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Investigation of the solubility and dispersion degree of calf skin collagen in ionic liquids

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Abstract

The dissolution of collagen in ionic liquids (ILs) was highly dependent on the polarity of ILs, which was influenced by their sorts and concentrations. Herein, the solubility and dispersion degree of collagen in two sorts of ILs, namely 1-ethyl-methylimidazolium tetrafluoroborate ([EMIM][BF$_4$]) with low polarity and 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]) with high polarity in a concentration range from 10% to 70% at 10 °C were investigated. When 150 mg of collagen was added to 30 mg of ILs, the minimum soluble collagen concentration was 0.02 mg/mL in 70% [EMIM][BF$_4$] with lowest polarity and the maximum was 3.57 mg/mL in 70% [EMIM][Ac] with highest polarity, which indicates that soluble collagen and insoluble collagen fibers were both present. For insoluble collagens, differential scanning calorimetry showed that the thermal-stability was weakened when increasing the ILs concentration and polarity, and the fiber arrangement was looser with a more uniform lyophilized structure, observed by atomic force microscopy and scanning electron microscopy. For soluble collagens, electrophoresis patterns and Fourier transform infrared spectroscopy showed that no polypeptide chain degradation occurred during dissolution, but the thermal denaturation temperature decreased by 0.26 °C~ 7.63 °C with the increase of ILs concentrations, measured by ultra-sensitive differential scanning calorimetry. Moreover, the aggregation of collagen molecules was reduced when ILs polarity was increased as determined by fluorescence measurements and dynamic light scattering, which resulted in an increased loose fiber arrangement observed by atomic force microscopy. If the structural integrity of collagen needs to be retained, then the ILs sorts and concentrations should be considered.

Keywords: Ionic liquids concentrations, Collagen solubility, Structural integrity, Dispersion degree, Aggregation state

1 Introduction

Ionic liquids (ILs), which are known as "supramolecular solvents", have appeared as novel solvents that permit the compounding of a series of conventional natural biomasses that are not easily soluble. ILs are molten salts that are composed of bulky organic cations and small inorganic anions [1, 2]. As promising alternatives to conventional organic solvents, ILs possess many unmatched favorable properties, such as miscibility with water and organic solvents, high tunability, low or negligible vapor

pressure, and thus, they are classified as "green solvents" [3–5]. Recently, progress has been rapid in the application of ILs as a novel solvent type. Fukaya et al. [6] found that 1-ethyl-3-methyl imidazolidic phosphonate ionic liquid had an excellent solubility in cellulose, and the dissolution rate of cellulose was 10% at 30 min and 45 °C. The solubility of chitosan with a molecular weight of 9.7 kDa could reach 12% in [C4mim][CH3COO] at 110 °C as reported by Wu et al. [7] Philips et al. [8] studied the solubility of silk fibroin in various ILs, and found that [BMIM]Cl had the highest solubility for protein fibers.

Because many reports have verified the possibility of dissolving natural polymer, such as cellulose, chitosan, and biological protein with ILs, researchers started to consider the application of the high polarity ILs in

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collagen dissolution. Collagen, as the most abundant fibrous protein in connective tissues of land-based mammals (e.g. calf skin) which are common raw materials in leather industry, has been widely applied in food, pharmaceutical, biochemistry and cosmetic products, because of its unusual combination of good biocompatibility, low antigenicity and controlled biodegradability [9–12]. These great biological properties of collagen are ascribed mostly to its unique triple helix conformation, which is stabilized by hydrogen bonds [13–15]. However, the molecular weight of ~ 300 kDa and the large numbers of hydrogen bonds, electrovalent bonds, hydrophobic bonds, and van der Waals forces prevent collagen from being dissolved in general solvents, but it is highly desirable that ILs provide a high transformation and ability to utilize collagen.

The applications of ILs in related collagen research have focused mainly on a solvent medium of blend [16–18], pretreatment solvent of collagen extraction [10, 19], or conditions of collagen dissolution and changes in regenerated-collagen properties [20–22]. Wang et al. [16] studied the dissolution performance of white-hide-powder from leather-making in $[C_6O_2(mim)_2][Br]_2$, and found that when the white hide powder solubility reached 8%, the dissolving time was 55 min at 120 °C. The triple helix structure of white-hide-powder was destroyed partially during $[C_6O_2(mim)_2][Br]_2$ dissolution. As Meng et al. [21] reported, native skin collagen fibers could dissolve in [BMIM]Cl at 100 °C for 6 h, but the triple helix structure of collagen was partially disturbed during the dissolution and regeneration. The collagen materials used in these studies were usually not highly purified collagen. Hu et al. [22] dissolved collagen in [EMIM][Ac] with a series of sodium salts at different temperatures (25, 30, 35, 40, and 45 °C) to evaluate [EMIM][Ac]/sodium-salt systems as suitable solvents for collagen. The [EMIM][Ac] solvent system broke most of the hydrogen bonds between the amino hydroxyl and ester oxygen of collagen. A higher temperature appeared to promote the intermolecular distance of collagen significantly and allowed ILs to destroy hydrogen bonds more easily. To prepare the collagen solution efficiently, these studies usually dissolved collagen in ILs at a higher temperature, which was easy to damage the triple-helix structure of collagen. Also, since complete dissolution of collagen in ILs was not achieved, the insoluble part of collagen had not been thoroughly studied. Furthermore, it should be noted that both the sorts and concentrations of ILs, which are closely related to their polarity, have great influences on the properties of collagen [10, 19, 23–28]. For example, the differences in composition of imidazolium-based ILs endowed by varying anions with different polarity namely acetate, hydrogen sulfate, dicyanamide, dimethyl phosphate, dihydrogen phosphate and sulfate, had resulted in different

destabilizing effects on the triple helical structure of collagen [25]; a notable decreasing trend in denaturation temperature of collagen fibers was discovered with the increase of ILs concentration from 0.1% to 10% (w/v) [10]. Therefore, it will be of importance and significance to study how the ILs sorts and concentrations would affect the structure and properties of collagen in the dissolution process. Moreover, the dispersion degree is a core property of collagen, affecting its functional performances as biomaterials, for instance, the viscoelastic behavior originated by the aggregation of collagen molecules [22]. Thus, the dispersion degree of collagen after the dissolving process should also be investigated.

Herein, we controlled the collagen dissolution at a lower temperature with more safety (~ 10 °C) and investigated the solubility and the dispersion degree of collagen fibrils in two types of 1-ethyl-methylimidazolium ([EMIM]) based ILs containing different anions ([EMIM] tetrafluoroborate ([EMIM][BF$_4$]) and [EMIM] acetate ([EMIM][Ac]), which are liquid state at room temperature), the ions of which exhibited good biocompatibility and mild polarity [22, 29]. [EMIM][Ac] was used in a concentration range of 10%–70%, while [EMIM][BF$_4$] was used solely in 70%, owing to its low polarity. The solubility of collagen in ILs was tested, and insoluble as well as soluble collagens were examined separately to determine their structural integrity and dispersion degree. The obtained results will help to understand the effect of ILs on the solubility and dispersion degree of collagen while maintaining its structural integrity and provide guidance for the application of ILs in collagen research.

2 Experimental
2.1 Materials
Native collagen used in this study was extracted from calf skin by 0.5 M acetic acid that contained 3% pepsin according to the method of Zhang et al. with a slight modification [30]. The ionic liquids (ILs), 1-ethyl-methylimidazolium tetrafluoroborate ([EMIM][BF$_4$]) and 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]) were purchased from Lanzhou Institute of Chemical Physics (Chinese Academy of Sciences, Lanzhou, China) with high purification (≥99%). [EMIM][Ac] was diluted in deionized water at concentrations of 30%, 50%, and 70% (w/w) respectively, while [EMIM][BF$_4$] was diluted in deionized water solely at 70% (w/w). 8-Anilino-1-naphthalenesulfonate (ANS) was from Sigma Chemicals (Sigma-Aldrich, Munich, Germany). Other chemicals were from Chengdu Kelong Industrial (Chengdu, China).

2.2 Processing collagen with ILs
Lyophilized collagen (150 mg) was added to a beaker with 30 g of [EMIM][BF$_4$] (70%) or different concentrations of [EMIM][Ac] (10%, 30%, 50%, 70%) to achieve an

ideal concentration of 5 mg/mL. All mixtures were stirred ceaselessly by magnetic stirrer for 48 h in water bath at 10 °C, and then centrifuged at 7900 rpm for 10 min by using a high-speed freezer centrifuge. The insoluble collagen fibrils (Col/ILs-fibrils10%, Col/ILs-fibrils30%, Col/ILs-fibrils50%, Col/ILs-fibrils70%, Col/ILs-fibrils70%B) were washed with distilled water several times, dissolved in 0.5 M acetic acid, and dialyzed against 0.1 M acetic acid for 3 days to remove ILs. The supernatant, which is the soluble collagen (Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, Col/ILs-soluble70%) was reserved for the following measurements. The obtained specimen was lyophilized by freeze drying and preserved in a desiccator before used.

2.3 Collagen solubility in ILs

The solubility of collagen (Col/ILs-fibrils70%B, Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, Col/ILs-soluble70%) were measured using a method recommended by Li et al. [31] The soluble collagen was diluted with 6 M HCl at a 1:5 ratio and mixed evenly. Briefly, diluents were added to ampere bottles three times and then sealed for hydrolysis for 24 h at 110 °C. The hydrolysates were transferred to a rotary evaporator and distilled in a vacuum with water bath at 60 °C. After evaporation, the solid was transferred to 25-mL volumetric flasks with citrate–acetate-buffer (pH 6). The sample solutions (300 μL) were mixed with 600 μL of isopropanol and 300 μL of liquid A (7% chloramine T solution:pH 6 citrate–acetate-buffer = 1:4). The absorbance was then measured at 558 nm, using an ultraviolet and visible spectrophotometer (Lambda 25, PerkinElmer, USA). The values of soluble collagen concentration (C) were calculated from:

$$C = \frac{y - 0.0042}{0.1687} \times v \times A \div 1000$$

where y is the absorbance, v is the volume dilution ratio, and A is the conversion coefficient of hydroxyproline concentration and collagen concentration. The standard curve of hydroxyproline is y = 0.1687x + 0.0042 (R^2 = 0.999).

2.4 DSC of insoluble collagen fibrils

The thermal stability of the lyophilized Col/ILs-fibrils70%B, Col/ILs-fibrils10%, Col/ILs-fibrils30%, Col/ILs-fibrils50% and Col/ILs-fibrils70% was detected by using DSC (Netzsch DSC 200PC, Bayern, Germany). The freeze-dried Col and collagen fibrils (~ 3 mg) were weighed into aluminum pans and sealed, then scanned in a nitrogen atmosphere from 35 to 90 °C at 2 °C/min. The measurements were performed in triplicate.

2.5 SEM of insoluble collagen fibrils

The morphologies of the lyophilized Col/ILs-fibrils70%B, Col/ILs-fibrils10%, Col/ILs-fibrils30%, Col/ILs-fibrils50% and Col/ILs-fibrils70% were observed by SEM (S-800, HITACHI, Japan) at 20 kV. Images of each sample were obtained at different spots to confirm the consistency of the morphology observed.

2.6 AFM measurements of insoluble and soluble collagen

The morphologies of the insoluble collagen fibrils (Col/ILs-fibrils70%B, Col/ILs-fibrils10%, Col/ILs-fibrils30%, Col/ILs-fibrils50% and Col/ILs-fibrils70%) and the soluble collagen (Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50% and Col/ILs-soluble70%) were observed by AFM with a pinpoint. The insoluble collagen fibrils were dispersed in 0.5 M acetic acid. The soluble collagen was dispersed in corresponding concentrations of [EMIM][Ac]. The corresponding concentration of collagen was used as a reference. 15 μL of each diluted samples was dropped on mica and then dried at 30 °C for 3 days. Particular attention was given to the fact that 1 day after drying, it was necessary to rinse the mica sheets of Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, and Col/ILs-soluble70% slowly with distilled water to remove ILs.

2.7 SDS–PAGE patterns of soluble collagen

SDS-PAGE was performed according to the method of Laemmli [32] with slight modifications. The lyophilized collagen was dissolved in 0.1 M acetic acid (Col). Col, Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, and Col/ILs-soluble70% were mixed with buffer solution and then boiled in water for 5 min. Then, each sample was loaded for each lane and electrophoresed by Mini-PROTEAN 3 Cell (Bio-Rad, California, USA). After electrophoresis, the gel was dyed by 0.25% coomassie brilliant blue R-250 and then destained with 7.5% acetic acid and 5% methanol.

2.8 FTIR of soluble collagen

All collagen samples were homogeneously triturated with 300 mg potassium bromide (KBr) and the mixtures were made into disks under a pressure of 20 MPa. The FTIR spectra were recorded by a Nicolet iS10 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) at the wavenumbers ranging from 4000 to 500 cm^{-1} with a resolution of 2 cm^{-1} at 25 °C.

2.9 US-DSC of soluble collagen

The thermal stability of the soluble collagen Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, and Col/ILs-soluble70% was determined by US-DSC (VP-DSC, Microcal, Northampton, USA) and matching concentrations of [EMIM][Ac] and Col were used as references. All

specimens were measured at 0.5 mg/mL and performed in triplicate.

2.10 Fluorescence measurements of soluble collagen

Fluorescence measurements of ANS in Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, and Col/ILs-soluble70% were measured by a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), as described by Kamyshny et al. [33] with a slight modification. The same concentration of soluble collagen (0.5 mg/mL) was prepared to evaluate the aggregation status of collagen molecules. Briefly, the ANS probe was prepared in 0.1 mol/L sodium phosphate buffer (pH 7.0) to obtain a 400 mmol/L transparent solution. Then, 10 mL sample solutions were mixed with 50 μL of ANS solution and the mixtures were homogenized. The fluorescence spectrum was recorded at 20°. The excitation wavelength was fixed at 432 nm and the emission spectra were measured from 450 to 750 nm. The slits were fixed at 5 nm.

2.11 DLS measurements of soluble collagen

A DLS technique that uses a Zetasize (Nano-ZS, Malvern Instruments Ltd., Malvern, UK) was employed for the soluble collagen Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, and Col/ILs-soluble70% to obtain information on the size distribution and average aggregation size. The sample solutions were first diluted to 1.0 mg/mL, then filtered through a 2-μm filter (Millipore, Billerica, USA) and measured in a polystyrene cuvette.

3 Results and discussion

3.1 Solubility of collagen in ILs

The dissolution behavior of collagen in ILs was investigated. Table 1 shows the concentration of soluble collagen in [EMIM][BF$_4$] and different concentrations of [EMIM][Ac] stirred continually for 48 h at 10 °C. The solubility of collagen in [EMIM][BF$_4$] was nearly zero, and the concentration of collagen dissolved in 10% [EMIM][Ac] was 1.94 mg/mL. When the [EMIM][Ac] concentration was increased from 10% to 70%, the concentration of collagen increased to 3.57 mg/mL, but was still lower than the input. This proved that even in 70%

Table 1 The concentration of soluble collagen in different ILs and concentrations

ILs and concentrations	concentration of soluble collagen (mg/mL)
70%[EMIM][BF$_4$]	0.02
10%[EMIM][Ac]	1.94
30%[EMIM][Ac]	2.61
50%[EMIM][Ac]	3.25
70%[EMIM][Ac]	3.57

[EMIM][Ac], the collagen did not achieve complete dissolution. A phenomenon of partial dissolution was resulted with the soluble collagen and insoluble collagen fibers, and the solubility of collagen was affected markedly by ILs sorts and concentrations. This could be likely due to the disrupting of intermolecular hydrogen bonds and ionic bonds in collagen, not only by the bulky [EMIM]$^+$ that have the capability to donate proton, but also by the proton-accepting anions including Ac$^-$ and BF$_4^-$. Because of the low polarity and minor proton-accepting ability of BF$_4^-$, there was no dissolution of collagen in [EMIM][BF$_4$]. For [EMIM][Ac], with the increase of concentration and polarity, the degree of rupturing of hydrogen bonds was deeper, resulting in the increase of solubility. The partial dissolution of collagen led to two forms of collagen in ILs, namely, soluble collagen and insoluble collagen fibrils.

3.2 Thermal analysis of insoluble collagen fibrils

The denaturation temperature (T$_d$) of collagen, which is related to the destruction of the native triple helix structure, can be determined by the endothermal peak of DSC [34, 35]. The thermal transition curves of lyophilized Col and insoluble collagen fibrils Col/ILs-fibrils70%B, Col/ILs-fibrils10%, Col/ILs-fibrils30%, Col/ILs-fibrils50% and Col/ILs-fibrils70% are shown in Fig. 1. It appears that the T$_d$ value of Col was 61.9 °C. But the T$_d$ values of insoluble collagen fibrils were 61.2 °C (70%B), 58.4 °C (10%), 57.5 °C (30%), 55.4 °C (50%), and 50.6 °C (70%), which were lower than that of Col, and which suggested that the insoluble collagen fibrils displayed a weaker thermal stability than the native ones. For Col/ILs-fibrils70%B derived from [EMIM][BF$_4$], the decrease was minor. On the contrary, collagen derived from [EMIM][Ac] faced a more

Fig. 1 DCS curves of the insoluble collagen fibers derived from ILs (**a**:Col, **b**:Col/ILs-fibrils70%B, **c**:Col/ILs-fibrils10%, **d**:Col/ILs-fibrils30%, **e**:Col/ILs-fibrils50%, **f**:Col/ILs-fibrils70%)
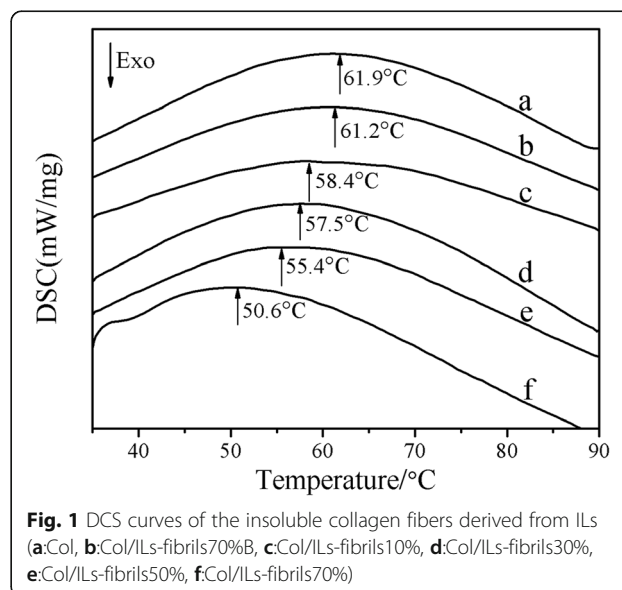
significant decrease with the increase of ILs concentration. The downward tendency of denaturation temperature may be related to the effect of ILs on the fracture of collagen hydrogen bonds. Hu et al. [22] reported that during the dissolving process of collagen in [EMIM][Ac], firstly [EMIM][Ac] will destroy hydrogen bonds between intramolecules or intermolecules; then, after removing [EMIM][Ac], some of the hydrogen bonds will be reestablished. Thus, the fiber arrangement became looser, followed by the ability of the insoluble collagen fibers to undergo heat denaturation was reduced. The degree of thermal-stability reduction or the degree of collagen dispersion was correlated with the different capability of ILs destroying hydrogen bonds orientated by polarity, more specifically, their sorts and concentrations.

3.3 SEM images of insoluble collagen fibrils
The lyophilized insoluble collagen fibrils Col/ILs-fibrils70%B, Col/ILs-fibrils10%, Col/ILs-fibrils30%, Col/ILs-fibrils50% and Col/ILs-fibrils70% were used to obtain morphologies via SEM (Fig. 2). The surface micromorphology of lyophilized collagen was homogeneous with a similar sheet structure and connected pores. The insoluble collagen fibrils retained a relatively homogeneous pore structure, but the surface morphology appeared to be looser, and its pore size was larger than that of native collagen. Moreover, the fibers arrangement became looser, initiated by the destruction of hydrogen bonds as previously discussed. However, the degree of dispersion degree varied in [EMIM][BF$_4$] and different

concentrations of [EMIM][Ac]. Collagen fibers derived from [EMIM][BF$_4$] were slightly looser than the native ones, while fibers treated by [EMIM][Ac] were much more sparsely distributed with the increase of ILs concentrations. Such differences might be attributed to the lyotropic action and reorientation of the hydration network of collagen induced by ILs with different ions and polarity [10, 23]. The high polarity can reduce the water associated with collagen, lower its viscosity and consequently increase the mobility of collagen fibers and become looser.

3.4 AFM images of insoluble collagen fibrils
Morphological changes of the insoluble collagen fibril molecules (Col/ILs-fibrils70%B, Col/ILs-fibrils10%, Col/ILs-fibrils30%, Col/ILs-fibrils50% and Col/ILs-fibrils70%) were observed by AFM in Fig. 3. This AFM examination suggests that the native collagen exhibited a representative fibrillar structure with fibers entangled and overlapped. The fibril arrangement of the collagen fibers derived from [EMIM][BF$_4$] was a little looser than the native one, while that of [EMIM][Ac] was much looser and presented a more sparse distribution of fiber network in the high concentrated samples. Although the partial interruption of the hydrogen bonds did not make the insoluble collagen fibrils achieve dissolution, the fiber arrangement became looser, and the porosity improved with the increase of ILs polarity and concentration. Overall, these changes in molecular morphologies
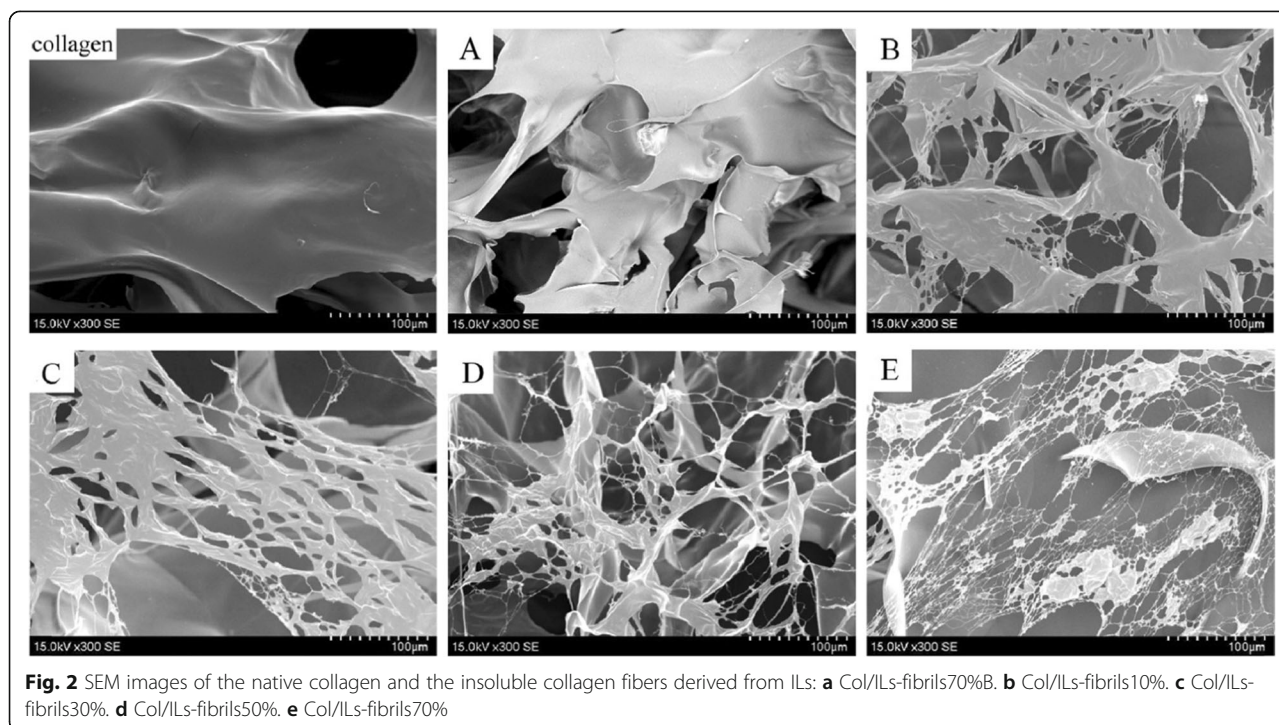

Fig. 2 SEM images of the native collagen and the insoluble collagen fibers derived from ILs: **a** Col/ILs-fibrils70%B. **b** Col/ILs-fibrils10%. **c** Col/ILs-fibrils30%. **d** Col/ILs-fibrils50%. **e** Col/ILs-fibrils70%
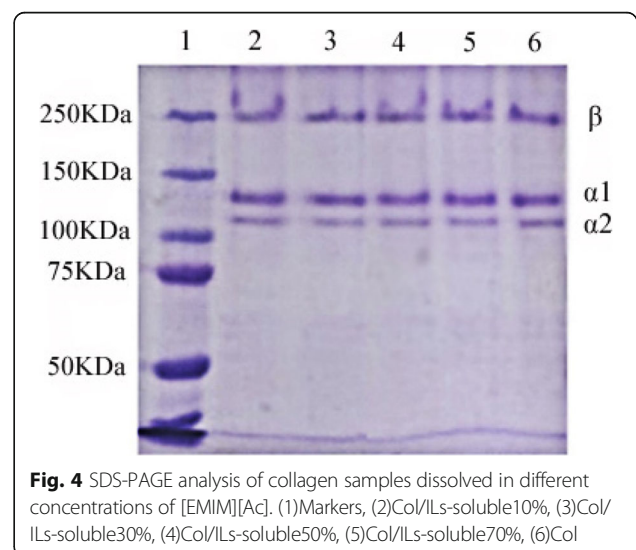
Fig. 3 AFM images of the native collagen and the insoluble collagen fibers derived from different ILs: **a** Col/ILs-fibrils70%B. **b** Col/ILs-fibrils10%. **c** Col/ILs-fibrils30%. **d** Col/ILs-fibrils50%. **e** Col/ILs-fibrils70%
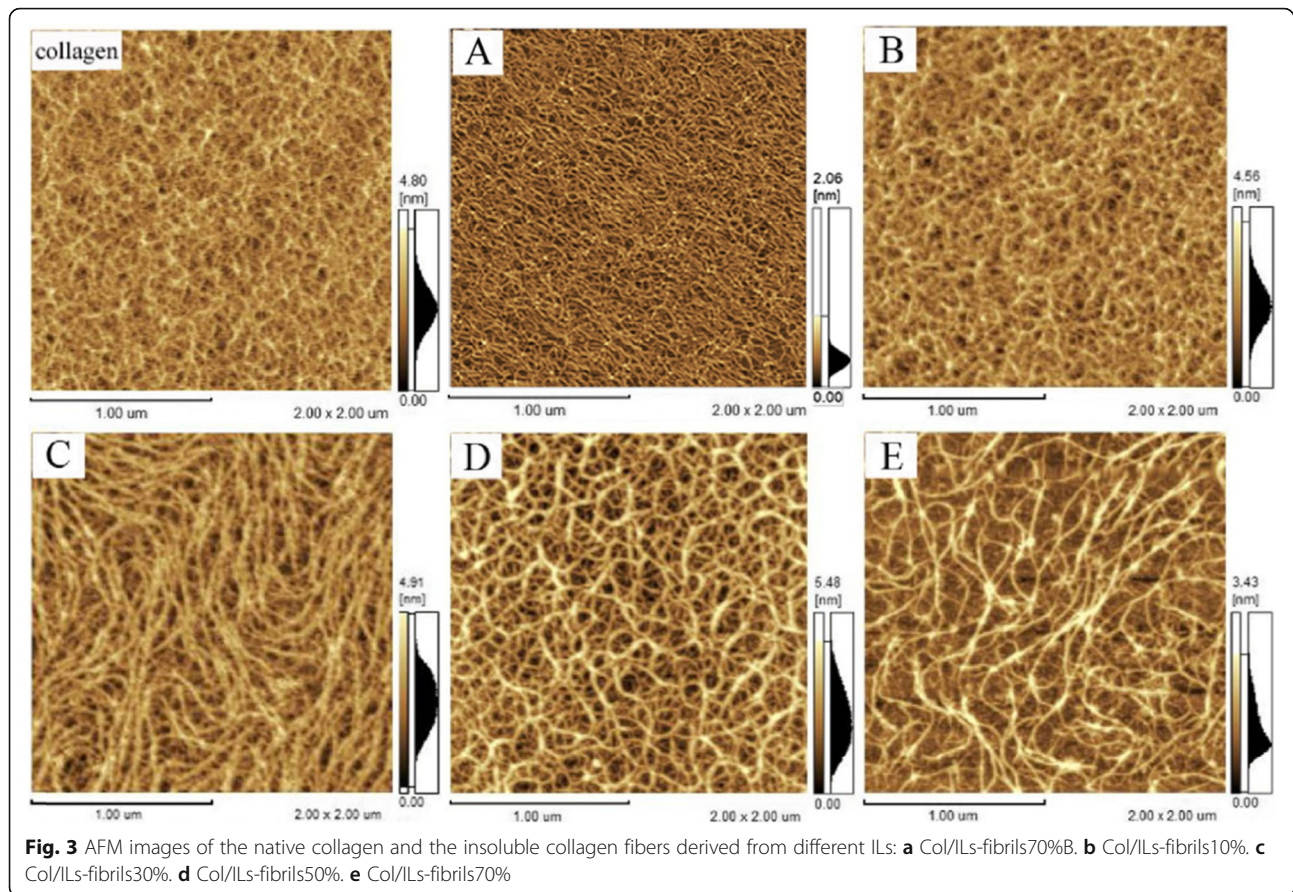
observed by AFM agree well with the surface morphologies aforementioned changes observed by SEM.

3.5 SDS-PAGE patterns of soluble collagen

Measurements of electrophoresis analysis were applied to estimate the molecular weight distribution of soluble collagen aggregation within the [EMIM][Ac] concentration from 10% to 70%. Figure 4 displays the SDS−PAGE patterns of Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, and Col/ILs-soluble70%, with Col as a comparison. All samples are consisted of two typical chains (α1 and α2) with molecular weight of approximately 100 kDa and one crosslinked component β chain (∼ 200 kDa), which is known as the dimer of α chains. Moreover, the band intensity of α1 was higher than α2 chain, which indicates the collagen might have a $(α1)_2α2$ structure. These results are in good agreement with the findings that report the characteristic electrophoretic bands of type I collagen. None of the electrophoretic bands existed at lower molecular weights (< 100 kDa), indicating no degradation of polypeptide chain happened during dissolution, although some collagen hydrogen bonds had been broken by [EMIM][Ac]. In order to identify the alterations between the native collagen samples and the Col/ILs samples, additional structural analysis should be conducted.

3.6 FTIR spectra analysis of soluble collagen

In addition, to further examine the triple helix conformation of collagen, the secondary structure of Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, and Col/ILs-soluble70%, with Col as a comparison was examined by FTIR. The spectra of all collagen samples

Fig. 4 SDS-PAGE analysis of collagen samples dissolved in different concentrations of [EMIM][Ac]. (1)Markers, (2)Col/ILs-soluble10%, (3)Col/ILs-soluble30%, (4)Col/ILs-soluble50%, (5)Col/ILs-soluble70%, (6)Col

shown in Fig. 5 exhibited the presence of five characteristic FTIR peaks including amide A, B, I, II, and III, which were typical for type I collagen [20]. The amide A peaks of $3400 \, cm^{-1}$ were related to the hydrogen-bonded -NH groups, while the amide B bands were found at wavenumbers of $2940 \, cm^{-1}$, reflecting C-H stretching [36]. The amide I bands at $1650 \, cm^{-1}$ were attributed to C=O stretching vibrations in the polypeptide backbone, and the amide II bands at $1550 \, cm^{-1}$ were initiated from N-H bending and C-H stretching. The bands at $1245 \, cm^{-1}$ representing amide III were dominated by the C-H vibrations [31]. Moreover, the absorption ratios between the amide III and $1450 \, cm^{-1}$ bands for all collagens were nearly 1.0, unambiguously suggesting that the triple-helical structure of collagen was preserved [22]. Overall, the FTIR analysis supported the structural integrity of collagen, conforming to the above SDS-PAGE results.

3.7 Thermal analysis of soluble collagen

The temperature dependence of the specific heat capacity (Cp) of soluble collagen dissolved in different concentrations of [EMIM][Ac] is shown in Fig. 6. The DSC curves show that every collagen samples exhibited a characteristic thermal denaturation peak. The minor endothermic peak (Tm_1) at low temperature was owing to a breakage of hydrogen bonds in the aggregates of collagen, which was apt to be neglected, and the major endothermic peak (Tm_2) at high temperature was initiated by the transition of triple helix to the random coil

state [37, 38]. Values of Tm_1 as well as Tm_2 for varying [EMIM][Ac] concentrations are given in Table 2. The Tm_2 values of Col/ILs-soluble10% was 42.47 °C, which was close to 42.73 °C of Col. With [EMIM][Ac] addition, the values of Tm_2 showed a decreasing tendency from 42.47 to 35.10 °C, which demonstrates the decrease of thermal stability from alterations to interactions in collagen molecules. [EMIM][Ac] exhibited no endothermic peak. For concentrations of 30% and 50%, multiple consecutive endothermic peaks exist, which may be related to the different impact of [EMIM][Ac] on the structure of collagen.

Compared with Col, the thermal denaturation temperature of Col/ILs-soluble70% was reduced by 7.63 °C, which is larger than the decrease of Col-regenerated-35 °C with 4.1 °C reported by Hu et al. [22] This showed the thermal stability decreased more significantly with [EMIM][Ac] concentration. Hydrogen bonds played an important role in the stabilization of triple-helix structure. The interchain hydrogen bonds were mediated by one water molecule, which likely reinforced the triple-helix at regions that lacked proline [39]. [EMIM][Ac] was deduced to break part of the intermolecular hydrogen bonds. Therefore, less water oriented hydrogen bonding in collagen molecules in [EMIM][Ac] were formed because of the increased hydrophobic interaction [40]. Therefore, the stability of the collagen helix weakened as the [EMIM][Ac] concentration increased and a continuous decrease in the values of the Tm_2 was
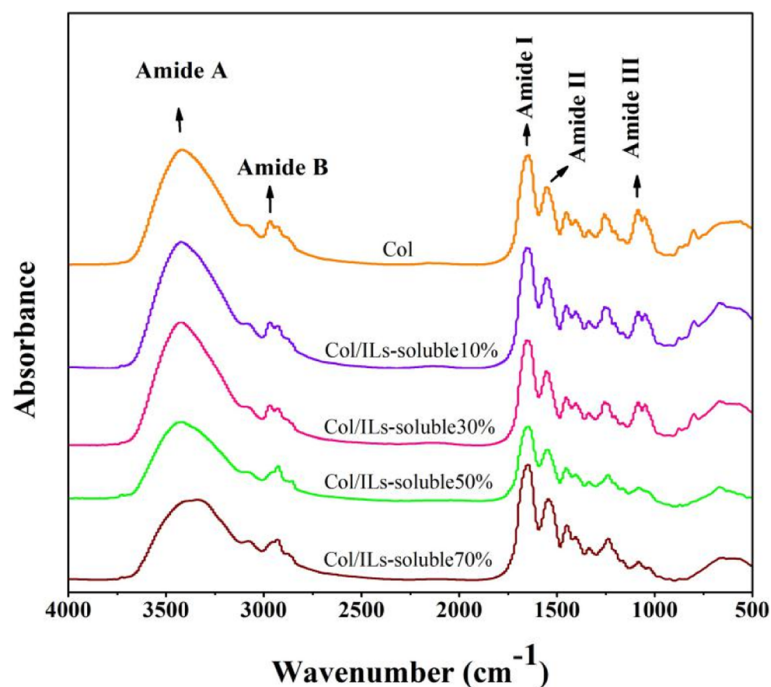

Fig. 5 FT-IR spectra of Col and collagen samples dissolved in different concentrations of [EMIM][Ac]
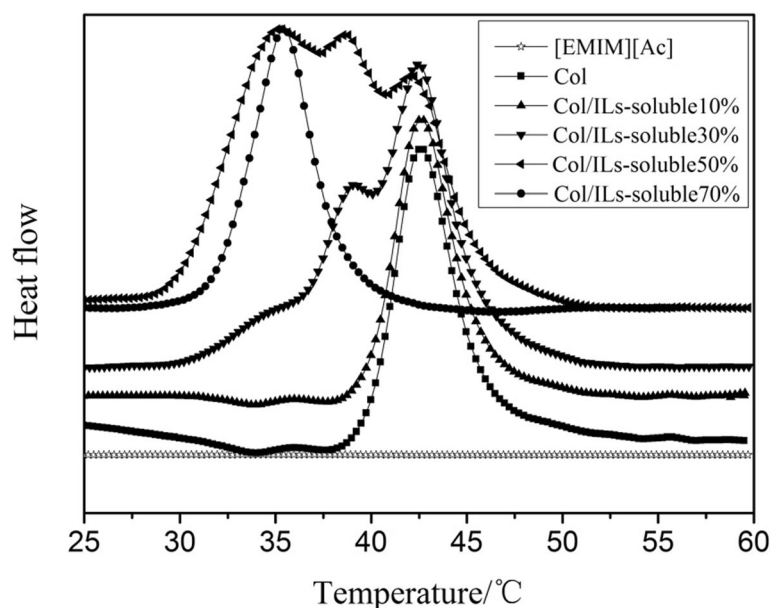
Fig. 6 US-DSC curves of Col, [EMIM][Ac] and collagen samples dissolved in different concentrations of [EMIM][Ac]

detected. Together with the results of SDS-PAGE, we can see that although there was no degradation in polypeptide chain during the process of dissolving, the thermal stability of collagen was reduced because of the decreased hydrogen bonds.

3.8 Fluorescence analysis of soluble collagen

ANS, which is a fluorescent hydrophobic probe, has high sensitivity to the environment polarity in solution [41]. The introduce of ANS to proteins causing the rise of hydrophobicity is equal to decrease in polarity, leading to the blue shift in maximum peak and an increase in fluorescence intensity [33]. The fluorescence emission spectra of ANS are shown in Fig. 7, displaying four main peaks at ~ 526.0, ~ 526.8, ~ 529.8, and ~ 530.6 nm in different soluble collagen solutions. With an increase in [EMIM][Ac] concentration, the fluorescence intensity reduced and a red shift resulted from 526.0 to 530.6 nm in the maximum peak, which indicates the hydrophobicity of the collagen solutions was reduced. Wu et al. [42]

reported that the increase of fluorescence intensity was because of the form of hydrophobic areas from the aggregation of collagen molecules. The results showed that with the increase of [EMIM][Ac] concentration, the polarity of the solutions increased, and the aggregation status of collagen molecules was reduced.

3.9 DLS analysis of soluble collagen

DLS has been reported to be a good method to measure the aggregate size [43]. Alterations in the sizes of the soluble collagen aggregates dissolved in different concentrations of [EMIM][Ac] are shown in Fig. 8. The distribution of size regions was bimodal, and comprised two populations of aggregates of 21–122 nm and 459–4150 nm, respectively, might attributing to different sizes of collagen aggregates. Differences in intensity and location of these two regions of distribution are also shown in Fig. 8. The two collagen-aggregate distribution regions tended to a smaller size, which demonstrates collagen aggregates with relatively smaller sizes could be more sensitive to the [EMIM][Ac] concentration and loosened more easily with an increase in [EMIM][Ac] concentration. The intensity of the first region increased markedly, whereas the second region decreased evidently, which indicates that more small aggregates were produced at a high [EMIM][Ac] concentration. The average sizes of the collagen aggregates of the Col/ILs-soluble10%, Col/ILs-soluble30%, Col/ILs-soluble50%, and Col/ILs-soluble70% were 1900.81, 1406.46, 983.16, and 334.32 nm, respectively. The size reduction might be caused by disaggregation of collagen aggregates because of the breaking of hydrogen bonds by [EMIM][Ac] and an

Table 2 Thermodynamic parameters of Col and collagen samples dissolved in different concentrations of [EMIM][Ac][a]

Samples	Tm_1/°C	Tm_2/°C
Col	35.97 ± 0.15	42.73 ± 0.12
Col/ILs-soluble10%	35.68 ± 0.09	42.47 ± 0.18
Col/ILs-soluble30%	34.09 ± 0.17	42.39 ± 0.10, 39.03 ± 0.14
Col/ILs-soluble50%	29.04 ± 0.13	42.31 ± 0.06, 38.55 ± 0.16, 35.36 ± 0.13
Col/ILs-soluble70%	27.65 ± 0.11	35.10 ± 0.13

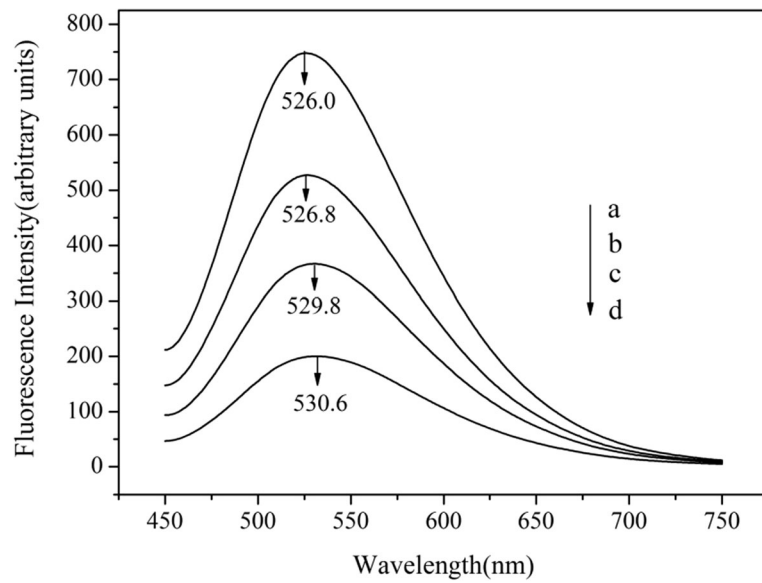[a]Values are average ± standard derivation ($N = 3$)

Fig. 7 Fluorescence spectra of ANS in collagen solutions at different [EMIM][Ac] concentrations. a:Col/ILs-soluble10%, b:Col/ILs-soluble30%, c:Col/ILs-soluble50%, d:Col/ILs-soluble70%

increase of electrostatic repulsion between each collagen chain.

3.10 AFM images of soluble collagen
AFM was performed to observe the molecule structures of the soluble collagen. Figure 9 exhibits the morphology changes of Col/ILs-soluble10%, Col/ILs-soluble30%, and Col/ILs-soluble50%, where native collagen was applied as comparison. Characteristic topographies in collagen fibers were observed. By inspection, with increasing [EMIM][Ac] concentration, the fiber arrangement tended to be loosened with larger pore sizes, and the fiber length was
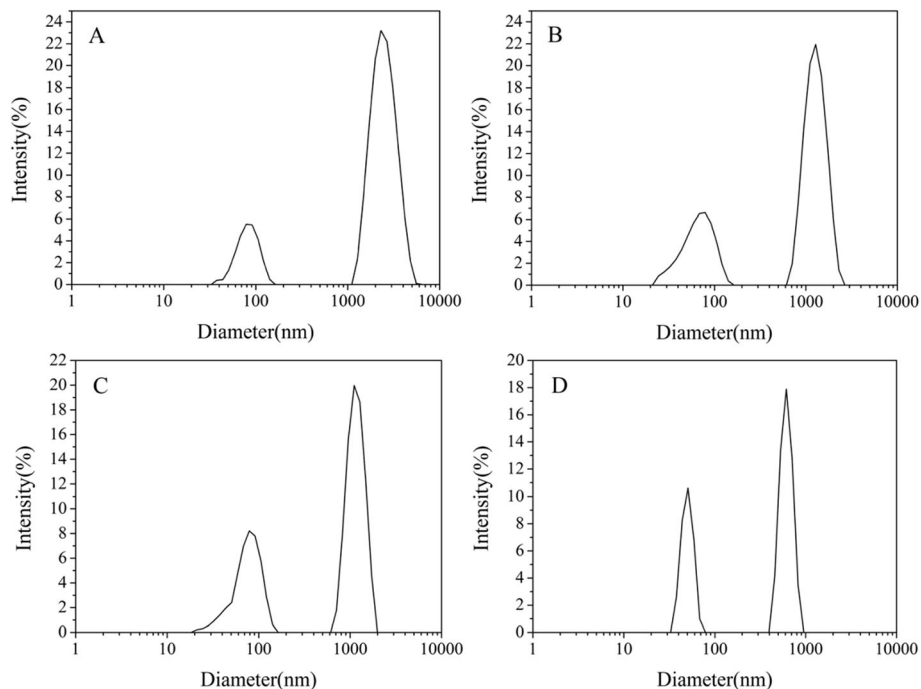

Fig. 8 The sizes and distribution of collagen aggregates determined by DLS dissolved in different concentrations of [EMIM][Ac] (**a**:Col/ILs-soluble10%, **b**:Col/ILs-soluble30%, **c**:Col/ILs-soluble50%, **d**:Col/ILs-soluble70%)
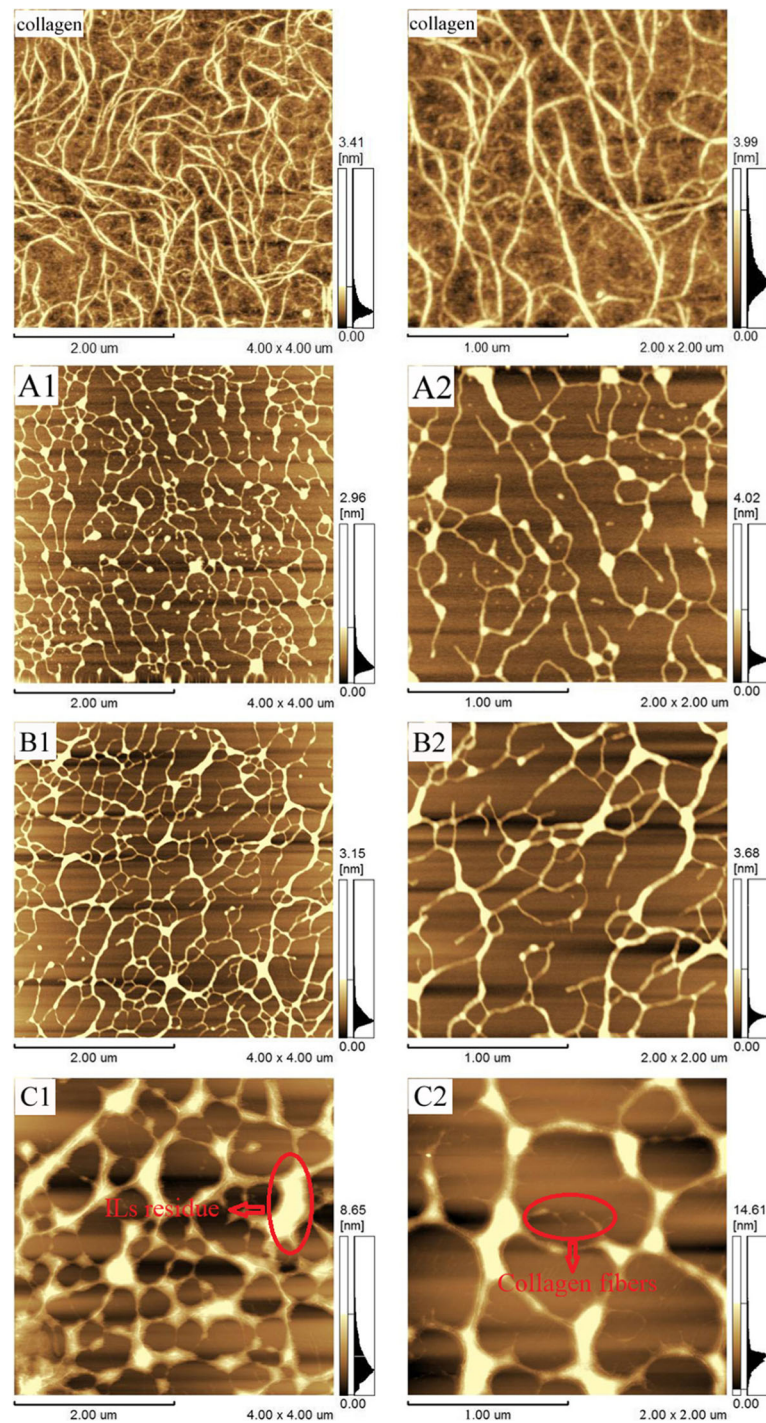
Fig. 9 AFM images of the native collagen and collagen samples dissolved in different concentrations of [EMIM][Ac]. Col/ILs-soluble10% (**A1**, **A2**), Col/ILs-soluble30% (**B1**, **B2**), Col/ILs-soluble50% (**C1**, **C2**), 4.00 × 4.00 μm (**A1**, **B1**, **C1**), 2.00 × 2.00 μm (**A2**, **B2**, **C2**)

shortened, exhibiting a more sparsely distributed fiber network. This result may indicate that the decreased intermolecular hydrogen bonds caused weakened intermolecular forces between collagen molecules in [EMIM][Ac]. Consequently, the results agreed well with the results for the

sizes and distribution of the collagen aggregates in different concentrations of [EMIM][Ac]. Note that the white spots in AFM images were ILs residue, and the thin strings were collagen fibers. Because of the high concentration of [EMIM][Ac], the Col/ILs-soluble70% sample

did not yield an AFM image because of ILs residue, although distilled water had been used to wash the sample many times.

4 Conclusions

The solubility and dispersion degree of collagen in 70% [EMIM][BF$_4$] and [EMIM][Ac] in concentrations from 10% to 70% were studied. Only insoluble collagen fibers were presented from [EMIM][BF$_4$], due to no dissolution of collagen occurred in [EMIM][BF$_4$]. Though dissolved in [EMIM][Ac], the total dissolution of collagen was not achieved and a phenomenon of partial dissolution resulted with the soluble collagen and insoluble collagen fibers across the entire [EMIM][Ac] concentration range. For the insoluble collagen fibers, although the partial interruption of hydrogen bonds did not make it dissolved, the entire fiber arrangement was looser and the thermal stability decreased with the increase of ILs polarity and concentration. For the soluble collagen, no degradation of polypeptide chain occurred during dissolution. Owing to the fact that more hydrogen bonds were broken with the increase of ILs concentration, the reduction in aggregation states of soluble collagen was more prominent, and resulted in a decreased thermal stability, a looser fiber arrangement, and shorter fiber lengths. Therefore, [EMIM][BF$_4$] is a good dispersion agent for collagen with no destruction to collagen structures, while [EMIM][Ac] is a good solvent and dispersion agent for collagen, depending on the concentration. The information obtained may be useful in the application of ILs in more collagen products with a suitable sort and concentration to retain the structural integrity of the collagen.

Abbreviations

[EMIM][Ac]: 1-ethyl-3-methylimidazolium acetate; [EMIM][BF$_4$]: 1-ethyl-methylimidazolium tetrafluoroborate; Col: Collagen; Col/ILs-fibrils10%: Insoluble collagen fibrils from 10% [EMIM][Ac]; Col/ILs-fibrils30%: Insoluble collagen fibrils from 30% [EMIM][Ac]; Col/ILs-fibrils50%: Insoluble collagen fibrils from 50% [EMIM][Ac]; Col/ILs-fibrils70%: Insoluble collagen fibrils from 70% [EMIM][Ac]; Col/ILs-fibrils70%B: Insoluble collagen fibrils from 70% [EMIM][BF$_4$]; Col/ILs-soluble10%: Soluble collagen in 10% [EMIM][Ac]; Col/ILs-soluble30%: Soluble collagen in 30% [EMIM][Ac]; Col/ILs-soluble50%: Soluble collagen in 50% [EMIM][Ac]; Col/ILs-soluble70%: Soluble collagen in 70% [EMIM][Ac]; ILs: Ionic liquids

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Not applicable

Authors' contributions

QL has equal contribution to the first author. SL designed and performed the experiments, analyzed the data and wrote the manuscript. QL performed the experiments of soluble collagen and was a major contributor in writing the manuscript. GL analyzed the data and improved the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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