Dissolution of Fibrous Protein in Ionic Liquids

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Dissolution of Fibrous Protein in Ionic Liquids

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Errata

p 29 2nd column, line 4: Add "400 MHz and 100 MHz, respectively."

p 94 line 16: Change "400 MHz" to "100 MHz"

p 104 2nd column, line 32: Add "400 MHz and 100 MHz, respectively."

p 112 line 10 and 13: Change "400 MHz" to "100 MHz"

General Declaration

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Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with the Monash University Doctorate Regulations 17.2 Doctor of Philosophy and master of Philosophy (MPhil) regulations, the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original papers published in peer reviewed journals, 1 submitted unpublished publication. The core theme of the thesis is 'Dissolution of Fibrous Proteins in Ionic Liquids". The ideas, developments and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Chemistry under the supervision of Professor Douglas R. MacFarlane and Assoc. Prof. Anthony F. Patti.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledgements input into team-based research.

My contribution to the work involved the following:

CHAPTER No	PUBLICATION TITLE	PUBLICATION STATUS	NATURE AND EXTENT OF CANDIDATE'S CONTRIBUTION
2	Dissolution of feather keratin in ionic liquids.	Published in Green Chemistry.	Initiation, key ideas, performed the experiments, synthesis, data analysis and interpretation, manuscript development and writing up. Contribution: 90 %
3	Dissolution and regeneration of wool keratin in ionic liquids.	Published in Green Chemistry.	Initiation, key ideas, performed the experiments, synthesis, data analysis and interpretation, manuscript development and writing up. Contribution: 90 %
4	Thermoplastic materials from the dissolution of feather keratin biopolymer in ionic liquids.	Submitted to Polymer.	Initiation, key ideas, performed the experiments, synthesis, data analysis and interpretation, manuscript development and writing up. Contribution: 90 %
5	Distillable protic ionic liquids for keratin dissolution and recovery.	Published in ACS Sustainable Chemistry and Engineering.	Initiation, key ideas, performed the experiments, synthesis, data analysis and interpretation, manuscript development and writing up. Contribution: 90 %

I have ordered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: 

…… ……

Azila Mohd Idris

School of Chemistry

Monash University

Date: 18 June 2014

I dedicate this thesis to
my family for their unconditional love
and endless support.
I love you all dearly.

TABLE OF CONTENTS

ABSTRACT

Keratin, one of the most abundant natural biopolymers, can be obtained from different sources such as feather, wool, hair, nail and horn. Due to the generation of large amounts of these biopolymers as a waste from textile, livestock and slaughter industries, it is of significance to recover these by-products and convert them into useful biomaterials. Organic solvents have been investigated in the past to dissolve keratin based products, but with limited success. Recently, ionic liquids have been used as an alternative to conventional organic solvents in biomass dissolution due to their unique physical and chemical properties; in this study, task specific ionic liquids have been developed and investigated as solvents for keratin based biopolymers (turkey feather and wool). Imidazolium (1-allyl-3-methylimidazolium chloride ([AMIM]Cl), 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-allyl-3-methylimidazolium dicyanamide ([AMIM][DCA])), and choline based ionic liquids ([choline][thioglycolate] and [bis-(2-ethylhexyl)][thioglycolate]) were synthesized, characterized and tested for feather and wool solubilisation. Apart from these aprotic ionic liquids, protic ionic liquids (such as hydroxyl ammonium based ionic liquids (eg dimethylethanolammonium formate (([DMEA][formate])) were also synthesized and employed in the dissolution of keratin. Overall, the most effective, were the imidazolium based ionic liquids containing dicyanamide or chloride anions, showing a significant solubility (up to 45 wt. % for feather and up to 20 wt. % for wool) for keratins. The dissolved

keratins were regenerated by precipitation from water and characterised by spectroscopic methods.

The influence of reducing agents and deep eutectic mixtures on keratin solubility was also investigated in this study. The addition of reducing agents to the ionic liquids increased the amount of dissolved keratin by 50-100 mg g^{-1}. Deep eutectic solvent mixtures were shown to be a potentially less expensive alternative solvent for dissolution of keratin biopolymers, however there was no significant increase in keratin solubility compared to ionic liquids.

The next stage of the study involved the production of regenerated keratin bio-materials in the form of films, fibers and gel. This process could form the basis of a commercial process. The method employed in this study involved the dissolution of keratin, casting the dissolved keratin onto a Teflon plate, soaking the plates into water to diffuse out the ionic liquid and drying the material for further mechanical testing.

The recycling of the protic ionic liquid was also investigated. After the dissolution and regeneration of the keratin biopolymer, the residual IL was recovered by distilling the mixture to remove the solvent. The ^{1}H NMR spectra of the protic ionic liquids before and after distillation showed no structural change which indicates the potential for recyclability of these materials. The distilled ionic liquids showed high yield and purity (~99% recovered).

ACKNOWLEDGMENTS

It is a pleasure to acknowledge all of individuals who have contributed towards completion of this thesis.

Above all, I would like to express my deep and sincere gratitude to my supervisors, Prof Doug Macfarlane and Assoc. Prof. Dr. Tony Patti for their excellent supervision and endless assistance over the whole duration of this research. Their expertise and wide knowledge have been of great value for me. Without their guidance and persistent help, this dissertation would not have been possible.

My appreciation also goes to Monash University Institute of Graduate Research (MIGR), Faculty of Science and School of Chemistry for providing a conducive working environment and well equipped laboratory facilities throughout the years.

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Thank you to my friends in Monash University and University of Malaya for being great friends and providing distractions outside my studies. Thank you for all the moments filled with love, care, laughter and tears.

Finally, none of this would have been be possible without the love of my parent and siblings. Their endless encouragement, concern and support throughout the whole period are much appreciated. There are no words to convey how much I love each and every one of them.

ABBREVIATIONS

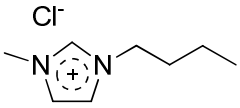
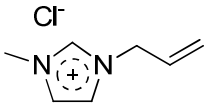
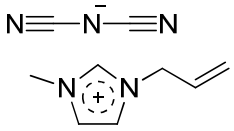
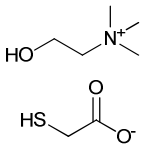
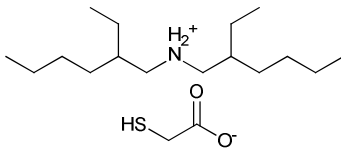
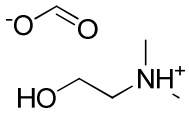
Instrumental Abbreviations:

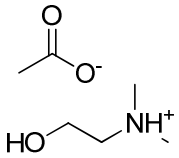
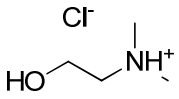
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon Nuclear Magnetic Resonance
MAS	Magic Angle Spinning
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy
PXRD	Powder X-ray Diffraction
TGA	Thermogravimetric Analysis
DSC	Differential Scanning Calorimetry
ESI-MS	Electrospray Ionisation Mass Spectrometry
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Acronyms and symbols:

FWHM	Full Width at Half Maximum
$CDCl_3$	Deuterated chloroform
CH_3CN	Acetonitrile
DMSO	Dimethyl sulfoxide
D_2O	Deuterium oxide
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublets of doublets

Eq	Equivalent
H_2O	Water
ILs	Ionic liquid(s)
J	Coupling constant
M	Mole per liter
m	Multiplet
min	Minute
mL	Milliliters
MW	Molecular weight
s	Singlet
t	Triplet
υ	Infrared absorption
δ	Chemical shift
wt %	Weight percent

Abbreviation	Full name	Structure
[BMIM]Cl	1-butyl-3-methylimidazolium chloride	
[AMIM]Cl	1-allyl-3-methylimidazolium chloride	
[AMIM][dca]	1-allyl-3-methylimidazolium dicyanamide	
[choline][thioglycolate]	[choline][thioglycolate]	
[bis-(2-ethylhexyl) ammonium][thioglycolate]	[bis-(2-ethylhexyl) ammonium][thioglycolate]	
[DMEA][HCOO]	2-hydroxy-*N*,*N*-dimethylethanaminium formate or dimethylethanolammonium formate	

Abbreviation	Full name	Structure
[DMEA][CH_3COO]	2-hydroxy-*N,N*-dimethylethanaminium acetate or dimethylethanolammonium acetate	
[DMEA]Cl	2-hydroxy-*N,N*-dimethylethanaminium chloride or dimethylethanolammonium chloride	

Chapter 1

Introduction

INTRODUCTION

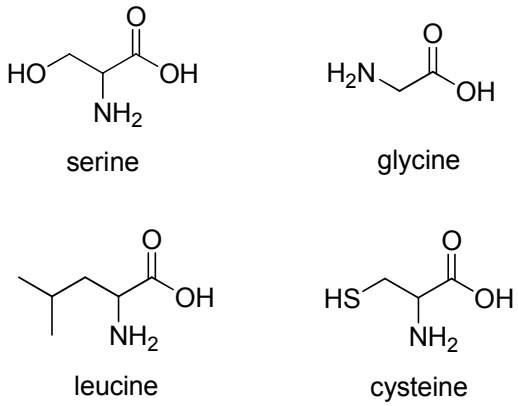
1.1 Keratin Biopolymers

Today, many of the world's consumer products are derived from crude oil. Concern about rising oil prices, reduction of crude oil reserves and the deposition of accumulated waste disposal has stimulated research on ways to substitute fossil fuel derived products with renewable bio-based materials. Most of the current research focuses on processing cellulosic fibers into useful products, due to abundant waste materials being available from processing tree and plant matter. Very few studies have focused on protein-based bio-waste, despite the large amount of waste from industries using such materials. One of the most abundant and renewable biomass materials in this class is the protein keratin.

Keratin is a fibrous protein that can be found in feather, wool, hair, nail, horn and animal claws.[1,2] Keratin from wool and feather is currently one of the most studied protein biomass materials. Large amounts of non-spinnable wool fiber are discarded as waste in the textile industry, while feather is abundantly available as waste from poultry processing.[3-6] Waste wool and feather are both difficult to degrade in natural conditions, and their accumulation is causing environmental and ecological problems.[7,8]

Wool is composed of 95% of keratin while feather contains 90% of keratin.[9,10] Compared with other fibrous proteins such as collagen and elastin, keratins are distinguished by their high cysteine content.[11] The most prevalent amino acids

found in the feather and wool keratin are serine, glycine, leucine and cysteine, which have the chemical structures as shown in Scheme 1.[12] Other amino acids found in keratin include aspartic acid, asparagine, glutamic acid, glutamine, threonine and arginine.[12]

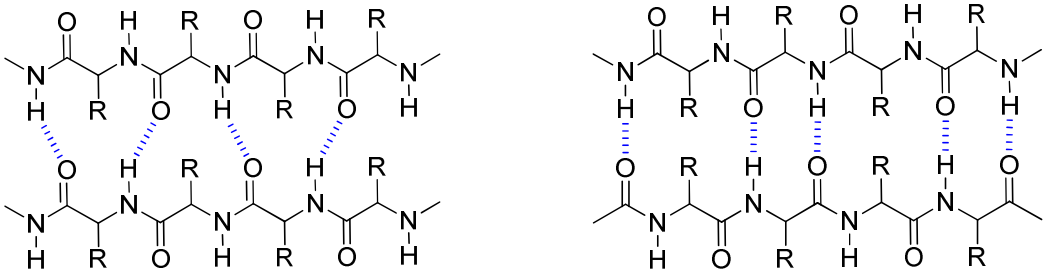


Scheme 1. Structure of serine, glycine, leucine and cysteine.

One of the main differences *between* feather and wool is also the cysteine content in their amino acid sequences; approximately 7% cysteine in feather compared with 11-17% in wool.[13,14] Cysteine is an amino acid that is able to form disulfide bonds (S-S) with other cysteine molecules via intra- or intermolecular interactions. The crosslinks of disulfide bonds (S-S) give high stiffness to the keratin.[14] Since wool possesses a higher amount of cysteine content compared to feather, wool keratin has a higher stability and lower solubility than feather keratin.[15,16]

The two main configurations in the secondary structure of feather and wool keratin are an α-helix and a β-sheet, that correspond to α-keratin and β-keratin structures.[10,11] The parallel and anti-parallel β-sheet structures are given in Scheme

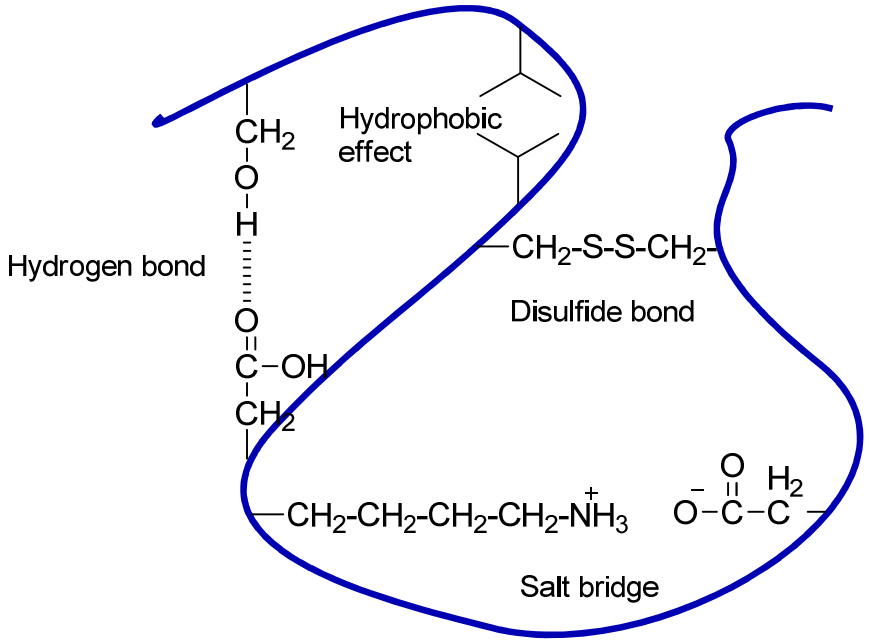
2. From the literature,[17-19] it has been reported that α-helix is stabilized by intramolecular hydrogen bonds between carbonyl (C=O) groups and adjacent amine (-NH-) groups on the next turn of the α-helix. The β-sheet is obtained by hydrogen bonding of the peptide chains that crosslink between peptide groups of opposite chains. The β-sheet structure can be formed in parallel (two strands in the same direction) or anti-parallel (two strands in the opposite direction) sheets.[17-19]



(a) β-sheet (parallel) (b) β-sheet (Anti-parallel)

Scheme 2. The structure of (a) parallel β-sheet and (b) anti-parallel β-sheet.

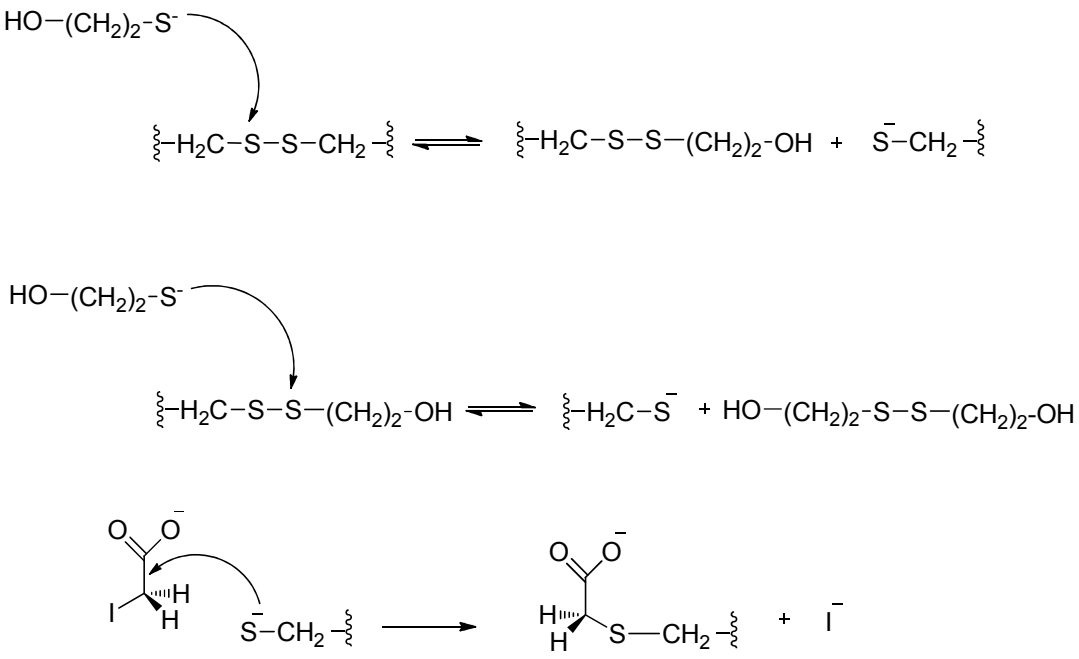
The stable three-dimensional conformation of polypeptide chains is also supported by a range of non-covalent interactions, including electrostatic forces, hydrogen bonds, hydrophobic forces and the covalent disulfide bonds.[20,21] These interactions result in increased strength of the protein and also provide stability to the polypeptide backbone.[20,21] The diagram below (Scheme 3) illustrates the various covalent and non-covalent interactions in keratin.



Scheme 3. Covalent and non-covalent interactions in keratin.

Like other cross-linked biopolymers, feather and wool fibers are difficult to dissolve in water, dilute acids and alkalis, and organic solvents due to the interactions and complex structures of the keratin.[22] Generally, in order to dissolve keratin fibers, the disulfide bridges and hydrogen bonds have to be broken. Several approaches have been reported to dissolve keratin from feather and wool fibers by reduction,[11,23-26] the Shindai method,[27] sulfitolysis[28,29] or oxidative sulfitolysis.[28,30] Reduction reactions are commonly carried out using thiols (R'-SH),[11,23-25] though a large excess of thiol is required.[23,24] Although many reagents are capable of reducing disulfide bonds, only a few have the required ability and reactivity to preserve and maintain a protein structure.[25,28] 2-Mercaptoethanol is known as a reducing agent that has the ability to cleave the disulfide cross-links without damage to the protein backbone.[25,28] Certain thiols, namely thioglycolic acid and 2-

mercaptoethanol, can dissolve keratin in solution with pH above 10. Nonetheless, the dissolution effectiveness reduces when the pH solution is between 7 and 10, except with the addition of a hydrogen bond breaking reagent such as urea.[23,24] Urea is also known as a swelling agent in keratin dissolution.[28] Schrooyen *et al.*[11] demonstrated keratin extraction from feather with a combination of 2-mercaptoethanol and urea. In order to obtain a stable keratin solution and prevent the oxidation of cysteine groups, alkylation was performed via nucleophilic substitution with hydrophilic compounds (eg. iodoacetamide, iodoacetic acid), resulting in water soluble derivatives of keratin.[11] The reaction is shown in Scheme 4.



Scheme 4. Reduction of cysteine with mercaptoethanol followed by alkylation with iodoacetate.[11]

Yamauchi *et al.*[26] reported that the combination of urea, 2-mercaptoethanol, water and surfactant is a good solvent mixture to extract keratin. The surfactant used for keratin extraction in that study was sodium dodecyl sulfate (SDS). SDS not only promotes the extraction of keratin, it also stabilizes the reduced protein solution. This extraction process is associated with the hydrogen bond breaking by urea, disulfide bond cleavage (from S-S bond to S-H group) by 2-mercaptoethanol, followed by formation of reduced keratin that produces micelles with the surfactant. The molecular weight of the reduced keratin obtained was around 52-69 kDa. However, the SDS could not be easily removed, even after several washings.[26]

Nakamura *et al.*[27] developed the 'Shindai method', which involved mixtures of thiourea, urea and a reducing agent to extract keratin from hair, wool, feather and nails. From gel electrophoresis analysis, the extracted keratin consists of protein fractions with molecular mass of 12-18 kDa, 40-60 kDa, 110-115 kDa and 125-135 kDa respectively. The use of thiourea caused increased extraction of keratin by ~3-5 fold, compared to conventional methods (without addition of thiourea). It was also observed that protein hydrolysis had not occurred during this procedure.[27]

Keratin can also be extracted using metabisulfite via a sulfitolysis reaction.[28,29] Sulfitolysis describes the cleavage of the disulfide bond by sulfite to produce reduced keratin (cysteine thiol) and Bunte salt residue.[28,29] The reaction is reversible, while oxidative sulfitolysis[28,30] is not reversible as it converts the

disulfide into two S-sulfonate anions. These reactions[28-30] are as shown in Scheme 5.

Sulfitolysis:

$$R\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}R + SO_3^{2-} \rightleftharpoons R\text{-}CH_2\text{-}S\text{-}SO_3^{-} + R\text{-}CH_2\text{-}S^{-}$$

Oxidative sulfitolysis:

$$R\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}R + 2SO_3^{2-} + H_2O \xrightarrow{[O]} 2R\text{-}CH_2\text{-}S\text{-}SO_3^{-} + 2OH^{-}$$

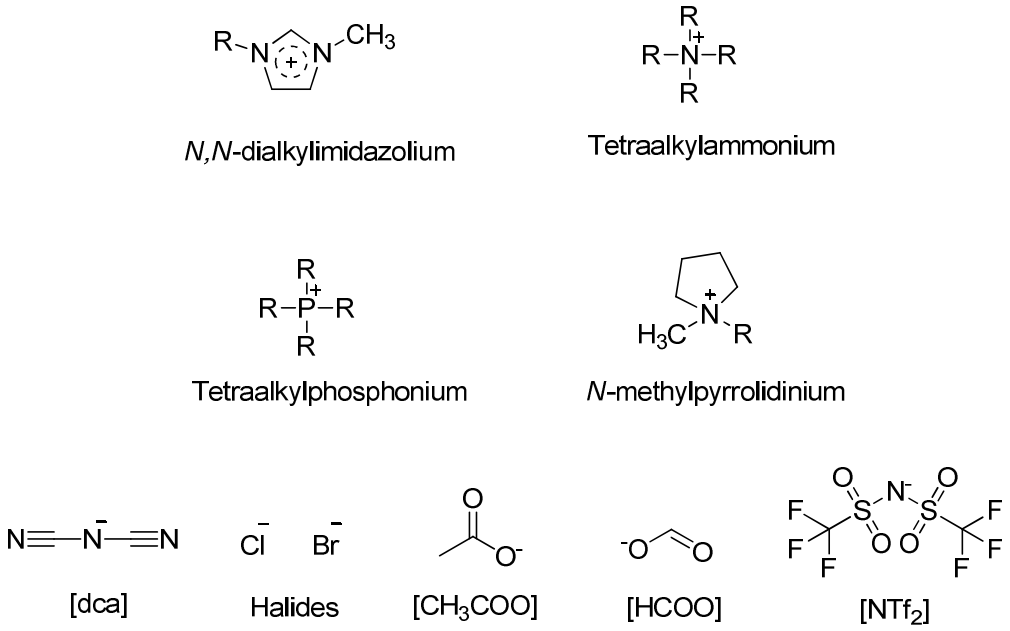
Scheme 5. Sulfitolysis with sulfite compared with oxidative sulfitolysis.[16,17,30]

The reagents used in these reactions described above are often toxic, difficult to recover and the waste solution could potentially cause a strong environmental impact. In order to overcome these problems, researchers have developed new dissolution techniques. The use of ionic liquids as solvents for the dissolution of keratin is one of several approaches that have potential and this is the subject of this thesis.

1.2 Ionic liquids as solvents for the dissolution of biopolymers

Ionic liquids (ILs) are generally defined as salts which are liquid below 100 °C and comprised entirely of cations and anions.[31-33] Ionic liquids can be divided into two groups, aprotic and protic ionic liquids.[34] Aprotic ionic liquids are generally the

combination of alkylated organic cations (imidazolium, pyridinium, etc.) and various anions (chloride, bromide, dicyanamide, etc.), typically formed through ion exhange.[34] Protic ionic liquids, on the other hand, are formed on proton transfer from a Bronsted acid to a Bronsted base.[32,35] Common aprotic cations include *N,N'*-dialkylimidazolium, tetraalkylammonium, tetraalkylphosphonium and *N*-methylpyrrolidinium, while common anions include dicyanamide [dca], halides (chloride and bromide), acetate [CH_3COO], formate [HCOO] and bis(trifluoromethylsulfonyl)amide [NTf_2]. These structures are as shown in Scheme 6.



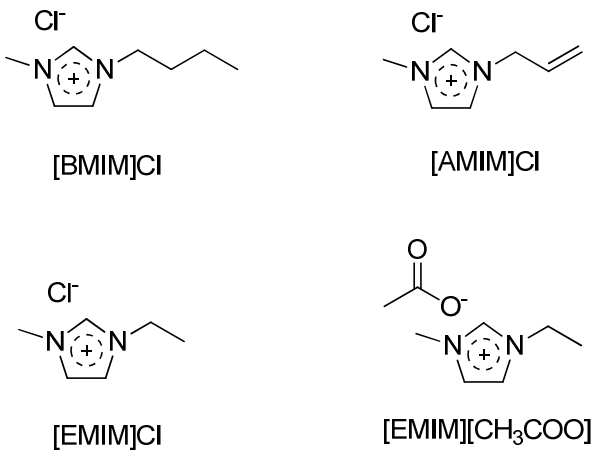
Scheme 6. Common cations and anions of aprotic ILs

In recent years, ionic liquids (ILs) have found a number of applications ranging from stabilisation of proteins,[36,37] fuel cell electrolytes,[38-40] solvents for organic

synthesis,[41,42] enzymatic biocatalysis,[43-45] and extraction/separation processes.[34,46,47] This is due to the availability of unique properties such as low vapour pressure, high thermal and chemical stability, non-flammability, relatively high electrical conductivity, ease of recycling and solvating properties for various kinds of materials including organic, inorganic and organometallic compounds.[34,48-51] The properties of ionic liquids can be designed by adjusting the structures of their cations and anions to tune specific properties such as their hydrophobic/hydrophilic and solvency behavior, in particular their solvency properties towards biopolymers.[52] Certain biopolymers such as cellulose, lignin, starch, wood, feather and wool are difficult to dissolve and have a very limited solubility in water and organic solvents; however, these biopolymers can be dissolved in ionic liquids.[53-59]

Dissolution of lignocellulosic biomass in ionic liquids has been studied extensively. Lignocellulosic biomass consists of carbohydrate macromolecules which can be divided into three main constituents, namely cellulose, hemicellulose and lignin.[60-63] Cellulose consists of repeating glucose units in a highly crystalline polymer due to the strong ability to form intra- and intermolecular hydrogen bonds. Hemicelluloses are highly branched polymers that are non-crystalline, comprised of hexose and pentose sugars. Hemicellulose forms hydrogen bonds with cellulose and covalent bonds with lignin. Lignins on the other hand, are an amorphous phenylpropanoid polymer which links to the hemicellulose and cellulose by hydrogen bonds and ester linkages.[60-63] 1-Butyl-3-methylimidazolium chloride [BMIM]Cl, 1-allyl-3-methylimidazolium chloride [AMIM]Cl, 1-ethyl-3-

methylimidazolium chloride [EMIM]Cl and 1-ethyl-3-methylimidazolium acetate [EMIM][CH_3COO] have been reported to be the most efficient ILs to dissolve lignocellulosic biomass (Scheme 7).[53,64,65] Rogers *et al.*[53] proposed that the dissolution of cellulose occurred due to the disruption of hydrogen bonds involving the hydroxyl groups of cellulose by the Cl^- anion. Up to 10 wt% cellulose was dissolved in [BMIM]Cl at 100 °C in conventional heating and 25 wt% in microwave heating.[53]



Scheme 7. The most efficient ionic liquids for the dissolution of lignocellulosic biomass.

Several ionic liquids have been found to selectively dissolve lignin and hemicelluloses.[45,64,66-69] Tan *et al.*[56] developed a method for lignin extraction from lignocellulosic materials using [EMIM] xylenesulfonate. Also, Pu *et al.*[70] demonstrated that lignin could be dissolved in 1-hexyl-3-methylimidazolium trifluoromethanesulfonate [HMIM][CF_3SO_3], 1,3-dimethylimidazolium methylsulfate [DMIM][$MeSO_4$] and 1-butyl-3-methylimidazolium methylsulfate

[BMIM][$MeSO_4$]. They also discovered that ionic liquids containing the large, non-coordinating anions [PF_6] and [BF_4] were unsuitable as solvents for lignin.[56,70]

Biswas *et al.*[54] revealed that starch can dissolve in [BMIM]Cl and 1-butyl-3-methylimidazolium dicyanamide [BMIM][dca] at up to 10% (wt/wt) concentrations at 80°C. These ionic liquids are able to cleave the hydrogen bonds between hydroxyl groups of the polysaccharide. From the literature,[71] the interaction between starch, [EMIM][CH_3COO] and water in a different ratio can produce pre-gelatinized starch and soluble starch with different viscosities. This can be used as a chemical modification of starch and an approach to the preparation of plasticized starch.[71] Lajunen *et al.*[72] showed that starch can be dissolved within 5 to 6 hours in 1-ethyl-3-imidazolium dimethylphosphate [EMIM][Me_2PO_4] and 2-hydroxyethylammonium formate [$NH_3CH_2CH_2OH$][HCOO].

In recent years, the solubility of protein and DNA based biopolymers in ionic liquids has also been receiving more research interest.[36,43,45,73-82] For example, Fujita *et al.*[73,74] reported that choline dihydrogenphosphate hydrated ionic liquid (ie a mixture of 20% (w/w) water, 80% (w/w) salt) was able to dissolve significant amount of cytochrome *c*. The protein was shown to maintain its structure and redox activity for up to 18 months of storage at room temperature. The effects of ionic liquids as solvents on the stability of DNA was reported by Vijayaraghavan *et al.*[75] They showed that DNA can be dissolved and exhibit long-term stability in choline

dihydrogenphosphate, choline lactate and choline nitrate hydrated ionic liquids. These ionic liquids were shown to stabilise the double-helical DNA structure. [75]

Several studies[4,79,82-84] have reported that [BMIM]Cl and [AMIM]Cl can act as solvents for wool and feather keratin. Xie *et al.*[4] prepared wool keratin/cellulose composite materials such as fibers and membranes using [BMIM]Cl as a solvent.[4] There was no residual fiber remaining in the membrane, demonstrating that the wool keratin had been completely dissolved. The thermal stability of the regenerated keratin was slightly higher than that of raw wool. In addition, compared with the raw wool, the regenerated wool keratin exhibited a β-sheet structure with the disappearance of the α-helix structure.[4] Several studies have investigated processing of materials based on dissolved keratin/IL solutions with the addition of other biopolymers or plasticisers.[79,85,86] Therefore, it seems that keratin proteins have the potential to be processable in ILs and converted into valuable products, however, much remains to be done to optimise this process and characterise the nature of the regenerated material.

An inexpensive alternative dissolution method has also been explored using mixtures known as deep eutectic solvents. Abbott *et al.*[87,88] reported that a eutectic mixture occurs in a mixture of a substituted quaternary ammonium salt such as choline chloride and urea, in a ratio of 1:2, producing a liquid at room temperature.[87,88] Biswas *et al.*[54] then reported that mixtures of choline chloride/zinc chloride and choline chloride/oxalic acid are excellent solvents for dissolution of

starch. Therefore, it appears that deep eutectic solvent systems have significant solvent properties in dissolving biopolymer that warrant further investigation.

1.3 Aims and Objectives of The Study

The dissolution of biopolymers using ionic liquids has offered new avenues in the investigation of the conversion and utilization of these biopolymers as cost effective and/or novel materials. Thus, the overall aim of this project was to develop and investigate the use of ionic liquids for the purpose of dissolving and processing keratin materials (e.g. wool and feathers).

The specific objectives of the project were:

1. To synthesise and characterise selected ionic liquids for the purpose of dissolution of keratin based natural products. The ionic liquids were based on:

   i. imidazolium salts of the various anions
   ii. hydroxyl ammonium ionic liquids

2. To test the ionic liquids for the dissolution of keratin based materials under a variety of conditions including:

   i. influence of reducing agents (to cleave disulfide bonds)
   ii. influence of binary solvents or deep eutectic solvent mixtures
   iii. the anion effect on dissolution

iv. the cation effect on dissolution

3. To determine the main properties supporting the dissolution process, for example the effect of the structure of the ionic liquids on their dissolving power.

4. To characterise the regenerated keratin obtained from IL dissolution.

5. To produce a reduced form of keratin biomaterial and evaluate the possibility of processing it into physical states such as fibers, using known techniques.

1.4 Overview of Thesis

This thesis is presented in a "Thesis including Published Works" format. It is made up of 4 journal articles (3 published and 1 submitted). Each chapter contains a general overview along with the journal article as the main body of work, followed by the supplementary information. Each paper contains a more detailed literature review relevant to the topic of the paper.

An overview summary of the chapters is as follows:

Chapter 2. *Dissolution of feather keratin in ionic liquids*

In this chapter, the dissolution and regeneration of feather in a series of ionic liquids are described. The work presented demonstrates that the imidazolium ionic liquids ([BMIM]Cl, [AMIM]Cl) and an ionic liquid containing thiol functionality ([choline][thiolgycolate]) were able to dissolve considerable amounts of feather keratin (~450 mg of feather in 1 g of IL). The characterisation of water soluble and water insoluble fractions that were obtained after regeneration of the keratin from the solution are also presented in the manuscript. The manuscript was published in *Green Chemistry*.

Chapter 3. *Dissolution and regeneration of wool keratin in ionic liquids*

In this chapter, the manuscript expands our studies of ionic liquid dissolution and the regeneration of keratin materials to wool keratin, which is available as a waste material from textile processing. The paper builds on previous observations of

dissolution in simple chloride ionic liquids to demonstrate >400 mg wool per 1 g IL solubility in a dicyanamide IL. The influence of reducing agent and deep eutectic solvents is also elaborated upon. The study was published in *Green Chemistry*.

Chapter 4. *Thermoplastic materials from the dissolution of feather keratin biopolymer in ionic liquids*

This chapter extends the work from Chapter 2 and Chapter 3. The manuscript, submitted to *Polymer*, discusses the utilization of keratin biomass (feather) through the dissolution and regeneration process. The mouldable gels and films obtained from the dissolved keratin feather/IL solution can be produced in any desired shape and size. Our vision is that this may be a source of high T_g polyamide materials to replace crude-oil derived polyamides.

Chapter 5. *Distillable protic ionic liquids for keratin dissolution and recovery*

In this chapter, a series of distillable ILs comprised of the *N,N*-dimethylethanolammonium cation with formate, acetate and chloride anions were synthesised. The manuscript, published in *ACS Sustainable Chemistry and Engineering,* discusses the dissolution and regeneration of keratin using protic ionic liquids that are relatively cheap and completely recoverable via distillation, for example it was shown that *N,N*-dimethylethanolammonium formate could be distilled with 99.6% recovery of the original IL.

Chapter 6. *Conclusions and future work*

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Chapter 2

Dissolution of feather keratin in ionic liquids

CHAPTER 2

Dissolution of feather keratin in ionic liquids

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2.1 Specific Declaration

PART B: Suggested Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Initiation, key ideas, performed the experiments, synthesis, data analysis and interpretation, manuscript development and writing up	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Douglas MacFarlane	Key ideas, proof reading and final drafting	-
Antonio Patti	Key ideas, proof reading and final drafting	-
R. Vijayaraghavan	Synthesis thioglycolate ionic liquids and proof reading	-
Usman Ali Rana	Assisted in ^{13}C CP MAS NMR experiment and in the discussion	-
Dale Fredericks	Assisted in gel electrophoresis experiment	-

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** **18/6/2014**
Main Supervisor's Signature	[redacted]	**Date** **18/6/2014**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

2.2 General Overview

In the slaughtering process, huge amounts of waste feathers are discarded from the poultry industries.[1-4] As a consequence, this abundant waste creates an environmental pollution issue due to its poor degradability.[5,6] Therefore, it is desirable to develop approaches to utilize this renewable resource as a source of keratin biopolymer. Potentially they could be transformed into useful polyamide materials. Table 1 summarizes the different types of ionic liquids that have been used to dissolve feather keratin in the literature.

Table 1: Dissolution of feather keratin in ionic liquids

Ionic liquids	Experimental condition of the dissolution process	Summary	References
[BMIM]Cl	100 °C, 48 hours	Solubility of 23%	Sun *et al.*[7]
[BMIM]Cl	80 °C, 24 hours	Solubility of 5%	Zhao *et al.*[8]
[AMIM]Cl	80 °C, 24 hours	Solubility of 8%	Zhao *et al.*[8]
[BMIM]Br	80 °C, 24 hours	Insoluble	Zhao *et al.*[8]
[HOEMIm][NTf_2]*	80 °C, 4 hours	Solubility of 8%, [HOEMIm][NTf_2] is a hydrophobic IL	Wang *et al.*[6]

*[HOEMIm][NTf_2] =1-hydroxyethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)amide

The main objective of this study was to develop a simple dissolution process for utilisation of feather using ionic liquids as solvent. In this chapter, we have investigated several anion and cation combinations of ionic liquids and the main properties involved in the dissolution process. In particular, we focused on imidazolium ionic liquids, [BMIM]Cl and [AMIM]Cl in this study due to the

known ability of these ILs to dissolve other biomass such as cellulose and lignin. The efficiency of chloride containing ionic liquids in dissolving biomass, is attributed to the capability of the chloride anion as a strong hydrogen acceptor to disrupt the hydrogen bonds in the macromolecular aggregates.

Reducing agents also have a known effect on keratin biopolymers, therefore ionic liquids containing thiol functionality ([choline][thiolgycolate] and [bis-(2-ethylhexyl)ammonium][thioglycolate]) were introduced to examine the effect of reducing agents on the solubility of the feather keratin. The rate of dissolution in ionic liquids was also studied in order to assess the effect of disulfide bond cleavage. Comparative experiments were performed on the dissolution of pure cystine in thioglycolate ionic liquids.

Three different temperatures, room temperature, 65 °C and 130 °C, were evaluated in order to explore its effect on the complete dissolution of feather.

Water soluble and water insoluble protein fractions were obtained on regeneration of the feather keratin/IL solution. These were characterized using a number of techniques. The percentage of secondary structures (α-helix and β-sheet) in the raw feather and in the water insoluble fraction were estimated by the deconvolution of the carbonyl peak of ^{13}C NMR MAS NMR spectra using the dmfit program.[9] The estimated molecular weight of keratin obtained in the water soluble fraction was identified by gel electrophoresis.

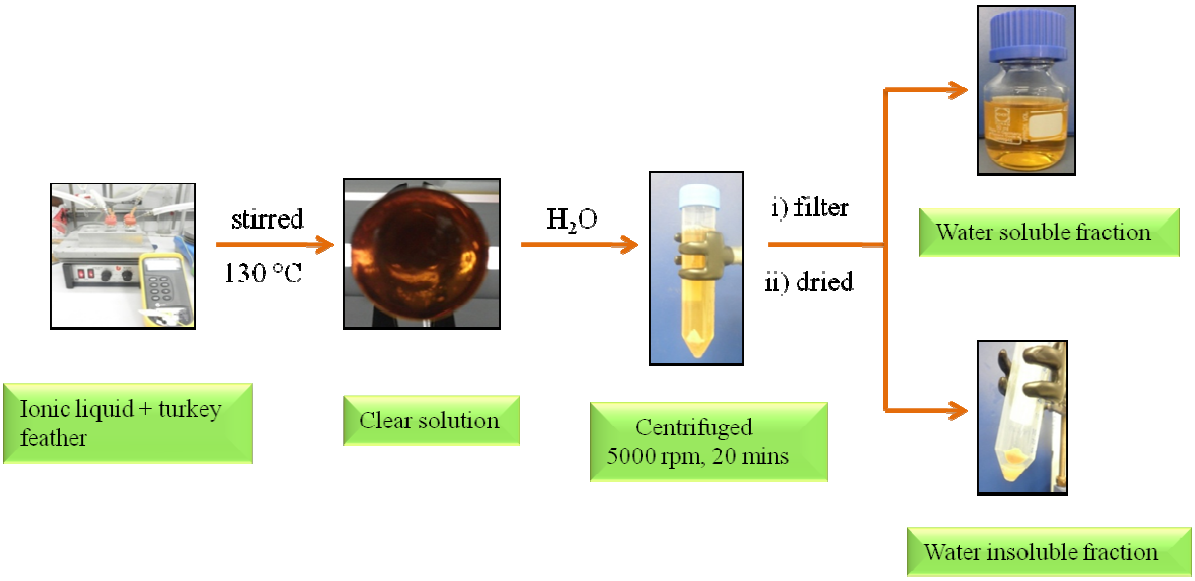
The details of this study have been published in the paper entitled " Dissolution of feather keratin in ionic liquids."[10]

2.3 Publication 1

Dissolution of feather keratin in ionic liquids

Azila Idris, R. Vijayaraghavan, Usman Ali Rana, Dale Fredericks, A. F. Patti and D. R. MacFarlane

Considerable amounts of keratin biopolymer can be dissolved in certain ionic liquids. In current studies, [BMIM]Cl, [AMIM]Cl and [choline][thioglycolate] were shown to have good potential as solvents for keratin dissolution. Water soluble and water insoluble fractions were obtained after regeneration of the keratin from the solution.



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Dissolution of feather keratin in ionic liquids†

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Keratin from various livestock industries is currently a waste material that has potential as a source of polyamide polymers that could replace fossil fuel derived materials if processing methods can be developed. In this work we have investigated methods for the dissolution and regeneration of keratin. Dissolution of keratin (from turkey feather) in ionic liquids was conducted under nitrogen at 130 °C for 10 hours. It was found that [BMIM]Cl, [AMIM]Cl and [choline][thioglycolate] could dissolve turkey feather keratin without addition of solvent or other chemicals. A significant percentage of solubility was obtained, up to 45% by weight. A water insoluble fraction was recovered by addition of water to the solution (~50%). The structure and properties of this regenerated, water insoluble fraction were investigated. Compared to the starting material, the regenerated keratin shows structural changes rather than chemical changes within the polypeptide chains. The remaining fraction, consisting of water soluble fragments, was characterised by gel electrophoresis.

1. Introduction

Keratin is a fibrous protein that can be found in feather, wool, human hair, finger nails, animal claws and horn.[1,2] These biopolymers are plentifully available as a by-product from poultry production and textile industries.[3–5] However this kind of protein is not easy to dissolve or extract because keratin is bound by strong internal interactions that stabilise the protein structure.[4,6] In order to utilise these biopolymers and convert them to usable materials there is a need for alternative solvents that are efficient in dissolving keratin and preserving, at least in part, the protein structure. The main objective of the present study therefore is to investigate the use of ionic liquids (ILs) in the dissolution and regeneration of keratin, in particular utilising the unique solvency characteristics and high temperature properties of ILs. Feathers are about 90% keratin[7] which contains about 7% of cysteine.[8,9] Feather proteins are insoluble in normal organic solvent due to the tight packing of the α-helix and β-sheet in the polypeptide chain. Additionally, a high degree of crosslinking of the polypeptide chain is affected by cysteine residues which participate in disulfide bridge formation.[6,8,10] In order to extract the keratin, cleaving the disulfide bonds can be achieved by reduction,[11–13] oxidation,[12–14] sulfitolysis[13,15] or oxidative sulfitolysis.[13,16] Even though many reagents have the capability to reduce the disulfide bonds, only a few, such as thiols[17] and ammonium bisulfite, have the required ability and reactivity to preserve and maintain the protein structure. However, these materials are difficult to recycle, often toxic and costly to produce.

Ionic liquids (ILs) are generally defined as salts which are liquid below 100 °C and comprised entirely of cations and anions.[18,19] They offer a unique combination of properties as solvents including in some cases low vapour pressure and high thermal stability.[20] The hydrophobicity/hydrophilicity of ionic liquids can be altered by manipulating the structures of the cations and anions.[21] In recent years, a number of ionic liquids have been identified as solvents for the dissolution of biopolymers such as cellulose, starch, wood, lignin, feather and wool. These are generally insoluble in organic solvents but show a reasonable solubility in certain ionic liquids.[4,22–28] Duck feather keratin, for example, was found to be soluble in the ionic liquids (1-butyl-3-methylimidazolium chloride [BMIM]Cl and 1-allyl-3-methylimidazolium chloride [AMIM]Cl).[29] The same ionic liquids have been found to be useful for wool dissolution by Xie *et al.*[4] [BMIM]Cl was also found to be useful in the synthesis of wool keratin/cellulose composite materials. The materials displayed a homogeneous structure and did not show a residual fiber structure.[4] Therefore, the dissolution of biopolymers using ionic liquids provide

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possibilities for the utilisation and conversion of biopolymers into useful materials with great potential in industrial applications.

In the current study we have used [BMIM]Cl, [AMIM]Cl, as well as novel ionic liquids containing a thiol functional group [choline][thioglycolate] and [bis-(2-ethylhexyl)ammonium]-[thioglycolate] as possible disulfide bridge reducing, or exchange reagents, to dissolve and regenerate the keratin in turkey feather. A combination of analytical techniques including ATR-FTIR, XRD, solid state NMR, TGA, DSC and gel electrophoresis has been used to investigate the extent of keratin solubility in these ionic liquids and the physiochemical properties of the regenerated keratin material. We show that a significant amount of keratin (approximately 45–50 wt%) can be dissolved in the above mentioned ionic liquids.

2. Experimental

Materials preparation

The cleaned turkey feathers used in the experiments were commercial materials supplied by Shamrockcraft, purchased from Spotlight, Clayton, Australia. Only barbs of the turkey feathers were used in the experiments. 1-Butyl-3-methylimidazolium chloride ([BMIM]Cl, 98% of purity) was purchased from Sigma Aldrich. All other ionic liquids were prepared in-house for this project as described further below. For the preparation of these ionic liquids, allylchloride (98%), *N*-methylimidazole (99%), choline hydroxide (20 wt% in H_2O), thioglycolic acid (98%) and bis-(2-ethylhexyl)amine (99%) were obtained from Sigma Aldrich. For NMR analysis, deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OD) purchased from Merck was used. Unless otherwise stated, all other organic solvents and reagents were used as received from commercial suppliers.

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

Fourier transform infrared spectra were obtained using a Bruker IFS Equinox FTIR system coupled with a Golden Gate single bounce diamond micro-Attenuated total reflectance crystal and a liquid nitrogen cooled Mercury/Cadmium Telluride detector. The FTIR was performed in the wavenumber range of 600 cm^{-1} to 4000 cm^{-1}. The spectra were recorded with a resolution of 4 cm^{-1} with 50 scans. Spectra were baseline corrected.

Powder X-ray diffraction (PXRD)

The powder X-ray diffraction (PXRD) patterns were obtained at 22 ± 2 °C using a Sietronics powder diffractometer. For each XRD experiment, approximately, 1 2 g of the finely ground sample was placed randomly on a locally designed flat brass sample holder fitted with an o-ring sealed covered Mylar sheet providing an airtight atmosphere. CuKα1 radiation (λ = 1.540 Å) was produced at 40 kV and 25 mA. The data were collected in the Brag–Brentano ($\theta/2\theta$) horizontal geometry using a 2θ-range of 5 to 50.0° (2θ) range with a step size of 0.02° 2θ and an accompanying scan rate of 0.5° min^{-1}.

Nuclear magnetic resonance spectroscopy (NMR)

^1H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker DPX-400 spectrometer. All samples were measured as solutions in deuterated chloroform and methanol. Chemical shifts are reported in ppm on the δ scale, and the coupling constant is given in Hz. Chemical shifts were calibrated on the solvent peak unless otherwise specified. Solid state 1D ^1H static NMR spectra of neat and regenerated samples were acquired at a Larmor frequency of 300 MHz on a Bruker AV-300 spectrometer. The ^{13}C CP MAS NMR spectra of these samples were acquired using a 4 mm rotor with Kel-f cap at 10 kHz spinning rate. The contact time in the CP MAS experiments was 2.4 ms with a recycle delay of 1 s and cw decoupling. The number of scans was ~90 000 to 100 000. In the case of 1D ^1H static NMR experiments, the pulse length was ~3.6 μs with a recycle delay of 5 s and 16 to 20 scans.

Thermogravimetric analysis (TGA)

The thermal stability of the original and regenerated materials were investigated by TGA using a Pyris 1 in a flowing dry argon atmosphere between 25 and 700 °C at a heating scan rate of 10 °C min^{-1}. The instrument was calibrated using four reference materials, alumel, perkalloy, iron and nickel. The samples were first dried under vacuum in an oven at a temperature of 70 °C. These samples were then loaded in ceramic pans and equilibrated for 15 minutes at the starting temperature of 25 °C before running each experiment.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was conducted on a DSC Q100 series from TA Instruments with 5–10 mg of sample in closed aluminium pans, at a ramp rate of 10 °C per minute. All samples were cooled to −150 °C, held for 5 minutes and heated to 200 °C. Thermal scans below room temperature were calibrated *via* the cyclohexane solid–solid transition and melting point at −87.0 °C and 6.5 °C respectively. Thermal scans above room temperature were calibrated using indium, tin, lead and zinc with melting points at 156.6 °C, 231.9 °C and 419.5 °C respectively. Transition temperatures are reported using the peak maximum of the thermal transition.

Electrospray ionisation mass spectrometry (ESI-MS)

Electrospray ionisation mass spectra were recorded on a Micromass Platform II API QMS Electrospray Mass Spectrometer. Samples dissolved in methanol were subjected to a suitable cone voltage, usually 25 V to 35 V. Measurements were made in both the positive and negative modes.

SDS-PAGE electrophoresis

Protein samples were diluted in 4× NuPAGE® loading buffer (Life Technologies) and electrophoresed using the Hoefer miniVE vertical electrophoresis system (Amersham Biosciences) in 4–12% Bis-Tris NuPAGE® gradient gels (Life

Technologies). Proteins were stained and visualised by silver staining.

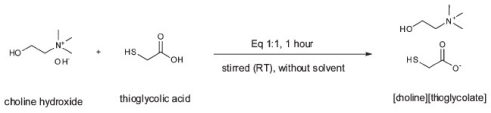
Preparation of ionic liquids

1-Allyl-3-methylimidazolium chloride [AMIM]Cl was prepared according to the literature[30] as described in more detail in the ESI.† Structural confirmation of the ionic liquids obtained was carried out by spectroscopic methods including ^{1}H NMR and mass spectroscopy (electrospray ionisation). As contaminants have been shown to affect the physical properties of ILs,[31,32] water content was determined by Karl Fischer titration.

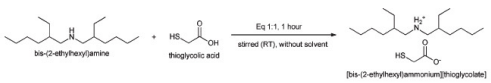
Apart from imidazolium salts, a series of thioglycolate ionic liquids were also synthesised. The reaction is a simple acid–base neutralisation which involved transferring a proton from a Bronsted acid to a Bronsted base without the use of any solvent.[19] In this investigation, bis-2-ethylhexylammonium hydroxide and choline hydroxide were combined with one equivalent of thioglycolic acid, respectively. These chemical reactions are highly exothermic, therefore an ice bath was used to control the temperature throughout the chemical reaction. Further details are as follows.

[Choline][thioglycolate]. Choline hydroxide (15.17 ml, 0.026 moles) was initially placed in a three-necked flask and equipped with a reflux condenser and a dropping funnel. An equimolar amount of thioglycolic acid (1.82 ml, 0.026 moles) was then added dropwise to the base while stirring rapidly in an ice bath. A viscous liquid (Scheme 1) was obtained after evaporation of the water by-product using a rotary evaporator. (4.57 g, 91%); ^{1}H NMR (ppm, 400 MHz, DMSO) δ_H: ^{1}H NMR (ppm, 400 MHz, DMSO) δ_H: 4.49 (1H, broad s, OH), 3.84 (2H, q, CH_2), 3.44 (4H, m, $2CH_2$), 3.12 (9H, s, $3CH_3$), 1.92 (1H, s, SH); ES-MS: ES^+ m/z 104.1 [choline]$^+$. ES^- m/z 90.9 [thioglycolate]$^-$. Water content (Karl Fischer): 18 034 ppm.

[Bis-(2-ethylhexyl)ammonium][thioglycolate]. Bis-(2-ethylhexyl)-amine (7.48 g, 30.9 mmoles) was loaded into a three-necked flask and equipped with a reflux condenser under vigorous stirring with a magnetic stirrer. An equimolar amount of thioglycolic acid (2.72 g, 29.5 mmoles) was then added dropwise to the flask. The flask was placed in an ice bath. Evaporation of water gave [bis-(2-ethylhexyl)ammonium][thioglycolate], a colourless liquid (Scheme 2) (9.8 g, 98%); ^{1}H NMR (ppm, 400 MHz, $CDCl_3$) δ_H: 7.23 (2H, broad s, $^+NH_2$), 3.57 (2H, s, CH_2), 2.74 (4H, d, J = 6.4 Hz, $2CH_2$), 1.73 (2H, m, 2CH), 1.27 (17H, m, $8CH_2$, SH), 0.804 (12H, m, $4CH_3$); ES-MS: ES^+ m/z 242.3 [bis-(2-ethylhexyl)ammonium]$^+$. ES^- m/z 90.9 [thioglycolate]$^-$. Water content (Karl Fischer): 8698 ppm.



Scheme 1 Preparation of [choline][thioglycolate].



Scheme 2 Preparation of [bis-(2-ethylhexyl)ammonium][thioglycolate].

Keratin solubility

The solubility experiments were conducted in glass vials, which were fitted into a heating block under an inert atmosphere of N_2. To quantify the solubility, small incremental amounts of feather material (5 wt%) were gradually added to the ionic liquid (1 g) and mechanically stirred until they were completely dissolved in the ionic liquid. Initially it was observed that the dissolution of turkey feather occurred rapidly. Then the dissolution rate decreased as the viscosity of the turkey feather solution increased. Complete dissolution was assumed to have occurred when the feather could not be visually detected and the ionic liquid solvent remained transparent in the glass vial. A laser beam was used to more sensitively detect the presence of small particles *via* light scattering. The dissolution was run at 130 °C for 10 hours.

3. Results and discussion

Dissolution of keratin in ionic liquids

Keratin was dissolved in two series of ionic liquids which consisted of the imidazolium and thioglycolate systems. These studies were undertaken in order to determine the main properties involved in the dissolution process, for example, the effect of the structure and composition of ionic liquids on their dissolving capability. Initially, the dissolution of turkey feather was attempted at room temperature and 65 °C. Partial dissolution was observed at 65 °C. At 130 °C, complete dissolution was clearly observed. This could be due to the keratin structures at some level becoming unfolded at the elevated temperature,[33] hence permitting more interaction between keratin and the ionic liquids. According to Xie *et al.*,[4] temperature has a strong effect on the solubility and at 130 °C, better solubility of wool keratin was noted. Therefore, based on this study, the temperature was maintained at 130 °C in order to minimize any potential protein degradation and prevent any decomposition of the ionic liquids. The keratin that dissolved completely in ionic liquids resulted in a viscous solution. There was no precipitation observed after the dissolution process or after cooling to room temperature.

Table S1 in the ESI† and Fig. 1 show the limiting solubility in weight percentage of keratin in [BMIM]Cl, [AMIM]Cl, [choline][thioglycolate]. The limiting solubility was found to be around 50 wt% in all of these cases. This was determined through the sequential addition of the feather material until the solution became too viscous for rapid dissolution at 130 °C. It is likely that the true solubility is even higher than indicated here. It is interesting to note that the chloride ion seems to be as effective as the thioglycolate ion in dissolving the keratin. In the chloride case, this high solubility is

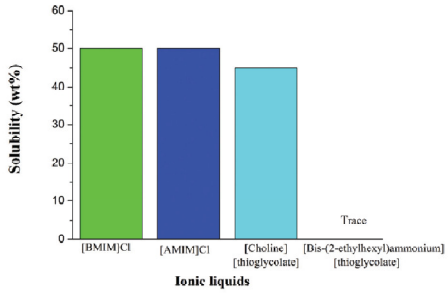


Fig. 1 Solubility of turkey feather keratin in ionic liquids.

presumably related to the ability of the ionic liquid chloride ion to disrupt hydrogen bonding in the keratin material, similar to the understanding that has emerged on the role of Cl^- in cellulose and other biopolymer dissolution in ionic liquids.[22,26,28,34] Gillespie and Lennox[35] demonstrated that alkaline thioglycolate solutions could be used to reduce the disulfide bonds in the dissolution of wool, however there was no significant increase in feather solubility observed in [choline][thioglycolate], compared to [BMIM]Cl and [AMIM]Cl. This suggests that disulfide cleavage is not a significant factor in the solubility *limit* of this type of keratin.

No dissolution was observed for turkey feather in [bis-(2-ethylhexyl)ammonium][thioglycolate]. This is probably due to the longer chain of the cation which can therefore only penetrate into the keratin network with difficulty. In addition, the viscosity of [bis-(2-ethylhexyl)ammonium][thioglycolate] is much higher compared to [choline][thioglycolate], thus decreasing the solvating properties of the ionic liquid. This compound is also the only classical protic ionic liquid of the group studied and may be prone to the proton transfer problems that have been widely discussed with respect to protic ionic liquids. The ΔpK_a of this acid–base pair is ~7.2 which has been suggested as being sufficient for strong proton transfer with regard to primary amines, but insufficient for many tertiary amine based ILs.[36]

Fig. 2 shows the keratin–[AMIM]Cl solution after returning to room temperature. At this point the solution was a very viscous, clear liquid.

Fig. 3 shows the rate of dissolution of turkey feather in [BMIM]Cl, [AMIM]Cl and [choline][thioglycolate]. Dissolution in [choline][thioglycolate] was visually slightly more rapid in the first ~10 minutes compared to the other two ionic liquids, suggesting that the initial cleavage of the disulfide bridges can accelerate the process but that the difference becomes minimal at later times. Since little is known about the disulfide bridge under these conditions, it is important to recognise that there are a number of cleavage reactions possible,[12–15,37,38] including exchange, homolytic cleavage as well as further oxidative processes if sufficient oxygen is present. In order to probe the reactions possibly taking place

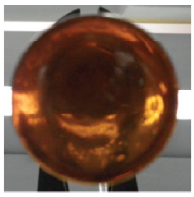


Fig. 2 Dissolved feather keratin in [AMIM]Cl.

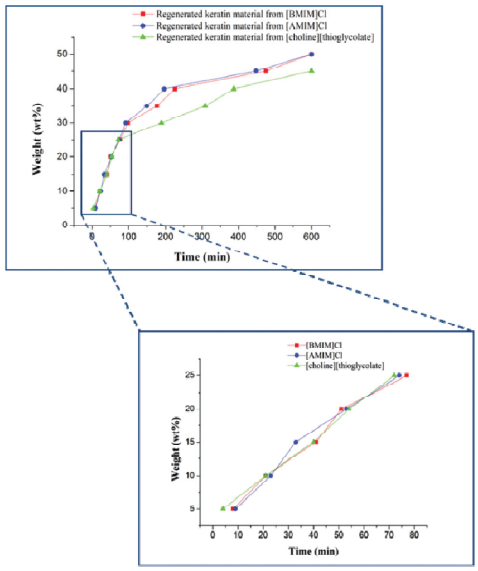


Fig. 3 Rate of dissolution of feather keratin in ionic liquids.

in the thioglycolate case, the interconversion of model compounds cystine and cysteine were studied in the thioglycolate ionic liquid. Neat cystine was added to choline thioglycolate ionic liquids and heated to 130 °C for about 5 hours. The resulting mixture was analysed by ^{13}C NMR, showing 2 new peaks in the region of 170 ppm. Cysteine dissolved in ionic liquid showed a single peak at 170 ppm. These observations seem to indicate partial cleavage or exchange of the cystine S–S bridge to form cysteine as well as the exchange product, as is also suggested by the NMR data of the regenerated keratin discussed further below.

Regenerated keratin from ionic liquids

After the keratin was dissolved, it was precipitated from the solution by addition of water at room temperature. The regenerated keratin was separated by centrifugation for 20 minutes at 5000 rpm, then dried under vacuum at 60 °C for 3 days. The

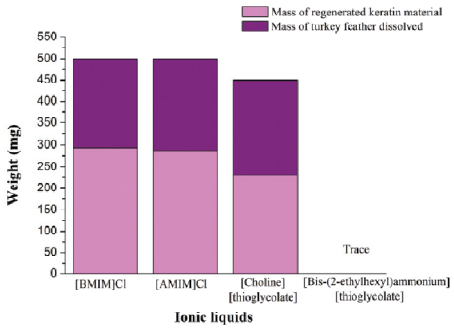


Fig. 4 Recovery of water insoluble fraction of IL dissolved keratin.

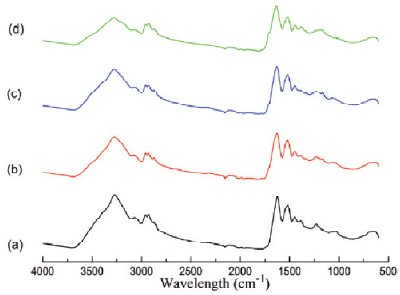


Fig. 5 ATR-FTIR spectra of (a) raw material and regenerated keratin material from (b) [BMIM]Cl, (c) [AMIM]Cl, and (d) [choline][thioglycolate].

regenerated keratin material was a fawn-coloured hard solid. Table S2 of the ESI† and Fig. 4 show the masses obtained of the regenerated aqueous-insoluble fraction. It has been reported that feather keratin consists of approximately 40% hydrophilic and 60% hydrophobic groups in the amino acid sequence.[8] Therefore, it was suggested that some of the dissolved keratin is insoluble in water and the other part of the dissolved material might be cleaved into smaller constituent polypeptide subunits which may be water soluble. Gel electrophoresis analysis was employed to further investigate the soluble component in the water–ionic liquid mixture produced during the regeneration step, as discussed further below.

Characterisation of regenerated keratin

ATR studies. In order to further understand the nature of the water insoluble keratin material regenerated from the ionic liquids, ATR-FTIR measurements were used. Infrared absorption spectra of the raw material and regenerated fraction (Fig. 5) showed characteristic absorption bands that are typically assigned to the peptide bonds (–CONH). These spectra provide information as to the orientation of the constituents and the conformation of the polypeptide chains. The Amide A vibrations of the peptide bond show medium absorption bands at 3273 cm^{-1} due to the N–H stretching. A strong absorption band was also observed at 1627 cm^{-1} and this was attributed to C=O (Amide I). A strong absorption peak at 1515 cm^{-1} was attributed to C–N stretching and N–H bending vibrations (Amide II). A weak band was observed at 1236 cm^{-1} which was due to the C–N and C–O stretching, N–H and O=C–N bending vibration (Amide III).[39,40] It can be seen that the spectra are similar to the original starting material and there are no signatures of new functional groups appearing in the regenerated material. However, in view of NMR results (below), it appears that there are chemical changes occurring due to the breaking of disulfide linkages and hydrogen bonds, which cannot be observed clearly in the ATR spectra. Therefore dissolution in these ionic liquids does not strongly affect the peptide bonds.

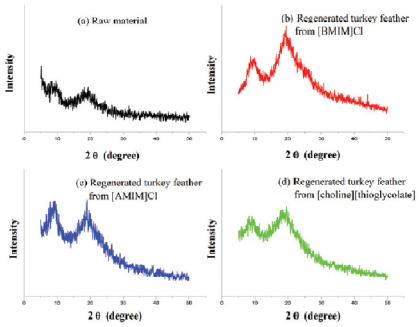


Fig. 6 XRD of (a) raw material and regenerated keratin material from (b) [BMIM]Cl, (c) [AMIM]Cl, and (d) [choline][thioglycolate].

XRD studies. Raw material and the regenerated keratin material were examined by X-ray diffraction (XRD). In Fig. 6, the XRD patterns are compared. These figures show crystallinity in the feather keratin fibers from the substantial 2θ peak at about 9° (0.98 nm) which has been assigned to both α-helix and β-sheet structures.[41,42] The peak at about 17.8° (0.51 nm) corresponds to the diffraction pattern of the α-helix whereas the peak at about 19° (0.47 nm) is typical of the β-sheet structure.[41,42] However, due to the overlapping signals at about 17.8° and 19° from the α-helix and β-sheet, both are unable to be unambiguously assigned. It can be seen from Fig. 6 that the regenerated keratin exhibits similar diffraction patterns, indicating regeneration of the crystallinity of the original keratin. However, the peaks at about 9° and 19° are both significantly stronger in the regenerated material, suggesting a greater content of the β-sheet structure. These results are consistent with the ^{13}C CP MAS NMR spectra discussed further below.

Solid state NMR studies. Solid state ^{13}C NMR was conducted on the raw material and the regenerated keratin material to investigate the local structure and dynamics of the keratin molecules. Fig. 7 displays the ^{13}C CP MAS NMR spectra

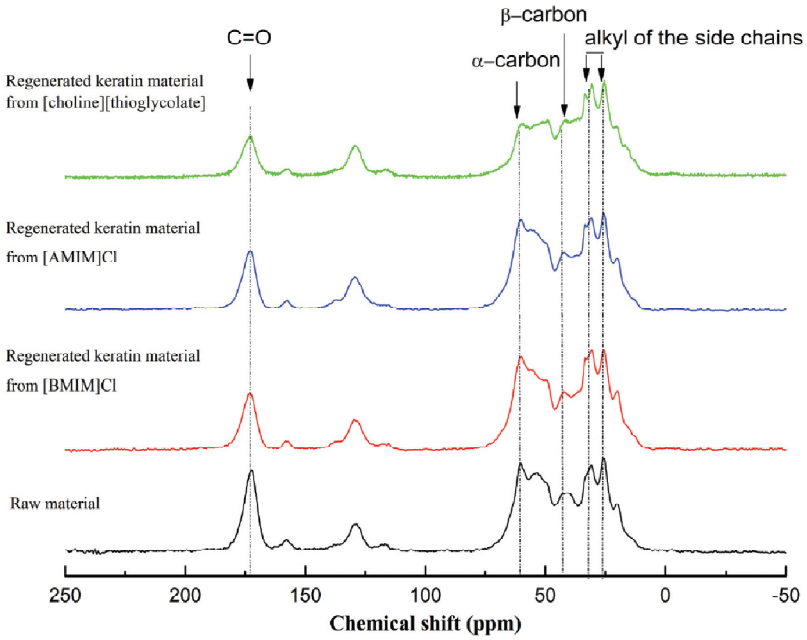


Fig. 7 The ^{13}C CP MAS NMR spectra of (a) raw material, (b) regenerated keratin material from [BMIM]Cl, (c) regenerated keratin material from [AMIM]Cl, and (d) regenerated keratin material from [choline][thioglycolate].

of keratin materials. All spectra in Fig. 7 display a distinct asymmetric peak with maxima centred between 172 and 174 ppm.

This peak corresponds to the carbonyl carbons present in keratin. The peak at 130 ppm is ascribed to the aromatic species present in the keratin.[43] All NMR spectra in Fig. 7 display this aromatic peak, which suggests that the aromatic group containing amino acid sequences are retained in the regenerated materials. The peak at 54 ppm is related to the α-carbons, while the one at 40 ppm is ascribed to the β-carbons present in leucine and cysteine residues.[44] The carbon peaks at about 30 ppm to 40 ppm are attributed to the carbon present in the proline, glutamic acid and glutamine residues. The NMR peaks at low chemical shift centred at 20 ppm, 25 ppm and 31 ppm, in addition to the peak as a shoulder at 17 ppm correspond to the alkyl groups of the side chains.[44] A peak at ~20 ppm is somewhat reduced in the thioglycolate case – this region is associated with the δ-carbon of leucine residues.[44]

Reductive cleavage of disulfide bridges associated with cysteine is expected to reduce the β-carbon signal at 40 ppm and produce a thiol signal around 25–29 ppm.[44] The two chloride cases show distinct peaks at 40 ppm that are similar to the raw material, suggesting substantial retention of disulfide bridges in these cases. In the thioglycolate case a peak remains evident, although less distinct. An exchange reaction in the thioglycolate case could produce a new S–S bridge which would also produce resonances near 40 ppm; therefore it appears likely that some sulfide exchange has taken place.

Since, the alkyl groups and the reduced cystine groups (*i.e.*, cysteine groups) display peaks in the same chemical shift range around 25–29 ppm, it is hard to distinguish the reduced cysteine uniquely in these spectra.

The two regenerated materials from [BMIM]Cl and [AMIM]-Cl display NMR spectra that are not very different from the original keratin, while the [choline][thioglycolate] material shows clear differences in the 50–65 ppm region. This is possibly because of disruption due to the reductive cleavage of the disulfide linkages.

The secondary structure of keratin, which consists of α-helix and β-sheets, produces a slightly different chemical shift of the carbonyl group in the NMR spectrum.[43,44,46] Thus the estimated percentage fraction of the α-helix and the β-sheet can be estimated by the deconvolution of the C=O peak. Fig. 8 displays the deconvoluted carbonyl NMR peaks of raw and regenerated materials. The full width at half maximum (FWHM) of the peak also provides information about the degree of motional freedom possessed by the nuclei. The higher value of full width at half maximum (FWHM) shows that the nuclei possess lower motional freedom. Previous studies showed that such deconvolutions usually result in two peaks, one at 175.6 ± 0.2 ppm, which is ascribed to the

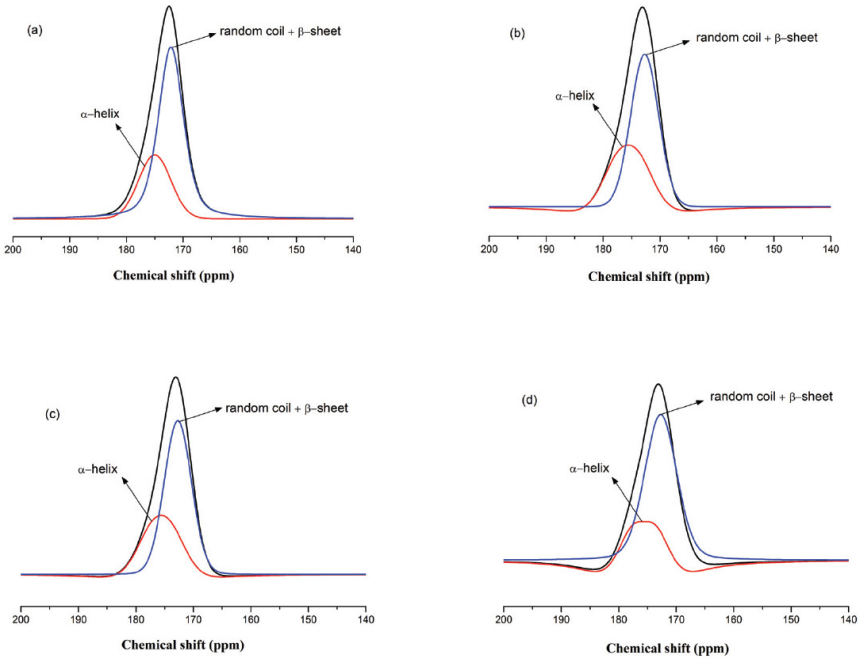


Fig. 8 The ^{13}C CP MAS NMR spectra of (a) raw material, (b) regenerated keratin material from [BMIM]Cl, (c) regenerated keratin material from [AMIM]Cl, and (d) regenerated keratin material from [choline][thioglycolate] which were fitted with Gaussian/Lorentzian fitting functions. The error in the fitting was ~3–5%.

α-helix, and the other at 172.7 ± 0.2 ppm which is related to the β-sheet molecular conformations, respectively.[43,44,46] However, another study ascribed the NMR peak at 172 ppm to the random coil structure as well.[47] Hence, we assume that the NMR peak ~172.0 ± 0.2 emerges from the nuclei of both β-sheet and random coil structures. In the present study, best fits for deconvoluting the carbonyl NMR peak for the raw material were achieved by using 175.0 ppm for the α-helix and 172.0 ppm for the random coil + β-sheet. On the other hand, for all of the regenerated materials, 175.6 ppm and 172.7 ppm respectively gave the best fits. Table 1 shows that, in the case of the raw and regenerated materials from [BMIM]Cl and [AMIM]Cl, the percentage fraction of the random coil + β-sheet was about double that of the α-helix and was unchanged in the regenerated material. Conversely, the percentage fraction of the random coil + β-sheet was much higher in the regenerated keratin from [choline][thioglycolate]. A plausible explanation of this observation is that the dissolution of turkey feather in reducing ionic liquids such as [choline][thioglycolate] results in a much stronger cleavage of the disulfide linkages and therefore promotes the formation of random coil and β-sheet structures. The NMR data in Table 1, which display a lesser fraction of the α-helix compared to the random coil + β-sheet for all materials, agree well with their corresponding X-ray diffraction spectra that also show a lesser fraction of the α-helix compared to the random coil + β-sheet.

Table 1 The percentage fraction of α-helix and random coil + β-sheet of raw material and regenerated keratin material, which were fitted using dmfit[a] program ^{13}C CP MAS NMR spectra

Peaks	% Fraction ± 5	Chemical shift (ppm) ± 0.2	FWHM (Hz) ± 20
Raw material			
α Helix	33	175.0	470
Random coil + β sheet	67	172.0	379
Regenerated keratin material from [BMIM]Cl			
α Helix	33	175.6	617
Random coil + β sheet	67	172.7	437
Regenerated keratin material from [AMIM]Cl			
α Helix	33	175.6	588
Random coil + β sheet	67	172.7	419
Regenerated keratin material from [choline][thioglycolate]			
α Helix	11	175.6	559
Random coil + β sheet	89	172.7	494

[a] The dmfit program was used for the deconvolution of NMR into various peaks and simulated fit.[45]

Thermal stability and phase behaviour

The thermal decomposition analyses of the raw material and regenerated keratin material were conducted by TGA (Fig. 9). Thermal stability of raw material and regenerated keratin showed that these materials were stable up to 200 °C, with the stability of regenerated keratin material being slightly higher

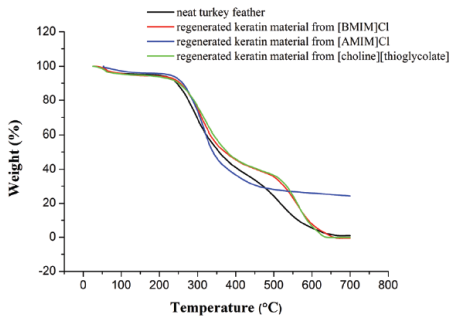


Fig. 9 Single heating scan TGA traces of keratin samples.

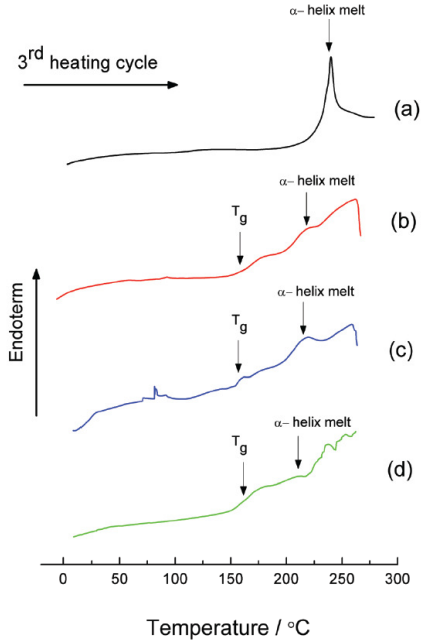


Fig. 10 DSC heating scan (3rd cycle) for (a) raw material and regenerated keratin material from (b) [BMIM]Cl, (c) [AMIM]Cl, and (d) [choline]-[thioglycolate].

than the raw turkey feather. There are two stages of decomposition that can be seen in all cases. A small weight loss involved in the first stage could be due to the evaporation of incorporated water near100 °C. Between 250 °C and 400 °C, the second stage involved with the degradation of the keratin materials.[10]

The phase behaviour of the keratin materials was studied by a differential scanning calorimeter (DSC). In order to ensure that no moisture was present in the samples, three consecutive heating and cooling cycles were carried out; the DSC traces in Fig. 10 are reported for the 3rd heating cycle. The DSC trace for the raw material {Fig. 10(a)} shows an exothermic peak around ~230 °C, which is usually assigned to α-helix disordering and decomposition (in some cases described as a "melt").[1] In the case of the regenerated keratin materials {Fig. 10(b)–(d)}, this peak was broadened and shifted to comparatively lower temperatures. These observations suggest the formation of some greater degree of disordered/amorphous phase and loss of the α-helix in all regenerated keratin materials compared to the raw keratin material. No obvious glass transition (T_g) was observed in the raw keratin material {Fig. 10(a)}, however in all regenerated materials, a prominent T_g was observed *ca.* 170 °C–180 °C. This tends to confirm the suggestion that these materials contain a higher degree of disordered material.

Characterisation of water soluble fraction

The development of methods to separate the water soluble fraction from the IL–water solution are underway and will be reported in a future publication. However, it was possible to gain some insight into the nature of the aqueous components as follows.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with a standard marker was employed to characterise and identify proteins in the water soluble fraction obtained during the regeneration of the keratin material. The protein bands were visualised by silver staining (Fig. 11). The presence of protein was confirmed and the majority of these proteins were in the molecular weight fraction range between 10 and 40 kDa. This observation indicates that upon dissolution in these three ionic liquids, there are proteins isolated that are water soluble. As 90% of the protein in feather samples is predicted to be keratin,[7] it is most likely that these water soluble polypeptides are keratin related fragments. The

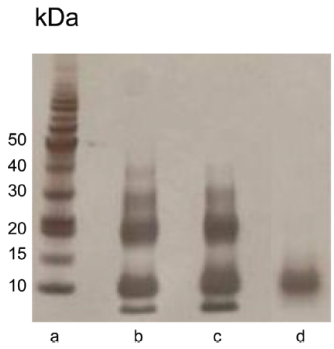


Fig. 11 SDS-PAGE pattern of (a) protein standard (b) water soluble protein in [BMIM]Cl, (c) water soluble protein in [AMIM]Cl, and (d) water soluble protein in [choline][thioglycolate].

most intense bands appeared at 10 kDa and 20 kDa in the [BMIM]Cl and [AMIM]Cl samples, whereas in [choline][thioglycolate] only one band appeared, at about ~10 kDa. The discreet bands differing by 10 kDa each are suggestive of monomeric, dimeric and possibly trimeric forms of the same protein sequence. This possibility is fuller supported by the disappearance of the longer multimeric fragments in the [choline][thioglycolate] that is thought to act as a reducing agent {Fig. 11(d)}. In addition, as the molecular weight of feather keratin monomer is ~10 kDa,[13,37,48] it is likely that these bands are representative of keratin monomer and dimers.

Conclusions

In summary, the solubility of feather keratin in ionic liquids is dependent on the ability of the anion and cation combination to disrupt the hydrogen bonding in the material. Several ionic liquids were shown to have good potential to be applied as solvents for feather dissolution. In particular, it was found that at 130 °C, turkey feather keratin is soluble in [BMIM]Cl, [AMIM]Cl and [choline][thioglycolate] up to 45 wt%. A regenerated keratin material representing up to 51% of the starting mass was obtained by precipitation from water. By ATR-FTIR, XRD and solid state NMR, it was observed that dissolution occurs without major chemical change of the polypeptide chain conformation, but did involve breakdown of the polymer chains into smaller segments and loss of some of the α-helix structure. The thioglycolate IL appeared to accelerate dissolution somewhat, causing a greater degree of fragmentation in the water soluble fraction and a greater degree of disruption of the α-helix structure, however it is clear that such a reducing agent is not a necessary component in dissolution. From the gel electrophoresis results, it was shown that the water soluble fraction is of lower molecular weight but still substantially polymeric. Further experimental studies of mechanical properties of a regenerated keratin biomaterial are in progress to evaluate the possibility of processing it into physical states such as fibers or films using known techniques.

Acknowledgements

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2.4 Supplementary Information

Supplementary Information to accompany:

Dissolution of feather keratin in ionic liquids

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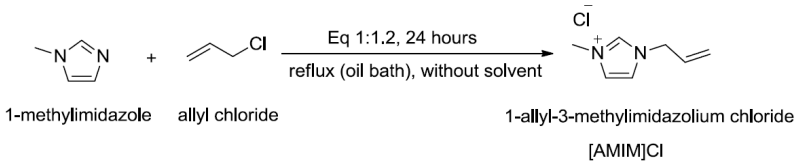
Supplementary Information

Preparation of ionic liquids

1-allyl-3-methylimidazolium chloride [AMIM]Cl was prepared according to the literature[1]



Scheme S1. Preparation of [AMIM]Cl

Allylchloride (30.8 ml, 0.378 moles) and *N*-methylimidazole (25.1 ml, 0.315 moles) were inserted in a three neck round bottom flask via a syringe. The three neck round bottom flask equipped with a reflux condenser and a magnetic stirrer was flushed with nitrogen for 10 mins beforehand. The mixture was stirred at 50°C to 60°C for 24 hours. Evaporation of excess allylchloride under *vacuo* gave the product, a viscous liquid **(Scheme S1)** displaying a slight amber colour (48.695 g, 97%);^{1}H NMR (ppm, 400 MHz, CD_3OD) δ_H: 9.00 (1H, s, ArH), 7.62 (2H, s, ArH), 6.08 (1H, m, CH), 5.43 (2H, m,CH_2), 4.87 (2H, d, CH_2), 3.96 (3H, s, CH_3); ^{13}C NMR (ppm, 400 MHz, $CDCl_3$) δ_C: 138.0, 132.1, 125.1, 123.6, 121.9, 52.8, 36.6; ES-MS: ES^+ *m/z* 123.1 $[AMIM]^+$. ES^- *m/z* 35.1 $[Cl]^-$. Water content (Karl Fischer): 16234.ppm

Dissolution of keratin in ionic liquids

Table S1. Solubility of turkey feather in ionic liquids

Ils	Condition/°C	Time/H	Solubility (wt% ± 1)	Appearance
[BMIM]Cl	130	10	50	soluble, viscous
[AMIM]Cl	130	10	50	soluble, viscous
[Choline][thioglycolate]	130	10	45	soluble, viscous
[Bis-2-ethylhexylammonium] [thioglycolate]	130	10	-	Insoluble

Regenerated keratin from ionic liquids

Table S2. Mass of regenerated turkey feather from dissolution in ionic liquids

Ils	Mass of turkey feather dissolved (mg)	Mass of regenerated turkey feather (mg)	Mass of regenerated turkey feather (% ± 1)
[BMIM]Cl	500.0	293.5	59
[AMIM]Cl	500.0	287.2	57
[Choline][thioglycolate]	450.0	230.1	51
[Bis-2-ethylhexylammonium] [thioglycolate]	-	-	-

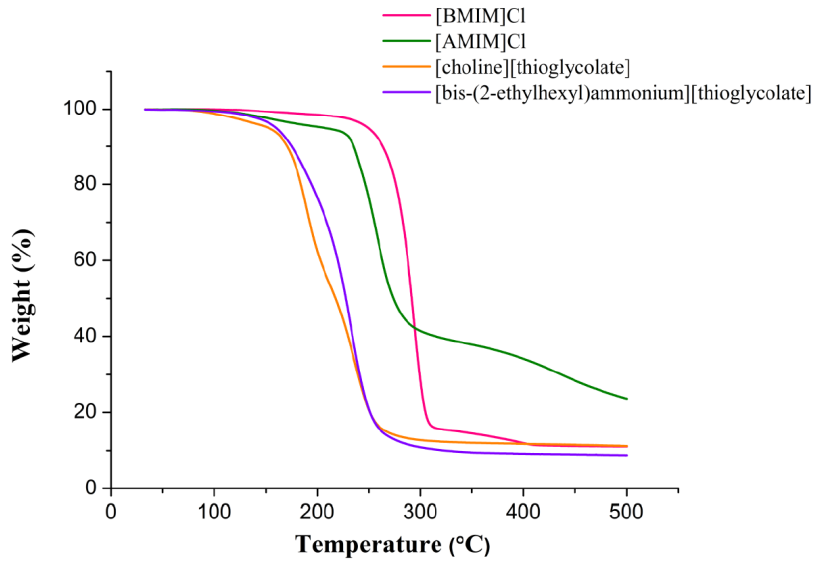


Figure S1. Single heating scan TGA traces of neat ionic liquids

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Chapter 3

Dissolution and regeneration of wool keratin in ionic liquids

CHAPTER 3

Dissolution and regeneration of wool keratin in ionic liquids

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3.1 Specific Declaration

PART B: Suggested Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Initiation, key ideas, performed the experiments, synthesis, data analysis and interpretation, manuscript development and writing up	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Douglas MacFarlane	Key ideas, proof reading and final drafting	-
Prof. Antonio Patti	Key ideas, proof reading and final drafting	-
R. Vijayaraghavan	Synthesis thioglycolate ionic liquid and proof reading	-
Usman Ali Rana	Assisted in ^{13}C CP MAS NMR experiment	-

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** **18/6/2014**
Main Supervisor's Signature	[redacted]	**Date** **18/6/2014**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

3.2 General Overview

In our previous manuscript (Publication 1 in Section 2.3), we have optimised various process parameters such as temperature, process time and managed to dissolve feather keratin using different ionic liquids ([BMIM]Cl, [AMIM]Cl and [choline][thioglycolate]). In this study, we have investigated dissolution aspects of another type of fibrous keratin protein, namely wool. The difference between feather and wool is the cystine content[1-3] and typically if the cystine content is greater in any fibrous protein, it is harder to dissolve.[4,5] In order to address this issue, we have employed dicyanamide ionic liquids in this study. From the literature,[6] it has been reported that the solubility of corn protein zein in 1-ethyl-3-methylimidazolium dicyanamide was almost double compared to other imidazolium based ionic liquids. It is hypothesised that this anion forms strong hydrogen bond interactions towards the polypeptide chains of the protein. In addition, the viscosity of 1-ethyl-3-methylimidazolium dicyanamide is much lower compared to the other ionic liquids, therefore it is likely to dissolve the keratin more rapidly. An overview of ionic liquids used in wool dissolution as reported in the literature is presented in Table 1.

Table 1: Dissolution of wool keratin in ionic liquids

Ionic liquids	Experimental condition of the dissolution process	Summary	References
[BMIM]Cl	130 °C, 10 hours	Solubility of 11 wt%	Xie *et al.*[7]
[BMIM]Cl	100 °C, 10 hours	Solubility of 4 wt%	Xie *et al.*[7]
[BMIM]Cl	130 °C, ~9 hours	Solubility of 15 wt%	Li *et al.*[8]
[BMIM]Cl	100 °C, 12 hours	Natural wool/cellulose	Hameed *et*

		acetate blend developed using [BMIM]Cl (5 wt% solution)	*al.*[9]
[AMIM]Cl	130 °C, 10 hours	Solubility of 8 wt%	Xie *et al.*[7]
[AMIM]Cl	130 °C, ~10 hours	Solubility of 21 wt%	Li *et al.*[8]
[BMIM]Br	130 °C, 10 hours	Solubility of 2 wt%	Xie *et al.*[7]
[BMIM][BF_4]	130 °C, 24 hours	Insoluble	Xie *et al.*[7]
[BMIM][PF_6]	130 °C, 24 hours	Insoluble	Xie *et al.*[7]

The solvent properties of deep eutectic solvents were also studied as an alternative to the ionic liquids. Abbot *et al.*[10] showed that the mixture of solid compounds forms a liquid (eutectic mixture) that possess a melting point lower than their individual starting compounds. Deep eutectic solvents can be tuned via the combinations of organic salts (quartenary ammonium salts) and complexing agent.[10-12] These solvents can be considered as inexpensive and easy to synthesise.[11,13] Several eutectic solvents such as choline chloride/urea, choline chloride/citric acid and choline chloride/succinic acid have been reported as excellent solvents for starch dissolution.[14,15]

Apart from the influence of the deep eutectic solvents in the dissolution process, the effect of reducing agent is also studied and discussed. Reducing agents have the ability to reduce the disulfide bonds in the protein. There are various types of reducing agent that are available commercially. However, not all of the reducing agents can simultaneously cleave the disulfide bonds and preserve the peptide bonds of the protein backbone. In this study, 2-mercaptoethanol was employed as

reducing agent because it has potential to cleave the disulfide crosslinks without destroying the backbone.[16,17]

As mentioned in the previous manuscript (Publication 1 in Section 2.3), two protein fractions (water soluble and water insoluble) were also obtained upon regeneration of wool keratin/IL solution. The regenerated wool keratin materials from different ionic liquids were analysed and characterised using ^{1}H NMR, ^{13}C NMR spectroscopy and mass spectrometry, XRD, ATR-FTIR and thermal analysis. The approximate molecular weights of the water soluble materials were determined by gel electrophoresis analysis.

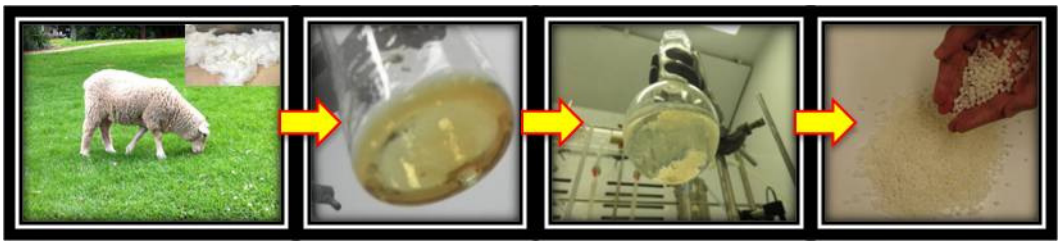
The details of this investigation have been published in the paper entitled "Dissolution and regeneration of wool keratin in ionic liquids."[18]

3.3 Publication 2

Dissolution and regeneration of wool keratin in ionic liquids

Azila Idris, R. Vijayaraghavan, Usman Ali Rana, A. F. Patti and
D. R. MacFarlane

Substantial dissolution of wool was obtained in [AMIM][dca] and [choline][thioglycolate] ionic liquids as well as deep eutectic solvent mixtures under a variety of conditions including the use of a reducing agent.



Green Chemistry

PAPER

Dissolution and regeneration of wool keratin in ionic liquids†

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Azila Idris,*[a,b] R. Vijayaraghavan,[a] Usman Ali Rana,‡[a] A. F. Patti[a] and D. R. MacFarlane[a]

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www.rsc.org/greenchem

Wool keratin, a natural biopolymer, is potentially an important *renewable* source of raw materials for the polyamide plastics industry. Large quantities of non-spinnable and short fibers of wool are discarded globally and hence are available as low value waste materials. In this study, we have investigated different solvents, including ionic liquids and deep eutectic mixtures, for the dissolution and processing of wool. The results show that substantial dissolution of wool (up to 475 mg wool per gram of solvent) can be obtained in the 1-allyl-3-methylimidazolium dicyanamide [AMIM][dca] ionic liquid at 130 °C. Our studies also indicated enhanced dissolution (an additional 50–100 mg g^{-1}) of wool upon the addition of a reducing agent to the ionic liquids. Water insoluble fractions (20–40%) were obtained on the addition of water to the dissolved wool. This regenerated fraction was characterized for structural and chemical changes and found to contain a larger fraction of β-sheets and random coils than the starting material. The water soluble fraction was characterised and the results indicated the presence of fragments of low molecular weight polypeptide chains.

1. Introduction

Biopolymers have gained significant attention as new alternatives to petroleum based materials. Although recent research focuses more on cellulosic fibers, it is also possible to consider biopolymer based protein fibers as potential renewable sources of polyamides.[1] Keratin is one of the most abundant biopolymers in this class and is available from a variety of sources. Wool keratin is one of these sources and large quantities of non-spinnable and short wool fibers are available globally as waste from the textile industry. These are currently discarded and, as a result, alternative uses of these materials are attracting the attention of researchers.[2,3]

Wool is a fibrous protein (approximately 95 wt% pure keratin[4]) that consists of about 11–17% cysteine.[5–7] This protein is insoluble in water, organic solvents, dilute acids, and alkalis and shows resistance to degradation in common solvents.[8] This is due to the tight packing of the α-helices and β-sheets present in the polypeptide structure of wool keratin. In addition, the presence of inter- and intramolecular bonding of polar and nonpolar amino acids and strong disulfide bonds in wool keratin provides conformational constraints to the polypeptide backbone and stability to the protein structure.[9,10] The diameter of the fibers varies significantly among different sheep breeds,[11,12] between 11.5 and 47 μm.[13]

Solubilisation of wool, which requires partial disruption of the keratin structure, is difficult but can be achieved in a number of ways, including reduction,[1,14,15] oxidation[1,15,16] and sulfitolysis of the disulfide bonds.[1,17,18] The reagents used in these reactions are, however, often toxic and difficult to recycle. These limitations have inspired researchers to develop new dissolution techniques, including the use of ionic liquids (ILs). Much has been published on the application of ILs as solvents for the dissolution of biopolymers such as cellulose, silk, starch, lignin and other polysaccharides.[19–26] Studies of keratin dissolution have been more limited[2,3,27,28] and mostly focused on feather keratin[27,29–31] because of the greater availability of feather raw material. One of the significant differences between the types of keratin is the difference in the cysteine content; feather and wool keratin types contain ~7% and 11–17% cysteine units in their amino acid sequences, respectively.[5–7] The higher cysteine content in wool makes this biomass more difficult to dissolve.[32,33] On the other hand, the higher cysteine content offers the potential of high glass transition (T_g) materials depending on whether the S–S bonding is preserved or reformed in the regenerated material.

Here we investigate a number of ionic liquids and deep eutectic solvents for the dissolution and regeneration of wool keratin with the ultimate aim of providing the basis for a

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process which could utilize waste wool fibres to produce useful materials. Deep eutectic solvents were also studied since these exhibit many remarkable solvent properties including for biopolymers,[34,35] and are easy to synthesize from inexpensive starting materials.[34,36,37] There are several additional variables yet to be explored to improve the solubilisation of wool, including the influence of a reducing agent for cleavage of the disulphide bonds; the dissolution experiments here were conducted with and without a reducing agent. Although many reagents are capable of reducing the disulphide bond, only a few such as thiols[38] and ammonium bisulfite have the required ability and reactivity to simultaneously preserve and maintain the peptide bonds of the protein structure. In this study, 2-mercaptoethanol was investigated for this purpose.[1,39] Structural elucidation of the materials obtained was carried out by ^{1}H NMR, ^{13}C NMR and mass spectroscopy (electron spray ionisation) along with XRD, ATR-FTIR, thermal analysis and gel electrophoresis.

2. Experimental

2.1 Materials

The medium Merino wool (approximately 23 μm diameter) used in these experiments was provided by Australian Wool Innovation Limited (AWI). It was cleaned and defatted using a 1 : 1 v/v mixture of hexane and dichloromethane in a Soxhlet extractor for 48 hours. The cleaned wool sample was dried in a vacuum oven at 70 °C for 48 hours. The remaining "bound" water fraction was quantified by thermogravimetric analysis (TGA) at approximately 10%. Since this bound water is difficult to remove prior to dissolution, our process design needed to recognize that residual water was always present. This is in distinct contrast to lignocellulose dissolution processes in ILs[26,40] in which low water content is an important aspect.

1-Butyl-3-methylimidazolium chloride ([BMIM]Cl, 98% purity) was purchased from Sigma Aldrich. 1-Allyl-3-methylimidazolium chloride, [AMIM]Cl and 1-allyl-3-methylimidazolium dicyanamide, [AMIM][dca] and choline thioglycolate were synthesized according to literature[27,41] procedures. For the preparation of these ILs, allylchloride (98%), *N*-methylimidazole (99%), choline hydroxide (20 wt% in H_2O), thioglycolic acid (98%), silver nitrate (99%) and sodium dicyanamide (96%) were obtained from Sigma Aldrich. For NMR analysis, deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OD) purchased from Merck were used. Unless otherwise stated, all organic solvents and reagents were used as received from commercial suppliers. Water contents, as indicated by mass loss around 100 °C in the TGA data (Fig. S1†), were below 1% for the chloride ILs, and between 1% and 5% for the thioglycolate and dca ILs, both of which are difficult to completely dry. However, it is important to note that since wool also contains difficult to remove bound water, our goal was to study a process that could be carried out under practical conditions and typical water contents.

2.2 Dissolution of wool

The solubility of wool fibre was determined at 130 °C for 10 hours in glass vials which were placed into a heating block under a N_2 atmosphere. In order to quantify the solubility, 50 mg wool fibers were sequentially added to the ionic liquid (2 g) and continually stirred with a magnetic stirrer until the fiber was observed visually to have completely dissolved (at 130 °C the solution was sufficiently fluid for this to be practical). In addition, a laser beam was used to identify the presence of small particles, through the observation of any light scattering from the solutions. In some cases, particularly at high wool contents, the rate of wool dissolution appeared to decrease markedly, probably due to the increasing viscosity of the solution; for this reason we describe these ultimate observations as "limiting solubility" to indicate that these are kinetically limited values rather than thermodynamic solubilities.

The regeneration of the dissolved wool keratin was achieved through the addition of water (30 ml) to the IL solutions. The precipitate was separated using a centrifuge at 5000 rpm for 25 minutes. The regenerated keratin obtained was washed 3× with water (40 ml) *via* centrifugation in order to remove any ionic liquid that may be retained in the regenerated material. Then, it was dried *in vacuo* for 3 days at 45 °C. The regenerated solid keratin obtained was a light yellowish brown color. The water–ionic liquid solution from the washing of the precipitate was then evaporated using a rotary evaporator to leave the water soluble keratin fraction and the ionic liquid. Thus far, we have not been able to separate the soluble fraction of keratin from the IL. However, we have been able to identify the soluble proteins using SDS-PAGE analysis as described below.

The powder X-ray diffraction (XRD) patterns were obtained at 22 ± 2 °C using a Sietronics powder diffractometer. For each XRD experiment, approximately 1–2 g of the finely ground sample was placed randomly on a locally designed flat brass sample holder fitted with an o-ring sealed Mylar sheet cover, providing an airtight atmosphere. CuKα1 radiation (λ = 1.540 Å) was produced at 40 kV and 25 mA. The data were collected in the Bragg–Brentano ($\theta/2\theta$) horizontal geometry using a 2θ-range of 5 to 50.0° with a step size of 0.02° and an accompanying scan rate of 0.5 ° min^{-1}.

Fourier transform infrared spectra were obtained using a Bruker IFS Equinox FTIR system coupled with a Golden Gate single bounce diamond micro-attenuated total reflectance crystal and a liquid nitrogen cooled Mercury/Cadmium Telluride detector. The FTIR was performed in the wavenumber range of 600 cm^{-1} to 4000 cm^{-1}. The spectra were recorded with a resolution of 4 cm^{-1} with 50 scans. Spectra were baseline corrected.

^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker DPX-400 spectrometer. All samples were measured as solutions in deuterated chloroform and deuterated methanol. Chemical shifts are reported in ppm on the δ scale, and the coupling constant is given in Hz. Chemical shifts were calibrated on the solvent peak unless otherwise specified. Solid state 1D ^{1}H static NMR spectra of neat and regenerated

samples were acquired at a Larmor frequency of 300 MHz on a Bruker AV-300 spectrometer. The ^{13}C CP MAS NMR spectra of these samples were acquired using a 4 mm rotor with Kel-f cap at a 10 kHz spinning rate. The contact time in the CP MAS experiments was 2.4 ms with a recycle delay of 1 s and cw decoupling. The number of scans was ~90 000 to 100 000. In the case of 1D ^{1}H static NMR experiments, the pulse length was ~3.6 μs with a recycle delay of 5 s and 16 to 20 scans.

Differential scanning calorimetry (DSC) was conducted on a DSC Q100 series instrument from TA Instruments with 5–10 mg of sample in closed aluminium pans, at a ramp rate of 10 °C per minute. All samples were cooled to −150 °C, held for 5 minutes and heated to 200 °C. Thermal scans below room temperature were calibrated *via* the cyclohexane solid–solid transition and melting points at −87.0 °C and 6.5 °C respectively. Thermal scans above room temperature were calibrated using indium, tin and zinc with melting points at 156.6 °C, 231.9 °C and 419.5 °C respectively. Transition temperatures are reported using the peak maximum of the thermal transition.

The thermal stability of the neat wool and regenerated materials was investigated by TGA using a Pyris 1 under a flowing dry argon atmosphere between 25 °C and 500 °C, at a heating scan rate of 10 °C min^{-1}. The samples were first dried under vacuum in an oven at a temperature of 70 °C. These samples were then loaded into aluminum crucibles and equilibrated for 15 minutes at the starting temperature of 25 °C before running each experiment.

Electrospray ionisation mass spectra were recorded on a Micromass Platform II API QMS Electrospray Mass Spectrometer. Samples dissolved in methanol were subjected to a suitable cone voltage, usually 25 V to 35 V. Measurements were made in both the positive and negative modes.

Protein samples in the water soluble fraction were diluted in 4× NuPAGE® loading buffer (Life Technologies) and electrophoresed using the Hoefer miniVE vertical electrophoresis system (Amersham Biosciences) in 4–12% Bis-Tris NuPAGE® gradient gels (Life Technologies). Proteins were stained and visualised by silver staining.

3. Results and discussion

3.1 Dissolution and regeneration of wool keratin in ionic liquids

Prior to starting the dissolution process, the stability of all ionic liquids used in this work was studied. The TGA data of all the ILs have been included in the ESI (Fig. S1†) and the ILs showed a small weight loss between 100 °C and 130 °C due to water loss. Thereafter, they were stable until decomposition above 200 °C. This temperature was chosen for the dissolution studies since better solubility was observed at this temperature in literature reports of wool dissolution by other methods.[2]

The apparent limiting solubility results for wool fibre in [BMIM]Cl, [AMIM]Cl, [choline][thioglycolate], and [AMIM][dca] are shown in Fig. 1. As the solutions become viscous, the dissolution rate slows down considerably; hence, it is likely that the true solubility limit is even higher than these results. The highest solubility was found to be 475 mg (wool) g^{-1} (IL) in [AMIM][dca]. Solubility in [choline][thioglycolate] was 225 mg g^{-1}. The results for [BMIM]Cl and [AMIM]Cl are in approximate agreement with the work of Li *et al.*[42] who observed 250 mg g^{-1} and 200 mg g^{-1} dissolution respectively. Xie *et al.*[2] observed significantly lower solubility for the chloride salts; however, neither of the papers identifies the type of wool and fiber diameter involved and it is likely that this factor will have some effect on the apparent rate and extent of solubility. From this it appears that the [dca] anion plays a significant role in effecting solubility compared to the other ionic liquids. It is notable that the viscosity of the solutions are lower in the dca case.[43] In the literature,[44] it was also reported that the high solubility of carbohydrates (glucose and xylose) in [BMIM][SCN] is due to the strong [SCN] hydrogen bond interactions; similar effects might be expected of the dca anion and hence the high solubility for keratin is observed here.

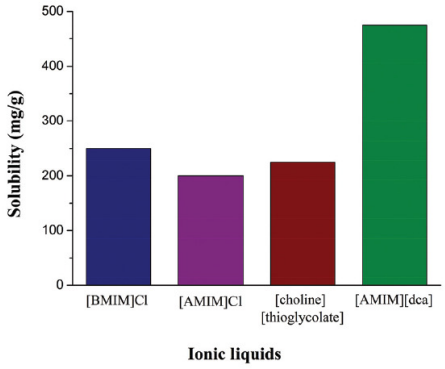

Fig. 1 Solubility of wool in ionic liquids.

It is interesting to compare the treatment conditions developed here with those that have emerged in the dissolution of lignocellulose materials. Despite the distinct difference in the chemical nature of the biopolymers involved (polypeptide in the case of keratin as opposed to polysaccharide and poly aromatic ether in the case of cellulose and lignin respectively) the dissolution conditions are quite similar. The conditions required in the lignocellulose case have been reviewed recently by Da Costa Lopes *et al.*[26] and broadly involve temperatures above 100 °C for multiple hours of treatment, similar to the times and temperatures that appear to be necessary for keratin dissolution.[2,27]

Fig. 2 shows the fraction of aqueous-insoluble material regenerated by precipitation from water in each case. It has been reported that wool keratin consists of approximately 40% hydrophilic and 60% hydrophobic groups in the amino acid sequence.[6] Therefore, if some degree of cleavage of the protein occurs then it can be expected that a fraction of the material will become soluble in water, as observed. The nature of this water soluble fraction that is generated is characterised further

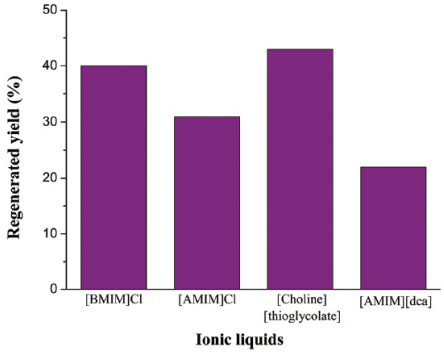

Fig. 2 Regenerated yield of aqueous insoluble wool keratin from ionic liquids.

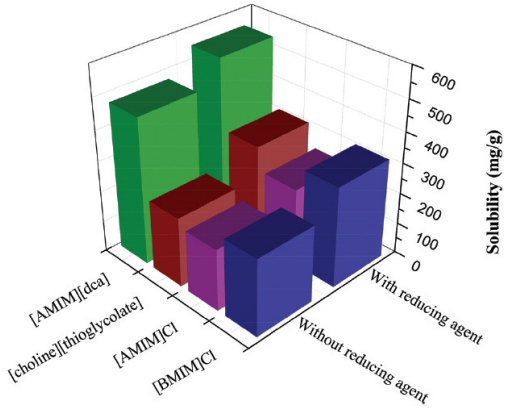

Fig. 3 Solubility of wool in ionic liquids, with and without a reducing agent.

below. The fraction of regenerated material that precipitated from the water–[AMIM][dca] mixture was found to be lower than other ILs, perhaps due to the ability of the dicyanamide anion to disrupt the hydrogen-bonding in the protein aggregates and this H-bonding is not easily reformed in some cases to regenerate the insoluble material.

3.2 Dissolution of wool in deep eutectic solvents

Deep eutectic mixtures were also studied as inexpensive alternatives to ionic liquids. Compositions and solubility characteristics of wool in the deep eutectic solvent mixtures are shown in Table 1. It can be seen that wool dissolved to a small extent in mixtures of choline chloride/urea and choline chloride/oxalic acid in a 1 : 2 mole ratio. No wool solubilisation was noted for calcium chloride/urea. Thus, although Biswas *et al.*[45] reported that mixtures of choline chloride/zinc chloride and choline chloride/oxalic acid are excellent solvents for dissolution of starch[45] and cellulose;[46] it appears that these solvents are not effective for keratin dissolution.

3.3 Influence of reducing agents

Fig. 3 shows the solubility of wool in ionic liquids with and without a reducing agent. In each case, the inclusion of a reducing agent resulted in an increase in limiting solubility. This action is thought to be due to the cleaving of the inter- and intramolecular disulphide bonds *via* a reduction reaction.[39,47] The breaking of disulphide bonds disrupts the tertiary and quaternary structures of the wool protein, as well as allowing the breakdown of the wool keratin into smaller fragments.[39]

Table 1 Solubility of wool keratin in deep eutectic solvent mixtures

Component A	Component B	Molar ratio (A : B)	Solubility (mg g^{-1} ± 12.5)
Choline chloride	Urea	1 : 2	120
Choline chloride	Oxalic acid	1 : 2	30
Calcium chloride	Urea	1 : 2	Insoluble

3.4 Characterisation of regenerated wool keratin

The X-ray diffraction (XRD) spectra of the raw wool and regenerated wool keratin materials are presented in Fig. 4. Two crystal structures are typically observed for raw wool;[48,49] an α-helix structure is manifested in peaks appearing at 2θ = 9° (0.98 nm) and 17.8° (0.51 nm) whereas the β-sheet structure shows peaks appearing at 2θ = 9° (0.98 nm) and 19° (0.47 nm). Compared to raw wool, the XRD patterns for the regenerated wool keratin samples (Fig. 4(c)–(e)) clearly show the disappearance of the peak at about 9°. This indicates that the crystallinity of these regenerated samples is significantly reduced by the dissolution and regeneration process. In the case of the regenerated material from [AMIM][dca] (Fig. 4(d)), an additional broad diffraction peak appeared, centered around 2θ = 27°. This observation suggests the formation of a disordered/amorphous material.[50]

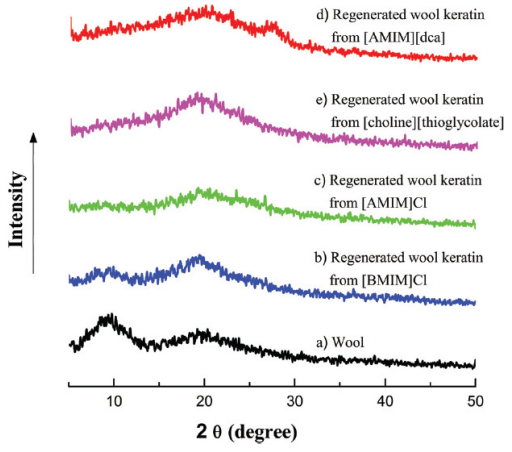

Fig. 4 XRD patterns of wool and regenerated wool keratin.

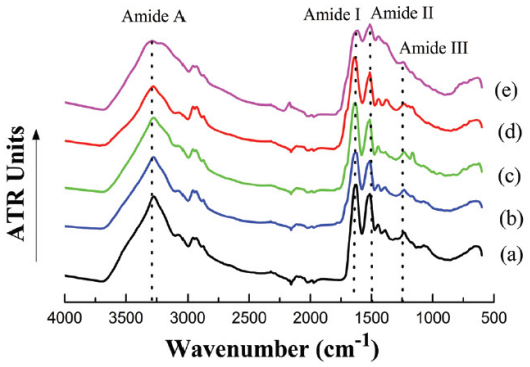

Fig. 5 ATR-FTIR of (a) wool and regenerated wool keratin from (b) [BMIM]Cl, (c) [AMIM]Cl, (d) [choline][thioglycolate], (e) [AMIM][dca].

In Fig. 5, the ATR-FTIR spectra of raw wool and regenerated keratin are compared. The spectra showed characteristic absorption bands ascribed predominantly to the peptide bonds (–CONH) and these have been labeled as Amide A, Amide I, Amide II and Amide III bands. A medium absorption band in the range of 3283–3273 cm^{-1} is attributed to N–H stretching (Amide A), while Amide I showed a strong absorption band that occurred in the range of 1640–1614 cm^{-1}. A strong band was observed in the range of 1516–1513 cm^{-1} and assigned to C–N stretching and N–H bending vibrations (Amide II). Another weak band was recorded in the range of 1234–1242 cm^{-1} indicating the C–N, C–O stretching, N–H and O=C–N bending vibrations (Amide III).[32,51,52] No additional bands were seen in the ATR-FTIR of the regenerated samples. However, the ^{13}C NMR results indicate that some chemical changes have occurred and these are described below.

^{13}C CP MAS spectra of the raw and regenerated keratin materials are shown in Fig. 6. The spectra show an asymmetric

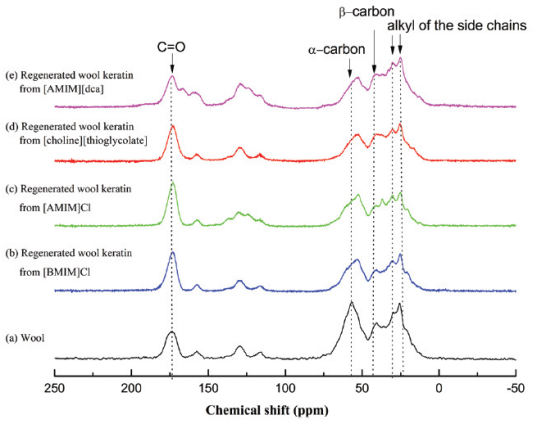

Fig. 6 The ^{13}C CP MAS NMR spectra of (a) wool and regenerated wool keratin from (b) [BMIM]Cl, (c) [AMIM]Cl, (d) [choline][thioglycolate], (e) [AMIM][dca].

peak with a maximum between 172 ppm and 174 ppm. This peak is attributed to the amide carbonyl carbons of the keratin protein. The peak at 175 ppm is assigned to the α-helix of keratin, while that at 172 ppm is attributed to the β-sheet molecular and random coil conformations.[53–55] The peak at around 130 ppm indicates the presence of aromatic group containing amino acids in the keratin.[53] The α-carbons were recorded between 52 ppm and 56 ppm, while the β-carbons (present in leucine and cross-linked cysteine residues) were observed at 40 ppm.[54] The carbon peaks recorded between 30 ppm and 40 ppm indicated the presence of proline, glutamic acid and glutamine residues. The NMR signal at low chemical shifts is associated with the alkyl groups of the side chains.[54] Cysteine groups were difficult to observe (25 ppm to 29 ppm) in the NMR spectra as they overlap with the chemical shift of the alkyl groups.

The α-carbon peak between 52 ppm and 56 ppm was broadened and formed a shoulder with its maximum moving to lower frequency in the regenerated keratin materials (Fig. 6(b)–(e)). The broadening of peaks in the regenerated keratin samples may be due to the ability of the ionic liquids to disrupt hydrogen bonding of the original keratin raw material, leading to the unfolding of the polypeptide chains. This would result in the formation of a greater fraction of β-sheet and random coil structures, in agreement with the XRD data discussed above.

Fig. 6(e) shows that the peaks from the carbonyl carbon in the material regenerated from [AMIM][dca] in the region of 170–180 ppm were significantly different from the raw wool and the other regenerated keratin materials. The additional peak around 172 ppm from random coil and β-sheet structures seems to suggest a greater degree of disruption of the protein during dissolution in this case. The same observations are reported by Ando *et al.*[56] in their solid state NMR study of wool keratin.

The phase behaviour of the raw wool and regenerated keratin materials was studied using differential scanning calorimetry (DSC), Fig. 7. The samples were subjected to three consecutive heating and cooling cycles in order to obtain reproducible results and the third cycles are reported. The DSC trace of the raw material (Fig. 7(a)) shows a peak around 230 °C which corresponds to α-helix disordering and decomposition[57] (some literature also describes this transition as a "melt"[58]). In the case of the regenerated keratin materials (Fig. 7(b)–(e)), no sharp peak was observed; this is consistent with the loss of crystallinity that was observed in the XRD data. Broad endothermic steps observed between 150 °C and 200 °C may indicate the glass transition in an amorphous fraction of the material.

The thermal stability of the materials was investigated by thermogravimetric analysis (TGA), (Fig. 8), indicating that the thermal stability of the original wool keratin has been retained. A two-step decomposition process was observed in all cases. The TGA curves show a small weight loss at 100 °C, which is due to the evaporation of bound water present in the keratin materials.[42,59,60] The decomposition between 250 °C

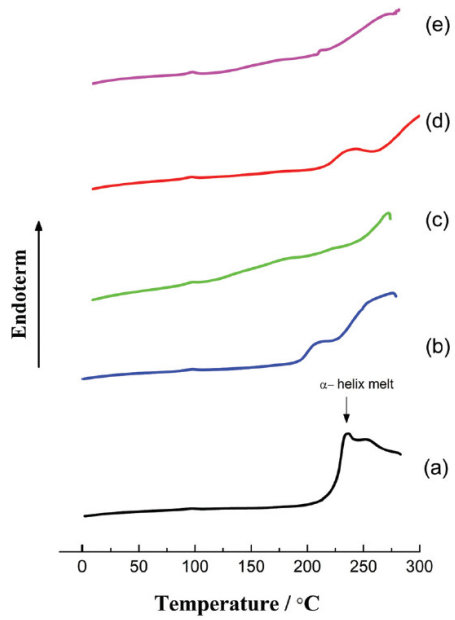

Fig. 7 DSC thermograms of (a) wool and regenerated wool keratin from (b) [BMIM]Cl, (c) [AMIM]Cl, (d) [choline][thioglycolate], (e) [AMIM][dca].

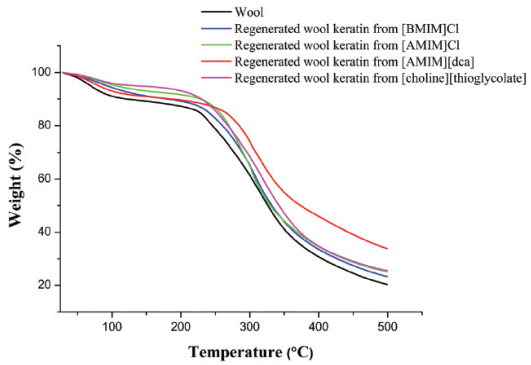

Fig. 8 TGA plot of wool and regenerated wool keratin.

and 400 °C could be initiated by the denaturation and degradation of the wool protein molecules.[42,59,60] It was also reported that volatile compounds including hydrogen sulphide and sulphur dioxide are released from wool due to the cleavage of the disulphide bonds that occurred between 230 °C and 250 °C.[60]

3.5 Characterisation of water soluble fraction

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with a standard marker was employed to characterize the residual water soluble fraction remaining after the regeneration of the insoluble keratin material. The protein band was visualized by silver staining. A typical gel pattern is shown in Fig. S2 in the ESI.† The bands that appear on the gel confirm the presence of water soluble protein fractions in the solution. Their molecular weights range from as low as 15 kDa to >120 kDa. In the electrophoresis separation patterns obtained by Tonin *et al.*[61] with their steam exploded wool, two main keratin fractions were obtained, described as low sulfur proteins and high sulfur proteins in the molecular weight range between 67 kDa–43 kDa and 28 kDa–11 kDa, respectively. In the case of the [BMIM]Cl, [AMIM]Cl and [AMIM][dca] samples here, this distinction is not clear, a broad range of molecular weights being present. In the [choline][thioglycolate] case, the low sulfur protein fraction appears to have been reduced in molecular weight. Since the continuous bands observed in gel electrophoresis were in the low molecular weight range, it was concluded that the ionic liquids had cleaved the protein into smaller polypeptide chains as also observed by Tonin *et al.*[61]

4 Conclusions

In summary, we have investigated a number of ionic liquids and deep eutectic mixtures as solvents for the dissolution of wool. The dissolution in deep eutectic solvents is poor compared to the ionic liquids and therefore it appears that the ionic medium and the nature of some of the ions must play a major role in the dissolution process. In particular, the highest limiting solubility (475 mg g^{-1}) was achieved in [AMIM][dca]. The addition of a reducing agent such as mercaptoethanol caused increased dissolution of the wool by 50–100 mg g^{-1}. The regenerated wool keratin retained the protein backbone, as observed by ATR measurements, while the crystallinity was substantially lost, as determined by XRD methods. The dissolution also produced a breakdown of the polypeptide chains into lower molecular weight fragments, some of which became water soluble as observed by gel electrophoresis. Further work relating to the processing of the regenerated materials into fibers and films as well as the recycling of the ILs is currently underway.

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3.4 Supplementary Information

Supplementary Information to accompany:

Dissolution and regeneration of wool keratin in ionic liquids

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Supplementary Information

Dissolution of wool in ionic liquids

Table S1. Solubility of wool in ionic liquids

ILs	Condition/°C	Time/H	Solubility (mg/g ± 12.5)	Appearance
[BMIM]Cl	130	10	250	soluble
[AMIM]Cl	130	10	200	soluble
[choline][thioglycolate]	130	10	225	soluble
[AMIM][dca]	130	10	475	soluble

Regenerated wool keratin material from ionic liquids

Table S2. Mass of regenerated wool keratin material from dissolution in ionic liquids

ILs	*Mass of wool keratin dissolved (mg)	Mass of regenerated wool keratin (mg)	Percentage wool keratin recovered (% ± 2)
[BMIM]Cl	500.0	200.0	40
[AMIM]Cl	400.0	124.0	31
[choline][thioglycolate]	450.0	195.0	43
[AMIM][dca]	950.0	208.0	22

* Mass of wool dissolved in 2 g of ILs

Table S3. Solubility of wool in ionic liquids with and without reducing agent

ILs	*Reducing agent		Condition/°C	Time/H	Solubility (mg/g ± 12.5)	Appearance
	with	without				
[BMIM]Cl		☞	130	10	250	soluble
[BMIM]Cl	☞		130	10	330	soluble
[AMIM]Cl		☞	130	10	200	soluble
[AMIM]Cl	☞		130	10	250	soluble
[choline][thioglycolate]		☞	130	10	230	soluble
[choline][thioglycolate]	☞		130	10	330	soluble
[AMIM][dca]		☞	130	10	480	soluble
[AMIM][dca]	☞		130	10	550	soluble

*Reducing agent used: 2-mercaptoethanol

Table S4. Solubility of wool in deep eutectic solvent mixtures*

Component A	Component B	Condition/°C	Time/H	Solubility (mg/g ± 12.5)	Appearance
choline chloride	Urea	130	10	120	soluble
choline chloride	oxalic acid	130	10	30	soluble
calcium chloride	Urea	130	10	-	insoluble

* Ratio by mol (1 Component A : 2 Component B)

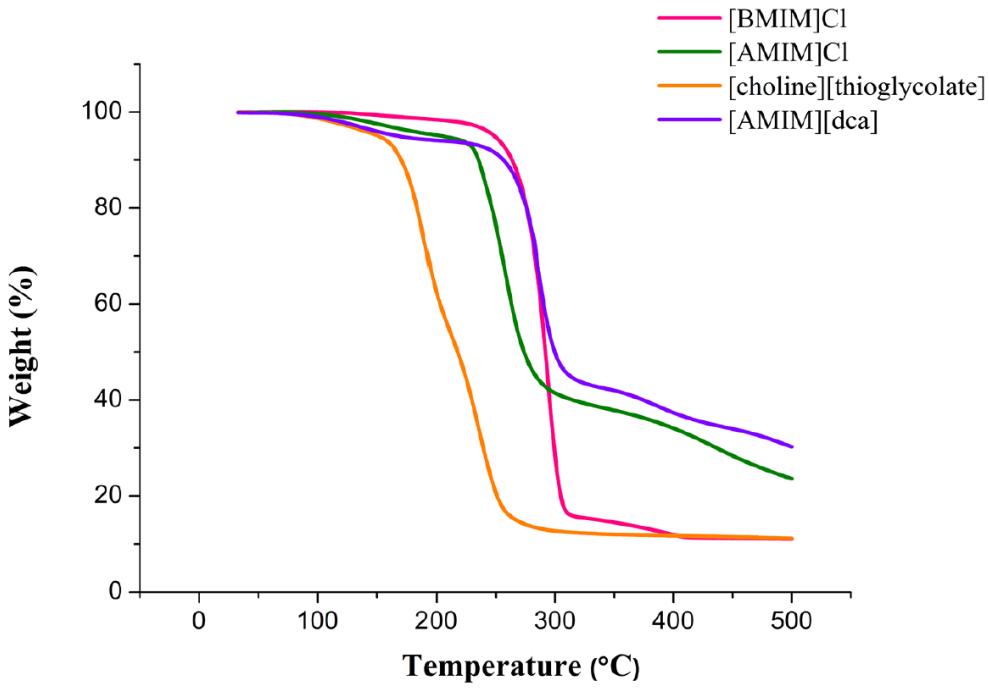

Figure S1. Single heating scan TGA traces of pure ionic liquids

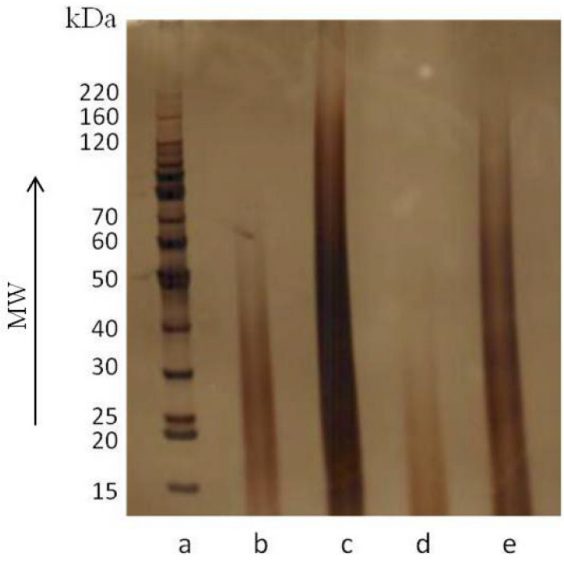

Figure S2. SDS-PAGE pattern of (a) protein standard (b) water soluble fraction from [BMIM]Cl, (c) water soluble fraction from [AMIM]Cl, (d) water soluble fraction from [choline][thioglycolate] and (e) water soluble fraction from [AMIM][dca].

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Chapter 4

Thermoplastic materials from the dissolution of feather keratin biopolymer in ionic liquids

CHAPTER 4

Thermoplastic materials from the dissolution of feather keratin biopolymer in ionic liquids

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Submitted to *Polymer*

June 2014

4.1 Specific Declaration

PART B: Suggested Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Initiation, key ideas, performed the experiments, synthesis, data analysis and interpretation, manuscript development and writing up	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Douglas MacFarlane	Key ideas, proof reading and final drafting	-
Antonio Patti	Key ideas, proof reading and final drafting	-
R. Vijayaraghavan	Key ideas, proof reading and final drafting	-

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** **18/6/2014**
Main Supervisor's Signature	[redacted]	**Date** **18/6/2014**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

4.2 General Overview

Studies on the dissolution of fibrous keratin (in particular feather and wool)[1,2] in ionic liquids were described in Publication 1 and 2 (Section 2.3 and 3.3). The follow up work here in Publication 3 (Section 4.3) was an extension of the work involving the production of the regenerated keratin biomass in ionic liquids and evaluates the possibility of processing it into physical states such as gels, films and fibers.

Due to the difficulty in processing and irregular breakage during sample preparation and mechanical analysis of wool, no further work was conducted on the wool. This chapter focused on feather keratin only. In the literature,[3,4,5] it has been reported that wool contains greater amounts of cysteine in the amino acid sequences, compared to feather. Intra- or intermolecular interactions of cysteine molecules can form cysteine bonds (S-S).[5] The crosslinks that form from the intermolecular S-S bonds give higher stiffness to the wool therefore making it more difficult to dissolve and process.[6]

From our previous studies,[1,2] [BMIM]Cl, [AMIM]Cl, [AMIM][dca] and [choline][thioglycolate] were shown to be effective solvents for dissolving fibrous keratin. In this study, [BMIM]Cl and [AMIM]Cl were chosen because these ILs gave the highest percentage of dissolution and regeneration of feather keratin compared to other ionic liquids that have been studied.[1]

Several approaches have been used to produce gels and films from feather keratin/IL solution. Our first approach involved spreading out the feather keratin/IL solution onto the surface and pressing between two glass plates to make a thin gel and film. This sandwiched plate was then soaked in distilled water to remove the IL. A thin gel or film, of flaky surface appearance, due to adherence to both surfaces was obtained. The use of Teflon mould was more successful. The gels and films that are formed by this approach without adding any plasticizers or additives can be produced in any desired shape and size. The feather keratin/IL solution was also spun into fiber.

The thermal, structural, electrical (conductivity) and mechanical properties of these materials were analyzed using DSC, XRD, ATR-FTIR and TGA along with ac impedance spectroscopy for the ionic conductivity study. Tensile testing was carried out on the soft gels and hard films in order to gain in-depth understanding of their physical properties. This understanding is crucial for the production of useful polyamide derived materials which can be applied in large scale biomass processing in the future. Conductivity measurements were also investigated to determine any potential of the regenerated keratin material as a gel electrolyte.

The details of this study have been submitted in the manuscript entitled "Thermoplastic materials from the dissolution of feather keratin biopolymer in ionic liquids."

4.3 Publication 3

Submitted to Polymer, June 2014

Thermoplastic materials from the dissolution of feather keratin biopolymer in ionic liquids

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Keywords: Thermoplastic, keratin, feather, ionic liquids, biopolymer, gel, film

ABSTRACT

Globally, animal feathers are discarded as a waste material, although they contain a potentially valuable fibrous protein keratin. The possibility of utilizing the feather keratin through the processing of the biomass into a variety of biomaterials could provide an alternative platform for the production of polyamide polymers that are

currently being produced from petroleum derived materials. This work investigates the properties thermoplastic materials (gel, film and fiber) prepared from regenerated keratin without any addition of plastisizer. Interestingly, ionic liquids used in this study as the processing solvent were also act as plasticizer wherein, the IL, modified the keratin structure by crosslinking with the keratin polymer strands, thus leading to a formation of keratin polymer-IL network. The thermal, structural, electrical (conductivity) and mechanical properties of these thermoplastic materials were investigated.

INTRODUCTION

In recent years, the development of thermoplastic materials based on the renewable biopolymers, has gained momentum amongst the researchers and technologists. These renewable sources have the potential to replace petroleum-based materials.[1] Natural polymers, derived from a wide range of proteins and polysaccharides such as wool, cellulose, chitin and silk, are widely studied due to their availability as renewable resources.[2-4] Feather is another most interesting protein receiving attention due to its abundance as a renewable resource.[5-9] Although some quantities of feathers, disposed as a waste from poultry industry,[10] have been effectively processed as a low-value animal feed,[1,11] large quantities of feathers are currently discarded as landfill which can cause serious problems for the environment.[1,11] Therefore, in order to reduce the environmental impacts while at the same time extract valued products from these feathers, we have recently been studying approaches to transform them into useful products.

Feather is a fibrous protein composed of amino acids connected by polyamide linkages.[12,13] It essentially contain 90 % keratin,[14] within which typically there are 7 % cysteine units[15] and 22 % of hydroxyl-amino acids such as serine, threonine and tyrosine.[11] The difference between feather and other fibrous proteins such as collagen and elastin, is the presence of a high content of cysteine.[16] The presence of cysteine (involved in disulfide bridges formation) gives strength to the protein structure and renders is water insoluble. The stability of the polypeptide chain in a triple helical structure is achieved by the presence of inter- and intramolecular bonding of polar and non-polar amino acids along with the hydrogen bonding in the secondary structure (α-helix and β-sheets).[12,13,17] These interactions make the feather difficult to dissolve in normal polar and apolar solvents.[16] Broadly speaking, in order to dissolve feathers, the disulfide bridges and hydrogen bonds have to be broken.

In the literature, several ionic liquids (ILs) have been shown to dissolve various carbohydrates such as, cellulose, starch and chitin.[4] Besides carbohydrates, it has also been shown that certain ILs can dissolve fibrous proteins such as wool and feather.[6,8,18-23] In cellulose dissolution, the anion of the IL interacts and forms hydrogen bonds with the hydroxyl groups of cellulose,[24,25] thus cleaving the intra- and inter-molecular hydrogen bonds of the biopolymer.[26] A similar phenomenon occurs in the case of fibrous protein dissolution, where the interaction of IL results in the cleavage of the hydrogen bond network present between the polypeptides chains.[18] According to Li *et al.*,[27] regenerated keratin film can be prepared from 10

wt% of dissolved wool in 1-allyl-3-methylimidazolium chloride [AMIM]Cl or 1-butyl-3-methylimidazolium chloride [BMIM]Cl. Several studies have investigated processing of materials based on dissolved keratin/IL solutions with the addition of other biopolymers or plasticisers.[18,22] Therefore, it can be concluded that keratin proteins are processable in ILs and have the potential to be converted into valuable products.

In our previous study[6] we showed that [AMIM]Cl and [BMIM]Cl dissolves a significant fraction of feather keratin; this was regenerated into soluble and insoluble fractions and characterized. In the present study, we have further investigated the possibility of processing the feather keratin-IL solution into physical states such as a mouldable gels, films and fibers. These materials might find application in a wide range of contexts either as a direct replacement for current polyamide polymers or as sustainable materials in new fields. For example, there is growing interest in gel/solid electrolyte materials for devices and sensors.[28] It is important to note that from acute oral toxicity studies, [BMIM]Cl is considered as toxic when swallowed with $LD_{50\ rat/oral}$ of 50-300 mg/kg and also to be slightly irritating to the skin.[29,30] Therefore it is unlikely to be suitable as gel electrolytes for body contact applications particularly when small amount of the ionic liquids are still retained in the gel electrolytes. Thus we have investigated the thermal, structural, electrical (conductivity) and mechanical properties of these materials, employing DSC, XRD, ATR-FTIR and TGA techniques. The ionic conductivity

study and tensile strength testing was carried out using ac impedance spectroscopy and dynamic mechanical analysis respectively.

EXPERIMENTAL

Materials preparation

The cleaned barbs of turkey feathers used in these experiments were commercially available from Spotlight, Clayton, Australia. 1-Butyl-3-methylimidazolium chloride ([BMIM]Cl, 98 % purity) was purchased from Sigma Aldrich. 1-Allyl-3-methylimidazolium chloride ([AMIM]Cl) was prepared as prescribed by previous literature methods[6,31] and given in the supplementary information. In order to prepare ionic liquids, allylchloride (98 %) and *N*-methylimidazole (99 %) were obtained from Sigma Aldrich. The water contents of [BMIM]Cl and [AMIM]Cl, as determined by Karl Fischer titration, were 1.0 and 3.6 % respectively. As discussed previously,[32] since the process envisaged involves a keratin that will carry a significant content of bound water, these ILs were used without further drying. By design some water evaporates off during the dissolution stage of the process at 130 °C. Deuterated methanol (CD_3OD) was purchased from Merck to carry out NMR analysis. Unless otherwise stated, all other organic solvents and reagents were used as received from commercial suppliers.

Solvent casting

A keratin gel and film were prepared by a solvent casting method using 400 mg feather per gram of IL. The dissolution involved the gradual addition of feather (approximately 50 mg) to the ionic liquid kept at 130 °C for 10 hours, resulting in a transparent and viscous solution as described previously.[6] The keratin/IL solution was cast onto a Teflon mould. The keratin gel was obtained by soaking the keratin/IL solution in distilled water, then soaking twice again with fresh batches of distilled water to diffuse out the IL. A soft keratin gel was obtained if the solution was soaked for 15 minute periods, but a hard and brittle film was obtained if soaked for 30 minute periods. The films obtained were dried at room temperature overnight and further dried under vacuum at 50 °C for 2 days.

Fiber spinning

Thin fibers were produced by spinning the solution of dissolved feather (380 mg feather per gram of IL) in [BMIM]Cl and [AMIM]Cl. Typically the keratin/IL spinning solution was heated to 100 °C for 30 minutes prior to use to reduce the viscosity of the IL as well as to ensure the solution was smoothly spun. The spinning solution was then transferred into a syringe equipped with a syringe pump (flow rate of ~50 µl/h) and a hole spinneret with a 150 µm diameter. The spun fiber was drawn into a coagulation bath containing distilled water kept at room temperature. In order to remove ionic liquids, the spun fiber was immersed in water for 30 minutes. The spun fiber was then dried at room temperature overnight.

Powder X-ray diffraction (PXRD)

The powder X-ray diffraction (PXRD) patterns were obtained at 22 ± 2 °C using a Sietronics powder diffractometer. For each XRD experiment, 1-2 g of the finely ground sample was placed randomly on a locally designed flat brass sample holder fitted with an o-ring sealed covered Mylar sheet providing an airtight atmosphere. CuKα1 radiation (λ = 1.540 Å) was produced at 40 kV and 25 mA . The data were collected in the Brag-Brentano (θ/2θ) horizontal geometry using a 2θ-range (of 5 to 50.0° (2 θ)) with a step size of 0.02° 2θ and an accompanying scan rate of 0.5 ° / min.

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Fourier transform infrared spectra were obtained using a Bruker IFS Equinox FTIR system coupled with a Golden Gate single bounce diamond micro-attenuated total reflectance crystal and a liquid nitrogen cooled mercury/cadmium telluride detector. The FTIR was performed in the wavenumber range of 600 cm^{-1} to 4000 cm^{-1}. The spectra were recorded (base line corrected) with a resolution of 4 cm^{-1} over 50 scans.

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) was carried out using a DSC Q100 series (TA Instruments) with 5-10 mg of sample weighed into a closed aluminium pan kept at a ramp rate of 10 °C per minute. All samples were cooled to -50 °C, held for 5 minutes and heated to 230 °C. Thermal scans below room temperature

were calibrated using the cyclohexane solid-solid transition and melting point at -87.0 °C and 6.5 °C respectively. Thermal scans above room temperature were calibrated using indium, tin and zinc (with melting points at 156.6 °C, 231.9 °C and 419.5 °C respectively). The glass transition temperature was determined at the midpoint of the heat-capacity change.

Thermogravimetric Analysis (TGA)

The thermal stability of the neat feather, regenerated keratin gels and films were investigated by TGA using a Mettler-Toledo TGA/DSC 1 in a flowing dry nitrogen atmosphere between 25 °C and 500 °C at a heating scan rate of 10 °C/min. The samples were first dried under vacuum (in an oven at a temperature of 60 °C). These samples were then loaded in aluminum crucibles and the data was analyzed using STARe DBV 10.00 software.

Ionic conductivity

Ionic conductivity of the soft keratin gel was measured using ac impedance spectroscopy employing a high frequency response analyzer by HFRA, Solartron 1296. A free-standing film was produced from dissolved feather in [BMIM]Cl (400 mg of feather per 1g of IL). The gel was cut into a round shape (0.082 mm thick and 13 mm in diameter). The gel was sandwiched between two stainless steel blocking electrodes and then placed in the conductivity barrel cell. The data were accumulated over a temperature range of 5 °C to 150 °C in 10 °C intervals, using a frequency range of 0.1 Hz to 10 MHz (ten points per decade) and a 30 mV amplitude. The temperature was controlled within 1 °C using a Eurotherm 2204e

temperature controller and a band heater with a cavity for the cell using a thermocouple type T, which was embedded in the cell.

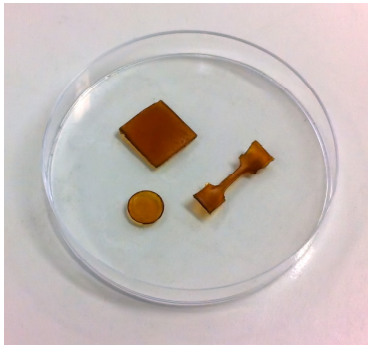
Tensile Testing

Tensile testing was performed using a dynamic mechanical analyser from TA Instruments, USA. The soft gels and hard films keratin obtained from [AMIM]Cl and [BMIM]Cl with 12-13 mm gauge length were stretched at a strain rate of 1 mm min^{-1} at ambient temperature. Stress-strain curves were plotted and the tensile strength, tensile strain and tensile modulus were calculated.

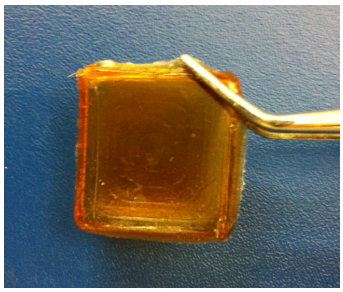
RESULTS AND DISCUSSION

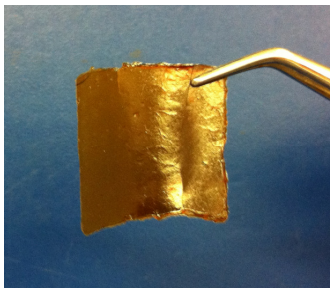
Thermoplastic materials

In our previous study,[6] the dissolution of feather at levels up to 500 mg of feather in 1g of IL in [AMIM]Cl and [BMIM]Cl was achieved. However, in the present study, the mouldable gels and films (Figure 1) were obtained by dissolving around 400 mg of feather per g IL without adding any plasticizers or additives.



a) Various shapes

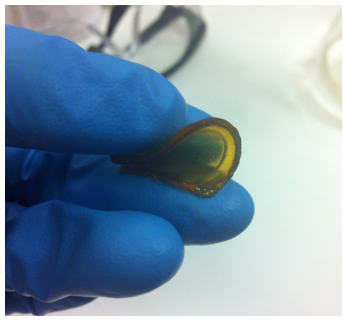




b) Soft keratin gel. **c)** Hard keratin film.

Figure 1. Mouldable, regenerated keratin gel and film.

The dissolved keratin feather/IL solution was found to be transparent and viscous, and became more viscous as it cooled to room temperature. Therefore, the solution needed to be heated (below 100 °C) to facilitate the easy transfer of the mixture onto the mould. After washing, depending on the amount of IL retained in the mouldable samples, flexible soft gel and hard films can be obtained (Figure 1 (b) and 1 (c)). Figure 2 shows the transparent and flexible nature of the soft gel.



a) Transparent. **b)** Offers flexibility.

Figure 2. Physical properties of the soft keratin gel.

The percentage of IL retained in the film as opposed to diffused into the aqueous phase were calculated by mass balance as shown in Table 1. The flexible gels had the highest retained IL content and accordingly the soft gels are more flexible than hard films. The gels and films were characterized for their thermal, spectral and mechanical properties.

Table 1: Percentage of retained IL in the materials

Regenerated keratin materials		IL content in materials (% w/w)
[AMIM]Cl	Soft gel	53
	Hard film	17
[BMIM]Cl	Soft gel	48
	Hard film	5

Characterization of regenerated thermoplastic keratin

Phase behaviour

The DSC curves of raw feather and regenerated keratin gels and films were shown in Figure 3. No glass transition (T_g) temperature was detected in the raw feather whereas T_gs were seen for the regenerated keratin gels and films produced from [AMIM]Cl and [BMIM]Cl. This observation confirms that these materials are amorphous in nature. It can be seen in Figure 3 that T_gs of the hard films shifted towards a higher temperature region (~160-170 °C) while the soft gels have quite low Tgs (~14-15 °C). This observation suggests that the IL is acting as a plasticizer, allowing the keratin backbone more degrees of freedom which reduces the T_g. The

plasticizing effect of IL on other matrices has been reported in the literature recently.[33-35]

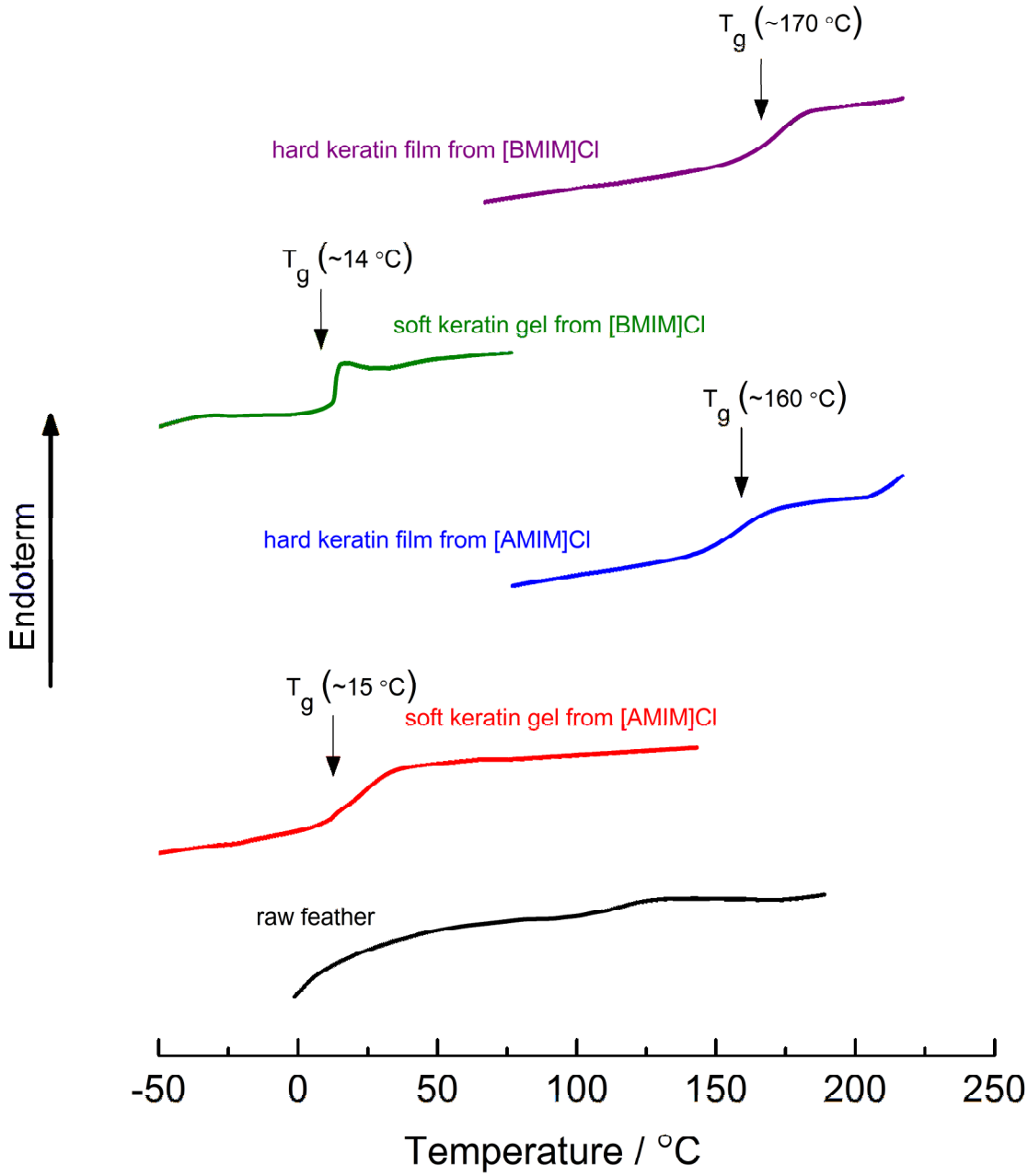


Figure 3. DSC thermograms of raw feather and regenerated keratin gels and films.

ATR – FTIR studies

The ATR-FTIR spectra of raw feather and regenerated keratin gels and films are presented in Figure 4. The spectra show the characteristic absorption bands for peptide linkages (-CONH-), as follows. The Amide A vibration, which is related to the N-H stretching, is observed as a medium absorption band in the range of 3273-3283 cm^{-1}. A strong band of Amide I (which absorbs in the range of 1617-1633 cm^{-1}) is related to the C=O stretching vibration, while another strong band (in the range of 1511-1546 cm^{-1}) is correlated to C-N stretching and N-H bending vibrations (Amide II). A weak band of Amide III, corresponds to C-N, C-O stretching, while the N-H and O=C-N bending vibrations were recorded in the range of 1224-1236 cm^{-1}.[36,37]

All the bands described above exist in the raw and regenerated keratin gels and films. This indicates that no major damage to the protein backbone occurs during the dissolution and regeneration process. However, the presence of IL residue in the soft keratin gels was detected in ATR-FTIR as an additional band (in the range of 1380-1390 cm^{-1}), which is likely related to C-N vibration on the cation.[38] This behaviour coincides with the lower T_g in the DSC thermogram as mentioned above, associated with the presence of a substantial amount of IL retained in the soft keratin gels. Apart from that, the band in the region of 3274-3411 cm^{-1} also shifted to a higher wavenumber, due to the formation of a hydrogen bond between the IL and the polypeptide chains of the keratin, which has also been observed by Wang *et al.*[39] in their blend film studies.

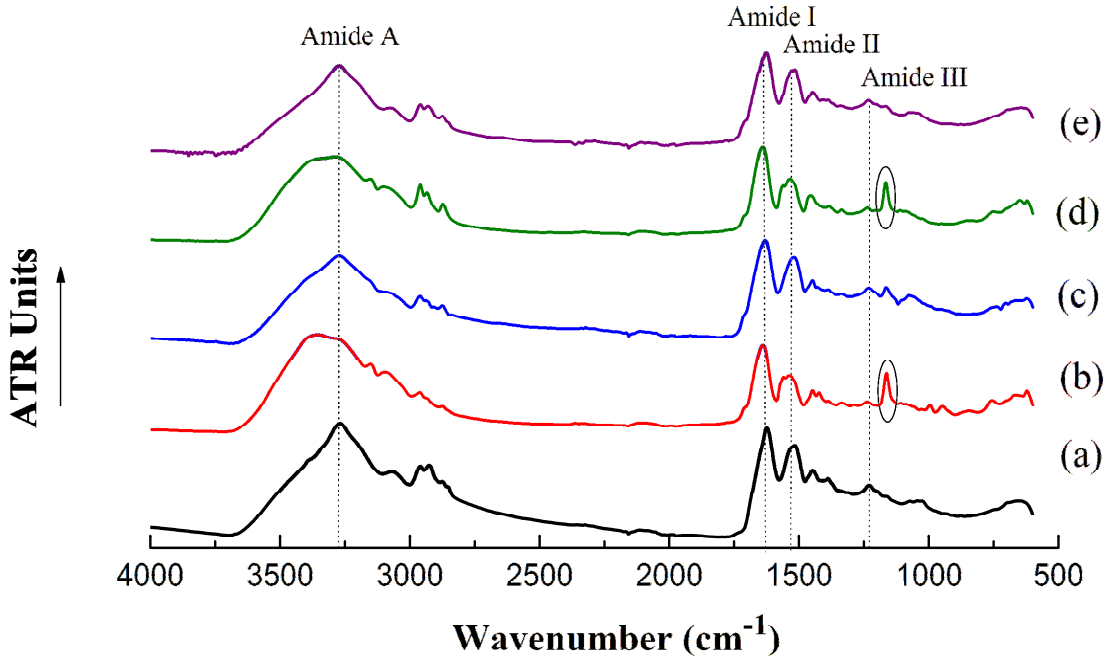


Figure 4. ATR-FTIR spectra of (a) raw feather and regenerated keratin of (b) soft gel from [AMIM]Cl, (c) hard film from [AMIM]Cl, (d) soft gel from [BMIM]Cl, (e) hard film from [BMIM]Cl.

XRD studies

XRD was performed to investigate structural changes of the raw feather and regenerated keratin gels and films. Figure 5 shows the XRD patterns of raw feather and regenerated gels and films obtained from [AMIM]Cl and [BMIM]Cl. Peaks appeared at 2θ = 9° (0.98 nm) and 17.8° (0.51 nm) corresponding to the diffraction pattern of the α-helix, while peaks at 2θ = 9° (0.98 nm) and 19° (0.47 nm) are related to the β-sheet structures.[40,41] These characteristic peaks of protein secondary structures are usually observed in raw feather.[5,42] Similar diffraction patterns were observed in the regenerated hard films, however the α-helix and β-sheet peaks have broadened, suggesting that the crystallinity of the hard films has decreased through the dissolution and regeneration process. In comparison, in the soft gels, significant

differences in the diffraction patterns were observed. The absence of a 2θ peak at 9° suggested that the crystallinity of the protein is not present and the soft gels remain more amorphous with the appearance of broad amorphous peaks at the higher values of 2θ (centered around ~27° in soft keratin gels from [AMIM]Cl).[18] The contrast between the two materials is interesting in that it suggests that the retention of the IL in the film continues to provide a solvating environment for the biopolymer such that it remains in the amorphous state. On removal of that IL component, some degree of crystallinity is reformed. The ultimate degree of crystallinity will no doubt be time and temperature dependent and we will investigate this aspect in our future work.

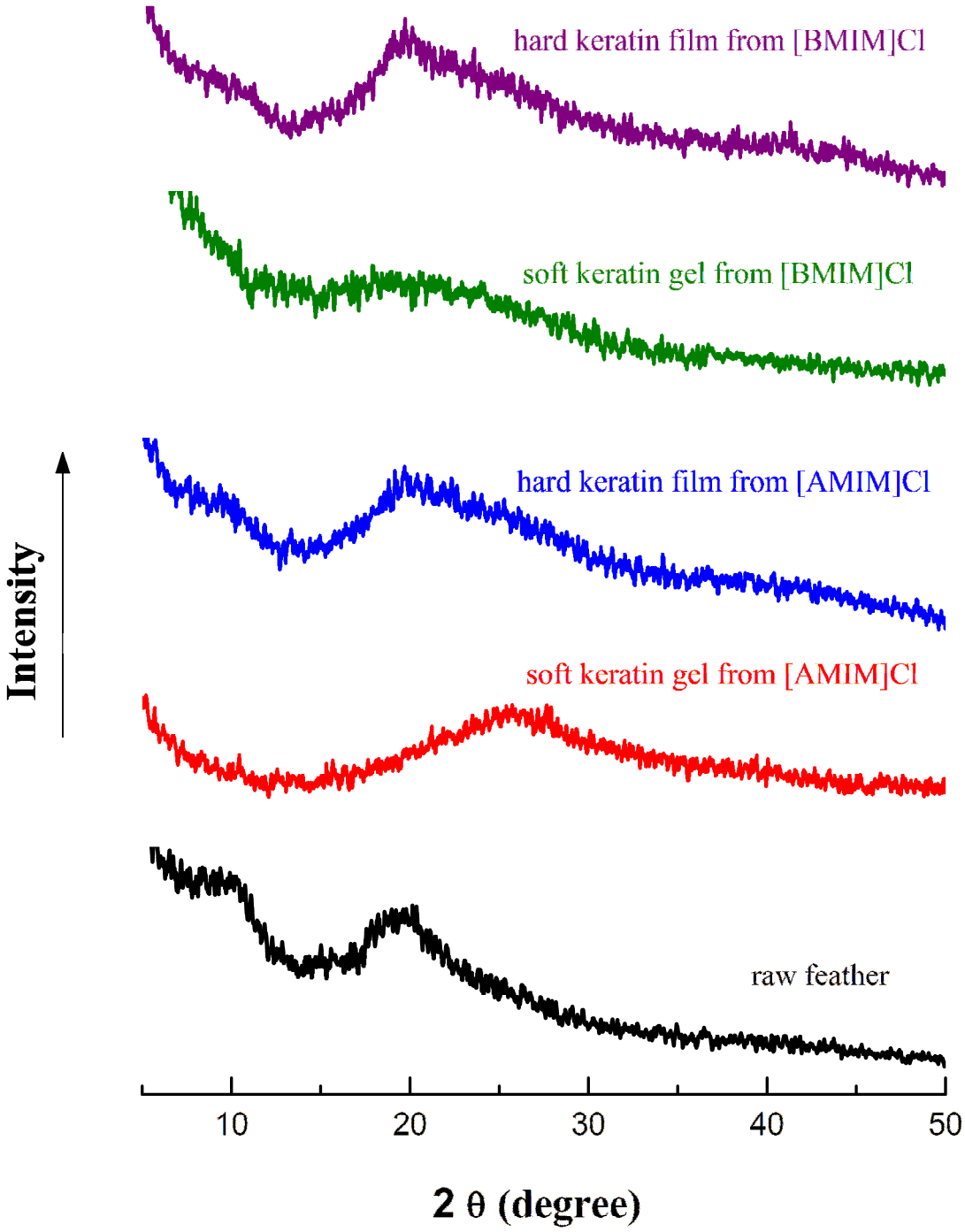


Figure 5. XRD of raw feather and regenerated keratin gels and films.

Thermal stability

TGA curves of raw feather and regenerated keratin gels and films produced from [BMIM]Cl and [AMIM]Cl are shown in Figure 6. Two stages of decomposition were observed in the thermograms. The initial weight loss beginning at ~100 °C and extending up to 200 °C, likely corresponds to the loss of water in the material.[10,13] The soft keratin gels, produced from both ILs, showed a greater weight loss (~10%) compared to the hard keratin films. The second stage of decomposition between 250 °C and 400 °C was attributed to the denaturation and degradation of peptide bridges and chain linkages in the keratin.[10,13,43] It has been reported that destruction of disulfide linkages in keratin (between 230 °C and 250 °C) leads to the liberation of volatile compounds, namely hydrogen sulfide, hydrogen cyanide and sulfur dioxide.[44,45]

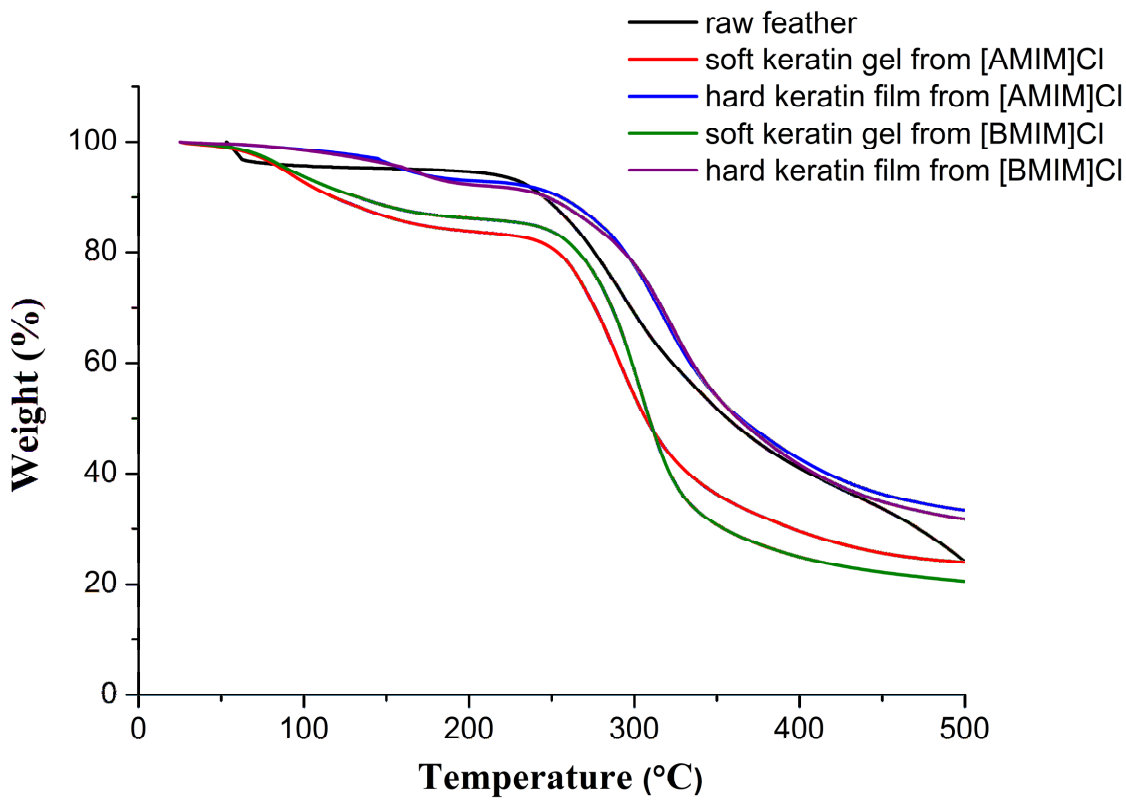


Figure 6. TGA curves of raw feather and regenerated keratin gels and films.

Mechanical properties

The stress-strain curves for the soft gels obtained from [AMIM]Cl and [BMIM]Cl are shown in Figure 7 and the data of mechanical parameters are summarized in Table 2. The tensile strengths of 0.34 ± 0.08 MPa and 0.60 ± 0.01 MPa with 26 ± 5 % stand 22 ± 8 % strain (at yield) were measured for the regenerated keratin gels from [AMIM]Cl and [BMIM]Cl, respectively. The yield strain is the point at the maxima of the curves in Figure 7. Meanwhile, the slightly higher mean values of strain at break (ε break) of 33 ± 5 % was measured for the soft gel from [AMIM]Cl while 25 ± 6 % was measured for the soft gel from [BMIM]Cl.

The tensile strength and tensile strain results for the soft gels (as shown in Table 2), are relatively low as expected for such gels, but are sufficient for gel electrolyte applications where the main requirement is to arrest any tendency towards liquid flow. It is well known that better mechanical properties, of these soft gels can be obtained by the incorporation of certain types of additives and fillers; for instance, Yamauchi *et al.*[46] described the inclusion of a crosslinker such as ethylene glycol diglycidyl ether (EGDE) and glycerol diglycidyl ether (GDE), to generate a tenacious and flexible film.[46] The formation of chemical crosslinking between additives and the keratin polymer matrix is known more generally to improve the mechanical properties of the blend. [47-50]

The hard films in this study were too brittle for routine analysis in the mechanical tester, due to their high T_gs and produced irregular breakage during sample preparation and set-up. However, data for the limited runs that were successful are included in Figure 7.

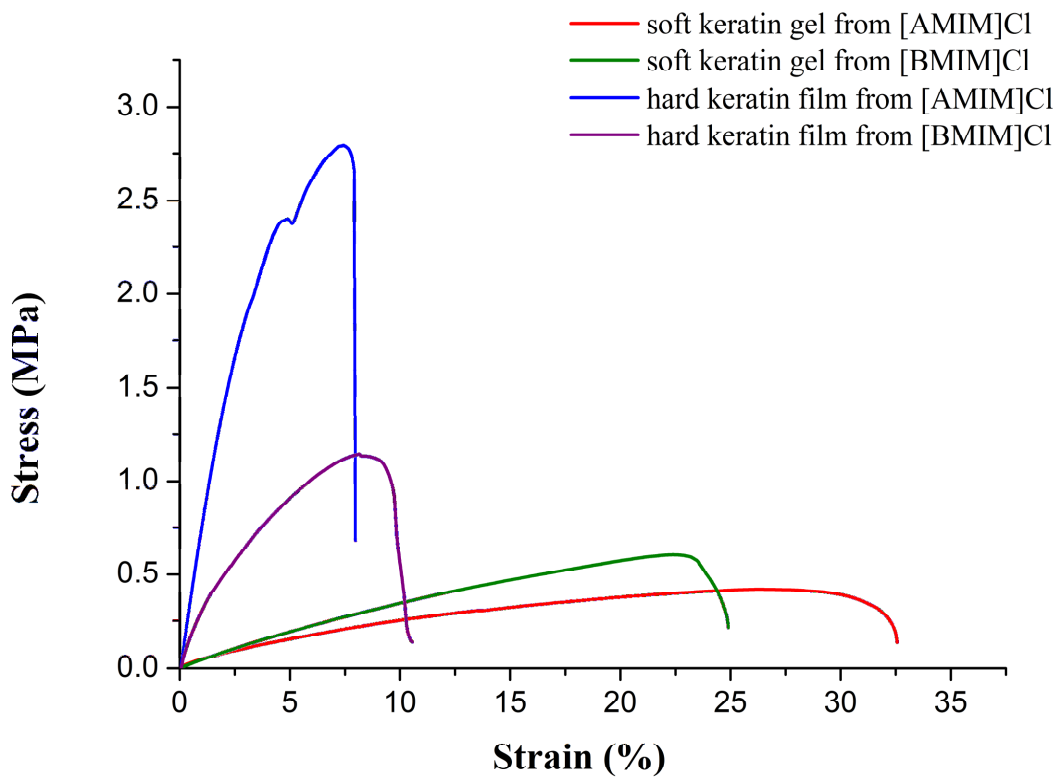


Figure 7. Stress-strain curves of regenerated soft keratin gels.

Table 2. Tensile properties of regenerated soft keratin gels.

Materials	Tensile strength σ (MPa)	Tensile modulus E (MPa)	Tensile strain ε yield (%)	Strain at break ε break (%)
Soft gel from [AMIM]Cl	0.34 ± 0.08	6 ± 2	26 ± 5	33± 5
Soft gel from [BMIM]Cl	0.60 ± 0.01	5 ± 1	22 ± 8	25 ± 6
Hard film from [AMIM]Cl	1.1	75	7	8
Hard film from [BMIM]Cl	0.6	34	8	11

Mean values of 2 replicates ± standard error

Ion Conductivity

An initial impedance spectroscopy experiment was carried out on the keratin gel processed from [BMIM][Cl], to indicate the potential of these materials as a gel electrolyte. This data is shown in Figure 11, expressed in an Arrhenius plot. From the Arrhenius plot, it can be seen that the conductivity increases steadily with temperature, though a change of slope exists at approximately 60 °C (3.0 K^{-1}). Above this temperature the conductivity lies in the range between 10^{-3} and 10^{-4} S/cm and as such is showing an impressive level of conductivity that make it of interest as a gel electrolyte, potentially for applications in sensors.

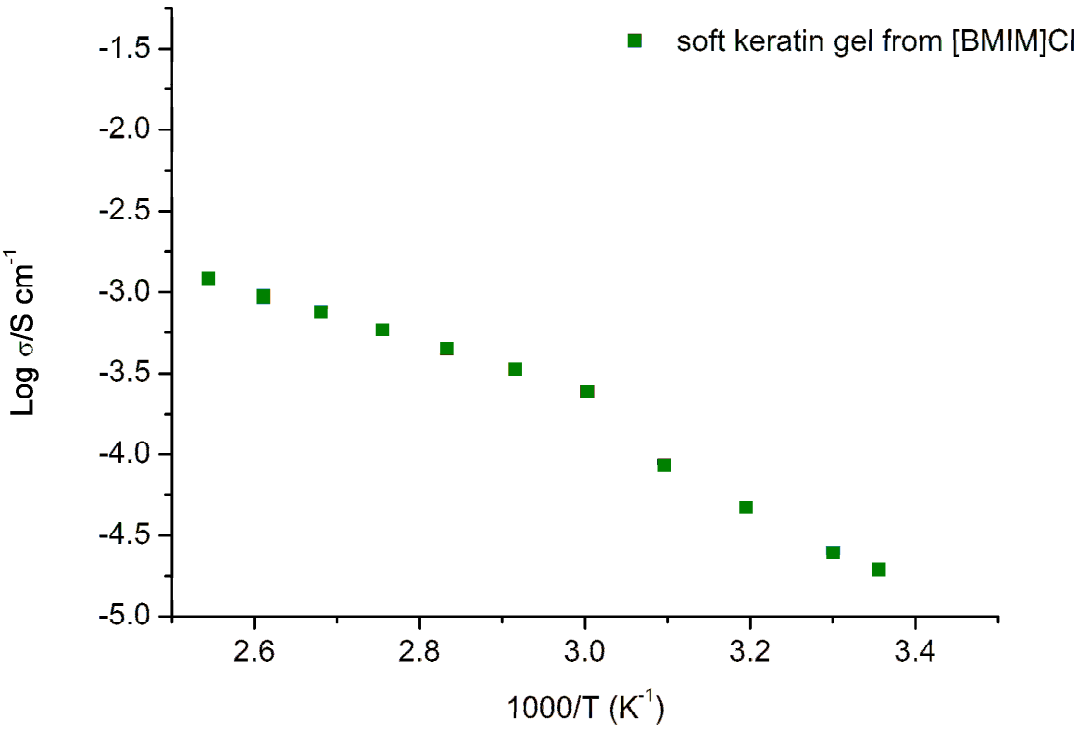


Figure 11. Arrhenius plot of conductivity for a soft keratin gel from [BMIM]Cl.

Fiber formation

The regenerated keratin fibers were produced from a solution of 50, 150 and 380 mg feather per gram of IL dissolved feather (without the addition of any filler/plasticizer) in [BMIM]Cl and [AMIM]Cl. It is interesting to note that only the 380 mg feather per gram of IL sample produced a continuous fiber (Figure S3 in supplementary information). We have also investigated different coagulation solvents (water, methanol, ethanol and a mixture of ethanol and water (ratio 1:1)) for fiber formation. It was found that water was the best coagulation solvent. The fibers were quite brittle and further study is underway to examine the effect of the addition of fillers and plasticizers to the fiber spinning mixture.

CONCLUSIONS

This study demonstrates that feather keratin dissolved in an ionic liquid medium can be formed/moulded into a desired shape and spun as a continuous fiber without addition of plasticizers. Some of the gels, films and spun fibers obtained in this study were of high T_g and hence brittle. It was concluded that the presence of residual concentrations of IL influences the physical and mechanical properties of the keratin gels and films. DSC, ATR-FTIR and XRD studies indicated the plasticizing effect of IL on the keratin polymer matrices. Therefore, it appears that ILs not only can serve as a solvent for dissolution of keratin, but also act as plasticizer and improve the flexibility of the regenerated materials. Based on the promising conductivity measurements, the soft keratin gels should be investigated further as gel electrolytes. In order to improve the mechanical properties of the

regenerated films, work is in progress investigating the addition of additives and plasticizers and the findings will be reported in the near future.

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ABBREVIATIONS

ILs, ionic liquids; [BMIM]Cl, 1-butyl-3-methylimidazolium chloride; [AMIM]Cl, 1-allyl-3-methylimidazolium chloride.

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4.4 Supplementary Information

Supplementary Information to accompany:

Thermoplastic materials from the dissolution of feather keratin biopolymer in ionic liquids

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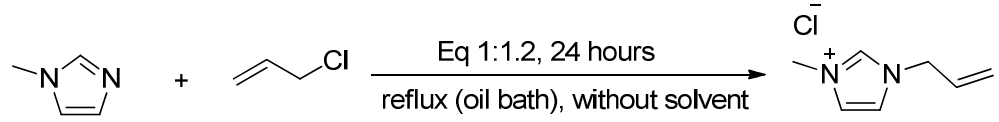
Preparation of ionic liquids

1-allyl-3-methylimidazolium chloride [AMIM]Cl was prepared according to the literature[6,28]



Scheme S1. Preparation of [AMIM]Cl.

Allylchloride (30.8 ml, 0.378 moles) and *N*-methylimidazole (25.1 ml, 0.315 moles) were inserted in a three neck round bottom flask via a syringe. The three neck round bottom flask equipped with a reflux condenser and a magnetic stirrer was flushed with nitrogen for 10 mins beforehand. The mixture was stirred at 50°C to 60°C for 24 hours. Evaporation of excess allylchloride under *vacuo* gave the product, a viscous liquid **(Scheme S1)** displaying a slight amber colour (48.695 g, 97%);^{1}H NMR (ppm, 400 MHz, CD_3OD) δ_H: 9.00 (1H, s, ArH), 7.62 (2H, s, ArH), 6.08 (1H, m, CH), 5.43 (2H, m,CH_2), 4.87 (2H, d, CH_2), 3.96 (3H, s, CH_3); ^{13}C NMR (ppm, 400 MHz, $CDCl_3$) δ_C: 138.0, 132.1, 125.1, 123.6, 121.9, 52.8, 36.6; ES-MS: ES^+ *m/z* 123.1 $[AMIM]^+$. ES^- *m/z* 35.1 $[Cl]^-$.

TGA Traces of [BMIM]Cl and [AMIM]Cl

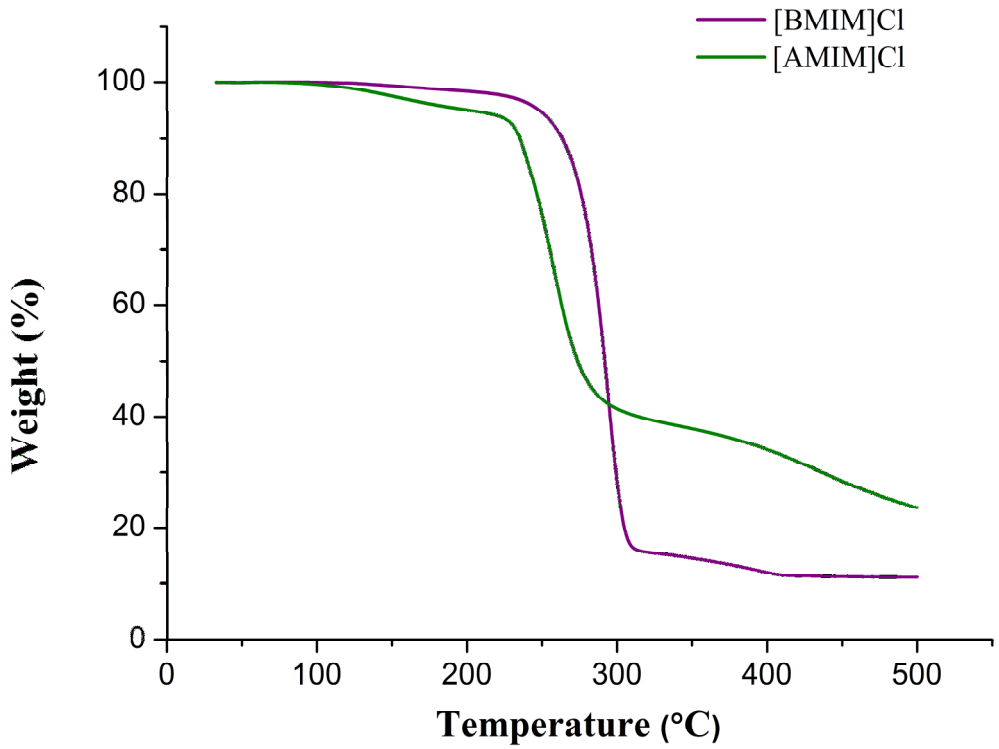


Figure S1. Single heating TGA traces of [BMIM]Cl and [AMIM]Cl.

ATR-FTIR spectra of [BMIM]Cl and [AMIM]Cl

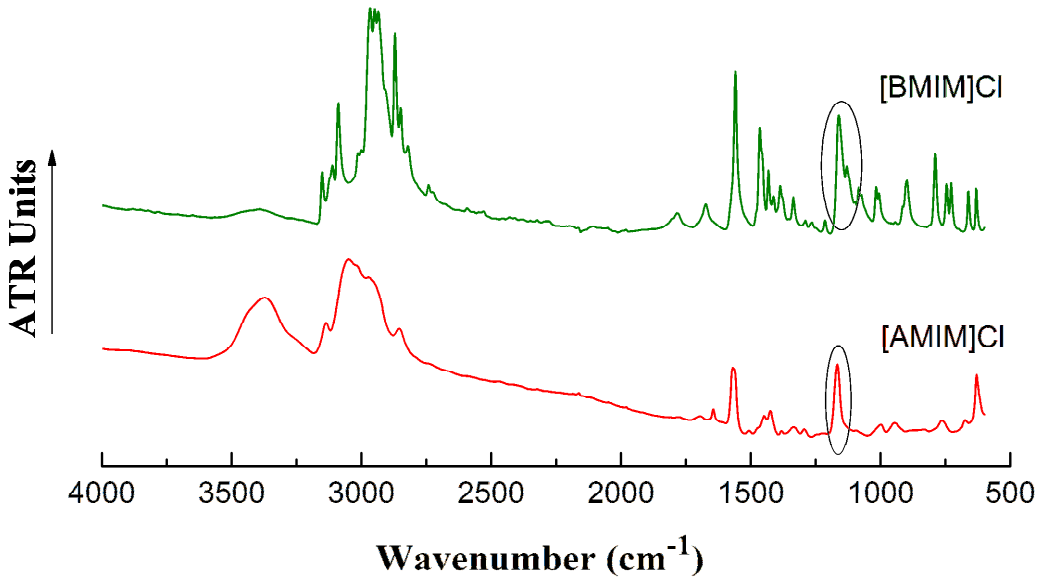


Figure S2. ATR-FTIR spectra of neat [BMIM]Cl and [AMIM]Cl.

Picture of fiber under optical microscope

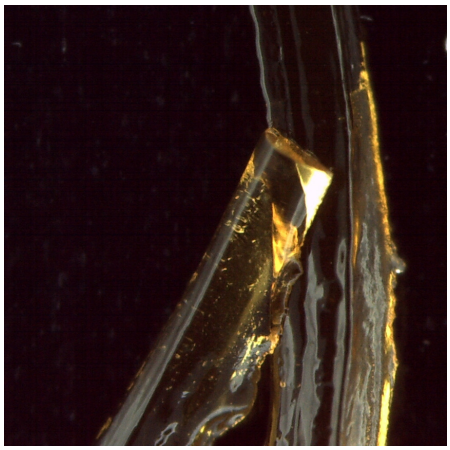


Figure S3. Regenerated keratin fiber from 38 wt% of dissolved keratin feather in [AMIM]Cl under optical microscope (20x magnification).

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Chapter 5

Distillable protic ionic liquids for keratin dissolution and recovery

CHAPTER 5

Distillable protic ionic liquids for keratin dissolution and recovery

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5.1 Specific Declaration

PART B: Suggested Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Initiation, key ideas, performed the experiments, synthesis, data analysis and interpretation, manuscript development and writing up	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Douglas MacFarlane	Key ideas, proof reading and final drafting	-
Antonio Patti	Key ideas, proof reading and final drafting	-
R. Vijayaraghavan	Key ideas, proof reading and final drafting	-

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** **18/6/2014**
Main Supervisor's Signature	[redacted]	**Date** **18/6/2014**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

5.2 General Overview

This final stage of my fibrous protein studies was built on previous observations of dissolution of keratin biopolymers in aprotic ionic liquids.[1,2] Although our previous publications[1,2] (Publication 1 and 2) report higher dissolution of keratin in aprotic ionic liquids, we could not easily recover and recycle the ionic liquids. Hence the main aim of the present study was to use protic ionic liquids that are relatively cheap and completely recoverable via distillation. Even though distillable ionic liquids are known, it was far from obvious that these would be good solvents for keratin. Accordingly, in the Publication 4 (Section 5.3), we describe the successful use of a distillable PIL in keratin dissolution.

A series of distillable protic ionic liquids based on *N,N*-dimethylethanolammonium cation with formate, acetate and chloride anion were synthesized. *N,N*-dimethylethanolammonium formate ([DMEA][HCOO]) was selected as the best candidate for the dissolution, regeneration and distillation experiments due to the stability shown in the TGA results of the neat protic ionic liquids.

In this study, the dissolution process was carried out at 100 °C as opposed to aprotic ionic liquids where 130 °C was used. The rationale for the lower dissolution temperature being used was based on the TGA experiments which revealed that at

higher temperatures (above 100 °C), significant mass losses were observed due to evaporation.

In the ATR-FTIR characterization of the regenerated product, the Amide bands have been deconvulated to provide conformational analysis (% fraction of α-helix, β-sheet, random coil and turns) using Fourier self-deconvolution and fitting using OPUS software. The estimated fractions of the various structures are presented in the supplementary information.

Two protein fractions (water soluble and water insoluble) were obtained after the dissolution and regeneration process. Since our focus in this publication is mainly on the regenerated material, rather than the water soluble residue, we have not emphasized characterization of the residue at this stage.

Finally, the formation of films was also investigated. Upon washing, a substantial amount of ionic liquid (34 %) was still retained in the film, while the remaining ionic liquid diffused into the water. Amorphous films of T_g ~93°C were obtained. It is thought that this may become a sustainable source of high T_g polymer materials to replace crude-oil derived polyamides.

The details of this investigation have been published in the paper entitled "*Distillable protic ionic liquids from keratin dissolution and recovery*."[3]

5.3 Publication 4

Distillable protic ionic liquids for keratin dissolution and recovery

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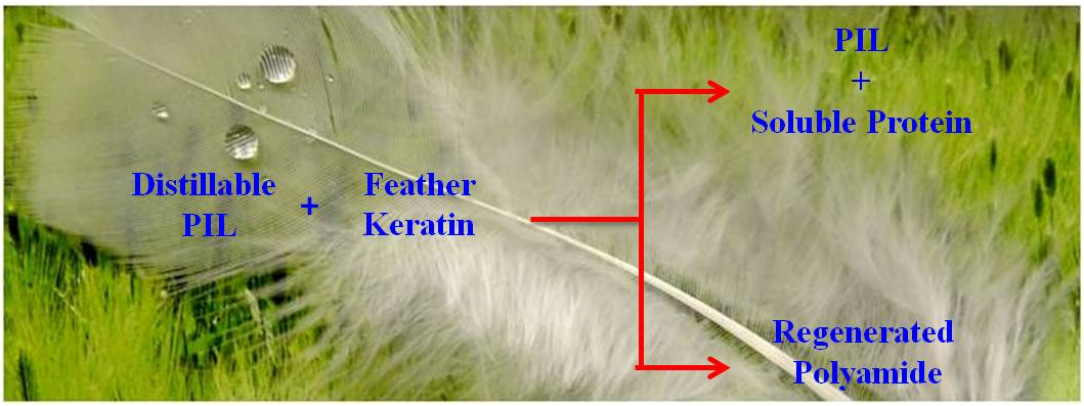
In current studies, distillable *N,N*-dimethylethanolammonium formate has been distilled with 99.6 % recovery of the original mass. The IL was also shown to dissolve 150 mg feather per gram of solvent. Two fractions were obtained after regeneration of the dissolved keratin which consist of water soluble (PIL + soluble protein) and water insoluble protein (regenerated polyamide).



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Distillable Protic Ionic Liquids for Keratin Dissolution and Recovery

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Supporting Information

ABSTRACT: Feathers, a form of keratin, are available in large quantities as a waste in many countries. They could be a potential source of polyamides if suitable methods for the dissolution and regeneration of the keratin were developed. A series of distillable *N,N*-dimethylethanolammonium cation-based protic ionic liquids (PILs) have been investigated in this work for use as processing solvents for this material. *N,N*-dimethylethanolammonium formate ([DMEA][HCOO]) is shown to dissolve keratin to an extent of 150 mg per gram of solvent. Recovery of the regenerated keratin material is easily achieved by the addition of methanol. We also demonstrate distillation and recovery of the ionic liquid from the dissolved keratin.

KEYWORDS: *Dissolution, Regeneration, Keratin, Feather, Protic ionic liquids*

INTRODUCTION

Ionic liquids have been a subject of interest in the past decade because of their attractive properties for a variety of physical, chemical, and biological applications.[1−7] Protic ionic liquids (PILs) are a relatively new subclass of ionic liquids formed on proton transfer from a Brønsted acid to a Brønsted base.[8,9] In recent years, PILs have found a number of applications ranging from stabilization of proteins to fuel cells.[10−14] There are several advantages of protic ionic liquids as compared to aprotic ionic liquids, including that they can be relatively cheaper and easier to prepare than their aprotic counterparts and that in some cases they can be distilled from a reaction mixture. Several PILs have previously been reported as distillable for recycling after use in the extraction of lignin and cellulose from biomass.[15,16] Our group recently employed *N,N*-dimethylammonium-*N′,N′*-dimethylcarbamate (DIMCARB) as a simple, inexpensive, distillable ionic liquid for the extraction of tannin from plant materials.[17] Nonetheless, the very different solvent environment offered by PILs means that solubility trends observed in the aprotic systems do not necessarily translate to a protic system. Thus, in the present study, we have investigated a number of inexpensive protic ILs as potential distillable and recycleable solvents for keratin dissolution.

Keratin, a fibrous protein, is abundantly available worldwide as a waste byproduct, feathers, from poultry production, and also in the form of short-fiber waste from wool processing in the textile industry.[18−20] Keratin exhibits a stable three-dimensional polypeptide structure consisting of a triple-helix of protein chains held together by a range of covalent (disulfide bonds) and noncovalent interactions.[21−25] Feather fiber (barbs) consists of 41% α-helix and 38% β-sheet, whereas the quill (shafts) has more β-sheet (50%) than α-helix (21%).[26,27] One of the main differences between feather and wool keratin is the cysteine content; approximately 7% cysteine in feather compared with ∼11−17% in wool.[22,28,29] Keratin represents, therefore, a potentially renewable source of polymer materials that should have similar properties to polyamides. The dissolution and processing of keratin in common solvents is difficult.[30] Known methods include alkali hydrolysis[31] and the use of sodium sulfide,[32] enzyme−alkaline treatment,[33] steam explosion,[34,35] and the Shindai method.[36,37] Several other methods involve the use of reducing agents such as dithiothreitol and 2-mercaptoethanol, which have the ability to cleave the disulfide bonds of the keratin.[38,39]

Recently, several research groups have attempted to dissolve keratins in aprotic ionic liquids with differing degrees of solubility.[19,40−43] We have shown in our previous study[41] that certain aprotic ionic liquids, with anions including thioglycolate, can dissolve feather to a substantial extent. The main drawback of these aprotic ILs is that they cannot be easily removed from the substrate; in addition, these ILs are expensive. In order to address these issues, PILs have been envisaged. The PILs are relatively less expensive and are potentially distillable.[15−17] Hence, in this study, distillable PILs based on the *N,N*-dimethylethanolammonium cation with several anions such as formate, acetate, and chloride have been investigated. On the basis of the viscosities and thermal stabilities of these ILs, dimethylammonium formate ([DMEA][HCOO]) was chosen for further investigation as a keratin dissolution solvent.

Though the chemical reaction for synthesizing a PIL is often a simple proton transfer, obtaining a pure salt is dependent on the strengths of the component acid and base, as expressed by

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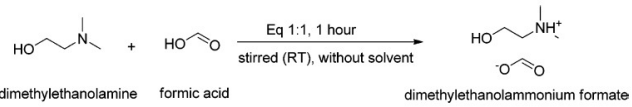
Scheme 1. Preparation of [DMEA][HCOO]



the difference in their pK_a^{aq} values.[44,45] Angell et al.[44,45] observed an empirical relationship with regard to extent of protonation such that only when ΔpK_a^{aq} was greater than 10 was complete proton transfer occurring. However, in many systems, this large value of ΔpK_a^{aq} is not achieved, and the properties of the PILs then largely depend on the degree of proton transfer from acid to base. More recent studies[46,47] have shown that in some cases full proton transfer can take place in the neat PIL at smaller values of ΔpK_a^{aq} if other H-bonding functional groups are present, e.g., −OH. Given their protic nature, the ions in PILs can typically take part in multiple hydrogen bond type interactions. Therefore, it is expected that they can form numerous hydrogen bonds with biopolymers such as keratin and thereby lead to their dissolution. Thus, in this study, we investigate the ability of a protic ionic liquid to dissolve feather keratin and characterize the soluble and insoluble fractions of the regenerated keratin obtained. Because [DMEA][HCOO] is known to be completely distillable, we also examine the recovery by distillation after the keratin regeneration process to explore the potential reusability of PILs in a keratin dissolution process.

EXPERIMENTAL SECTION

Materials Preparation. The cleaned turkey feathers used were supplied by Shamrockcraft and purchased from Spotlight, Clayton, Australia. For consistency with previous work, only barbs that were cut from the shafts of the turkey feather were used in the experiments (a likely industrial version of this process would involve a shredding operation prior to dissolution). The barbs were dried in a vacuum oven at 50 °C for 48 h. The remaining "bound" water fraction in the barb was quantified by TGA at approximately 5%. The ILs are commercially available, but for this work, they were prepared in house. For the preparation of the ionic liquid, *N,N*-dimethylethanolamine (99.5%) and formic acid (99%) were obtained from Sigma-Aldrich Co., LLC. For NMR analysis, deuterated chloroform ($CDCl_3$) purchased from Merck Pty., Ltd. was used. Unless otherwise stated, all other organic solvents and reagents were used as received from commercial suppliers. Water contents, as indicated by mass loss around 100 °C in the TGA, were below 3% for the formate IL and between 5% and 8% for acetate and chloride ILs, respectively.

Powder X-ray Diffraction (PXRD). Powder X-ray diffraction (PXRD) patterns were obtained at 22 ± 2 °C using a Sietronics powder diffractometer. For each XRD experiment, approximately 1−2 g of finely ground sample was placed randomly on a locally designed flat brass sample holder fitted with an O-ring sealed Mylar sheet providing an airtight atmosphere. Cu $K\alpha_1$ radiation (λ = 1.540 Å) was produced at 40 kV and 25 mA. The data were collected in the Brag-Brentano ($\theta/2\theta$) horizontal geometry using a 2θ-range of 5° to 50.0° with a step size of 0.02° 2θ and an accompanying scan rate of 0.5°/min.

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR). Fourier transform infrared spectra were obtained using a Bruker IFS Equinox FTIR system coupled with a Golden Gate single-bounce diamond micro-attenuated total reflectance crystal and a liquid nitrogen-cooled mercury/cadmium telluride detector. FTIR measurements were taken in the wavenumber range of 600 to 4000 cm^{-1}. The spectra were recorded with a resolution of 4 cm^{-1} with 50 scans and were baseline corrected.

Differential Scanning Calorimetry (DSC). Differential scanning calorimetry (DSC) was conducted on a DSC Q100 series from TA Instruments with 5−10 mg of sample in closed aluminum pans at a scan rate of 10 °C/min. All samples were cooled to −150 °C, isothermed for 5 min, and then heated to 280 °C. Thermal scans below room temperature were calibrated via the cyclohexane solid−solid transition and melting point at −87.0 and 6.5 °C, respectively. Thermal scans above room temperature were calibrated using indium, tin, and zinc with melting points at 156.6, 231.9, and 419.5 °C respectively. Transition temperatures are reported using the peak maximum of the thermal transition.

Thermogravimetric Analysis (TGA). The thermal stabilities of the neat feather and regenerated materials were investigated by TGA using a Mettler-Toledo TGA/DSC 1 in a flowing dry nitrogen atmosphere between 25 to 500 °C at a heating scan rate of 10 °C/min. The samples were first dried under vacuum in an oven at a temperature of 60 °C. These samples were then loaded in aluminum crucibles, and the data was analyzed using STARe DBV 10.00 software.

Electrospray Ionization Mass Spectrometry (ESI-MS). Electrospray ionization mass spectra were recorded on a Micromass Platform II API QMS electrospray mass spectrometer. Samples dissolved in methanol were subjected to a suitable cone voltage, usually 25−35 V. Measurements were made in both the positive and negatives modes.

SDS-PAGE Electrophoresis. Protein samples were diluted in 4× NuPAGE loading buffer (Life Technologies) and electrophoresed using the Hoefer miniVE vertical electrophoresis system (Amersham Biosciences) in 4−12% Bis-Tris NuPAGE gradient gels (Life Technologies). Proteins were stained and visualized by silver staining.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^1H NMR and ^{13}C NMR spectra of the PILs were recorded at 400 MHz on a Bruker DPX-400 spectrometer as solutions in deuterated chloroform and dimethyl sulfoxide. Chemical shifts are reported in ppm on the δ scale, and the coupling constant is given in Hz. Chemical shifts were calibrated on the solvent peak unless otherwise specified. The solid state ^{13}C CP-MAS NMR spectra of the regenerated samples were acquired using a 4 mm rotor with a Kel-f cap at a 10 kHz spinning rate. The contact time in the CP MAS experiments was 2.4 ms with a recycle delay of 1 s and cw decoupling. The number of scans was ∼90,000−100,000. In the case of 1D ^1H static NMR experiments, the pulse length was ∼3.6 μs with a recycle delay of 5 s and 16−20 scans.

Preparation of Ionic Liquids. Synthesis of *N,N*-dimethylethanolammonium formate, [DMEA][HCOO], is shown in Scheme 1. Synthesis of other PILs used the same process with their respective acids (Supporting Information).

N,N-Dimethylethanolammonium formate was prepared (Scheme 1) by neutralization of *N,N*-dimethylethanolamine with formic acid. The base was initially placed in a three-necked flask and equipped a dropping funnel. An equimolar amount of acid (34.0 g, 0.74 mol) was then added dropwise to the base (65.96 g, 0.74 mol) while stirring rapidly in an ice bath. The mixture was stirred for 1 h and yielded the colorless liquid as final product: (99.5 g, 99.5%). ^1H NMR (ppm, 400 MHz, $CDCl_3$) δ_H: 9.39 (2H, s, ^+NH, OH), 8.42 (1H, s, $HCOO^-$), 3.78 (2H, q, CH_2), 2.94 (2H, t, CH_2), 2.65 (6H, s, 2 CH_3). ^{13}C NMR (ppm, 400 MHz, $CDCl_3$) δ_C: 168.4, 60.3, 56.3, 43.4. ES-MS: ES^+ m/z 90.0 $[DMEA]^+$. ES^- m/z 45.0 $[HCOO]^-$.

Solvent Casting. Films and molded samples of the regenerated feather keratin were obtained by a solvent casting technique. The [DMEA][HCOO]/keratin solution (150 mg/g) was cast onto a Teflon plate and then soaked in water and washed three times with fresh water to remove the [DMEA][HCOO]. A transparent keratin film was obtained after drying at room temperature. Further drying was achieved under vacuum at 40 °C for 2 days.

RESULTS AND DISCUSSION

Stability and Distillation of PILs. To determine their useful temperature ranges, TGA scans were carried out to determine the thermal stability range and decomposition temperatures of the synthesized PILs. The TGA scan for [DMEA][HCOO] (Figure 1) shows little volatility up to 100

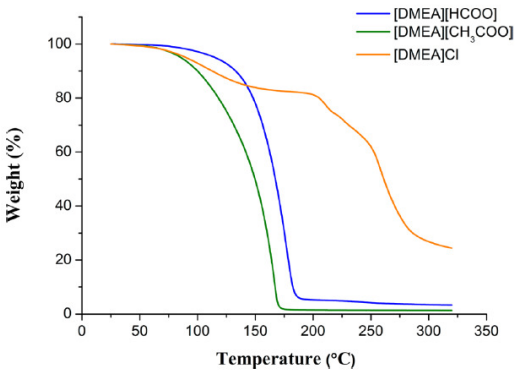


Figure 1. TGA curve of protic ionic liquids.

°C, and hence, this temperature was selected for this study. Beyond this, the PIL loses mass rapidly due to distillation. The [DMEA][CH_3COO] PIL begins to lose mass from around 65 °C and is therefore potentially too volatile to effectively dissolve feather keratin. Because our TGA results reveal a continuous mass loss from 65 °C onwards, there could be a possibility of shift in the proton transfer equilibrium of the acid−base mixture, leading to evaporation of one of the constituents. The [DMEA]Cl, which is quite hygroscopic, loses ∼15% mass around 100 °C probably due to water loss and then is stable to >200 °C. However, the mass loss beyond 200 °C in this case appears to be decomposition, and therefore, this PIL is not suitable as a distillable solvent. Thus, [DMEA][HCOO] was chosen as the best candidate for further study in this work.

To confirm that this IL is distillable, it was distilled at 122 °C at 0.5−0.6 mbar with almost no residue and complete recovery of the PIL; 99.6% of starting mass was recovered in the distillate. NMR spectra of the IL before and after distillation, indicating no substantial change in the material, are included in Figures S3 and S4 of the Supporting Information.

Dissolution of Feather in Protic Ionic Liquids. The feather dissolution was carried out in a two-necked round-bottomed flask fitted with a reflux condenser, and the process was carried out at 100 °C for 7 h. The solubility of feather was quantified by adding small incremental amounts of feather (approximately 150 mg) to the ionic liquid (6 g) with mechanical stirring until the point when the feather could not be visually seen and the ionic liquid solvent remained transparent in the flask. A laser beam was used to detect the presence of small particles via scattering, and if none were present, further amounts were added stepwise until the materials was observed to not properly dissolve further. This probably represents a "practical" solubility limit that is somewhat below the true solubility at this temperature. The solubility results and a picture of the dissolved keratin are shown in the Supporting Information. [DMEA][HCOO] was found to dissolve feather up to 150 mg/g. This value is lower than the solubility reported for aprotic ionic liquids[41] but is nonetheless sufficient for potential practical processes. The decrease in solubility could be due to the lower dissolution temperature used in the present study due to the risk of volatilisation and decomposition of the PILs at higher temperatures.

Regenerated Feather Keratin Components from [DMEA][HCOO]. The dissolved keratin was added to excess methanol to obtain the regenerated keratin. The insoluble fraction (regenerated keratin) was washed several times with fresh methanol. The regenerated keratin was separated by centrifuging (20 min, 1500 rpm) the reaction mixture, and after removal of the liquid fraction, it was dried in vacuum at 60 °C. Up to 63% (±1%) of the starting mass was regenerated by this means, which is similar to that obtained in the aprotic systems where the regenerated keratin was typically 51−59%.[41] The dried regenerated keratin material is shown in Figure 2.

Figure 2. Regenerated feather keratin from [DMEA][HCOO].

The soluble fraction of feather keratin remaining upon the addition of methanol also contains the PIL. Initially, the mixture was distilled at 45 °C to remove methanol, and then the IL was distilled at 118 °C under reduced pressure at 0.75 mbar. The distilled ionic liquid was analyzed by ^{1}H NMR, and the spectra of neat and distilled ILs are provided in the Supporting Information. The spectra show that there was no difference between the IL spectrum after distillation indicating that pure IL was obtained. The NMR of the methanol recovered was also unaltered.

In contrast to our previous study,[41] by this distillation method, we are able to separate the soluble protein from the IL through distillation. A small amount of dark resinous material remained after distillation, which was collected and analyzed for the presence of dissolved proteins using the gel electrophoresis technique. The electrophoresis separation pattern shows the presence of water-soluble proteins with molecular weights up to about 20 kDa (Figure S5, Supporting Information). In our previous study on dissolution of feather keratin in aprotic ILs,[41] the majority of the soluble proteins obtained were in the molecular weight range between 10−40 kDa. The presence of soluble protein in the lower molecular weight range here suggests that further breakdown of the protein into smaller polypeptide chains has occurred in this process.[41] The greater degree of fragmentation in the present case is probably due to the higher processing temperatures (122 °C at 0.5−0.6 mbar during distillation) in contrast to the previous work.[41] Having been rendered water soluble by this dissolution and recovery

process, it is likely that this proteinaceous material could be used as an animal food source.

Characterization of Regenerated Feather Keratin. *XRD Studies.* The water insoluble fraction of the regenerated feather keratin was characterized by XRD to study the crystallinity of the materials, and the results are shown in Figure 3. Both neat

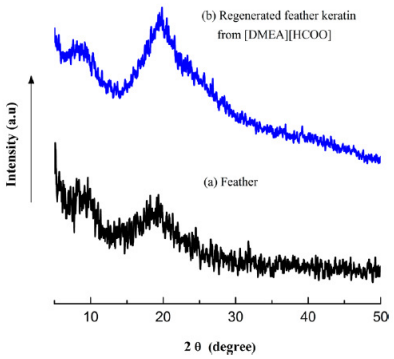


Figure 3. XRD of (a) feather and (b) regenerated feather keratin from [DMEA][HCOO].

feather and regenerated feather keratin show diffraction characteristic of the α-helix appearing at 2θ = 9° (0.98 nm) and 17.8° (0.51 nm) and of the β-sheet appearing at 9° (0.98 nm) and 19° (0.47 nm).[48,49] This observation indicates that some crystallinity is retained after the regeneration process.

ATR-FTIR Studies. ATR-FTIR analysis was used to study conformational changes in the polypeptide chains. The ATR-FTIR spectra of raw feather and regenerated keratin from [DMEA][HCOO] are shown in Figure 4. A medium

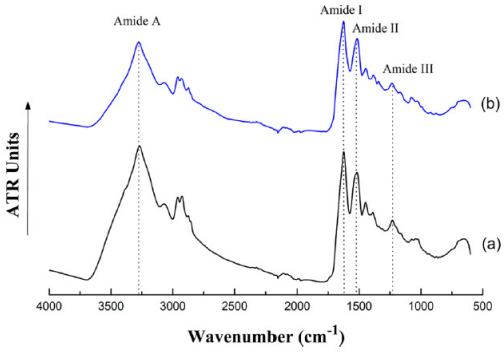


Figure 4. ATR-FTIR of (a) feather and (b) regenerated feather keratin from [DMEA][HCOO].

absorption band was observed in the range of 3270−3275 cm^{-1} indicating a characteristic N−H stretching (Amide A). The Amide I vibrations show a strong absorption band at 1627 cm^{-1}, which is attributed to C═O. A strong band was also observed in the range of 1515−1517 cm^{-1} indicating the C−N stretching and N−H bending vibrations (Amide II). The Amide III vibrations show a weak absorption band at 1233 cm^{-1} due to the C−N, C−O stretching, N−H, and O═C−N bending vibrations.[50,51] As described in the Supporting Information, the Amide I region can be deconvoluted to provide further information about the structures present (Figure S2). The percentage fraction of α-helix is observed from this to decrease after dissolution and regeneration.

Solid-State NMR Studies. A comparison of the ^{13}C CP MAS spectra of raw feather and regenerated keratin from [DMEA]-[HCOO] is presented in Figure 5. The most downfield signal is

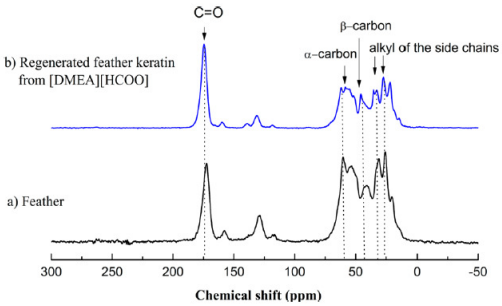


Figure 5. ^{13}C CP MAS NMR spectra of (a) feather and (b) regenerated feather keratin.

assigned to the asymmetric peak of amide carbonyl carbon in the keratin protein with the maxima centered between 172 and 175 ppm. The peak recorded at 175 ppm is attributed to the α-helix of keratin, while the one at 172 ppm is related to the β-sheet molecular and random coil conformations.[52−54] A peak at 130 ppm indicates the presence of aromatic group containing amino acid sequences. The peaks at about 54 and 40 ppm are assigned to the α-carbon and β-carbons present in leucine and cysteine residues, respectively.[53] Meanwhile, the presence of proline, glutamic acid, and glutamine residues are indicated by the observation of the peaks around 30−40 ppm.[53] In the upfield region, the absorption peaks at about 20−30 ppm are due to the carbon resonance of the alkyl groups of the side chains.[53] As the carbon peaks of the alkyl and cysteine groups appear in the same chemical shift region (25−29 ppm), it is not easy to differentiate between these two groups. The NMR spectrum of the regenerated keratin is very similar to the raw feather, although a small difference could be observed as a broadening of the peak at about 54 ppm that corresponds to the α-carbon. The broadening of this peak is believed to be due to the breaking up of intramolecular hydrogen bonding in the protein aggregates. There are also small changes in relative intensities of the broad peaks in the range of 30−75 ppm; these could be due to concentration effects as well as disrupted hydrogen bonding.

Thermal Stability and Phase Behavior. DSC curves of the third heating cycle of keratin materials are shown in Figure 6. Three sequential heating and cooling cycles were performed to remove all the water that is bound to the keratin material. In Figure 6a, the peak around 230 °C is generally described as a melting of α-helix crystallites[55] (also proposed as a α-helix disordering and decomposition in some of the literature[56,57]). In Figure 6b, the large peak that begins at 150 °C and peaks around 180 °C may relate to evaporation of retained water and/or degradation as indicated in the TGA data in Figure 7.

The TGA curve of the raw material shows stability up to 200 °C. In contrast, the regenerated feather keratin begins to

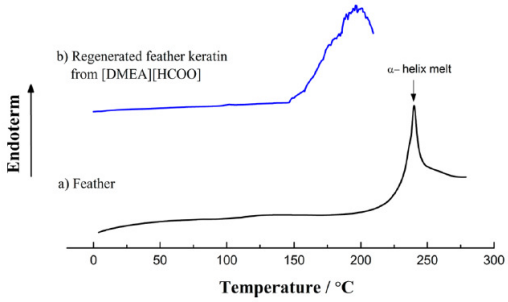


Figure 6. DSC curves of (a) feather and (b) regenerated feather keratin.

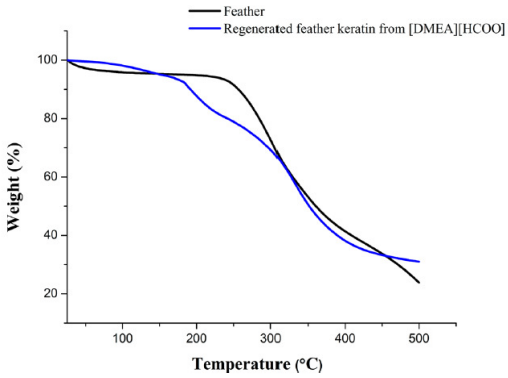


Figure 7. TGA plot of feather and regenerated feather keratin.

degrade at lower decomposition temperatures. This shows that the stability of the raw feather is influenced by the presence of strong cross-linking between the keratin fibers, whereas in the regenerated keratin the hydrogen bonded cross-linking networks are disrupted somewhat, leading to lower stability. The TGA of these materials show two steps of mass loss. The first step occurs close to 100 °C corresponding to the evaporation of water bound to the material. The second step involves the keratin degradation that is understood to be associated with the rupture of the helical conformation and disulfide bond breakage.[23,58]

Film Formation. The dissolved feather keratin ([DMEA][HCOO]/keratin solution) was solvent cast onto Teflon plates and molds to prepare films and molded shapes as shown in Figure 8. The formation of these films was obtained without using any fillers or any cross-linking agents. After washing, this material was characterized by DSC (Figure S6, Supporting Information). A glass transition, T_g, is now observed at 93 °C in these films, suggesting that the solvent-casting-based regeneration method increases the amorphous phase fraction in the material. XRD and FTIR data for the films were similar to the regenerated keratin material (Figures S7 and S8, Supporting Information).

CONCLUSIONS

The distillable PIL, *N,N*-dimethylethanolammonium formate, has been demonstrated to dissolve feather keratin at 100 °C.

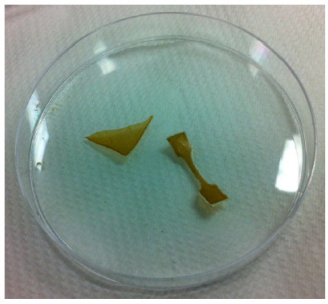


Figure 8. Films prepared from dissolved feather in [DMEA][HCOO].

Regeneration of the keratin was achieved by precipitation from methanol. This fraction is water insoluble. A water/methanol soluble fraction (~37%) initially remains mixed within the IL. Recovery (99%) of the IL was then achieved by distillation at 122 °C, demonstrating the value of a protic IL in this context. The regenerated material could also be prepared as a film and exhibited a T_g of ~93 °C. Further work is examining the mechanical properties of these materials in various film and molded forms.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, Fourier self-deconvolution (FSD) of the infrared spectra, SDS page, ^{1}H NMR of neat and distilled IL, DSC, XRD, and FTIR of feather and keratin films. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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5.4 Supplementary Information

Supplementary Information to accompany:

Distillable protic ionic liquids for keratin dissolution and recovery

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Supporting Information to accompany:

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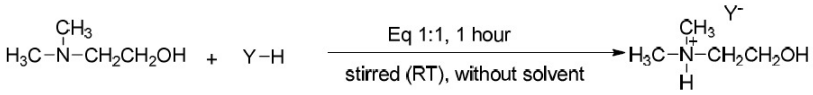
Number of Figures: 8

Preparation of ionic liquids [DMEA][CH_3COO] and [DMEA]Cl



Y-H	Y^-
CH_3COOH	CH_3COO^-
HCl	Cl^-

Scheme S1: Preparation of [DMEA][CH_3COO] and [DMEA]Cl.

[DMEA][CH_3COO]: (19.80 g, 99%); ^{1}H NMR (ppm, 400 MHz, $CDCl_3$) δ_H: 8.67 (2H, s, $^+$NH, OH), 3.74 (2H, q, CH_2), 2.80 (2H, t, CH_2), 2.54 (6H, s, 2 CH_3), 1.95 (3H, s, CH_3); ^{13}C NMR (ppm, 400 MHz, $CDCl_3$) δ_C: 178.1, 60.6, 57.2, 43.8, 22.8; ES-MS: ES^+ *m/z* 89.9 $[DMEA]^+$. ES^- *m/z* 59.0 [acetate] $^-$.

[DMEA]Cl: (18.67 g, 93%); ^{1}H NMR (ppm, 400 MHz, DMSO) δ_H: 10.40 (1H, s, $^+$NH), 3.71 (2H, q, CH_2), 3.10 (2H, t, CH_2), 2.49 (6H, s, $2CH_3$); ^{13}C NMR (ppm, 400 MHz, DMSO) δ_C: 58.3, 55.2, 42.4; ES-MS: ES^+ *m/z* 90.0 $[DMEA]^+$. ES^- *m/z* 35.0 $[Cl]^-$.

Dissolution and regeneration of feather keratin in ionic liquid

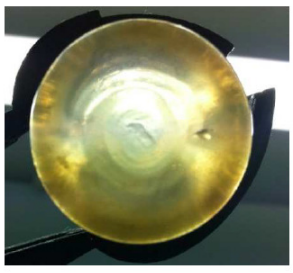


Figure S1. Dissolved feather keratin in [DMEA][HCOO].

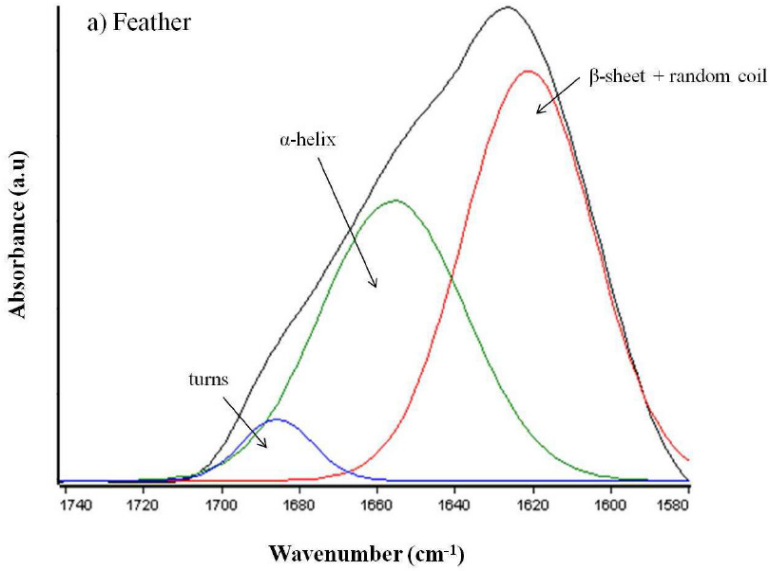
Table S1. Solubility of feather in protic ionic liquid.

IL	Condition/°C	Time/h	Solubility (mg feather / g(PIL))	Appearance
[DMEA][HCOO]	100	7	150	soluble

Table S2. Mass of regenerated feather keratin from dissolution in ionic liquid.

IL	Mass of feather dissolved (mg)	Mass of regenerated keratin material (mg)	% Recovery (%)
[DMEA][HCOO]	900	563	63

Fourier self-deconvolution (FSD) of the infrared spectra (Amide I region)



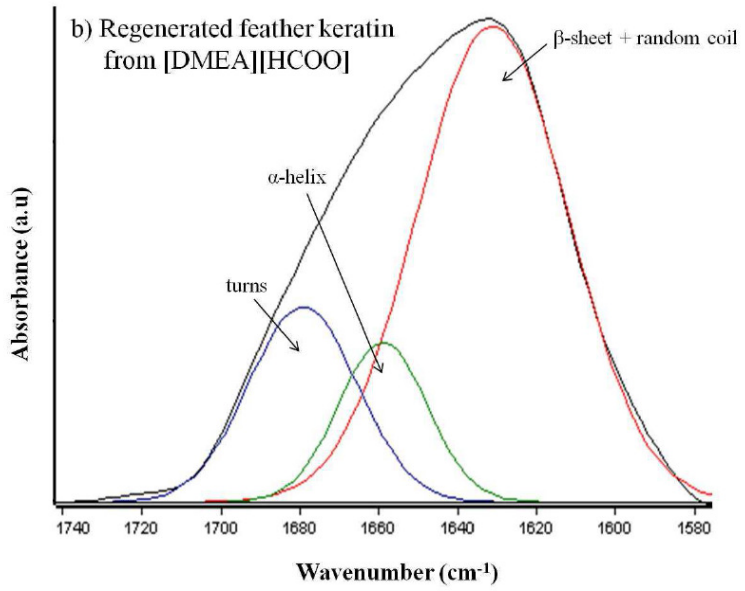


Figure S2: The deconvolution of FTIR spectra in Amide I region of a) feather b) regenerated keratin feather from [DMEA][formate] which were fitted using Gaussian fitting function.

Table S3. The percentage fraction of α-helix and β-sheet + random coil of feather and regenerated keratin feather, which were deduced after Fourier self-deconvolution and fitted using OPUS software.[1,2]

Material	Wavenumber (cm^{-1})	Assignment	% Fraction
Feather	1621	β-sheet + random coil	55
	1656	α-helix	37
	1685	Turns	8
Regenerated feather keratin from [DMEA][HCOO]	1631	β-sheet + random coil	57
	1659	α-helix	19
	1679	Turns	24

Figure S2 shows the deconvoluted Amide I FTIR bands of feather and regenerated feather from [DMEA]HCOO]. The secondary structure in protein which consists of α-helix, β-sheet, random coil and turns can be estimated quantitatively by the deconvolution of the Amide I band region (~1580cm^{-1} and 1720cm^{-1}).[1,2] Table S3 displays the assignment of each component that represent the secondary of protein structure. From Table S3, it can be seen that the percentage fraction of α-helix decreased after dissolution and regeneration process. The probable explanation of this observation could be due to the ability of the protic IL to to disrupt the hydrogen bonded network of keratin. We also believe that some of the alpha helix fraction were converted in the formation of higher degree of random coil and β-sheet fraction during the dissolution and regeneration process.

^{1}H NMR of neat and distilled IL

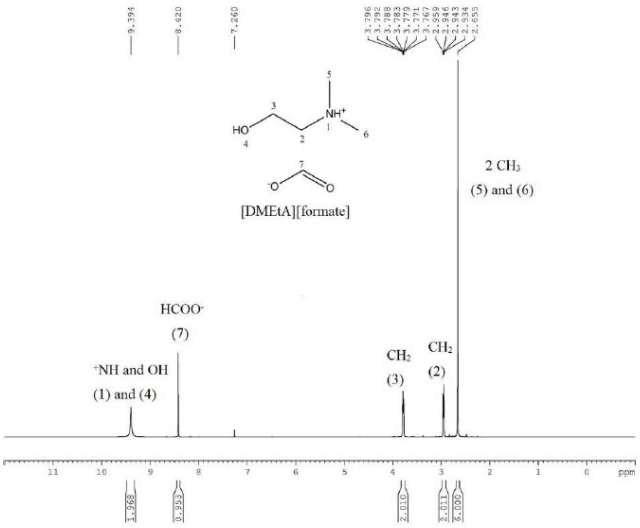


Figure S3. NMR spectrum of neat [DMEA] HCOO].

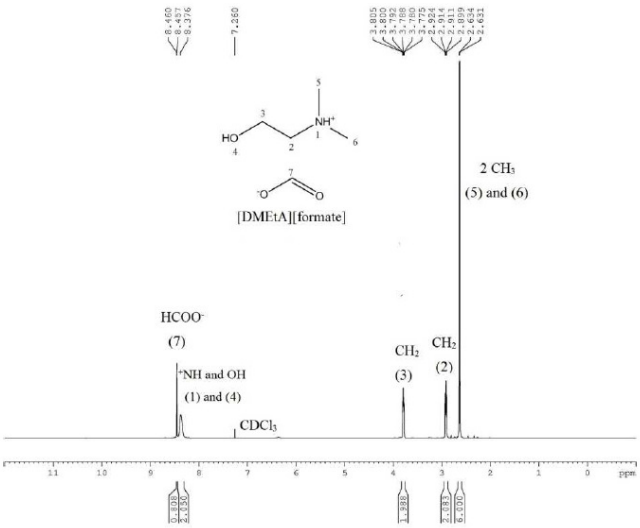


Figure S4. NMR spectrum of recovered [DMEA][HCOO] after distillation.

The spectra are substantially similar. However, the NH/OH peak has moved; D_2O confirming that this is an exchangeable proton. Therefore, shift is likely due to differing amounts of residual water in the sample (or the $CDCl_3$ solvent).

SDS Page

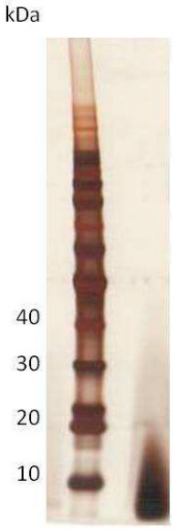


Figure S5. SDS-PAGE pattern of (a) protein standard and (b) water soluble protein remaining in dark resin after distillation.

DSC traces of feather and keratin film

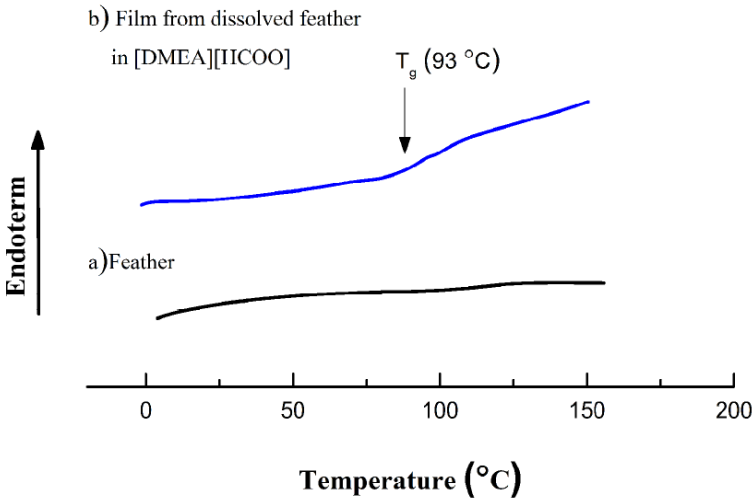


Figure S6. DSC of a) feather and b) film

XRD of feather and keratin film

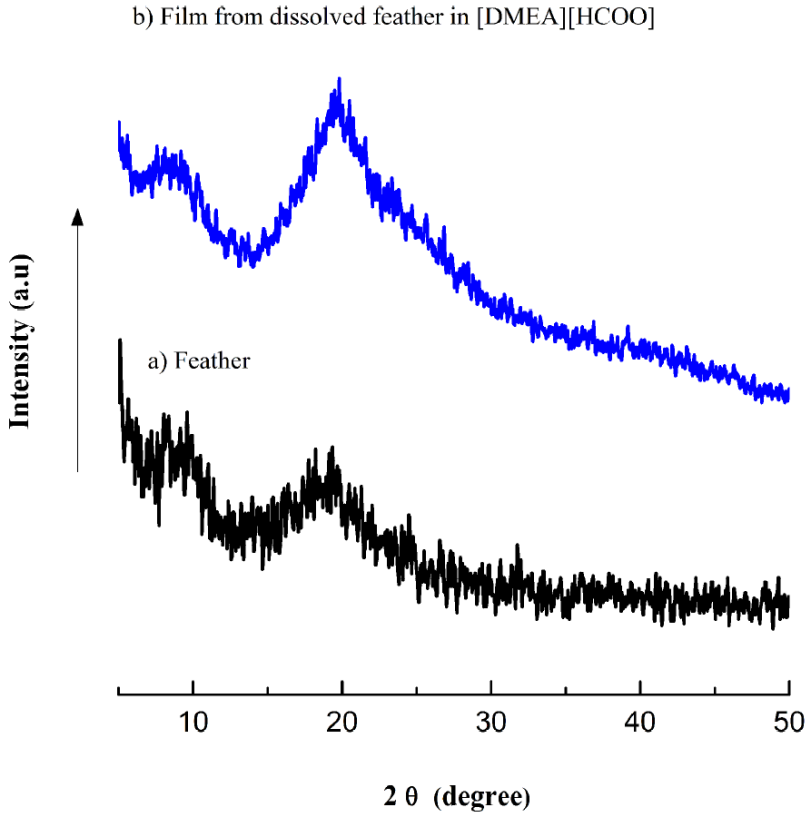


Figure S7. XRD of (a) feather and (b) film from dissolved feather in [DMEA][HCOO].

FTIR of feather and keratin film

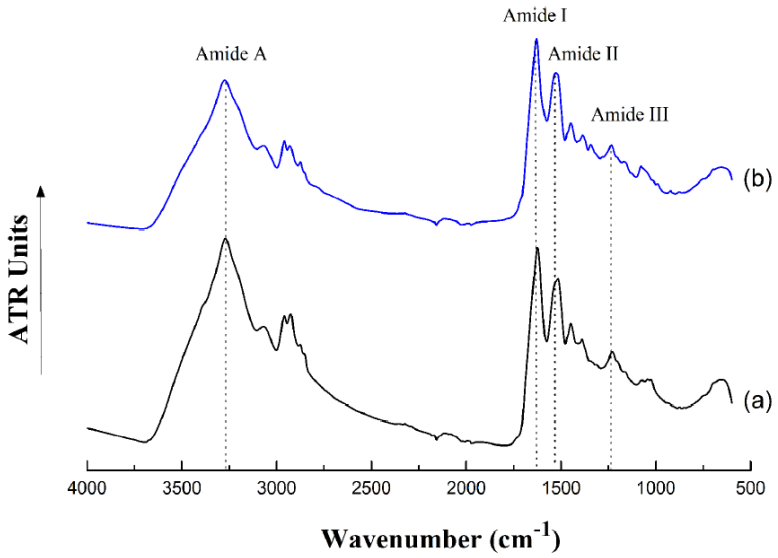


Figure S8. ATR-FTIR of (a) feather and (b) film from dissolved feather in [DMEA][HCOO].

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5.5 References

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Chapter 6

Conclusions and future work

6.1 Conclusions

In summary, this study successfully confirmed the hypothesis that certain ionic liquids, namely [BMIM]Cl, [AMIM]Cl, [choline][thioglycolate], [AMIM][dca], and [DMEA][HCOO] have good potential to be applied as solvents for keratin biopolymer dissolution.

The key outcomes from this study were as follows:

(i) Ionic liquids were synthesised and applied to dissolve and regenerate keratin biopolymers, in particular feather and wool. The properties of the regenerated keratin material were evaluated. The best ILs for dissolution of feather keratin were found amongst the chloride ion containing ionic liquids, while the dca anion was the most effective IL to dissolve wool. Up to 50 wt. % solubility was achieved for feather and 47 wt. % for wool dissolution. The deep eutectic solvents were also studied in wool dissolution as an alternative to ILs; these solvents did not improve upon the solubility, but may offer a less expensive alternative. The addition of a reducing agent also increased the solubility of keratin by 50-100 mg g^{-1}.

(ii) The utilization of the regenerated keratin was investigated with the production of soft gels, hard films and fibers from the dissolved feather keratin. This study demonstrated that the regenerated material can be

moulded into various shapes as gels, films and fibers. The characterizations of these materials showed that retention of some IL in the gels and films offers flexibility to the materials.

(iii) It was also found that protic ionic liquids can offer high keratin solubility. These protic ionic liquids can be easily distilled off from the reaction mixture. For example, the distillable PIL, *N*,*N*-dimethylethanolammonium formate ([DMEA][HCOO]), was shown to dissolve feather keratin at 100 ºC. On distillation, the recovery of the PIL was high – 99 % of the initial mass was recovered by distillation at 122 °C under vacuo. The ease of recovery of these PILs provide great potential for recycling.

6.2 Future Work

The following are some suggestion for future work:

The investigation of other keratin sources such as hair, horn and nails could also be investigated. These biopolymers are also abundant as waste materials that can contributes to environmental pollution, therefore it would be valuable to utilise these wastes and convert these biopolymers into new materials with innovative properties and profitable applications.

The protic ILs exhibited sufficiently high solubility for most processing applications and therefore further investigation and optimization of the recyclability and dissolution efficiency with different type of protic ILs is warranted. The challenges that arise from the distillation process are to ensure that the protic ILs are stable with a prolonged and repeated heating without decomposition.

The dissolution of keratin under ultrasound irradiation could also be explored. The application of ultrasound irradiation is being increasingly used in the extraction of various materials, for example, the increased of dissolution of cellulose in ILs has been proven with the assistance of ultrasound irradiation. Therefore, the impact of ultrasound irradiation on keratin dissolution is worth examination.

In our present work on preparing final physical forms (films and fibers) from keratin-IL solutions, these materials were prepared by casting the keratin-IL solution without using any additives of chemical crosslinkers. One of the limitations that we encountered was the materials obtained were brittle. In order to reinforce the keratin films, more work can be carried out using fillers, cross-linking agents, or composites to blend with in the keratin solution or regenerated keratin material. This will enhance the mechanical strength, improve the mechanical properties and also create the desired properties in the film, fibers and gels that have characteristics relevant to various specific applications.