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Intracellular pH-sensitive supramolecular amphiphiles based on host–guest recognition between benzimidazole and β-cyclodextrin as potential drug delivery vehicles†

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Intracellular pH-sensitive supramolecular block amphiphiles based on the host–guest interaction between benzimidazole (BM) modified poly(ε-caprolactone) (BM-PCL) and cyclodextrin (β-CD) terminated dextran (Dex-β-CD) were designed. The supramolecular block amphiphiles could further self-assemble into supramolecular micelles and exhibit pH-sensitive behaviour in acidic aqueous solution when the pH value was below 6. Doxorubicin (DOX), a model anticancer drug, was effectively loaded into the supramolecular micelles *via* hydrophobic interactions. The DOX release from all DOX-loaded micelles was accelerated in acid conditions mimicking the endosomal/lysosomal compartments. The enhanced intracellular DOX release was observed in HepG2 cells. DOX-loaded intracellular pH-sensitive supramolecular micelles showed higher cellular proliferation inhibition towards HepG2 cells than pH-insensitive micelles. These features suggested that the supramolecular micelles could efficiently load and deliver DOX into tumor cells and enhance the inhibition of cellular proliferation *in vitro*, providing a powerful mean for delivering and releasing cargoes at the tumor sites.

Introduction

Stimuli responsive micelles, which show sharp intelligent response to intracellular environmental stimuli, such as pH,[1] redox,[2,3] temperature,[4] enzymes[5] and so on, are promising drug carriers. The stimuli responsive micelles are noted for their site-specific targeting release of payloads modulated by the specific microenvironments of intracellular space, leading to aggressive anticancer activity and maximal chemotherapeutic efficacy with fewer side effects.[6] Of all the intelligent micelles, pH-sensitive micelles have received greater attention for drug delivery. The pH targeting approach is regarded as a more general strategy than conventional specific tumor cell surface targeting approaches, because the acidic tumor microclimate is most common in solid tumors.

Compared with conventional covalent amphiphile micelles, supramolecular amphiphile micelles formed by non-covalent interactions have attracted extensive attention because of their convenient synthesis procedure.[7] In the past few years, a variety of non-covalent interactions, such as multiple hydrogen bonding, host–guest recognition, hydrophobic interaction, π–π stacking, metal–ligand coordination and integrated non-covalent interactions, have been employed to drive the building blocks together to fabricate supramolecular copolymers.[8–12] Meanwhile, the natures of non-covalent interactions and the structures of building blocks endow the resulting supramolecular copolymers with responsiveness to surrounding environmental stimuli, such as pH,[13] temperature,[14] light,[15,16] redox[17] and electric field.[18] However, up to now, pH-sensitive amphiphilic supramolecular copolymers, especially those responsive at intracellular pH, have been seldom reported.

It is known that benzimidazole (BM) exhibits pH-sensitive host–guest interaction with β-cyclodextrin (β-CD).[19] At the physiological pH (~7.4), the BM stalk has a hydrophobic nature and can bind to the β-CD molecule *via* host–guest interactions. When the BM stalk is protonated under acidic conditions (pH < 6) mimicking the endosomal/lysosomal compartments, the BM/β-CD binding constant decreases dramatically and thus causes the dissociation of β-CD from the BM stalk.[20] Based on these special properties, we designed pH-sensitive supramolecular block amphiphiles composed of benzimidazole (BM) modified poly(ε-caprolactone) (BM-PCL) and β-cyclodextrin (β-CD) terminated dextran (Dex-β-CD). The supramolecular block amphiphiles could self-assemble into micelles at physiological pH ~7.4 and exhibited pH-sensitivity in acidic

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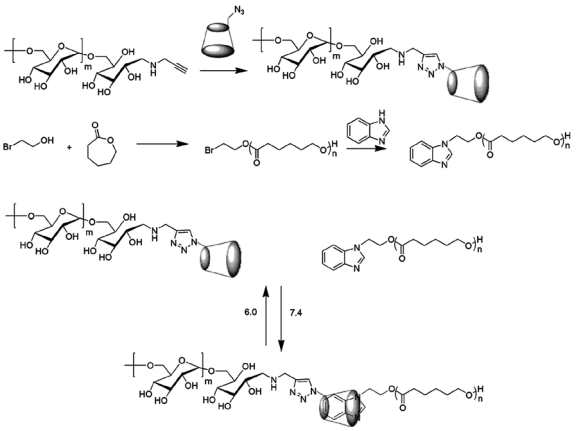
conditions (pH < 6). Based on the unique pH-dependent supramolecular properties, the novel supramolecular micelles could serve as a promising system for the intelligent anti-tumor drug delivery (Scheme 1), because they could offer a favorable platform to construct an efficient drug delivery system for cancer therapy.[21,22]

Results and discussion

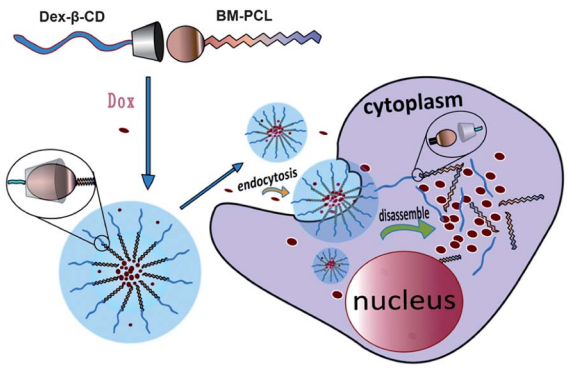
Preparation and characterization of the supramolecular amphiphile micelles

In the present work, pH-sensitive Dex-β-CD/BM-PCL supramolecular copolymer was synthesized (Scheme 2). The chemical structures of Dex-β-CD and BM-PCL were confirmed by ^{1}H NMR (Fig. S1 and S2†) and FT-IR. As shown in Fig. 1, the characteristic signals of triazole ring protons at 7.9 (j) indicated the successful synthesis of the desired Dex-β-CD. Fourier transform infrared spectroscopy (FT-IR) analysis of Dex-β-CD was also performed (Fig. 2). The disappearance of the azide peak and α-alkyne peak at 2100 cm^{-1} demonstrated that azido-functionalized β-CD had been completely consumed during the reaction with alkyne-terminated dextran, suggesting the successful synthesis of dextran-*block*-β-CD. In addition, GPC profiles of alkyne dextran and Dex-β-CD exhibited in Fig. S3† were also confirmed the successful synthesis of dextran-*block*-β-CD. As shown in Fig. 3, The molecule weight of PCL-Br was calculated to be 6000 g mol^{-1} from the integration ratio between the proton of methylene of initiator at 3.4 ppm (a, Br–CH_2CH$_2$–) and the proton of ε-CL unit appearing at 1.3 ppm (f, –CH$_2$CH_2CH$_2$–). The peaks at 3.4 and 4.4 ppm assigned to the methylene protons close to bromine (a, Br–CH_2CH$_2$– and b, Br–CH$_2$CH_2) disappeared completely, while the peaks of benzene protons at 7.3 and 7.8 ppm (i and j, –C$_6H_4$) and that ascribed to methylene protons linked to BM at 3.9 ppm (k, –NCH_2CH$_2$–) were observed. Hence, the ^{1}H NMR results indicated the successful synthesis of BM-PCL. The molecular weights of PCL-Br and BM-PCL from GPC are shown Table S1.†

In order to demonstrate the successful association between BM-PCL and Dex-β-CD, the blends of BM-PCL and Dex-β-CD with different ratios were dissolved in dimethyl sulfoxide (DMSO) and then nine times the amount phosphate buffered saline (PBS) was added to the solution. The fluorescence of the complex in DMSO/PBS (1 : 9, v/v) was studied. BM-PCL showed distinct dual fluorescence in the polar solvents at 290 and 580 nm. The higher emission at 290 nm was assigned to the neutral form of BM. The other lower emission band (580 nm) arose from the anionic state of BM which was due to the intermolecular proton transfer between solute and solvent in the excited state.[23] As shown in Fig. 4A and S5,† when the concentration of Dex-β-CD was below 1.2 mg mL^{-1}, the addition of Dex-β-CD resulted in a marked decrease in fluorescence intensity of BM-PCL, which was attributed to the association of BM-PCL and Dex-β-CD. It was noteworthy that this tendency was in contrast to the previously reported results in which the fluorescence intensity of BM increased after associating with β-CD.[17] It could be reasonably explained that the fluorescence intensity of BM molecules was enhanced due to the formation of hydrophobic surroundings by



Scheme 2 Synthetic route for Dex-β-CD/BM-PCL copolymer.



Scheme 1 Schematic illustration of DOX loading and intracellular microenvironment triggered release from DOX-loaded Dex-β-CD/BM-PCL micelle.

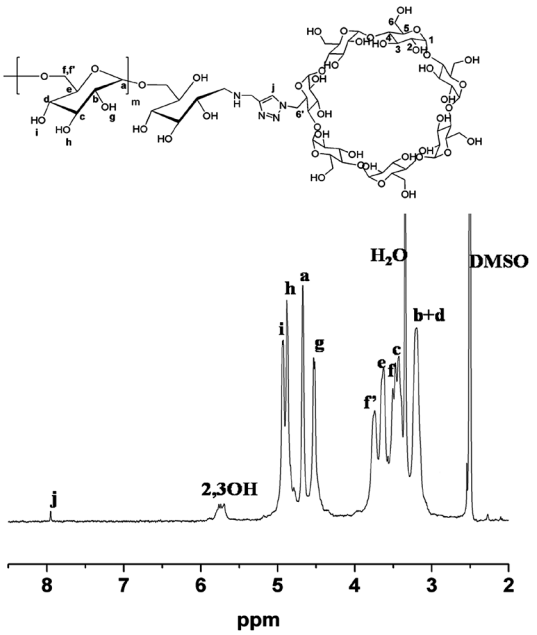


Fig. 1 ^{1}H NMR spectrum of Dex-β-CD in DMSO-d$_6$.

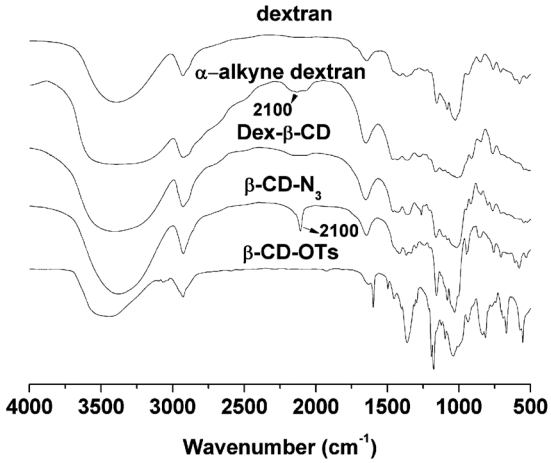


Fig. 2 FT-IR spectra of β-CD-OTs, β-CD-N_3, Dex-β-CD, α-alkyne dextran and dextran.

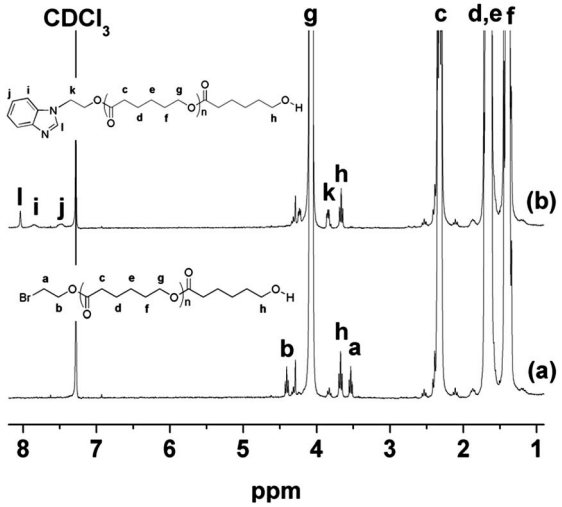


Fig. 3 ^{1}H NMR spectra of Br-PCL (a) and BM-PCL (b) in $CDCl_3$.

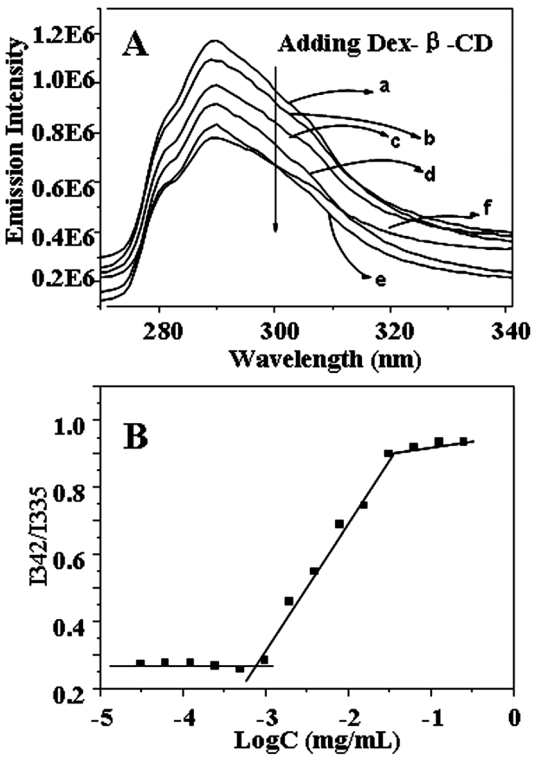


Fig. 4 Fluorescence emission spectra of BM-PCL in DMSO/PBS (1 : 9, v/v) solutions with different Dex-β-CD concentrations (λ_{ex} = 240 nm). The concentration of BM-PCL was set at 0.5 mg mL^{-1}, while the concentrations of Dex-β-CD were 0 mg mL^{-1} (a), 0.2 mg mL^{-1} (b), 0.5 mg mL^{-1} (c), 0.8 mg mL^{-1} (d), 1.2 mg mL^{-1} (e) and 1.6 mg mL^{-1} (f) (A); intensity ratios of I_{342}/I_{335} from pyrene excitation spectra as a function of the concentration of Dex-β-CD/BM-PCL copolymer in PBS at pH 7.4 (B).

PCL. When Dex-β-CD was added, the fluorescence intensity decreased because a less hydrophobic environment was formed by the association of BM group with β-CD. To confirm this inference, the fluorescent properties of the BM/β-CD complex and PCL/BM blend in DMSO/PBS (1 : 9, v/v) were studied (Fig. S6 and S7†). The addition of β-CD to the BM aqueous solution resulted in a slight increase in fluorescence intensity due to the entrance of BM to the hydrophobic cavity of β-CD (Fig. S6†). Conversely, there was a dramatic increase in fluorescence intensity after adding a small amount of PCL to the BM solution in DMSO/PBS (1 : 9, v/v). And when the concentration of PCL was further increased, the fluorescence intensity would decrease rapidly due to the precipitation of PCL as observed in Fig. S7.† These results suggested that BM wrapped in PCL suspended nanoparticles was in more hydrophobic surroundings than in the cavity of β-CD.

Based on the above results the Dex-β-CD/BM-PCL copolymer micelle was prepared by dissolving equal amounts of Dex-β-CD and BM-PCL in 5.0 mL of DMSO and dropped into PBS at 7.4. After dialyzing against deionized water (MWCO 3.5 kDa) for 12 h to remove DMSO the micelle solution was obtained and the CMC value was investigated by a widely reported pyrene-probe-based fluorescence technique. The plot of fluorescence intensity ratio I_{342}/I_{335} *versus* log *c* of the copolymers is shown in Fig. 4B. Under neutral pH conditions (at pH 7.4), the CMC value of Dex-β-CD/BM-PCL is 0.82 μg mL^{-1}. The assembly behavior indicates the association between BM-PCL and Dex-β-CD.

To demonstrate the pH-sensitivity of the Dex-β-CD/BM-PCL micelles, the dissociation of Dex-β-CD/BM-PCL supramolecular copolymer in response to pH change was studied *via* testing the change of emission intensity at 580 nm as a function of time. As indicated in Fig. 5A, the Dex-β-CD/BM-PCL copolymer slowly dissociated when the pH decreased below 6.4. And sharper disassociation occurred when the pH was reduced below 6.0, because the BM group dissociated from β-CD and was exposed to the hydrophilic surroundings. The results suggested that the Dex-β-CD/BM-PCL supermolecular copolymer micelle was stable under physiological (pH 7.4) and tissular pH (pH 6.8) conditions while dissociated at intracellular pH (pH < 6.0) indicating great potential as an intracellular targeted drug delivery platform.

It revealed that the representative Dex-β-CD/BM-PCL micelles had a spherical morphology with an average diameter

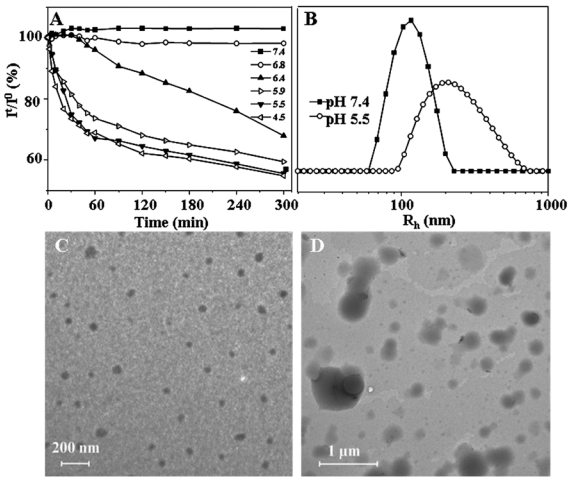


Fig. 5 Normalized fluorescence emission intensity (λ = 580 nm) of Dex-β-CD/BM-PCL copolymer in PBS solutions as a function of time at different pH values (A). The hydrodynamic radii (R_h) of Dex-β-CD/BM-PCL micelles in PBS at pH 7.4 and pH 5.5 (B). TEM micrographs of Dex-β-CD/BM-PCL at pH 7.4 (C) and pH 5.5 (D), respectively.

of around 90 nm at pH 7.4 from TEM measurements (Fig. 5C). In contrast, the average diameter increased drastically at pH 5.5 (Fig. 5D). The hydrodynamic radii (R_h) measured by DLS at pH 7.4 and 5.5 were around 112 ± 9.8 and 259 ± 90.2 nm, respectively (Fig. 5B). The smaller sizes obtained from TEM observations as compared to those determined by DLS should be assigned to the dehydration of the micelles in the process of TEM sample preparation. The shrinkage of the Dex shell might also contribute to this difference. The pH-responsive behavior of the micelle was obviously exhibited. The increase in size and size distribution of the micelle was attributed to the aggregation of the hydrophobic BM-PCL chain after the disassociation of the BM/β-CD complex at low pH.

In vitro DOX loading and stimuli triggered release

To verify the feasibility of using the self-assembled supramolecular micelle for intracellular drug delivery in cancer chemotherapy, doxorubicin (DOX) was used as a model drug and loaded into the micelles. A pH-insensitive dextran-*b*-poly(ε-caprolactone) (Dex-PCL) copolymer was synthesized for comparison (Fig. S4, S8 and S9†). The *in vitro* release behaviors were investigated at pH 5.5 and 7.4, mimicking the pH in late endosome, and blood or normal tissue, respectively. As shown in Fig. 6A, up to 90% of DOX was released from DOX-loaded Dex-CD/BM-PCL micelles in PBS at pH 5.5 in 24 h. On the other hand, the DOX release rate was obviously much lower at pH 7.4. The different release behaviors were likely to result from the acid-triggered disassembly of the Dex-β-CD/BM-PCL micelle that was formed on the basis of pH-dependent interaction between BM and β-CD. In contrast, there was no obvious difference in the release behaviors of the pH-insensitive DOX-loaded Dex-PCL micelles at pH 5.5 and 7.4.

It is necessary to evaluate the potential toxicity of polymeric materials for drug delivery applications. The *in vitro* cytotoxicity of the Dex-β-CD/BM-PCL supramolecular copolymer to HepG2

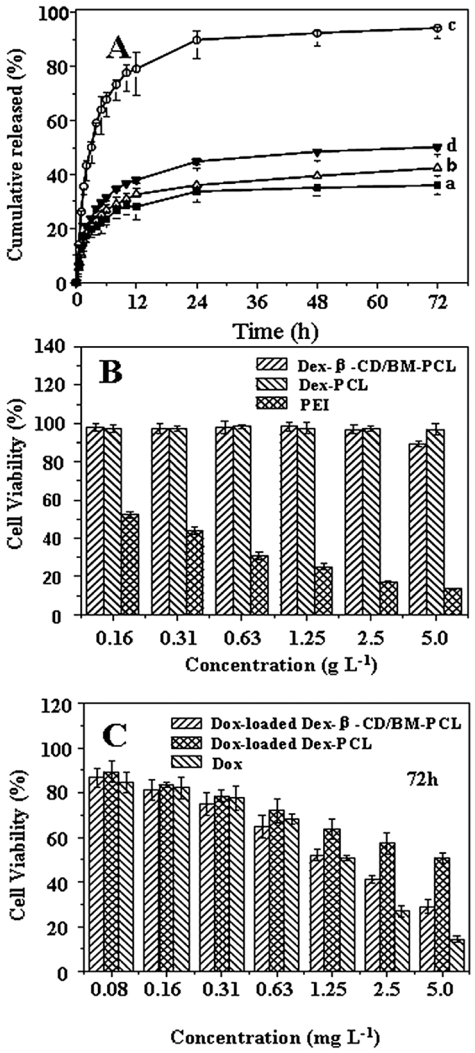


Fig. 6 *In vitro* DOX release profiles for DOX-loaded Dex-β-CD/BM-PCL micelles in PBS at 37 °C and pH 7.4 (a) and 5.5 (c), respectively, as well as those for DOX-loaded Dex-PCL micelles at pH 7.4 (b) and 5.5 (d) (A). Cytotoxicity of Dex-β-CD/BM-PCL and Dex-PCL micelles towards HepG2 cells after incubation for 72 h (B). Cytotoxicities of DOX-loaded Dex-β-CD/BM-PCL micelles, DOX-loaded Dex-PCL micelles, and free DOX towards HepG2 cells after incubation for 72 h (C).

cells was evaluated by a MTT assay. As shown in Fig. 6B, the viabilities of HepG2 cells treated with Dex-β-CD/BM-PCL micelles for 72 h were over 85% at all test concentrations up to 5.0 mg mL^{-1}. The results suggested that Dex-β-CD/BM-PCL has low cytotoxicity and can be safely used as biocompatible carriers for drug delivery.

The *in vitro* cellular proliferation inhibitions of DOX-loaded Dex-β-CD/BM-PCL and Dex-PCL micelles against HepG2 cells were also estimated by a MTT assay. As shown in Fig. 6C, in contrast to DOX-loaded Dex-PCL, DOX-loaded Dex-β-CD/BM-PCL exhibited significantly higher growth inhibition efficiency compared to HepG2 cells (Fig. S10 and S11†). The results revealed that the faster DOX release from DOX-loaded Dex-β-CD/BM-PCL micelles was triggered by endosomal pH as compared to the pH-insensitive DOX-loaded Dex-PCL micelles, leading to enhanced inhibition of cell proliferation.

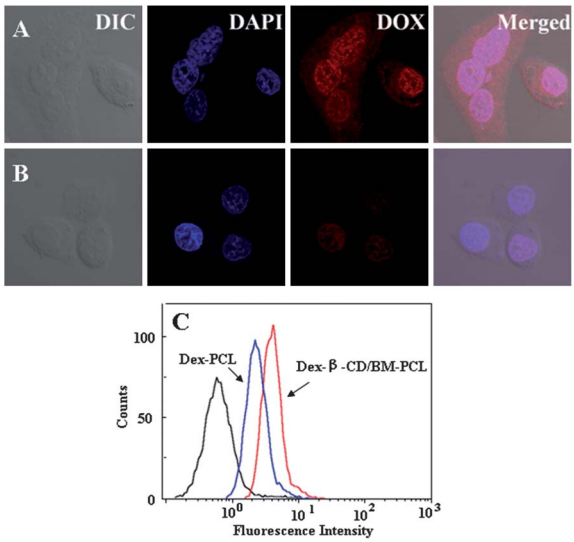


Fig. 7 Representative CLSM images of HepG2 cells incubated with DOX-loaded Dex-β-CD/BM-PCL micelles (A) and DOX-loaded Dex-PCL micelles (B) for 3 h. For each panel, the images from left to right show a differential interference contrast (DIC) image, cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlays of the three images. Flow cytometry profiles of HepG2 cells incubated with DOX-loaded Dex-β-CD/BM-PCL and Dex-PCL micelles for 3 h (C).

The cellular uptake and intracellular release behaviors of DOX-loaded Dex-β-CD/BM-PCL and Dex-PCL micelles were followed by CLSM and flow cytometry toward HepG2 cells. As expected, stronger intracellular DOX fluorescence was observed in the cells after incubation with DOX-loaded Dex-β-CD/BM-PCL micelles for 3 h, in contrast to those incubated with DOX-loaded Dex-PCL micelles (Fig. 7A and B). The drug release triggered in the intracellular environment was further confirmed by flow cytometric analyses. As shown in Fig. 7C, the flow cytometric histogram for the cells incubated with DOX-loaded Dex-β-CD/BM-PCL micelles was shifted clearly in the direction of high fluorescence intensity as compared with that incubated with DOX-loaded Dex-PCL micelle. The mean fluorescence intensity of DOX in HepG2 cells incubated with DOX-loaded Dex-β-CD/BM-PCL and Dex-PCL micelles were 4.0 and 2.4 respectively. Thus, the enhanced fluorescence intensity in the HepG2 cells incubated with DOX-loaded Dex-β-CD/BM-PCL micelles should be attributed to the enhanced intracellular DOX release induced by acid-trigged disassociation of the supermolecular micelle.

Conclusion

In summary, supramolecular amphiphilic Dex-β-CD/BM-PCL block copolymer was prepared through host–guest recognition between terminal BM and β-CD. The supramolecular amphiphilic micelles exhibited intracellular pH-sensitivity in acidic conditions mimicking the endosomal/lysosomal compartments. DOX-loaded micelle exhibited faster DOX release behavior in HepG2 cells than those pH-insensitive micelles. Moreover, higher cellular proliferation inhibition efficacy was achieved. These features of the DOX-loaded Dex-β-CD/BM-PCL micelle revealed the promising application in intelligent drug delivery.

Experimental section

Materials

Dextran (Dex, M_n = 6 kDa, Sigma), propargylamine (98%, Sigma), sodium cyanoborohydride (95%, Sigma), sodium azide (Sigma), benzimidazole (BM, 98%, Fluka), *N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetriamine (PMDETA, 99%, Sigma), polyethyleneimide (PEI, M_w = 25 kDa, Sigma) and *N*,*N*-diisopropylethylamine (99.5%, Sigma) were used as received. 2-Bromoethanol was purified by vacuum distillation over CaH_2. Mono-6-deoxy-6-(*p*-tolylsulfonyl)-β-cyclodextrin (β-CD-OTs) was purchased from Shandong Zhiyuan Chemical Reagent Co. and used as obtained.

Characterizations

^{1}H NMR spectra were recorded on a Bruker AV 400 NMR spectrometer in dimethyl sulfoxide-d_6 (DMSO-d_6) or deuterated chloroform ($CDCl_3$). FT-IR spectra were recorded on a Bio-Rad Win-IR instrument using the potassium bromide (KBr) method. Transmission electron microscopy (TEM) measurements were performed on a JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 kV. A drop of the micelles solution (0.1 g L^{-1}) was deposited onto a 230 mesh copper grid coated with carbon and allowed to dry in air at 25 °C before measurements. Dynamic laser scattering (DLS) measurements were performed on a WyattQELS instrument with a vertically polarized He–Ne laser (DAWN EOS, Wyatt Technology). The scattering angle was fixed at 90°. X-ray diffraction measurement was carried out on a Bruker D8 Advance X-ray diffractometer using Cu Kα radiation in the scattering angle range of 2θ = 10–30° at a scan speed of 4° min^{-1}.

Synthesis of α-alkyne dextran

Dex (2.7 g, 0.454 mmol) was dissolved in 2% (w/v) acetate buffer (pH 5.0) in a flask at 50 °C. Propargylamine (2.5 g, 45.4 mmol) and sodium cyanoborohydride (2.85 g, 45.4 mmol) were added under stirring. The mixture was stirred at 50 °C for 96 h. The solution was concentrated by a Rotavapor, and then dialysed against deionized water for 4 days, and the product was collected by lyophilization (yield: 72%).[24]

Synthesis of mono-6-deoxy-6-azido-β-cyclodextrin (β-CD-N_3)

β-CD-N_3 was synthesized according to the previous literature.[25] β-CD-OTs (1.0 g, 0.78 mmol) was suspended in 10.0 mL of water. After heating to 80 °C, NaN_3 (0254 g, 3.9 mmol) was added. The reactive mixture was stirred at 80 °C for 18 h. The reactive solution was then cooled to room temperature and precipitated in 80.0 mL of acetone. The crude product was redissolved in 5.0 mL of water and then precipitated in 40.0 mL of acetone again. The white solid was dried under vacuum at 60 °C for 2 days (yield: 91%).

Synthesis of Dex-β-CD

α-Alkyne Dex (0.6 g, 0.1 mmol), β-CD-N_3 (0.24 g, 0.2 mmol) and PMDETA (40 μL, 0.2 mmol) were dissolved in 30.0 mL of dried

DMSO. The mixture was stirred for 10 min and degassed by three freeze–thaw cycles and transferred to another Schlenk flask containing CuBr (30.0 mg, 0.2 mmol) *via* N_2-purged syringe. The Schlenk flask was placed in an oil bath at 60 °C for 72 h. The reaction medium was then dialyzed against deionized water (MWCO 10 kDa) for 4 days and the product was collected by lyophilization (yield: 76%).

Synthesis of BM-PCL

2-Bromoethanol (0.1 g), ε-caprolactone (ε-CL, 5 g, 0.043 mol) and stannous octoate (0.017 g, 0.1 mol% with respect to ε-caprolactone) were added into a glass ampoule with a magnetic bar. The reaction was continued in an oil bath at 120 °C for 24 h. The product was collected by precipitation in 10-fold diethyl ether. Then, the product was redissolved into trichloromethane and precipitated thrice in diethyl ether. After vacuum drying for 24 h, PCL-Br was obtained with 70% yield. Secondly, the obtained PCL-Br (2.0 g, 0.33 mmol) was dissolved in anhydrous DMF (20.0 mL), into which 0.4 g (3.3 mmol) of BM and 0.58 mL (3.3 mmol) of *N*,*N*-diisopropylethylamine were added. The solution was stirred at 70 °C under inert atmosphere for 24 h. The solution was concentrated by a Rotavapor, and then the product was redissolved into trichloromethane and washed three times with deionized water. The solution was dried with $MgSO_4$ and precipitated in diethyl ether. After vacuum drying for 24 h, BM-PCL was obtained with 76% yield.

Synthesis of Dex-β-CD/BM-PCL copolymer

Dex-β-CD (0.72 g, 0.1 mmol) and BM-PCL (0.6 g, 0.1 mmol) were dissolved in 5.0 mL of DMSO and dropped into PBS at 7.4. The solution was dialyzed against deionized water (MWCO 3.5 kDa) for 12 h to remove DMSO and the product was obtained by lyophilization.

Synthesis of Dex-PCL copolymer

Mono azido-terminated PCL (PCL-N_3) was synthesized firstly. PCL-Br (1.2 g, 0.2 mmol) was dissolved in 10.0 mL of DMF. After heating to 80 °C, NaN_3 (0.126 g, 2 mmol) was added. The reactive mixture was stirred at 80 °C for 18 h. The reactive solution was then cooled to room temperature and precipitated in 80.0 mL of diethyl ether. The crude product was redissolved in trichloromethane and washed at least twice with deionized water. The solution was dried with $MgSO_4$ and precipitated in diethyl ether. After vacuum drying for 24 h, PCL-N_3 was obtained with 74% yield. PCL-N_3 (0.6 g, 0.1 mmol), α-alkyne Dex (1.2 g, 0.2 mmol) and PMDETA (40 μL, 0.2 mmol) were dissolved in 30.0 mL of dried DMSO. The mixture was stirred for 10 min and degassed by three freeze–thaw cycles and transferred to another Schlenk flask containing CuBr (30 mg, 0.2 mmol) *via* N_2-purged syringe. The Schlenk flask was placed in an oil bath at 60 °C for 72 h. The reaction medium was dialyzed against deionized water (MWCO 10 kDa) for 4 days and the product was obtained by lyophilization (yield: 78%).

In vitro drug loading and release

Doxorubicin (DOX) was used as a model drug for *in vitro* drug loading and release. DOX-loaded Dex-β-CD/BM-PCL micelle was prepared by a simple dialysis technique. Typically, Dex-β-CD (10.0 mg), BM-PCL (10.0 mg) and drug (4.0 mg) were mixed in 2.0 mL of DMSO. The mixture was stirred at room temperature for 24 h and then added dropwise into 20.0 mL of PBS at pH 7.4. The DMSO was removed by dialysis against water at pH 8.0 for 24 h. The dialysis medium was refreshed five times and the whole procedure was performed in the dark. Then, the solution was filtered and lyophilized. The DOX-loaded Dex-PCL micelle was prepared similarly as DOX-loaded Dex-β-CD/BM-PCL micelle. To determine the drug loading content (DLC) and drug loading efficiency (DLE), the drug-loaded micelle was dissolved in DMSO and analyzed by fluorescence measurement (Perkin-Elmer LS50B luminescence spectrometer) using a standard curve method ($\lambda_{ex} = 480$ nm). The DLC and DLE of drug-loaded micelles were calculated according to eqn (1) and (2), respectively:

$$\text{DLC (wt\%)} = \text{amount of drug in micelle/amount of drug loaded micelle} \times 100 \quad (1)$$

$$\text{DLE (wt\%)} = \text{amount of drug in micelle/total amount of feeding drug} \times 100 \quad (2)$$

In vitro drug release profiles of drug-loaded micelles were investigated in PBS at pH 5.5 and 7.4, respectively. The pre-weighed freeze-dried DOX-loaded micelles were suspended in 20.0 mL of release medium and transferred into a dialysis bag (MWCO 3500 Da). The release experiment was initiated by placing the end-sealed dialysis bag into 150.0 mL of release medium at 37 °C with continuous shaking at 70 rpm. At predetermined time intervals, 2.0 mL of external release medium was taken out and an equal volume of fresh release medium was replenished. The amount of released DOX was determined by using fluorescence measurement ($\lambda_{ex} = 480$ nm). The release experiments were conducted in triplicate.

Intracellular drug release

The cellular uptake and intracellular release behaviors of DOX-loaded micelles were observed by confocal laser scanning microscopy (CLSM) and flow cytometric analyses toward HepG2 cells.

CLSM

HepG2 cells were seeded in 6-well plates at a density of 2×10^5 cells per well in 2.0 mL of complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, supplemented with 50 IU mL^{-1} penicillin and 50 IU mL^{-1} streptomycin, and cultured for 24 h. After the culture media were removed, the cells were incubated at 37 °C for additional 3 h with DOX-loaded micelles at a final DOX concentration of 5.0 mg L^{-1} in complete DMEM. Then, the culture medium was removed and cells were washed with PBS thrice. Thereafter, the cells were fixed with 4% paraformaldehyde for 30 min at room

temperature, and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue) for 20 min. CLSM images of cells were obtained through confocal microscope (Olympus FluoView 1000).

Flow cytometric analyses

HepG2 cells were seeded in 6-well plates at 2×10^5 cells per well in 2.0 mL of complete DMEM, and cultured for 24 h. The cells were then washed by PBS and incubated at 37 °C for additional 3 h with DOX-loaded micelles at a final DOX concentration of 5.0 mg L^{-1} in complete DMEM. Thereafter, the culture medium was removed and the cells were washed with PBS thrice and treated with trypsin. Then, 1.0 mL of PBS was added to each culture well, and the solutions were centrifuged for 4 min at 3000 rpm. After the removal of supernatants, the cells were resuspended in 0.3 mL of PBS. Data for 1×10^4 gated events were collected, and analysis was performed by flow cytometer (Beckman, California, USA).

Cell viability assays

The relative cytotoxicities of micelles against HepG2 cells were evaluated *in vitro* by a standard MTT assay. The cells were seeded in 96-well plates at 1×10^4 cells per well in 200.0 mL of complete DMEM and incubated at 37 °C in 5% CO_2 atmosphere for 24 h. The culture medium was then removed and micelle solutions in complete DMEM at different concentrations (0–5.0 g L^{-1}) were added. The cells were subjected to MTT assay after being incubated for an additional 72 h. The absorbance of the solution was measured on a Bio-Rad 680 microplate reader at 490 nm. Cell viability (%) was calculated based on eqn (3):

$$\text{Cell viability (\%)} = A_{\text{sample}}/A_{\text{control}} \times 100 \quad (3)$$

where, A_{sample} and A_{control} represent the absorbances of the sample and control wells, respectively.

The cytotoxicities of DOX-loaded micelles against HepG2 cells were also evaluated *in vitro* by a MTT assay. Similarly, cells were seeded into 96-well plates at 1×10^4 cells per well in 200.0 μL of complete DMEM and incubated for 24 h. After washing cells with PBS, 180.0 μL of complete DMEM and 20.0 μL of DOX-loaded micelle solutions in PBS were added to form culture media with different DOX concentrations (0–5.0 mg L^{-1} DOX). The cells were subjected to MTT assay after being incubated for 24, 48 and 72 h. The absorbance of the solution was measured on a Bio-Rad 680 microplate reader at 490 nm. Cell viability (%) was also calculated based on eqn (3).

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