A Facile Chemoenzymatic Synthesis of Amphiphilic Miktoarm Star Copolymers from a Sugar Core and Their Potential for Anticancer Drug Delivery

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Abstract

Miktoarm star copolymers, which are in the simplest form of branching, are particularly of interest to biomedical applications. Nevertheless, their synthesis is facing troubles of seeking inner cores with heterogeneous functional groups as well as tedious protection and deprotection. Herein, ethyl- β -D-glucopyranoside, a sugar molecule containing homogeneous groups, was directly employed as a core to synthesize an amphiphilic miktoarm star copolymer via a chemoenzymatic approach without complicated decoration and protection of the core. Because of the high regioselectivity originated from enzymatic catalysis, the resultant copolymers with well-regulated molecular architecture were synthesized, which can self-assemble into highly-stable nanoaggregates in aqueous media. The copolymers were nontoxic in preliminary *in vitro* cytotoxicity assays and able to carry doxorubicin into tumor cells, indicating their promising application as a new biocompatible platform for drug delivery.

Key Words

Miktoarm star copolymer; Chemoenzymatic catalysis; Drug delivery

1. Introduction

Star polymers with a number of arms connected to a central core have gained worldwide attention, especially in the fields of materials and biomedicine, because of their unique topological structures and special physical/chemical properties, including low solution viscosity, small hydrodynamic radius, high drug loading and excellent cellular uptake properties.¹⁻⁷ Star polymers are generally divided into two types. One is regular star polymers having the same arm segments while the other is asymmetric (also called miktoarm or heteroarm) star polymers.^{4, 5, 8, 9} The miktoarm star polymers are typically built in two ways (“core first”¹⁰⁻¹³ and “arm first”¹⁴⁻¹⁶). As for the arm first approach, well-defined polymer arms are often fabricated prior to covalent linking to a central core; while for the core first strategy, a multifunctional core is urgently required, from which arms are directly grown.^{2, 17, 18} The core first manner holds advantages of precise control of arm number and moderate polymerization reaction,¹⁹ and therefore various methods are devoted to prepare the miktoarm star polymers via this approach, including “click chemistry” reaction, ring-opening polymerization (ROP), atom transfer radical polymerization (ATRP), living anionic polymerization, etc. For example, Yilmaz and coworkers²⁰ used the alkyne-azide cycloaddition click reaction to build PEG arm on cholic acid (CA), followed by initiating ROP of ϵ -caprolactone on the three hydroxyl groups of CA to develop an AB₃ miktoarm star copolymer. Oriol et al.²¹ exploited “click chemistry” and ATRP methods to build a AB₃ miktoarm star polymer with three identical PEG arms and one 4-isobutyloxyazobenzene side chain polymethacrylate arm. Tsukruk et al.²² and Dumas et al.²³ employed the combination of anionic polymerization of ethylene oxide and ATRP of styrene to synthesize multi-arm asymmetric (PEO)_n-(PS)_m ($n + m \leq 4$) and PS(PEO)₃ star copolymers. Particularly, it should be noted that the reaction cores have to contain different functional groups, which can meet the requirements of diverse reactions. In addition, tedious protection and deprotection have to be performed for the cores to avoid side reactions. Therefore, how to search or design multifunctional cores with selective reactivity is a trouble for the synthesis of

miktoarm star polymers.

Oligosaccharides are promising candidates as the cores of miktoarm star polymers because of the existence of several hydroxyl groups. However, up to now, most reports are merely focused on the construction of symmetric star polymers on the basis of these oligosaccharide cores.^{24, 25} It is worth mentioning that there are both primary and secondary hydroxyl groups on oligosaccharides. As well known, enzymatic catalysis has significant advantages, such as mild reaction condition as well as high regioselectivity.²⁶⁻³⁰ The ROP of lactone can only occur at the primary hydroxyl group of oligosaccharides by using lipase as the catalyst.³¹ Inspired by these findings, we started to consider whether amphiphilic miktoarm star copolymers can be built by taking advantage of enzymatic catalysis, which is able to avoid troubles of decorating multifunctional cores as well as their complicated protection/deprotection.

In this work, we selected a natural sugar, ethyl- β -D-glucopyranoside, as the core, which contained one primary as well as three secondary hydroxyl groups. At first, the ROP of ϵ -caprolactone selectively occurred at the primary hydroxyl group by using Novozyme 435 as the catalyst, and diblock copolymers of PEG and PCL were subsequently grown on the secondary hydroxyl groups in a chemical catalysis manner. Amphiphilic miktoarm star copolymers, A(AB)₃, with different molecular structures were synthesized simply via the combined chemoenzymatic method, and their self-assembly behavior, drug loading and delivery property were also investigated.

2. Experimental section

2.1 Materials

Ethyl- β -D-glucopyranoside (EGP, purity $\geq$ 98%) was received from Chembest (Shanghai, China). ϵ -Caprolactone (ϵ -CL, purity $\geq$ 99%) was provided from Acros (Beijing, China) and distilled under reduced pressure prior to use. Methoxypolyethylene glycol (M_n : 2000 and 5000), tin 2-ethylhexanoate (Sn(Oct)₂), and vinyl acetate (VA) were purchased from Sigma-Aldrich (Shanghai, China). Novozyme 435 was supplied by Novo Nordisk Bioindustrials, Inc., in China. Succinic anhydride was obtained from Bodi Chemical Co. Ltd. (Tianjin, China).

N,N-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)-pyridine (DMAP) were obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China). Doxorubicin hydrochloride (DOX•HCl, purity≥98%) was supplied by Meilun Biology Technology Co. Ltd. (Dalian, China). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Beyotime Institute of Biotechnology. Other chemicals were purchased from Kelong Chemical Industries Reagent Co. Ltd. (Chengdu, China).

2.2 Measurements

¹H NMR and DEPT-135 NMR spectra were recorded on a Bruker Advance 400 and 600 spectrometer (Bruker, Germany) using tetramethyl silane (TMS) as an internal reference. Molecular weight and its distribution were measured by a HLC-8320 gel permeation chromatography (GPC, Tosoh Co., Japan) equipped with two columns (TSKgel SuperHZM-M and TSKgel SuperHZ3000). Tetrahydrofuran (THF) was used as the mobile phase with a flow rate of 0.6 mL/min at 40 °C. [The molecular weights of samples were determined using a calibration curve based on linear polystyrene standards.](#) Hydrodynamic diameter of micelles were measured at 37 °C by a Zetasizer Nano ZS90 (Malvern Instruments, Ltd., U.K.) with a He-Ne laser (633 nm) and 90° collecting optics. UV-Vis spectra were recorded on a Varian Cary® 50 UV-Vis spectrometer (Varian Co., USA). Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrometer (Varian Co., USA). Morphology of the micelles was investigated by a Hitachi H600 transmission electron microscope (TEM) at operated voltage of 75 kV. Samples were dropped on carbon-coated copper grids and stained with 0.2% (w/v) phosphotungstic acid.

2.3 Synthesis of 6-*O*-poly(ε-caprolactone)-ethyl-β-D-glucopyranoside (EGP-PCL)

EGP-PCL was synthesized via an enzyme-catalyzed method (Scheme 1). Briefly, EGP (1 g) and Novozyme 435 (0.8223 mg) were placed in a round-bottom flask. ε-CL (7.69 ml) was then added by a syringe under argon atmosphere. The ROP reaction was carried out at 70 °C for 48 h, followed by dissolving the crude product in chloroform after cooling to room temperature. The Novozyme 435 was removed by filtration and the filtrate was precipitated in cold diethyl ether to get the product (yield:

85%). ^1H NMR (CDCl_3 , δ , ppm): 1.25-1.31 (t, 3H, H_2), 1.34-1.47 (m, 2H, H_c), 1.56-1.73 (m, 4H, H_b+H_d), 2.27-2.37 (t, 2H, H_a), 3.64-3.70 (t, 2H, H_e), 4.03-4.13 (t, 2H, H_e). DEPT-135 NMR (DMSO , δ , ppm): 60.98 (C_e), 63.93 (C_e), 64.05 (C_6), 64.43 (C_1), 70.55 (C_4), 73.75 (C_2), 74.00 (C_5), 76.89 (C_3).

2.4 Synthesis of A-*b*-A₃ type star polymer (PCL_3 -*b*-PCL-VA)

Firstly, EGP-PCL (2 g) and Novozyme 435 (1 g) were added in 20 mL THF under stirring. VA (0.38 mL) was added via a syringe to seal the terminal hydroxyl group of EGP-PCL (Scheme 1). The reaction was performed at 30 °C for 8 h. After that, the catalyst was removed by filtration and the filtrate was added dropwise in cold diethyl ether to remove unreacted VA from the product (yield: 84.7%). ^1H NMR (CDCl_3 , δ , ppm): 1.25-1.31 (t, 3H, H_2), 1.34-1.47 (m, 2H, H_c), 1.56-1.73 (m, 4H, H_b+H_d), 2.07 (s, 3H, H_f), 2.27-2.37 (t, 2H, H_a), 4.03-4.13 (t, 2H, H_e). DEPT-135 NMR (DMSO , δ , ppm): 63.93 (C_e), 64.05 (C_6), 64.43 (C_1), 70.55 (C_4), 73.75 (C_2), 74.00 (C_5), 76.89 (C_3).

PCL_3 -*b*-PCL-VA was synthesized by ROP of ϵ -CL in the presence of $\text{Sn}(\text{Oct})_2$ as the catalyst. EGP-PCL-VA (0.4 g), ϵ -CL (1.27 mL), and $\text{Sn}(\text{Oct})_2$ (0.25 mL, 0.1 mol/L) were mixed in a round-bottom flask and heated to 110 °C. After 48 h reaction, the product was dissolved in chloroform and precipitated with excess cold diethyl ether (yield: 89%). ^1H NMR (CDCl_3 , δ , ppm): 1.34-1.47 (m, 2H, H_c), 1.56-1.73 (m, 4H, H_b+H_d), 2.07 (s, 3H, H_f), 2.27-2.37 (t, 2H, H_a), 3.64-3.70 (t, 2H, H_e), 4.03-4.13 (t, 2H, H_e).

2.5 Synthesis of (PEG-PCL)₃-EGP-PCL-VA [A(AB)₃ type] star copolymer

Before the synthesis of A(AB)₃ copolymer, mPEG should be endowed with carboxyl group at chain terminal according to previous reports (Scheme 1).³²⁻³⁴ In brief, mPEG-2000 (5 g), succinic anhydride (0.3753 g), DMAP (0.3665 g), and TEA (0.42 mL) were mixed in 40 mL CHCl_3 . After reacting at room temperature for 24 h, the solution was precipitated with diethyl ether. Product was obtained after filtration and treatment with hydrochloric acid (10% in v/v) in CHCl_3 . Moreover, mPEG with the molecular weight of 5000 (mPEG-5000) was also reacted with succinic anhydride under the same condition to synthesize carboxyl-terminated derivative of mPEG.

As a following step, the mPEG derivative (0.4 mmol) was mixed with PCL₃-*b*-PCL-VA (0.5 g), DCC (0.1862 g) and DMAP (0.044 g) in 8 mL anhydrous CHCl₃. The reaction was performed at room temperature for 36 h. After removing the by-product, dicyclohexylcarbodiurea, by filtration, the filtrate was added in cold methanol to clear unreacted mPEG derivative away. The formed precipitates were collected by centrifugation. ¹H NMR (CDCl₃, δ , ppm): 1.34-1.47 (m, 2H, H_c), 1.56-1.73 (m, 4H, H_b+H_d), 2.06 (s, 3H, H_f), 2.26-2.38 (t, 2H, H_a), 2.62-2.67 (m, 4H, H_k), 3.40 (s, 3H, H_g), 3.54-3.60 (t, 2H, H_i), 3.60-3.75 (m, 4H, H_h), 4-4.11 (t, 2H, H_e), 4.22-4.28 (t, 2H, H_j).

2.6 Preparation of blank and DOX-loaded micelles

A general dialysis method was used to prepare A(AB)₃ micelles. Briefly, 2 mg of star copolymer was dissolved in 1.0 mL DMSO. The resultant solution was added into 7 mL deionized water under vigorous stirring. After 30 min, the solution was transferred into a dialysis bag (molecular weight cut-off 3500 Da) and dialyzed against deionized water for 24 h. The deionized water was replaced every 4 h within the first 12 h period and every 6 h within the following 12 h.

To prepare DOX-loaded micelles, 2 mg DOX•HCl was dissolved in 1 mL DMSO in the presence of TEA for 1 h, while 10 mg star copolymer was dissolved in another 4 mL DMSO. The two solutions were mixed together after 30 min, followed by injection into 35 mL deionized water under vigorous stirring. DMSO and unloaded DOX were removed by dialysis treatment for 24 h.

2.7 Critical Micelle Concentration (CMC) Measurements

CMC of copolymers was determined by a fluorescence spectroscopy using pyrene as a probe. The sample concentration was varied from 1.0×10^{-4} to 0.2 mg/mL, and the final pyrene concentration was kept at 6×10^{-7} mol/L. The measurements were carried out at room temperature under an emission wavelength of 372 nm. The CMC value was calculated from the inflection point of polymer concentration where the relative excitation fluorescence intensity ratio (I_{337}/I_{333}) was changed.

2.8 Drug loading and *in vitro* drug release

To determine the drug loading, 2 mg freeze-dried DOX-loaded micelles were

dissolved in 10 mL DMSO, which was then measured by a UV-Vis spectrometer at 485 nm. From the standard curve generated before, the drug loading content (DLC) and efficiency (DLE) were calculated by the following equations:

$$\text{DLC (\%)} = \frac{\text{weight of loaded drug}}{\text{weight of drug loaded micelles}} \times 100 \quad (1)$$

$$\text{DLE (\%)} = \frac{\text{weight of loaded drug}}{\text{total weight of freed drug}} \times 100 \quad (2)$$

In vitro drug release study was investigated in PB at pH 7.4. The lyophilized DOX-loaded micelles (4 mg) were dispersed in PB (2 ml, pH 7.4) and transferred into a dialysis bag (MWCO of 3500 Da). Then the dialysis bag was immersed into PB (20 ml, pH 7.4) at 37 °C with a speed of 100 rpm. At predetermined time intervals, a 4 mL buffer solution outside the dialysis bag was taken out and an equal volume of fresh PB was added. Dox release contents were determined by measuring the fluorescence absorbance of the release DOX at 480 nm.

2.9 Cell culture and viability assay

Human cervical cancer cells (HeLa) were received from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS, penicillin (100 IU mL⁻¹), and streptomycin (100 µg mL⁻¹) under a humidified atmosphere that contained 5% CO₂ at 37 °C.

The cell viability of the micelles was evaluated by a standard MTT assay. The cells were seeded at 5×10^3 per well in 96-well plate and incubated for 24 h to allow cell attachment. Then the culture medium was removed, and the blank and DOX-loaded micelles solutions at different concentrations were added. After 24 h or 48 h, 20 µL of MTT solution (0.5 mg mL⁻¹) was added to the cells, and the cells were incubated for another 4 h. Afterwards, the medium was removed and 200 µL of DMSO was added to dissolve formazan crystals. The absorbance of the solution was measured on a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., USA) at a test wavelength of 570 nm and a reference wavelength of 630 nm. Cell viability (%) was calculated based on the following equations:

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

where A_{sample} and A_{control} represent the absorbance of the sample and control wells,

respectively.

2.10 Cellular uptake

The Leica DM2500 inverted fluorescence microscope (Leica Microsystems Ltd., Germany) was employed to examine the intracellular distribution of DOX. The cells were seeded in 6-well plates at a density of 2×10^4 cells per well in 2 mL of DMEM containing 10% FBS supplemented with 50 U mL⁻¹ penicillin and 50 U mL⁻¹ streptomycin, and incubated for 24 h. The cells were then incubated with free DOX and DOX-loaded micelles at a final DOX concentration of 8 µg mL⁻¹ in DMEM for 4h. At each prescribed time, the culture media were removed and the cells were washed with PBS three times. Later, the cells were fixed with 4% (w/v) paraformaldehyde aqueous solution for 30 min at room temperature and were stained with 4',6-diamidino-2-phenylindole (DAPI) for 20 min. Fluorescence microscopy images of cells were obtained by using an inverted fluorescence microscope.

To analyze the cellular uptake of free DOX and DOX-loaded micelles quantitatively, HeLa cells were seeded in 6-well plates at 3×10^5 cells per well and cultured for 24 h. Then the cells were then incubated with free DOX and DOX-loaded micelles at a final DOX concentration of 8 µg mL⁻¹. After 8h, the cells were washed twice with PBS and detached using a trypsin–EDTA solution. The mean fluorescent intensity of DOX was determined using a flow cytometer (ACEA NovoCyt 451141011480, Hangzhou, China).

3. Results and discussion

3.1 Synthesis of A(AB)₃ star copolymers

Hydroxyl group is known as a general reaction site for ROP. To prevent occurrence of the reaction on the all hydroxyl groups of EGP at the same time, Novozyme 435, an efficient catalyst for the ROP of lactones,^{35, 36} was employed to selectively catalyze the polymerization of ε-CL at the 6-primary hydroxyl group. Fig. 1a illustrates the ¹H NMR spectrum of EGP-PCL. The integral ratio of H_{2'} and H_{e'} was determined to be about 3:2, indicating that only one PCL was connected on each EGP molecule. To further confirm where the PCL chain was linked, DEPT-135 NMR measurement was

conducted on EGP and EGP-PCL. As for EGP (Fig. 2a), positive peaks were observed within 70~78 ppm for C₂~C₅, and negative peaks at 61.53 ppm and 64.28 ppm for C₆ and C_{1'}, respectively. Compared with the DEPT-135 NMR spectrum of EGP, all peak signals of EGP-PCL (Fig. 2b) remained at the same positions except C₅ and C₆, which shifted from 77.26 ppm and 61.53 ppm to 74.00 ppm and 64.04 ppm, respectively. In addition, two new peaks belonging to PCL appeared at 60.98 ppm and 63.93 ppm. Therefore, it can be concluded that PCL was merely connected on the 6-primary hydroxyl group rather than other secondary hydroxyl groups. From the integration ratio of H_e versus H_{e'}, the molecular weight of EGP-PCL was calculated to be 2.78×10^3 g/mol.

Scheme 1. General procedure for the synthesis of miktoarm polymers A(AB)₃.

Figure 1. ^1H NMR spectra of (a) EGP-PCL and (b) EGP-PCL-VA.

Figure 2. DEPT-135 NMR spectra of (a) EGP and (b) EGP-PCL.

As designed, after construction of A arm (PCL homopolymer), three B arms (diblock copolymer composed by PEG and PCL) were linked at the residual

secondary hydroxyl groups. To avoid the linking of B arms on the terminal hydroxyl group of PCL chain, the group was protected prior to generating B arms. Novozyme 435 was still exploited to selectively catalyze the protection reaction of EGP-PCL with vinyl acetate. From the ^1H NMR spectrum of EGP-PCL-VA shown in Fig. 1b, the original signal of H_{e} at 3.65 ppm was found to disappear and a new peak attributing to the H_{f} of VA appeared at 2.06 ppm. In addition, the integral ratio of H_{d} and H_{f} was close to 1:1, and all peaks of EGP-PCL-VA except H_{e} still remained unchanged compared with EGP-PCL. These results strongly indicated VA was merely reacted on the terminal hydroxyl group of PCL chain of EGP-PCL and the three secondary hydroxyl groups were well preserved for the sake of following construction of B arms.

Figure 3. ^1H NMR spectra of (a) star $\text{PCL}_3\text{-EGP-PCL-VA}$ and (b) $\text{A(AB)}_3\text{-1}$.

Because there existed only secondary hydroxyl groups on the macro-initiator, for which Novozyme 435 showed a very limited catalytic ability, $\text{Sn}(\text{Oct})_2$ was selected as the catalyst to generate B arms. Fig. 3a shows ^1H NMR spectrum of $\text{PCL}_3\text{-EGP-PCL-VA}$, from which the peak attributing to the terminal CH_2 groups of

PCL chains was determined at 3.65 ppm again. This indicated the successful polymerization of ε -CL on the residual hydroxyl groups of EGP-PCL-VA. As we know, the three secondary hydroxyl groups are close to each other (steric hindrance), and it is generally difficult to obtain a “completely substituted” product. From the integral area ratio of H_f versus H_e , the degree of substitution of EGP-PCL-VA was calculated to be 2.79 and molecular weight of resultant PCL₃-EGP-PCL-VA to be 1.20×10^4 g/mol. From GPC measurement (Fig. S1), moreover, a unimodal curve was detected for PCL₃-EGP-PCL-VA with the M_n of 1.66×10^4 g/mol.

Table 1. Molecular characterizations of prepolymers and resultant copolymers

Sample	$M_{n(NMR)}^a$ (g/mol)	$M_{n(GPC)}^b$ (g/mol)	M_w/M_n^b
EGP-PCL-VA	2780	4235	1.49
PCL ₃ -EGP-PCL-VA	12000	16044	1.45
mPEG ₂₀₀₀ -COOH	2100	2183	1.04
mPEG ₅₀₀₀ -COOH	5100	7218	1.05
A(AB) ₃ -1	17490	22844	1.50
A(AB) ₃ -2	23990	32315	1.37

^a calculated from ¹H NMR measurement;

^b calculated from GPC.

On the other hand, to endow the PCL₃-EGP-PCL-VA with an amphiphilic feature, PEG was selected as the hydrophilic component. At first, polyethylene glycol methyl ether (mPEG) with M_n of 2000 was reacted with succinic anhydride, generating terminal carboxyl groups. As a following step, mPEG-COOH was conjugated with PCL₃-EGP-PCL-VA in the presence of DCC and DMAP. As shown in Fig. 3b, characteristic peaks belonging to PEG (3.4 and 3.69 ppm) were observed for resultant product, A(AB)₃-1. From the integral area ratio of H_g versus H_f , the degree of substitution of mPEG₄₅-COOH was calculated as 2.60. In addition, the GPC curve of A(AB)₃-1 was unimodal (Fig. S1), and the molecular weight of A(AB)₃-1 was found

to be higher than that of PCL₃-EGP-PCL-VA (Table 1). The results meant the successful introduction of PEG onto PCL₃-EGP-PCL-VA. To modulate the amphiphilicity of resultant star copolymer, another PEG prepolymer with M_n of 5000 was selected. From ¹H NMR spectrum of A(AB)₃-2 (Fig. S2), the substitution degree of mPEG₁₁₃-COOH was calculated as 2.35. The molecular weight of the resultant miktoarm copolymer, A(AB)₃-2, was summarized in Table 1 from the GPC and ¹H NMR results (Fig. S1 and Fig. S2).

3.2 Self-assembly of A(AB)₃ star copolymers

The miktoarm copolymers can self-assemble into nano-aggregates in aqueous solution because of their amphiphilic features. CMC was investigated by fluorometry in the presence of pyrene as the probe. Particularly, both A(AB)₃-1 and A(AB)₃-2 exhibited quite low CMC values, that is, 0.9 and 3.45 µg/mL respectively (Fig. S3), which were lower than the linear PEG-*b*-PCL copolymers with similar hydrophilic/hydrophobic ratios.³⁷⁻⁴⁰ Similar to the linear copolymers,^{6, 41} the both two miktoarm star polymers formed typical spherical micelles in water with diameters less than 100 nm (Fig. 4). A further evaluation was conducted to determine the exact hydrated diameter and its distribution of micelles by DLS analysis (Fig. 5 and Table S1). The hydrated diameter of micelles was decreased with the increase in the length of PEG, which can be attributed to the change of hydrophilic/hydrophobic ratio, the diffusion behavior of PEG block in aqueous medium and hydrophobic interaction among PCL blocks.⁴²⁻⁴⁵ Similar results about the dependence of micelle size on copolymer structure can be found in previous reports.^{40, 44, 46, 47} In particular, it should be noted that A(AB)₃-2 micelles were more likely to aggregate than A(AB)₃-1 micelles from TEM results. Two reasons are proposed: 1) the smaller size of A(AB)₃-2 micelles (Fig. 5) results in a larger specific surface, which are favorable to the aggregation of nanoparticles; 2) uniform spherical morphology of A(AB)₃-2 micelles (Fig. 4b) is another contributor for the interaction among the different nanoparticles.

Figure 4. TEM images of (a) A(AB)₃-1 micelles and (b) A(AB)₃-2 micelles. The scale bar represents 100 nm.

Figure 5. Hydrated diameters of blank and DOX-loaded star copolymer micelles.

3.3 Drug loading and delivery property

Because of the self-assembly feature of A(AB)₃ star copolymers in aqueous solution, their potential application of drug transportation was investigated. DOX, a broad-spectrum antitumor agent, was selected as a model drug. In particular, the loading contents of DOX were achieved to be 8.8% and 9.1% for A(AB)₃-1 and A(AB)₃-2 micelles, and the corresponding loading efficiencies were 52.8% and 54.6%.

Compared with blank micelles, the diameter of DOX-loaded micelles became obviously larger (shown in Fig. 5). However, their average diameters were still limited within 200 nm (Table S1), which was of importance for an efficient drug delivery.^{48, 49} Moreover, a high size stability of drug-loaded micelles was urgently required during the circulation *in vivo*. Therefore, the dependence of diameter of DOX-loaded micelles on storage period was evaluated accordingly. The diameter of DOX-loaded micelles remained nearly unchanged during four-week period (Fig. 6), indicating that no aggregation among micelles or precipitation was happened.

Fig. 7 presents the DOX release profiles from miktoarm star copolymer micelles. In contrast to free DOX with a fast release rate as well as a high cumulative drug release within 5 h (about 90%), the release rate of encapsulated DOX in A(AB)₃ micelles was obviously lowered. The cumulative DOX release was achieved at 50% and 60% within 12 h for A(AB)₃-1 and A(AB)₃-2, respectively, showing a sustained release behavior. The difference of release rate between the two samples can be attributed to the length of PEG block as well as the size of micelles. The payload was just physically kept in the cores of micelles, and so the burst release of DOX was detected in this work. In contrast to previous reports, the DOX release rate for the A(AB)₃ system was higher than those for linear copolymers as well as comparable to those for other star copolymers,^{6, 50, 51} which indicated the topological structure of copolymers played a very important role in the controlled release of drugs.

Figure 6. Size stability of DOX-loaded micelles.

Figure 7. *In vitro* release profile of DOX from A(AB)₃-1 and A(AB)₃-2 micelles in PBS (pH=7.4) at 37 °C (mean±standard deviation; n=3).

Cellular uptake behavior of DOX-loaded micelles was investigated using an inverted fluorescence microscope. After incubation HeLa cells with free DOX and

DOX-loaded micelles for 4 h respectively, red fluorescence of DOX was detected either in nuclei or in cytoplasm (Fig. 8). In detail, free DOX exhibited a quick accumulation towards cell nuclei as reported frequently.⁵²⁻⁵⁴ Compared with that, both miktoarm copolymers could carry DOX into cell quickly but not all loaded-DOX entered in nuclei within the period. It was worth noting that the fluorescence of DOX was stronger in the cells after incubation with drug-loaded A(AB)₃-2 micelles, compared with the A(AB)₃-1 ones, which was attributed to the difference of particle size between them. As a further investigation, flow cytometry was employed to quantitatively determine the cellular uptake of DOX-loaded micelles. As shown in Fig. 9, the fluorescence intensity of DOX in HeLa cells incubated with free DOX was 1.5-fold and 0.8-fold higher than those incubated with drug-loaded A(AB)₃-1 and A(AB)₃-2 micelles, respectively, which was in good agreement with the fluorescence microscopy analysis.

The *in vitro* therapeutic of free DOX and DOX-loaded micelles was subsequently evaluated against HeLa cells by MTT assay. After 24 h of incubation, the drug-loaded micelle system exhibited an obvious proliferation inhibition of HeLa cells (Fig. 10). Compared with free DOX, the cytotoxicity of DOX-loaded micelles was lower, however, their difference became less pronounced after 48 h incubation at an equivalent DOX concentration higher 8 µg/mL (Fig. S4). The corresponding half maximal inhibitory concentration (IC₅₀) was 0.26 µg/mL, 3.2 µg/mL, and 1.8 µg/mL for free DOX, DOX-loaded A(AB)₃-1 and A(AB)₃-2 micelles, respectively. The lower IC₅₀ value determined for DOX-loaded A(AB)₃-2 micelles was mainly resulted from their smaller particle size as well as better cellular uptake behavior; some similar results can be found in previous reports.⁵⁵⁻⁵⁷ Biocompatibility of the miktoarm copolymers was evaluated against the cells. Except at the comparably high concentration of 128 µg/mL, the cell viability was determined at almost 90% after incubation with blank micelles for 48 h (Fig. 11), indicating their low toxicity towards HeLa cells.

Figure 8. Fluorescence microscopy images of intracellular tracking of free DOX, DOX-loaded A(AB)₃-1 micelles and DOX-loaded A(AB)₃-2 micelles in HeLa cells after 4 h incubation. The scale bar represents 100 μm.

Figure 9. DOX fluorescence intensity of HeLa cells incubated with free DOX, DOX-loaded A(AB)₃-1 micelles and DOX-loaded A(AB)₃-2 micelles at 37 °C for 8 h.

Figure 10. Cytotoxicity of free DOX, DOX-loaded A(AB)₃-1 micelles and DOX-loaded A(AB)₃-2 micelles against HeLa cells at different DOX concentrations for 24h (mean ± standard deviation; n=3).

Figure 11. Viabilities of HeLa cell lines after incubation of empty A(AB)₃-1 micelles and A(AB)₃-2 micelles for 48 h (mean ± standard deviation; n=3).

4. Conclusion

In summary, this work presented for the first time to our knowledge the synthesis and characterization of amphiphilic A(AB)₃ miktoarm star copolymers by an enzyme-assisted catalysis method. In comparison to the traditional synthetic methods of miktoarm star copolymers, the troubles of core decoration and protection are accurately avoided under the manipulation of Novozyme 435 with high regioselectivity. The resultant copolymers can form highly stable micelles with adjusted diameters in water. Furthermore, the micelles exhibited good biocompatibility and efficient drug transportation property toward cells. The results mean that the versatile synthesis method described herein can provide a new way to construct more novel amphiphilic miktoarm star copolymers that are desirable for biomedical applications.

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