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Precursor-involved and Conversion Rate-controlled Self-assembly of a 'Super Gelator' in Thixotropic Hydrogels for Drug Delivery

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Enzymatic hydrogelation is a totally different process to the heating-cooling gelation process, in which the precursors of the gelators can be involved during the formation of self-assembled structures. Using thixotropic hydrogels formed by a super gelator as our studied system, we demonstrated that the enzyme concentration/conversion rate of enzymatic reaction had a strong influence on the morphology of resulting self-assembled nanostructures and the property of resulting hydrogels. The principle demonstrated in this study not only helps to understand and elucidate the phenomenon of self-assembly triggered by enzymes in biological systems, but also offers a unique methodology to control the morphology of self-assembled structures for specific applications such as controlled drug release.

Keywords self-assembly, hydrogel, enzymatic reaction

Introduction

Small molecular hydrogels (SM hydrogels)[1-8] attract extensive research interests recently because they mimic the extra-cellular matrix (ECM) and can provide biocompatible environments for the cells, proteins, and other bioactive molecules.[9-12] They have been widely used in the fields of cell culture,[13-16] detection of bioactive molecules,[17,18] drug release,[19,20] *etc*. The formation of a three dimensional (3D) matrix that can hold water molecules within them is crucial for the production of an SM hydrogel. To generate such kind of 3D matrix, external stimuli are needed to trigger the self-assembly of small molecules, including temperature change,[21,22] pH adjustment,[23,24] charge screening,[15] sonication,[25,26] and chemical reactions.[27] Recently, an enzymatic reaction was found to be a unique method to produce SM hydrogels since the enzymes worked efficiently in mild conditions.[28-31] Comparing with other methodologies used for the formation of SM hydrogels, enzymatic hydrogelation is more complicate due to the presence of the precursors of SM hydrogelators, diffusion of the enzyme substrates and products, and the conversion rate of enzymatic reactions, which is similar to the cases in biological systems such as the formation of β-amyloid fiber. We believed that both the existence of precursor and the concentration of enzyme should play important roles in the morphology of resulting nanostructures, thus leading to different mechanical and optical property of the SM hydrogels.

Experimental

Chemicals

Fmoc-OSu, *L*-Tyr-OBu-*t* and other Fmoc-amino acid were obtained from GL Biochem (Shanghai). Chemical reagents and solvents were used as received from commercial sources. Alkali phosphatase was purchased from Takara (Dalian, P. R. China) (D2250) Bio. Inc. Commercially available reagents were used without further purification, unless noted otherwise. All the reagents such as DMF and DCM were used as received unless noted.

General methods

^{1}H NMR and ^{31}P NMR (Bruker ARX 400) were used to characterize the synthesized compounds. ESI-MS spectrometric analyses were performed at the Thermo Finnigan LCQ AD System. Conversion of compound **1** was carried out by LCMS-20AD (Shimadzu). AFM was done on a Veeco multimode V system with the tip of Veeco RTESP. Emission spectra were

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detected on a Perkin-Elmer LS-55 luminance spectrometer at excitation wavelength of 272 nm. Rheology test was done on an AR 2000ex (TA instrument) system, 40 mm parallel plates were used during the experiment at the gap of 500 μm.

Hydrogel formation

2.0 mg compound **1** and 1 equiv. of Na_2CO_3 (to neutralize the phosphoric acid on **1**) were dissolved in 1.0 mL PBS buffer (pH=7.4), and then 1.0, 3.0, 5.0, and 7.0 μL of alkaline phosphatase (30 U/μL) was added (final enzyme concentration=30, 90, 150, 210 U/mL) to form gel I, gel II, gel III and gel IV, respectively. The gel with 0.192 wt% of **2**, 0.008 wt% of **1**, and 1 equiv. of Na_2CO_3 was formed by a heating-cooling cycle as gel V.

Releasing of ciprofloxacin from gel I and gel III

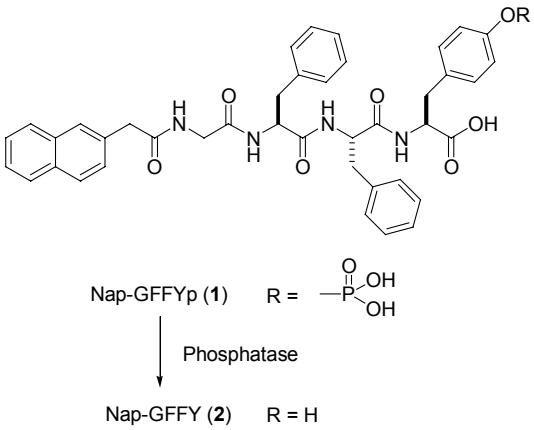
After 1.0 mL of gel I and gel III were formed by the enzyme (1 h after the addition of the phosphatase), 0.1 mL of PBS buffer solution of ciprofloxacin at different concentration of 3, 5, 8, and 15 mg/mL was added to each gel, respectively. 60 seconds' vortexes were applied to the resulting mixtures, and then the solutions were kept at room temperature overnight (gelation was observed less than 1 h for both gels after the vortex (determined by invert-tube method), which meant that the addition of ciprofloxacin helped the recovery of the gels). 2.0 mL of PBS buffer was added to the top of both gels. 10 μL of upper PBS solutions at different time points were taken out for UV absorbance measurement at 277 nm (the PBS solutions on gels without ciprofloxacin were used as controls. And actually the UV absorbance intensity of the control samples is near zero). And then the releasing profiles were calculated. The experiments were repeated for three times.

Results and Discussion

Gelation properties

To investigate the influence of enzyme concentration on the property of the resulting hydrogels, the gels formed by treating PBS (pH = 7.4) solutions of Nap-GFFYp (**1** in Scheme 1) with different amounts of phosphatase were studied. Four kinds of enzyme concentrations (30, 90, 150, 210 U/mL) were used to trigger the formation of SM hydrogels (gel I, gel II, gel III and gel IV, respectively, initiate concentration of **1**=0.2 wt% in all gels).[32] It was found that the conversion percentages of **1** in all hydrogels after being incubated for 1 h were similar—about 96% of **1** being converted to **2** (Nap-GFFY in Scheme 1). And a gel with 96 wt% of **2** and 4 wt% of **1** was prepared by a heating-cooling cycle (gel V) as a control. The minimum gelation concentration of **1** was about 0.08 wt% by the enzyme, which meant that one molecule of **1** could gel more than 54000 H_2O molecules after the enzymatic conversion. This result also indicated that Nap-GFFY could be used as a general moiety for the construction of molecular gelators with excellent gelation ability.[33]

Scheme 1 Chemical structure of the precursor of an SM hydrogelator and schematic hydrogelation catalyzed by phosphatase



We also studied the relationship between the conversion ratio and the gel/sol phase of our system with different concentrations of **1** and the enzyme. As shown in Figure 1, samples with lower initiate concentrations of **1** needed higher conversion percentages of **1** to **2** for the formation of the hydrogels. For examples, the conversion percentage of **1** to **2** was 75%, 58%, 46%, 42%, and 39% for gel I with 0.10, 0.15, 0.25, 0.30, and 0.50 wt% of **1**, respectively. Comparing the results from the samples with the same initiate concentrations of **1** but treated with different amounts of the enzyme, samples treated with higher concentrations of the enzyme obviously needed higher conversion percentages of **1** to **2** for the formation of the hydrogels. Though the reasons remained unclear for why samples treated with higher concentrations of the enzyme needed higher conversion ratios for gelation, this result implied that higher concentrations of the enzyme (higher conversion rates) would lead to weaker hydrogels with smaller G' values.

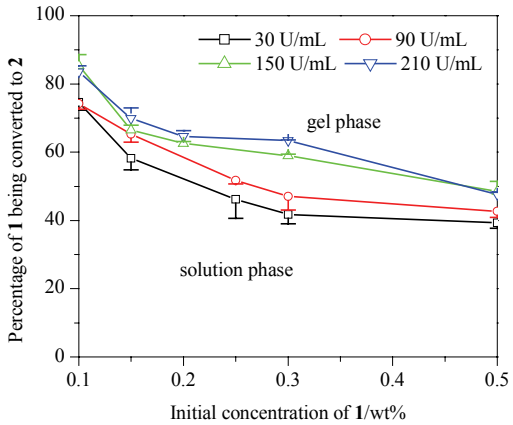


Figure 1 A diagram to illustrate the relationship between the gel/sol phase and the percentage of conversion of **1** to **2** for samples with different initiate concentrations of **1** and treated with different concentrations of the enzyme.

Rheology

The dynamic time sweep was used to investigate the conversion rate of enzymatic gelations by different amounts of enzyme. As shown in Figure 2, less time was needed for the gelation of PBS solutions containing 0.2 wt% of **1** treated with higher amounts of phosphatase—the gelation time was about 3 min, within 1.5 min, and within 1 min for gel I, gel II/gel III, and gel IV, respectively.[32] The gelations were nearly complete after being incubated for 1 h, indicated by both the values of G' and G'' reaching plateaus. However, as shown in the supporting information, the final values of G' (elasticity) of the gels were different—about 360, 250, 130, and 100 Pa for gel I, gel II, gel III, and gel IV, respectively.[32] These results indicated that less amount of enzyme (lower conversion rate) led to gels with higher elasticity and *vice versa*.

The dynamic strain sweep at the value of frequency near zero (0.1 Hz in our experiments) provided useful information on the yielding property of the hydrogels. As shown in Figure 3A and 3C, though gel I formed by a less amount of the enzyme (lower conversion rate) possessed a bigger value of the elasticity than that of gel III, gel I exhibited a weaker tolerance to the external force than that of gel III. The critical strain value of gel I was about 12.2%. However, this value for gel III was larger than *ca.* 30%. These results indicated that gels formed by higher amounts of the enzyme could show better resistant properties to external stress than those formed by lower amounts of the enzyme.

To our surprise, the incorporation of the ciprofloxacin into the gels could enhance the elasticity of the gels. As shown in Figure 3, gel I containing 1.5 wt% of the ciprofloxacin exhibited a bigger value of G' (about 1060 Pa in Figure 3B) than that of gel I without the ciprofloxacin (about 360 Pa in Figure 3A). However, gel I with ciprofloxacin showed a smaller critical strain value (11.3%) than the one without ciprofloxacin (12.2%). For gel III, we also observed a slightly enhancement of elasticity for the gel with the ciprofloxacin—about 190 Pa and about130 Pa for gel III with and without 1.5 wt% of the ciprofloxacin, respectively. However, gel III with the drug showed a weaker resistance to the external stress than gel III itself—the critical strain value for gel III with the drug was about 12%, while this value for gel III itself was about 30%. These results implied that the incorporation of the ciprofloxacin would make the nanostructures in the gels more rigid and brittle.

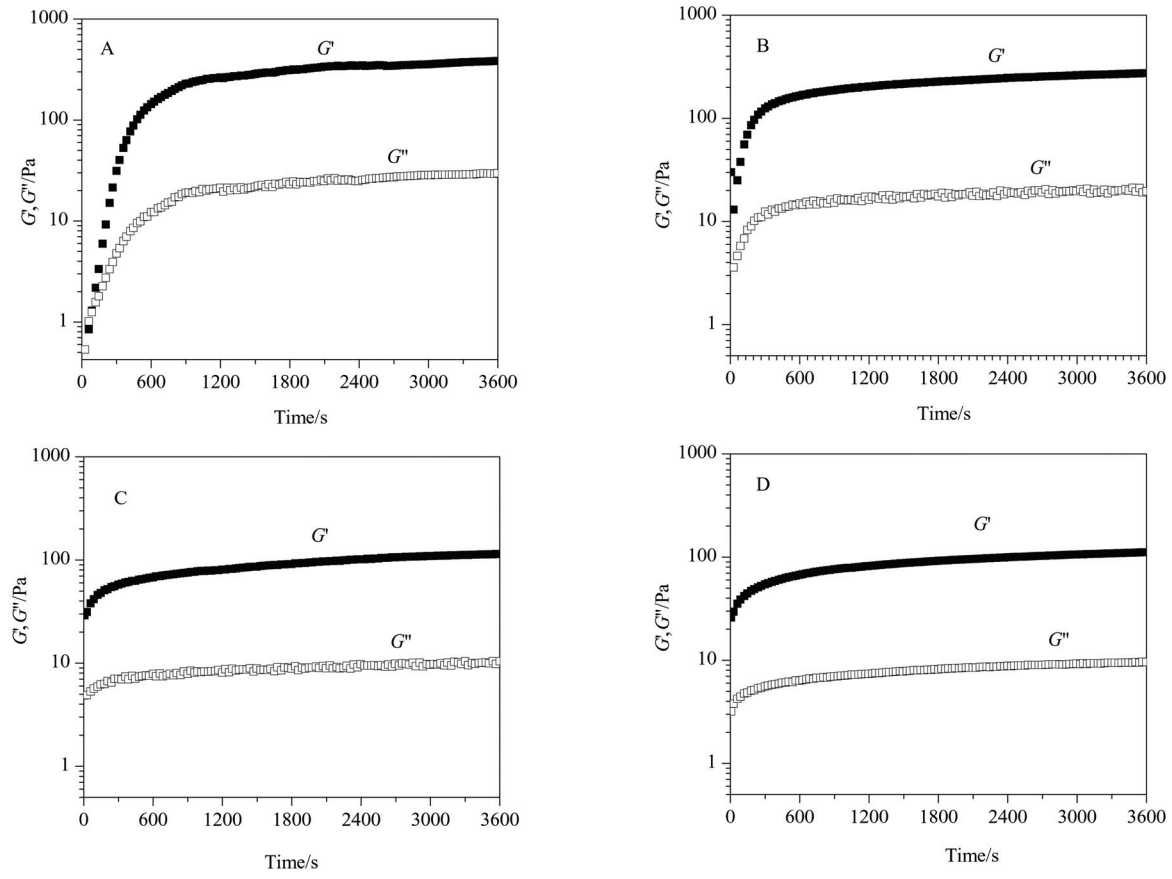


Figure 2 Dynamic time sweep of PBS buffer solutions containing 0.2 wt% of **1** with different concentrations of the phosphatase. (A) 30, (B) 90, (C) 150, and (D) 210 U/mL, strain＝2% and frequency＝2 rad/s.

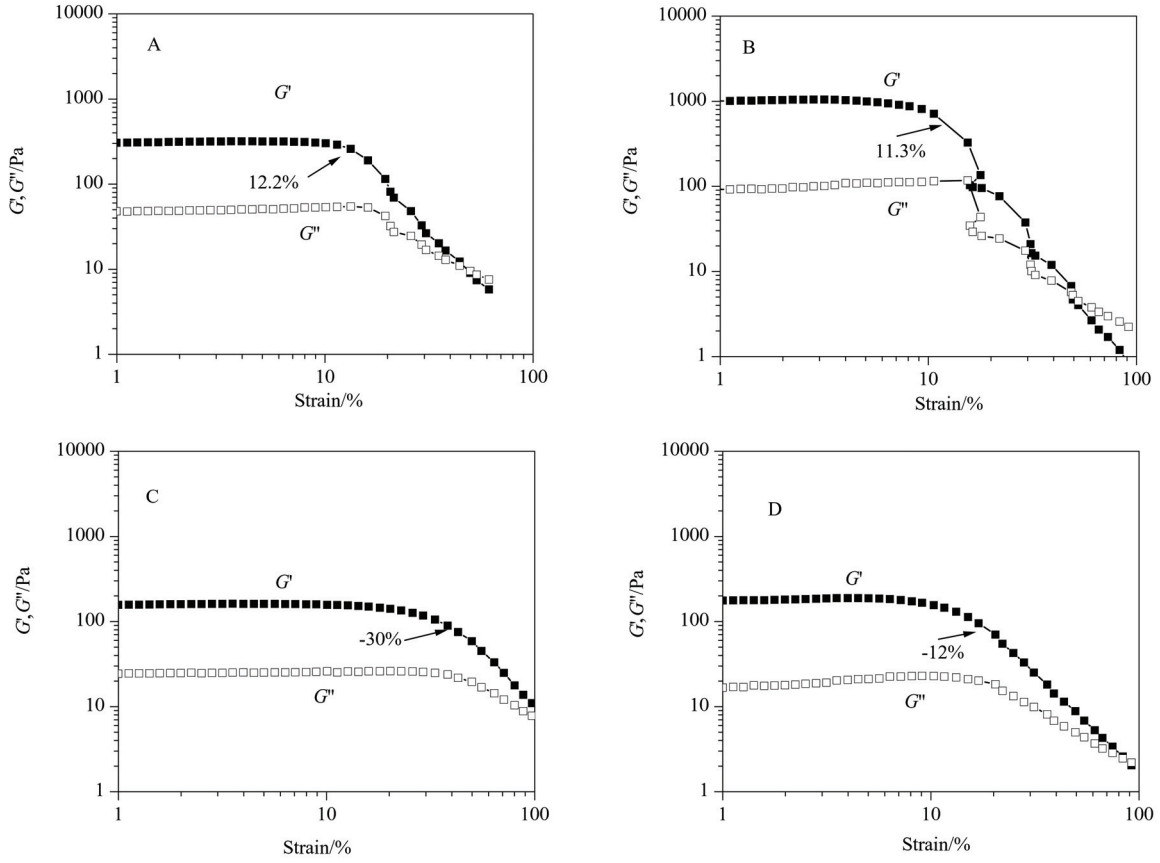


Figure 3 Dynamic strain sweep of (A) gel I formed by treating a solution containing 0.2 wt% of **1** with 30 U/mL of the phosphatase, (B) gel I with 1.5 wt% of the ciprofloxacin, (C) gel III formed by treating a solution containing 0.2 wt% of **1** with 150 U/mL of the phosphatase, and (D) gel III with 1.5 wt% of the ciprofloxacin, frequency＝0.1 Hz (the arrows indicate critical strain values).

Morphology

Atomic force microscopy (AFM) was used to characterize the nanostructures within the gels (Figure 4). Nanospheres with diameter of about 25—45 nm and nanofibers with width of 25—60 nm and length of ＞1 μm were observed in gels I—III. However, the ratio between the nanosphere and the nanofiber was obviously different—there was higher percentage of nanospheres in gel I and higher percentage of nanofibers in gel III, suggesting that a less amount of the enzyme (a lower conversion rate) led to the smaller aggregate of nanosphere and a high concentration of the enzyme (a higher conversion rate) led to the bigger aggregate of nanofibers. This result correlated well with the results obtained by the rheological measurement in Figure 3A and 3C that gel III possessed a better resistance to external stress than gel I. This phenomenon was also partially proved by the AFM image of gel IV, in which nanofibers (18—50 nm) were dominated. Interestingly, helical nanofibers and no nanospheres could be seen in gel V formed by the heating-cooling cycle. Comparing with the nanofibers in gels formed by the enzymatic reaction (gel I—IV) and the heating-cooling cycle (gel V), their size was more uniform in gel I—IV (18—60 nm) than that in gel V (15—100 nm). These results indicated that enzymatic reaction led to more ordered structures in this system. It also suggested that the resulting nanostructures might be controlled by the conversion rate of the enzymatic reaction.

Emission

Fluorescence microscopy was also used to characterize the process of the enzymatic hydrogelation and the interaction between naphthalene groups on **1** and **2** in solution and in gel phase. As shown in Figure 5A, **1** in solution exhibited distinct peaks at 327 and 336 nm, which correspond to the ^1L_b transition of naphthalene.[34] The shoulder peak at 350—400 nm suggested that **1** formed small aggregates in solution phase.[34] The emission peak from ^1L_b transition of naphthalene kept a red-shift by time after the addition of the enzyme. And this red shift was nearly finished at 60 min post incubation, which was consistent with the rheological measurements in Figure 2. The more pronounced peaks at 350—400 nm from the gels suggested that naphthalene groups were restricted in gel phase. Comparing the emission pattern of gels formed by different amounts of enzyme at 60 min, they showed slight differences—their emission peaks were centered at 347.9, 346.1, 345.7, and 345.3 nm for gel I, gel II, gel III, and gel IV, re-

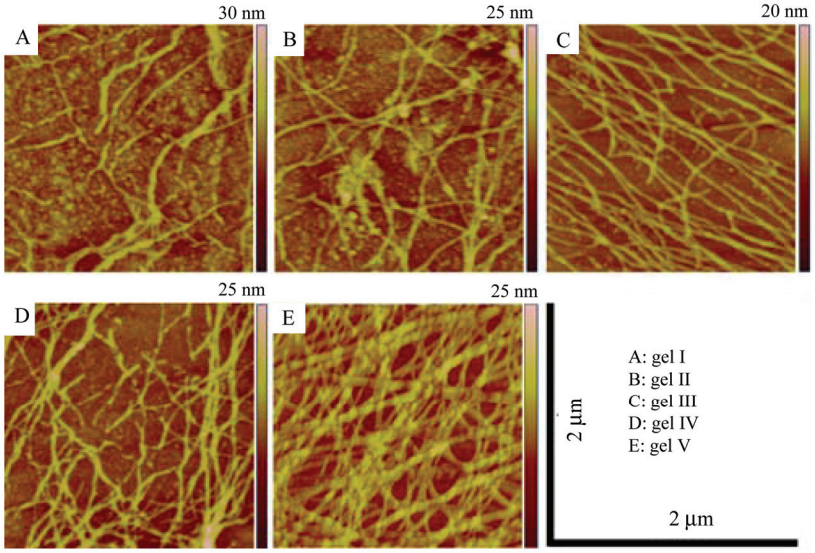


Figure 4 AFM images of different hydrogels (AFM was done on a Veeco multimode V system with the tip of Veeco RTESP).

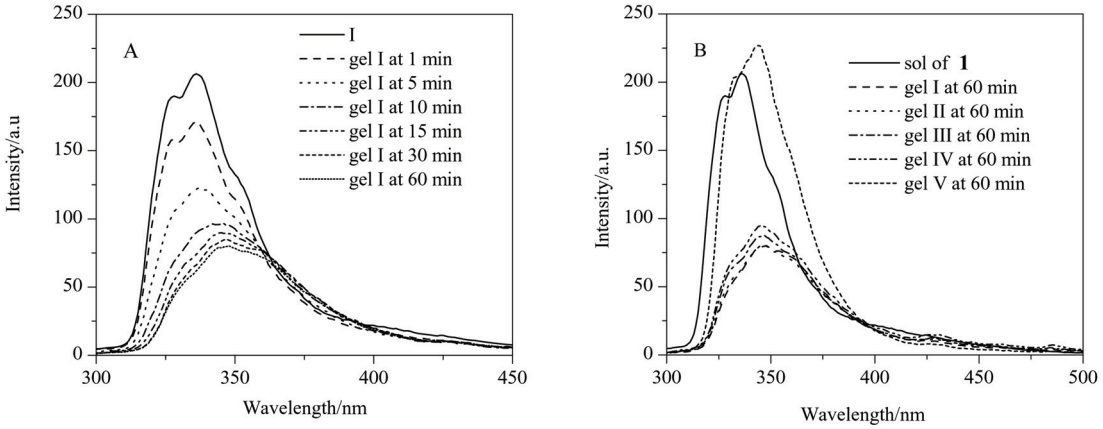


Figure 5 Emission spectra ($\lambda_{excitation}$=272 nm) of (A) sol. of **1** and gel **I** at different time points and (B) sol. of **1** and gel **I**—**V** at 60 min.

spectively, which also suggested that lower conversion rate would lead to more efficient packing of naphthalene groups in the gel phase. The emission pattern of gel V at 60 min was similar to gels formed by enzyme at short time such as gel I at 1 min, which implied that the heating-cooling process led to the most irregular molecular arrangements in the gel phase. And this result was consistent with the fact that gel V had the lowest elasticity within all the gels.[32]

Drug release from thixotropic hydrogels

We also found that gels I—IV were thixotropic, which meant that they could change to liquid-like fluids upon shaking probably due to the weak matrixes formed by nanospheres and nanofibers. As shown in Figure 6A, gel I could change to a slightly opaque solution by a gently hand-shaking for seconds. And it took long time for the resulting solution to recover to its original gel stage (about 6 h at room temperature). The dynamic time sweep was used to study the recovery of partially breakdown gel I (Figure 6B)—only about 40% of the mechanical strength was achieved after 45 min, which also meant that gel I possessed a slow recovery property. However, its recovery property could be improved by the incorporation of the ciprofloxacin. As shown in the supporting information, about 82% of the initial elasticity of the gel I with the drug was achieved after 45 min and this value was higher than 90% at the time point of 60 min. Since thixotropic hydrogels had been successfully applied for the delivery of cell-gel constructs and therapeutic agents,[35,36] the possibility of the gels in this study for drug delivery was studied. The drug molecule of ciprofloxacin was homogeneously incorporated into gel I and gel III (1 h post hydrogenation) by the vortex for 1 min and being kept at room temperature overnight. And then the releasing profile of the drug was measured. Since the loading amount of the drug was very important to the resulting releasing profile, we encapsulated the ciprofloxacin in both gel I and gel III at four kinds of concentrations (0.3, 0.5, 0.8, and 1.5 mg/mL). As shown in Figure 6C and 6D, both gels exhibited higher released percentages of the ciprofloxacin when the loading amounts of the drug were lower. For example, the releasing percentage of ciprofloxacin from gel I was

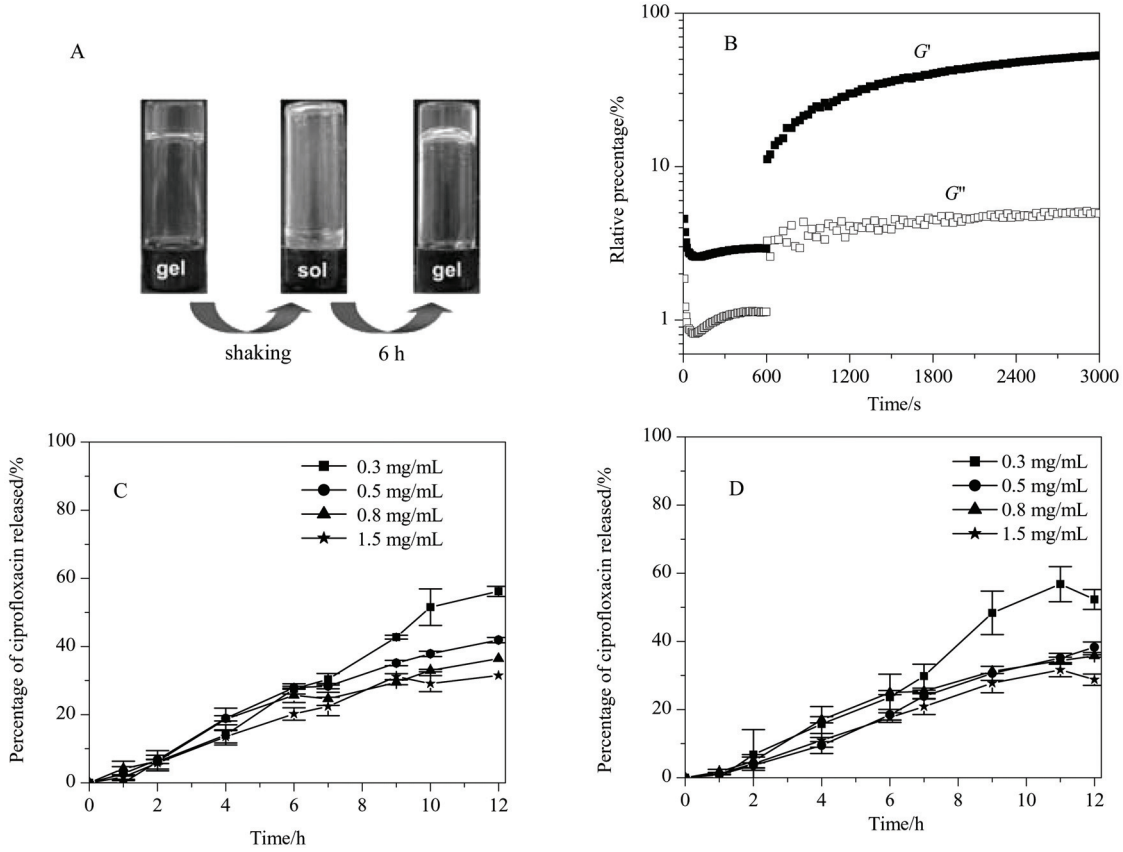


Figure 6 (A) from left to right: Optical images of gel I, the solution from gel I obtained by hand-shaking for 10 s, and the recovered gel I 6 h post shaking, (B) the recovery of gel I from partially breakdown by external shear force (the gel was first applied with the large strain of 50% for 10 min and then the recovery was probed at the strain of 2% and frequency of 2 rad/s), and releasing profile of ciprofloxacin from (C) gel I and (D) gel III with different concentration of Ciprofloxacin.

about 56%, 42%, 36%, and 31% when the concentration of ciprofloxacin in it was 0.3, 0.5, 0.8, and 1.5 mg/mL, respectively. Comparing the releasing profiles of both gels in Figure 6C and 6D, gel III possessed a slightly faster releasing rate than gel I when the concentration of the ciprofloxacin within them was the same—the releasing percentage of ciprofloxacin from gel III was about 58%, 46%, 37%, and 33% when the concentration of ciprofloxacin in it was 0.3, 0.5, 0.8, and 1.5 mg/mL, respectively probably due to the larger total surface area of the nanospheres in gel I than that of the nanofibers in gel III (gels with larger total surface areas could have higher affinities to the component within them). Though there was only a small difference between the releasing profiles from both gels, this result suggested that using different concentrations of enzyme to control the morphology of resulting nanostructures in hydrogels might provide a novel strategy for controlled drug release.

Conclusions

Based on above information, we proposed a possible mechanism of the precursor-involved and conversion rate-controlled self-assembly—the precursor was involved during the self-assembly of **2** due to their similar chemical structures;[37] upon low enzyme concentration/ slow conversion rate, the local concentration of **2** generated by the enzyme was low and it had a big chance to be surrounded by surfactant-like **1** to form nanospheres; while a high concentration of the enzyme/ high conversion rate would lead to a high local concentration of **2** that tended to form aggregates by themselves. It meant that both the existence of precursors and the conversion rate of enzymatic reactions had big influences on the resulting self-assembled nanostructures in enzyme-triggerd self-assembled systems. The result of drug releasing profile suggested that the morphology of nanostructures on hydrogels also had influences in the affinity of the hydrogels to drug molecules. The study in this paper might offer a simple method of using different amounts of enzyme to control the morphology of self-assembled nanostructures. And our system might provide a simple platform to control the activity of encapsulated cells,[37] drug molecules, *etc*.

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