

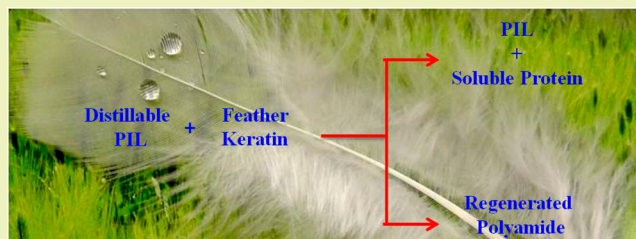
Distillable Protic Ionic Liquids for Keratin Dissolution and Recovery

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S 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 recyclable 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 hydrolysis³¹ 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 study⁴¹ 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.

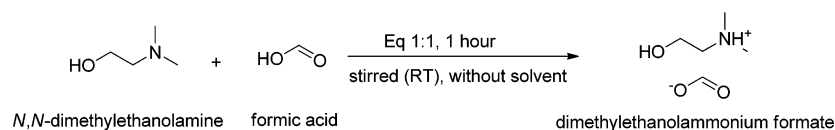
Though the chemical reaction for synthesizing a PIL is often a simple proton transfer, obtaining a pure salt is dependent on the strengths of the component acid and base, as expressed by

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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., $-\text{OH}$. 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. $\text{Cu K}\alpha_1$ radiation ($\lambda = 1.540$ 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 $0.02^\circ 2\theta$ and an accompanying scan rate of $0.5^\circ/\text{min}$.

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 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 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 $\sim 90,000$ – $100,000$. 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: $\text{ES}^+ m/z$ 90.0 [DMEA] $^+$. $\text{ES}^- m/z$ 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

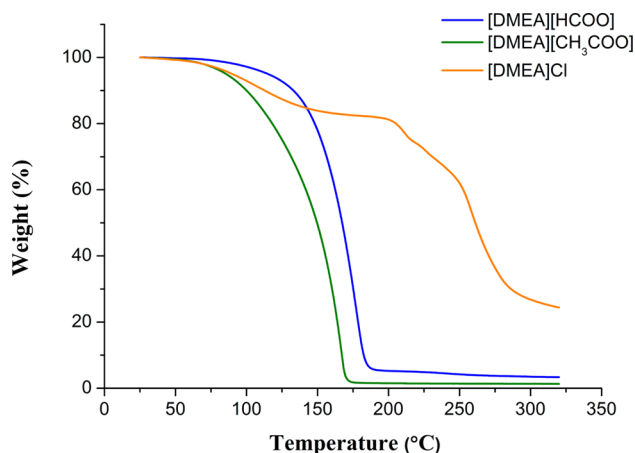


Figure 1. TGA curve of protic ionic liquids.

°C, and hence, this temperature was selected for this study. Beyond this, the PIL loses mass rapidly due to distillation. The [DMEA][CH₃COO] 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 liquids⁴¹ 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 ¹H 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

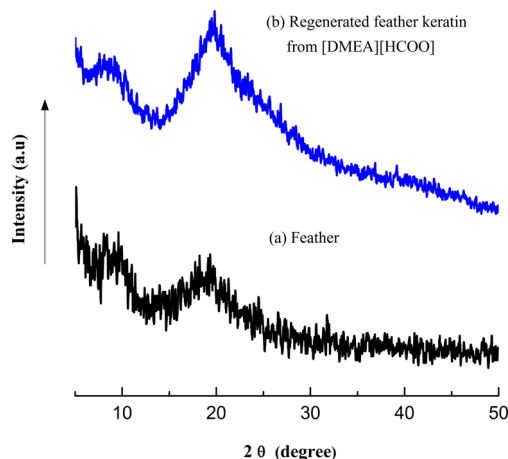


Figure 3. XRD of (a) feather and (b) regenerated feather keratin from [DMEA][HCOO].

feather and regenerated feather keratin show diffraction characteristic of the α -helix appearing at $2\theta = 9^\circ$ (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

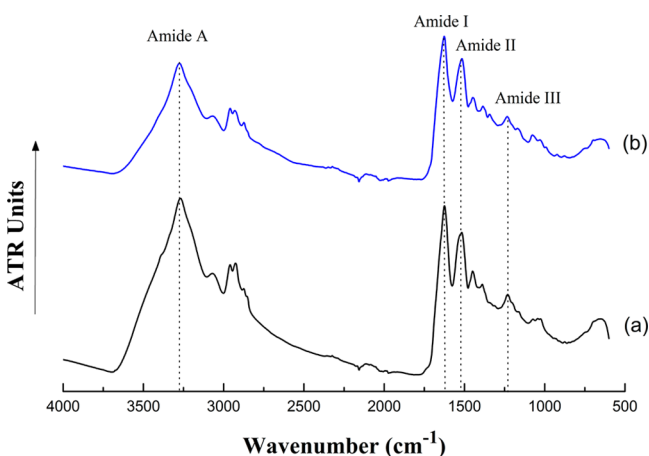


Figure 4. ATR-FTIR of (a) feather and (b) regenerated feather keratin from [DMEA][HCOO].

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

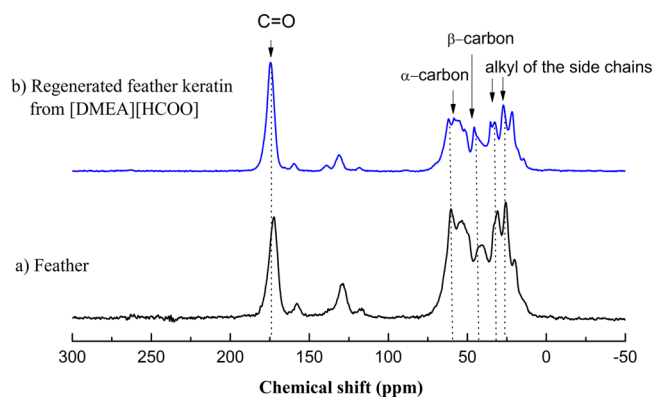


Figure 5. ^{13}C CP MAS NMR spectra of (a) feather and (b) regenerated feather keratin.

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 $^\circ\text{C}$ is generally described as a melting of α -helix crystallites⁵⁵ (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 $^\circ\text{C}$ and peaks around 180 $^\circ\text{C}$ 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 $^\circ\text{C}$. In contrast, the regenerated feather keratin begins to

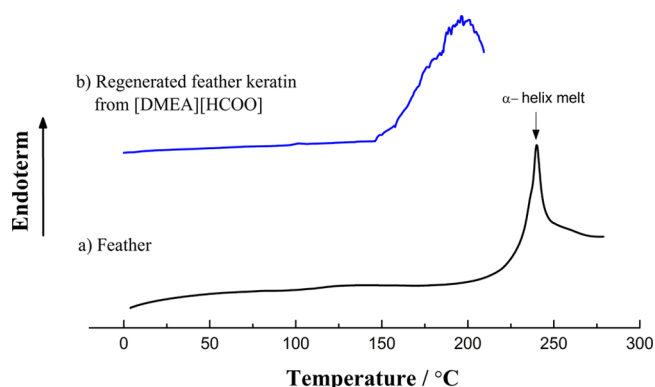


Figure 6. DSC curves of (a) feather and (b) regenerated feather keratin.

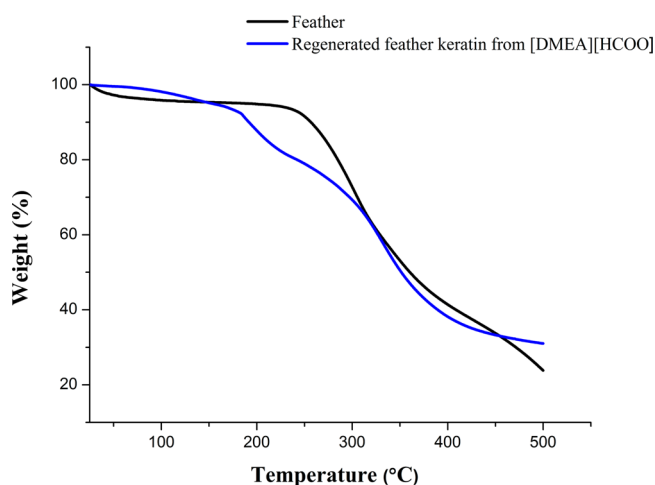


Figure 7. TGA plot of feather and regenerated feather keratin.

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, ¹H 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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