1-Butyl-3-Methylimidazolium Acetate as an Alternative Solvent for Type I Collagen

by

Jie Liu,[1] Zhou Xu,[1] Yi Chen,[1]* Haojun Fan[2]* and Bi Shi[2]

[1]*Key Laboratory of Leather Chemistry and Engineering of Ministry of Education, Sichuan University,* CHENGDU 610065, P.R. China

[2]*National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University,* CHENGDU 610065, P.R. China

Abstract

Low solubility and undesirable denaturation in conventional solvents continue to represent a significant challenge for efficient extraction, accurate characterization and versatile processing of collagen. In the present study, a room temperature ionic liquid (IL), 1-butyl-3-methylimidazolium acetate ([BMIM]Ac), was synthesized, and then evaluated as an alternative solvent for type I collagen. Real-time polarizing optical microscope observation indicated complete disintegration of hierarchical structure of collagen aggregates as solubilized in [BMIM]Ac at 25°C. The solubility reached up to approximately 8.0 wt.% at 25°C, more than ten times higher than that in conventional dilute acetic acid. In comparison with dilute acetic acid and recently reported chloridion ILs, high solubility of collagen in [BMIM]Ac at room temperature was ascribed to loose binding between $[BMIM]^+$ and acetate, as well as stronger proton-accepting ability of [BMIM]Ac, which enabled rupture of those intermolecular hydrogen bonds and ionic bonds that stabilized collagen aggregates. However, such bond-rupturing effect was found selective at room temperature. As demonstrated by Fourier transform infrared, circular dichroism, atomic force microscope, and ultrasensitive differential scanning calorimetry analysis, [BMIM]Ac did not destroy the special triple-helical structure of tropocollagen molecules that had been identified as being of importance for the functional and bioactive properties of collagen. According to these results, the discovery of [BMIM]Ac as an ideal solvent for collagen may open up new possibilities for the chemistry and engineering of collagen, which has long been established as a readily accessible and renewable resource with many unique properties.

Introduction

Collagen is the major structural and functional element of connective tissues and is also found in large quantities in the interstitial tissues of virtually all parenchymal organs, where it maintains the structural integrity of tissues and contributes to various physiological functions.[1] Structurally speaking, all members of the collagen family are composed of three polypeptide chains, each one of which is structured in a left-handed, polyproline II-type (PPII) helix and then twisted together into a special right-handed triple helix. By virtue of low immunogenicity, good biocompatibility, favorable biodegradability, and the ability to stimulate tissue regeneration,[2-4] naturally occurring collagen has currently found a broad spectrum of applications that cover from functional cosmetics for skin rejuvenation, through dietary supplement promoting muscle growth, to advanced biomedical devices with haemostatic or repair function.

However, tropocollagen molecules tend to form microfibrils and fibrils *in vivo* with unusual strength and stability through self-aggregation and cross-linking, and thus they can only swell, but hardly dissolve in most solvents.[5,6] Indeed, the lack of good solvent has long been a significant challenge for efficient extraction, accurate characterization, and large-scale processing of collagen, which decisively impedes the industrial motivation to develop novel collagen-derived devices for versatile utilization. In general, few commonly employed solvents for collagen thus far include neutral salt solution, dilute organic acid, 2,2,2-trifluoroethyl (TFE), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFP). Despite their potency to rupture the bonds that stabilize the hierarchical structure of collagen aggregates, collagen dissolution in these

*Corresponding authors' e-mail: chenyi_leon@scu.edu.cn, fanhaojun@scu.edu.cn.

Manuscript received January 22, 2014, accepted for publication March 27, 2014.

solvents still suffers from certain well-recognized disadvantages, including low solubility, environmental risks, as well as occurrence of denaturation.[7-9] For instance, the solubility of collagen in dilute acetic acid usually does not exceed 0.75 wt%, or the solution loses fluidity as a result of gel effect.[7] In the case of organic TFE and HFP, the preparation process is known to be laborious, requires specialized equipment and also can be hazardous. In addition, these organic solvents have been classified as toxicants to reproductive system, bladder and upper respiratory tract, so the biomedical community raises concerns over potential cytotoxicity from any residual solvents that conceivably affects the biological performance of collagen.[8] More importantly, it has been found that upon being dissolved in TFE and HFP, collagen will lose up to 99% of the special triple-helical structure that has been identified as being important in many specific biological interactions as well as being a structural element.[9] Obviously, undesirable denaturation in those solvents remarkably compromises the very properties that have established collagen as a leading natural biomaterial.

Over the past decades, ionic liquids (ILs), consisting of an inorganic or organic anion paired with a bulky, highly asymmetric and diffusely charged organic cation, have emerged at an exponential rate as a potentially green replacement for volatile organic solvents, owing to their negligible vapor pressure, wide operating temperature range, nonflammability, excellent thermal/chemical stability, high ionic conductivity, and low toxicity.[10] More advantageously, the physicochemical properties of ILs strongly depend on the species of cation and anion, as well as the length of the lateral alkyl groups on the heterocyclic rings, and thereby altering these structural parameters allows the viscosity, solvation, catalytic activity, hydrophobicity and melting points of ILs to be finely tailored for particular application. These unique properties have made ILs popular throughout industry and academia, particularly as solvents for organic synthesis, catalysis and also as media for extraction processes.[11-13] As early as in 2007, R.A. Mantz *et al*. accidentally found that type I collagen could be dissolved in 1-ethyl-3-methylimidazolium chloride ([EMIM]Cl), an imidazolium IL with ethyl and methyl as substituents, above the melting point of [EMIM]Cl (T_m = 87°C), and the solubility reached as high as 1.3 wt%.[14] However, being aware of thermal denaturation of collagen (i.e. loss of the triple helicity of tropocollagen molecules) as a result of overheating up to 87°C, R.A. Mantz *et al*. appeared quite conservative to recommend [EMIM]Cl as a viable solvent for collagen. Inspired by such pioneering work, Z. Meng *et al*. explored the feasibility of preparing collagen/cellulose composite with different forms using 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) as a medium. For effective dissolution, it was also found the dissolving process must be carried out at a temperature higher than 100°C, inevitably leading to thermal denaturation of collagen. Thus, how to maintain the triple helicity of tropocollagen molecules while maximizing the solubility seems crucial for the continuing attraction of ILs as promising alternative solvents for collagen fibers.

In our previous study, the temperature-dependent solubility of bovine collagen in [BMIM]Cl was investigated in details.[16] It was found that bovine collagen was insoluble in [BMIM]Cl unless the mixture was heated up to 60°C. However, concerns over possible denaturation of collagen at high dissolving temperature remained unresolved. As continuous work, a room temperature IL, 1-butyl-3-methylimidazolium acetate ([BMIM]Ac), was synthesized in the present study, and then used as a medium for dissolution of bovine collagen. Due to stronger proton-accepting ability of acetate than chlorine anion, a maximum of 8.0 wt% of bovine collagen could be efficiently dissolved in [BMIM]Ac at room temperature (25°C), and the special right-handed triple helix of tropocollagen molecules was found to be retained. To systematically evidence this finding, Fourier transform infrared (FTIR), circular dichroism (CD), atomic force microscope (AFM), and ultrasensitive differential scanning calorimetry (US-DSC) analysis were carried out to investigate the structural difference between native collagen and the one regenerated from [BMIM]Ac. Based on these results, we aim to provide an alternative solvent for dissolving collagen under mild condition, which selectively maintains the delicate triple helix of tropocollagen molecules while achieving higher solubility relative to conventional solvents. Also, the present study may establish crucial fundamental for efficient extraction and versatile processing of naturally occurring collagen using ILs on an industrial scale.

EXPERIMENTAL PROCEDURES

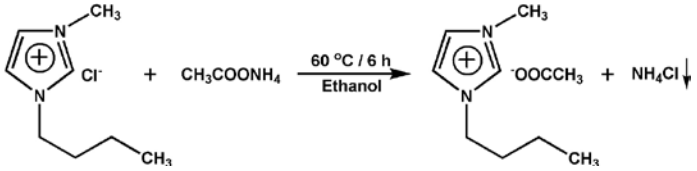
Materials

Type I collagen was extracted from limed bovine split wastes discharged from leather industry according to a previously published protocol by Z. Zhang *et al*.[17] The native collagen obtained was stored at 4°C before use. As indicated by SDS-PAGE analysis, the native collagen exhibited a molecular weight of about 300 kDa with narrow distribution. In addition, [BMIM]Cl (purity ≥ 99.0%) in the form of white powder was purchased from Cheng Jie Chemical Engineering Co. Ltd. (Shanghai, China). Ammonium acetate (NH_4Ac), acetic acid (HAc), and anhydrous ethanol of analytically pure grade were obtained from Kelong Chemical Engineering Co. Ltd. (Chengdu, China).

Synthesis of [BMIM]Ac

[BMIM]Ac was synthesized in-house via an ion exchange method as previously described by Y. Liu *et al*.[18] In brief, [BMIM]Cl (17.4 g; 0.1 mol) and NH_4Ac (7.7 g; 0.1 mol) were

dissolved in 100 ml anhydrous ethanol, and the mixture was continuously stirred at 60°C under N_2 atmosphere for 6 h. As the ion exchange reaction proceeded, NH_4Cl in the form of white solid was precipitated from the mixture. After filtration, the filtrate was treated with 5 Å molecular sieve for 48 h, and then dried at 60°C to remove ethanol until a constant mass. The resultant [BMIM]Ac was a viscous, slightly amber liquid. The synthesis procedure mentioned above was depicted in Scheme 1.



Scheme 1. Synthesis procedure of [BMIM]Ac.

Dissolution of Collagen in [BMIM]Ac

The dissolution of type I collagen in [BMIM]Ac was carried out in a round-bottom, three-necked separable flask equipped with a mechanical stirrer, a nitrogen inlet, and a thermometer. The solubility was determined in a step-wise procedure that involved attempting to dissolve as much collagen as possible into 10 g [BMIM]Ac at 25°C. Accordingly, small incremental amounts of collagen were added into 10 g [BMIM]Ac, which was mechanically stirred at 25°C, until the added collagen could not be visually detected. The solubility was expressed as the maximum percentage, by mass, of collagen that was dissolved into the IL medium.

To regenerate collagen from IL for structural characterization, collagen solution in [BMIM]Ac was first cast on a leveled polytetrafluoroethylene plate to a thickness of 50 μm using a Gardner knife. It was then immersed into a precipitating bath (de-ionized water), in which diffusion of precipitating agent into and [BMIM]Ac out of the collagen solution resulted in regeneration of collagen as a thin film. Before analysis, the semitransparent collagen film was dried at 25°C under vacuum condition until a constant mass.

Polarizing Optical Microscope (POM) Observation

The dissolving process of collagen in [BMIM]Ac was observed on a Leica DMLP POM (LEICA, Germany) equipped with a temperature-controlled hot stage. Immediately after mixing, a drop of [BMIM]Ac containing 8.0 wt% of collagen was sandwiched between a freshly cleaved microscope slide and a coverslip. This assembly was then mounted onto the hot stage of the POM that was thermostated at 25°C throughout the experiment. At a fixed time interval, the extent of collagen dissolution in [BMIM]Ac was observed and photographed.

FTIR Analysis

Using film technique, FTIR spectra of collagen films regenerated from [BMIM]Ac were collected at 25°C on a Nicolet iS10 FTIR spectrometer (Thermo Scientific, United States), over a wavenumber range from 500 to 4000 cm^{-1} after 32 scans at 4 cm^{-1} resolution.

CD Measurement

The collagen regenerated from [BMIM]Ac was dissolved in 0.05 M acetic acid to form a 0.1 mg/ml solution. CD measurement was carried out at 25°C using a Research-Grade Circular Dichroism Spectrometer (AVIV model 400). The CD spectra were collected from 190 to 250 nm with a bandwidth of 1 nm under nitrogen atmosphere. The scan speed was set to 20 nm/min and a slit width of 1 nm was used with a time constant of 1 s. The CD spectrum of 0.05 M acetic acid was used as a reference. The reported spectra were expressed in terms of molar ellipticity as a function of wavelength.

AFM Observation

A stoichiometric amount of collagen regenerated from [BMIM] - Ac was re-dissolved in 0.5 M acetic acid at 4°C to obtain a 10 μg/ml collagen solution. Then, 6 μl of the sample solution was dropped onto a freshly cleaved mica substrate, and thoroughly dried at 25°C under vacuum condition until a constant mass. Morphologies of the collagen deposited on the mica substrate were observed at 25°C on a Shimadzu SPM-9600 multimode AFM (Japan) equipped with a Dimension 3100 Nanoscope IV controller and a silicon TESP cantilever using a tapping mode.

DSC Measurement

DSC measurement was performed on an ultrasensitive differential scanning calorimeter (US-DSC) from Microcal Inc. (Northampton, MA) at an external pressure of ca. 160 kPa. Native collagen or the regenerated one from [BMIM]Ac was dissolved in 0.5 M acetic acid at 4°C to obtain a 0.5 mg/ml collagen solution. After filtered through a 0.2 μm Millipore (Bradford, MA) syringe filter, the sample solution was injected into the sample compartment for degassing for 30 min at 25°C using Thermovac, the degassing accessory from Microcal Inc. (Northampton, MA). Finally, the sample solution was heated at a constant heating rate of 1°C/min from 25°C to 55°C, using 0.5 M acetic acid as a reference.

RESULTS AND DISCUSSION

Dissolution Mechanism of Collagen in [BMIM]Ac

It is well known that type I tropocollagen molecules *in vivo* self-aggregate into large microfibrils, and then fibrils, through numerous intermolecular cross-links, which is responsible for the insolubility of collagen fibers in most solvents.[5,6] To achieve efficient dissolution therefore, an ideal solvent must be capable of rupturing those intermolecular cross-links, mostly hydrogen bonds and ionic bonds, which stabilize the hierarchical structure of collagen aggregates. However, such

bond-rupturing effect must be selective, i.e. those intramolecular hydrogen bonds that are responsible for the triple-helical stability of tropocollagen elements should remain intact during the dissolution process, or the functional and bioactive properties of collagen will be compromised. Owing to their asymmetrically composed and oriented structure, type I collagen microfibrils and fibrils exhibit both intrinsic and form birefringence under polarized optical microscope, serving as an efficient indicator to monitor the dissolution of collagen fibers in [BMIM]Ac. The real-time POM images recording the dissolving process of bovine collagen in [BMIM]Ac at 25°C were illustrated in Figure 1. Lyophilized native collagen was employed as control. Immediately after mixing, collagen fibers in [BMIM]Ac were still positively birefringent as the native ones (see Figure 1 a and b), indicating a quasi-crystalline, three-dimensional orientation within the collagen aggregates at this moment. As the dissolving time prolonged, the positive birefringence gradually decreased and disappeared completely after 24 h (see Figure 1 c and d). This observation implied complete disintegration of hierarchical structure of collagen aggregates as solubilized in [BMIM]Ac.

Using a step-wise procedure that involved attempting to dissolve as much collagen as possible into a fixed amount of [BMIM]Ac, the solubility of type I collagen in [BMIM]Ac was determined to be 8.0 wt% at 25°C, much higher than that in dilute acetic acid, which usually did not exceed 0.75 wt%.[7] In addition to solubility, a distinct discrepancy in appearance could also be observed between saturated solutions of collagen in [BMIM]Ac and dilute acetic acid, respectively. As demonstrated in Figure 2 (b), 0.75 wt% collagen solution in 0.5 M dilute acetic acid exhibited a highly viscous and semi-gel state at 25°C. The lack of fluidity in this case was believed to be related to weak interaction between collagen aggregates and the solvent. Even in a fairly dilute solution, acetic acid molecules only partially ionize as other weak acids. Low concentrations of acetate anions and hydrogen cations can only disrupt weak ionic bonds between tropocollagen molecules, while numerous hydrogen bonds largely responsible for the hierarchical structure of collagen aggregates remain intact. Therefore, although partially de-crosslinked, the microfibrils and fibrils were still interdigitated in dilute acetic acid, and thereby formed a viscous semi-gel by swelling. Considering low solubility and fluidity, extraction, characterization, and processing of naturally occurring collagen using dilute acetic acid as a medium seemed inconvenient. In comparison with dilute acetic acid, the cations in [BMIM]Ac are large and asymmetric, leading to loose binding between $[BMIM]^+$ and acetate. In other words, [BMIM]Ac are composed entirely of ionized $[BMIM]^+$ and acetate.[11,12] Consequently, large numbers of $[BMIM]^+$ and acetate not only destroy the ionic bonds between two oppositely charged groups on adjacent tropocollagen molecules, but also break hydrogen bond cross-links owing to the strong capability of $[BMIM]^+$ and acetate to donate and accept proton, respectively. Almost complete disintegration of hierarchical structure of collagen aggregates allows higher concentrations of collagen to be dissolved in [BMIM]Ac than in traditional solvent systems, and the solution remains fluid even saturated by 8.0 wt% collagen (see Figure 2 a). In addition, [BMIM]Ac contain polarizing-prone aromatic cations ($[BMIM]^+$) that can also shield the formed acetate-collagen complexes, further contributing to the dissolution capability of [BMIM]Ac for collagen.

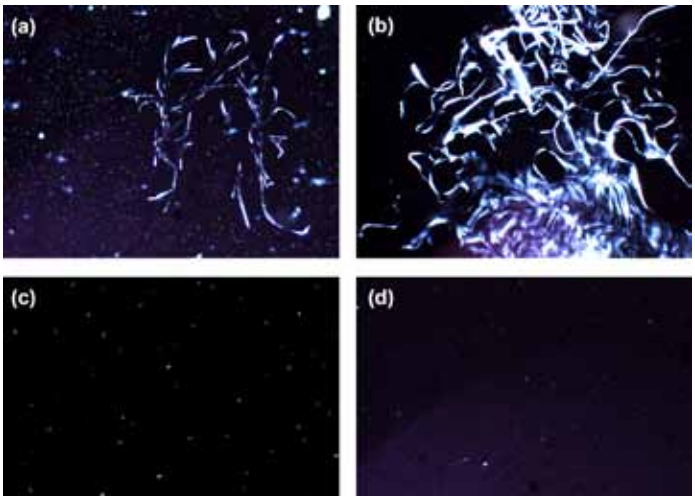


Figure 1. POM images concerning (a) lyophilized native collagen at 25°C; the dissolution of bovine collagen in [BMIM]Ac at 25°C for (b) 0 h; (c) 16 h; and (d) 24h.

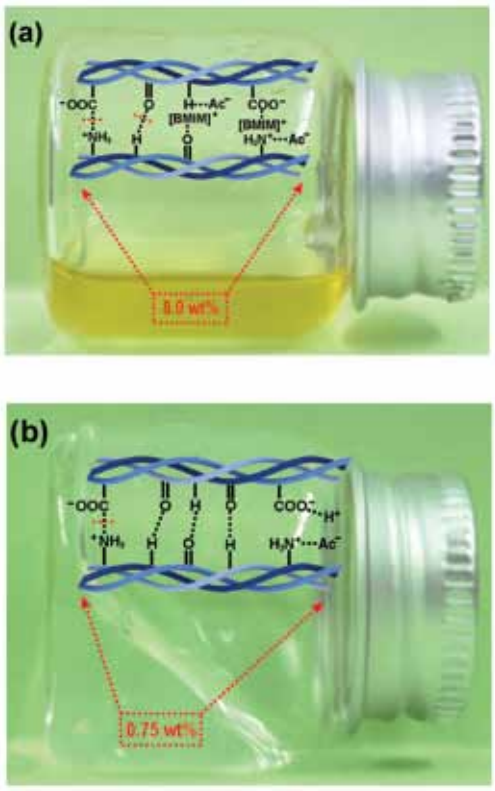


Figure 2. Solubility and appearance of saturated solutions of collagen at 25°C in (a) [BMIM]Ac; and (b) 0.5 M dilute acetic acid.

On the other hand, it was noteworthy that the dissolution of bovine collagen in [BMIM]Ac was achieved at a quite mild temperature (25°C), much lower than that as previously reported in chloridion ILs. To explain such significant improvement, the Kamlet-Taft β parameter, which is an index of the proton-accepting ability of a solvent should be considered. In general, the higher the β parameter, the stronger proton accepting ability of a solvent possesses. According to previous report, the β parameter of [BMIM]Ac was determined to be 1.20 because of the presence of acetate, almost 1.5 times higher than that of [BMIM]Cl (β=0.83).[19] Therefore, [BMIM]Ac exhibit stronger proton accepting ability to disturb the extensive and well-organized intermolecular hydrogen-bonding network of collagen aggregates, thus allowing collagen dissolution at a much lower temperature than [BMIM]Cl. In this way, thermal denaturation of collagen during the dissolving process was advantageously avoided.

Triple-helical Structure of Collagen Regenerated from [BMIM]Ac

In addition to high solubility, an ideal solvent should not destroy the special triple-helical structure of tropocollagen molecules that is the foundation of many important and specific biological functions of collagen. In the present study, the collagen dissolved in [BMIM]Ac was regenerated by using de-ionized water, and the change in triple helicity was systematically characterized by different methods. The

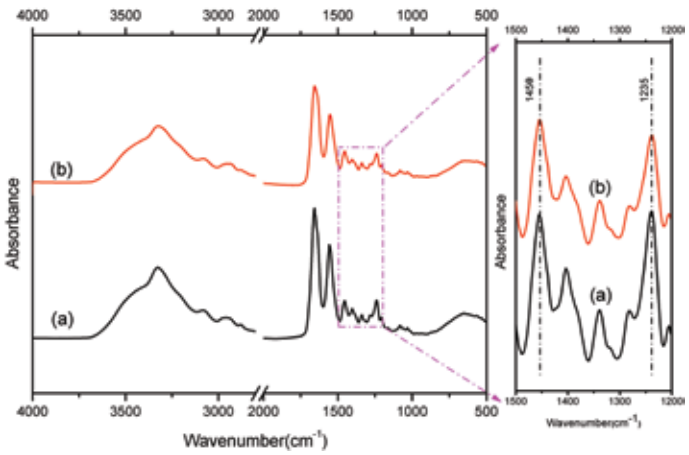


Figure 3. FTIR spectra of (a) native collagen; and (b) collagen regenerated from [BMIM]Ac.

FTIR spectra of native collagen and collagen regenerated from [BMIM]Ac were displayed in Figure 3. In general, the FTIR absorption band at 1235 cm^{-1} corresponds to the C-N bond in-plane vibration (amide III) and the N-H stretch (amide I), which is associated with the triple-helical structure of tropocollagen molecules. The absorption band at 1450 cm^{-1} can be ascribed to the pyrrolidine ring vibration of proline and hydroxyproline, which is not affected by changes in collagen secondary structure. Thus, it is widely accepted that the intensity ratio of FTIR absorption at 1235 and 1450 cm^{-1} ($I_{1235/1450cm^{-1}}$) can be used as a sensitive indicator of the integrity of triple-helical structure in collagen.[20-22] Typically, an $I_{1235/1450cm^{-1}}$ close to unity indicates intact triple-helical structure, whereas an $I_{1235/1450cm^{-1}}$ significantly lower than unity suggests the triple helicity has been completely destroyed (e.g., typical $I_{1235/1450cm^{-1}}$ for denatured collagen was reported to be as low as 0.60).[20-22] Based on the FTIR spectra in Figure 3, the $I_{1235/1450cm^{-1}}$ values of native collagen and collagen regenerated from [BMIM]Ac were calculated, and the results were listed in Table I. Clearly, the $I_{1235/1450cm^{-1}}$ of regenerated collagen from [BMIM]Ac was determined to be 0.97, quite comparable to that of native collagen (1.0). This result indicated that the triple-helical structure was preserved in the regenerated collagen. In other words, although [BMIM]Ac was capable of rupturing those intermolecular hydrogen bonds and ionic bonds that stabilized the hierarchical structure of collagen aggregates, it hardly damaged the intramolecular hydrogen bonds that were important for the triple-helical stability of tropocollagen molecules.

In addition, quantitative analysis of molar ellipticity changes in CD spectra before and after collagen dissolution in [BMIM]Ac could also provide valuable information regarding the integrity of triple-helical structure. CD spectroscopy is the

TABLE I
Intensities of FTIR absorption band (at 1235 and 1450 cm^{-1}) and molar ellipticity values (at 223 and 197 nm) of native collagen and collagen regenerated from [BMIM]Ac.

	Intensity of FTIR absorption band (I)			Molar ellipticity (E)		
	$I_{1235cm^{-1}}$	$I_{1450cm^{-1}}$	$I_{1235/1450cm^{-1}}$	E_{223nm}	E_{197nm}	Rnp
Native collagen	0.805	0.815	1.01	11.67	-76.72	0.152
Regenerated collagen from [BMIM]Ac	0.852	0.827	0.97	10.12	-71.74	0.141

most widespread technique used for estimating the secondary structures of proteins and polypeptides in solution. This technique can be used to distinguish between unordered (random coil) and ordered (triple helix) structures. CD detects wavelength-dependent differences in the absorption of right and left circularly polarized light by optically active molecules such as collagen. The CD spectrum of unordered collagen is usually characterized by a single band below 200 nm, whereas triple-helical structure usually presents one positive band at 223 nm, along with one negative band at 197 nm.[23,24] It has been well established that the absolute ratio value of molar ellipticity at 223 nm over molar ellipticity at 197 nm (Rpn) can be used to characterize the integrity of triple-helical conformation of collagen in solution.[23,24] As illustrated in Figure 4, the CD spectra of native collagen and collagen regenerated from [BMIM]Ac were plotted for comparison. As for native collagen, the CD spectra exhibited a positive peak at around 223 nm and a negative peak at around 197 nm, which indicated a typical triple-helical conformation. After dissolution in [BMIM]Ac, the CD spectrum of the collagen showed similar pattern to that of the native one, suggesting the preservation of triple helicity. The molar ellipticity at 197 and 223 nm for both native collagen and collagen regenerated from [BMIM]Ac were listed in Table I. Clearly, the Rnp value of the regenerated collagen was quite close to that of the native reference. This result proved that dissolution in [BMIM]Ac did not destroy the triple-helical structure of tropocollagen molecules.

As already recognized, the triple helicity of tropocollagen molecules is the basis of the supermolecular structure of collagen fibers. After solvent evaporation, the dissolved tropocollagen elements with intact triple helix are capable of self-assembling into fibrillar structure through intermolecular interactions again. However, if the triple-helical structure of tropocollagen elements has been destroyed, such self-assembling will not be possible. The morphologies of regenerated collagen from [BMIM]Ac were investigated by AFM technique, and the results were illustrated in Figure 5. The molecular morphologies of native collagen and gelatin after solvent (0.5 M dilute acetic acid) evaporation were employed as negative and positive control, respectively. By comparison, the regenerated collagen still exhibited fibrillar structure as the negative control, where curved collagen fibers overlapped with one another. In the case of gelatin however, no fibrillar structure could be observed after solvent evaporation. The absence of fibrillar structure in gelatin could be ascribed to complete destruction of triple-helical structure. These results indicated again that dissolution in [BMIM]Ac did not influence the triple-helical structure that stabilized tropocollagen molecules. Therefore, it could be speculated that the functional and bioactive properties of collagen were not compromised after dissolution in [BMIM]Ac.

The thermal stability of native collagen and collagen regenerated from [BMIM]Ac, both dissolved in 0.5 M dilute acetic acid, was measured by US-DSC, and the thermograms obtained were displayed in Figure 6. Generally speaking, the heat transformation of collagen in the temperature range of

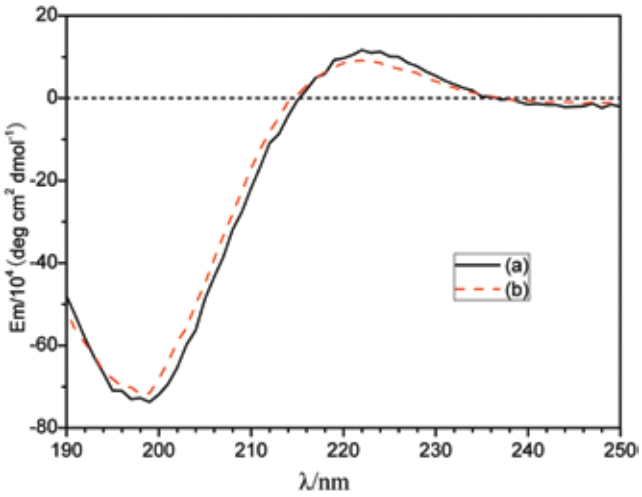


Figure 4. CD spectra of (a) native collagen; and (b) collagen regenerated from [BMIM]Ac.

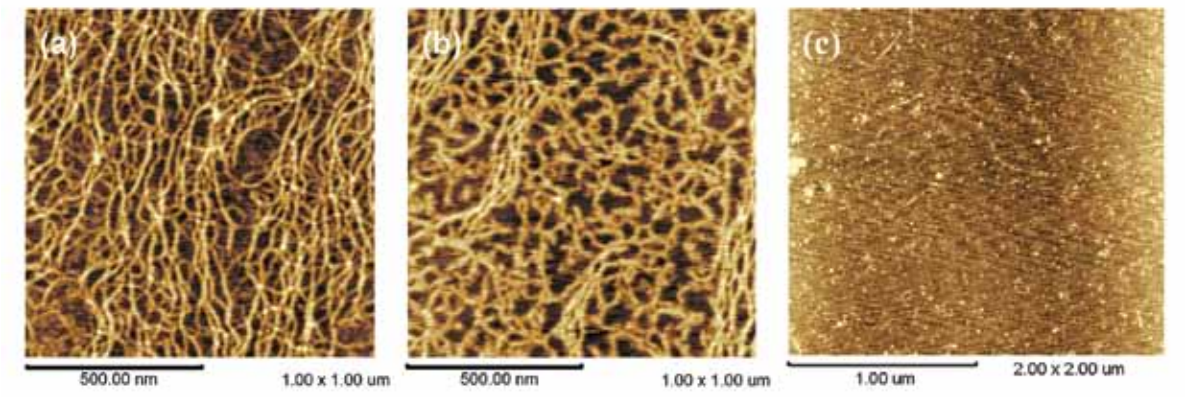


Figure 5. AFM height images of (a) native collagen; (b) collagen regenerated from [BMIM]Ac; and (c) gelatin after solvent evaporation.

35-40°C is attributable to the collapse of triple-helical structure into random coils.[25] This endothermic peak is normally assigned as T_d. According to Figure 6, the T_d values of native collagen and collagen regenerated from [BMIM]Ac were determined to be 38.3°C and 39.4°C, respectively, which were very close to each other. This indicated that the triple helicity of the regenerated collagen from [BMIM]Ac was comparable to that in the native one.

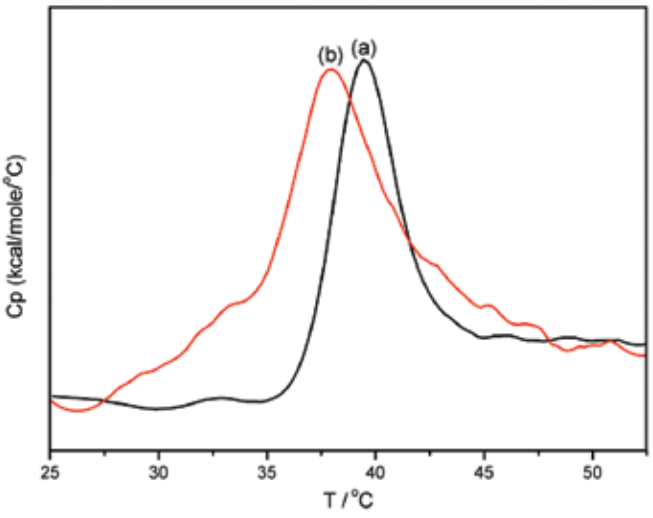


Figure 6. US-DSC thermograms of (a) native collagen; and (b) collagen regenerated from [BMIM]Ac.

CONCLUSIONS

Owing to loose binding between $[BMIM]^+$ and acetate, as well as stronger proton accepting ability of [BMIM]Ac, type I collagen can be dissolved in [BMIM]Ac with much higher solubility and at much lower temperature than in dilute acetic acid or chloridion ILs. The solvation process involves selective rupturing of intermolecular hydrogen bonds and ionic bonds that stabilize collagen aggregates. However, the intramolecular triple-helical structure of tropocollagen molecules remains intact. Such high efficiency and selectivity is promising for versatile application of collagen, which has been greatly limited over the past decades by lack of an ideal medium for collagen extraction, characterization and processing.

ACKNOWLEDGEMENT

The authors wish to acknowledge financial support from National Natural Science Foundation of China (51273128 and 21206096), New Teachers' Fund for Doctor Stations, Ministry of Education of China (20120181120116), and Fundamental Research Funds for the Central Universities, China.

REFERENCES

1. Shoulders, M.D., Raines, R.T.; Collagen structure and stability. *Annu. Rev. Biochem.* **78**, 929-958, 2009
2. Gao, D.G., Ma, J.Z., Lv, B., Zhang, J.; Collagen modification using nanotechnologies: a review. *JALCA* **108**, 392-400, 2013
3. Brown, E., Latona, R., Taylor, M.; Effects of pretanning processes on collagen structure and reactivity. *JALCA* **108**, 23-29, 2013
4. Matyasovsky, J., Sedliacik, J., Cervinkova, D., Jurkovic, P., Duchovic P., Gajtanska, M.; Polymer bio-composites based on collagen. *JALCA* **107**, 323-335, 2012
5. Rabinovich, D.; Everything you wanted to know about collagen models-but were too afraid to ask!. *JALCA* **106**, 242-254, 2011
6. Gautieri, A., Vesentini, S., Redaelli, A., Buehler, M.J.; Hierarchical structure and nanomechanics of collagen microfibrils from the atomistic scale up. *Nano Lett.* **11**, 757-766, 2011
7. Lai, G., Li, Y., Li, G.; Effect of concentration and temperature on the rheological behavior of collagen solution. *Int. J. Biol. Macromol.* **42**, 285-291, 2008
8. Lin, H.Y., Kuo, Y.J., Chang, S.H., Ni, T.S.; Characterization of electrospun nanofiber matrices made of collagen blends as potential skin substitutes. *Biomed. Mater.* **8**, 1-9, 2013
9. Zeugolis, D.I., Khew, S.T., Yew, E.S.Y., Ekaputra, A. K., Tong, Y.W., Yung, L.Y.L., Hutmacher, D.W., Sheppard, C., Raghunath, M.; Electro-spinning of pure collagen nano-fibres-just an expensive way to make gelatin?. *Biomaterials* **29**, 2293-2305, 2008
10. Rogers, R.D., Seddon, K.R.; Ionic liquids-solvents of the future?. *Science* **302**, 792-793, 2003
11. Earle, M.J., Seddon, K.R.; Ionic liquids. Green solvents for the future. *Pure Appl. Chem.* **72**, 1391-1398, 2000
12. Seoud, O.A.E., Koschella, A., Fidale, L.C., Dorn, S., Heinze, T.; Applications of ionic liquids in carbohydrate chemistry: a window of opportunities. *Biomacromolecules* **8**, 2629-2647, 2007
13. Welton, T.; Room-temperature ionic liquids. Solvents for synthesis and catalysis. *Chem. Rev.* **99**, 2071-2083, 1999
14. Mantz, R.A., Fox, D.M., Green III, J.M, Fylstra, P.A., De Long, H.C., Trulove, P.C.; Dissolution of biopolymers using ionic liquids. *Z. Naturforsch.* **62a**, 275-280, 2007
15. Meng, Z., Zheng, X., Tang, K., Liu, J., Ma, Z., Zhao, Q.; Dissolution and regeneration of collagen fibers using ionic liquid. *Int. J. Biol. Macromol.* **51**, 440-448, 2012
16. Xia, C., Liu, S., Chen, Y., Fan, H., Shi, B.; Dissolution characteristics of protein in an ionic liquid. *JSLTC* **97**, 11-17, 2013
17. Zhang, Z., Liu, W., Li, D., Li, G.; Physicochemical properties of succinylated calfskin pepsin-solubilized collagen. *Biosci. Biotechnol. Biochem.* **71**, 2057-2060, 2007

18. Liu, Y., Li, M., Lu, Y., Gao, G.H., Yang, Q., He, M.Y.; Simple, efficient and recyclable palladium catalytic system for Heck reaction in functionalized ionic liquid network. *Cata. Commun.* **7**, 985-989, 2006
19. Lopes, A.M.D.C., Joao, K.G., Morais, A.R.C., Lukasik, E.B., Lukasik, R.B.; Ionic liquids as a tool for lignocellulosic biomass fractionation. *Sustainable Chemical Processes* **1**, 3-33, 2013
20. Madhan, B., Subramanian, V., Rao, J.R., Nair, B.U., Ramasami, T.; Stabilization of collagen using plant polyphenol: role of catechin. *Int. J. Biol. Macromol.* **37**, 47-53, 2005
21. Figueiro, S.D., Goes, J., Moreira, R.A., Sombra, A.S.B.; On the physico-chemical and dielectric properties of glutaraldehyde crosslinked galactomannan-collagen films. *Carbohyd. Polym.* **56**, 313-320, 2004
22. Andrews, M.E., Murali, J., Muralidharan, C., Madhulata, W., Jayakumar, R.; Interaction of collagen with corilagin. *Colloid. Polym. Sci.* **281**, 766-770, 2003
23. Consonni, R., Zetta, L., Longhi, R., Toma, L., Zanaboni, G., Tenni, R.; Conformational analysis and stability of collagen peptides by CD and by ^{1}H- and ^{13}C-. *Biopolymers* **53**, 99-111, 2000
24. Usha, R., Ramasami, T.; Structure and conformation of intramolecularly cross-linked collagen. *Colloids and Surfaces B: Biointerfaces* **41**, 21-24. 2005
25. Mu, C., Li, D., Lin, W., Ding, Y., Zhang, G.; Temperature induced denaturation of collagen in acidic solution. *Biopolymers* **86**, 282-287, 2007