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Core–Shell Nanosized Assemblies Mediated by the α–β Cyclodextrin Dimer with a Tumor-Triggered Targeting Property

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ABSTRACT In this paper, the α–β cyclodextrin dimer is designed *via* "click" chemistry to connect the hydrophilic and hydrophobic segments to form self-assembled noncovalently connected micelles (NCCMs) through host–guest interactions. A peptide containing the Arg-Gly-Asp (RGD) sequence was introduced to NCCMs as a target ligand to improve the cell uptake efficacy, while PEGylated technology was employed *via* benzoic-imine bonds to protect the ligands in normal tissues and body fluid. In addition, two fluorescent dyes were conjugated to different segments to track the formation of the micelles as well as the assemblies. It was found that the targeting property of NCCMs was switched off before reaching the tumor sites and switched on after removing the poly(ethylene glycol) (PEG) segment in the tumor sites, which was called "tumor-triggered targeting". With deshielding of the PEG segment, the drugs loaded in NCCMs could be released rapidly due to the thermoinduced phase transition. The new concept of "tumor-triggered targeting" proposed here has great potential for cancer treatment.

KEYWORDS: α–β cyclodextrin dimer · host–guest interaction · tumor-triggered targeting · drug delivery

Polymer inclusion complexes (PICs)[1,2] formed by host–guest interactions have been extensively investigated to construct supramolecular architectures. Cyclodextrin (CD)[3–5] and its derivatives are most widely used to induce the host–guest interactions because of their capability to form inclusion complexes selectively with hydrophobic guest molecules in aqueous solutions. For example, Jiang et al.[6–9] introduced various types of CD derivates to construct noncovalently connected micelles (NCCMs) by hydrogen bonds between shells and cores. Ritter et al.[10] reported the formation of specific host–guest interactions between the β-CD dimer and polymers with adamantyl groups. In the biomedical field, the hydrophobic inner cores of NCCMs serve as nanocontainers for hydrophobic drugs and the hydrophilic outer shells provide the colloidal stability. These NCCMs,[11,12] especially stimuli-responsive micelles that are sensitive to external stimuli like temperature, light, or pH, have been widely studied.

It is known that there exist slight differences in temperature/pH between normal cells ($T = 37$ °C, pH = 7.4) and malignant cells ($T > 37$ °C, pH < 6.8). The primary challenge in cancer treatment is to deliver drugs efficiently into tumor cells and prevent normal tissues from harm. Motivated by this, micelles with a targeting property through incorporation of tumor target ligands have been extensively investigated.[13,14] However, to our best knowledge, in these studies, the target ligands are directly linked to the surface of the micelles. Due to the extracellular barriers, it is still a challenge to deliver the drug-loaded micelles to the target cell population and to realize receptor-mediated cell uptake by injection or systematic delivery.

Here, we fabricated a novel stimuli-responsive NCCM with a tumor switchable cell targeting property. As shown in Scheme 1, a peptide containing the Arg-Gly-Asp (RGD) sequence is introduced as a target ligand to improve the cell uptake efficacy because RGD receptors are overexpressed on the surface of tumor cells. PEGylated technology is used to protect the ligand in normal tissues and body fluid. The PEG side chains are connected to the main chains through benzoic-imine bonds, which are stable under basic conditions and hydrolyze when the pH value is below 6.8, such as the extracellular environment of solid tumors.[15] Once the micelles reach tumor sites, the PEG chains in the outer layer of the micelles will be removed and the exposed RGD targeting groups can realize cell targeting. In other words, the RGD cell targeting

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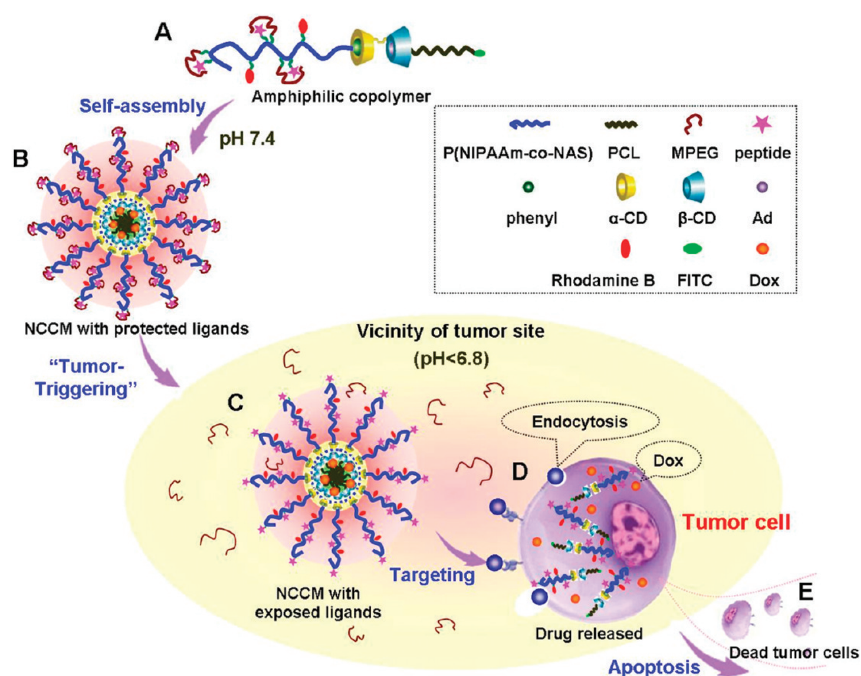

Scheme 1. Formation of shell−core micelles with switchable tumor cell triggered targeting: (A) Diblock copolymer connected by α−β cyclodextrin dimer; (B) Noncovalently connected micelle (NCCM) with protected target ligands at pH = 7.4; (C) Tumor-triggered de-shielding to switch on the targeting property through removal of PEG segments at pH < 6.8; (D) Endocytosis of NCCM and drug release after destruction of the shell−core structure of NCCM at $T >$ LCST; (E) Apoptosis of tumor cells.

is switched off before reaching the tumor sites and switched on in the tumor sites, which is called "tumor-triggered targeting".

It is also well know that practical applications of stimulus-responsive micelles are still limited and clinical success remains relatively rare because of the small temperature or pH variation between pathological and normal tissues that could not stimulate the transition of conventional thermosensitive polymers. Micelles with high sensitivities are urgently needed. So another purpose of this study is to endow high thermoresponsibility to the micelles through adjusting the transition temperature after a pH-induced structural change. In normal tissues, the PEGylated micelles have a lower critical solution temperature (LCST) of 38 °C, and the segments containing *N*-isopropylacrylamide (NIPAAm) and *N*-acroyloxysuccinimide (NAS) units are hydrophilic.[16−19] On the arrival to tumor sites, due to the removal of the PEG segments, the LCST of the non-PEGylated micelle decreases to 35.5 °C, and these segments containing NIPAAm units suddenly become hydrophobic, which would make the loaded drug release from micelle sufficient.

In our stimuli-responsive NCCMs, the α−β cyclodextrin dimer is used as a "bridge" to connect the hydrophilic and hydrophobic segments (Scheme 1A). Additionally, by using two fluorescent dyes at different segments, that is, rhodamine B is conjugated to the hydrophilic segment and fluorescein isothiocyanate (FITC) is introduced to the end of the hydrophobic segment, the formation of the micelle as well as tracking of the assemblies could be easily achieved.

RESULTS AND DISCUSSION

The α−β cyclodextrin dimer was synthesized *via* click reaction and the detailed synthesis route was shown in Figure 1. The hydrophilic P(NIPAAm-*co*-NAS) (M_n 27000 g mol^{-1}, Table 1) segment having a phenyl group was synthesized *via* the reversible addition−fragmentation chain transfer (RAFT) polymerization. The hydrophobic segment, poly(ε-caprolactone) with a terminal adamantyl group (Ad-PCL, M_n 5100 g mol^{-1}) was prepared by using the terminal hydroxyl group of 1-adamantanol to initiate the ring-opening polymerization of CL. Furthermore, the peptide was synthesized manually, employing a standard Fmoc chemistry by the solid phase peptide synthesis (SPPS) method. Functional MPEG (M_n 2000 g mol^{-1}) was obtained by using 4-formylbenzoic acid to connect MPEG and ethylenediamine. The chemical structures of some intermediate products and the polymers are confirmed by ^{1}H NMR (Figure S2 in Supporting Information).

The core−shell assemblies are formed *via* host−guest interaction mediated spontaneous assem-

TABLE 1. Molecular Weights of Polymers Determined by GPC

	M_n	M_w	M_z	PDI
P(NIPAAm-*co*-NAS)	27000	34000	42000	1.25
Ad-PCL	5100	6400	7900	1.25

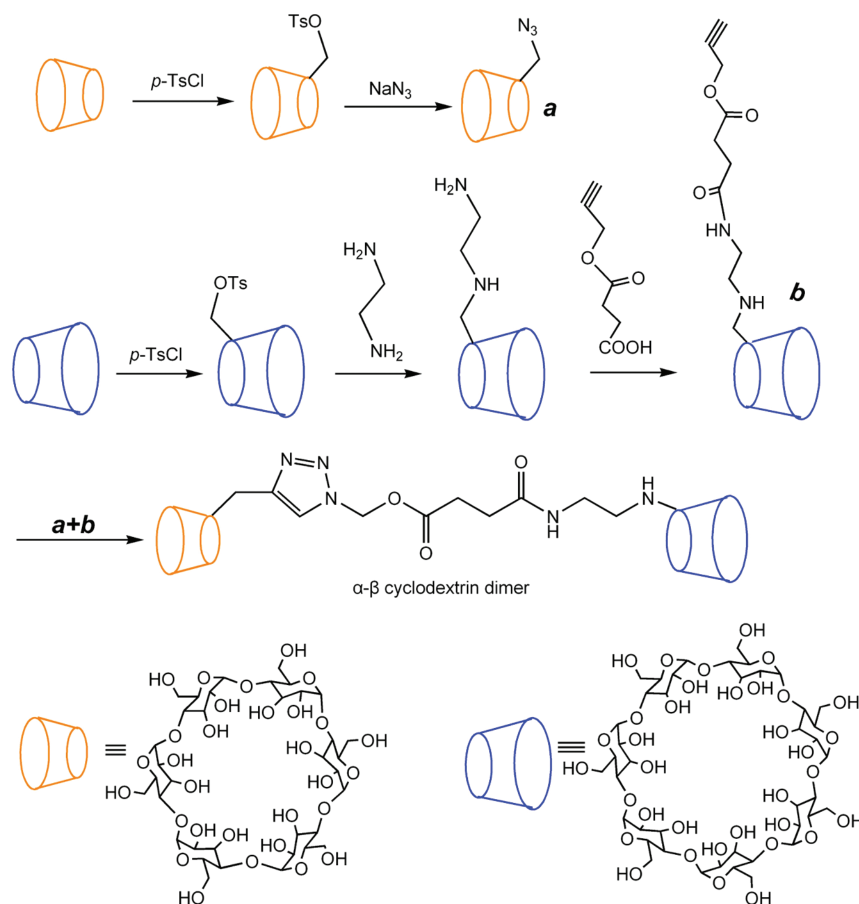

Figure 1. Synthesis of the α−β cyclodextrin dimer.

bly in an aqueous solution (Scheme 1B). The hydrophilic segments and the hydrophobic segments are connected through the host−guest interaction between the CD dimer and the guest molecules, that is, adamantyl and phenyl in polymer chains. As we know, in accordance to the geometric compatibility, different kinds of cyclodextrins are able to accommodate different molecules. The terminal adamantyl group in the hydrophobic Ad-PCL segment would selectively link with β-CD,[20] and α-CD is preferred to accommodate the phenyl group in P(NIPAAm-*co*-NAS) chain. It is worth mentioning that, according to the water solubility difference of α-CD and β-CD, soluble α-CD is designed to attach the hydrophilic segment, while the poorly soluble β-CD links with the hydrophobic segment.

After the self-assembly, the freeze-dried sample was characterized by X-ray diffraction (XRD) to identify the inclusion associations between phenyl/adamantyl groups and the α−β cyclodextrin dimer. As shown in Figure 2, the most intense diffraction peak in the spectra of the freeze-dried sample is at $2\theta = 30.78$, which is substantially different from those of pure α-CD and β-CD, indicating the existence of the threaded phenyl/adamantyl groups within the CD. Furthermore, the 2D $^{1}H-^{1}H$ gCOSY spectrum of micelles in $CDCl_3$ (Figure 3) exhibits significant correlation peaks between H_b of CD with those of the terminal phenyl and adamantyl moieties, also demonstrating the formation of micelles through host−guest interactions.

The formation of the micelles is further demonstrated by the changes in the fluorescent properties. In DMSO, the diblock copolymer is well dissolved and no micelle formed. The color of the solution is close to orange at 0.6 mg/mL (Figure $4A_{1a}$) and the fluorescence intensity of FITC moieties decreases gradually with descending concentration (Figure $4A_2$). In an aqueous solution, when the concentration of the self-assembly system reaches a certain value (CMC), the PCL-based hydrophobic segments self-assemble to form the hy-

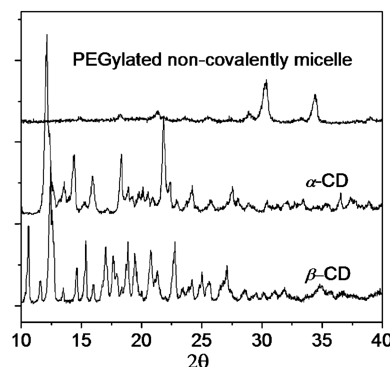

Figure 2. X-ray diffraction patterns of pure α-CD, β-CD, and freeze-dried PEGylated NCCMs connected by the α−β cyclodextrin dimer.

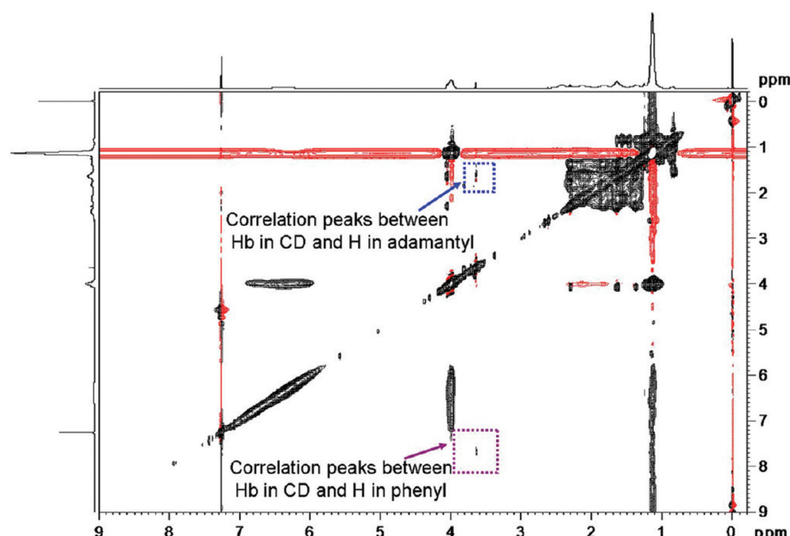

Figure 3. $^1H-^1H$ **gCOSY spectrum of freeze-dried NCCMs after removal of the MPEG segment under acidic conditions.**

drophobic core of micelles, as shown in Figure $4B_{1a}$. The green FITC moieties are buried in the core and the color of the micelle-contained solution turned to yellow at 0.6 mg/mL. The rapid change from curve b to c (Figure $4B_2$) indicates the formation of shell−core micelles and the CMC value would be between 0.15 and 0.3 mg/mL.

The micelle formation was studied by using pyrene as a hydrophobic fluorescent probe (Figure 5). Concomitant with the increase in fluorescence intensity, a red-shift from 337 to 340 nm could be observed. This is ascribed to the micellization because pyrene is preferentially partitioned into the hydrophobic core of the micelles with a change of the photophysical properties. From the pyrene excitation spectra, the CMC value is determined to be around 0.26 mg/mL, which is consistent with the changes in fluorescent properties of the assemblies.

TEM was also used to elucidate the morphology characteristics of micelles. As shown in Figure 6, the copolymers are able to self-assemble into shell−core micellar nanoparticles with an average size of around 100 nm at different pH values. The shell and core of the micelles are distinct in the images. Based on these results, we can conclude that the core−shell assemblies have been successfully constructed through host−guest interactions mediated by the α−β cyclodextrin dimer.

We further investigated the endocytosis behavior of the obtained micelles under different conditions. At pH 7.4, the faint fluorescence in Figure 7A,B indicates that very few nanoparticles could be endocytosed by tumor cells because the benzoic-imine bonds do not cleave from the hydrophilic block and the target ligands are shielded by PEG corona. This implies the micelles will not interact with the normal tissues in human physi-

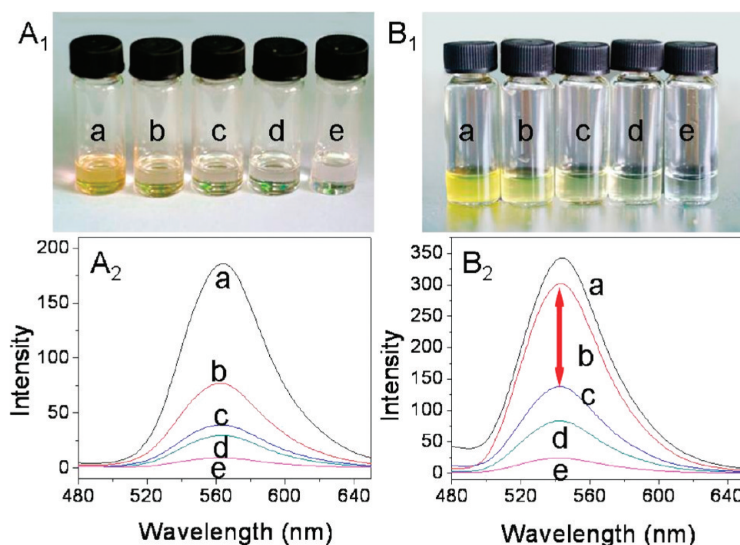

Figure 4. Optical images (A_1: DMSO, B_1: H_2O) and emission spectra of FITC (A_2: DMSO, B_2: H_2O) of the self-assembly systems with different concentrations: (a) 0.6 mg/mL; (b) 0.3 mg/mL; (c) 0.15 mg/mL; (d) 0.075 mg/mL; (e) 0.015 mg/mL.

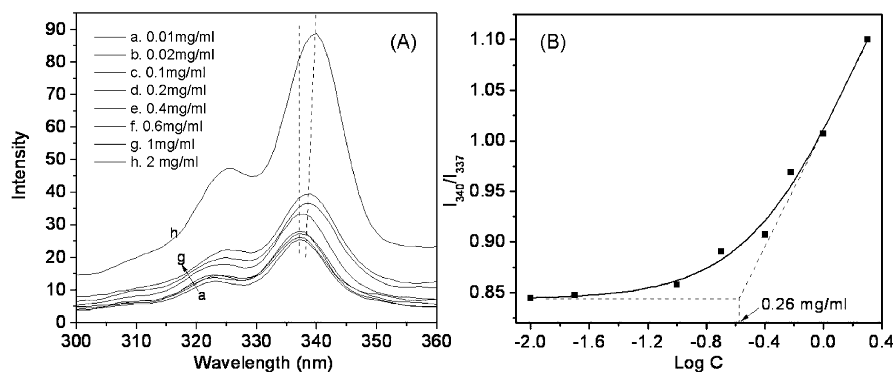

Figure 5. (A) Excitation spectra of pyrene at λ_{em} = 390 nm with increasing concentration. (B) Plot of the intensity ratio I_{340}/I_{337} *vs* log C.

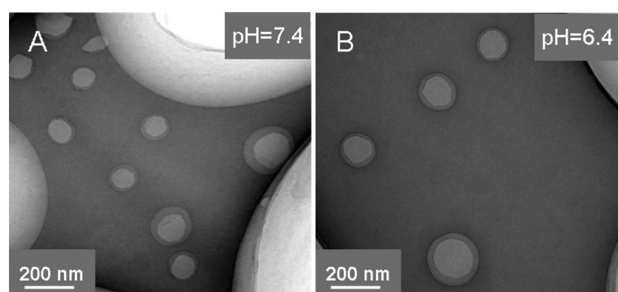

Figure 6. TEM images of micelles with (A) and without (B) PEG segments at different pH values.

ological pH and will not have side effects even at a high temperature. However, at pH 6.8, the fluorescence with a high intensity could be observed in most cells (Figure 7C,D), indicating that the endocytosis of nanoparticles is greatly improved and the temperature hardly affects the internalization of NCCMs. This is attributed to the hydrolysis of benzoic-imine bonds, resulting in the exposure of target ligands (Scheme 1C). With the exposed target ligands, the nanoparticles would be easily endocytosed by tumor cells (Scheme 1D). Based on these observations, we can infer that our micelles with a switchable targeting property could be selectively uptaken by solid tumor.

It should be noted that another important purpose to incorporate PEG segment is to adjust the LCST of the polymeric assemblies. The deshielding of PEG segment chains strongly affect the thermosensitive property of the supramolecular complexes. After cleaving of benzoic-imine bonds, the LCST of the micelles shifts from 38 to 35.5 °C so that the thermoresponse behavior of micelles becomes acute. That is, in normal conditions (37 °C, pH = 7.4), the PEGylated micelles are stable. When the PEGylated micelles contact tumor cells (pH < 6.8), due to the removal of the PEG segments, the LCST of nonPEGylated micelles becomes 35.5 °C (Figure 8), which means that the micelle structure will be destroyed and the loaded drugs in the micelles would be rapidly released (Scheme 1D).

To evaluate the antitumor effect, HeLa cells were treated with Dox-loaded micelles as compared with blank micelles. The cell viability with blank micelles (Figure 9a) is above 95% when the concentration is below 1.0 mg/mL, indicating that the blank carriers have a low cytotoxicity. After coincubated with Dox-loaded micelles for 4 h at pH 7.4, the cell viability is around 95% and changes little with the increasing concentration (Figure 9b) at 37 °C, manifesting that the drug-loaded micelles could not be endocytosed by tumor cells. This result further confirms that the micelles do not exhibit apparent cytotoxicity to the normal tissues (37 °C, pH 7.4).

As expected, when the pH is below 6.8, the cell viability decreases with the increasing concentration of

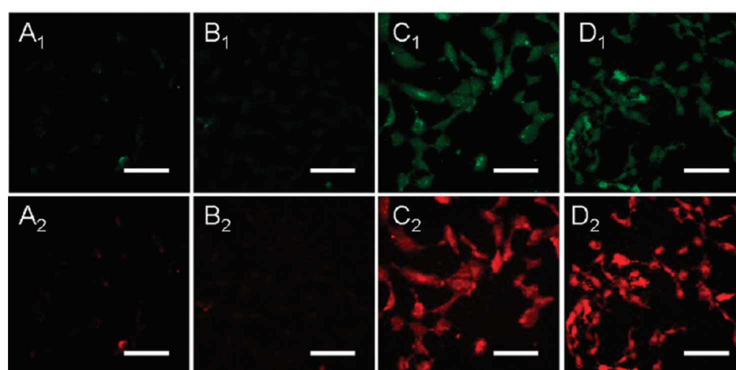

Figure 7. Confocal microscopy images of HeLa cells treated by rhodamine B loaded and FITC labeled micelles for 4 h at different conditions: (A) pH = 7.4, 37 °C; (B) pH = 7.4, 39 °C; (C) pH = 6.4, 37 °C; (D) pH = 6.4, 39 °C (scale bar: 80 μm).

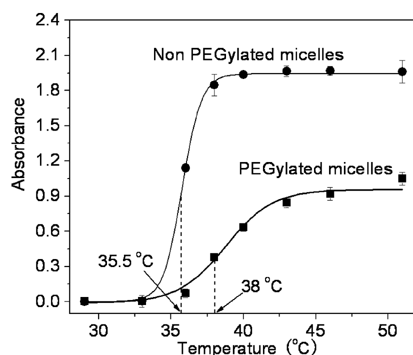

Figure 8. Determination of LCSTs of the PEGylated micelles and nonPEGylated micelles based on the optical absorbance in aqueous media at various temperatures.

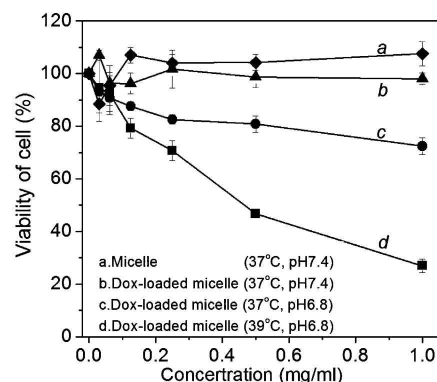

Figure 9. Viability of HeLa cells after being incubated with blank micelles and Dox-loaded micelles for 4 h.

drug-loaded micelles (Figure 9c,d), due to the fact that the endocytosis efficacy of the micelles would be much improved because of the deshielding of PEG segment. At 37 °C, we can see that 72% of the cells (Figure 9c) are kept alive even at a high concentration. The results illuminate that, although the endocytosis by tumor cells is greatly enhanced, most of the loaded drugs are still trapped in the micelles. When the temperature is changed to 39 °C, the structure of the micelle is destroyed and the loaded drugs would be released quickly from the micelles. As a result, the cell viability decreases rapidly and only 26% of the cells survive (Figure 9d). From these results, we can also draw a conclusion that the micelles could be endocytosed by tumor cells (<pH 6.8), release the loaded drug in tumor cells (>37 °C), and then kill them. The higher the temperature of the solid tumor, the higher the mortality of the drug-loaded NCCMs.

CONCLUSIONS

In summary, the α−β cyclodextrin dimer, synthesized *via* click chemistry, was used to connect the hydrophilic and hydrophobic segments to form self-assembled NCCMs. Endocytosis experiments confirm that the novel shell−core NCCMs with switchable tumor-triggered targeting property could be selectively uptaken by tumor cells, and then the drugs loaded in micelles could be released rapidly due to the thermoinduced phase transition. Importantly, the new concept, tumor-triggered targeting, can optimize the cure efficiency and minimize the size effect to normal tissues in tumor treatment.

EXPERIMENTAL SECTION

Materials. *N*-Isopropylacrylamide (NIPAAm) and *N,N,N′,N′,N′*-pentamethyl diethylenetriamine (PMDETA) were purchased from Acros and used as received. α-Cyclodextrin (α-CD), fluorescein isothiocyanate (FITC), and *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC) were obtained from Sigma-Aldrich and used as received. Dulbecco's modified Eagle medium (DMEM) and dimethyl sulfoxide (DMSO) were obtained from GIBCO Invitrogen Corporation. Doxorubicin hydrochloride (Dox · HCl) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd. (China). Rhodamine B was obtained from Tianjin Chemical Reagent Co. (Tianjin, China) and used as received. *N,N′*-Dicyclohexylcarbodimide (DCC), 4-(dimethylamino) pyridine (DMAP), sodium azide (NaN_3), copper(II) sulfate pentahydrate, and sodium ascorbate were purchased from Shanghai Reagent Chemical Co. (China) and used directly. ε-Caprolactone (CL), *N,N′*-dimethylformamide (DMF) and tetrahydrofuran (THF) were obtained from Shanghai Chemical Reagent Company and used after distillation. *N,N′*-Azobisisobutyronitrile (AIBN) was purchased from Shanghai Chemical Reagent Company and used after recrystallization with 95% ethanol. *N*-Hydroxysuccinimide (NHS) was purchased from Shanghai Chemical Reagent Company and *N*-acroyloxysuccinimide (NAS) was synthesized according to the reported procedure.[21] 2-(Phenylcarbonothioylthio) acetic acid (CTA) was synthesized as the previous literature.[14] All other reagents and solvents were used without further purification.

Synthesis of Mono-6-(*p*-tolylsulfonyl)-β-cyclodextrin (TsO-β-CD). A total of 10 g of nature β-CD was dissolved into 240 mL of deionized water under stirring, and later, 2.6 g *p*-toluenesulfonyl chloride was added in at a very low rate (1/3 mL/min) to ensure that the substitution occurred at the C6 position. After vigorous agitation for about 2−3 h at room temperature, 40 mL of 2.5 M sodium hydroxide solution was dropped. The obtained suspension was filtered and ammonium chloride (12.0 g) was added into the filtrate solution until its pH value was adjusted to 8. The resultant solution was cooled down to approximately 4 °C and kept overnight. The precipitation was collected by vacuum filtration and washed by acetone three times. Finally, to remove unreacted *p*-TsCl and β-CDs, the crude product was recrystallized at 60 °C at least three times. The final yield was 4.5 g after being dried at 50 °C for 48 h under vacuum drying (yield: 28%).

Synthesis of Mono-6-deoxy-6-EDA-β-CD (EDA-β-CD). To synthesize EDA-β-CD, the pure mono-6-OTs-β-CD reacted with nucleophilic reagent ethylenediamine by the same method as described in ref 22. A total of 10.0 g OTs-β-CD was dissolved into 60 mL of anhydrous ethylenediamine, and the nucleophilic reaction was carried out at 80 °C for 48 h. The resultant solution was cooled down to room temperature, poured into a large amount of acetone, and the precipitation was dissolved in water/methanol (3:1 v/v). The precipitating and dissolving procedures were repeated twice to sufficiently wash the product. At last, the product was dried at 50 °C for 2 days, resulting in a dry weight of 3.2 g (yield: 32%).

Synthesis of 4-Oxo-4-(prop-2-ynyloxy) Butanoic Acid. 4-Oxo-4-(prop-2-ynyloxy) butanoic acid was synthesized according to the published literature.[23] Propargyl alcohol (4.76 g, 0.085 mol), succinic anhydride (10.6 g, 0.116 mol), pyridine (9.18 g, 0.116 mol), and TEA (8.46 g, 0.116 mol) were dissolved in 100 mL of dry 1,4-dioxane. The mixture was stirred at room temperature for 24 h and then evaporated under vacuum. The crude product was dissolved in CH_2Cl_2 and washed with cold 1 M HCl. The organic

phases were dried over $MgSO_4$, filtered, and evaporated. The product was isolated as a brown solid (yield: 42%).

Synthesis of Alkyne-Modified EDA-β-CD. 4-Oxo-4-(prop-2-ynyloxy) butanoic acid (0.37 mmol), and DCC (1.1 mmol) were dissolved in 20 mL of dry DMF and reacted at 0 °C for 24 h. The insoluble precipitation was filtered. Then EDA-β-CD (0.25 mmol) and NHS (1.1 mmol) was added to the aforementioned solution. The mixture was stirred and reacted at room temperature for 24 h. The product was poured into a large amount of acetone, filtered, and dried at 50 °C for 48 h under vacuum drying (yield: 21%).

Synthesis of Azide-Modified α-CD (NaN_3-α-CD). Mono-6-(*p*-tolylsulfonyl)-α- cyclodextrin (TsO-α-CD) was synthesized according to the method we have mentioned above. In brief, 10 g of nature α-CD was dissolved into 20 mL of deionized water under stirring, and later, 2.6 g *p*-toluenesulfonyl chloride was added. Other conditions were the same as that for the synthesis of TsO-β-CD. To synthesized azide-modified α-CD, NaN_3 (1 g), and TsO-α-CD (1.3 g) was dissolved in 15 mL of deionized water purged by nitrogen. The reaction was carried out at room temperature for at least 8 h under a nitrogen atmosphere. The resultant solution was poured into large amount of acetone to obtain the NaN_3-α-CD. The product was dried at 50 °C for 2 days under vacuum drying (yield: 29%).

Synthesis of the α−β Cyclodextrin Dimer by "Click" Chemistry. Alkyne-modified β-cyclodextrin (0.1315 g) and azide-modified α-cyclodextrin (0.09978 g) were dissolved in 15 mL of H_2O. Then PMDETA (80 μL), $CuSO_4$ aqueous solution (100 μL 0.06 mol/L), and sodium ascorbate aqueous solution (100 μL 0.06 mol/L) were added to initiate the click reaction between alkyne and azide groups. The yield was about 75%.

Modification of MPEG (Amino-Oriented PEG) (i). 4-Formylbenzoic acid (1.5 g) and DCC (3.09 g) were dissolved in THF. The mixture was stirred at room temperature for a day. Precipitated dicyclohexylurea (DCU) was removed by filtration. Then, DMAP (1.9 g) and MPEG (2 g) were added to the solution. After a 24 h reaction at room temperature, the product was precipitated in an excess of hexane twice and dried under vacuum after filtration. The obtained product (2.0 g), ethylenedimine (1.6 mL), was reacted in DMSO at 40 °C. The product was precipitated in an excess of hexane twice and dried under vacuum after filtration (yield: 35%).

Modification of Rhodamine B (Amino-Oriented Rhodamine) (ii). Rhodamine B (0.48 g, 1 mmol) and DCC (0.3 g) were dissolved in 15 mL of THF. The mixture was stirred at room temperature under nitrogen for a day. Precipitated dicyclohexylurea (DCU) was removed by filtration. Then, DMAP (0.3 g) and ethylenediimine (1.5 mL) in 5 mL of THF were added to the solution. After a 24 h reaction at room temperature, the product was precipitated from acetone two times and dried under vacuum after filtration (yield: 27%).

Synthesis of Functionalized Peptide (NH_2-GRGDS-COOH) (iii). The peptide was synthesized manually in 0.6 mmol scale on the 2-chlorotrityl chloride resin, employing a standard Fmoc chemistry by solid phase peptide synthesis (SPPS) method.[24] The coupling of the first residue used 4 equiv of Fmoc-protected amino acid relative to resin substitution degree with 6 equiv of DIEA in a DMF solution. Other amino acid couplings were carried out with 4 equiv of Fmoc-protecting amino acid, 4 equiv of HBTU, and 6 equiv of DIEA for 4 h. Through the synthesis, the Fmoc protecting groups were deprotected with 20% (v/v) piperidine/DMF twice. Cleavage of the peptide was performed in a mixture of TFA, deionized water, and TIS in the ratio of 95:2.5:2.5. After 2 h stirring at room temperature, the cleavage mixture was collected. Excess TFA was removed by rotary evaporation, the remaining viscous peptide solution was precipitated with cold ether, the resulting white product was collected and vacuum-dried, then dissolved in distilled water, and freeze-dried (yield: 92%).

Synthesis of P(NIPAAm-*co*-NAS) (iv) by RAFT. The synthesis of P(NIPAAm-*co*-NAS) was synthesized according to the literature[25] with some modifications. NIPAAm (3.42 g, 30 mmol), NAS (78 mg, 0.45 mmol), AIBN (1.64 mg, 0.01 mmol), and CTA (21.2 mg, 0.1 mmol) were dissolved in 10 mL of THF. The solution was degassed by bubbling with nitrogen for 1 h at room temperature. Then the polymerization was carried out at 70 °C for 48 h. The product was precipitated in an excess of diethyl ether twice and dried under vacuum after filtration. The product was further purified by dissolving in distilled water and dialyzing against distilled water for 1 week using a dialysis membrane with a molecular weight cut off (MWCO) of 8000−10000 g/mol (Shanghai Chemical Reagent Co., China). The final product was harvested by freeze-drying. The yield was about 95%.

Synthesis of Ad-PCL (v). Ad-PCL was synthesized by ring-opening polymerization of CL by using −OH terminal groups of 1-adamantanol. The typical polymerization procedures were as follows. 1-Adamantanol (0.3 g), stannous 2-ethyl hexanoate ($SnOct_2$, 20.5 mg), and CL (1.2 g) were added into a glass ampule with a magnetic bar. The ampule was sealed under vacuum (<10 Pa), and then the ampule was immersed in an oil bath at 120 °C for 7 h. The product was precipitated in an excess of diethyl ether twice and dried under vacuum after filtration. The final product was harvested by freeze-drying. The yield was about 25%.

Modification of P(NIPAAm-*co*-NAS). When an amide condensation reaction between amino groups and NAS was used, **i** (10 mg), **ii** (10 mg), and **iii** (1 mg), were introduced to modify the P(NIPAAm-*co*-NAS) (0.11 g) in distilled water (12 mL) at 25 °C for 24 h. TEA was added into the mixture to adjust the pH value to 7−8. The product was purified with the same process.

Synthesis of Ad-PCL-FITC. To track the micelles, FITC was further conjugated to Ad-PCL according to the previous report.[26] Briefly, Ad-PCL (0.2 g) was immersed in 20 mL of DMSO, and then 5 mg of FITC was added. After being stirred for 48 h at room temperature, the reaction mixture was purified by dialysis against distilled water for at least 10 days, the water was refreshed twice every day, and lyophilized to obtain FITC-labeled micelles. The prepared micelles were designated as Ad-PCL-FITC (yield: 87%).

Formation of Micelles. The α−β cyclodextrin dimer (18 mg, 0.008 mmol) and the modified P(NIPAAm-*co*-NAS) (0.12 g, 0.004 mmol) were added into 15 mL of H_2O. Then Ad-PCL-FITC (40 mg, 0.008 mmol) was dissolved in 14 mL of DMSO and added dropwise to the aforementioned solutions with vigorous stirring for 24 h. The product was further purified by dissolving in distilled water and dialyzing against PBS (pH = 7.4) for 1 week. The final product was harvested by freeze-drying. The yield was about 35%.

^1H NMR. ^1H NMR spectra were recorded on a Mercury VX-300 spectrometer at 300 Hz and 2D $^1H-^1H$ gCOSY spectrum was recorded at 600 Hz.

Gel Permeation Chromatography (GPC). GPC was used to determine the molecular weights of polymers. A dual detector system consisting of a MALLS device (DAWN EOS, Wyatt Technology) and an interferometric refractometer (Optilab DSP, Wyatt Technology) was used. The columns used were styragel HR1 and HR4. The concentration of each copolymer was kept constant at 10 mg/mL and THF (chromatographic grade) was used as the eluent at a flow rate of 0.3 mL/min. The MALLS detector was operated at a laser wavelength of 690 nm. The molecular weight of copolymers was shown in Table 1.

XRD. Wide-angle X-ray scattering (WAXS) characterization was performed with an X-ray diffractometer Shimadzu XRD-6000 equipped with a Cu Kα radiation source (λ = 0.154 nm, 2 kW).

Measurement of Critical Micelle Concentration (CMC). Fluorescence spectra were recorded on a LS55 luminescence spectrometer (Perkin-Elmer) and pyrene was used as a hydrophobic fluorescent probe.[27] Aliquots of pyrene solutions (6×10^{-6} M in acetone, 1 mL) were added to containers, and the acetone was allowed to evaporate. Aliquots of 10 mL of aqueous solutions at different concentrations were then added to the containers containing the pyrene residue. It should be noted that all the aqueous sample solutions contained excess pyrene residue at the same concentration of 6×10^{-7} M. The solutions, which contain P(NIPAAm-*co*-NAS), Ad-PCL, and α−β cyclodextrin dimer with a particular ratio, with various concentrations from 1×10^{-2} to 2.0 g/L were added to each flask. Emission wavelength was carried out at 390 nm, and excitation spectra were recorded, ranging from 300 to 360 nm. The excitation and emission bandwidths were 3 and 5 nm, respectively. From the pyrene excitation spectra, the intensity ratio I_{340}/I_{337} was analyzed as a function of the polymer concentration. A CMC value was determined from the intersection of the tangent to the curve at

the inflection with the horizontal tangent through the points at low concentration.[28]

Transmission Electron Microscopy (TEM). At room temperatures, a drop of micelle suspension (~1.0 mg/mL) was placed on a copper grid with Formvar film and dried before observation on a JEM-100CXa TEM at an acceleration voltage of 80 keV.

Determination of LCST. Optical absorbance of polymers in aqueous solutions (1.0 mg/mL, distilled water was used as the solvent) at various temperatures were measured at 500 nm with a Lambda Bio40 UV-vis spectrometer (Perkin-Elmer) to determine its LCST. The sample cell was thermostatted in a refrigerated circulator bath at different temperatures prior to measurements. The LCST was defined as the temperature exhibiting a 50% increase of the total increase in optical absorbance.

Cell Internalization. HeLa cells were used to evaluate the cell internalization of the micelles. The cells were allowed to grow to ~70% confluence in LabTek chamber slide system (4.0×10^4 cells/plate). Culture medium was replaced, 1 mL of fresh DMEM containing fluorescence-labeled nanoparticles (1 mg/mL) was added into each well, and the pH value of the solution was adjusted to pH 6.4. After being incubated with cells at 37 and 39 °C for 4 h, respectively, medium was removed and cells were washed with PBS solution three times. Then, 1 mL of PBS solution was added in each well. As a control, the same procedure was carried out at pH 7.4. The fluorescent images of HeLa cells were observed under excitation at 488 nm (green fluorescence) and 543 nm (red fluorescence) using confocal laser scanning microscopy (Leica TCS SP2AOBS, Germany).

Drug Loading. Dox · HCl (6 mg) was stirred with 2 mL of TEA in 12 mL of DMSO overnight to obtain the Dox. And then freeze-dried micelle particles (40 mg) were dissolved in the solution. The solution was put into a dialysis tube and subjected to dialysis against 2 L of PBS (pH = 7.4) at 25 °C for 4 days, and the distilled water was refreshed every 4 h during this period. The final drug-loaded micelles were collected by freeze-drying. The UV absorbance of the dialysis solution was used to determine the amount of unloaded Dox, which was used to calculate the encapsulation efficiency (EE%). The concentration of drug was determined by UV-vis spectroscopy at 497 nm.[29] Here, the EE% is defined as

$$\text{EE\%} = \frac{(\text{total amount of Dox}) - (\text{unloaded amount of Dox})}{\text{total amount of Dox}} \times 100\%$$

The EE% was found to be around 26.1%.

In Vitro Cytotoxicity Measurement. HeLa cells were seeded into a 24-well plate (6.0×10^4 cells/well) containing 1 mL of DMEM. After incubation for 24 h (37 °C, 5% CO_2), the culture medium was removed, and DMEM containing Dox-loaded carrier particles containing a particular amount of drug were added in each well. The pH value of some wells was adjusted to 6.8 with 1 M HCl. The cells were coincubated with Dox-loaded nanoparticles at 37 °C for 4 h. Then the DMEM medium containing carriers was replaced with 1 mL of fresh DMEM, and the cells were further incubated at 37 and 39 °C, respectively, for 24 h. Finally, 60 μL of MTT solution (5 mg/mL) was added to each well, respectively. After incubation for 4 h, the MTT medium was removed from each well and 600 μL of DMSO was added. At last, the mixture was transferred into a 96-well plate. The optical density (OD) was measured.

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Supporting Information Available: Detailed chemical structures and ^{1}H NMR data. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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