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# Synthesis and Properties of $CO_2$-Switchable Dex-*g*-PAHMA Copolymers†

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Dextran graft poly((*N*-amidino)hexyl methacrylamide) (Dex-*g*-PAHMA) copolymers were synthesized by free radical polymerization in aqueous solution and characterized. Dex-*g*-PAHMA copolymers can self-assemble into micelles with the PAHMA rich core and dextran rich shell in aqueous media. The $CO_2$ sensitivity of the micelles was investigated by dynamic light scattering (DLS), conductivity and Zeta potential. The results confirmed that the Dex-*g*-PAHMA copolymer micelles have reversible $CO_2$ sensitivity. The micelles can be used as doxorubicin (DOX) carriers and DOX molecules mainly locate in the core of the micelles. MTT assay indicated that the Dex-*g*-PAHMA graft copolymers are non-toxic to cells in the copolymer concentration range of 5-1000 μg $mL^{-1}$. Whereas the relative cell viability is greatly reduced with the increase of copolymer concentration for DOX-loaded micelles. The DOX-loaded micelles can be endocytosed by MCF-7 cells and the DOX can release from micelles and diffuse into the cell nucleus. The Dex-*g*-PAHMA copolymers have the promising applications as drug carriers for cancer therapy.

## Introduction

Polymers that can response to mild environmental stimuli have attracted increasing attentions in recent years.[1-12] These polymers have great potential applications as drug carriers,[9] gene delivery,[10] biosensors,[2,8] smart coatings,[8] intelligent surfaces,[3,8] surfactants,[8] *etc.* The triggers generally includes temperature,[7,8] pH,[6-8] redox,[9] light,[2] ionic strength,[11] glucose,[13] electric field,[14] and so on. Recently, $CO_2$ stimulus responsive polymers have been attracting increasing attentions,[15-21] due to that $CO_2$ is a kind of nontoxic, cheap, abundant, and environmentally friendly chemical reagent.[22,23] In principle, polymers bearing tertiary amine or carboxylic acid side groups could have the property of $CO_2$ responsiveness.[16]

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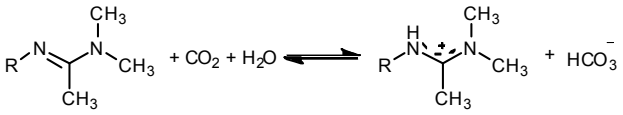
† Electronic supplementary information (ESI) available: The optimization of the polymerization parameters, the determination of CMC of the graft copolymers, the cytotoxicity of Dex-*g*-PHMA, cell uptake of DOX-loaded FITC-Dex-*g*-PAHMA micelles, and the release of DOX from Dex-*g*-PAHMA micelles. See DOI: 10.1039/x0xx00000x

The inspiration of $CO_2$ sensitive polymers is originated from the $CO_2$ switchable solvents, solute, and surfactants.[24-26] Most of the $CO_2$-swticthable compounds have amidino groups in their molecular structure. The amidino group can react with $CO_2$ in the presence of water to form cationic amidinium bicarbonate (Scheme 1), by which to switch the hydrophobicity of the compounds.[25-28] The properties of polymeric materials with amidino groups can be adjusted by using $CO_2$ and $N_2$ (or argon), which avoids the accumulation of additives, especially ions, in the system and offers new opportunities for understanding polymer physics and fabrication new polymer materials for the applications in separation and biology. By introducing amidino groups into polystyrene chains, a novel polymer with reversible neutral-charged-neutral properties in DMF/$H_2O$ was synthesized, by which the dynamics of polyelectrolytes in salt-free dilute solutions was investigated.[20] The solubility of homo amidine-based polymers can be tailored by bubbling with $CO_2$ and $N_2$.[15] Moreover, vesicles prepared from amidine-containing block copolymers show the reversible $CO_2$ stimulus responsive volume expansion in aqueous solution, by which the release of the loaded-drug in the vesicles can be tailored by $CO_2$.[17,29] Recently, polymers containing tertiary amine groups, such as poly(*N,N*'-diethylamino ethyl methacrylate) (PDEAEMA) and poly(*N*-(3-di-methylamino propyl)methacrylamide) (PDMAPMA) have also been used as $CO_2$-responsive blocks[16,30-32] for the preparation of $CO_2$-switchable hydrogels,[32-34] nanoparticles,[35] smart surface[3,18,36] and polymeric surfactants.[37-40]



**Scheme 1**. The reaction of amidine with $CO_2$ and water.

$CO_2$-switchable polymers in literatures are mainly based on synthetic polymers. Natural polymers, such as polysaccharides and proteins, have many advantages including biodegradable, biocompatible, and low toxicity, which have the promising applications in the fields of biology.[41,42] The combination of the advantages of natural polymers with the $CO_2$-switchable properties could broaden the applications of natural polymers. Dextran, a water-soluble, biodegradable, nontoxic natural polymer that can be enzymatic digested in human body,[9,41,42] has been used in pharmacy, food industry, and biotechnology.[41] In previous work, dextran graft copolymers have been synthesized and the biodistribution and blood clearance time can be adjusted by varying the molecular weight and the composition of the side chains.[42,43] The dextran graft copolymers have the promising applications as the drug carrier for radiation therapy.

It is well-known that tumour tissues have a mild acidic environment (pH ~6.5) compared to those in blood and normal tissues (pH ~7.4), which is due to the lactic acid produced by acidic intracellular organelles and hypoxia.[44-46] The mild acidic pH in tumour tissues and endosome and lysosome of cells (pH 5–6) provides a potential trigger for a pH dependent transition of a pH sensitive carrier, and could lead to the rapid drug release at the target sites.[47,48] $CO_2$ can be dissolved in aqueous media to form carbonic acid, a weak acid. The concentration of $CO_2$ in saturated aqueous solution is about 0.033 mol $L^{-1}$ at room temperature and atmospheric pressure, and the pH of the solution is 5.6, close to the pH in tumour tissues and endosome and lysosome of cells. Therefore, a $CO_2$-response group, such as amidino group, is also a pH sensitive group. In this work, amidine-containing side chains, poly((*N*-amidino)hexyl methacrylamide) (PAHMA), were introduced to dextran backbone by free radical polymerization using $K_2S_2O_8$/$NaHSO_3$ as the initiator. $K_2S_2O_8$/$NaHSO_3$ is a redox initiator system that is low in toxicity and suitable to be used in vivo of the resultant graft copolymers.[49] The purpose of this work is to prepare $CO_2$ sensitive dextran graft copolymers for being used as drug carriers that response to biological environments.

## Experimental

### Materials

Dextran (Fluka, $M_w$ = 100 kDa) was purified as follows. Dextran was first dissolved in water and filtered to remove insoluble impurities. The solution was then dialyzed (cut-off molecular weight, COMW, 14 kDa) against deionized water (replaced every 12 h) for 48 h. The resultant solution was lyophilized to obtain the purified dextran.

Methacryloyl chloride (Aladdin Chemical Co. Ltd., 95%), *N*-Boc-1,6-diaminohexane (Alfa Aesar, 98%), and *N,N*-dimethylacetamide dimethyl acetal (Alfa Aesar, 90%) were used as received. Triethylamine (TEA, Beijing Chemical Works, 99%) was dried over $CaH_2$ and distilled under reduced pressure before use. Doxorubicin hydrochloride (DOX) (Beijing Huafeng United Technology Co.) was used as received. All the other chemicals and solvents were supplied by local chemical suppliers and used as received. Water from Milli-Q Reference Water Purification System (Millipore) was used for the reaction and purification of the graft copolymers.

### Synthesis of Dex-*g*-PAHMA

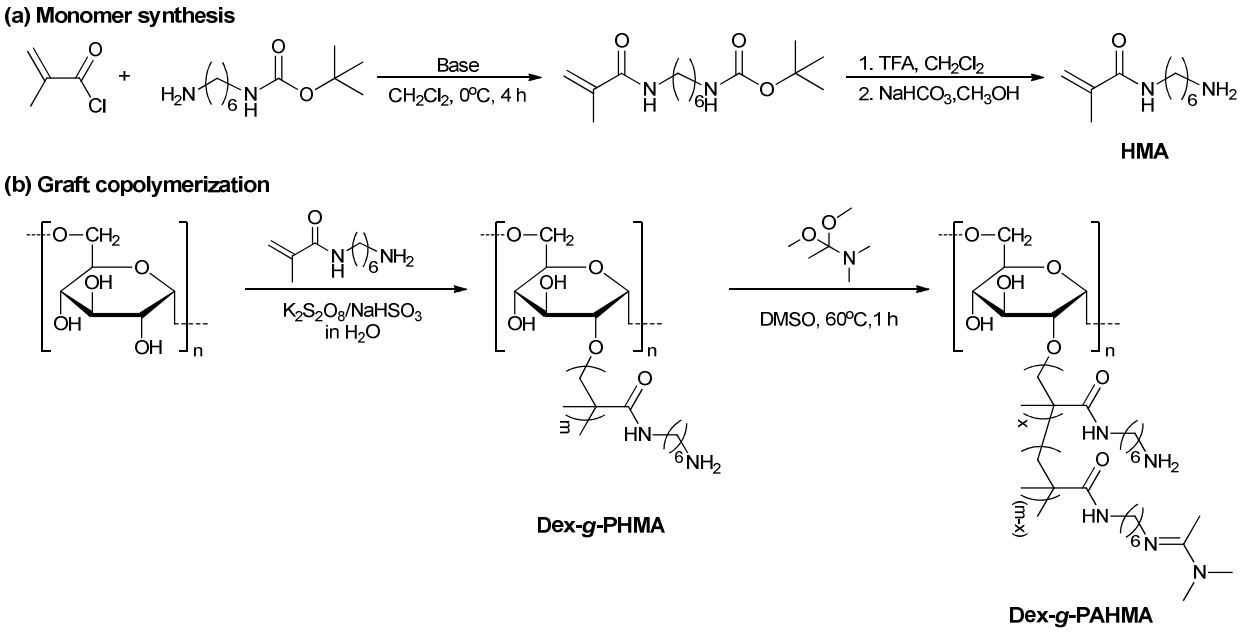
The synthesis route of dextran graft poly((*N*-amidino)hexyl methacrylamide) (Dex-*g*-PAHMA) is show in Scheme 2. The monomer, (*N*-amido)hexyl methacrylamide (HMA), was synthesized by two steps. *N*-Boc-hexyl methacrylamide (Boc-HMA) was first synthesized and then Boc groups were deprotected to obtain HMA (Scheme 2a). In details, *N*-Boc-1,6-hexanediamine (6.72 mL, 30.0 mmol) and triethylamine (4.2 mL, 30.0 mmol) were dissolved in 80 mL of $CH_2Cl_2$ and the flask was then transferred to an ice bath at 0°C. Then methacryloyl chloride (3.06 mL, 30.0 mmol) solution in $CH_2Cl_2$ (15 mL) was added dropwise. The reaction mixture was kept in the ice bath at 0 °C for 4 h and then poured in excess cold diethyl ether. The precipitates were removed by filtration. Boc-HMA was obtained by distilling the filtrate and then dried in vacuum to constant weight (8.43g, yield: 99%). $^1$H NMR (δ, ppm, $d_6$-DMSO): 7.87 (t, 1H, C-C(=O)-N***H***-), 6.76 (t, 1H, O-C(=O)-N***H***-), 5.61 (s, 1H, C***H***$_2$=C-), 5.29 (s, 1H, C***H***$_2$=C-), 3.06-3.08 (m, 2H, -C(O=)C-NH-C***H***$_2$ -), 2.87-2.89 (m, 2H, O-C(=O)-NH-C***H***$_2$-), 1.83 (s, 3H, -C=C-C***H***$_3$), 1.36-1.42 (m, 13H, -C(O=)C-NH-CH$_2$-C***H***$_2$-, -O(O=)C-NH-CH$_2$-C***H***$_2$-, -C-(C***H***$_3$)$_3$), 1.22 (m, 4H, -CH$_2$-CH$_2$-C***H***$_2$-C***H***$_2$-CH$_2$-CH$_2$-).

The deprotection of Boc was carried out as follows. Boc-HMA (0.284 g, 1.0 mmol) was dissolved in 4 mL $CH_2Cl_2$, and 1 mL trifluoroacetic acid (13.5 mmol) was added. The solution was stirring at room temperature for 4 h. Then the mixture was distilled and yellow viscous liquid was obtained. The liquid was diluted with 15 mL $CH_3OH$, and excessive $NaHCO_3$ was added to neutralize the residual acid until pH to 8. The solution was stirring at room temperature for 5 h and filtrated, and the filtrate was distilled to obtain HMA (light yellow. 0.182g, yield: 99%). $^1$H NMR (δ, ppm, $D_2O$): 5.63 (s, 1H, C***H***$_2$=C-), 5.40 (s, 1H, C***H***$_2$=C-), 3.20-3.24 (m, 2H, O=C-NH-C***H***$_2$-), 2.92-2.96 (m, 2H, -C***H***$_2$-NH$_2$), 1.89 (s, 3H, -C=C-C***H***$_3$), 1.60-1.64 (m, 2H, O=C-NH-CH$_2$-C***H***$_2$-,), 1.51-1.54 (m, 2H, -C***H***$_2$-CH$_2$-NH$_2$), 1.34-1.38 (m, 4H, -CH$_2$-CH$_2$-C***H***$_2$-C***H***$_2$-CH$_2$-CH$_2$-).

Dextran graft poly((*N*-amido)hexyl methacrylamide) (Dex-*g*-PHMA) was first synthesized. Briefly, dextran (250 mg), $K_2S_2O_8$ (8.1 mg, 0.03 mmol) and $NaHSO_3$ (2.1 mg, 0.02 mmol) were dissolved in 6 mL water. The solution was deoxygenized by bubbling with $N_2$ at room temperature for 30

min and 4 mL HMA aqueous solution with desired HMA concentration was then added slowly under $N_2$ atmosphere. The reaction mixture was transferred into an oil bath at 50 °C to perform the graft copolymerization for certain time. The reaction mixture was then dialyzed (COMW: 14 kDa) against water (replaced every 12 h) for 48 h to remove the remained initiators and homopolymers. The resultant solutions were lyophilized to result purified Dex-*g*-PHMA. Yield: 54%. $^1$H NMR (δ, ppm, $D_2O$): 4.95 (s, 1H, $H_1$ from ring of dextran), 3.40-4.10 (m, 9H, dextran), 3.12 (m, 2H, O=C-NH-C***H***$_2$-), 3.03 (m, 2H, -C***H***$_2$-$NH_2$), 1.70 (m, 4H, O=C-NH-$CH_2$-C***H***$_2$-, -C***H***$_2$-$CH_2$-$NH_2$), 1.52 (m, 2H, -C***H***$_2$-C-), 1.39 (m, 4H, -$CH_2$-$CH_2$-C***H***$_2$-C***H***$_2$-$CH_2$-$CH_2$-), 0.80-1.20 (m, 3H, -C-C***H***$_3$).

The content of PHMA in Dex-*g*-PHMA copolymers can be adjusted by varying the feeding ratio of HMA, reaction time, temperature, and the initiator concentration. The details for the optimization of the reaction are provided in ESI. The amino groups in Dex-*g*-PHMA side chains were then converted into amidino groups to result Dex-*g*-PAHMA according to Scoggins' procedure.[26] Briefly, Dex-*g*-PHMA (100 mg) was dissolved in 10 mL DMSO at 60°C to obtain light yellow homogeneous solution. Dimethyl acetamide dimethyl acetal (200 times molar amounts of primary amine) was added and the mixture was stirred at 60 °C for 1 h. The methanol byproduct and solvent were removed by dialysis using a dialysis bag (COMW: 14 kDa) and then lyophilized to obtain result Dex-*g*-PAHMA. Yield: 95%. $^1$H NMR (δ, ppm, $D_2O$): 4.97 (s, 1H, $H_1$ from ring of dextran), 3.40-4.00 (m, 9H, dextran), 3.11-3.23 (m, 4H, O=C-NH-C***H***$_2$-, -C***H***$_2$-N=C-), 2.29 (s, 6H, -N(C***H***$_3$)$_2$), 1.99 (s, 3H, -N=C-C-C***H***$_3$), 1.60 (m, 4H, O=C-NH-$CH_2$-C***H***$_2$-, -C***H***$_2$-$CH_2$-$NH_2$), 1.50 (m, 2H, -C***H***$_2$-C-), 1.33 (m, 4H, -$CH_2$-$CH_2$-C***H***$_2$-C***H***$_2$-$CH_2$-$CH_2$-), 0.90-1.13 (m, 3H, -C-C***H***$_3$).




**Scheme 2.** Synthesis of Dex-*g*-PAHMA Copolymers.

### 2.3. Preparation of Dex-*g*-PAHMA copolymers micelles and Dox-loaded micelles

Dex-*g*-PAHMA micelles in aqueous solutions were prepared as follows. Dex-*g*-PAHMA (30 mg) was dissolved in DMSO (3 mL) and added dropwise into 6 mL Milli-Q water under ultrasonic agitation. The resultant micellar solutions were dialyzed (COMW: 14 kDa) against water (replaced every 12 h) for 24 h to remove DMSO. The resultant micellar solutions were about 15 mL after dialysis, corresponding to the polymer concentration of 2 mg $mL^{-1}$.

The DOX-loaded Dex-*g*-PAHMA micelles were prepared as follows. Dex-*g*-PAHMA (15 mg) and DOX (7.5 mg) were mixed with 1.5 mL DMSO and stirred at 25 °C for 3 h to obtain completely dissolved solution. The solution was then added dropwise into 3 mL Milli-Q water under ultrasonic agitation and stirred for 2 h. The micellar solution was dialyzed (COMW: 14 kDa) against 2000 mL Milli-Q water (replaced every 12 h) for 24 h to remove DMSO and free DOX. DOX-loaded micellar solution was adjusted to 15 mL after dialysis to result solutions with polymer concentration of 1 mg $mL^{-1}$. The loading content ($C_{DOX}$) and encapsulation efficiency ($E_e$) of DOX in the micelles were estimated by,[50]

$$C_{DOX}(\%) = (W_{DOX,L}/W_M) \times 100 \quad (1)$$

$$E_e(\%) = (W_{DOX,L}/W_{DOX,F}) \times 100 \quad (2)$$

where $W_{DOX,L}$, $W_{DOX,F}$ and $W_M$ are the mass of DOX in the micelles, the feeding DOX, and the micelles, respectively. The

concentration of DOX was estimated by measuring the absorbance at λ = 481 nm on a Shimadzu UV-1601PC spectrophotometer, and then $W_{DOX,L}$ was estimated by compared with the calibration curve.

Fluorescence quenching studies of DOX were carried out on Perkin Elmer LS 55 fluorescence spectrometer and the excitation wavelength was 500 nm. Samples of free DOX (10 μg $mL^{-1}$) and DOX-loaded Dex-*g*-PAHMA ($DS_{Am}$ = 0.513, 0.1 mg $mL^{-1}$) micellar solutions were checked. KI was used as the fluorescence quenching agent. The level of $I^{-1}$ (KI) varied from 0.0 to 0.50 M. The ionic strength of the solutions was kept constant by adding NaCl.

### 2.4. Characterizations

$^1$H NMR spectra were measured on a Bruker Advance NMR (400 MHz) instrument at 25°C. FTIR spectra were recorded on a Bruker-Equinox 55 FTIR spectrometer at a resolution of 2 $cm^{-1}$ using KBr pellet. Elemental analysis was performed on a Flash EA 1112 Elemental Analyzer. Dynamic light scattering (DLS) were performed on an ALV/SP-150 spectrometer equipped with an ALV-5000 multi-τ digital time correlator and a solid-state laser (ADLS DPY 425II, output power ca. 400 mW at λ = 632.8 nm) as the light source. All the samples were filtered through a Millipore Millex-FH nylon membrane (0.45 μm) before measurements. All data were collected at the scattering angle of 90° at 25 °C and the hydrodynamic radius (<$R_h$>) and its distribution were estimated by the CONTIN program. Transmission electron microscope (TEM) observation was carried out on a 2200FS TEM (JEOL, Japan) at an acceleration voltage of 200 kV. The samples for TEM observation were prepared by dropping a small drop of the Dex-*g*-PAHMA micellar solutions on carbon film supported on copper grid and air dried. Zeta potentials of the Dex-*g*-PAHMA micellar solutions were measured on a Malvern Zetasizer ZS (Malvern Instr., UK). The conductivity of the copolymers was measured on a Lei-ci conductivity meter (DDS-307A) at 25°C. Prior to the conductivity measurement, the solution was bubbled with $N_2$ for 1 h till a stable conductivity was reached.

### 2.5. In vitro cytotoxicity of Dex-*g*-PAHMA copolymers

The cytotoxicity of Dex-*g*-PAHMA and Dex-g-PHMA copolymers was estimated by MTT assay using MCF-7 cell (European Collection of Cell Cultures, UK). Cells were cultivated in DMEM containing 10% FBS and 1% antibiotics (penicillin and streptomycin) at 37 °C in 5% $CO_2$. The cells were then counted and seeded into 96-well plates at $5\times10^4$ cells per well in 200 μL of the culture medium. After incubated for 24 h, the culture medium was removed and 200 μL medium containing graft copolymers or DOX-loaded micelles was added into the wells. The cells were then incubated for another 24 h. The culture medium was then removed and wells were washed thoroughly with PBS three times. Then, 100 μL culture medium and 10 μL (5 mg $mL^{-1}$) MTT assay solution in PBS were added into each well and the cells were incubated for another 4 h. The unreacted MTT was removed carefully and 150 μL DMSO was added to dissolve the blue formazan crystals. The absorbance was then measured by a microplate reader (MULTISKAN MK3, Thermo Electron Corporation) at a wavelength of 570 nm for each cell. The relative cell viability (%) was calculated by comparing the optical density at 570 nm with untreated cells.

### 2.6. Cell uptake of DOX-loaded Dex-*g*-PAHMA micelles

MCF-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, US) supplemented with 10% fetal bovine serum (FBS, Invitrogen, US) at 37 °C with 5% $CO_2$ in a 95% humidified atmosphere in an incubator. The cells were subcultured every 48 h. Before testing, the cells were harvested by trypsinization using a 0.05% (w/v) trypsin/0.03% (w/v) EDTA solution. The cellular uptake experiments were monitored by confocal laser scanning microscopy. The DOX-loaded Dex-*g*-PAHMA micelles were labelled by FITC. The FITC-Dex-*g*-PAHMA was synthesized by adding 40 μL DMSO solution of FITC (1 mg $mL^{-1}$) into Dex-*g*-PAHMA aqueous solution (1 mg $mL^{-1}$). The solution was stirred overnight at 4°C for 8 h in dark and terminated by adding 200 μL ammonium chloride aqueous solution (5 mol $L^{-1}$). Then the solution was purified by dialyzing for 48 h and lyophilized to give an orange powder. The DOX-loaded FITC-Dex-*g*-PAHMA micelles were prepared as described above. MCF-7 cells were plated on microscope slides in a culture dish ($5 \times 10^4$ cells per well) using 1 mL of DMEM media supplemented with 10% FBS in a humidified atmosphere of 5% $CO_2$ at 37 °C for 24 h. Cells were washed with PBS three times and incubated for an additional 1, 3, 8, 12, and 24 h in DMEM containing DOX-loaded FITC-labelled Dex-*g*-PAHMA micelles with a final polymer concentration of 500 μg $mL^{-1}$. Then, the culture medium was removed and the cells were washed with PBS three times. The cells were fixed using 4% paraformaldehyde for 30 min at 37 °C and rinsed with PBS three times. The cell nuclei were stained with DAPI for 15 min and washed with PBS three times. Fluorescence images of the cells were obtained using a FV 1000-IX81 confocal laser scanning microscope (Olympus, Japan).

## 3. Results and Discussion

### 3.1. Synthesis Dex-*g*-PHAMA copolymers

Various initiators have been used in the synthesis polysaccharides graft copolymers by free radical copolymerization,[51-54] among which $K_2S_2O_8$/$NaHSO_3$ redox pair has been proven to be effective and nontoxic initiator system, and the resultant graft copolymers are suitable for being used in vivo.[49,51] Therefore, $K_2S_2O_8$/$NaHSO_3$ redox pair was used as the initiator in this work. During the synthesis procedure, free radicals produced by the initiator first transferred to the hydroxyl groups on the dextran chains,[49] by which the monomer HMA can be initiated and propagated to



form the graft chains. The synthesis route of Dex-*g*-PAHMA copolymers is shown in Scheme 2. The successful synthesis of the compounds at each step was checked by $^1$H NMR. Fig. 1 shows the $^1$H NMR spectra of dextran, the monomers and the graft copolymers. On the $^1$H NMR spectrum of the monomer HMA (Fig. 1a), the signals at 1.89, 5.40, and 5.63 ppm attribute to the protons of the methacryl groups linked to 1,6-hexanediamine.On the $^1$H NMR spectrum of the Dex-*g*-PHMA, protons of the PHMA side chains appeared at the chemical shift of δ=3.12 (m, 2H), 3.03(m, 2H), 1.70 (m, 4H), 1.52 (m, 2H), 1.39 (m, 4H), 0.80–1.20 (m, 3H) ppm (Fig. 1c) besides the typical peaks from protons of dextran backbone at δ= 3.40–4.10 and 4.95 (Fig. 1b). On the $^1$HNMR spectrum of the graft copolymer Dex-*g*-PAHMA, the peaks at δ = 2.29 and 1.99 ppm come from the protons on the methyl of -N($CH_3$)$_2$ and -N=C-$CH_3$ of PAHMA, respectively, confirmed the formation of amidino groups in the graft copolymers. Fig. 2 shows the FTIR spectra of dextran and the graft copolymers. The absorption peaks at 1634, 1537 and 1681 cm$^{-1}$ correspond to N-H bending vibration ($\delta_{N-H}$) of primary amine groups, N-H bending vibration ($\delta_{N-H}$) and C=O stretching vibration ($\nu_{C=O}$) of secondary amide groups, respectively (Fig. 2b), which also confirmed the successful synthesis of the dextran graft copolymers Dex-*g*-PHMA. The appearance of the absorption peak of the $\delta_{C=N}$ of amidino group at 1647 cm$^{-1}$ also indicated the successful synthesis Dex-*g*-PAHMA (Fig. 2c). In addition, elemental analysis which shows the presence of nitrogen atoms in the graft copolymers (Tables 1), also confirms the successful synthesis of the dextran graft copolymers.

The content of PHMA side chains in the graft copolymers, defined as the average -$NH_2$ groups per glucopyranose unit ($DS_{-NH2}$), can be estimated by $DS_{-NH2} = A_2/4A_1$, where $A_1$ and $A_2$ are the integrated areas of the protons on dextran backbone (δ 4.95 ppm) and the methylene of -C- NH-$CH_2$- and -$CH_2$-$NH_2$ groups of the PHMA side chains (δ 3.03–3.12 ppm) on the $^1$H NMR spectra, respectively. The molecular weight of Dex-*g*-PHMA copolymers was calculated by $M_w = M_{w,Dex} + 184nDS_{-NH2}$, where $M_{w,Dex}$ is the molecular weight of dextran and 184 is the molar mass of HMA, and $n$ is the average numbers of glucose unit per dextran chain. The synthesis parameters were optimized (Fig. S1 and Table S1, ESI). The optimal parameters for the graft copolymerization were selected as follows, the concentration of the initiator pair of $K_2S_2O_8$ and $NaHSO_3$ were selected at 3 and 2 mmol L$^{-1}$, respectively. The copolymerization was carried out at 50 °C for 24 h. The graft ratio of Dex-*g*-PHMA ($G$), defined as the mass content of the side chains in the graft copolymers, is predetermined by the mass of feeding ratio of the monomer HMA to dextran. Three Dex-*g*-PHMA copolymers with the graft ratio of $G$ = 8.0%, 23.3%, 45.2% were used for the further investigations in the following parts. The details for the reaction parameters and the resultant copolymers are listed in Table 1.

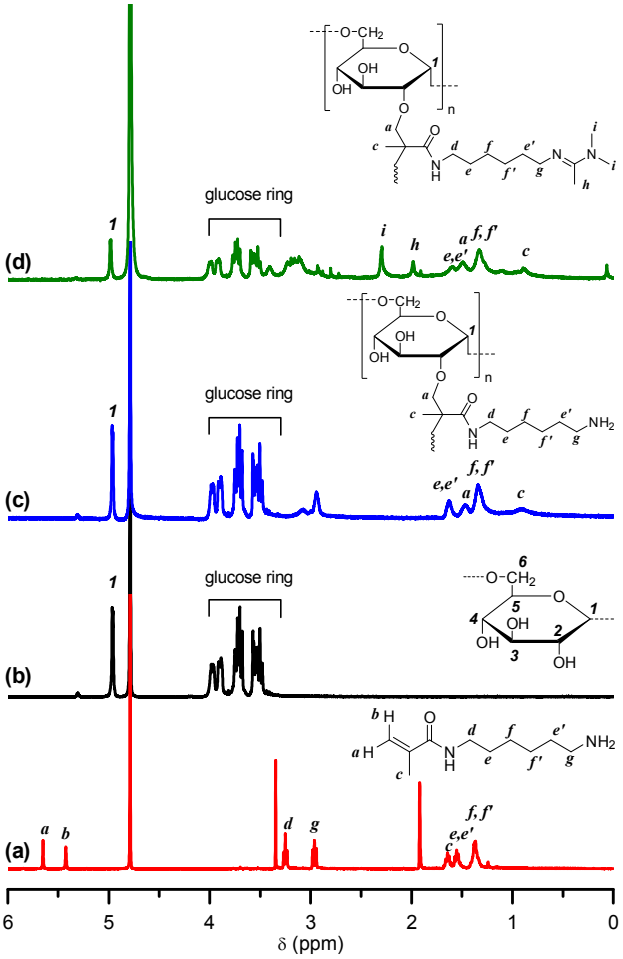



**Fig. 1** $^1$HNMR spectra of (a) the monomer HMA, (b) dextran, (c) Dex-*g*-PHMA, and (d) Dex-*g*-PHAMA in $D_2O$ at 400 MHz.

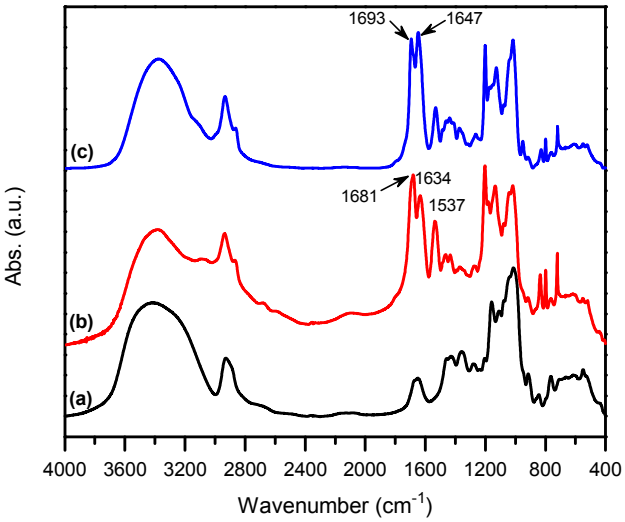



**Fig. 2** FTIR spectra of (a) dextran, (b) Dex-*g*-PHMA and (c) Dex-*g*-PAHMA.

For the purpose to convert the amino groups into amidino groups completely, 200 times of dimethyl acetamide dimethyl acetal to the primary amine groups in Dex-*g*-PHMA (in mole) was use in the amidino formation reaction. The details of the obtained Dex-*g*-PAHMA copolymers are listed in Table 1. The average amidino groups per glucopyranose unit $DS_{Am}$, can be estimated by $DS_{Am} = A_3/6A_1$, where $A_1$ and $A_3$ are the integrated areas of the protons on dextran backbone (δ 4.95 ppm) and tertiary amine of amidino (δ 2.29 ppm) on the $^1$HNMR spectra. The $DS_{Am}$ of the Dex-*g*-PAHMA prepared from Dex-*g*-PHMA with graft ratio of 8.0%, 23.3%, 45.2% are 0.064, 0.216 and 0.513, respectively, corresponding to the conversion efficiency of the amino groups to amidino groups of 82.9%, 80.7%, and 70.8% (Table 1).

**Table 1.** Three graft copolymers Dex-g-PAHMA with different $DS_{Am}$.

| Sample | Dex (mg) | $H_2O$ (mL) | HMA (mg) | $G$ (wt%)[a] | Dex-*g*-PHMA | | | Dex-*g*-PAHMA | | | | $E_e$ (wt%) | $C_{DOX}$ (wt%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $DS_{-NH2}$[a] | N%[b] | $M_{w,\ PHMA}$ (kDa)[c] | $DS_{Am}$[a] | Am (%)[d] | N%[b] | $N_{Am}$[e] | | |
| 1 | 500 | 8 | 500 | 8.0 | 0.077 | 1.06 | 109 | 0.064 | 82.9 | 1.15 | 39.4 | 2.0 | 1.0 |
| 2 | 250 | 4 | 500 | 23.3 | 0.268 | 3.20 | 130 | 0.216 | 80.7 | 4.01 | 133.3 | 8.8 | 4.4 |
| 3 | 250 | 4 | 1250 | 45.2 | 0.725 | 5.39 | 182 | 0.513 | 70.8 | 6.64 | 316.5 | 18.9 | 9.4 |

[a] $G$(%), $DS_{-NH2}$ and $DS_{Am}$ were calculated by $^1$H NMR. [b] N% is the content of N element in graft copolymers measured by element analysis. [c] $M_w$ is the average molar mass of PHMA that calculated by $^1$H NMR referring to the molar mass of dextran. [d] The conversion of the -$NH_2$ groups into amidino groups. [e] The average numbers of amidino groups per dextran chain.

### 3.2. Micellization and pH sensitivity of Dex-*g*-PAHMA copolymers in aqueous solution

Dex-*g*-PAHMA copolymers have the hydrophilic dextran backbones and the relatively hydrophobic PAHMA side chains with hexyl methacrylamide pendants and amidine ends. Such graft copolymers can self-assemble into micellar structure due to the typical amphiphilic feature, in which the relatively hydrophobic PAHMA side chains mainly locate in the core of the micelles and the relatively hydrophilic dextran backbone prefer to stay outside. The micellization behaviours of Dex-*g*-PAHMA copolymers were investigated by using pyrene as fluorescent probe (Fig. S2, ESI). The critical micelle concentration (CMC) of Dex-*g*-PAHMA copolymers with $DS_{Am}$ of 0.064, 0.216, 0.513 are about 11.7, 8.5, and 10.8 μg $mL^{-1}$, respectively. The CMC of graft copolymers with different $DS_{Am}$ is quite similar to each other, which could be attributed to that the difference in the hydrophobicity between the dextran backbone and the PAHMA side chains is relatively smaller than those copolymers with typical amphiphilic characteristics. The polymer concentration in all the following measurements is above CMC in present work. The average hydrodynamic radius (<$R_h$>) shows a double distribution mode with the peak position at around 10 and 100 nm in aqueous solution (Fig. 3), which suggests that there are single chains or smaller aggregations in the micellar solutions. The <$R_h$> of the micelles increases from 80 to 110 nm for Dex-*g*-PAHMA with $DS_{Am}$ of 0.064, 0.216, and 0.513.

Fig. 4 shows <$R_h$> distribution of Dex-*g*-PAHMA ($DS_{Am}$ = 0.513) in aqueous solution at different pH. At pH=5.8, the <$R_h$> of Dex-*g*-PAHMA micelles is about 340 nm. The larger <$R_h$> attributes to the electrostatic repulsion between the partial protonation of the PAHMA chains in the core of the micelles. Increased pH to 7.5, the deprotonated PAHMA side chains in the core of the micelles collapse and the <$R_h$> of reduce to about 102 nm. The <$R_h$> of micelles increase to about 261 nm at pH 10.0, which is due to the aggregation of the collapsed micelles.[55,56] TEM images indicate that the micelles have a spherical morphology. The diameter of the micelles is smaller than that estimated by DLS, which is due to that the TEM samples were observed in dried state and the micelles should be shrunk during the drying procedure. TEM results also indicate that the micelles at pH 7.5 are smaller than those at pH 5.8 and 10.0, which is consisted with DLS results.

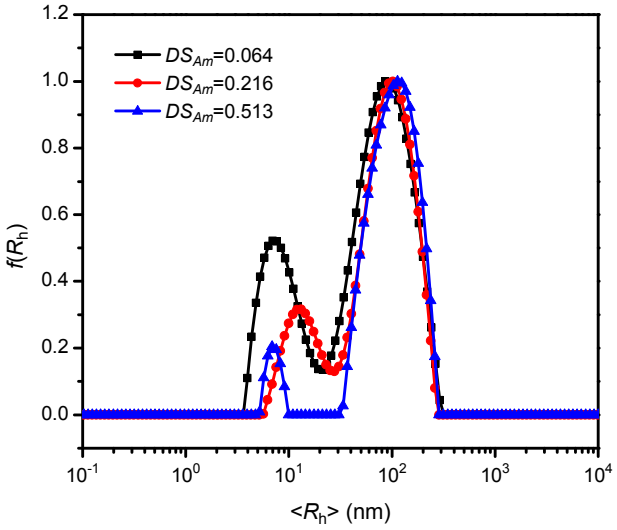



**Fig. 3** Hydrodynamic radius distribution of Dex-g-PHAMA with different graft ratios in aqueous solutions at 25 °C. The initial concentration of the solutions was 2 mg $mL^{-1}$. pH=7.6.

### 3.3. $CO_2$ sensitivity

The amidino groups in Dex-*g*-PAHMA can react with $CO_2$ to form cationic amidinium bicarbonate in the presence of water, which offers the $CO_2$ sensitivity of the copolymers.[25-28] Fig. 5 shows the $<R_h>$ of Dex-*g*-PAHMA and Dex-*g*-PHMA copolymers in aqueous solution. The as-prepared micellar solutions were first bubbled with $N_2$ for 30 min to expel $CO_2$. Then the micellar solutions were bubbled with $CO_2$ for 30 min, $<R_h>$ of the micelles increased. This change of $<R_h>$ is reversible and can be recovered by further bubbled the solutions with $N_2$ (Fig. 5). The dissolution of $CO_2$ in the aqueous micellar solutions will cause the amidino groups to form charged bicarbonate,[17,26] which will lead to the swelling of the PAHMA core of the micelles. The $CO_2$ induced swelling of the micelles is reversible. When the micellar solutions were bubbled with $N_2$ to remove $CO_2$ in the solutions, the micelles were shrunk and $<R_h>$ recovered (Fig. 5). Moreover, the $CO_2$ stimuli induced swelling of the micelles depends on the content of the PAHMA in the Dex-*g*-PAHMA copolymers. The increase in $<R_h>$ of the micelles becomes more obviously with the increase in the content of PAHMA side chains in the graft copolymers (Fig. 5). For comparison, $<R_h>$ of the Dex-*g*-PHMA copolymers has no obvious changes during all the procedures.

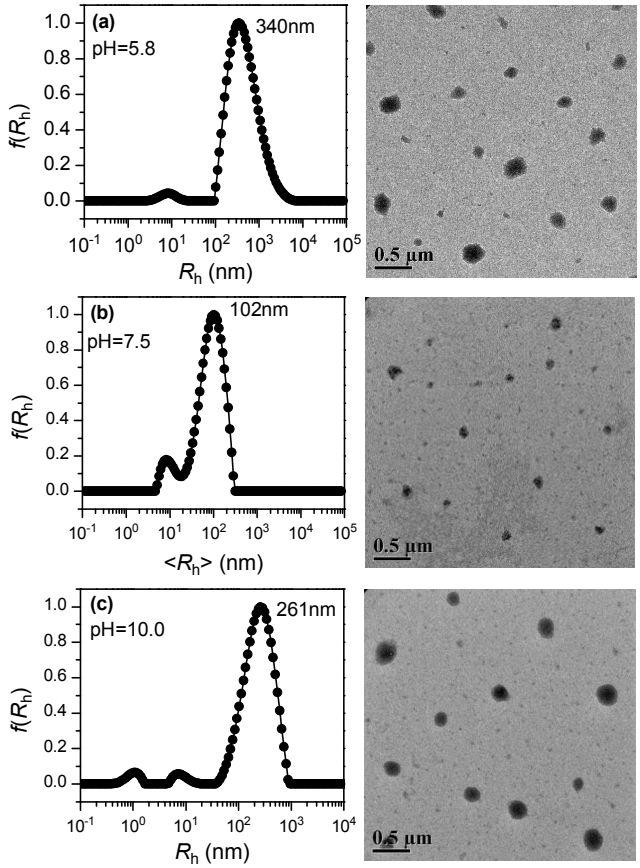



**Fig. 4** Hydrodynamic radius distribution and TEM images of Dex-g-PAHMA with $DS_{Am}$ = 0.513 in aqueous solution at pH of 5.8 (a), 7.5 (b), and 10.0 (c). Polymer concentration was 2 mg $mL^{-1}$.

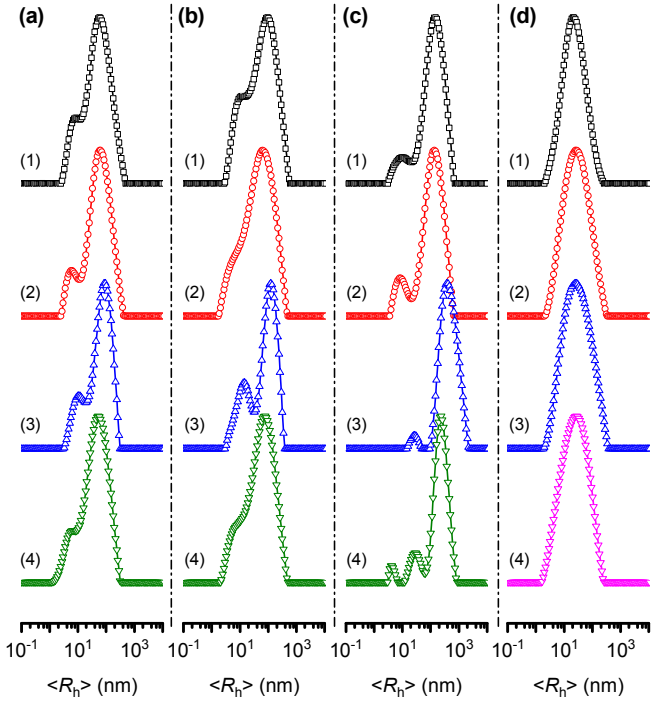



**Fig. 5** The $<R_h>$ of (1) as-prepared micelles, bubble with (2) $N_2$ for 30 min, (3) then bubbled with $CO_2$ for 30 min and (4) then bubble with $N_2$ for 30 min for Dex-*g*-PAHMA with $DS_{Am}$ of (a) 0.064, (b) 0.216, (c) 0.513, and (d) Dex-*g*-PHMA with graft ratio of 45.2% in aqueous solutions at 25 °C. The polymer concentration was kept at 2 mg $mL^{-1}$.

Fig. 6 shows the conductivity profiles of Dex-*g*-PAHMA aqueous solutions during alternatively bubbling with $CO_2$ for 20 min and $N_2$ at 25°C for 30 min. The results show that the solution of the copolymer with higher graft ratio has the higher initial conductivity at the same polymer concentration, which is due to higher content of protonated amidino group. For the solution prepared from Dex-*g*-PAHMA with $DS_{Am}$ of 0.064, 0.216, and 0.513, the solution conductivity of increased from 28.0, 73.5, and 134.1 μS $cm^{-1}$ to 48.3, 97.0, and 161.2 μS $cm^{-1}$ after the solutions were bubbled with $CO_2$ for 20 min. For comparison, the conductivity of Dex-*g*-PHMA solution increased only about 12 μS $cm^{-1}$ when bubbled with $CO_2$ due to the partial protonation of primary amine groups. The conductivity of the copolymer aqueous solutions can be totally recovered after the solutions were bubbled with $N_2$ for 30 min. This procedure can be repeated for several times (Fig. 6), indicates the good reversibility and repeatability of the $CO_2$ sensitivity of the graft copolymers.

Zeta potential experiments also demonstrate the reversible $CO_2$ sensitivity of the Dex-*g*-PAHMA copolymers in aqueous solution (Fig. 7). The Zeta potential of the freshly prepared micellar solutions of copolymers with $DS_{Am}$ of 0.064, 0.216, and 0.513 are 20.4, 30.0, and 49.4 mV, respectively, confirmed that the relatively good dispersion stability of micellar solutions. When the micellar solutions were bubbled with $CO_2$, the Zeta potential of micellar solutions increased to 25.7, 38.1 and 62.5 mV for copolymers with $DS_{Am}$ of 0.064, 0.216, and 0.513 respectively. The increase in Zeta potential of the

micellar solutions attributes to the conversion of the amidino groups to positively charged bicarbonate salt state of the PAHMA side chains in the system.[17] For comparison, The Zeta potential of Dex-*g*-PHMA (*G*=45.2%) aqueous solution remained at about 55 mV during the bubbling cycles using $CO_2$ or $N_2$. Zeta potential experiments also confirmed the reversible procedure of the $CO_2$ sensitivity of the Dex-*g*-PAHMA copolymers in aqueous solution. The Zeta potential of the micellar solutions can be recovered by bubbled with $N_2$ (Fig. 7). Above results confirmed the reversible $CO_2$ sensitivity of the of the Dex-*g*-PAHMA copolymers in aqueous solution.

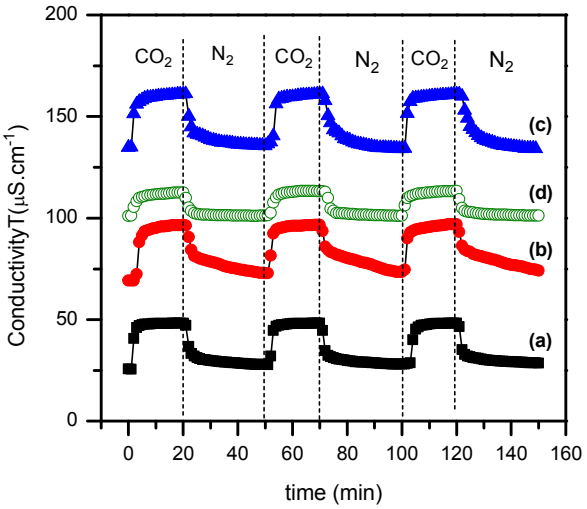



**Fig. 6** Conductivity of Dex-*g*-PAHMA aqueous solutions being bubbled alternatively with $CO_2$ (20 min) and $N_2$ (30 min) at 25 °C for 3 cycles. The Dex-*g*-PAHMA with $DS_{Am}$ of (a) 0.064, (b) 0.216, and (c) 0.513. (d) Dex-*g*-PHMA (*G* = 45.2%) was used as control. The polymer concentration was 5 mg $mL^{-1}$ for all the solutions.

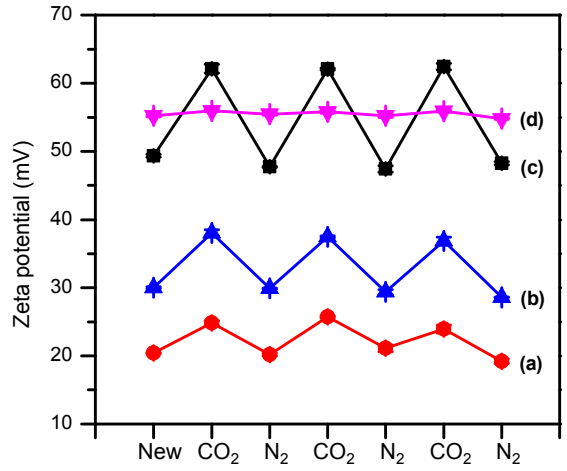



**Fig. 7** The changes of Zeta potential of dextran graft copolymers with $DS_{Am}$ of (a) 0.064, (b) 0.216, and (c) 0.513 when bubbling alternatively with $CO_2$ and $N_2$ for 30 min in aqueous solutions at 25 °C for cycles. (d) Dex-*g*-PHMA (*G* = 45.2%) was used as control. Polymer concentration was 2 mg $mL^{-1}$. The error bars were estimated by three parallel experiments.

### 3.4. Cytotoxicity and cellular uptake of DOX-loaded Dex-*g*-PAHMA copolymers micelles

The loading properties of Dex-*g*-PAHMA micelles for DOX, a widely used drug for cancer therapy, were investigated. The loading efficiency and drug content are listed in Table 1. The DOX content in the micelles increased with the increasing content of PAHMA in the graft copolymers. The location of DOX in Dex-*g*-PAHMA micelles was checked by fluorescence quenching of DOX using Stern-Volmer plot.

$$F_0/F = 1 + K_Q [I^{-1}] \quad (3)$$

where $F_0$ and $F$ are the maximal fluorescence intensity of DOX in the solutions without and with $I^{-1}$ ions. $K_Q$ is the collisional quenching constant that represents the environmental polarity of the accessible fluorophors.[59] Fig. 8 shows the Stern-Volmer plots of free DOX and DOX-loaded Dex-*g*-PAHMA micellar solutions. The linear fitting resulted the $K_Q$ of 98.0 and 26.3 $M^{-1}$ for free DOX and DOX-loaded Dex-*g*-PAHMA micellar solutions, respectively. The results indicate that the free DOX molecules in an aqueous medium are readily quenched by $I^{-1}$ ions. Whereas the DOX molecules loaded in Dex-*g*-PAHMA micelles cannot be readily accessed by $I^{-1}$ ions. The results confirm that the DOX molecules mainly locate inside the cores of Dex-*g*-PAHMA micelles.[60]

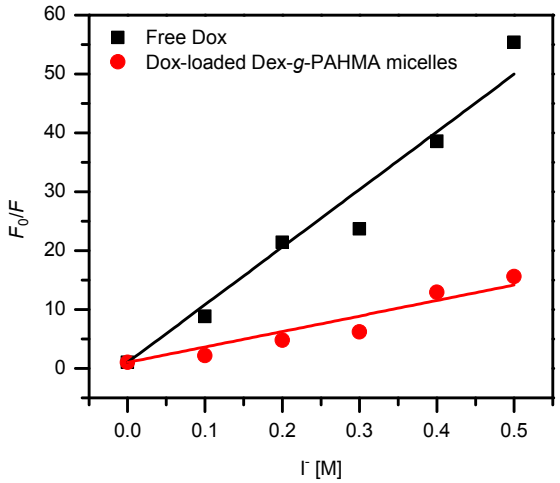



**Fig. 8** Stern-Volmer plots of free DOX and DOX-loaded Dex-*g*-PAHMA ($DS_{Am}$ = 0.513) micelles in aqueous solution.

The cytotoxicity of the polymeric materials is one of the key benchmarks for being used as drug carriers. Fig. 9 shows the cell viability of Dex-*g*-PAHMA copolymers and DOX-loaded Dex-*g*-PAHMA micelles estimated by MTT assay using MCF-7 cells after incubation for 24 h. Copolymers solutions in the concentration range of 5–1000 μg $mL^{-1}$ were used to treat the cells. It was found that the relative cell viability is gradually reduced with the increasing concentration for Dex-*g*-PHMA (Fig. S3, ESI). The results are reasonable due to that the amino

groups are toxic to cells.[57,58] The relative cell viability was obviously improved for Dex-*g*-PAHMA copolymers (Fig. 9a). The cell viability is still around 90% at the polymer concentration of 1000 μg $mL^{-1}$. The results indicate that amidino groups are less toxicity than that of amino groups. The results confirm that the Dex-*g*-PAHMA copolymers are non-toxic to cells in the concentration range up to 1 mg $mL^{-1}$.

The relative cell viability of DOX-loaded Dex-*g*-PAHMA micelles is shown in Fig. 9b. At the polymer concentration of 5 and 10 μg $mL^{-1}$, there is no obvious cytotoxicity to cells, which is reasonable due to that the DOX concentration in the culture media is below 1 μg $mL^{-1}$. When the polymer concentration of the DOX-loaded micelles is above 50 μg $mL^{-1}$, the relative cell viability gradually decreases with polymer concentration (Fig. 9b). For DOX-loaded micellar solutions with polymer concentration in the range of 50–1000 μg $mL^{-1}$, the DOX concentration is in the range of 0.56–11.3, 2.4–48.3, and 4.6–92.0 μg $mL^{-1}$ for micellar solutions prepared from Dex-*g*-PAHMA copolymers with $DS_{Am}$ of 0.064, 0.216, and 0.513, respectively. Higher DOX concentration in the cell culture medium will kill more cells during the incubation, which resulted in the decrease in relatively cell viability after incubating the cells for 24 h. Micelles prepared from Dex-*g*-PAHMA copolymers with higher $DS_{Am}$ have better cell-killing efficiency. The results suggest that the Dex-g-PAHMA copolymers could be used as the efficient DOX carriers for cancer therapy.

Fig. 10 shows the representational cellular uptake results of FITC-labelled Dex-*g*-PAHMA ($DS_{Am}$ = 0.513) micelles on MCF-7 cell line. The cellular uptake results of micelles prepared from Dex-*g*-PAHMA copolymers with different $DS_{Am}$ are provided in ESI (Fig. S4). For Dex-*g*-PAHMA with higher PAHMA content ($DS_{Am}$ = 0.513), only few red fluorescence of DOX can be observed in the cell cytoplasm, while strong green fluorescence of FITC labelled copolymers can be observed on the cell membranes after incubation for 1 h (Fig. 10a). The Dex-*g*-PAHMA micelles are positively charged as that indicated in Fig. 7, which results the strong electrostatic interaction between the micelles and the cell membrane. After incubation for 3 h, strong green fluorescence can be observed in the cytoplasm of MCF-7 cell, which indicates that a large amount of the copolymer micelles had been endocytosed by the cells (Fig. 10b). With the incubation time expanded to 8 h, strong yellow fluorescence can be visualized in cytoplasm of MCF-7 cell, which resulted from the overlap of the green and red fluorescence of the FITC labelled Dex-*g*-PAHMA and DOX, respectively. Moreover, the green fluorescence on the cell membrane disappeared (Fig. 10c). The cellular uptake of the micelles prepared from Dex-*g*-PAHMA copolymers with relatively lower PAHMA content ($DS_{Am}$ = 0.216 and 0.064) (Fig. S4, ESI) is much lower than that of micelles prepared from Dex-*g*-PAHMA copolymer with higher PAHMA content ($DS_{Am}$ = 0.513) at the same time intervals, which could be due to the lower content of amidino groups in these copolymers and the electrostatic interaction between the micelles and cell membrane become weaker. The results indicate that the Dex-*g*-PAHMA micelles can be efficiently taken up by MCF-7 cells. In addition, the red fluorescence increases significantly in nucleus region of the cells after incubation for 24 h (Fig. 10e), which is due to that the endosomes internalized the micelles are in acidic environment (pH 5–6),[47] which could stimulate the quick release and diffusion of the loaded-DOX from the micelles into the nucleus. Above results confirm that the DOX-loaded Dex-*g*-PAHMA micelles can be efficiently endocytosed by MCF-7 cells and the DOX can release from micelles and diffuse into the cell nucleus. The release of DOX in vitro was also checked under $CO_2$ or $N_2$ bubbling conditions and in aqueous solutions at pH of 7.4 and 5.5 (Fig. S5, ESI). It was found that only part of the loaded DOX can be released in all the cases. When the micellar solution was bubbled with $N_2$, only about 3.1% of the loaded-DOX was released in 60 h. Whereas, about 11.5% of the loaded-DOX can be released from the micelles within 60 h when the solution was bubbled with $CO_2$ (Fig. S5a, ESI). Moreover, about 12.6% and 8.9% of the

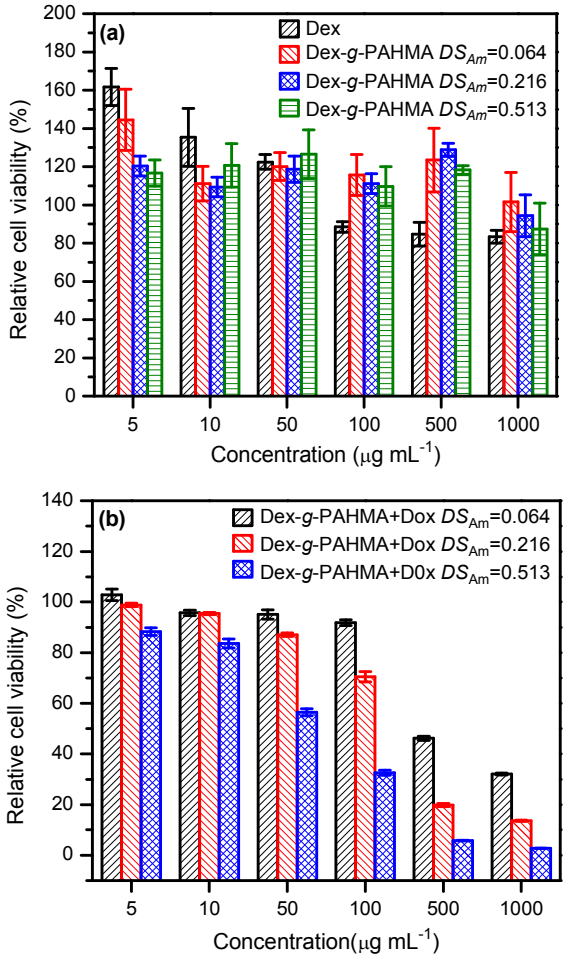



**Fig. 9** Relative cell viability of MCF-7 cells after being cultured for 24 h in (a) Dex-*g*-PAHMA and (b) DOX-loaded Dex-*g*-PAHMA solutions with different copolymer concentration . MCF-7 cells incubated without copolymers were used as the control and the cell viability was determined by MTT assay. Each point is the mean of three parallel measurements.

loaded-DOX in the Dex-*g*-PAHMA micelles can be released within 120 h at pH of 5.5 and 7.4, respectively (Fig. S5b, ESI). The lower release efficiency of the loaded-DOX in the Dex-*g*-PAHMA micelles could be due to that the strong interactions between the DOX molecules and the Dex-*g*-PAHMA copolymers. Although the release of the loaded-DOX is relatively low, MCF-7 cells can be killed efficiently as indicated in Fig 9b. In the future, the molecular structure of the graft copolymers can be designed for promising drug carrier based on the present work.

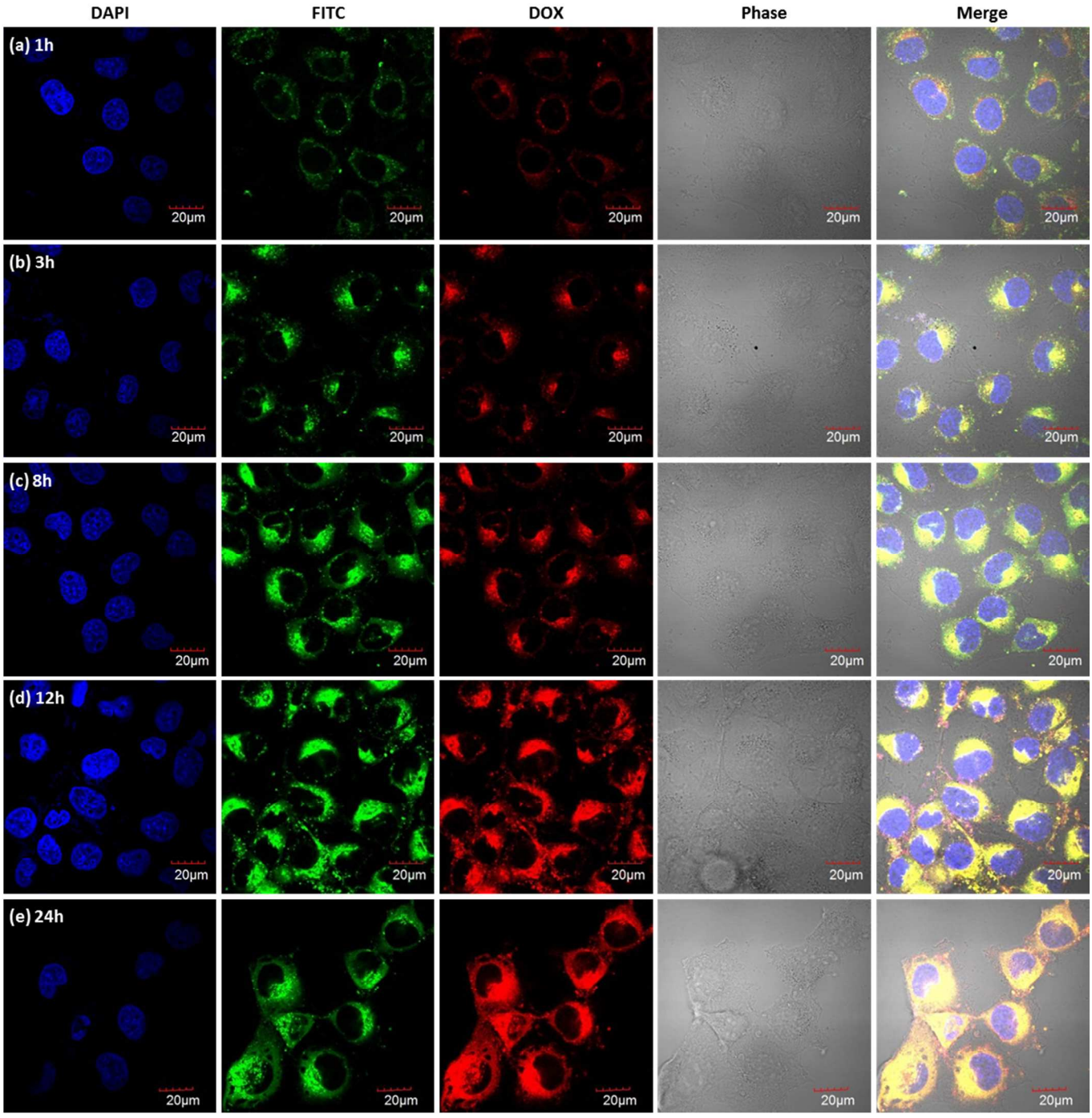



**Fig. 10** Representative CLSM images of MCF-7 cells incubated with DOX-loaded FITC-Dex-*g*-PAHMA micelles ($DS_{Am}$ = 0.513) for (a) 1 h, (b) 3 h, (c) 8 h, (d) 12 h and (e) 24 h, respectively. Cell nuclei were stained with DAPI.

## 4. Conclusion

Dex-*g*-PAHMA copolymers with different $DS_{AM}$ were successfully synthesized by free radical polymerization in aqueous solution. The graft copolymers can self-assemble into micelles with PAHMA rich core stabilized with the dextran rich shell in aqueous solution. The Dex-*g*-PAHMA micelles have the reversible $CO_2$ sensitivity due to the protonated of amidino groups on the PAHMA side chains in the presence of $CO_2$ in aqueous media. DOX can be loaded in the copolymers micelles and the drug content increased with the increasing $DS_{Am}$ of the Dex-*g*-PAHMA copolymers. The Dex-g-PAHMA copolymers have no cytotoxicity in the polymer concentration range of 5-1000 μg $mL^{-1}$. The relative cell viability was greatly reduced with the increase in copolymer concentration for the DOX-loaded micelles. The DOX-loaded micelles can be efficiently endocytosed by MCF-7 cells and the DOX release from micelles and diffuse partially into the cell nucleus. The Dex-*g*-PAHMA micelles have promising applications as drug carriers for cancer therapy.

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# Synthesis and Properties of $CO_2$-Switchable Dex-*g*-PAHMA Copolymers

Ning Che, Saina Yang, Hongliang Kang, Ruigang Liu,* Zhuang Li, Zhijing Liu, Pingping Li, Xiaozhong Qu,* and Yong Huang*

$CO_2$-Switchable Dex-*g*-PAHMA copolymers were synthesized and characterized. The properties of the graft copolymers and the cytotoxicity and cellular uptake of DOX-loaded Dex-*g*-PAHMA copolymers micelles were investigated.