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Evaluation of 1-Ethyl-3-Methylimidazolium Acetate Based Ionic Liquid Systems as a Suitable Solvent for Collagen

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ABSTRACT: Collagen, a prominent biopolymer, which is famous for its excellent biological activity, has been used extensively for tissue engineering applications. In this study, a novel solvent system for collagen was developed with an ionic liquid, 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]), solvent system. A series of sodium salts were introduced into this solvent system to enhance collagen's dissolution procedure. The results show that the solubility of collagen was significantly influenced by the temperature and sodium salts. The solubility reached up to approximately 11% in the [EMIM][Ac]/Na_2HPO_4 system at 45°C. However, the structure of the regenerated collagen (Col-regenerated) may have been damaged. Hence, we focused on the structural integrity of the collagen regenerated from the [EMIM][Ac] solvent system by the methods of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Fourier transform infrared spectroscopy, ultrasensitive differential scanning calorimetry, atomic force microscopy, X-ray diffraction, and circular dichroism because its signature biological and physicochemical properties were based on its structural integrity. Meanwhile, a possible dissolution mechanism was proposed. The results show that the triple-helical structure of collagen regenerated from the [EMIM][Ac] solvent system below 35°C was retained to a large extent. The biocompatibility of Col-regenerated was first characterized with a fibroblast adhesion and proliferation model. It showed that the Col-regenerated had almost the same good biological activity as nature collagen, and this indicated the potential application of [EMIM][Ac] in tissue engineering. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2013

KEYWORDS: biopolymers and renewable polymers; ionic liquids; properties and characterization

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INTRODUCTION

Recently, the most bountiful structural protein, collagen, which accounts for approximately one-third of all proteins in all vertebrate bodies, has become a cynosure because of its unusual combination of excellent biological activity, nice biocompatibility, and lower antigenicity.[1–6] Collagen is present in the form of elongated fibers in various tissues, the building blocks of which are rodlike triple helices; these fibers are mostly stabilized by intramolecular hydrogen bonds between glycine and hydroxyproline in adjacent chains.[4,7] Generally, native collagen can be applied to pharmaceutical, cosmetics, biomedical, and biotechnological industries as swollen hydrogels, sparse fibers in a latticelike organization, sponges in a puffy state, porous membranes, and so on.[1,3,4,8] However, the applications of collagen are limited because of its inherent insoluble and intractable nature.[4,9] The triple-helix conformation within collagen molecules, which is the prerequisite and foundation that supports collagen's biological activity, prevents it from being dissolved to some extent.[4,9] Furthermore, the solubility of collagen is limited in general solvents, for instance, acetic acid (3 wt %),[10] under the precondition of maintaining the basic triple-helix configuration within the collagen molecules. The solubility of collagen in hexafluoroisopropanol can easily reach up to 10 wt %; however, Zeugolis and coworkers[4,7] demonstrated that the electrospinning of collagen out of fluoroalcohols results in the creation of gelatin, a protein derived from denatured collagen, and this is characterized by destroyed α chains, disrupted triple-helical and fibrillar structures, and a lack of internal structure or configurational order. Because most of the collagen products need to undergo a procedure of lyophilization or electrospinning to be shaped into its required features after it they are dissolved in an acidic solution or fluoroalcohols, workers have to face diseases (i.e., blepharoedema, chronic pharyngitis, allergic dermatitis) when they are constantly exposed to harmful acidic or fluoroalcohol vapors. Therefore, it is highly desirable that we develop more friendly and more efficient solvent systems for collagen.

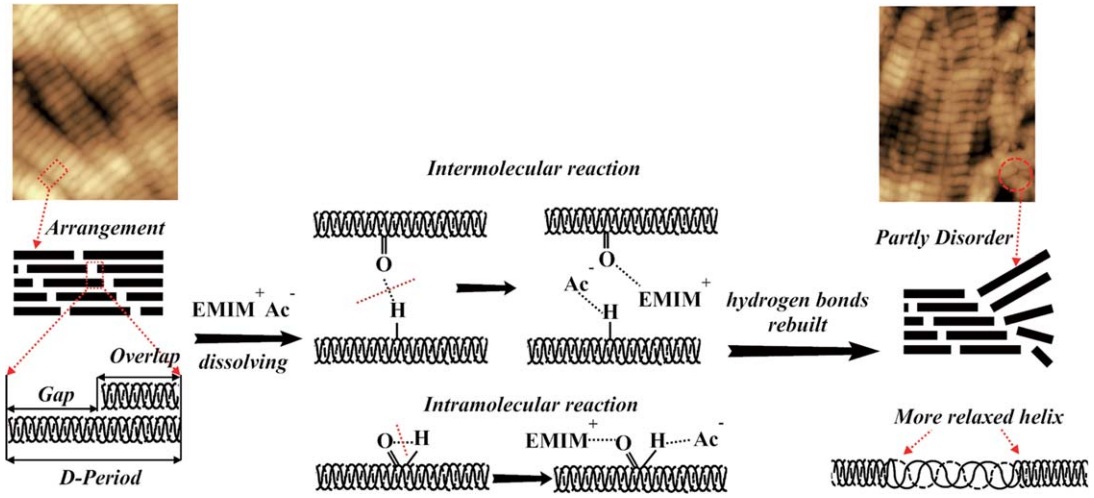

Figure 1. Schematic illustration showing the possible interactions between the [EMIM][Ac] and collagen molecules. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Recently, ionic liquids (ILs) have appeared as a novel class of green solvents that permit the compounding a series of conventional, not easily soluble natural biomasses. ILs are considered to be organic salts composed of large molecular ions and are generally liquids at room temperature.[11–13] As promising alternatives to conventional organic solvents, they possess many unmatched favorable properties, for instance, good solubility, negligible vapor pressure, excellent thermal stability, a wide liquidus range, low flammability, and the ability to be easily recycled.[14,15] In recent years, progress in the application of ILs as a novel kind of solvent has been quite rapid, and many researchers have reported on the possibility of manufacturing natural biopolymer fibers, membranes, or hydrogels with ILs.[15,16] Most of these studies have been related to cellulose, chitosan, cork, and silk, whereas another kind of important natural biopolymer, collagen, has been neglected to a certain degree, even though one of the most recent articles[9] referred to the interaction between native skin collagen fibers and 1-butyl-3-methylimizazolium chloride. The results obtained in this study were quite inspiring and encouraging; however, there are some potential worries with regard to this series of halogen-containing ILs. On one hand, the hydrolysis products of halogen-containing ILs could cause considerable corrosive damage to steel-made and glass reactors in some cases if they take part in the reaction. On the other hand, halogen-containing ILs are hard to biodegrade and could cause environmental problems.[17] Hence, halogen-free ILs are more attractive to chemists these days. More important, in view of the great potential of ILs as solvents for biopolymers and their further application, the determination of the biocompatibility and special biological activity of recycled biopolymers has not yet been fully carried out and is strongly recommended.

Fortunately, a series of halogen-free ILs have been explored, particularly for methylimidazolium-based IL solvent systems. Recent studies have shown that a natural polymer cellulose/chitosan homogeneous solution can be obtained with 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]).[18,19] Despite the widespread use of ILs in the dissolution of natural biopolymers such as chitosan, cellulose, silk, and wool, the use of [EMIM][Ac] in the dissolution of collagen and the biocompatibility evaluation of recycled collagen has not yet been well studied. Hence, in this study, we selected [EMIM][Ac] as a common solvent to manufacture collagen membranes or sponges. In light of the previous discussion, our hypothesis was that through the dissolution and regeneration of collagen in the [EMIM][Ac] solvent system, [EMIM][Ac] would first disrupt the hydrogen bonds between intermolecules or intramolecules; then, the hydrogen bonds would be rebuilt after the removal of [EMIM][Ac], as described in Figure 1. To verify our hypothesis, the interaction between collagen and [EMIM][Ac], especially the dissolving behavior of collagen in [EMIM][Ac] in the presence of sodium salts and the structural integrity of regenerated collagen (Col-regenerated) was studies with Fourier transform infrared (FTIR) spectroscopy, DSC, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), X-ray diffraction (XRD), and atomic force microscopy (AFM) analysis. Furthermore, the biocompatibility and biological activity of collagen prepared from the collagen/[EMIM][Ac] blended system were first evaluated.

EXPERIMENTAL

Materials

The acid-soluble collagen used in this study was self-prepared from porcine skin according to our previous report and was lyophilized in a freeze dryer (Labconco Freeze Dry System Freezone, 6 L) and preserved as a sponge at 4°C before use.[2] To verify the extracted collagen's purity and structural integrity, the electrophoretic patterns of collagen were examined by SDS–PAGE (Bio-Rad Powerpac 300).[20] The room-temperature IL, [EMIM][Ac], was prepared and purified with a procedure described in the literature (Figure 2).[15] *N*-Methylimidazole [analytical reagent (AR)], chloroethane (AR), and gelatin (AR) were purchased from Shanghai Jingchun Industrial Co., Ltd. (Shanghai, China) and were used as received. Na_2SO_4, NaCl,

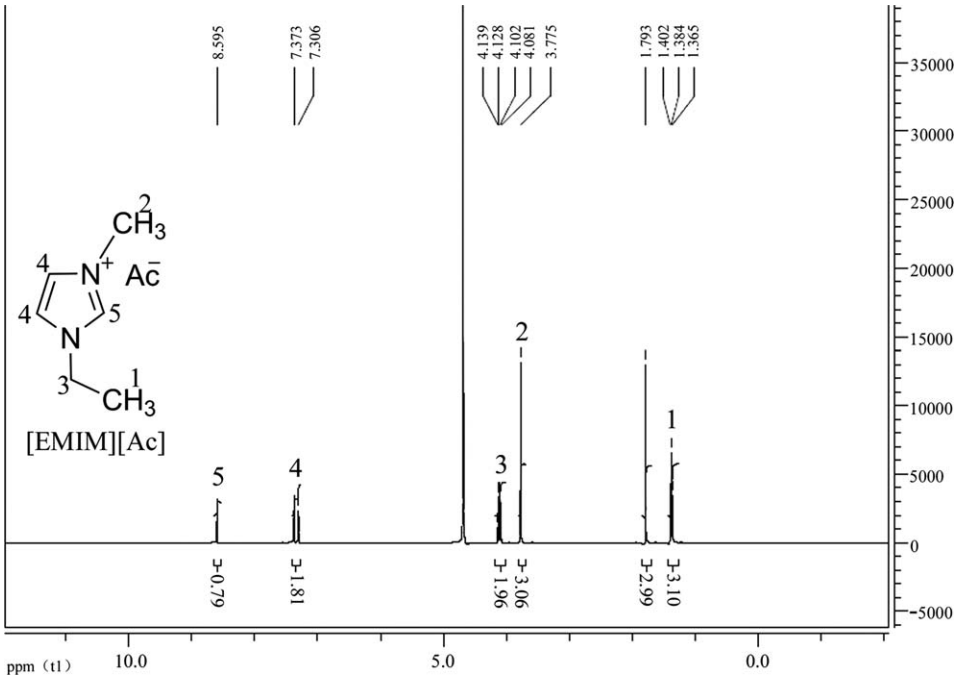

Figure 2. ^{1}H-NMR spectra of [EMIM][Ac].

Na_3PO_4, Na_2HPO_4, and $NaHSO_4$ were obtained from Chengdu Kelong Industrial Co., Ltd. (Chengdu, China).

Preparation of the [EMIM][Ac]/Sodium Salt Solvent Systems

Concentrations of 1.0% (w/w) of Na_2HPO_4, Na_2SO_4, NaCl, Na_3PO_4, and $NaHSO_4$ were dissolved in [EMIM][Ac] to prepare the [EMIM][Ac]/sodium salt solvent systems. To prevent moisture absorption, all of the solvent mixtures were prepared right before use.

Dissolution of the Collagen in the [EMIM][Ac]/Sodium Salt Solvent Systems

First, 5.0 g of dried [EMIM][Ac]/sodium salt was added to a 20-mL colorimetric tube, and the tube was sealed with parafilm. Then, the tube was immersed in an oil bath (DF-101S, Gongyi Instrument Factory, China) with an initial temperature of 25°C. After that, the collagen specimen was added to the tube with an initial mass fraction of 0.5%, and the mixture was stirred all of the time. An additional amount of 0.1% collagen was added to the mixture each time until the solution became optically unclear under a polarization microscope (NikonIv100, Nikon, Japan). The solubility of collagen (expressed in grams per 100 g of IL) in the [EMIM][Ac]/sodium salt solvent systems at the given temperature (25, 30, 35, 40, and 45°C) was determined when the collagen specimen began to precipitate. All of the previous dissolution experiments were carried out during the period of 24 h.

Preparation of the Col-regenerated Specimens

After the complete dissolution of collagen in the [EMIM][Ac]/sodium salt solvent system, it was convenient to prepare the Col-regenerated sponges through the addition of water or ethanol to the mixtures. In a typical recovery procedure, collagen was dissolved in 5.0 g of [EMIM][Ac]/Na_2HPO_4 at 25 and 35°C with a concentration of 3 and 6 wt %, respectively. Then, the blended solutions were transferred to a 100-mL beaker containing 15 mL of distilled water. Subsequently, the beaker sealed with parafilm was stirred for 1 h at room temperature (21 ± 1°C). After that, the precipitated collagen was separated by filtration through a ceramic funnel with Nylon filter paper *in vacuo*. The collagen specimen was thoroughly washed three times to remove residual [EMIM][Ac]. Finally, the collagen specimen containing water was frozen at −30°C for 5 h and then lyophilized at low-chamber vacuum (0.05 bar). The Col-regenerated specimen was preserved as sponges at 4°C before use (Figure 3).

Characterization of the Col-regenerated Specimens

Electrophoretic Analysis. Electrophoretic patterns of the collagen specimens were obtained via SDS–PAGE (Bio-Rad Powerpac 300) with a discontinuous Tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl)/glycine buffer system with 7.5% resolving gel and 4% stacking gel with a slight modification.[21] First, all the collagen specimens were subjected to acid solubilization in 0.5*M* acetic acid or pepsin digestion in 0.1 mg of pepsin per milliliter of 0.5*M* acetic acid. The concentration of all of the collagen solutions was 2 mg/mL in either the acid solubilization or pepsin digestion case. The obtained suspensions were centrifuged for 15 min at 4°C; then, the supernatants were mixed with a sample buffer (1 mol/L Tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl), pH 6.8, containing 1% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.01% bromophenol blue) at a volume ratio of 1:1. After that, the mixed solution was boiled for 3 min, and 20-μL treated samples were injected into gel wells and run for approximate 70 min. The gel was stained for about 30 min with 0.25% Cooomassie brilliant blue R250 solution and destained with a 7.5% acetic acid/5% methanol solution until the bands were clear.

FTIR Spectroscopy. FTIR spectra were obtained from tablets containing 2 mg of collagen specimens in approximately 100 mg of potassium bromide (KBr) with an FTIR spectrophotometer

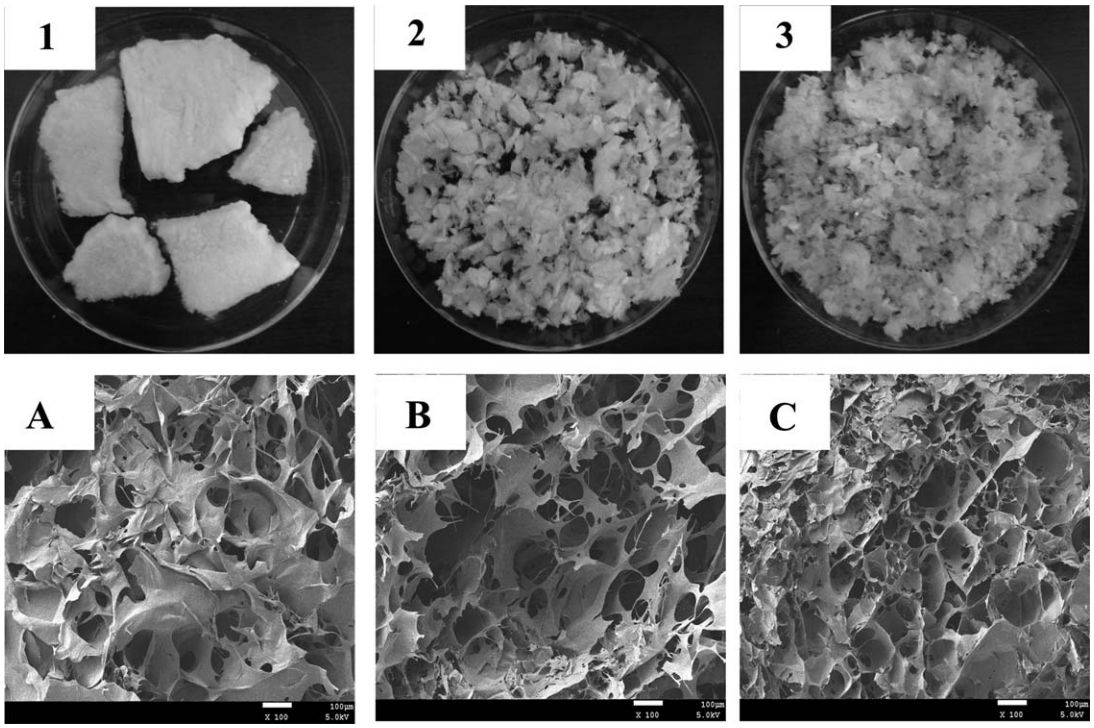

Figure 3. Surface topography of the collagen specimens: (1,A) COL-before, (2,B) COL-regenerated-25°C, and (3,C) COL-regenerated-35°C.

(Spectrum One, PerkinElmer, Inc., Waltham, MA) for structural analysis. All of the spectra were collected in the range 4000–400 cm^{-1} and were recorded in transmission mode with a 4 cm^{-1} resolution and 32 scans. All measurements were performed in a dry atmosphere at room temperature (21 ± 1°C).

Ultrasensitive Differential Scanning Calorimetry (US-DSC) Measurements. The denaturation temperature (T_d) of the collagen specimens was determined on a US-DSC microcalorimeter (Microcal, Northampton, MA) with the matching acetic acid buffer as the reference. The collagen solutions, with a concentration of 0.5 mg/mL, were degassed for 30 min at ambient temperature (21 ± 1°C) before the tests. All of the scans were carried out at a constant heating rate of 1°C/min at temperatures ranging from 25 to 60°C.

Measurement of the Intrinsic Viscosity. The intrinsic viscosity of the collagen solutions was measured with an Ubbelohde capillary viscometer in a constant-temperature bath at 25.0 ± 0.1°C according to our previous literature.[22] Collagen specimens were first dissolved in 0.5*M* acetic acid (0.5 mg/mL); then, a 10-mL collagen solution was diluted to yield four lower concentrations made by the addition of an appropriate amount of 0.5*M* Acetic acid (HAc) to the stock solutions. The relative viscosities of all of the collagen solutions were calculated by the division of the flow times of the solutions by that of the pure solvent. All of the experiments were performed in the range $1.1 < \eta_r < 2.5$ to provide typical data points (η_r means the solution-solvent viscosity ratio).

AFM. Briefly, the collagen specimens were first dissolved in 0.5*M* acetic acid with a concentration of 50 μg/mL; then, 20 μL of the aforementioned collagen solution was dropped onto a fresh mica substrate; this was followed by drying in a desiccator for at least 24 h at room temperature (21 ± 1°C).

Meanwhile, to examine the self-assembly ability of all of the collagen specimens, the collagen specimens were first dissolved in 0.5*M* acetic acid at a 2 mg/mL concentration. Then, the fiber formation buffer (comprised of 10 m*M* sodium phosphate and 130 m*M* sodium chloride at pH 7.4) was prewarmed for 30 min at room temperature and mixed with one of the collagen solutions (ratio of fiber formation buffer to collagen solution = 10:1). Subsequently, 20 μL of collagen solution was dropped onto a fresh mica substrate, and the mixtures were incubated for 24 h at room temperature (21 ± 1°C). After that, the mica substrate was gently washed twice with distilled water and dried. The following procedure was the same as mentioned previously.

The AFM analyses were performed in air at room temperature on a Dimension 3100 Nanoscope IV equipped with silicon TESP cantilevers (Shimadzu SPM-9600, Japan) in tapping mode. All of the obtained images were processed with Nanoscope analysis software, with either flattening or line fitting according to the relief characteristics with the minimal polynomial order needed.

XRD Analysis. The XRD analysis pattern of the collagen specimens was recorded on an X'Pert Pro X-ray diffractometer (Philips, The Netherlands) with Cu Kα radiation (wavenumber (λ) = 0.1541 nm) at 40 kV. The 2θ range was from 5 to 50°, and the scanning rate was 2 min^{-1}.

Circular Dichroism (CD) Measurements. The lyophilized collagen samples were dissolved in 0.1*M* acetic acid to a concentration

of 0.5 mg/mL and scanned in a wavelength range from 185 to 250 nm at 25°C. The molar ellipticity was recorded with a CD apparatus (model 400, AVIV)

Measurement of the Col-Regenerated Specimens' Cytotoxicity and Biological Activity. ***Cell isolation and culture.*** Primary fibroblasts were isolated from the New Zealand rabbit, as described in our previous report,[23] and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in humidified 95% air/5% CO_2. The culture medium was changed every 3 days, and the fibroblasts were used at passages 3 to 8.

Adhesion of the fibroblasts cultured in plastic tissue culture plates coated by gelatin or collagen solutions. First, sterilized collagen specimens (either before dissolution in [EMIM][Ac] or regenerated) and gelatin were dissolved in phosphate-buffered saline (PBS) at a concentration of 10 μg/mL at 4°C overnight. Subsequently, 24-well plastic tissue culture plates were coated with either collagen or gelatin solutions at room temperature. Then, fibroblasts were seeded in the culture plates at the same concentration of 1×10^5 cells per well and cultured for 4, 8, and 12 h, whereas an uncoated culture plate was used as the reference. To remove unadhered fibroblasts, the cells were washed gently with PBS; then the cells were digested with trypsin (0.25 wt %) for approximately 2 min to obtain a cell suspension and were counted with a hemacytometer. The attachment ratio of the fibroblasts on a protein-coated culture plate was calculated with the following formula:

$$\text{Attachment ratio}\,(\%) = \left(\frac{\text{Number of cells adhering on the plates}}{\text{Number of cells seeded on the plates}}\right) \times 100\%$$

The values are expressed as the mean plus or minus the standard deviation ($n = 5$).

Proliferation of fibroblasts cultured in the protein-coated plastic tissue culture plates. The mitochondria activity of the fibroblasts seeded in the protein-coated plastic tissue culture plates was determined by colorimetric assay, which detected the conversion of 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT; Sigma) to formazan.[24] In brief, the surface of each plastic tissue culture plate (24-well) was first coated with either collagen or gelatin solution (10 μg/mL in PBS). The fibroblasts were then seeded into the 24-well plates at a density of 1×10^4 cells/well and cultured for 1, 3, 5, and 7 days at 37°C in an atmosphere of 5% CO_2, whereas the cells cultured in the uncoated plastic tissue culture plate were used as a reference. The cells on the surface of the coated plates were recorded with microscopy at day 3. During a period of 7 days, 50 μL/well MTT solution (1 mg/mL in test medium) was added to the 24-well plates and incubated at 37°C for 4 h to form the formazan crystals. After that, 400 μL/well dimethyl sulfoxide was added to the plates and mixed thoroughly to dissolve the dare blue crystals. Subsequently, the solutions in 24-well plates were transferred into a 96-well plate at 150 μL per well. Finally, the 96-well plate was read on a microplate reader (model 550, Bio-Rad Corp.) at a wavelength of 492 nm. All of the experiments were performed with five replicates.

Table I. Solubility of Collagen in the [EMIM][Ac]/Sodium Salt Solvent Systems

	Solubility (wt %) at different temperatures				
Solvent system	25°C	30°C	35°C	40°C	45°C
[EMIM][Ac]	3.1	3.9	4.6	5.1	7.4
[EMIM][Ac]/Na_2HPO_4	3.9	5.5	6.5	7.8	10.5
[EMIM][Ac]/Na_2SO_4	2.9	3.5	4.3	4.7	6.9
[EMIM][Ac]/NaCl	3.0	3.7	4.1	4.9	6.7
[EMIM][Ac]/Na_3PO_4	3.1	3.9	4.8	5.4	8.8
[EMIM][Ac]/$NaHSO_4$	3.7	4.9	5.8	7.4	10.1

Statistical Analysis

Statistical analysis was conducted with a one-way analysis of variance on SPSS 19.0. The differences between groups were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Influence of the Temperature and Various Sodium Salts on the Solubility of Collagen in [EMIM][Ac]

The solubility of collagen in various [EMIM][Ac]/sodium salt solvent systems was investigated at different temperatures (25, 30, 35, 40, and 45°C). Table I shows that the solubility of collagen was effected by the addition of a small amount of sodium salt into [EMIM][Ac]. The addition of Na_2HPO_4 and $NaHSO_4$ caused a more observable promotion of the solubility than that of the other sodium salts, from 7.4 to 10.5 and 10.1% at 45°C, respectively. On the other hand, the addition of Na_2SO_4 and NaCl decreased the solubility of collagen, from 7.4 to 6.9 and 6.7% at 45°C, respectively. The possible reason was that collagen is a typical kind of ampholyte amino acid, the solubility of which mainly depends on the pH value of the solvent system. When the pH value deviated either higher or lower from the isoelectric point of collagen, the collagen fibers tended to swell and dissolve. Na_2HPO_4 and $NaHSO_4$, the sodium salts of the weak acid and strong acid, respectively, could bring about a change in the pH value of the solvent system, and this was beneficial for dissolving the collagen samples, whereas the neutral salts, NaCl and Na_2SO_4, were more likely to maintain the conformation of the collagen samples.

On the other hand, increasing the temperature was also found to promote the solubility of collagen in the IL solvent system. As described in Figure 1, within the collagen fibril, collagen molecules with a length of 300 nm and a width of 1.5 nm were staggered with respect to their neighbors by multiples of *D*. The conformation of collagen was stabilized by the presence of strong intermolecular and intramolecular hydrogen bonds, ionic bonds, van der Waals' forces, and hydrophobic bonds between the polar and nonpolar groups. Meanwhile, the insolubility of collagen was mainly due to the structural organization within the fibril, where the axial and lateral organization and topology of the collagen molecules ensured strong intermolecular interactions and crosslinkage. The [EMIM][Ac] solvent system was deduced to break most of the hydrogen bands within the

adjacent collagen molecules and even some parts of the hydrogen bands within the collagen triple helixes. Thus, the collagen fibrils tended to open up more and swell. The hydrogen bands between the amino hydroxyl of collagen and the ester oxygen were thoroughly interrupted and replaced by two kinds of stronger hydrogen bonds, that is, the acetate (Ac^-) associated with the amino hydroxyl and 1-ethyl-3-methylimidazolium ($EMIM^+$) complexes with the collagen ester oxygen. An increase in the dissolution temperature significantly promoted the intermolecular distance of collagen and then reduced the hydrogen bond strength between the collagen molecules. A higher temperature seemed to be easier for the cations and anions of IL to create new strong hydrogen bonding between the collagen molecules, as reported in other works.[9]

Structural Characterization of the Col-Regenerated from the [EMIM][Ac]/Sodium Salt Solvent Systems

Because the Col-regenerated was lyophilized twice, the surface morphology of the Col-regenerated seemed to be looser than that of the original collagens specimen (Figure 3). However, we needed to do more chemical composition analysis to identify the changes in the structure between the original collagen specimens and the Col-regenerated specimens.

Figure 4 shows the SDS–PAGE patterns of the COL-regenerated from dissolution in the [EMIM][Ac]/sodium salt system at various temperatures (25, 30, 35, 40, and 45°C) and the collagen before dissolution (COL-before) as the reference. It was obvious that the SDS–PAGE patterns of the collagen specimens either before or after dissolution in the IL solvent system all consisted of at least two typical α chains (α_1 and α_2) with a molecular weight of approximate 100 kDa. However, from the SDS–PAGE patterns, both COL-before and COL-regenerated-25°C/35°C still contained intermolecular and intramolecular crosslinked components, β chains (ca. 200 kDa) and γ chains (ca. 300 kDa), which are known as the dimer and trimer of α chains, respectively. Moreover, patterns with higher molecular weights than

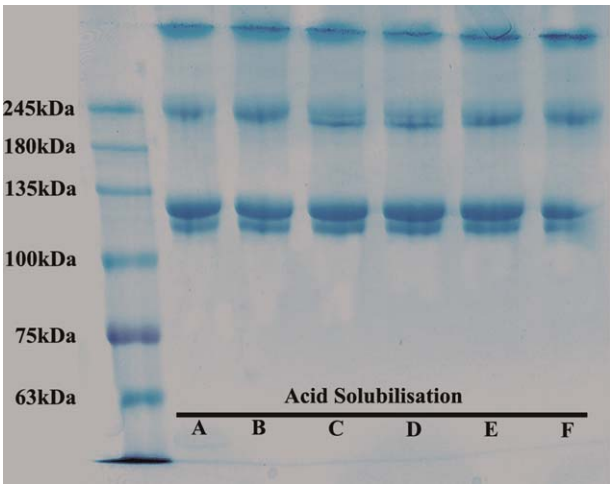

Figure 4. SDS–PAGE analysis of the acid-solubilized materials: (a) COL-before, (b) COL-regenerated-25°C, (c) COL-regenerated-30°C, (d) COL-regenerated-35°C, (e) COL-regenerated-40°C, and (f) COL-regenerated-45°C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

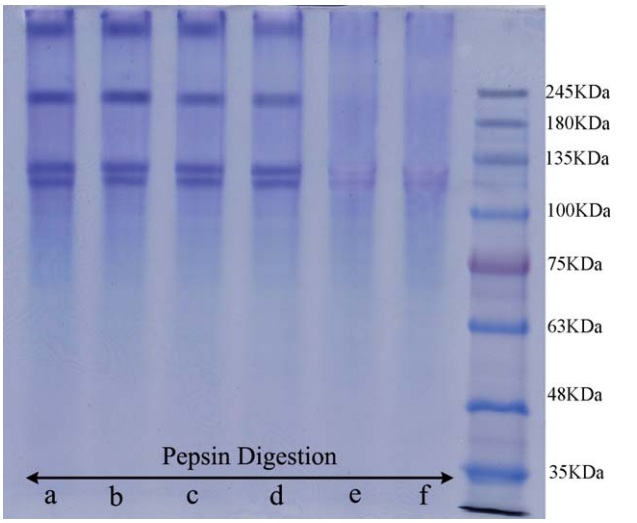

Figure 5. SDS–PAGE analysis of the pepsin-digested materials: (a) COL-before, (b) COL-regenerated-25°C, (c) COL-regenerated-30°C, (d) COL-regenerated-35°C, (e) COL-regenerated-40°C, and (f) COL-regenerated-45°C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

100 kDa in the two groups (COL-regenerated-45°C/40°C) were not so obvious as that of the COL-before and COL-regenerated-25°C/35°C. This may further indicate that the structure of collagen specimens regenerated from the IL solvent system was partially destroyed.

Although Figure 4 indicates the extensive losses of the Col-regenerated, that is, COL-regenerated-40/45°C lost part of its triple-helix structure, it does not allow us to decide whether the α chains shown in Figure 4 were derived from intact or denatured triple helices. Hence, pepsin was involved in the SDS–PAGE testing because of its special characteristics and could destroy globular proteins easily, but it could not attack an intact collagen type I triple helix. It was apparent that almost no collagen was detectable, and this demonstrated that COL-regenerated-40/45°C was completely destroyed (Figure 5). Some interhydrogen or intrahydrogen bonds within the collagen specimens may have been broken; this is the key foundation to maintaining the conformation of collagen molecules. To some extent, this phenomenon of hydrogen bonds broken within collagen molecule in solutions at higher temperatures was more likely to be the denaturation process, which was in agreement with many studies. This is discussed further in the thermal stability section. Because the structural integrity of collagen specimens is so important, temperatures of 35 and 25°C were chosen to perform the collagen regeneration process in the latter experiments (Figure 3). We further focused on the whether the structure of collagen would be destroyed or not at 25 and 35°C in the IL solvent system.

It is commonly accepted that the secondary backbone conformation of collagen can be reflected in the FTIR spectra. Figure 6 shows the quintessential FTIR spectra of COL-before and COL-regenerated samples. As mentioned before, collagen with intact conformation possesses a special triple-helix structure, which can be characterized by its feature amide bonds in FTIR

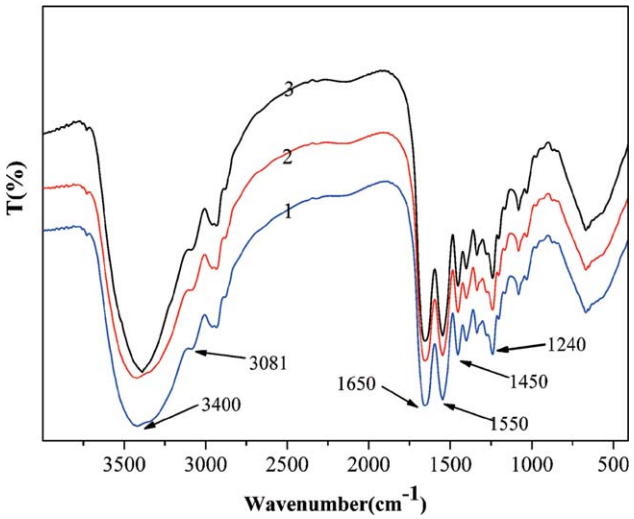

Figure 6. FTIR spectra of the collagen specimens: (1) COL-before, (2) COL-regenerated-25°C, and (3) COL-regenerated-35°C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

spectra. Normally, the amide A and B bonds at approximately 3400 and 3081 cm^{-1} are primarily correlated to the stretching vibrations of N—H groups. The amide I bond is usually found at about 1650 cm^{-1} and mostly results from the stretching vibrations of C═O groups, whereas the amide II bond at about 1550 cm^{-1} is composed of N—H bending vibrations coupled to the C—N stretching vibrations. The amide III absorbance at approximate 1240 cm^{-1} is mainly associated with the C—N and N—H bending vibrations and wagging vibrations of CH_2 groups in the glycine backbone and proline side chains.[4] It seems as if the peaks were obtained at similar wave numbers for the three collagen samples. However, distinctions could still be found at 3400 cm^{-1}, in which the amide A bond appeared to be sharp after dissolution in the IL solvent systems at both 25 and 35°C. This may have arisen from the partial disruption of hydrogen bonds within collagen molecules, resulting from the interaction between collagen and [EMIM][Ac] molecules, as revealed in the illustration graph (Figure 1).

Table II. FTIR Absorption Ratios of A_{1240} to A_{1450} for the Collagen Specimens Both before and after Dissolution in the [EMIM][Ac]/Na_2HPO_4 Solvent System

	COL-before	COL-regenerated-25°C	COL-regenerated-35°C
A_{1240}/A_{1450}	1.00	0.99	0.95

Meanwhile, the absorbance peak at about 1240 cm^{-1} was affected by the changes in the triple-helical structure of collagen, and therefore, the FTIR absorption ratios of amide III to 1450 cm^{-1}, denoted as A_{1240}/A_{1450}, could be used to quantify the denaturation of the collagen sample.[3] Table II shows that the ratio of the COL-before and COL-regenerated samples decreased slightly from approximately 1 (0% denaturation) to 0.95, whereas the ratio for gelatin, in which the triple-helix conformation was thoroughly destroyed (100% denaturation), was about 0.6.[8] According to the results in Table II, it is conceivable that the triple-helical conformation of Col-regenerated was mostly retained.

Figure 7 shows the AFM morphological changes of COL-regenerated-25°C/35°C induced by the IL solvent system, where COL-before was used as a comparison. Typical topographies in the collagen fibrils were observed (Figure 7); the COL-before samples presented a typical fibrillar structure, and each fibril overlapped with one another, whereas the entangled fibrous network topographies of the COL-regenerated samples were observed for the first time. The fibrils arrangement was much looser than that in the COL-before membranes and showed a more sparsely distributed network in the COL-regenerated-35°C membrane. This may have indicated that higher temperatures caused the partial collapse in the conformation of collagen in the IL solvent system at 35°C. A higher concentration (2 mg/mL) was applied to the mica substrate for the self-assembly experiments. The axially ordered fibrillar structure with characteristic cross-striated banding pattern formed by the quarter stagger arrangement of collagen molecules was clearly observed (Figure 8). As described in the literature,[4,25] not only the typical D period was found,

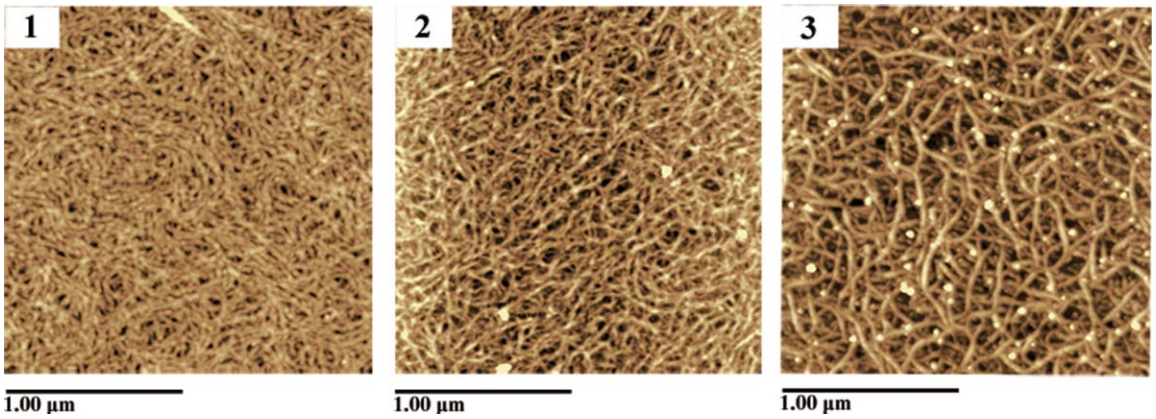

Figure 7. AFM images of the collagen samples: (1) COL-before, (2) COL-regenerated-25°C, and (3) COL-regenerated-35°C. All of the collagen concentrations were 50 μg/mL in 0.5*M* Acetic acid (HAc). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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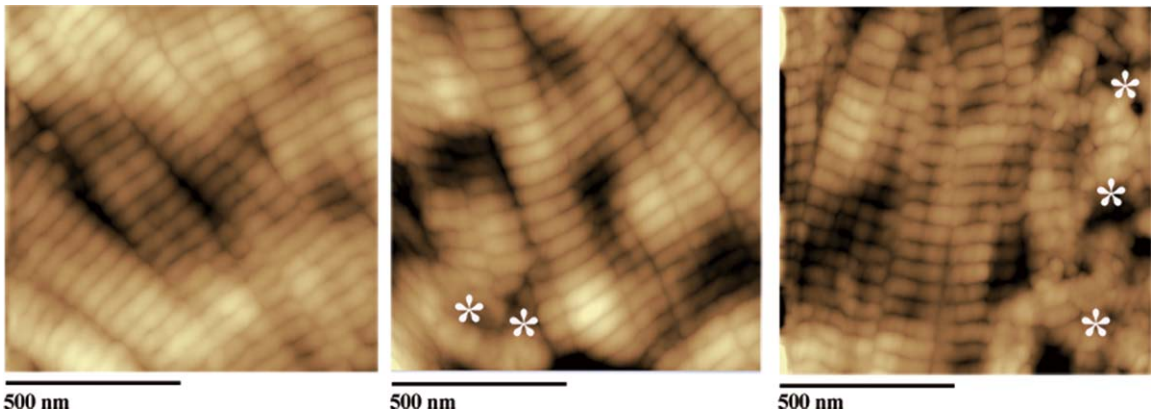

Figure 8. AFM images of the self-assembled collagen samples: (1) COL-before, (2) COL-regenerated-25°C, and (3) COL-regenerated-35°C. Each one exposed the typical D-band periodicity (≈65 nm). All of the collagen concentrations were 2 mg/mL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

but also the compact and regular arrangement were observed in the collagen samples with structural integrity. Furthermore, the arrangement of collagen fibrils appeared to be a picture just like the perfect crystal structure, whereas the COL-regenerated-35°C membrane also represented disfigurement somewhere and indicated the possibility of a disruption in the conformation of the COL-regenerated-35°C samples to some extent. However, most of the arrangements still remained compact and clear.

The thermal stability of both COL-before and COL-regenerated was assessed by US-DSC analysis (Figure 9). Generally, the heat transformation of collagen is considered to be the collapse of the collagen triple-helical structure into random coils, and the main endothermic peak is normally assigned to T_d. There is also usually a slightly endothermic peak in the range from about 25 to 35°C that is apt to be neglected. This peak value is always interpreted as the pretransition temperature (T_p); the triple-helical structure within collagen samples tends to be looser and more relax but still maintains its special triple-helical configuration, and this seems to just be ready for the denaturation process.[8,26] The T_d values of COL-before and COL-regenerated-25°C were 41.6 and 39.8°C, respectively, and were close to each other, whereas T_d of COL-regenerated-35°C was approximately 37.5°C and was slightly lower than those of the previous two collagen samples. In addition, T_p of the collagen samples treated in the [EMIM][Ac]/sodium solvent system at 35°C was found to be lower, about 3°C lower, than that of COL-before. It was speculated that the ionic groups that is, $EMIM^+$ and Ac^-, could break the hydrogen bonds between the adjacent polypeptide chains of the collagen molecules, as shown in the illustration graph, when the collagen samples were dissolved in the IL solvent system. After being extracted from the system, the hydrogen bonds could be reconstructed, but there were still some hydrogen bonds that could not reform between adjacent collagen molecules. This may have been the reason that T_p of COL-regenerated-35°C was lower than that of both COL-before and COL-regenerated-25°C. However, T_d of COL-regenerated-25°C was still kept around 40°C, and this was considered to be acceptable under its lower requirement for use.

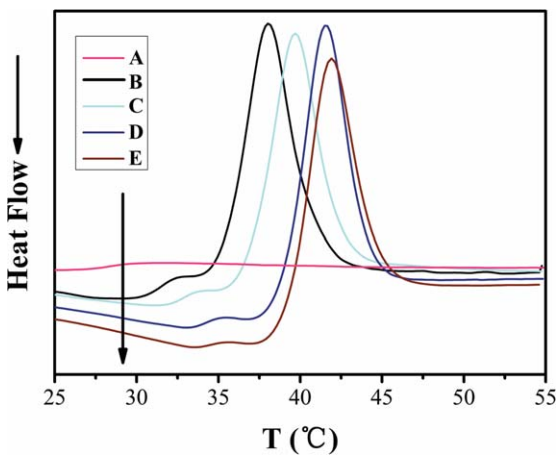

Figure 9. US-DSC patterns of the collagen solutions: (A) gelatin, (B) Col-regenerated-35°C, (C) Col-regenerated-25°C, (D) original collagen, and (E) Sigma type I collagen. (T=Temperature) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

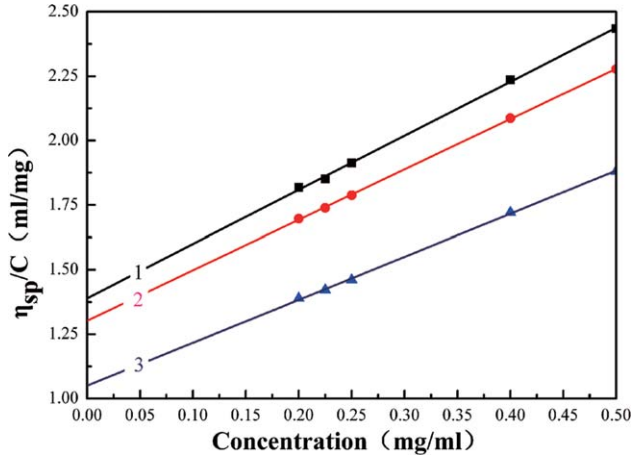

Figure 10. Intrinsic viscosity (η_{sp}) of the collagen samples: (1) COL-before, (2) COL-regenerated-25°C, and (3) COL-regenerated-35°C. (C=Concentration) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

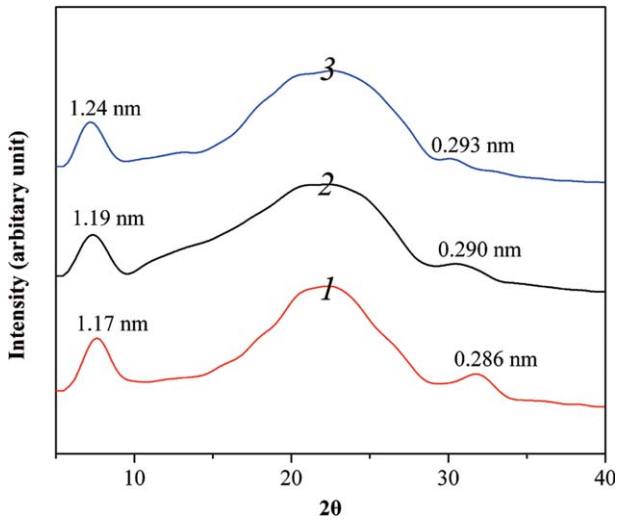

Figure 11. XRD diagrams of the collagen samples: (1) COL-before, (2) COL-regenerated-25°C, and (3) COL-regenerated-35°C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Collagen, as a special natural biopolymer, can also be characterized by the measurement of intrinsic viscosity. Figure 10 shows the intrinsic viscosities of both the COL-before and COL-regenerated (COL-regenerated-25°C and COL-regenerated-35°C) specimens on the basis of the measurement of the reduced viscosities of the collagen solutions in a concentration range of 0.20–0.50 mg/mL at 25°C. The intrinsic viscosity of both the COL-before and COL-regenerated-25°C remained almost the same, about 1.39 and 1.30 mL/mg, respectively. This result correlated well with the value (1.40 mL/mg) reported in our previous work,[20] and this further indicated that the conformation of collagen during the regeneration process was still retained to a large extent. In addition, it was obvious that the intrinsic viscosity of COL-regenerated-35°C was smaller than that of both COL-before and COL-regenerated-25°C; this indicated that the structure of COL-regenerated-35°C was destroyed to a certain degree. Apparently, the lower temperature seemed to be more suitable for the manufacture of Col-regenerated with a higher structural integrity, which is considered important for collagen's biological activity.

The XRD diagrams (Figure 11) of both the COL-before and COL-regenerated specimens yielded first a sharp peak around 1.2 nm; this indicated an intermolecular lateral packing distance between the collagen molecular chains; a second broad peak resulted from the diffuse scattering, and a third peak resulted from the unit height, which is typical of the triple-helical structure (~0.29 nm). The third peak around 0.29 nm was associated with the helical rise per residue distance; this is considered important to the conformational integrity of collagen.[27] Figure 11 shows that the axial rise per residue distance of COL-regenerated-35°C stayed almost the same, around 0.29 nm, compared to that of the COL-before and COL-regenerated-25°C specimens; this indicated that collagen dissolved in the IL solvent system at a lower temperature did not cause any further disruptions. However, the mean center-to-center distance between the collagen fibrils reflected by the peak around 1.2 nm in the COL-regenerated-35°C specimen appeared to be more broadened than that in the COL-before and COL-regenerated-25°C specimens. This broadening may have been a result of an increase in the disorder, a reduction in the crystallite size, or a combination of the two; this was consistent with the results revealed in Figure 8.

Figure 12 shows the typical CD patterns of all of the collagen specimens, that is, the original collagen and COL-regenerated-25°C and COL-regenerated-35°C. As is known to us, the denaturation of the collagen or its helix destruction usually results in the disappearance of the positive band around 221 nm, characteristic of a collagen triple helix. The CD pattern of COL-regenerated-25°C showed almost the same tendency as that of the original collagen; this may have indicated that its structural integrity still remained intact to a large extent. Compared to the original collagen, the COL-regenerated-35°C had a decreased molar ellipticity at 221 and 197 nm compared to both the original collagen and COL-regenerated-25°C. This result further indicates that a higher temperature could have destroyed the conformation of collagen when it was dissolved in the [EMIM][Ac]/sodium salt solvent system. However, the absorbances at 221 and 197 nm of COL-regenerated-35°C were still much higher than those of either gelatin or collagen regenerated from hexafluoroisopropanol.

Analysis of the Cytocompatibility of Col-Regenerated from the [EMIM][Ac]/Sodium Salt System

Because the Col-regenerated could be used as a material for tissue engineering and ILs themselves are reported to be more toxic than common solvents,[28] it was very important to evaluate the biocompatibility of all of the Col-regenerated samples.

Fibroblasts attached to the plastic tissue culture plates coated by gelatin or collagen solutions were observed (Figure 13). There was more fibroblast attachment to the collagen-coated plates, with either the COL-before or COL-regenerated samples. The collagen coating of the plates resulted in a significantly

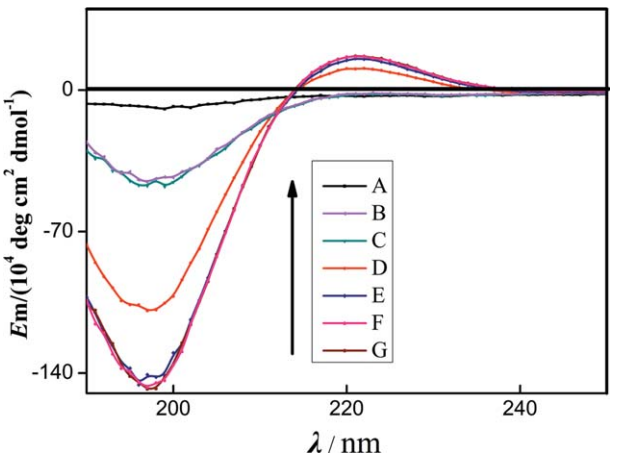

Figure 12. CD spectra of the collagen solutions: (A) Sigma type I collagen, (B) original collagen, (C) Col-regenerated-25°C, (D) Col-regenerated-35°C, (E) gelatin, (F) collagen regenerated from hexafluoroisopropanol, and (G) Col-regenerated-45°C. (Em=Molar ellipticity) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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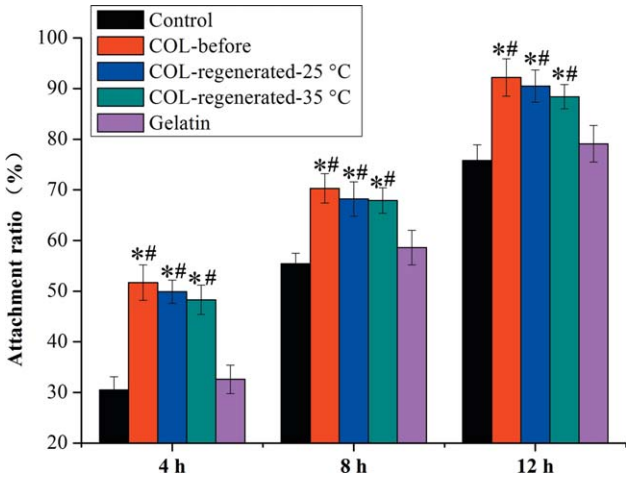

Figure 13. Attachment ratio of the fibroblasts on the protein-coated culture plates at different time intervals (4, 8, and 12 h). $^{*}p < 0.05$ relative to the control and $^{\#}p < 0.05$ relative to the gelatin groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

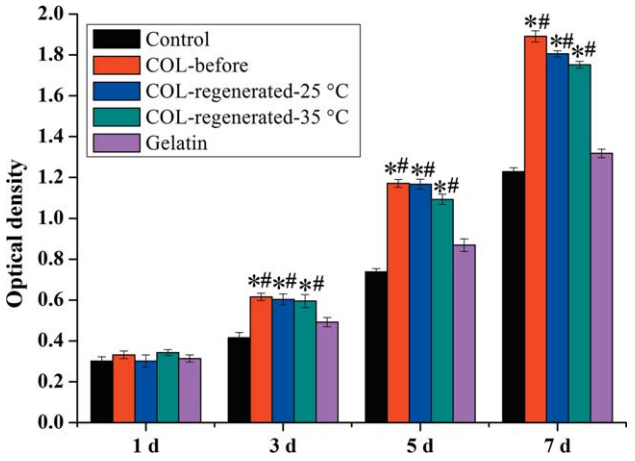

Figure 14. Proliferation of the fibroblasts on the protein-coated culture plates over a period of 7 days. $^{*}p < 0.05$ relative to the control and $^{\#}p < 0.05$ relative to the gelatin groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

promoted fibroblast attachment ratio compared to that in the control group. The ratios of the collagen-coated plates (i.e., with COL-before, COL-regenerated-25°C, and COL-regenerated-35°C samples) were 92.2, 90.5, and 88.4%, respectively, whereas the denaturation product of collagen, gelatin, appeared to have no ability to improve fibroblast attachment compared to the control, and there was no significant difference between the two groups in the ratio of attachment. After 12 h, the attachment ratios of both the control and gelatin groups just remained at 75.8 and 79.1%, respectively. On the one hand, because [EMIM][Ac] can dissolve easily in water, most of this IL could be washed out when the Col-regenerated specimens had undergone sufficient washing. On the other hand, in

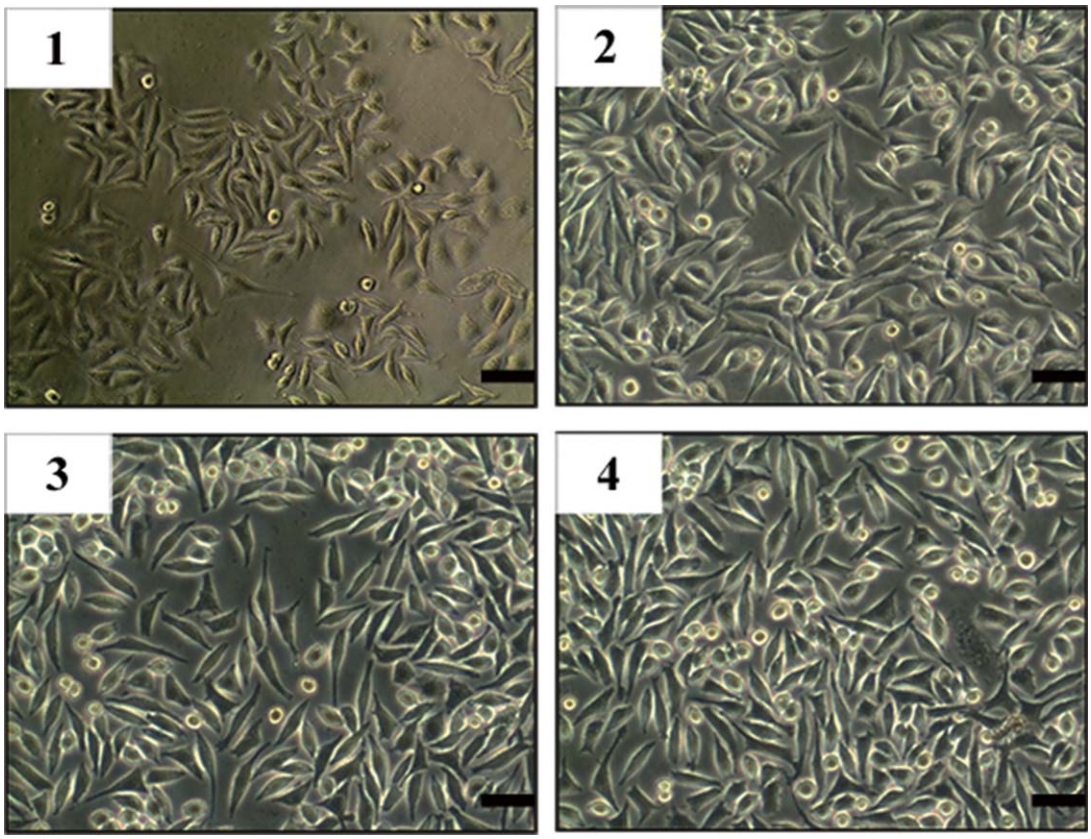

Figure 15. Morphology of the fibroblasts on the protein-coated culture plates at day 3: (1) control group, (2) COL-regenerated-35°C, (3) COL-regenerated-25°C, and (4) COL-before (scale bar = 10 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

agreement with the results discussed previously, although collagen regenerated from the IL solvent system was demonstrated to be partially denatured, the main conformation of collagen molecules still retained. As is known, type I collagen is the most significant and abundant fibrillar collagen involved in the promotion of the activation of cell membrane proteases for collagenolysis; this in turn leads to cell migration and/or adhesion.[4] This biological activity of collagen is based on its structure and conformational integrity, especially the integrity of its triple-helical structure.[29] If the chains within collagen molecules are cleaved thoroughly, the denaturation product of collagen, gelatin, will lose the corresponding biological features of collagen itself, as revealed in Figure 13.

MTT is a pale yellow substrate that produces a dark blue formazan product when incubated with living cells.[24,30] It has been widely accepted as a characterization method for cell attachment and proliferation because the formation of formazan can only occur in active mitochondria.[31] Figure 14 shows the effect of various coated collagen specimens on the viability of fibroblasts as measured by the MTT assay. When fibroblasts were cultured in either gelatin or collagen-coated plates for the 1st day, there was no statistically significant increase in the formation of formazan. During this period, fibroblasts were totally committed to adhering onto the bottom of the culture plates, and the proliferation of fibroblasts had not yet started, whereas from the 3rd day, the formation of formazan attained the statistically significant high level ($p < 0.05$) for all of the collagen-coated groups (COL-before, COL-regenerated-25°C, and COL-regenerated-35°C). The microscopy images of the samples at day 3 (COL-before, COL-regenerated-25°C, and COL-regenerated-35°C) were also observed, as shown in Figure 15, whereas the uncoated culture plate was used as the reference. The optical density for both the COL-regenerated-25°C and COL-regenerated-35°C group was slightly lower than that of COL-before, but no significant difference was observed among the three groups. As demonstrated previously in the structural characterization section, the main conformation of collagen regenerated from the [EMIM][Ac] solvent system still remained, even though the distances between collagen molecules increased. Associated with the results of fibroblast adhesion and proliferation, we drew the conclusion that collagen that underwent the procedure of being dissolved and regenerated from the IL solvent system still maintained its special biological activity to promote the seeded cells' growth.

CONCLUSIONS

[EMIM][Ac] is a viable solvent for the dissolution of collagen extracted from porcine skin. The solubility of collagen in this IL solvent system was significantly affected by the temperature and the addition of sodium salts. Collagen could be regenerated from the IL solvent system by a water precipitator, and the integrity conformation of the Col-regenerated specimens was evaluated by FTIR spectroscopy, SDS–PAGE, DSC, XRD, and AFM. The results show that collagen dissolved in the IL solvent system at a low temperature still maintained nearly the same conformation, that is, a typical triple-helix structure, as that of native collagen. The temperature may have been an important factor that affected the collagen structural integrity in the IL solvent system. Nevertheless, the main structure of collagen after dissolution in the IL solvent system at a higher temperature (35°C) was still retained to some extent. Meanwhile, the most important biological characteristic, the biocompatibility of COL-regenerated, was estimated with fibroblast adhesion and proliferation. The results suggest that the COL-regenerated did promote fibroblast adhesion and proliferation compared to the gelatin and control groups. This phenomenon may have further indicated that the structure of COL-regenerated maintained most of its conformation because the structural integrity was the foundation of its biological activity. In addition, further studies are underway to better determine the interactions between IL and collagen during the electrospinning process to broaden the IL's application.

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