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Polypeptide dendrimers: Self-assembly and drug delivery

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Amphiphilic dendritic poly(glutamic acid)-*b*-polyphenylalanine copolymers were synthesized using generation 3 dendritic poly(glutamic acid) as the macroinitiator in the ring-opening polymerization of NCA-Phe. The block copolymers self-assembled micelles with polyphenylalanine segments as core and dendritic poly(glutamic acid) segments as shell. The biocompatibility of the micelles was studied. The release of the anticancer drug doxorubicin from the micelles was investigated *in vitro*. The results showed that the sustaining release of the drug could last for 60 h. The micellar drug release system was efficient in inhibiting the proliferation of HepG2 liver cancer cells, 75% cancer cells were killed under appropriate *in vitro* incubation.

self-assembly, dendritic polypeptide block copolymer, drug delivery

1 Introduction

Molecular self-assembly is becoming a widely adopted approach in biomaterials research for constructing functional materials and improving their performances [1–3]. Synthetic polypeptides are important polymeric materials that have inherent biocompatibility. They can mimic the conformational structures of natural proteins and impose specific properties and functions through selective self-assembly processes. Weak physical interactions within polypeptide chains can readily lead to different morphologies such as spherical, short rod-shaped, worm-shaped and vesicular [4–7]. Exploitations of their special functions have been extensively pursued in physics, chemistry and biology [4, 8, 9]. Synthetic polypeptides derived from natural amino acids become important biomedical materials for drug delivery and tissue repair due to their excellent biocompatibility and biodegradability. Polypeptides self-assembled drug/gene carriers were easily modified with targeting and stimuli-sensitive functions [10–12]. Self-assembly polypeptides hydrogels have been reported to perform effects on tissue regeneration [13–15].

Dendritic polypeptides contain unique properties including protein-like structure, water solubility, regular structure, a large amount of branches and peripheral functional groups, nano-scaled size and unitary molecular weights. In comparison with amphiphilic linear copolymer micelles, the dendritic copolymer micelles have better stability and also have advantages in fabricating targeting and stimuli-sensitivity with the functionalization of a large amount of peripheral groups. For instance, phase behavior of dendritic block copolymers is significantly different from the linear block copolymers [17]. With the functionalization of the peripheral groups, the solubility of dendritic block copolymers was improved, thus led to the improvement of the physical and chemical properties of their aggregates [18]. The worm-like nanoparticles were fabricated by self-assembled dendron/linear/dendron triblock copolymers and used as vectors for drug delivery [19].

In this paper, a novel self-assembly micellar system containing a dendritic polypeptide is reported. Generation 3 dendritic poly(glutamic acid) was synthesized by a convergent-divergent method [20]. It was used to initiate the ring-

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opening polymerization of L-phenylalanine *N*-carboxyanhydride (NCA-Phe) to obtain amphiphilic poly(glutamic acid)-polyphenylalanine block copolymers [21]. The dendron-linear block copolymers self-assembled into core-shell structured micelles. Anticancer drug doxorubicin was loaded in the micelles. The self-assembly of the dendron-linear block copolymers was investigated by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and dynamic light scattering (DLS). The drug release was studied *in vitro*. Confocal laser scanning microscopy (CLSM) was used to investigate the cellular uptake of the micelles in HepG2 cells and the *in vitro* anticancer effect was evaluated by CCK-8 assays.

2 Experimental

2.1 Materials

Protected amino acids Boc-Glu-COOH, *H*-Glu(OBzl)-OBzl and *N*-Cbz-Phe were purchased from GL Biochem Ltd. (Shanghai). 1-Hydroxybenzotriazole hydrate (HOBt) and 1-ethyl-3-[3-(dimethylamino) propyl] carbodiimide (EDC) were purchased from GL Biochem Ltd. (Shanghai) and used as received. *N,N*-Diisopropylethylamine (DIEA) and trifluoroacetic acid (TFA) were purchased from Asta Tech Pharmaceutical (Chengdu, China). Doxorubicin hydrochloride was obtained from Zhejiang Hisun Pharmaceutical. Methylene chloride (DCM), tetrahydrofuran (THF), dimethyl formamide (DMF), ethyl acetate and *n*-hexane were purified before use. Dulbecco's modified Eagle's medium (DMEM) was used for cytotoxicity test.

2.2 Instruments

^{1}H NMR spectra were performed on a Bruker Avance II NMR spectrometer at 400 MHz with $CDCl_3$ and DMSO-d_6 as solvents. FTIR spectra were recorded on a FTIR PE Spectrometer. Time of flight mass spectrometry Bruker Autoflex III was used to determine the molecular weight of the polymers on the substrate of DHB. Dynamic light scattering (DLS) experiments were performed on a Malvern Zetasizer Nano ZS at 25 °C. The absorbance of doxorubicin was detected by fluorescence spectrum. The morphologies of the particles were observed on scanning electron microscopy (JSM-5900LV) and Transmission Electron Microscope (JEM-100CX). The cellular uptake was observed by confocal laser scanning microscopy (Leica TCP SP5), and doxorubicin was excited at 488 nm with emissions at 595 nm.

2.3 The synthesis of generation 3 dendritic poly(glutamic acid)

Synthesis of generation 2 dendritic poly(glutamic acid)

Boc-Glu-COOH and excess H-Glu (OBzl)-OBzl were dissolved in CH_2Cl_2 with HOBT/EDC in N_2 atmosphere. DIEA was dropwise added and the condensation reaction was lasted for 24 h. The mixture was washed with aqueous solutions of $NaHCO_3$, $NaHSO_4$ and NaCl for several times.

The organic phase was dried with $MgSO_4$ for 12 h. After concentration, the product was recrystallized in CH_2Cl_2/hexane. Compound **1** in Scheme 1 was filtrated and dried.

Synthesis of generation 3 dendritic poly(glutamic acid)

The *tert*-butoxycarbonyl of generation 2 dendritic poly (glutamic acid) was treated with TFA, and the mixture was stirred for 6 h to remove *tert*-butoxycarbonyl groups. The solvent and TFA were removed in vacuum. The rest of the product was treated with anhydrous diethyl ether. The solid product was precipitated (product **2**, Scheme 1). After adding excess of Boc-Glu-COOH to repeat step 2.3.1, generation 3 dendritic poly(glutamic acid) was obtained. Generation 3 dendritic poly(glutamic acid) (product **3**, Scheme 1) was purified by silica gel column.

2.4 Amphiphilic dendritic poly(glutamic acid)-*b*-polyphenylalanine copolymer

Preparation of macroinitiator G3-Glu-NH_2

Generation 3 dendritic poly(glutamic acid) was deprotected with TFA in N_2 atmosphere to obtain product **4** (Scheme 1). The pH value was adjusted by NaOH methanol solution, the mixture was dried under reduced pressure. The left solid was dissolved in CH_2Cl_2 and stirred for 1 h, then filtered the solid out. The macroinitiator was received after the removal of solvent.

Synthesis of L-Phenylalanine N-carboxyanhydride (NCA-Phe)

The anhydrous CBZ-phenylalanine (Cbz-Phe) was dissolved in anhydrous THF at 40 °C with the protection of nitrogen. Then $SOCl_2$ was distilled into the reaction system and the mixture was stirred for 4 h. Excess anhydrous hexane was added into the solution, the mixture was kept at 40 °C for 12 h. White precipitate was obtained after filtration. The precipitate was dissolved in anhydrous ethyl acetate and recrystallized with hexane to get needle-like crystals.

Synthesis of dendritic poly(glutamic acid)-b-polyphenylalanine copolymers

Dry macroinitiator and L-Phe-NCA with the molar ratio of 1:10 were accurately weighted and added into reaction flask. After the removal of atmosphere, the flask was purged with nitrogen. DCM/DMF mixed solvents were added at room temperature and Product **5** in Scheme 1 was generated after 24 h. The protected OBzl groups were removed in the presence of Pd/C and hydrogen under 0.8 MPa.

2.5 Determination of critical micelle concentration (CMC)

Critical micelle concentration of the dendritic block co-

polymer was determined by fluorescence using pyrene as probe. The fluorescence excitation wavelength was fixed at 490 nm, and the fluorescence absorbance values of I_{338} and I_{335} were tested for the calculation of critical micelle concentration.

2.6 *In vitro* drug release

The dendritic block copolymer and doxorubicin were dissolved in methanol. Deionized water was dropwise added into the solution under ultrasonic condition to prepare drug loaded micelles. Methanol was removed by reduced pressure. The micelles were dialyzed to remove the free drug at 4 °C for 24 h, the doxorubicin-loaded self-assembled micelles were obtained after freeze-dry.

The doxorubicin-loaded micelles were accurately weighed and dissolved in organic solvent. According to the standard absorption curve of free doxorubicin, the drug loading was calculated by the determination of absorption values at the fluorescence wavelength of 485 nm.

The doxorubicin-loaded micelles were weighed and dissolved in phosphate buffer (pH 7.4). 1 mL of doxorubicin-loaded micelle solution was added into dialysis tubes (MWCO 2000). The tubes were submerged in 25 mL phosphate buffer at 37 °C and shaken. At given time intervals, 1 mL of outside buffer solution was taken for analysis, and fresh medium of 1 mL was supplemented. The absorption at 485 nm was investigated.

2.7 Cytotoxicity test

Cell culture

NIH/3T3 cells (a mouse embryonic fibroblast cell line) and HepG2 (a human hepatocellular carcinoma cell line) were cultivated in DMEM (Dulbecco's modified Eagle's medium, Invitrogen) supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum (FBS, Hyclone) and 1% (*v/v*) penicillin-streptomycin solution at 37 °C in 5% CO_2.

Cytotoxicity assays

The relative cytotoxicity of micelles was evaluated by a cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). NIH/3T3 cells were seeded in three 96-well plates for 24 h before experiment (plate 1 with a cell density of 1×10^4 cells per well for 12 h test, plate 2 with a cell density of 5×10^3 cells per well for 24 h test, and plate 3 with a cell density of 3×10^3 cells per well for 48 h test). The medium was removed and replaced by the pre-prepared growth media containing the final micellar concentrations of 150, 100 and 50 μg/mL, respectively. After incubated for 12, 24 and 48 h, the cells were washed by PBS buffer and replaced by fresh media containing 10 μL CCK-8. The plates were incubated at 37 °C for an additional 2 h. The absorbance was measured at the wavelength of 492 nm using a microplate reader 550 (Bio-Rad). both live and dead cells were imaged by a Leica TCP SP5 fluorescence scope.

2.8 Cellular uptake of micelles

The cellular uptake experiments in human hepatocellular carcinoma cells HepG2 were performed using confocal laser scanning microscope (CLSM). The cells were seeded in 35 mm glass culture dishes at a density of 1×10^4 cells/well and adhered for 24 h. The culture medium was removed. Free Dox and Dox-loaded micellar solutions (Dox concentration: 10 μg/mL) were added together with culture medium. After incubated at 37 °C for 2 h, the culture medium was carefully removed and the cells were washed with PBS for three times. CLSM images were observed with inverted CLSM (Leica TCP SP5, Germany). Dox was excited at 485 nm with emissions at 595 nm.

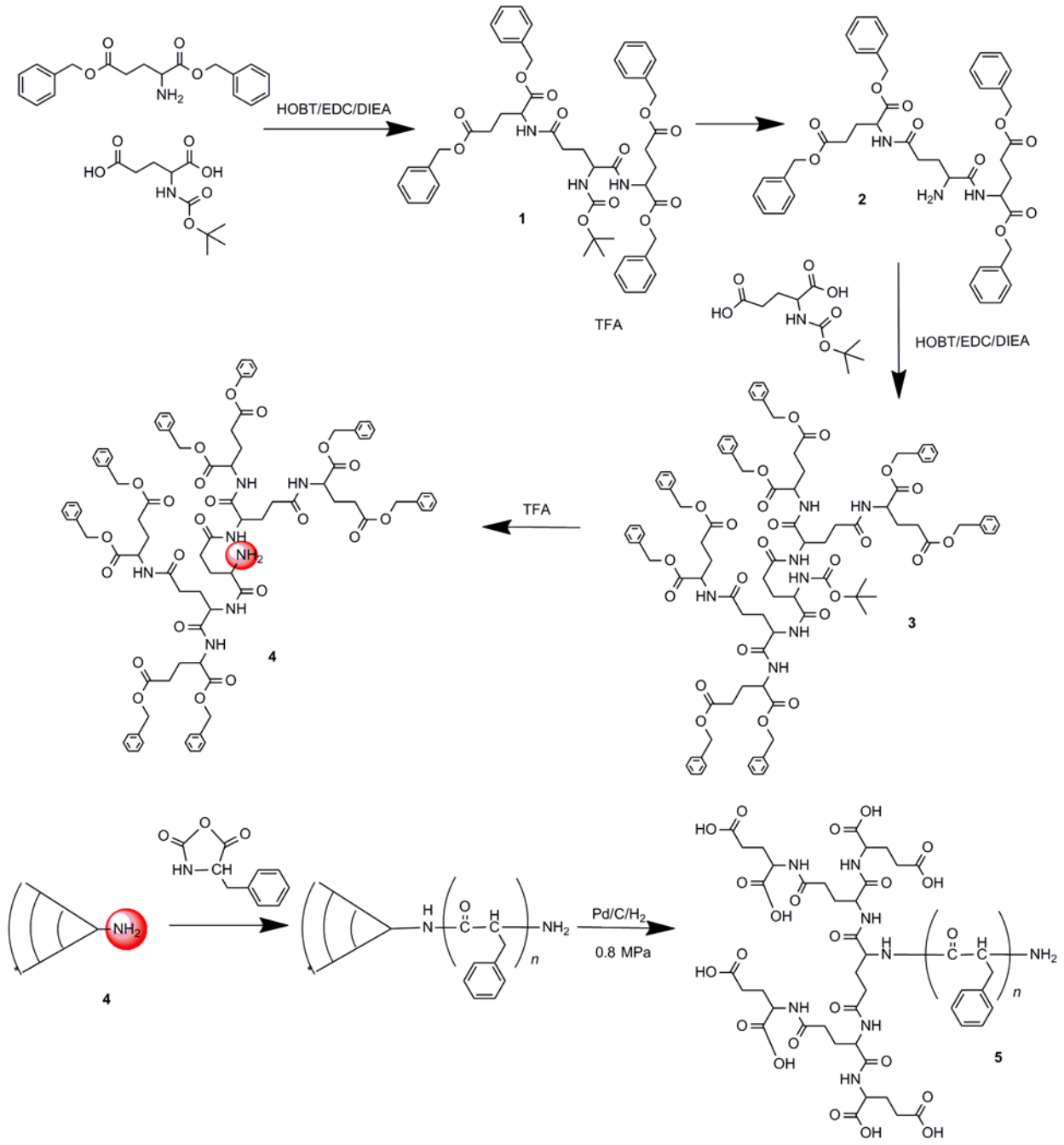
2.9 *In vitro* cytotoxicity

Cytotoxicity test was used to evaluate *in vitro* antitumor activity of the drug loaded micelles. The cytotoxicity of the drug loaded micelles was measured by CCK-8 assay kit using Dox·HCl and Dox as controls. Briefly, HepG2 cells harvested in a logarithmic growth phase were seeded in three 96-well plates (plate 1 with a concentration of 1×10^4 cells per well for 12 h test, plate 2 with a concentration of 5×10^3 cells per well for 24 h test, and plate 3 with a concentration of 3×10^3 cells per well for 48 h test). After being incubated for 24 h, the medium was removed and free medium was added with drug loaded micelles. The Dox concentrations in the wells were from 5 to 15 μg/mL. At the prescribed time intervals (12, 48 and 72 h), the cells were washed by PBS solution for three times, and replaced with fresh culture media, 10 μL of CCK-8 kit was added and the plate was incubated for another 2 h at 37 °C. Finally, the plates were shaken for 10 min, and the absorbance of the product was measured at 492 nm by a microplate reader.

3 Results and discussion

The synthesis of dendritic poly(glutamic acid)-*b*-polyphenylalanine copolymers is shown in Scheme 1. Generation 3 dendritic poly(glutamic acid) was further used as a macroinitiator to initiate the ROP of NCA-Phe to produce dendron-linear block copolymers. The synthesis of monomers and polymerization were carried out under rigorously anhydrous conditions to avoid side reactions.

The key products in the synthetic process were analyzed using ^{1}H NMR (400 MHz). As shown in Figure 1(a), the integral ratio of NCA-Phe ($CDCl_3$) proved that the product was obtained. In infrared spectrum, there are the characteristic absorptions of the NCA-Phe anhydride structure appearing at the 1856 and 1776 cm^{-1}, which are the further evidence of the NCA-Phe. In Figure 1(b), the peaks from



Scheme 1 Synthesis of dendritic poly(glutamic acid)-polyphenylalanine block copolymers.

1.75 to 2.75 ppm are the protons in the skeleton of G3-Glu (DMSO-d_6 as solvent); the chemical shifts at 5.1 ppm and the peaks from 7.2 to 7.4 ppm are attributed to the characteristic peaks of benzyl protection. According to Figure 1(c), the integral ratio and position of the protons indicated that the dendritic poly(glutamic acid)-*b*-polyphenylalanine copolymers were successfully synthesized. Infrared spectroscopy showed that after the hydrophilic segment put off after benzyl protection, the absorption of –OH in carboxyl group from 3550 to 3500 cm^{-1} was significantly enhanced. Meanwhile, the time of flight mass spectrometry results indicated that the strongest signal at *m/z* 2409.23 was true of the designed molecular weight.

The self-assembly of the dendritic poly(glutamic acid)-*b*-polyphenylalanine copolymer micelles is shown in Scheme 2. The segment of dendritic poly(glutamic acid) was hydrophilic due to the large amount of peripheral carboxyl groups, polyphenylalanine segment was hydrophobic [22]. Hydrophobic doxorubicin was loaded in the core of micelles.

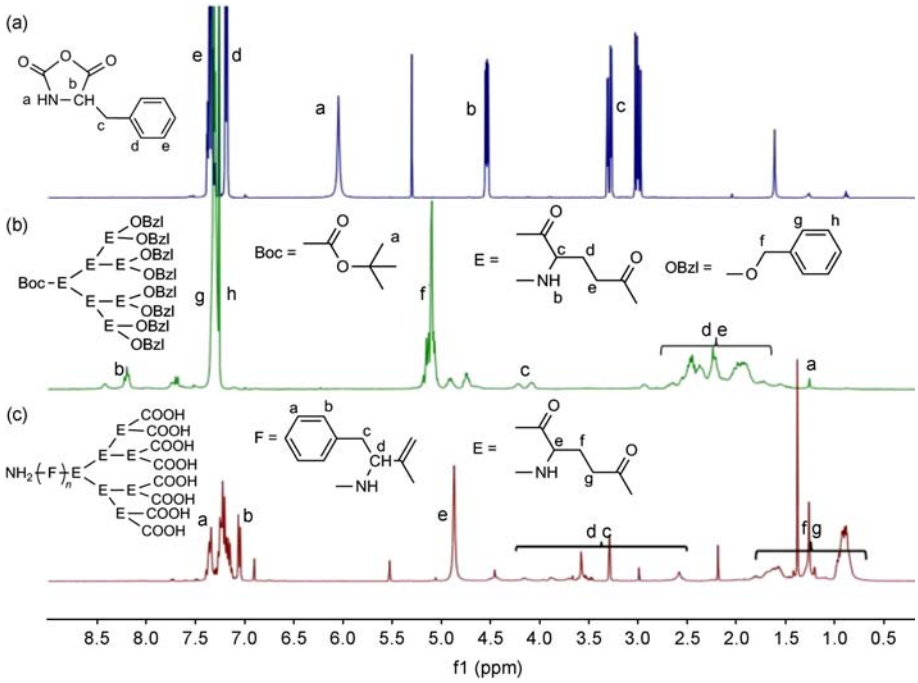
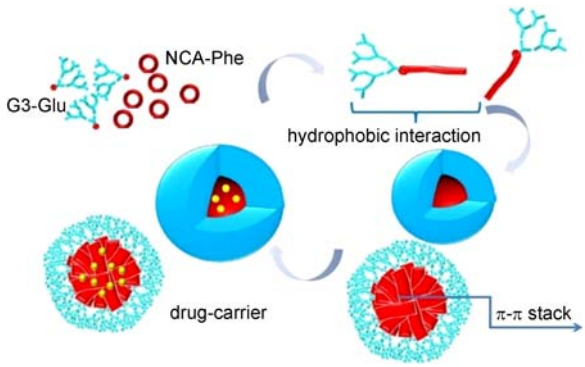


Figure 1 ^{1}H NMR spectrum of NCA-Phe (a), dendron-G3-Glu (b), dendron-linear block copolymers (c).



Scheme 2 Self-assembly of dendritic poly(glutamic acid)-*b*-polyphenylalanine copolymers and drug loaded.

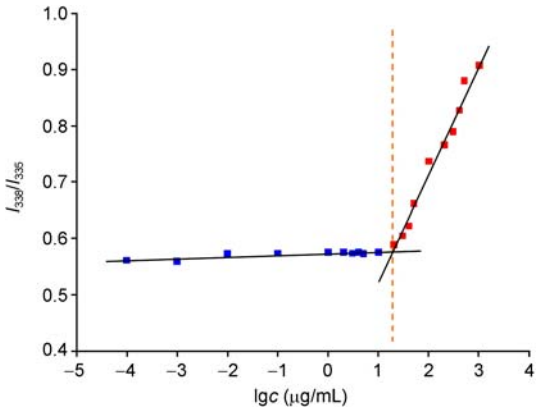


Figure 2 Critical micelle concentration of dendron-linear block copolymers.

The block copolymers self-assembled into core-shell structured micelles. The critical micelle concentration (CMC) is an important parameter to characterize the micellization. The CMC shows the critical concentration at which the amphiphilic block copolymers assembled spontaneously. The CMC of the micelles was tested using pyrene as a fluorescent probe. Figure 2 shows the relationship between the fluorescence intensity ratios (I_{338}/I_{335}) and the block copolymer concentrations. The intersection value of the two trend lines corresponded to the CMC, which was 19.50 μg/mL.

The size distributions of the blank and doxorubicin-loaded micelles were investigated by dynamic light scattering (DLS). Figure 3 illustrated the size distributions of both blank and drug loaded micelles, the concentration of the micelles was 50 μg/mL. The polydispersity indexes of blank and doxorubicin-loaded micelles were 0.320 and 0.394, respectively. Interestingly, the mean diameter of the drug loaded micelles was comparable to that of blank micelles. A possible reason was that the hydrophobic and π-π stacking effects between doxorubicin and polyphenylalanine in drug loaded micelles compressed the size of the micelles, which led to it comparable to that of blank micelles.

TEM was employed to investigate the structure of both blank and drug loaded micelles. As shown in Figure 4(a) and 4(b), there was little discrepancy in the morphology between blank and drug loaded micelles, the drug loaded micelles were more explicit in core-shell structure. The possible reason was that the doxorubicin enhanced the hydrophobic effect of micelles due to the additional conjugation effect between polyphenylalanine and doxorubicin [22, 23]. Figure 4(c) is the SEM micrograph of blank micelles, the

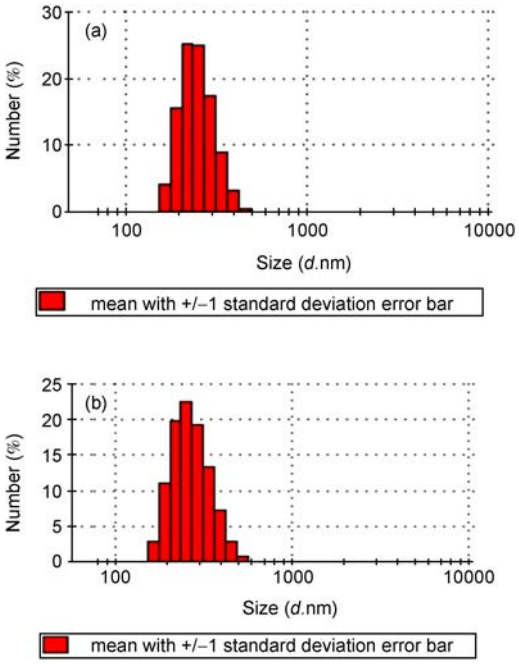


Figure 3 Distributions of micellar sizes. (a) Self-assembly of dendron-linear block copolymer; (b) drug loaded nanoparticles.

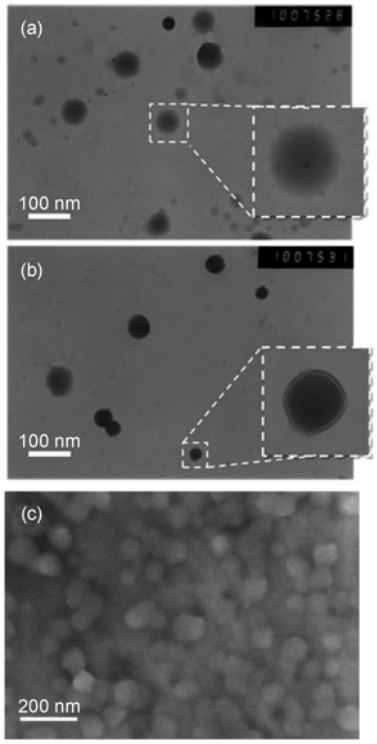


Figure 4 (a) TEM micrographs of 100 μg/mL self-assembly systems; (b) TEM micrographs of drug loaded nanoparticles; (c) SEM micrographs of 100 μg/mL dendron-linear block copolymers self-assembly systems.

concentration of the block copolymers was 100 μg/mL. The micelles were monodisperse spherical particles with the sizes ranging from 80 to 100 nm.

The release profile of the doxorubicin-loaded micelles is presented in Figure 5. It was carried out in PBS solution (pH 7.4) at 37 °C. The sustaining release time of the doxorubicin was more than 60 h, it indicated an attractive release performance. Both free doxorubicin hydrochloride and doxorubicin were used as controls. No release effect was observed in free doxorubicin, it precipitated in PBS for the hydrophobicity. In the control of free doxorubicin hydrochloride, more than 90% of the drug was released within 2 h. In contrast, however, only less than 20% drugs were released in the micellar system over the same period, it clearly showed the favorable behavior in controlled release.

The biocompatibility of the self-assembled micelles was evaluated in NIH/3T3 cells. The CCK-8 assay was employed to investigate the cytotoxicity via concentration and time. As shown in Figure 6, different concentrations of self-assembled blank micelles exhibited high cell viability at 12, 24 and 48 h, respectively. It indicated that the cell viabilities were not obviously affected by concentration and time. To some extent, the low concentration of the micelles seemed to promote the cell proliferation.

The cellular uptake of the micelles was studied by CLSM.

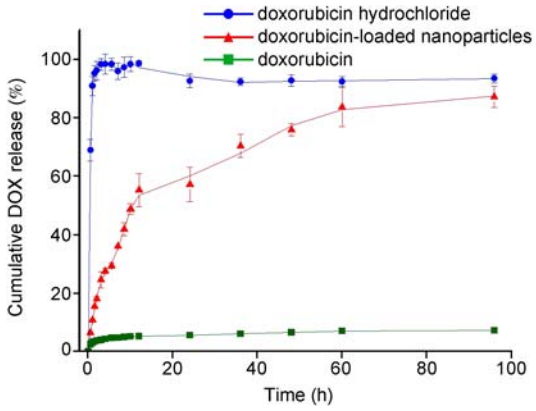


Figure 5 Release profiles of the Dox·HCl , Dox and Dox-loaded micelles (37 °C, pH 7.4).

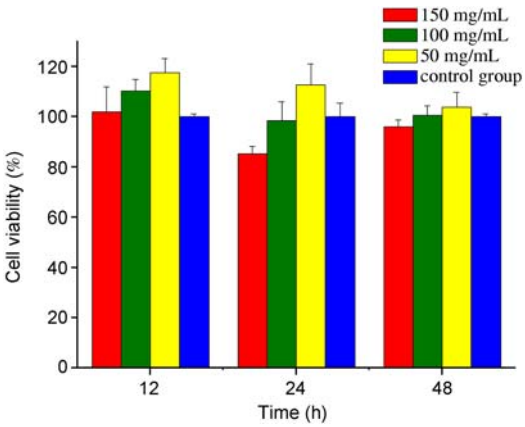


Figure 6 The cell viability of 3T3 cells after incubation with blank micelles at 37 °C for 12, 24 and 48 h.

HepG2 cells were incubated with drug loaded micelles for 2 h, the concentration of doxorubicin was 10 mg/L. It was found that free doxorubicin hydrochloride rapidly diffused into the nucleus of HepG2 cells (Figure 7(a)). In Figure 7(b), the diffusion of free doxorubicin to the nucleus of HepG2 cells was slow, nearly no doxorubicin was observed in cells.

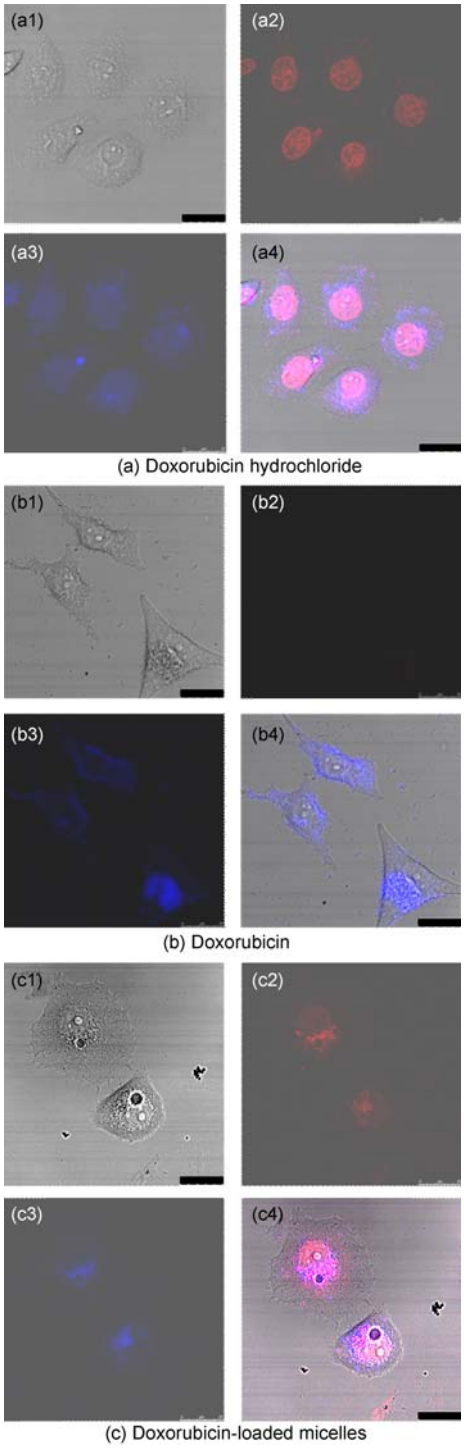


Figure 7 Confocal microscopic images of HepG2 cells incubated with free DOX·HCl, DOX and DOX-loaded nanoparticles (DOX concentration 10 mg/L).

Figure 7(c) showed that the drug loaded micelles were mainly distributed in the cytoplasm and some of them were in nucleus. These results indicated that dendron-linear self-assembled micelles could be a promising candidate for doxorubicin delivery via phagocytosis.

The *in vitro* anticancer effects of the drug-loaded micelles were investigated. The influences of time and concentration are considered in Figure 8. In Figure 8(a), the concentration of drug was 10 μg/mL, the viabilities of HepG2 cells were measured at 12, 24 and 48 h. In the free doxorubicin hydrochloride sample, more than 60% cells were killed at 12 h. Less than 30% cells were killed at 48 h in the free hydrophobic doxorubicin sample. Doxorubicin-loaded micelles revealed optimistic effect in the inhibition of the proliferation of HepG2 cells. When the drug concentration was increased to 15 μg/mL (Figure 8(b)), doxorubicin-loaded micelles showed a high anticancer activity with the killed tumor cells being as high as 75%.

Figure 9 reveals the images of HepG2 cells incubated with doxorubicin hydrochloride (Figure 9(a)), free doxorubicin (Figure 9(b)), doxorubicin-loaded micelles (Figure 9(c)) and blank group (Figure 9(d)) for 48 h with the drug concentration of 10 μg/mL. After the HepG2 cells were incubated in a cell culture plate for 48 h, the HepG2 cells increased significantly (Figure 9(d)). In the doxorubicin hydrochloride

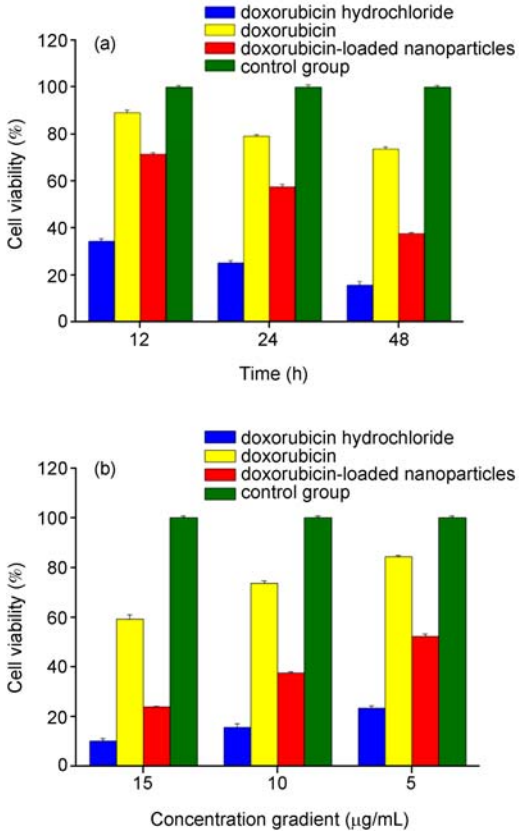


Figure 8 *In vitro* cytotoxicity of HepG2 cells. (a) Time effect; (b) concentration effect.

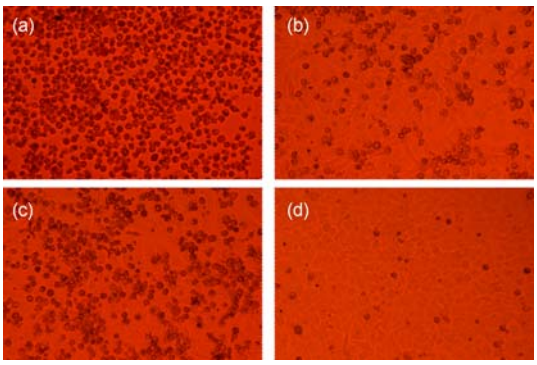


Figure 9 Images of HepG2 cells incubated with drug-loaded micelles for 48 h.

sample, the cells changed their morphology greatly; most of the HepG2 cells detached from the plates and shrank to spherical. Plenty of killed cells were floated in the medium. Less killed cells were observed in drug loaded micelles. Most of the cells attached well in the free doxorubicin sample. The images verified that the micelles were suitable for anticancer drug delivery.

4 Conclusions

Amphiphilic dendron-linear block copolymers were synthesized with the combination of convergent-divergent method and ring-opening polymerization. The synthetic copolymers were biocompatible. These dendron-linear copolymers self-assembled into core-shell structured micelles in water. The CMC of the micelles was as low as 19.50 μg/mL. Anticancer drug doxorubicin was loaded in the micelles. The mean diameters of both blank and drug loaded micelles were comparable at around 250 nm. The release profiles, cellular uptake and the *in vitro* anticancer effects studies revealed that the sustaining drug release time of the micellar system was longer than 60 h. The drugs released from micelles could diffuse into nucleus efficiently to kill cancer cells. The dendron-linear copolymer micelles are promising carriers for anticancer drug delivery.

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