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A facile and versatile strategy to efficiently synthesize sulfonated poly(butylene succinate), self-assembly behavior and biocompatibility†

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A series of amphiphilic and anionic copolyesters of sulfonated poly(butylene succinate) (SPBS) with sulfonate groups distributing randomly along the biodegradable backbone, were synthesized via addition of sodium hydrogen sulphite to carbon-carbon double bonds on the backbone of poly(butylene succinate-*co*-butylene fumarate) (PBSF). Content of hydrophilic sulfonate groups can be facilely regulated by changing initial feed ratio. The structures of PBSF and SPBS were systematically characterized by NMR and GPC. The negatively charged micelles self-assembled from SPBS were prepared by dialysis method and characterized by NMR, DLS and TEM. *In vitro* cytotoxicity assay indicates the SPBS micelles possess excellent biocompatibility. The biocompatibility of SPBS micelles increases with increasing content of sulfonate groups. This work provides a broad new method to facially synthesize novel anionic copolyesters with high efficiency and controllable anion content. These copolyesters are highly promising as drug delivery carriers for cancer therapy.

Introduction

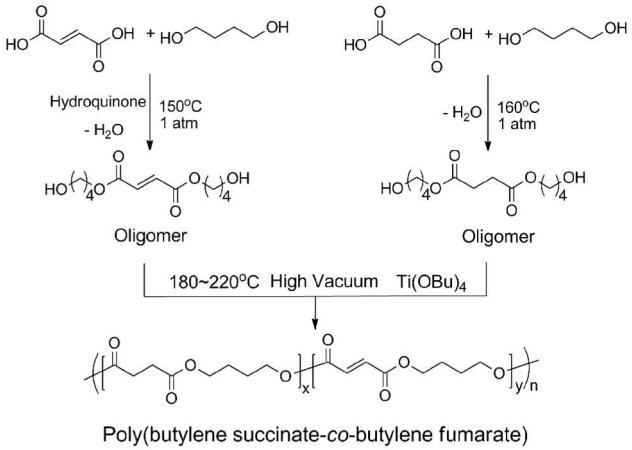
Aliphatic polyesters such as poly(lactic acid), polycaprolactone and poly(butylene succinate) are widely used as reabsorbable sutures, tissue-engineering scaffolds, and drug delivery vehicles, because of their nonimmunogenicity, biodegradability and biocompatibility. Besides biodegradability and biocompatibility, it is also crucial for biomaterials to be appropriately functionalized to interact with cellular environment. Most of the aliphatic polyesters, however, are highly hydrophobic and lack of reactive functional groups that can be linked by biomolecules. Thus, their biomedical applications are seriously limited.

During the past two decades, great efforts have been made to deal with these problems. Functional groups such as hydroxyl, carboxyl, amino and sulfonate groups were introduced to obtain amphiphilic copolyesters.[1-19] The introduction of functional pendant groups not only improves hydrophilicity and degradability of biodegradable polyesters, but also provides a versatile access to conjugate a variety of bioactive molecules such as RGD peptide, folic acid and biotin. Among these functional groups, the charged ones, such as carboxyl, amino and sulfonate groups, have attracted great interest, because the introduction of charged groups allows the preparation of positively or negatively charged nanoparticles, which have been well applied in the field of drug delivery.[20,21] The charges on nanoparticle surface not only enhance the stability of particle and prevent the particles from aggregating in solution via the electrostatic repulsion, but also determine the cellular uptake efficiency and mechanism, and *in vivo* fate of nanoparticle.[22] Especially, negatively charged nanocarriers have shown potential protein resistance and long circulation for *in vivo* experiments, thus deliver the anti-cancer drugs more efficiently to the tumor sites.[22-24] Furthermore, negatively charged nanoparticles also display remarkably high loading contents and loading efficiencies for cationic peptides and drugs due to the electrostatic interactions.[25] Nevertheless, as reported in these works, the synthesis of anionic polyesters usually involved the synthesis and polymerization of functional groups substituted monomers. The synthesis of functionalized monomers usually led to long reaction time[2,6-8,14,15] and low overall yield[2,6,14] due to complex and multistep reactions. [2,6-10,14,16] Especially, polyesters containing more than 20 mol % of anion groups could not be reached through this approach.[7,15-19] Thus, it is highly significant to develop a novel and facile strategy for efficient synthesis of anionic aliphatic polyesters.

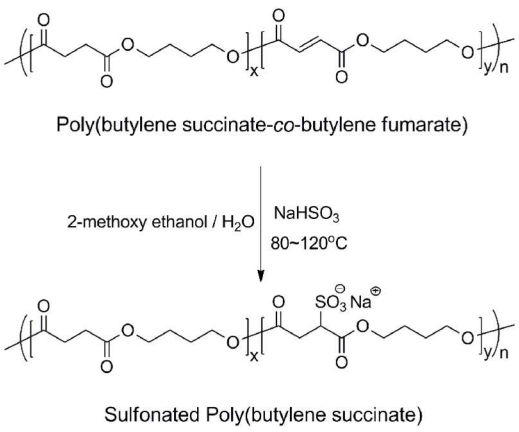
During previous works, we reported a series of novel unsaturated poly(butylene fumarate) and its copolyesters with poly(butylene succinate).[26-28] The copolyesters possess excellent biodegradability, thermal properties and mechanical properties. In addition, the abundant carbon-carbon double bonds on the backbone provide many reactive sites for further modification and introduction of functional groups. But to the best of our knowledge, its functionalization is limited to dihydroxylation.[29] Recently, Alemdar and coworkers successfully prepared amphiphilic and sulfonated polyesters via addition of sodium hydrogen sulphite to carbon-carbon double bonds of the unsaturated poly(ethylene succinate-*co*-ethylene maleate/fumarate).[30] However, the critical micelle concentration (CMC) reaches up to 2.9–3.4 g L^{-1} due to the low molecular weights (M_n: 1650–1950). This might cause the self-

assembly micelles unstable during circulation, and thus greatly restrain their applications in drug delivery system.

Herein, we present a new facile and versatile strategy to synthesize novel anionic biodegradable polyesters with high molecular weights and low CMC that could be used for drug delivery. First of all, high-molecular-weight copolyesters of poly(butylene succinate-*co*-butylene fumarate) (PBSF) were synthesized via the copolycondensation of succinic acid, fumaric acid and 1,4-butanediol. Then sulfonated poly(butylene succinate) (SPBS) were synthesized via addition of sodium hydrogen sulphite to carbon-carbon double bonds on the backbone of poly(butylene succinate-*co*-butylene fumarate) in the mixed solvent of 2-methoxyethanol and water. The negative charge ratios of sulfonate on the backbones can be facilely adjusted by variation the feed ratio of fumaric acid to succinic acid. Our work provides a rapid and versatile method to efficiently synthesize novel anionic copolyesters on a multigram scale with high sulfonate content. The chemical structures of unsaturated copolyesters and novel anionic polyesters were systematically characterized by NMR and GPC. The self-assembly behavior of the amphiphilic anionic polyesters in aqueous solution were also detailedly examined by NMR, DLS and TEM. The biocompatibility of the self-assembled micelles was evaluated by *in vitro* cytotoxicity assay.



Scheme 1 Synthesis routes of PBF oligomers, PBS oligomers and random copolyesters of PBSF.



Scheme 2 Addition of sodium hydrogen sulphite to carbon-carbon double bonds of PBSF.

Experimental part

Materials

Fumaric acid and 1,4-butanediol were purchased from Alfa Aesar (USA) and BASF (German), respectively. Succinic acid was purchased from Anqinghexing Chemical Corp. (China). Hydroquinone was obtained from Xilong Chemical Corp. (China). Nile Red (99%) was bought from Across (Belgium). Other reagents and solvent were analytical grade, and purchased from Beijing Chemical Reagents Corp. (China) and used without purification.

Synthesis of sulfonated poly(butylene succinate) (SPBS)

As shown in Scheme 1 and Scheme 2, novel anionic polyesters of SPBS were prepared via a two-step synthetic strategy. PBSF40 (the number 40 designates the molar ratio of fumarate to succinate in the copolyester) and its sulfonate-functional anionic polyester of SPBS40 were chosen as the representative for the synthesis and characterization.

Synthesis of poly(butylene succinate-*co*-butylene fumarate) (PBSF)

The random copolyesters of PBSF were synthesized from succinic acid, fumaric acid and 1,4-butanediol with different feed ratios by a two-step reaction of esterification and polycondensation. Titanium(IV) butoxide ($Ti(OBu)_4$) and hydroquinone were used as catalyst and free radical inhibitor, respectively. The detailed process for the synthesis of PBSF is described in the Supporting Information. ^{1}H NMR, δ (400 MHz, $CDCl_3$, TMS, ppm): 6.85 (–C**H**=C**H**–, s); 4.25–4.22 (–CH=CH–COO–C**H**$_2$–, t); 4.15–4.12 (–CH$_2$–CH$_2$–COO–C**H**$_2$–, t); 3.71–3.66 (–C**H**$_2$OH, m); 2.62 (–COC**H**$_2$–, s); 1.80–1.58 (–C**H**$_2$–CH$_2$O–, m).

Synthesis of SPBS via addition of sodium hydrogen sulphite to carbon-carbon double bonds

5 g of PBSF40 was placed into a glass flask equipped with a reflux condenser and dissolved in 20 mL of ethylene glycol monomethyl ether under nitrogen atmosphere, and then mixed with 20 mL of 10% $NaHSO_3$ solution. The mixture was heated to 110 ^{o}C and maintained for 7 h until the solution turned to be clear. Thereafter, the reaction mixture was evaporated with a rotary evaporator under reduced pressure to remove the mixed solvents of 2-methoxyethanol and water. The product left in the flask was dissolved in methanol and filtered, then precipitated into acetone, repeatedly. The remaining solid product was dried in vacuum oven at 60 ^{o}C for 24 h to give SPBS40 with pendant sodium sulfonate groups. ^{1}H NMR, δ (400 MHz, DMSO-d_6, ppm): 4.03–4.02 (–C**H**$_2$O–, d); 3.67–3.64 (>C**H**–SO$_3$Na, d); 2.95–2.78 (–C**H**$_2$–CH<, m); 2.56 (–CO–C**H**$_2$–, s); 1.61 (–C**H**$_2$–CH$_2$O–, s).

Characterization

^{1}H NMR spectra were recorded on a AV-400 nuclear magnetic resonance spectrometer (Bruker) using $CDCl_3$, D_2O or DMSO-d_6 as the solvent at ambient temperature. The molecular weight and molecular weight distribution were determined by Gel

permeation chromatography (Waters 1515) equipped with three Waters Styragel columns (HT5, HT4 and HT3) and a differential refractometer detector. The measurements were taken at 45 °C. And $CHCl_3$ was used as the eluent at a flow rate of 1.0 mL min^{-1}. The number and weight average molecular weight was calculated by using a calibration curve with monodisperse polystyrene as standards. The average diameter and size distribution of the micelles were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS. The scattering angle was kept at 173° and the wavelength was set as 633 nm during the whole experiment. Number-average hydrodynamic diameter was adopted and all data were averaged over three measurements. Transmission electron microscopy (TEM) measurements were performed on a Hitachi HT7700 TEM operating at 80 kV. TEM samples were prepared by drying a drop of a dilute aqueous solution of micelles onto a carbon-coated copper grid and allowed to dry at room temperature. The grid was stained with a 6% solution of uranyl acetate for 15 min and then dried until analysis. Fluorescence spectra were obtained using a Hitachi F-4500 spectrofluorometer at room temperature.

Critical micelle concentration (CMC)

CMC of the micelles were determined using Nile Red as fluorescence probe. The micelles loaded with Nile Red were diluted for the CMC determination, the concentration of the micelles was varied from 0.5 mg mL^{-1} to 1×10^{-5} mg mL^{-1}. Fluorescence measurements were taken at an excitation wavelength of 550 nm and the emission monitored from 580 nm to 720 nm. Excitation and emission slit widths were both maintained at 10 nm and spectra were accumulated with a scan speed of 240 nm min^{-1}.

In vitro cytotoxicity assay

The cytotoxic effects of SPBS micelles were determined by the MTT assay. Before determination, the cells were first counted and seeded in 96-well plates at 5×10^3 cells per well in 100 μL of corresponding medium. After a 24-hour culture, the culture medium was removed and replaced with 100 μL of medium containing different concentrations of SPBS micelles (1-1000 μg mL^{-1}). Phosphate buffered saline was chosen as control. The cells were incubated for another 24 hours. Following this, the culture medium was removed and wells were washed with phosphate buffered saline. Then, 100 μL of 5 mg mL^{-1} MTT assay stock solution in phosphate buffered saline were added to each well. After incubating the cells for 4 hours, the medium containing unreacted MTT was removed carefully. The obtained blue formazan crystals generated by live cells were dissolved in 150 μL of DMSO, and the absorbance at wavelength of 570 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 570 nm with control wells.

Result and discussion

Synthesis and characterization of PBSF

The dihydroxyl-terminated PBSF was synthesized by esterification and polycondensation of succinic acid, fumaric acid and 1,4-butanediol, using $Ti(OBu)_4$ as catalyst. Hydroquinone was introduced to prevent the radical crosslinking of carbon-carbon double bonds. To achieve a faster reaction rate and less crosslinking density, the polycondensation temperature was increased from 180 °C to 220 °C with decreasing feed ratio of PBF oligomers. The synthetic approach is shown in Scheme 1.

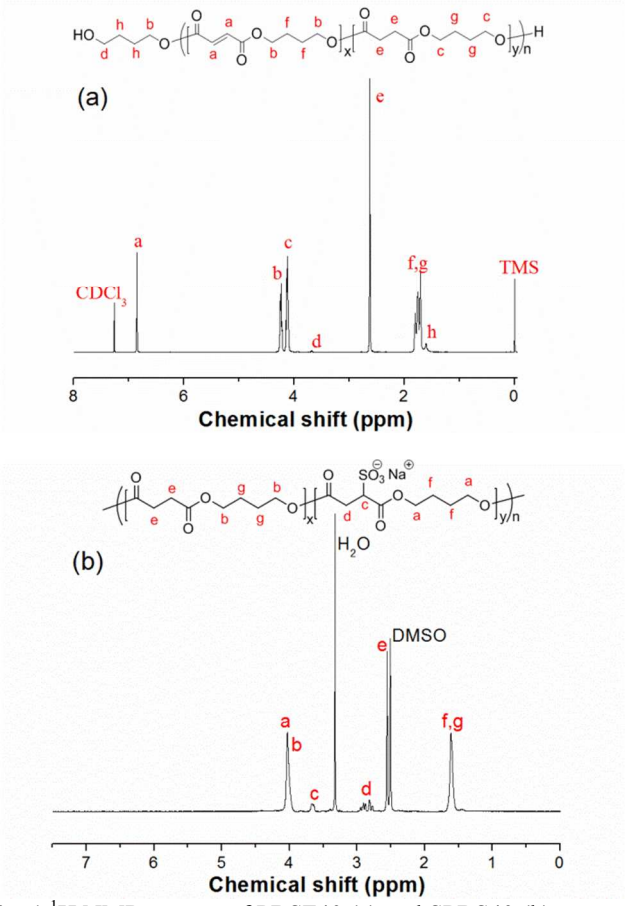


Fig. 1 ^{1}H NMR spectra of PBSF40 (a) and SPBS40 (b).

The chemical structures of synthesized hydroxyl-terminated PBSF were confirmed by ^{1}H NMR spectra. Fig. 1a shows the ^{1}H NMR spectrum and corresponding assignments of PBSF40. The typical signals occurring at 6.85 ppm and 2.62 ppm belong to the unsaturated protons in —C**H**=C**H**— (a) with *E*-conformation on fumarate and saturated protons of —C$\mathbf{H_2}$—C$\mathbf{H_2}$— (e) on succinate, respectively.[26] The comonomer composition of PBSF copolymers was calculated from the integral ratio of H^a to H^e, as summarized in Table 1. The content of butylene fumarate repeating units in PBSF40, for example, calculated to be 39 mol %, is slightly lower than that in feed, demonstrating the lower polymerization activity of fumaric acid compared with succinic acid.

Moreover, no additional resonance signal occurs at 6.31 ppm, the typical signal of protons on —CH=CH— with *Z*-conformation as reported,[31] suggesting that no isomerization of C=C conformation takes place during the melt polymerization.[27] Therefore, it is reasonable to deduce that esterification and polycondensation just proceeded in the way as designed. The average molecular weight and molecular weight distribution of the copolyesters were determined by GPC, and the results were also summarized in Table 1. It can be found that the weight-average molecular weights of PBSF are higher than 5×10^4, which is high enough for application.

Table 1 Composition, average molecular weight of the synthesized polyesters

sample	BS/BF molar ratio		$M_n \times 10^{-4}$ (g mol^{-1})[b]	$M_w \times 10^{-4}$ (g mol^{-1})[b]	M_w/M_n[b]
	feed ratio	resultant ratio[a]			
PBSF20	80/20	80/20	3.61	7.34	2.03
PBSF40	60/40	61/39	4.26	8.43	1.98
PBSF60	40/60	41/59	3.47	5.90	1.69
PBSF80	20/80	21/79	3.35	6.28	1.87
PBSF100	0/100	0/100	2.98	5.01	1.68

[a]Determined by ^{1}H NMR. [b]Determined by GPC.

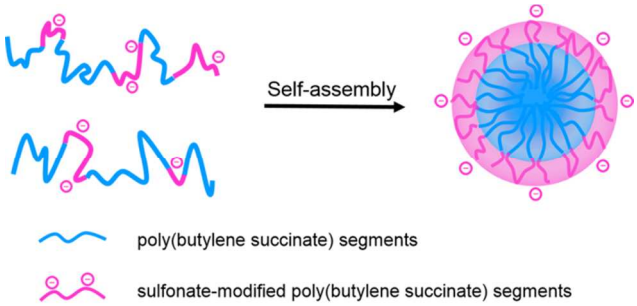
Synthesis and characterization of SPBS

Amphiphilic anionic biodegradable polyesters were synthesized by modification of hydrophobic copolyesters PBSF with hydrophilic sulfonate groups, via addition of sodium hydrogen sulphite to carbon-carbon double bonds on fumarate.[30] The synthesis route is shown in Scheme 2.

The chemical structure of SPBS40 was confirmed by ^{1}H NMR using DMSO-d_6 as solvent. The ^{1}H NMR spectrum and corresponding assignments are presented in Fig. 1b. The signal at 6.85 ppm, attributed to carbon-carbon double bonds on fumarate, completely disappears in the spectrum of SPBS40. And new signals at 2.78-2.95 ppm and 3.64-3.67 ppm with an integral ratio of 2:1 are attributed to the protons of —C**H**$_2$—CH< (d) and >C**H**—SO_3Na (c) of sulfonated succinate moieties. The ^{1}H NMR results confirm the successful synthesis of amphiphilic anionic biodegradable polyesters of SPBS. However, molecular weight and molecular weight distribution of SPBS could not be determined by GPC due to the strong adsorption of sulfonate groups on column.[32,33]

Self-assembly of SPBS and determination of CMC

It is well-known that amphiphilic polymers can self-assemble into nano-objects with different shapes in a selective solvent, which have found tremendous applications in drug delivery system.[34-38] Similarly, it is also found that the synthesized SPBS could self-assemble into micelles in aqueous solution. Self-assembly micelles of SPBS were prepared by dialysis method as described in literature.[39] Scheme 3 illustrates the assumed structure of the micelles.



Scheme 3 Schematic for the self-assembly of sulfonated poly(butylene succinate) micelle in aqueous solution.

The self-assembly behavior of SPBS in aqueous solution was preliminarily confirmed by ^{1}H NMR. Fig. 1S presents ^{1}H NMR spectra for SPBS in DMSO-d_6 and D_2O, respectively. Since both the hydrophobic segments of poly(butylene succinate) and the hydrophilic segments of sulfonated poly(butylene succinate) are soluble in DMSO-d_6, the amphiphilic random copolyesters behave as unimers in the solution. Thus, resonance signals associated with both segments are visible and strong in the ^{1}H NMR spectrum. On the contrary, with a hydrophobic core of poly(butylene succinate) sterically stabilized by a hydrophilic corona of sulfonated poly(butylene succinate), the amphiphilic random copolyesters in D_2O tend to self-assemble into micelles, which limit the segmental motion. Therefore, the resonances of all segments are significantly attenuated. The ^{1}H NMR data provide direct spectroscopic evidence for the formation of the core-corona structure in aqueous solution.

Fluorescence probing technique was employed to further confirm the self-assembly behavior of micelles, using Nile Red as hydrophobic fluorescence probe. Typical fluorescence emission spectra of Nile Red at different concentrations of SPBS20 in aqueous solution are displayed in Fig. 2a. It can be found that the fluorescence intensity enhances dramatically with increasing concentration of micellar solution, indicating the successful formation of micelles and the encapsulation of Nile Red into the hydrophobic core of micelles. Fig. 2b demonstrates the curve of emission intensity at 620 nm as a function of concentration of SPBS20. The CMC of SPBS20 is obtained from the intersection of the two tangent lines as shown in Fig. 2b. For the drug delivery application, CMC is a very important parameter describing the thermodynamic stability of the micelles. According to Rapoport, intravenous injections of micellar solutions are usually associated with extreme dilutions, e.g. 25-fold dilution at bolus injection, by circulating blood.[40] CMC of polymer micelles is much lower than that of surfactant micelles. The low CMC prevents micelles from being prematurely destroyed before they reach the targets. According to Fig. 2b, the CMC of SPBS20 is around 0.057 g L^{-1}, and it is a relative low value and comparable to that of amphiphilic block copolymer.[35] As mentioned above, it is desired in the field of drug delivery. The SPBS with more than 40% content of sulfonate groups show greater hydrophilicity, which leads to an unstable structure of micelles and rapid release of Nile Red. Thus, their CMC and size were not measured.

Table 2 Characterization of SPBS micelles

nanoparticle	CMC (g L^{-1})	D_h[a] (nm)	D_{TEM}[b] (nm)	PDI[a]	Zeta potential in water (mV)[a]
SPBS20	0.057	154 ± 9	41 ± 3	0.081 ± 0.015	-39.9 ± 1.1
SPBS40	0.069	169 ± 6	47 ± 3	0.066 ± 0.019	-34.8 ± 1.8

[a]Mean ± standard deviation, determined by DLS. [b]Determined by TEM.

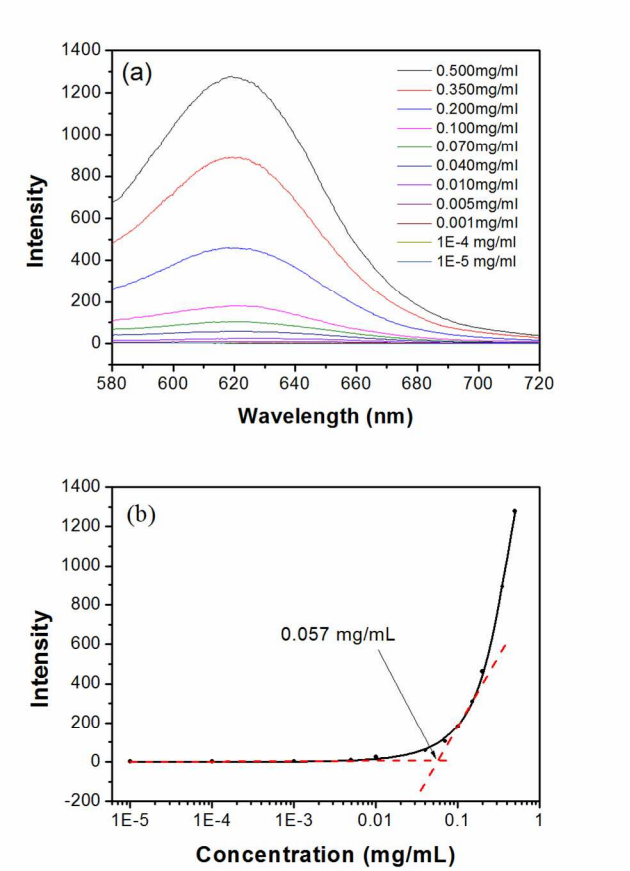


Fig. 2 Fluorescence emission spectra of Nile Red in SPBS micelles of varying concentrations (a) and plot of the emission intensity at 620 nm as a function of concentration of SPBS micelles (b).

The hydrodynamic diameters (D_h) and surface charges of the SPBS micelles were investigated by dynamic light scattering. As depicted in Fig. 3a and Table 2, the D_h of SPBS20 and SPBS40 micelles are 154 ± 8 nm and 169 ± 5 nm, respectively. Besides, the polydispersity of SPBS micelles is very low (PDI<0.1). It indicates that micelles are near uniform distributed in aqueous solution. The measurement results of Zeta potential for SPBS20 (-39.9 ± 1.1 mV) and SPBS40 (-34.8 ± 1.8 mV) suggest that the particle surfaces are negatively charged. The negative charges on particle surfaces, arising from sulfonate groups, may protect nano-particles against aggregation and reduce the interaction with serum components thus prolong circulation time of nano-particles *in vivo* and facilitate their accumulation at the tumor sites.

The morphology of micelles prepared from aqueous solution of SPBS was further investigated by TEM and the results are shown in Fig. 3b. It can be found that SPBS40 micelles appear as a spherical morphology with average diameter of 47 ± 3 nm. All the results above confirm the successful formation of stable SPBS micelles in aqueous solution.

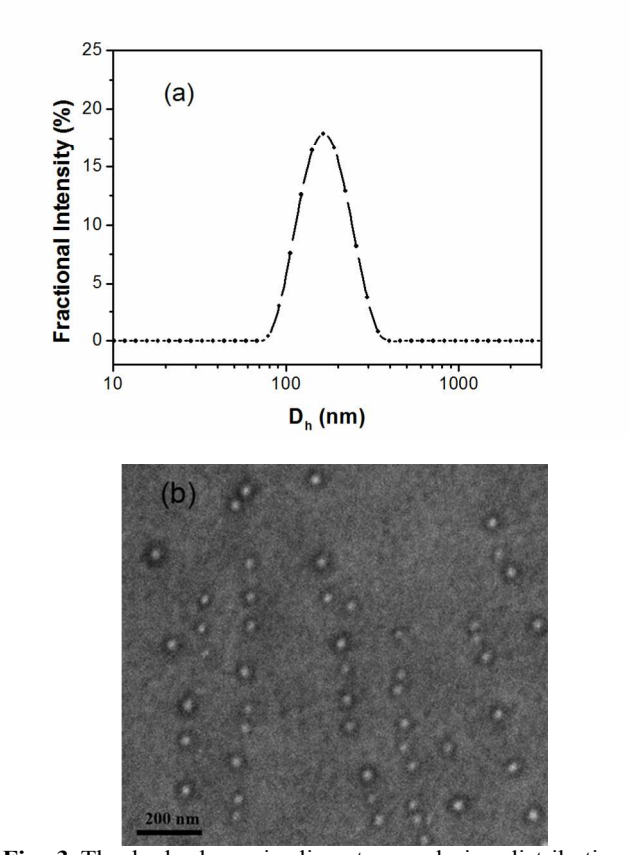


Fig. 3 The hydrodynamic diameters and size distributions of SPBS20 micelles determined by DLS (a) and TEM micrograph of SPBS40 micelles (b).

In vitro cytotoxicity of SPBS micelles

The cytotoxicity of the novel anionic copolymers was evaluated by MTT assay against 293T cell. Fig. 4 shows the viabilities of the 293T cells after treated with different concentrations of SPBS micelles for 24 h. Over 85% cells are viable with micelle concentrations from 1 μg mL^{-1} to 100 μg mL^{-1} for SPBS20–100. Even at a high copolymer concentration of 1000 μg mL^{-1}, the viabilities of 293T cells are still higher than 75% for SPBS40–100. As far as SPBS20 is concerned, the viabilities decrease to 53%. These results imply that the introduction of sulfonate groups indeed enhances the biocompatibility of aliphatic copolyesters. The novel anionic copolymers are relatively

nontoxic to the cells and possess excellent biocompatibility. All the above findings provide strong evidences that the novel biodegradable anionic copolyesters of SPBS have great potential for drug delivery applications.

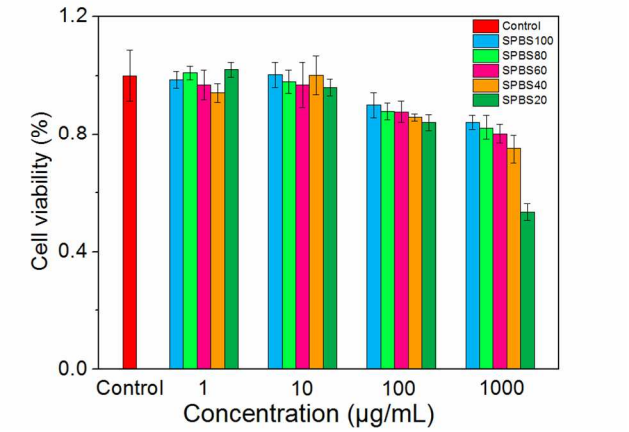


Fig. 4 Cytotoxicity of SPBS micelles with different concentrations after incubation for 24 h.

Conclusions

In summary, a facile and versatile strategy to synthesize anionic polyesters bearing sulfonate groups is presented. Novel amphiphilic anionic copolyesters composed of biodegradable backbone have been successfully synthesized through esterification and polycondensation of succinic acid, fumaric acid, 1,4-butanediol, and subsequent modification of hydrophobic copolyester backbone with hydrophilic sulfonate groups. The content of hydrophilic sulfonate groups could be facilely regulated. The resulting amphiphilic copolymers are able to self-assemble into micelles in aqueous solution with relatively low CMC (0.057–0.069 g L^{-1}) and average size of 154–169 nm. The micelles formed by amphiphilic copolymers are negatively charged on corona. The negative charges on the corona provide strong electrostatic repulsion between micelles and make the micelles stable in aqueous solution, which is very useful for drug delivery application. Cell viability test shows that SPBS micelles possess excellent biocompatibility. The novel amphiphilic anionic biodegradable copolyesters are highly promising as drug delivery carriers for cancer therapy. Further studies concerning modification via targeting ligands and *in vitro* drug release are now under investigation in our lab.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Typical procedure for the synthesis of poly(butylene succinate-co-butylene fumarate) (PBSF) copolyester in bulk; Micelles preparation and their encapsulation of Nile Red; ^{1}H NMR spectra of SPBS40 in DMSO-d_6 and D_2O. See DOI: 10.1039/b000000x/

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