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ARTICLE

Real Time Quantification of the Chemical Cross-Link Density of a Hydrogel by *in situ* UV-vis Spectroscopy

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In this paper, we prepare a hydrogel in a green way via the redox reaction between phenol groups and EosinY by visible light irradiation. Eosin Y shows significant absorption at 515 nm while the reduced product does not. A series of control experiments show that almost 100% of the consumed Eosin Y is reacted with phenol groups to form cross-link points. On the basis of the two results, the chemical cross-link density can be easily determined quantitatively by UV-vis spectroscopy. With this method, specimens from the state of the starting solution to solid gel can be measured *in situ* and in real time without any pretreatment. The result obtained by this new method is proved by the traditional rheological measurements.

Introduction

The cross-link density is a crucial feature of gel-forming materials because it can influence many material properties. For instance, the rate of drug release, the mechanical strength of gels and their responses to external stimuli can be controlled by cross-link density.[1-7] To build an accurate correlation between cross-link density and the related properties of gels, it is essential to have quantitative determination of cross-link density. However, such determination in a simple and effective way is still a challenge.

So far, many methods have been utilized to determine the cross-link density of gels or rubbers. As a classical method, the swelling theory developed by Flory has been extensively utilized to determine the cross-link density of gels or rubbers.[8] The parameters in equations, which show strong dependence on environmental conditions, are not easy to be determined exactly.[9-11] Besides, this method is not ideal for the measurement of hydrogels from biomacromolecules because the chains between the cross-link points are *not* Gaussian chains, and the elastic moduli are 3 - 10 times larger than that predicted using rubber elasticity theory.[12-14] By utilizing the differences of the characteristic peaks between the starting sample and the cross-linked one, Fourier transform infrared spectroscopy (FTIR)[15-17] and nuclear magnetic resonance (NMR)[18-20] can be used to determine the cross-link density.[21-24] Dynamic torsional vibration method (DTVM) can also be utilized to study the cross-link density of rubber or resin.[25,26]

Apart from methods above, it has been reported that UV-vis spectra can be used to study the properties of hydrogels under various conditions.[27-29] Once combing with other measurements like FTIR, NMR, differential scanning calorimetry (DSC),[30,31] gel permeation chromatography (GPC)[32-34] and high performance liquid chromatography (HPLC),[35,36] UV-vis spectra can be used to determine the cross-link density of gels or rubbers.[37-40]

In general, all of the methods mentioned above share the same feature: They all measure the samples after the formation of gels or rubbers,[41-43] rather than measuring samples *in-situ*, the moment cross-link structures are generated.

In our opinion, if a system is properly designed, the cross-link density of the system can be determined *in-situ* and in a simple way while the cross-link reaction is ongoing. In this study, we provide an example to demonstrate the feasibility of designing such system. Interestingly, we find that a dye molecule as a reactant in the cross-link reaction would lose its UV-vis spectral feature after the reaction. Meanwhile, the consumed dye molecule is used to form cross-link structures at the efficiency of almost 100%. Thus, UV-vis spectra as a simple and efficient method can be utilized to investigate the hydrogel formation process, and more importantly, the increase of cross-link density *in-situ* and in real time.

Experimental section

Materials

Sodium alginate (hereafter refers as SA, *Mw ca.* 60 000) was purchased from Sigma Aldrich Corp. Tyramine hydrochloride

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was supplied by Acros Corp. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC•HCl) and N-hydroxysulfosuccinimide (NHS) were purchased from Shanghai Medpep Ltd. Eosin Y (hereafter refers as D) was purchased from Accela ChemBio Ltd. Deionized (DI) water (>15 MΩ cm) was used when water was involved. Other chemicals were analytically pure and used without further treatment.

The modification of sodium alginate

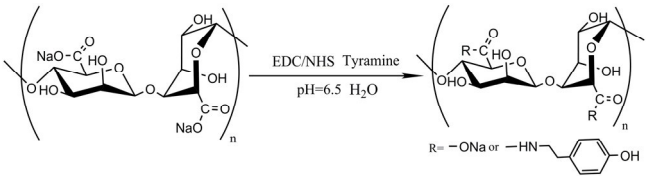

Fig. 1 Synthetic scheme for the modification of sodium alginate with tyramine residues.

SA was treated via EDC-NHS chemistry in mild conditions to obtain tyramine modified SA (hereafter refers as MSA),[44,45] see Fig. 1. In brief, purified SA (2.0 g) was dissolved in a 200 mL aqueous solution (pH 4, adjusted by HCl) of 0.3 M NaCl. NHS (2.88 g, 25 mmol) was dissolved in the above SA solution, and then EDC·HCl was added at a molar ratio of 1:1 to NHS. The aqueous solution of tyramine (2.0 g, 14.6 mmol) was introduced into the above mixture dropwise while maintaining the pH at 6.5. The reaction was conducted at room temperature for 72 h. After that, the resultant was precipitated with ethanol and then redissolved in DI water. Then the solution was treated by dialysis (MWCO 3500) against DI water for 2 days and precipitated by ethanol again. The precipitate was dried in a vacuum oven at 40 °C for 24 h, and finally MSA was obtained.

Determination of the concentration of phenol groups in the MSA solutions

The average grafting ratio of tyramine residues to the carboxyl groups of SA was determined to be 8%, according to the UV-vis spectra of SA and MSA (Fig. 2).[44] The concentration of phenol groups in the solution can be obtained as follows:

$$[\text{Phenol group}] = \frac{wt}{M_0} \times 0.08 \qquad \text{(Eq. 1)}$$

where *wt* is the concentration of MSA in the solution (g/mL), M_0 is the molecular weight of the sugar residues in SA (216 g·mol^{-1}) and the constant (0.08) is the grafting ratio of MSA.

Preparation of MSA-based hydrogels and *in situ* UV-vis measurements

Hydrogel samples were prepared by the addition of D solution to MSA solution (both in PBS, pH = 7.0) at predetermined concentrations. After vortexing for about 20s, the mixture was immediately transferred to quartz cuvettes (1 cm × 1 cm) and exposed to green light (LED with an emission of $\lambda = 515$ nm, Model: MR16, *TKS Co.*) at a distance of 4 cm. After the irradiation for a certain period, the light was switched off and UV-vis absorbance (Lambda 35, Perkin-Elmer) of such samples at 515 nm were recorded.

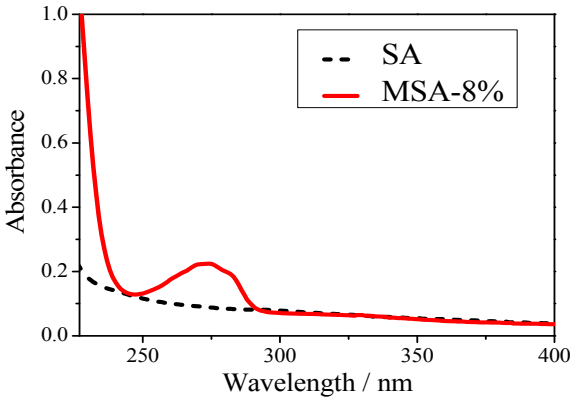

Fig. 2 The UV-vis spectra of 0.1 wt.% aqueous solutions of SA and MSA.

Rheological analysis

Rheological experiments were carried out with an AR2000ex rheometer (TA Instruments) using parallel plates (25 mm diameter, 0°) configuration at 25 °C in the oscillatory mode. In brief, for the preparation of the samples, the PBS solution of MSA with D was injected into a polystyrene mold, and the LED was fixed at a distance of 4 cm from the mold. After irradiation with a certain time, the sample was mounted into the opaque chamber for rheological measurement. The evolution of the storage modulus (*G'*) was recorded as a function of frequency from 0.01 to 1 Hz at 0.2% strain.

Gelation time determination

It is well known that the viscosity of polymer solutions increases as the concentrations increase, which would greatly influence the gelation process. Therefore, the concentration of MSA in this work was set to a constant while that of D was varied. The gelation time was determined by the test tube inverting method, see Fig. S6.

Results and discussion

Eosin Y (D) as an indicator of cross-link density

Eosin Y (D) shows significant absorption at 515 nm (A_{515}). However, upon visible light irradiation, this peak would disappear gradually due to the redox reaction between phenol groups (from MSA) and D, see Fig. 3. Thus, D can be used as the indicator of this photoreaction, and the reaction can be monitored by observing the changes of absorbance of D in UV-vis spectra. The changes of A_{515} (ΔA_{515}) could be utilized to investigate the chemical cross-link density of MSA as well.

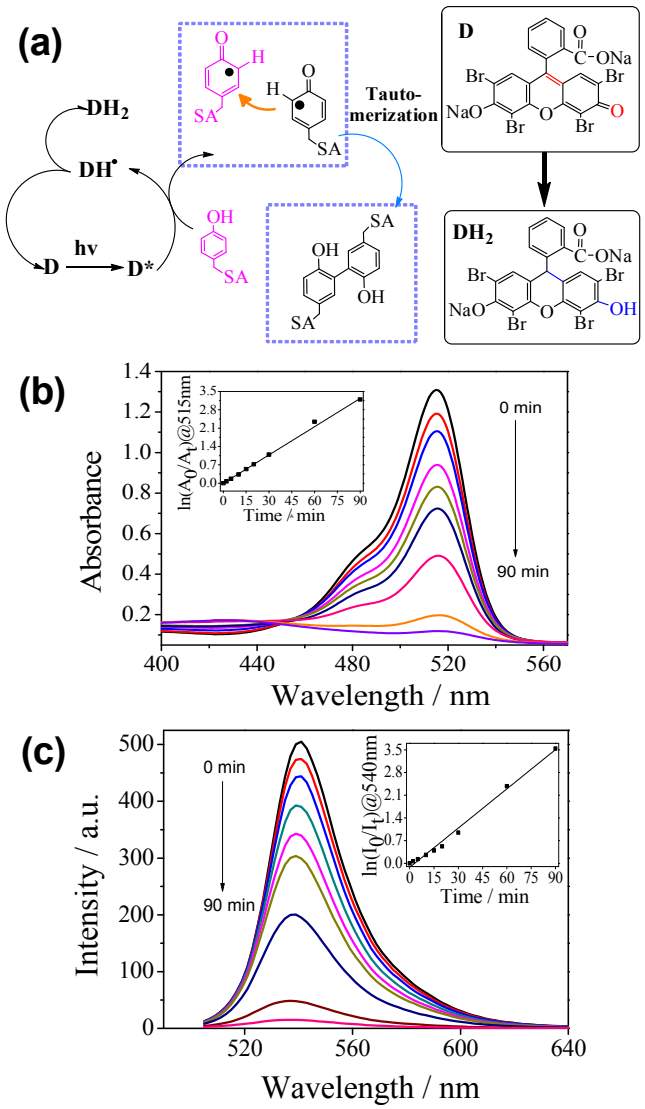

Fig. 3 (a) The schematic for the reaction of D molecules with phenol groups triggered by green light, and the chemical structures of D and DH_2, respectively. (b) The UV-vis and (c) fluorescent (λex = 490 nm) spectra of D and MSA in 0.1 M PBS buffer (pH = 7.0) with different irradiation time. The concentration of D and MSA were 13.4 μM, 0.025 wt.%, respectively, and the irradiation intensity was 3 W. Insets: The plot of $\ln(A_0/A_t)$ (at 515 nm) and $\ln(I_0/I_t)$ (at 540 nm) vs irradiation time, respectively.

For example, in solutions with a low concentration of MSA (0.025 wt.%), the concentration of phenol groups is about 100 μM, which is much higher than that of D used in this work, whose maximum concentration is 24.4 μM. Thus, in this study, the degree of this chemical reaction only depends on the concentration of D.

It is reported that the triplet D is able to oxidize amines, resulting in α - amino radicals, which can initiate the polymerization of methacrylate or acrylate groups.[46-48] In this study, with the absence of amines, the redox reaction involving phenol groups and D is realized (Fig. 3a). In brief, upon light irradiation, the oxidation of phenol groups results in two kinds of radicals, i.e., phenol radical and semi-reduced dye (DH•, see Table S1 in ESI). And then, the phenol radicals could proceed to form cross-linked products with the same species nearby,[44,49,50] and DH• is decayed by a process which returns to normal D and DH_2, respectively.[51,52] As shown in Fig. 3b and 3c, the decrease of both UV-vis absorbance and fluorescent emission intensity were observed with the increase of irradiation time, which could be associated with the conversion from D to DH_2. Due to the structural change (Fig. 3a), the latter molecule lost the spectral feature.[53] The decrease of the absorbance in a monotonic fashion indicates that each absorbance corresponds to only one specific concentration of the products. Importantly, the absence of side reactions (see Table S1 in ESI) makes it possible to quantitatively estimate the chemical cross-link density of the hydrogels from the UV-vis spectra data.

Investigating the kinetics of the reaction between phenol groups and D

A_{515} of these systems were monitored to investigate the kinetics of the reaction between phenol groups and D. In detail, MSA was dissolved in 0.1 M PBS (pH 7.0) buffer solutions at the concentration of 0.5 wt.%, 13.4 μM of D was sequentially added to the mixed solution. The homogenous solutions (obtained by vortexing for about 20s) were transferred into quartz cuvettes (1 cm × 1 cm × 4.5 cm) and exposed to green LED with identical intensities at a distance of 4 cm. A_{515} were measured after irradiation with a series of interval irradiation time.

The photoreaction between D and phenol groups is accomplished in a multiple-step process (see Table S1 in ESI for details), and it can be summarily represented as Reaction 1 below:

$$D + 2\ SA\text{-}Ph \rightarrow DH_2 + SA\text{-}Ph\text{-}Ph\text{-}SA \quad (1)$$

where SA-Ph and SA-Ph-Ph-SA denote MSA and the cross-link points of MSA hydrogels, respectively.

It is helpful to note that the phenol-phenol linkages (Fig. 3a) are the only chemical cross-link points in the MSA hydrogels. Thus, the concentration of SA-Ph-Ph-SA is actually the chemical cross-link density of the hydrogel. The concentration of SA-Ph-Ph-SA should be directly and stoichiometrically related with the consumption of D. However, there are two other possible pathways to consume D during the gelation process, including the possible reaction between dissolved O_2 and D (Reaction IX in Table S1) and the possible reaction between SA and D (see Fig. S1 for details). A series of control experiments are conducted under the same conditions to investigate whether these pathways have remarkable influence on the consumption of D.

In the first control experiment, A_{515} of D solution is measured before and after the light irradiation. If D reacts with the dissolved O_2 (Reaction IX in Table S1 of ESI), it would lead to a marked decrease of A_{515}. However, as shown in Fig. S2a,

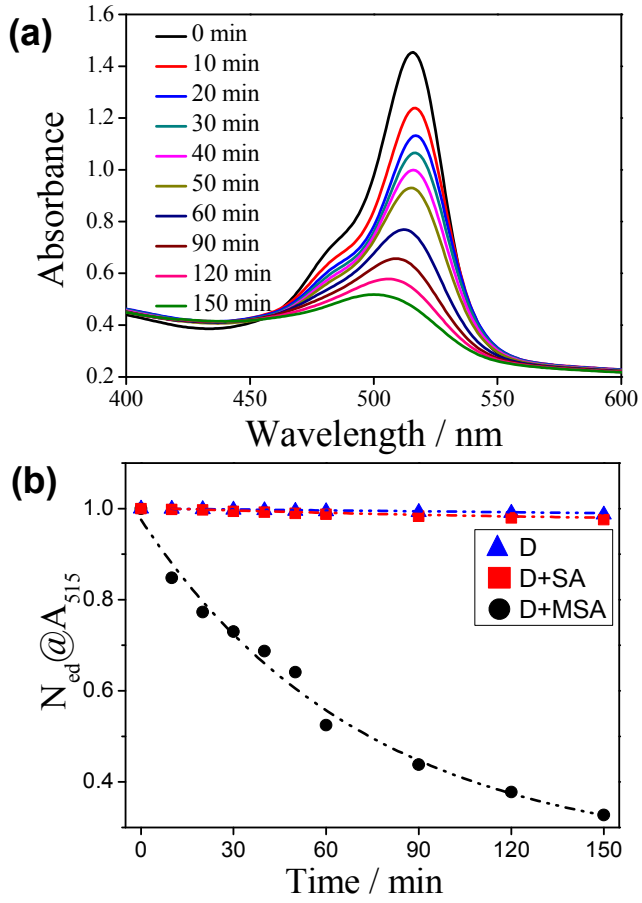

Fig. 4 The UV-vis spectra of D at 515nm in 0.1 M PBS buffer, (a) MSA with D (MSA 0.5 wt.%, D 13.4 μM and 3 w LED irradiation) vs irradiation time, (b) Normalized UV-vis absorbance at 515 nm (N_{ed}@A_{515}) of the data in Fig. S2a (blue triangle), Fig. S2b (red square) and Fig. 4a (black circle).

the oxidation of D by the dissolved O_2 can be ignored since A_{515} does not have a remarkable change (from 100% to 98.96%) after the light irradiation of 150 min (see also Fig. 4b).

In the second control experiment, A_{515} of mixed solution of D and SA is measured before and after the light irradiation. A similar result to the first control experiment is observed in this case (from 100% to 97.56%), indicating that the dissolved O_2 in the solution should explain the decrease of A_{515}. Thus, SA has no marked impact upon the consumption of D as well (see Fig. S2b). According to the two control experiments above, these two possible pathways are excluded. Thus, almost 100% of the consumed D is reacted with phenol groups (Reaction 1). In this reaction, D molecule is reduced into DH_2 by taking one H atom from a phenol group, and the diphenyl structure (SA-Ph-Ph-SA) is promptly formed. The investigation of the kinetics further demonstrates that Reaction 1 is an irreversible process (Fig. S3), and the reaction rate is mainly determined by the process of photo excitation of singlet dye. The effects of other factors on the rate constant of Reaction 1 are not in the scope of the

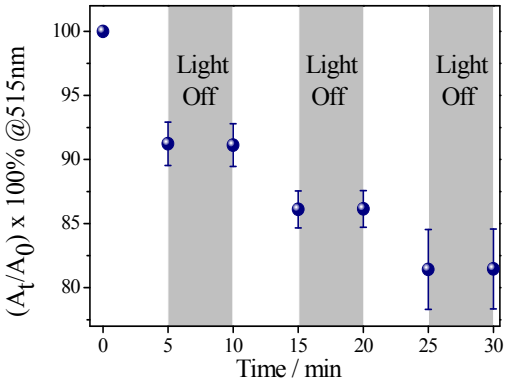

Fig. 5 Influence of intermittent irradiation on the decrease of A_{515} of D. Conditions: MSA 0.5 wt.%, D 4.5 μM and 3w irradiation (n=3).

present study and will not be discussed in detail.

Fig. 4a shows that for the case of D with MSA (Reaction 1), an obvious decrease of A_{515} (from 100% to 32.76%) in 150 min can be observed (see also Fig. 4b). According to Reaction 1, the increase of chemical cross-link density can be calculated by tracing the decrease of A_{515} (Fig. 4a), which will be discussed in details in the next section.

Apart from the above discussions, it can be observed from Fig. 5 that A_{515} decreased with green light irradiation but stayed constant in dark. This result indicates that both the initiation and termination of the cross-link reaction (between D and MSA) can be easily controlled in a remote control manner and the UV-vis measurements have no significant effect on the cross-link reaction.

The determination of the chemical cross-link density

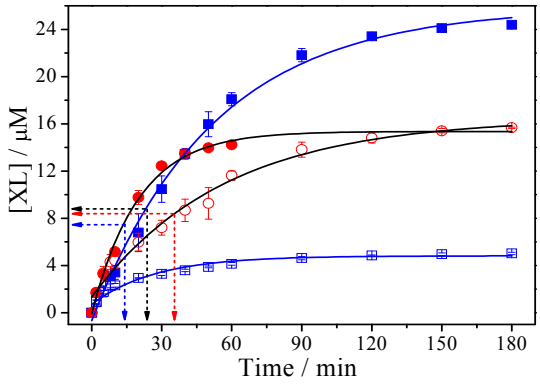

Fig. 6 The cross-link density vs irradiation time in different conditions. Blue solid squares: D 24.4 μM and 3w LED irradiation. Blue open squares: D 4.5 μM and 3w LED irradiation. Red open circles: D 13.4 μM and 3w LED irradiation. Red solid circles: D 13.4μM and 6w LED irradiation. The concentrations of MSA for all samples were 0.5 wt.%. The arrows point out the gelation time of the three profiles of different conditions (n=3). An enlarged figure is shown as Fig. S5 in ESI.

To determine the chemical cross-link density of the MSA hydrogels, the experiment was performed under the identical conditions, except that a higher concentration of MSA (0.5 wt.%) was used for the preparation of the hydrogels. Based on the changes of A_{515} ($\Delta A_{515} = A_0 - A_t$, where A_0 and A_t are the A_{515} at the initial state and the state after a certain time of irradiation, respectively) and the standard curve (Fig. S4), the variation of [D] could be obtained. Accordingly, the chemical cross-link density, [XL], of MSA hydrogels can be estimated by Eq. 2:

$$[XL] = \Delta A_{515}/0.0627 \qquad \text{(Eq. 2)}$$

where the constant (0.0627) is the slope of the standard curve of D in PBS buffer (Fig. S4). Thus, the chemical cross-link density of the hydrogels with different irradiation time can be determined, see Fig. 6.

Compared with the swelling method, the rheological measurement is more applicable to confirm the above result from the UV-vis measurement because of the degradability of MSA at prolonged time and uncertainty of the Flory-Huggins interaction parameter between solvent and MSA. The $[XL]_r$, which is the cross-link density obtained by the rheological measurement of hydrogels, is estimated as a function of the storage modulus from rheological data as follows:[54,55]

$$[XL]_r = (G'/RT)\times\alpha \qquad \text{(Eq. 3)}$$

Here, *G'* is the storage modulus from the rheological measurements (Pa), *R* is the gas constant (8.314 Pa m^3 / (mol K)), *T* is the temperature (K), and all other related factors are denoted as a coefficient, α.[54,55] In this study, all the measurements are performed under the same conditions (i.e., the same sample of MSA, pH values and ionic strength of the solutions). Thus, the value of α for each experiment should be identical. It is helpful to note that, Eq. 3 is a simplified equation that intra-chain cross-linking is not considered. The ratio of $[XL]_r$ of the two systems, r, can be calculated by Eq. 4:

$$r = [XL]_{r,1}/[XL]_{r,2} = G_1'/G_2' \qquad \text{(Eq. 4)}$$

Here, $[XL]_{r,1}$ and $[XL]_{r,2}$ are the chemical cross-link density of hydrogels by the rheological measurement in different [D], and G_1', G_2' are the corresponding storage modulus, respectively. Table S2 shows that the storage modulus of the blank sample (i.e., [XL] = 0) is 1.3 ± 1.3 Pa. For the two systems with different concentrations of D (13.4 μM and 24.4 μM), the storage moduli are 51.2 ± 2.9 Pa and 97.9 ± 1.8 Pa, respectively. According to Eq. 4, r is calculated to be 1.91, which is close to the ratio of [XL] of the two systems in the same conditions by UV-vis method, 1.75. This result supports our proposal that UV-vis spectroscopy is reliable to determine the [XL] of hydrogels in a simple way.

For the systems with different concentrations of D (13.4 μM and 24.4 μM), the gelation time, which is determined by test tube inverting method (Fig. S6 in ESI), are 35 ± 7 min and 23 ± 4 min, respectively (Fig. 6). This result indicates that the increase of [D] would facilitate the formation of hydrogels. Moreover, the decrease of the gelation time from 35 ± 7 min to 14 ± 1 min can be observed by increasing the irradiation intensity (see Fig. 6 and S5). Furthermore, the gelation time can also be determined by rheological measurement, which is similar to that by the test tube inverting method, see Fig. S6. According to the curves in Fig. 6 and S5, the cross-link densities at the gelation time for three different conditions were comparable (7.5 μM, 8.4 μM and 8.8 μM, respectively). Therefore, it is estimated that for this kind of hydrogel, the cross-link density at the gelation time is 8.2 ± 0.6 μM. This result was supported by the failure of gelation with a lower concentration of D at 7.0 μM.

Conclusions

In conclusion, we illustrate a simple way to estimate the chemical cross-link density *in situ* and in real time by tracing the change of A_{515} in the sample. With this method, specimens from the state of the starting solution to solid gel can be studied under mild conditions without any pretreatments. The cross-link density obtained by this approach is very close to that obtained by the traditional rheological measurement, which demonstrates that the simple method is reliable. It is greatly anticipated that, besides the decrease of UV-vis peaks in this work, other spectral changes before and after cross-link reaction (such as the generation of new peaks, increase of absorbance, etc.) can also be utilized to determine the cross-link density of the gels by a similar method. On the other hand, starting from two biocompatible and nontoxic materials (MSA and Eosin Y), the hydrogels are prepared in physiological conditions by the irradiation of visible light that is not harmful to living cells (compared with UV light or gamma-ray). Thus, the hydrogels in the current study are prepared by a green method. These advantages may predict the promising applications in the biological related areas.

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