

Resorcinarene-centered Amphiphilic Star-block Copolymers: Synthesis, Micellization and Controlled Drug Release*

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Abstract Eight-arm star-shaped poly(ϵ -caprolactone)-block-poly(ethylene glycol)s (SPCL-*b*-PEG) have been prepared by a combination of controlled ring-opening polymerization (CROP) and coupling reaction. First, eight-arm star-shaped poly(ϵ -caprolactone)s (SPCL) with a resorcinarene core were synthesized using octamethyl tetraundecylresorcinarene octaacetate as octa-initiator and yttrium tris(2,6-di-*tert*-butyl-4-methylphenolate) [Y(DBMP)₃] as catalyst. Then the coupling reaction was carried out between SPCLs and carboxyl-terminated methoxy poly(ethylene glycol)s (mPEG-COOH) in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), resulting in eight-arm star-shaped SPCL-*b*-PEGs with controlled molecular weight and well-defined architecture. Furthermore, these amphiphilic eight-arm SPCL-*b*-PEGs could self-assemble into micelles with low critical micellar concentrations (CMC), which was characterized by fluorescent spectroscopy. Moreover, indomethacin loaded micelles with high drug loading content and high encapsulation efficiency can be prepared, which is probably due to the highly branched architecture. The morphologies of micelles were characterized by transmission electron microscopy (TEM), which exhibited diverse nanostructures as the drug loading contents varied. *In vitro* drug release of indomethacin from SPCL-*b*-PEG micelles was carried out in PBS, from which a sustained release behavior was observed. SPCL-*b*-PEG micelles did not show significant cytotoxicity at copolymer concentrations up to 1000 mg/L, making them very promising for drug delivery.

Keywords: Star block copolymer; Resorcinarene; Micelles; Drug delivery.

INTRODUCTION

The preparation of polymeric micelles self-assembled from amphiphilic copolymers as drug carriers has attracted considerable attention in recent years^[1–4]. Poly(ϵ -caprolactone) (PCL) and poly(ethylene glycol) (PEG) are U.S. Food and Drug Administration (FDA) approved polymers and have been widely used in biomedical applications^[5–13]. Amphiphilic block copolymers composed of hydrophobic PCL segments and hydrophilic PEG segments could self-assemble into nanosized micelles, which have been considered as suitable candidate carriers for drug delivery, due to their biodegradability, biocompatibility, good drug permeability, nontoxicity and so on^[14–21]. Polymeric micelles prepared from PCL-*b*-PEG block copolymers can prevent the absorption of proteins and enzymes effectively and have long residence time in circulation^[22].

The formation of polymeric micelles is thermodynamically favorable only above CMC. When the concentration is below the CMC, the micellar structure becomes unstable and dissociates into free chains, which is the most important concern for *in vivo* applications^[23]. Compared with linear amphiphilic block copolymers, star-shaped amphiphilic block copolymers can form more stable micelles in aqueous solutions with lower CMC

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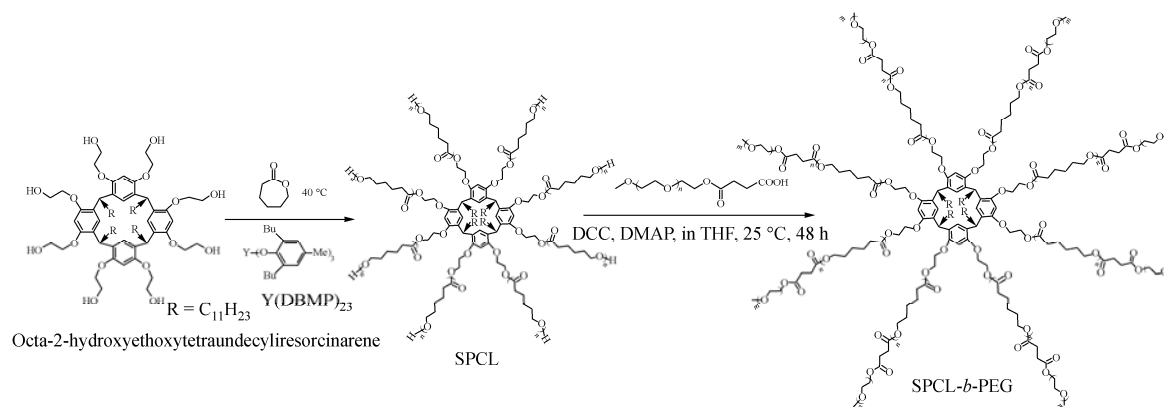
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than their linear analogues, due to the unique chemical structure, that is, several amphiphilic arms are covalently attached in one core^[24–30].

For the synthesis of star-shaped copolymers, the design and synthesis of multifunctional cores are of great interest. Recently, many macrocyclic compounds, such as cyclodextrins^[31–39], calixarenes^[40–43], resorcinarenes^[44–49] and their derivatives have been used as core moieties to prepare star polymers for their well-defined architecture and reduced hindrance. Moreover, the strong supramolecular interaction between macrocyclic compound and guest molecules leads to the formation of stoichiometric inclusion complexes, which can be considered as an important drug delivery system^[20, 50–55].

In recent decades, seeking a facile preparation for star-shaped amphiphilic copolymer with low CMC is urgent. Meanwhile, another important factor is the biocompatibility of the amphiphilic copolymer. In this paper, well-defined star block copolymers consisting of eight PCL-*b*-PEG arms were facily synthesized by “core-first” method utilizing a resorcinarene derivative as core moiety, as shown in Scheme 1. Indomethacin was chosen as a model drug to fabricate drug delivery systems with these amphiphilic star block copolymers. The physicochemical properties, biocompatibility and *in vitro* drug release behaviors of this novel drug delivery system were investigated in detail.



Scheme 1 Synthetic route of eight-arm SPCL-*b*-PEG from a resorcinarene core

EXPERIMENTAL

Materials

ϵ -Caprolactone (CL, Acros) was distilled under reduced pressure before use. Tetrahydrofuran (THF, Shanghai Chemical Reagent Co.) was distilled from Na-K alloy in the presence of benzophenone. Carboxyl-terminated mPEG (mPEG-COOH, $M_n = 1000, 2000$), octa-2-hydroxyethoxytetraundecylresorcinarene and Y(DBMP)₃ were synthesized according to our previous report^[56]. Other reagents were purchased from Shanghai Chemical Reagent Co. and used as received.

Measurements

Proton nuclear magnetic resonance spectroscopy (¹H-NMR) spectra were recorded on a Bruker Avance DMX500 spectrometer in CDCl₃ with tetramethylsilane as an internal standard. The molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC). The GPC system consisted of a Waters degasser, a Waters 1525 HPLC pump with 717 plus autosampler, Waters 2410 RI detector and columns: Styragel, HT 3, HT 4 and HT 5. The calibration was performed with commercial polystyrene standards. THF was used as the mobile phase at a flow rate of 1.0 mL/min at 40 °C. The hydrodynamic diameter and size distribution of micelles were determined by dynamic light scattering (DLS) at 90° angle to the incident beam and at 25 °C on a Brookhaven 90 Plus particle size analyzer. All micellar solutions had a final polymer concentration of 1.0 mg/mL and were filtered through 0.22 μ m filter. Fluorescence excitation spectra were

recorded on a Hitachi F-4500 fluorescence spectrometer at 390 nm emission wavelength and 2.5 nm slit width. TEM images were obtained using a JEM-1230 microscope operating at an acceleration voltage of 60 kV. A drop of 1.0 mg/mL micellar solution was placed onto the surface of Formvar-carbon film-coated copper grids. Excess solution was quickly wicked away with a filter paper. All grids were finally negatively stained by 2 wt% phosphotungstic acid. Ultraviolet spectrophotometric (UV) measurements were performed on a UV-2550 ultra-violet-visible spectrophotometer.

Synthesis of SPCL

SPCLs were synthesized according to our previous work^[47]. Typically, 0.146 g of octa-2-hydroxyethoxytetraundecylresorcinarene (0.8 mmol of OH group) was transferred into a 20 mL flame-dried ampoule under argon atmosphere. Then 0.3 mmol of Y(DBMP)₃ in 3.0 mL of THF was injected into the ampoule and aged for 30 min at 30 °C. Finally, 1.85 g of CL (16.2 mmol) was added to the ampoule *via* a syringe. The ampoule was put into a water bath at 40 °C. After 4 h, the mixture was poured into a large amount of methanol, and the polymer precipitated from methanol, filtered and dried in vacuum to constant weight.

Synthesis of SPCL-*b*-PEG

A typical example is given below: 198 mg of SPCL ($M_n = 19800$, 0.08 mmol OH), 96.8 mg of mPEG-COOH (0.088 mmol), 24.9 mg of DCC (0.12 mmol) and 4.9 mg of DMAP (0.04 mmol) were dissolved in 40 mL of anhydrous THF, and the reaction was performed at room temperature for 48 h under argon atmosphere. The reaction byproduct dicyclohexylcarbodiurea was removed by filtration. After evaporating most of the solvent, the mixture of the star block copolymer and the excess unreacted mPEG-COOH was precipitated by addition of cold diethyl ether. Pure SPCL-*b*-PEG was finally recovered by dialysis (dialysis membrane, MWCO 14000) against double distilled water and lyophilization.

Preparation of Micelles

As an example, 25 mg of SPCL-*b*-PEG was dissolved in 5 mL of THF, and then distilled water (*ca.* 5 mL) was added dropwise to the solution with vigorous stirring. The micelle solution was stirred overnight and dialyzed against distilled water over 24 h to remove THF. The final concentration of the micelle solution was adjusted to 1.0 mg/mL.

Critical Micelle Concentrations (CMC) of SPCL-*b*-PEG Micelles

The CMCs of SPCL-*b*-PEG micelles were determined by fluorescence measurements using pyrene as a fluorescent probe. The concentration of the micellar solutions ranged from 1.0×10^{-6} mg/mL to 1.0 mg/mL. The pyrene concentration in the solution was 6.0×10^{-7} mol/L.

Preparation of Indomethacin Loaded Micelles

25 mg of SPCL3-*b*-PEG and the calculated amount of indomethacin were dissolved in 5 mL of THF, and then distilled water (*ca.* 5 mL) was added dropwise to the solution under vigorous stirring to induce the hydrophobic indomethacin incorporated into the micellar core. The micelle solution was stirred overnight and dialyzed against distilled water over 24 h to remove THF. The unloaded indomethacin precipitate was filtered through a 0.22 μ m filter, and the solid-state drug-loaded star block copolymers were recovered by lyophilization. The drug-loading content of the samples was characterized by ¹H-NMR spectra. The loading content and the encapsulation efficiency were calculated using Eqs. (1) and (2), respectively:

$$\text{Loading content (\%)} = W_l/W_p \times 100\% \quad (1)$$

$$\text{Encapsulation efficiency (\%)} = W_l/W_{10} \times 100\% \quad (2)$$

where W_l is the weight of loaded indomethacin measured by ¹H-NMR spectra, W_p is the weight of copolymer, and W_{10} is the weight of indomethacin in feed.

Indomethacin Release from SPCL-*b*-PEG Micelles

In vitro release profiles of indomethacin from the SPCL-*b*-PEG micelles were investigated by the dialysis method. Concretely, 4 mL of indomethacin loaded SPCL-*b*-PEG micellar solution was introduced into a dialysis bag (cut off M_n : 14 kDa) against 20 mL PBS buffer solution (pH 7.4, 20 mmol/L). At desired time intervals, 4 mL of release medium was taken out and replenished with an equal volume of corresponding fresh medium. The amount of released indomethacin was determined by UV measurements at 320 nm. The release experiments were conducted in triplicate, and the results are presented as the average data with standard deviations.

In vitro Cytotoxicity

The *in vitro* relative cytotoxicity of SPCL3-*b*-PEG1k against HeLa cells was evaluated by a Cell Counting Kit-8 (CCK-8) assay. Appropriate amounts of SPCL3-*b*-PEG1k micelles were separately dissolved in PBS to obtain their extracts with concentration of 1000, 500, 100, 50, 10, 5, 1 and 0 $\mu\text{g/mL}$, respectively. HeLa cells were seeded into 96-well plates at a density of 10000 cells per well in 180 μL of culture medium. 6 h later, 20 μL extracts of various concentrations were added to the well. Cells were exposed to the gel precursor for 48 h. Before 100 μL fresh medium and 10 μL CCK-8 reagent were added, each well was washed three times with PBS. After a further incubation of 3 h, the optical density (OD) of the wells was measured at 450 nm with a Microplate Spectrophotometer (SpectraMax M5).

RESULTS AND DISCUSSION

Synthesis and Characterization

Eight-arm SPCLs were synthesized according our previous report. The $^1\text{H-NMR}$ spectrum of SPCL1 is displayed in Fig. 1(A), in which the signals of methyl groups (H^i) and methylene groups (H^f) due to octafunctional core were clearly detected at around $\delta = 0.87$ and 1.26 respectively. Moreover, the integral ratio of H^i and H^f is exactly 3/4, which demonstrates the well-defined eight-arm structure of the SPCLs. The molecular weights obtained from $^1\text{H-NMR}$ spectra are close to the theoretic values calculated by conversion and monomer/initiator ratio, as summarized in Table 1. The GPC measurements show that the SPCLs prepared have single and reasonably narrow molecular weight distributions.

Table 1. Synthesis of eight-arm SPCLs by controlled ring-opening polymerization of CL using a resorcinarene derivative as initiators and $\text{Y}(\text{DMBP})_3$ as a catalyst ^a

Polymer	[CL]/[Initiator] (molar ratio)	Conv. (%)	M_n, Cal^b (g/mol)	M_n, NMR^c (g/mol)	M_n, SEC^d (g/mol)	MWD ^d
SPCL1	92	93.8	11300	11500	18700	1.39
SPCL2	162	93.1	18700	19800	27000	1.47
SPCL3	304	95.2	34500	36500	58000	1.45

^a Polymerization conditions: $[\text{Initiator}]/[\text{Y}] = 0.375$, $[\text{CL}]_0 = 2.0 \text{ mol/L}$, 40 $^\circ\text{C}$, 4 h, in THF; ^b Calculated from the feeding molar ratio of $[\text{CL}]/[\text{Initiator}]$ and conversion; ^c Calculated from $^1\text{H-NMR}$ spectra; ^d Measured by GPC calibrated with polystyrene standards

The resorcinarene-centered eight-arm star-block copolymers were synthesized by the coupling reaction of pre-synthesized SPCLs and mPEG-COOH in the presence of DCC and DMAP. Figure 1(B) exhibits the $^1\text{H-NMR}$ spectrum of the star block copolymer SPCL1-*b*-PEG1k. Compared to the $^1\text{H-NMR}$ spectrum of its precursor (SPCL1, Fig. 1A), the signals due to the methylene protons (H^e , $\delta = 3.65$) and methyl protons (H^f , $\delta = 3.38$) in PEG block, as well as that of succinate junction (H^h), could be clearly detected. The coupling efficiency between mPEG-COOH and SPCLs could be estimated from relative integral values of the methylene protons (H^e) of PEG and that of PCL (H^b) blocks, based on their theoretical molecular weights. Figure 2 shows typical GPC traces of star block copolymers (SPCL3-*b*-PEG1k and SPCL3-*b*-PEG2k) in comparison with that of SPCL3. After the coupling reaction with mPEG-COOHs, The SEC traces shift to the higher molar mass region and still exhibit fairly narrow unimodal molecular weight distributions, which indicates the successful synthesis of the copolymers with well-defined eight-arm star block architecture. The molecular weights and molecular weight distributions of the star block copolymers are summarized in Table 2.

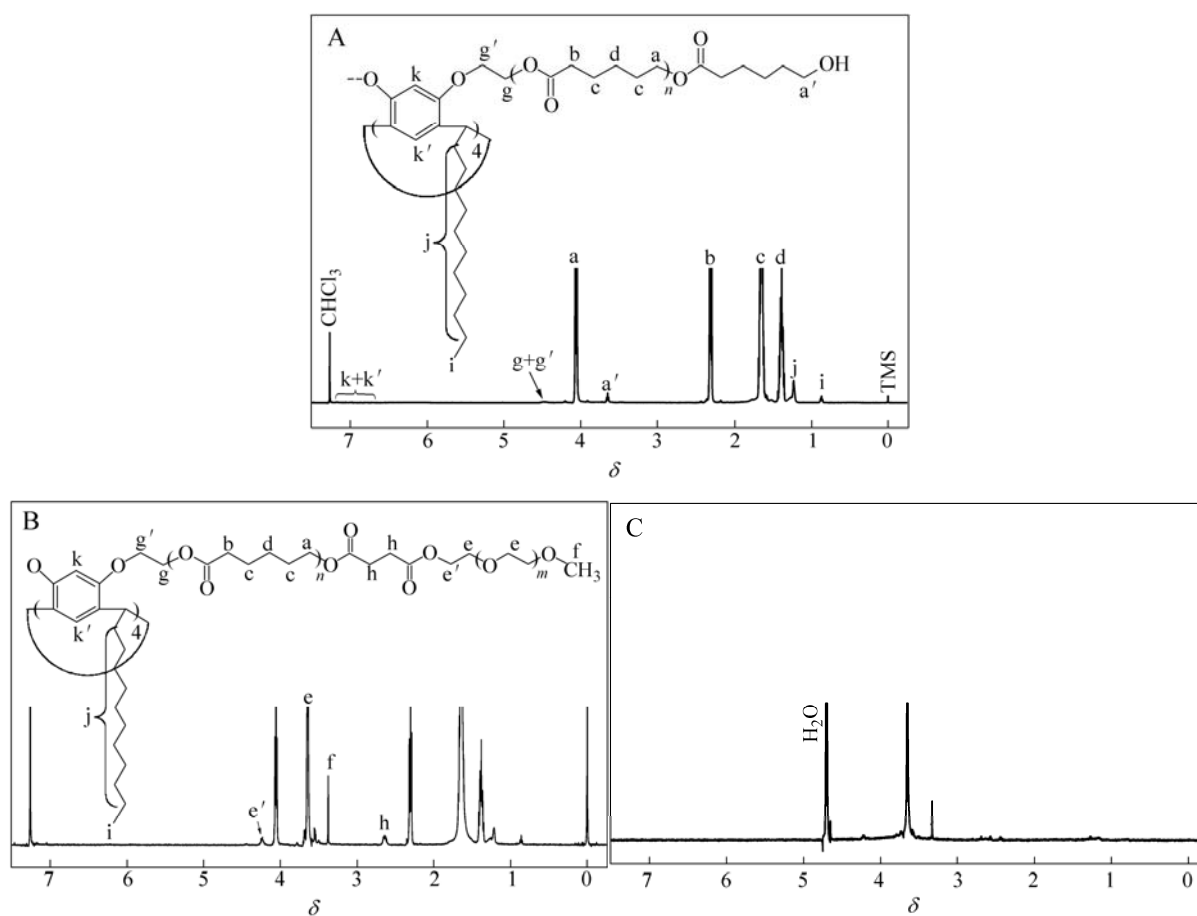


Fig. 1 ^1H -NMR spectra of (A) SPCL1 in CDCl_3 , (B) SPCL1-*b*-PEG1k in CDCl_3 and (C) SPCL1-*b*-PEG1k in D_2O

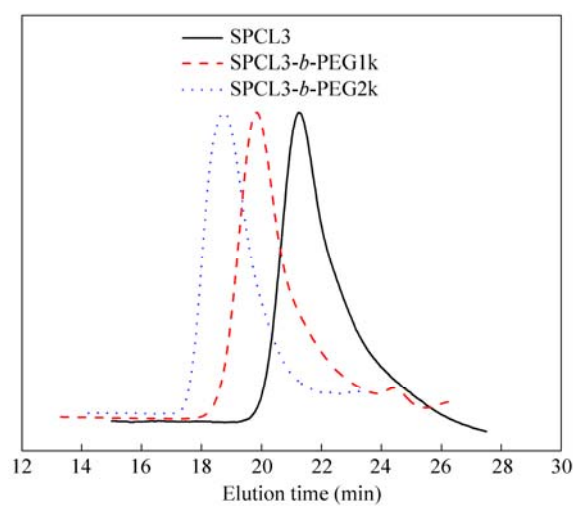


Fig. 2 GPC curves of SPCL3, SPCL3-*b*-PEG1k and SPCL3-*b*-PEG2k

Table 2. Eight-arm SPCL-*b*-PEGs by coupling reaction between mPEG-COOH and eight-arm SPCLs

Polymer	M_n , SEC (g/mol) ^a	MWD ^a	CE (%) ^b	Diameter (nm) ^c	CMC (mg/L)
SPCL1- <i>b</i> -PEG1k	38400	1.25	99.1	16.0	1.845
SPCL1- <i>b</i> -PEG2k	49100	1.33	98.1	19.5	2.793
SPCL2- <i>b</i> -PEG1k	41500	1.31	98.8	17.9	0.801
SPCL2- <i>b</i> -PEG2k	57700	1.38	96.5	21.8	1.730
SPCL3- <i>b</i> -PEG1k	81200	1.45	94.3	27.9	0.579
SPCL3- <i>b</i> -PEG2k	93500	1.41	89.8	32.8	1.140

^a Determined by GPC calibrated with polystyrene standards; ^b Coupling efficiency, calculated from the relative integral values of the methylene protons (H^c) of PEG and PCL (H^a) blocks; ^c Number-average mean diameters of SPCL-*b*-PEG micelles by DLS

Micellization

The amphiphilic SPCL-*b*-PEGs could undergo self-assembly into polymeric micelles in aqueous solutions. As summarized in Table 2, the diameters of these micelles increased with the increasing of the chain length of PCL and PEG blocks. The SPCL-*b*-PEG micelles in solid state were recovered by lyophilization, which were dissolved in D₂O and characterized by ¹H-NMR as shown in Fig. 1(c). Only the signals of PEG block were detected, which indicates the core-shell structure of the micelles. The signals of water insoluble resorcinarene moieties and PCL blocks were shielded by the PEG shell. The critical micelle concentrations (CMC) of amphiphilic SPCL-*b*-PEGs were measured by a fluorescence technique using pyrene as a probe. Pyrene will preferentially incorporate into hydrophobic microdomains showing strong fluorescence intensity while weak fluorescence intensity and a shift of excitation peak in a polar environment (aqueous solution). In addition, the sharp rise in intensity ratio of peaks at 338 and 333 nm of pyrene in the excitation spectra indicates the on-set of micellization (CMC) for SPCL-*b*-PEGs. Figure 3 shows the relationship of the intensity ratios (I_{338}/I_{333}) as a function of copolymer concentration at room temperature. It can be obviously observed that the CMC values of these amphiphilic star block copolymers were very low (ranging from 0.579 mg/L to 2.793 mg/L), decreasing as the chain length of PEG decreased and that of PCL increased (Table 2), which was probably due to the highly branched architecture. These results indicate that the micellar aggregates of SPCL-*b*-PEGs in the aqueous solution are stable even with very low concentration, making them suitable candidate carriers for drug delivery.

Drug Loading and Controlled Drug Release

Indomethacin was used as a model drug to evaluate the drug loading content and drug encapsulation efficiency of SPCL3-*b*-PEG1k, which owns the lowest CMC in this work. As shown in Table 3, drug loading content increased and encapsulation efficiency decreased, concomitant with the increase of drug in feed. For instance, drug loading content increased from 8.8% to 17.2% and encapsulation efficiency decreased from 88.1% to 57.3%, when theoretical indomethacin loading enhanced from 10.0% to 30.0%. The micelles show high drug loading content and encapsulation efficiency to indomethacin, which is probably due to the highly branched architecture. The morphologies of the drug loaded micelles, as well as the micelles without drug, were investigated by TEM. It can be seen from Fig. 4(a), SPCL3-*b*-PEG1k without indomethacin mainly forms spherical micelles with an average diameter of about 15 nm at room temperature, which is in good accord with DLS result in Table 2. However, larger aggregates were formed when indomethacin molecules were encapsulated into the micelles, as summarized in Table 3. Furthermore, SPCL3-*b*-PEG1k with an indomethacin loading content of 8.8% formed near-spherical micelles in aqueous solution (Fig. 4b). Nevertheless, worm-like and stick-shaped micelles were observed when the loading content of indomethacin increased to 13.5% and 17.2% respectively (Figs. 4c and 4d). It should be noted that the sizes of indomethacin loaded micelles measured by TEM are not in accordance with the DLS data in Table 3, which should be attributed to the fact that the basic equations for calculating the hydrodynamic diameter in DLS measurements are originated from the correlation function cumulant analysis, based on the assumption that the particles are noninteracting spheres and not anisotropic objects.

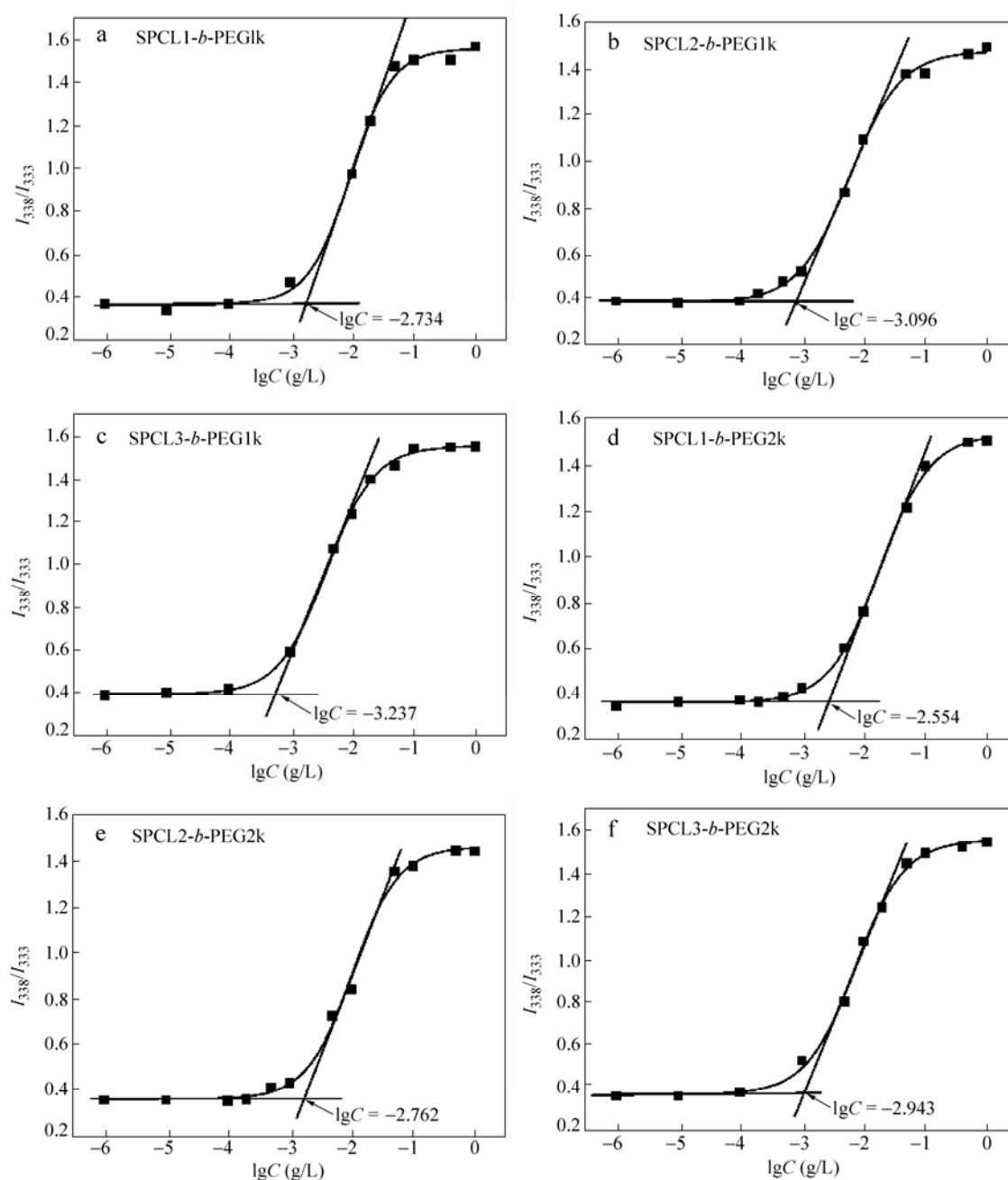


Fig. 3 Plots of the intensity ratio I_{338}/I_{333} from pyrene excitation spectra versus concentration of SPCL-*b*-PEGs in double-distilled water

Table 3. The effect of indomethacin/polymer ratio on LC, EE and particle size of SPCL3-*b*-PEG1k micelles

Drug-loaded micelles	Drug/Polymer ^a	LC (%) ^b	EE (%) ^c	Diameter (nm) ^d
DLM-1	1/10	8.8	88.1	31.2
DLM-2	2/10	13.5	67.4	36.8
DLM-3	3/10	17.2	57.3	57.3

^a Indomethacin/polymer weight ratio in feed; ^b Loading content, determined by $^1\text{H-NMR}$; ^c Encapsulation efficiency, determined by $^1\text{H-NMR}$; ^d Number-average mean diameters of drug-loaded micelles, determined by DLS

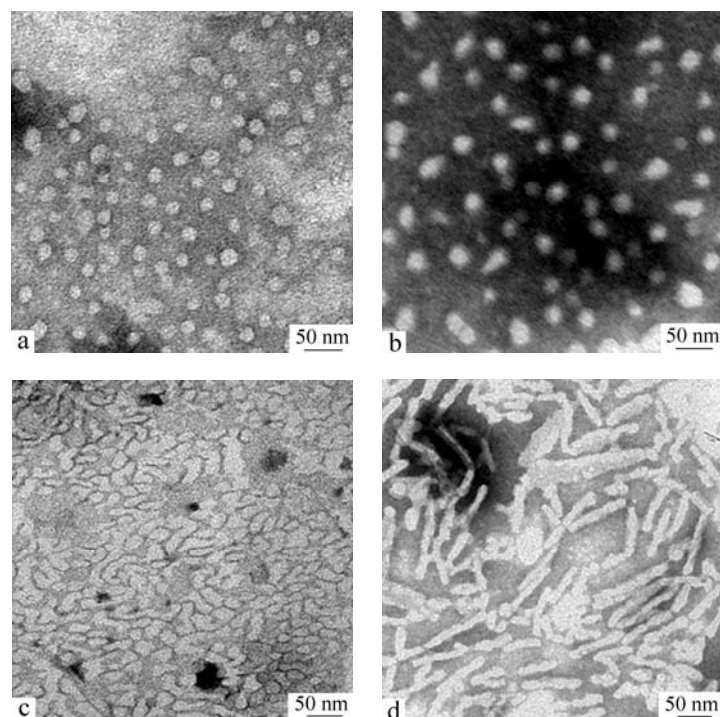


Fig. 4 TEM images of (a) SPCL3-*b*-PEG1k micelles, (b) DLM-1, (c) DLM-2 and (d) DLM-3

In vitro drug release of indomethacin from SPCL3-*b*-PEG1k micelles was carried out in PBS (pH 7.4) at 37 °C as shown in Fig. 5. It was observed that the release profiles of indomethacin loaded SPCL-*b*-PEG micelles were typical two phase release profiles. The release rate is quite fast in the first 12 h, which might be attributed to the initial burst of expelled indomethacin from the micelle shells. In the second stage, a usually more constant stage with low release rate was dependent on indomethacin's diffusion from micelle cores, which could be prolonged to nearly 80 h. These results indicate the strong interaction in the micelles between indomethacin molecules and PCL blocks, as well as the resorcinarene core, led to a decreased rate of drug release from micelles.

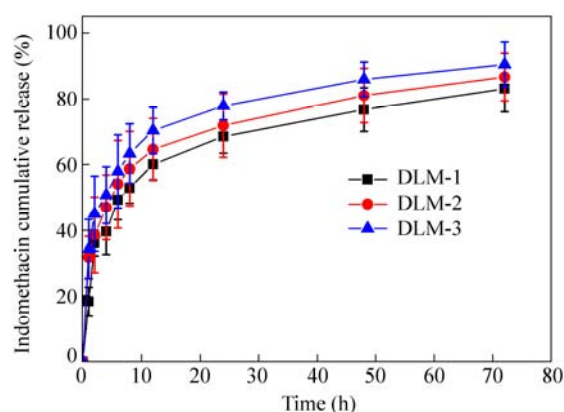


Fig. 5 *In vitro* cumulative release of indomethacin from SPCL3-*b*-PEG1k polymeric micelles at pH 7.4 with different drug loading contents

***In vitro* Cytotoxicity**

As drug carriers, the biocompatibility of copolymer micelles should be considered as a crucial issue. Low cytotoxicity is one of the indices for biocompatible copolymer micelles. The cytotoxicity of the SPCL3-*b*-PEG1k micelles was evaluated through CCK-8 assay at a series of micellar concentrations ranging from 1 mg/L to 1000 mg/L using Hela cells for 24 h and 48 h respectively (Fig. 6). The presence of all the samples tested did not significantly inhibit cell growth, which suggested that SPCL-*b*-PEG micelles were almost nontoxic against Hela cells. Even when the polymer concentration reached to 1 mg/mL, the relative cell viability remained above 90% after 48 h of incubation, which should be ascribed to the biocompatibility of both PCL and PEG blocks.

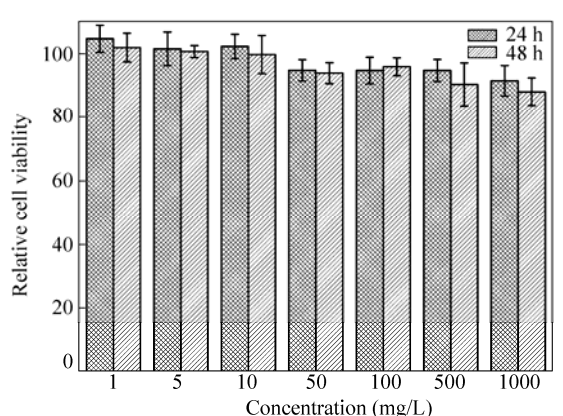


Fig. 6 Relative cell viability of SPCL3-*b*-PEG1k measured by CCK-8 assay

CONCLUSIONS

Resorcinarene-centered amphiphilic eight-arm star block copolymers (SPCL-*b*-PEG) with controlled molecular weight and well-defined architecture have been successfully synthesized. These amphiphilic star block copolymers could self-assemble into micelles which were used as nanocarriers for hydrophobic drugs, such as indomethacin. Moreover, high drug loading content and high drug encapsulation efficiency were achieved, due to the strong hydrophobic interaction between PCL blocks and drugs, as well as the highly branched architecture, which also leads to a sustained release behavior. The star copolymers exhibit no apparent cytotoxicity based on CCK-8 assay. With these excellent properties, this kind of star block copolymer micelles has potential applications as versatile nanocarriers for controlled release of hydrophobic drugs.

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