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# Effect of hydroxypropyl methylcellulose on collagen fibril formation *in vitro*

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ABSTRACT

Collagen and hydroxypropyl methylcellulose (HPMC) were mixed to obtain blends and the effect of HPMC on collagen self-assembly was studied. As deduced from atomic force microscopy (AFM), the amount of nuclei in collagen–HPMC solutions was changed with the addition of HPMC. Under physiological conditions, the kinetics curves of fibril formation showed that the turbidity of blends at 313 nm was higher than that of native collagen. More HPMC was involved in the hydrogel network for blends with higher HPMC/collagen. However, both the thermal stability and the storage moduli of hydrogels, which was evaluated by UV and rheological measurements respectively, reached the maximum just when HPMC/collagen = 0.25. Furthermore, it was showed by AFM that denser fibrils with smaller diameter would be obtained as HPMC/collagen < 0.25, while more addition of HPMC (HPMC/collagen > 0.25) would bring about fibrils with larger diameter. However, HPMC did not significantly affect the characteristic D-periods of the fibrils for all blends.

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## 1. Introduction

Collagen is an abundant structural protein in animals, which accounts for one third of the total protein and is the major component of the extracellular matrix. Collagen has been applied in many fields such as medicine, cosmetics, food and chemical industry due to its diversity and complexity, together with its weak antigenecity, biodegradability, biocompatibility and bioactivity [1]. Especially, collagen has been extensively applied to make three-dimensional scaffolds for tissue engineering purposes through the self-assembly of collagen monomers *in vitro* under physiological conditions. The tissue engineering scaffolds were required to possess certain viscoelasticity and strength to resist gel contraction [2,3]. Fibril size was shown to be crucial for proper interaction of the scaffold with cell and tissue formation (*i.e.* for cell morphology, adhesion, migration, proliferation, and differentiation) [2]. However, the relatively weak properties such as mechanical properties and thermal stabilities limit the application of collagen hydrogel, therefore, many natural biopolymers are introduced to obtain composite scaffolds with improved properties [4,5].

As a natural biopolymer, polysaccharide is commonly employed to obtain various composite scaffolds with collagen. Polysaccharide was found to influence the process of collagen fibril formation from a kinetic perspective. For instance, Vogel et al. [6] reported that the small dermatan sulfate proteoglycan of bovine tendon exhibited a unique ability to inhibit the fibrillogenesis of both type I and type II collagen *in vitro*. Wood [7] and Keech [8] found that chondroitin-4-sulfate, chondroitin-6-sulfate and keratin sulfate slightly accelerated collagen aggregation, whereas dermatan sulfate and hyaluronic acid did not have this effect. Sang et al. [9] suggested that the electrostatic complexation between collagen and alginate played a crucial role in the aggregation of collagen–alginate blends. Furthermore, Wang et al. [10] investigated the influence of Chi/Col ratio on the manner of collagen self-assembly. It should be noted that most of these studies focused on the effect of anionic polysaccharide such as dermatan sulfate proteoglycan, keratin sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and alginate on fibril formation of collagen in the blends. Nevertheless, the effect of nonionic polysaccharide on collagen fibril formation has rarely been reported. Honya [11] indicated that the nonionic materials and dextrans, increased the rate of collagen fibril formation.

Hydroxypropyl methylcellulose (HPMC), which is a typical nonionic polysaccharide, is a hydrophilic cellulose ether derivative and has been extensively used as a matrix for drug delivery [12,13] as well as used in adhesives and cosmetics [14]. HPMC contains lots of hydroxypropyl, which could form hydrogen bonding with carboxyl and hydroxyl groups of collagen [15]. Thus, we were interested in the influence of HPMC on the processes of type I collagen self-assembly *in vitro*. Such information will be essential to the design of new biomass composites from natural biopolymers such as collagen and HPMC.

Herein HPMC was added into collagen solutions to make blends with various HPMC/collagen ratios, and then the kinetics curves of

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self-assembly for collagen in the blends were obtained by turbidity measurements. The properties as mentioned above, including thermal stability and viscoelasticity of hydrogels and the diameter of fibrils, were then measured by turbidity measurement, dynamic frequency sweep and atomic force microscopy (AFM). By combining these results, the effect of HPMC on the self-assembly behavior of collagen was explored.

## 2. Materials and methods

### 2.1. Materials

Type I collagen was extracted from calf skin by the method described by Zhang et al. [16]. Briefly, the supernatants extracted from the delimed and neutralized bovine split pieces by 0.5 M acetic acid containing 3% pepsin (EC 3.4.23.1, 1:10,000, Sigma Chemical Co.) were collected by refrigerated centrifugation at 9000 × *g*. After centrifuging, the supernatants were salted out, and then the precipitate was re-dissolved in 0.5 M acetic acid. After dialysis in 0.01 M acetic acid, the collagen solution was stored at 4 °C for the following experiments. HPMC (purchased from Hercules, USA) with a methoxy group content of 27.0–30.0% and with a hydroxypropoxy group content of 4.0–7.5% was dissolved in deionized water to achieve a concentration of 8 mg/ml.

### 2.2. Preparation of collagen–HPMC blended solutions

Collagen–HPMC blended solutions were prepared according to the method of Tenni et al. [17] with some modifications. Briefly, collagen and HPMC were mixed at 4 °C using a wide range of HPMC/collagen ratios 0:1, 0.125:1, 0.25:1, 0.5:1, 1:1, 2:1 and 3:1 (by weight), and then mixed with chilled phosphate buffer containing NaCl, from which the ratio dihydrogen phosphate/monohydrogen phosphate was 1:11.5. After mixing, collagen–HPMC blends containing buffer (10 mM phosphate, 100 mM NaCl, pH ∼7.2) were obtained. Samples were named as CH(0), CH(0.125), CH(0.25), CH(0.5), CH(1), CH(2) and CH(3), respectively, according to the HPMC/collagen ratios. The final collagen concentration in all samples was kept at 1 mg/ml. HPMC solution with a concentration of 1 mg/ml was prepared by the same methods.

Collagen concentration in these samples was increased to 2 mg/ml in order to obtain hydrogels with enough gel strength which were only applied for rheological measurements.

### 2.3. Atomic force microscopy (AFM) of collagen–HPMC blended solutions

The solutions of CH(0), CH(0.125), CH(0.25), CH(0.5), CH(1) were diluted to obtain the collagen concentration of 25 μg/ml, and HPMC was also diluted to be 25 μg/ml, 10 μl of which was dropped quickly and evaporated on mica, and then dried at room temperature (∼20 °C) for 2 days. The surface morphology of the dried samples was observed by AFM (SHIMADZU SPM 9600, Japan) in the dynamic mode at room temperature (∼20 °C). Each sample was scanned with a scan rate of 1 Hz.

### 2.4. Turbidity measurement of collagen–HPMC assemblies

The assembly kinetics of samples CH(0), CH(0.125), CH(0.25), CH(0.5), CH(1), CH(2) and CH(3) was studied by monitoring the real-time turbidity changes at 313 nm on a UV spectrometer (Perkin Elmer Lambda 25, USA). These cold mixtures were placed in a quartzose cuvette (1 cm) and immediately transferred to the spectrophotometer. Fibril formation was initiated by increasing temperature from 4 to 37 °C in less than 1 min, and then monitored by recording absorbance which is essential measurement of turbidity at 313 nm. The temperature fluctuation was controlled within ±0.1 °C. The rate of fibril formation was calculated from the time required to reach the midpoint of the final turbidity ($t_{1/2}$). In addition, the absorbance of HPMC solution was also measured at 313 nm as a control.

### 2.5. The quantitation of HPMC in hydrogels

The amount of HPMC remained in fibrillar hydrogels was determined by elution according to the method of Sang et al. [9] with some modifications. CH(0.125), CH(0.25) and CH(0.5) were incubated at 37 °C for 60 min, and then the resultant hydrogels were used to determine the content of HPMC in the collagen–HPMC fibrillar hydrogels by diphenylamine colorimetric method. Firstly hydrogels were soaked in 40 ml deionized water for 2, 5, 8 and 11 days, and then the following quantitative experiments should be conducted in dark as follows: 2 ml eluant of each sample at the designated soaking intervals was extracted to comparison tubes, and then diphenylamine solution was subsequently added. After mixing, the comparison tubes were heated in a boiling water bath for 30 min and finally cooled to room temperature in ice bath. The absorbance of the analytical solutions was measured at 635 nm on a spectrometer (Perkin Elmer Lambda 25, USA). The HPMC content in the eluant was calculated using a standard curve, therefore the content of HPMC remained in the hydrogels could be determined.

### 2.6. Turbidity measurement of hydrogels at 46 °C

The samples CH(0), CH(0.125), CH(0.25), CH(0.5) and CH(1) were incubated at 37 °C for 60 min. After this step, the absorbance change for hydrogels at 46 °C was recorded for 30 min at 313 nm on the UV spectrometer. The value of reduction in turbidity, which was related to the thermal stability of hydrogels, was calculated according to the followed equation:

$$\text{Reduction in turbidity}\,(\%) = \left(1 - \frac{A_f}{A_0}\right) \times 100 \quad (1)$$

where the subscripts $A_0$ and $A_f$ stand for the initial absorbance and final absorbance, respectively.

### 2.7. Measurement of dynamic viscoelasticity of hydrogels

The samples CH(0), CH(0.125), CH(0.25), CH(0.5) and CH(1) with final collagen concentration of 2 mg/ml were incubated for 60 min at 37 °C, and then dynamic frequency sweeps for all of the hydrogels were immediately tested on a Rheometer System (Malvern Instruments Gemini 200, UK). All tests were performed from 0.01 to 10 Hz at 30 °C at a constant strain of 5%. The temperature was controlled by a Peltier temperature controller, with an accuracy of ±0.1 °C.

### 2.8. Atomic force microscopy (AFM) of collagen–HPMC assemblies

The samples CH(0), CH(0.125), CH(0.25), CH(0.5) and CH(1) were incubated for 60 min at 37 °C, and then hydrogels of 15 μl was dropped on mica and dried at room temperature (∼20 °C) for 2 days. The surface morphology of the dried samples was observed by AFM (SHIMADZU SPM 9600, Japan) in the dynamic mode at room temperature (∼20 °C). Each sample was scanned with a scan rate of 1 Hz. For each condition, at least 20 fibrils were randomly picked to measure fibril diameters [18].

## 3. Results and discussion

Fig. 1 is the AFM images of air-dried collagen–HPMC blended solutions. As shown in Fig. 1, there were obvious differences of AFM images between native collagen and collagen–HPMC blends both

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