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Dual-Stimuli-Responsive Nanoassemblies as Tunable Releasing Carriers

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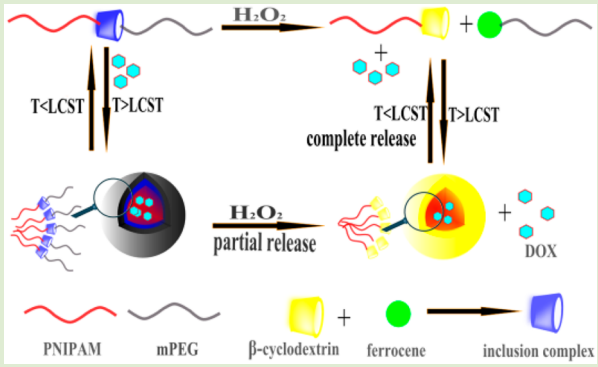
Supporting Information

ABSTRACT: Two end-decorated homopolymers, methoxy polyethylene glycol-ferrocene (mPEG-Fc) and poly(*N*-isopropylacrylamide)-*β*-cyclodextrin (PNIPAM-*β*-CD), were further orthogonally self-assembled into stable micelles in aqueous solution by controlling the temperature of the solution via terminal host–guest interactions. Because of the H_2O_2 cleavable CD/Fc connection and thermoresponsive PNIPAM, an H_2O_2 and thermo dual-controlled drug release based on this system was also achieved. Interestingly, the cytotoxicity evaluation of mPEG-Fc/PNIPAM-*β*-CD indicated good biocompatibility. Compared with free doxorubicin, the doxorubicin-loaded supramolecular micelles exhibited equal cellular proliferation inhibition toward A549 cells. This supramolecular complex is thus anticipated to serve as a promising new type of alternative drug-delivery system.



Stimuli-responsive polymer micelles have attracted significant attention in a broad range of fields, including drug delivery,[1] sensor systems,[2] and nanodevices.[3] Over the past few decades, numerous stimuli-sensitive micelles that respond to various stimuli,[4] such as pH,[5,6] temperature,[7,8] light,[9] redox species,[10] enzymes,[11] and ultrasound,[12,13] have been reported. In recent years, multisensitive micelles have become increasingly prevalent because they mimic the behavior of natural responsive materials and have high efficiency.[14−16] In particular, the fabrication of multisensitive micelles with precisely tunable properties is considered to be a very important future direction.

The most common approaches toward multisensitive micelles with tunable properties are self-assembly processes of block copolymers, which typically consist of both multisensitive segments and cleavable linkages.[17,18] Another effective alternative strategy to produce multisensitive micelles is the "block copolymer-free" strategy. In these micelles, the sensitive components are connected by reversible noncovalent bonds.[19−23] Molecular recognition systems or host−guest interactions have been recognized as one of the most important classes of noncovalent interactions for inducing self-assembly because of their excellent properties, which include specific molecular recognition, reversibility, and precise size controllability.[24−27] Compared to the conventional strategy, this approach has the advantages of synthetic simplicity and the reversibility feature of micelles. During the past decade, numerous stimuli-sensitive noncovalently connected micelles (NCCMs), including multiresponsive systems, have been reported.[28−32] However, precisely controlling the size and release of the encapsulated molecules in response to multiple stimuli remains a challenge.

Herein, we designed a type of dual-sensitive micelle by connecting the thermally responsive poly(*N*-isopropylacrylamide) (PNIPAM) chain with the methoxy polyethylene glycol (mPEG) chain through redox-switchable inclusion complexation between the *β*-cyclodextrin (*β*-CD) and ferrocene (Fc) groups. The resulting micelles exhibited not only reversible self-assembly behavior but also tunable release of encapsulated molecules in response to a single stimulus or to combinations of stimuli (Scheme 1).

To synthesize mPEG-Fc, methoxy poly(ethylene glycol) was functionalized with Fc via an *N*,*N*′-dicyclohexylcarbodiimide (DCC) coupling reaction in 85.3% yield. The homopolymer, PNIPAM-*β*-CD, was prepared by the free-radical polymerization of NIPAM in anhydrous DMF at 60 °C for 12 h using mono-6-thio-*β*-CD as the chain transfer reagent and AIBN as the initiator. The reaction afforded a well-defined and nearly monodisperse product in 52.7% yield (M_w = 4125 g/mol, M_w/M_n = 1.33, details of the mPEG-Fc and PNIPAM-*β*-CD syntheses as well as their characterization results are given in the Supporting Information).

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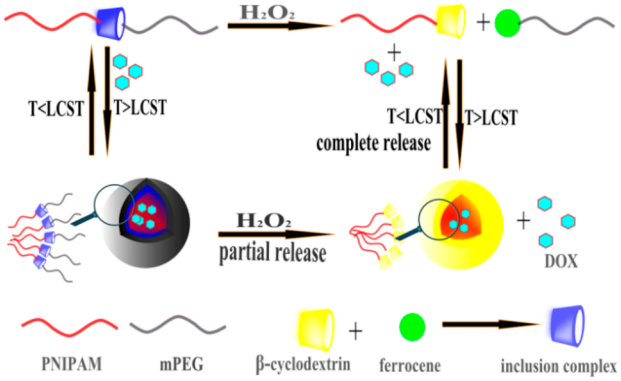
Scheme 1. Illustration of the Dual-Stimuli-Responsive Assembly and Disassembly of the mPEG-Fc/PNIPAM-β-CD Micelles



β-CD is known to interact with Fc to form a 1:1 inclusion complex; however, it cannot form an inclusion complex with oxidized Fc because of the mismatch between the host and guest.[33,34] Therefore, H_2O_2 was chosen as an oxidant for the investigation of the interactions between PNIPAM-β-CD and mPEG-Fc. As shown in the 2D NOESY spectrum of the mPEG-Fc/PNIPAM-β-CD supramolecular complex in Figure 1, cross-peaks attributed to dipolar interactions between the

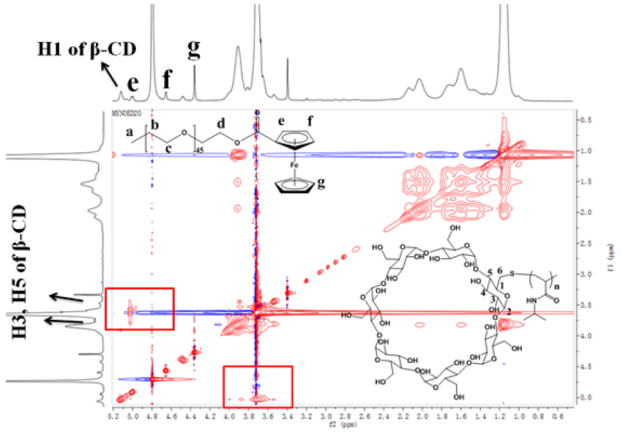


Figure 1. 2D NOESY NMR spectrum of mPEG-Fc and PNIPAM-β-CD in the absence of H_2O_2 (solvent: D_2O).

signals in the range 3.65−3.46 ppm, which are assigned to the inner protons (the 3- and 5-H protons) located in the cavities of β-CD, and the signals at 4.99 and 4.38 ppm, which are ascribed to the Fc moieties, are clearly observed. These peaks strongly indicate that the Fc moieties are deeply embedded in the cavities of β-CD and that the mPEG-Fc/PNIPAM-β-CD supramolecular complex was successfully obtained through host−guest interactions. However, in the presence of H_2O_2, no apparent correlation between β-CD and Fc was observed, indicating that the β-CD-Fc^+ inclusion complexes dissociated in response to the addition of oxidant (Figure S13, Supporting Information).

The supramolecular mPEG-Fc/PNIPAM-β-CD was anticipated to exhibit thermoresponsive behavior at temperatures near the low critical solution temperature (LCST) of PNIPAM.[35] Figure S15a (Supporting Information) shows the temperature-dependent optical transmittance of the mPEG-Fc/PNIPAM-β-CD solution. At temperatures below 31 °C, the mPEG-Fc/PNIPAM-β-CD complexes dissolved very well in aqueous solution, and the transmittance of the solution was greater than 91.4%. As the temperature was increased from 31 to 34 °C, the transmittance of the solution decreased abruptly to approximately 24.5%, suggesting the formation of aggregates. Additionally, this thermosensitive behavior was completely reversible (Figure S15b, Supporting Information). Moreover, variable-temperature ^{1}H NMR spectroscopy was used to monitor the dehydration of the thermoresponsive block under various temperature conditions. As shown in Figure 2,

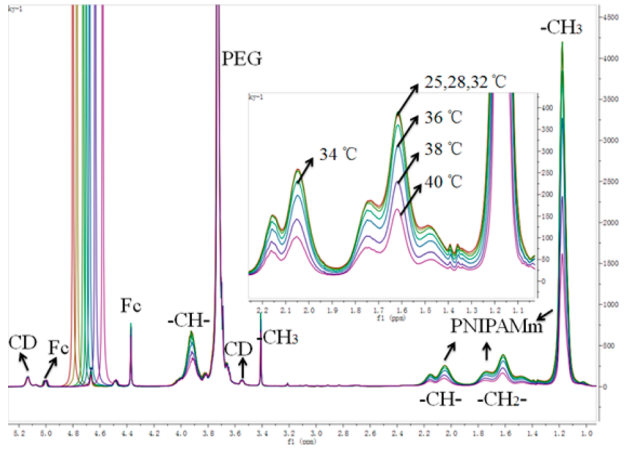


Figure 2. Variable-temperature ^{1}H NMR spectra of the mPEG-Fc/PNIPAM-β-CD complex in D_2O.

at temperatures below the LCST of PNIPAM, the proton signals assigned to both the mPEG-Fc block and the PNIPAM-β-CD block are clearly discernible (spectra recorded at 32, 28, and 25 °C), indicating that mPEG-Fc/PNIPAM-β-CD complexes are molecularly soluble in D_2O. When the temperature was increased from 32 to 40 °C, the intensity of four typical proton signals at δ 1.04, 3.84, 1.97, and 1.44 ppm, which were attributed to the PNIPAM block, were significantly attenuated, whereas the characteristic signals of the mPEG-Fc block remained approximately the same as those in the spectra collected at 25, 28, and 32 °C. These results indicate the beginning of the soluble-to-insoluble phase transition of the PNIPAM block at temperatures above the LCST of the PNIPAM segment and the formation of the core−corona micelles containing a dehydrated PNIPAM core and a water-soluble mPEG corona. These results suggest that the mPEG-Fc/PNIPAM-β-CD complex turns from a double hydrophilic structure to PNIPAM-core micelles as the environmental temperature is elevated above the LCST of PNIPAM.

The critical micelle concentration (CMC) of mPEG-Fc/PNIPAM-β-CD at 37 °C was 0.31 mg/mL (Figure S16, Supporting Information). Dynamic light scattering (DLS) measurements showed that mPEG-Fc/PNIPAM-β-CD aggregated into particles approximately 218 nm in diameter with a very narrow size distribution (Figure 3a). Transmission electron microscopy (TEM) revealed that the mPEG-Fc/PNIPAM-β-CD aggregates were uniform, spherical nanoparticles approximately 200 nm in diameter, consistent with the diameters detected by DLS (Figure 3c).

After the addition of H_2O_2, the size distribution of the micelles remained very narrow; however, the average Dh decreased to approximately 148 nm (Figure 3b). TEM images also showed that the size of the micelles obviously decreased to

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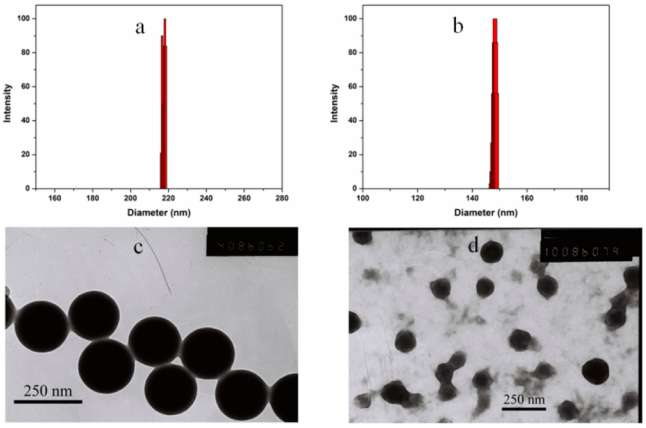

Figure 3. mPEG-Fc/PNIPAM-β-CD formed micelles measured by (a) DLS and (c) TEM (scale bar = 250 nm) at 37 °C. After the addition of H_2O_2 as an oxidant, mPEG-Fc/PNIPAM-β-CD formed micelles, which were measured by (b) DLS and (d) TEM (scale bar = 250 nm) at 37 °C.

approximately 130 nm after the addition of H_2O_2 (Figure 3d). These subordinate micelles were attributed to the self-assembly of PNIPAM-β-CD.

In the presence/absence of H_2O_2, the mPEG-Fc/PNIPAM-β-CD solution was centrifuged at 37 °C at 14 000 rpm. The aggregates were composed of PNIPAM-β-CD and mPEG-Fc simultaneously in the absence of H_2O_2 (Figure S18a, Supporting Information). However, in the presence of H_2O_2, the aggregates only consisted of PNIPAM-β-CD (Figure S18b, Supporting Information). The results indicate that the mPEG-Fc/PNIPAM-β-CD micelles dissociated into smaller PNIPAM-β-CD micelles after the addition of oxidant. The expected structure of the PNIPAM-β-CD micelles is a hydrophobic PNIPAM core surrounded by hydrophilic β-CD.

The responsive micelles are good candidates for the encapsulation and release of molecules. As shown in Figure 4a, thermal stimulus caused a burst of DOX release in the first 15 h, and approximately 91.5% of the DOX was released after 48 h. In contrast, the addition of the oxidant resulted in only 48.5% release of DOX over a period of 48 h. As previously discussed, because of the phase transition of the PNIPAM, the mPEG-Fc/PNIPAM-β-CD micelles completely dissolved in water as the temperature decreased to less than 32 °C. As a result, most of the encapsulated molecules were released. However, the addition of oxidant at 37 °C did not result in complete disruption of the micelles but rather caused the mPEG-Fc/PNIPAM-β-CD micelles to dissociate into smaller PNIPAM-β-CD micelles. The oxidation of Fc and the dissociation of the β-CD/Fc inclusion occurred at the micelle corona−core interface. As a result, at 37 °C, only 50.9% of the DOX was released in response to the redox over a period of 48 h, where greater than 49.1% of the DOX remained entrapped in the core of the PNIPAM-β-CD micelles. Thus, release of encapsulated molecules was slow and incomplete. Without thermal or redox stimuli, only 28.1% of the DOX was released, which may have been caused by the dissolution and diffusion of DOX. Figure 4b shows the two-step release behavior of DOX. In the first step, DOX release was triggered by redox. mPEG-Fc/PNIPAM-β-CD micelles dissociated to PNIPAM-β-CD micelles in the presence of H_2O_2 at 37 °C, causing partial release of DOX. Approximately 28.9% of the DOX was released, but most of the DOX was still entrapped in the core of

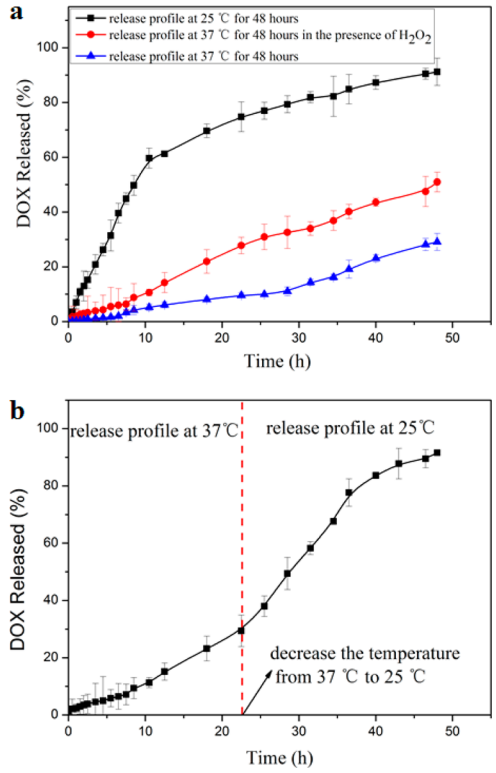

Figure 4. Amount of DOX released as a function of time over a period of 48 h under (a) single or no stimulus and (b) dual stimuli.

PNIPAM-β-CD micelles. The second step of the release was controlled by a combination of oxidant and temperature. When the environmental temperature was lowered to 25 °C after the redox-triggered release, the PNIPAM-β-CD micelles dissociated into hydrophilic polymers due to the hydrophobic-to-hydrophilic transition of the PNIPAM. As a result, DOX entrapped in the core of PNIPAM-β-CD micelles was released. After an additional 24 h, the total RE of the DOX in response to both stimuli was 90.7%, which is quite similar to that observed in response to only temperature.

It is known that H_2O_2 plays an important role in cancer development, and several tumor cell lines even could constitutively produce large amounts of H_2O_2 during their growth process. So, it is important to note the use of the micellar delivery systems with redox-sensitive behavior to result in a decrease in the cellular levels of H_2O_2 and in the release of antitumor drugs to kill cancer cells, while these systems are accumulated in the tumor sites via the enhanced permeability and retention (EPR) effect.[36,37] In this study, the release amount of DOX could be controlled by the concentration of H_2O_2 (Figure S17, Supporting Information).

The mPEG-Fc/PNIPAM-β-CD micelles developed in this study could release the encapsulated drug (1) slowly and partially in response to the H_2O_2 concentration in the cancer cells or (2) quickly and completely through application of either simple ice packs or deeply penetrating cryoprobes, which are used clinically to freeze tissues such as tumors.[38,39] In addition, two-step release could also be realized by a combination of the effects of redox and temperature.

The diverse responsiveness to external stimuli of mPEG-Fc/PNIPAM-β-CD micelles provides an opportunity to fine-tune the release properties of guest molecules to each stimulus independently or to a combined effect of multiple stimuli. This

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system has implications in areas such as on-demand drug delivery, cell and tissue imaging, and clinical diagnosis.

The cytotoxicities of mPEG-Fc, β-CD-PNIPAM, mPEG-Fc/β-CD-PNIPAM complex, DOX-loaded micelles, and DOX were investigated in A549 cancer cell lines using MTT assays. As shown in Figure 5a, mPEG-Fc, β-CD-PNIPAM, and the

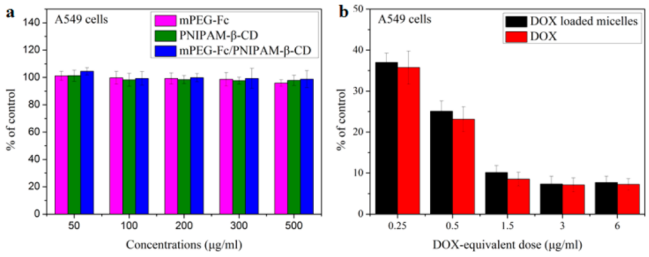

Figure 5. In vitro cytotoxicity of (a) mPEG-Fc, β-CD-PNIPAM, mPEG-Fc/β-CD-PNIPAM complex and (b) DOX-loaded micelles and free DOX to A549 lung cancer cells. Values represent the average ± s.d. (n = 6).

mPEG-Fc/β-CD-PNIPAM complex used for in vitro cytotoxicity were nearly nontoxic to the cells even when the tested concentration reached 500 μg/mL; these results indicate that the mPEG-Fc/β-CD-PNIPAM inherited the excellent biocompatibility of PEG and PNIPAM. The activity of DOX-loaded micelles is shown to be equal to that of DOX at concentrations as low as 0.25−6 μg/mL (Figure 5b), indicating that the DOX-loaded micelles maintained the excellent anticancer activity of DOX.

In summary, we have designed and fabricated a type of noncovalently connected copolymer in which PNIPAM-β-CD and mPEG-Fc chains are connected by inclusion interaction between β-CD and Fc. These mPEG-Fc/PNIPAM-β-CD complexes can self-assemble into micelles in aqueous solution at temperatures above the LCST of PNIPAM and are capable of encapsulating hydrophobic molecules such as DOX. The supramolecular block copolymer mPEG-Fc/β-CD-PNIPAM is nontoxic even at high concentrations of approximately 500 μg/mL.

The mPEG-Fc/PNIPAM-β-CD micelles were demonstrated to disassemble under the effects of oxidant or temperature via different mechanisms: (1) the oxidant affords the dissociation of β-CD-Fc, hence mPEG-Fc/PNIPAM-β-CD micelles dissociate into PNIPAM-β-CD micelles; (2) at temperatures below the LCST, the hydrophobic PNIPAM becomes hydrophilic, hence no assembly occurs. This feature provides a method of fine-tuning the release kinetics of encapsulated molecules. The encapsulated molecules can be released through three models according to the demand of the application: quick and complete release, relatively slow and partial release, or two-step release. Fabrication of multisensitive systems with fine-tunable release properties is considered to be a very important future direction. This study demonstrates that connecting sensitive components by reversible noncovalent bonds is an effective and simply way to obtain nanocarriers with diverse and tunable release kinetics.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of the synthesis, turbidity tests, and CMC measurements. Standard curve plotted by the ΔA485 nm of the standard versus the DOX concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval of the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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