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ARTICLE TYPE

Self-assembled Micelles of PEG-Poly(Disulfide Carbamate Amine) Copolymers for Intracellular Dual-Responsive Drug Delivery †

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A group of poly(ethylene glycol)-poly(disulfide carbamate amine) (PEG-SSPCA) diblock copolymers are designed, prepared and successfully applied for intracellular dual-responsive drug delivery. PEG-SSPCA copolymers can be obtained by polycondensation reaction between 2, 2'-dithiodiethanol bis(*p*-nitrophenyl carbonate) and a mixture of amino-terminal PEG (M_w=5k) and tertiary amine-containing primary diamine. The copolymers self-assemble to form stable nanoscale micelles under a physiological condition and the micelles may perform rapid destabilization in an acidic or reductive condition. The micelles based on the copolymer having 1,4-bis(3-aminopropyl) piperazine (BAP) residue (termed as PEG-SSBAP) can carry anti-cancer drug, doxorubicin (Dox) with the drug loading content of 5.7±1%. *In vitro* accumulative drug release test of Dox-loaded PEG-SSBAP micelles manifests slow drug release in a physiological condition and accelerated drug release in an acidic or reductive environment, but sufficient drug release in an acidic plus reductive environment. Confocal laser scanning microscopy imaging indicates that Dox-loaded PEG-SSBAP micelles are capable of delivering and liberating Dox into cellular nucleus. In vitro, PEG-SSBAP micelles are of low toxicity against different cancer cells at a high concentration of 400 $\mu g \cdot mL^{-1}$. However, Dox-loaded PEG-SSBAP micelles exert marked cytotoxicity against the cancer cells. In vivo, intravenous administration of the Dox-loaded micelles at a medium Dox dose of 2.5 $mg \cdot Kg^{-1}$ induces considerable growth inhibition of HepG2 tumor xenografted in nude mice with anti-cancer efficacy comparable to that of free Dox-chemotherapy but negligible systemic toxicity. PEG-SSPCA block copolymer represents an efficient nano-carrier for controlled drug release and cancer therapy.

Introduction

The development of amphiphilic block polymers and their self-assembly into micelles for controlled release of anti-cancer drugs have received an enormous attention in nano-pharmaceutics over the past two decades.[1] A lot of researches have demonstrated that drug-loaded nanomedicine may offer improved circulation time, passive targeting (enhanced permeation and retention effect) of nanomicelles to solid tumors in tumor-bearing mouse model,[2] and considerably alleviated side effects in patients. However, efficient intracellular liberation of anti-cancer drugs from polymeric nanomicelles remains a massive challenge, because most of the drugs like doxorubicin (Dox) must be transferred into the nucleus of cancer cells to intercalate with gene, thus causing the cell death. This issue has been addressed in the design of amphiphilic block copolymers which can not only load anti-cancer drugs in their nanomicelles but also actively release the drugs in response to an intracellular micro-environment. For instance, the micelles based on amphiphilic poly(ethylene glycol) (PEG)-coupled copolymers with a pH-responsive chemical linker such as imidazole, imine and hydrazone can mediate efficient drug release in acidified endosomes/lysosomes.[3] Besides, the micelles based on disulfide-linked amphiphilic copolymers possess unique ability to conduct reduction-responsive drug release in cytoplasm and nucleus via the disulfide cleavage due to intracellular reductive environment (e.g. 10–20 mM glutathione).[4] In order to attain a satisfied anti-cancer efficacy, it is indispensable for drug-loaded micelles to liberate their cargoes in those complicated (acidic and reductive) inracellular environment. For that reason, those micelles having a single-responsive drug release ability could not perform the most sufficient drug release in an efficient way because they have to undergo different translocation, that is, in an acidic or reductive compartment or both two. Alternatively, dual-responsive micelles would be an option to offer intracellular sufficient drug release.

Most of current studies generally present polymeric nanomicelles with a single-responsive (acidic/reductive) drug release ability.[5] Only few researches have focused on the nanomicelles capable of mediating both acid and reduction dual-responsive drug release. One method for such micelles is the design of dual-responsive amphiphilic polymers. For example, Chen *et al*. prepared PEG-grafted reducible poly(amino ester)s (RPAE-PEG) by Michael-type addition of a disulfide-containing diacrylate and a mixture of amino-terminated PEG and 4, 4'-trimethylene dipiperidine.[6] The authors showed that Dox-loaded RPAE-PEG micelles completely liberated Dox in an acidic and reductive complicated environment. Bahadur *et al*. produced a disulfide-linked PEG-

grafted poly[(2-(pyridin-2-yl-disulfanyl)ethyl acrylate)] (PDSG) having disulfide bond and pyridine residue in the side chain.[7] PDSG nanoparticles conducted faster drug release in an acidic and reductive complex environment compared to an acidic environment. Besides PEG-grafted amphiphilic copolymers, amphiphilic PEG-coupled di- or tri-block polymers are exploited for dual-responsive drug release. For example, Yu *et al*. reported on PEG-polyurethane-PEG block copolymers with disulfide and tertiary amine in the polyurethane block (PRS-PU).[8] PRS-PU micelles could load Dox and liberate the drug in response to an acid and reduction condition at higher release rate than the case in an acidic or physiological condition. More recently, Chen *et al*. developed a PEG-disulfide-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) block polymer (PEG-SS-PTMBPEC) with an acid and reduction dual-responsive ability,[9] and they found that PEG-SS-PTMBPEC micelles were able to mediate maximized drug release in an acidic and reductive complex condition compared to an acidic or reductive condition alone. A similar phenomenon was also illustrated by Chu *et al*., who indicated that dual-responsive micelles from PEG-disulfide-poly[(benzyl-l-aspartate)-co-(N-(3-aminopropyl) imidazole-l-aspartamide)] block copolymers afforded the most sufficient drug release in an acidic and reductive complex environment.[10] All the researches reflect that dual-responsive micelles are more efficient for sufficient drug release than single-responsive counterparts.

The development of amphiphilic di-block copolymers hold a high promise for intracellular dual-responsive drug release since they are prone to the formation of well-structured nanomicelles having hydrophobic core and hydrophilic shell. In this study, we present a facile approach to generate a group of new amphiphilic diblock copolymers with PEG and reducible poly(carbamate) blocks (Fig. 1a). The poly(carbamate) block has disulfide linkage and tertiary amino residue in polymeric repeating units, thus being denoted as poly(disulfide carbamate amine) (SSPCA). It was hypothesized that the copolymers (termed as PEG-SSPCA) could self-assemble to form micelles which are practical for acid and reduction dual-responsive intracellular drug release (Fig. 1b). Herein, chemical structure, composition and molecular weight of the PEG-SSPCA copolymers were respectively characterized by FT-IR, ^{1}H NMR, and gel penetration chromatography. The characteristics of PEG-SSPCA micelles in terms of size, drug-loading content, and dual-responsive drug release were investigated. Anti-tumor activity of Dox-loaded PEG-SSPCA micelles was evaluated against various cancer cells *in vitro* and HepG2 tumor-bearing nude mouse.

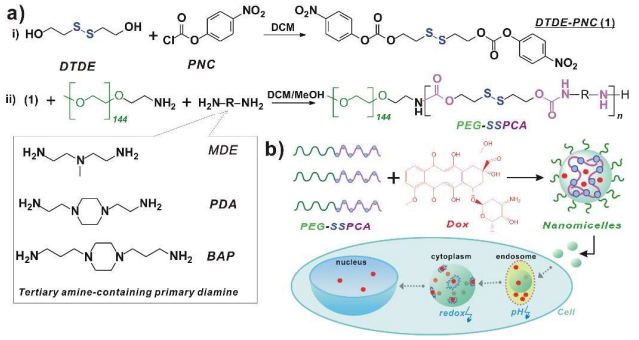

Figure 1. a) Schematic synthesis of disulfide-based DTDE-PNC compound and preparation of PEG-SSPCA diblock copolymers; b) Self-assembly of PEG-SSPCA copolymers into micelles for anti-cancer drug, doxorubicin (Dox), loading and subsequent pH- and reductive-dual-responsive intracellular release.

Experimental Section

Materials

All the chemicals were directly used without further purification. 1, 4-bis(3-aminopropyl)piperazine (BAP), dithiothreitol (DTT), 2, 2'-dithiodiethanol (DTDE), *p*-nitrophenyl chloroformate (PNC) Cysteamine, and α-methoxy-ω-amino poly(ethylene glycol) (PEG-NH_2, M_w=5 kDa) were ordered from Sigma-Aldrich (USA). N-methyl-2,2'-diaminodiethylamine (MDE) piperazine-1,4-diethylamine (PDA) and doxorubicin (Dox)/HCl were ordered from J&K. Anhydrous dichloromethane (DCM) was obtained by overnight drying in CaH_2 and distillation at vacuum. Dialysis tube with molecular weight cut-off (MWCO) was ordered from Spectra/Por Co.(USA).

Synthesis of 2, 2'-dithiodiethanol bis(p-nitrophenyl carbonate) (DTDE-PNC)

DTDE (4 g) and anhydrous pyridine (6 g) in DCM (20 mL) were firstly dissolved in a brown, round-bottom flask. PNC (10 g) in anhydrous DCM (20 mL) were then dropwise added in nitrogen atmosphere at 0 °C within 2 h. The reaction was proceeded for 4 h at 0 °C and 20 h at room temperature. After rotary evaporation of DCM, the residue was purified in silica column using DCM as a mobile phase (yield: 35%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.3 (4H, *o*-aromatic proton), 7.4 (4H, *m*-aromatic proton), 4.6 (4H, 2×SSCH_2C$\underline{H_2}$); 3.1 (4H, 2×SS$\underline{CH_2}$CH_2).

Synthesis of PEG-poly(disulfide carbamate amine) (PEG-SSPCA) diblock copolymer

PEG-SSPCA copolymers were synthesized by polycondensation reaction between DTDE-PNC and a mixture of a primary diamine and PEG-NH_2 at a mole ratio of 9/1 (Scheme 1a). In a typical synthesis for PEG-SSPCA with BAP residue (denoted as PEG-SSBAP), DTDE-PNC (0.54 g, 1 mmol), BAP (0.2 g, 0.9 mmol) and PEG-NH_2 (0.56 g, 0.1 mmol) were charged into a round flask and dissolved in anhydrous DCM (5 mL) and methanol (1 mL) as a solvent. The reaction continued for 5 days at room temperature under nitrogen atmosphere. Next, an excess amount of BAP (0.02 g) was added into the reaction for another 2 days. After the removal of solvent by rotary evaporation, the crude product was dissolved in acidic water (pH~5.0) and purified by ultrafiltration (10k MWCO) with deionzed water (pH~5). PEG-SSBAP was obtained as a white powder after freeze-drying (yield: 0.62 g).

Chemical Characterization

Chemical composition of chemicals and polymers synthesized in this work was characterized by ^{1}H NMR spectrometer (500 MHz, Varian Inova) and FT-IR spectrometer (Bruker Tensor 27). Number-average molecular weight (M_n) and polydispersity index (PDI) of PEG-SSPCA copolymers were tested by gel permeation chromatography (Viscoteck, Malvern, UK) equipped with one MBMMW column and Triple Detection System (S100, Malvern, UK), with DMF/LiBr (0.02 M) elute at a flow rate of 1 mL•min^{-1} and column temperature of 50°C. Acid–base titration of PEG-SSPCA polymers was done by previous method and protonation degree was calculated.[11] Particle size and surface charge of PEG-

SSPCA nanomicelles were measured by dynamic light scattering (DLS) analysis at 25 ºC with Nanosizer NS90 (Malvern, UK). The shape of micelles was observed under transmission electron microscopy (TEM) (JEM 200CX, JEOL Corp.).

Micelle formation and critical micelle concentration (CMC)

In brief, micelles of PEG-SSPCA copolymers were prepared by dropwise addition of phosphate buffer (10 mL) (pH 7.4, 50 mM) into PEG-SSPCA (1 mg•mL^{-1}) in DMSO (1 mL) and TEA (10 μL), followed by dialysis (25k MWCO) against deionzed water (2×5 L); CMC was determined using a pyrene probe method. Briefly, after incubating different concentration of PEG-SSPCA copolymers (5×10^{-4}–0.5 mg•mL^{-1}) with the pyrene (0.125 μg•mL^{-1}) as a probe in phosphate buffer (2 mL, pH 7.4) for overnight at room temperature, fluorescence spectra analysis were run with a fluorescence spectrometer (L55, PerkinElmer, USA) at excitation wavelength of 330 nm. The ratio of emission fluorescence density at 372 nm and 383 nm (*i.e.* I_{372}/I_{383}) was calculated. By plotting these ratio (*y*-axis) as the function of the polymer concentrations (*x*-axis) and extrapolating two tendency lines, CMC was found as *x*-axis value of cross-point of the two lines.

Destabilization characterization of PEG-SSPCA micelles in an acidic and/or reductive environment

As an example, effect of an acid or reduction condition on PEG-SSBAP micelles was examined by tracking their size change. The powder of PEG-SSPCA micelles was dissolved in a phosphate buffer (pH 7.4, pH 6.5, and pH 5.5, 20 mM) in the absence or presence of DTT (0.5 mM) for 12 h at room temperature. The size was then measured by DLS analysis.

Drug loading of PEG-SSPCA micelles and drug release test

Loading of Dox into PEG-SSPCA micelles was performed by a dialysis method. In brief, Dox/HCl salt (1 mg) and triethylamine (10 μL) were dissolved in DMSO (1 mL) for stirring overnight. PEG-SSPCA copolymers (10 mg) were also dissolved in DMSO (1 mL) containing triethylamine (10 μL). Next, the solution of Dox and copolymer were mixed homogenously and dropwise added into deionzed water (10 mL) in a round-botton flask for stirring over 2 h. The resulting solution was finally purified by dialysis (10k MWCO) in deionzed water (2×5 L). The residue solution was filtered through a membrane (0.45 μm, Millipore) and a red powder was eventually obtained after freezing-drying. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated with the equations: DLC=(weight of loaded drug/weight of drug-loaded micelles)×100%; DLE=(weight of loaded drug/weight of drug in feed)×100%.

Dox release from Dox-loaded PEG-SSBAP micelles was studied at pH 7.4 and pH 6.5 in the absence and presence of 0.5 mM DTT. In brief, the powder of Dox-loaded micelles was dissolved in deionzed water (1 mL) at 10 mg•mL^{-1}. The micelle solution was transferred into a dialysis bag (10k MWCO), immersed in 30 mL of phosphate buffer (pH 7.4, 20 mM) or acetate buffer (pH 6.5, 20 mM) with or without 0.5 mM DTT. The release system was incubated in the dark at 37 °C and in a shaker at 300 rpm. At time intervals, external buffer (3 mL) was used for analysis and 3 mL of fresh buffer was added into the system. The amount of released Dox was tested by Ultraviolet and Visible Spectroscopy (UV-Vis, PerkinElmer, USA) and calculated with a standard curve obtained from a series of standard Dox solution at different concentrations from 0.01 to 0.1 mg•mL^{-1}.

Trafficking of Dox in cancer cells

Intracellular trafficking of Dox-loaded micelles was observed under confocal laser scanning microscopy (CLSM) with z-stack scanning mode. Briefly, MCF-7 cells or SKOV-3 cells at 5×10^4 density were seeded in a 24-well cover glass (VWR International, USA) for 24 h incubation at 37 ºC and humid 5% CO2. Dox-loaded PEG-SSBAP micelles were co-incubated with the cells in complete culture medium. At 1, 4 or 24 h, the cells were washed with fresh PBS, fixed with cold acetone, stained with DAPI (1%) for 10 min. Red fluorescence imaging in the cells was visualized by CLSM at z-stack scanning (Nikon A1R, Tokyo, Japan).

Anti-cancer efficacy of PEG-SSBAP micelles.

Anti-cancer activity of Dox-loaded PEG-SSBAP micelles was evaluated in different cancer cells *in vitro* (HepG2, SKOV-3 and MCF-7). For instance, the cells (1×10^4 cell) in a 96-well plate were incubated with the micelles at Dox concentrations (0.001-10 μg•mL^{-1}) in complete culture medium for 24 h. Cell viability was then detected by AlamarBlue assay (Invitrogen) according to the manufacture's protocol. The viability of untreated cells (blank) was taken to be 100%. Anti-cancer activity of Dox alone at the same concentrations was tested as a control. Cytotoxicity of PEG-SSBAP copolymer at varied concentrations (5-400 μg•mL^{-1}) was tested against the cells after co-incubating PEG-SSBAP with the cells in complete culture medium for 24 h. Also, cytotoxicity of degradation products of PEG-SSBAP, *i.e.* BAP and cysteamine, was tested against the cells with the same protocol.

Animal experiment was done under the approval by Institutional Animal Care and Use Committee of Tongji University. HepG2 tumor-xenografted mouse was constructed by a routine method, that is, subcutaneous injection of 5×10^6 HepG2 cells into flank region of 4-6-week male Balb/c nu/nu mice (SLAC, Shanghai, P.R. China). As the tumor size was about 200 mm^3, Dox-loaded PEG-SSBAP micelles or Dox in PBS buffer (300 μL, 5% glucose) was injected into the mice every four days for four times by tail vein with the Dox dose of 2.5 mg•Kg^{-1} (n=3-4). Tumor size was measured with a caliper every four day and the tumor volume was calculated by the formula: (length) × (width)^2/2. Meanwhile, mouse weight was measured with balance every four day. After sacrifice of the mice, their tissues (liver, lung, spleen, and kidney) and tumor were harvested for hematoxylin-eosin (H&E) staining. The tumor was weighted and its section was analyzed by TUNEL and Ki-67 staining with the standard protocols.

Statistical analysis

Statistical analysis was calculated by student's t-test. A difference at $P<0.05$ was considered to be statistically significant.

Results

Synthesis and characterization of PEG-poly(carbamate amine) diblock copolymers

In this work, a series of new PEG-poly(disulfide carbamate amine) (denoted as PEG-SSPCA) diblock copolymers were prepared by polymerization reaction of 2, 2'-dithiodiethanol

bis(*p*-nitrophenyl carbonate) (DTDE-PNC) and a mixture of amino-terminated PEG (PEG-NH_2) and a teritary amine-containing primary diamine (Fig. 1), followed by the reaction termination with an excess amount of the diamine. Herein, three diamines were applied to afford PEG-SSPCA copolymers with varied structures in the poly(carbamate) block. The crude product was totally dissolved in acidic aqueous solution (pH ~5.0) and purified by ultrafiltration process, in order to eliminate low molecular-weight polymers and non-conjugated PEG. Three copolymers were thus obtained as HCl-salt form after freeze-drying. Solubility test in DMF and water revealed that these copolymers were soluble at a tested polymer concentration of 1 mg•mL^{-1}. ^{1}H NMR spectra analysis showed that these PEG-SSPCA copolymers were in good accordance with their expected chemical compositions (Fig. S1). Moreover, from the spectra, the signals at δ 7.3 and δ 8.3, attributed to the aromatic protons of *p*-nitrophenyl residue, could not be detected, showing that the PEG-SSPCA copolymers comprise primary amine terminal group and *p*-nitrophenyl residue should be totally consumed in termination reaction. FT-IR analysis of PEG-SSBAP revealed two signals at 1700 cm^{-1} and 1530 cm^{-1}, an indicative of carbonyl group (C=O) of carbamate bond in the copolymer (Fig. S2). GPC measurement indicated that these PEG-SSPCA copolymers possessed a single-mode chromatography peak (Fig. S3) and their number-average molecular weights (M_n) were in line with theoretical M_n (Table 1). A relatively narrow molecular weight distribution (PDI=1.4-1.6) of these copolymers was likely ascribed to the removal of low-molecular-weight polymers by ultrafiltration process. Acid-base titration of these copolymers (Fig. S4a) further showed that they had higher buffering capacity (Table 1) between the range of pH 5.1-7.4 as compared to brancehd polyethylenimine (42-68% *vs*. 24%), suggesting that they could mediate a faciliated endsomal escape by the "proton sponge" effect.[12] The above results confirm that PEG-SSPCA copolymers are generated sucessfully through a facile polycondensation reaction.

Table 1. Characteristics of PEG-SSPCA diblock copolymers

Sample code [a]	Theoretical M_n (kDa)	Tested M_n (kDa) [b]	PDI [b]	Buffering capacity [c] (%)
PEG-SSMDE	17.3	19.0	1.4	68
PEG-SSPDA	19.3	19.5	1.6	45
PEG-SSBAP	20.3	21.1	1.4	42

[a] the copolymers are named after diamine used in polycondensation reaction
[b] determined by GPC analysis. [c] estimated by acid-base titration curve

Formation of PEG-SSPCA nanomicelles for drug loading and release *in vitro*

To ascertain whether PEG-SSPCA copolymers can form micelles, the solution of these copolymers was analyzed by dynamic light scattering (DLS) and transition electronic microscopy (SEM), respectively. It was found that these copolymers self-assembled to form nanosized micelles with the particle size in the range of ~141-214 nm in diameter (Table 2). The size distribution of these PEG-SSPCA micelles was relatively narrow (PDI~0.12-0.26) and a single-peak mode. Furthermore, the structure of poly(carbamate) block has an effect on particle size of PEG-SSPCA. PEG-SSBAP micelles had the smallest size, likely due to hydrophobic property of BAP residue. Fig. 2a shows typical size distribution peak of PEG-SSBAP micelles by DLS analysis and spherical morphology observed under TEM. Fluorescence spectra analysis of varied concentrations of PEG-SSPCA in PBS solution having a pyrene probe provides the critical micelle concentration (CMC) of these copolymers (Fig.S5, Table 2), reflecting hydrophobic region in PEG-SSPCA micells. CMC values of PEG-SSPCA copolymers can be regulated by the structure of amino residue. PEG-SSBAP has the smallest CMC (24.5 μg•mL^{-1}) among three copolymers and thus applied for further studies.

Table 2. Characteristics of the micelles of PEG-SSPCA diblock copolymers

Sample code	CMC (μg • mL^{-1})[a]	Size of micelles (nm)/ PDI [b]	Surface charge of micelles (mV) [b]	Size of Dox-loaded micelles (nm)/ PDI [b]	DLC (%)[c]	DLE (%)
PEG-SSMDE	52.3	127.2/ 0.14	+0.46	131.2/ 0.24	3.4	34.8
PEG-SSPDA	76.9	214.2/ 0.16	+0.83	202.1/ 0.32	3.9	40.3
PEG-SSBAP	24.5	108.8/ 0.12	+0.70	111.6/ 0.17	5.7	65.4

[a] determined with pyrene as a fluorescence probe
[b] determined by DLS analysis at 0.25 mg·mL^{-1} polymer concentration; PDI means polydispersity index
[c] theoretical drug loading content (DLC) was set at 10%

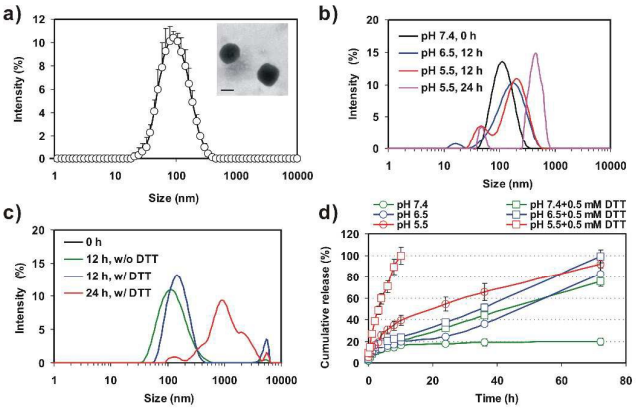

Figure 2. DLS analysis of PEG-SSBAP micelles, destabilization and cumulative release of anti-cancer drug, Dox, in response to an acidic and/or reductive condition. a) Size distribution of PEG-SSBAP micelles (inserted: TEM image of the nanomicelles, scale bar: 100 nm); b) DLS analysis showing the size change of PEG-SSBAP micelles at different pH value; c) DLS analysis showing the size change of PEG-SSBAP micelles in a reductive condition; d) Cumulative Dox release kinetics from PEG-SSBAP micelles at a pH (7.4, 6.5 or 5.5) and/or reduction (0.5 mM DTT) condition.

The ability of PEG-SSPCA micelles in responsive to intracellular environments was evaluated by detecting particle size of PEG-SSBAP micelles in an acidic or reductive condition. As shown in Fig. 2b, the size of the PEG-SSBAP micelles always keeps steady 12 h in PBS buffer at pH 7.4. However, when pH environment was decreased from 7.4 to 6.5 and 5.5, the size of the micelles increased markedly from ~141 to ~192 nm and ~225 nm, which suggests an acid-responsive ability of PEG-SSBAP micelles. Also, elongating incubatin time from 12 h to 24 h, the size of the micelles increased from ~225 nm to ~458 nm. This acid response behavior is attributed to the protonation activity of tertiary amines in poly(carbamate amine) block. This point was further supported

by acid-base titration analysis of PEG-SSBAP, which indicates that apparent protonation degree of tertiary amine increases from 44% to 62% with decreasing pH value from 7.4 to 5.5 (Fig. S4b). In addition to acid response feature, PEG-SSBAP micelles have a reduction responsive profile. For example, after co-incubating the micelles with 0.5 mM DTT (1 mM thiols to mimetic intracellular reductive condition) for 12 h, the size of the micelles significantly augmented from ~141 nm to ~186 nm (Fig. 2c). This reduction response is more pronounced after 24 h co-incubation, following with larger size of ~1127 nm. Overall, these results manifest that PEG-SSBAP micelles can be relatively stable in a physiological condition but destabilized in an acidic or reductive environment, indicating that the micelles may be intracellularly destabilized for a responsive drug release.

To examine drug loading of PEG-SSCPA micelles, an anti-cancer drug, doxorubicin (Dox), was encapsulated into the micelles by a traditional dialysis method. DLS analysis revealed that the size of Dox-loaded PEG-SSCPA micelles (except for PEG-SSPDA) was slightly larger than that of PEG-SSCPA micelles alone (Table 2) but the size distribution became wide (PDI=0.17-0.32). Probably, an incorporation of Dox into hydrophobic region of SSCPA block causes the increment of the size. Drug loading content (DLC) and drug loading efficiency (DLE) were tested, respectively (Table 2). PEG-SSBAP showed the highest ability to load Dox, *i.e.* 5.7% of DLC at a moderate DLE (65.4%). Besides, from four independent batches, DLC of PEG-SSBAP is 5.7±0.5% whic is comparable or higher than that of those dual-responsive copolymers in previous reports such as RPAE-PEG (3.47%)[6], PRS-PU (3.42%)[7], PDSG (4.88%)[8], and disulfide-linked PEG-polycaprolactone (4.5%)[13].

Next, Dox release from Dox-loaded PEG-SSBAP micelles was evaluated in an acidic and/or reductive environment to mimetic an intracellular micro-environment (Fig. 2d). In a physiological condition (pH 7.4), the PEG-SSBAP micelles afforded slow drug release with only ~20% of accumulative drug release within 72 h. However, the drug release rate was enhanced as pH environment was adjusted to pH 6.5 (an endosomal pH). In this case, ~82% of loaded Dox released from the micelles within the same time. As the micelles were incubated at pH 5.5 (a lysosomal pH), the drug release rate could be promoted. For example, during 10 h, ~40% of Dox release from the micelles was detected at pH 5.5, but only ~20% of Dox release at pH 6.5. This enhanced drug release may be attributed to the destabilization of PEG-SSBAP micelles along with decreasing acidic pH from 6.5 to 5.5 (Fig. 2b). Furthermore, efficient drug release from the micelles was found in a reductive condition (0.5 mM DTT) as a result of the destabilization of the micelles by disulfide cleavage (Fig. 2c). Notably, whatever in an acidic or reductive condition was enhanced but insufficient drug release found, *i.e.* ~80% of accumulative Dox release within 72 h. However, in an acid and reduction environment, for instance, pH5.5 plus 0.5 mM DTT, ~100% of accumulative Dox release from PEG-SSBAP micelles could be found within 10 h (Fig. 2d), suggesting that such dual-responsive micelles are more efficient for complete drug release inside the cells.

Intracellular distribution of Dox-loaded PEG-SSBAP micelles in MCF-7 cells was observed under CLSM at z-stack mode through detecting red fluorescence signal emitted from Dox. Fig.3 shows intracellular distribution of the micelles (or free Dox as a control) in the cells 1 h or 24 h after incubating the micelles (or Dox) with the cells. Those cells co-incubated with Dox-loaded PEG-SSBAP micelles had red fluorescence signal in the nucleus and cytoplasm 1 h after the co-incubation (Fig.3a). However, the fluorescence intensity was much weaker compared to that detected in the cells treated by free Dox (Fig.3c). This difference may be ascribed to the fact that Dox-loaded PEG-SSBAP micelles have to undergo slow endocytosis as a result of their almost neutral surface charge but free Dox may enter the cells by rapid molecule diffusion. A similar phenomenon was also observed in SKOV-3 cells, where Dox-loaded PEG-SSBAP micelles were dominantly found in the cytoplasm 1 h after the co-incubation while free Dox located in the nucleus (Fig.S6). The accumulation level of Dox in MCF-7 cells markedly augmented 4 or 24 h after incubating the micelles with the cells (Fig. 3b&c). Besides, red fluorescence aggregates could be observed in the cytoplasm probably because a part of the micelles located in the endosome/lysosome. Support for this point was also found by CLSM imaging, where (Dox) red fluorescence could be detected in the lysosome (Fig. S7). Because the micelles revealed red fluorescence signal in the nucleus 24 h after their co-incubation with the cells (Fig. 3c&e), PEG-SSBAP micelles had the ability to deliver and release Dox into the cells. Most likely, the micelles can liberate Dox by an intracellular destabilization in an acidic or reductive micro-environment, thereby inducing Dox location in the nucleus. A futher study may focus on intracellular location of the micelles by endosome staining with a fluorescence probe, endocytotic pathways and detailed mechanism underlying endosomal escape of the micelles and Dox diffusion.

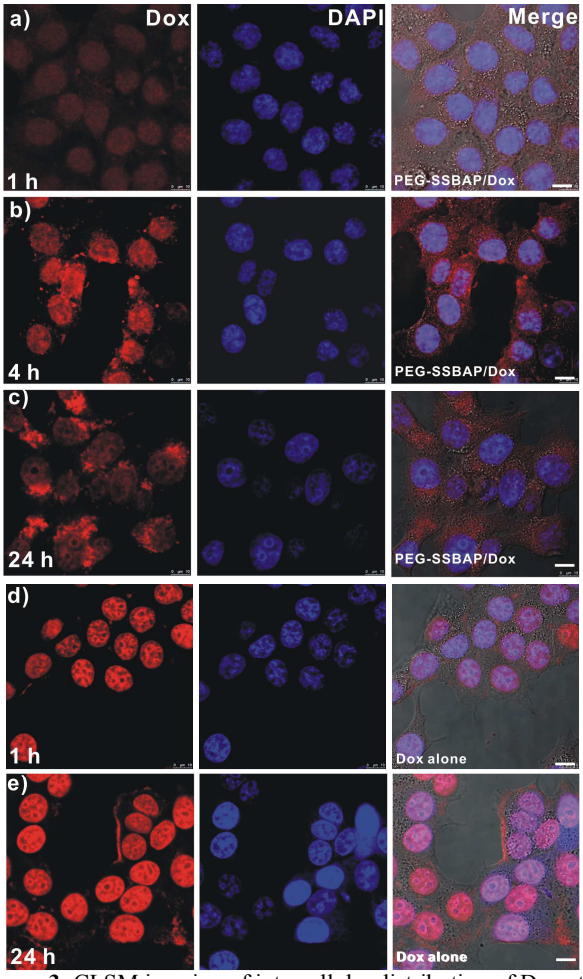

Figure 3. CLSM imaging of intracellular distribution of Dox at a) 1 h, b) 4 h or c) 24 h-post incubation of Dox-loaded PEG-SSBAP micelles with MCF-7 cells. The distribution is also shown at d) 1 h- or e) 24 h-post incubation with free Dox (as a control).

Anti-cancer efficacy of PEG-SSBAP micelles *in vitro*

Since Dox-loaded PEG-SSBAP micelles can liberate Dox slowly in a physiological environment but efficiently in an intracellular environment, the micelle is an appropriate carrier for controlled drug release against cancer. An in vitro study revealed negligible cytotoxicity of PEG-SSBAP micelle itself in MCF-7, HepG2 and SKOV-3 cells at a high concentration of 400 μg•mL^{-1} (Fig. 4a), reflecting good cyto-compatibility of PEG-SSBAP micelles. Also, BAP and cysteamine as degradation productions of PEG-SSBPA displayed low cytotoxicity with above 80% cell viability against MCF-7, HepG2 and SKOV-3 cells at a high concentration of 400 μg•mL^{-1} (Fig.S8). By contrast, Dox-loaded PEG-SSBAP micelles afforded cytotoxicity against the cells (Fig. 4b-d). Furthermore, antic-cancer activity of the micelles should be cell line-type dependent. For example, at a Dox concentration of 5 μg•mL^{-1}, Dox-loaded micelles afforded significant growth repression in SKOV-3 and HepG2 cells (~60% of cell viability) but only moderate growth inhibition in MCF-7 cells (~90% of cell viability). Besides cell type, the sensitivity of Dox concentration to cancer cells is another factor to affect anti-cancer activity. This may rationally interpert that, in HepG2 cells, the micelles afforded comparable anti-cancer efficacy to free Dox at a Dox concentration of 1-10 μg•mL^{-1} (Fig. 4d). However, at a low Dox concentration of 0.1-1 μg•mL^{-1}, in all these cancer cells, antic-cancer efficacy of Dox-loaded PEG-SSBAP micelles was inferior to that obtained with free Dox. This is probably because that free Dox can rapidly diffuse into the nucleus and intercalates into DNA for killing cells, but the micelles need longer time to transfer and unload Dox into the nucleus. This hypothesis is also supported by the Dox distribution data (Fig. 3d&e), where free Dox has a higher Dox accumulation level in the nucleus of MCF-7 cells when compared to Dox-loaded PEG-SSBAP micelles at 1 h or 24 h.

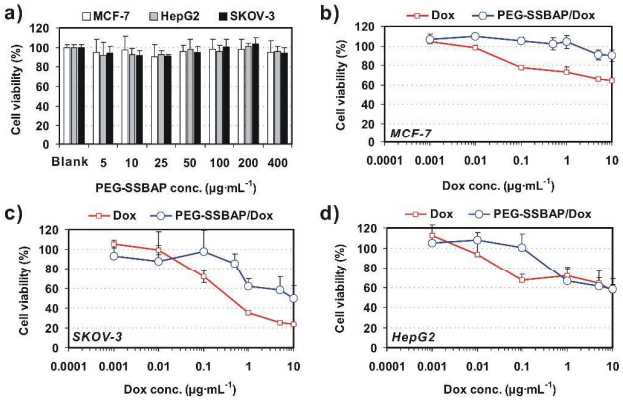

Figure 4. Cell viability assay after the incubation of PEG-SSBAP micelles or Dox-loaded PEG-SSBAP micelles with cancer cells. a) Cell viability as a function of PEG-SSBAP concentration ranging from 5 to 400 μg•mL^{-1}; b-d) Cell viability as a function of Dox concentration ranging from 0.001 to 10 μg•mL^{-1} against MCF-7, SKOV-3 and HepG2 cells.

In vivo anti-cancer efficacy of PEG-SSBAP/Dox micelles

Encouraged by *in vitro* anti-cancer efficacy, *in vivo* chemotherapy of HepG2 tumor using Dox-loaded PEG-SSBAP micelles was investigated in a HepG2 tumor-bearing Balb/c nude mouse model. The Dox-loaded micelles exerted significant growth repression of the tumor by intravenous injection every 4 day for 4 times during 12 days (Fig.5a). Besides, within day 12 to 28 without the therapy, a slow tumor growth was an indicative of durative anti-cancer effect. In comparison with free Dox group (Fig. 5a), the micelle group showed comparable tumor inhibition effect during 28-day therapy. Also, the tumor mass between Dox-loaded PEG-SSBAP micelle group and free Dox group was similar (Fig. 5b). Further TUNEL and Ki-67 staining assays of the tumor sections revealed that the mechanism underlying this anti-tumor activity of the micelles is due to remarkable apoptosis in the tumor cells and growth inhibition (Fig. S9). These results thus reflect that Dox-loaded PEG-SSBAP micelles are efficient for cancer therapy. A discrepancy between the Dox-loaded micelle and free Dox group is systemic toxicity. When using a medium dose of 2.5 mg·Kg^{-1}, free Dox induced detectable body weight lose as an indicative of toxicity to the mouse (Fig. 5c). However, Dox-loaded PEG-SSBAP micelles did not afford this adverse effect. Besides, H&E staining assay revealed no damage of organ tissues of the mice in the micelle group (Fig. S10), showing good *in vivo* compatibility of the micelle formulation. Overall, the above data manifest high feasibility of Dox-loaded PEG-SSBAP micelles in cancer therapy.

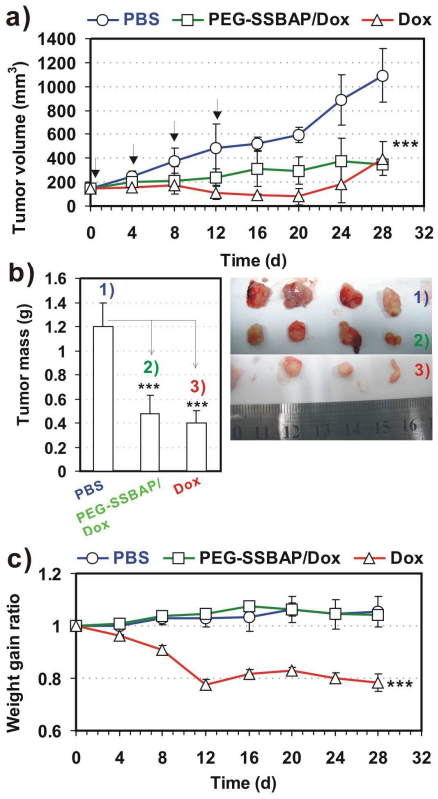

Figure 5. Chemotherapy of HepG2 tumor bearing in nude mice using Dox-loaded PEG-SSBAP micelles. a) Tumor growth curve after intravenous injection of Dox-loaded PEG-SSBAP micelles at a medium dose of 2.5 mg·Kg^{-1} every four days for four times (see arrow). PBS or free Dox were used as controls (n=3-4); b) The mass of the tumor harvested at day 28 after chemotherapy with Dox-loaded PEG-SSBAP micelles or free Dox (positive control) or PBS (negative control). The tumors harvested are also shown; c) Mouse weight gain ratio as a function of therapy time within 28 days using Dox-loaded PEG-SSBAP micelles, free Dox (positive control) or PBS (negative control) (***P<0.001: PEG-SSBAP/Dox *vs*. Dox).

Discussion and Conclusion

The development of bio-responsive nano-carriers for controlled and intracellular drug release is indispensable for sucessful cancer therapy. In recent years, a vesatile of nano-carriers that are able to response to intracellular micro-environments such as acid, redox, oxidation and enzyme have been studied.[14] However, most of these nano-carriers reported only have a single-responsive feature, inducing a single responsive drug release. Given that complicated intracellular environments, dual- or multi-responsive drug release is undoubtedly applicable to achieve efficient and sufficient drug release.[5] This background leads to the focus on acid and redutive dual-responsive drug release system because most of anti-cancer drugs normally undergo the translocation in acidified endosomes or lysosomes and then in reductive nucleus for killing cancer cells. Therefore, polymeric micelles having a unique acid and reduction dual-responsive ability should be suited drug release systems.

The design of amphiphlic block polymers with intracellular dual-responsive functional groups is a practical approach to gain such dual-responsive micelles. Those previous studies usually reported on ester or amide-based block copolymers installed with disulfide linkage and protonable amine[6, 10] or acid-liable group[15, 16]. Herein, We develope poly(carbamate)-based diblock copolymers (Fig. 1). A unique feature of the PEG-SSPCA copolymers is that disulfide, carbamate and protonable tertiary amino groups are well arranged in the polymeric repeated units. To the best of our knowledge, this is the first report on poly(carbamate amine) copolymers.

In PEG-SSPCA copolymers, PEG block is highly hydrophilic but SSPCA block is hydrophobic due to hydrophobic carbamate bond and unprotonated amine residues. The amphiphlic copolymers are thus prone to self-assembly into well-defined micelles, which are confirmed by DLS and TEM analyses (Fig. 2, Table 1). Probably, hydrogen bonding between carbamate groups also contributes for highly hydrophobic region of SSPCA core of the micelles. This question should be examined in a further study. The drug loading ability of the PEG-SSPCAs may be adjusted by varying chemical structure of diamines (Table 2). As such, a relatively high Dox loading content (5.7%) may be obtained when BAP is applied. Because tertiary amino group and disulfide linkage respectively endow acid- and reduction-reponsive properties for PEG-SSPCA copolymers, their micelles display dual-responsive destabilization features, causing an accelerated and sufficient drug release in an intracellular environment (Fig. 2). These features thus imply that PEG-SSBAP micelle is a suited nano-carrier for intracellular drug release against cancer.

Although acid and reduction dual-responsive diblock copolymer micelles are designed and investigated for cancer therapy *in vitro*, their further *in vivo* therapy is only reported in few references.[17] Probably, such dual-responsive micelles reported previously have a low drug loading content (e.g. <5%), impeding *in vivo* practice. Hererin, by optimizing the structure of diamine residue in SSPCA block, PEG-SSBAP is found to possess high Dox loading content of 5.7%. A future work may rationally adjust length ratio between hydrophobic SSBAP chain and hydrophilic PEG chain, in order to improve the drug loading content. Besides, *in vitro* anti-cancer tests corroborate that Dox-loaded PEG-SSBAP micelles have comparable anti-cancer ability to free Dox for killing human HepG2 cells (Fig.4). These results reveal that the micelles have a high feasibility for chemotherapy. By intravenous injection into HepG2 tumor-bearing Balb/c mice at a medium drug dose (2.5 mg·Kg^{-1}), the micelles indeed exert marked inhibition of the tumor growth along with minor systemic toxicity (Fig.5). A rational explanation on this low toxicity is that the micelles afford slow Dox release in a physiological condition (Fig.2d), leading to a low level of free Dox accumulation in heart and in turn minimizing the cardiotoxicity of free Dox.[18] Besides, low toxicity of BAP contributes good bio-compatibility *in vivo*. It was shown that BAP had no detectable side effect on male rats at intravenous injection dose of 20 mg·Kg^{-1}·d^{-1} for 14 days.[19] Herein, the amount of BAP in PEG-SSBAP for *in vivo* study is *ca*. 14.8 mg·Kg^{-1}·d^{-1}, which is lower than this safe dose. Thus, it may be deduced that BAP as degradation product of PEG-SSBAP likely affords minor toxicity. Bio-safety assessment of PEG-SSBAP should be done in future work.

In conclusion, we have confirmed that novel PEG-poly(disulfide carbamate amine) diblock copolymers can be generated that have multiple disulfide linkage, carbamate linkage and amino group. These copolymers self-assemble into nanosized micelles and their

size may be regulated by the structure of the amino residue. The micelles based on PEG-poly(disulfide carbamate 1, 4-bis(3-aminopropyl)piperazine) (PEG-SSBAP) may carry anti-cancer drug, doxorubicin, and sufficiently liberate the drug in an acidic and reductive environment. Moreover, doxorubicin-loaded PEG-SSBAP micelles afford marked anti-cancer efficacy *in vitro* and *in vivo* with minor cytotoxicity and systemic toxicity. This study offers new insight on biodegradable poly(carbamate) copolymers and their micelles as nano-carriers for use in nanomedicine.

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Notes and references

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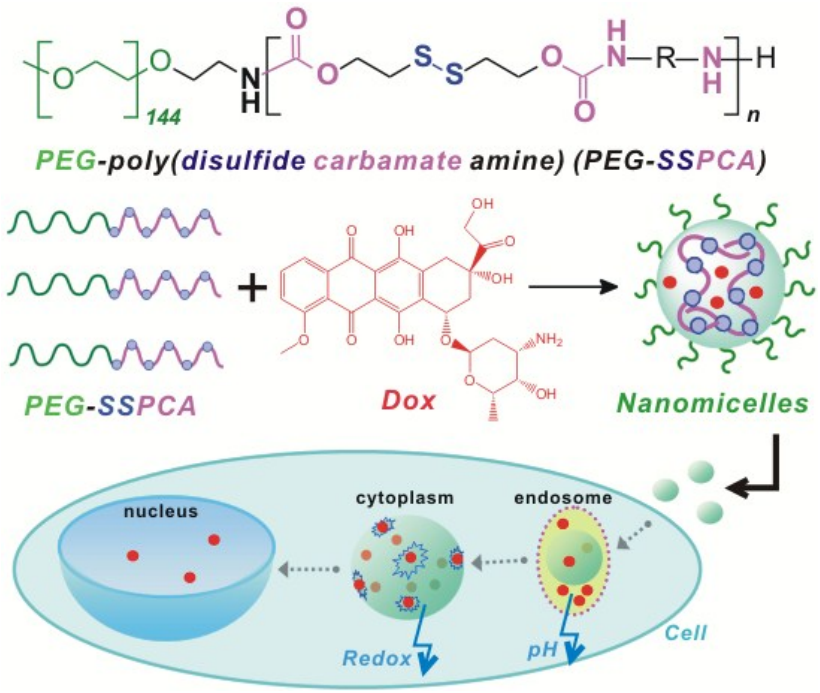

Self-assembled nanomicelles based on biodegradable poly(carbamate) copolymers are applicable for pH and redox dual-responsive release of doxorubicin (Dox) and cancer chemotherapy