Original Article

ESTIMATION OF TYPE I COLLAGEN STRUCTURE DISSOLVED IN INORGANICAL ACIDS FROM CIRCULAR DICHROISM SPECTRA

ESTIMATIVA DA ESTRUTURA DE COLÁGENO TIPO I DISSOLVIDA EM ÁCIDOS INORGÂNICOS A PARTIR DE ESPECTROS DE DICROÍSMO CIRCULAR

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ABSTRACT: Usually, weak inorganic acids have been used to disperse collagen as green solvents for fabricating kinds of biomaterials all the time. However, it is an open question how much the dissolving process preserves or alters the native structure of collagen till now. Herein, we have examined the effect of three different solvents (HAc, HCl, H_3PO_4) on the secondary structures of collagen, based on circular dichroism (CD) spectra of collagen from 185 to 260 nm together with CDNN programs. We have found that collagen almost completely preserved its triple helical structure in the three inorganic acids at pH=3.0 or so, which demonstrated that it was the concentration of free H^+ in the above three solutions whose pH was fixed at 3.0 that can maintain an proper amount of surface charge on the collagen colloidal particles and appropriately loose the three-helix structure, which can not only lead to a better dispersion behavior, but also maximize the preservation of the integrity of the collagen structure. Although the fractions of kinds of secondary structures in collagen were different from all the three solvents based on CDNN data, which gave very similar results for each other. These results was tested for the first time in this work to estimate the secondary structures for collagen in the different common inorganic acids, which provides a new avenue for green collagen solvents to prepare collagen-based composite with well triple-helical structure for tissue engineering.

KEYWORDS: type I collagen. inorganic acid. the triple helical structure. CD.

INTRODUCTION

Scaffolds comprised of type I collagen offer distinct advantages when selected as biomaterials for use across a broad spectrum of regenerative medicine applications (SCHARNWEBER et al., 2003, SUN et al., 2013). Collagen type I is a heterotrimer, consisting of triple helical fibrils made of poly-peptide chains with carboxyl groups, interconnected by covalent and hydrogen bonding (GHEZZI et al., 2012). The triple helical structure is very important to the biological activity of collagen as it protects it from being broken down by proteases and is important for cell adhesion and the assembly of ECM as well as its good mechanical properties. Hence, collagen and collagen-based biocomposites have been designed with the purpose of optimizing the performance of the material by choosing suitable solvent for collagen, and research has shown positive results when collagen is dispersed in organic solvents, such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (TANG et al., 2016), inorganic acids (H_3PO_4, HAc) (ZHOU et al., 2013, BURCK et al., 2017, DIPPOLD et al., 2016) and phosphate-buffered saline (PBS) and ethanol (ZHOU et al., 2015). For

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example, tens of papers dealt with collagen to prepare collagen or collagen-based composite, mostly by dissolving it in 1,1,1,3,3,3-hexafiuoro-2-propanol (HFIP) and 2,2,2-trifiuoroethanol (TFE) (RASHTRAPAL et al., 2017). However, it was demonstrated that fluoroalcohols denature the molecular structure of collagen via hydrophobic and hydrophilic interactions that unfold the triple helix and produce an ‘‘open-helical structure’’ (DONG et al., 2011). Besides, Hirano et al. described the production of chitosan/collagen fibers produced by wet spinning from an aqueous 2% acetic acid-methanol solution spun into an aqueous 5% ammonia solution containing 40-43% ammonium sulfate (SRIPRIYA et al., 2011). Likewise, Fofonoff and Bellhave reported a method for forming collagen fibers by wet spinning from an aqueous 0.05% acetic acid (0.5M) solution (ACKERMAN et al., 1999). Overall, weak inorganic acids have been typically dispersed collagen in order to improve their bio-compatibility, while maintaining the specific triple helical structure of the original collagen. However, the effect of usually used inorganic acids on the structure of collagen type I are not clear till now, which need to be fully demonstrated from CD spectra. In the present study, we investigated the effect of three different common solvent systems on the triple helical structure of collagen and the secondary structure of collagen estimation were applied to the CD spectra using CDNN programs over the spectral range of 185-260 nm to obtain detailed quantitative information about the triple helical fraction of collagen in different common inorganic acids solvents.

MATERIAL AND METHODS

Materials

Lyophilized type-I collagen from calf skin was a generous gift of the Tianjin Sannie Bio-engineering Technology Co., Ltd, China. Hydrochloric acid (HCl, 32%) , acetic acid (HAc, CH_3COOH) and phosphoric acid (H_3PO_4, 85.0%～90.0%) were purchased from Sigma-Aldrich and used as received.

Preparation of collagen solutions.

The samples were prepared by dissolving collagen in three different solvent systems as followed: 10% w/v collagen solution was prepared by dissolving collagen in HAc, HCl, and H_3PO_4, and the pH of three different inorganic acids was altered from 0.5 to 4.0 at almost 0.5 interval to study the effect of inorganic acids solvent on the secondary structure of collagen.

Characterization

Circular Dichroism (CD) spectropolarimetry was employed to characterize the structure of collagen in solutions. 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 amount of triple helix was calculated according to the following equation (BURCK et al., 2013, DONG et al., 2011):

$$\%_{TH} = \frac{\theta_{obs}-\theta_D}{\theta_H-\theta_D}\times 100 \qquad \text{----------(1)}$$

where $\%_{TH}$ is the percentage of folded protein, θ_{obs}, θ_H, and θ_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. 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).

RESULTS AND DISCUSSION

HAc system

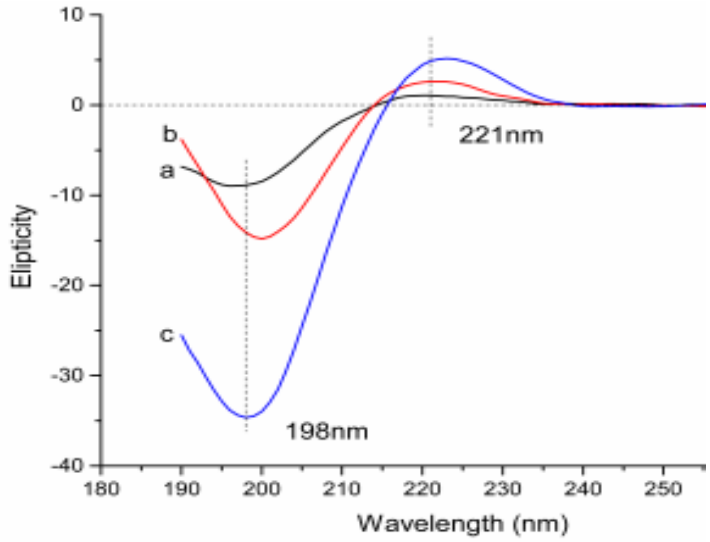

Figure 1. CD spectra of native collagen dissolved in different HAc solvents (a) pH=4.0, (b) pH=3.5, (c) pH=3.0.

Characterization of the triple helix structure of collagen in HAc 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 (ACKERMAN et al., 1999), and the results are shown in Fig.1 and Fig.2. Native collagen displays a characteristic CD spectrum with a negative peak at around 198 nm, across-over at 214 nm and a positive peak at around 220 nm, that corresponds to the triple helix structure, whose intensity decreases upon denaturation (TRONCI et al., 2013). As shown in Fig.1, when the pH value of HAc solution is from 3.0 to 4.0, the CD spectra of native collagen in HAc solution are almost the same, with a typical positive peak at around 221 nm and a typical negative peak at around 198 nm. Obviously, it seems to be different in the intensity of the peak, which is actually caused by the different concentration of collagen in the HAc solution. Therefore, the results showed that the natural structure of collagen was less damaged in the three kinds of HAc solutions. According to equation (1), the triple helix of the native collagen in HAc solution at pH=3.0 was still preserved up to 88.6%.

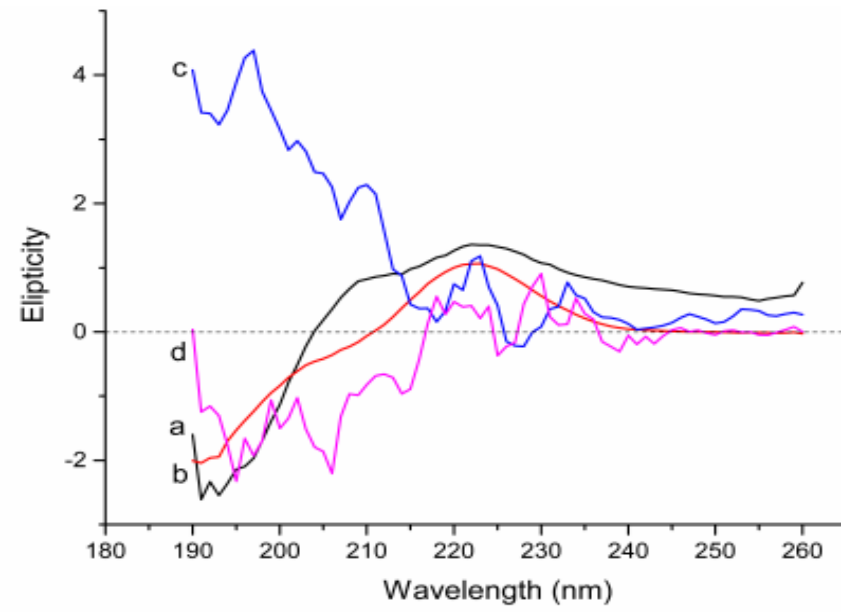

Figure 2. CD spectra of native collagen dissolved in different HAc solvents (a) pH=2.5, (b) pH=2.0, (c) pH=1.5, (d) pH=1.0.

But if the pH value of HAc solution is lower than 3.0, the CD spectra of native collagen in HAc solution are markedly different as presented in Fig.2, which means that the triple helical structure of native collagen is gradually destroyed. Consequently, when the pH value of HAc decreases to 2.0, the positive peak totally disappeares, which is the characteristic of the random conformation of polypeptide chains. Therefore, when the pH of the solution is lower than 3.0, too much H^+ can force collagen molecules irreversibly unwinded into random polypeptide chains, which means 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. Among the most cited inorganic acid, HAc system at pH 3.0 is the most used to dissolve collagen with well preservation of triple helices because of its low cost and low toxicity. This was in line with our previous results (QI et al., 2015) that the solvent whose pH was kept at 3.0 unchanged could provide the desired hydrogen ions to be absorbed on the surface of collagen colloidal particles, and therefore not only had a better dispersion behavior, but also maintained the more native structure unchanged than others samples.

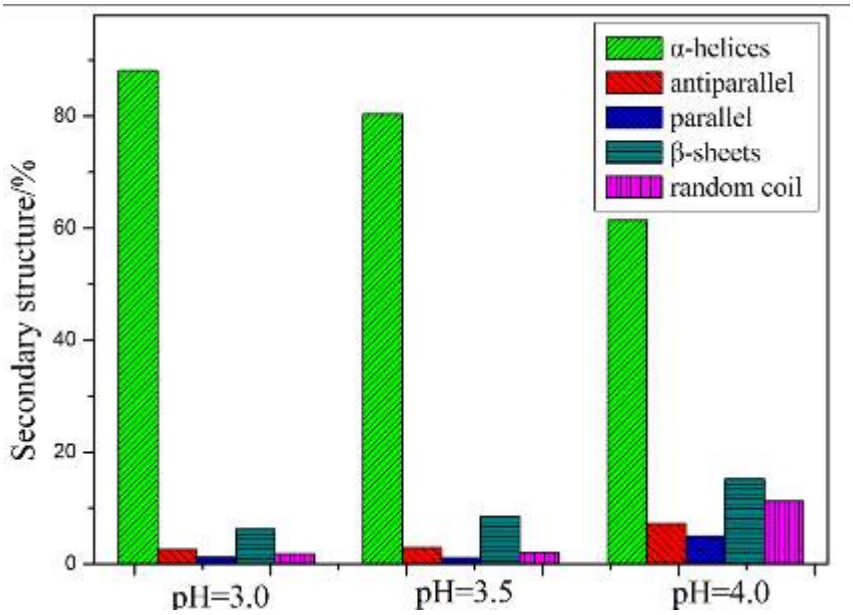

Figure 3. Different secondary structure level of native collagen in HAc solution at different pH

In addition, CD utilizes the differential absorption of left and right handed circular polarized light in an asymmetric environment to assess secondary structure. The amide bonds of a protein in highly ordered conformations such as α-helices, β-sheets, or the PP-II helix exhibit characteristic spectral line shapes due to the specific orientations of the chromophores contained in the protein backbone (ZHANG et al., 2014). Fig.3 shows the different secondary structure level of native collagen in HAc solutions at high different pH values, which are estimated from CD data using CDNN software. When the pH value is lower than 3.0, the secondary structure level of native collagen in HAc solutions can not be correctly measured, due to the disordered CD spectra obtained from collagen in HAc solution at low pH value. When the pH of HAc solution is 3.0, the α-helices fraction in collagen molecule is highest up to 89.2%. When the pH values are increased to 3.5 and 4.0, the α-helices fraction decreases to 80.4 and 61.4, respectively. However, the random coil fraction gradually increases with the increasing of the pH value. This was not surprising that α-helices in the peptide chains was formed by the hydrogen bonds between amide hydrogen and carbonyl oxygen. In aqueous systems, the amide hydrogen and carbonyl oxygen on the peptide bond can not only form internal hydrogen bonds, but also hydrogen bonds with water molecules, and the latter could lead to an increase in random coil fraction (HE et al., 2011).

HCl system

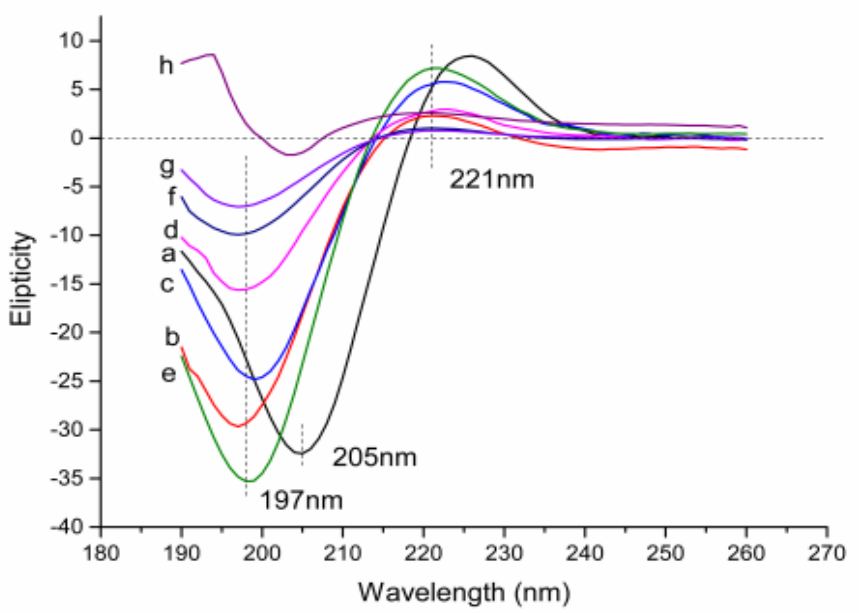

Figure 4. CD spectra of native collagen dissolved in different HCl solvents (a) pH=4.0, (b) pH=3.5, (c) pH=3.0, (d) pH=2.5 (e) pH=2.0, (f) pH=1.5, (g) pH=1.0,(h) pH=0.5.

Figure 4 shows the CD spectra of collagen in HCl sulution at different pH values. As expected, when the pH of the HCl solution is 3.0, collagen, in the absence of solution, gives a sinusoidal CD spectrum consisting of a positive band at 221 nm, and a negative band with a peak at approximately 197 nm, which is characteristic of the collagen triple-helical structure. When the pH is adjusted to 3.5 and 4.0, the peak position of the positive peaks shift from the initial 221 nm to 223 nm and 226 nm, and the negative peak position from the initial 198 nm are translated to 199 nm and 205 nm, respectively. The reason for the changes in the positive and the negative peaks were not clear. One possibility was that the increase of pH in HCl solution, resulted in the decrease of collagen concentration in the solution. When the pH value of HCl solution is lower than 2.0, there is a decrease in molar ellipticity at 221 and 197 nm as the pH of the solvent increased. When the pH value of HCl solution decrease to 1.0, the positive peak about 221 nm totally disappeares, which is the characteristic of the random conformation of polypeptide chains and is possibly due to the acid hydrolysis of collagen in the presence of excess H^+ in HCl solution. When HCl solvent at a certain pH to dissolve collagen can be combined with the basical molecules in the collagen molecules as a consequence of breaking the intermolecular and intramolecular ionic bonds as well as hydrogen bonds and other non-covalent crosslinking bonds in collagen molecules, and then destroy the non-helical crystalline region of collagen (SARKAR et al., 2014), the absence of covalent bonds between triple helices make collagen soluble in HCl solutions, thus allow its dissolution. Hence, the pH value of HCl solvent is critical to the preservation of collagen triple helices. This results demonstrated that the moderate acid environment at a proper pH value could appropriately loose the three-helix structure, which not only led to a better dispersion behavior, but also maximized the preservation of the integrity of the collagen structure. However, when the pH value of HCl solvent is too low, collagen is extensively denatured after the disolving process and collagen in solution preserved only a very small amount of triple helix even totally acidolysis to peptide.

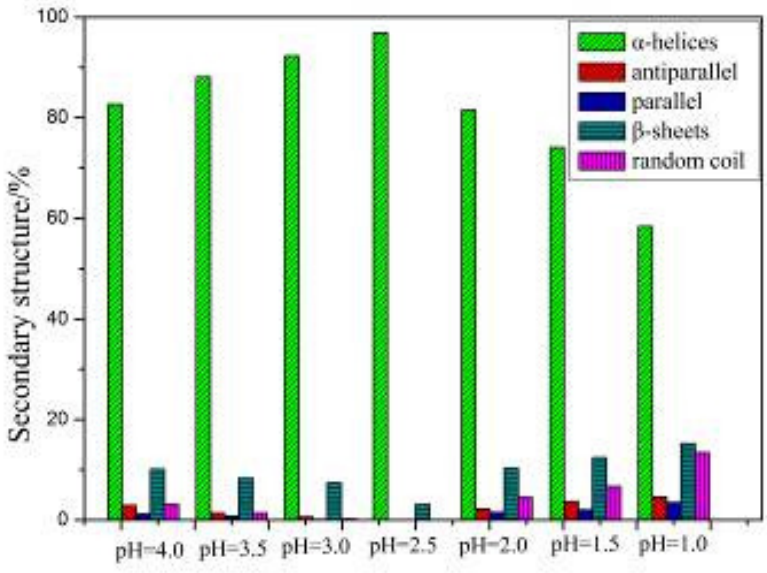

Figure 5. Different secondary structure level of native collagen in HCl solution at different pH

The composition of the secondary structure of the collagen in HCl system are presented in Fig.5, which shows that the helix content reach the highest up to 98.2% at pH=2.5, whereas the random crimp content is the lowest to zero. It is worth noting that the helix content estimatd with CDNN estimates is significantly higher than the data using equation (1) (85.69%). One possible reason was that there is a difference between the input conditions and the real experimental values. In addition, this experiment was based on the hypothesis that the collagen peptide chains were monomeric (GHEZZI et al., 2013), without considering more complex forms of polymerization. Meanwhile, ignoring the interaction of the free peptide chains in collagen molecules. As can be seen from Fig.5, the fractions of α-helices and random coil have not been always increased or reduced, but with a very similar trend for all seven samples at different pH. Especially to deserve to be mentioned, HCl molecules, unlike HAc, can be completely ionized in solutions and chloride ions has little interaction with collagen molecules. These considerations indicated that only hydrogen ions in HCl solutions could affect the dispersing process of collagen. However, it was pointed out that it was somewhat difficult to make direct comparison among secondary structure fractions of collagen in the different solvents. Indeed, for this kind of samples, secondary structure fractions depended on many factors such as experiment conditions and so on.

H_3PO_4 system

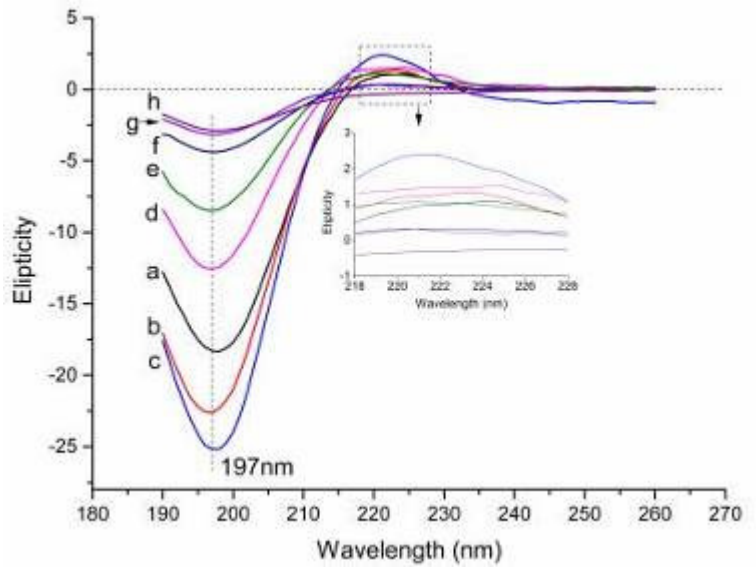

Figure 6. CD spectra of native collagen dissolved in different H_3PO_4 solvents (a) pH=4.0, (b) pH=3.5, (c) pH=3.0, (d) pH=2.5 (e) pH=2.0, (f) pH=1.5, (g) pH=1.0,(h) pH=0.5.

CD spectra of native collagen in H_3PO_4 solution are presented as in Fig.6. It can be seen from Fig.6 that native collagen spectra in H_3PO_4 solvent at different pH gave spectra that are more similar to the spectrum of collagen in HCl solution than to the collagen in HAc solution. However, there was a significant difference between the solution spectra in H_3PO_4 and HCl at higher pH values (between 3.0 and 4.0). That was collagen in H_3PO_4 at pH =3.0 had a positive ellipticity at wavelength of ~221 nm and a negative band at wavelength of ~197 nm, which was accepted as an indicator of triple helical structure. According to equation (1), the fraction of triple helix can be caculated as 88.25% for collagen in H_3PO_4 solvent at pH 3.0, 82.68% at pH 3.5, and 78.32% at pH 4.0. But if the pH value of H_3PO_4 solution is lower than 1.0, for example, at pH =0.5, denatured collagen only exhibits the negative peak, which is characteristic of randomly arranged a-chains. As it was known, the diluted H_3PO_4 system (0.06M) was a commonly used to dissolved collagen for preparing collagen-based biomaterials, pH of that can be calculated to be about 2.1. Accordingly, in Fig.7 collagen sample at pH 2.0 shows a weak positive and negative peak at 221 nm and 197nm respectively, which are the indication of the triple helical structure existence about 77.6% (using equ.1). Therefore, the calculated triple helical fraction by our work for the first time indeed provide information that 0.06M H_3PO_4 can be a better solvent for dissolving collagen.

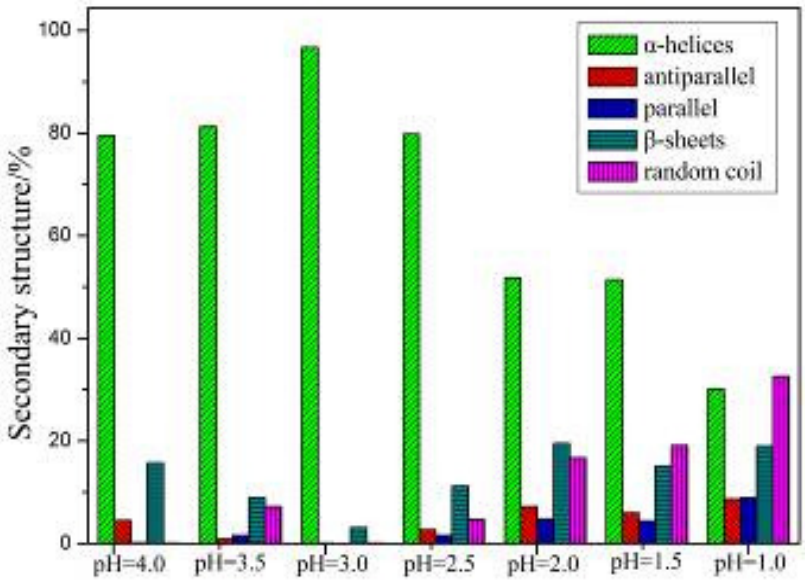

Figure 7. Different secondary structure level of native collagen in H_3PO_4 solution at different pH

Figure 7 shows the composition of the secondary structure of the collagen in the H_3PO_4 system using CDNN. From Fig.7, we still can see that caculations at pH 3.0 gave more α-helices fractions compared to the other pH values, but with very similar results for the other two solvent systems. In particular CD spectra enabled to calculate an amount ofα-helices of 97% in the collagen solution produced from H_3PO_4 at pH 3.0. It is important to noted that collagen is easily activated by the phosphorylation reagent, and therefore the absorbed H_3PO_4 molecule on the side chain of the collagen can further penetrate into the triple helix of the collagen molecule, which are beneficial to causing the original chain to swell, resulted in a increases of the length of collagen molecular chain. One of the difference between H_3PO_4 and HAc is that its ionic strength is large and the increase in ionic strength can enhance the interaction of the polymers and weaken the interactions between polymers and solvent (PERDIGAO et al., 1996).

CONCLUSIONS

Type I collagen solution from weak inorganic acid solvents can be prepared to designe kinds of collagen-based biomaterials, but the effect of the common inorganic acid solvents on the secondary structure of collagen has not been reported in detail till now. In this study, we investigated the structure of collagen in different three inorganic acid solvents using CD spectroscopy in details. The experimental results were compared with the structure of native collagen, and data evaluation procedures to determine the α-helices fraction in the collagen solution were established. We demonstrated that collagen was completely denaturation in HAc solvents with low pH (< 3.0). Whereas collagen was completely denaturation in both HCl and H_3PO_4 solvents with low pH (< 1.5), the native structure was basically preserved with high pH (>3.0), and partially preserved with pH between 1.5 and 3.0. It should be noted that the moderate acid environment at a proper pH value can appropriately loose the three-helix structure, which can not only lead to a better dispersion behavior, but also maximize the preservation of the integrity of

the collagen structure. Then, CDNN programs was performed to test the effect of different solvents on the secondary structure of collagen, all of the three solvents gave the fractions of different secondary structures which are almost identical to each other and relatively independent of the pH values. When the pH of the solution was lower than 3.0, too much H^+ would force collagen molecules irreversibly unwinded into random polypeptide chains, and the unfolded fraction increased. By comparing fractions of different secondary structures of collagen in different solvents, we demonstrated that it is not just the pH value, but also the kind of inorganic acid will result with certain amount of the secondary structure in the end product and sacrifice over the native structure. Our results have provided clues to the development of a better solvent for type I collagen in the future, which promote the widespread use of collagen into biomedical applications such as suturing, wound dressing and wound closure.

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RESUMO: Habitualmente, os ácidos inorgânicos fracos têm sido usados para dispersar colágeno como solventes verdes para fabricar tipos de biomateriais o tempo todo. No entanto, é uma questão aberta quanto o processo de dissolução preserva ou altera a estrutura nativa do colágeno até agora. Aqui, examinamos o efeito de três solventes diferentes (HAc, HCl, H3PO4) nas estruturas secundárias de colágeno, com base em espectros de dicroísmo circular (CD) de colágeno de 185 a 260 nm em conjunto com programas CDNN. Descobrimos que o colágeno preservou quase completamente sua estrutura helicoidal tripla nos três ácidos inorgânicos a pH = 3,0 ou mais, o que demonstrou que foi a concentração de H+ livre nas três soluções acima cujo pH foi fixado em 3,0 que pode manter uma boa quantidade de carga superficial sobre as partículas coloidais de colágeno e destrói adequadamente a estrutura de três hélices, o que não só pode levar a um melhor comportamento de dispersão, mas também maximizar a preservação da integridade da estrutura de colágeno. Embora as frações de tipos de estruturas secundárias em colágeno fossem diferentes de todos os três solventes com base em dados CDNN, que deram resultados muito semelhantes entre si. Estes resultados foram testados pela primeira vez neste trabalho para estimar as estruturas secundárias para o colágeno nos diferentes ácidos inorgânicos comuns, o que fornece uma nova alternativa para solventes de colágeno verdes para preparar compósitos à base de colágeno com a estrutura helicoidal tripla para engenharia de tecidos.

PALAVRAS-CHAVE: Colágeno tipo I. Ácido inorgânico. Estrutura tripla helicoidal. CD

REFERENCES

ACKERMAN, M. S.; BHATE, M.; SHENOY, N.; BECK, K.; RAMSHAW, J. A.; BRODSKY, B. Sequence dependence of the folding of collagen-like peptides single amino acids affect the rate of triple-helix nucleation. **J Biol Chem.** v. 274, p. 7668-7673, 1999. https://doi.org/10.1074/jbc.274.12.7668

BURCK, J.; ARAS, O.; BERTINETTI, L.; ILHAN,C. A.; ERMEYDAN, M. A.; SCHNEIDER, R.; ULRICH, A. S.; KAZANCI, M. Observation of triple helix motif on electrospun collagen nanofibers and its effect on the physical and structural properties. **Journal of Molecular Structure,** v. 1151, p. 73-80, 2018. https://doi.org/10.1016/j.molstruc.2017.09.030

BURCK, J.; HEISSLERR, S.; GECKLE, U.; ARDAKANI, M. F.; SCHNEIDER, R.; ULRICH, A. S. Resemblance of Electrospun Collagen Nanofibers to Their Native Structure. **Langmuir, v.** 29, p. 1562-1572, 2013. https://doi.org/10.1021/la3033258

DIPPOLD, D.; CAI, A.; HARDT, M.; BOCCACCINI, A.R.; HORCH, R.; BEIER, J. P.; SCHUBERT, D. W. Novel approach towards aligned PCL-Collagen nanofibrous constructs from a benign solvent system. **Materials Science and Engineering: C.** v. 72, p. 278-283, 2017. https://doi.org/10.1016/j.msec.2016.11.045

DONG, Z. X.; WU, Y. Q.; CLARK, R. L. Thermodynamic Modeling and Investigation of the Formation of Electrospun Collagen Fibers. **Langmuir.** V. 27, p. 12417-12422, 2011. https://doi.org/10.1021/la201859c

GHEZZI, C. E.; RISSE, P.; MARELLI, B. An airway smooth muscle cell niche under physiological pulsatile flow culture using a tubular dense collagen construct. **Biomaterials,** v. 34, p. 1954-1966, 2013. https://doi.org/10.1016/j.biomaterials.2012.11.025

HE, L.; MU, C.; SHI, J. Modification of collagen with a natural cross-linker, procyanidin. **International Journal of Biological Macromolecules,** v. 48, p. 354-359, 2011. https://doi.org/10.1016/j.ijbiomac.2010.12.012

PERDIGAO, J.; LAMBRECHTS, P.; VAN M. B. Morphological field emission-SEM study of the effect of six phosphoric acid etching agents on human dentin[J]. **Dent Mater,** v. 12, p. 262-271, 1996. https://doi.org/10.1016/S0109-5641(96)80033-9

QI, P. W.; ZHOU, Y. Y.; WANG, D. L.; HE, Z. H.; LI, Z. J. A new collagen solution with high concentration and collagen native structure perfectly preserved. **RSC Adv.,** v. 5, p. 87180-87186, 2015. https://doi.org/10.1039/C5RA14423J

RASHTRAPAL, Z.; MUKTY, S. Nanofibrous poly(3-hydroxybutyrate-co-3-hydroxyvalerate) /collagen/graphene oxide scaffolds for wound coverage. **Materials Science and Engineering: C.** v. 80, p. 129-134, 2017. https://doi.org/10.1016/j.msec.2017.05.138

SRIPRIYA, R.; KUMAR, R.; BALAJI, S.; KUMAR, M. S.; SEHGAL, P. K. Characterizations of polyanionic collagen prepared by linking additional carboxylic groups. **React Funct Polym.** v. 71, p. 62-69, 2011. https://doi.org/10.1016/j.reactfunctpolym.2010.11.005

SCHARNWEBER, D.; BORN, R.; FLADE, K.; ROESSLER, S.; STOELZEL, M.; Worch H. Mineralization behaviour of collagen type I immobilized on different substrates. **Biomaterials,** v. 25, p. 2371-2380, 2005.

https://doi.org/10.1016/j.biomaterials.2003.09.025

SARKAR, B.; OLEARY, L. E.; HARTGERINK, J. D. Self-Assembly of Fiber-Forming Collagen Mimetic Peptides Controlled by Triple-Helical Nucleation [J]. **Journal of the American Chemical Society,** v. 136, p. 14417-14424, 2014. https://doi.org/10.1021/ja504377s

SUN, H. L.; ZHU, F.; HU, Q. G.; KREBSBACH, P. H. Controlling stem cell-mediated bone regeneration through tailored mechanical properties of collagen scaffolds. **Biomaterials,** v. 35, p. 1176-1184, 2014. https://doi.org/10.1016/j.biomaterials.2013.10.054

TANG,Y. F.; CHEN, L.; ZHAO,K.; WU, Z.X.; WANG,Y.; TAN, Q.C. Fabrication of PLGA/HA (core)-collagen/amoxicillin (shell) nanofiber membranes through coaxial electrospinning for guided tissue regeneration. **Composites Science and Technology.** v.125, p.100-107, 2016. https://doi.org/10.1016/j.compscitech.2016.02.005

TRONCI, G.; DOYLE, A.; RUSSELL, S. J.; WOOD, D. J. Triple-helical collagen hydrogels via covalent aromatic functionalisation with 1,3-phenylenediacetic acid. **J. Mater. Chem. B,** v. 1, p. 5478-5488, 2013. https://doi.org/10.1039/c3tb20218f

ZHANG, X. J.; ZHANG, Y. M.; WANG, Z. Q.; LI, Q. J.; LI, B. X. The effect of non-growth factors on chondrogenic differentiation of mesenchymal stem cells. **Cell Tissue Bank.** v. 15, p. 319-327, 2014. https://doi.org/10.1007/s10561-013-9403-z

ZHOU, Y. Y.; LIU, Q.; ZHAO, Z. H.; WANG, W. Q.; ZHENG, L. G.; LI, Z. J. Preparation and Characterization of Phosphorylated Collagen and Hydroxyapatite Composite as a Potential Bone Substitute. **Chem. Lett.,** v. 42, p. 83-85,2013. https://doi.org/10.1246/cl.2013.83

ZHOU, Y. Y.; YAO, H.C.; WANG, J. S.; WANG, D. L.; LIU, Q.; LI, Z. J. Greener synthesis of electrospun collagen/hydroxyapatite composite fibers with an excellent microstructure for bone tissue engineering. **Int. J. Nanomed.,** v. 10, p. 3203-3215, 2015.