Stimuli-Responsive Polymers

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Reversible Surface Switching of Nanogel Triggered by External Stimuli**

Xiaochen Shen, Leyang Zhang, Xiqun Jiang, Yong Hu, and Jian Guo*

In biology, a number of organisms such as viruses and toxins can change their conformation and composition of surface membrane protein in response to a pH change, leading to an enhanced ability to overcome biological membrane barriers and an effective internalization in cells.[1,2] To mimic such behaviors that occur in biological systems, a variety of stimuli-responsive polymeric systems have been developed in the size range from macroscale to nanoscale.[3,4] However, the pH-responsive behavior of most of those polymeric systems is observed in the form of dimension changes and integration/disintegration processes. Herein, we report the preparation of a novel nanogel comprising two biocompatible components, namely chitosan and ethylenediaminetetraacetic acid, that shows novel surface switching of both composition and charge in response to pH changes in the medium (Figure 1). Moreover, the pH-dependent surface switch process of this nanogel is fully reversible, and the particle integrity is maintained in the entire pH range owing to the gel nature of the system.

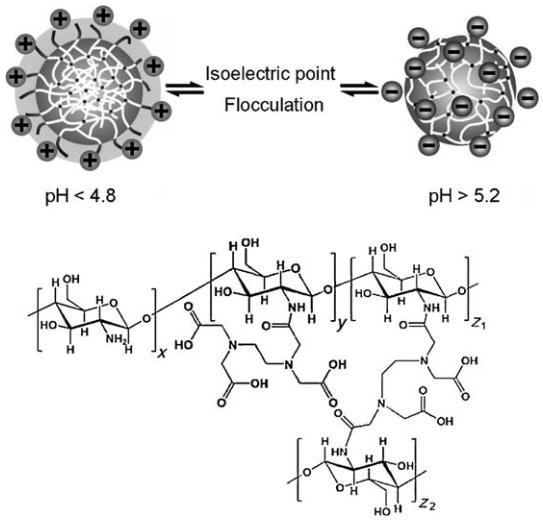

Figure 1. Top: Schematic representation of the surface switch of the nanogel triggered by pH changes; bottom: chemical structure of the chitosan-EDTA nanogel.

Generally, a double-hydrophilic water-soluble AB diblock copolymer can self-assemble to form micelles in aqueous medium with the A block forming the micellar core and the B block forming the shell.[5,6] In some cases, the surface reversal in both charge and composition of the micelles can be achieved by a "flip–flop" of the core and shell through a pH trigger and is also referred to as a "schizophrenic" character.[7–9] However, in most cases, a disassembly process is required for the inversion to take place. In this study, we used chitosan (CS) and ethylenediaminetetraacetic dianhydride (EDTAA) as starting materials to prepare a novel nanogel composed of chitosan and ethylenediaminetetraacetic acid (CS-EDTA nanogel), which can show another form of the switch at the nanogel surface in response to pH changes; this type of switch differs from the "flip–flop" switch exhibited in some diblock copolymer assemblies. CS is a biocompatible and biodegradable biopolymer consisting of *N*-acetylglucosamine units and glucosamine units and is often used as a biomaterial.[10] When combined with EDTA, an intestinal permeation enhancer in oral administration,[11] CS-EDTA nanogel carriers may have applications in biomedical and pharmaceutical areas.

The CS-EDTA nanogel was synthesized by amide linkage between the anhydride groups of EDTAA and the amino groups of water-soluble CS, which has a molecular weight of 5.4 kDa and a degree of deacetylation of 97.5%, in methanol/water (80:20 v/v) with an apparent pH value of 4–5 at room temperature. The reaction was heterogeneous, and the water-insoluble EDTAA was suspended in the solvent mixture. As the reaction proceeded, precipitation occurred. After reaction for 24 h, the wet precipitate was collected and dispersed in 0.01M NaOH solution to dissolve the unreacted EDTAA. At this step, a light-blue color was observed in the suspension. Unreacted CS, EDTAA, and macroscopic aggregates were then removed by centrifugation and subsequent dialysis against basic aqueous solution, acidic aqueous solution, and pure water. The hydrodynamic diameter of the resulting sample was 80.0 nm (pH 2) and 73.5 nm (pH 11) as determined by dynamic light scattering (DLS; Figure 2a). The FT-IR spectra confirmed the formation of amide linkages based on the absorption peak at 1736 cm^{-1}, assigned to the stretching vibration of C=O in the carboxyl group of reacted EDTA, and at 1684 and 1630 cm^{-1}, assigned to the amide groups. The degree of substitution was determined to be 71.5% by using 2,4,6-trinitrobenzenesulfonic acid,[12] meaning that 71.5% of the anhydroglucose units in CS were linked with the EDTA moiety. Combined with the result of element analysis, it was calculated that 6.6% of the attached EDTA moieties served

[*] X. Shen, Dr. L. Zhang, Prof. X. Jiang, Dr. Y. Hu, Dr. J. Guo
Laboratory of Mesoscopic Chemistry and
Department of Polymer Science & Engineering
College of Chemistry & Chemical Engineering
Nanjing University, Nanjing, 210093 (China)
Fax: (+86) 25-8331-7761
E-mail: jiangx@nju.edu.cn

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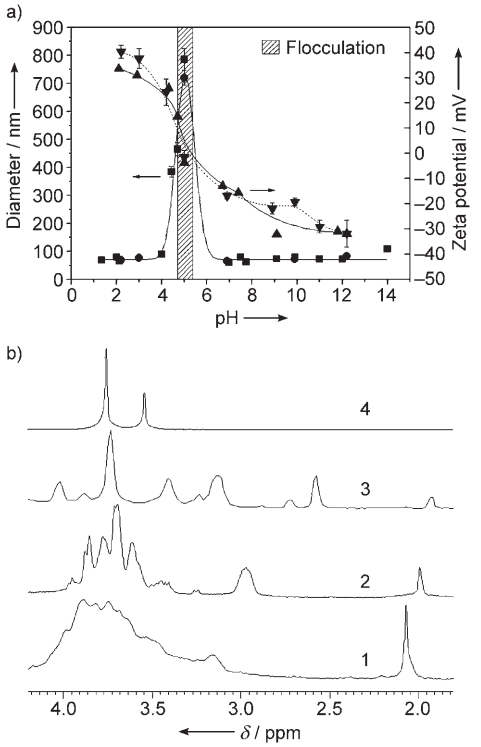

Figure 2. a) Variation of intensity-average hydrodynamic diameter (■ increasing pH; ◆ decreasing pH) and ζ potential (▲ increasing pH; ▼ decreasing pH) of the nanogel as a function of pH variation. The hashed area represents the region in which the flocculation of the nanogel occurs; b) ^{1}H NMR spectra of the nanogel in D_2O: 1) the nanogel at pH 2; 2) chitosan; 3) the nanogel at pH 11; 4) Na_4EDTA.

as the cross-linking agent. Fluorescence spectroscopy with pyrene as the probe indicated that there was no concentration-dependent unimer–assembly transition for the aqueous solution of sample both at pH 2.9 and 11.8, which suggests that the sample exhibits high stability and confirms again that the sample has a gel structure (details are given in the Supporting Information).

From the chemical structure of the sample, it is expected that the residual free amino groups in the CS chains can be protonated in acidic medium and possess positive charge, while the carboxyl groups of the pendent EDTA moieties in the CS chains can be deprotonated in basic medium and possess negative charge. Furthermore, the existence of three or two intrinsic pK_a values of the EDTA moiety provides the nanogel with a wider pH-responsive range. This result was confirmed by ζ-potential and DLS measurements, as shown in Figure 2a. It can be seen that the nanogel shows a positive potential below pH 4.8, and its potential approaches zero in the pH range 4.8–5.2. With increasing pH value, the ζ potential further decreases and becomes negative above pH 5.2. This result is quite similar to the behaviors observed in the cases of amphoteric polyelectrolytes and their gels.[13,14] Obviously, the positive ζ potential at low pH indicates that the surface of the nanogel consists of the protonated amino groups of the CS chains, while the negative potential above pH 5.2 suggests that at the nanogel surface the deprotonated EDTA moieties attached to the CS chains prevail. Thus, the transition area from positive to negative potential can be assigned to the isoelectric-point region of the nanogels, where they flocculate owing to the neutralization of the positive and negative charges. However, the flocculated nanogels could be easily redispersed by either an increase or a decrease in pH of the medium. It was also found that the size and ζ potential of the CS-EDTA nanogel are reversibly changed by a descending and ascending series of pH-variation cycles, and the particle integrity of the nanogels is finely maintained in the entire pH-variation range. Since 71.5% of the amino groups in the CS are substituted by EDTA moieties, it is expected that the nanogel size in the high-pH region would be larger than that in the low-pH region owing to the electrostatic intranetwork chain repulsion arising from the "net" charges. However, the hydrodynamic diameters of the CS-EDTA nanogel at low- and high-pH regions are almost the same, for example, 80.0 nm at pH 2 and 73.5 nm at pH 11.

To correlate these behaviors with the structure of the nanogel in low- and high-pH medium and to determine whether the pH-dependent ζ-potential variation arises solely from the acid–base chemistry associated with the amino/carboxyl groups in the system, or whether a change in surface structure of the nanogel also makes some contribution, ^{1}H NMR studies in D_2O at pH 2.0 and pH 11.0 were conducted. By comparing with the spectra of CS and Na_4EDTA in Figure 2b, it is clear that at pH 2 the characteristic signals of CS in the nanogel at $\delta = 3.0$ ppm (C2 of chitosan) and $\delta = 3.60$–3.85 ppm (C3–C6 of chitosan) are prominent, suggesting that the CS segments with protonated amino groups are highly solvated and are located at the outermost surface of the nanogel. In contrast, at pH 11 the signals of EDTA in the nanogel at $\delta = 3.41$ and 3.75 ppm become prominent and the signals from the CS segments are significantly reduced, suggesting that the deprotonated EDTA moieties in the nanogel network are forced outside of the nanogel surface and the CS segments with deprotonated amino groups go inside the nanogels. Considering that the cross-linking density of the nanogel is only about 6.6% and the CS subchains in the nanogel network are relatively rigid, it is reasonable to conclude that the NMR signals of the nanogel at pH 2 come from the dangling CS chains with protonated amino groups, which act as a shell at the nanogel surface, while the prominent EDTA signals at pH 11 are attributed to deprotonated EDTA moieties located in the CS chains in the nanogel network, and the shell composed of the dangling CS chains with deprotonated EDTA moieties does not seem to be present at the nanogel surface as based on the absence of sufficient CS signal (Figure 2b). An additional static light scattering (SLS) study of the nanogels in both low- and high-pH aqueous solutions yielded an average radius of gyration (R_g) of 98 nm (pH 2) and 70 nm (pH 11), thus suggesting further that the nanogel particles in acidic medium have a more extended chain conformation than in basic medium owing to the presence of dangling CS chains at the particle surface. Thus, the surface structure of the nanogel, including the composition and the charge, switches upon pH change of the medium, as illustrated in Figure 1. It should be noted that such a pH-dependant surface switch of nanogels does accompany an intermediate flocculation state but not a

disassembly state. Such an intermediate state may be viewed as a start position of the switch to a gel structure in which either the gel network is surrounded by a shell consisting of dangling CS chains with protonated amino groups (in low-pH medium) or the gel network consisting of CS linked with deprotonated EDTA units is swelled and expanded to the outermost surface of the nanogel particles, while the shell of the dangling CS chains is absent in this case (in high-pH medium).

Visualization by atomic force microscopy (AFM) provides the morphology of the nanogels in the solid state. A spherical shape with a mean diameter of about 60 nm is observed in the dried state at both pH 2 (Figure 3a) and pH 12 (data not shown). To fix the surface structure of the nanogel, the CS-EDTA nanogels were secondarily cross-linked by glutaraldehyde at pH 2 and by 2,2′-(ethylenedioxy)bis(ethylamine) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide at pH 12, respectively. The secondary cross-linking reaction using glutaraldehyde in an acidic medium should mainly take place between the dangling CS chains with protonated amino groups at the surface of the nanogel, while the reaction with diamine in a basic medium should link the carboxylic acid groups located at the surface of the nanogel network. In contrast to the featureless spherical morphology over a wide pH range for the secondarily cross-linked CS-EDTA nanogel using glutaraldehyde, TEM reveals that the structure of the sample that was secondarily cross-linked using diamine in basic medium has distinct features. As shown in Figure 3b, after the secondary cross-linking and dialysis against basic aqueous solution with pH 12, the chain-packing density at the center of the nanogel particles is markedly reduced owing to the interior swelling and surface fixation. In addition, the dark ring observed in the nanogel particles can be ascribed to the dangling CS chains and other CS segments with deprotonated amino groups on the basis of their hydrophobility. More interestingly, when such nanogel particles were dialyzed against aqueous solution with pH 6, the morphology of the sample becomes particle-in-hollow particle shape. The formation of such structures may be due to the surface fixation and the interior shrinkage of the nanogel particles arising from the deswelling, both of which lead to a reduced chain-packing density in the space between the cross-linked surface and the densified central particle region. These results further corroborate the conclusion drawn from ^{1}H NMR measurements, that is, that in low-pH aqueous medium, the CS-DETA gel network is surrounded by a shell consisting of dangling CS chains with protonated amino groups. Conversely, in high-pH aqueous medium, especially in the case of relatively strong basic medium, the swelled gel network goes to the outside and the CS segments with amino groups go to the inside of the nanogels, which results in a gel network at the outermost surface of the nanogel particles.

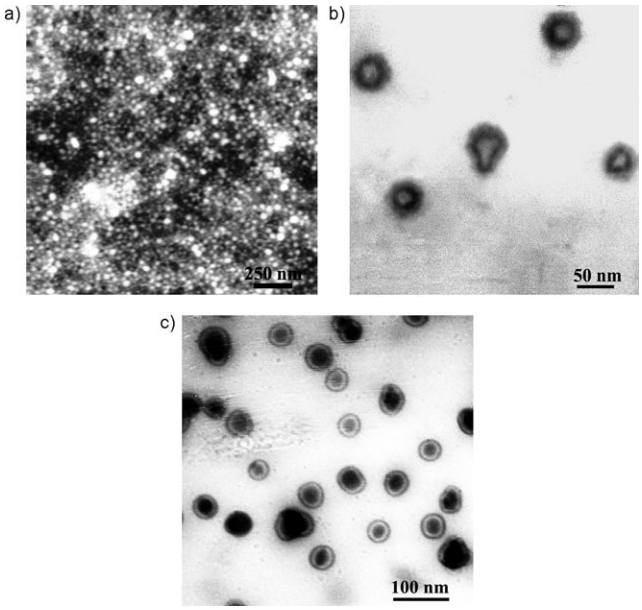

Figure 3. a) AFM image of the nanogel at pH 2. b) TEM image of nanogels which were secondarily cross-linked at pH 12; c) TEM image of nanogels in (b) dialyzed against aqueous solution at pH 6.

The formation mechanism of the nanogels can be explained as follows. As the water-insoluble EDTA moieties gradually attach covalently to the CS chains and in the meanwhile a small degree of intermolecular cross-linking takes place, which also generates the branched CS chains, the CS chains become insoluble and precipitate out of the system around the isoelectric point. The precipitated CS cannot continue the reaction with EDTAA, thus preventing the formation of a macroscopic gel. Thus, when the precipitate is redispersed into a lower- or higher-pH aqueous medium, CS-EDTA nanogels are formed and stabilized by either protonated amino groups in acidic aqueous medium or deprotonated carboxyl groups at the surface of the nanogels in basic aqueous medium. On the other hand, it is also worth mentioning that since the reaction is heterogeneous and the apparent pH value of the aqueous solution is 4 to 5 (owing to the partial hydrolysis of EDTAA), the CS segments with attached EDTA molecules tend to aggregate to form a less soluble phase when the substitution reaches a certain degree. Thus, the cross-linking by EDTAA may preferably take place in the less soluble phase. From this point of view, the cross-linking density would be higher in the CS segments in which substitution is more than that of the CS segments with less substitution. The CS segments with less substitution would be highly solvated and extend into the medium, which may be the origin of the dangling chitosan chains on the surface of the nanogels in acidic medium, and the nanogel surface is only a swollen network (of glucosamine units that are covalently attached to EDTA molecules) in basic medium.

With high particle integrity in the entire pH range and the ability to reversibly switch surface structure upon pH change, the prepared nanogel may serve as a good prototype for synthetic polymer systems that mimic some external-stimuli-responsive organisms. Moreover, the stability and unique switch behavior can also be exploited in the facile encapsulation of pH-sensitive anticancer agents in aqueous medium. For example, highly water-insoluble camptothecin (CPT), an anticancer agent, would open its labile E ring at neutral pH or

above, thereby resulting in the water-soluble carboxylate form with low antitumor activity and high toxicity.[15] However, the carboxylate can be converted into the water-insoluble lactone form again by acidification of the medium. Thus, by taking advantage of the novel switch properties exhibited by the nanogel, we can encapsulate water-soluble lactone-ring-opened CPT in basic aqueous medium and then convert the drug into the lactone form of CPT by acidifying the medium to enhance the encapsulation of the lactone form of CPT in the carrier. This process has been demonstrated to be feasible. The fluorescence emission spectrum of a CPT-loaded nanogel shows that the position of the band maximum is $\lambda = 432$ nm for loaded CPT in acidic aqueous solution (pH 5.5) and is in good agreement with the value reported,[16] thus indicating that the loaded CPT is indeed in the lactone form of the drug (Figure 4a). Interestingly, the fluorescence intensity of the drug-loaded nanogel in Figure 4a, in which the CPT-loaded nanogel concentration was diluted by a factor of 25 with distilled water to avoid exceeding the upper limit of the fluorescence spectrometer, is still much stronger than that of the saturated CPT aqueous solution, suggesting that the CS-EDTA nanogel acts as a solubilizer to remarkably enhance the solubility of lactone-formed CPT in acidic aqueous solution. On the basis of the solubility of CPT in acidic aqueous solution and the concentration of the CPT-loaded nanogel in aqueous solution, the CPT loading amount in the nanogel was determined to be over 12% (by nanogel weight), which is much higher than that in polymeric micelles.[17] The release profile of CPT from the nanogel in neutral aqueous solution at 37°C shows that the CPT is continuously released in vitro over 24 h (Figure 4b). Yet the release is fast because of the fast exchange of water molecules between the hydrophilic nanogel and aqueous medium, and because of the increased solubility of CPT in a neutral aqueous medium.

In summary, we have found that the CS-EDTA nanogel has a pH-dependent switch character in aqueous medium, and is demonstrated to be a very promising candidate in the encapsulation and delivery of therapeutic agents. Although all the "schizophrenic" systems to date do share one common feature—that they all contain two chemically opposite components, one acidic and the other basic, in their structures—the structure of the nanogel prepared in this work is unique in that it is irregular and weakly cross-linked as compared to the well-defined structure of the reported block copolymer-based assemblies. This irregular and weakly cross-linked gel structure with surface switch character may better resemble the structures of most organisms than block copolymer assemblies, and provides a preliminary insight into the design of smart materials which mimic natural organisms both in structure and in function. The abundant reactive carboxyl and amino groups present at the surface of the nanogels are subject to subsequent binding of targeting agents to provide additional functions, which may further unleash the application potential of such nanogel systems.

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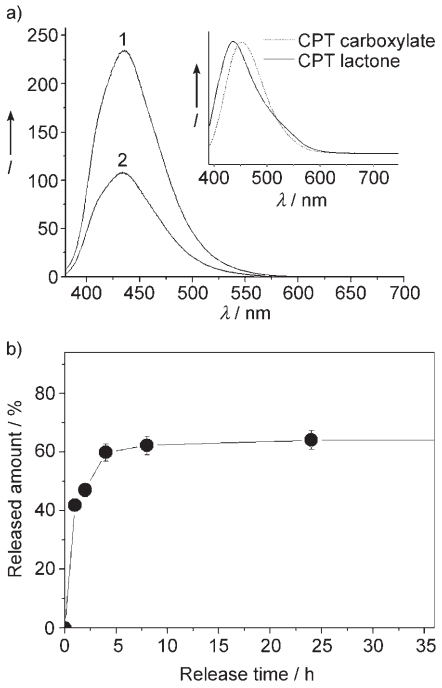

Figure 4. a) Fluorescence spectra of CPT-loaded nanogel in aqueous solution in which the nanogel concentration was diluted by a factor of 25 with distilled water (1) and saturated CPT aqueous solution (2); the pH value of both solutions is 5.5. The inset shows the spectra of two CPT forms, lactone and carboxylate. b) Release profile of CPT-loaded nanogel at 37°C in water at neutral pH.

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