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Research Paper

Doxorubicin-Loaded Polymeric Micelles Based on Amphiphilic Polyphosphazenes with Poly(*N*-isopropylacrylamide-co-*N*, *N*-dimethylacrylamide) and Ethyl Glycinate as Side Groups: Synthesis, Preparation and *In Vitro* Evaluation

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Purpose. To construct novel Doxorubicin-loaded polymeric micelles based on polyphosphazenes containing *N*-isopropylacrylamide copolymers and evaluate their various properties as well as *in vitro* anticancer effect.
Methods. These amphiphilic graft polyphosphazenes PNDGP were synthesized via thermal ring-opening polymerization and subsequent two-step substitution reaction of hydrophilic and hydrophobic side groups. Micellization behavior in an aqueous phase was confirmed by fluorescence technique, DLS and TEM. Doxorubicin (DOX) was physically loaded into micelles by dialysis or O/W emulsion method. CLSM and MTT test were applied to observe intracellular drug distribution and determine cytotoxicity of drug-loaded micelles on Hela and HepG2 cells lines, respectively.
Results. A series of PNDGPs with controlled substitution ratios were obtained. Poly(NIPAm-co-DMAA) can act as hydrophilic segments in micellular system since its LCST was over 37°C when PNIPAm was copolymerized with DMAA. The CMC value was decreased with the increase of Glyet content. In addition, more hydrophobic group content introduced into the polymer would facilitate DOX encapsulation into the micelle. DOX-loaded micelle could achieve comparative cytotoxicity as free drug via endocytosis and succedent drug release into cytoplasm of cancer cells.
Conclusions. The results suggest that these polymers might be used as potential carriers of hydrophobic anti-tumor drug for cancer therapy.

KEY WORDS: cytotoxicity; doxorubicin; micelle; polyphosphazene.

INTRODUCTION

Recently, self-assemblies such as liposome, microemulsion and polymeric micelle have attracted growing interest. Particularly, much attention is now being paid to polymeric micelles as nanocontainers for drug targeting system (1). Due to its unique core–shell structure, polymeric micelles have demonstrated longevity in the bloodstream and effective tumor accumulation after their systemic administration. Since Bader *et al*. proposed it as drug carrier in 1984, polymeric micelles have become one of the most promising modalities of drug carriers. Polymeric micelles have proven to be excellent carriers for delivering hydrophobic drugs especially antitumor drugs (2). For example, PEG-block-poly(aspartic acid) [PEG-b-P(Asp)] copolymers chemically conjugated with DOX can spontaneously form polymeric micelles with a diameter of 15–60 nm (3). This polymeric micelle can efficiently incorporate free DOX into the inner hydrophobic core, and the optimized formulation called NK911 is now being studied in a phase II clinical trial.

Polyphosphazenes are a relatively new class of polymers, quite distinct from all the other biodegradable polymers synthesized so far, due to their synthetic flexibility and versatile adaptability for applications (4). Both *in vitro* and *in vivo* studies have indicated that polyphosphazenes have tremendous potential as matrices for proteins and other drugs (5,6). Allcock group first developed amphiphilic block polyphosphazenes through the transformation of the phosphoraneimine ($Cl_3P{=}NSiMe_3$) or congeners (7). This solution-state "living" cationic polymerization allows access to polyphosphazene with controlled molecular weight and narrow polydispersity. But the demand of polymerization activity would limit the scope of available monomers. Till now there is no report about the application in drug delivery of amphiphilic polyphosphazenes synthesized by living polymerization. Song *et al*. reported a series of polyphosphazene-based copolymers bearing hydrophilic α-amino-ω-methoxy-PEG (AMPEG) and hydrophobic amino acid ester as side groups, which exhibited reversible sol–gel properties but not micellization behavior (8,9). Our attention, however, has been focusing on drug nanocarriers constructed from amphiphilic graft polyphosphazenes.

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Poly(*N*-isopropylacrylamide) (PNIPAm) is a well-known water-soluble polymer, showing reversible hydration–dehydration conversion in response to a small solution temperature change. A temperature showing the hydration–dehydration change is called the lower critical solution temperature (LCST). The LCST of PNIPAm in water is known as 32°C (10). Liu *et al.* demonstrated that by copolymerization with a more hydrophilic monomer, the LCST of PNIPAm can be improved (11). More interestingly, the LCST is regulated with the different ratio of the two monomers (12–15). In our study, NIPAm was copolymerized with *N*,*N*-dimethylacrylamide (DMAA) to obtain poly (NIPAm-co-DMAA) with a higher LCST, which can avoid the hydration–dehydration change under physiological condition (37°C). Thus the water-solubility of poly(NIPAm-co-DMAA) can be utilized as hydrophilic chains to construct micelles which should be soluble under physiological condition as drug carriers.

On the other side, poly(ethylene glycol) (PEG) was the most popular hydrophilic segments used in polymeric micellar system due to its good water-solubility and biocompatibility. However, the commercial PEG are divided into limited species with various molecular weight. Comparatively, the molecular weight of polyacrylamide derivates can be regulated by controlling the amount of chain transfer agent during polymerization. Also other properties such as hydrophilicity can be easily tuned by adding different comonomer. This point will be advantageous to design drug delivery system.

Since the characteristic structure of poly(dichlorophosphazene) provides great alternatives for the change of both hydrophilic and hydrophobic components, the final graft copolymers with special properties for drug delivery and other biomedical applications can be tailored conveniently (4,16,17). Our group proposes to synthesize a series of amphiphilic graft polyphosphazene containing poly(NIPAm-co-DMAA) by changing the ratio of hydrophilic segment to the hydrophobic segment. *In vitro* evaluation indicated that this newly synthesized copolymer can also form micelle with similar core/shell structure as that constructed from block copolymers, where hydrophobic groups form the core, while hydrophilic poly (NIPAm-co-DMAA) chains serve as corona/shell.

Herein we report the synthesis of these novel amphiphilic polyphosphazenes bearing hydrophilic poly(NIPAm-co-DMAA) and hydrophobic ethyl glycinate (Glyet) as side groups. The drug loading and other related properties were investigated in detail. In addition, *in vitro* cytotoxicity evaluation of DOX-loaded micelles were performed.

MATERIALS AND METHODS

Materials

N-Isopropylacrylamide (NIPAm) was purchased from Sigma-Aldrich and recrystallized from *n*-hexane for two times. *N*,*N*-Dimethylacrylamide (DMAA) was also obtained from Sigma-Aldrich and was purified via reduced-pressure distillation. Phosphonitrilic chloride trimer was purchased from Sigma-Aldrich and was purified via sublimation at 80–90°C. *N*, *N*-Azobis(isobutyronitrile) (AIBN) was purified by recrystallization twice in ethanol. 2-Aminoethanethiol hydrochloride (2-AET.HCl) and aluminium chloride(99%), obtained from Acros Organics, were used as received. Glycine ethyl ester hydrochloride was purchased from Alfa Aesar and was used without further purification. DOX was kindly supplied by Haikou Manfangyuan Chemical Company in China. Fetal bovine serum (FBS), RPMI-1640 medium (RPMI), trypsin–EDTA (0.5% trypsin, 5.3 mM EDTA tetra-sodium), and penicillin–streptomycin (100 U/mL) were purchased from JiNuo biotechnology Company in China. The other reagents were commercially available and used without further purification.

Polymer Synthesis

Synthesis of Amino-Terminated Poly(NIPAm-co-DMAA)

NIPAm and DMAA (molar ratio 6.5:3.5) were copolymerized in the presence of a chain initiator (AIBN) and a chain transfer agent (2-AET.HCl) to introduce an amino group at one terminal of the copolymer. The dissolution of NIPAm (20 g, 177 mmol) and DMAA (11.7 g, 118 mmol) in methanol (100 mL) preceded the addition of AIBN (0.5 g, 3.1 mmol) and 2-AET.HCl (4.2 g, 36.9 mmol). The mixed solution was degassed by bubbling with nitrogen for 30 min. The reaction mixture was refluxed for 16 h under nitrogen. The polymerization was terminated by flowing air into this reaction flask. Upon completion, the product was precipitated from diethyl ether. The product was purified by reprecipitation twice from tetrahydrofuran (THF)-diethyl ether using a slow liquid–liquid diffusion method. After dried in vacuo, the resultant white powder was obtained. The copolymer composition was determined from the peak integration ratio in ^{1}H-NMR spectra of methane protons (–NHCH(CH_3)$_2$) in NIPAm units at 4.0 ppm. to methyl protons (–N(CH_3)$_2$) in DMAA units at 3.0 ppm. The molecular weight was measured by S element analysis.

Synthesis of Amphiphilic Polyphosphazenes (PNDGPs)

The synthesis of copolymer was accompanied by the sequent substitution of side groups to the main chain. Poly (dichlorophosphazene) was synthesized by thermal ring-opening polymerization using 3.0% aluminum chloride as a catalyst and the polymer was purified by dissolving in toluene and precipitation from dry petroleum ether (18). PNDGPs were synthesized as follows. Poly(dichlorophosphazene) (0.6 g, 0.01 mol P–Cl bonds) was dissolved in THF (20 mL) first. Then poly(NIPAm-co-DMAA) (9.14 g, 2.68 mmol)/THF (50 mL) solution containing dry triethylamine (TEA, 0.42 mL, 3.0 mmol) was added dropwise and the polymer solution kept stirring magnetically at room temperature. After 24 h, an excess amount of Glyet (1.03 g, 0.01 mol) in 20 mL THF together with 1.39 mL (0.01 mol) of freshly distilled TEA was added slowly into the reaction mixture, which was stirred for further 48 h. All the reactions were carried out under a dry nitrogen atmosphere. The resultant suspension was filtered and, after the filtrate was concentrated, it was poured into diethyl ether to obtain a precipitate. The precipitate was dialyzed in distilled water for 2 days to remove the residual reactants. The dialyzed solution was freeze-dried to obtain the copolymeric product (a white powder).

Copolymer Characterization

FT-IR spectra were measured on JASCO FT/IR-4000 spectrometer. ^{1}H-NMR spectra were recorded on an Avence

DMX500 spectrometer using DMSO-d^6 as the solvent. The molecular weights of PNDGPs were determined using a size exclusion chromatograph (GPC) equipped with a Waters 515 HPLC Pump, a Waters StyragelTM HT3 GPC column (300× 7.8 mm) and a Waters 2410 refractive index detector. THF was used as solvent with a flow rate of 1.5 mL/min at 40°C and narrow disperse polystyrene as calibration standards. Copolymer composition, i.e. the molar ratio of poly(NIPAm-co-DMAA) to Glyet, was calculated according to the ^{1}H-NMR. The LCST of poly(NIPAm-co-DMAA) and copolymer solutions was measured by turbidity method. UV-visible Spectrophotometer (Beijing PuXi General Instrument Co., Beijing, China) with a TC-1 temperature control was used to trace the phase transition by monitoring the temperature dependent transmittance at 500 nm. The polymer concentration was 5 mg/mL, and the heating rate was 0.02°C/min. The critical micelle concentration (CMC) of the copolymer was determined by fluorescence technique using pyrene as probe. Fluorescence measurements were conducted using an FP-6500 fluorescence spectrophotometer with the excitation wavelength of 339 nm and emission wavelength of 390 nm.

Incorporation of DOX into Polymeric Micelles

DOX-loaded polymeric micelles were prepared by dialysis method and emulsion method, respectively. In the dialysis method, the copolymer (20 mg) was dissolved in *N*,*N*-dimethylformamide (DMF) (2 mL), and a corresponding DOX·HCl (6 mg or 8 mg) with TEA (3 mol eq. to DOX·HCl) was added into the polymer solution. The mixture was stirred at room temperature overnight. Then the final mixture was dialyzed against distilled water using a dialysis membrane (molecular weight cut off (MWCO)=12,000–14,000) for 8 h. During the first 3 h, the water was exchanged three times (every hour) and then twice during the following 5 h. In the O/W emulsion method, briefly, DOX·HCl (6 mg or 8 mg) was dispersed in chloroform ($CHCl_3$) (4 mL) in the presence of TEA (3 mol eq. to DOX·HCl) to form the oil phase. And a graft copolymer (20 mg) was dissolved in distilled water (40 mL) to be the water phase. Under magnetically stirring, the oil phase was added to the water phase dropwise. Then the mixture was stirred overnight in darkness to remove chloroform.

Characterization of DOX-Loaded Micelles

Transmission Electron Microscopy (TEM) Images

TEM images were obtained using a JEM 1230 operating at an acceleration voltage of 80 kV. Samples were prepared by dipping a TEM grid into the sample solution of 0.5 wt.% and extra solution was blotted with filter paper. The water was evaporated at room temperature for 2 h before TEM observation.

Determination of Drug Content in Polymeric Micelles

Four or five milligrams of lyophilized polymeric micelles were dissolved in 10 mL DMF. The drug content in the resultant polymeric micelles was determined via UV-visible Spectrophotometer at 483 nm. Then the absorbance (A) of DOX in DMF was introduced into the standard curve ($A=18.9\times C+0.0069R=0.9999$) to calculate the DOX concentration (C, in mg/mL).

Drug Release from Polymeric Micelles

Five milligrams of lyophilized polymeric micelles containing DOX dissolved in 1 mL 1/15 M PBS (pH 7.4, 6.5 or 5.5) was contained in dialysis bag with a MWCO of 12,000, which was placed into 20 mL PBS. At appropriate intervals, 5.0 mL was removed from the outer aqueous solution and replaced by fresh release medium. The released drug was quantified spectrophotometrically at 483 nm. The test was performed in a 37°C incubator–shaker at 100 rpm.

Cell Culture

HepG2 obtained from the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences (IBCB, Shanghai, China), were routinely cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C, 5% CO_2 and 95% humidity.

Confocal Laser Scanning Microscopy

The analysis of intracellular distribution of DOX was carried out on HepG2 cells grown on a six-well plate. The DOX content in micelle was adjusted to be equivalent to DOX solution (20 μg/mL). The drug-treated cells for 0.5 h and 4 h incubation were washed three times with PBS pH 7.4 and then the cells were fixed with parapolyformaldehyde in PBS for 0.5 h. All specimens for the detection of DOX were

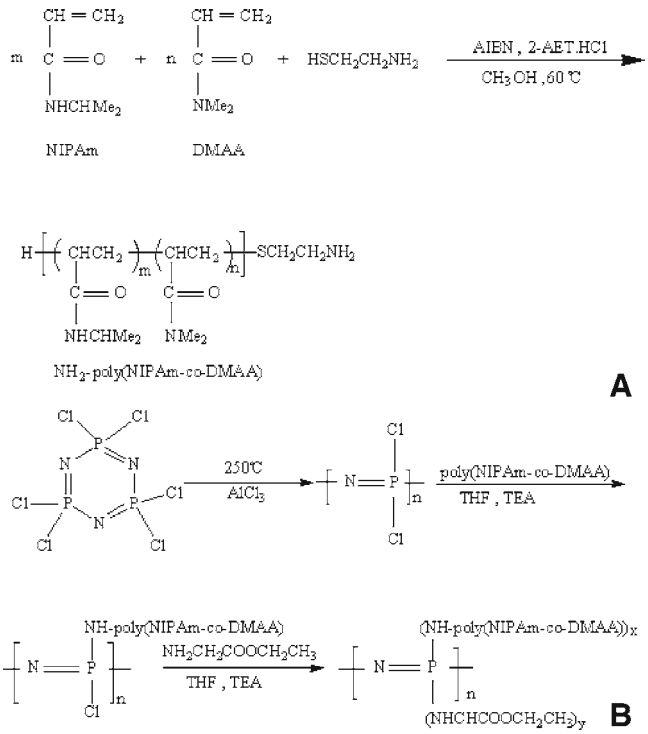


Fig. 1. **A** The synthesis route of the hydrophilic segment of NH_2 terminated poly(NIPAm-co-DMAA), **B** the synthesis route of the amphiphilic graft polyphosphazene poly(NIPAm-co-DMAA)/Glyet-PPPs (PNDGPs) after two-step substitution reaction.

Table I. Copolymer Characterization

Polymer	Molar ratio (Theor. value) x[b]	Molar ratio (Theor. value) y[c]	Molar ratio (Exper. value[a]) x	Molar ratio (Exper. value[a]) y	M_n	M_w	LCST (°C)	CMC (g/L)
Poly(NIPAm-co-DMAA)					3,400[d]		39	–[e]
PNDGP-1	1.16	0.84	1.2	0.8	12,000	19,400	38.7	0.281
PNDGP-2	0.44	1.56	0.6	1.4	10,800	18,600	38.4	0.178
PNDGP-3	0.28	1.72	0.3	1.7	10,000	20,900	38.3	0.0324

[a] Calculated by ^{1}H NMR
[b] Mole of Poly(NIPAm-co-DMAA) in PNDGP
[c] Mole of Glyet in PNDGP
[d] Determined by sulfur elemental analysis
[e] Not found

examined under a confocal microscope (Leica TCS SP, Germany) and the excitation and emission wavelengths were 488 and 560 nm, respectively.

Flow Cytometry

The analysis of intracellular uptake of DOX was carried out on HepG2 cells grown on a six-well plate. The DOX content in micelle was adjusted to be equivalent to DOX solution (20 μg/mL). The drug-treated cells for 0.5, 2 and 4 h incubation were rinsed twice with 1 mL PBS (pH 7.4) to remove any free DOX or micelles, detached with trypsin–EDTA for 1 min, then terminated by RPMI-1640 containing 10% FBS and the cell suspension was centrifuged at 1,000 rpm for 3 min. Supernatants were discarded, and the particles were re-suspended with 1 mL PBS (pH 7.4). The suspension was re-centrifuged and the particles were suspended with proper volume of PBS. The final suspension was introduced into a flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488-nm argon ion laser. The data presented are the mean fluorescent signals for 10,000 cells.

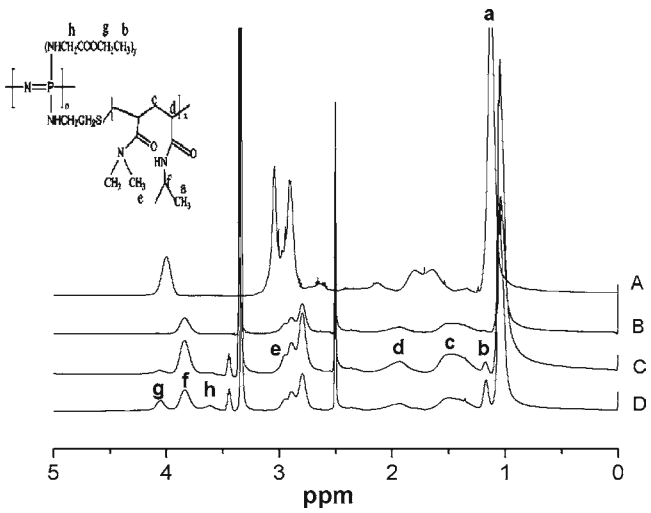


Fig. 2. ^{1}H-NMR spectra of *A* poly(NIPAm-co-DMAA) in $CDCl_3$, and poly(NIPAm-co-DMAA)-g-PPPs in DMSO-D^6: *B* PNDGP-1, *C* PNDGP-2, *D* PNDGP-3 with TMS as an internal standard at 500 MHz.

Cytotoxicity of DOX Loaded Micelles

HepG2 cells (5×10^4 cells/mL) harvested from growing cells as a monolayer were seeded in a 96-well plate in 100 μL of RPMI 1640 medium 24 h prior to the cytotoxicity test. DOX solution and DOX loaded micelles were added to the 96-well plate with different DOX concentrations (100–20,000 ng/mL), and incubated for 24 h. Chemosensitivity was assessed using tetrazolium salt MTT assay. 31.5 μL MTT PBS (pH 7.4) solution (5 mg/mL) was added to each well. The plate was incubated for an additional 4 h, and then 200 μL DMSO was added to each well. The absorbance of each well was read on a microplate reader using a test wavelength of 570 nm and a reference wavelength 630 nm.

RESULTS AND DISCUSSION

Synthesis and Characterization of PNDGPs

Polyphosphazenes can be synthesized by a number of different methods. The most widely developed way is based on the ring-opening polymerization of hexachlorocyclotriphosphazene, $(NPCl_2)_3$, to poly (dichlorophosphazene) (4). This method was employed in our research due to its easy performance and commercially available hexachlorocyclotriphosphazene, $(NPCl_2)_3$. Graft copolymers were obtained along the synthetic routes shown in Fig. 1.

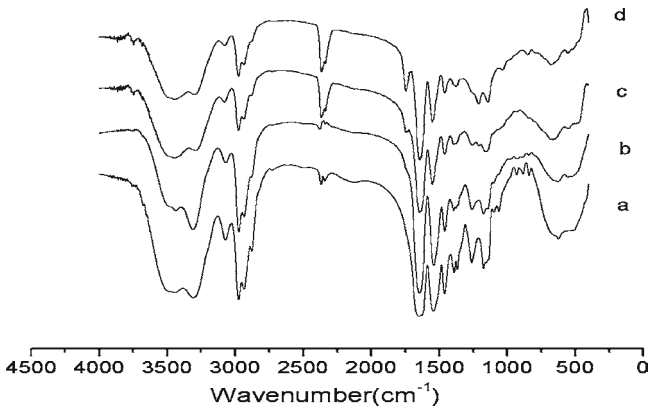


Fig. 3. FT-IR spectra of *a* poly(NIPAm-co-DMAA), *b* PNDGP-1, *c* PNDGP-2, *d* PNDGP-3.

Table II. The Effect of Preparation Methods on Actual Drug Loading Content of Micelles Based on PNDGP-2 and PNDGP-3

Polymer	DOX/polymer (*w*/*w*)	Loading content (%)	
		Dialysis method	Emulsion method
PNDGP-2	3:10	0.57	19.5
	4:10	0.80	20.7
PNDGP-3	3:10	20.5	20.7
	4:10	26.1	26.5

In this work, three water-soluble graft polymers were synthesized and their characterization is listed in Table I. Amino-terminated random copolymer, Poly(NIPAm-co-DMAA), was synthesized by radical polymerization and its M_n measured by S elemental analysis was 3,400. The formation of copolymer was validated via FT-IR and ^{1}H-NMR in d^6-DMSO. Their ^{1}H-NMR spectra are shown in Fig. 2 and the peak assignments are also present in the same figure. It can be observed that the more Glyet content in the polymer (PNDGP-1<PNDGP-2<PNDGP-3, the stronger the intensity of b, g, h peaks which were attributed to Glyet groups. The molar ratios of poly (NIPAm-co-DMAA) to Glyet calculated by ^{1}H-NMR analysis were 1.16:0.84, 0.44:1.56, 0.28:1.72 for PNDGP-1, PNDGP-2 and PNDGP-3, respectively, which agrees well with their theoretical molar ratios (1.2:0.8, 0.6:1.4 and 0.3:1.7, respectively). Also the molar ratio of NIPAm to DMAA in poly (NIPAm-co-DMAA) determined by ^{1}H-NMR analysis was 2:1, which was close to the theoretical molar ratios 6.5:3.5 of two monomers. The M_n and M_w of polymers were determined by GPC measurement and the values listed in the Table I were just as a reference since the nature of polymer used as a standard (polystyrene) was rather different from PNDGPs.

Fig. 3 shows the typical FT-IR spectra of poly (NIPAm-co-DMAA) and graft polyphophazenes with characteristic bands at 1,642 cm^{-1} (amide I) and 1,564 cm^{-1} (amide II). PNDGPs were prepared by a nucleophilic substitute reaction of amino groups and P–Cl bonds in polydichlorophosphazene backbone, and residual P–Cl bond was cosubstituted by GlyEt. Infrared spectra of the graft copolymers were consistent with the expected structure: characteristic P=N "stretching" absorptions between 1,320 and 1,100 cm^{-1} and absorption at 925 cm^{-1} due to P–N and C–N stretching vibration. The band at 1,740 cm^{-1} demonstrated the introduction of Glyet into the polyphosphazene backbone and the peak intensity was increased with the increasing content of Glyet, as the peak intensity of PNDGP-3 is stronger than that of PNDGP-2, and the peak of Glyet of PNDGP-1 cannot be seen obviously in the spectra due to the excessive amount of hydrophilic segment introduced.

Our investigation aims to eliminate the phase separation behavior of graft polyphosphazenes under normal body temperature 37°C by adding hydrophilic monomer, DMAA, copolymerized with NIPAm. The LCST was measured by turbidimetry using UV-visible Spectrophotometer. The transmittance at 500 nm was recorded as a function of temperature. The LCST was defined as the temperature with a transmittance of 50%. Table I exhibits the UV–vis light transmittance measurements of 0.5% Poly(NIPAm-co-DMAA) and PNDGPs solution in water when temperature ranging from 20°C to 40°C. The aqueous solution became cloudy near its LCST, and the polymer began to precipitate as the temperature increased above LCST. PNIPAm itself shows a temperature-induced phase transition at 32°C, and its copolymerization with DMAA increased the LCST to about 39.2°C. This relatively higher LCST ensured the application of poly(NIPAm-co-DMAA) as a hydrophilic chain to form a micelle under the normal physiological condition. It was really demonstrated that the LCSTs of aqueous PNDGPs solutions were over 37°C though they were little lower than that of poly (NIPAm-co-DMAA) which was due to the introduction of Glyet, a hydrophobic group, into the copolymer.

The critical micelle concentration (CMC) is a measurement describing the physical properties of the micelles associated with stability. Amphiphilic polymer molecules can self-assembly into an ordered structure via the hydrophobic interaction, in which a core formed by hydrophobic groups was surrounded by hydrophilic corona. Amphiphilic copolymers with low CMC are desirable, as the stability of their micelles remains unaffected under conditions of extreme dilution, like the case in the physiological environment, after administration. The most widely used way to determine the

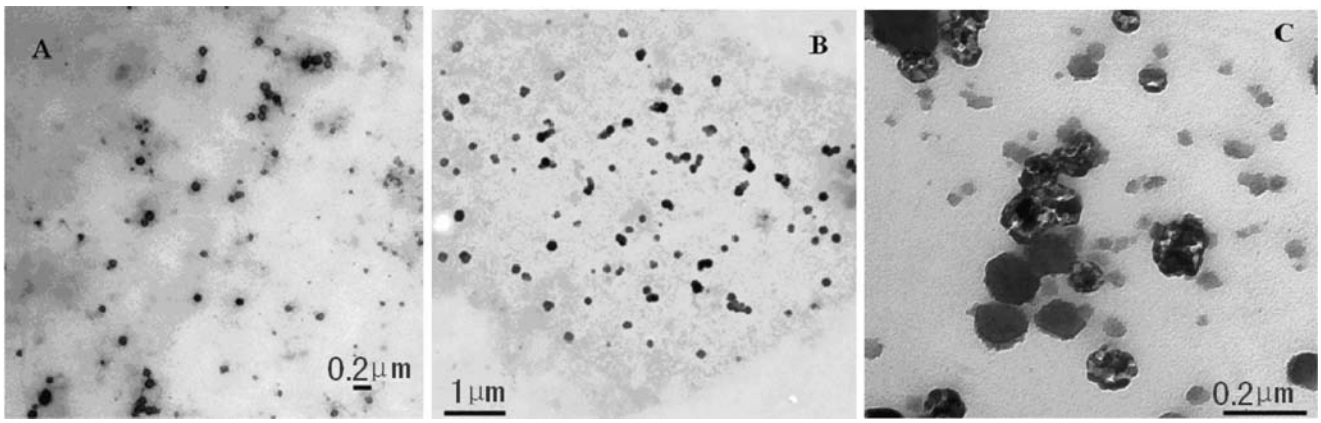


Fig. 4. TEM micrograph of DOX loaded micelles of **A** DOX-loaded micelles based on PNDGP-2 prepared by emulsion method with LC of 20.7%; **B** DOX-loaded micelles based on PNDGP-3 prepared by emulsion method with LC of 20.7%, zoom out 20,000 times; **C** the same as **B** but zoom out 120,000 times.

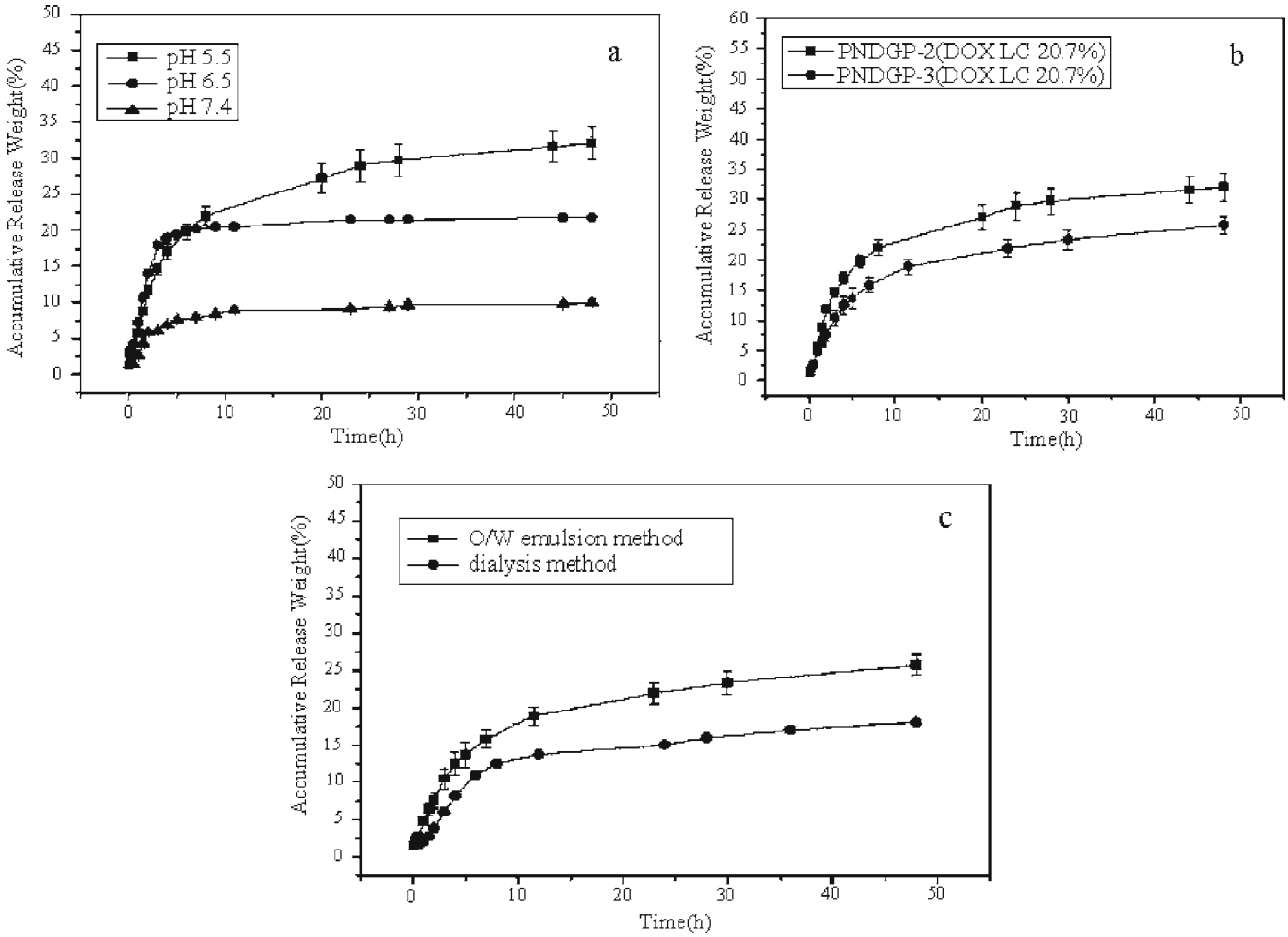


Fig. 5. **a** *In vitro* release profiles of DOX from PNDGP-2 micelles (loading content 20.7%) in PBS of different pH (5.5,6.5,7.4), **b** *in vitro* release profiles of DOX from micelles prepared by emulsion method based on different polymers (PNDGP-3 micelle with the LC 20.7% and PNDGP-2 with the LC 20.7%) in the same release medium (PBS, pH 5.5), **c** *in vitro* release profiles of DOX from micelles based on PNDGP-3 prepared by emulsion method (LC 20.7%) and dialysis method (LC 20.5%) (PBS, pH 5.5).

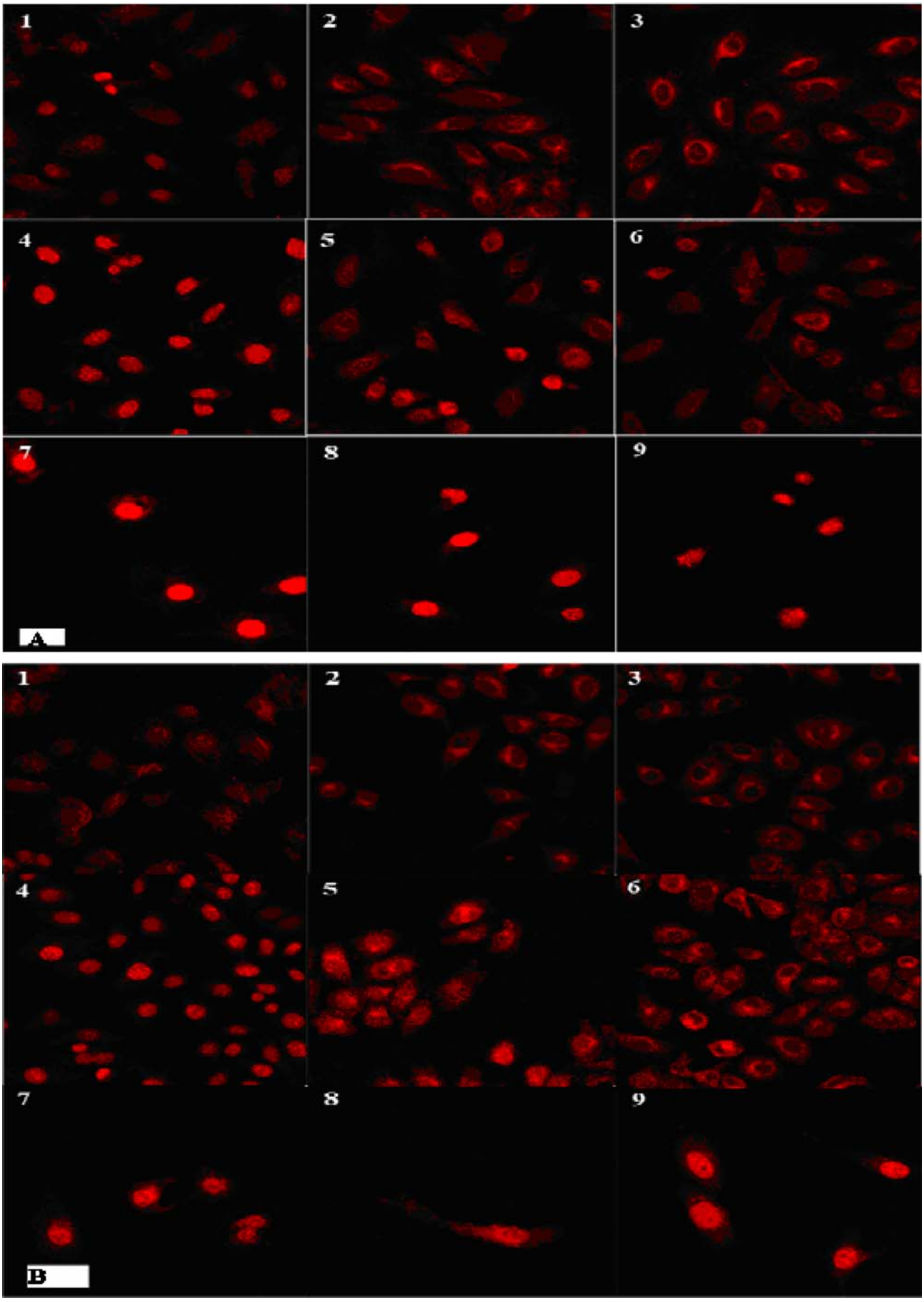
CMC of polymeric micelle is a hydrophobic fluorescence probe method that is sensitive to changes in the vicinal polarity of microenvironment (19), and therefore, it was adopted in our experiments to study the hydrophobic microenvironment of PNDGP aggregates in aqueous solution (20). The final computed CMC of PNDGP-1, PNDGP-2 and PNDGP-3 are 0.281, 0.178, and 0.0324 g/L, respectively, which increased with the increase of hydrophilic segments content in the copolymers as previously reported. Therefore it was validated that the coexistence of Glyet and poly (NIPAm-co-DMAA) in the copolymer was responsible to the micelle formation.

Characterization of DOX-Loaded Micelles

Doxorubicin is a chemotherapy drug that is given as a treatment for many different types of cancer. There are several methods explored for loading DOX into micelles such as dialysis method (21,22) and emulsion method (23–25). In our investigation DOX was physically loaded into micelles tried by dialysis method and O/W emulsion method respectively. As seen from Table II, the relatively high Loading Content (LC) could be achieved by O/W emulsion method for both PNDGP-2 and PNDGP-3. The dialysis method, however, seems inefficient for PNDGP-2 whose drug loading content was very low as 0.57% and 0.80% for 3:10 and 4:10 of DOX/polymer respectively. Compared with dialysis method, micelle formation was found relatively slower during emulsion and solvent evaporation process, which facilitated DOX encapsulation into the micelle. On the other side, PNDGP-3 bears more hydrophobic groups which can conjugate more DOX via stronger hydrophobic interaction and larger accumulation space than PNDGP-2. The detailed study will be declared in another paper.

All the DOX-loaded micelles prepared by these two methods can be reconstituted completely in distilled water and most of the micelles had an average number diameters

Fig. 6. Confocal laser scanning microscopy images of Hela cells (**A**) and HepG2 cells (**B**) after exposure of 0.5 h (*1–3*), 4 h (*4–6*) and 24 h (*7–9*), from *left* to *right* DOX solution, DOX-loaded micelles (DOX/PNDGP-2=4:10 with the loading content of 20.7%), micelles (DOX/PNDGP-3=3:10 with the loading content of 20.7%).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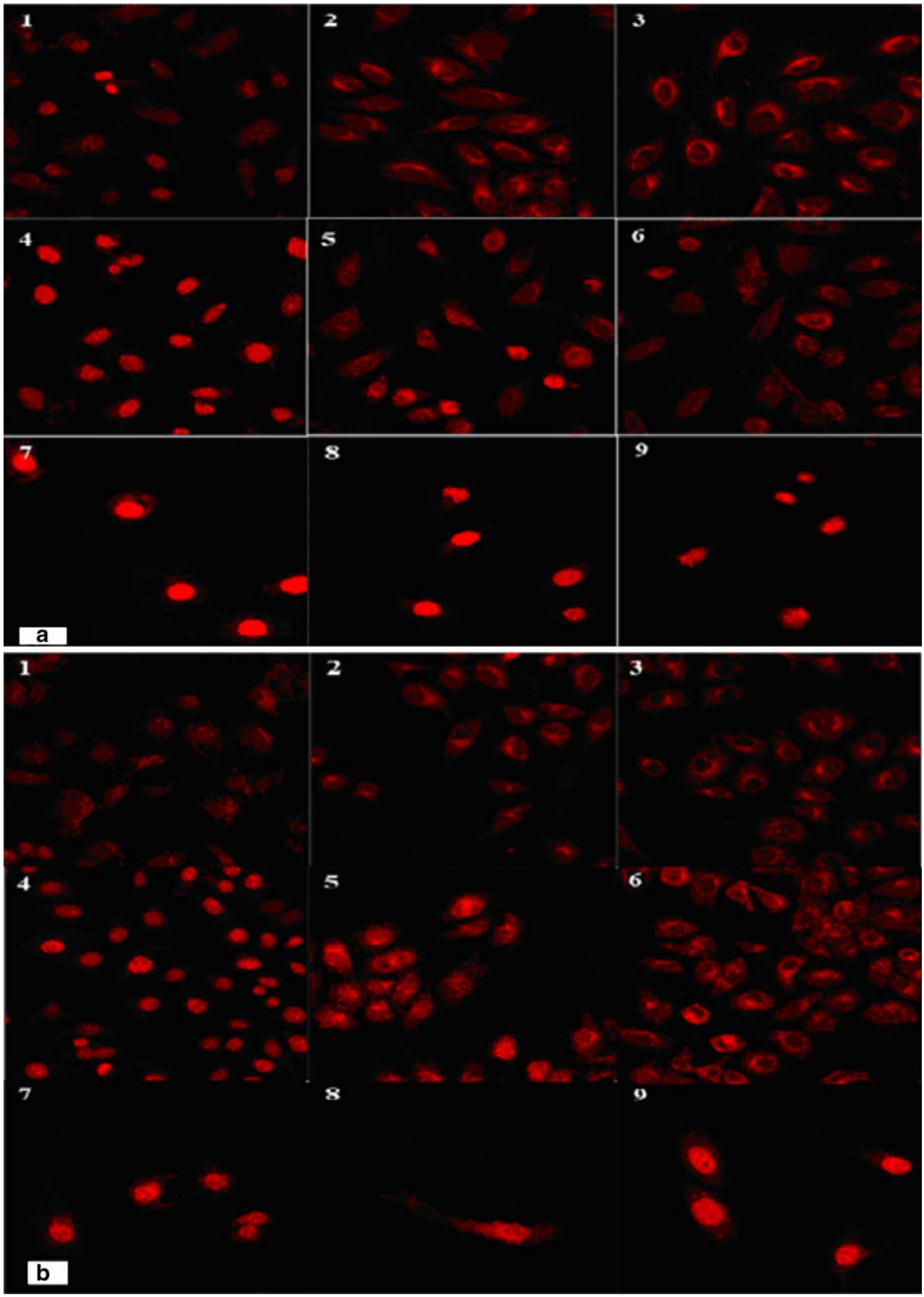
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Table III. The Values of Fluorescence Intensity After Incubation for 0.5, 2 and 4 h Against HepG2 Cells

	Intensity values	
Exposure time (h)	DOX solution	DOX-loaded micelle
0.5	28.57	22.41
2	52.17	46.81
4	94.49	71.02

under 150 nm. The morphologies of some DOX-loaded polymeric self-assemblies were displayed in Fig. 4. It can be seen that the graft copolymers mainly formed spherical micelles with an average diameter of about 100 nm.

In Vitro Drug Release

The samples used for the vitro drug release were prepared by O/W emulsion method and dialysis method. Fig. 5a shows the *in vitro* release profiles of DOX from PNDGP-2 micelles (DOX/PNDGP-2=4:10 with LC 20.7%) in PBS of different pH (5.5, 6.5, 7.4) simulating different physiological surrounding *in vivo*. The result revealed that the accumulative release weight was accelerated by decreasing pH, as can be seen, the DOX-loaded micelles had a more rapid release speed and much more release weight in pH 5.5 and 6.5 than in pH 7.4. As is known, there is a NH_2 group in the structure of DOX. With the decrease of pH, the proportion of protonated DOX increased, which thus enhanced its solubility in the outer medium (23). Accelerated drug release at weak acidic solution is considered to be an advantage in antitumor drug delivery system. Since DOX-loaded micelles were relatively stable in the blood circulation at pH 7.4 while DOX can be released at tumor tissue where local pH was reported to be lower than that of normal tissue (26). This drug release character can reduce the toxicity of antitumor drug to normal tissue and improve antitumor therapy effect. Fig. 5b exhibits the *in vitro* release profiles of DOX from micelles based on PNDGP-2 and PNDGP-3 with the same loading content (20.7%) in the same release medium (PBS, pH 5.5). It was revealed that accumulative release amount of micelles increased when decreasing hydrophobic segment content in the polymer. DOX release from hydrophobic inner core was associated with the hydrophobic force between DOX and hydrophobic segments as well as the solubility of DOX in the outer environment. The slower release in the PNDGP-3 micelles with more hydrophobic segment content can be attributed to the stronger hydrophobic interaction between hydrophobic domain and drug. Also in Fig. 5c, *in vitro* DOX release of micelles prepared by O/W emulsion method was a bit quicker than that prepared by dialysis method, which was supposed to be related to the diverse drug distribution caused by different micelle formation procedures. Further validation to this assumption was to be needed.

◀ **Fig. 7.** The fluorescence intensity of different samples after incubation for 0.5, 2 and 4 h against HepG2 cells: **a** DOX solution and **b** PNDGP-3 (DOX/PNDGP-3=3:10 prepared by emulsion method with the loading content of 20.7%).

The Cellular Uptake of DOX-Loaded Micelles

CLSM was used to study the internalization of DOX-loaded micelle into the Hela cells and HepG2 cells. As shown in Fig. 6, drug intracellular distribution of the DOX-loaded micelles is quite different from that of DOX solution. After 0.5 h of incubation with the DOX solution against two kinds of cells, strong fluorescence was observed in cell nuclei in addition to the very weak fluorescence in cytoplasm. In contrast, DOX fluorescence was observed only in cytoplasm rather than the cell nuclei at 0.5 h incubation time for both two types of cells. When two kinds of cells were exposed to DOX solution for 4 and 24 h, more intense DOX-fluorescence was observed in the nuclei, and still no strong fluorescence was detected in the cytoplasm. It is interesting to find that strong DOX fluorescence was observed in the nuclei rather than in cytoplasm when cells were incubated with DOX-loaded micelles for 4 and 24 h. However, the DOX fluorescence in the cell nuclei of DOX-loaded micelles based on PNDGP-3 was weaker than that of micelles based on PNDGP-2 at 4 h, which may be ascribed to the slower DOX release from PNDGP-3 micelles as can be seen in the Fig. 5b. However, the CLSM data (Fig. 6) indicated much faster intracellular drug diffusion from micelles than the *in vitro* release. As can be seen in Fig. 5a, a little drug (~20%) released from the micelles at 4 h in the pH 5.5 PBS, and even after 50 h about 70% of drug didn't release outside yet. In contrast, the DOX delivered by micelles could reach cell nucleus in 4 h and it had been completely concentrated in nucleus in 24 h (Fig. 6), which is similar to DOX solution. The mechanism of much faster intracellular drug diffusion from micelles than its release *in vitro* is still unclear and it needs elaborate research to illustrate the mechanism. Maybe the intracellular condition is far more complicated than that of *in vitro* release medium, for example there are dozens of enzymes in the lysosome. However, the similar result was also reported by Ko *et al.* (27). In their research, the CLSM results indicated that the DOX molecules released from the polymeric micelles were mostly localized in the nuclei after incubation for 30 min, while the *in vitro* release profile showed that the DOX released from micelles was only about 25% at 30 min and about 75% still remained in the micelles. Confocal microscopy results not only demonstrate that DOX-loaded micelle is an efficient vehicle to transport DOX into the cytoplasm, but also reveal that the internalization mechanism of micelle is different from that of DOX solution. Similar results were reported by Shuai and coworkers in the MCF-7 cell incubation with mPEG-b-PCL micelle (28). Moreover, Kataoka group reported the similar results in the SBC-3 cell incubation with PEG-p(Asp-Hyd-DOX) micelle. In their study, dot-shaped fluorescence was observed within cytoplasm and was considered to be micelles trapped in the endocytic vesicles. Based on these reports, DOX fluorescence in punctuated dot-shape from cytoplasm exhibited the characteristic for DOX-micelles uptake via endocytosis but not via diffusion, and then being localized in acidic endocytic compartments. For those micelles located inside endosomes, DOX transported with PNDGP micelles will be released in a controlled and pH-modulated manner from the micelle particles. Due to the *in vitro* DOX-release from the micelles was still a slow process even at pH 5.5 (Fig. 5b), the DOX transported by these micelles will not enter

the nucleus as quickly as the DOX solution, as indicated by the CLSM measurement. Being consistent with the CLSM results, the flow cytometry results elucidated the mechanisms further. Fig. 7 shows the DOX fluorescence intensity of DOX solution and DOX-loaded PNDGP-3 micelles after incubation for 0.5, 2 and 4 h, and Table III displays the corresponding values of fluorescence intensity. The DOX-loaded PNDGP-3 micelles showed only a bit lower cellular retention compared to DOX solution. Based on the drug release characteristic, at least 82% DOX remained in micelles at 4 h, namely little drug released from the micelles outside the cells. Therefore, it could be concluded that DOX in micelles was internalized into cells via endocytosis of micelles rather than via passive diffusion as the free DOX solution.

In Vitro Cytotoxicity of DOX-Loaded Micelles

The cytotoxicity of DOX-loaded micelles compared to that of DOX solution was determined by the cell growth inhibition assay. Fig. 8A and B shows the cytotoxicity of blank polymer against Hela cells and HepG2 cells. The results appear that PNDGP-2 and PNDGP-3 are biocompatible with Hela and HepG2 cell lines. Fig. 8C and D shows the cytotoxic effects of DOX solution and DOX-loaded micelles against Hela cells and HepG2 cells, respectively. Time-dependent cytotoxicity on Hela cells and HepG2 cells was observed for both free and micelle-encapsulated DOX. Compared to DOX solution, micelle-delivered DOX showed effective but a little less potent cytotoxicity in the Hela and HepG2 cells. The lower potency of micelle-delivery DOX can be due to a time-consuming DOX-release from micelles and delayed nuclear uptake in Hela and HepG2 cells, which are consistent with *in vitro* DOX-release and CLSM studies. However, also can be seen from Fig. 8C and D, the cytotoxicity of DOX-loaded micelles based on PNDGP-2 was a little higher than that based on PNDGP-3, and it also is consistent with the *in vitro* release and CLSM results. Generally, the advantage of polymeric micelles is mainly focused on *in vivo* circulation

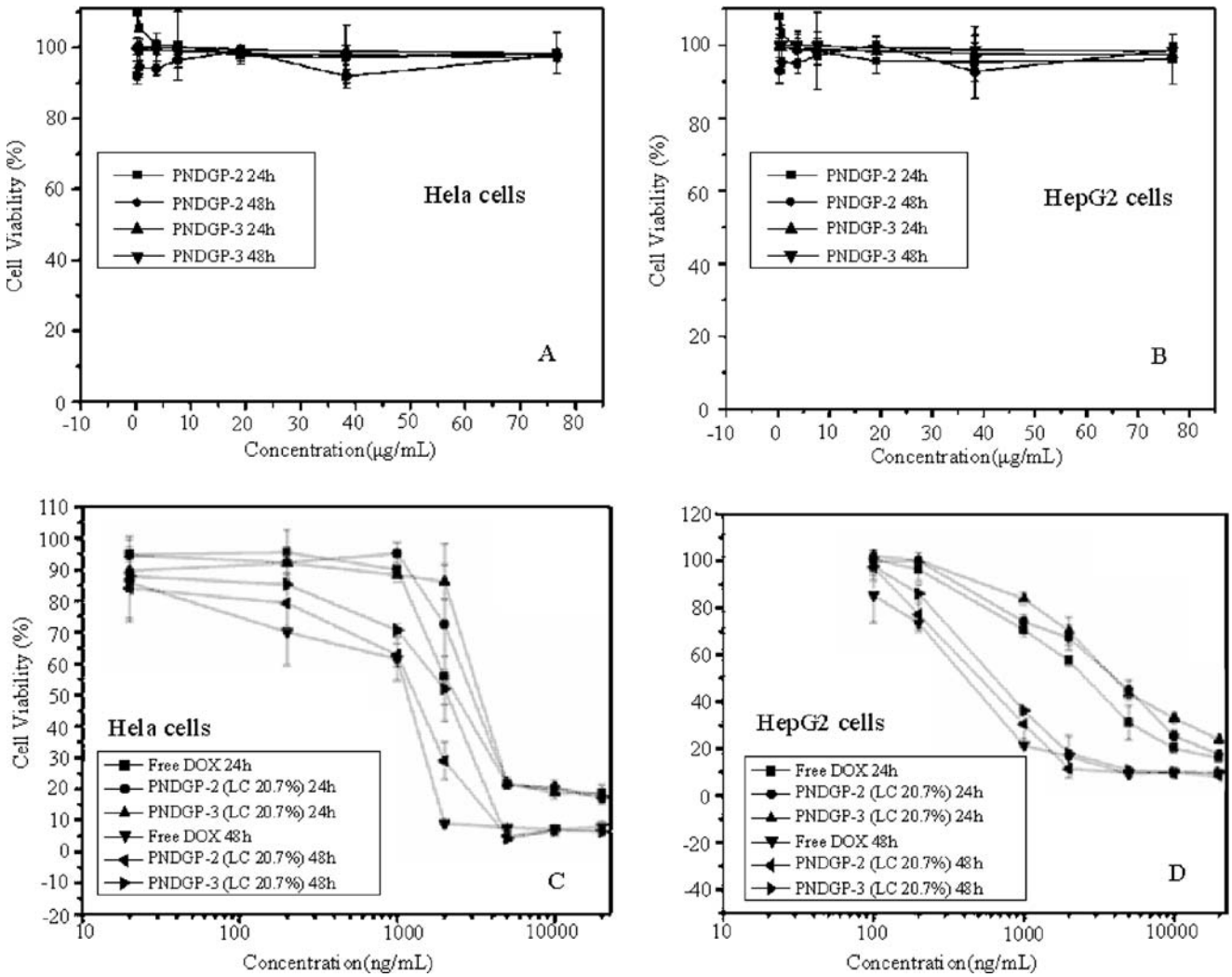

Fig. 8. The cytotoxicity of **A** PNDGP-2 and PNDGP-3 blank micelles against Hela cells, **B** PNDGP-2 and PNDGP-3 blank micelles against HepG2 cells after 24 and 48 h incubation, **C** DOX solution and DOX-loaded micelle (DOX/PNDGP-2=4:10 with the LC of 20.7% and DOX/PNDGP-3=3:10 with the LC of 20.7% prepared by emulsion method, respectively)against Hela cells, **D** the same sample as **C** against HepG2 cells after 24 and 48 h incubation.

and tumor-targeting properties. The primary *in vivo* pharmacokinetics has been carried out in mice, which demonstrated that the DOX-loaded micelles exhibited quite long circulating ability in blood. The *in vivo* evaluation on anti-tumor effect in tumor-bearing nude mice are under going. All those achievement will be published systematically elsewhere in future.

CONCLUSIONS

As NIPAm was copolymerized with DMAA at molar ratio of 6.5:3.5, poly(NIPAm-co-DMAA) became a typical thermosensitve polymer with LCST of 39.2°C. In this study, however, the water solubility of poly(NIPAm-co-DMAA) under the normal physiological condition was utilized to construct amphiphilic graft polyphosphazenes containing hydrophobic ethyl glycinate at the controlled substitution ratios, and consequently to form micelles in an aqueous solution with tunable CMC. This series of novel polyphosphazenes exhibited good biocompatibility with Hela cells and HepG2 cells, while DOX-loaded micelles could achieve comparative cytotoxicity as free drug via endocytosis and succedent drug release into cytoplasm of cancer cells. These results suggested that NIPAm copolymer with relatively high LCST has potentials to act as hydrophilic segments in micellar drug carriers. The *in vivo* evaluation of DOX-loaded micelles on tumor-bearing mice is under going.

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REFERENCES

1. N. Nishiyama, and K. Kataoka. Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. *Pharmacol. Therapeut.* 112:630–648 (2006). doi:10.1016/j.pharmthera.2006.05.006.
2. A. N. Lukyanov, and V. P. Torchilin. Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs. *Adv. Drug Deliver. Rev.* 56:1273–1289 (2004). doi:10.1016/j.addr.2003.12.004.
3. Y. Matsumura, T. Hamaguchi, T. Ura, K. Muro, Y. Yamada, Y. Shimada, K. Shirao, T. Okusaka, H. Ueno, M. Ikeda, and N. Watanabe. Phase I clinical trial and pharmacokinetic evaluation of NK911, a micelle-encapsulated doxorubicin. *Brit. J. Cancer.* 91:1775–1781 (2004). doi:10.1038/sj.bjc.6602204.
4. H. R. Allcock. Polyphosphazenes: new polymers with inorganic backbone atoms. *Science*. 193:1214–1219 (1976). doi:10.1126/science.193.4259.1214.
5. A. K. Andrianov, S. Cohen, K. B. Visscher, L. G. Payne, H. R. Allcock, and R. Langer. Controlled-release using ionotropic polyphosphazene hydrogels. *J. Control. Rel.* 27:69–77 (1993). doi:10.1016/0168-3659(93)90058-D.
6. S. M. Ibim, A. A. Ambrosio, D. Larrier, H. R. Allcock, and C. T. Laurencin. Controlled macromolecule release from poly (phosphazene) matrices. *J. Control. Rel.* 40:31–39 (1996). doi:10.1016/0168-3659(95)00136-0.
7. Y. Chang, S. C. Lee, K. T. Kim, C. Kim, S. D. Reeves, and H. R. Allcock. Synthesis and micellar characterization of an amphiphilic diblock copolyphosphazene. *Macromolecules*. 34:269–274 (2001). doi:10.1021/ma992036l.
8. B. H. Lee, Y. M. Lee, Y. S. Sohn, and S. C. Song. A thermosensitive poly (organophosphazene) Gel. *Macromolecules*. 35:3876–3879 (2002). doi:10.1021/ma012093q.
9. B. H. Lee, and S. C. Song. Synthesis and characterization of biodegradable thermosensitive poly(organophosphazene) gels. *Macromolecules.* 37:4533–4537 (2004). doi:10.1021/ma0305838.
10. H. G. Schild. Poly(*N*-isopropylacrylamide): experiment, theory and application. *Prog. Polym. Sci.* 17:163–249 (1992). doi:10.1016/0079-6700(92)90023-R.
11. H. Y. Liu, and X. X. Zhu. Lower critical solution temperatures of *N*-substituted acrylamide copolymers in aqueous solutions. *Polymer.* 40:6985–6990 (1999). doi:10.1016/S0032-3861(98)00858-1.
12. I. C. Barker, J. M. Cowie, T. N. Huckerby, D. A. Shaw, I. Soutar, and L. Swanson. Studies of the "smart" thermoresponsive behavior of copolymers of *N*-isopropylacrylami-de and *N*,*N*-dimethylacrylamide in dilute aqueous solution. *Macromolecules.* 36:7765–7770 (2003). doi:10.1021/ma034250m.
13. F. Kohori, K. Sakai, T. Aoyagi, M. Yokoyama, M. Yamato, Y. Sakurai, and T. Okano. Control of adriamycin cytotoxic activity using thermally responsive polymeric micelles composed of poly (*N*-isopropylacrylamide-co-*N*,*N*-dimethylacrylamide)-b-poly(d,l-lactide). *Colloid. Surface. B.* 16:195–205 (1999). doi:10.1016/S0927-7765(99)00070-3.
14. M. Nakayama, T. Okano, T. Miyazaki, F. Kohori, K. Sakai, and M. Yokoyama. Molecular design of biodegradable polymeric micelles for temperature-responsive drug release. *J. Control. Rel.* 115:46–56 (2006). doi:10.1016/j.jconrel.2006.07.007.
15. X. M. Liu, Y. Y. Yang, and K. W. Leong. Thermally responsive polymeric micellar nano-particles self-assembled from cholesteryl end-capped random poly (*N*-isopropylacrylami-de-co-*N*,*N*-dimethylacrylamide): synthesis, temperature-sensitivity, and morphologies. *J. Colloid Interf. Sci.* 266:295–303 (2003). doi:10.1016/S0021-9797(03)00691-X.
16. R. X. Zhang, X. J. Li, L. Y. Qiu, X. H. Li, M. Q. Yan, Y. Jin, and K. J. Zhu. Indomethacin-loaded polymeric nanocarriers based on amphiphilic polyphosphazenes with poly (*N*-isopropylacrylamide) and ethyl tryptophan as side groups: preparation, *in vitro* and *in vivo* evaluation. *J. Control. Rel.* 116:322–329 (2006). doi:10.1016/j.jconrel.2006.09.013.
17. J. X. Zhang, L. Y. Qiu, X. L. Wu, Y. Jin, and K. J. Zhu. Temperature-triggered nanosphere formation through self-assembly of amphiphilic polyphosphazene. *Macromol. Chem. Phys.* 207:1289–1296 (2006). doi:10.1002/macp.200600139.
18. L. Y. Qiu, and K. J. Zhu. Novel biodegradable polyphosphazenes containing glycine ethyl ester and benzyl ester of amino acethydroxamic acid as cosubstituents: syntheses, characterization, and degradation properties. *J. Appl. Polym. Sci.* 77:2987–2995 (2000). doi:10.1002/1097-4628(20000923)77:13<2987::AID-APP24>3.0.CO;2-F.
19. K. C. Dowling, and J. K. Thomas. A novel micellar synthesis and photophysical characterization of water-soluble acrylamide-styrene block copolymers. *Macromolecules*. 23:1059–1064 (1990). doi:10.1021/ma00206a025.
20. M. Wilhelm, C. L. Zhao, Y. C. Wang, R. L. Xu, M. A. Winnik, J. L. Mura, G. Riess, and M. D. Croucher. Poly(styrene-ethylene oxide) block copolymer micelle formation in water a fluorescence probe study. *Macromolecules*. 24:1033–1040 (1991). doi:10.1021/ma00005a010.
21. F. Kohori, M. Yokoyama, K. Sakai, and T. Okano. Process design for efficient and controlled drug incorporation into polymeric micelle carrier systems. *J. Control. Rel.* 78:155–163 (2002). doi:10.1016/S0168-3659(01)00492-8.
22. S. Q. Liu, Y. W. Tong, and Y. Y. Yang. Incorporation and *in vitro* release of doxorubicin in thermally sensitive micelles made from poly(*N*-isopropylacrylamide-co-*N*,*N*-dimethylacrylamide)-b-poly (d,l-lactide-co-glycolide) with varying compositions. *Biomaterials*. 26:5064–5074 (2005). doi:10.1016/j.biomaterials.2005.01.030.
23. K. Kataoka, T. Matsumoto, M. Yokoyama, T. Okano, Y. Sakurai, S. Fukushima, K. Okamoto, and G. S. Kwon. Doxorubicin-loaded poly(ethylene glycol)-poly(beta-benzyl-l-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance. *J. Control. Rel.* 64:143–153 (2000). doi:10.1016/S0168-3659(99)00133-9.
24. G. Kwon, M. Naito, M. Yokoyama, T. Okano, Y. Sakurai, and K. Kataoka. Block copolymer micelles for drug delivery: loading and release of doxorubicin. *J. Control. Rel.* 48:195–201 (1997). doi:10.1016/S0168-3659(97)00039-4.

25. S. Cammas-Marion, and P. Guerin. Design of malolactonic acid esters with a large spectrum of specified pendant groups in the engineering of biofunctional and hydrolyzable polyesters. *Macromol. Symp.* 153:167–186 (2000). doi:10.1002/1521-3900(200003)153:1<167::AID-MASY167>3.0.CO;2-Q.
26. K. Ulbrich, T. Etrych, P. Chytil, M. Jelinkova, and B. Rihova. HPMA copolymers with pH-controlled release of doxorubicin—*in vitro* cytotoxicity and *in vivo* antitumor activity. J. Control. Rel. 87:33–47 (2003). doi:10.1016/S0168-3659(02)00348-6.
27. J. Ko, K. Park, Y. Kim, M. S. Kim, J. K. Han, K. Kim, R. W. Park, I.S. Kim, H. K. Song, D. S. Lee, and I. C. Kwon. Tumoral acidic extracellular pH targeting of pH-responsive MPEG-poly(β-amino ester) block copolymer micelles for cancer therapy. J. Control. Rel. 123:109–115 (2007). doi:10.1016/j.jconrel.2007.07.012.
28. X. Shuai, H. Ai, N. Nasongkla, S. Kim, and J. Gao. Micellar carriers based on block copolymers of poly(-caprolactone) and poly(ethylene glycol) for doxorubicin delivery. J. *Control. Rel.* 98:415–426 (2004). doi:10.1016/j.jconrel.2004.06.003.