NOVEL METHOD FOR PREPARING FISH COLLAGEN GELS WITH EXCELLENT PHYSICOCHEMICAL PROPERTIES VIA THE DEHYDRATION OF ETHANOL

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Abstract. Fish collagen has been considered to be an alternative for mammalian collagen, however, physicochemical properties of fish collagen-based materials such as gels are so far not adequate for actual application. In the present study, we prepared two types of fish collagen gels with sufficient elasticity: i) dehydrated fibrillogenesis collagen gels (DFCG), which were fabricated via collagen self-assembly followed by immersion in different concentrations of ethanol solutions, and ii) dehydrated cross-linking collagen gels (DCCG), which were fabricated via collagen self-assembly and simultaneous cross-linking followed by immersion in ethanol solution. Furthermore, the physicochemical properties of DFCG and DCCG were analyzed by atomic force microscopy, differential scanning calorimetry and dynamic viscoelastic measurements. The microstructure of DFCG was consisted of characteristic D-periodic collagen fibrils and insusceptible of ethanol concentrations (20-100% (v/v)). However, the thermal stabilities and mechanical properties of DFCG distinctly increased with the increase of ethanol doses, possibly ascribing that ethanol with higher polarity might dehydrate partial free water of DFCG and strengthen the interactions of hydrogen bond. Especially, for the gel treated by 100% (v/v) ethanol, T_d increased by 32.7 °C and G' was 55-folds than those of undehydrated gel (43.1 °C and 239 Pa). In the case of DCCG, the formation of collagen fibrils was depended on the concentrations of *N*-hydroxysuccinimide adipic acid derivative (NHS-AA), which was converted to [NHS-AA]/[NH_2] ratios (calculated by the [active ester group] of NHS-AA and [ε-NH_2] of lysine and hydroxylysine residues of collagen). As the ratio= 0.05, the characteristic D-periodic fibrils were still formed and the treatment of 60% (v/v) ethanol increased the T_d (52.5 °C) and G' (7388 Pa) values of the gel compared with those of uncross-linked gel (49 °C and 2064 Pa, respectively), majorly resulting from the effects of covalent cross-linking bonds and hydrogen bonds. However, when the ratio= 0.2, the collagen self-assembly was intensively inhibited and the dehydration of free water within gel structure in the absence of thick fibrils led to the shrinkage of the gel and an obvious decrease in T_d (42 °C) and G' (432 Pa). Although the [NHS-AA]/[NH_2] ratio further increased to 0.8, the thermal stability and elasticity of the gel enhanced mildly suggesting that the presence of thick fibrils formed via the self-assembly was significantly crucial for reinforcing the gels.

Key words: fish collagen gel, self-assembly, cross-linking, thermal stability, dynamic viscoelasticity

1 Introduction

Hydrogels based on the self-assembly of natural proteins such as silk fibroin, elastin and collagen, which are three-dimensional, hydrophilic, polymeric networks capable of absorbing large amounts of water or biological fluids, have been widely applied to biomedical areas due to their inherent biodegradability and biocompatibility, tunable rigidity and toughness, flexible environmental responsiveness and abundant resources. [1-5] Collagen, as the major constituent of the extracellular matrix, is an attractive candidate for biomedical applications.[2,6] Generally, collagen such as type I collagen with an approximate molecular weight of 300 kDa, which is composed of two α1 chains and one α2 chain in a right-handed triple helix, can undergo self-assembly via noncovalent bonds including hydrogen bonding, hydrophobic interaction, and electrostatic interaction to form the gels with an entangled network of thick D-periodic fibrils under physiological conditions.[1,7-9] Recently, the fish collagen has been a potential alternative for the collagen from the mammals due to its

natural abundant resources, high yield, and absence of infections such as bovine spongiform encephalopathy, transmissible spongiform encephalopathy and foot-and -mouth disease.[10-12] However, the biomedical applications of the gels based on fish collagen are limited because of low denaturation temperature (T_d) and poor gelling properties, etc. [13,14] For example, the T_d values of shark, salmon and grass carp collagen solution are approximately 30, 19 and 36 °C, respectively, which result in the low T_d values of their fibrillogensis gels at 37, 28 and 39 °C, respectively.[13,15,16] To effectively reinforce the physicochemical properties of fish collagen gels has attracted more attention of researchers.

The physiochemical properties of fibrillogenesis collagen gels depend on the thickness, length, and three-dimensional density of the collagen fibrils.[17,18] In the last decades, a variety of chemical and physical methods based on different principles to reinforce the collagen gels were broadly developed. Firstly, there are many active amino residues (-NH_2) and/or carboxyl residues (-COOH) on the side chains of collagen molecule, which can be covalently cross-linked by adding cross-linking agents. Increasing the cross-linking number modifies the network architecture, thus reducing the distance between joints and creating denser and stiffer networks.[18] In our previous study, we have investigated the physiochemical properties of fish collagen gels prepared by collagen self-assembly and simultaneously cross-linking with N-hydroxysuccinimde adipic acid derivative (NHS-AA), which could form long bridges between contiguous ε-NH_2 of collagen molecules with low toxicity.[7,19] The results showed that the introduction of NHS-AA could inhibit the collagen self-assembly and the T_d and G' of the gel (47 °C and 420.7 Pa, respectively) increased by 8 °C and 198.3 Pa than those of uncross-linked gel due to the formation of abundant covalent cross-linking bonds. Secondly, the blending of collagen with natural and synthetic polymers (*e.g.*, chitosan, hyaluronic acid, alginate and poly(ethylene glycol), etc.) were also used to reinforce the gels by the introduction of non-covalent bonds including hydrogen bonds, electrostatic interactions, hydrophobic interactions.[20-24] Thirdly, the effects of physical parameters such as compressing, extension, and irradiation (*e.g.*, UV irradiation, and gamma-ray irradiation and electron-beam irradiation, etc.) have been widely used to induce the intermolecular cross-linking of collagen.[17,25,26] Nevertheless, the mechanical properties of the cross-linked collagen gels by chemical and physical methods are still not adequate to meet the demand for application, especially in the clinical fields of orthopedics, cardiovascular surgery, and neurosurgery.[27]

It is important to note that the water enwrapped in the network structure plays a crucial role for reinforcing thermal stability and mechanical properties of the collagen gels because the water can not only affect the stability of polymeric backbone by non-covalent bonds, but also influence three-dimensional density of collagen fibrils. Recently, Mori et al. constructed the collagen gels with sufficient mechanical strength and elasticity by EDC/gamma ray cross-linking and sequentially heating for 30 min at 80 °C.[17] They found that the density of collagen fibrils significantly increased due to the reduction of water during the heating process and the collagen gels sequentially cross-linked by 125 mM EDC after heating exhibited the highest G' value (7010 Pa), which was approximately 158-folds higher than that of uncross-linked gel without heating (G', 44.1 Pa). However, there were partial collagen fibrils that were not cross-linked and were easily denatured during the heating process because of its low thermal stability. Furthermore, another method that was proceed by repeating the cycle of gel formation, cross-linking with EDC, freeze-drying, and heating, was developed to prepare collagen gels with high mechanical strength in the previous study described by Mori et al.[27] The collagen concentration could be significantly increased using repeated gel-formation inside the micropores of lyophilized collagen sponge, leading to the network structure consisting of densely packed fibrils. Especially, the collagen gel prepared by repeating the cycle for 3 times in which the heating was done only once after 125 mM EDC cross-linking in the first cycle (3-cycled collagen gel) exhibited an extremely high G' value (40200 Pa), which was approximately 911-folds higher than uncross-linked collagen gel. However, when 3-cycled collagen gel was sequentially heated once, the G' value decreased to 38500 Pa, attributing

that the uncross-linked collagen was denatured by heating. Although the dehydration by heating and gel-formation/lyophilization cycle can effectively reinforce the gels, it is worthy to note that their serious effects would denature partial collagen and break the original network structure of the gels consisting of periodic fibrils. The water-holding capacity of the gels depends on the capillary, osmotic and hydration interactions.[28] The bound water is important to sustain the stability of the collagen fibrils by maintaining the triple helix structures with the hydrogen bonding with collagen, in which the loosely-bound water can be removed by chemical dehydration with polar solvents such as methanol, ethanol and *n*-propanol.[29-31] Moreover, the replacement of polar solvents to the water could also establish stronger hydrogen bonds between the solvent and collagen to enhance the stability of network structure. Therefore, to study the effects of the dehydration of polar solvents on the preparation of the fish collagen gels with excellent physiochemical properties are extremely significant for expanding the medical applications of fish collagen.

In the present study, in order to establish a method to prepare the fish collagen gels with excellent physiochemical properties, we prepared two types of fish collagen gels: i) dehydrated fibrillogenesis collagen gels (DFCG), which were fabricated via collagen self-assembly followed by immersion in different concentrations of ethanol solutions; and ii) dehydrated cross-linking collagen gels (DCCG), which were fabricated via collagen self-assembly and simultaneously cross-linking followed by immersion in ethanol solution. Then, the microstructure, thermal stabilities, and dynamic viscoelasticity of collagen gels were analyzed.

2 Materials and methods

2.1 Materials

Southern catfish skin was purchased from a local market in Chengdu and was fleshing prior to cutting into small pieces. Then, the fish skins were throughly washed using deionized water and stored at -20 °C until used. The pepsin collagen was extracted according to the method as described in a previous paper.[7] Briefly, the supernatants were extracted from the fish skins using 0.5 M acetic acid containing 1% (w/w) pepsin (EC 3.4.23.1, 1, 1:10000, Sigma–Aldrich, MO, USA) at 4 °C for 3 days after degreasing and decoloring procedures. Then, the precipitates were collected via adding 0.7 M NaCl into the supernatants and dissolved in 0.1 M acetic acid prior to dialyzed against 0.01 M acetic acid for 4 days. Finally, the collagen solution was finally lyophilized by a freezer dryer (Alpha 1-2 LDplus, Christ, Osterode, Germany). The purity and molecular weight of collagen were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) measurement. The SDS-PAGE pattern displayed two α bands (110 kDa for α1 and α2) and one β band (250 kDa), which are typical bands of type I collagen. NHS-AA was synthesized according to the method previously described by Chen et al.[32] In brief, 2 M NHS and 1 M adipic acid were mixed in acetone for 15 min. Then, 2.2 M EDC was added into the mixed solution and reacted for 1 day at 20 °C. The acetone was removed using rotary evaporation prior to drying in vacuum at 50 °C. The structure of synthesized NHS-AA was evaluated by a fourier transform infrared spectrometer (FTIR) (Nicolet iS 50, Thermo Fisher Scientific, Waltham, USA). The FTIR spectra showed the typical peaks of ester group, which were at 1065, 1210 and 1739 cm^{-1}.

2.2 Preparation of DFCG and DCCG

Lyophilized collagen was dissolved in 10 mM phosphate-buffered saline solution (PBS, pH 7.4) containing 125 mM NaCl and centrifuged at 10000×g for 10 min at 4 °C to remove bubbles. For the preparation of DFCG, 5 mg/mL collagen solution was incubated at 30 °C for 5 h and then immersed in different concentrations of ethanol solutions (0, 20, 40, 60, 80 and 100% (v/v), respectively) for

24 h at a liquor ratio of 1:10 (w/v). Ethanol solutions were changed once every 2 h. The obtained gels were named DFCG(0), DFCG(20), DFCG(40), DFCG(60), DFCG(80) and DFCG(100). In the case of preparing DCCG, the collagen self-assembly and cross-linking of NHS-AA were simultaneously carried out, and then the gels were dehydrated by the treatment of ethanol solution. NHS-AA concentrations were converted to active ester groups of NHS-AA as molar ratios to the calculated ε-NH_2 in hydroxylysine and lysine residues ([NHS-AA]/NH_2).[7] 0.044, 0.175, 0.35 and 0.7 mM NHS-AA solubilized in dimethyl sulfoxide (DMSO) were respectively mixed with PBS-solubilized collagen solutions to obtain a series of 5 mg/ mL collagen solutions with different [NHS-AA]/NH_2 ratios (0.05, 0.2, 0.4 and 0.8, respectively). Both uncross-linked and cross-linked samples were immediately incubated at 30 °C for 5 h to gel and immersed in 60% (v/v) ethanol solution at a liquor ratio of 1:10 (w/v) for 24 h. Ethanol solutions were replaced once every 2 h. The obtained collagen gels were called DCCG(0), DCCG(0.05), DCCG(0.2), DCCG(0.4) and DCCG(0.8).

2.3 Atomic force microscopy (AFM) measurements

The slices cut from DFCG and DCCG by scissors were dropped onto freshly cleaved mica substrates. Subsequently, the samples were air dried in a desiccator for 72 h at 20 °C. The surface topography of DFCG and DCCG was detected by an atomic force microscope (Dimension 3100 Nanoscope IV, Shimadzu Corporations' SPM 9600, Kyoto, Japan). The height images were recorded in the soft tapping mode using silicon cantilevers with a force constant of 42 N/m and a scanning rate of 1 Hz. Every image was obtained at three various points to confirm the consistency of the observed morphologies.

2.4 Differential scanning calorimetry (DSC) measurements

DSC measurements was conducted to evaluate the thermal stability of collagen gels using DSC 200PC (Netzesch, Selb, Germany). Approximate 8 mg DFCG and DCCG were accurately weighed and sealed in an aluminum pans while a sealed aluminum pan containing same solvent with the sample was used as the control. The temperature ranged from 30 to 90 °C with a heating rate of 5 °C/min under a nitrogen atmosphere. The transition temperature of endothermic peak in the DSC curves was taken as the denaturation temperature (T_d) of collagen gels. [33]

2.5 Dynamic viscoelasticity measurements

DFCG and DCCG disks with a diameter of 35 mm were prepared for dynamic viscoelasticity measurements, which were carried out using a Rheometer System Mars III (Hakke, Karlsruhe, Germany) with a parallel plate (φ = 35 mm) at a constant strain of 2% within the linear viscoelastic region. The temperature was controlled at 20 °C using a Peltier temperature controller with an accuracy of ± 0.1 °C by a circulatory water bath. The storage modulus (G') and loss modulus (G'') were recorded as a function of frequency at a range from 0.01 to 10 Hz. Triplicate experiments were conducted for each sample and mean value was calculated.

3 Results and discussion

3.1 Appearance of the gels

Appearance of two types of collagen gels before and after dehydration of ethanol is shown in Fig. 1. As shown in Fig. 1, the fibrillogenesis gels without the dehydration of ethanol looked opalescent and shrank slightly to form turbid gels after dehydration using different concentrations of ethanol.

For the gels prepared by the self-assembly and simultaneously cross-liking, the appearance was dependent on the concentration of NHS-AA. Without the dehydration of ethanol, the appearance of gels changed from opalescent state to transparent state. The results were in agreement of those of the previous study.[7] Upon the dehydration of ethanol, DCCG shrank slightly when the [NHS-AA]/NH_2 ratio lower than 0.05, while it shrank distinctly and changed opalescent when the [NHS-AA]/NH_2 ratio higher than 0.2, compared with the untreated collagen gels.

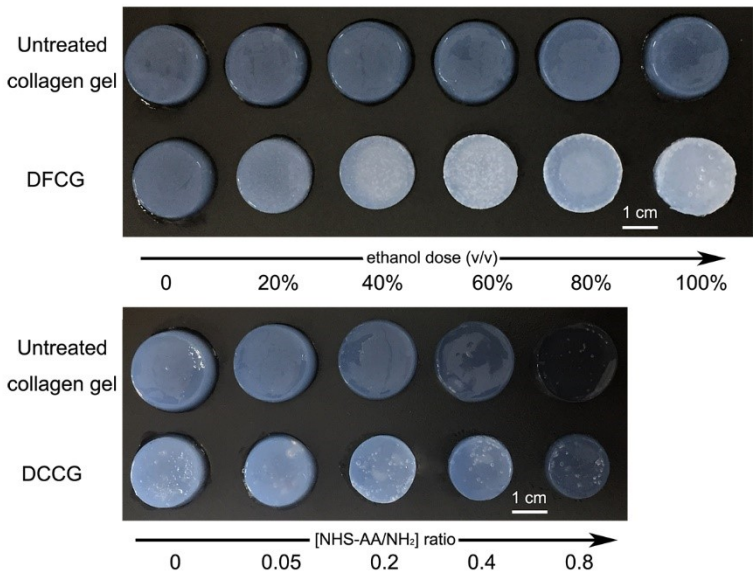

Fig. 1. Appearance of the DFCG and DCCG. Untreated and treated collagen gels are shown in the upper and lower rows, respectively.

In order to elucidate the variation on appearance of the gels, the microstructure of the gels was explored. The AFM images of DFCG and DCCG are shown in Fig. 2. As shown in Fig. 2A, the network structure of DFCG(0) was consisted of abundant D-periodic fibrils, which were formed by the self-assembly of collagen molecules through hydrogen bonding, hydrophobic and electrostatic interactions.[6,9] The increase of ethanol concentration almost not changed the microstructure of DFCG, in which the diameter of fibrils was similar. The explanation was probably that the structure consisted of thick D-periodic fibrils was stable and different concentrations of ethanol only replaced free water and partial bound water within the structure of the gels owing to its higher polarity. Upon the addition of NHS-AA, the collagen self-assembly was slightly inhibited when the [NHS-AA]/NH_2 ratio lower than 0.05, but was distinctly hindered when the [NHS-AA]/NH_2 ratio higher than 0.2.[7] For DCCG(0.5), the diameter of fibrils slightly decreased (Fig. 2B), but the gel was still constructed by the collagen self-assembly and had good stability to resist the dehydration of ethanol. However, as the [NHS-AA]/NH_2 ratio exceeded 0.2, the formation of gels was predominately depended on the covalent bonds between collagen molecules and NHS-AA, and exhibited an entangled network of tenuous fibrils (Fig. 2B), which could not resist the dehydration of ethanol. Furthermore, the shrinkage of the gels resulted in higher density of fibrils within the gels, which would hinder the transmittance of light and make DCCG(0.8) to become turbid.

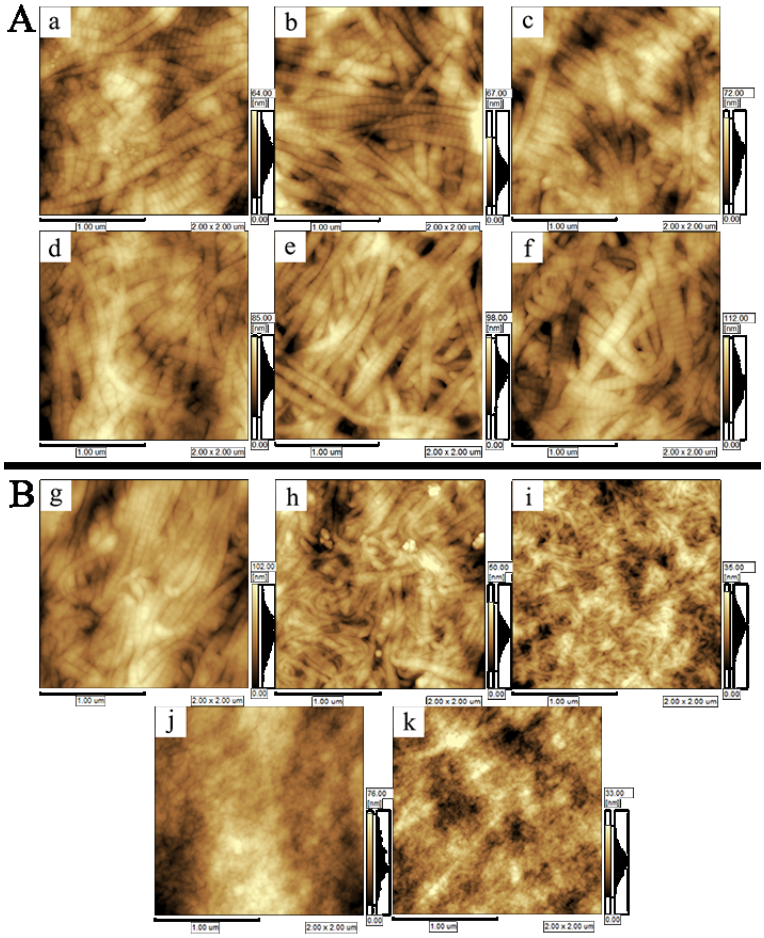

Fig. 2. AFM images of DFCG (A) and DCCG (B). The images from a to f are the fibrillogenesis collagen gels treated under the doses of ethanol of 0, 20, 40, 60, 80 and 100% (v/v), respectively. The images from g to k are the collagen fibrillogenesis and simultaneous cross-linking of NHS-AA treated by 60% (v/v) ethanol with the [NHS-AA]/NH_2 ratios of 0, 0.05, 0.2, 0.4 and 0.8, respectively.

3.2 DSC measurements

The potential application of fish collagen materials was restricted owing to its low thermal stability. The triple helix structure of collagen molecules is stabilized by intra-chain hydrogen bonds and cross-linking bonds. The thermal transition curves of DFCG and DCCG as detected by DSC are shown in Fig. 3. Both the self-assembly and cross-linking could improve the thermal stability of collagen. For DFCG, the dehydration of ethanol promoted the increase of thermal stability, which might be attributed that the different concentrations of ethanol replaced the water in the gels and stronger hydrogen bonds between collagen molecules and ethanol molecules were formed due to higher polarity of ethanol than water. Therefore, the T_d values increased with increasing ethanol concentrations, especially, the T_d value of DFCG(100) distinctly increased by 32.7 °C compared with that of DFCG(0) (Fig. 3A). For the collagen gels formed by the self-assembly and simultaneously cross-linking, the effects of NHS-AA on the thermal stability were similar to the results in our previous study. When the [NHS-AA]/NH_2 ratio was 0.05, the collagen self-assembly was slightly inhibited and the introduction of cross-linking bonds reinforced the structure of gels; as the [NHS-AA]/NH_2 ratio increased to 0.2, the collagen self-assembly was seriously hindered, but the cross-linking degree was low resulting in the decrease of thermal stability; as the [NHS-AA]/NH_2 ratio higher than 0.2, the increase of covalent cross-linking bonds promoted the increase of thermal

stability. Furthermore, compared with the gels without the dehydration of ethanol, the thermal stability of all DCCG samples increased. Interestingly, DCCG(0.05) exhibited a higher T_d value (52.5 °C) , however, the T_d value of the gel formed at a [NHS-AA]/NH_2 ratio of 0.05 decreased by 2.7 °C than that of the gel formed at a [NHS-AA]/NH_2 ratio of 0.8 without the dehydration of ethanol. This phenomenon might be originated that the triple helix structure of partial collagen molecules denatured by 60% ethanol solutions when NHS-AA dosages was higher ([NHS-AA]/NH_2 ≥ 0.4). Consequently, the presence of thick D-periodic fibrils was more significant than the cross-linking for preparing the collagen gels with high thermal stability for application by the dehydration of ethanol.

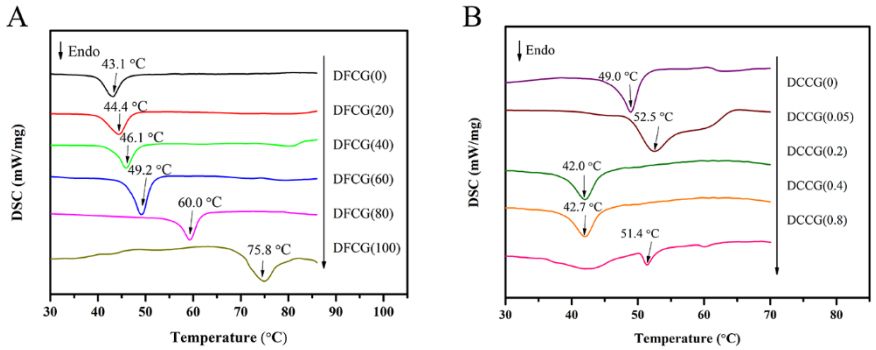

Fig. 3. DSC curves of DFCG (A) and DCCG (B). The curves from a to f are the fibrillogenesis collagen gels treated under the doses of ethanol of 0, 20, 40, 60, 80 and 100% (v/v), respectively. The curves from g to k are the collagen fibrillogenesis and simultaneous cross-linking of NHS-AA treated by 60% (v/v) ethanol with the [NHS-AA]/NH_2 ratios of 0, 0.05, 0.2, 0.4 and 0.8, respectively.

3.3 Dynamic viscoelasticity measurements

The technique of dynamic viscoelasticity measurement was frequently used capable of resolving the structural properties of materials into an elastic and a viscous response using the parameters of G' and G'', respectively.[34] The values of G' and G'' of two types of collagen gels as a function of frequency at a range from 0.01 to 10 Hz are displayed in Fig. 6. For both DFCG and DCCG, the values of G' and G'' were nearly constant over the region of dynamic frequency and the values of G' were higher than that of G'', characterizing high elasticity of these two types of collagen gels.[19,35] The G' value at frequency of 1 Hz was assumed to be the shear modulus (G) value. The number of network points per cubic meter (v) might be calculated using the following formula:[17,27]

$$G = vkT$$

where T is the absolute temperature (293 K) and k is the Boltzmann constant (1.38×10^{-23} J/K).

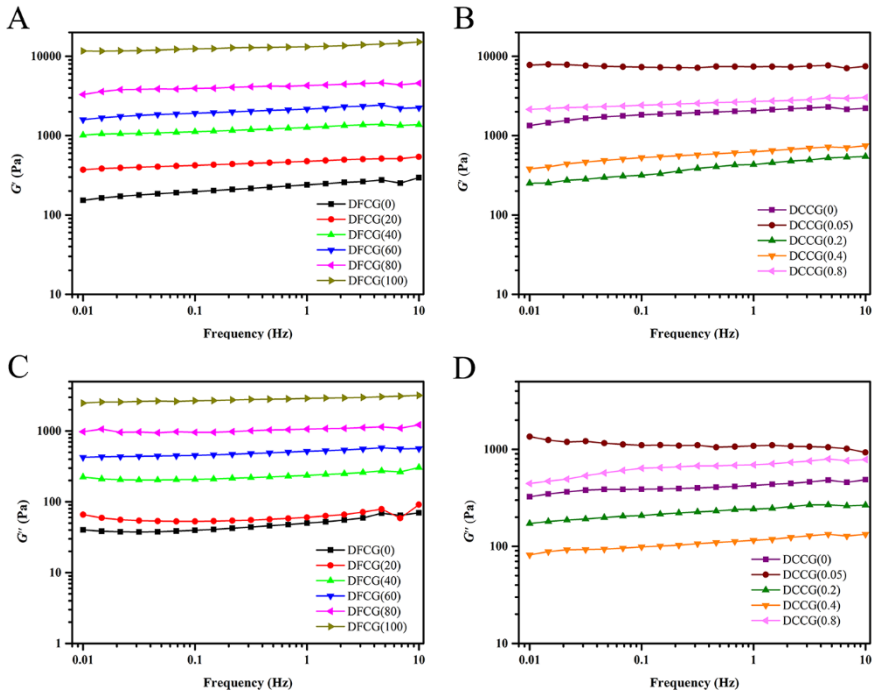

Fig. 4. The storage modulus (G') and loss modulus (G'') of the fibrillogenesis collagen gels dehydrated by ethanol at different concentrations (A and C) and the collagen fibrillogenesis and simultaneous cross-linking of NHS-AA treated by 60% (v/v) ethanol (B and D), respectively.

The values of *G*' and *G*'' at frequency of 1 Hz as well as *v* value are summarized in Table 1. As shown in Fig. 6 and Table 1 and Table 2, the *G*', *G*'' and *v* values of DFCG increased with the increase of ethanol concentrations. In the case of DFCG, ethanol might majorly replace free water and partial bound water within the gel structure and stronger hydroxy bonds among ethanol, bound water and collagen molecules were established. Therefore, the network structure of the gels was reinforced, which was indicated by increasing *v* values. Furthermore, slightly shrinkage of the gels owing to the exchange between ethanol and free water also improved the density of fibrils. As a result, when ethanol concentration was 100%, the values of *G*' and *G*'' were 13142.6 and 2893.8 Pa, respectively, which were approximately 55-folds than those of DFCG(0). For DCCG, the influence of NHS-AA dosages on dynamic viscoelasticity of the gels was in agreement of the results of DSC measurements. When the [NHS-AA]/[NH_2] ratio was 0.05, the *G*' value was approximately 2-folds higher than that of DCCG(0) due to the introduction of the covalent cross-linking bonds in the presence of thick fibrils. As the [NHS-AA]/[NH_2] ratio higher than 0.2, the collagen self-assembly was distinctly inhibited. Although there were abundant covalent cross-linking bonds among collagen molecules, free water was removed during the infiltration of 60% ethanol solutions, illustrating that the covalent cross-linking bonds could not maintain the stability of free water within the gel structure. Additionally, the triple helix might be broken during the process of ethanol permeation. Therefore, DCCG(0.8) exhibited lower *G*' and *v* values than those of DCCG(0).

Table 1. The values of *G*' and *G*'' at frequency of 1 Hz as well as *v* values of the fibrillogenesis collagen gels dehydrated by ethanol at different concentrations.

Samples	*G'* (Pa)	*G''* (Pa)	*v* (× 10^{23}, number/m^3)
DFCG(0)	239.2 ± 31.4	49.9 ± 10.7	0.59
DFCG(20)	474.4 ± 58.1	60.2 ± 12.6	1.17
DFCG(40)	1265.9 ± 103.4	236.2 ± 29.4	3.13
DFCG(60)	2162.9 ± 176.3	516.5 ± 61.2	5.35
DFCG(80)	4285.0 ± 249.5	1066.0 ± 76.4	10.59
DFCG(100)	13142.6 ± 528.2	2893.8 ± 185.1	32.48

Table 2. The values of *G*' and *G*'' at frequency of 1 Hz as well as *v* values of the collagen fibrillogenesis and simultaneously cross-linking of NHS-AA treated by 60% (v/v) ethanol.

Samples	*G'* (Pa)	*G''* (Pa)	*v* (× 10^{23}, number/m^3)
DCCG(0)	2064.3 ± 164.8	424.8 ± 46.5	5.10
DCCG(0.05)	7388.0 ± 319.7	1085.5 ± 94.3	18.26
DCCG(0.2)	432.2 ± 60.3	242.0 ± 29.5	1.07
DCCG(0.4)	626.9 ± 84.5	115.6 ± 17.2	1.54
DCCG(0.8)	2699.18 ± 192.8	692.12 ± 92.7	6.67

4 Conclusions

In summary, two types of fish collagen gels were prepared by the dehydration of ethanol in the present study and we proved that the presence of thick D-periodic fibrils was distinctly significant to fabricate the gels with excellent physiochemical properties. For dehydrated fibrillogenesis gel, the three-dimensional structure of the gels was well maintained after the dehydration of different concentrations of ethanol, and stronger hydroxy bonds among ethanol, bound water and collagen molecules were established to improve the thermal stability and elasticity of the gels. The properties of the dehydrated cross-linking gels were dependent on the NHS-AA dosages. Low concentrations of NHS-AA ([NHS-AA]/[NH_2] ratio= 0.05) almost not affected the formation of thick D-periodic fibrils, and the gel exhibited the largest values of T_d and *G*' compared with the dehydrated fibrillogensis gel owing to the introduction of covalent cross-linking bonds. As the [NHS-AA]/[NH_2] ratio higher than 0.2, thermal stability and elastic modulus of dehydrated cross-linking gels increased with the increase of NHS-AA dosage, however, the values of T_d and *G*' were lower than those of the gel formed when the [NHS-AA]/[NH_2] ratio was 0.05 due to the absence of thick fibrils.

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