Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information

Synergetic enhancement of antitumor efficacy with charge-reversal and reduction-sensitive polymer micelles

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Experimental

Materials

Dicyclohexyl carbodiimide (DCC, ≥95%), triethylamine (TEA), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), cuprous bromide (CuBr, ≥98.5%) and 2,2'-bipyridine (BPy, ≥99.5%) were purchased from Sinopharm Chemical Reagent Co., Ltd. 2,2'-Dithio glycol (SS-DOH, 90%) was purchased from Acros Company. Dimethylaminopyridine (DMAP) were purchased from J&K scientific Co., Ltd. 2-Bromo-2-methyl-propionic acid (98%) was purchased from Sahn Chemical Technology (Shanghai) Co., Ltd. Stannous octoate ($Sn(Oct)_2$, 95%) was purchased from Shanghai Aladdin biological technology Co.,Ltd. D,L-Lactide (D,L-LA, ≥99.0%) was purchased from Shanghai Dibo Chemical Technology Co., Ltd. Dithiothreitol (DTT, >98%) was purchased from Merck. Doxorubicin hydrochloride (DOX.HCl) was purchased from Shanghai Yingxuan Company. 2-Aminoethanol hydrochloride (98%), 2,3-dimethyl-maleic anhydride (DMMA, 96%) and succinic anhydride (SA, 96%) were procured from TCI (China).

DMEM (Dulbecco's Modified Eagle's Medium) medium, trypsin, L-glutamine, double-antibody (penicillin -streptomycin) and Hochest 33258 were purchased from Gibco Company. FBS (Fetal bovine serum, FBS) and Bovine serum albumin (BSA)

were purchased from Hyclone Company. MTT was purchased from Sigma. Human cervical carcinoma cells (HeLa) were procured from China Center for Type Culture Collection (Wuhan), which were cultured in DMEM in the presence of 5% CO_2 at 37°C before used. Rats were purchased from Centre-south Hospital of Wuhan University. Confocal (Bodega dish) (diameter 20 mm) was purchased from Nest Company.

HO-SS-iBuBr, HO-CC-iBuBr and 2-aminoethyl methacrylate hydrochloride (AEMA) were synthesized according to the previous literatures [1-3, 4].

SD rats (250 g) were from the Laboratory Animal Centre, Wuhan University. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and the procedures were approved by the Wuhan University of China Animal Care and Use Committee.

Characterization

^1H NMR was detected by Mercury VX-300 type NMR spectrometer at 300 MHz, $CDCl_3$ or DMSO-d_6 as a solvent, tetramethylsilane (TMS) as the internal standard. Gel permeation chromatography (GPC) measurements were carried out by using a Waters 2690D HPLC equipped with Styragel HR3 column. Samples were detected with a Waters 2410 differential refractive index detector. DMF was used as the mobile phase at a flow rate of 0.3 mL min-[1]. The column temperature was 40 °C. Poly(methyl methacrylate) was used as the standard. Fluorescence properties were measured by Hitachi F-4500 fluorescence spectrometer. The absorbance of hemoglobin at 540 nm was detected by Perkin-Elmer Lambda 35 UV/VIS Spectrophotometers. The size and distribution of polymeric micelles were detected by Malvern Zetasizer Nano ZS. The morphology of the polymer micelles were investigated by JEM-100CX II transmission electron microscopy with an accelerating voltage of 100 KV. The cytotoxicity was tested through microplate reader (Bio-Rad 550, Hercules, CA, USA) at 570 nm. Cell fluorescence photo was taken by EZ-C1 (Nikon, Japan) confocal laser scanning microscopy (CLSM). Intracellular fluorescence intensity was measured by flow cytometry (CyAN-ADP, Beckman).

Synthesis of PLA-SS-iBuBr and PLA-CC-iBuBr

PLA-SS-iBuBr was synthesized by ring opening reaction as follows: HO-SS-iBuBr (90.1 mg, 0.3 mmol), D,L-LA (3.0 g, 20.8 mmol), $Sn(Oct)_2$ (0.4 mL, 0.04 mmol) and toluene (2 mL) were added to a 25 mL Schlenk flask. The resulting mixture was completely dehydrated and deoxygenated through freeze-pump-thaw cycles for three times. In the presence of argon, the mixture kept stirring for 24 h at 120 °C in oil bath. Then the mixture was cooled and exposed to air to stop the reaction. The product was purified by precipitation in methanol (D,L-LA can dissolve in methanol) and then dried in a vacuum oven overnight at 40 °C with a yield of 86%.

The synthesis method of PLA-CC-iBuBr is similar to that described above. The white powdery product was obtained with a yield of 81%.

Synthesis of PLA-SS-PAEMA and PLA-CC-PAEMA

Synthesis of PLA-SS-PAEMA was conducted by ATRP reaction as follows: PLA-SS-iBuBr (0.5 g, 0.114 mmol), AEMA (0.564 g, 3.42 mmol), bipyridine (34.97 mg, 0.228 mmol), and THF/H_2O (2mL, 1:1) were added to a 25 mL schlenk flask. Then CuBr (15.97 mg, 0.114 mmol) was added to the above mixture which was deoxygenated through three times freeze-pump-thaw cycles before. Under argon atmosphere, the polymerization started at 50 °C in oil bath after the mixture thawed. The reaction was terminated by cooling and exposing the mixture to air after 8h. The copper complex was removed by passing the polymer solutions through a basic alumina column. The pure product could be obtained by precipitation in the mixture of hexane/ethyl acetate (1:1, v/v) for three times and then dried in a vacuum oven at 40 °C for 48 h.(yield, 61%).

PLA-CC-PAEMA was synthesized in accordance with the method described above. PLA-SS-iBuBr would be substituted for PLA-CC-iBuBr during the synthesis and then the target product could be gotten with a yield of 65%.

Synthesis of PLA-SS-PAEMA/DMMA , PLA-CC-PAEMA/DMMA and PLA-SS-

PAEMA/SA

The synthesis procedures of PLA-SS-PAEMA/DMMA an**d PLA-SS-PAEMA/SA** were described as follows: Excess amount of DMMA (431.3 mg, 3.42 mmol) or SA (342.2 mg, 3.42 mmol), PLA-SS-PAEMA (640 mg), triethylamine (1 mL) and pyridine (1 mL) were dissolved in DMSO (10 mL). Then, the resulting mixture kept stirring for 24 h at 40 °C. The purification of the products were conducted by the dialysis in deionized water (MW cut off 3500). PLA-SS-PAEMA/DMMA or PLA-SS-PAEMA/SA could be obtained by lyophilization with the yield of 68% and 57%, respectively.

PLA-CC-PAEMA/DMMA could be synthesized in a similar method with a yield of 72%.

Critical micelle concentration (CMC)

The critical micelle concentration (CMC) was measured by a fluorescence method. Pyrene was chosen as the fluorescent probe to measure the CMC of the amphiphilic polymers, which exhibited selective distribution between the hydrophilic and hydrophobic phase. The polymer solution was diluted to a serial of different concentrations of polymeric aqueous solution, which ranged from 3.3×10^{-6} to 0.5 mg/mL. Then 3 mL of the resulting solutions with various concentrations were separately added into the vials containing pyrene. The final concentration of pyrene was fixed 6.26×10^{-7} mol/L). After that the samples stayed in dark for 24 h at room temperature. The emission spectra of pyrene in each sample were detected by fluorescence spectrometer with the excitation wavelength of 334 nm and slit width of 5 nm. The emission spectra within 350-450 nm were recorded. The CMC could be obtained according to the curve of fluorescence intensity ratio (I_{394} / I_{378}) against the logarithm of the polymeric micelle concentration.

Preparation of polymeric micelles

The polymer micelles were prepared by the dialysis method. Briefly, the polymer (20 mg) was first dissolved in 2 mL DMSO. Then deionized water (5 mL) was added to

the resulting organic solution slowly with vigorous stirring. The solution kept stirring overnight and then was transferred into dialysis tube (MW cut off 3500). The organic solvent was removed by further dialysis against deionized water for 24 h with the exchange of fresh water every 3 h. The obtained polymeric micelles were characterized by DLS and TEM and taken for further study.

DOX-loaded polymer micelles were also prepared by the method described above. Polymer (20 mg), DOX.HCl (4 mg) and twice the number of moles of TEA were dissolved in 2 mL DMSO. The mixture continued to stirring overnight after deionized water (5 mL) was dropwise added. The DOX-loaded polymeric micelles were obtained after the resulting mixture solution was dialyzed against deionized water for 2 days with refreshing water every 3 h.

Transmission electron microscopy (TEM)

The morphologies of the polymer micelles were investigated by JEM-100CX II transmission electron microscopy with an accelerating voltage of 100 KV. In order to prepare the TEM samples, a drop of the polymeric micelles solution was deposited onto a carbon-coated copper grid and then the water evaporated slowly in air. Subsequently, the prepared films on the grid were stained with phosphomolybdic acid (1 % w/v) for 30 s. Then the samples could be photographed after dried by airing.

Charge-conversion and reduction-sensitivity

To detect surface charge-conversion of the polymeric micelles, the micelle solutions were diluted to 0.1 mg/mL with the PBS of pH 7.4 or 6.5, respectively, and then incubated at 37 °C. The zeta potentials of the resulting solutions were monitored by Malvern Zetasizer Nano ZS at the designed time intervals.

Protein adsorption

In order to investigate the effect of different pH values (pH 5.0, 6.5 and 7.4) on protein adsorption of the polymeric micelles, bovine serum albumin (BSA) was used as the model protein. The polymer micelles were separately incubated with BSA in

PBS buffer with different pH values (0.01 M, pH 5.0, 6.5 and 7.4). The final concentration of the polymeric micelles and BSA were fixed at 0.20 and 0.25 mg/mL, respectively. After incubation at 37 °C for designed time interval, the aliquots of samples were withdrawn and centrifuged at 16000 rpm for 15 min to precipitate the BSA protein adsorption micelles. The supernatant were collected for the quantitative analysis of the BSA concentration. The commercial BCA Protein Assay Kits were applied to analyze the content of BSA in the resulting supernatant. The supernatant (20 μL) and BCA reagent (200 μL) were added into the 96-well plate with co-incubation at 37 °C for half an hour. The adsorption intensities of the samples were detected by the micro plate reader at 570 nm. Then the residual concentrations of BSA in the samples were calculated according to a prepared standard calibration curve.

Hemolysis assay

The blood compatibility of the polymeric micelles was evaluated by hemolysis assay according to the previous literatures[5-6]. Firstly, the fresh whole blood obtained from rats was collected in anticoagulation tubes containing heparin. Then the resulting plasma and buffy coat were removed after the whole blood was centrifuged at 750 rpm for 10 min, obtaining the red blood cells washed with PBS 7.4 for three times. Subsequently, the red blood cells were resuspended in PBS 7.4 at 2% hematocrit. Then 0.2 mL polymeric micelles with various concentrations were separately added in the tubes, which contained 0.8 mL 2% red blood cells suspension and 1.0 mL PBS 7.4. The deionized water and PBS 7.4 were utilized as the positive control and negative control, respectively. After incubation for 2 h at 37 °C, the above mixtures were centrifuged at 1500 rpm for 15 min. Finally, the supernatant was separately withdrawn for analysis of the hemolysis by detecting the absorbance of hemoglobin at 540 nm using UV absorption spectroscopy. The hemolysis rate was calculated using the following equation:

$$\text{Hemolysis (\%)} = (A_{Sample} - A_{Negative}) / (A_{Positive} - A_{Negative}) \times 100$$

A_{Sample} refers to the absorbance of sample. $A_{Positive}$ and $A_{Negative}$ represent the

absorbance of the positive control and negative control, respectively.

In vitro drug release

The release of DOX from the polymer micelles was investigated in PBS buffer with different pH and different DTT concentration. 1 mL of DOX-loaded polymer micelles solution was transferred into dialysis tube (MW cutoff 3500) and then immerged into 30 mL of PBS buffer. After that they were incubated at 37 °C in an incubator shaker (100 rpm). At the designed time intervals, 5 mL of the external buffer of each sample was withdrawn and subsequently supplemented by equivalent fresh buffer. The concentrations of released DOX were measured by fluorescence spectrometer with the excitation wavelength of 485 nm, emission wavelength of 590 nm and slit width of 3nm. On the basis of the pre-established calibration curve, the cumulative amounts of the released drug were calculated. The tests of each sample were repeated for three times for the statistical evaluation of results.

In vitro cytotoxicity

MTT assay was used to investigate the cytotoxicity of DOX-free micelles and DOX-loaded micelles against HeLa cells at pH 6.5 and 7.4. Briefly, HeLa cells were seeded in 96-well plates with the density of 10000 cells per well in 100 μL DMEM medium of pH 7.4 or 6.5, respectively. In the presence of 5% CO_2 atmosphere, the resulting plates were incubated for 24 h at 37 °C. Then the culture media were replaced with fresh media at different pH containing various concentrations of DOX-free micelles or DOX-loaded micelles. The cells were kept incubation for 48 h at 37 °C. Then 20 μL of MTT (5mg/mL) was added to each well and subsequently incubated for 4 h. After that, the media were removed and 150 μL of DMSO were added to each well to dissolve the produced formazan crystals. The absorbance of the solution in each well was detected at 570 nm using a micro plate reader. The cell viability was calculated by the equation:

Cell viability = $100 \times (A_{test} - A_0)/(A_{control} - A_0)$

A_{test} and $A_{control}$ refer to the mean absorbance of the treatment groups and the control

groups, respectively. A_0 represent the mean absorbance of the cells without MTT.

CLSM observation

HeLa cells were seed in the confocal laser dishes (diameter = 20 mm) at the proper density in 2mL DMEM (pH 7.4) and incubated for 24 h at 37 °C. After incubation, the culture media were replaced by fresh DMEM media with pH 6.5 or 7.4 containing DOX-loaded micelles with final DOX concentration of 2.0 μg/mL in each dish. At the designed time intervals (4 h or 24 h), the media were removed and washed with PBS 7.4 for three time. Then the cells were fixed with 4 w/v% formaldehyde for 10 min. After that, the cell nuclei were stained with Hochst 33258 for another 10 min. Finally, the samples were observed by CLSM with excitation at 408 and 488 for Hoechst 33258 and DOX, respectively.

Flow cytometry

HeLa cells were seed in 6-well plates at the proper density in 2 mL DMEM (pH 7.4) and incubated for 24 h at 37 °C in 5% CO_2 humidified atmosphere. After that the original media were removed and washed with PBS for three times and replaced by fresh DMEM media with pH 6.5 or 7.4 containing DOX-loaded micelles with the final DOX concentration of 2.0 μg/mL in each well. The resulting cells were incubated at 37 °C for 4 h or 24 h, and then washed with PBS for three times. Subsequently the cells were harvested by trypsin treatment. The collected cells were suspended in PBS and centrifuged at 1000 rpm for 10 min. Then the supernatants were removed and the cells were washed. The above washing and centrifugation procedure was repeated for another two times. After that the cells were suspended in 500 μL of 4 w/v% formaldehyde and detected by flow cytometry. The results were analyzed using Flow Jo 7.6 software.

CLSM Observation of Endocytosis Inhibition.

Firstly, HeLa cells were seeded in confocal laser dishes at a density of 3×10^4 cells/dish in 2 mL DMEM and incubated for 24 h at 37 °C. After that three kinds of

endocytosis inhibitors were pre-cultured with the cells for 30 min at proper concentrations (5 mM MBCD, 5 μM cytochalasin D and 0.45 M sucrose). Then the above media were replaced by fresh DMEM containing the DOX loaded PLA-SS-PAEMA/DMMA micelles with the final drug concentration of 2.0 μg/mL in each dish at different pH values (pH 7.4 or pH 6.5). After incubation for 24 h, the resulting cells were treated and observed similarly.

Flow Cytometry Analysis of Endocytosis Inhibition.

HeLa cells were seed in 6-well plates at a density of 4×10^4 cells/well in 2 mL DMEM. Which were incubated for 24 h at 37 °C in 5% CO_2 atmosphere. Subsequently, three different endocytosis inhibitors were separately added to above wells at the safe concentrations and cultured for 30 min. Then the above media were replaced by fresh DMEM containing the DOX loaded PLA-SS-PAEMA/DMMA micelles with the final drug concentration of 2.0 μg/mL in each well at different pH values. After incubation for 24 h, the resulting cells were treated and measured similarly.

Table S1 Properties of DOX-free and DOX-loaded polymeric micelles

Sample	DOX-free micelles			DOX-loaded micelles at 10:2 (w/w)[c]			
	Size [a] (nm)	PDI [a]	CMC [b] (μg/mL)	Size [a] (nm)	PDI [a]	DLC (%)	EE (%)
PLA-SS-PAEMA/DMMA	100	0.123	6.0	109	0.103	10.6	59.5
PLA-CC-PAEMA/DMMA	92	0.186	5.0	107	0.104	9.8	54.1
PLA-SS-PAEMA/SA	101	0.163	6.3	137	0.114	10.2	56.5

[a] Micelle size was measured by DLS.
[b] Measured using pyrene as the fluorescence probe.
[c] Feed ratio of polymer to DOX.

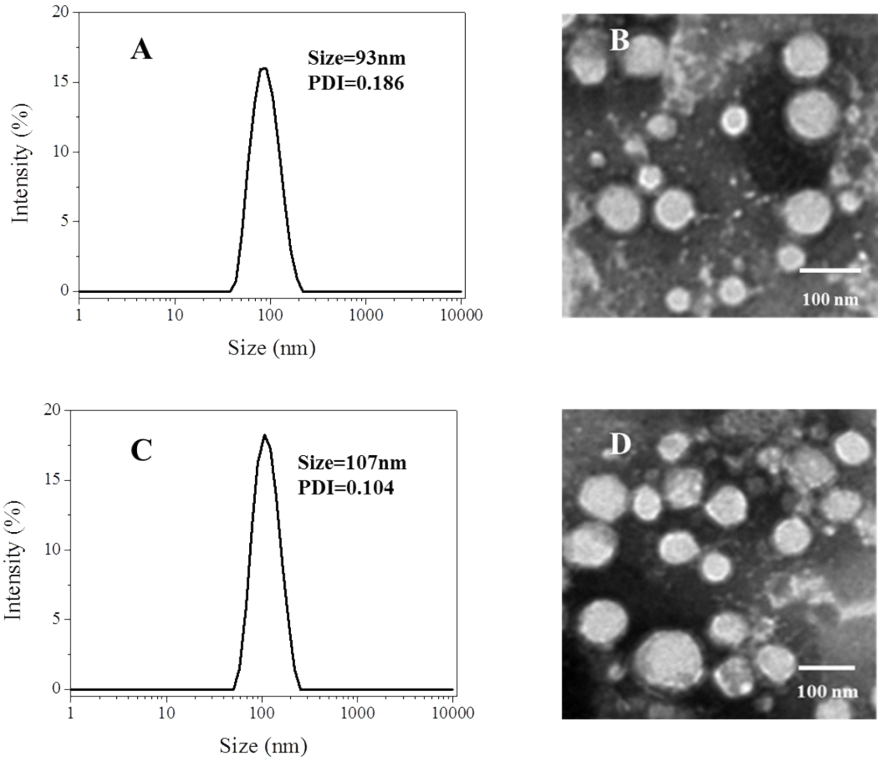

Fig. S1 Size distribution of the blank micelles (A) and drug-loaded micelles (C) of the polymer PLA-CC-PAEMA/DMMA, measured by DLS analysis. TEM images of the blank micelles (B) and drug-loaded micelles (D) of the polymer, scale bar: 100 nm.

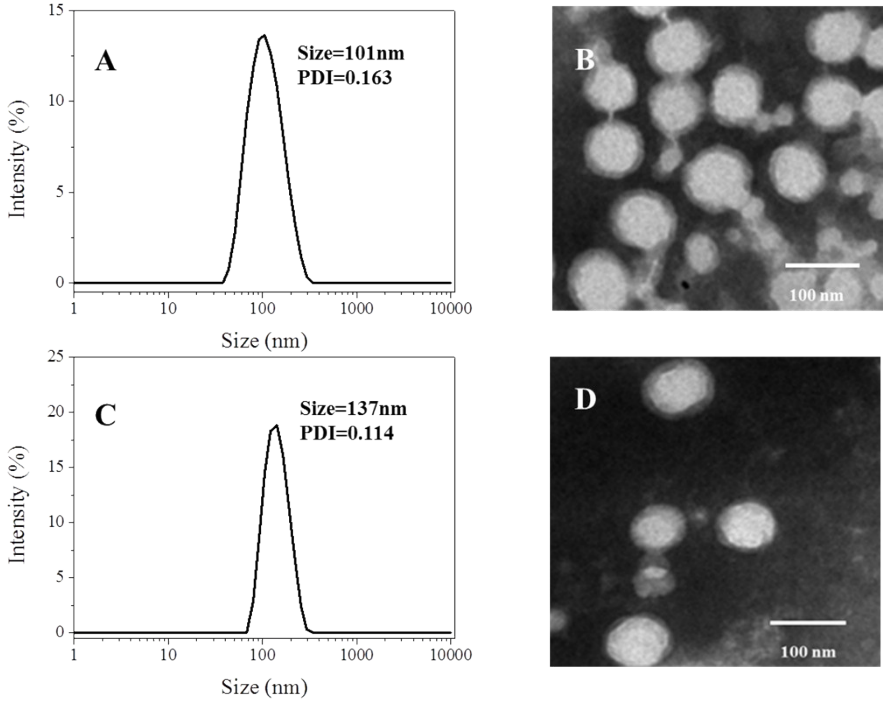

Fig. S2 Size distribution of the blank micelles (A) and drug-loaded micelles (C) of the polymer PLA-SS-PAEMA/SA, measured by DLS analysis. TEM images of the blank micelles (B) and drug-loaded micelles (D) of this polymer, scale bar: 100 nm.

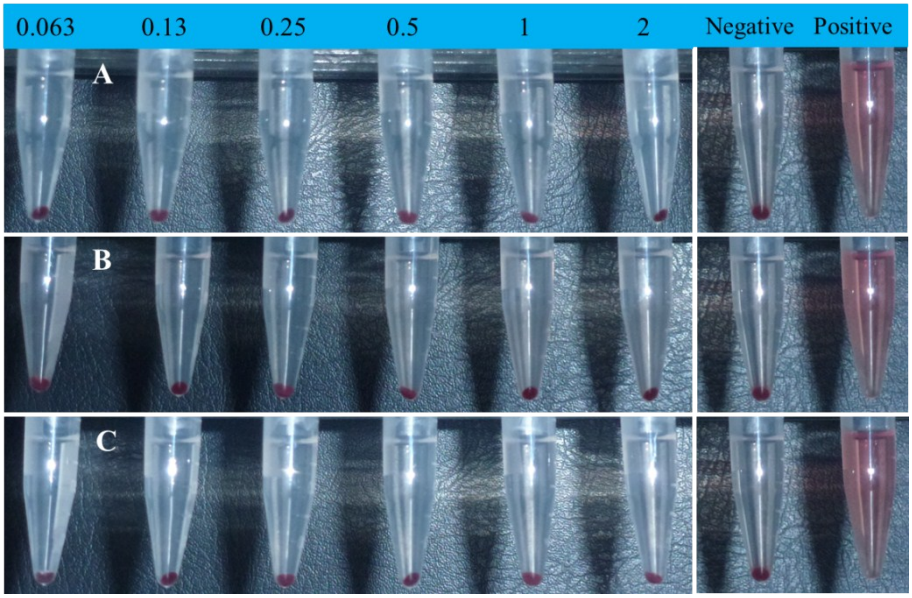

Fig. S3 The optical photographs of hemolysis assay for red blood cells treated with polymeric micelles of varying concentrations (mg/mL), compared to the positive control (deionized water) and negative control (PBS 7.4). A: PLA-SS-PAEMA/DMMA, B: PLA-CC-PAEMA/DMMA, and C: PLA-SS-PAEMA/SA.

References

1. B. K. Sourkohi, A. Cunningham, Q. Zhang and J. K.Oh，*Biomacromolecules,* 2011, **12**, 3819.
2. N. R. Ko, K. J. Yao, C. B. Tang and J. K.Oh, *Polym. Chem.*, 2013, **51**, 3071.
3. Q. Zhang, S. Aleksanian, S. M. Noh and J. K. Oh. *Polym. Chem*., 2013, **4**, 351.
4. R. Narain and S. P. Armes, *Biomacromolecules*, 2003, **4**, 1746.
5. C. Altmeyer, T. K. Karam, N. M. Khalil and R. M. Mainardes. *Mat. Sci. Eng. C*. 2016, **60**, 135.
6. Y.Y. Guo, P. Zhang, Q. Y. Zhao, K. M. Wang and Y. X. Luan. *Macromol. Biosci.*, 2016, **16(3)**, 420.