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PAPER

Disulfide cross-linked cholic-acid modified PEG-poly(amino acid) block copolymer micelles for controlled drug delivery of doxorubicin

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Reduction responsive biodegradable core-cross-linked micelles are developed form lipoic acid (LA) and cholic acid (CA) decorated poly(ethylene glycol)-*b*-poly($_L$-glutamic acid) (PEG-pGlu(EDA-LA)-CA) block copolymers and investigated for intracellular doxorubicin (DOX) release. The amphiphilic polymers can self-assemble into nano-sized core-shell micelles that are easily cross-linked in the presence of a catalytic amount of dithiothreitol (DTT). The cross-linked micelles (CLM) show excellent stability against extensive dilution and high salt concentration but rapid dissociation and drug release in reductive environments. Confocal laser scanning microscopy further demonstrates that DOX was delivered and released into the nuclei of HeLa cells following 8 h incubation with DOX-loaded CLM. MTT assays reveal that DOX-loaded CLM had similar anti-tumor activity as non-cross-linked micelles (NCLM) for HeLa cells following 48 h incubation, while blank micelles were practically nontoxic up to a tested concentration of 1.0 mg·mL^{-1}. These reduction responsive core-cross-linked micelles have great potential for drug delivery in cancer chemotherapy.

Introduction

Over the past few decades, polymeric micelles assembled from amphiphilic block copolymers have attracted significant attention in the field of drug delivery.[1,2] These nanocarriers offer several unique features, such as enhancing the aqueous solubility and bioavailability of antitumor drugs, prolonging the circulation time, preferential accumulation at the tumor sites by the enhanced permeability and retention (EPR) effect, and reducing systemic side effects. However, a limitation with self-assembled micelles is their low structural stability in *vivo*, resulting in disassociation of the micelles and premature drug release. A powerful approach to circumvent this limitation is to stabilize micelles by covalent cross-linking in shell or core of micelles.[3,4] Reversing the cross-linking with stimuli-responsive linkages has attracted great interest, and some stimuli-responsive cross-linked micelles have been reported. These stimuli responsive linkages have been designed to be responsive to a change in the environment, such as temperature, ionic strength, pH, enzyme, or light.[5-7] For example, Liu's group reported the first example of thermo-responsive core cross-linked micelles for selective rationmetric fluorescent detection of Hg^{2+} ions based on well-defined double hydrophilic block copolymer.[8] Chen's group prepared pH-sensitive micelles based on PEG-*block*-acetalated-dextran (PEG-*b*-AC-Dex) and used them for acid-triggered intracellular release of anticancer drug DOX.[9] Hu reported photo-triggered release of caged camptothecin prodrugs from dually responsive shell cross-linked micelles based on two types of amphiphilic diblock copolymers of P(CL-*g*-CPT)-*b*-P(OEGMA-*co*-MAEBA)-CPT and PCL-*b*-P(OEGMA-*co*-MAEBA-*co*-FA).[10] Covalent cross-linking can enhance the stability of micelles and control the drug release by using stimuli-responsive linkages.

Among them, cross-linking with disulfides is an area of great importance and can be used for bioactive agents including drugs, genes, and proteins.[11-13] This kind of cross-linking is based on the thiol-disulfide exchange reaction which plays an important role in the metabolic activity of living cells. Glutathione (GSH) is the most abundant thiol-containing tripeptide in living cells with a concentration around (0.5-10)×10^{-3} M. In contrast, the extracellular concentration of GSH is very low (approximately (2-20)×10^{-6} M). Furthermore, it was also reported that the concentration of GSH in tumor tissues is higher than that in normal tissues.[14] Taking advantages of the dramatic variation of GSH concentration, the drug delivery systems containing disulfide bonds would be very stable in the extracellular environment and could rapidly release the therapeutic agents intracellularly through cleavage of the disulfide bonds by GSH.

Recently, various methods have been developed for the formation of disulfide-cross-linked micelles, including using disulfide-containing cross-linkers,[15,16] oxidizing free thiol groups in shell or core of polymer block,[17,18] and using lipoic acid (LA) conjugates.[19-22] Using LA conjugates to get disulfide-cross-linked micelles is more favorable because the five-member ring of lipoic acid is apt to ring-opening polymerization catalyzed by catalytic

amount of DTT under aqueous condition. In addition, LA is a naturally occurring compound produced by human body. Zhong and other groups reported some reduction-responsive cross-linked nanocarriers with disulfide bonds based on LA conjugates, such as poly(ethylene glycol)-poly(ε-caprolactone) that contains two lipoyl functional groups at their interface (PEG-L_2-PCL),[23] dextran-lipoic acid derivatives (Dex-LA),[21] hydrophobic modification of 1.8 kDa PEI with lipoic acid (PEI-LA),[24] water-soluble poly(ethylene glycol)-*b*-poly(*N*-2-hyaroxypropyl methacrylamide)-lipoic acid (PEG-*b*-PHPMA-LA) block copolymers conjugates,[20] lipoic acid and cis-1,2-cyclohexanedicarboxylic acid (CCA) decorated poly(ethylene glycol)-*b*-poly ($_L$-lysine) (PEG-P(LL-CCA/LA) block copolymers,[25] and starch-*g*-poly(ethylene glycol) (starch-*g*-PEG) modified with LA.[19] These studies demonstrate that disulfide-cross-linking method can elegantly resolve the problem caused by dissociation upon dilution and intracellular drug delivery.

Polymeric micelles self-assembled from amphiphilic block polymer poly(ethylene glycol)-poly(amino acids) (PEG-PAA) are effective vehicles for drug and gene delivery in biomedical application, which has been proved by some studies that both PEG and PAA components are the most facile components for the design of micelles. [26,27] Our aim is to develop new nanocarriers based on PEG-PAA block polymers which was modified with small moleculars to introduce resposibility and also improve the stability and drug loading content.

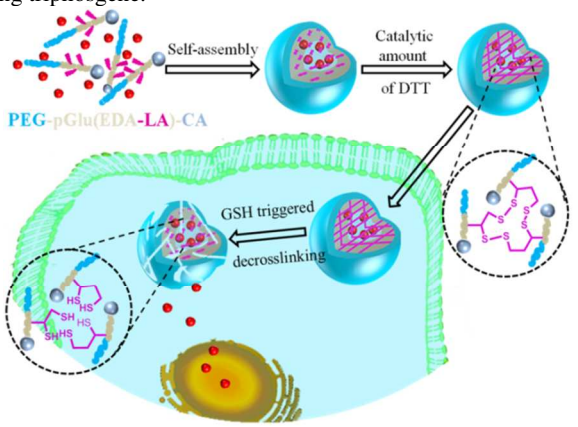
In this work, we design reduction-sensitive reversibly core-cross-linked micelles based on cholic acid (CA) and lipoic acid decorated poly(ethylene glycol)-poly($_L$-glutamic acid) (PEG-pGlu(EDA-LA)-CA) block copolymers for active loading and triggered intracellular release of DOX. PEG and poly($_L$-glutamic acid) (PBLG) were selected as building blocks for our reversibly cross-linked micelles due to their inherently non-cytotoxicity, immunogenicity, and biodegradability, which had been practically used for efficient intracellular delivery of antitumor drugs such as DOX, paclitaxel (PTX), 4-amino-2-trifluoromethyl-phenyl retinate (ATPR) and camptothecin.[28-31] PEG-pGlu(EDA-LA)-CA could self-assembled into micelles with the PEG shell and the PBLG inner core. The PEG shell can stabilize the micelles in blood circulation and reduce uptake at reticuloendothelial sites, and the PBLG core is a reservoir of hydrophobic anticancer drugs and can be cross-linked by introducing lipoic acid. We use lipoic acid to achieve reduction sensitivity, which can be easily cross-linked in the presence of a catalytic amount of DTT. Cholic acid is a main bile acid produced in the human and has been successfully applied in drug delivery.[32, 33] We integrated CA to improve drug loading content because CA can form part of the micelle core to encapsulate hydrophobic drugs.[34] As far as we know, PEG-PAA micelles which have enhaced stability, improved drug loading content and responsive intracellular drug release have not been reported. The resulting disulfide-cross-linked micelles while possessing enhanced stability under the extracellular environment, which are prone to rapid de-cross-linking and drug release under an intracellular reductive condition.

Experimental section

Materials

α-Methoxy-*ω*-amine-poly(ethylene glycol) (*m*PEG-NH_2, M_n = 5000 g mol^{-1}) was supplied by Suzhou PegBio Co., Ltd., *γ*-benzyl-$_L$-glutamate (BLG) was purchased from GL Biochem Ltd., cholic acid (CA, 98%) and 1,3-dicyclohexyl carbodiimide (DCC, 99%) were supplied by Alfa Aesar, lipoic acid (LA, 98%) was from Acros, 1,4-dithio-$_{D,\ L}$-threitol (DTT, 99%) was supplied by Merck, *N*-hydroxysuccinimide (NHS, 98%) was purchased from Sigma-Aldrich, doxorubicin hydrochloride (DOX·HCl, 99%) was purchased from Beijing Zhongshuo Pharmaceutical Technology Development Co., Ltd.. Triphosgene was purchased from Shanxi Jiaocheng Jingxin Chemical Factory and re-crystallized with chloroform prior to use. Tetrahydrofuran (THF) was dried by refluxing over sodium wire and distilled prior to use. Methylene dichloride (DCM), *N,N*-dimethyl formamide (DMF), ethylenediamine, triethylamine were refluxed by CaH_2 and distilled before use.

Roswell Park Memorial Institute medium (RPMI-1640, Thermo Fisher Scientific), fetal bovine serum (FBS, Gibco), 24-well and 96-well plates were obtained from Corning Costar (Shanghai, China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Biosharp) , 4',6-diamidino-2-phenylindole (DAPI, Roche). All other chemicals were used as received. *γ*-benzyl-$_L$-glutamate N-carboxy-anhydride(BLG-NCA) was synthesized according to the Fuchs-Farthing method using triphosgene. [35]



Scheme1 Illustration of the preparation of DOX-loaded PEG-pGlu(EDA-LA)-CA core-cross-linked micelles for reduction responsive intracellular drug release.

Characterization

The ^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using chloroform-*d*. The molecular weight and polydispersity of copolymers were determined by a PL GPC 50 instrument equipped with Jordi GPC columns (10E4, 2M) following a differential refractive-index detector (PL-RI). The measurements were performed using THF with 0.05 mol·L^{-1} LiBr as the eluant at a flow rate of 1 mL·min^{-1} at 30 ℃ and a series of narrow polystyrene standards for the calibration of the columns. The size and zeta-potential of micelles were determined by a Zetasizer Nano-ZS from Malvern Instruments. Transmission electron microscopy (TEM) measurement was performed on a FEI Tecnai G2 T12 operated with an accelerating votage of 200 kV. Confocal laser scanning

microscopy (CLSM) images were acquired using a FV1000 microscope. The absorbance of each well in MTT assays was measured using a Synergy H4 Hybrid Multi-Mode Microplate Reader. The fluorescence measurements of doxorubicin were performed using F-4600 FL Spectrophotometer.

Synthesis of PEG-*b*-PBLG diblock copolymers

PEG-*b*-PBLG block copolymers were synthesized by ring-opening polymerization of BLG-NCA using PEG-NH_2 as a macroinitiator. The following is a typical example on synthesis of PEG-*b*-$PBLG_{15}$ copolymer. Under a nitrogen atmosphere, to a solution of BLG-NCA (1.05 g, 4 mmol) in DMF (2 mL) under stirring was quickly added the stock solution of PEG-NH_2 (1.00 g, 0.2 mmol, monomer to initiator ratio of 17) in DMF (4 mL). The reaction mixture was stirred for 48h at 40 ℃. The resulting PEG-*b*-$PBLG_{15}$ copolymer was isolated by repeated precipitation in cold diethyl ether and dried in *vaccum*. Yield: 85.0%.

Synthesis of PEG-PBLG-CA

PEG-PBLG-CA was synthesized by amidation of PEG-*b*-PBLG with cholic acid. Briefly, cholic acid (0.33g, 0.036 mmol) and *N*-hydroxysuccinimide (NHS) (0.027g, 0.23 mmol) were dissolved in a mixture of dry THF (4 mL) and dry acetonitrile (1 mL), then *N*,*N*'-dicyclohexylcarbodiimide (DCC) (0.04g, 0.19 mmol) in dry THF (2 mL) was added dropwise to the resulting homogeneous solutionat 10-15 ℃. The mixture was stirred at 25 ℃ for 18 h and the precipitated *N*, *N*-dicyclohexyl urea was removed by filtration. After 0.5 g PEG-*b*-PBLG was added to the solution, the reaction was allowed to proceed for 24 h at room temperature. The solution was precipitated into cool ethyl alcohol. The product was recovered by filtration, washed with cool ethyl alcohol and dried at room temperature in *vaccum* for 24 h to obtain purified PEG-PBLG-CA. Yield: 68.5%.

Synthesis of PEG-pGlu(EDA)-CA

PEG-PGlu(EDA)-CA copolymer was obtained through aminolysis with excess ethylenediamine.[36] Briefly, PEG-PBLG-CA (0.33g, 0.55 mmol of benzyl ester) was dissolved in DMF (10 mL) at 40℃, then ethylenediamine (1.84 mL, 2.75 mmol) was added to the solution. The mixture was stirred overnight at 40℃. The resulting solution was added dropwise into 10% aqueous acetic acid solution (10 mL). The neutralized solution was dialyzed against 0.01M hydrochloric acid solution (× 3) and distilled water (×3). The white powder, PEG-pGlu(EDA)-CA was obtained as hydrochloric acid salt after lyophilization. Yield: 92.0%.

Synthesis of PEG-pGlu(EDA-LA)-CA

PEG-P(BLG-EDA)$_{15}$-CA (0.10 g, 0.18 mmol) was dissolved in anhydrous DCM (11 mL) and DMF (2 mL). To this solution was added *α*-lipoic acid (0.052 g, 0.25 mmol), DCC (0.052 g, 0.25 mmol), NHS (0.029 g, 0.25 mmol) and triethylamine (0.056 g, 0.55 mmol). The mixture was stirred at room temperature for 48 h, and then filtrated to remove dicyclohexylurea. The polymer was purified by precipitation in diethylether twice and dried in *vaccum* at room temperature for 24 h. Yield: 84.8%.

Formation and characterization of micelles

The micelles were prepared by solvent extrange method. Briefly, the synthesized block polymer of PEG-pGlu(EDA-LA)-CA(1 mg) was dissolved in DMSO (1 mL). This solution was added to 2 mL of doubly distilled water at a rate of 0.5 mL·min^{-1} under moderate stirring at room temperature. The resultant PEG-pGlu(EDA-LA)-CA suspension was extensively dialyzed against deionized water for 48 h (Spectra/Pore, MWCO 3500 Da).

Critical micelle concentration (CMC) was investigated by steady-state fluorescence methodology using pyrene as a probe on a F-4600 fluorescences pectrophotometer at room temperature. The concentration of polymer for fluorescence investigation was varied from 1.0×10^{-4} to 0.15 mg·mL^{-1} and the final concentration of pyrene was fixed at 0.6 μM. The fluorescence spectra were recorded with the excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm was monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

Dynamic Light Scattering (DLS) was performed on samples of polymer micelles to determine the size of the structures at a concentration of 0.2mg·mL^{-1}. All experiments were performed at 25 ℃ and equilibrated for 2 min. Prior to analysis, samples were passed through a 0.45 μm filter.

Samples for TEM were prepared by depositing a drop of the micelle solution (0.1 mg·mL^{-1}) on a 300-mesh carbon-coated copper grid followed by staining with phosphotungstic acid (1 wt.%).

Preparation of cross-linking micelles

The cross-linked PEG-pGlu(EDA-LA)-CA micelles were prepared under nitrogen atmosphere at room temperature using a catalytic amount of DTT as reported previously.[23] Briefly, the prepared micelles solution was bubbledwith N_2 for 20 min, and then 10 mol% DTT relative to the amount of lipoyl units wasadded to the solution under a N_2 atmosphere. After stirring for 24 h at room temperature, the micelles solution was dialyzed against distilled water for 24 h. The colloidal stability of the cross-linked micelles and the non-cross-linked micellesagainst large volume dilution and high salt concentration was investigated using DLS.

The size change of cross-linked micelles in responsive to a reductive environment was monitored by DLS measurement. Briefly, cross-linked micelles solution in a vial was bubbled with N_2 for 20 min, and then predetermined amount of DTT was added to yield the final DTT concentration of 10 mM. The vial was sealed and placed in a shaking bed at 200 rpm and 37℃. The size of the micelles was determined by DLS at different time intervals.

Encapsulation of DOX

DOX was loaded into micelles by dropwise addition of distilled water to a DMSO solution of DOX and PEG-pGlu(EDA-LA)-CA (Theoretical drug loading contents = 10-15 wt.%) under stirring at room temperature, followed by dialysis against distilled water for 24 h (Spectra/Pore, MWCO 3500 Da). The water was refreshed 5 times. The whole procedure was performed in the dark. The DOX-loaded micelles were cross-linked as described above using a catalytic amount of DTT (10 mol% relative to the lipoyl units).

The amount of DOX was determined using fluorescence (FL 4600) measurement (excitation at 480 nm and emission at 555 nm). For determination of drug loading content (DLC), the DOX-

loaded micelles were lyophilized and dissolved in DMSO and analyzed with fluorescence spectroscopy, wherein calibration curve was obtained with DOX/DMSO solutions with different DOX concentrations.

Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following formula:

DLC(wt.%) = (weight of loaded drug/total weight of polymer and loaded drug) × 100%

DLE(%) = (weight of loaded drug/weight of drug in feed) × 100%

In vitro release of DOX

Release of DOX from PEG-pGlu (EDA-LA)$_{15}$-CA and PEG-pGlu(EDA-LA)$_{26}$-CA micelles was monitored by dialysis. Briefly, 0.5 mLDOX-loaded micelle solution, either cross-linked or non-cross-linked, was transferred to a dialysis cassette (MWCO 12,000) by syringe. The cassette was suspended in a sealed container with 30 mL of release medium, *i.e.* PB (10 mM, pH 7.4), or PB (10 mM, pH 7.4) containing 10 mM DTT. Containers were kept shaking (200 rpm) at 37 ℃, and at select time points, 9 mL aliquots were removed from the external media and replaced with fresh buffer. The amount of DOX released was determined by using fluorescence (FL 4600) measurement (excitation at 480 nm). The release experiments were conducted in triplicate and the results presented were the average data with standard deviations.

MTT assays

The cytotoxicity of DOX-loaded PEG-PGlu(EDA-LA)-CA micelles was evaluated by MTT assays using Hela cells. The cells were seeded in a 96-well culture plate (6×10^3 cells/well) using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% $_L$-glutamine, antibiotics penicillin (100IU·mL^{-1}), and streptomycin (100μg·mL^{-1}), and incubated at 37 ℃ and 5% CO_2 for 24 h. The medium was removed and replaced by 80 μL of fresh medium containing 10% FBS. 20 μL of DOX-loaded cross-linked or non-cross-linked micelles in PB buffer (10 mM, pH 7.4) was added to yield final anticancer drug concentrations of 0.1, 0.5, 1, 5, 10, 20 and 40 μg·mL^{-1}. The cells were incubated at 37 ℃ and 5% CO_2 for pre-determined time. Then, the medium was aspirated and replaced by 100 μL of fresh medium. 10 μL of a stock solution containing 5 mg·mL^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in PBS was added and incubated for another 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. Data are presented as average ± SD (n = 4). The cytotoxicity of blank cross-linked and non-cross-linked micelles was determined in a similar way.

Intracellular release of DOX

The cellular uptake and intracellular release behaviors of DOX-loaded cross-linked micelles were observed by confocal laser scanning microscopy (CLSM) using Hela cells. The cells were plated on microscope slides in a 24-well plate at a density of 5×10^4 cells per well using RPMI-1640 medium supplemented with 10% FBS, 1% $_L$-glutamine, antibiotics penicillin (100 IU·mL^{-1}), and streptomycin (100 μg·mL^{-1}). The cells were incubated with prescribed amounts of DOX-loaded micelles or free DOX at 37 ℃ and 5% CO_2-containing atmosphere. After incubation for 4 or 8 h, the culture medium was removed and the cells on microscope plates were washed three times with PBS. The cells were fixed with 4% (w/v) paraformaldehyde for 30 min at 25 ℃ and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue) for 15 min, and then the cells on microscope plates were washed three times with PBS again. CLSM images of cells were obtained using confocal microscope (FV1000).

Results and discussion

Synthesis of PEG-pGlu(EDA-LA)-CA

The synthesis pathway of PEG-pGlu(EDA-LA)-CA conjugates is shown in Scheme 2. PEG-*b*-PBLG copolymers were synthesized through ring-opening polymerization of BLG-NCA initiated from the terminal primary amine group of PEG-NH_2 as reported previously.[38] Two compositions of block copolymers containing different *β*-benzyl-$_L$-glutamic (BLG) were synthesized by increasing the amount of BLG-NCA with respect to PEG. The chemical structures of representative PEG-pGlu(EDA-LA)$_{15}$-CA copolymer was confirmed by ^{1}H NMR. As shown in Fig. 1a, ^{1}H NMR showed characteristic signals of PEG (δ 3.36 and 3.62) and PBLG (δ 2.09-2.51, 3.91, 5.10, 7.31 and 7.50). Using the ratio of the area of the PEG peak (δ 3.62) to the area of the BLG peak (δ 5.10), the degree of polymerization (DP) of PBLG was determined to be 15 and 26, respectively. The GPC trace in Fig. 2 was mono-modal and quite symmetric, revealing a number average molecule weight (M_n) of 8400 g mol^{-1} and polydispersity index (PDI, M_w/M_n) of 1.02 in Table 1. In comparison with that of *m*PEG-NH_2, the GPC trace of *m*PEG-*b*-PBLG exhibited a clear shift to the higher M_n region, indicating an almost quantitative initiating efficiency. ^{1}H NMR (Fig. 1a) and GPC analyses confirmed the successful synthesis of *m*PEG-*b*-PBLG with high purity and narrow polydispersity. PEG-PBLG$_{15}$-CA copolymers were synthesized by esterification reaction of PEG-*b*-PBLG$_{15}$ with CA activated by DCC. The structure of PEG-PBLG$_{15}$-CA was also verified by ^{1}H NMR spectra. As shown in Fig. 1b, the proton signals (δ 1.2-1.3) corresponding to CA could be observed in the spectra. The aminolysis reaction of PEG-PBLG-CA was reacted in presence of ethylenediamine, ^{1}H NMR indicated that the signal of benzyl esters (δ 5.10) completely disappeared after aminolysis in Fig. 1c . ^{1}H NMR showed that DP of BLG block was not changed. Then LA was conjugated onto PEG-PBLG$_{15}$-CA through amide bonds formed via the reaction of the amino group of PEG-pGlu(EDA)-CA with LA activated by DCC. Fig. 1d was ^{1}H NMR spectrum of PEG-pGlu(EDA-LA)-CA, and the grafting of LA was confirmed by the appearance of peaks (δ 1.86-1.90) attributed to protons of five-member in LA. The molecular weight and composition of the resulting block copolymer could be adjusted by changing the feed ratio. Thus, ^{1}H NMR and GPC measurements demonstrated the successful synthesis of PEG-pGlu(EDA-LA)-CA block copolymers.

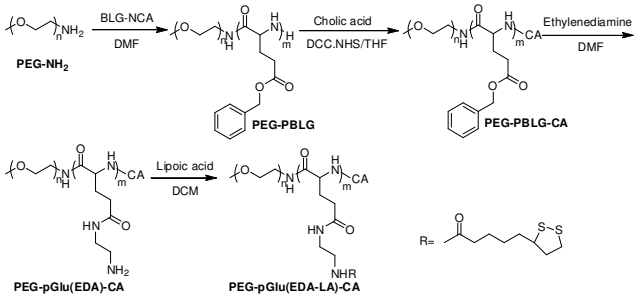
Micelle formation and core-cross-link of micelles

Micelles were formed from self-assembly of PEG-pGlu(EDA-LA)-CA in aqueous solution by solvent exchanged method. The received micelles possessed two domains: the PEG blocks formed the outer corona, the hydrophobic PBLG blocks formed the inner core, and the ending CA was acted as a part of the inner core. Dynamic light scattering (DLS) measurements showed that the size of PEG-pGlu(EDA-LA)-CA micelles increase from 122.6 to 144.7 nm with the increase of PBLG from 15 units to 26 units in PEG-pGlu(EDA-LA)-CA copolymers. The critical micelle concentration (CMC) was an important physical parameter used to character the self-aggregation ability of amphiphilic conjugates. The critical micelle concentration (CMC) was estimated to be

Table 1 Molecular characteristics of PEG-pGlu(EDA-LA)-CA

Copolymer	M_n(kg·mol^{-1})		M_n (GPC)^b	M_w/ M_n^{b}
	Design	^{1}H NMR^a		
PEG-pGlu(EDA-LA)$_{15}$-CA	5.0-7.1-0.4	5.0-5.4-0.4	8400	1.02
PEG-pGlu(EDA-LA)$_{26}$-CA	5.0-10.0-0.4	5.0-9.3-0.4	13900	1.03

^{a}Calculated by ^{1}H NMR spectra.
^{b}The polymers of PEG-*b*-PBLG was determined by GPC measurement in THF.



Scheme 2 Synthesis of PEG-pGlu(EDA-LA)-CA.

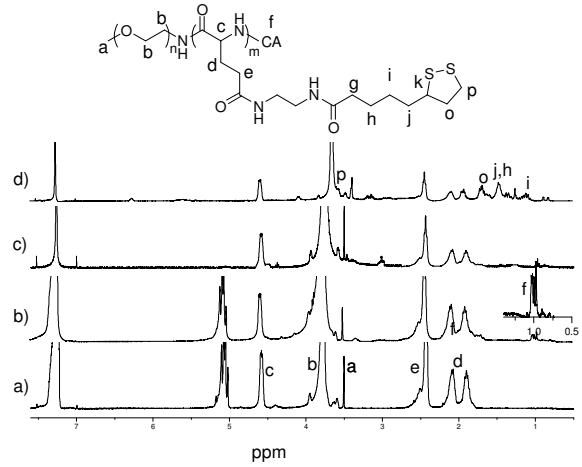


Fig. 1 ^{1}H NMR spectra of (a) PEG-*b*-PBLG, (b) PEG-PBLG-CA, (c) PEG-pGlu(EDA)-CA, (d) PEG-pGlu (EDA-LA)-CA in C*D*Cl$_3$.

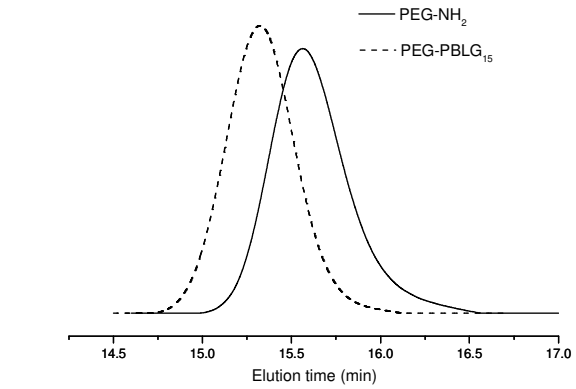


Fig. 2 Gel permeation chromatograms (GPC) of PEG-NH_2 and PEG-*b*-$PBLG_{15}$ (Polystyrene standard; eluant, THF; temperature, 30^{o}C; RI detection)

35 mg·L^{-1} for PEG-pGlu(EDA-LA)$_{15}$-CA block copolymer by fluorescence measurements using pyrene as probe. It was clear that the CMC decreased with the increase of the PBLG, which may attributed to the elongated hydrophobic chain.

Then, the disulfide cross-linked micelles were prepared by thiol-disulfide exchange in the presence of 10 mol% DTT relative to the lipoyl units in the micelles, similar to some previous reports.[19-21] Under the DTT reduction, the lipoyl rings of PBLG middle core were opened and the disulfide bonds were reformed between different lipoyl units, leading to core cross-linking of PEG-pGlu(EDA-LA)-CA micelles. DLS measurements showed that cross-linked micelles were well dispersed and slightly smaller compared with parent non-cross-linked micelles (Table 2). The TEM micrograph showed that the core-cross-linked micelles took a spherical morphology with an average radius around 100 nm in Fig. 3. In contrast, the hydrodynamic radius measured by DLS was 115 nm in Fig. 3. The smaller size from TEM observations should be due to the dehydration of the micelles in the TEM sample preparation process.

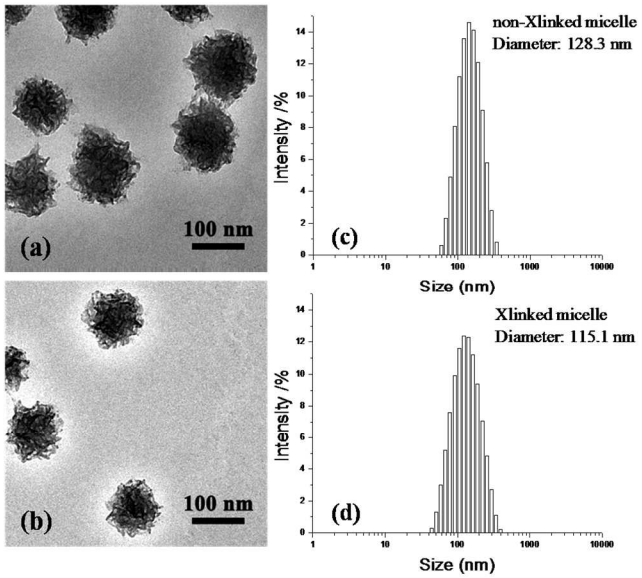


Fig. 3 Characterization of non-cross-linked and cross-linked PEG-pGlu (EDA-LA)$_{15}$-CA micelles: a) TEM images of micelle of PEG-pGlu (EDA-LA)$_{15}$-CA; b)TEM images of cross-linked micelle of PEG-pGlu (EDA-LA)$_{15}$-CA; c) DLS measurementsof micelle of PEG-pGlu (EDA-LA)$_{15}$-CA; d) DLS measurements of cross-linked micelle of PEG-pGlu (EDA-LA)$_{15}$-CA.

Furthermore, the structural stability of the cross-linked PEG-pGlu(EDA-LA)-CA micelles against extensive dilution and concentrated salt conditions was investigated using DLS

measurements in ESI, Fig. S2. In particular, core-cross-linked micelles following 100-fold dilution (C < CMC) illustrated only slight increase in hydrodynamic size and remained a narrow size distribution, while the size of parent non-cross-linked micelles increased and some large aggregates turned up under otherwise the same circumstance as showed in Fig. S2. The size of core-cross-linked micelles had little change under physiological and high salt conditions (2 M), but non-cross-linked micelles showed large aggregates even under physiological salt (0.15 M NaCl)

Table 2 Characteristics of non-cross-linked and cross-linked PEG-pGlu(EDA-LA)-CA micelles

Entry Polymers	Non-cross-linked Micelles				Cross-linked Micelles		
	Size[a] (nm)	PDI[a]	Zeta[a] (mV)	CMC[b] (mg·L^{-1})	Size[a] (nm)	PDI[a]	Zeta[a] (mV)
PEG-pGlu(EDA-LA)$_{15}$-CA	128.3	0.159	-12.3	35	115.1	0.182	-4.07
PEG-pGlu(EDA-LA)$_{26}$-CA	144.7	0.238	-9.36	11	134.3	0.241	-2.09

[a]Determined by DLS using Zetasizer Nano-ZS (Malvern Instruments) at 25 ℃ in water.
[b]Determined by fluorescence measurement using pyrene as a fluorescence probe (pyrene final concentration is 0.6 μM).

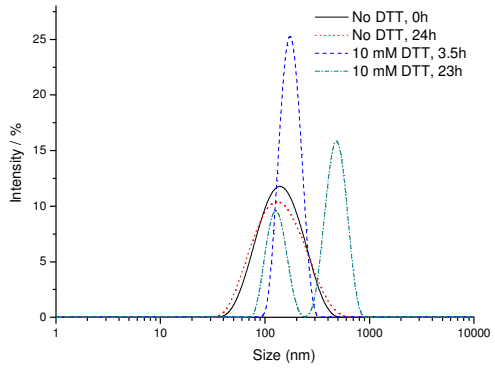


Fig.4 Change of size distribution of cross-linked PEG-pGlu (EDA-LA)$_{15}$-CA micelles in response to 10 mM DTT at 37 □.

in Fig.S2. These results combined showed that core-cross-linking could greatly improve the stability of micelles.

It is well-known that the disulfide linkages are stable under normal physiological conditions but respond to reductive conditions via reversible cleavage into free thiols. To investigate whether the cross-linked micelles can de-cross-link under a reductive intracellular environment, the size change of core-cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles in response to 10 mM DTT was monitored by DLS measurement over time at 37 □ (Fig. 4). The results showed that DTT lead swelling of reduction-sensitive core-cross-linked micelles to large aggregates with a diameter over 254 nm in 3.5 h and over 657 nm with multimodal distribution in 23 h in Fig.4. In contrast, little change of core-cross-linked micelle size was observed over 24 h in the absence of DTT under otherwise the same conditions. These results confirmed that cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles are apt to rapid dissociation under a reduction condition imitating that of the cytoplasm and cell nucleus.

Loading and in vitro release of DOX

Doxorubicin (DOX) is one of the most widely used antitumor drugs against a broad range of solid malignant tumors, which can insert into DNA and then inhibite the biosynthesis of bioactive macromolecules. In the current study, DOX was used as a model drug and loaded to evaluate the in vitro drug release behavior of drug-loaded PEG-pGlu(EDA-LA)-CA micelles. DOX was loaded into the micelles through a dialysis method, and the drug loading contents (DLC) and drug loading efficiencies (DLE) were determined to be 6.0-8.4% and 48.2-77.1%, respectively in Table 3. Meanwhile, we also synthesized copolymer PEG-pGlu(EDA-LA)$_{15}$ as a control to compared DLC and DLE with copolymer PEG-pGlu(EDA-LA)$_{15}$-CA. The results showed that PEG-pGlu(EDA-LA)-CA had a higher DLE than PEG-pGlu(EDA-LA) due to the presence of the ending CA (Table 3). For example, the DLE of PEG-pGlu(EDA-LA)$_{15}$-CA was 77.1% at a theory loading content of 10.0%, while the DLE of PEG-pGlu(EDA-LA)$_{15}$ was 70.2% at the same theory loading content. Hence, we can confirm that the conjugation of CA to PEG-b-PBLG has effectively increased the DLC and DLE of PEG-pGlu(EDA- LA) micelles. This is also supported by the fact that CA has a rigid hydrophobic cyclopentenophenanthrene nucleus structure and the hydrophobic CA has been successfully applied in drug delivery.[30, 31]

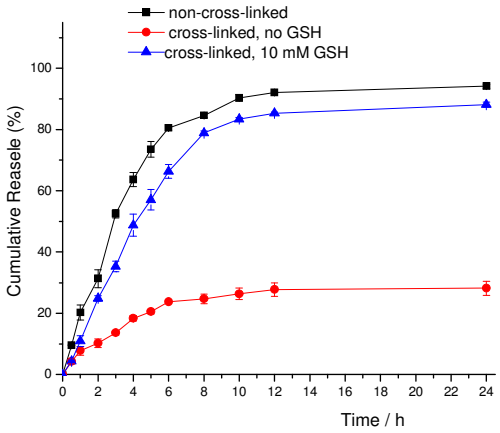


Fig.5 DOX release from core-cross-linked PEG-pGlu (EDA-LA)$_{15}$-CA micelles in PB buffer (10 mM) at 37 □. Non-cross-linked micelles were used as a control. Data are presented as mean ± SD (n = 3).

Table 3 Characteristics of DOX-loaded cross-linked PEG-pGlu(EDA-LA)-CA micelles[a]

Copolymers	DLC (wt.%) theory	Non-cross-linked Micelles		Cross-linked Micelles		DLE[b] (%)	DLC[c] (wt.%)
		Size[a] (nm)	PDI[a]	Size[a] (nm)	PDI[a]		
PEG-pGlu (EDA-LA)$_{15}$	15.0	115.9	0.226	94.82	0.226	43.8	6.6
	10.0	112.3	0.372	107.1	0.234	70.2	7.0

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PEG-pGlu(EDA-LA)$_{15}$-CA	15.0	137.4	0.196	112.8	0.183	56.1	8.4
	10.0	133.1	0.238	119.5	0.223	77.1	7.7
PEG-pGlu(EDA-LA)$_{26}$-CA	15.0	154.9	0.197	149.7	0.187	48.2	7.2
	10.0	106.6	0.286	97.28	0.227	60.3	6.0

[a]Determined by DLS using Zetasizer Nano-ZS.
[b]DLE (%) = (weight of loaded drug/weight of drug in feed) × 100%, DLE was determined by fluorescence measurements.
[c]DLC (%) = (weight of loaded drug/weight of polymer in feed) × 100%.

The drug release behavior of the DOX-loaded PEG-pGlu(EDA-LA)$_{15}$-CA micelles was investigated at a low micelle concentration of 35 μg·mL^{-1} at 37 °C with or without the presence of GSH. The accumulative drug release profiles as a function of time are plotted in Fig. 5. The results showed that at physiological reductive conditions only ca. 28.2% DOX was released from DOX-loaded core-cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles in 24 h, likely because of their particularly sturdy core-cross-linked structure. It was found that the drug release was significantly enhanced by addition of GSH. As shown in Fig.5, the cumulative release of DOX from the drug loaded core-cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles was increased to 88.1% in 24 h in the presence of 10 mM GSH, and gave a release profile similar to that for DOX-load non-cross-linked micelles. These results clearly indicated that reduction-triggered drug release is rapid and nearly complete.

Cellular uptake of DOX loaded micelles and intracellular distribution of DOX

HeLa cells were used to investigate the cellular uptake and intracellular drug release behaviors of DOX-loaded cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles using CLSM. Free DOX and DOX-loaded cross-linked micelles of polymer PEG-pGlu(EDA-LA)$_{15}$-CA were incubated with HeLa cells for 4 h or 8 h at 37 □, respectively. Cell nuclei were stained with DAPI to determine whether DOX enters the nuclei. The CLSM results were shown in Fig.6. As shown in Fig.6, although the drug began to be accumulated inside the cells for both free drug and DOX-loaded PEG-pGlu(EDA-LA)$_{15}$-CA micelles in 4 h incubation, the drug intracellular distribution of the drug-loaded micelles was quite different from that of free drug. After 4 h of incubation with the free drug, the fluorescence of DOX in HeLa cells was observed in cell nuclei, and no obvious fluorescencewas detected in the cytoplasm as depicted in Fig.6a-6c, suggesting that DOX quickly diffuses into the cytoplasm through the cell membrane and transports into the nuclei. The DOX fluorescence in the cells treated with DOX-loaded micelles was much weaker than treated with free DOX and the fluorescence of the DOX-loaded micelles was displayed both in cell nuclei and cytoplasm after 4 h incubation in Fig.6d-6f, indicating that the fast internalization of micelles occurs and only part of DOX is released from the micelles in this period of time. However, the stronger fluorescence was observed in the nuclei after 8 h incubation. This is clearly due to the fact that more DOX is released from the DOX-loaded micelles with longer incubation time and subsequently enter the nuclei as shown in Fig. 6g-6i.The seresults were in accordance with our expectation that disulfide bonds are cleaved in the intracellular compartments such as the cytosol and the cell nucleus due to presence of comparatively high concentrations of reducing glutathione tripeptides (2-10 mM). These intracellular drug release observations showed that reduction sensitive cross-linked PEG-pGlu(EDA-LA)-CA micelles can promote intracellular drug release fast, thus, drug could reach the nuclei of cells and kill cancer cells.

Anti-tumor activity of DOX-Loaded core-cross-linked micelles.

PEG and PBLG are polymers with low cytotoxicity and good biocompatibility, making them suitable for various applications in biomedical fields such as drug carriers, tissue engineering as well as stimuli-responsive materials. Here, the in vitro cytotoxicity of block copolymers against HeLa cells was evaluated by MTT assay. The HeLa cells were incubated with different concentrations of polymer micelles in 24 h. As shown in Fig. 7, the experimental results revealed that the PEG-pGlu(EDA-LA)$_{15}$-CA micelles are nontoxic up to the highest testing concentration of 1.0 mg·mL^{-1}, and different concentrations of the polymer results obtained are greater than 92% cell viability. The results revealed that the cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles had low cytotoxicity and could be safely used as drug delivery carriers.

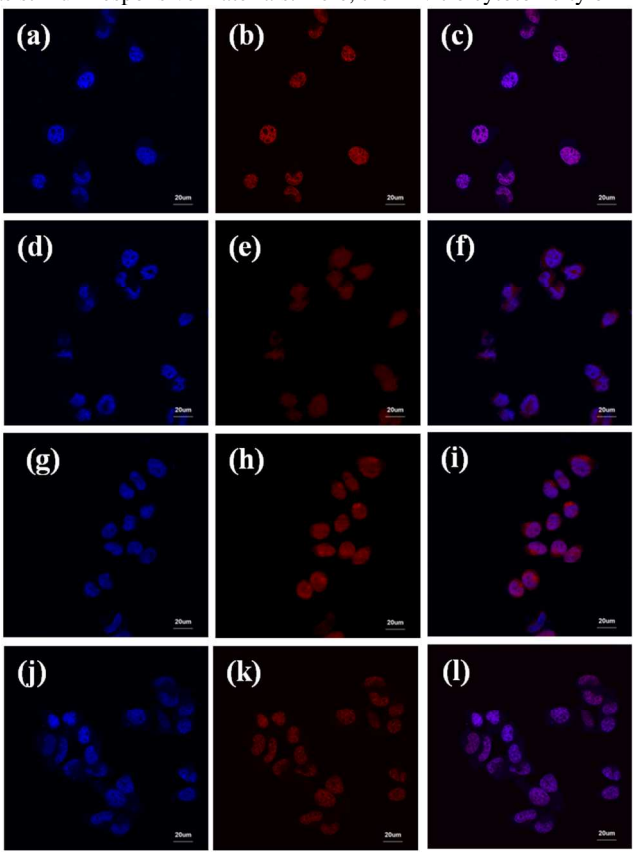

Fig.6 CLSM images of HeLa cells incubated with DOX-loaded cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles (dosage: 25 μg DOX equiv./mL). For each panel, the images from left to right show cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlays of the two images. The scale bars correspond to 20 μm in all the images. a,b,c) Free DOX, 4 h incubation; d,e,f) DOX-loaded cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles, 4 h incubation; g,h,i) DOX-loaded cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles, 8 h incubation; j,k,l) Free DOX, 8 h incubation.

The antitumor activity of the DOX-loaded cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles against Hela cells were further tested by MTT assay, with free DOX and DOX-loaded non-cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles as the control. As shown in Fig. 8, the free DOX displayed the highest cytotoxicity at the same DOX concentration and the desulfide cross-linked micelles had slightly lower toxicity than the non-cross-linked micelle. For instance, at a drug dosage of 20 μg·mL^{-1}, cell viabilities of 34.2, 33.5 and 28.3% were observed for HeLa cells following 48h incubation with DOX-loaded cross-linked micelles, non-cross-linked micelles and free drug, respectively. The half maximal inhibitory concentration (IC_{50}) value of DOX-loaded cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles was determined to be 5.2 μg·mL^{-1} for HeLa cells, which was a little higher than those observed for the non-cross-linked counterparts (3.5μg·mL^{-1}). It should also be noted that the antitumor activity of DOX-loaded cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles is higher than that reported for DOX-loaded degradable block copolymer micelles (6.96 μg·mL^{-1},[38] 6.7 μg·mL^{-1} [20]). These results strongly support that cross-linking has no significant adverse effect on the intracellular drug release of PEG-pGlu(EDA-LA)-CA micelles, likely due to fast de-cross-linking of micelles triggered by the intracellular level of glutathione.

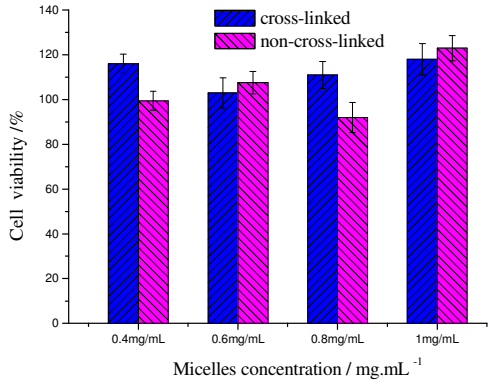

Fig.7 MTT assay of PEG-pGlu(EDA-LA)$_{15}$-CA at different micelle concentrations. HeLa cells were incubated for 24 h with micelles. Data are presented as the average ± SD (n=4).

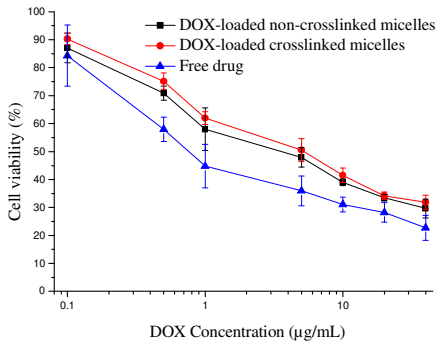

Fig.8 Antitumor activity of the DOX-loaded cross-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles in Hela cells. DOX-loaded non-corss-linked PEG-pGlu(EDA-LA)$_{15}$-CA micelles and free DOX were used as controls. Data are presented as the average ± SD (n=4).

Conclusions

In this study, we have demonstrated that a novel reduction responsive core-cross-linked micelles self-assembled from PEG-pGlu(EDA-LA)-CA block polymers efficiently release entrapped DOX into cancer cells, resulting in favourable anti-tumor activity. The introduction of lipoic acid and cholic acid into PEG-poly(amino acid)s copolymer micelles rendered their potential as an efficient targeted nanocarrier: (i) good biocompatibility and biodegradability of the empty micelles and high cytotoxicity of DOX-loaded micelles to HeLa cells; (ii) superior stability underphysiological conditions but rapid dissociation and drug release under reductive environments; (iii) high drug-loading content and superior reduction sensitivity in response to intracellular glutathione, that leading to rapid delivery of DOX into the cell. Therefore, these intelligent core-cross-linked micelles have tremendous potential for intelligent intracellular drug delivery in clinical tumor-targeted chemotherapy.

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Notes and references

1 Y.-C. Chen, C.-L. Lo and G.-H. Hsiue, *J. Biomed. Mater. Res.*, 2014, **102**, 2024.
2 C. Deng, Y. Jiang, R. Cheng, F. Meng and Z. Zhong, *Nano Today*, 2012, **7**, 467.
3 S. Samarajeewa, A. Ibricevic, S. P. Gunsten, R. Shrestha, M. Elsabahy, S. L. Brody and K. L. Wooley, *Biomacromolecules*, 2013, **14**, 1018.
4 S. Wu, H. Kuang, F. Meng, Y. Wu, X. Li, X. Jing and Y. Huang, *J. Mater. Chem.*, 2012, **22**, 15348.
5 Y. Li, K. Xiao, W. Zhu, W. Deng and K. S. Lam, *Adv.Drug Delivery Rev.*, 2014, **66**, 58.
6 C. S. Kim, B. Duncan, B. Creran and V. M. Rotello, *Nano Today*, 2013, **8**, 439.
7 X. He, J. Li, S. An and C. Jiang, *Therapeutic delivery*, 2013, **4**, 1499.
8 X. Wan, T. Liu and S. Liu, *Langmuir : the ACS journal of surfaces and colloids*, 2011, **27**, 4082.
9 Z. Zhang, X. Chen, L. Chen, S. Yu, Y. Cao, C. He and X. Chen, *ACS Appl. Mater. Interfaces*, 2013, **5**, 10760.
10 X. Hu, J. Tian, T. Liu, G. Zhang and S. Liu, *Macromolecules*, 2013, **46**, 6243.
11 Y. Li, T. Liu, G. Zhang, Z. Ge and S. Liu, *Macromol.Rapid Comm.*, 2014, **35**, 466.
12 J. Zhao, J. Liu, S. Xu, J. Zhou, S. Han, L. Deng, J. Zhang, J. Liu, A. Meng and A. Dong, *Acs Appl. Mater. Interfaces*, 2013, **5**, 13216.
13 T. Xing, B. Lai and L. Yan, *Macromol. Chem. Phys.*, 2013, **214**, 578.
14 S. Chakravarthi, C. E. Jessop and N. J. Bulleid, *EMBO Rep.*, 2006, **7**, 271.
15 H. Wang, L. Tang, C. Tu, Z. Song, Q. Yin, L. Yin, Z. Zhang and J. Cheng, *Biomacromolecules*, 2013, **14**, 3706.
16 Z. Zhang, L. Yin, C. Tu, Z. Song, Y. Zhang, Y. Xu, R. Tong, Q. Zhou, J. Ren and J. Cheng, *ACS Macro. Lett.*, 2012, **2**, 40.
17 Y.-C. Wang, Y. Li, T.-M. Sun, M.-H. Xiong, J. Wu, Y.-Y. Yang and J. Wang, *J. Macromol.Rapid Comm.*, 2010, **31**, 1201.
18 Y. Li, K. Xiao, J. Luo, W. Xiao, J. S. Lee, A. M. Gonik, J. Kato, T. A. Dong and K. S. Lam, *Biomaterials*, 2011, **32**, 6633.
19 A. Zhang, Z. Zhang, F. Shi, J. Ding, C. Xiao, X. Zhuang, C. He, L. Chen and X. Chen, *Soft Matter*, 2013, **9**, 2224.
20 R. Wei, L. Cheng, M. Zheng, R. Cheng, F. Meng, C. Deng and Z. Zhong, *Biomacromolecules*, 2012, **13**, 2429.
21 Y. L. Li, L. Zhu, Z. Liu, R. Cheng, F. Meng, J. H. Cui, S. J. Ji and Z. Zhong, *Angew. Chem., Int. Ed.*, 2009, **48**, 9914.
22 L. Wu, Y. Zou, C. Deng, R. Cheng, F. Meng and Z. Zhong, *Biomaterials*, 2013, 34, 5262.
23 Y. Xu, F. Meng, R. Cheng and Z. Zhong, *Macromol. Biosci.*, 2009, **9**, 1254.

24 M. Zheng, Y. Zhong, F. Meng, R. Peng and Z. Zhong, *Mol. Pharm.*, 2011, **8**, 2434.
25 L. Wu, Y. Zou, C. Deng, R. Cheng, F. Meng and Z. Zhong, *Biomaterials*, 2013, **34**, 5262.
26 F. Costanza, S. Padhee, H. Wu, Y. Wang, J. Revenis, C. Cao, Q. Li and J. Cai, *Rsc Advances* 2014**, 4**, 2089.
27 H. Lu, J. Wang, Z. Song, L. Yin, Y. Zhang, H. Tang, C. Tu, Y. Lin and J. Cheng, *Chemical Communications* 2014**, 50**, 139.
28 Y. Ma, X. Jiang and R. Zhuo, *J. Polym. Sci. Pol. Chem.*, 2013, **51**, 3917.
29 J. Ding, C. Xiao, C. He, M. Li, D. Li, X. Zhuang and X. Chen, *Nanotechnology*, 2011, **22**.
30 Q. Wu, F. Du, Y. Luo, W. Lu, J. Huang, J. Yu and S. Liu, *Colloids and Surfaces B-Biointerfaces*, 2013, **105**, 294.
31 J. Tang, X. Wang, T. Wang, F. Chen and J. Zhou, *J. Eur. J. Pharm. Sci.*, 2014, **51**, 157.
32 W. Wang, D. Cheng, F. Gong, X. Miao and X. Shuai, *Adv. Mater.*, 2012, **24**, 115.
33 X. Zeng, W. Tao, L. Mei, L. Huang, C. Tan and S. S. Feng, *Biomaterials*, 2013, **34**, 6058.
34 Y. Xiong, J. Qi and P. Yao, *Macromol. Biosci.*, 2012, **12**, 515.
35 H. Y. Tian, C. Deng, H. Lin, J. R. Sun, M. X. Deng, X. S. Chen and X. B. Jing, *Biomaterials*, 2005, **26**, 4209.
36 Y. Lee, S. Fukushima, Y. Bae, S. Hiki, T. Ishii and K. Kataoka, *J. Am. Chem. Soc.*, 2007, **129**, 5362.
37 J. Yue, R. Wang, S. Liu, S. Wu, Z. Xie, Y. Huang and X. Jing, *Soft Matter*, 2012, **8**, 7426.
38 S. J. Lee, K. H. Min, H. J. Lee, A. N. Koo, H. P. Rim, B. J. Jeon, S. Y. Jeong, J. S. Heo and S. C. Lee, *Biomacromolecules*, 2011, **12**, 1224.