Polymer Nanostructures

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Facile, Template-Free Synthesis of Stimuli-Responsive Polymer Nanocapsules for Targeted Drug Delivery**

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Nanometer-sized hollow polymer capsules, or polymer nanocapsules, have received much attention in recent years because of their potential applications in many areas, including drug delivery, encapsulation, and imaging.[1] In particular, their potential as drug-delivery vehicles has been well recognized.[2,3] However, to be a promising drug-delivery vehicle to enhance drug efficacy, and at the same time to minimize undesired side effects, nanocapsules should be 1) biocompatible; 2) readily synthesized; 3) easily functionalizable, in particular for the introduction of various functional moieties, such as targeting ligands on the surface; and 4) able to deliver and release drugs efficiently in targeted cells.[2,3] In particular, to control the intracellular release of drugs, stimuli-responsive nanocapsules were studied that can release loaded cargos in response to external stimuli, such as pH change,[4] reducing agents,[5] and heat.[6] However, despite considerable efforts, developing polymer nanocapsules that satisfy all of these conditions remains challenging.

Although there are several methods for synthesizing hollow polymer capsules, they usually require either a template[7] or a preorganized structure to shape the core–shell structure,[8] and then removal of the core to form a hollow capsule.[9] Recently, we developed a new strategy for synthesizing polymer nanocapsules that requires neither the use of a template or a preorganized structure, nor core removal.[10,11] More specifically, the thiol-ene photopolymerization (click reaction)[12] of $(allyloxy)_{12}$cucurbit[6]uril,[13,14] a rigid disk-shaped molecule that has a cavity and twelve polymerizable allyl groups at its periphery, and dithiols directly produced polymer nanocapsules which had a very thin (essentially single-monomer-thick) shell with a two-dimensional (2D) polymer network. Furthermore, as the shell is made of cucurbit[6]uril (CB[6]), a nontoxic molecule with a cavity that can recognize and bind polyamines, such as spermine, very tightly ($K \approx 10^9$ to $10^{12}\,M^{-1}$) through host–guest interactions,[15] a wide variety of tags or functional moieties, such as targeting ligands and imaging probes can be easily introduced onto the surface of the polymer nanocapsules in a noncovalent, nondestructive, and modular manner simply by treating the nanocapsules with tag-polyamine conjugates.[10,16]

This success prompted us to develop CB[6]-based, "smart" polymer nanocapsules using the same strategy, which can not only deliver entrapped drugs to target cells, but also release them inside the cells in a controlled manner after internalization. In particular, for the intracellular release of entrapped drugs, we wanted to develop reductively labile polymer nanocapsules that can be collapsed to release loaded cargos in cytosol, which is known to be a highly reducing environment because of the presence of naturally occurring reducing agents, such as glutathione (GSH).[5,17] With this in mind, we chose to incorporate disulfide bridges, which can be readily cleaved in a reducing environment, into the 2D polymer network of polymer nanocapsule shells. However, it is difficult to introduce disulfide bridges into the polymer network using our previous direct synthetic approach, which was based on thiol-ene photopolymerization, because disulfide bonds are reversibly cleaved to thiyl radicals under UV irradiation.[12,18] Therefore, whilst keeping the same general strategy, we explored other synthetic methods that could afford the spontaneous formation of hollow polymer nanocapsules. After a number of unsuccessful attempts, we discovered that a polymerization technique based on amide bond formation is compatible with our direct approach to polymer nanocapsules. This direct approach not only produced hollow polymer nanocapsules directly, but also allowed the incorporation of new functionalities into polymer nanocapsules. Herein, we report the facile, template-free synthesis of a stimuli-responsive polymer nanocapsule that can be collapsed to release loaded cargos in a reducing environment. The reductively labile polymer nanocapsule, once noncovalently decorated with a targeting ligand, can deliver an encapsulated model drug into cytosol after internalization into target cells, thus demonstrating its potential as a targeted drug delivery vehicle.

In a typical experiment, simple stirring of a mixture of **1**[19] and **2** (1:6 mole ratio) in a chloroform/methanol solution 1:1 (v/v) in the presence of a catalytic amount of triethylamine at room temperature for a day, followed by dialysis, directly

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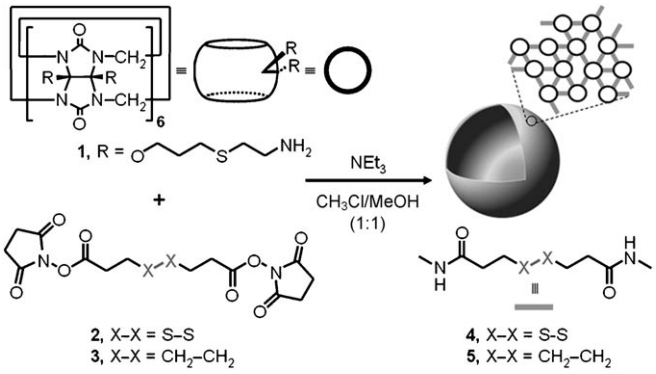
produced polymer nanocapsule **4** in 84% yield based on **1** (Scheme 1). The resulting polymer nanocapsule **4** was characterized by scanning and transmission electron microscopy (SEM and TEM, respectively). The SEM images showed that



Scheme 1. Synthesis of polymer nanocapsules **4** and **5**, with and without disulfide bridges, respectively.

4 has an average diameter of (70 ± 20) nm (Figure 1 a). High-resolution TEM (HRTEM) studies revealed that **4** had a hollow interior, surrounded by a thin shell that had an average thickness of (2.0 ± 0.3) nm (Figure 1 b), thus indicating that the polymer nanocapsule **4** was successfully generated. The FT-IR spectrum of **4** (see the Supporting Information, Figure S1) revealed two characteristic peaks for the CB[6] unit at 1760 and 1458 cm^{-1} (C=O and C–N stretching vibrations, respectively), as well as intense amide peaks at 1650 and 1540 cm^{-1}, which corresponded to the amide I (C=O stretching) and amide II (N–H bending) vibrational modes, respectively;[20] this confirmed the formation of a polymer network containing CB[6] that was linked by amide bonds through polymerization.[21]

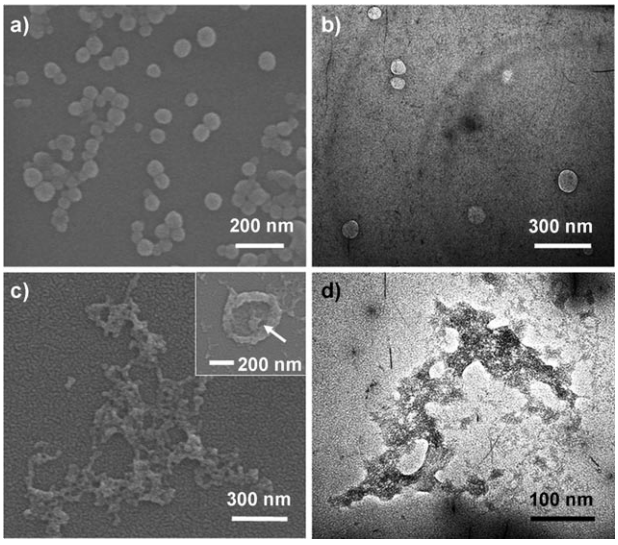


Figure 1. a) SEM and b) HRTEM images of polymer nanocapsule **4**; c) SEM and d) HRTEM images of **4** after treatment with DTT for 30 minutes.

The disulfide bridges that were incorporated into the polymer network of **4** are easily cleaved to afford the corresponding thiols using reducing agents, such as dithiothreitol (DTT).[5,17] The reductively labile nature of polymer nanocapsule **4** was confirmed by SEM and HRTEM studies. The morphological change of **4**, monitored by SEM and HRTEM, showed that the polymer nanocapsule started to lose its spherical shape and to form aggregates upon treatment with DTT. After 30 minutes, most polymer nanocapsules (**4**) had collapsed and aggregated (Figure 1 c,d). On the other hand, the morphology of **5**, polymer nanocapsules without disulfide bridges, did not change even after prolonged treatment with excess DTT. These results clearly indicate that the disulfide bridges in the polymer network of nanocapsule **4** were quickly cleaved, thereby leading to the collapse of the polymer nanocapsule.

Having established the reductively labile nature of polymer nanocapsule **4**, we decided to investigate the reduction-triggered release of encapsulated fluorescent dyes from the nanocapsule by fluorescence spectroscopy. First, a polymer nanocapsule (CF@**4**) that had encapsulated carboxyfluorescein (CF) in its interior, was synthesized by carrying out the same polymer nanocapsule formation reaction as described above in the presence of CF (3×10^{-4} M), followed by dialysis. The average size of the CF@**4** nanocapsules was slightly larger than that of **4**, with an average diameter of (90 ± 30) nm, as confirmed by SEM studies. More importantly, the procedure of incorporating the fluorescent dye into the polymer nanocapsule did not significantly affect its spherical shape of a hollow interior surrounded by a thin polymer shell, as revealed by SEM and HRTEM studies (see the Supporting Information, Figure S2). Approximately 700 CF molecules are estimated to be entrapped inside a nanocapsule that has a diameter of 90 nm based on the initial concentration of CF in the reaction medium.

The CF-encapsulated nanocapsule (CF@**4**) was redispersed in a 5% methanol/PBS buffer solution and the emission intensity of CF was measured; the emission intensity was a significantly lower value compared to that of free CF, presumably owing to self-quenching of the encapsulated dye. Stirring of the solution for 3 hours resulted in little change in the emission intensity (< 5%; Figure 2), thus indicating that no significant portion of encapsulated CF molecules was released from the polymer nanocapsule. In contrast, when the dispersion CF@**4** was treated with 100 mM DTT, the emission intensity of CF quickly increased initially, before increasing more slowly, and finally reached a plateau over 3 hours (Figure 2); these results indicated the release of the entrapped fluorescent dye molecules from the polymer nanocapsule.[6,22] In a control experiment, the CF-encapsulated polymer nanocapsule that did not contain disulfide bridges (CF@**5**) did not give any significant change in fluorescence intensity upon treatment with 100 mM DTT (Figure 2). Taken together, these results indicated that the release of the encapsulated dye molecules from polymer nanocapsule **4** upon treatment with DTT was not due to passive diffusion or swelling of the nanocapsule, but rather to rupturing of the polymer nanocapsule that was triggered by the reducing agent.[17a]

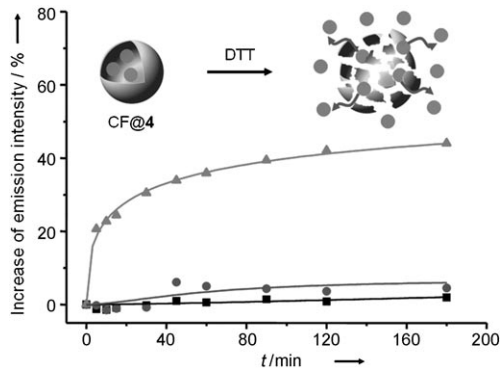
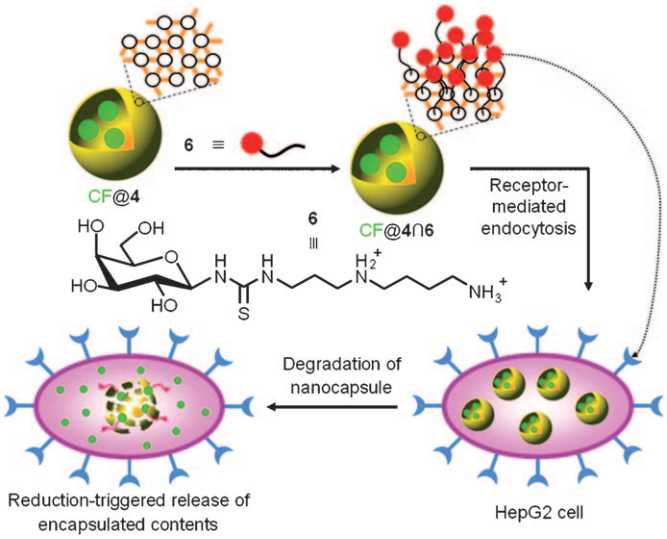


Figure 2. Increase in the emission intensity at 516 nm as a function of time for CF@**4**, stirred in 5% methanol/PBS in the presence (▲) and absence (●) of 100 mM DTT, and CF@**5** stirred in 5% methanol/PBS in the presence of 100 mM DTT (■).

To illustrate the potential utility of the reductively labile polymer nanocapsule as a targeted drug-delivery vehicle, we studied the targeted delivery of the nanocapsule to cancerous cells and release of an encapsulated model drug inside the targeted cells (Scheme 2). We first prepared CF-encapsulating polymer nanocapsules CF@**4** and CF@**5**, in which CF was used as a model drug and imaging probe. Then, the surface of the nanocapsules was decorated with a targeting ligand in a noncovalent manner by taking advantage of the fact that the shell of the polymer nanocapsules were made of CB[6], which can bind polyamines with extremely high affinity.[10] In this study, we chose HepG2 hepatocellular carcinoma cells with over-expressed galactose receptors as target cells. By treatment of CF@**4** and CF@**5** with galactose-spermidine conjugate **6**, the targeting ligand was readily introduced onto the surface of the dye-encapsulated polymer nanocapsules to produce CF@**4**∩**6** and CF@**5**∩**6**, respectively.



Scheme 2. Schematic representation of the noncovalent surface modification of the CF-encapsulating polymer nanocapsule (CF@**4**) with **6** through host–guest interactions, the receptor-mediated endocytosis, and the autonomous triggered release of the encapsulated CF to cytosol.

After incubating CF@**4**, CF@**4**∩**6**, and CF@**5**∩**6** with HepG2 cells in separate media, their internalization and reduction-triggered release of the encapsulated fluorescent dyes inside the targeted cells were monitored by confocal microscopy. There was no significant increase of the fluorescence signal inside the cells for CF@**4** and CF@**5**∩**6** after incubation for 1 hour, whilst a sharp increase in the fluorescence signal was observed for CF@**4**∩**6** (Figure 3). The low-fluorescence of CF@**4**, which did not contain the targeting ligand, was presumably due to little or slow internalization of

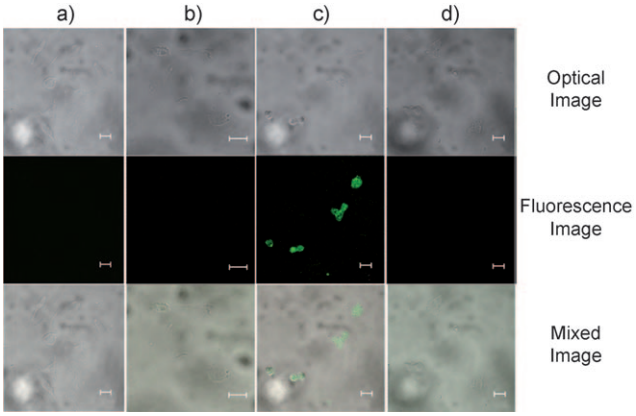


Figure 3. a) Confocal microscopy images of untreated HepG2 cells; b) cells treated with CF@**4**; c) cells treated with CF@**4**∩**6**; and d) cells treated with CF@**5**∩**6**. Scale bars: 20 μm.

the polymer nanocapsule. The encapsulated CF@**5**∩**6** nanocapsules, in which the nanocapsule did not contain disulfide bridges, was successfully internalized into the cells by receptor-mediated endocytosis; however, the entrapped dye molecules remained trapped inside the nanocapsule, owing to the lack of cleavable disulfide bridges, thus resulting in no significant increase in fluorescence because of the self-quenching of the entrapped dye molecules. On the other hand, the markedly increased fluorescence signal observed for CF@**4**∩**6** clearly indicated that after facile internalization into HepG2 cells, the disulfide bridges were ruptured in the reducing intracellular environment to release the encapsulated dye molecules into the cytosol.

In conclusion, we have developed a template-free synthetic approach to stimuli-responsive polymer nanocapsules that has potential applications for targeted drug delivery. The reductively labile polymer nanocapsules that are composed of CB[6] and disulfide bridges allows not only the facile, noncovalent surface modification for targeting, but also the release of encapsulated cargo in response to a predefined redox stimulus in an intracellular environment. We anticipate that the cargo release profile of the nanocapsule can be tuned by controlling the number of disulfide bridges in the polymer network of the nanocapsule. In addition to various targeting ligands, other functional moieties, such as imaging probes and antifouling groups can be easily introduced onto the surface in a modular manner, thus suggesting that such stimuli-responsive polymer nanocapsules can be utilized as versatile platforms for targeted drug delivery and controlled release.

Experimental Section

Polymer nanocapsule **4**: Linker **2** (7.2 mg, 18.0 μmol) was added to a solution of **1** (10.2 mg, 3.0 μmol) in a 1:1 (v/v) mixture of chloroform and methanol (30 mL). After addition of a catalytic amount of triethylamine (150 μL), the reaction mixture was stirred at room temperature for a day. The product was purified by dialysis using a 1:1 (v/v) mixture of chloroform and methanol for 2 days to give a colloidal solution of polymer nanocapsule **4**, which was used for further experiments. Removal of solvent in vacuo afforded polymer nanocapsule **4** for characterization (8.6 mg, 84%). Elemental analysis calcd for **4** $[(C_{94}H_{161}N_{35}O_{24}S_{11})(C_6H_6O_2S_2)_{2.5}(CH_4O)_9(H_2O)_{10}]_n$: C 41.41, H 6.83, N 14.33, S 14.99; found: C 40.66, H 5.77, N 14.11, S 14.18. For further characterization of **4** and other experimental details, see the Supporting Information.

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[21] The elemental analysis data (N/S ratio) of **4** indicated that the ratio of **1** and **2** incorporated into **4** is 1:2.5, which suggests that approximately 5 of 12 amine terminal groups of **1** participate in the polymer network of **4** by forming amide bonds.

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