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A new collagen solution with high concentration and collagen native structure perfectly preserved

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Collagen, the most abundant protein in mammals, is widely used for making biomaterials. Usually, organic solvents, such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), or acids (H₃PO₄, HAc ) have been used to disperse collagen to make collagen-based biomaterials. However, the native structure of collagen has been often seriously damaged and the concentration of collagen in solutions was constantly relatively low, which greatly limited its application. In this research, we have firstly made a detailed study of the effect of H⁺ and HAc on collagen dispersing in terms of Zeta potential, particle size and Circular Dichroism (CD), after that a benign sodium acetate/acetic acid buffer solution is proposed to disperse collagen. The results showed that the collagen solution from NaAc/HAc buffer solution at pH=3.0 had the suitability of both high concentration (100mg/mL) and perfect native structure preservation (up to 94%). We demonstrated that it was the constant concentration of free H⁺ in the NaAc/HAc buffer solution whose pH was fixed at 3.0 that can maintain the amount of surface charges on the collagen colloidal particles unchanged, which makes collagen dispersed well even in high dose. As an application, the collagen solution from NaAc/HAc solution at pH 3.0 was successfully electrospun into nanofibers, and the obtained collagen nanofibers still can keep as much as 57% of its native structure. This study indicated that the novel buffer solution of NaAc/HAc at pH 3.0 would be commonly used in the processing of collagen for a variety of biomaterials based on collagen solution, which had great potential for use in tissue engineering.

1. Introduction

Collagen is the most important structural element in native extracellular matrices, and presents in the form of elongated fibers in various tissues. These collagen fibers are made of rod-like triple helices, stabilized by intramolecular hydrogen bonds between Gly and Hyp in adjacent chains, which not only provide a structural support for cells but also act as an important regulator of cell behavior. Therefore, collagen or collagen-based materials is currently one of the most favored biomaterials for wound healing and biological implants. Most of the applications require collagen firstly to be dispersed to a high concentration and the triple-helical structure to be preserved in the final collagen-based materials. For collagen dispersing, the widely used solvents are non-strong acids with low concentration such as H₃PO₄ and HAc. However, the concentration of collagen in the obtained solutions is relatively low (lower than 10 mg/mL), which affects the manufacture of collagen-based biomaterials and limits its application in some aspects such as electrospinning and wet spin. In recent years, tens of papers dealt with collagen to prepare collagen or collagen-based composite nanofibers, mostly by dissolving it in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 2,2,2-trifluoroethanol (TFE). Although the concentration of collagen can be greatly increased, HFIP and TFE, resulted in an apparent loss of the triple helical configuration of collagen, apart from their corrosive nature. Besides, Luciana et al have put forward the ionic liquid as a solvent to dissolve collagen at 100 °C, which only emphasized the utility of the green solvent in the dissolution of collagen, but ignored the preservation of triple helices of collagen. In addition, Wnek et al have studied a phosphate buffer saline (PBS)/ethanol system as a solvent for collagen to electrospin collagen nanofibers, however, a large quantity of salts introduced by PBS had not been further removed, which largely affected the property of collagen nanofibers. Up to now, much efforts have been made to disperse or dissolve collagen in solvents, but to find an appropriate solvent that can both preserve the native structure of collagen and improve the content of collagen in solution at the same time is still a crucial issue.

In this research, dispersing collagen in HClO₄ solutions at different pH was firstly performed to investigate the effect of H⁺ on the dispersing process of collagen, and then a most commonly used weak acid, HAc, was employed to study the effect of HAc on collagen dispersing. Characterizations of collagen solution were performed by means of Zeta potential and particle size analysis, and the preservation of triple helices of obtained collagen samples was observed via Circular Dichroism (CD) and Fourier-Transform Infrared Spectroscopy (FTIR). By analyzing the effect of H⁺ and HAc on the dispersing process of collagen, a benign sodium acetate/acetic acid solvent...
system at pH=3.0 was proposed to disperse collagen, which had the suitability of both high collagen concentration and perfectly preserved collagen native structure(94%). To assess the capacity of collagen in high concentration to be processed into biomaterials, collagen solution from NaAc/HAc solution was successfully electrospun into nanofibers without any other additions, and the collagen fibers obtained can maintain as much as 57% of the native structure. Our work provided an effective method for the dispersion of collagen, by which not only high collagen concentration can be attained, but also the collagen native structure perfectly preserved. The results would be useful for the development of collagen-based biomaterials.

2. Materials and methods

2.1 Materials

Lyophilized type-I collagen from calf skin was a generous gift of the Tianjin Sannie Bio-engineering Technology Co., Ltd, China. Acetic acid (HAc, CH$_3$COOH), sodium acetate (NaAc, CH$_3$COONa) and perchloric acid (HClO$_4$; ≥ 99.9%) were purchased from Sigma-Aldrich and used as received.

2.2 Preparation of collagen solutions.

NaAc/HAc buffer solution was prepared by dissolving 1.2 g CH$_3$COONa in 22.5 mL CH$_3$COOH and 27.5 mL H$_2$O, and the pH was adjusted with HAc. Collagen solution was prepared by dispersing 1.0 g lyophilized collagen in 10 mL NaAc/HAc buffer solution and stirring at room temperature for 2 h.

2.3 Electrospinning Procedure.

Collagen solution from NaAc/HAc at pH 3.0 was directly used for electrospinning. The spinning solution was fed from a 10 mL syringe with a 6-gauge blunt-tip needle attached. The syringe was mounted onto a syringe pump (LongerPump LSP02-1B, Hebei, China), and the needle was connected to a high-voltage power supply (Dingtong High Voltage Power Supply, DPS-100(50 kV/50w), Dalian, China). Under 22 kV voltage, the fluid jet was injected out at a rate of 1.0 µL/min and the resultant nanofibers were collected on an aluminum foil which was put at 15 cm distance down from the needle. The electrospinning process was continuously performed for 4 h at room temperature (15-20°C) with a relative humidity of about 30%. The obtained products were dried 30°C in an oven overnight and used to observe the morphology of nanofibers by SEM and TEM.

2.4 Characterization of samples.

The particle size and the Zeta potential of collagen dispersion (1mg/mL) were determined with BDL-A Surface Potential-Particle Sizer (Shanghai Technical Super-vision Bureau). Circular Dichroism (CD) spectropolarimetry was employed to characterize the structure of collagen in solutions. Samples of collagen in NaAc/HAc buffer solution were prepared as followed: collagen in NaAc/HAc solution was firstly freeze-dried, after that dissolved in 0.05 M acetic acid (0.1 mg/mL) and equilibrated at 20°C for 12 h. CD spectra were recorded at 20°C over the wavelength interval of 190-280 nm with a path length of the cuvette for 1 mm (Chirascan, Applied Photophysics Ltd.). A scan speed of 1 nm/s was used with an average of three scans per sample. The collagen nanofiber solutions after electrospinning were prepared in a similar way by dissolving the weighed-in material in 0.05 M acetic acid. The amount of triple helix was calculated according to the following equation: $\%\chi_{III} = \frac{\theta_{obs} - \theta_D}{\theta_H - \theta_D} \times 100$ (1)

where $\%\chi_{III}$ is the percentage of folded protein, $\theta_{obs}$, $\theta_H$, and $\theta_D$ represent the ellipticity values measured at 221 nm for the sample, and for the collagen solutions at 10°C and 90°C, respectively, in 0.05 M HAc. Chemical bonding state of collagen was analyzed by FTIR using a Thermo Scientific (Nexus470) spectrometer. Morphological characterization of the electrospun nanofibers was performed using a scanning electron microscope (SEM, JSM5600LV, JEOL, Japan) with a beam voltage at 10 kV. And transmission electron microscopy (TEM) was obtained on a Tecnai G2 F20 S-TWIN electron microscope.

3. Results and discussion

3.1 The effect of H$^+$ on dispersing process of collagen in solutions

As is known, the original crystalline supramolecular structure of collagen fibers are self-assembled by tropocollagen molecules through secondary bonds. Generally acids can promote the dispersion process of collagen from fibers to colloidal particles by destroying the secondary bonds between tropocollagen molecules. However, the triple helix of tropocollagen molecules can be simultaneously unfolded by excess acids. To study the effect of acids on collagen dispersing process, we have firstly dispersed collagen into HClO$_4$ solutions at different pH from 2.0 to 4.5. In fact, perchloric acid can be considered fully ionized, and perchlorate ions have large spherical structure, so they are not easy to be absorbed on the surface of collagen molecules. These considerations indicate that only hydrogen ions in perchloric acid solutions can affect the dispersing process of collagen. The Zeta potential and particle size of collagen colloidal particles in HClO$_4$ solutions at different pH values are displayed in Fig.1. It can be observed that the size of collagen colloidal particles decreased when the pH of the solution varied from 4.5 to...
be adsorbed onto the surface of collagen molecules as displayed in the equation (2), and the rest are free in the solvents.

\[ R - NH_2 + H^+ \rightarrow R - NH_3^+ \quad \ldots \ldots (2) \]

Where R- represents the main part of collagen molecules, and -NH2 represents all the adsorption sites for hydrogen ions on the surface of collagen molecules.

When the concentration of collagen is increased, the pH of the solution will also increase, which will result in an increase in the amount of hydrogen ions in the collagen molecules. The increased amount of hydrogen ions will cause the collagen molecules to unfold, resulting in an increase in the adsorption level of hydrogen ions on the surface of collagen colloidal particles and can be presented as equation (3).

\[ \frac{[R - NH_3^+]}{[R - NH_2]} = K[H^+] \quad \ldots \ldots (3) \]

Where K represents the equilibrium constant, which is only related to temperature, and \([R-NH_3^+]/[R-NH_2]\) represents the adsorption level of hydrogen ions on the surface of collagen colloidal particles.

From the equations it can be seen that the adsorption level of hydrogen ions on the surface of collagen colloidal particles absolutely depends on the concentration of free hydrogen ions in the solution, when the temperature is kept constant. The more free hydrogen ions are provided by the solvent, the more hydrogen ions can be adsorbed onto the surface of collagen colloidal particles. Therefore, the Zeta potential increases with the increase in the concentration of free hydrogen ions in solutions.

Although it can be seen that the dispersing behavior of the collagen colloidal particles become better as the pH of the solution decreased (Fig.1). However, the native structure may be destroyed when the concentration of H+ is too high. A more detailed characterization of the triple helix structure of collagen in HClO4 solutions with different pH was carried out by means of CD spectroscopy, which utilized the differential absorption of left and right handed circular polarized light in an asymmetric environment to assess secondary structure. The results were shown in Fig.2. The triple helical structure in native collagen gave rise to a typical positive peak at around 221 nm and this peak value decreased when the pH of the collagen solution was decreased, which mean that the triple helical structure of native collagen was gradually destroyed. Consequently, when the concentration of HClO4 reached 1.0 mol/l, the positive peak totally disappeared, which was the characteristic of the random conformation of polypeptide chains. Therefore, when the pH of the solution was lower than 3.0, too much H+ would force collagen molecules irreversibly unwound into random polypeptide chains, which mean that collagen molecules lost their biological activity. So the concentration of acids is always limited to a low extent when they are chosen to be solvents for dispersing collagen.

3.2 The effect of HAc on dispersing process of collagen in solutions

![Fig. 2 CD spectra of native collagen dissolved in HClO4 solutions at different pH values.](Image)

From the above analysis, it can be seen that H+ plays a very
important role in the dispersing process of collagen. Actually, HAc solution with a low concentration (usually 0.3%), is widely used to disperse collagen, which can not only disperse collagen into homogeneous solution, but also keep collagen native structure unchanged.\textsuperscript{22} So we studied the dispersion process of collagen in HAc solutions with different pH from 2.0 to 4.5 and the results were displayed in Fig.3. As Fig.3 demonstrated, the size of collagen colloidal particles decreased with the pH of the solutions decreasing, and the smallest size of collagen colloidal particles was about 20 nm when the pH of the solution was 3.0. Unlike that in HClO\textsubscript{4} solutions, the size of collagen colloidal particles increased with the further decrease of the pH in HAc solutions. At the same time, it was clear that the collagen colloidal particles had the highest zeta potential value when the pH of the HAc solution was 3.0, and the results were in well consistent with the particle size analysis, both indicating that the collagen colloidal particles dissolved in HAc solutions had the best dispersion behavior when the pH of the solution was 3.0. When the pH was higher than 3.0, the dispersing behavior of collagen in HAc solutions was conform well with that in HClO\textsubscript{4} solutions. When the pH was lower than 3.0, the observed differences of Zeta potential and particle size of collagen colloidal particles between in HClO\textsubscript{4} solutions and HAc solutions may be due to the effect of CH\textsubscript{3}COOH in the solution.

Table 1. The concentration of H\textsuperscript{+} and HAc in HAc solution with different pH values

<table>
<thead>
<tr>
<th>solution</th>
<th>pH</th>
<th>c(H\textsuperscript{+})/ c(HAc)</th>
<th>c(H\textsuperscript{+})/</th>
<th>c(HAc)/c(H\textsuperscript{+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAc</td>
<td>4.0</td>
<td>1×10\textsuperscript{-4}</td>
<td>5.7×10\textsuperscript{-2}</td>
<td>5.7</td>
</tr>
<tr>
<td>HAc</td>
<td>3.0</td>
<td>1×10\textsuperscript{-3}</td>
<td>5.7×10\textsuperscript{-2}</td>
<td>57</td>
</tr>
<tr>
<td>HAc</td>
<td>2.0</td>
<td>1×10\textsuperscript{-2}</td>
<td>5.7×10\textsuperscript{-2}</td>
<td>570</td>
</tr>
</tbody>
</table>

HAc molecules, unlike HClO\textsubscript{4}, are incompletely ionized in solutions and can also be absorbed onto the surface of collagen molecules. The concentrations of H\textsuperscript{+} and HAc molecule in HAc solutions with different pH can be calculated based on the ionization constant (K\textsubscript{a}=1.8 × 10\textsuperscript{-5}, 25\textdegree{}C) and were displayed in Table 1. It can be seen that when the pH of the HAc solution was higher than 3.0, for example at 4.0, the ratio of the concentration of HAc and H\textsuperscript{+} was 5.7, which was relatively low, and the absorption process of H\textsuperscript{+} was the main process. Hence, the change trends of Zeta potential and particle size of collagen colloidal particles in HAc solutions were in line with that in HClO\textsubscript{4} solutions. However, when the pH of the HAc solution was lower than 3.0, such as 2.0, the ratio of the concentration of HAc and H\textsuperscript{+} was 570, which was relatively high, and the absorption process of HAc can not be ignored. We speculated that it was the HAc molecules absorbed onto the surface of the collagen colloidal particles that occupied some of the absorption sites, which decreased the left absorption sites for H\textsuperscript{+} to absorb. So the absorption level of H\textsuperscript{+} was decreased and this could be a major cause of the decrease of the Zeta potential and the increase of the particle size of collagen colloidal particles.

According to the above analysis, it can be seen that collagen colloidal particles had the best dispersing behavior when the pH of the HAc solution was kept at 3.0. The CD spectra of native collagen in HAc solution (pH=3.0) at different temperatures were detected and showed in Fig.4. Between 10–30\textdegree{}C, the CD spectra of native collagen in HAc solution with pH=3.0 were almost the same, with a typical positive peak at around 221 nm and a typical negative peak at around 198 nm. Above 35\textdegree{}C, the typical positive peak at around 221 nm gradually decreased when the temperature of the collagen solution was increased, which mean that the triple helical structure of native collagen was gradually destroyed. Therefore, it can be concluded that collagen in HAc solution at pH=3.0 not only had a better dispersion behavior, but also maintained the native structure unchanged.

3.3 The proposal of NaAc/HAc buffer solution at pH=3.0 for collagen dispersing

Based on the above analysis, it is reasonable to suppose that if there were a solution whose pH is kept at 3.0 unchanged to some extent with the sustainable increase of collagen concentration, the absorption level of hydrogen ions on the surface of collagen colloidal particles would not be changed and the size of the collagen colloidal particles would be kept unchanged. This was verified by the fact that NaAc/HAc buffer solution whose pH was fixed at 3.0 as a solvent can disperse collagen well with a high concentration and the optical photograph of collagen solutions with different concentration were displayed in Fig.5.
It was found that when the concentration of collagen increased from 5 mg/mL to 20 mg/mL in HAc solution at pH=3.0, the flowing sol would turn into solidified glue, which could not flow back down when the bottle was laid upside down, as shown in Fig.5b. In contrast, in NaAc/HAc buffer solution at pH=3.0, the collagen solution still had a good fluidity, even the concentration of collagen was increased from 20 mg/mL to 100 mg/mL, which are apparent from Fig.5c and Fig.5d. This phenomenon coincided with the above analysis and it was due to the fact that the free H$^+$ consumed by collagen new added can be supplemented by the H$^+$ ionized by HAc molecules in NaAc/HAc buffer solution at pH 3.0, resulting in a steady absorption level to a certain degree. So the collagen colloidal particles dispersed in the buffer solution were always in the best dispersing behavior.

3.4 An example for the application of collagen solution

Based on collagen solution with such a high concentration, there must be various applications in the fabrication of collagen-based biomaterials. As an example, collagen nanofibers can be prepared from the collagen solution without any other additions via electrospinning. Fig.6 showed the SEM (a) and TEM (b) images of the electrospun nanofibers from the collagen solution (100 mg/mL), and the fibers randomly oriented and interconnected into fibrous meshes with the fiber diameter in the nanometer range. TEM image revealed the collagen nanofibers had a more uniform morphology without any sign of beads formation.

![SEM and TEM images of nanofibers obtained by electrospinning](image)

Fig.6 SEM and TEM images of nanofibers obtained by electrospinning

Based on collagen solution with such a high concentration, there must be various applications in the fabrication of collagen-based biomaterials. As an example, collagen nanofibers can be prepared from the collagen solution without any other additions via electrospinning. Fig.6 showed the SEM (a) and TEM (b) images of the electrospun nanofibers from the collagen solution (100 mg/mL), and the fibers randomly oriented and interconnected into fibrous meshes with the fiber diameter in the nanometer range. TEM image revealed the collagen nanofibers had a more uniform morphology without any sign of beads formation.

![FTIR spectra of thermal denatured collagen obtained at 90 °C (a), native collagen(b), collagen dissolved in NaAc/HAc buffer solution(c), and collagen nanofibers(d).](image)

Fig.7 FTIR spectra of thermal denatured collagen obtained at 90 °C (a), native collagen(b), collagen dissolved in NaAc/HAc buffer solution(c), and collagen nanofibers(d).

In order to general analysis the degree of triple helix preservation, FTIR absorption ratio of amide III to 1450 cm$^{-1}$ band ($A_{III}/A_{1450}$) was determined in three samples$^{[32-33]}$ which decreased from about 1.0 for native collagen, to about 0.997 for collagen dissolved in NaAc/HAc solution, 0.925 for collagen nanofibers and 0.843 for thermal denatured collagen. These results provide evidence of the preserved triple helix integrity following dissolving in NaAc/HAc solution of native collagen, and a little of denaturation for the collagen nanofiber as well as denaturation to a great extent for the collagen nanofiber. A more detailed characterization of collagen triple helix structure of the above four samples was carried out by means of CD spectroscopy.

![CD spectra of native collagen(a), native collagen dissolved in NaAc/HAc solution(b), nanofibers after electrospinning (c) and denatured collagen(d).](image)

Fig.8 showed the CD spectra of native collagen, collagen in NaAc/HAc solution, and collagen nanofibers as well as denatured collagen. It can be seen that, the CD spectrum of collagen dispersed in NaAc/HAc buffer solution (100 mg/mL) had the similar positive peak at around 221 nm to that of native collagen. According to equation (1), it was calculated that 94% of the triple helix of the native collagen has been preserved. It is the first time that a collagen solution could keep 94% the triple helix of the native structure with such a high concentration in the best of our knowledge. In contrast, the positive peak of collagen nanofibers at around 221 nm was relatively decreased. This should be caused by the process of electrospinning. However, 57% of the triple helix of the native collagen was still preserved after
electrospinning, and this had made greater progress than the previous reported work.12

4. Conclusions
In the present study, a new solvent, NaAc/HAc buffer solution at pH 3.0, was firstly proposed to disperse collagen, which can make the collagen well dispersed even the concentration of collagen up to 100 mg/mL in solution. The constant concentration of free H+ in the NaAc/HAc buffer solution with pH=3.0 can maintain the amount of surface charge on the collagen colloidal particles unchanged, which make collagen dispersed well even in high dose. More to be point, this solvent can make collagen in solution keep a high triple-helical structure about 94%. Furthermore, Collagen nanofibers can be prepared easily via electrospinning, and even after electrospinning, the obtained nanofibers could contained a relatively high amount of triple helix about 57%. Therefore, this solvent opens a new avenue for collagen to prepare collagen-based composite with well triple-helical structure for tissue engineering.

Notes and references
3. Teo WE, Ramakrishna S. Nanotechnology 2006;17:89–106.