 105) resulted in the largest suppression of the Soret band is in accord with literature reports of cytochrome *c* destabilisation in dicyanamide-containing pyridinium and choline ionic liquids.[143] Furthermore, the bistriflamide anion (IL 109) also produced a significant decrease in the Soret absorption, despite the fact that literature reports abound for biocatalytic reactions in bistriflamide ionic liquids.[2,63,69,71,72,144] These results further demonstrate that various enzymes are affected differently even by the same ions hence accurate predictions of enzymatic behaviour in ionic liquids are difficult to formulate.[6]

The significant decrease in the Soret absorption between buffer solution and hydrophobic ionic liquids may also be a result in part of the solubility of myoglobin in ionic liquids. The presence of a Soret band near 409 nm in each case indicates that some of the protein is dissolved, however it is improbable that all of the protein present in the buffer solution was fully dissolved in the ionic liquids after removal of the water since these ionic liquids contain the tetradecyl(trihexyl)phosphonium cation and are hydrophobic, a characteristic which often leads to poor solubility of biomolecules.[51,145] Typical preparations of myoglobin in IL 109 contained visible aggregates of myoglobin after removal of the water. The other ionic liquid solutions may have consisted of dissolved protein as well as a suspension of finely divided particles not easily discernable by eye. Due to the differences in solubility of myoglobin in these ionic liquids, a comparison of the denaturing effects of different anions based on the UV-Vis spectra may not prove useful. Previous reports have shown that suspensions of proteins in organic solvents[32] as well as in ionic liquids[6] are catalytically active and thermally stable,

hence future development of myoglobin-catalysed reactions in these hydrophobic phosphonium ionic liquids may be successful since at least a portion of the protein is shown to be in its native conformation in the ionic liquids.

The fluorescence studies of myoglobin in solutions containing varying amounts of ionic liquids are not reliable for several reasons. Firstly, fluorescence measurements are solvent-dependent. Protein fluorescence is affected by variables such as solvent polarity and solvent viscosity,[146] which are highly variable for ionic liquids and especially aqueous solutions of ionic liquids. Secondly, fluorescence emissions can be quenched by resonance energy transfer[147] involving other fluorophores present in solution as is believed to be the case with IL 105, IL 106, and IL 109. In addition, the results of the fluorescence measurements are not consistent with the UV-Vis data, which are more reliable since such solvent effects are minimal. The results of the steady-state fluorescence measurements of myoglobin in neat ionic liquids afford three conclusions. First, IL 169 appears to denature the protein more severely than the other ionic liquids, which is consistent with the UV-Vis data; however, the relative amount of unfolding between ionic liquid concentrations cannot be ascertained via fluorescence since the solvent composition and properties change significantly with increasing amounts of ionic liquid. Second, tryptophan fluorescence of myoglobin in neat IL 101 exhibits a significant emission peak indicating unfolding of myoglobin, which is consistent with literature reports of the denaturation of other proteins in chloride-containing ionic liquids.[6,53,142] As mentioned before, however, there are reports of successful biocatalysis in chloride ionic liquids.[57] Third, not all ionic liquids are useful media for fluorescence

measurements, though fluorescence studies of enzymes in other ionic liquids have been reported.[53,142,148-151]

Myoglobin in the low-spin form (with azide as the sixth axial ligand) was selected for the EPR studies so that conformational changes of the protein could be easily monitored by changes in the observed g-values. EPR of myoglobin-azide in buffer compared to the ionic liquids demonstrated that the hydrophilic ionic liquids (IL 106 and IL 169) caused significant denaturation of the protein whereas the hydrophobic ionic liquids (IL 101, IL 105, IL 109) resulted in only partial denaturation (demonstrated by the presence of both the high-spin and low-spin signals) of myoglobin-azide.

The EPR spectra of myoglobin-azide in all ionic liquids except IL 106 exhibit both an axial high-spin Fe(III) species and a rhombic low-spin Fe(III) species as is shown in Figure 3.15. Measured g-values of the low-spin complex (2.8, 2.2, 1.7) indicate that the signal arises from the myoglobin-azide species and is consistent with the EPR spectrum of myoglobin-azide in buffer solution as well as literature reports.[117] The EPR spectra indicate that two species (high-spin and low-spin) are present in the hydrophobic ionic liquids IL 101, IL 105 and IL 109, which is evidence that at least some of the myoglobin-azide added to the ionic liquids is not denatured. These findings are consistent with the UV-Vis data reported earlier in this thesis. Myoglobin-azide in IL 106, on the other hand, only exhibits the axial high-spin spectrum at g = 5.9, which may correspond to the dissociated heme from the protein or a high-spin myoglobin species. UV-Vis data of the protein in IL 106 demonstrate that the species in solution is most likely free heme, though a 5-coordinate myoglobin species may be possible. Myoglobin-azide in IL 169 exhibits a heme-NO species in addition to the two species present for the other ionic

liquids. The generation of nitric oxide will be discussed further later in this chapter. These results indicate that the hydrophobic ionic liquids are less denaturing than the hydrophilic ionic liquids.[93,142]

The high-spin signal may arise from several sources: (1) complete loss of the heme from the protein; (2) loss of the histidine coordination to the iron, but with the heme still held in hydrophobic pocket of the protein; or (3) axial ligand exchange. One scenario of significant denaturation would be the complete loss of the heme from the protein and the observed signal would arise from a high-spin 5-coordinate iron-porphyrin species.[152] In this instance complete unfolding of the protein and subsequent release of the heme might be occurring.

Another situation involving the generation of a 5-coordinate iron-porphyrin species may involve the loss of histidine ligation to the iron, but with the heme group remaining in the pocket of the folded protein. This likely is not the case since the coordination of the histidine to the iron is known to be a strong interaction accounting for a major fraction of the heme-binding energy to folded myoglobin.[110,153,154] Removal of the proximal histidine residue leads to a decrease in the affinity of apomyoglobin for heme and an increase in heme dissociation (~100 fold as compared to native myoglobin).[153] Based on refolding experiments in which dilution of solutions of high concentrations of denaturant did not result in the refolding of myoglobin to its native state but lower concentrations did result in the native form, it was determined that at lower concentrations of denaturants the histidine may still be coordinated to the heme.[110,153,155] This point was further demonstrated through gel filtration where the heme was only shown to be separated from the protein at high denaturant concentrations (> 5 M Gu-

HCl).[110] These results indicate that the iron-histidine coordination is strong since high concentrations of denaturants are required to cause breaking of the iron-histidine bond. Therefore it is not likely that the protein would be folded but the iron-histidine coordination broken.

Yet another possible source of the high-spin species may be the replacement of azide, an intermediate strength ligand, by another more favourable ligand. De Rosa *et al.*[156] have shown that myoglobin affinity for azide can be modulated by the presence of other anions in solution via interactions with the distal residue at the heme site. Additionally, it was shown that the anions were not binding competitively to the iron causing displacement of the azide ligand. Considering these findings it is more likely that ionic liquids would cause alterations in myoglobin conformation than binding directly to the iron displacing the azide ligand. Most of the ionic liquid anions used in this study would be too large to fit into the binding pocket of folded myoglobin in order to displace azide. Furthermore, to demonstrate whether chloride was able to replace azide, as in the chloride ionic liquid, solutions of sodium chloride in buffer with myoglobin-azide were measured by EPR and did not show displacement of azide by chloride, which would result in a high-spin EPR spectrum (data not shown). Ligands that produce high-spin Fe(III) species include weak field ligands such as water, fluoride, and chloride.[117] In the samples discussed the only species present in solution are myoglobin, sodium azide, phosphate buffer, water, and ionic liquid. Therefore it may be considered that if the protein is intact and the azide anion is displaced from the iron, water may be replacing it as the axial ligand. Literature reports of aquo-metmyoglobin demonstrate similar $g_{\perp}$

values (5.86 - 6.0)[117] to those of myoglobin in ionic liquids, however the high-spin value is similar for many species.

EPR studies of heat denaturation of oxyhemoglobin conducted by Hollocher[157] were found to involve restructuring of the protein through a three step process to give a rhombic low-spin EPR spectrum which was reasoned to be due to a complex between heme and denatured protein. The EPR spectrum observed for heat denatured myoglobin resembled that of a hemin-imidazole complex.[157] Additionally, through amino acid mutations it has been shown by Ikeda-Saito *et al.*[139] that 5-coordinate high-spin myoglobin species lacking the distal water ligand give an axial high-spin Fe(III) EPR spectrum at 77 K.

To examine whether the high-spin species could be the free heme, EPR measurements of hemin chloride (iron protoporphyrin IX chloride) were carried out in the ionic liquids. Hemin chloride exhibits a similar high-spin Fe(III) species ($g_\perp$ = 5.9) to myoglobin-azide ($g_\perp$ = 5.8) in all ionic liquids, indicating that the high-spin species may indeed be free heme. Differentiation between high-spin heme and a high-spin derivative of myoglobin is best performed at very low temperatures,[118] which was not available at the time of these measurements. Importantly, the absence of a signal at g = 4.3, which is typical for rhombic, non-heme high-spin Fe(III),[158,159] indicates that the iron is still bound to the porphyrin macrocycle. Future work would include very low-temperature EPR measurements (~ 4 K), since low-temperature EPR facilities have recently become available. In addition, electron-nuclear double resonance (ENDOR) studies may reveal the nature of the high-spin species through identification of the axial ligand.

The observation that hydrophilic solvents tend to cause greater denaturation of proteins than hydrophobic solvents has been reported for both organic solvents[31,33,160] and ionic liquids.[93,142] This is consistent with the observation that IL 106 and IL 169 significantly denature myoglobin given that they are both very miscible with water (see Table 2.4). As such, these ionic liquids may strip the protein of tightly bound water molecules that are essential for the protein to maintain its structure and activity.[33] On the other hand, hydrophobic ionic liquids do not remove essential water from the protein and allow it to remain locked in a native-like conformation.[33]

Another explanation for the observation that hydrophilic ionic liquids tend to denature proteins more strongly than hydrophobic ionic liquids may be related to the structure of the ionic liquids. Dupont[161] reasoned that due to the hydrogen-bonded network structure of imidazolium ionic liquids, small amounts of water may be present as pockets of water in the bulk ionic liquid solution. Similarly, phosphonium ionic liquids were also postulated to form an extensive hydrogen-bonded network based on the crystallographic data of a similar phosphonium salt.[3] In light of these findings, it is probable that in hydrophobic ionic liquids the enzyme is present in aqueous pockets in the hydrogen-bonded network, especially at high concentrations of ionic liquid.[151] Less significant denaturation of myoglobin would result as the individual ions cannot interact with the protein since they are hydrogen-bonded to each other. Alternatively, the ions of hydrophilic ionic liquids in aqueous solution are hydrated and hydrogen bond preferentially with bulk water rather than other ions, which results in the dissociation of the ionic liquid into individual anions and cations at low ionic liquid concentrations.[162] At high concentrations of hydrophilic ionic liquids the situation may be more consistent with

the hydrophobic ionic liquids where the ionic liquid ions interact more with each other and less with water molecules. This suggestion may explain why lower concentrations of hydrophilic ionic liquids more aggressively denature the protein since the ions are more able to interact directly with the protein when not participating in a hydrogen-bonded network as in hydrophobic ionic liquids.

Some attempts have been made in the literature to explain the effect of ionic liquids on enzyme behaviour using the Hofmeister Series[51,52,145,162] however many exceptions to the rule have also been reported.[6,151] Phosphonium cations have not been described on the chaotropic-kosmotropic scale and ionic liquid cations have previously been shown to not fit into the Hofmeister Series.[6] Therefore a discussion of these results on the basis of the ion positions in the Hofmeister Series is not useful.

Denaturation of Myoglobin by Known Denaturants

To compare the denaturation of myoglobin by ionic liquids, denaturation of myoglobin by known denaturants guanidine-hydrochloride and methanol were carried out and analyzed by UV-Vis and fluorescence spectroscopic methods.

The effects of ionic-liquid induced denaturation of myoglobin have been compared to the denaturation events of known denaturing agents guanidine-hydrochloride and methanol. Denaturation of myoglobin by guanidine-hydrochloride has been well documented in the literature reports in which the heme was shown via UV-Visible spectroscopy to be released from the protein into solution where it aggregated resulting in a decreased and blue shifted absorption band.[111,112] The UV-Vis spectra of myoglobin in solutions containing different concentrations of guanidine-hydrochloride indicate that significant unfolding, caused by ionic interactions with charged amino acid

residues, at concentrations greater than 2.0 M results in a complete loss of the protein Soret absorption and a small broad absorption at 397 nm. The release of heme into solution and subsequent aggregation of the free heme was observed by UV-Vis to be similar to the denaturation caused by IL 169.

Myoglobin denaturation by various alcohols has been studied by many research groups in order to understand the helical structure observed at higher concentrations of alcohol.[104,107,108,113] The absorption spectra demonstrated a shift of the Soret band to 399 nm at concentrations greater than 50% and the presence of a large shoulder near 360 nm. Interestingly, the denaturation in methanol was not accompanied by a large decrease in the observed Soret absorption which indicates that the free heme does not aggregate in methanol as it does in aqueous solution. The spectra of myoglobin denatured by IL 106 are reminiscent of the methanol-denatured spectra in that the absorption of the free heme is large, which is consistent with the heme being monomeric. It is important to note that while methanol and guanidine-hydrochloride denature myoglobin through different mechanisms it is unlikely that IL 106, though demonstrating a UV-Vis spectrum similar to methanol-denatured myoglobin, would cause denaturation of the protein through the same mechanism. Alcohols are thought to denature proteins through preferential solvation of hydrophobic residues whereas salts denature proteins by charge-charge interactions and hydrogen bonding (discussed in Section 3.1.2). The denaturation of myoglobin observed in phosphonium ionic liquids could be a result of both types of interactions, but one may be more prevalent than the other.

3.4.2 Reactivity Studies of Myoglobin in Aqueous Solutions of IL 169 and IL 106

The relationship between enzyme conformation and activity is well established: denatured proteins do not catalyse reactions as effectively as native proteins. Klyachko and Klibanov[97] demonstrated the potential for non-enzymic heme proteins such as hemoglobin and myoglobin to exhibit peroxidase-like activity in aqueous solutions of organic solvents. Previous reports have indicated that the solvent composition can affect the degree of oxidation of dibenzothiophene to its sulfoxide (single oxidation) and the sulfone (double oxidation), Scheme 3.2.[97] Many of these studies were focused on hemoglobin reactivity. It is expected that hemoglobin would exhibit similar reactivity to myoglobin since the individual monomers of hemoglobin (a dimer of dimers) closely resemble myoglobin. Indeed, a comparison of heme-protein reactivities demonstrated that hemoglobin and myoglobin oxidised dibenzothiophene to similar extents (99% conversion and 95%, respectively),[97] therefore, the hemoglobin studies can be compared to the myoglobin studies presented in this thesis. The oxidation of dibenzothiophene catalysed by myoglobin was chosen as a model reaction to demonstrate the potential for phosphonium ionic liquids as reaction media for biocatalytic reactions. This is the first account of a myoglobin-catalysed reaction in ionic liquids and the second report of biocatalysis in phosphonium ionic liquids.[27]

Total Conversion of Dibenzothiophene

Literature reports of the heme-protein catalysed oxidation of dibenzothiophene in organic solvents demonstrate that hemin chloride is not an effective catalyst, with only 9% conversion of dibenzothiophene compared to 95% and 99% conversion for myoglobin and hemoglobin respectively.[97] The extent of the oxidation of

dibenzothiophene in solutions of IL 169 correspond with earlier spectroscopic observations that indicate the protein is significantly denatured in the ionic liquid. The optimal concentration was determined spectroscopically to be 25% IL 169 and the reactivity of the solution containing 25% IL 169 produced the greatest amount of conversion of dibenzothiophene to its oxidation products (total conversion, 40%). Other concentrations of IL 169 produced only minimal oxidation of dibenzothiophene. Further experiments would determine the extent of oxidation of dibenzothiophene in solutions of IL 169 mediated by hemin chloride, however in IL 106 it was demonstrated that hemin chloride does not catalyse the oxidation reaction.

Significant oxidation of dibenzothiophene occurs in solutions containing 25% (v/v) IL 106 (58% total conversion). The previous spectroscopic results indicated that the protein was greatly denatured in nearly all solutions containing IL 106 with the observation that the greatest amount of native protein was present in the solution containing 25% IL 106. The oxidation of dibenzothiophene in 25% IL 106 is consistent with the finding that greater amounts of native myoglobin are present in this solution. Control reactions demonstrated that hydrogen peroxide and myoglobin are both required for oxidation to occur since insignificant amounts of conversion were found in the absence of oxidant or in the presence of hemin chloride rather than myoglobin.

Klyachko and Klibanov[97] studied the degree of oxidation of dibenzothiophene in various solvents in order to compare the effect of different types of solvents on the oxidation reaction. The best solvents for the hemoglobin-catalysed oxidation reaction were found to be protic, acidic solvents such as alcohols and amides.[97] Protic solvents act as a source of H^+, which is required for the generation of the hemoglobin species that

catalyses the oxidation reaction. Alcohols, being more protic than ionic liquids, should therefore, facilitate greater total oxidation of dibenzothiophene. This was observed for the oxidation of dibenzothiophene in 25% 1-propanol (83% total conversion) compared to 25% IL 169 (40% total conversion) and 25% IL 106 (58% total conversion).

The study of solvent composition by Klyachko and Klibanov[97] also demonstrated the ability of hemoglobin to catalyse the reaction in nearly neat (99% (v/v)) organic solvent.[97] It was shown that a drastic decrease in conversion occurred upon increasing the organic solvent content to 99% (v/v), the maximal degree of dibenzothiophene oxidation in 99% 1-propanol was merely 11% (down from 99% in 25% (v/v) 1-propanol). The best organic solvent was determined to be 1,2-propanediol which gave 36% conversion of dibenzothiophene to its oxidation products. This study only reported the maximal degree of dibenzothiophene oxidation, no mention of the amounts of single oxidation (sulfoxide) and double oxidation (sulfone) products was made for the different organic solvents.

Comparison of the Oxidation Products

A comparison to the reaction in 1-propanol shows that myoglobin produced mostly dibenzothiophene sulfoxide with minimal conversion to dibenzothiophene sulfone. Interestingly, nearly equal amounts of the sulfoxide and the sulfone were measured for the 25% IL 106 solution. From these preliminary results it appears that IL 106 mediates the second oxidation of dibenzothiophene (Scheme 3.2), which is in contrast to literature accounts for other solvents.[97,163] The mechanism for this double oxidation in IL 106 is not completely understood.

Previous literature reports examined the extent of the oxidation by hemoglobin in organic solvent at several pH values in order to determine the optimal pH for maximal

conversion of dibenzothiophene to its oxidation products.[97] It was established that the best pH for complete conversion (90% sulfoxide, 10% sulfone) was 5.2, increasing or decreasing the pH resulted in decreased oxidation. Interestingly at pH 7.0, though a decreased total oxidation was observed, a shift in the oxidation products was found such that 40% of the oxidation products were dibenzothiophene sulfone (compared to 10% previously). The pH of the ionic liquid solutions was not measured but a shift to higher pH could explain the observation that nearly one-half of the total oxidation products for IL 106 was the sulfone. At 25% IL 106, the buffering capacity of the acetate-phosphate buffer could have been exceeded. No explanation was offered in the literature regarding the shift to the sulfone at higher pH in organic solvents.

Klyachko and Klibanov[97] demonstrated that the oxidation reaction ceases after a single oxidation of dibenzothiophene not because the oxidation is complete but because the heme moiety is destroyed by hydrogen peroxide. This was shown by the addition of new protein to the reaction mixture which led to continued oxidation of the substrate. These results imply that the second oxidation event, to dibenzothiophene sulfone, may occur if the destruction of the heme could be prevented. Considering this implication and the observation that myoglobin in a solution of 25% IL 106 catalysed almost equal amounts of single- and double-oxidation, IL 106 may be preventing the destruction of the heme group leading to increased oxidation to the sulfone. Further experiments are required to confirm that IL 106 is stabilising the protein towards hydrogen peroxide.

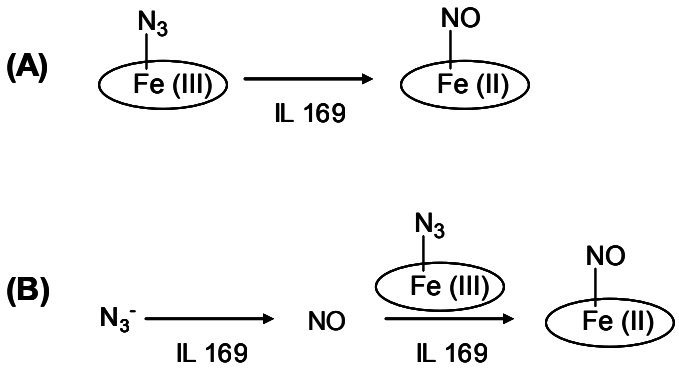
Oxidation of dibenzothiophene by heme proteins in the presence of organic solvents was initially investigated because dibenzothiophene is poorly soluble in water.[97] To study the dependence of the reaction at higher concentrations of dibenzothiophene,

high concentrations of organic solvent were required. It was determined that at higher concentrations of dibenzothiophene the oxidation is faster, however maximal conversion is less. These results cannot be compared to the oxidation in 25% 1-propanol since the total oxidation was shown to be lower in greater organic solvent concentrations. Dibenzothiophene was more soluble in the ionic liquids used in this study than in 1-propanol. Therefore, ionic liquids are potentially more useful solvents for enzymatic dibenzothiophene oxidation than many organic solvents since inadequate solubility of reactants can hinder reactions in some cases.

There are several limitations to the application of phosphonium ionic liquids, as well as other ionic liquids, as reaction media for biocatalysis. First, extraction of various products from the ionic liquid is strenuous. In order to analyse the reaction by HPLC the products had to be extracted into organic solvent. Determination of a solvent system for product extraction that was not miscible with the ionic liquids was time-consuming and varied for different compounds. In some cases, removal of residual ionic liquid from the extractant required further purification by column chromatography. Second, different reaction conditions are often needed for individual ionic liquids as solvent properties such as viscosity vary widely.

3.4.3 Generation of Nitric Oxide in IL 169

The generation of heme-nitric oxide in IL 169 is very interesting and perplexing. Iron porphyrin complexes are well known as nitric oxide (NO) traps.[164] There are two possible reactions that may be occurring (Scheme 3.3) to generate the heme-NO species:

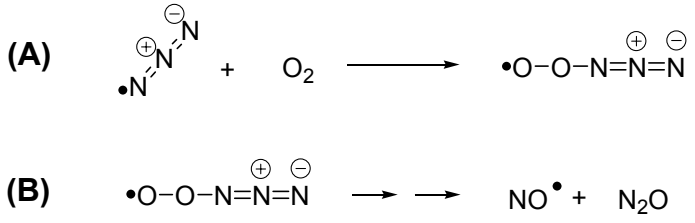


Scheme 3.3 Possible reactions involving generation of nitric oxide (NO). (A) Heme-N_3 is converted to Heme-NO directly; (B) N_3 reacts to form NO which is trapped by a heme-like species to generate Heme-NO.

The first possible reaction, Scheme 3.3 (A), involves the reaction of azide while it is bound to the iron ion, thus converting heme-azide to heme-NO. A reduction of Fe(III) to Fe(II) would occur in conjunction with the conversion of azide to nitric oxide. Literature reports indicate that the reaction of heme proteins and various iron-porphyrin based models with nitric oxide is more common.[165] Therefore, path (B), displacement of azide by nitric oxide is more likely. The nitric oxide most likely forms from a reaction of azide in the ionic liquid. Nitric oxide is then trapped by the high-spin Fe(III) species in solution, thereby producing the Fe(II) nitric oxide species (Scheme 3.3 (B)) observed near g = 2 in the EPR spectra. The suggested mechanism for the reduction of the metal ion involves two molecules of nitric oxide. Addison *et al.*[166] proposed that one molecule of nitric oxide binds to the oxidised form of the protein and a second nitric oxide molecule reduces the Fe(III)-NO complex to Fe(II)-NO.

Kalyanaraman *et al.*[167] proposed that nitric oxide could be generated by the oxidation of azide to the azidyl radical in the presence of hydrogen peroxide and heme proteins. To demonstrate their hypothesis, the spin trap DMPO was used to capture the

short-lived azidyl radical, which was proposed to react with molecular oxygen to produce nitric oxide upon disproportionation of the dioxygenylazidyl radical (Scheme 3.4). DMPO spin trapping was employed in solutions of azide and myoglobin-azide in IL 169 to determine whether the azidyl radical was the source of the generated nitric oxide, but this species was not detected.



Scheme 3.4 Generation of nitric oxide from the azidyl radical proposed by Kalyanaraman *et al.*[167] (A) Reaction of azidyl radical with molecular oxygen to form dioxygenylazidyl radical; (B) Disproportionation of dioxygenylazidyl radical to form nitric oxide and nitrous oxide.

In this work there is no evidence from the spin trapping experiments for the azidyl radical. EPR results are consistent with a peroxyl (·OOX) or superoxide ($OO^{\cdot -}$) spin adduct, which can be generated from: (1) an unidentified oxygen-based radical; or (2) the dioxygenylazidyl radical (NNNOO·) proposed by Kalyanaraman.[167] Molecular oxygen may react with a transient radical species found in the ionic liquid to form a more stable peroxyl-based radical upon storage of the ionic liquid under aerobic conditions, or an oxygen-based radical may already be present. Previous investigations have demonstrated that an oxygen-based radical species, determined by DMPO spin trapping to be a peroxyl radical, was present in another phosphonium ionic liquid in which the anion was decanoate.[81,168] These studies found that addition of molecular oxygen produced a greater concentration of the observed radical. Phosphonium ionic liquids are known to stabilise

radical species and consequently prevent dimerization thereby making it feasible that a radical may be produced and remain stabilised in the ionic liquid.[8]

Based on the results of the DMPO spin trapping experiments in neat IL 169 it is apparent that a small amount of an oxygen-based radical is present in the original ionic liquid. The source of this radical is unknown. A reaction cannot be occurring between the unknown radical and azide since the radical was trapped by DMPO before azide was added to the solution. After azide was added, the amount of trapped species appeared to increase. These findings point toward a reaction between the ionic liquid or a non-radical impurity and sodium azide. EPR of the solution of myoglobin-azide in DMPO-IL 169 seem to indicate two things: (1) the concentration of the trapped radical species increases on addition of the myoglobin-azide solution, which also contains an excess of sodium azide; and (2) the suppression of nitric oxide production upon removal of the intermediate radical species via DMPO spin-trapping. Different trapped species in neat ionic liquid and in solutions containing azide and myoglobin-azide may coincidentally produce the same EPR spectra since the substituent (X) of the peroxyl radical (·OOX) is too far from the unpaired electron to produce observable hyperfine interactions.

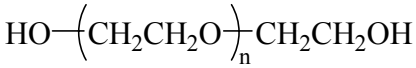
Owing to the observation that the azidyl radical was not trapped in the solutions, studies of the reaction of myoglobin-azide in IL 169 under an inert atmosphere were undertaken to determine whether the source of oxygen for the production of nitric oxide was molecular oxygen or from some other source. The anaerobic studies indicated that molecular oxygen was not involved in the generation of nitric oxide, however, complete removal of oxygen from the ionic liquids by either the freeze-pump-thaw method or simply cycling under inert atmosphere and vacuum may not have been achieved. Since

the anaerobic studies indicated that molecular oxygen was possibly not involved, ^{31}P NMR studies of the ionic liquid and concentrated azide were performed to establish whether a reaction between the ionic liquid and azide was occurring. It was expected that if the ionic liquid was reacting with azide, a reduction in the integration of one of the peaks would be accompanied by the occurrence of a new peak corresponding to a phosphorus-containing degradation product. ^{31}P NMR studies indicate degradation of the diethyl phosphate anion as demonstrated by a decrease in the integrated area of the peak after azide addition, which could be the source of the oxygen atom for nitric oxide. However, the ^{31}P results are inconclusive since no new peak was observed in the spectrum even though a large window, 3900 ppm, was used. Future experiments using ionic liquid containing isotopically oxygen labelled diethyl phosphate would establish whether the ionic liquid anion is the source of the nitric oxide oxygen atom.

4 PEO-MODIFIED MYOGLOBIN

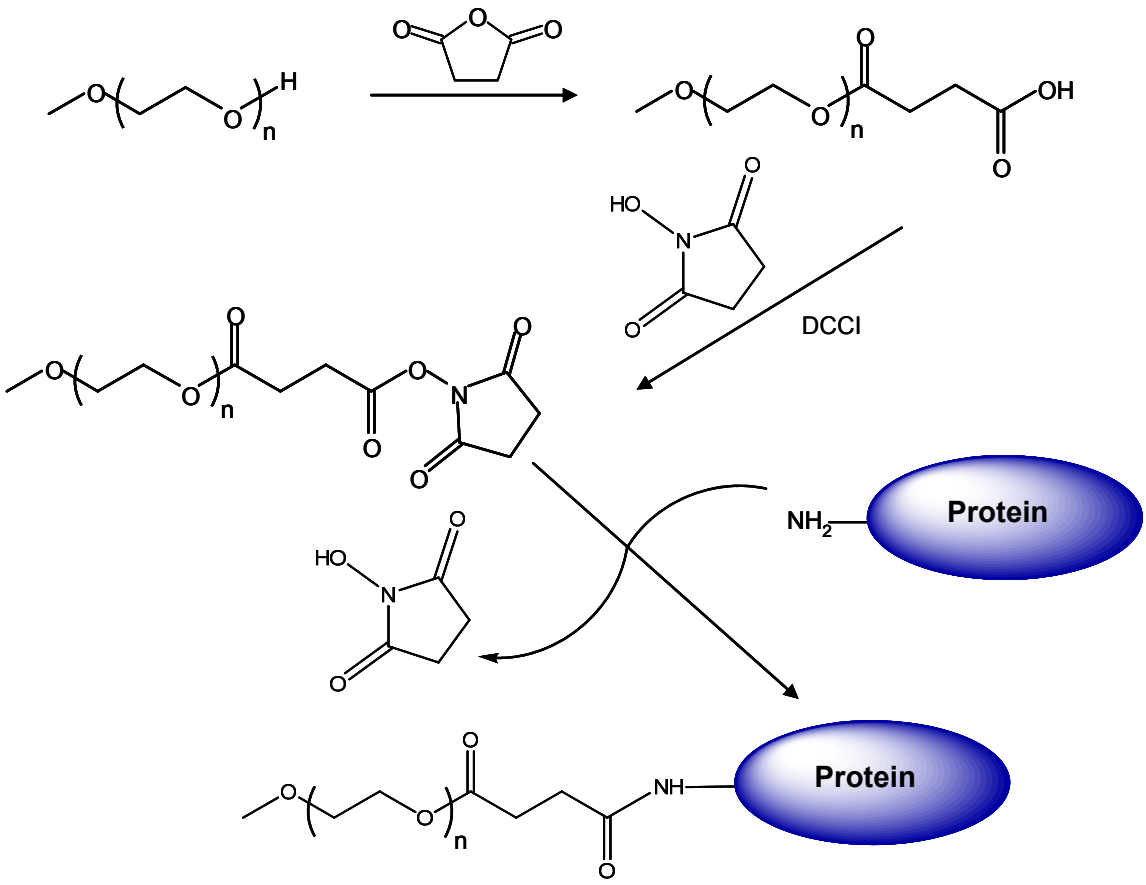
4.1 Introduction

Covalent modification with polymers is a well established method used to increase the solubility and stability of proteins in non-aqueous media. Polyethers such as poly(ethylene glycol), also known as poly(ethylene oxide) (PEO),



are polar linear polymers that are commonly available in a variety of molecular weights and are soluble in water and many organic solvents. The hydroxyl group of the polymer can be readily activated or derivatised to react with proteins and other molecules. Owing to the solubility characteristics and non-toxicity of PEO, PEO-modified proteins have been used in an assortment of biotechnical and biomedical applications.[169]

Numerous techniques and coupling agents have been used for the covalent modification of proteins due to the range of amino acid functionalities available.[170] Common reagents for the covalent modification of amino groups include esters such as *N*-hydroxysuccinimide. *N*-hydroxysuccinimide-activated PEO reacts with primary amines (Scheme 4.1), usually lysine residues, on the surface of a protein to produce the polymer-modified protein through formation of an amide bond.[171,172]



Scheme 4.1 Preparation of *N*-hydroxysuccinimide-activated PEO and coupling to primary amine of protein.

This chapter describes the synthesis and characterisation of PEO-modified myoglobin. Studies on the denaturation of the modified protein were performed by UV-Visible, fluorescence, and EPR spectroscopy. It was anticipated that the covalent modification of myoglobin would protect the protein from the denaturation that was observed for the unmodified protein in ionic liquids. A comparison between the UV-Vis and fluorescence measurements of unmodified and PEO-modified myoglobin revealed that PEO modification gave improved stabilisation of the protein at high concentrations of ionic liquid. The modification did not appear to protect the protein from denaturation

caused by small molecules such as the common denaturants guanidine-hydrochloride and methanol.

4.2 Experimental

4.2.1 Preparation of PEO-Myoglobin

Activated poly(ethylene oxide) was prepared by coupling *N*-hydroxysuccinimide to poly(ethylene oxide) according to literature methods.[173] Succinic anhydride (10.0034 g, 0.1000 mol) was dissolved in dichloroethane (500 mL) with pyridine (10.0 mL) and was stirred for 45 minutes. Polyethylene glycol monomethyl ether (PEO, average molecular weight 5,000 g/mol) (99.9940 g, 0.020 mol) was added and the solution was stirred for a further 20 minutes to fully dissolve the PEO. After transferring to a round-bottom flask, the solution was refluxed at 90 ^{o}C for 36 hours under nitrogen gas. The solvent was then removed by evacuation for a period of two days to yield a pale pink solid. Dissolution of the solid residue in distilled water (200 mL) produced a dark pink-red solution. The solution was extracted with ether until the ether layer was no longer cloudy (4 × 60 mL). Chloroform (2 × 80 mL) was used to extract the aqueous layer with the upper aqueous layer being dark pink-red and the lower organic layer being clear, pale yellow. The chloroform was removed under vacuum to yield PEO-succinate which was verified using ^{1}H NMR spectroscopy.

PEO-succinate (78.2232 g, 0.01569 mol) was dissolved with stirring in acetonitrile (400 mL) at 37 ^{o}C. To the PEO-succinate solution was added *N*-hydroxysuccinimide (1.9805 g, 0.0172 mol), the solution was stirred at 37 ^{o}C for 90 minutes. Dicyclohexylcarbodiimide (DCCI) (3.5595 g, 0.0172 mol) was dissolved in acetonitrile at 40 ^{o}C. The PEO-succinate solution was cooled to 0 ^{o}C in an acetone-ice

bath while the dicyclohexylcarbodiimide solution was added drop-wise. The resulting cloudy solution was then incubated for 24 hours at room temperature. A white precipitate was removed by filtration. The filtrate was collected and the solvent removed under vacuum. Dimethyl formamide (600 mL) was added to dissolve the solid. Cold benzene was added (200 mL), then the resulting solution was added drop-wise to petroleum ether (800 mL) at 0 ^{o}C. Collection of the product by filtration gave a solid that was left to dry at room temperature overnight. The filtrate was added to cold petroleum ether (400 mL) and was left on ice to precipitate overnight. Solid product was collected and added to the previously collected product. Precipitation was twice repeated to yield *N*-hydroxysuccinimide-activated PEO.

PEO-myoglobin was synthesized according to the procedure of Ohno *et al*.[173] Activated PEO (2.3588 g, 4.50×10^{-4} mol) was dissolved in buffer (120 mL) (0.05 M phosphate, pH 9.0, 0.85% NaCl) with stirring for 15 minutes. Myoglobin (0.1574 g, 9.26×10^{-6} mol) was added to the solution with gentle stirring for 1 hour. The solution was then divided among four 50 mL centrifuge tubes and lyophilised over 48 hours. The contents of each tube were dissolved in 2.5 mL buffer (0.05 M phosphate, pH 9.0, 0.85% NaCl). The PEO-Mb solution was passed through a Sephadex G-100 column (2.5 cm × 13.5 cm), and eluted with buffer until the fractions contained no Soret band in the UV-Vis absorption spectrum, indicating the absence of myoglobin. Fractions were collected starting from the elution of the brown myoglobin solution. Each fraction was checked by UV-Vis for the presence of excess activated PEO (λ_{max} at 261 nm). Fractions with minimal PEO, as determined by UV-Vis, were combined. The combined fractions were divided between two 50 mL centrifuge tubes and lyophilised for 48 hours.

After each separation using the column the gel was washed with buffer, and after two PEO-Mb samples were separated from unreacted PEO, the PEO on the column was removed with 0.2 M NaOH solution. UV-Vis was used to determine whether all unreacted PEO was eluted from the column, as indicated by the absence of the absorption band at 261 nm.

4.2.2 UV-Visible and Fluorescence of PEO-Myoglobin

PEO-myoglobin in aqueous solutions of IL 106 and IL 169 was prepared as previously described for the unmodified protein (Section 3.2.1). Protein concentration was determined by UV-Vis spectroscopy using the molar extinction coefficient of 188,000 $M^{-1}cm^{-1}$ given in literature.[106]

4.2.3 EPR Experiments in Phosphonium Ionic Liquid

Purified PEO-myoglobin was dissolved in a minimal amount of buffer (20 mM phosphate, pH 7.0). Excess potassium ferricyanide ($K_3Fe(CN)_6$) was added to oxidise all iron to the +3 oxidation state. The oxidant and excess PEO were removed with a Sephadex PD-10 desalting column (GE Healthcare). PEO-myoglobin was passed through the column in 2.5 mL aliquots and was eluted with 3.5 mL of buffer. A total volume of 40 mL was concentrated to 6.5 mL using Amicon Ultra-4 concentrators (Millipore), molecular weight cut-off 10 kDa. Protein concentration was determined to be 2.06 mM via UV-Vis absorption of the Soret band (λ_{max} 409 nm, Absorbance = 0.7761; molar extinction coefficient 188,000 $M^{-1}cm^{-1}$).[106] Sodium azide (0.2605 g, 4.1×10^{-3} mol) was added to the solution, mixed and allowed to equilibrate at room temperature for 15

minutes. The colour of the PEO-myoglobin solution changed from dark brown to deep red on addition of azide.

Ionic liquid (1.5 mL) was added to a side-arm round-bottom flask containing a stirbar. With the stirplate set to a low setting, PEO-MbN_3 solution (750 μL) was added to the flask. The solution was allowed to mix with stirring for 15 minutes. Samples were evacuated overnight with gentle stirring for 18 hours to remove water.

A buffer solution of the modified protein containing 20% (v/v) glycerol as a glassing agent was prepared by mixing the PEO-MbN_3 solution (750 μL) with glycerol (300 μL) and buffer (450 μL). An aliquot of each solution was placed in a quartz EPR tube and stored under liquid nitrogen.

4.3 Results

4.3.1 SDS-PAGE of PEO-Myoglobin

Degree of PEO-Modification for UV-Vis and Fluorescence Measurements

Myoglobin contains 19 lysine residues. According to literature reports, the lysine residues are all located on the surface of myoglobin and can react with the activated polymer.[171] Hence a fully modified myoglobin molecule should contain 20 PEO chains, formed by the modification at each lysine residue as well as the amino terminus of the protein, and have an approximate molecular weight of 117 kDa. The extent of protein modification by the addition of activated-PEO to myoglobin was determined by SDS-PAGE[171] using 10% SDS gels (Figure 4.1). Each PEO polymer chain has a molecular weight of approximately 5 kDa, hence addition of each chain to myoglobin results in a new band separated by 5 kDa. However, the PEO is not completely monodisperse, leading to some broadening of the bands in the SDS-PAGE. This broadening effect

increases as the degree of modification increases since the molecular weight dispersion of PEO is additive.

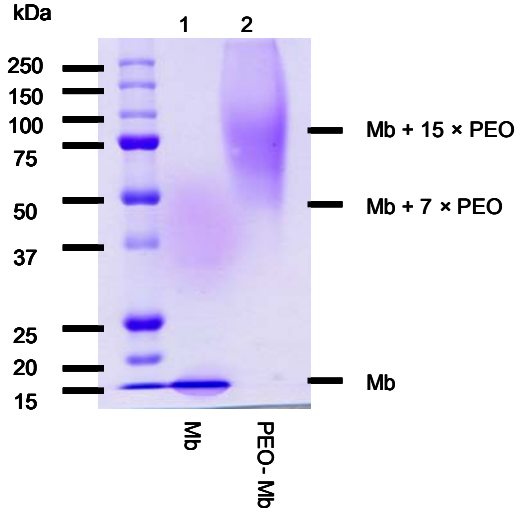


Figure 4.1 SDS-PAGE of PEO modification of myoglobin for UV-Vis and fluorescence studies (1) and native myoglobin; and (2) PEO-myoglobin.

As shown in Figure 4.1, lane 1 contains the native myoglobin control solution. Horse heart myoglobin has a molecular weight of 16951.49 Da[174] and so appears near the lowest position on the molecular weight marker. Lane 2 contains the modified protein. It is evident from the gel that very little unmodified myoglobin is present after modification. The more intense bands located between 50 kDa and 75 kDa indicate that the majority of the modifications were between 7 and 15 PEO chains per myoglobin molecule.

Degree of PEO-Modification for EPR Measurements

Two new batches of PEO-myoglobin were prepared for EPR experiments. The extent of protein modification by the addition of activated-PEO to myoglobin was determined using SDS-PAGE (Figure 4.2) according to literature methods.[171] As shown in Figure 4.2, lane 1 contains the native myoglobin control solution. As described above,

myoglobin appears near the lowest molecular weight marker. Lane 2 in the gel contains the first batch of the modified protein that was prepared. It can be seen from the gel that only a small amount of unmodified myoglobin is present after modification. The faint band between 29 kDa and 37 kDa, corresponds to three PEO chains (32 kDa). Distinct darker bands between 37 kDa and 86 kDa indicate that the protein was primarily covalently modified by 5 (42 kDa) to 14 PEO chains (87 kDa), with the greater concentration being between 7 and 14 polymer chains per myoglobin molecule. A band near the 116 kDa marker indicates that up to 20 PEO chains were attached to the protein (117 kDa). The broadening of the bands at higher molecular weights is due to decreased resolution for higher mass proteins as well as distribution in the molecular weight of the polymer. A second sample of modified myoglobin was prepared and run in lane 3. The results show that the modification of myoglobin in the second batch is very similar to the first preparation of PEO-myoglobin, indicating consistency in the preparations. Lane 4 contains the combined solutions of the two batches.

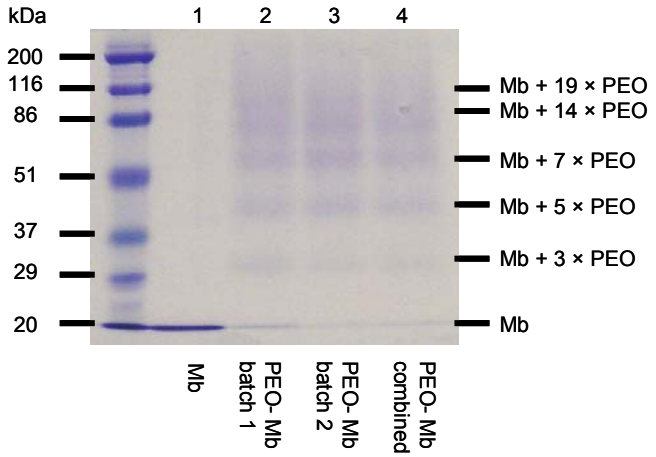


Figure 4.2 SDS-PAGE of PEO modification of myoglobin for EPR studies (1) native myoglobin; (2) PEO-myoglobin, first preparation; (3) PEO- myoglobin, second preparation; (4) combined fractions.

The two preparations of PEO-myoglobin were combined and used for subsequent EPR experiments. A better quality gel was produced for the EPR samples since a rocking platform was used for the staining and destaining process unlike for the UV-Vis and fluorescence gel in which the individual bands on the gel appear smeared.

4.3.2 UV-Visible and Fluorescence Studies of PEO-Myoglobin

PEO-Myoglobin in Aqueous IL 169

The UV-Visible absorption spectra of 4.1 μM PEO-myoglobin in buffer (pH 7.0) containing varying amounts of IL 169 (Figure 4.3) show a marked change in the absorption in the Soret region compared to PEO-myoglobin in buffer only. PEO-modified myoglobin in buffer with 0% (v/v) IL 169 exhibits a Soret band at 409 nm consistent with previous measurements and literature reports for unmodified myoglobin.[106] Addition of 10% (0.25 M) IL 169 while maintaining the same protein concentration results in almost complete loss of the protein Soret band (shifted to approximately 400 nm) joined with the occurrence of a stronger band at 366 nm. The absorption of the Soret band of PEO-myoglobin in 25% (0.62 M) and 50% (1.25 M) IL 169 is greater than any other amount and is less blue shifted than 10% IL 169 with maximum wavelengths, λ_{max}, at 402 nm and 404 nm respectively. The slight shift in λ_{max} indicates a small conformational change around the heme. A concentration of 75% (1.88 M) IL 169 gave an absorption whose intensity was midway between the most denatured (10% IL 169) and the least denatured (50% IL 169) with no significant shift in λ_{max} (403 nm) from the other IL 169 solutions. PEO-myoglobin in 90% (2.25 M) IL 169 shows a maximum absorption at λ_{max} 404 nm, which is significantly less than the best-tolerated amounts of IL 169 but slightly better than the almost completely denaturing 10% IL 169. All spectra, except for the solution of

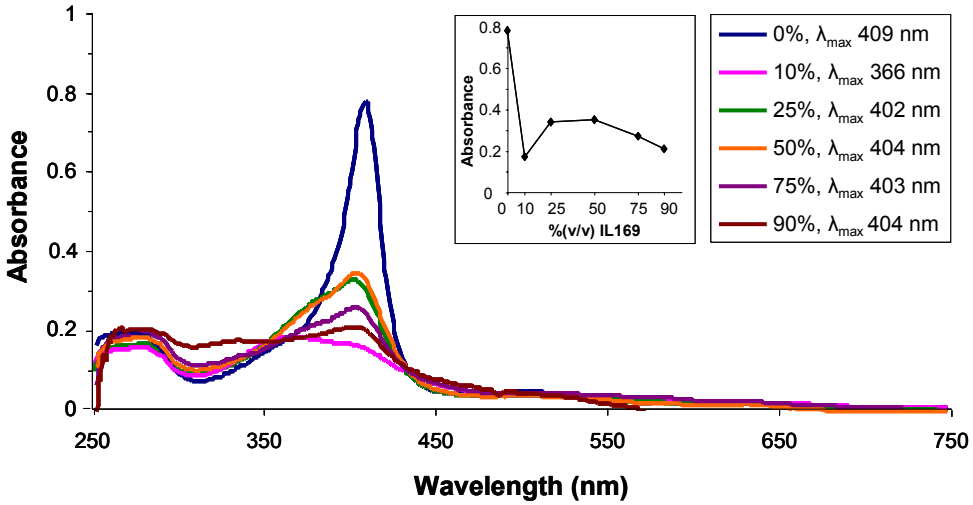


Figure 4.3 **UV-Vis spectra of 4.1 µM PEO-myoglobin in aqueous solutions of IL 169 (% v/v). (Inset) Absorbance versus concentration for peaks ~409 nm.**

PEO-myoglobin in 90% IL 169, also features a shoulder near 370 nm, which, as discussed previously for unmodified myoglobin, may have been present but less visible in the initial buffer solution. The considerable decrease in the protein Soret absorption as compared to the original solution of PEO-myoglobin in buffer (Figure 4.3) and the presence of a band near 370 nm upon addition of the ionic liquid are consistent with a decrease in heme binding to the protein[107-109] however the presence of two bands indicates that heme-containing myoglobin is present in the solution. The slight shift in λ_{max} could be an effect of the solvent or an indication of slight perturbations to the heme environment.

Comparison between these data and those of the unprotected protein in aqueous solutions of IL 169 (Figure 3.6 of Section 3.3.1) demonstrates that the PEO-protection of myoglobin significantly enhances the stability of the protein especially for 50% IL 169. At higher concentrations of IL 169, the protected protein appears less denatured than the

unprotected protein as can be seen in the larger Soret absorption for PEO-myoglobin than unmodified myoglobin.

PEO-Myoglobin in Aqueous IL 106

Similar to unmodified myoglobin in IL 106, aqueous solutions of 4.0 μM PEO-myoglobin containing varying amounts of IL 106 show very different UV-Vis absorption spectra than for IL 169. The solution containing 10% (0.27 M) IL 106 demonstrated no shift in the λ_{max} from PEO-myoglobin in buffer (Figure 4.4). However, a substantial decrease in the Soret absorption is evident thus indicating that partial denaturation of the modified protein occurs at this concentration of ionic liquid. In addition, the shoulder at about 370 nm seen in the spectra for PEO-myoglobin in IL 169 is also observed in the spectrum for 10% IL 106. The greatest shift in λ_{max} (399 nm) is exhibited in solutions of 25% (0.68 M) IL 106 and the absorption is significantly less than the modified protein in buffer. As previously discussed for the unmodified protein in IL 106, the large blue shift of λ_{max} is consistent with free heme; however it is also possible that the band results from the loss of the distal water ligand resulting in a 5-coordinate high-spin myoglobin species.[138-140] Increasing the amount of IL 106 resulted in greater heme loss than with lower amounts of ionic liquid as is evidenced by the increase of the heme Soret absorption at about 401 nm (with an experimental uncertainty of ± 2 nm). A 50% (1.35 M) solution of IL 106 appears to denature the protein more significantly as the absorption of the free heme Soret band is much greater than for 25% IL 106, λ_{max} of the Soret band is at 401 nm. PEO-myoglobin in a solution containing 75% (2.03 M) IL 106 appears to result in the most heme loss. The λ_{max} is at the same wavelength (within experimental uncertainty: ± 2 nm) for the 50% and 75% solutions (401 nm and 402 nm

respectively). The spectrum for 90% (2.23 M) IL 106 exhibits a broader peak than for the other solutions and the smaller Soret band indicates that there is possibly less denaturation for 90% IL 106 than 50% and 75% IL 106. There is no significant shift in λ_{max} on changing the concentration of IL 106 from 50% to 75% to 90%. Essentially, for PEO-myoglobin it appears that less IL 106 is better for the modified protein, as it was for the unmodified protein, since the protein Soret band is the strongest at the lowest concentration (0.27 M) of ionic liquid.

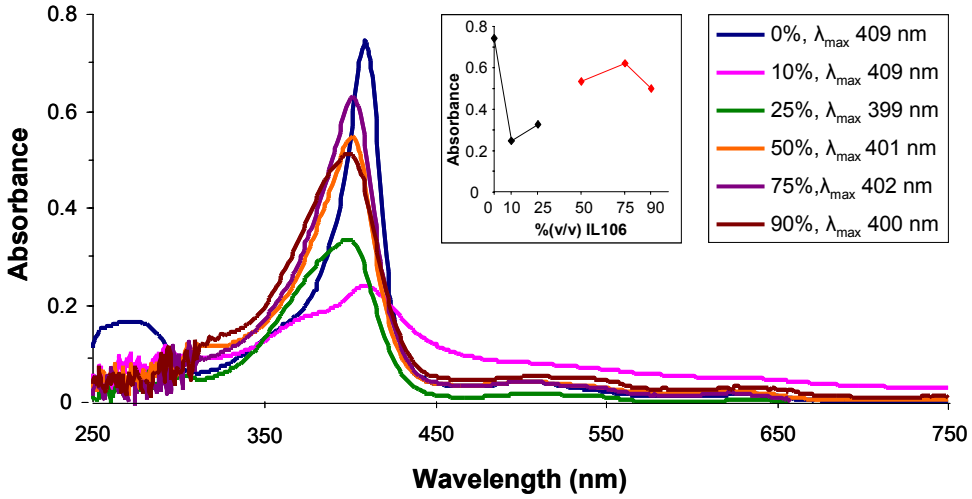


Figure 4.4 **UV-Vis spectra of 4.0 µM PEO-myoglobin in aqueous solutions of IL 106 (% v/v). (Inset) Absorbance versus concentration, black points: peaks ~ 409 nm; red points: blue shifted peaks.**

These results, when compared with the UV-Vis of the unmodified protein in aqueous solutions of IL 106 (Figure 3.7, Section 3.3.1), show that higher concentrations of IL 106 may be more tolerated by the protected protein than the unprotected protein. For example, at 90% IL 106 the Soret band of PEO-myoglobin is broader than for the unmodified protein. The broadness of the band may indicate two species, free heme and native modified protein, which would demonstrate that PEO-myoglobin is not completely

denatured by high concentrations of IL 106 as was observed for the unmodified protein. Alternatively, PEO-modification may be causing broadening of the Soret bands.

Denaturation of PEO-Myoglobin in Solutions of Guanidine-Hydrochloride or Methanol

The denaturant guanidine-hydrochloride (Gu-HCl) commonly unfolds proteins at concentrations in the region of 2.0 M or greater.[111] UV-Visible spectra of a 4.2 μM solution of PEO-myoglobin in buffer (pH 7.0) with different amounts of Gu-HCl are shown in Figure 4.5. Concentrations up to 0.5 M exhibit very little effect on the Soret band (λ_{max} 409 nm) of the modified protein. A 1.0 M concentration of Gu-HCl caused a decrease in the interaction between the heme and the modified protein resulting in a suppression of the Soret absorption, though no shift in λ_{max} is apparent. Complete loss of the Soret band occurs at concentrations of 2.0 M and 3.0 M. Concomitant with the decrease in the absorption is a blue shift of the maximum observed in the spectrum to 395 nm and further to 388 nm for 2.0 M and 3.0 M Gu-HCl respectively. The unfolding of modified myoglobin and the expulsion and aggregation of heme at higher concentrations of Gu-HCl are consistent with literature reports for the unmodified protein.[111,112,175]

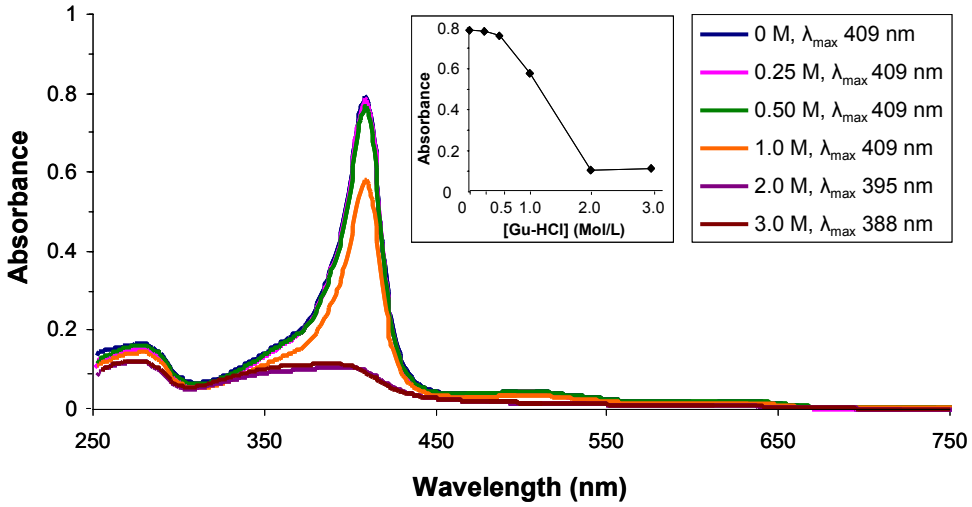


Figure 4.5 UV-Vis spectra of 4.2 µM PEO-myoglobin in aqueous solutions of Gu-HCl. (Inset) Absorbance versus concentration for peaks ~ 409 nm.

Protection of myoglobin by covalent modification with PEO resulted in an unexpected decrease in the protein stability in the presence of Gu-HCl. Comparison of the PEO-myoglobin results with those of the native protein in Section 3.3.1 (Figure 3.8) show that PEO modification caused a destabilisation of myoglobin in solutions containing 1.0 M Gu-HCl. This destabilisation is indicated by the decrease of the PEO-myoglobin Soret absorption as compared to the buffer solution of the modified protein. Native myoglobin in solutions of 1.0 M Gu-HCl did not exhibit a decrease in absorption compared to the buffer protein solution.

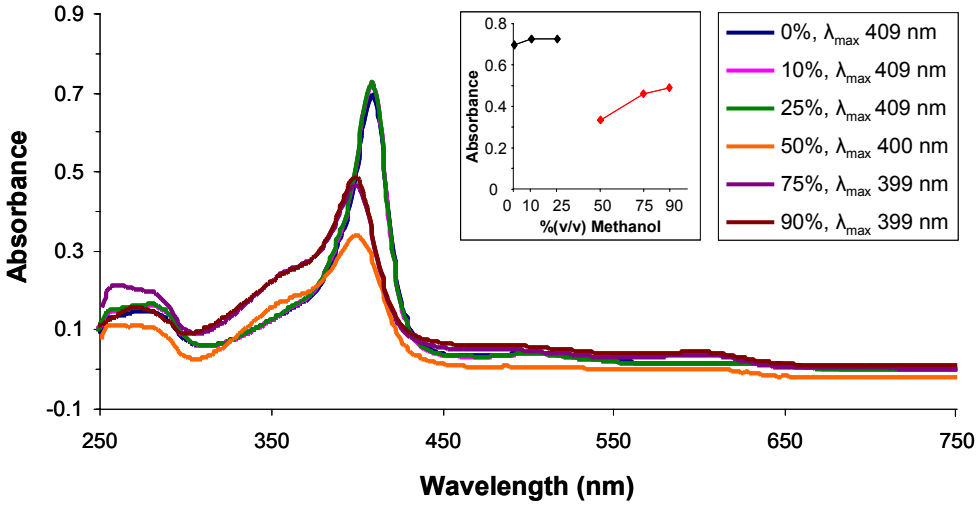


Figure 4.6 **UV-Vis spectra of 3.7 µM PEO-myoglobin in aqueous solutions of methanol (% v/v). (Inset) Absorbance versus concentration, black points: peak ~ 409 nm; red line: blue shifted peaks**

Methanol was also used to demonstrate denaturation of a 3.7 µM solution of PEO-myoglobin by a known denaturant (Figure 4.6). No significant unfolding was observed for the solutions of PEO-myoglobin containing up to 25% (6.3 M) methanol, there is also no shift in the λ_{max} at 409 nm compared to the modified protein in buffer. The spectra are similar for 50% (12.5 M), 75% (18.75 M) and 90% (22.5 M) methanol. The Soret band was blue shifted to 400 nm, 399 nm and 399 nm, which are the same within experimental error, for 50%, 75% and 90% methanol respectively, and is consistent with free heme in methanol (λ_{max} 398 nm) as previously reported for PEO-myoglobin in methanol by Wiwatchaiwong *et al.*[140] In addition, the absorptions at λ_{max} for each are nearly equivalent. These results indicate that with 50% methanol the maximum amount of unfolding has occurred and higher concentrations do not cause any further damage to the protein.

In comparing the UV-Vis spectra of the modified myoglobin with the unmodified protein spectra (Section 3.3.1, Figure 3.9) it is apparent that PEO modification again

causes destabilisation of the protein. The data presented in Figure 4.6 show that at 50% methanol the level of denaturation is similar to higher concentrations of methanol, whereas the unmodified protein in a solution containing 50% methanol exhibited only partial denaturation.

Fluorescence Studies of PEO-Myoglobin

Steady-state fluorescence was measured for the modified myoglobin solutions utilising tryptophan as the fluorophore. A reference solution of PEO-myoglobin in buffer (pH 7.0) was used for each set of measurements because the concentration of the protein varied slightly between sets of solutions. All spectra shown are an average of four measurements. The fluorescence measurements indicate that a solution of 4.1 μM PEO-myoglobin is significantly unfolded in the presence of IL 169 (Figure 4.7). At 10% (v/v) IL 169, there is no shift in the maximum wavelength (λ_{max} 328 nm) however significant unfolding occurs in comparison to the reference PEO-myoglobin solution in buffer as demonstrated by the increase in intensity for PEO-myoglobin in 10% IL 169. Increasing the amount of ionic liquid in solution to 25% produced the greatest fluorescence intensity and a shift of λ_{max} to 331 nm. PEO-myoglobin in 50% IL 169 demonstrated an emission intensity equal to that of 10% IL 169 however a red shift of the λ_{max} (from 328 nm in buffer to 335 nm in 50% IL 169) was observed. The fluorescence intensity was even less for 75% (λ_{max} 337 nm) and 90% (λ_{max} 338 nm) IL 169 accompanied by an additional red shift of λ_{max} indicating that at these amounts of ionic liquid the modified protein is less unfolded than the lower concentrations of IL 169.

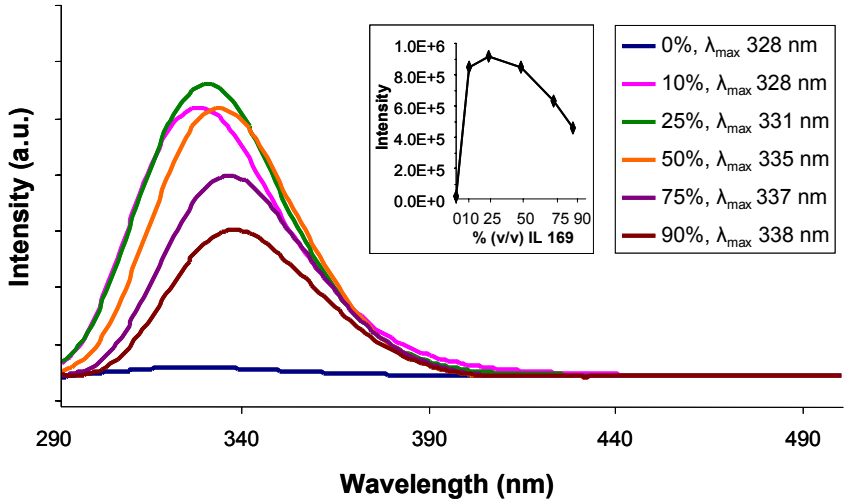


Figure 4.7 Fluorescence emission spectra of 4.1 µM PEO-myoglobin in aqueous solutions of IL 169 (% v/v). (Inset) Intensity versus concentration.

The fluorescence of the unmodified and PEO-modified protein demonstrates that both forms of the protein are significantly unfolded in aqueous solutions of IL 169. However, there is one difference between the two spectra: PEO-modified myoglobin, according to the fluorescence measurements, is less denatured by 10% IL 169 than 25%, which is in contrast to the unmodified protein (Figure 3.11, Section 3.3.1). As previously discussed, the fluorescence measurements are unreliable for specific unfolding trends due to solvent effects, which may account for the differences in observations between UV-Vis and fluorescence.

Fluorescence measurements were also performed for the solutions containing varying amounts of IL 106, but as described earlier (Section 3.3.1) IL 106 is not a useful medium for fluorescence measurements. The anion of IL 106 contains an aromatic ring which is believed to cause quenching of the tryptophan fluorescence.[115,176] This conclusion is based on the negative fluorescence peaks observed when the background

solution spectrum is subtracted from the protein solution spectrum. All fluorescence intensities were significantly less than the reference solution of PEO-myoglobin.

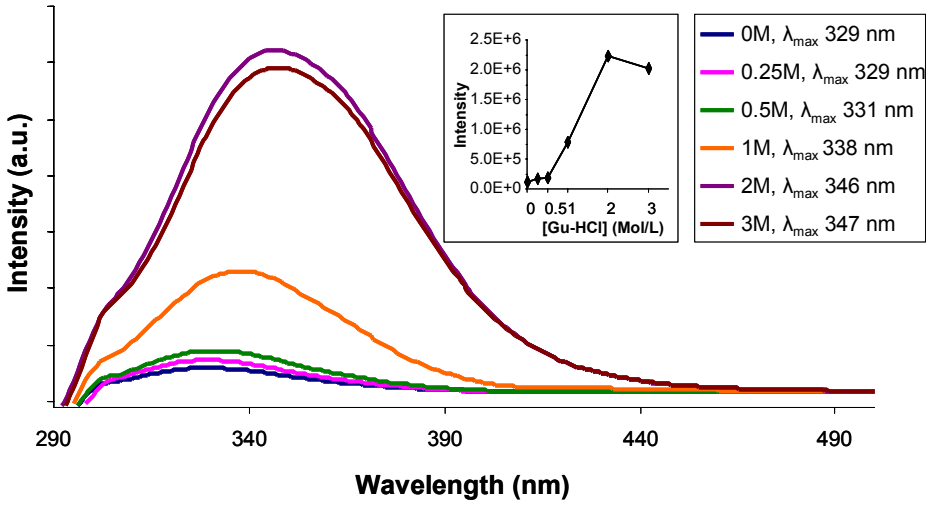


Figure 4.8 Fluorescence emission spectra of 4.2 µM PEO-myoglobin in aqueous solutions of Gu-HCl. (Inset) Intensity versus concentration.

Fluorescence measurements of Gu-HCl denaturation of 4.2 µM PEO-myoglobin (Figure 4.8) were measured for comparison with the solutions of PEO-myoglobin in ionic liquids. The reference solution of PEO-myoglobin in buffer (pH 7.0) shows the least emission and a λ_{max} at 329 nm indicating that the tryptophan residues are enclosed in a hydrophobic environment not accessible to the solvent molecules. Addition of 0.25 M Gu-HCl causes a small increase in the fluorescence intensity with no corresponding shift in λ_{max} (329 nm). The fluorescence of PEO-myoglobin with 0.50 M Gu-HCl is also very similar to the reference solution of PEO-myoglobin in buffer. Differences between the fluorescence of 0 M to 0.50 M Gu-HCl are minimal. An increase in emission for PEO-myoglobin in 1.0 M Gu-HCl is accompanied by a shift of λ_{max} to 338 nm, further indicating that the fluorophores are at least partially exposed to polar solvent molecules. Finally, concentrations of 2.0 M and 3.0 M Gu-HCl afford the greatest emissions as well

as the largest solvent-imposed red shifts (λ_{max} 346 nm and 347 nm, respectively) signifying the most unfolding of the protein. The conclusion that high concentrations (> 2.0 M) of Gu-HCl cause complete unfolding of the protein are consistent with literature reports for unmodified myoglobin,[111] but there are no previous reports of PEO-myoglobin denaturation by Gu-HCl.

Comparison of the fluorescence measurements of PEO-myoglobin and myoglobin (Figure 3.12, Section 3.3.1) in solutions containing Gu-HCl indicate that PEO-myoglobin is less stable toward the denaturant than unmodified protein. Consistent with the UV-Vis data, PEO-myoglobin in 1.0 M Gu-HCl exhibits partial denaturation, as demonstrated by a fluorescence emission intermediate between folded (0 M Gu-HCl) and completely denatured protein (3 M Gu-HCl). Whereas, myoglobin in 1.0 M Gu-HCl gave a fluorescence emission similar to the native, folded protein in buffer solution.

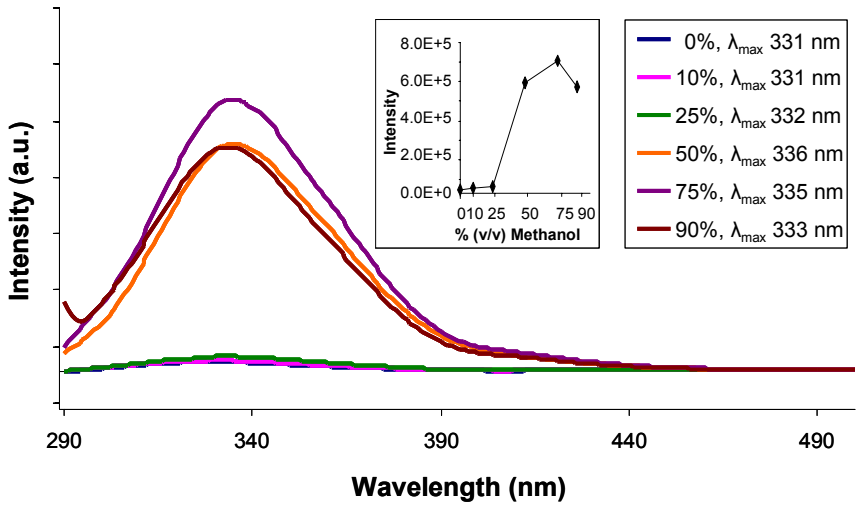


Figure 4.9 Fluorescence emission spectra of 3.7 µM PEO- myoglobin in aqueous solutions of methanol (% v/v). (Inset) Intensity versus concentration.

The fluorescence of PEO-myoglobin (3.7 µM) in solutions containing varying amounts of methanol (Figure 4.9) indicate that for up to 25% (v/v) methanol there is no

significant unfolding of the protein. The λ_{max} for 0%, 10%, and 25% methanol are 331 nm, 331 nm, and 332 nm respectively, implying that the tryptophan residues are not exposed to the polar solvent. At 50% methanol the emission intensity increases and a shift in λ_{max} to 336 nm occurs. Further unfolding of the protein occurs at 75% methanol as the maximum amount of observed unfolding occurs though no further shift in λ_{max} (335 nm) is seen from that of 50% methanol (λ_{max} 336 nm). PEO-myoglobin in a solution with 90% methanol exhibits a fluorescence intensity comparable to that of 50% methanol. However a smaller red shift of the maximum (λ_{max} 333 nm) from the reference PEO-myoglobin solution is observed.

Comparison of these data with the unmodified protein (Section 3.3.1, Figure 3.13) indicates that a greater amount of unfolding occurs at 50% methanol for PEO-myoglobin than for myoglobin. Additionally, there is a larger difference between the emissions observed for PEO-myoglobin in 75% and 90% methanol than for myoglobin. These results point towards destabilisation of PEO-myoglobin as compared to native myoglobin in aqueous solutions of methanol.

4.3.3 EPR Experiments in Phosphonium Ionic Liquids

The X-band EPR spectra of PEO-myoglobin-azide (PEO-MbN_3) in buffer and phosphonium ionic liquids at 77 K show that the modified protein behaves very differently from the unmodified protein (Figure 4.10). PEO-MbN_3 in buffer is dominated by a typical rhombic low-spin spectrum for MbN_3 (g-values = 2.78, 2.21, 1.72; indicated by •) but also shows contributions from other paramagnetic species. Two weak signals appear in the low-field region with g-values 6.02 (indicated by ◇) and 5.36 (indicated by ❖) due to high-spin Fe(III) species. An unidentified signal near g ≈ 2 appears to display

some partially resolved hyperfine interactions (Figure 4.11). The sharp signal at g = 2.00 is a background radical signal from the dewar (indicated by *).

An aliquot of the protein buffer solution in IL 101 at the same protein concentration as the solution in buffer produced an EPR spectrum (Figure 4.10) exhibiting a large signal with a g-value of 5.8 (indicated by ◇) characteristic of high-spin (S = 5/2) Fe(III) states of the protein[117] or free heme. Two smaller signals appear at g = 4.26 (△) and 3.67 (■, Figure 4.10). Non-heme iron in the high-spin Fe(III) state is expected at about g = 4.3, hence one of the signals can be identified as such; the other signal remains unidentified. The signal at g ≈ 2 was present in the original buffer spectrum and does not arise from the addition of PEO-MbN_3 to IL 101.

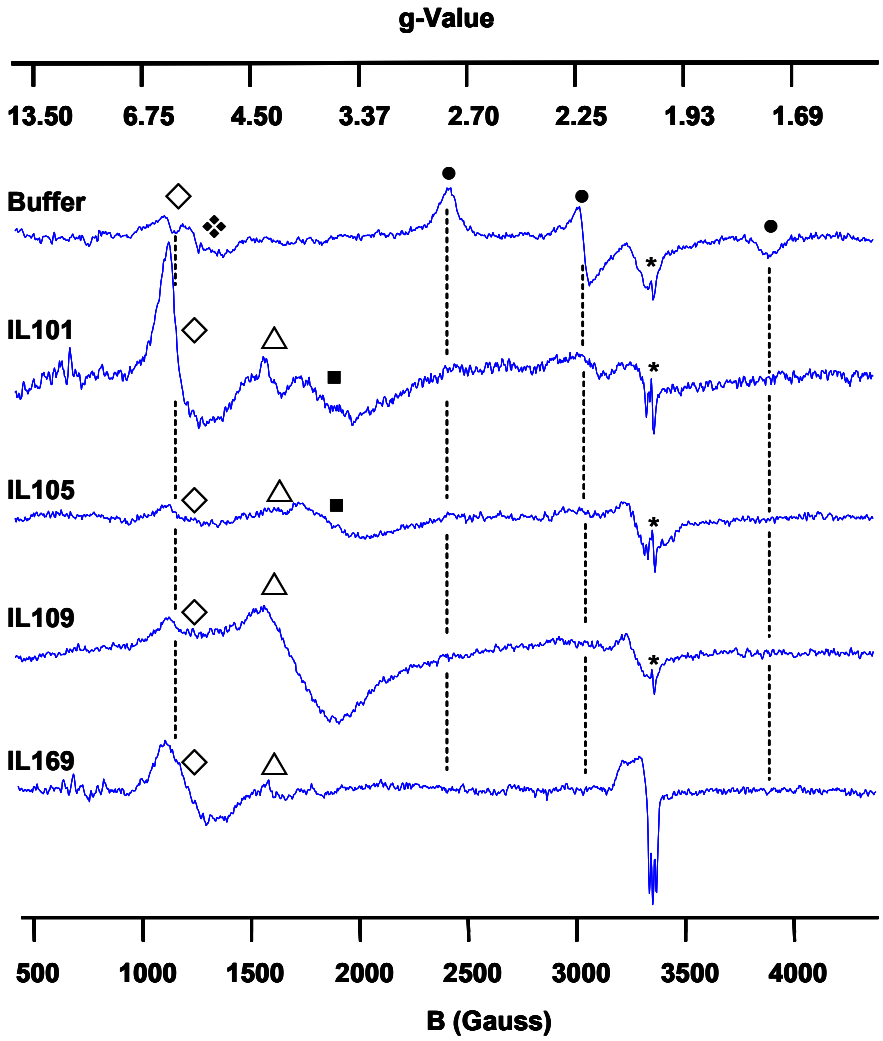


Figure 4.10 **X-Band EPR Spectra of 1 mM PEO-MbN_3 in buffer and neat ionic liquids at 77 K. (*Experimental parameters*) Microwave frequency = 9.4 GHz, modulation amplitude = 8 G, microwave power = 2.00 mW, receiver gain = 1×10^4, time constant = 40.96 ms, scan time = 40.96 s, average of 10 scans.**

The spectrum of PEO-MbN_3 in IL 105 (Figure 4.10) exhibits similar species to that of IL 101 but in different relative amounts. For example, in IL 105 the high-spin Fe(III) signal at g = 5.8 (indicated by ◇) is much more comparable in size to that at g = 3.6 (■) than for the analogous signals in IL 101. There is also present the species with g-value ≈ 2 from the original buffer solution. The rhombic low-spin spectrum was not observed in this ionic liquid solution.

In contrast, modified myoglobin in IL 109 (Figure 4.10) exhibits a large, broad signal near g = 4.3 (indicated by △) with a linewidth of 350 G which is the main feature of the spectrum with a much smaller signal in the high-spin Fe(III) region (g = 5.8, ◇). The signal at g ≈ 2 observed in the other solutions is also present however with no apparent low-spin azide spectrum.

PEO-myoglobin-azide in IL 169 produced two significant paramagnetic species (Figure 4.10). A high-spin species similar to that found in the other ionic liquid solutions appears at g = 5.8 (indicated by ◇) and a species previously identified in the unmodified protein EPR spectrum as heme-NO is centred at a g-value of 2.01 with nitrogen hyperfine coupling constant a_N = 16.3 G, which is comparable to the previously observed heme-NO adduct (a_N = 16.8 G) (Figure 3.17).

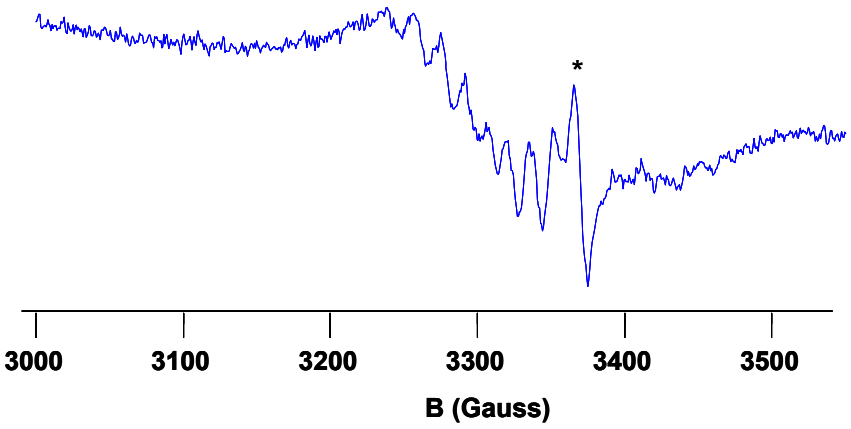


Figure 4.11 Expanded EPR spectrum of PEO-MbN_3 in buffer or ionic liquids in the g = 2 region showing 9-line spectrum at 77 K. (* denotes free radical impurity in dewar). (*Experimental parameters*) Microwave frequency = 9.446 GHz, modulation amplitude = 8 G, microwave power = 2.00 mW, receiver gain = 2 × 10^4, time constant = 40.96 ms, scan time = 40.96 s, average of 20 scans.

A closer inspection of the paramagnetic species at g ≈ 2 reveals a 9-line spectrum that is not fully resolved (Figure 4.11) with an average hyperfine coupling constant of

14 G. The relatively large derivative signal to the right (indicated by *) is a background radical signal from the dewar. A 9-line spectrum would be produced in the case of a porphyrin-based radical in which four nitrogen (I = 1) atoms were interacting with the unpaired electron. However, literature accounts of a porphyrin π-cation radical are not consistent with the observed spectrum since the coupling constants for these radicals range between 1.4 G to 1.6 G.[177,178] The occurrence at g ≈ 2 suggests that the species is an S = ½ system and is probably an organic-based rather than metal-based radical. An extensive review of the literature provided no comparable EPR spectrum.

4.4 Discussion

Covalent modification with polymeric molecules such as poly(ethylene oxide) (PEO) has been well established in the literature as a method for increasing solubility and stability of proteins and other biomolecules.[179-183] Heme proteins such as hemoglobin[180,181], myoglobin,[140,171,173,179,181] cytochrome *c*[184,185] and cytochrome *P*-450[182] have previously been modified with PEO chains of varying lengths and studied for their redox properties and have been used in biosensors, bioreactors, and biochips. These studies indicated that the modification of the protein did not affect the redox properties but increased solubility and stability in electrolytic solvents while preventing denaturation. PEO modification of myoglobin in this study was applied to protect the protein from denaturation in ionic liquids by providing a protective layer between the ionic liquid ions and the protein. UV-Vis and fluorescence spectroscopic methods were used to analyse the effectiveness of the modification against denaturation by ionic liquids as well as common denaturants.

4.4.1 Stabilisation of Myoglobin by PEO Modification Studied by UV-Visible and Fluorescence Spectroscopy

PEO-Myoglobin in Aqueous Solutions of Ionic Liquids

In general, the addition of PEO to myoglobin resulted in improved stability of the protein in aqueous solutions of ionic liquids. A comparison with the unmodified protein indicates that, as expected, covalent modification of myoglobin by PEO enhances its stability in higher concentrations of IL 169 and IL 106.

The blue shift of the Soret band in IL 106 for PEO-myoglobin from the original PEO-myoglobin in buffer solution, as previously mentioned for the unmodified protein, could be due to the free heme or a change in the heme coordination environment, such as loss of the distal water ligand.[138-140] Wiwatchaiwong *et al*.[140] found that PEO-myoglobin dissolved in liquid PEO_{200} (a PEO polymer with an average molecular weight of 200 g/mol) resulted in the loss of the distal water ligand as demonstrated by UV-Vis and Raman spectroscopies. Further studies, such as Raman spectroscopy, could determine the coordination environment of the heme, specifically the identity of the distal ligand.

PEO-Myoglobin in Aqueous Solutions of Guanidine-Hydrochloride and Methanol

Spectroscopic studies of PEO-modified myoglobin with denaturing agents such as guanidine-hydrochloride and methanol were performed in order to demonstrate whether the modified protein was protected against common denaturants. UV-Vis and fluorescence studies, however, revealed that the modification did not protect myoglobin from being denatured, but destabilised the protein in comparison to unmodified myoglobin.

UV-Vis and fluorescence studies of modified-myoglobin showed that lower concentrations of denaturants (1.0 M Gu-HCl or 50% methanol) caused greater denaturation than for the unmodified protein. These findings indicate that perhaps modification of the protein by PEO polymer chains leads to an increase in the interactions between Gu-HCl or methanol and the protein which may lead to increased denaturation of the protein at lower concentrations of denaturant. Denaturation of PEO-modified proteins by Gu-HCl has not been previously reported in the literature. UV-Vis of the denaturation of PEO-myoglobin in anhydrous methanol was previously shown to be consistent with that of free heme,[140] which is consistent with the results presented in this work.

4.4.2 Stabilisation of Myoglobin by PEO Modification Studied by EPR Spectroscopy

The EPR of PEO-modified myoglobin-azide in buffer and ionic liquids demonstrates that the modification procedure does change the behaviour of the protein. Examining the spectrum of PEO-myoglobin in buffer indicates that a significant amount of porphyrin has been released from the protein even before introduction into the ionic liquids. Lyophilisation was used to dehydrate the samples for storage. It may be the process of lyophilisation that caused the damage to the protein as evidenced by the partially resolved signal near g = 2 in the buffer solution. A literature precedent has been established for denaturation of proteins during the lyophilisation process.[39,40] Prevention of the structural damage to proteins may be achieved in future experiments by using a lyoprotectant (such as sugars) that is co-lyophilised with the protein to minimize the impact of the freeze-drying method.[32] The occurrence of an EPR signal near g = 4.3

indicates the presence of non-heme iron in the ferric oxidation state.[158] The iron signal at g = 4.3 is additional evidence that the protein has been aggressively denatured and stripped of the metal ion.[186] Furthermore, literature reports of EPR studies of freeze-dried hemoglobin has shown that a signal at g = 4.3 occurs after the lyophilisation process indicating degradation of the heme.[187]

Interestingly, the signal at g = 4.3 is most evident for IL 101, which was not expected to cause the greatest amount of denaturation based on the data obtained for unmodified myoglobin in the ionic liquids. An EPR spectrum was unable to be obtained for PEO-myoglobin in IL 106 due to unavoidable instrumentation issues. Absent from the spectra involving PEO-myoglobin-azide in ionic liquids is a discernable rhombic low-spin signal arising from the azide species, indicating that the modified protein is most likely significantly denatured in the ionic liquids. These findings are in contrast those of Ohno *et al.*[184] who found that PEO-modified cytochrome *C* was easily dissolved in imidazolium ionic liquids and remained redox active. Other reports of modified proteins in ionic liquids do not address denaturation.[182,188] The high-spin axial signal observed in the EPR of PEO-myoglobin in the ionic liquids may arise from either free iron-heme[152] or a 5-coordinate myoglobin species,[139] as previously discussed (Section 3.4.1).

A reaction between azide and PEO is unlikely and would not be consistent with the observation of a rhombic PEO-myoglobin-azide EPR signal in buffer, which is not observed in the spectra in ionic liquids. To explain this, it may be proposed that the activated PEO is reacting in some way with the ionic liquid, but this seems unlikely since phosphonium ionic liquids have been demonstrated to be chemically stable solvents.[4] It is more likely that the azide, activated PEO, and ionic liquids are all involved in the

denaturation of PEO-myoglobin-azide as observed in the EPR spectra, since UV-Vis measurements showed no indication of a reaction between the ionic liquid and the modified protein.

The studies of PEO-modified myoglobin with common denaturants suggest that the PEO modification may facilitate denaturation of the protein through increased interaction of the denaturant with the modified protein. However, PEO modification appears to protect the protein from complete denaturation at higher concentrations of ionic liquids compared to the unmodified protein. Future work regarding PEO-modified myoglobin should include the reactivity of the modified protein and comparison to the reactivity of unmodified myoglobin.

5 SUMMARY AND FUTURE DIRECTIONS

The goal of this thesis was to demonstrate that phosphonium ionic liquids could be useful reaction media for biocatalysis. The research was broken into four sections: first, preparation of new biocompatible phosphonium ionic liquids; second, characterisation of myoglobin in ionic liquids; third, biocatalysis by myoglobin in the ionic liquids; and fourth, protection of the protein by covalent modification.

New phosphonium ionic liquids were prepared using two general methods: silver-salt metathesis and acid-base neutralisation. It was shown that silver-salt metathesis provided the greatest amount of anion exchange. In addition, this thesis reveals that preparation of ionic liquids by sodium-salt metathesis is limited. Indeed, the results of this thesis demonstrate that new ionic liquids are best prepared using soluble silver salts.

The work presented in this thesis demonstrates the potential of phosphonium ionic liquids for biocatalysis. This report is the first account of a myoglobin-catalysed reaction in ionic liquids. Studies of myoglobin in commercially available phosphonium ionic liquids demonstrated that denaturation of the protein occurs. Despite this, the results of reactivity studies show that the use of these ionic liquids for biocatalysis is not only feasible but can promote distinct reactivity compared to organic solvents. Biocatalytic oxidation of dibenzothiophene was shown to yield varying amounts of total conversion depending on the water to ionic liquid ratio. Furthermore, different ratios of oxidation products were observed for two water-miscible ionic liquids, IL 106 and IL 169. Since spectroscopic studies of myoglobin in hydrophobic ionic liquids IL 101, IL 105, and IL

109 indicate less denaturation than in the hydrophilic ionic liquids IL 106 and IL 169, further studies of the catalytic activity of myoglobin in the hydrophobic ionic liquids is expected to show improved conversion of dibenzothiophene as compared to IL 106 and IL 169. However, owing to the immiscibility of the hydrophobic ionic liquids with water, new experimental protocols will be required.

Modification of myoglobin by poly(ethylene oxide) was shown to destabilise the protein toward common denaturants guanidine-hydrochloride and methanol. In the ionic liquids, however, the modification was shown to protect the protein and demonstrated improved stability, especially at higher ionic liquid concentrations. Further studies of PEO-myoglobin reactivity in the ionic liquids could demonstrate whether the modification has an effect on the reactivity of the protein.

Future studies could include extension of these experiments to other heme proteins such as hemoglobin and cytochrome *c*. Hemoglobin is structurally similar to myoglobin, being essentially a tetramer of myoglobin-like monomers, hence these studies could easily be expanded to hemoglobin. A more catalycially capable heme enzyme, cytochrome *P-450*, could also be studied for this reaction since its catalytic mechanism contains a peroxide shunt. Furthermore, various heme proteins have been reported to mediate the oxidation of dibenzothiophene,[97] hence a comparison of the reactivities of the proteins in ionic liquids could be made.

Since the results of this thesis indicate that phosphonium ionic liquids are suitable reaction media for biocatalysis, more industrially-applicable enzyme-mediated reactions should be explored to determine whether enhanced reactivity or selectivity of enzymes such as α-chymotrypsin or lipases could be achieved. Ionic liquids are known for their

ease of catalyst recycling. Development of appropriate procedures for enzyme recovery and recycling may advance the future use of phosphonium ionic liquids as reaction media for biocatalysis.

APPENDICES

Appendix A: Electron Paramagnetic Resonance

Due to the intrinsic spin angular momentum ("spin") of electrons they have magnetic moments. The magnetic moment of a single electron, μ_z, due to spin is given by:

$$\mu_z = g_e \beta_e m_s$$

Where g_e is the free electron g-value, β_e is the electronic Bohr magneton, and m_s is the magnetic spin quantum number for a single electron. The Zeeman effect describes the interaction of the electron's magnetic moment with an applied magnetic field, B_o. The energy of this interaction is given by:

$$E_{Zeeman} = -\mu_z B_o = g_e \beta_e m_s B_o$$

This forms the basis of magnetic resonance spectroscopy, where the energy level separation is probed by radiation of a frequency that satisfies the resonance condition:

$$\Delta E = h\nu = g_e \beta_e B_o$$

h is Planck's constant and ν is the frequency in Hz; g_e and β_e are defined as above. The effect of the Zeeman interaction on the energy levels is shown in Figure A.1.

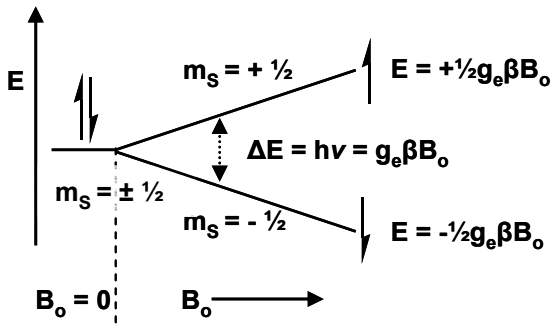


Figure A.1 Energy level diagram as a function of applied magnetic field showing electronic Zeeman interaction for a single free electron (m_s = ±1/2). In the absence of an applied magnetic field (B_o) the energies of the states are degenerate, however on application of B_o the degeneracy is removed and states of different energies are observed.

In EPR spectroscopy, the frequency is held constant as the magnetic field is swept, hence the measurable parameter is the magnetic field at which transitions occur. Similar to NMR where chemical shift is used to denote the position of a resonance independent of the magnitude of the applied magnetic field, the g-value is used in EPR to describe the position of a resonance independent of the frequency of the instrument. The observed g-value is calculated using the instrument frequency and the measured magnetic field, B_o:

$$g = \frac{h\nu}{\beta_e B_o}$$

The local field felt by an unpaired electron varies due to interactions with the local environment and this is quantified in terms of the g-value. The amount that this varies from the free electron g-value, g_e, reflects how much the local field differs from the applied field due to the effects of spin-orbit coupling and the local ligand field.

Differences in the symmetry of the local environment of an unpaired electron yield three cases: isotropic, in which the system is symmetric in the x, y, and z-directions; axial, where two directions (x and y) are equivalent; and rhombic, in which each direction

is unique. These symmetries give rise to the three different powder EPR patterns shown in Figure A.2.

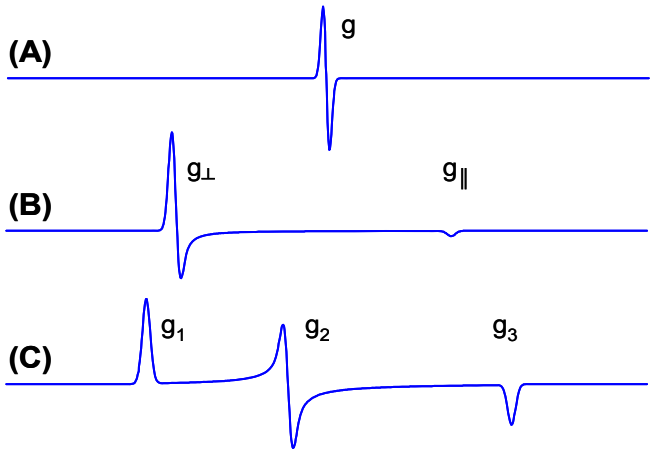


Figure A.2 EPR spectra observed for three local symmetry cases: isotropic, axial, and rhombic. (A) Isotropic symmetry, $g_x = g_y = g_z$; (B) Axial symmetry, $g_x = g_y \neq g_z$ and $g_\perp$ corresponds to $g_x = g_y$, $g_\parallel$ corresponds to g_z; (C) Rhombic symmetry, $g_x \neq g_y \neq g_z$, typically denoted g_1, g_2 and g_3.

In addition to the Zeeman effect, unpaired electrons can interact with other unpaired electrons and spin-bearing nucei. The effect of an interaction between an unpaired electron and an $I = 1/2$ nucleus on the energy levels is shown in Figure A.3. Interactions of electrons with nuclei are known as hyperfine (HF) interactions and are observed as extra splittings in the EPR spectrum (shown as solid-arrow transitions in Figure A.3). Interactions between unpaired electrons, known as zero-field splittings, often lead to large shifts in the observed g-values. Individual energy levels for a system showing these interactions can be described by an equation of the form:

$$E = M_S g_e \beta_e B_o + M_I g_N \beta_N B_o + A M_S M_I + (D M_S^2)$$

For a given system, S is the effective spin and M_S is the spin magnetic quantum number. In the nuclear Zeeman term, M_I is the nuclear magnetic quantum number, and g_N and β_N are the nuclear g-factor and nuclear magneton, respectively. The third term of the

equation describes the hyperfine interaction where A is the hyperfine coupling constant describing the strength of the interaction. The last term in brackets, zero-field splitting, is only required for S > 1/2.

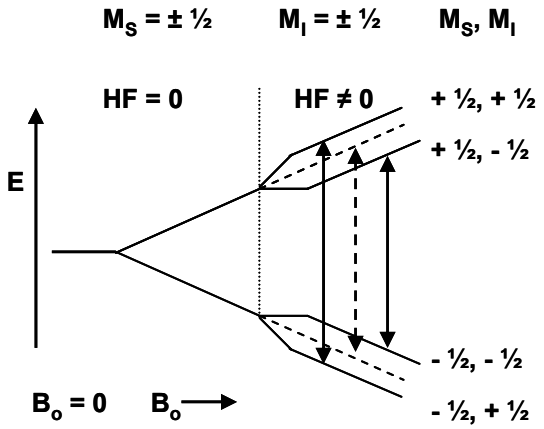


Figure A.3 Energy level diagram as a function of applied magnetic field (B_o) showing electronic Zeeman interaction for an electron (S = 1/2, $M_S = \pm 1/2$) and additional splitting of energy levels for an interaction with a nucleus ($I = 1/2$, $M_I = \pm 1/2$). In the absence of an applied magnetic field (B_o) the electron spin states are degenerate, however on application of B_o the degeneracy is removed and states of different energies are observed. Dashed arrow indicates allowed transition for an electron. Solid arrows indicate transitions for an electron interacting with a nucleus. Each transition is observed as a peak in the EPR spectrum.

Myoglobin, the metalloprotein studied in this research, contains iron. When oxidised (met-myoglobin), all iron is in the Fe(III) state (d^5), which allows for the protein to be studied by EPR spectroscopy since it is paramagnetic. Typically, iron in myoglobin is in an octahedral crystal field therefore, depending on the strength of the ligands, either a high-spin (S = 5/2) or low-spin (S = 1/2) electron configuration is expected (Figure A.4). High-spin S = 5/2 Fe(III) spectra are typically of axial symmetry, while the low-spin spectra are usually rhombic.

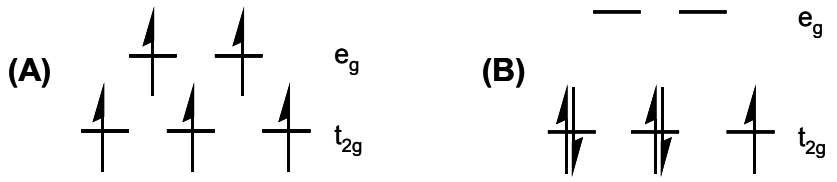


Figure A.4 d-orbital splitting for Fe(III) in an octahedral crystal field. (A) High-spin, S = 5/2; (B) Low-spin, S = 1/2.

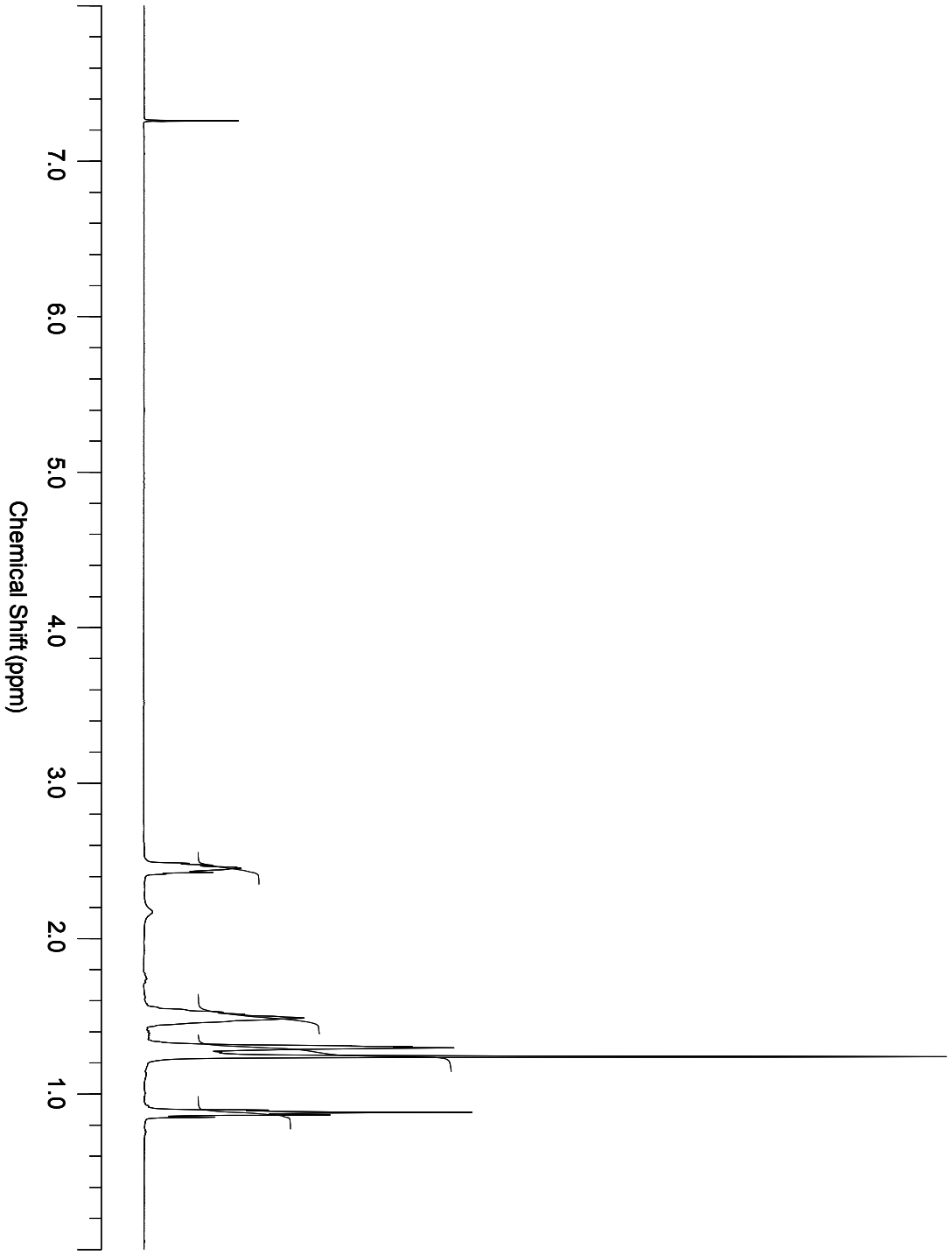
Another important difference between these species is their usual g-values. Low-spin Fe(III) generally shows g-values comparatively close to the free electron value, that is, around 2. By contrast, due to the effects of zero-field splitting, high-spin Fe(III) shows a $g_{\perp}$ dramatically shifted from this region at around g = 6, and $g_{\parallel}$ at g = 2. Thus, it is straightforward to distinguish between the two cases.

Appendix B: NMR Spectroscopy Parameters

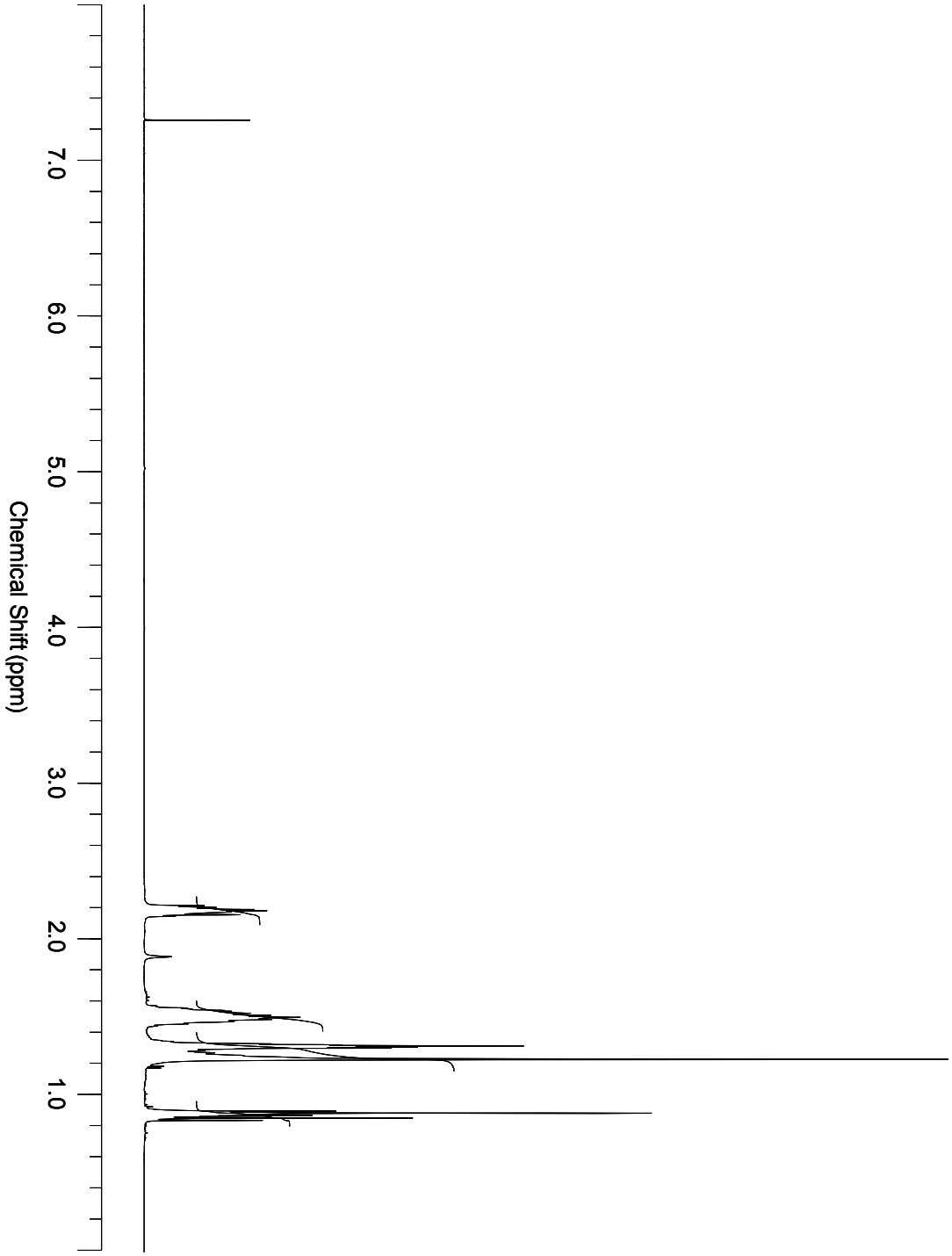
Spectrometer	Bruker AVANCE II 600 MHz					Varian Inova 500 MHz
	BBO		CPTCI	CPQNP		
	^{31}P	^{31}P	^{13}C	^{1}H	^{31}P	^{1}H
Frequency (MHz)	242.94	242.94	150.95	600.13	242.93	499.77
^{1}H Decoupling Sequence	waltz16	N/A	waltz16		waltz16	
Pulse Sequence	zgpg	zg30	zgpg	zg30	zgpg30	s2pul
Number of Points	98034	262144	131072	131072	65536	15128
Sweep Width (ppm)	201.9	3920.3	238.9	14.03	395.8	16.01
Recycle Delay (D1) (s)	3	2	2	2	2	1
Offset (O1) (ppm)	50.0	-10.0	100.0	5.50	-50.0	6.00
^{1}H Decoupling Offset (O2) (Hz)	2400.5	-12146.89	2401.3		2400.5	
90° Pulse (p1) (μs)	13.8	13.8	11	15.9	11	5.8

Appendix C: ^{1}H NMR Spectra of Cytec Ionic Liquids

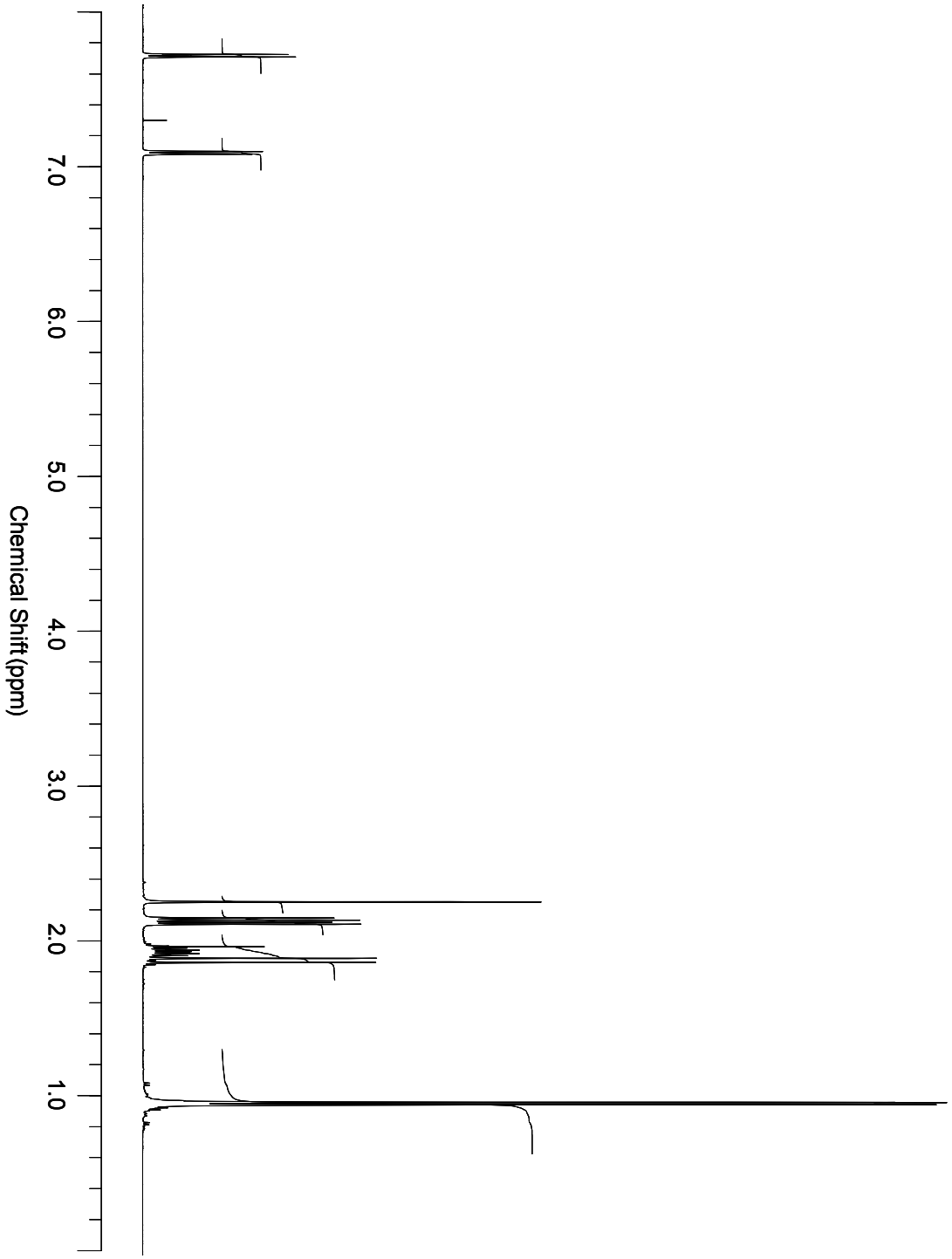
IL 101: Tetradecyl(trihexyl)phosphonium Chloride



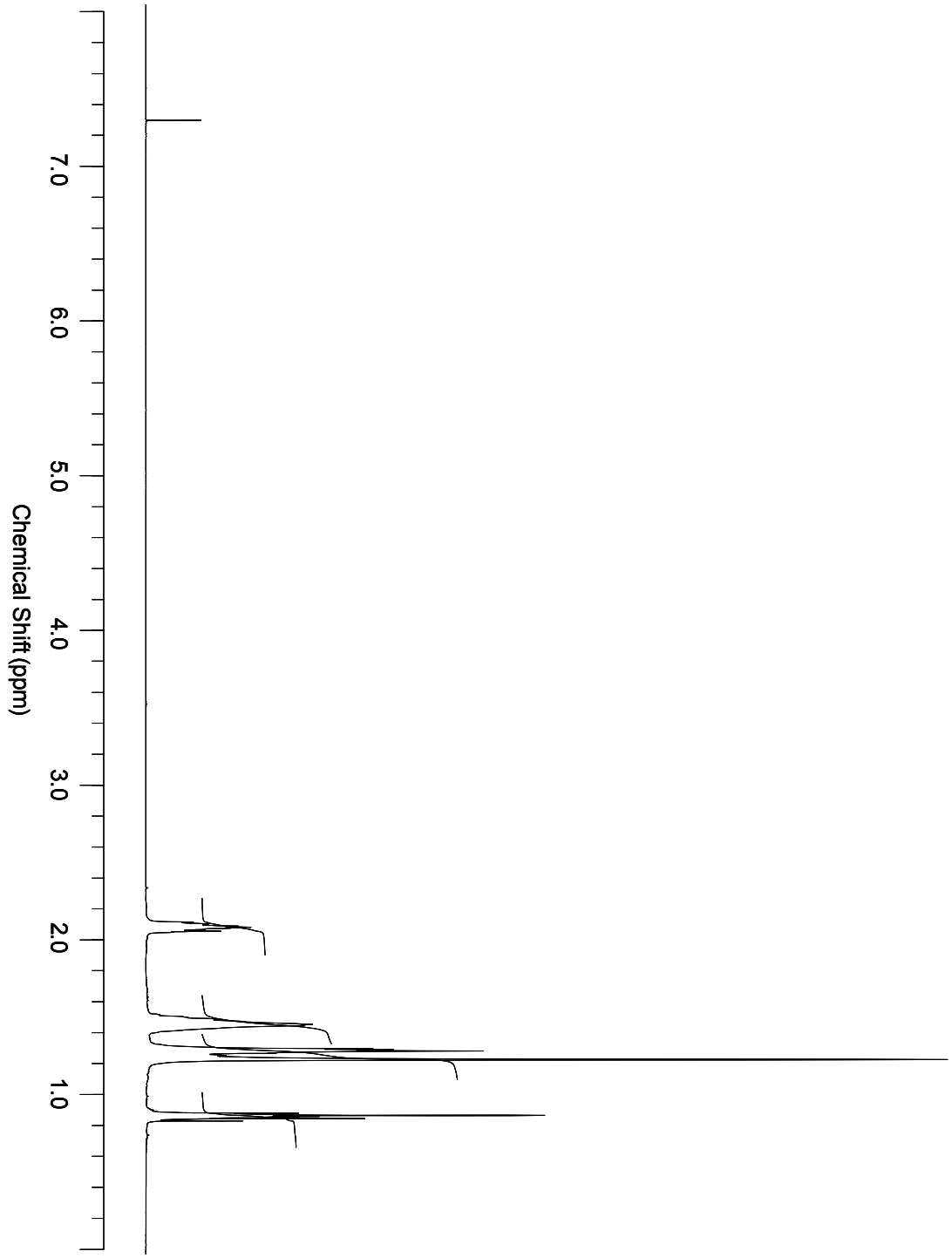
IL 105: Tetradecyl(trihexyl)phosphonium Dicyanamide



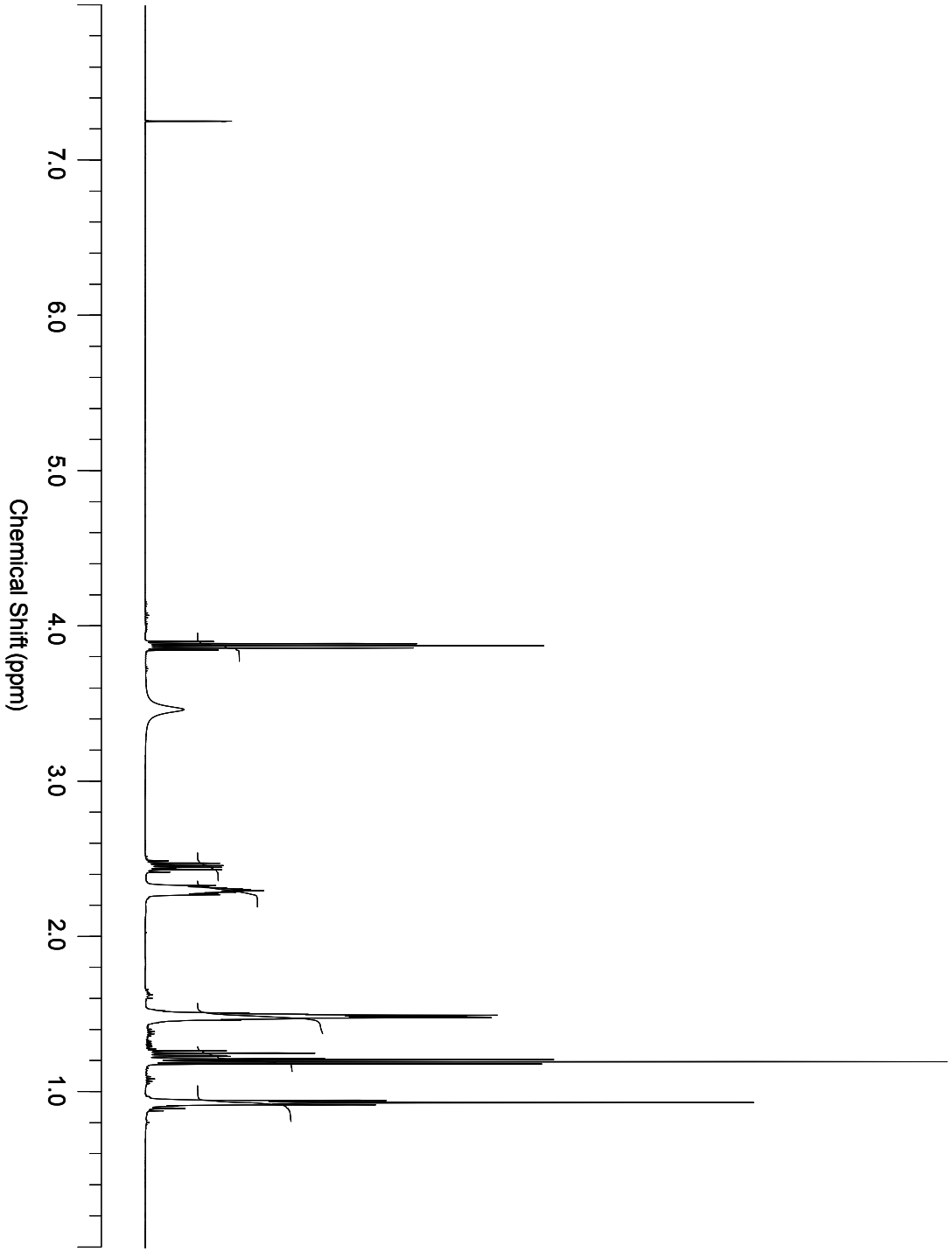
IL 106: Methyl(triisobutyl)phosphonium *p*-Toluene Sulfonate



IL 109: Tetradecyl(trihexyl)phosphonium Bistriflamide



IL 169: Ethyl(tributyl)phosphonium Diethylphosphate



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