Polymer Degradation and Stability 130 (2016) 264–270

Contents lists available at ScienceDirect

Polymer Degradation and Stability

journal homepage: www.elsevier.com/locate/polydegstab

The microstructure and stability of collagen hydrogel cross-linked by glutaraldehyde

Zhenhua Tian [a, b], Wentao Liu [a, b], Guoying Li [a, b, *]

[a] The Key Laboratory of Leather Chemistry and Engineering of Ministry of Education, Sichuan University, Chengdu 610065, PR China
[b] National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu 610065, PR China

ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form
8 June 2016
Accepted 15 June 2016
Available online 23 June 2016

Keywords:
Collagen hydrogel
Glutaraldehyde
Cross-linking
Microstructure
Stability

ABSTRACT

Collagen hydrogels, which are comprised of fibrils and possess three-dimensional network structure, were prepared via self-assembly of collagen molecules and sequentially incubated in glutaraldehyde (GTA) solutions with different [CHO]/[NH_2] ratios (0–9). The cross-linking degree of outer and inner parts of hydrogels was similar, demonstrating the homogeneous reaction. Based on the results of atomic force microscopy, differential scanning calorimetry and dynamic rheological measurements, it was conjectured that the stability of hydrogels were closely associated with the structural changes of collagen fibrils. When the [CHO]/[NH_2] ratios $\leq$3, cross-linking preferentially occurred between adjacent fibrils; therefore, the fibrils presented in pairs and then densely agglomerated. As a result, the thermal denaturation temperature (from 47.1 to 73.6 °C) and elastic modulus (from 108.32 to 1618.55 Pa) increased drastically, accompanied by a distinct decrease in enzymatic degradation degree (from 93.69 to 26.91%). The effective binding ratio (*EB*) of aldehyde groups reduced from 72.66 to 43.92%. Moreover, hydrogels turned into yellow and yellowness (Δb^*) increased from 0 to 1.68. When the [CHO]/[NH_2] ratio reached 3, the arrangement of fibrils became very compact; therefore, although the GTA dosage was multiplicative ([CHO]/[NH_2] = 9), there was only a relatively small improvement in thermal stability and anti-enzymolysis. Furthermore, the *EB* value was only 16.40%, indicating that intensive self-polymerization of GTA molecules caused a large consumption of aldehyde groups, accompanied by a remarkable yellow-stain ($\Delta b^* = 2.73$). These data on the stability of cross-linked hydrogels could be helpful for the design and fabrication of materials based on collagen hydrogels.

1. Introduction

Hydrogels are attractive materials as scaffolds in tissue engineering, wound dressings and carriers for drug delivery systems due to their highly swollen network structure, ability to encapsulate bioactive molecules and efficient mass transfer [1]. A variety of hydrophilic polymers, including natural, synthetic and natural/synthetic hybrid polymers, are usually used to construct hydrogel via chemical or physical cross-linking. Recently, more and more researchers pay attention to the natural polymeric hydrogels with weak antigenicity, good biodegradability, biocompatibility and abundant availability [2–4], such as collagen hydrogels. It has been well known that collagen molecules (collagen solution) could self-assemble in a head-to-tail and laterally quarter-staggered arrangement to form fibrils (collagen hydrogel) *in vitro* under physiological conditions [5,6]. The reconstituted collagen fibrils, which are held together by non-covalent interactions (hydrogen bonding, hydrophobic and electrostatic interactions), are not stable — the collagen molecules are free to slide, and the fibril is easily dissociated by variations in temperature, ionic strength, pH or collagenase [7–9]. As a result, although the native collagen hydrogel possesses a three-dimensional network structure, it shows weak mechanical strength, insufficient thermal stability and fast biodegradation rate, which cannot meet the demand *in vivo* and *in vitro* applications and limit further use of materials based on collagen hydrogels. In general, the stability of the collagen hydrogel consisting of reconstituted fibrils is closely related to the contents of cross-linking bonds and water. It is well known that the exogenous chemical cross-linking bonds are always introduced via cross-linking agents in order to stabilize the three-dimensional network structure and improve the physicochemical properties of collagen

* Corresponding author. The Key Laboratory of Leather Chemistry and Engineering of Ministry of Education, Sichuan University, Chengdu 610065, PR China.
E-mail address: liguoyings@163.com (G. Li).

http://dx.doi.org/10.1016/j.polymdegradstab.2016.06.015
0141-3910/

gel [10,11]. According to extensive studies [12,13], for collagen fibrils, the hydrated water molecules not only act as a load transfer medium by forming water bridges between the triple helix, but also play a role of a lubricating medium in preventing the stickslip motion; as a result, the hydration has an adverse effect on the mechanical properties of collagen fibrils. Consequently, the reduction of water content and the introduction of covalent cross-linking bonds are effective methods to improve the physicochemical properties of collagen gel. In the previous studies [14,15], in order to prepare the collagen gels with high mechanical strength, the researchers treated collagen gel consisting of reconstituted fibrils by cross-linking with chemical cross-linking agent (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and sequential heating (30 min at 80 °C), and found that the cross-linked collagen gels shrank and their viscoelasticity increased drastically due to the reduction of water content during heating process. It was also found that the three-dimensional network structure of the uncross-linked collagen gel collapsed due to the denaturation of collagen fibrils; similarly, the uncross-linked fibrils in the cross-linked gels were also denatured owing to heating so that they exhibited a gelatin-like structure. It can be concluded that the collagen gel with high mechanical strength prepared by heating treatment must be firstly reinforced via chemical cross-linking. Therefore, the present work focused on how the cross-linking agent affects the microstructure and physicochemical properties of collagen hydrogel.

Glutaraldehyde (GTA) has been widely applied as a preferred cross-linking agent due to its low cost, high reactivity and high solubility in aqueous solution [16]. It is generally assumed that the primary reaction of GTA with collagen is the interaction between aldehyde groups of GTA and ε-amino groups of lysine or hydroxylysine of collagen, resulting in the formation of a Schiff base type compound [17]. With regard to the reaction of collagen fibril with GTA, McPherson et al. [18] examined the physicochemical characterizations of collagen fibrous suspensions treated with various concentrations of GTA (0–1.0%) and found that the insolubility, viscosity, thermal stability and anti-enzymolysis were ameliorated with the increased GTA concentration. In a later study, 1% insoluble collagen fibrous dispersions (pI ~5.1) were cross-linked with GTA at the [CHO]/[NH_2] ratios of 0.08 and 0.5, and the increases in the viscosity and storage modulus indicated more resistance to deformation and flow of the GTA-crosslinked collagen dispersions [19]. These investigations were mainly focused on the properties of collagen fibrous suspensions rather than collagen hydrogels. Cheung et al. [20] treated the collagen hydrogels consisting of reconstituted collagen fibrils (1.5 mg/mL) with different concentrations of GTA ([CHO]/[NH_2] = 0–12), and inferred that the intermolecular cross-linking predominantly was formed among collagen fibrils; as a result, the solubility of the cross-linked hydrogel in CNBr or collagenase solutions decreased.

To further explore the relationships between the microstructure (viz. the structures of collagen fibrils) and the stability of collagen hydrogel, in the present work, collagen hydrogels, which possess a three-dimensional network structure [21], were prepared via the self-assembly of collagen molecules. Sequentially, the hydrogels were immersed in phosphate buffer solutions containing various GTA dosages. The morphological characteristics of collagen fibrils and the stability of collage hydrogels (e.g. thermal stability, anti-enzymolysis and viscoelasticity) were examined in this study.

2. Materials and methods

2.1. Materials

Collagen was extracted from calf skin using 0.5 mol/L acetic acid containing 1% pepsin (EC 3.4.23.1, 1:10000, Sigma Chemical Co.) according to the previously described method of Zhang et al. [22]. The supernatant of extracted solution was collected by centrifugation (10000*g*, 10 min) at 4 °C and then salted out in 3 mol/L NaCl solution followed by centrifugation. The precipitated collagen was again dissolved in 0.5 mol/L acetic acid and salted out by adding NaCl to a final concentration of 0.7 mol/L. The precipitate was dissolved in 0.5 mol/L acetic acid, and then dialyzed against 0.1 mol/L acetic acid for 3 days to remove NaCl. Finally, the collagen solution was lyophilized with a freeze dryer (Labconco Freeze Dryer FreeZone 6 Liter, USA) at −50 °C for about 2 days and stored at 4 °C for no more than 3 months.

2.2. Preparation of the cross-linked collagen hydrogels

Lyophilized collagen was dissolved in phosphate buffer saline (PBS, 10 mmol/L phosphate, 100 mmol/L NaCl, pH 7.4) in ice bath to obtain 5 mg/mL collagen solution. The native collagen hydrogels were prepared by incubating the collagen solutions at 37 °C for 3 h and sequentially immersed in PBS with different glutaraldehyde (GTA) dosages for 24 h at room temperature (~25 °C). The GTA dosages were converted to the molar ratios ([CHO]/[NH_2]) of aldehyde groups to ε-amino groups of lysine or hydroxylysine residues, of which the content was approximate 100 in one type I collagen molecule [23]. The final [CHO]/[NH_2] ratios used were 0, 0.06, 0.6, 1.5, 3 and 9. These resultant collagen hydrogels were named as GC(0), GC(0.06), GC(0.6), GC(1.5), GC(3) and GC(9).

2.3. Cross-linking degree

In order to justify whether the reaction is homogeneous throughout the collagen hydrogels, the cross-linking degree of inner and outer parts of hydrogel was determined respectively by trinitrobenzensulfonic acid (TNBS) assay, a spectrophotometric method of Duan et al. [24,25]. Approximately 0.15 g collagen hydrogel, 2.00 mL borax buffer (pH 10.00) and 2.00 mL freshly prepared 0.1% (v/v) TNBS were mixed in a glass tube. After reacting in the dark at 50 °C for 60 min, 4.00 mL 6 mol/L HCl was subsequently added into the mixture and incubated again at 60 °C for 90 min. The absorbance (*A*) of the mixed solution was recorded at 340 nm using a UV spectrophotometer (PerkinElmer Lambda 25, Waltham, MA). All the samples were measured in triplicate. The cross-linking degree of the cross-linked collagen hydrogels was calculated according to the following formula:

$$\begin{aligned} \textit{Cross}-\textit{linking degree}\ (\%) &= \frac{CDI + CDO}{2} \\ &= \frac{(1 - A_{ic}/A_{in}) + (1 - A_{oc}/A_{on})}{2} \times 100 \end{aligned} \quad (1)$$

where *CDI* and *CDO* represent the cross-linking degree of inner and outer parts of hydrogel, respectively; the subscripts ic and oc stand for the inner and outer parts of the cross-linked collagen hydrogels, respectively; similarly the subscripts in and on stand for the inner and outer parts of the native collagen hydrogel, respectively.

2.4. The effective binding ratio of aldehyde groups with amino groups

The GTA concentration in the effluent was obtained by the standard curve method. The GTA solution was scanned from 200 to 600 nm using a UV spectrophotometer and the PBS was used as the blank sample. The absorption peak only appeared at 235 nm, which was consistent with the peak of GTA reported in previous literature