On the structure alteration of crosslinkable gelatin coupled with methacrylic acid and its hydrogel

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(Received: 18 February, 2011; published: 22 January, 2012)

Abstract: Physical structures of a crosslinkable gelatin derivative (GM) were studied in terms of alteration of apparent molecular weight, triple helix content and mechanical strength. The GM with a substitution degree (DS) of 49% and 79% was prepared by grafting methacrylic acid (MA), which was able to form injectable hydrogel by photoinitiating polymerization. The zeta potential was increased along the increase of DS. After modification, the apparent number-average molecular weight (Mn) detected by gel permeation chromatography was decreased to about 2/3 of gelatin, while the apparent weight-average molecular weight (Mw) was changed within a small range. Differential scanning calorimetry and circular dichroism (CD) revealed that ability of triple-helix formation of GM was decreased along with the increase of DS and decrease of GM concentration. After photo-crosslinking, the sol-gel transition of GM49 physical-chemical hydrogel still existed, but completely disappeared for its chemical hydrogel. The physical-chemical hydrogel showed a larger storage modulus at 20°C than at 37°C as a result of additional physical crosslinking.

Keywords: injectable; hydrogel; gelatin; physical structure; biomaterials

Introduction

Collagen exists widely in extracellular matrix (ECM) of bone, cartilage, skin and other connective tissues. Although the isolated collagen has good biocompatibility and degradability in vivo, it shows more or less antigenicity in some cases [1, 2]. Gelatin is a hydrolysis product of collagen. While the merits of collagen are remained, the possible antigenicity is eliminated as a result of heat denaturation [3, 4]. Similar as the collagen, the gelatin contains a lot of bioactive sequences such as Arg-Gly-Asp (RGD) peptide, which can be recognized by cell receptors [5]. The gelatin is also much cheaper and easier to be dissolved in water. These characteristics lead to its widespread applications in tissue engineering and drug delivery [6-10] in various formats such as membranes, scaffolds, microspheres or nanoparticles, fibers and hydrogels.

Due to its large solubility in water and fast degradability both in vitro and in vivo, modifications of the gelatin molecules are required in many cases especially in tissue
engineering and drug delivery. These modifications include physical, chemical and biological treatments to modify the conformation, structure, and consequently functional properties of gelatin [11, 12]. The major physical modification is heat treatment and the effect of heat on gelatin has received much attention [11]. The chemical modifications with improved functional properties is very prevalent in biomedical application, among which acylation with acid, anhydrides and amino compounds are used very commonly [12]. For example, photocurable gelatin was obtained by acylation with 4-vinylbenzoic acid [12]. The gelatin also was derivatized with methacrylamide side groups and was subsequently cross-linked by radical polymerization via photoinitiation [13]. Thiolated gelatin was synthesized by acylation with dithiothreitol [14]. The crosslinking can improve the mechanical properties and resist its dissolution in water [9, 10, 15]. Although these modifications endow the gelatin with new functions to satisfy some special purpose, the structural change brought by the modification is still uncertain and thus deserved to study.

We previously prepared the gelatin scaffolds [16], microcarriers and hydrogels [17]. For example, in order to obtain injectable hydrogel a crosslinkable gelatin (GM) was synthesized by grafting methacrylic acid (MA) via amidation under the catalyzation of water soluble carbodiimide [17]. The GM hydrogel made by photoinitiating polymerization has good comprehensive performance and cell compatibility, thus has a great potential to be used in tissue engineering, for example, cartilage regeneration. However, the structural alteration of the gelatin molecule, which is important for its biomedical applications, is not disclosed yet. In this work, the structural change of gelatin is characterized in terms of charging property, molecular weight, sol-gel transition and rheological performance by means of differential scanning calorimeter (DSC) and circular dichroism (CD) etc.

**Results and discussion**

Detail characterizations of the molecular structures of gelatin before and after MA coupling were performed previously, confirming the consumption of -NH$_2$ groups of gelatin as a result of amidation with MA [17]. This enables the resulted gelatin (GM) to chemically crosslink to form a hydrogel. Table 1 summarizes the basic parameters of gelatin and GM. Since the MA molecule has C element but not N element, MA modification caused increase of C/N molar ratio of the GM, according to which degree of MA substitution (DS) was calculated. Here a low (GM49, DS 49%) and high (GM79, DS 79%) DS of GMs was synthesized, respectively. The zeta potential slightly increased with the increase of DS, because under catalysis of EDC condensation between the -COOH and -OH of gelatin occurred simultaneously [18].

**Tab. 1.** Basic parameters of GM with different DS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C/N molar ratio</th>
<th>DS (%)</th>
<th>Zeta potential</th>
<th>$M_n(\times 10^4)$</th>
<th>$M_w(\times 10^5)$</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>3.270</td>
<td>/</td>
<td>-13.0</td>
<td>6.2</td>
<td>1.8</td>
<td>2.84</td>
</tr>
<tr>
<td>GM49</td>
<td>3.333</td>
<td>49%</td>
<td>-8.2</td>
<td>4.0</td>
<td>1.4</td>
<td>3.59</td>
</tr>
<tr>
<td>GM79</td>
<td>3.372</td>
<td>79%</td>
<td>-4.2</td>
<td>4.1</td>
<td>1.9</td>
<td>4.59</td>
</tr>
</tbody>
</table>
After MA modification, the apparent number-average molecular weight ($M_n$) decreased to about $2/3$ of gelatin, while the apparent weight-average molecular weight ($M_w$) changed within a relatively small range and the polydispersity became a little bit larger (Fig. 1, Table 1).

![Fig. 1. GPC curves of gelatin, GM49 and GM79.](image)

During the process of amidation and esterification in the MA substitution reaction, crosslinking between the gelatin molecules would increase the molecular weight. Even intramolecular crosslinking of a gelatin molecule should make the chain become more rigid, leading to larger coil size determined by GPC. On the other hand, the hydrophilicity of the gelatin should become worse after MA substitution as a result of consumption of the hydrophilic groups, leading to decrease of hydrodynamic size or in other words, the molecule becomes more compact. The interplay between these factors may finally decide the apparent molecular weight of the GM determined by GPC. Since the apparent molecular weight was decreased, the latter factor should surpass the former, implying that the intermolecular crosslinking during the MA substitution is not severe.

The gelatin solution can transfer to hydrogel by simply cooling, the sol-gel transition of which was characterized by DSC, as shown in Fig. 2a. This transition was observed in gelatin and GM49 solution, but not in GM79 solution. The melt temperature ($T_m$) of GM49 (~25 °C) was apparently lower than that of gelatin (~28 °C), and enthalpy was smaller too. The gelatin chain is composed of Gly-Xaa-Yaa peptide, and proline and hydroxyproline are often in Xaa and Yaa position [19, 20]. This structure makes the molecular chain to be the left hand helical conformation. Three left hand helical chains compose the triple-helix structure, which is stabilized by hydrogen bonds between the molecules. At a high temperature, the gelatin molecules are in random coil state, while at a low temperature the coil chain can partially reverse to triple-helical segment. Therefore, physical crosslinkages are formed on cooling, leading to the sol-gel translation of gelatin solution, i.e. formation of the physical gel [9, 10, 19-22]. The $T_m$ of the gel is correlated with stability of the triple-helix structure. With a longer triple-helix structure, the $T_m$ is also higher [20]. The enthalpy reflects the extent of renatured triple helices [22, 23]. According to the
result of zeta potential, MA modification reduces the amido groups, carboxyl groups and hydroxyl groups, which are important in formation of the hydrogen bonds. Therefore, MA modification reduced both the length and extent of the renatured triple-helix, leading to both T_m and enthalpy decrease.

![DSC curves](image)

**Fig. 2.** DSC curves of (a) 15% gelatin, GM49 and GM79 physical gel; (b) GM49 physical gel with different polymer concentration; (c) 15% GM49 chemical gel and physical-chemical gel.

Next, the influence of GM concentration on the sol-gel transition was investigated by DSC. Fig. 2b shows that T_m of the 20% GM physical gel (~31 °C) was higher than that of the 5%, 10% and 15% GM concentration (~26 °C). Moreover, the enthalpy was enlarged from 11.3 J/g (5%) to 19.5 J/g (20%) too. The higher concentration endows a larger opportunity to form interchain hydrogen bonds among the polymer chains, leading to a longer length and larger extent of renatured triple-helix structure. The folding kinetics of gelatin is strongly dependent on temperature and concentration, and is explained previously by Gornall using a concentration-temperature superposition mechanism, i.e. increasing the concentration has the same effect on the renaturing of gelatin as decreasing temperature [19]. Our result on the GM hydrogel can be similarly explained by this mechanism.

CD spectroscopy can disclose the molecular confirmation of proteins [24]. The CD spectra of gelatin, GM49 and GM79 are compared in Fig. 3. A peak at 222 nm was recorded for all the samples, which is assigned to the triple-helix structure [25]. The intensity of the peak was weakened along with the sequence of gelatin, GM49 and GM79, demonstrating the gradual decrease of the triple-helix structure percentage as a result of the MA modification. This is basically consistent with the DSC results (Fig. 2), except that the minor amount of triple helix in GM79 was sensitively determined by the CD spectroscopy but not by the DSC.
According to the results of DSC and CD, the structural change of gelatin, GM49 and GM79 on cooling and heating is depicted in Scheme 1. Cooling is beneficial of formation of the triple-helix structure, while heating causes the reverse process. This transition occurs in the GM49 and GM79 too. However, the helix length and triple-helix content are shorter and smaller after MA modification. Moreover, the higher substitution degree causes larger denaturation of the gelatin molecule, in which the triple-helix structure of GM79 is not detectable as that of GM49 by DSC.

![Fig. 3. CD spectra of gelatin, GM49 and GM79.](image)

According to the above results, GM49 was chosen for the next experiments in consideration of its more similarity to gelatin. Here two different kinds of hydrogels were prepared: physical-chemical hydrogel and chemical hydrogel. The GM chemical hydrogel was formed by photoinitiating polymerization, in which the double carbon bonds were mostly polymerized [17]. The sol-gel transition was still observed in the physical-chemical hydrogel, but disappeared completely in the chemical hydrogel, as shown in Fig.2c. It is likely that the covalent crosslinking of the chemical hydrogel restricts the movement of gelatin chains, thus the triple-helix (i.e. renature of the GM49 hydrogel) is hardly formed, or at least its content is under the detection limit of DSC [9, 10]. By contrast, the physical-chemical hydrogel was prepared by pre-incubation of the GM 49 at 28 °C for 3d before covalent crosslinking. This incubation provides long enough time for formation of the large enough triple helix structure.

Finally, the rheological and swelling properties of these two GM hydrogels were studied and the results are summarized in Table 2.

**Tab. 2.** The rheological properties and swelling ratio of GM49 hydrogel.

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Temperature, °C</th>
<th>G', kPa</th>
<th>G'', ×10²Pa</th>
<th>Swelling ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical-chemical gel</td>
<td>37</td>
<td>2.5±0.4</td>
<td>5.3±0.9</td>
<td>13.4±3.2</td>
</tr>
<tr>
<td>Chemical gel</td>
<td>37</td>
<td>2.4±0.6</td>
<td>5.2±0.3</td>
<td>14.5±5.3</td>
</tr>
<tr>
<td>Physical-chemical gel</td>
<td>20</td>
<td>7.3±0.3</td>
<td>4.0±0.2</td>
<td>12.1±4.9</td>
</tr>
</tbody>
</table>
The swelling ratio is mainly decided by the chemical crosslinking nature of the hydrogel, thus was not affected by the hydrogel type and measuring temperature. At 37 °C, both the storage modulus and the loss modulus were also similar for the physical-chemical hydrogel and GM chemical gel. However, the storage modulus of the GM physical-chemical gel was larger at 20 °C than at 37 °C, but the loss modulus was not changed significantly. As mentioned above, at 20 °C the renatured triple-helix structure of physical-chemical hydrogel is formed, which enhances the hydrogel strength as a result of additional physical crosslinking [9, 10].

**Scheme 1.** Physical gelation of gelatin and GM of different MA substitution degree.

**Conclusions**

Two crosslinkable gelatin derivatives with a DS of 49% (GM49) and 79% (GM79) were prepared by MA grafting, whose zeta potentials were increased along with the increase of DS. Their apparent Mₙ detected by GPC was decreased to about 2/3 of gelatin, while Mₘ was changed within a relatively small range. The Tₘ and enthalpy of sol-gel transition detected by DSC were both decreased after the MA modification. A higher GM concentration resulted in larger enthalpy and higher Tₘ too. CD spectroscopy confirmed the triple-helix structure, whose content was decreased along with the sequence of gelatin, GM49 and GM79. After crosslinking by photoinitiating polymerization, the sol-gel transition of GM49 physical-chemical hydrogel still existed, but completely disappeared for the chemical hydrogel. There were no significant difference for the GM49 physical-chemical hydrogel and chemical hydrogel in terms of storage modulus and loss modulus as well as swelling ratio at 37 °C and 20 °C. However, the strength of the physical-chemical hydrogel was improved at 20 °C than at 37 °C.

**Experimental part**

**Materials**

Gelatin was purchased from Shanghai Medicine and Chemical Company, China. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Fetal bovine serum (FBS) was purchased from Sijiqing biotech. Co., China. 2-Hydroxy-1-[4-(hydroxyethoxyphenyl]-2-methyl-1-propanone (Irgacure 2959) was obtained from
Ciba Specialty Chemicals. Methacrylic acid (MA) was purified via distillation under reduced pressure. All other chemicals were of analytical grade and used as received.

**Synthesis of crosslinkable gelatin (GM)**

Gelatin was modified by MA via amidation under the catalyzation of EDC to obtain crosslinkable GM. Briefly, 500 mg EDC (2.1mmol) or 160 mg EDC (0.67mmol) was added into 100mL 1% gelatin solution containing 800μL MA (9.4mmol) under magnetic agitation. The reaction was maintained for 8 h at room temperature. In order to remove the unreacted MA and other small molecular weight products, the resulted mixture was sealed in a membrane with a cut off molecular weight of 10kDa and dialyzed in large amount of triple-distilled water for 3d. Finally, the solution was freeze-dried to obtain the GM. The degree of MA substitution (DS) of GM was quantified using the results of elemental analysis (Eager 300) [17]:

\[ R(C/N \text{ molar ratio}) = (C/12)/(N/14) \] (1)

\[ DS = [(R_{GM} - R_{GELATIN}) \times 100\% / 4] \times 31 \] (2)

where \( R_{GM} \) and \( R_{GELATIN} \) were obtained from respective GM and gelatin according to equation (1).

The molecule weight of gelatin and GM was characterized by gel permeation chromatography (GPC, WATERS-515), using poly(ethylene glycol) (PEG) as a standard. Zeta potential of gelatin and GM was measured by dynamic light scattering (Delsa™ Nano C, A53878) at 37 °C.

**Hydrogel formation**

200 μL gelatin or GM solution with variable concentrations was kept at 28 °C for 3 d, and then were stored at 4 °C overnight to obtain the physical gelatin or GM hydrogel, respectively. 200μL 15% GM solution containing 0.05% (w/v) Irgacure2959 (UV initiator) was injected in 1mL polypropylene test tube, which was incubated at 28 °C for 3d and then irradiated by 365nm UV light with a power of ~10mW/cm² for 20min at room temperature (~20 °C) to obtain the GM physical-chemical hydrogel. 200μL 15% GM solution containing 0.05% (w/v) Irgacure2959 was irradiated for 20min at room temperature directly to obtain the GM chemical hydrogel.

**Differential scanning calorimeter (DSC)**

The sol-gel transition of gelatin and GM physical gel was determined by differential scanning calorimetry (DSC, Perkin Elmer Pyris 1 calorimeter). 30-40 mg gelatin or GM physical gel was sealed in aluminum pan at 5 °C, and scanned by a speed of 5 °C /min until 65 °C.

**Circular dichroism (CD)**

Triple-helix structure of the gelatin and GM was determined by circular dichroism (CD, JASCO J-815). 0.5% gelatin or GM solution was added into the cuvette, and then scanned from 215 to 300 nm at 10 °C.

**Rheological test**

The hydrogel with a column shape (25mm diameter, 0.22mm height) prepared in a mold was placed in a parallel plate mode for the rheological measurement by a
strain-controlled ARES rheometer (Advanced Rheometric Expansion System, Rheometric). Dynamic oscillatory mode was used to measure the storage and loss modulii. All tests were performed with a fixed strain of 1%.

**Swelling ratio**

The hydrogel was freeze-dried and weighed \(W_0\). The dried hydrogel was hydrated in water at 37 °C or 20 °C. 24 h later the hydrogel was taken out and blotted with filter papers to remove the excess water on the outside of the hydrogel, and then weighed \(W_1\). The swelling ratio of the hydrogel is defined as \(W_1/W_0\).

**Acknowledgements**

This study is financially supported by the Natural Science Foundation of China (20934003), Ph.D. Programs Foundation of Ministry of Education of China (2009010110049), the Science and Technology Program of Zhejiang Province (2009C14003) and the Major State Basic Research Program of China (2005CB623902).

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