Polyethylene Glycol as a Preservative for Pigskin and Its Interaction with Collagen

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Abstract

Herein, an immersing method for the pigskin preservation by using polyethylene glycol (PEG) with different molecular weights (M_W = 200, 600, 6000) was developed and the influence of PEG on the conformation of type I collagen was investigated. Only PEG200 followed dehydration and rehydration patterns similar to that of salt curing and exhibits reasonably good preservative and bloodstains removal effects. Zeta potential analyses show that the collagen-PEG solutions have good dispersions stability. FT-IR and US-DSC results indicate that collagen triple helixes are kept integrated in the presence of PEG, and the highly hydrated feature of PEG helps to maintain the stability of the protein conformation. Atomic Force Microscope (AFM) images show that the collagen fibrils become more dispersed with the increasing M_W of PEG. The present work gives positive insight to partially replacing sodium chloride for the preservation of raw hide and skin, as well as the development of PEG-collagen biomaterials.

Introduction

In the manufacture of leather, the grade of finished leather significantly depends on the quality of raw hide and skin. Since the main constituents of animal skins consist of 60~70 wt% water and 25~30 wt% protein,[1] untreated skins are vulnerable to microbial attack and so they putrefy.[2] Therefore, proper preservation process is essential especially during storage and transportation. The methods can be physical, chemical, and biocidal, and their principles lie in the bactericidal effect or bacteriostatic action on the putrescible hide and skin.[3] In fact, the most common approach used in worldwide tanneries is still salting curing with sodium chloride (NaCl) at the level of 40-50% (based on green weight of skins), because it is very economical and safe for workers. However, heavy use of salts results in about 40% of chlorides pollution and 55% of total dissolved solids (TDS) in the composite tannery effluent.[4] The increasing new environmental regulations have developed stricter policy and standards for the chloride content in wastewater.[5] Moreover, salt curing has other weaknesses such as the "red heat" of raw hide and skin induced by halophilic bacteria.[6]

The alternatives to salt curing have thus been intensively proposed in recent years,[7] ranging from inorganic silicate,[8] organic preservatives such as natural plant extractive,[9] to bacteriocins.[10] Among them, polyethylene glycol (PEG) has attracted attention due to its good biocompatibility and biodegradability. As a neutral, non-toxic and water soluble polymer, PEG belongs to the rare synthetic polymers approved by FDA for the use of internal injection.[11] And it has been widely used for organ preservation,[12] specimen fixation, and wood surface stabilization[13] due to its strong dehydration. Furthermore, PEG and their derivatives are the most commonly used anti-biofouling material owing to their abilities to resist proteins and bacteria adhesion.[14] The possibility of PEG for hide preservation was first explored by the group of Rao *et al* in 2009.[3,15] So far, the limited researches show that the efficacies of PEG for preservation are varied depending on the molecular weight (M_W) of PEG, dosage and application methods.[16-18] It is noted that any preservation process should be reversible without altering the structure and properties of raw hides, especially the matrix protein, collagen.[7] However, present publications mainly focus on PEG curing process and final leather properties. The underlying preservative principles and the effect of PEG on the conformation of hide collagen are still unclear.

Unlike the reported PEG-based dry coating preservation methods for raw hides or skins,[15-18] herein we use PEG with different M_W for the curing of pigskin by an immersing method so as to minimize PEG use. Their bacteriostatic properties have

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Manuscript received May 25, 2018, accepted for publication August 7, 2018.

been examined by disk agar diffusion method and compared with the same concentration of NaCl solutions. The interactions between PEG molecules and type I collagen are further investigated. The aim of this work is to explore the practicable eco-friendly preservative approach and the underlying mechanism.

Experimental

Materials

The fresh pigskins were obtained from local slaughter house. Type I Collagen used in this work was extracted from the fresh adult bovine achilles tendon using 0.5 M acetic acid with pepsin, following the similar procedure as previously reported.[19] Polyethylene glycol (PEG, M_W = 200~20000 g mol^{-1}) from Kelong Chemical Reagent Company (Chengdu, China) were used as received. Staphylococcus aureus (*S. aureus*, ATCC 25923) and Escherichia coli (*E. coli*, ATC 25922) were obtained as indicators of experimental microbes. Other reagents were of analytical grade and used as received.

Preservation Test with PEG

PEG with different molecular weights (PEG200, PEG600 and PEG6000) were employed for curing the pigskins. In detail, the pigskins were cut into equal pieces of 3 cm × 3 cm each after surface degreasing and trimming treatments. Then the samples were weighed and immersed in PEG aqueous solutions at different concentrations 10, 20, 30 and 40 wt%, based on the total solution, respectively. For comparison, another group of samples were cured with 40 wt% salt solutions (NaCl). At regular intervals, the samples were taken out and weighed after gentle removal of surface water with filter paper. The dehydration process of the fresh skins and the rehydration of the dried cured skins were both recorded by measuring the moisture contents of the samples with time, similar to the standard procedure,[20] and calculated by the following equation.

$$\text{Moisture (\%)} = (M_t - M_0) / M_t \times 100\% \quad (1)$$

Where M_0 represents the lyophilized dry weight of each sample, M_t represents the weight of the cured samples at a designated time. The dehydration and rehydration assays for each sample were carried out in triplicate.

Inhibition Zone Test of PEG

The antibacterial activity of PEG was evaluated using Gram-negative bacterium *E. coli* and Gram-positive bacterium *S. aureus* as the test cultures by disk diffusion method.[21] The paper disks with a diameter of 18 mm were immersed in 40% PEG solutions (w/v) or 40% NaCl solution for 4 h, respectively. And the paper impregnated with deionized water (≥ 18.2 MΩ·cm, Ulupure, China) was prepared as blank control group. All the disks were sterilized by UV irradiation for 1 h before test. Then, the sterilized samples were placed onto nutrient agar plates which have been inoculated with overnight aged active bacteria (200 uL, 10^6 UF/mL). The following incubations were kept at 37°C for 24 h. Three samples were prepared in parallel (n = 3) for each experiment.

Preparation of PEG-collagen Samples

PEG with different M_W was respectively added into aqueous collagen solutions in acetic acid under gentle stirring. The mass ratios of PEG to collagen (m_{PEG}/m_{col}) were varied from 0, 20, 30 to 50 wt%. The obtained PEG-collagen samples were designated as PEGx-Col, where x represents the corresponding M_W of PEG. Subsequently, the samples were measured by zeta potential, FTIR, US-DSC and AFM observation in order to explore the interactions between PEG and collagen. All the samples were freshly prepared before measurements.

Zeta Potential Measurement

Zeta potential (ζ-potential) of the PEG-collagen solutions was measured by Zetasizer Nano ZS (Malvern Co., UK). The final concentration of collagen in the solution (C_{col}) was 0.4 mg/mL (pH = 3.0) and the m_{PEG}/m_{col} is 50 wt%. The solutions were centrifuged at 6000 rpm for 5 min before test. Each measurement was recorded from an average of nine replicates.

Fourier Transform Infrared Spectroscopy (FT-IR) Measurement

The PEG-collagen samples for FT-IR measurement were prepared by casting the mixed liquors onto a polytetrafluoroethylene (PTFE) plate and freeze-drying at –20°C by a vacuum freezing drier (Alpha 1-2 LD, Christ, Germany) for 24 h. FT-IR spectra were obtained on a Bruker VECTOR-22 IR spectrometer using KBr disk method. Each sample was scanned for 64 times in the range of 400-4000 cm^{-1} with a spectral resolution of 4 cm^{-1}.

Ultra-Sensitive Differential Scanning Calorimeter (US-DSC)

0.5 mg/mL collagen solutions with different contents of PEG (m_{PEG}/m_{col} = 20 wt% and 30 wt%, respectively) were measured on a VP-DSC micro-calorimeter (Microcal, Northampton, USA) with the matching acetic acid solution (pH 3.0) as the reference. The sample and reference solutions were degassed at 4°C for 12 h and equilibrated at 10°C for 2 h before scanning. All the scans were carried out from 20 to 60°C at a constant heating rate of 1°C/min. The peak fitting was performed as our previous report[19]. The phase transition temperature (T_p) was taken as the maximum centered at the transition. The enthalpy change (*DH*) during the transition was calculated from the area under each peak.

Atomic Force Microscope (AFM) observation

10 μL diluted PEG-collagen solutions (C_{col} = 6×10^{-6} g/mL, m_{PEG}/m_{col}= 20 wt%) were dropped onto a freshly cleaved mica

substrate. After dried in a desiccator for 12 h at room temperature, the samples were scanned on a Dimension 3100 Nanoscope IV with Silicon TESP cantilevers a in a tapping mode (SPM-9600, Shimadzu, Japan). All samples were characterized by AFM for obtaining height and phase images. For each sample, the analyses were made at three different points to confirm the consistency of the observed morphology.

Results and Discussion

The skin samples cured by immersing in NaCl or PEG aqueous solutions at different concentrations are shown in Figure 1a. It can be seen that PEG200 is more effective in the removal of the bloodstains than higher M_W PEG. Combined with putrid smell of the test samples, PEG6000 shows negligible preservative effect for fresh skins in solution. Whereas, PEG200 at 30-40% concentrations exhibited reasonably good decay resistance, and the resultant skins were soft and clean, similar to NaCl solutions preserved specimens.

The moisture contents of the skin samples in the curing process are shown in Figure 1b. Owing to the osmotic pressure from high concentrations of PEG or salt,[22] water in free form can be removed from the skins, namely, dehydration. It shows that the moisture content of the salt cured sample first decreases at the early stage, then increases and tends to be stable after 8 h. While for PEG curing, the dehydration effect is obviously greater, and the moistures of the samples at equilibrium are apparently lower than saline cured samples. Moreover, such effect increases with the increasing M_W. Nevertheless, only PEG 200 follows a dehydration pattern similar to that of salt curing, which was also reported by J. R. Rao.[15]

It is known that the good preservative effects of the salt are not only related to its dehydration of hide and skin, but also depended on the ability to rehydration of preserved ones to fresh state. The latter is so important for leather making, and in fact, soaking is normally the initial and crucial step in the beamhouse. Figure 1c shows the rehydration processes of NaCl or PEG cured samples. Compared with higher M_W PEG, PEG200 cured sample has the best rehydration effect, close to the salted skins.

On the whole, the preservation by immersing method indicates that PEG200 with lower M_W has better preservative and cleaning effect at medium concentrations than PEG600 and PEG6000. The reason is possibly that it can penetrate into skin fibers and further exclude the water and bloodstains in the skins, as reflected in the initial stage of dehydration process (Figure 1b). Such behavior is more like small molecular NaCl than high M_W PEG. As the penetration is triggered by the concentration gradient, the skin will rehydrate to some degree. In contrast,

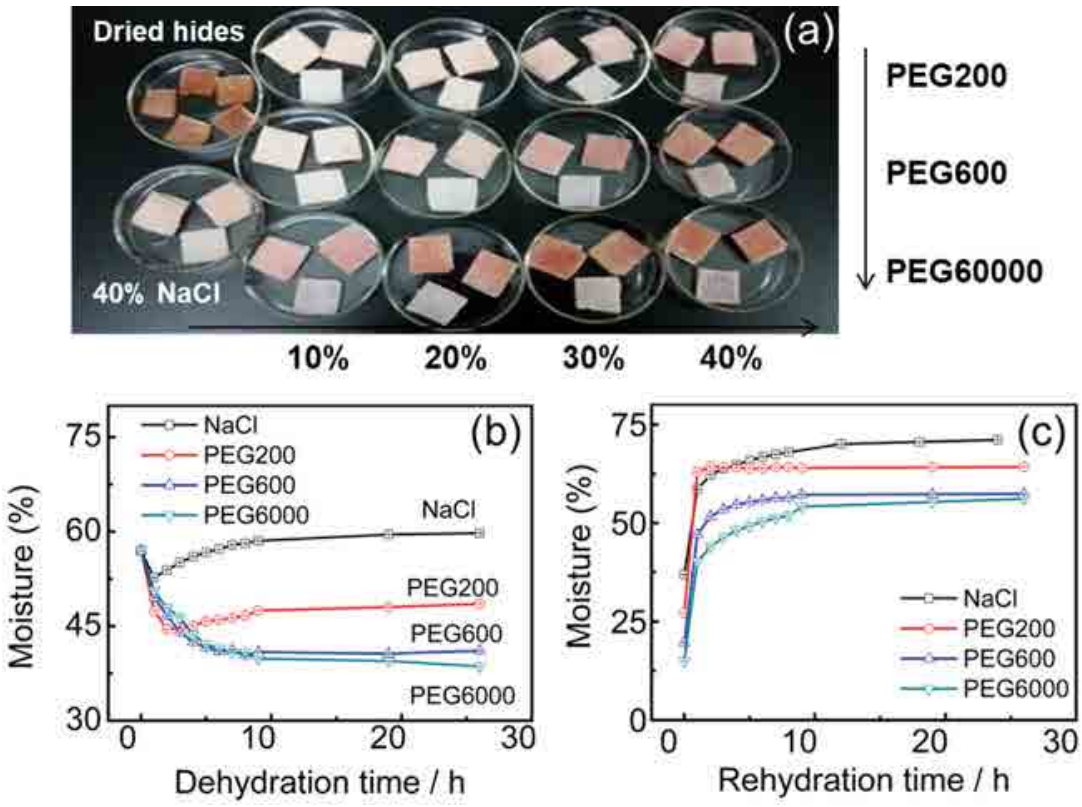

Figure 1. (a) appearance of the preserved samples (n=3); (b) time dependence of the moisture contents of the pigskins immersed in 40 wt% of NaCl and PEG with varied M_W; (c) rehydration profiles of the preserved pigskins;

PEG with higher M_W mainly tends to absorb onto the skin surfaces and acts as filtering membrane due to lower penetrability and higher viscosity,[23] thus impeding the decontamination of bloodstains in skins. Therefore, high M_W PEG can be used as preservatives by dry-coating method based on dramatic dehydration effect.[16]

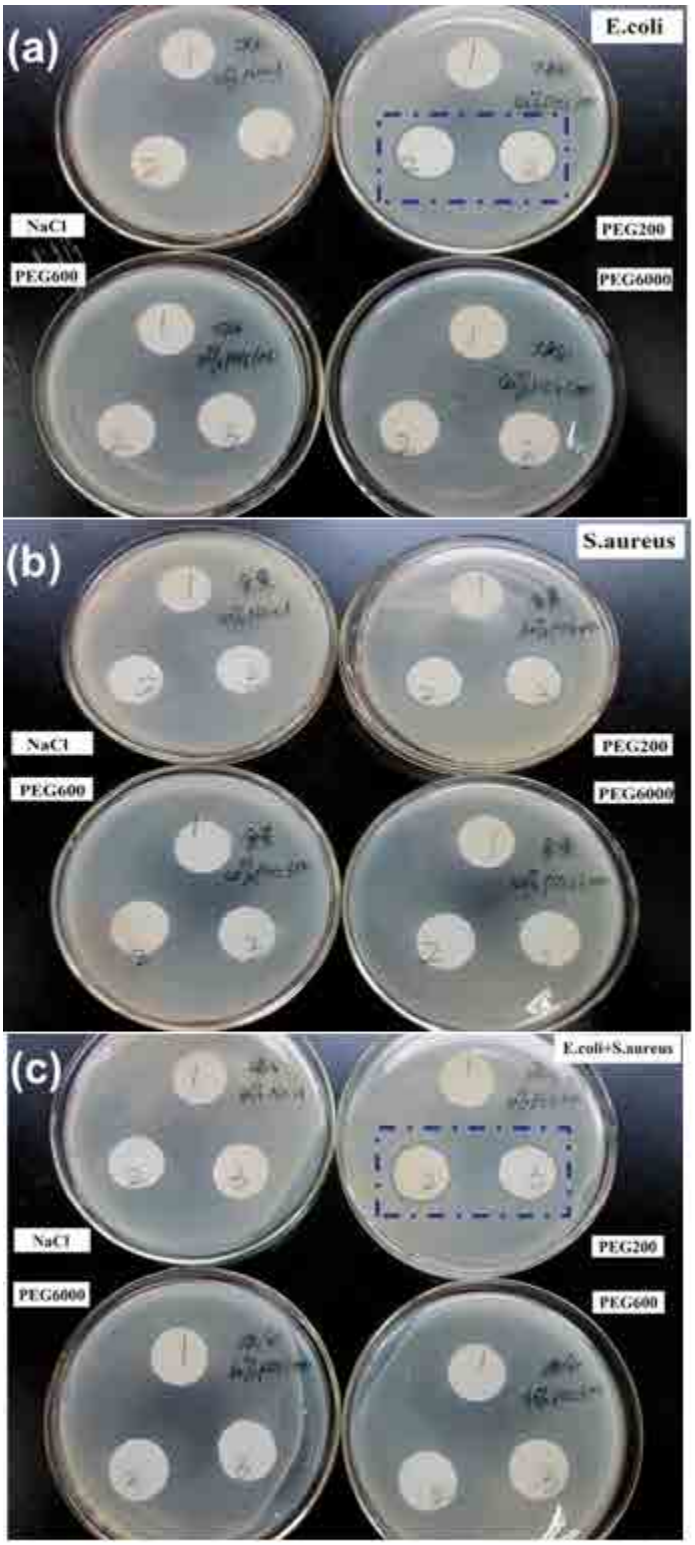

Figure 2. Antibacterial properties of NaCl and PEG with different M_W: (a) gram-positive bacterium *E. coli*; (b) gram-negative bacterium *S. aureus*; (c) Mixed bacteria of *E. coli* and *S. aureus*. Disk 1: control group; Disk 2-3: parallel experiment of samples treated with 40 wt% preservatives.

The antibacterial properties of PEG with different M_W were evaluated by disk agar diffusion method as shown in Figure 2. According to available national standard for the evaluation of textiles, the specimen with its inhibition band width above 1 mm is considered to exhibit good antibacterial activity.[24] Among all the tests, only the paper disks soaked with PEG200 show the inhibition band width (~2 mm) higher than the standard value, typically for both *E. coli* and *S. aureus*, as well as the mixed bacteria. Whereas no clear inhibition zones can be observed for PEG600 and PEG1000, indicating that the bacteriostatic property of PEG is indeed related to the molecular size. Agreeing with above preservative results, PEG200 with lower M_W shows better bacteriostatic performance.

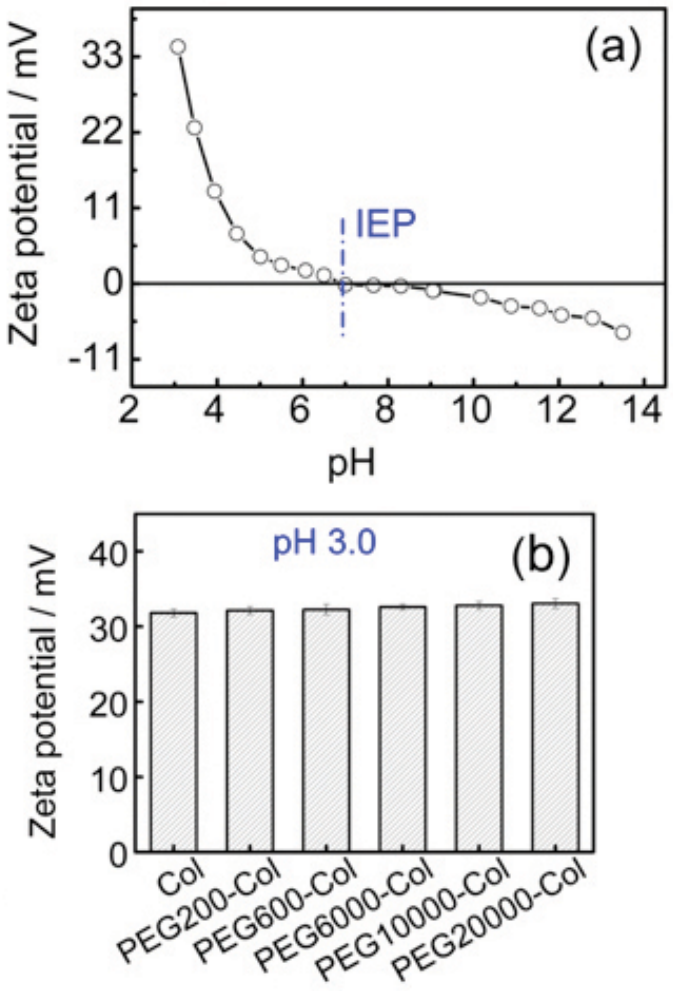

Figure 3. (a) Zeta potentials of collagen at different pH; (b) zeta potentials of PEG-collagen solutions at pH 3.0, C_{col} = 0.4 mg/mL, m_{PEG}/m_{col} = 50 wt%.

However, at the same concentration of 40 wt% as that of PEG200, sodium chloride soaked disks show weak bacteriostatic effect, with the inhibition band width ~0.7 mm. It may be the reason why traditional salt-curing or brine preservation of raw hide and skin commonly needs extremely high salinity, thus resulting in chloride pollution and soil salinization.

The zeta potentials of the collagen and PEG-collagen solutions are shown in Figure 3. In acetic acid solution at pH 3.0, the collagen is positively charged (ζ ~31.8 mV) and far away from its isoelectric point (IEP) at pH ~6.9 (Figure 3a). The high zeta potential value indicates its good dispersion stability, as the surface charges can effectively prevents aggregation of the collagen molecules.[25] After adding PEG at pH 3.0, the dispersions remain rather stable and the zeta potential value of ~32 mV is almost unchanged, irrespective of molecular size (Figure 3b). The results indicate that the neutral polymer PEG neither affects the ampholytic polyelectrolyte characterization of collagen,[7] nor destructs the conformation of the collagen in water due to its high hydrophilicity. In one word, PEG does not influence the stability of the collagen solutions, even at high mass ratio.

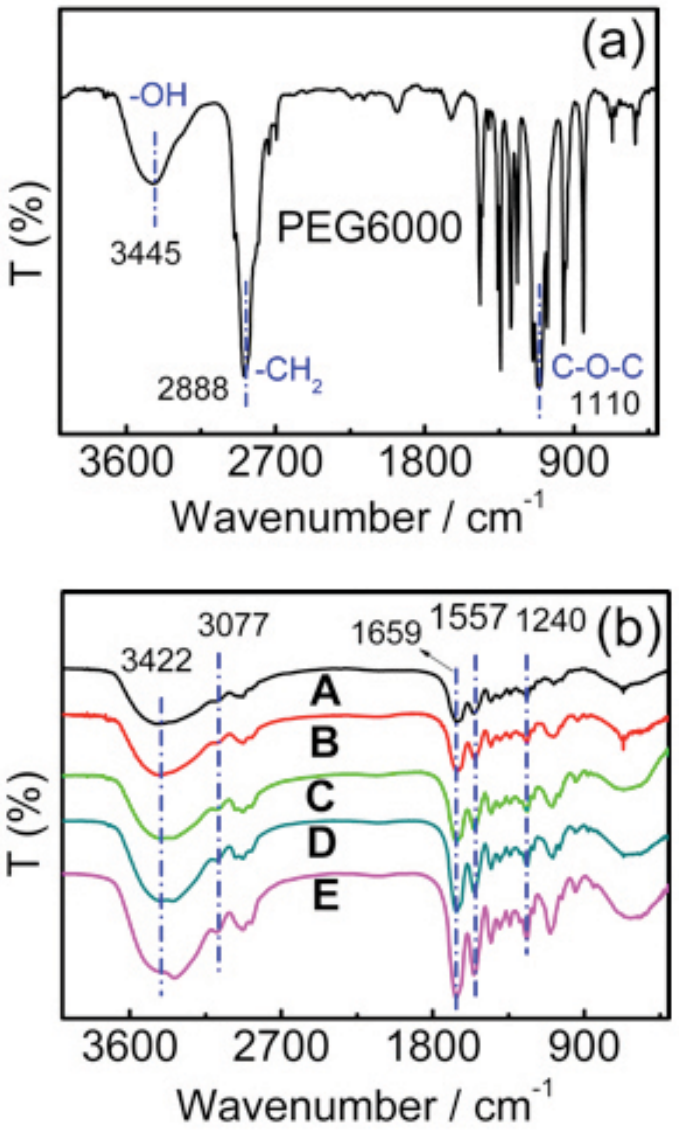

Figure 4. FTIR spectra of (a) PEG and (b) PEG-collagen: (A) collagen, (B) PEG200-Col, (C) PEG600-Col, (D) PEG6000-Col and (E) PEG20000-Col, m_{PEG}/m_{col} = 20 wt%.

Figure 4 shows typical FT-IR spectra of PEG, collagen and PEG-collagen. The characteristic bands of PEG are very similar to previous reports.[26] The peak at 3445 cm^{-1} is mainly assigned to the stretching vibrations of hydroxyl groups (–OH). The peak at 2888 cm^{-1} and 1110 cm^{-1} is attributed to the stretching vibrations of alkyl (R–CH_2) and ether bond (C-O-C), respectively. As for collagen, the positions of feature amide bands which characterize its special triple helix conformation[27] are showed in Table I. The amide A and B bands at ~3420 cm^{-1} and ~3077 cm^{-1} respectively, are mainly associated with the stretching vibrations of N–H groups. The amide I band at ~1659 cm^{-1} is dominantly attributed to the stretching vibrations of peptide C=O groups. The amide II absorbance at ~1557 cm^{-1} arises from the N–H bending vibrations coupled to C–N stretching vibrations. The Amide III band centered at 1240 cm^{-1} is assigned to the C–N stretching and N–H bending vibrations from amide linkages, as well as wagging vibrations of CH_2 groups in the glycine backbone and proline side chains. It is clear that the positions of amide B, I, II, and III bands do not change with the M_W of PEG, revealing that the triple helix conformation is not affected by PEG[28,29]. Notably, the position of amide A band of PEG-collagen shifts to lower wavenumbers, indicating that additional hydrogen bonds have been introduced in PEG-collagen.[30]

Figure 5 and Table II show the temperature dependence of the specific heat capacity (*Cp*) of type I collagen in 0.5 M acetic acid in the presence of PEG. As reported previously, a bimodal phase

Table I
Amide bands position in the FTIR spectra of collagen and PEG-collagen.

Samples (m_{PEG}/m_{col} = 20 wt%)	Amide bands (cm^{-1})				
	A	B	I	II	III
Collagen	3422	3077	1659	1557	1240
PEG200-Col	3420	3078	1660	1554	1240
PEG600-Col	3415	3076	1655	1560	1240
PEG6000-Col	3341	3077	1659	1555	1241
PEG20000-Col	3335	3078	1661	1554	1241

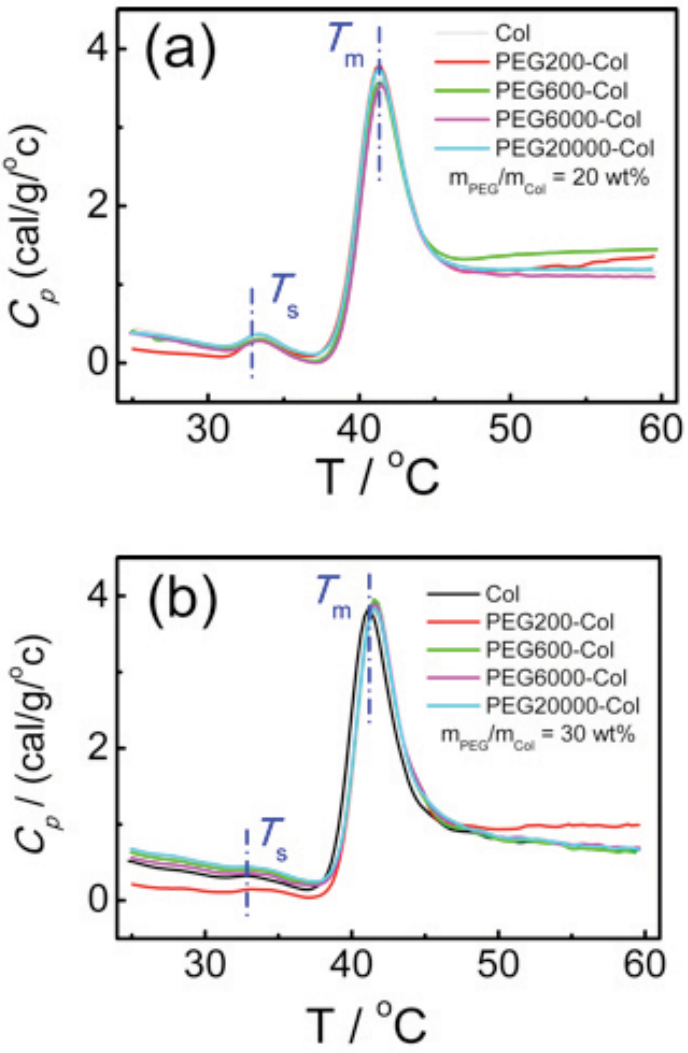

Figure 5. The specific heat capacity (C_p) of type I collagen in 0.5 M acetic acid (C_{col} = 0.5 mg/mL) in the presence of PEG with different M_W: (a) m_{PEG}/m_{col} = 20 wt%; (b) m_{PEG}/m_{col} = 30 wt%.

Table II
The phase transition temperature (T_m) and enthalpy change (ΔH) of collagen and PEG-collagen solutions.

Sample	m_{PEG}/m_{col} = 20 wt%		m_{PEG}/m_{col} = 30 wt%	
	T_m (°C)	DH (cal/g)	T_m(°C)	DH(cal/g)
Collagen	41.12	12.50	41.12	12.50
PEG200-Col	41.11	12.55	41.43	12.57
PEG600-Col	41.18	12.59	41.52	12.64
PEG6000-Col	41.41	12.45	41.54	12.47
PEG20000-Col	41.25	12.61	41.53	12.45

transition with a major peak at T_m ~ 41 °C, a shoulder peak at T_s ~ 32 °C and a total enthalpy change (DH ~12 cal/g) are observed for the collagen.[19] After the introduction of PEG, the bimodal shape does not change even at higher M_W and concentrations. It is known that the change in enthalpy corresponding to the major transition (DH_m) is mainly derived from the breakage of the intra-helix hydrogen bonds which stabilizes the triple helix of collagen molecules.[19] The enthalpy change concerning the shoulder (DH_s) is related to the defibrillation with the breaking

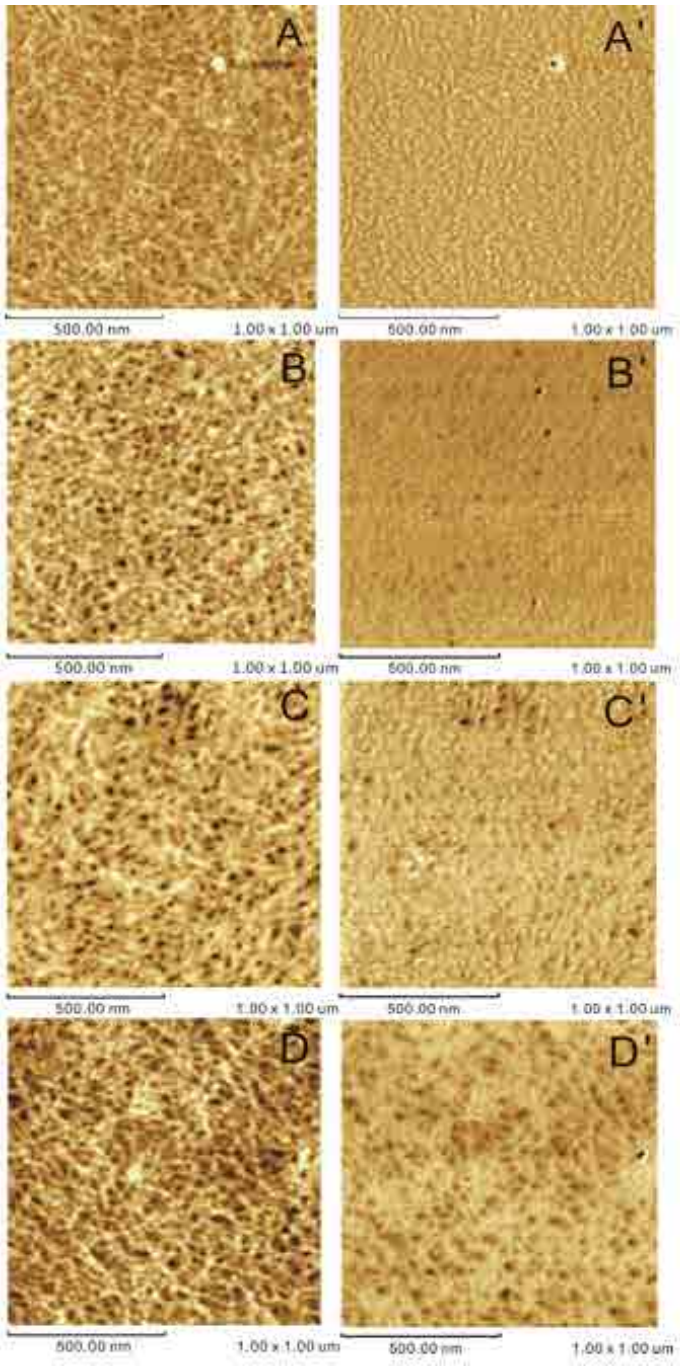

Figure 6. AFM height (left) and phase images (right) of PEG-collagen: (A) collagen; (B) PEG200-Col; (C) PEG4000-Col; (D) PEG6000-Col (C_{col} = 6×10^{-6} g/mL, m_{PEG}/m_{col} = 20 wt%)

of hydrogen bonds between triple helices. Herein, the unchanged *DH* values further prove that PEG does not impact the hydrogen bonds in intra- or inter-triple helices of the collagen. And the slight increase in T_m may imply the presence of PEG molecules at higher concentrations (Figure 5b) is favorable for stabilizing the triple helix conformation of collagen. Combining the above FI-IR results, such stabilizing effect of PEG on the collagen can be due to the hydration of PEG in aqueous solution. It is known that water molecules can readily form a tightly hydration layer (water barrier) on hydrophilic PEG surface mainly via hydrogen bond interactions,[31] which is possibly related to the additional hydrogen bonds observed in FT-IR spectra. And the collagen molecule also has a highly hydrated surface to stabilize its folded structure. As such, the presence of the hydrophilic PEGs helps to maintain the stability of the protein conformation.

Figure 6 presents the AFM morphological changes of collagen induced by PEG with different M_W. The pure collagen exhibits a typical fibrillar structure on mica substrate, randomly curved and overlapped as reported.[32] In the presence of PEG, the fibrillar morphologies are maintained without apparent cross-linking or aggregation, supporting our US-DSC results and other research literatures.[16] Whereas, As the M_W of PEG increases, the collagen fibrils become more isolated. It is very likely attributed to the adsorption of high M_W of PEG on the collage[33] due to the increased hydrophobic interaction as well as van de Waals force,[34,35] as reflected in the dispersion of collagen fibrils. In contrast, the short chain PEG shows little tendency to affect protein because of its high hydrophilicity.

The interactions between PEG and the collagen are schematically shown in Figure 7. On the one hand, both PEG and collagen are preferentially hydrated respectively, which contributes to the conformation stability of collagen.[30,36] On the other hand, since high M_W PEG exhibits more amphiphilic instead of highly hydrophilic characteristic in low M_W PEG, the increased

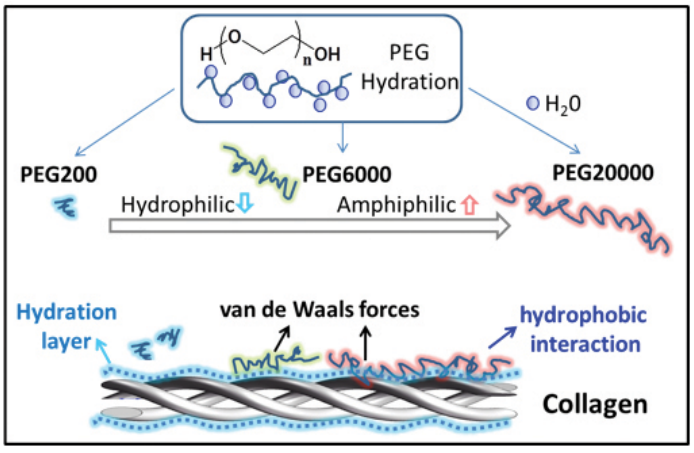

Figure 7. Schematic illustration of the interactions between PEG and collagen.

hydrophobic and van de Waals interactions between collagen and higher M_W PEG may lead to the adsorption of PEG on the collagen,[34-36] and thus promote the dispersion of collagen fibrils. The present understanding of PEG in the influence of collagen conformation is significant for the development of collagen-PEG composite biomaterials.[37]

Conclusions

The preservation by immersing method indicates that PEG200 has good preservative and bloodstains removal effects for pigskins at medium concentrations, and its dehydration and rehydration pattern is similar to salt curing. Inhibition zone tests show that the bacteriostatic performance of PEG agrees with preservative results. The interactions studies reveal that collagen-PEG solutions exhibit good dispersion **s**tability, and PEG has a stabilizing effect on the triple helix conformation of collagen. AFM results indicate that the collagen fibrils become more dispersed with the increasing M_W of PEG. Based on this study, it suggests that PEG does not affect the conformational structures of collage fibers, and PEG200 can be used as a preservative used either alone or in the combination with sodium chloride for pigskins in the leather industry.

Acknowledgement

The financial support of National Natural Science Foundation (NNSF) of China (21476148), Innovation Team Program of Science & Technology Department of Sichuan Province (Grant 2017TD0010), and National Key R&D Program of China (2017YFB0308402) are gratefully acknowledged.

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