The background of the entire page is a close-up photograph of numerous water droplets of various sizes. The droplets are clear and spherical, reflecting light, and are set against a vibrant blue background. The density of the droplets is higher in the upper half of the image, creating a textured, bubbly appearance, while the lower half shows fewer, more isolated droplets.

Biodegradable Polycarbonate-Based Stimuli-Responsive Nanosystems for Intracellular Drug Delivery

Wei Chen

**BIODEGRADABLE POLYCARBONATE-BASED
STIMULI-RESPONSIVE NANOSYSTEMS FOR
INTRACELLULAR DRUG DELIVERY**

Wei Chen

2013

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Biodegradable polycarbonate-based stimuli-responsive nanosystems for intracellular drug delivery

Wei Chen

PhD thesis with references, with summaries in English and Dutch
University of Twente, Enschede, The Netherlands

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BIODEGRADABLE POLYCARBONATE-BASED STIMULI-RESPONSIVE NANOSYSTEMS FOR INTRACELLULAR DRUG DELIVERY

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Chapter 1

General Introduction

1.1 Background

The past decade has witnessed the development of nanotechnology for drug delivery since it provides efficient approaches to deliver potent chemotherapeutics including low molecular weight drugs, as well as macromolecules such as proteins or genes to the diseased tissue [1-3]. Biocompatible and biodegradable nanosystems such as micelles, nanoparticles, nanocapsules, polymersomes and polymer conjugates have been mostly investigated as therapeutic agent formulations, since these systems have several advantages in drug delivery (Figure 1.1), including (i) a remarkable enhancement of the solubility of anti-cancer drugs in aqueous systems; (ii) prolonging the drug circulation time, especially for therapeutic agents like proteins, peptides, and nucleic acid drugs preventing enzymatic degradation; (iii) targeting to tumor tissues *via* the enhanced permeability and retention (EPR) effect; and (iv) improvement of the drug bioavailability [4-6]. Also, the release of therapeutic agents from nanoparticles can be controlled by modulating polymer characteristics, to achieve desired therapeutic levels in diseased tissue with optimal drug efficacy [7-9]. Further, nanoparticles can be delivered to distant target sites by conjugation of a specific ligand which can guide them to the target tissue or organ [10].

Figure 1.1. Novel strategies to address the extracellular and intracellular barriers of drug-loaded biodegradable micelles for targeted, safe and efficient cancer therapy [5].

Biodegradable polymers have been widely used in the biomedical fields because of their biocompatibility and biodegradability [11]. Among numerous biodegradable polymeric materials applied in drug delivery applications, aliphatic polycarbonates have been one of the most interesting materials due to their biocompatibility, nontoxic degradation products, and the absence of autocatalytic degradation processes. In addition, polycarbonate degradation will not lead to increased levels of acidity, which may occur during polyester degradation, and which may be hazardous to loaded drugs or healthy tissues [12, 13]. For the further development of biotechnology and nanotechnology, very often common polycarbonates such as poly(trimethylene carbonate) (PTMC) cannot satisfy the requirements for particular applications, due to their high hydrophobicity, improper degradation profile, and/or lack of reactive centers in the polymer chain for the covalent immobilization of bioactive molecules such as drugs, peptides and proteins. In recent years, the design of functional cyclic carbonate monomers has received more and more interest, and various functional aliphatic polycarbonate-based homopolymers and copolymers containing *e.g.* hydroxyl, carboxyl, and amine pendant groups have been reported [14-16]. These functional polymers on one hand show improved physiochemical properties such as enhanced hydrophilicity and biodegradability, and on the other hand facilitate drug conjugation or further derivatization. There are few reports on nanosystems based on functional biodegradable polycarbonate-based polymers for targeted and triggered intracellular drug release. It is anticipated that advanced biodegradable nanosystems based on these polycarbonate-based polymers are highly promising for safe and efficient cancer treatment.

1.2 Aim of the study

The aim of this study is to prepare functional biodegradable polycarbonate-based polymers that facily form nanocarriers for intracellular drug delivery. Nanoparticles should be delivered to target sites with a biospecific ligand that also induces efficient cellular uptake, and furthermore ideally the therapeutic agents should be released from polymeric nanoparticles by a local trigger, allowing the desired therapeutic efficacy in the target tissue. Aliphatic polycarbonates and their copolymers, due to their low toxicity, biocompatibility and biodegradability, have been widely studied as biomaterials for drug delivery applications. Design of functional cyclic carbonate monomers has driven wider interest in this area leading to the creation of functional degradable polymers containing versatile pendants, providing interesting platforms for targeted and triggered intracellular release of potent chemotherapeutics.

1.3 Outline of the thesis

In this thesis advanced biodegradable polycarbonate-based nanocarriers used as controlled intracellular drug delivery systems for anticancer therapy are described. The synthesis of copolymers, preparation of nanocarriers, loading and *in vitro* release of drug, as well as cellular uptake and antitumor activity of drug loaded nanoparticles were studied in detail. Parts of this thesis have been published elsewhere or have been submitted for publication [17-19].

In **Chapter 2** a literature overview is presented focusing on advanced biodegradable nanocarriers based on designed functional cyclic carbonate monomers for controlled drug and gene delivery. This review aims to contribute to the understanding of the current status in this area.

In **Chapter 3** the synthesis of a new monomer, pyridyl disulfide-functionalized cyclic carbonate (PDSC), is presented. This monomer was used for the facile preparation of reduction-sensitive polycaprolactone-*graft*-SS-poly(ethylene glycol) graft copolymer (PCL-*g*-SS-PEG) via combination of ring-opening polymerization (ROP) of PDSC with ϵ -caprolactone (ϵ -CL) and a thiol-disulfide exchange reaction with thiolated methoxy polyethylene glycol (PEG-SH). In this study, the synthesis and ROP of PDSC monomer, synthesis and self-assembly of PCL-*g*-SS-PEG graft copolymer, loading and reduction-responsive release of doxorubicin (DOX), as well as intracellular release and antitumor activity of DOX-loaded graft copolymer micelles were investigated.

In **Chapter 4** the preparation of biodegradable polycaprolactone-*graft*-SS-galactose (PCL-*g*-SS-Gal) copolymer is described. This copolymer was prepared by a combination of ROP of ϵ -CL with PDSC, and post-polymerization modification with thiolated galactose (Gal-SH) via the thiol-disulfide exchange reaction. Gal-shielded redox-sensitive nanoparticles based on biodegradable PCL-*g*-SS-Gal amphiphilic copolymer were developed for hepatoma-targeted intracellular delivery of DOX. The synthesis and self-assembly of Gal-bearing graft copolymers, loading and *in vitro* release of DOX, as well as targeting liver cancer cells, cellular uptake and antitumor activity of DOX-loaded polymeric nanoparticles were studied.

Chapter 5 describes novel redox and pH-responsive biodegradable micellar nanoparticles based on poly(ethylene glycol)-SS-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) block copolymer (PEG-SS-PTMBPEC) for dually triggered intracellular release of DOX in cancer cells. It was assumed that endocytosis of drug loaded PEG-SS-PTMBPEC micelles by cancer cells would first lead to partial release of DOX due to hydrolysis of acetal bonds at endosomal pH, followed by complete release of DOX in the cytosol due to cleavage of the disulfide bonds in response to a high glutathione (GSH) concentration. In this study, the synthesis, *in vitro* drug release and antitumor activities of DOX-loaded PEG-SS-PTMBPEC micellar nanoparticles were investigated and the results were compared with those obtained using DOX-loaded poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PEG-PTMBPEC) counterparts, not containing disulfide bonds.

In **Chapter 6** novel reversibly crosslinked pH-responsive micelles based on poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate-*co*-pyridyl disulfide carbonate) PEG-P(TMBPEC-*co*-PDSC) block copolymer for dually activated intracellular release of DOX in cancer cells are presented. In the current approach micelles were designed that can be stabilized by reversible crosslinking using PDSC units with minimal drug leakage during circulation while quickly and completely releasing the payload after arriving at the tumor site as well as in the intracellular environment. The synthesis, stability, *in vitro* drug release and tumor cell killing activity of DOX-loaded reversibly crosslinked PEG-(PTMBPEC-*co*-PDSC) micellar nanoparticles were investigated.

In **Chapter 7** the preparation of non-toxic and biodegradable glycomicelles by a one-pot sequential

ROP of acryloyl cyclic carbonate monomer (AC) and ϵ -CL in dichloromethane (DCM), followed by sequential post-polymerization modification with Gal-SH and 2-(2-methoxyethoxy)ethanethiol ((EO)₂-SH) through Michael-type conjugate addition reaction is described. The glycomicelles had a tunable galactose density and were investigated for active hepatocyte-targeted drug delivery. The synthesis and self-assembly of biodegradable block glycopolymers, loading and *in vitro* release of DOX, as well as the uptake of DOX-loaded glycomicelles by asialoglycoprotein receptor (ASGP-R) positive HepG2 liver cancer cells and their toxicity towards these cells have been investigated.

In **Chapter 8** *in situ* forming reduction-sensitive degradable nanogels from poly(ethylene glycol)-poly(2-(hydroxyethyl) methacrylate-*co*-acryloyl carbonate) (PEG-P(HEMA-*co*-AC)) block copolymers using cystamine as a crosslinker are presented. These nanogels were developed for facile loading and triggered intracellular release of proteins. The pendant cyclic carbonate units will facilitate crosslinking of PEG-P(HEMA-*co*-AC) with cystamine under aqueous conditions via a ring-opening reaction. In this study, the preparation of *in situ* forming reduction-responsive degradable nanogels, loading and reduction-triggered release of cytochrome c (CC), intracellular protein release as well as anti-tumor activity of CC-loaded nanogels were investigated.

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Chapter 2

Advanced Biodegradable Nanocarriers Based on Designed Functional Cyclic Carbonate Monomers for Controlled Drug and Gene Delivery

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Abstract

Biodegradable polymeric nanocarriers have emerged as one of the most promising platforms for targeted and controlled drug delivery due to their excellent biocompatibility, prolonged circulation time, enhanced accumulation in tumors, and *in vivo* degradability. Aliphatic polycarbonates, on the account of their low toxicity, biocompatibility and biodegradability, have been widely studied as biomaterials for drug delivery applications. Furthermore, the design of functional cyclic carbonate monomers has driven wider interest in this area leading to the creation of functional degradable polymers containing versatile pendants by ring-opening polymerization. In this review, biodegradable functional polycarbonate-based polymers bearing diverse functionalities such as hydroxyl, carboxyl, amine/urea, alkene, alkyne, halogen, azido and sugars, will be discussed with respect to their use for the preparation of intelligent nanocarriers for controlled drug and gene delivery. It is concluded that functional biodegradable polycarbonate-based polymers are very promising for use in advanced multifunctional drug carriers, which can be applied for targeted, safe and efficient cancer treatment and for gene delivery.

2.1 Introduction

In the past decade, ever-growing efforts have been devoted to develop nanotechnology for drug delivery since it offers a suitable approach for transporting low molecular weight drugs including peptides, as well as macromolecules such as proteins or genes by either localized or targeted delivery to the diseased tissue [1-3]. Nanotechnology focuses on formulating therapeutic agents in biocompatible and biodegradable nanocarriers such as micellar systems, nanoparticles, nanocapsules, polymersomes and polymer conjugates. These nanosized polymeric nanosystems have multifaceted advantages in drug delivery, including (i) a remarkable enhancement of the solubility of anti-cancer drugs in aqueous systems; (ii) prolonging the drug circulation time, especially for therapeutic agents like proteins, peptides, and nucleic acid drugs preventing enzymatic degradation; (iii) passive targeting to tumor tissues *via* the enhanced permeability and retention (EPR) effect; and (iv) improvement of the drug bioavailability [4-6]. In addition, triggered release of therapeutic agents from nanoparticles can be induced by modulating polymer characteristics, allowing the desired therapeutic efficacy in the target tissue [7-10]. Furthermore, nanoparticles can be delivered to tissue or organ target sites via conjugation with a biospecific ligand [11-13].

Among numerous biodegradable polymeric materials used in drug delivery applications, aliphatic polycarbonates are one of the most interesting materials due to their biocompatibility, nontoxic degradation products, and the absence of autocatalytic degradation processes [14-16]. For example, based on their favorable material properties, copolymers of carbonates such as trimethylene carbonate (TMC) with some other cyclic monomers such as lactide (LA), glycolide (GA), and ϵ -caprolactone (ϵ -CL) have already found application as sutures and in controlled drug delivery systems [17-19]. In addition, materials based on poly(trimethylene carbonate) (PTMC) are known to degrade *in vivo* by surface erosion in contrast to the bulk degradation behavior observed with poly(ester)s [20]. Furthermore, polycarbonate degradation will not lead to increased levels of acidity, which may occur during polyester degradation, and which may be hazardous to loaded drugs or healthy tissues.

However, in the practice of drug delivery, very often PTMC homopolymers and their copolymers cannot satisfy the requirements for particular applications, due to their high hydrophobicity, improper degradation profile, and/or lack of reactive centers in the polymer chain for the covalent immobilization of bioactive molecules such as drugs, peptides and proteins. Furthermore, the ever advancing tissue engineering and regenerative medicine technology demands development of complex and tunable biologically active biomaterials. In the past decade, design of functional cyclic carbonate monomers has received more and more interest, and various functional aliphatic polycarbonate-based homopolymers and copolymers containing *e.g.* hydroxyl, carboxyl, and amine pendant groups have been reported [21-23]. These functional polymers on one hand show improved physiochemical properties such as enhanced hydrophilicity and biodegradability, and on the other hand facilitate drug conjugation or further derivatization. Herein, we review up-to-date novel functional polycarbonate-based biodegradable nanocarriers for controlled drug and gene delivery. It is anticipated that with the favorable properties of

biodegradable polycarbonate-based polymers we are able to create advanced multifunctional drug carriers for targeted, safe and efficient cancer treatment.

2.2 Polycarbonates containing hydroxyl functional groups or derivatives

Hydroxyl functional groups were commonly introduced into biodegradable polymers via homopolymerization of benzyloxy [24-26] or acetal [27-30] protected cyclic carbonate or copolymerization of these functional cyclic carbonates with other cyclic monomers (*i.e.* LA, ϵ -CL, and TMC), followed by deprotection with Pd/C or hydrolysis at mild acidic conditions (Scheme 2.1). Recently, biodegradable hyperbranched polycarbonates were synthesized by ring-opening polymerization (ROP) of hydroxyl-bearing cyclic carbonate, using its own hydroxyl group as the initiator, to produce hydroxyl functional groups at the outer sphere of the hyperbranched polymer [31, 32]. These OH-enriched poly(2-hydroxyl trimethylene carbonate) (PHTMC) demonstrated high cell-biocompatibility as determined with the MTT assay [33]. It has been well known that the degradation rate of aliphatic polycarbonates is considerably lower than that of most aliphatic polyesters, which restricts their use as short-term implant biomaterials. Due to the improved hydrophilicity and the autocatalytic effect of the hydroxyl groups, PHTMC has a much faster degradation rate than that of the non-functionalized PTMC analog, with a similar structure differing merely by the absence of pendant hydroxyl groups [28, 33, 34]. It is assumed that the fast degradation also involves an intra-molecular nucleophilic attack by the pendant hydroxyl groups on the carbonate linkages of the main chain [35].

Well-defined amphiphilic biodegradable block copolymers comprising of a functionalized polycarbonate hydrophobe and poly(ethylene glycol) (PEG) undergo phase separation in aqueous medium, leading to the formation of nanosized core-shell micellar structures, which represent a major advance in drug delivery applications. For example, Allen *et al.* reported amphiphilic diblock copolymers with various block compositions of PEG as the hydrophilic block and poly(5-benzyloxy-trimethylene carbonate) (PBTMC) as the hydrophobic block [36]. Poly(ethylene glycol)-*block*-poly(5-benzyloxy-trimethylene carbonate) (PEG-*b*-PBTMC) copolymers self-assembled into micelles in water with a narrow size distribution. These micelles were biodegradable and noncytotoxic. Subsequently, they investigated PEG-*b*-PBTMC (5k-*b*-4.8k) micelles as a delivery system for the hydrophobic anti-cancer agent ellipticine [37]. Ellipticine-loaded micelles have a spherical morphology and an average diameter of 96 nm with a loading efficiency of 95%. It was found that approximately 32% of the total drug loaded within the micelles was released over a 24-h incubation period. It was confirmed that the anti-cancer activity of ellipticine was retained following formulation in the PEG-*b*-PBTMC micelles.

Scheme 2.1. Synthesis of functional polycarbonate-based polymers containing hydroxyl groups and derivatives.

In order to establish the formation of pH-sensitive micellar systems for controlled drug release, acetal or ketal groups can be used as acid sensitive protecting groups [38]. In our group, we designed a novel acid-labile acetal-containing cyclic carbonate monomer, 2,4,6-trimethoxybenzylidenepentaerythritol carbonate (TMBPEC), which was used to develop pH-sensitive degradable micelles [39]. The results showed that the acetals in poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidenepentaerythritol carbonate) (PEG-PTMBPEC) block copolymer micelles were prone to fast hydrolysis at mildly acidic pH, which transformed the hydrophobic PTMBPEC block into hydrophilic poly(pentaerythritol carbonate) (PPEC) block, resulting in markedly enhanced intracellular drug release. Interestingly, when the M_n 's of PEG-PTMBPEC copolymer blocks were tailored to 1.9 k-6.0 k, pH-sensitive biodegradable polymersomes

could be prepared [40], which could simultaneously encapsulate hydrophobic (paclitaxel, PTX) and hydrophilic (doxorubicin hydrochloride, DOX·HCl) drugs (Figure 2.1). Furthermore, *in vitro* release studies demonstrated that the release of PTX and DOX·HCl from these polymersomes was highly pH-dependent. Very recently, we also developed redox and pH dual-responsive biodegradable nanoparticles from poly(ethylene glycol)-SS-poly(2,4,6-trimethoxybenzylidenepentaerythritol carbonate) block copolymer (PEG-SS-PTMBPEC) for dual-triggered intracellular release of doxorubicin (DOX) [41]. Based on the relatively fast drug release from PEG-PTMBPEC nanoparticles at mildly acidic pH, an even enhanced drug release was observed when 10 mM glutathione (GSH) was used at pH 5.0 and 94.2% of DOX was released from the dual-responsive biodegradable nanoparticles in 10 h. Interestingly, DOX release was obviously enhanced by 2 or 4 h incubation at pH 5.0 and then at pH 7.4 with 10 mM GSH (mimicking the intracellular pathway of endocytosed micellar drugs). MTT assays using HeLa and RAW 264.7 cells revealed that DOX-loaded PEG-SS-PTMBPEC nanoparticles had a significant anti-tumor activity.

Figure 2.1. Illustration of pH-sensitive degradable polymersomes based on PEG-PTMBPEC diblock copolymer for triggered release of both hydrophilic and hydrophobic anticancer drugs. In comparison, pH-sensitive degradable micelles/nanoparticles are typically applied for release of hydrophobic drugs only [40].

The benzyloxy group is inert during most polymerization conditions and is therefore often used to protect hydroxyl functional groups which may be incompatible in a ROP process. After deprotection of the benzyl groups, aliphatic polycarbonate-based biodegradable copolymers containing pendant hydroxyl groups can be used for the covalent immobilization of bioactive molecules such as drugs, peptides and proteins [42-44]. For example, Jing *et al.* developed two types of DOX-conjugated prodrugs based on amphiphilic PEG-*b*-poly(l-lactide-*co*-2,2-dihydroxymethylpropylene carbonate) PEG-*b*-P(LLA-*co*-DHP) block copolymer, using dihydroxyl-functionalized carbonate units to accomplish conjugation of DOX *via* a carbamate link or an acid-labile hydrazone link, respectively [45]. Both DOX-conjugated prodrugs

exhibited pH-dependent release behavior, and the micelles with a hydrazone link showed better pH-sensitivity than those with a carbamate link. Furthermore, the tumor-targeting ligand folic acid (FA) was successfully conjugated to PEG-*b*-P(LLA-*co*-DHP) polymer *via* an ester linkage also through the hydroxyl-functionalized carbonate units using the same carrier. The *in vitro* uptake of these carriers by cells was followed by confocal laser scanning microscopy (CLSM) and flow cytometry and showed an enhanced internalization of FA-containing tumor-targeted micelles by human ovarian cancer cell line SKOV-3 as compared to the control without FA. These targeted and pH sensitive micelles constructed *via* conjugation through the hydroxyl-functionalized carbonate units could be a promising drug delivery system for cancer therapy. They also reported a lactose mediated liver-targeting effect as shown by *ex vivo* imaging technology [46]. Rhodamine B (RhB) as the fluorescence probe was conveniently conjugated to amphiphilic PEG-*b*-P(LLA-*co*-DHP) block copolymer *via* an ester link through the hydroxyl-functionalized carbonate units, to prepare two kinds of (RhB)-bearing micelles (lactose-free and lactose-containing micelles) based on PEG-P(LLA-*co*-DHP/RhB) copolymer. The ability of the lactosylated copolymer to target over-expressed asialoglycoprotein receptors (ASGP-R) in the liver of mice was tested. Micelles of a mixture of lactose-containing copolymer (Lac-PEG-PLLA) and PEG-P(LLA-*co*-DHP/RhB) copolymer and a model drug, were prepared and administered by tail vein injection. Fluorescence-based *in vivo* imaging showed preferential accumulation of the lactosylated micelles in the liver (Figure 2.2). In contrast, micelles without lactose residue were distributed more evenly among liver and other organs. The hydroxyl-functionalized carbonate units provided a promising platform for facile conjugation of the fluorescence probe in micelles which were subsequently used for *ex vivo* imaging.

Figure 2.2. Schematic representation of multifunctional copolymer micelles targeting lactose- or galactose-receptors on mammalian liver cells [46].

Recently, Feng *et al.* developed a facile catalyst-free method to prepare hyperbranched hydroxyl-enriched aliphatic polycarbonate by self-condensing ring-opening polymerization (SCROP) strategy [47]. PEG was successfully attached to multi-arm hyperbranched copolymer *via* urethane links through the hydroxyl groups at the outer sphere of the hyperbranched (5-ethyl-5-hydroxymethyl-1,3-dioxan-2-one) (denoted as HEHDO-star-PEG). HEHDO-star-PEG micelles showed excellent stability with respect to micellar size upon dilution, and displayed good cell-biocompatibility. A high drug loading content of DOX as well as a sustained release pattern of DOX with the HEHDO-star-PEG based delivery system was achieved.

2.3 Polycarbonates containing carboxyl functional groups

Pendant carboxyl groups along polycarbonate-based polymers provide useful sites for further modification to fabricate biomaterials bearing other functional/reactive groups. Benzyl ester groups often used to protect carboxyl functional groups which may be incompatible in a ROP process are inactive at most polymerization conditions [48-50] (Scheme 2.2).

Scheme 2.2. Synthesis of functional polycarbonate-based polymers containing carboxyl groups and derivatives.

As well known, drug-loaded micellar systems *in vivo* require sufficient extracellular stability during blood circulation upon systemic administration. To this end, Mahato and coworkers prepared an amphiphilic PEG-*b*-poly(2-methyl-2-carboxytrimethylene carbonate-*graft*-dodecanol) (PEG-*b*-PCD) lipopolymer, by conjugating hydrophobic lauryl alcohol onto the parent PEG-*block*-poly(5-benzoyloxycarbonyl-trimethylene

carbonate) (PEG-*b*-PBTMCC) copolymer after deprotection by removing the benzyl groups [51]. PEG-*b*-PCD micelles had a remarkably higher stability than PEG-*b*-poly(2-methyl-2-carboxytrimethylene carbonate) (PEG-*b*-PTMCC) micelles and the critical micelle concentration (CMC) of PEG-*b*-PCD micelles could even be as low as 10^{-8} M, which was 50-fold lower than that of the counterpart PEG-*b*-PTMCC. The strong hydrophobic inter-chain interaction within the core domain of the micelles is believed to help to protect the aggregates from structural collapse. It is surmised that the stability improved micelles would be potentially more suitable with respect to longer systemic circulation times and enhanced therapeutic efficacy of loaded drugs.

Mahato *et al.* synthesized novel biodegradable polyethylene glycol-*block*-poly(2-methyl-2-benzoxycarbonyl-propylene carbonate-*co*-l-lactide) copolymers (PEG-*b*-P(BTMCC-*co*-LLA)) for systemic micellar delivery of bicalutamide [52]. Biodegradable PEG-*b*-P(BTMCC-*co*-LLA) micelles had an improved drug loading efficiency as compared to PEG-PLLA counterparts without carbonate units. The drug loading of micelles formulated with PEG-*b*-P(BTMCC-*co*-LLA) copolymer containing 20 mol% carbonate was about 4-fold higher than that of micelles based on the PEG-PLLA copolymer and the solubility of bicalutamide increased from 5 mg/mL in water to 4000 mg/mL in aqueous micellar systems. The bicalutamide-loaded PEG-*b*-P(BTMCC-*co*-LLA) micelles showed significant inhibition of LNCaP cell growth in a dose-dependent manner similarly to the efficacy of free drug. They further used PEG-*b*-P(BTMCC-*co*-LLA) micelles to encapsulate a newly potent 5-indolyl derivative, (2-(1 H-Indol-5-yl) thiazol-4-yl) 3,4,5-trimethoxyphenyl methanone (LY293), to treat resistant melanoma [53]. The results showed that LY293-loaded micelles exhibited excellent efficacy and induced apoptosis of melanoma cells with an IC_{50} of 12.5 nM and 25 nM for A375 and B16F10 cells, respectively. Recently, they also reported the targeting of hedgehog (Hh) and epidermal growth factor receptor (EGFR) signaling in pancreatic cancer by cyclopamine (CYA, Hh inhibitor) and gefitinib (GEF, EGFR inhibitor) which were simultaneously and efficiently loaded into PEG-*b*-P(BTMCC-*co*-LLA) micelles.[54]. Combination therapy showed a synergistic effect against L3.6pl cells but an additive effect against MIA PaCa-2cells. Caspase 3 and 7 activity was also increased when this combination therapy was used, indicating apoptotic cell death. Furthermore, the combination therapy decreased tumor growth rate in L3.6pl-derived xenograft mouse tumors (Figure 2.3). The same authors also developed amphiphilic PEG-poly(2-methyl-2-benzoxycarbonyl-propylene carbonate) (PEG-*b*-PBTMCC) copolymers for micellar delivery of rapamycin [55]. Rapamycin was effectively incorporated into PEG-*b*-PBTMCC micelles and drug loading increased with increasing hydrophobic core length, with a maximal drug loading content of 10 wt.% (w/w, drug/polymer) and a drug loading efficiency of about 85%. However, the drug release from PEG-*b*-PBTMCC micelles was relatively sluggish.

Figure 2.3. Anticancer activity of CYA, GEF, and their combination loaded micelles on the growth of tumors derived from L3.6pl cells in nude mice. (A and B) Tumor volumes and body weights, (C) pictures of tumors excised from the mice, and (D) tumor weights at the end of the study [54].

As far as the pharmaceutical application is concerned, a number of studies have further exploited nanoparticle systems formulated with functional poly(2-methyl-2-carboxytrimethylene carbonate-*co*-*D,L*-lactide) (P(TMCC-*co*-DLLA)) [56, 57]. Shoichet's group described an elegant route to immuno-polymeric micelles based on P(TMCC-*co*-DLLA)-*graft*-furan-terminated PEG copolymer (P(TMCC-*co*-DLLA)-*g*-PEG-furan) *via* amido links through functional carboxyl groups on carbonate units [58]. Furan groups on the outer PEG shell of the micelles were then reacted with a maleimide-modified anti-HER2 (a therapeutic antibody used to treat breast cancer) by Diels-Alder cycloaddition. The anti-HER2 immuno-micelles specifically bound to HER2-over-expressing cells, demonstrating the potential of this procedure to create bioactive immuno-micelles. As only a few anti-HER2 antibodies on the surface of micelles were necessary for targeting, thousands of PEG-furan chains were available for coupling to a DOX-maleimide conjugate in a subsequent step [59]. The resulting DOX-conjugated immuno-micelles represented an entirely new method for localized co-delivery of chemotherapeutics and antibodies. Flow cytometric analysis showed that the conjugated DOX maintained its biological function and induced similar apoptotic progression in SKBR-3 cells as free DOX, in which the combined DOX and anti-HER2 nanoparticle were more efficacious than the nanoparticle formulation with either DOX or anti-HER2 alone (Figure 2.4). It should be also noted that the combined DOX-anti-HER2 nanoparticle was significantly more cytotoxic against SKBR-3 cells than against healthy HMEC-1 cells, suggesting the

benefit of nanoparticle-conjugated DOX for cell type-specific targeting. Similarly, self-assembled P(TMCC-*co*-LA)-*g*-PEG-N₃ micelles containing azido functional groups on the outer shell were modified with alkyne-bearing KGRGDS peptides using the “Click Chemistry” reaction [60]. The GRGDS nanoparticle system that selectively bound with cells expressing the integrin receptor could be suitable for targeted drug delivery.

Figure 2.4. The preparation of co-labeled antibody- and DOX- polymeric nanoparticles (DOX-conjugated immuno-nanoparticles) using the same Diels-Alder (DA) chemistry as used for covalent surface modification [59].

Jing’s group has also developed antitumor drug docetaxel and tripeptide arginine-glycine-aspartic acid (RGD) conjugated amphiphilic biodegradable copolymers based on PEG-(PTMCC-*co*-LLA) through the functional carboxyl groups [61, 62]. The resulting copolymers showed high cytotoxic activity against HeLa cancer cells, as well as superior cell adherence. Using the carboxyl functional pendants on the amphiphilic biodegradable PEG-(PTMCC-*co*-LLA) copolymer, pirarubicin (THP) was successfully conjugated onto the copolymer *via* hydrazone, ester, and amide bonds, respectively [63]. The *in vitro* THP release showed that hydrazone linked conjugate micelles displayed the highest pH sensitivity, compared to the other two conjugate micelles, in which 40% THP released from “hydrazone” micelles at pH 5.0 in 40 h, while less than 10% released at pH 7.4 during the same time. The *in vitro* cell results showed that all the three polymer-THP conjugates displayed higher cell-uptakes and better antitumor activities against mouse mammary adenocarcinoma EMT6 cells than free THP at 4 h. The *in vivo* antitumor activity of the micelles in Balb/c mice models bearing EMT6 tumors were compared with that of free THP. It should be noted that micelles with a hydrazone linkage had the highest anti-tumor activity *in vivo*, while those with an amide linkage had the lowest activity. Based on the biodegradable triblock PEG-*b*-PTMCC-*b*-PLLA copolymer, Jing and coworkers developed dopamine conjugated copolymer (PEG-*b*-P(TMCC-*g*-dopamine)-*b*-PLLA),

which could be self-assembled to micelles and further stabilized only by bubbling air to crosslink the outer layer of the micellar core due to oxidation of the dopamine pendants [64]. These cross-linked micelles were able to load DOX with superior loading capacity of up to 19.5 wt.% (w/w, drug/micelle) with high drug loading efficiency (97.5%).

Cisplatin, carboplatin, oxaliplatin and other platinum(II)-based drugs are widely employed in cancer chemotherapy and have greatly improved the prognosis for ovarian, lung and especially testicular cancer [65, 66]. Jing *et al.* selected three amphiphilic biodegradable PEG-*b*-P(TMCC-*co*-LLA) copolymers as a drug carrier for the active anticancer drug of oxaliplatin (diaminocyclohexane platinum, DACH-Pt) to form a PEG-*b*-P(LLA-*co*-TMCC/Pt) complex [67]. Folic acid (FA)-containing multifunctional micelles were successfully prepared by co-assembly of PEG-*b*-P(LLA-*co*-TMCC/Pt) and FA-PEG-PLLA copolymer. The *in vivo* blood clearance of platinum after injection of DACH-Pt-micelles or oxaliplatin into the mice showed that micelles had a longer blood circulation time. The *in vivo* biodistribution of Pt and antitumor activity also showed that the micelles with FA moieties exhibited greater antitumor efficacy than those without FA or free oxaliplatin.

Polycarbonates provide an attractive option for use as gene delivery vectors owing to their biocompatibility and ease of incorporating functional moieties. Yang's group used oligoethyleneimines (OEIs) with different chain lengths (triethylenetetramine, tetraethylenepentamine or pentaethylenhexamine) to conjugate onto a series of COOH-functionalized polycarbonates *via* DIC/NHS chemistry to develop the biodegradable cationic polycarbonate as gene carrier [68]. The resulting OEI-containing polycarbonates could form nanoparticles upon simple dissolution in water and were able to condense DNA. *In-vitro* gene transfection studies further demonstrated that selected amine-functionalized polycarbonates could mediate efficient luciferase expression in HEK293, HepG2 and 4T1 cell lines at levels that were comparable, or even superior, to the polyethylenimine (PEI) standard. Interestingly, Chen *et al.* conjugated different kinds of OEI onto the amphiphilic biodegradable PEG-*b*-PTMCC copolymer *via* EDC/NHS chemistry [69], similar to Yang's method [68]. The introduction of PEG could improve solubility, reduce aggregation, decrease cytotoxicity, and possibly decrease opsonization by serum proteins in the bloodstream. Two types of PEG-*b*-P(TMCC-*g*-OEI) (OEI: 600 and 1800) could efficiently condense DNA into nanosized particles (100-140 nm) at weight ratios of polymer/DNA above 10:1. *In vitro* experiments showed that especially the polymer with OEI-1800 exhibited lower cytotoxicity and higher gene transfection efficiency than PEI-25K in CHO and COS-7 cell lines in the absence as well as in the presence of serum.

2.4 Polycarbonates containing amino, amine, and urea functional groups

Cyclic carbonates with nitrogen-containing functional groups may have pendants with amide, amino, amido and urea functionalities [70-74]. Cyclic carbonates with pendant amides include those with pendant carbamic acid benzyl ester (Z) groups and carbamic acid tert-butyl ester (Boc) functional groups (Scheme 2.3), which are used to protect functional amino groups like primary amines during the polymerization. For

example, Jing *et al.* developed degradable poly(ester-co-carbonate)s with benzyloxycarbonyl protected amino groups, which could be removed by catalytic hydrogenation to afford the corresponding poly(ester-co-carbonate)s with free amino groups [72]. They further developed amphiphilic amino-bearing biodegradable copolymer of PEG-*b*-P(LLA-co-serinol carbonate) (PEG-*b*-P(LLA-co-CA)) [75]. Through the pendant amino groups on the carbonate units, NHS-activated folic acid (FA) and fluorescein isothiocyanate (FITC) were successfully conjugated onto the copolymer, to prepare PEG-*b*-P(LA-co-CA/FA) and PEG-*b*-P(LA-co-CA/FITC) conjugates, respectively. Self-assembled micelles based on these block copolymers could serve as targeting moieties and fluorescent probes due to the presence of FA and FITC groups. Very recently, Feng *et al.* prepared a new cyclic carbonate monomer, 2-dimethylaminotrimethylene carbonate (DMATC), which could be polymerized by ring-opening to a water-soluble polycarbonate (PDMATC) with dimethylamino pendant groups using Novozym-435 as a catalyst [76].

Interestingly, Feng and coworkers designed a novel cyclic carbonate monomer, 6,14-dimethyl-1,3,9,11-tetraoxa-6,14-diaza-cyclohexadecane-2,10-dione ((ADMC)₂), and synthesized the corresponding polycarbonate, PADMC, via Novozym-435 lipase or tin octoate (Sn(Oct)₂) catalyzed ring-opening polymerization [73]. The copolymerization of (ADMC)₂ and ϵ -CL provided amphiphilic biodegradable P(ADMC-co-CL) copolymers containing tertiary amine groups in the backbone [77]. Copolymers with a higher ADMC content degraded far faster in phosphate-buffered saline (PBS) solution (pH 7.4, 100 mM) at 37 °C. Polymer surfaces made from biodegradable copolymers containing ADMC promoted cell adhesion and proliferation. Furthermore, P(ADMC-co-CL)-based microspheres exhibited a controlled release of the drug ibuprofen, with a higher release rate at acidic conditions. Recently, they reported a novel amphoteric aliphatic polycarbonate bearing both amine and carboxyl groups synthesized by one-step enzymatic copolymerization of three cyclic carbonate monomers (TMCC, (ADMC)₂ and TMC) in the absence of protection-deprotection chemistry [78]. The simultaneous introduction of amine and carboxyl functionalities provided copolymers with pH-tunable self-aggregation, leading to well-dispersed positively or negatively charged nanoparticles in a controlled manner. Based on the strong buffering capacity of the tertiary amine groups in PADMC, as well as the fast degradation of PADMC, they also developed an amphiphilic triblock copolymer of PCL-PADMC-PCL, which was used to prepare micellar carriers for pH-sensitive release of prednisone acetate [79]. PEG is mostly considered as the hydrophilic block in copolymers for micelle formulation and the flexible PEG outer shell can protect drug-loaded micelles from removal from the circulation by the reticulo-endothelial system (RES) (so-called stealth effect) [80]. Feng and coworkers introduced a PEG segment into the previously mentioned poly(ADMC-co-CL) copolymer to design functional drug carriers based on PEG-*b*-poly(ADMC-co-CL) for fast pH-responsive drug release [81]. The morphology of the drug carriers as well as the drug release patterns were pH-tunable. The copolymers could readily self-assemble into micelles (~100 nm) and exhibited high stability during 80 h incubation in different media. Ibuprofen-loaded PEG-*b*-poly(ADMC-co-CL) micelles showed an accelerated ibuprofen release at acidic conditions due to the protonation of tertiary amine groups in ADMC units.

Scheme 2.3. Synthesis of functional polycarbonate-based polymers containing amino groups and derivatives.

Based on the preparation of hyperbranched hydroxyl-enriched aliphatic polycarbonate by SCROP strategy [31], Feng *et al.* reported a pH-sensitive macro and nano-hydrogel constructed from a cationic hyperbranched polycarbonate, P(EHDO-co-ADMC), functionalized with many hydroxyl and amine groups [82]. This nanohydrogel could stabilize poorly water-soluble molecules such as Nile red in a neutral pH environment. Due to the ionization of the tertiary amine groups in the ADMC units a fast change in the size and morphology of the nanohydrogels took place across a narrow pH range from 7.4 to 6.6.

Urea groups can bind carbonyl derivatives such as DOX and PTX, and their isosteres (sulfonates, phosphonates, phosphates, etc.) [83, 84]. Therefore micelles containing urea groups provide a high loading efficiency for these anticancer drugs. Yang's group developed a supramolecular micellar drug delivery system, in which the micellar core was constructed by urea-modified polycarbonate that allowed for the formation of hydrogen bonding within the core domain, which would in turn strengthen the core cohesion and stabilize the micellar structure [85, 86]. Amphiphilic block copolymers were synthesized by organocatalytic ring-opening copolymerization of urea-functionalized cyclic carbonates (UMTC) with ethoxycarbonyl cyclic carbonate (EMTC) using PEG as a macroinitiator. In the copolymer structure, EMTC units served as the nonfunctional building components to tailor the urea content in the hydrophobic block. The function of incorporated urea groups was evidenced by the fact that the CMC decreased significantly with increasing UMTC content. The hydrogen-bonding function of the urea groups considerably improved the kinetic stability of both drug-free and DOX-loaded micelles. As expected, higher urea contents also led to a significant enhancement in DOX loading, as well as a slightly decreased drug release rate, due to the hydrogen-bonding interaction between matrix and drugs. More importantly, all polymer matrices were non-cytotoxic, while DOX-loaded micelles were shown to kill HepG2 human liver carcinoma cell lines efficiently.

To further fine tune the critical properties of drug-loaded micelles, Yang *et al.* prepared mixed polymeric micelles of PEG-*b*-PTMCC/PEG-*b*-PUMTC loaded with DOX [87]. The mixed micelles demonstrated higher kinetic stability than single micelles prepared of either PEG-*b*-PTMCC or PEG-*b*-PUMTC, due to hydrogen bonding between the carboxylate groups of the polycarbonate and the urea-derivatized polycarbonate (Figure 2.5). In this particular case, hydrogen bonding can occur between urea-urea, carboxylate-carboxylate, urea-carboxylate or between any of the two groups and DOX. They also found that the molecular structure of acid- and urea-functionalized polycarbonate block copolymers (*i.e.* acid as the middle block or the end block or random distribution) in the PEG-polycarbonate amphiphilic copolymers has an effect on the micellar properties, such as CMC, particle size and size distribution, kinetic stability and drug loading capacity [88]. The polymers with carboxyl and urea groups placed in the random form formed micelles with better size distribution (two size populations), and their DOX-loaded micelles were more stable than micelles in which the groups were placed in the block form. It was found that PEG-*b*-P(TMCC-co-UMTC) with 8 acid/urea groups in the random form had the optimum properties. MTT assays demonstrated that the polymer was non-toxic towards HepG2 and HEK293 cells, while DOX-loaded micelles were potent against HepG2 cells with an IC₅₀ of 0.26 mg/L, comparable to that of free DOX (IC₅₀: 0.20 µg/mL). In addition, intravenous injection of DOX-loaded micelles yielded lower

DOX concentrations in the heart tissue of mice as compared to the injection of the free DOX formulation.

Figure 2.5. Biodegradable supramolecular nanostructures based on acid/urea-functionalized mixed micelles designed for high cargo loading capacity and kinetic stability [87].

The kinetic stability of micelles is an important factor that influences micelle behavior in the blood stream, and it determines how fast the micelles will dissociate into individual polymer molecules [89]. The micelles may be still stable in the blood stream for a certain period of time even at polymer concentrations below its CMC. To this aim, Yang and coworkers developed two kinds of mixed micelles based on biodegradable diblock copolymers containing acid or urea-functionalized polycarbonates as the hydrophobe and PEG with two different M_n 's (*i.e.* PEG_{5k}-b-PTMCC/PEG_{5k}-b-PUMTC and PEG_{10k}-b-PTMCC/PEG_{10k}-b-PUMTC), to investigate the effect of the kinetic stability on biodistribution and antitumor activity of drug-loaded biodegradable polymeric micelles [90]. DOX-loaded PEG_{5k} mixed micelles had higher kinetic stability than DOX-loaded PEG_{10k} mixed micelles due to the higher hydrophobicity of the PEG_{5k} block copolymers. A sustained DOX release from the mixed micelles was observed without an obvious initial burst release, and DOX-loaded mixed micelles effectively suppressed the proliferation of HepG2 and 4T1 cells. More importantly, *in vivo* studies conducted with a 4T1 mouse breast cancer model demonstrated that the mixed micelles were preferably transported to the tumor with the PEG_{5k} mixed micelles accumulating in the tumor more rapidly and to a larger extent than the PEG_{10k} mixed micelles. The DOX-loaded PEG_{5k} mixed micelles with higher kinetic stability than the PEG_{10k} mixed micelles inhibited tumor growth more effectively than free DOX and DOX-loaded PEG_{10k} mixed micelles without causing significant body weight loss or cardiotoxicity.

2.5 Polycarbonates containing alkene and alkyne functional groups

The introduction of pendant unsaturated groups (*i.e.* alkene and alkyne) in polycarbonates prepared by the ring-opening of cyclic carbonate monomers has received increased attention in the past few years (Scheme 2.4). Such functionalities, in which no protection/deprotection steps are needed during the polymerization, can provide opportunities of further post-polymerization modification *via* highly efficient and orthogonal reactions, such as Michael addition [91-94], radical thylation [95-97], epoxidation [98, 99], and thermal or UV-crosslinking [100-103].

Micelle crosslinking can efficiently inhibit micelle disassociation and premature drug release in unexpected locations after intravenous injection. Crosslinking of the hydrophobic core of micelles based on block copolymers with hydrophilic PEG blocks and hydrophobic poly(ester-carbonate) blocks containing pendant allyl or acryloyl-groups was realized by heating in the presence of azobisisobutyronitrile (AIBN) or by UV irradiation of the micelles in the presence of a biocompatible photoinitiator, respectively. For example, Jing *et al.* prepared biodegradable micelles based on amphiphilic block PEG-*b*-P(LLA-*co*-allyl carbonate) copolymer (PEG-*b*-P(LLA-*co*-MAC)) and improved their stability by crosslinking of the double bonds using radical polymerization initiated with AIBN [104]. The crosslinked micelles had similar sizes and a narrow size distribution compared to their uncrosslinked precursor. In our group, we reported the preparation of interfacially photo-crosslinked PEG-PLA and PEG-PCL micelles based on PEG-*b*-poly(acryloyl carbonate)-*b*-poly(d,l-lactide) (PEG-PAC-PLA) or PEG-*b*-poly(acryloyl carbonate)-*b*-PCL (PEG-PAC-PCL) block copolymers [101, 102]. This interfacial crosslinking approach has uniquely combined advantages of core and shell crosslinking, which on one hand allows the crosslinking reaction to take place at high micelle concentrations without inter-micellar crosslinking, and on the other hand has little influence on the properties of the micellar core and shell. The interfacially crosslinked micelles exhibited excellent stability with minimal PTX release at low micellar concentrations. MTT assays showed that FA-conjugated crosslinked PEG-PAC-PLA micelles had a high anti-cancer activity to KB cells over-expressing folate receptors [101]. Notably, *in vivo* studies using human hepatoma-bearing nude mice revealed that galactose-decorated PTX-loaded crosslinked PEG-PAC-PCL micelles inhibited the growth of the human hepatoma more effectively than PTX-loaded crosslinked micelles without galactose decoration as well as galactose-decorated PTX-loaded non-crosslinked micelles [102], confirming that micelle stabilization plays a critical role in targeted tumor therapy. In a more recent study, we have prepared core-crosslinked pH-sensitive degradable micelles from PEG-*b*-P(TMBPEC-*co*-acryloyl carbonate) (PEG-*b*-P(TMBPEC-*co*-AC)), which combined pH-sensitive TMBPEC units and acryloyl-functionalized carbonate (AC) units as the hydrophobe (Figure 2.6) [105]. Similarly, the micelles were crosslinked by UV irradiation using AC units. The *in vitro* release studies showed that PTX leakage from core-crosslinked PEG-*b*-P(TMBPEC-*co*-AC) micelles was minimal at pH 7.4 even at low micelle concentrations, while fast PTX release was observed at endosomal pH due to hydrolysis of acetal bonds [39]. MTT assays revealed that PTX-loaded crosslinked pH-sensitive degradable micelles retained high antitumor activity comparable to PTX-loaded non-crosslinked counterparts, supporting efficient drug release from PTX-loaded crosslinked micelles inside tumor cells. These core-crosslinked pH-responsive biodegradable micelles have elegantly addressed the extracellular stability versus intracellular drug release dilemma of micellar anticancer drugs.

Figure 2.6. Illustration of photo-crosslinked pH-sensitive degradable micelles based on PEG-*b*-P(TMBPEC-*co*-AC) block copolymer. PTX-loaded crosslinked pH-sensitive degradable micelles exhibit superior extracellular stability and minimal drug leakage on dilution while “actively” release PTX under mildly acidic conditions mimicking that of the endo/lysosomal compartments [105].

Polycarbonates containing alkene pendants have been mostly functionalized *via* an efficient reaction of thiols and alkenes (“thiol-ene”), which proceeds either *via* the free radical addition of thiols to carbon-carbon double bonds or *via* the Michael-addition of thiols to electron-deficient carbon-carbon double bonds. For example, Huang *et al.* reported that biodegradable PEG-*b*-P(LLA-*co*-MAC) copolymer containing allyl functional groups could be modified with thioglycerol *via* the thiol-ene reaction, followed by conjugation with nucleobases (adenine and thymine) to prepare amphiphilic biodegradable PEG-*b*-P(LLA-*co*-MAC/adenine) and PEG-*b*-P(LLA-*co*-MAC/thymine) copolymers [106, 107]. It was found that PEG-*b*-P(LLA-*co*-MAC/thymine) copolymers could form stable nanoparticles by the addition of 9-hexadecyladenine (A-C16) due to the formation of hydrogen bonds [106]. The *in vitro* DOX release profile showed that with the increase of A-C16 content, the DOX release at pH 7.4 decreased, while a higher release rate was observed at an acidic pH of 5.0. Importantly, DOX-loaded nanoparticles exerted significant cytotoxicity against MDA-MB-231 cells. Recently, they further found that the incorporation of nucleobases into the hydrophobic segment of the amphiphilic PEG-*b*-P(LLA-*co*-MAC/adenine) and PEG-*b*-P(LLA-*co*-MAC/thymine) copolymers could be used for physical crosslinking of the core of the formed micelles, which then had a lower CMC due to hydrogen bonding interaction between adenine and thymine [107]. The *in vitro* drug release profile also showed that the incorporation of nucleobases significantly restricted DOX release at pH 7.4, due to the compact crosslinked structure of the micelles,

while, a much faster release rate was observed at pH 5.0, because of the dissociation of hydrogen bonds between nucleobases. Therefore, the complementary multiple hydrogen bonds of nucleobases provided a convenient tool to stabilize the micelle structures by forming core-crosslinked structures, and could be further applied for controlled intracellular drug delivery. Our group reported that biodegradable micelles for controlled drug release could be prepared from PCL-g-poly(2-hydroxyethyl methacrylate) (PCL-g-PHEMA) graft copolymers by combination of ROP and RAFT reactions, in which the PCL macro-RAFT agent was synthesized by modification of P(CL-co-AC) copolymers *via* Michael addition with cysteamine, followed by coupling reaction with 4-cyanopentanoic acid dithionaphthalenoate (CPADN) [108]. These graft polymeric micelles had a relatively low CMC and showed pH-responsivity in *in vitro* DOX release. MTT assays with HeLa cells demonstrated that PCL-g-PHEMA micelles were practically non-toxic, while DOX-loaded micelles retained high anti-tumor activity with a low IC₅₀ of 1.47-1.74 µg DOX equiv. mL⁻¹. Very recently, we also developed cysteamine or mercaptopropionic acid-modified PEG-*b*-P(TMC-co-AC) copolymers, which could be self-assembled to biodegradable polymersomes containing an ionizable membrane [109]. These polymersomes enabled highly efficient loading and rapid release of proteins at endosomal pH. It should be noted that the presence of various amine groups might also facilitate endosomal escape, further increasing anti-cancer activity.

He *et al.* reported that PEI with low M_n (M_n : 423, 800 and 1800 g/mol) was successfully coupled to the allyl epoxidized activated PMAC polymers for the purpose of gene transfection [110, 111]. *In vitro* experiments using 293T cells demonstrated much lower cytotoxicity and a 3-10 fold enhanced transfection efficiency of all the three poly(5-methyl-5-allyloxycarbonyl-1,3-dioxan-2-one)-*graft*-polyethylenimine (PMAC-g-PEI) samples compared to that of 25k PEI [110]. It was proposed that the enhanced gene transfection efficiency was associated with the fast degradation behavior of the polymer since it will facilitate the intracellular escape of DNA from the DNA-polymer polyplexes. To probe the possible mechanism of PMAC-g-PEI mediated transfection, slowly degradable dimethyl carbonate (DTC) units were introduced into the backbone to tailor the degradation rate, which was considered to be an important factor to influence the transfection efficiency *in vitro* [111]. Polymers with the highest DTC contents (up to 45 mol%) provided systems with the highest transfection efficiency and cell-biocompatibility. This was explained by the following considerations: firstly, the degradation rate of the vectors should be low enough to sufficiently protect DNA from DNase degradation; secondly, DNA should be released at an appropriate site to become biologically active and acquire high protein expression in the nucleus.

2.6 Polycarbonates containing halogen and azido functional groups

Cyclic carbonates carrying halogen groups are an attractive class of monomers as these groups allow subsequent nucleophilic substitution and introduction of novel functionalities even after polymerization [70, 112-115]. Halogen-functional polycarbonates could be modified with sodium azide (NaN₃) in DMF at various temperatures to obtain azido-functional polycarbonates (Scheme 2.5). For example, Shen *et al.* reported that biodegradable amphiphilic PEG-grafted poly(ester-carbonate) copolymers were synthesized

by a combination of ROP and “click” chemistry [116], in which the poly(ester-carbonate) backbone containing pendant bromo-functional carbonate units were completely converted into azido groups, which permitted a “click” reaction with alkyne-terminated PEG by Huisgen 1,3-dipolar cycloaddition. They further developed well-defined ibuprofen-grafted seven-arm amphiphilic star (P(CL-*co*-carbonate)-*b*-PEG)s based on a β -cyclodextrin (β -CD) core by the combination of controlled ROP, esterification coupling reactions and “click” reactions [117]. Similarly, alkyne functionalized ibuprofen was grafted onto the star copolymers by copper(I)-catalyzed “click” reaction *via* the azido-functional carbonate units derived from bromide pendant groups.

Scheme 2.5. Synthesis of functional polycarbonate-based polymers containing halogen or azido groups.

Azido-functional polycarbonate homopolymers and copolymers can also be synthesized *via* ROP of azido-functional cyclic carbonate monomers. Zhuo *et al.* prepared amphiphilic block-graft

PEG-*b*-P(DTC-azido carbonate-*g*-palmitate) copolymers (PEG-*b*-P(DTC-ADTC-*g*-Pal)) by ROP of DTC and ADTC with PEG as an initiator, followed by the click reaction between propargyl Pal and azido groups on the polymer chains [118]. The self-assembled biodegradable micelles were rather stable, had a high drug-loading capacity due to the graft structure, and displayed a sustained release of methotrexate. Jing's group also developed azido-carrying biodegradable polymers, which could be post-functionalized with alkynyl compounds *via* micro-wave assisted click chemistry [119]. The pendant functional groups were used for conjugating the anticancer drugs gemcitabine or PTX and the fluorescent dye RhB. All the polymer-drug conjugates had a pronounced antitumor activity against SKOV-3 and HeLa cell lines.

Polycarbonate homopolymers and copolymers bearing pendant halogen groups can also be modified with tertiary amines such as trimethylamine or N,N,N',N'-tetramethylethylenediamine, to provide polymers with positive charge for efficient gene delivery. For example, Yang and coworkers described that functional polycarbonates containing alkyl halide side chains, were further modified with bis-tertiary amines to facilitate gene binding and endosomal escape [120]. Reporter gene expression efficiencies in HepG2, HEK293, MCF-7 and 4T1 cell lines were relatively high even in the presence of serum. In a further study, they prepared triblock cationic polycarbonate-*b*-PEG-*b*-cationic polycarbonate copolymers and diblock PEG-*b*-cationic polycarbonate copolymers and compared these polymers with a non-PEGylated cationic polycarbonate control to investigate the influence of their structure on key aspects of gene delivery [121].

Figure 2.7. Proposed mechanism of DNA condensation by (a) diblock PEG-*b*-cationic polycarbonate and (b) triblock cationic polycarbonate-*b*-PEG-*b*-cationic polycarbonate [121].

Among the polymers with similar molecular weights and N content, the triblock copolymer exhibited the most favorable physicochemical (*i.e.*, DNA binding, size, zeta-potential, and *in vitro* stability) and

biological (*i.e.*, cellular uptake and luciferase reporter gene expression) properties (Figure 2.7). Importantly, the various cationic polycarbonate/DNA complexes are biocompatible, inducing minimal cytotoxicity and hemolysis.

2.7 Polycarbonates containing sugar functional groups

Scheme 2.6. Synthesis of functional polycarbonate-based polymers containing sugar groups.

Carbohydrates are usually biocompatible and often can be degraded in the body. These compounds are also able to have specific interactions with proteins (lectins) making them very desirable for targeted drug delivery systems [122]. Introduction of these groups into polycarbonate backbones or side chains presents an attractive method for targeted drug delivery. As the hydroxyl groups in sugar-functional cyclic carbonates are incompatible with the ring-opening process, the hydroxyl groups require protection prior to the polymerization of the cyclic carbonate [123-127]. Yang and coworkers prepared biodegradable amphiphilic glycopolymer-PTMC block copolymers by a multi-step procedure, starting with the synthesis of protected sugar-containing (D-glucose, D-galactose, and D-mannose) cyclic carbonate monomers (Scheme 2.6), followed by sequential ring-opening copolymerization of sugar-containing carbonate monomers and TMC and deprotection using formic acid [128]. Self-assembled glycopolymer micelles with a high density of galactose in the shell were interacting more strongly with ASGP-R positive HepG2 liver

cancer cells than with ASGP-R negative HEK293 cells. DOX-loaded galactose-containing micelles had a higher toxicity for HepG2 cells than for HEK293 cells because of their enhanced uptake by HepG2 cells. In a most recent study, Yang and coworkers developed cationic polymers of PEI modified with mannose-functionalized carbonate units for the delivery of a plasmid encoding granzyme B (GrB) inhibitor, proteinase inhibitor-9 (PI-9) to prevent GrB-induced apoptosis of the target cells [129]. PEI with partial primary amine groups substituted by the carbohydrate had similar gene binding ability compared to unmodified PEI, leading to almost 100% transfection efficiency of a GFP-reporter plasmid in HEK293T human embryonic kidney cells. Furthermore, modification of PEI resulted in a decrease in the cytotoxicity of PEI/DNA complexes. These carbamate-mannose modified PEI/PI-9 encoding plasmid complexes were regarded to have potential clinical utility in the prevention of chronic transplant rejection and inflammatory disease caused by GrB.

2.8 Polycarbonates containing other functional groups

Polycarbonates bearing several other functionalities have been also reported for controlled drug delivery (Scheme 2.7). Recently, cyclic carbonate containing oligoethylene glycol (MTC-PEG) was successfully developed and well-defined amphiphilic biodegradable block-graft copolymers of PCL-*b*-P(DTC-*co*-(MTC-PEG)) with polyethylene glycol methyl ether pendant chains were facilely prepared by ROP [130]. The amphiphilic block-graft copolymers could be self-assembled to stable micelles with narrow size distribution. Interestingly, it was found that PTMC derivatives by introducing a PEG domain into the block copolymer or into the side chain along the polymer main chain could induce temperature responsiveness [131, 132]. Yang *et al.* reported water-soluble, thermoresponsive biodegradable block copolymers synthesized by the ROP of cyclic carbonate monomers derived from 2,2-bis(methylol)propionic acid (bis-MPA) with hydrophilic (MTC-PEG) and hydrophobic (MTC-C₂ and MTC-C₁₂) groups for application as nanocarriers in medicine [133]. Aqueous solutions of the polymers displayed temperature-responsiveness with lower critical solution temperatures (LCST's) in the range of 36-60 °C, depending on the M_n of hydrophilic PEG chains, composition of copolymers, molar ratios of hydrophilic to hydrophobic monomers in the corona, and the hydrophobic core. For example, PTX release from the TRC350-10/30/60 micelles (the ratio of MTC-C₂/MTC-PEG/MTC-C₁₂ in the copolymer was 10/30/60 and the M_n of PEG was 350 g/mol) with an LCST of 36 °C in PBS was faster, and cellular uptake of the micelles was higher at body temperature (*i.e.* 37 °C) as compared to temperatures below the LCST. Paclitaxel-loaded micelles killed HepG2 human liver carcinoma cells more efficiently at body temperature as compared to free paclitaxel and paclitaxel-loaded micelles at temperatures below the LCST.

Scheme 2.7. Synthesis of functional polycarbonate-based polymers containing other functionalities.

In a recent report by Yang *et al.*, a novel cyclic carbonate with a pendant cholesterol group (MTC-Chol), was homopolymerized (DP 11) or copolymerized with TMC using PEG as a macroinitiator to prepare biodegradable micelles for controlled release of PTX [134]. The Chol-containing micelles (the

molar ratio of Chol/TMC units of 1/3) had a relatively high PTX loading capacity (15 wt.%) due to hydrophobic interaction with cholesterol. PTX-loaded micelles were able to kill cancer cells much more effectively compared to free PTX. Near-infrared fluorescence (NIRF) imaging studies showed that these micelles allowed effective passive targeting, and were preferably accumulated in tumor tissue with limited distribution to healthy organs.

Amphiphilic poly(ester-carbonate)s bearing disulfide bonds (S–S) for triggered intracellular drug release have been rarely reported to the best of our knowledge. The S–S bonds are relatively stable under physiological conditions as well as in extracellular tissues, while rapid degradation takes place intracellularly due to the much higher GSH concentration [135]. Jing *et al.* reported amphiphilic biodegradable copolymers containing free thiol groups obtained after deprotection of 2,4-dinitrophenyl-protected carbonate units (MTC-DNPT) [136]. The copolymers could be self-assembled into micelles in aqueous solution and crosslinked by oxidation of free thiol groups in the micellar core to enhance the stability. The *in vitro* release behavior indicated that DOX release from micelles was inhibited by micelle core-crosslinking, while the release rate was greatly accelerated by treatment with GSH analogous to intracellular GSH levels in tumor cells. DOX-loaded crosslinked micelles showed higher cellular proliferation inhibition towards GSH pretreated MCF-7 cells than towards non pretreated cells. In a very recent study from our group, a new cyclic carbonate monomer containing the pyridyl disulfide group (PDSC) was successfully synthesized [137]. A similar structure of a carbonate monomer containing a PDS group has also been reported by Hedrick *et al* [70]. Interestingly, we prepared PDS-functionalized PCL polymers by copolymerization of ϵ -CL and PDSC monomers, followed by the disulfide exchange reaction between PDS-functionalized PCL and thiolated PEG (PEG-SH) to afford PCL-g-SS-PEG graft copolymers [137]. The graft copolymers self-assembled into robust reduction-sensitive micelles with a low CMC in water and *in vitro* DOX release studies revealed significant reduction responsiveness (Figure 2.8). DOX-loaded micelles induced pronounced anti-tumor activity to HeLa cells with an IC_{50} close to that for free DOX. These results indicate that bio-reducible graft copolymer micelles can be used as drug carriers for intelligent drug delivery.

Figure 2.8. Synthesis of functional PCL containing pendant pyridyl disulfide groups and facile access to reduction-sensitive biodegradable graft copolymer micelles for intracellular drug release [137].

2.9 Conclusions

Recent years have witnessed a rapid development of biodegradable nanocarriers based on functional polycarbonate-based polymers for the advancement of targeted and controlled drug and gene delivery. Functional polycarbonate-based polymers have overcome many drawbacks which limited the application area for the common polyester materials. It is evident from this review article that recently introduced novel functionalities in polycarbonate-based polymers allowed the preparation of novel nanocarriers, which are bioresponsive (*i.e.* pH, temperature, and redox) and which have improved stability (*i.e.* crosslinking and hydrogen bonding) and the ability to conjugate therapeutic agents, targeting ligands and fluorescence probes. Moreover functionalized polycarbonate based polymers could be successfully applied as gene delivery systems. The innovative design and synthesis of functional polycarbonate-based polymers have provided promising platforms for developing intelligent biodegradable nanocarriers for specific and efficient cancer and gene therapy.

2.10 Abbreviations

AC: acryloyl carbonate; A-C16: 9-hexadecyladenine; (ADMC)₂: 6,14-dimethyl-1,3,9,11-tetraoxa-6,14-diaza-cyclohexadecane-2,10-dione; ADTC: 2,2-bis(azidomethyl) trimethylene carbonate; AIBN: azobisisobutyronitrile; ASGP-R: asialoglycoprotein receptor; Boc: carbamic acid tert-butyl ester; CA: serinol carbonate; CAB: N-benzoxycarbonylserinol carbonate; CAC: critical association concentration; β -CD: β -cyclodextrin; ϵ -CL: ϵ -caprolactone; CLSM: confocal laser scanning microscopy; CMC: critical micelle concentration; CPADN: dithionaphthalenoate; DACH-Pt: diaminocyclohexane platinum; DBTC: 5,5-dibromomethyl trimethylene carbonate; DHP: 2,2-dihydroxymethylpropylene carbonate; DIC: N,N'-diisopropylcarbodiimide; DMATC: 2-dimethyl-aminotrimethylene carbonate; DOX: doxorubicin; DOX·HCl: doxorubicin hydrochloride; DP: degree of polymerization; DTC: dimethyl trimethylene carbonate; DTT: 1,4-dithiothreitol; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EGFR: epidermal growth factor receptor; EMTC: 5-methyl-5-ethoxycarbonyl-1,3-dioxan-2-one; EPR: enhanced permeability and retention; FA: folic acid; FITC: fluorescein isothiocyanate; GA: glycolide; GrB: granzyme B; GSH: glutathione; Hh: hedgehog receptor; HEHDO: hyperbranched (5-ethyl-5-hydroxymethyl-1,3-dioxan-2-one); IC₅₀: half maximal inhibitory concentration; IGFC: 1,2,5,6-Di-O-isopropylidene-3-O-MCDO-D-glucufuranose; IGPC: 1,2,3,4-Di-O-isopropylidene-3-O-MCDO-D-galactopyranose; IMFC: 2,3,5,6-Di-O-isopropylidene-3-O-MCDOD-mannofuranose; LA: lactide; LCST: lower critical solution temperature; LY293: (2-(1 H-Indol-5-yl) thiazol-4-yl) 3,4,5-trimethoxyphenyl methanone; MAC: 5-methyl-5-allyloxycarbonyl-1,3-dioxan-2-one; MTC-C₂: ethyl trimethylene carbonate; MTC-C₁₂: dodecyl trimethylene carbonate; MTC-Chol: cholesteryl 2-(5-methyl-2-oxo-1,3-dioxane-5-carboxyloxy)ethyl carbamate; MTC-DNPT: 2,4-dinitrophenyl-protected carbonate; MTC-PEG: poly(ethylene glycol) trimethylene carbonate; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NHS: N-hydroxysuccinimide; NIRF: Near-infrared fluorescence; OEI: oligoethyleneimine; PAC: poly(acryloyl carbonate); PADMC: poly(6,14-dimethyl-1,3,9,11-tetraoxa-6,14-diaza-cyclohexadecane-2,10-dione); P(ADMC-co-CL): poly(6,14-dimethyl-1,3,9,11-tetraoxa-6,14-diaza-cyclohexadecane-2,10-dione-co-caprolactone); Pal: palmitate;

P(BC-*co*-LA): poly(2-methyl-2-benzoxycarbonyl-propylene carbonate-*co*-lactide); PBTMCC: poly(5-benzyloxycarbonyl-trimethylene carbonate); PBS: phosphate-buffered saline; PBTMC: poly(5-benzyloxy-trimethylene carbonate); PCD: poly(2-methyl-2-carboxytrimethylene carbonate-*graft*- dodecanol); PCL-*g*-PHEMA: poly(ϵ -caprolactone)-*graft*-poly(2-hydroxyethyl methacrylate); PDMATC: poly(2-dimethylaminotrimethylene carbonate); PDSC: pyridyl disulfide cyclic carbonate; PEG: poly(ethylene glycol); PEG-PAC-PLA: PEG-*block*-poly(acryloyl carbonate)-*block*-poly(d,l-lactide); PEG-PAC-PCL: PEG-*block*-poly(acryloyl carbonate)-*block*-PCL; PEG-*block*-P(LLA-*co*-MAC): PEG-*block*-P(LLA-*co*-allyl carbonate); P(EHDO-*co*-ADMC): poly(5-ethyl-5-hydroxymethyl-1,3-dioxan- 2-one-*co*-6,14-dimethyl-1,3,9,11-tetraoxa-6,14-diaza-cyclohexadecane-2,10-dione); PEI: polyethylenimine; PHTMC: poly(2-hydroxyl trimethylene carbonate); P(LA-*co*-DHP): poly(lactide-*co*-2,2-dihydroxymethyl- propylene carbonate); PMAC: poly(5-methyl-5-allyloxycarbonyl-1,3-dioxan-2-one); PMAC-*g*-PEI: poly(5-methyl-5-allyloxycarbonyl-1,3-dioxan-2-one)-*graft*-polyethylenimine; PPEC: poly(pentaerythritol carbonate); PTMBPEC: poly(2,4,6-trimethoxybenzylidenepentaerythritol carbonate); PTMC: poly(trimethylene carbonate); PTMCC: poly(2-methyl-2-carboxytrimethylene carbonate); P(TMCC-*co*-LA): poly(2-methyl-2-carboxytrimethylene carbonate-*co*-d,l-lactide); PTX: paclitaxel; PUMTC: poly(5-methyl-5-phenylureidoethylcarboxyl-1,3-dioxan-2-one); RAFT: reversible addition-fragmentation chain transfer; RES: reticulo-endothelial system; RGD: arginine-glycine-aspartic acid tripeptide; RhB: rhodamine B; ROP: ring-opening polymerization; SCROP: self-condensing ring-opening polymerization; THP: pirarubicin; TMBPEC: 2,4,6-trimethoxybenzylidenepentaerythritol carbonate; TMC: trimethylene carbonate; UMTC: 5-methyl-5-phenylureidoethylcarboxyl- 1,3-dioxan-2-one.

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Chapter 3

Functional Poly(ϵ -caprolactone)s via Copolymerization of ϵ -Caprolactone and Pyridyldisulfide-Containing Cyclic Carbonate: Controlled Synthesis and Facile Access to Reduction-Sensitive Biodegradable Graft Copolymer Micelles*

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Abstract

Pyridyldisulfide-functionalized cyclic carbonate (PDSC) monomer was obtained in four straightforward steps from 3-methyl-3-oxetanemethanol and exploited for facile preparation of functional poly(ϵ -caprolactone) (PCL) containing pendant pyridyldisulfide (PDS) groups via ring-opening copolymerization with ϵ -caprolactone. The results showed that PDS-functionalized PCL polymers were prepared with controlled molecular weights and functionalities. The exchange reaction between PDS-functionalized PCL and thiolated poly(ethylene glycol) (PEG-SH) at a PEG-SH/PDS molar ratio of 2/1 afforded PCL-g-SS-PEG graft copolymers in high yields. Dynamic light scattering (DLS) analyses showed that PCL-g-SS-PEG copolymer self-assembled into micelles with a diameter of 110~120 nm and a low polydispersity (PDI) in phosphate buffer (pH 7.4, 10 mM). PCL-g-SS-PEG micelles while sufficiently stable under physiological conditions were prone to rapid shell-shedding and aggregation under a reductive condition. Doxorubicin (DOX) was loaded into PCL-g-SS-PEG micelles with a decent drug loading content of 10.1 wt.%. Notably, *in vitro* release studies revealed that ca. 82.1 % DOX was released in 12 h in a reductive environment analogous to that of the intracellular compartments such as cytosol and the cell nucleus whereas only ca. 17.5 % DOX was released in 24 h under non-reductive conditions. Confocal microscopy indicated that DOX was delivered into the nuclei of HeLa cells following 8 h incubation with DOX-loaded PCL-g-SS-PEG micelles. MTT assays using HeLa cells demonstrated that DOX-loaded PCL-g-SS-PEG micelles retained high anti-tumor activity with low IC₅₀ (half maximal inhibitory concentration) of 0.82-0.95 μ g DOX equiv./mL while blank PCL-g-SS-PEG micelles were non-toxic up to a tested concentration of 1.0 mg/mL. This study presents a versatile and controlled synthesis of PDS-functionalized biodegradable polymers and reduction-sensitive biodegradable graft copolymer micelles that are of particular interest for active intracellular drug release.

3.1 Introduction

Aliphatic polyesters and polycarbonates, such as poly(ϵ -caprolactone) (PCL), polylactide (PLA), poly(lactide-co-glycolide) (PLGA), and poly(trimethylene carbonate) (PTMC) are among the most important synthetic biodegradable materials [1-3]. They have been widely applied for absorbable orthopedic devices, [4] microparticles for controlled protein release [5, 6], cell and tissue scaffolds [7, 8], drug-eluting stents [9], as well as nanoparticles for targeted and controlled drug release [10, 11]. However, these traditional biodegradable polymers are lacking reactive groups, which limit their application for the development of advanced drug delivery systems and bioactive tissue scaffolds. In the past decade, various functional aliphatic polyesters and polycarbonates with pendant hydroxyl [12, 13], carboxyl [14, 15], amine [16, 17], allyl [18-20], alkyne/azide [21-24], acryloyl [25-27], and maleimide [28] groups have been reported. In particular, the design of functional cyclic carbonate monomers has received recent interest [29, 30]. We have designed 2,4,6-trimethoxybenzyl acetal, (methyl)acryloyl, or vinyl sulfone functionalized cyclic carbonate monomers that provided versatile access to pH-sensitive degradable micelles and polymersomes [31, 32], photo-crosslinked biodegradable micelles [33-35], biodegradable injectable hydrogels [36], and functional biodegradable polymers and coatings [37].

In recent years, taking advantage of the high redox potential in the cytoplasm and nuclei of cancer cells, reduction-sensitive degradable micelles and nanoparticles have been actively developed for efficient intracellular anti-cancer drug release [38, 39]. The intracellular doxorubicin (DOX) level and anti-tumor activity of DOX-loaded reduction-sensitive shell-sheddable micelles were significantly enhanced as compared to reduction-insensitive controls. It should be noted that pyridyldisulfide (PDS) is the most versatile thiol-disulfide exchange functional group used for the construction of disulfide linkages and crosslinks in bioconjugate chemistry [40]. For example, Hoffman *et al.* designed poly(methacrylic acid-co-butyl acrylate-co-pyridyldisulfide acrylate) random copolymer for facile conjugation and active intracellular release of thiol-containing therapeutic oligopeptide drugs [41]. Thayumanavan *et al.* developed reduction-responsive polymeric nanogels based on a random copolymer containing oligoethyleneglycol and PDS units as side-chain functionalities, in which PDS was used to form disulfide crosslinks as well as to introduce TAT peptide or FITC on nanogel surfaces [42, 43].

In this paper, we report the synthesis of novel functional PCL via ring-opening copolymerization of ϵ -CL and pyridyldisulfide-functionalized cyclic carbonate (PDSC) monomer (Scheme 3.1). The post-polymerization modification with thiolated PEG by the thiol-disulfide exchange reaction yielded reduction-sensitive, amphiphilic, biodegradable graft copolymers that are self-assembled into stable micellar nanoparticles in water. It is interesting to note that graft copolymer micelles offer several advantages over block copolymer micelles, for instance, they often exhibit enhanced stability with low critical micelle concentration (CMC) [44-46], micellar core and surface properties can be broadly adjusted by backbone length, graft density and graft length, and presence of many hydrophilic grafts per macromolecule enables conjugation of tunable density targeting ligands for optimal tumor-targeting. However, there are few reports on biodegradable graft copolymer micelles based on hydrophobic

biodegradable polymers grafted with hydrophilic polymers [21, 44, 47], likely due to challenging synthesis. In this study, the synthesis and ring-opening copolymerization of PDSC monomer, synthesis and self-assembly of PCL-g-SS-PEG graft copolymer, loading and reduction-responsive release of DOX, as well as intracellular release and antitumor activity of DOX-loaded graft copolymer micelles were investigated.

Scheme 3.1. Synthesis of functional PCL containing pendant pyridyldisulfide groups and facile access to reduction-sensitive biodegradable graft copolymer micelles for intracellular drug release.

3.2 Experimental section

Materials. 3-Methyl-3-oxetanemethanol (97%, Alfa), hydrobromic acid (40%, SCRC), sodium hydrosulfide hydrate (68%, Acros), 2-2'-dipyridyl disulfide (98%, Alfa Aesar), triethylamine (Et₃N, Alfa Aesar, 99%), stannous octoate (Sn(Oct)₂, 95%, Sigma), and doxorubicin hydrochloride (DOX·HCl, 99 %, Beijing Zhong Shuo Pharmaceutical Technology Development Co.Ltd.) were used as received. ε-Caprolactone (ε-CL, 99%, Alfa Aesar) was dried over CaH₂ and distilled under reduced pressure prior to use. Thiolated PEG (PEG-SH, *M_n* = 5.0 kg/mol) was synthesized according to a previous report [48]. Tetrahydrofuran (THF) and toluene were dried by refluxing over sodium wire under an argon atmosphere prior to distillation. Isopropanol was dried by refluxing over CaH₂ under an argon atmosphere. Ethyl chloroformate was freshly distilled before use.

Preparation of pyridyldisulfide carbonate (PDSC). Pyridyldisulfide carbonate (PDSC) was synthesized in four steps (Scheme 3.1). The first two steps were carried out according to our previous report [37]. Briefly, HBr (40%, 40 mL) was dropwise added into a solution of 3-methyl-3-oxetanemethanol (10.20 g, 100.0 mmol) in THF (100 mL) under stirring at 0 °C. The reaction mixture was warmed to room temperature and stirred for another 5 h. The reaction mixture was then diluted with H₂O (150 mL) and extracted with diethyl ether (4 × 150 mL). The organic phase was dried over anhydrous MgSO₄ and concentrated to give the desired product (bromo-diol) as a white solid. Yield: 17.57 g (96%). ¹H NMR (400 MHz, CDCl₃): δ 3.68 (s, 4H, -C(CH₂OH)₂), 3.55 (s, 2H, -CH₂Br), 2.13 (s, 2H, -(OH)₂), 0.93 (s, 3H, -CH₃).

Next, bromo-diol (9.15 g, 50.0 mmol) was added under stirring to a solution of NaSH (8.40 g, 150.0 mmol) in DMF (150 mL). The reaction mixture was stirred at 75 °C for 20 h. The solvent was removed by rotary evaporation, the residue was dissolved in 150 mL of deionized water and extracted with ethyl acetate (3 $\times$ 150 mL), and the organic phase was dried over anhydrous MgSO₄ and concentrated to yield mercapto-diol as a yellowish oil. Yield: 3.54 g (52%). ¹H NMR (400 MHz, CDCl₃): δ 3.64 (d, 4H, -C(CH₂OH)₂), 2.67 (d, 2H, -CH₂SH), 2.27 (s, 2H, -(OH)₂), 1.31 (t, 1H, -SH), 0.85 (s, 3H, -CH₃).

Then, to a stirred solution of 2,2'-dithiodipyridine (8.68 g, 39.0 mmol) and a catalytic amount of glacial acetic acid (0.5 mL) in methanol (75 mL) was added dropwise a solution of mercapto-diol (3.54 g, 26.0 mmol) in methanol at room temperature. The reaction mixture was stirred at room temperature for additional 16 h. The solvent was evaporated to yield crude product as a yellow oil. The product was purified by column chromatography using silica gel as stationary phase and a mixture of ethyl acetate/petroleum ether (v/v = 1/3) as eluent. Yield: 4.14 g (65%). ¹H NMR (400 MHz, CDCl₃): δ 8.47, 7.62, 7.41 and 7.17 (m, pyridyl protons), 3.67 (d, 4H, -C(CH₂OH)₂), 3.25 (s, 2H, -CH₂SS-Py), 0.84 (s, 3H, -CH₃).

Finally, to a stirred solution of pyridyldisulfide-diol (4.14 g, 16.9 mmol) and ethyl chloroformate (3.4 mL, 35.5 mmol) in dried THF (150 mL) at 0 °C was added dropwise a solution of Et₃N (5.4 mL, 39.1 mmol) in THF. The reaction was allowed to proceed for 4 h at 0 °C. The reaction mixture was filtered, the filtrate was concentrated under reduced pressure, and the residues were crystallized in THF/diethyl ether to yield a white solid. Yield: 3.66 g (50%). ¹H NMR (400 MHz, CDCl₃): δ 8.51, 7.65, 7.54 and 7.14 (m, pyridyl protons), 4.38-4.15 (d, 4H, -C(CH₂O)₂CO), 3.08 (s, 2H, -CH₂SS-Py), 1.23 (s, 3H, -CH₃). Elemental Analysis: Calcd. for C₉H₁₂O₅: C, 48.69; N, 5.16; H, 4.83. Found: C, 48.70; N, 5.02; H, 4.75. TOF-MS (*m/z*): calcd for C₁₁H₁₃NO₃S₂ 271.36; found 271.37.

Ring-opening copolymerization. The ring-opening copolymerization of ϵ -CL and PDSC was carried out in toluene at 100 °C using isopropanol as an initiator and Sn(Oct)₂ as a catalyst. The following is a typical example. In a glove-box under a nitrogen atmosphere, to a stirred solution of ϵ -CL (0.757 g, 6.64 mmol) and PDSC (0.200 g, 0.74 mmol) in toluene (8 mL) was quickly added isopropanol stock solution (0.18 mL, 0.2 M) and Sn(Oct)₂ stock solution (0.20 mL, 100 mM). The reaction vessel was sealed and placed in an oil-bath thermostated at 100 °C with magnetic stirring for 24 h. A sample was taken for determination of the monomer conversion using ¹H NMR. The resulting P(CL-co-PDSC) copolymer was isolated by precipitation in cold diethyl ether and dried *in vacuo* at room temperature.

Synthesis of PCL-g-SS-PEG by thiol-disulfide exchange reaction. To a solution of P(CL-co-PDSC) 5.4 % (50 mg, 20 μ mol of PDS groups) and PEG-SH (200 mg, 40 μ mol) in DCM under a nitrogen atmosphere was added a catalytic amount of acetic acid. The reaction was allowed to proceed with stirring for 48 h at room temperature. The resulting polymer was isolated by precipitation from cold diethyl ether, washed with excess ethanol, and dried *in vacuo* at room temperature.

Characterization. ¹H NMR spectra were recorded on the Unity Inova 400 operating at 400 MHz. CDCl₃ was used as solvent and the chemical shifts were calibrated against residual solvent signals. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation

chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using CHCl_3 as the eluent at a flow rate of 0.5 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns.

Micelle formation and critical micelle concentration. Micelles were typically prepared under stirring by dropwise addition of 2 mL phosphate buffer (10 mM, pH 7.4) to 0.2 mL amphiphilic PCL-g-SS-PEG copolymer solution (0.5 wt.%) in THF at room temperature. The resulting solution was stirred overnight under reduced pressure to allow complete evaporation of THF. The size and size distribution of the micelles were determined by dynamic light scattering (DLS). The micellar suspension was filtered through a 450 nm syringe filter before measurements. Measurements were carried out at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He-Ne laser using back-scattering detection.

The critical micelle concentration (CMC) was determined using pyrene as a fluorescence probe. The concentration of graft copolymer was varied from 2.0×10^{-5} to 0.2 mg/mL and the concentration of pyrene was fixed at 1.0 μM . Fluorescence spectra were recorded using a FLS920 fluorescence spectrometer and an excitation wavelength of 330 nm. Fluorescence emissions at 372 and 383 nm were monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

Redox-triggered change of micelle sizes. The change in size, size distribution and light scattering of the micelles in response to 10 mM DTT was followed by DLS at 37 °C. The micelles were prepared as above. PCL-g-SS-PEG micellar suspension (0.2 mg/mL) was divided into two aliquots. To one of the aliquots (1.0 mL) was added 10 μL of DTT (1.0 M), which gave a final DTT concentration of 10 mM. The samples were gently stirred at 37 °C. The changes in micelle size and PDI were monitored over time by DLS.

Loading and redox-triggered release of DOX. DOX was loaded into micelles by dropwise addition of 2 mL phosphate buffer (10 mM, pH 7.4) to a mixture of 0.2 mL of copolymer solution in DMF (5 mg/mL) and 10, 20 or 40 μL of DOX solution in DMSO (5 mg/mL) under stirring at room temperature, followed by stirring for 1 h and dialysis against phosphate buffer (10 mM, pH 7.4) with a molecular weight cut-off (MWCO) of 3500 Da at room temperature in the dark for 6 h.

The release profiles of DOX from PCL-g-SS-PEG micelles were studied using a dialysis tube (MWCO 12 000 Da) at 37 °C in two different media, *i.e.* PB (100 mM, pH 7.4) or PB (100 mM, pH 7.4) with 10 mM DTT. In order to acquire sink conditions, drug release studies were performed at low drug loading contents (ca. 2.9 wt.%) and with 0.6 mL of micelle suspension dialysis against 20 mL of the same medium. At desired time intervals, 5.0 mL release media was taken out and replenished with an equal volume of fresh media. The amount of DOX released was determined by using fluorescence (FLS920) measurement (excitation at 480 nm). The release experiments were conducted in triplicate. The results presented are the average data. For determination of the drug loading content, DOX-loaded micellar suspensions were freeze-dried, then dissolved in DMSO and analyzed with fluorescence spectroscopy. A

calibration curve was obtained using DOX/DMSO solutions with different DOX concentrations. To determine the amount of DOX released, calibration curves were run with DOX/phosphate buffer solution (pH 7.4, 100 mM) with different DOX concentrations. The emission at 600 nm was recorded. Release experiments were conducted in triplicate. The results are presented as the average $\pm$ standard deviation.

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

$$\text{DLC (wt.\%)} = (\text{weight of loaded drug} / \text{total weight of polymer and loaded drug}) \times 100\%$$

$$\text{DLE (\%)} = (\text{weight of loaded drug} / \text{weight of drug in feed}) \times 100\%$$

Confocal microscopy of HeLa cells incubated with DOX-loaded micelles. HeLa cells were plated on microscope slides in a 24-well plate (5×10^4 cells/well) using DMEM medium containing 10 % FBS. The cells were incubated with prescribed amounts of DOX-loaded PCL-g-SS-PEG **2** micelles or free DOX at 37 °C and 5 % CO₂. After incubation for 4 and 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% paraformaldehyde and the cell nuclei were stained with DAPI. Fluorescence images of cells were obtained with a Nikon Digital Eclipse C1si Confocal Laser Scanning Microscope (CLSM, Nikon).

MTT assays. The cytotoxicity of PCL-g-SS-PEG micelles and DOX-loaded PCL-g-SS-PEG micelles was studied by MTT assays using HeLa cells. Cells were seeded onto a 96-well plate at a density of 1×10^4 cells per well in 100 μ L of Dulbecco's Modified Eagle medium (DMEM) containing 10% FBS and incubated for 24 h (37 °C, 5% CO₂). The medium was replaced by 90 μ L of fresh DMEM medium containing 10% FBS, and then 10 μ L samples of various concentrations (0.5-10 mg/mL) of the micelle suspensions in phosphate buffer (10 mM, pH 7.4) were added. The cells were incubated for another 48 h, the medium was aspirated and replaced by 100 μ L of fresh medium, and 10 μ L of MTT solution (5 mg/mL) was added. The cells were incubated for 4 h, and then the medium was aspirated and replaced by 150 μ L of DMSO to dissolve the resulting purple crystals. The optical densities at 570 nm were measured using a BioTek microplate reader. Cells cultured in DMEM medium containing 10% FBS (without exposure to micelles) were used as controls.

3.3 Results and discussion

3.3.1 Synthesis of PDSC monomer

Pyridyldisulfide-functionalized cyclic carbonate (PDSC) was prepared in four straightforward steps: (i) 3-methyl-3-oxetanemethanol was treated with HBr to afford bromo-diol; (ii) bromo-diol was reacted with sodium hydrosulfide to yield mercapto-diol; (iii) thiol-exchange reaction between mercapto-diol and excess 2-2'-dipyridyl disulfide gave rise to pyridyldisulfide-diol; and (iv) similar to the reported procedure for other cyclic carbonate monomers [27, 31] cyclization of pyridyldisulfide-diol using ethyl chloroformate in dilute anhydrous THF solution at 0 °C with dropwise addition of triethylamine furnished PDSC monomer in decent yields (Scheme 3.2). PDSC monomer could be readily recrystallized from dry THF/diethyl ether to yield pure white crystals. ¹H NMR spectrum of pyridyldisulfide-diol showed signals of pyridine protons

at δ 8.47, 7.62, 7.41 and 7.17, as well as resonances at δ 3.67, 3.25 and 0.84 attributable to methylene protons next to hydroxyl groups, methylene protons neighboring the disulfide bond, and methyl protons, respectively (Figure 3.1A). ^1H NMR of PDSC revealed that signals assignable to the methylene protons next to the carbonate were clearly detected at δ 4.15–4.38 while signals at δ 3.67 owing to methylene protons next to hydroxyl groups shifted to δ 4.15–4.38 (Figure 3.1B). The integral ratio of resonances at δ 4.15–4.38 and δ 8.51, 7.65, 7.54 and 7.14 (pyridine protons) was close to the theoretical value of 1:1, indicating successful synthesis of PDSC monomer. The structure of PDSC was further confirmed by mass and elemental analyses. Hedrick et al. reported a versatile synthesis of functionalized cyclic carbonate monomers through a pentafluorophenyl ester intermediate, which after reacting with S-2-pyridyl-S'-2-hydroxyethyl disulfide could yield 2-(pyridin-2-yl-disulfanyl)ethyl 5-methyl-2-oxo-1,3-dioxane-5-carboxylate [49]. However, the ring-opening (co)polymerization of pyridyldisulfide-functionalized carbonate monomer to synthesize biodegradable polymers with pendant PDS groups and further reduction-sensitive amphiphilic biodegradable graft copolymers has not yet been reported.

Scheme 3.2. Synthetic pathway for pyridyldisulfide-functionalized cyclic carbonate monomer (PDSC). conditions: (i) 40% hydrobromic acid, 0 °C, 5 h, THF (yield: 96%); (ii) sodium hydrosulfide, 75 °C, overnight, DMF (yield: 52%); (iii) 2,2'-dithiodipyridine, catalytic amount of glacial acetic acid, room temperature, overnight, methanol (yield: 65%); (iv) ethyl chloroformate, Et_3N , 0 °C, 4 h, THF (yield: 50%).

Figure 3.1. ^1H NMR spectra (400 MHz, CDCl_3) of pyridyldisulfide-diol (A) and PDSC monomer (B).

3.3.2 Synthesis of functional PCL containing pendant PDS groups

Scheme 3.3. Synthesis of $\text{P}(\text{CL-co-PDSC})$ copolymer and reduction-sensitive PCL-g-SS-PEG graft copolymer. Conditions: (i) ring-opening copolymerization of ϵ -CL and PDSC using isopropanol as an initiator and $\text{Sn}(\text{Oct})_2$ as a catalyst in toluene at 100°C ; and (ii) thiol-disulfide exchange reaction with thiolated PEG using a catalytic amount of acetic acid in DCM at room temperature.

The aim of this study was to provide a versatile approach to PDS-functionalized biodegradable polymers and reduction-sensitive biodegradable graft copolymers by post-polymerization modification with thiol-containing molecules. To prove our concept, we synthesized PDS-functionalized PCL as an example (Scheme 3.3). The results showed that PDSC was readily copolymerized with ϵ -CL in toluene at 100°C using isopropanol as an initiator and $\text{Sn}(\text{Oct})_2$ as a catalyst to afford PDS-functionalized PCL in good yields (Table 3.1). ^1H NMR showed that signals characteristic of both CL units (δ 4.04, 2.30, 1.64 and 1.38) and PDSC units (δ 8.46, 7.65, 7.10, 4.05, 3.02 and 1.10) were clearly detected (Figure 3.2). Importantly, signals due to pyridine protons remained at δ 8.46, 7.65 and 7.10, indicating that PDS functional groups were intact during copolymerization and subsequent work-up procedures. The copolymer compositions could be determined by comparing signals at δ 2.30 (methylene protons next to carbonyl in CL units) and 3.02 (methylene protons next to disulfide bond in PDSC units). The results showed that $\text{P}(\text{CL-co-PDSC})$ copolymers with PDSC contents (F_{PDSC}) ranging from 2.5 to 10.3 mol.% were obtained at PDSC monomer feed ratio (f_{PDSC}) varying from 5 to 20 mol.% (Table 3.1). F_{PDSC} values though lower than the feed ratios increased linearly with increasing f_{PDSC} , denoting good control over PDS functionality. The number-average molecular weights (M_n) estimated from ^1H NMR end group analysis by comparing the integrals of peaks at 2.30 (methylene protons next to carbonyl in CL units) and 3.02 (methylene protons next to disulfide bond in PDSC units) with δ 1.22 (methyl protons of isopropyl ester end group) were close to the theoretical values (Table 3.1). Gel permeation chromatography (GPC) measurements using polystyrene as standards

showed that these P(CL-*co*-PDSC) copolymers had moderate polydispersities (PDI) of 1.33-1.61 and M_n increased with increasing monomer-to-initiator ratios (Table 3.1). Therefore, copolymerization of ϵ -CL and PDSC provides PDS-functionalized PCL with controlled molecular weights and functionalities.

Table 3.1. Synthesis of Pyridyldisulfide-Functionalized PCL.^a

Entry	[M]/[I]	f^b (%)	F^c (%)	$M_n (\times 10^3)$			PDI GPC ^e
				Design	¹ H NMR ^d	GPC ^e	
1	100	5	3.2	12.2	12.0	18.6	1.42
2	100	10	5.2	13.0	12.6	19.2	1.40
3	200	5	2.5	24.4	20.1	30.9	1.55
4	200	10	5.4	25.9	22.3	29.6	1.52
5	200	20	10.3	29.1	21.4	29.8	1.33
6	400	5	2.7	44.7	-	55.5	1.61

^a The copolymerization was carried out in toluene at 100 °C using isopropanol as an initiator and Sn(Oct)₂ as a catalyst at a total monomer-to-initiator ratio of 100/1, 200/1 and 400/1; ^b Molar fraction of PDSC monomer in feed; ^c Molar fraction of PDSC units in the resulting copolymer determined by ¹H NMR; ^d Estimated by ¹H NMR end-group analysis; ^e Determined by GPC (eluent: chloroform, flow rate: 0.5 mL/min, standards: polystyrene).

Figure 3.2. ¹H NMR spectrum (400 MHz, CDCl₃) of P(CL-*co*-PDSC) copolymer (Table 3.1, Entry 4).

3.3.3 Synthesis of PCL-g-SS-PEG graft copolymer by thiol-disulfide exchange reaction

It is well-known that the pyridyldisulfide group has a high reactivity towards thiol-containing molecules via the thiol-disulfide exchange reaction to form disulfide linkages or crosslinks [50]. Here, two P(CL-co-PDSC) copolymers with similar PDS functionality (5.2-5.4 %) but different M_n values (12.6 and 22.3 kg/mol, Table 3.1, Entries 2 and 4) were chosen to react with thiolated PEG ($M_n = 5000$). The reaction was performed at a PEG-SH/PDS molar ratio of 2/1 in DCM at room temperature for 48 h using a catalytic amount of acetic acid (Scheme 3.3). Free PEG was removed by washing with excess ethanol. ^1H NMR displayed that new signals corresponding to methylene protons of PEG appeared at δ 3.65 while peaks due to pyridine groups at δ 8.46, 7.65 and 7.10, were diminishing (Figure 3.3). The comparison of integrals of resonances at δ 3.36 (methoxyl protons of PEG) and δ 1.02 (methyl protons of PDSC units) indicated that ca. 86-89 % pyridyldisulfide groups have reacted with thiolated PEG to afford amphiphilic PCL-g-SS-PEG graft copolymers (Table 3.2). The M_n of these two copolymers was calculated by ^1H NMR to be 35.6 and 66.0 kg/mol (denoted as PCL-g-SS-PEG **1** and **2**, respectively). GPC curves of both graft copolymers remained unimodal with a PDI of 1.95-2.02 (Table 3.2). The M_n determined by GPC was lower than that calculated from ^1H NMR, likely due to the graft structure. The deviation of M_n could be also partly because polystyrene was used as a standard for GPC measurements. It is evident that PCL-g-SS-PEG graft copolymers can be readily prepared from PDS-functionalized PCL via the thiol-disulfide exchange reaction.

Figure 3.3. ^1H NMR spectrum (400 MHz, CDCl_3) of PCL-g-SS-PEG copolymer (Table 3.2, Entry 2).

Table 3.2. Synthesis of PCL-g-SS-PEG graft copolymer.

PCL-g-SS-PEG copolymer	P(CL- <i>co</i> -PDSC)		PEG conjugation ^a (%)	$M_n (\times 10^3)$		PDI GPC ^c	CMC ^d (mg/L)
	F_{PDSC} (%)	M_n ($\times 10^3$)		$^1\text{H NMR}^b$	GPC ^c		
1	5.2	12.6	86	35.6	28.6	2.02	0.92
2	5.4	22.3	89	66.0	43.9	1.95	0.87

^a Percentage of PEG conjugation determined by $^1\text{H NMR}$; ^b Determined by $^1\text{H NMR}$ according to the integral ratio of resonances at δ 3.36 (methoxyl protons attributed to PEG) and δ 1.02 (methyl protons attributed to initial PDSC units); ^c Determined by GPC (eluent: chloroform, flow rate: 0.5 mL/min, standards: polystyrene); ^d Critical Micelle Concentration (CMC) determined using pyrene as fluorescence probe.

3.3.4 Formation and reduction-triggered disruption of micelles

Micelles of PCL-g-SS-PEG copolymers were prepared by the solvent exchange method. Dynamic light scattering (DLS) measurements showed that PCL-g-SS-PEG copolymers formed micelles with average sizes of 110-120 nm and low polydispersities (PDIs) of 0.09-0.12 (Figure 3.4A). The critical micelle concentration (CMC) was determined using pyrene as a probe in 10 mM PB at pH 7.4 (Figure 3.4B). The results showed that PCL-g-SS-PEG **1** and **2** had particularly low CMC values of 0.92 and 0.87 mg/L, respectively (Table 3.2), supporting that amphiphilic graft copolymers form particularly stable micelles.

Figure 3.4. Size distribution of PCL-g-SS-PEG graft copolymer micelles determined by DLS (A) and the fluorescence intensity ratio I_{372}/I_{383} of pyrene as a function of PCL-g-SS-PEG **2** concentration (B).

The reduction-sensitivity of PCL-g-SS-PEG **2** micelles was studied using DLS by monitoring the change of micelle sizes in response to 10 mM dithiothreitol (DTT) in PB buffer (pH 7.4, 10 mM). The results showed that micelles were quickly destabilized by DTT to form large aggregates of about 600 nm in 4 h (Figure 3.5A), accompanied with decrease of light scattering intensity and increase of PDI (Figure

3.5B). These results are in line with our previous observations for PEG-SS-PCL micelles [48]. In contrast, no change in micelle sizes and PDIs was discerned after 24 h in the absence of DTT under otherwise the same conditions. DTT-triggered cleavage of disulfide bonds in PCL-g-SS-PEG **2** micelles was also confirmed by GPC in that PCL-g-SS-PEG **2** micelles following treatment with DTT gave two distributions corresponding to P(CL-co-PDSC) copolymer (Table 3.1, Entry 4) and PEG, respectively.

Figure 3.5. Change of particle sizes (A) and PDI and light scattering intensity (B) of PCL-g-SS-PEG **2** micelles in time in response to 10 mM DTT.

3.3.5 Loading and triggered release of DOX

DOX was loaded into PCL-g-SS-PEG micelles at theoretical drug loading contents (DLC) of 4.8, 9.1 and 16.7 wt.%. Interestingly, PCL-g-SS-PEG **1** and **2** micelles exhibited similar DOX loading levels and particle sizes (Table 3.3), indicating that the molecular weight of the amphiphilic graft copolymer had little influence on the drug loading behavior. The drug loading efficiencies (DLE) ranged from 54.1 to 71.1 %, which decreased with increasing DLC. Moreover, DOX-loaded PCL-g-SS-PEG micelles had low PDIs of 0.12-0.23 and particle sizes ranging from 56.2 to 116.1 nm depending on DOX loading levels. The micelle size became smaller at a low drug loading level likely due to the existence of effective interactions between the micellar core and DOX.

The *in vitro* release of DOX from PCL-g-SS-PEG **2** micelles was investigated using a dialysis tube (MWCO 12000 Da) and PB buffer (pH 7.4, 100 mM) at 37 °C either in the presence or absence of 10 mM DTT. Interestingly, the results showed that DOX was released rapidly in response to 10 mM DTT, in which ca. 62.1 % and 82.0 % DOX was released in 4 and 12 h, respectively (Figure 3.6). In contrast, minimal drug release (ca. 17.5 %) was observed within 24 h in the absence of 10 mM DTT under otherwise the same conditions. This redox-triggered drug release behavior has been reported for various shell-sheddable micelles [48, 51-54]. This fast drug release is likely because micelles following shedding of PEG shells are destabilized and form drug diffusion channels. These reduction-sensitive biodegradable graft copolymer micelles are highly interesting as “smart” vehicles for active intracellular drug release because they can be

easily prepared, have a good stability and show a rapid redox-triggered drug release behavior.

Table 3.3. DOX loading content and loading efficiency of polymeric micelles.

Polymer	DLC (wt.%)		DLE (%)	Size (nm)	PDI
	theory	determined ^a			
PCL-g-SS-PEG 1	4.8	3.3±0.07	68.2±1.5	56.2±0.5	0.13
	9.1	5.7±0.09	60.3±1.6	68.7±1.5	0.17
	16.7	9.8±0.12	54.1±2.0	116.1±2.6	0.23
PCL-g-SS-PEG 2	4.8	3.4±0.06	71.1±1.4	58.9±0.4	0.12
	9.1	5.8±0.09	61.5±1.7	64.8±1.3	0.14
	16.7	10.1±0.13	55.9±2.1	108.2±2.1	0.20

^a Drug loading content for DOX was determined by fluorescence measurements.

Figure 3.6. Reduction-triggered release of DOX from PCL-g-SS-PEG 2 micelles in phosphate buffer (pH 7.4, 100 mM). The release of free DOX from the dialysis tube was used as a control.

3.3.6 Intracellular drug release and anti-tumor activity of DOX-loaded PCL-g-SS-PEG micelles

The cellular uptake and intracellular drug release behavior of DOX-loaded PCL-g-SS-PEG micelles were studied using CLSM. The cell nuclei were stained with DAPI (blue). Interestingly, the results showed that DOX-loaded PCL-g-SS-PEG micelles delivered and released DOX into the perinuclei and nuclei regions following 4 h incubation (Figure 3.7A), indicating fast internalization of micelles and rapid release of DOX inside cells. At a longer incubation time of 8 h, DOX was fully delivered into the nuclei of HeLa

cells (Figure 3.7B). It is remarkable to note that DOX-loaded PCL-g-SS-PEG micelles displayed similar intracellular DOX concentrations in time as free DOX (Figure 3.7C).

Figure 3.7. CLSM images of HeLa cells following 4 or 8 h incubation with DOX-loaded PCL-g-SS-PEG 2 micelles or free DOX (10 $\mu\text{g/mL}$). For each panel, the images from left to right show cell nuclei stained by DOX fluorescence in cells (red), DAPI (blue), overlays of both images. The scale bars correspond to 25 μm in all the images. (A) DOX-loaded PCL-g-SS-PEG 2 micelles, 4 h; (B) DOX-loaded PCL-g-SS-PEG 2 micelles, 8 h; (C) free DOX, 4 h.

Figure 3.8. (A) Cytotoxicity of PCL-g-SS-PEG micelles to HeLa cells following 48 h incubation; (B) Viabilities of HeLa cells following 48 h incubation with DOX-loaded PCL-g-SS-PEG micelles and free DOX as a function of DOX dosage. All the data are presented as the average $\pm$ standard deviation ($n = 4$).

MTT assays revealed that both PCL-g-SS-PEG 1 and 2 micelles were non-toxic to HeLa cells with cell viabilities more than 98 % up to a tested concentration of 1.0 mg/mL (Figure 3.8A), confirming that

these biodegradable graft copolymer micelles have excellent biocompatibility. DOX-loaded PCL-g-SS-PEG micelles, however, displayed pronounced anti-tumor activity towards HeLa cells following 48 h incubation (Figure 3.8B). DOX-loaded PCL-g-SS-PEG **1** and **2** micelles had low IC₅₀ (half maximal inhibitory concentration) of 0.95 and 0.82 µg DOX equiv./mL, respectively, which was close to that observed for free DOX (IC₅₀ = 0.46 µg/mL). It should further be noted that the anti-tumor activity of graft copolymer micellar drugs could further be enhanced by installing a tumor-targeting ligand like aptamer, peptide, and antibody fragment that facilitates efficient and specific tumor cell uptake of micelles. These redox-sensitive biodegradable graft polymer micelles with excellent biocompatibility, low CMC and intracellular redox-responsive drug release are highly promising for targeted cancer therapy.

3.4 Conclusions

We have demonstrated that pyridyldisulfide-functionalized poly(ϵ -caprolactone) can be conveniently prepared with controlled molecular weights and functionalities by ring-opening copolymerization of ϵ -caprolactone with pyridyldisulfide cyclic carbonate monomer. To our knowledge, this represents a first report on the synthesis of biodegradable polymers containing pendant multiple pyridyldisulfide groups. These pyridyldisulfide-functionalized biodegradable polymers provide a direct access to novel reduction-sensitive amphiphilic biodegradable graft copolymers that readily form micelles in water. Interestingly, these graft copolymer micelles exhibit excellent biocompatibility, enhanced stability with a low critical micelle concentration, and redox-triggered drug release behavior. DOX-loaded micelles have shown pronounced anti-tumor activity to HeLa cells. This study has provided a proof of concept that pyridyldisulfide-functionalized biodegradable polymers can be prepared and used for the construction of redox-responsive amphiphilic biodegradable graft copolymers and micelles that mediate efficient intracellular delivery of anti-cancer drugs.

3.5 References

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Chapter 4

Galactose-Shielded Reduction-Sensitive Nanoparticles for Hepatoma Targeted Intracellular Delivery of DOX*

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Abstract

Galactose-shielded reduction-sensitive nanoparticles based on biodegradable poly(ϵ -caprolactone)-graft-SS-galactose (PCL-g-SS-Gal) amphiphilic copolymer were developed for hepatoma-targeted intracellular delivery of DOX. PCL-g-SS-Gal copolymers prepared via a combination of ring-opening copolymerization (ROP) of ϵ -caprolactone (ϵ -CL) and pyridyl disulfide carbonate (PDSC), and post-polymerization modification with thiol-containing Gal (Gal-SH) via the thiol-disulfide exchange reaction, were self-assembled into nanoparticles with a diameter of approximately 80 nm and a narrow PDI as determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM). PCL-g-SS-Gal nanoparticles while relatively stable under physiological conditions were prone to rapid shell-shedding and aggregation under a reductive condition. Doxorubicin (DOX) was loaded into PCL-g-SS-Gal nanoparticles with a decent drug loading content of 12.0 wt.%. Notably, *in vitro* release studies revealed that ca. 80.3% DOX was released in 24 h in a reductive environment whereas only ca. 20.7 % DOX was released in 24 h under non-reductive conditions. Minimal DOX was released from PCL-g-Gal reduction-insensitive nanoparticles under otherwise the same conditions. Flow cytometry and confocal microscopy revealed an efficient uptake of PCL-g-SS-Gal nanoparticles by HepG2 cells via asialoglycoprotein receptor (ASGP-R) mediated endocytosis followed by a rapid intracellular release of DOX. The cytotoxicity of DOX-loaded PCL-g-SS-Gal nanoparticles was much higher as compared to DOX loaded non reduction sensitive micelles, confirming the importance of the reduction sensitivity of the micelles for intracellular drug delivery. These results suggest that redox-sensitive PCL-g-SS-Gal nanoparticles are a promising platform for tumor-targeted intracellular drug release.

4.1 Introduction

Biodegradable nanoscale drug carriers such as polymeric micelles/nanoparticles have attracted growing attention based on their potential applications in cancer therapy [1-6]. These systems could dramatically improve the bioavailability of anticancer drugs by enhancing the drug solubility in aqueous systems, prolonging blood circulation time and improving the passive accumulation at tumor tissue *via* the enhanced permeability and retention (EPR) effect [7-9]. However, despite the fact that significant progress has been made in the past decade, few of the targeting systems have achieved optimal results, which is mostly due to inefficient uptake by the targeted tumor cells and/or a poor intracellular trafficking pathway [10, 11].

Although micellar drugs have been delivered and accumulated into solid tumors by passive tumor-targeting, most of these systems do not exert therapeutic effects because of poor cellular uptake. In contrast to passive drug delivery, active targeting based on specific ligand-receptor interactions has recently received significant attention [12, 13]. In this respect polymers bearing pendant carbohydrates are particularly useful for drug delivery applications that require the targeting of carbohydrate-binding proteins known as lectins [14]. In recent years, many nanocarriers decorated with β -D-galactose, *N*-acetylgalactosamine, and lactose ligands have been designed for the development of liver targeted drug delivery, due to the presence of the asialoglycoprotein receptor (ASGP-R) on mammalian hepatocytes that specifically recognizes these carbohydrates [15-17]. It has also been reported that glycopolymers with the appropriate density and relative spatial arrangements of the carbohydrate residues could improve the binding affinity toward proteins due to polyvalent interactions, which has been known as the “glycoside cluster effect” [18, 19]. Yang’s group [20] has demonstrated that biodegradable Gal-decorated micelles based on amphiphilic Gal-functionalized glycopolymers exhibited significant cellular uptake and anti-tumor efficacy using ASGP-R positive HepG2 cells. However, a lack of sensitivity of structural change of the micelles in response to intracellular signals may induce only incomplete drug release and inefficient cancer therapy.

In recent years, the design of novel bio-responsive nanocarriers that release drugs in response to an intracellular signal, in particular acidic pH and redox potential, has received great interest [21-24]. Comparing with pH-sensitive nanocarriers that promote drug release in endosomes, redox-responsive polymeric micelles and nanoparticles are intended to disassemble and release drugs into the cytosol which contains a 100 to 1000-fold higher concentration of reducing GSH tripeptide (approximately 2-10 mM) than the extracellular fluids (approximately 2-20 μ M) [25, 26]. These reduction-sensitive nanoparticles usually contain typical disulfide linkage(s) in the main chain [27-30], at the side chain [31-33], or in the cross-linker [34-37]. We reported that reduction-sensitive shell-sheddable biodegradable micelles based on poly(ethylene glycol)-SS-poly(ϵ -caprolactone) (PEG-SS-PCL) or dextran-SS-PCL copolymers efficiently released DOX under reductive conditions (10 mM dithiothreitol, DTT) and achieved significantly enhanced anticancer efficacy [38, 39]. The presence of an intrinsic intracellular redox potential has stimulated the design of novel and versatile redox-sensitive polymeric nanoparticles to promote intracellular drug release. Active tumor-targeting of stimuli-responsive nanoparticles based on

biodegradable amphiphilic glycopolymers has been rarely reported up to now.

In this study, Gal-shielded redox-sensitive nanoparticles based on biodegradable PCL-g-SS-Gal amphiphilic copolymer were developed for hepatoma-targeted intracellular delivery of DOX (Scheme 4.1). The biodegradable PCL-g-SS-Gal copolymer was synthesized by a combination of ring-opening polymerization (ROP) of ϵ -caprolactone (ϵ -CL) with pyridyl disulfide carbonate (PDSC), and post-polymerization modification with thiol-containing galactose (Gal-SH) via the thiol-disulfide exchange reaction. We have successfully developed biodegradable PCL-g-SS-PEG graft copolymer prepared by the combination of ROP and thiol-disulfide exchange reaction, which provided a versatile access to biodegradable reduction-sensitive micelles that exhibited enhanced stability with a low critical micelle concentration, redox-triggered drug release behavior and remarkable anti-tumor activity [40]. Here, the introduction of the disulfide bond as the linkage between the hydrophilic Gal and hydrophobic main chain can promote intracellular drug release by cleavage of the linkage. Synthesis and self-assembly of biodegradable Gal-bearing graft copolymers, loading and *in vitro* release of DOX, as well as targeting liver cancer cells, cellular uptake and antitumor activity of DOX-loaded polymeric nanoparticles were investigated.

Scheme 4.1. Illustration of the self-assembly, accumulation at tumor tissue and intracellular trafficking pathway of redox-sensitive PCL-g-SS-Gal nanoparticles. The intracellular trafficking pathway includes steps of receptor-mediated cellular internalization, reduction triggered micelle disassembly, and drug release.

Materials and methods. Triethylamine (Et_3N , 99%, Alfa Aesar), dithiothreitol (DTT, 99%, Merck), stannous octoate ($\text{Sn}(\text{Oct})_2$, 95%, Sigma), lactobionic acid (LBA, 97 %, Acros) and doxorubicin hydrochloride ($\text{DOX}\cdot\text{HCl}$, 99 %, Beijing Zhong Shuo Pharmaceutical Technology Development Co.Ltd.) were used as received. ϵ -Caprolactone ($\epsilon\text{-CL}$, 99%, Alfa Aesar) was dried over CaH_2 and distilled under reduced pressure prior to use. Toluene was dried by refluxing over sodium wire under an argon atmosphere prior to distillation. Isopropanol was dried by refluxing over CaH_2 under an argon atmosphere. Acryloyl cyclic carbonate monomer (AC) and pyridyldisulfide cyclic carbonate monomer (PDSC) were synthesized according to our previous reports [40, 41].

Characterization. ^1H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz. CDCl_3 and $\text{DMSO-}d_6$ were used as solvents and the chemical shifts were calibrated against residual solvent signals. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using CHCl_3 as the eluent at a flow rate of 0.5 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns.

Synthesis of P(CL-co-PDSC) and P(CL-co-AC) functional copolymers. The ring-opening copolymerization of $\epsilon\text{-CL}$ with PDSC or AC was carried out in toluene at 100 °C using $\text{Sn}(\text{Oct})_2$ as a catalyst and isopropanol as an initiator. The following is a typical example of the synthesis of P(CL-co-PDSC) copolymer according to our previous report. In the glove-box, under a nitrogen atmosphere to a stirred solution of $\epsilon\text{-CL}$ (42 mg, 0.369 mmol), PDSC (400 mg, 1.476 mmol) and isopropanol stock solution (60 μmol , 1 mM) in toluene (4 mL) was quickly added $\text{Sn}(\text{Oct})_2$ stock solution (20 mg, 50 μmol). The reaction vessel was sealed and placed in an oil-bath thermostated at 100 °C. The polymerization was allowed to proceed with magnetic stirring for 24 h and terminated by acetic acid. The P(CL-co-PDSC) copolymer was isolated by precipitation from cold diethyl ether and dried under vacuum at room temperature. AC-functionalized PCL copolymer was synthesized in a similar method.

Synthesis of thiol-containing galactose (Gal-SH). To a stirred solution of lactobionic acid (LBA, 3 g, 8.37 mmol) in deionized water (20 mL) was added EDC (3.2 g, 16.74 mmol) and NHS (1.9 g, 16.74 mmol). The pH of the solution was adjusted to 5 and then the solution was stirred for 4 h at room temperature. Under N_2 protection, 2-mercaptoethylamine hydrochloride (2.4 g, 20.93 mmol) was added into the mixture and the pH of the solution was adjusted to 7.4 by an aqueous solution of sodium hydroxide (1 M). The reaction was allowed to proceed for another 24 h. The solution was then treated with DTT (2.0 g, 12.98 mmol) for 5 h. The crude product was isolated by precipitation in isopropanol and washed with methanol to yield Gal-SH. The thiol functionality was determined via the Ellman assay (functionality of SH = 80 %).

Synthesis of PCL-g-SS-Gal and PCL-g-Gal graft copolymers. To a solution of P(CL-co-PDSC) (50 mg, 0.144 mmol of PDS group) and Gal-SH (375 mg, 0.72 mmol of SH group) in DMSO (25 mL) under a nitrogen atmosphere was added a catalytic amount of acetic acid, and the reaction was allowed to proceed with magnetic stirring for 48 h at 40 °C. The reaction was monitored by determining the amount of the pyridinethione reaction product using UV analysis. The polymer solution was purified by dialysis against

deionized water with a molecular weight cut-off (MWCO) of 3500 at room temperature for 48 h, to remove excess Gal-SH and pyridinethione. The aqueous suspension was finally freeze-dried to yield colorless powder.

PCL-g-Gal graft copolymer was synthesized in a similar method by Michael-type conjugate addition reaction in DMSO at room temperature using a catalytic amount of Et₃N [41].

Nanoparticle formation and critical association concentration (CAC). PCL-g-SS-Gal and PCL-g-Gal copolymer nanoparticles were prepared by the solvent exchange method. For example, to a stirred DMSO solution (200 μ L) of PCL-g-SS-Gal (5.0 mg/mL) was dropwise added 2.0 mL of phosphate buffer (PB, 10 mM, pH 7.4), and then the mixture was dialyzed against PB (10 mM, pH 7.4) for 8 h with a MWCO of 3500 at room temperature to remove the organic solvent. The size of the nanoparticles was determined using dynamic light scattering (DLS) and transmission electron microscopy (TEM). Suspensions of the nanoparticles were filtered through a 450 nm syringe filter before measurements. Measurements were carried out at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He-Ne laser using back-scattering detection. TEM was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μ L of a 0.2 mg/mL suspension of the nanoparticles on the copper grid followed by staining with phosphotungstic acid.

The critical association concentration (CAC) was determined using pyrene as a fluorescence probe. The concentration of the polymeric nanoparticles was varied from 2.0×10^{-5} to 0.5 mg/mL and the concentration of pyrene was fixed at 1.0×10^{-6} M. Fluorescence spectra were recorded using a FLS920 fluorescence spectrometer and an excitation wavelength of 330 nm. Fluorescence emissions at 372 and 383 nm were monitored. The CAC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

Size change of nanoparticles in response to reduction-stimulus. The change in size of the nanoparticles in response to disulfide bond cleavage was followed by DLS measurements at 37 °C. Samples with a polymer concentration of 0.5 mg/mL were prepared as above-mentioned. A PCL-g-SS-Gal nanoparticle suspension (0.5 mg/mL) was divided into two aliquots of 1 mL, and 10 μ L of DTT solution (1.0 M) was added into one of the two aliquots with a final DTT concentration of 10 mM. The samples were slowly stirred at 37 °C under a nitrogen atmosphere and the change in the nanoparticle size was monitored over time by DLS. Similar experiments were carried out with PCL-g-Gal nanoparticles used as the reduction-insensitive control (0.5 mg/mL).

Incorporation and triggered release of DOX. DOX was loaded into nanoparticles by dropwise addition of 5.0 mL PB (10 mM, pH 7.4) to a mixture of 500 μ L of copolymer solution in DMSO (5 mg/mL) and 25, 50 or 100 μ L of DOX solution in DMSO (5.0 mg/mL) under stirring at room temperature, followed by ultrasonication for 0.5 h and dialysis against PB (10 mM, pH 7.4) with a MWCO of 3500 at room temperature in the dark for 8 h. The dialysis medium was changed every hour.

The release profiles of DOX from nanoparticles in PB (100 mM, pH 7.4) at 37 °C with or without DTT were measured. The above prepared DOX-loaded nanoparticle suspensions (containing 0.5 mg/mL nanoparticles and 25 μ g/mL DOX) were divided into two aliquots. Each aliquot was immediately

transferred to a dialysis tube with a MWCO of 12000-14000. The dialysis tube was immersed into 20 mL of the appropriate buffer and the media were shaken at 37 °C. At desired time intervals, 5 mL of the release medium was taken out for measuring the fluorescence and replenished with an equal volume of fresh medium. To avoid oxidation of DTT, the release media were perfused with nitrogen gas. The concentration of DOX was determined by fluorescence (FLS920) measurements (excitation at 480 nm). For determination of the drug loading content, DOX-loaded nanoparticle suspensions were freeze-dried, then dissolved in DMSO and analyzed with fluorescence spectroscopy. A calibration curve was obtained using DOX/DMSO solutions with different DOX concentrations. To determine the amount of DOX released, calibration curves were run with DOX/PB solutions with different DOX concentrations at pH 7.4. The emission at 600 nm was recorded. Release experiments were conducted in triplicate. The results are presented as the average $\pm$ standard deviation.

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

$$\text{DLC (wt.\%)} = (\text{weight of loaded drug} / \text{total weight of polymer and loaded drug}) \times 100\%$$

$$\text{DLE (\%)} = (\text{weight of loaded drug} / \text{weight of drug in feed}) \times 100\%$$

CLSM of cells incubated with DOX-loaded nanoparticles. HepG2 cells were plated on microscope slides in a 24-well plate (5×10^4 cells/well) using DMEM medium containing 10% FBS. After 24 h incubation, the medium was replaced by 450 μ L of fresh DMEM and 50 μ L of prescribed amounts of DOX-loaded nanoparticles or free DOX. To confirm the binding of nanoparticles to the asialoglycoprotein receptor (ASGP-R) over-expressed on HepG2 cells surface, 100 μ L of free LBA (10 mg/mL) was added 4 h before the addition of DOX-loaded nanoparticles to block ASGP-R on the surface of the HepG2 cells. After incubation for 2 and 4 h with the DOX-loaded nanoparticles or free DOX, the culture medium was removed and the cells on microscope plates were washed three times with PBS. The cells were fixed with 4% paraformaldehyde and the cell nuclei were stained with DAPI. Fluorescence images of cells were obtained with Confocal Laser Scanning Microscope (Leica, TCS-SP2).

Cellular uptake-quantitative analysis by flow cytometry. HepG2 cells were seeded onto 6-well plates at 1×10^5 cells per well for 24 h using DMEM medium containing 10% FBS. After 24 h incubation, the medium was replaced by 0.9 mL of fresh DMEM and 0.1 mL of prescribed amounts of DOX-loaded nanoparticles or free DOX. Similarly, to investigate whether nanoparticles were specifically taken up by HepG2 cells through ASGP-R mediated endocytosis, cells were incubated with free-LBA (2 mg/mL) for 4 h before the addition of DOX-loaded nanoparticles. After incubation at 37 °C for 4 h, the cells were digested by 0.25 w/v% trypsin/0.03 w/v% EDTA. The suspensions were centrifuged at 1500 rpm for 5 min at 25 °C, pelleted in eppendorf tubes, washed twice with cold PBS, and then resuspended in 500 μ L of PBS. Fluorescence histograms were recorded with a BD FACSCalibur (Beckton Dickinson) flow cytometer and analyzed using Cell Quest software. We analyzed 10 000 gated events to generate each histogram. The gate was arbitrarily set for the detection of DOX fluorescence.

MTT assay. The cytotoxicity of blank or DOX-loaded nanoparticles was studied by the MTT assay using HepG2 cells and MCF-7 cells. Cells were seeded onto a 96-well plate at a density of 1×10^4 cells per

well in 100 μL of Dulbecco's Modified Eagle medium (DMEM) containing 10% FBS and incubated for 24 h (37 $^{\circ}\text{C}$, 5% CO_2). The medium was replaced by 90 μL of fresh DMEM medium containing 10% FBS, and then 10 μL samples of various concentrations of the nanoparticle suspensions in PB (10 mM, pH 7.4) were added. The cells were incubated for another 48 h, and then 10 μL of MTT solution (5 mg/mL) was added. The cells were incubated for 4 h, and the medium was aspirated and replaced by 150 μL of DMSO to dissolve the resulting purple crystals. The optical densities at 570 nm were measured using a BioTek microplate reader. Cells cultured in DMEM medium containing 10% FBS (without exposure to nanoparticles) were used as controls.

To evaluate whether the high drug efficacy was specially caused by ASGP-R mediated endocytosis, HepG2 cells seeded in a 96-well plate at a density of 1×10^4 cells per well for 24 h were incubated with (+LBA) or without (-LBA) free LBA (2 mg/mL) 4 h before the addition of DOX-loaded nanoparticles or free DOX. The ASGP-R on HepG2 cell surface can be blocked in DMEM medium containing free LBA. The cytotoxicity studies for DOX-loaded PCL-g-SS-Gal nanoparticles, DOX-loaded PCL-g-Gal nanoparticles and free DOX (drug dosage of 10 $\mu\text{g/mL}$) were carried out in a similar way. The medium was replaced by 90 μL of fresh DMEM medium and 10 μL of DOX-loaded nanoparticles or free DOX. After an additional 4 h incubation, the medium was replaced by 100 μL of fresh DMEM medium and the cells were cultured for another 48 h. After that, the same procedure was carried out as above-mentioned. 10 μL of MTT solution (5 mg/mL) was added and the cells were incubated for 4 h. The culture medium was aspirated and replaced by 150 μL of DMSO to dissolve the resulting purple crystals. The optical densities at 570 nm were measured using a BioTek microplate reader. Cells cultured in DMEM medium containing 10% FBS (without exposure to nanoparticles) were used as controls.

4.3 Results and discussion

4.3.1. Synthesis of PCL-g-SS-Gal amphiphilic copolymer

The ring-opening copolymerization of $\epsilon\text{-CL}$ and PDSC can provide a versatile approach to PDS-functionalized biodegradable copolymers, which can yield reduction-sensitive, amphiphilic, biodegradable graft copolymers by modification with thiol-containing molecules *via* the thiol-disulfide exchange reaction (Scheme 4.2). The biodegradable P(CL-*co*-PDSC) copolymer was synthesized in toluene at 100 $^{\circ}\text{C}$ using $\text{Sn}(\text{Oct})_2$ as a catalyst and isopropanol as an initiator proceeding for 24 h. ^1H NMR spectra show the corresponding signals which were attributed to CL units (δ 4.04, 2.30, 1.64 and 1.38) and PDSC units (δ 8.48, 7.70, 7.12, 4.05, 3.02 and 1.10) (Figure 4.1a). The copolymer composition determined by comparing signals at δ 3.02 (methylene protons next to the disulfide bond in PDSC units) and 2.30 (methylene protons next to the carbonyl in CL units) was approximately 60 mol% CL, which was close to the theoretical design (Table 4.1). The number-average molecular weights (M_n) estimated from ^1H NMR end group analysis by comparing the integrals of peaks at 2.30 (methylene protons next to carbonyl in CL units) and 3.02 (methylene protons next to the disulfide bond in PDSC units) with δ 4.34 (methine protons of isopropyl ester end group) were close to the theoretical value (Table 4.1). Gel permeation

chromatography (GPC) using polystyrene as standards showed that these P(CL-co-PDSC) copolymers had moderate polydispersities (PDI) of 1.58. The post-polymerization modification of P(CL-co-PDSC) was performed at a Gal-SH/PDS molar ratio of 5/1 in DMSO at 40 °C for 48 h using a catalytic amount of acetic acid (Scheme 4.2). After 48 h reaction, the produced amount of pyridinethione determined by UV spectroscopy was close to the original amount of pyridyldisulfide groups in the P(CL-co-PDSC) copolymer [42], which indicated that the thiol-disulfide exchange reaction had taken place quantitatively. ¹H NMR revealed that signals assignable to Gal were clearly detected at δ 3.4-3.8 and 4.4-5.4 while signals at δ 8.48, 7.70, 7.12 owing to pyridine protons disappeared (Figure 4.2). It is evident that PCL-g-SS-Gal graft copolymers can be readily prepared from PDS-functionalized PCL via the thiol-disulfide exchange reaction.

Scheme 4.2. Synthesis of PCL-g-SS-Gal graft copolymer by combining ring-opening polymerization and thiol-disulfide exchange reaction. Conditions: (i) toluene, 100 °C, 24 h; (ii) DMSO, 40 °C, 48 h.

Table 4.1. Synthesis of biodegradable P(CL-co-PDSC) and P(CL-co-AC) copolymers.^a

Entry	Copolymer	[M]/[I]	f ^b (%)	F ^c (%)	$M_n (\times 10^3)$ kg/mol			PDI
					Design ^d	¹ H NMR ^e	GPC ^f	
1	P(CL-co-PDSC)	30	70	60	6.7	6.4	6.9	1.58
2	P(CL-co-AC)	30	70	62	5.2	5.8	6.7	1.46

^a The ring-opening copolymerization was carried out in toluene at 100 °C using isopropanol as an initiator and Sn(Oct)₂ as a catalyst; ^b Molar ratio of functional monomers (PDSC or AC) in feed; ^c Molar fraction of functional units determined by ¹H NMR; ^d Calculated by the following formula: $M_n = M_{n(CL)} \times ([M]/[I]) \times (1-f) + M_{n(PDSC \text{ or } AC)} \times ([M]/[I]) \times f$; ^e Determined by end group analysis; ^f Determined by GPC (eluent: chloroform, flow rate: 0.5 mL/min, standards: polystyrene).

Gal-grafted P(CL-co-AC) copolymer, which was used as a reduction-insensitive control, was synthesized by combining the ring-opening polymerization and Michael-type addition according to our previous report (Scheme 4.3). The backbone of P(CL-co-AC) copolymer had a similar composition with respect to AC-functional components, as compared to the composition of PDS-functional pendants in P(CL-co-PDSC) copolymer (Figure 4.1b). The results showed that P(CL-co-AC) copolymer had a moderate PDI of 1.46 and an M_n of 5.8 kg/mol, close to that of P(CL-co-PDSC) (Table 4.1). After post-polymerization modification with Gal-SH via Michael-type addition reaction, the double bonds in P(CL-co-AC) copolymer were quantitatively converted, which confirmed the successful synthesis of P(CL-co-AC)-g-Gal copolymer.

Figure 4.2. ^1H NMR spectra (400 MHz, CDCl_3) of functional biodegradable copolymers of P(CL-co-PDSC) (A) and P(CL-co-AC) (B).

Figure 4.2. ^1H NMR spectrum (400 MHz, $\text{DMSO-}d_6$) of PCL-g-SS-Gal copolymer.

Scheme 4.3. Synthesis of PCL-g-Gal graft copolymer by combining ring-opening polymerization and Michael-type addition reaction. Conditions: (i) toluene, 100 °C, 24 h; (ii) DMSO, 40 °C, 48 h.

4.3.2 Nanoparticle formation and critical association concentration (CAC)

Reduction-sensitive nanoparticles were prepared by the solvent exchange method. The size of the nanoparticles was determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS showed that PCL-g-SS-Gal formed nanoparticles with sizes of approximately 80 nm and a narrow size distribution (Figure 4.3a). TEM revealed a homogeneous distribution of spherical particles in accord with the DLS results (Figure 4.3b). The control, PCL-g-Gal reduction-insensitive nanoparticles, had a particle size of 100 nm with a narrow size distribution as measured by DLS (Figure 4.3a). The size of PCL-g-Gal nanoparticles was a little larger than that of PCL-g-SS-Gal nanoparticles. The critical association concentration (CAC) was determined by fluorescence measurements using pyrene as a probe. The results showed that these graft copolymers had CAC values of 4.63 for PCL-g-SS-Gal and 4.25 mg/L for PCL-g-Gal, respectively. Such low CAC values ensure that the self-assembled nanoparticles retain their original structure under highly diluted conditions before arriving to the targeting sites.

Figure 4.3. Size distribution of PCL-g-SS-Gal and PCL-g-Gal nanoparticles determined by DLS (A) and TEM image of PCL-g-SS-Gal nanoparticles (B).

4.3.3 Reduction-triggered disassembly of nanoparticles

To investigate the reduction-triggered disassembly of nanoparticles, the change in size distribution of PCL-g-SS-Gal nanoparticles in the presence of 10 mM DTT was followed by DLS. As shown in Figure 4.4A, fast aggregation was observed for PCL-g-SS-Gal nanoparticles, in which the size of the nanoparticles increased from 80 nm to 300 nm in 3 h, reaching over 500 nm after 8 h, while the average size of PCL-g-SS-Gal nanoparticles was essentially unchanged in the absence of DTT. These aggregates were most probably formed by reductive cleavage of the intermediate disulfide bonds, which results in shedding of the Gal shells. In the control experiment, wherein 10 mM DTT was added, the size distribution of PCL-g-Gal reduction-insensitive nanoparticles had no obvious change throughout the experimental time (Figure 4.4B). This was in line with our previous report that shell-sheddable PCL-g-SS-PEG micelles,^[40] as well as PEG-SS-PCL and Dextran-SS-PCL micelles,^[38, 39] were capable to rapidly release DOX in the

presence of 10 mM DTT mimicking the intracellular reductive conditions.

Figure 4.4. Size change of nanoparticles in response to reductive environment (10 mM DTT) followed by DLS measurement. (A) PCL-g-SS-Gal reduction-sensitive nanoparticles, (B) PCL-g-Gal nanoparticles as reduction-insensitive control.

4.3.4 Entrapment and *in vitro* release of DOX

DOX-loaded nanoparticles were prepared by dialysis of a polymer/DOX solution in DMSO against PB (pH 7.4, 10 mM). The theoretical drug loading contents were set at 5, 10 and 20 wt.%. Interestingly, PCL-g-SS-Gal and PCL-g-Gal nanoparticles exhibited similar DOX loading levels and particle sizes (Table 4.2), may be due to the similar structure of the copolymers. The drug loading efficiencies (DLE) remained 50.1~56.0 % with varying DLC (drug loading contents) from 5% to 20%. Moreover, DOX-loaded PCL-g-SS-PEG micelles had low PDIs of 0.16-0.26 and particle sizes ranging from 85.2 to 140.7 nm depending on DOX loading levels.

Table 4.2. DOX loading content and loading efficiency of nanoparticles.

Copolymer	DLC(wt%)		DLE (%)	Size (nm) ^b	PDI ^b
	Theory	Determined ^a			
PCL-g-SS-Gal	5	2.5	50.1	85.2 ± 1.4	0.16
	10	5.8	56.0	92.4 ± 1.5	0.18
	20	12.0	55.0	105.3 ± 3.2	0.22
PCL-g-Gal	5	2.6	51.2	113.6 ± 2.6	0.21
	10	5.4	50.8	126.4 ± 2.4	0.19
	20	11.5	53.5	140.7 ± 3.5	0.26

^a Determined by fluorescence measurements; ^b Determined by DLS measurements

The *in vitro* release of DOX from PCL-g-SS-Gal nanoparticles was investigated using a dialysis tube (MWCO 12000–14000) in PB (pH 7.4, 100 mM) with 10 mM DTT or without DTT. To avoid the precipitation of DOX due to fast release, drug release studies were performed at low drug loading contents (polymer concentration of 0.5 mg/mL with DLC of 4.7 wt.%) and with 0.5 mL of a suspension of nanoparticles, which was dialyzed against 20 mL of the same medium. The results show that PCL-g-SS-Gal nanoparticles release DOX rapidly in the presence of 10 mM DTT. For example, ca. 50% of DOX was released in 5 h and up to 80% of DOX was released in less than 20 h (Figure 4.5). It should be noted that the rate of DOX release from PCL-g-SS-Gal nanoparticles in the presence of 10 mM DTT was clearly faster than that without DTT due to the reductive cleavage of intermediate disulfide linkage between hydrophilic pendants and hydrophobic backbone. This is in line with the previous observation that PCL-g-SS-Gal nanoparticles are rapidly destabilized in the presence of 10 mM DTT (Figure 4.4A). It was also observed that a minimal amount of drug was released (less than 20%) from PCL-g-Gal reduction-insensitive nanoparticles within 24 h in PB (pH 7.4, 100 mM) either in the presence or without 10 mM DTT. It should be further noted that for all the nanoparticles no burst release of DOX was observed. Redox-triggered drug release behavior has been reported for various shell-sheddable micelles. The fast drug release is likely because nanoparticles following shedding of Gal shells become destabilized, aggregate and form drug diffusion channels.

Figure 4.5. Cumulative release of DOX from PCL-g-SS-Gal nanoparticles in the absence or presence of 10 mM DTT at 37 °C in PB (pH 7.4, 10 mM). PCL-g-Gal nanoparticles were used as the reduction-insensitive control.

4.3.5 Cellular uptake of nanoparticles and intracellular release of DOX

To evaluate the targeting of PCL-g-SS-Gal nanoparticles to liver tumor cells, we studied the cellular uptake of DOX entrapped in the nanoparticles using HepG2 cells over-expressing ASGP-R, which can bind galactose residues strongly, inducing ASGP-R mediated endocytosis of Gal-containing nanoparticles. The cellular uptake of DOX was analyzed quantitatively *via* flow cytometric analysis. The results showed that after 4 h incubation the cellular uptake of DOX entrapped in PCL-g-SS-Gal nanoparticles was much higher

(approximately 10 fold increase) towards free HepG2 cells (without treatment with lactobionic acid: - LBA) as compared to that of lactobionic acid-blocked HepG2 cells (+ LBA) (Figure 4.6A/4.6B). This indicated that the cellular uptake of DOX had increased significantly by ASGP-R mediated endocytosis. Interestingly, the cellular uptake of DOX by free HepG2 cells (- LBA) after 4 h incubation was more than 20 times higher for PCL-g-SS-Gal nanoparticles than for PCL-g-Gal nanoparticles (Figure 4.6A). Although PCL-g-Gal nanoparticles could also well interact with the HepG2 cells over-expressing ASGP-R, the relatively low uptake of DOX by the cells was attributed to the slow release of DOX from the reduction-insensitive PCL-g-Gal nanoparticles, which was in line with the *in vitro* release results (Figure 4.5). With respect to the result with LBA-blocked HepG2 cells (+ LBA), the lowest cellular uptake of DOX using PCL-g-Gal nanoparticles was due to the combination of inferior endocytosis and a low drug release rate. It should be noted that the cellular uptake of DOX using PCL-g-SS-Gal reduction-sensitive nanoparticles was comparable to that of free DOX (Figure 4.6A), which adequately demonstrated the reduction sensitivity and liver tumor-targeting properties of PCL-g-SS-Gal nanoparticles.

Figure 4.6. Cellular uptake and intracellular release of DOX into HepG2 cells following 4 h incubation using free HepG2 cells (A, C) and LBA-blocked HepG2 cells (B, D) pretreated with free LBA (2 mg/mL) for 4 h before adding samples. (A, B) Flow cytometric analysis of DOX-loaded nanoparticles and free DOX at a DOX concentration of 5 μg/mL and cell counts of 10000; (C, D) CLSM images of HepG2 cells incubated with DOX loaded nanoparticles and free DOX (5 μg/mL). For each panel, the images from left to right showed cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red) and overlays of the two images. The scale bars correspond to 25 μm in all the images.

To further investigate the feasibility of PCL-g-SS-Gal nanoparticles for intracellular release of DOX, HepG2 cells were incubated with DOX loaded PCL-g-SS-Gal and studied with confocal laser scanning microscopy (CLSM). Remarkably, after just 4 h incubation with PCL-g-SS-Gal nanoparticles an intensive DOX fluorescence was observed, which was mainly present in the cell nucleus, similarly as for free DOX (Figure 4.6C). For the reduction-insensitive PCL-g-Gal nanoparticles, there was little DOX fluorescence found in the cells, as well as for PCL-g-SS-Gal nanoparticles towards the LBA-blocked cells (+ LBA) after 4 h incubation (Figure 4.6C/4.6D). The results indicate that reduction-sensitive nanoparticles provide an effective approach for rapid transport of drug into the cytosol and nucleus, as compared to PCL-g-Gal reduction-insensitive nanoparticles. In addition, intracellular fluorescence of DOX released from PCL-g-SS-Gal nanoparticles was rarely observed after 4 h incubation, when the HepG2 cells were pretreated with free-LBA to block the over-expressed ASGP-R, suggesting a lack of cellular uptake of nanoparticles. It was confirmed that cellular uptake of DOX-loaded PCL-g-SS-Gal nanoparticles was enhanced remarkably by ASGP-R mediated endocytosis, which was in line with results from flow cytometric analysis. It was remarkable to note that DOX-loaded PCL-g-SS-Gal nanoparticles induced a similar intracellular DOX concentration as free DOX (Figure 4.6C).

4.3.6 Anti-tumor activity of DOX-loaded PCL-g-SS-Gal micelles

MTT assays using ASGP-R positive HepG2 cells and ASGP-R negative MCF-7 cells revealed that both reduction-sensitive PCL-g-SS-Gal nanoparticles and reduction-insensitive PCL-g-Gal nanoparticles were practically nontoxic (cell viabilities $\geq 95\%$) up to a tested concentration of 1.0 mg/mL (Figure 4.7A and 4.7B), confirming that these degradable Gal-containing nanoparticles are not cytotoxic. The anti-tumor activity of free DOX and DOX-loaded nanoparticles was tested with HepG2 and MCF-7 cells. We found that after 48 h incubation at the same DOX concentration, PCL-g-SS-Gal nanoparticles suppressed the proliferation of both HepG2 and MCF-7 cells much more efficiently than PCL-g-Gal nanoparticles. This was attributed to the much faster DOX release from PCL-g-SS-Gal nanoparticles at the intracellular reductive conditions. For example, DOX-loaded PCL-g-SS-Gal nanoparticles had relatively low IC_{50} (the concentration of DOX that causes 50 % inhibition of maximal cell growth) values of 0.10 and 0.58 $\mu\text{g DOX equiv./mL}$ for HepG2 (Figure 4.7C) and MCF-7 cells (Figure 4.7D), respectively, which were substantially lower than those obtained with the reduction-insensitive nanoparticles based on PCL-g-Gal under otherwise the same conditions ($IC_{50} = 5.6$ and $10.2 \mu\text{g DOX equiv./mL}$ for HepG2 and MCF-7 cells, respectively). As mentioned before, the cell killing efficacy of DOX loaded reduction sensitive nanoparticles for HepG2 cells was substantially higher than for MCF-7 cells (*i.e.* IC_{50} of DOX-loaded PCL-g-SS-Gal nanoparticles: 0.10 and 0.58 $\mu\text{g DOX equiv./mL}$ for HepG2 and MCF-7 cells, respectively). This can be explained by the overexpression of ASGP-R on HepG2 cells and not on MCF-7 cells, inducing accelerated cellular uptake of DOX-loaded Gal-containing nanoparticles *via* receptor-mediated endocytosis. It is also important to note that the HepG2 cell killing efficacy of DOX loaded PCL-g-SS-Gal nanoparticles ($IC_{50} = 0.10 \mu\text{g DOX equiv./mL}$) was even higher than that of free DOX ($IC_{50} = 0.24 \mu\text{g DOX equiv./mL}$).

Figure 4.7. Cytotoxicity of blank and DOX-loaded nanoparticles by MTT assay using HepG2 cells (A, C) and MCF-7 cells (B, D) following 48 h incubation. (A, B) Blank PCL-g-SS-Gal and PCL-g-Gal nanoparticles at different concentrations; (C, D) DOX-loaded PCL-g-SS-Gal nanoparticles, DOX-loaded PCL-g-Gal nanoparticles and free DOX. Data are presented as the average $\pm$ standard deviation ($n = 4$).

The anti-tumor activity of DOX-loaded nanoparticles was further evaluated by endocytosis of the nanoparticles by HepG2 cells for 4 h, followed by incubation with fresh culture medium for another 48 h. The cellular uptake of DOX-loaded nanoparticles can take place efficiently by HepG2 cells via receptor-mediated endocytosis. The cell killing efficacy of DOX loaded PCL-g-SS-Gal nanoparticles using free HepG2 cells was much better than for LBA-blocked cells, which is in agreement with the results from flow cytometric analysis and intracellular release of DOX. For instance, the cell viability of free and LBA-blocked HepG2 cells incubated with DOX-loaded PCL-g-SS-Gal nanoparticles was 35.9 and 65.5%, respectively (Figure 4.8). After 4 h incubation of HepG2 cells with DOX-loaded reduction-sensitive PCL-g-SS-Gal nanoparticles and a subsequent 48 h incubation with medium, the cell viability (35.9%) was much lower than when reduction-insensitive nanoparticles were used (cell viability of 71.0%). It should be noted that the delivery of DOX to HepG2 cells by reduction-sensitive nanoparticles was even more

effective than using free DOX. It is concluded that these Gal-shielded reduction-sensitive biodegradable nanoparticles are highly promising for tumor-targeted cancer therapy.

Figure 4.8. Cytotoxicity of DOX-loaded PCL-g-SS-Gal nanoparticles, DOX-loaded PCL-g-Gal nanoparticles and free DOX. DOX dosage was 10 $\mu\text{g/mL}$. The cells were incubated with free LBA (+ LBA) or without free LBA (- LBA) for 4 h, then the medium was replaced by 80 μL of fresh DMEM medium and 20 μL of DOX-loaded nanoparticles or free DOX. After 4 h incubation, the medium was replaced by 100 μL of fresh DMEM medium and the cells were cultured for another 48 h. Data are presented as the average $\pm$ standard deviation ($n=4$, Student's t test, *** $p < 0.001$).

4.4 Conclusions

We have demonstrated the successful synthesis of biodegradable PCL-g-SS-Gal copolymer, which has been prepared by a combination of ROP and post-polymerization modification *via* thiol-disulfide exchange reaction. Self-assembled reduction-sensitive nanoparticles based on PCL-g-SS-Gal copolymer while relatively stable under physiological conditions are prone to rapid shell-shedding and aggregation under reductive conditions. The *in vitro* release of DOX from PCL-g-SS-Gal nanoparticles shows that this drug is rapidly released in response to an intracellular-mimicking reductive environment in contrast to the minimal drug release under non-reduction conditions. The most important finding of this study is that PCL-g-SS-Gal nanoparticles provide an efficient approach for rapid cellular uptake *via* ASGP-R mediated endocytosis and fast transport of DOX into the cytosol, which significantly increases the anti-tumor efficacy of DOX against HepG2 cells. We are convinced that these Gal-shielding reduction-sensitive PCL-g-SS-Gal nanoparticles are highly promising for tumor-targeted intracellular drug delivery, affording enhanced cancer chemotherapy.

4.5 References

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Chapter 5

Redox and pH-Responsive Degradable Micelles for Dually Activated Intracellular Anticancer Drug Release*

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Abstract

Redox and pH dual-responsive biodegradable micelles were developed based on poly(ethylene glycol)-SS-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PEG-SS-PTMBPEC) copolymer and investigated for intracellular doxorubicin (DOX) release. PEG-SS-PTMBPEC copolymer with an M_n of 5.0–4.1 kg/mol formed micellar particles with an average diameter of 140 nm and a low polydispersity of 0.12. DOX was loaded into PEG-SS-PTMBPEC micelles with a decent drug loading content of 11.3 wt.%. The *in vitro* release studies showed that under physiological conditions only ca. 24.5 % DOX was released from DOX-loaded micelles in 21 h. The release of DOX was significantly accelerated at pH 5.0 or in the presence of 10 mM glutathione (GSH) at pH 7.4, in which 62.8 % and 74.3 % of DOX was released, respectively, in 21 h. The drug release was further boosted under 10 mM GSH and pH 5.0 conditions, with 94.2 % of DOX released in 10 h. Notably, DOX release was also facilitated by 2 or 4 h incubation at pH 5.0 and then at pH 7.4 with 10 mM GSH, which mimics the intracellular pathways of endocytosed micellar drugs. Confocal microscopy indicated that DOX was delivered and released into the nuclei of HeLa cells following 8 h incubation with DOX-loaded PEG-SS-PTMBPEC micelles, while DOX was mainly located in the cytoplasm for reduction-insensitive PEG-PTMBPEC controls. MTT assays revealed that DOX-loaded PEG-SS-PTMBPEC micelles had higher anti-tumor activity than reduction-insensitive controls, with low IC_{50} of 0.75 and 0.60 $\mu\text{g/mL}$ for HeLa and RAW 264.7 cells, respectively, following 48 h incubation. PEG-SS-PTMBPEC micelles displayed low cytotoxicity up to a concentration of 1.0 mg/mL. These redox and pH dual-bioresponsive degradable micelles have appeared as a promising platform for targeted intracellular anticancer drug release.

5.1 Introduction

In the past decades, biodegradable nanoparticles and micelles have emerged as one of the most promising nanosystems for the controlled and targeted delivery of potent anticancer drugs [1-6]. The PEGylated nanoparticles and micelles have demonstrated several interesting features including prolonged circulation time, better pharmacological profiles, enhanced accumulation in the tumor sites via the enhanced permeability and retention (EPR) effect, decreased adverse effects, and improved drug tolerance [7]. It is noted, however, that current nanosystems often suffer sluggish drug release at the tumor site as well as in the cancer cells that has become a critical account for the compromised treatment benefits in the clinical settings.

In recent years, intracellular environment-sensitive nanosystems that release payloads in response to an intrinsic biological signal, in particular endosomal pH and cytoplasmic glutathione (GSH), have been designed and explored for enhanced cancer therapy [2, 8-11]. For example, pH-sensitive micelles have been developed based on acid labile ortho ester [12, 13], hydrazone [14, 15], cis-aconityl [16, 17], and acetal [18, 19] bonds. Fréchet et al. reported that trimethoxybenzylidene acetals exhibit a great pH sensitivity [18, 20, 21]. Taking advantage of rapid pH-sensitive hydrolysis of trimethoxybenzylidene acetals, we have designed poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PEG-PTMBPEC) block copolymer and prepared pH-responsive biodegradable micelles and polymersomes [22, 23]. The *in vitro* drug release studies showed that release of paclitaxel (PTX) and/or DOX was highly pH-dependant. More recently, we have prepared pH-sensitive degradable chimaeric polymersomes based on trimethoxybenzylidene acetals for high loading and triggered release of doxorubicin hydrochloride [24], as well as trimethoxybenzylidene acetal-modified low molecular weight polyethylenimine for active intracellular DNA release and improved *in vitro* transfection [25]. It should be noted, nevertheless, that intracellular drug release from these pH-responsive degradable nanocarriers, though improved, is not fast due to the small difference in endosomal and physiological pH. For example, ca. 36 % of PTX and 58 % of DOX was released from PEG-PTMBPEC micelles in 12 h at pH 5.0 and 37 °C [23].

In contrast to pH-sensitive micelles that promote drug release in endosomes, redox-responsive micelles and nanoparticles are intended to disassemble and release drugs into the cytosol which contains 100 to 1000-fold higher concentration of reducing GSH tripeptide (approximately 2-10 mM) than the extracellular fluids (approximately 2-20 μ M) [26, 27]. We recently found that reduction-sensitive shell-sheddable biodegradable micelles based on poly(ethylene glycol)-SS-poly(ϵ -caprolactone) (PEG-SS-PCL) or dextran-SS-PCL copolymers efficiently released DOX under a reductive condition containing 10 mM dithiothreitol (DTT) in phosphate buffer at pH 7.4 and achieved significantly enhanced anticancer efficacy [28, 29]. This reduction-sensitive shell-shedding approach has been adopted by several different groups to boost the intracellular drug release from various micellar anticancer drugs [30-36]. The highly hydrophobic and slow degradation nature of core-forming polyesters like PCL, however, might hamper quick and complete drug release inside the cancer cells.

In this study, we report on novel redox and pH-responsive micellar nanoparticles based on

PEG-SS-PTMBPEC block copolymer for dually-triggered intracellular release of DOX in cancer cells (Scheme 5.1). It was assumed that DOX-loaded PEG-SS-PTMBPEC micelles, like DOX-loaded PEG-PTMBPEC counterparts, following endocytosis would result in partial release of DOX in mildly acidic endosomal compartments due to the hydrolysis of acetal bonds. Once DOX-loaded PEG-SS-PTMBPEC micelles escaped from endosomes, cleavage of the intervening disulfide bonds in response to a high GSH concentration would lead to further thorough release of DOX into the cytosol. Notably, core-forming PTMBPEC block following partial acetal hydrolysis might facilitate reduction-triggered drug release inside the cells owing to improved hydrophilicity of the micellar core. It should be noted that we and other groups have reported pH and reduction dual-sensitive nanoparticles based on polybases with low pKa such as poly(2-(diethyl amino)ethyl methacrylate) that become water soluble at low pH instead of pH induced degradation[37-39]. In this paper, the synthesis, *in vitro* drug release and antitumor activity of DOX-loaded PEG-SS-PTMBPEC micellar nanoparticles were investigated and the results were compared with those obtained using DOX-loaded PEG-PTMBPEC counterparts.

Scheme 5.1. Illustration of reduction and pH-sensitive biodegradable micelles based on PEG-SS-PTMBPEC copolymer for dually activated intracellular release of anticancer drugs.

5.2 Experimental Section

Materials. Dicyclohexyl carbodiimide (DCC, 99 %, Alfa Aesar), N-hydroxysuccinimide (NHS, 98 %, Alfa Aesar), cystamine dihydrochloride (98 %, Alfa Aesar), glutathione (GSH, 99%, Roche), *p*-nitrophenyl chloroformate (NPC, 97%, Alfa Aesar), cystamine dihydrochloride (cystamine-2HCl, >98%, Alfa Aesar),

zinc bis[bis(trimethylsilyl)amide] (97 %, Aldrich), and doxorubicin hydrochloride (DOX·HCl, 99 %, Beijing Zhong Shuo Pharmaceutical Technology Development Co.Ltd.) were used as received. Allyl alcohol (98%, Alfa Aesar) was distilled under reduced pressure prior to use. 2,2'-Azobisisobutyronitrile (AIBN, 98 %, J&K) was re-crystallized twice from hexane and methanol, respectively. Methoxy poly(ethylene glycol) (PEG, $M_n = 5.0$ kg/mol) was purchased from Fluka and dried by azeotropic distillation from anhydrous toluene. Dichloromethane (DCM) was dried by refluxing over CaH₂ under an argon atmosphere. Mono-2,4,6-trimethoxybenzylidene -pentaerythritol carbonate (TMBPEC) was synthesized according to our previous report [23].

Synthesis of PEG-Cystamine (PEG-Cys). PEG-Cys was obtained as reported previously [40]. Briefly, to a solution of cystamine·2HCl (1.8 g, 8 mmol) and Et₃N (2.02 g, 20 mmol) in DMSO (15 mL) at room temperature (r.t.) was dropwise added a DMSO solution of PEG-NPC (2.0 g, 0.4 mmol) that was prepared by treating PEG ($M_n = 5.0$ kg/mol) with NPC in DCM. The reaction mixture was stirred at r.t. for 20 h. PEG-Cys was isolated by extensive dialysis against water (MWCO 1000) followed by lyophilization. Yield: 92%. ¹H NMR (400 MHz, CDCl₃): δ 2.95 (-CH₂-SS-CH₂-), 3.19 (-CH₂NH₂), 3.38 (-OCH₃), 3.56 (-CH₂-NH-C(O)-), 3.64 (PEG), 4.23 (-CH₂-O-C(O)-).

Synthesis of Allyl-PTMBPEC. In the glove-box under a nitrogen atmosphere, to a stirred solution of TMBPEC (0.50 g, 1.47 mmol) and allyl alcohol (5.8 mg, 0.1 mmol) in DCM (4.0 mL) was quickly added zinc bis[bis(trimethylsilyl)amide] (20 mg, 0.05 mmol). The reaction vessel was sealed and placed in an oil-bath thermostated at 50 °C. The polymerization was allowed to proceed with magnetic stirring for 4 days. The resulting Allyl-PTMBPEC polymer was isolated by twice precipitation from cold diethyl ether and dried at r.t. *in vacuo*. Yield: 80 %. ¹H NMR (400 MHz, CDCl₃): δ 3.77-3.82 (Ar-OCH₃), 4.10 (-CH(OCH₂)₂), 4.63 (allyl-CH₂-), 4.74 (-O)C-O-CH₂CCH₂-O-C(O)-, 5.30 (vinyl protons), 5.98 (Ar-CH-), 6.08 (aromatic protons).

Synthesis of HOOC-PTMBPEC. Under a N₂ atmosphere, to a solution of Allyl-PTMBPEC (0.40 g, 0.1 mmol) in THF (10 mL) was added 3-mercaptopropanoic acid (0.212 g, 2 mmol) and AIBN (0.082 g, 0.5 mmol). The mixture was stirred for 24 h at 65 °C. The resulting polymer was isolated by precipitation from diethyl ether and dried at r.t. *in vacuo*. Yield: 95 %. ¹H NMR (400 MHz, CDCl₃): δ 1.95 (-CH₂-CH₂-O-C(O)-), 2.76 (-CH₂-COOH), 2.95 (-CH₂-S-CH₂-), 4.25 (-CH₂-CH₂-O-C(O)-).

Synthesis of amphiphilic PEG-SS-PTMBPEC block copolymer. Under a N₂ atmosphere, to a stirred solution of HOOC-PTMBPEC (70 mg, 17 μmol) and NHS (8.0 mg, 70 μmol) in DCM (10 mL) in an ice bath was dropwise added a DCC (21 mg, 102 μmol) solution in DCM. The reaction was allowed to proceed at r.t. for 20 h. Then, a solution of PEG-Cys (70 mg, 14 μmol) in DCM was added. The reaction was continued for another 28 h at r.t.. The polymer was isolated by precipitation from cold diethyl ether and washed with ethanol for five times. Finally, PEG-SS-PTMBPEC block copolymer was dried at r.t. *in vacuo*. Yield: 95 %. ¹H NMR (400 MHz, CDCl₃): δ, 2.80~3.0 (-CH₂-SS-CH₂-, -CH₂-S-CH₂-), 3.64 (PEG), 3.77-3.82 (Ar-OCH₃), 4.10 (-CH(OCH₂)₂), 4.74 (-O)C-O-CH₂CCH₂-O-C(O)-, 5.98 (Ar-CH-), 6.08 (aromatic protons).

Characterization. ¹H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at

400 MHz using deuterated chloroform (CDCl_3) as a solvent. The chemical shifts were calibrated against residual solvent signals of CDCl_3 . The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using THF as the eluent at a flow rate of 1.0 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The size of the micelles was determined by dynamic light scattering (DLS) at 25 °C using a Zetasizer Nano-ZS (Malvern Instruments) equipped with a 633 nm He-Ne laser. The micelle suspensions were filtered through a 450 nm syringe filter before measurements. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of a 0.2 mg/mL suspension of the micelles on the copper grid followed by staining with phosphotungstic acid.

Micelle formation and critical micelle concentration (CMC). Micelles were prepared under stirring by dropwise adding 2.0 mL of phosphate buffer (PB, 10 mM, pH 7.4) to 0.20 mL of block copolymer solution in THF (0.5 wt.%) at r.t.. The resulting suspension was stirred overnight under reduced pressure to thoroughly remove THF.

CMC was determined using pyrene as a fluorescence probe. The concentration of block copolymer varied from 2.0×10^{-5} to 0.2 mg/mL and the concentration of pyrene was fixed at 1.0 μM . Fluorescence spectra were recorded using a FLS920 fluorescence spectrometer and an excitation wavelength of 330 nm. Fluorescence emissions at 372 and 383 nm were monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

pH-Dependent hydrolysis of acetal groups in the micelles. The hydrolysis of acetal groups in PEG-SS-PTMBPEC micelles was followed by UV/vis spectroscopy by measuring the absorbance at 290 nm, according to our previous reports [22, 23]. PEG-SS-PTMBPEC micelle suspension (0.2 mg/mL) prepared as described above was divided into four aliquots of 2.0 mL, which were adjusted into four different conditions: *i.e.* (i) PB buffer (100 mM, pH 7.4), (ii) acetate buffer (100 mM, pH 5.0), (iii) PB buffer containing 10 mM GSH (100 mM, pH 7.4), and (iv) acetate buffer containing 10 mM GSH (100 mM, pH 5.0). The suspensions were shaken at 37 °C. At the desired time intervals, 80 μL aliquots were removed and diluted with 3.5 mL PB buffer (100 mM, pH 7.4) and the absorbance at 290 nm was measured. At the end, all the samples were completely hydrolyzed by the addition of two drops of concentrated HCl and measured again to determine the absorbance at 100 % hydrolysis, which was used to calculate the extent of acetal hydrolysis.

pH and redox-triggered change of micelle sizes. The change of micelle sizes in response to pH and/or redox was followed by DLS measurements at 37 °C. PEG-SS-PTMBPEC micelle suspension (0.2 mg/mL) was divided into four aliquots of 1 mL, which were adjusted into four different conditions: *i.e.* (i) PB buffer (100 mM, pH 7.4), (ii) acetate buffer (100 mM, pH 5.0), (iii) PB buffer containing 10 mM GSH (100 mM, pH 7.4), and (iv) acetate buffer containing 10 mM GSH (100 mM, pH 5.0). The suspensions were gently stirred at 37 °C and micelle sizes were monitored in time by DLS. PEG-PTMBPEC micelles were used as a reduction-insensitive control.

Loading and release of DOX. DOX-loaded micelles were prepared by dropwise adding 5.0 mL of PB buffer (10 mM, pH 7.4) to a mixture of 0.5 mL of copolymer solution in DMF (5.0 mg/mL) and 25, 50 or 100 μ L of DOX solution in DMSO (5.0 mg/mL) under stirring at r.t. followed by ultrasonication for 0.5 h and extensive dialysis against PB buffer (10 mM, pH 7.4) (MWCO 3500) at r.t. in the dark.

The *in vitro* release of DOX from PEG-SS-PTMBPEC micelles was investigated at 37 °C under five different conditions, *i.e.* (i) PB buffer (100 mM, pH 7.4), (ii) acetate buffer (100 mM, pH 5.0), (iii) PB buffer containing 10 mM GSH (100 mM, pH 7.4), and (iv) acetate buffer containing 10 mM GSH (100 mM, pH 5.0), and (v) 2 or 4 h in acetate buffer (100 mM, pH 5.0) followed by PB buffer containing 10 mM GSH (100 mM, pH 7.4). DOX-loaded micelle suspension was divided into six aliquots and immediately transferred to a dialysis tube with a MWCO of 12000-14000. The dialysis tube was immersed into 20 mL of appropriate buffer (0.1 M) and shaken at 37 °C. At desired time intervals, 5.0 mL of the release medium was taken out and replenished with an equal volume of fresh medium. To avoid oxidation of GSH, the release media were perfused with nitrogen gas. The concentration of DOX was determined by fluorescence (FLS920) measurements (excitation at 480 nm).

To determine drug loading content (DLC), DOX-loaded micelle suspensions were freeze-dried, dissolved in DMSO and analyzed with fluorescence spectroscopy. A calibration curve was obtained using DOX/DMSO solutions with different DOX concentrations. To determine the amount of DOX released, calibration curves were run with DOX/corresponding buffer solutions with different DOX concentrations at pH 5.0 and 7.4, respectively. The emission at 600 nm was recorded. The release experiments were conducted in triplicate. The results are presented as the average $\pm$ SD. The DLC and drug loading efficiency (DLE) were calculated according to the following formula:

$$\text{DLC (wt. \%)} = (\text{weight of loaded drug} / \text{total weight of polymer and loaded drug}) \times 100 \%$$

$$\text{DLE (\%)} = (\text{weight of loaded drug} / \text{weight of drug in feed}) \times 100\%$$

MTT assay. The cytotoxicity of empty and DOX-loaded PEG-SS-PTMBPEC micelles and PEG-PTMBPEC micelles was studied by the MTT assay using HeLa and RAW 264.7 cells. Cells were seeded onto a 96-well plate at a density of 1×10^4 cells per well in 100 μ L of Dulbecco's Modified Eagle medium (DMEM) containing 10% FBS and incubated for 24 h (37 °C, 5% CO₂). The medium was removed and replenished by 80 μ L of fresh DMEM medium containing 10% FBS. 20 μ L of micelle suspensions at different concentrations in PB buffer (10 mM, pH 7.4) were added. The cells were incubated for another 48 h, the medium was aspirated and replaced by 100 μ L of fresh medium, and 10 μ L of MTT solution (5 mg/mL) was added. The cells were incubated for 4 h, and then 100 μ L of DMSO was added to dissolve the resulting purple crystals. The optical densities at 570 nm were measured using a BioTek microplate reader. Cells cultured in DMEM medium containing 10% FBS (without micelles) were used as controls.

Confocal microscopy observation of HeLa cells incubated with DOX-loaded micelles. HeLa cells were plated on microscope slides in a 24-well plate (5×10^4 cells/well) using DMEM medium containing 10 % FBS. The cells were incubated with prescribed amounts of DOX-loaded micelles or free DOX at 37 °C and 5 % CO₂. After incubation for 4 and 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% paraformaldehyde and the cell nuclei were stained with Hoechst 33342. Fluorescence images of cells were obtained with a Nikon Digital Eclipse C1si Confocal Laser Scanning Microscope (Nikon).

5.3 Results and Discussion

The present study was set to develop reduction and pH dual-bioresponsive biodegradable micelles and to investigate their intracellular anti-cancer drug release behavior in cancer cells. To this end, PEG-SS-PTMBPEC block copolymer micelles were designed, in which pendant acetal bonds in the core-forming PTMBPEC block are prone to hydrolysis under endosomal pH conditions while the intermediate disulfide bond linking hydrophilic PEG and hydrophobic PTMBPEC blocks is subject to reductive degradation in the cytosol due to its high reducing potential. We hypothesized that these micellar nanoparticles upon endocytosis would swell due to acetal hydrolysis, triggering partial drug release in endosomes. The swollen nanoparticles following further trafficking to the cytoplasm would rapidly collapse leading to complete release of payloads. Thus, reduction and pH dual-bioresponsive biodegradable micelles might induce synergistic effects in enhancing drug release and anti-tumor activity in target cells.

5.3.1 Synthesis of amphiphilic PEG-SS-PTMBPEC copolymer

PEG-SS-PTMBPEC block copolymer was prepared by the coupling reaction between PEG cystamine (PEG-Cys) and carboxyl PTMBPEC (HOOC-PTMBPEC) using carbodiimide chemistry (Scheme 5.2). PEG-Cys was synthesized by activating the hydroxyl end group of PEG ($M_n = 5.0$ kg/mol) with *p*-nitrophenyl chloroformate (NPC) followed by treatment with an excess of cystamine in DMSO (Scheme 5.3A). ^1H NMR indicated quantitative transformation of the hydroxyl end by cystamine, as revealed by an integral ratio of close to 4:3 between signals at δ 2.95 (methylene protons of cystamine moiety neighboring to the disulfide bond) and 3.38 (methoxy protons of PEG) (Figure 5.1). HOOC-PTMBPEC was obtained by ring-opening polymerization of TMBPEC using allyl alcohol as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst followed by conjugation with 3-mercaptopropanoic acid in the presence of AIBN in THF (Scheme 5.3B). ^1H NMR showed that PTMBPEC with an allyl functional group was successfully synthesized, as revealed by presence of signals at δ 5.30 and 4.6 attributable to vinyl protons and ethylene protons of allyl moieties, respectively (Figure 5.2A). ^1H NMR end group analysis suggested that allyl-PTMBPEC had a M_n of 4.1 kg/mol, which was close to the theoretical value (4.3 kg/mol). Figure 5.2B shows that the allyl groups were quantitatively converted into carboxylic groups following treatment with 3-mercaptopropanoic acid as revealed by the complete disappearance of vinyl protons and detection of new signals at δ 2.95 and 2.76 assignable to the methylene protons next to the thioether and methylene protons neighboring to the carboxylic groups, respectively.

Scheme 5.2. Synthesis of PEG-SS-PTMBPEC by the coupling reaction between PEG-Cys and HOOC-PTMBPEC in DCM at r.t. for 48 h in the presence of DCC and NHS.

Scheme 5.3. Synthesis of PEG-Cys (A) and HOOC-PTMBPEC (B). Conditions: (i) *p*-nitrophenyl chloroformate, pyridine, DCM, 0 °C; (ii) cysteamine dihydrochloride, Et₃N, DMSO, r.t.; (iii) zinc bis[bis(trimethylsilyl)amide], DCM, 50 °C; and (iv) 3-mercaptopropionic acid, AIBN, DMF, 65 °C.

Table 5.1. Synthesis of PEG-SS-PTMBPEC and PEG-PTMBPEC block copolymers.

Copolymer	Conv. ^a (%)	<i>M_n</i> (kg/mol)			PDI (GPC ^b)	CMC ^c (mg/L)
		Design	¹ H NMR	GPC ^b		
PEG-SS-PTMBPEC	86	5.0-5.0	5.0-4.1	12.8	1.16	0.81
PEG-PTMBPEC	84	5.0-5.0	5.0-3.9	12.3	1.09	0.64

^a Determined by ¹H NMR; ^b Determined by GPC (eluent: DMF containing 1 wt.% LiBr, flow rate: 0.8 mL/min, standards: polystyrene); ^c Critical micelle concentration determined using pyrene as a fluorescent probe.

Figure 5.1. ^1H NMR spectrum (400 MHz, CDCl_3) of PEG-Cys.

The coupling reaction of PEG-Cys and HOOC-PTMBPEC was carried out in DCM at r.t., in the presence of DCC and NHS. The resulting PEG-SS-PTMBPEC block copolymer was isolated by precipitation in diethyl ether followed by extensive washing with ethanol to remove excess PEG. The results of the synthesis are given in Table 5.1. ^1H NMR clearly showed signals characteristic of both PEG and PTMBPEC blocks (Figure 5.2C). The integral ratio between resonances at δ 5.98 (acetal protons of PTMBPEC) and 3.38 (methoxy protons of PEG) was close to the theoretical value of 4:1, indicating equivalent coupling of PEG and PTMBPEC. Importantly, gel permeation chromatography (GPC) measurements revealed a unimodal distribution with an M_n of 12.8 kg/mol (polystyrene standards) and a low polydispersity index (PDI) of 1.16 (Table 5.1), confirming successful synthesis of well-defined PEG-SS-PTMBPEC block copolymer.

PEG-PTMBPEC block copolymer, which was used as a reduction-insensitive control, was synthesized according to our previous report [23], using methoxy PEG-OH ($M_n = 5.0$ kg/mol) as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst in CH_2Cl_2 at 50°C . The results showed that PEG-PTMBPEC had a low PDI of 1.09 and a M_n of 5.0-3.9 kg/mol, close to that of PEG-SS-PTMBPEC (Table 5.1).

Figure 5.2. ^1H NMR spectra (400 MHz, CDCl_3) of allyl-PTMBPEC (A), HOOC-PTMBPEC (B), and PEG-SS-PTMBPEC (C).

5.3.2 Micelle formation and pH-triggered acetal hydrolysis

Micelles were prepared by the solvent exchange method. Dynamic light scattering (DLS) measurements showed that PEG-SS-PTMBPEC formed micellar nanoparticles with an average diameter of ca. 140 nm and a narrow size distribution in phosphate buffer (PB, 10 mM, pH 7.4) (Figure 5.3A). TEM revealed a homogeneous distribution of spherical nanoparticles with sizes in accordance with those determined by DLS (Figure 5.3B). The critical micelle concentration (CMC) was determined to be 0.81 and 0.64 mg/L for PEG-SS-PTMBPEC and PEG-PTMBPEC block copolymers, respectively, by fluorescence measurements using pyrene as a probe (Table 5.1).

The hydrolysis of acetals in PEG-SS-PTMBPEC micelles was studied at 37 °C at pH 7.4 or pH 5.0, either in the absence or presence of 10 mM GSH. The extent of acetal hydrolysis was determined according to our previous reports [22, 23], using UV/vis spectroscopy by monitoring the absorbance at 290 nm, which is the characteristic absorbance of the hydrolysis product, 2,4,6-trimethoxybenzaldehyde. The results showed that the hydrolysis rate of acetals in PEG-SS-PTMBPEC micelles was highly pH-dependant (Figure 5.4). Rapid hydrolysis took place at pH 5.0 with a half-life of 6.5 h, while negligible hydrolysis

(less than 10 %) was observed at pH 7.4 even after 24 h. It should be noted that a similar acetal half-life of 6.0 h was observed for reduction-insensitive PEG-PTMBPEC micelles at pH 5.0 [23], indicating that introduction of disulfide bond has little influence on pH-sensitive degradation of acetals in micelles. The results showed that presence of GSH (10 mM) had little effect on the rate of acetal hydrolysis in micelles (Figure 5.4), implying that PEG shedding caused by cleavage of the disulfide bonds does not alter acetal hydrolysis kinetics.

Figure 5.3 Size distribution of PEG-SS-PTMBPEC micelles determined by DLS (A) and TEM after staining with phosphotungstic acid (B).

Figure 5.4 pH-dependant hydrolysis of acetals in PEG-SS-PTMBPEC micelles with or without 10 mM GSH.

5.3.3 Redox and pH-induced size change of PEG-SS-PTMBPEC micelles

The size change of PEG-SS-PTMBPEC micelles in response to acidic pH and 10 mM GSH was studied by DLS. The results showed that PEG-SS-PTMBPEC micelles swelled from 140 nm to about 300 nm in 6 h and to over 700 nm in 16 h at pH 5.0 (Figure 5.5A), which was similar to that observed for

PEG-PTMBPEC micelles (Figure 5.6A). This swelling of micelles is due to increased hydrophilicity of the micellar core as a result of acetal hydrolysis [22, 23]. In contrast, no change of micelle size was observed over 24 h at pH 7.4. Interestingly, significant change of micelle sizes was also observed for PEG-SS-PTMBPEC micelles at pH 7.4 in the presence of 10 mM GSH, wherein micelle sizes increased from 140 nm to 450 nm in 6 h and to over 1000 nm in 16 h (Figure 5.5B). This reduction induced fast aggregation behavior has been reported for other reduction-sensitive shell-sheddable micelles such as PEG-SS-PCL and dextran-SS-PCL micelles [28, 29]. In comparison, no obvious size change was discerned for reduction-insensitive PEG-PTMBPEC micelles in 16 h under otherwise the same conditions (Figure 5.6B).

Figure 5.5. pH and redox-induced size change of PEG-SS-PTMBPEC micelles in time followed by DLS. (A) pH 5.0; (B) pH 7.4.

Figure 5.6. Size change of PEG-PTMBPEC micelles in response to acidic pH (pH 5.0) and reductive environment (10 mM GSH) followed by DLS. (A) pH 5.0 with or without 10 mM GSH; (B) pH 7.4 with or without 10 mM GSH.

It should be noted that the fastest size change of PEG-SS-PTMBPEC micelles was observed at pH 5.0 in the presence of 10 mM GSH, in which large aggregates with a diameter over 1000 nm were formed in 6 h (Figure 5.5A). These results indicate that redox and pH dual-sensitive micelles likely induce synergistic effects for intracellular drug release. It should further be noted that no precipitation was observed under these conditions, most probably due to enhanced hydrophilicity of core-forming PTMBPEC following acetal hydrolysis [41].

5.3.4 Loading and *in vitro* release of DOX

DOX is a potent anticancer drug applied for the treatment of varying types of solid malignant tumors in the clinic [42]. DOX was loaded into PEG-SS-PTMBPEC and PEG-PTMBPEC micelles at theoretical drug loading contents (DLC) of 4.8, 9.1 and 16.7 wt.%. The results showed similar drug loading levels for PEG-SS-PTMBPEC and PEG-PTMBPEC micelles, with drug loading efficiencies (DLE) in the range of 58.4 to 66.6 % (Table 5.2). The average diameters of PEG-SS-PTMBPEC micellar particles decreased from 140.2 to 130.3 nm following loading of 3.2 wt.% DOX, and then increased to 186.2 and 196.8 nm with further increasing DOX loading contents to 6.3 and 11.3 wt.%, respectively (Table 5.2). Analogous trends were also observed for DOX-loaded PEG-PTMBPEC micelles (Table 5.2).

Table 5.2. Characteristics of DOX-loaded PEG-SS-PTMBPEC and PEG-PTMBPEC micelles.

Copolymer	DLC (wt.%)		DLE (%)	Size (nm)	PDI
	theory	determined ^a			
PEG-SS-PTMBPEC	4.8	3.2	66.2	130.3 ± 0.2	0.12
	9.1	6.3	66.6	186.2 ± 0.3	0.15
	16.7	11.3	64.2	196.8 ± 1.2	0.25
PEG-PTMBPEC	4.8	3.1	64.3	132.5 ± 0.7	0.21
	9.1	6.0	62.5	154.2 ± 0.6	0.28
	16.7	10.5	58.4	180.6 ± 1.6	0.30

^a DOX loading content determined by fluorescence measurements.

The *in vitro* release of DOX from PEG-SS-PTMBPEC micelles was investigated at 37 °C under different conditions, *i.e.* (i) pH 7.4, (ii) pH 5.0, (iii) pH 7.4 and 10 mM GSH, (iv) pH 5.0 and 10 mM GSH, and (v) 2 or 4 h at pH 5.0 followed by pH 7.4 and 10 mM GSH (mimicking the intracellular trafficking pathway). The results showed that at physiological pH only ca. 24 % DOX was released from DOX-loaded micelles in 21 h (Figure 5.7A). The release of DOX was significantly accelerated at pH 5.0 with 62.8 % of DOX released in 21 h under otherwise the same conditions, likely due to pH-induced acetal hydrolysis [23]. The release of DOX was also boosted under a reducing environment containing 10 mM GSH at pH 7.4, in which 74.3 % of drug was released in 21 h. This redox-triggered drug release behavior has been reported for shell-sheddable PEG-SS-PCL and dextran-SS-PCL micelles [28, 29]. Notably, the fastest and most

complete drug release was observed at pH 5.0 in the presence of 10 mM GSH wherein 94.2 % of DOX was released in 10 h (Figure 5.7A). This is in line with the observation that PEG-SS-PTMBPEC micelles were rapidly destabilized under pH 5.0 and 10 mM GSH condition (Figure 5.5A). In contrast, DOX release from reduction-insensitive PEG-PTMBPEC micelles was not influenced by presence of GSH both at pH 5.0 and pH 7.4 (Figure 5.8). In order to simulate the intracellular trafficking process, drug release studies were performed at pH 5.0 (mimicking the acidic endosomal compartments) for 2 or 4 h and then at pH 7.4 and 10 mM GSH condition (mimicking the reductive environment of cytosols), which has interestingly resulted in faster and more thorough DOX release from PEG-SS-PTMBPEC micelles (ca. 91.5% of DOX released in a total time of 21 h) than at either pH 5.0 or pH 7.4 and 10 mM GSH condition (Figure 5.7B). These results clearly indicate that redox and pH dual-sensitive degradable micelles present synergistic effects in drug release. This is probably because partial hydrolysis of acetals in the micellar core in response to acidic pH not only promotes partial DOX release but also improves the hydrophilicity of the micellar core that further facilitates ensuing reduction-triggered drug release.

Figure 5.7. pH and/or redox-triggered release of DOX from PEG-SS-PTMBPEC micelles at 37 °C. (A) DOX release at pH 7.4 or 5.0 in the presence or absence of 10 mM GSH; and (B) DOX release at pH 5.0 for 2 or 4 h followed by pH 7.4 and 10 mM GSH (mimicking the intracellular trafficking pathway).

Figure 5.8. *In vitro* release of DOX from PEG-PTMBPEC micelles under different conditions at 37 °C.

5.3.5 Intracellular DOX release and antitumor activity

Figure 5.9. CLSM images of HeLa cells following 4 or 8 h incubation with DOX-loaded micelles and free DOX (30 $\mu\text{g/mL}$). For each panel, the images from left to right showed DOX fluorescence in cells (red), cell nuclei stained by Hoechst 33342 (blue), and overlays of both images. The scale bars correspond to 20 μm in all the images. (A) DOX-loaded PEG-SS-PTMBPEC micelles, 4 h; (B) DOX-loaded PEG-SS-PTMBPEC micelles, 8 h; (C) DOX-loaded PEG-PTMBPEC micelles, 4 h; (D) DOX-loaded PEG-PTMBPEC micelles, 8 h; and (E) free DOX, 8 h.

The cellular uptake and intracellular drug release profiles of DOX-loaded PEG-SS-PTMBPEC micelles were investigated by CLSM using HeLa cells. Notably, strong DOX fluorescence was observed in the cytoplasm and nuclei of HeLa cells following 4 h incubation with DOX-loaded PEG-SS-PTMBPEC micelles (Figure 5.9A). The DOX fluorescence became even stronger in the cell nuclei at a longer

incubation time of 8 h (Figure 5.9B), which was similar to that observed for HeLa cells following 8 h incubation with free DOX (control experiments) (Figure 5.9E). The uptake of DOX by the cell nuclei is crucial because DOX has to intercalate with DNA to induce cell death [43]. In contrast, little DOX fluorescence was observed for HeLa cells incubated for 4 h with DOX-loaded PEG-PTMBPEC micelles (reduction-insensitive control) under otherwise the same conditions (Figure 5.9C). The DOX fluorescence though enhanced after 8 h incubation was found mainly in the perinuclei region of cells (Figure 5.9D). The different intracellular drug release behavior of PEG-SS-PTMBPEC and PEG-PTMBPEC micelles is most likely due to fast DOX release from PEG-SS-PTMBPEC micelles after escape from endosomes as a result of reduction-triggered shell-shedding in the cytosol. In comparison, drug release from PEG-PTMBPEC micelles would be slow in the cytosol (neutral pH), as indicated in Figure 5.8. These results have demonstrated that pH and reduction dual-responsive PEG-SS-PTMBPEC micelles mediate more efficient intracellular anticancer drug release than PEG-PTMBPEC micelles.

MTT assays using HeLa and RAW 264.7 cells revealed that PEG-SS-PTMBPEC and PEG-PTMBPEC micelles were practically non-toxic (cell viabilities $\geq 92\%$) up to a tested concentration of 1.0 mg/mL (Figure 5.10), confirming that these degradable micellar particles have good biocompatibility. DOX-loaded PEG-SS-PTMBPEC micelles and DOX-loaded PEG-PTMBPEC micelles, however, displayed significant anti-tumor activity towards HeLa and RAW 264.7 cells following 48 h incubation (Figure 5.11). It should be noted that DOX-loaded PEG-SS-PTMBPEC micelles had low IC_{50} (half inhibitory concentration) values of 0.75 and 0.60 $\mu\text{g DOX equiv./mL}$ for HeLa and RAW 264.7 cells, respectively, which were lower than those obtained with the reduction-insensitive counterparts under otherwise the same conditions ($IC_{50} = 1.38$ and $0.94 \mu\text{g DOX equiv./mL}$ for HeLa and RAW 264.7 cells, respectively). This higher anti-tumor activity of PEG-SS-PTMBPEC micelles is in accordance with the confocal microscopy observations that PEG-SS-PTMBPEC micelles mediate fast intracellular drug release as compared to corresponding PEG-PTMBPEC micelles. Interestingly, the anti-tumor activity of DOX-loaded PEG-SS-PTMBPEC micelles, which was approaching that of free DOX ($IC_{50} = 0.46$ and $0.42 \mu\text{g DOX equiv./mL}$ for HeLa and RAW 264.7 cells, respectively), was much higher than that reported for other DOX-loaded degradable block copolymer micelles (27 $\mu\text{g/mL}$ [44], 15 $\mu\text{g/mL}$ [45], 3.2 $\mu\text{g/mL}$ [46]). It should further be noted that the anti-tumor activity of DOX-loaded PEG-SS-PTMBPEC micelles might be further enhanced by installing a targeting ligand such as folic acid, aptamer, peptide, and antibody fragment that facilitates efficient and specific cellular uptake of micelles. These redox and pH-sensitive degradable micelles with dually activated intracellular drug release properties are highly promising for targeted cancer therapy.

Figure 5.10. Cytotoxicity of PEG-SS-PTMBPEC and PEG-PTMBPEC micelles. (A) HeLa cells; (B) RAW 264.7 cells. The cells were incubated with micelles for 48 h. Data are presented as the average $\pm$ standard deviation ($n = 4$).

Figure 5.11. Anti-tumor activity of DOX-loaded PEG-SS-PTMBPEC micelles, DOX-loaded PEG-PTMBPEC micelles and free DOX as a function of DOX dosages. (A) HeLa cells; (B) RAW 264.7 cells. The cells were incubated with DOX-loaded micelles or free DOX for 48 h. Data are presented as the average $\pm$ standard deviation ($n = 4$).

5.4 Conclusions

We have demonstrated that reduction and pH dual-responsive degradable micelles based on PEG-SS-PTMBPEC copolymer efficiently deliver and release doxorubicin into cancer cells, resulting in superior anti-tumor activity. This represents a first design and study of degradable block copolymer micelles that respond to endosomal pH as well as cytoplasmic glutathione for enhanced intracellular anticancer drug release. These intelligent micelles have several interesting features: (i) they have low cytotoxicity and are degradable; (ii) they show decent drug loading levels; (iii) they are sufficiently stable with low drug release (only about 24 % drug release in 21 h) under physiological conditions (pH 7.4, 37

°C); and (iv) they promote fast and maximum drug release inside the cancer cells in that drug release is activated during the whole intracellular trafficking process, *i.e.* not only in the mildly acidic endosomal compartments but also under the highly reducing cytoplasms and cell nuclei. These reduction and pH dual-responsive biodegradable micelles provide an interesting platform for targeted and controlled intracellular release of potent chemotherapeutics.

5.5 References

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Chapter 6

Reversibly Core-Crosslinked pH-Sensitive Biodegradable Micelles for Dually Triggered Intracellular Release of DOX*

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Abstract

It is still a major challenge to design stable extracellular micellar drug delivery systems which show a rapid intracellular drug release. Here, reduction-sensitive reversibly core-crosslinked pH-responsive biodegradable micelles were developed based on poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate-*co*-pyridyl disulfide carbonate) (PEG-SS-P(TMBPEC-*co*-PDSC)) copolymers and investigated for intracellular doxorubicin (DOX) release. Micelles self-assembled from PEG-P(TMBPEC-*co*-PDSC) copolymer had an average diameter of 58.6 nm and were readily crosslinked by disulfide bond formation via the PDSC units after treatment with half the stoichiometrical amount of dithiothreitol (DTT). DOX was loaded into both crosslinked and non-crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles with a drug loading content of ~12.0 wt.%. *In vitro* release studies showed that under physiological conditions only ca. 19.9 % DOX was released from the reversibly crosslinked micelles in 24 h at a relatively low micelle concentration of 40 µg/mL. The release of DOX was accelerated at pH 5.0 or in the presence of 10 mM glutathione (GSH) at pH 7.4, in which 64.2 % and 44.1 % of DOX was released, respectively, in 24 h. The drug release was further boosted at pH 5.0 and 10 mM GSH, with 98.8 % of DOX released in 12 h. Notably, DOX release was also facilitated by 4 h incubation at pH 5.0 followed by incubation at pH 7.4 with 10 mM GSH, mimicking the intracellular conditions during the uptake of the micellar drugs via endocytosis. Confocal microscopy indicated that DOX was delivered and released into the nuclei of HeLa cells following 12 h incubation with DOX-loaded reversibly crosslinked micelles, while DOX was mainly located in the cytoplasm when irreversibly crosslinked poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate-*co*-acryloyl carbonate) (PEG-P(TMBPEC-*co*-AC)) control micelles were used. MTT assays revealed that DOX-loaded reversibly crosslinked micelles had much higher tumor cell killing activity than irreversibly crosslinked controls, with low IC₅₀ values of 1.65 and 1.14 µg/mL for HeLa and RAW 264.7 cells, respectively, following 48 h incubation. Both crosslinked and non-crosslinked micelles not loaded with DOX had a low cytotoxicity for both types of cells up to a concentration of 0.8 mg/mL. The reversibly crosslinked pH-sensitive biodegradable micelles with superior extracellular stability and dually-activated intracellular drug release provide a novel platform for tumor-targeting drug delivery.

6.1 Introduction

The past decade has witnessed an exploding development in biodegradable polymeric micelles for controlled delivery of hydrophobic anti-cancer drugs such as paclitaxel (PTX) and doxorubicin (DOX) [1-3]. The micelles have been shown to greatly improve drug water solubility as well as increase drug accumulation at the tumor sites via the enhanced permeability and retention (EPR) effect, resulting in better drug bioavailability and reduced side effects [4-6]. However, therapeutic effects of current micellar drugs are far from optimal due to the presence of several practical challenges including low drug loading levels, poor *in vivo* stability leading to premature drug release and reduced tumor targetability, and slow/incomplete drug release in tumor tissue and/or inside the tumor cells [7-9].

In recent years, stimulus-responsive nanosystems that release payloads in response to an intrinsic biological signal have been designed and explored for enhanced cancer therapy [10-13]. For example, pH-sensitive polymers containing various acid-labile groups such as ortho ester, hydrazone, cis-aconityl, and acetal have been widely studied for drug delivery applications, since the pH in the environment of tumor tissue is often 0.5-1.0 pH units lower than in normal tissue, whereas the pH in the intracellular endosomal/lysosomal compartments is only 4.0-6.5 [14]. Fréchet *et al.* reported that trimethoxybenzylidene acetals are rapidly hydrolysed at slightly acidic pH and that these entities can be incorporated in drug delivery systems for pH triggered drug release [15, 16]. We have designed poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PEG-PTMBPEC) block copolymers and prepared pH-responsive biodegradable micelles and polymersomes [17, 18]. More recently, we have prepared pH-sensitive degradable chimaeric polymersomes based on trimethoxybenzylidene acetals for high loading and triggered release of doxorubicin hydrochloride [19]. It should be however noted that self-assembled polymeric nanocarriers, often have inadequate *in vivo* stability, which leads to premature burst drug release following *i.v.* injection, resulting in drug loss not only during storage, but also in the blood circulation causing increased side effects [20, 21].

To improve the stability and to inhibit premature drug release, either the micellar core or the shell could be crosslinked. It has been demonstrated that crosslinked biodegradable micelles have advantageous properties like high drug loading efficiency, superior stability upon dilution, prolonged circulation time, and enhanced drug accumulation at the tumor site [22-24]. Recently, we have addressed the extracellular stability versus intracellular drug release dilemma of micellar anticancer drugs by designing amphiphilic biodegradable poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate-*co*-acryloyl carbonate) (PEG-*b*-P(TMBPEC-*co*-AC)) block copolymers to prepare photo-crosslinked pH-sensitive biodegradable micelles via the AC units [25]. It should however be noted that irreversible photo-crosslinking may induce incomplete drug release from crosslinked compartments, and also will generate non-degradable polyacrylate components in the carriers. In recent years, much attention has been given to the development of reversibly crosslinked polymeric micelles by which complete drug release can be obtained by de-crosslinking of the micelles via external stimuli [26]. Crosslinking via disulfide bonds, which are prone to cleavage in the intracellular environment due to the presence of a high reduction potential in the cytoplasm as well as in the cell nucleus, is an attractive approach to construct reversibly

crosslinked nanocarriers for triggered drug release [27, 28]. A substantial number of reversibly crosslinked micelle systems using disulfide bonds have been prepared using cystamine and its derivatives as the reducible crosslinkers [29-33]. The reduction-sensitive reversibly crosslinked nanosystems can be also produced by oxidizing free thiol groups in the shell-forming block or in the core-forming polyphosphoester, polycarbonate, polyacrylate, polypeptide and dendritic blocks [34, 35]. In our group, we have developed disulfide-crosslinked micelles by conjugation of lipoic acid which can be self-crosslinked by ring-opening polymerization with a catalytic amount of DTT [36-38]. In more recent years, self-crosslinking of pyridyl disulfide (PDS) units attached to polymers has emerged as a promising approach to develop reversibly crosslinked nanosystems. The PDS unit is highly reactive, but specific to thiols and provides disulfide-crosslinked structures by the thiol-disulfide exchange reaction under mild conditions [39-41]. For instance, Thayumanavan *et al.* developed reduction-sensitive self-crosslinked nanogels based on a random copolymer containing pendant oligoethyleneglycol and PDS side chains. The disulfide-crosslinked nanogels are of particular interest for intracellular protein release in that they on one hand possess superior colloidal stability and on the other hand are prone to rapid de-crosslinking and dissociation inside cells [42, 43].

In this study, we report on novel reversibly crosslinked pH-responsive micelles based on poly(ethylene glycol)-*b*-poly(mono-2,4,6-trimethoxy benzylidene-pentaerythritol carbonate-*co*-pyridyl disulfide carbonate) (PEG-P(TMBPEC-*co*-PDSC)) block copolymer for dually-triggered intracellular release of DOX in cancer cells (Scheme 6.1), in which the core-forming TMBPEC units are acid-labile components and PDS functionalities can be used to form reduction-sensitive core-crosslinked structures. We have already shown that dual activation of the intracellular release of DOX from micelles based on PEG-SS-PTMBPEC block copolymers worked synergistically in killing tumor cells [44]. In the current approach micelles were designed that can be stabilized by reversible crosslinking using PDSC units with minimal drug leakage during circulation while quickly and completely releasing payloads after arriving at the tumor site as well as in the intracellular environment. The synthesis, stability, *in vitro* drug release and tumor cell killing activity of DOX-loaded reversibly crosslinked PEG-(PTMBPEC-*co*-PDSC) micellar nanoparticles were investigated and the results were compared with those obtained using DOX-loaded irreversibly crosslinked poly(ethylene glycol)-*b*-poly(mono-2,4,6-trimethoxy benzylidene-pentaerythritol carbonate-*co*-acryloyl carbonate) (PEG-P(TMBPEC-*co*-AC)) micelles.

Scheme 6.1. Illustration of reversibly-crosslinked pH-sensitive biodegradable micelles for pH and reduction responsive intracellular release of DOX in tumor cells.

6. 2 Experimental Section

Materials. Glutathione (GSH, 99 %, Roche), zinc bis[bis(trimethylsilyl)amide] (97 %, Aldrich), doxorubicin hydrochloride (DOX·HCl, 99 %, Beijing Zhong Shuo Pharmaceutical Technology Development Co.Ltd.), 1,4-dithio-D,L-threitol (DTT, 99 %, Merck), and potassium persulfate (KPS, 99.5 %, Aladdin) were used as received. Methoxy poly(ethylene glycol) (PEG, $M_n = 5.0$ kg/mol) was purchased from Fluka and dried by azeotropic distillation from anhydrous toluene. Dichloromethane (DCM) was dried by refluxing over CaH_2 under an argon atmosphere. Mono-2,4,6-trimethoxybenzylidene-pentaerythritol carbonate (TMBPEC), pyridyl disulfide cyclic carbonate (PDSC), and PEG-P(TMBPEC-co-AC) copolymer were synthesized according to our previous reports [17, 25, 45].

Synthesis of PEG-P(TMBPEC-co-PDSC) copolymer. The ring-opening polymerization of TMBPEC and PDSC was carried out in DCM at 50 °C, using methoxy PEG as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst. Briefly, in the glove-box under a nitrogen atmosphere, to a stirred solution of PEG (250 mg, 50 μmol), TMBPEC (200 mg, 0.588 mmol) and PDSC (200 mg, 0.738 mmol) in DCM (3.0 mL) was quickly added zinc bis[bis(trimethylsilyl)amide] (10 mg, 26 μmol). The reaction vessel was sealed and placed in an oil-bath thermostated at 50 °C. The polymerization was allowed

to proceed with magnetic stirring for 4 days. The resulting polymer was isolated by twice precipitation from cold diethyl ether and dried at r.t. *in vacuo*. Yield: 78.2 %. ^1H NMR (400 MHz, CDCl_3): PEG: δ 3.38, 3.64; TMBPEC moieties: δ 3.82, 3.99, 4.75, 5.97, 6.08; PDSC moieties: δ 1.08, 2.92, 4.09, 7.64, 7.07, 8.45. M_n (^1H NMR) = 10.9 kg/mol, M_n (GPC) = 10.2 kg/mol, PDI = 1.34.

Characterization. ^1H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform (CDCl_3) as a solvent. The chemical shifts were calibrated against residual solvent signals of CDCl_3 . The average molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using CHCl_3 as the eluent at a flow rate of 0.5 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns.

Micelle formation and critical micelle concentration (CMC). Micelles were prepared by the solvent exchange method. Typically, under stirring 2.0 mL of phosphate buffer (PB, 10 mM, pH 7.4) was dropwise added into 0.20 mL of block copolymer solution in DMF (0.5 wt.%) at room temperature. The resulting suspension was dialyzed against PB (10 mM, pH 7.4) for 8 h to thoroughly remove DMF. The size of the micelles was determined by dynamic light scattering (DLS) at 25 °C using a Zetasizer Nano-ZS (Malvern Instruments) equipped with a 633 nm He-Ne laser. The micelle suspension was filtered through a 450 nm syringe filter before measurements. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of a 0.2 mg/mL suspension of the micelles on a copper grid followed by staining with phosphotungstic acid.

The CMC was determined using pyrene as a fluorescence probe. The concentration of block copolymer was varied from 2.0×10^{-5} to 0.5 mg/mL and the concentration of pyrene was fixed at 2.0 μM . Fluorescence spectra were recorded using a FLS920 fluorescence spectrometer and an excitation wavelength of 330 nm. Fluorescence emissions at 372 and 383 nm were monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

Reversible crosslinking of micelles. PEG-P(TMBPEC-*co*-PDSC) micelles were prepared as above-mentioned with a concentration of 0.50 mg/mL. The crosslinking of the micelles was accomplished by a simple thiol-disulfide exchange reaction. In brief, to 2 mL of this micellar solution (0.50 mg/mL, 0.92 μmol of pyridyl disulfide groups) was added DTT (0.46 μmol) solution in ethanol. The mixture was perfused with nitrogen for 10 min and stirred magnetically at 50 °C for 8 h. The crosslinking process was monitored by determining the amount of the pyridinethione byproduct using UV analysis [42]. Reversibly crosslinked micelles (RCMs) were isolated by dialysis against PB (10 mM, pH 7.4). The size distribution and stability of crosslinked micelles were studied using DLS.

pH and redox-triggered size change of crosslinked micelle. The change of the size of the crosslinked micelles in response to pH and/or redox was followed by DLS measurements at 37 °C. Reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelle suspension (0.50 mg/mL) prepared as

above-mentioned was diluted to a micellar concentration of 40 µg/mL with PB (10 mM, pH 7.4), and then divided into four aliquots of 1 mL, which were adjusted to four different conditions: *i.e.* (i) PB (100 mM, pH 7.4), (ii) acetate buffer (100 mM, pH 5.0), (iii) PB containing 10 mM GSH (100 mM, pH 7.4), and (iv) acetate buffer containing 10 mM GSH (100 mM, pH 5.0). The suspensions were gently stirred at 37 °C and micelle sizes were monitored in time by DLS. Irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles prepared using KPS (5 wt.% of the polymer) as the thermal initiator at 50 °C for 8 h were used as a reduction-insensitive control.

Loading and release of DOX. DOX-loaded micelles were prepared by dropwise adding 2.0 mL of PB (10 mM, pH 7.4) into a mixture of 200 µL of copolymer solution in DMF (5.0 mg/mL) and various volumes of DOX solution in DMSO (5.0 mg/mL, theoretical drug loading content (DLC) was 5, 10, and 20 wt.%, respectively) under stirring at room temperature, followed by stirring for 0.5 h and adding DTT solution (50 mol% of PDS) in ethanol. Nitrogen was passed through the mixture for 10 min and then the mixture was stirred magnetically at 50 °C for 8 h. The final mixture was extensively dialyzed against PB (10 mM, pH 7.4) with a MWCO of 3500 at room temperature in the dark for another 8 h.

The *in vitro* release of DOX from reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles with a low concentration (40 µg/mL containing 6 µg/mL of DOX) was investigated at 37 °C under five different conditions, *i.e.* (i) PB (100 mM, pH 7.4), (ii) acetate buffer (100 mM, pH 5.0), (iii) PB containing 10 mM GSH (100 mM, pH 7.4), and (iv) acetate buffer containing 10 mM GSH (100 mM, pH 5.0), and (v) 4 h in acetate buffer (100 mM, pH 5.0) followed by PB containing 10 mM GSH (100 mM, pH 7.4). A DOX-loaded micellar suspension was divided into five aliquots and immediately transferred to a dialysis tube with a MWCO of 12000-14000. The dialysis tube was immersed into 20 mL of appropriate buffer (100 mM) and shaken at 37 °C. At desired time intervals, 5.0 mL of the release medium was taken out and replenished with an equal volume of fresh medium. To avoid oxidation of GSH, the release media were perfused with nitrogen gas. DOX release from the thermal-crosslinked PEG-P(TMBPEC-*co*-AC) micelles was used as the irreversibly crosslinked control. The concentration of DOX was determined by fluorescence (FLS920) measurements (excitation at 480 nm, emission at 600 nm).

To determine the DLC, DOX-loaded micelle suspensions were freeze-dried, dissolved in DMSO and analyzed with fluorescence spectroscopy. A calibration curve was obtained using DOX/DMSO solutions with different DOX concentrations. To determine the amount of DOX released, calibration curves were run with DOX/corresponding buffer solutions with different DOX concentrations at pH 5.0 and 7.4, respectively. The emission at 600 nm was recorded. The release experiments were conducted in triplicate. The results are presented as the average ± standard deviation. The DLC and drug loading efficiency (DLE) were calculated according to the following formula:

$$\text{DLC (wt.\%)} = (\text{weight of loaded drug} / \text{total weight of polymer and loaded drug}) \times 100 \%$$

$$\text{DLE (\%)} = (\text{weight of loaded drug} / \text{weight of drug in feed}) \times 100 \%$$

Confocal microscopy of RAW264.7 cells incubated with DOX-loaded crosslinked micelles.

RAW264.7 cells were plated on microscope slides in a 24-well plate (5×10^4 cells/well) using DMEM medium containing 10 % FBS. The cells were incubated with prescribed amounts of DOX-loaded micelles

or free DOX at 37 °C and 5 % CO₂. After incubation for 6 and 12 h, the culture medium was removed and the cells on the microscope plates were washed three times with PBS. The cells were fixed with 4 % paraformaldehyde and the cell nuclei were stained with Hoechst 33342. Fluorescence images of cells were obtained with a Confocal Laser Scanning Microscope (Leica, TCS-SP2).

MTT assay. The cytotoxicity of blank and DOX-loaded reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles and irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles for HeLa and RAW 264.7 cells was studied using the MTT assay. Cells were seeded onto a 96-well plate at a density of 1×10^4 cells per well in 100 μ L of Dulbecco's Modified Eagle medium (DMEM) containing 10 % FBS and incubated for 24 h (37 °C, 5 % CO₂). The medium was removed and replenished by 90 μ L of fresh DMEM medium containing 10 % FBS. 10 μ L of micelle suspensions at different micelle concentrations in PB (10 mM, pH 7.4) were added. The cells were incubated for another 48 h, the medium was aspirated and replaced by 100 μ L of fresh medium, and 10 μ L of MTT solution (5 mg/mL) was added. The cells were incubated for another 4 h, and then 100 μ L of DMSO was added to dissolve the resulting purple crystals. The optical densities at 570 nm were measured using a BioTek microplate reader. Cells cultured in DMEM medium containing 10 % FBS (without micelles) were used as controls.

6. 3 Results and Discussion

6.3.1 Synthesis of amphiphilic PEG-P(TMBPEC-*co*-PDSC) copolymer

PEG-P(TMBPEC-*co*-PDSC) copolymer was prepared by ring-opening copolymerization of two cyclic carbonate monomers, TMBPEC and PDSC, using PEG ($M_n = 5.0$ kg/mol) as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst in CH₂Cl₂ at 50 °C (Scheme 6.2). We have also developed block copolymers of PEG-PTMBPEC and PEG-P(TMBPEC-*co*-AC) using analogous methods [17, 18, 25]. The ¹H NMR spectrum showed besides signals attributable to PEG (δ 3.38, 3.64) also peaks due to TMBPEC moieties (δ 3.82, 3.99, 4.75, 5.97, 6.08) and PDSC moieties (δ 1.08, 2.92, 4.09, 7.64, 7.07, 8.45) (Figure 6.1). The average molecular weight (M_n) of the P(TMBPEC-*co*-PDSC) block was determined to be 5.9 kg/mol with a molar ratio of TMBPEC and PDSC close to 1, by comparing the integrals of signals at δ 5.98 (acetal protons of TMBPEC moieties) and 2.92 (methylene protons neighboring to the disulfide bond of PDSC moieties) to 3.64 (methylene protons of PEG), respectively. There were on average about ten PDSC units per molecule. GPC revealed that the resulting copolymer had a low polydispersity of 1.34 and an M_n of 10.8 kg/mol, close to that determined by ¹H NMR (10.9 kg/mol), confirming the controlled synthesis of PEG-P(TMBPEC-*co*-PDSC) diblock copolymer. PEG-P(TMBPEC-*co*-AC) copolymer was prepared to be used for the preparation of the control irreversibly crosslinked micelles (IRCMs). The M_n of the P(TMBPEC-*co*-AC) block was evaluated to be 5.0 kg/mol with a molar ratio of TMBPEC and AC approaching 1.25 as determined by ¹H NMR.

Scheme 6.2. Synthesis of PEG-P(TMBPEC-*co*-PDSC) block copolymer by ring-opening copolymerization in DCM in CH_2Cl_2 at 50 °C using PEG-OH ($M_n = 5.0 \text{ kg/mol}$) as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst.

Figure 6.1. ^1H NMR spectrum (400 MHz, CDCl_3) of PEG-P(TMBPEC-*co*-PDSC) block copolymer

6.3.2 Micelle formation and crosslinking

Micelles were prepared by the solvent exchange method. DLS measurements showed that PEG-P(TMBPEC-*co*-PDSC) formed micellar nanoparticles with an average diameter of 58.6 nm and a narrow size distribution in PB (10 mM, pH 7.4) (Figure 6.2A). TEM revealed a homogeneous distribution of spherical nanoparticles with sizes in accordance with those determined by DLS (Figure 6.2B). The critical micelle concentration (CMC) of PEG-P(TMBPEC-*co*-PDSC) copolymers was ca. 1.46 mg/L as determined by fluorescence measurements using pyrene as a probe.

Figure 6.2. Size distribution for PEG-P(TMBPEC-*co*-PDSC) micelles determined by DLS (A) and TEM (B).

The reversible core-crosslinking of PEG-P(TMBPEC-*co*-PDSC) micelles was achieved by a simple thiol-disulfide exchange reaction, which provides disulfide bond crosslinks, which can be cleaved by reduction. DLS result revealed that the size and PDI of PEG-P(TMBPEC-*co*-PDSC) micelles before and after crosslinking were almost the same (Figure 6.3A). The progress of the crosslinking reaction was conveniently monitored by following the release of the reaction product 2-pyridothione by UV absorption at 343 nm [42]. It is assumed that formation of a single disulfide bond will require cleavage of two PDS units and produce two 2-pyridothione molecules. Considering the mechanism of the reaction, the addition of 50 mol% DTT (relative to the PDS groups) could result in the complete formation of the disulfide bonds *via* cleavage of PDS units and thiol-disulfide exchange, assuming 100 % reaction efficiency. The final crosslinking efficiency was determined to be ca. 86 % based on the ratio of released amount of 2-pyridothione to the total theoretical amount of 2-pyridothione (Figure 6.3B).

Figure 6.3. Size distribution of non-crosslinked and crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles determined by DLS (A) and UV-vis absorption spectra of 2-pyridothione (produced by the treatment of pyridyl disulfide groups with DTT) (B).

The colloidal stability of core-crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles against extensive dilution and organic solvent dilution was investigated using DLS measurements. Notably, crosslinked micelles following 1000-fold dilution showed only a slight increase in hydrodynamic diameter and maintained a low PDI, while significant aggregation was observed for the non-crosslinked micelles under otherwise the same conditions (Figure 6.4A). Moreover, unlike non-crosslinked micelles that were completely dissociated into unimers upon addition of 4-fold THF, crosslinked micelles just swelled (Figure 6.4B). These results confirmed that micelles were successfully crosslinked by the thiol-disulfide exchange reaction and that the colloidal stability of crosslinked micelles had been significantly improved. The colloidal stability of irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles prepared using KPS (5 wt.% of the polymer) as the thermal initiator was also remarkably increased with respect to that of the non-crosslinked micelles, similar to the UV core-crosslinked PEG-P(TMBPEC-*co*-AC) micelles as reported by us previously [25].

Figure 6.4. Stability of non-crosslinked micelles (NCLM) and crosslinked micelles (CLM) prepared from PEG-P(TMBPEC-*co*-PDSC) after dilution (1000 times) (A) and addition of a 4-fold volume of THF (B) as measured by DLS. The initial micelle concentration was 0.50 mg/mL.

6.3.3 Redox and pH-induced size change of crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles

The size change of crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles in response to acidic pH and 10 mM GSH was studied by DLS. Reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles swelled from 58 nm to about 180 nm in 30 h at pH 5.0 (Figure 6.5A), which was similar to that observed for irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles (Figure 6.6A). The size change could be explained by minor swelling of the the core-crosslinked micelles due to the increased hydrophilicity of the micellar core as a result of acetal hydrolysis. No change of the size of both crosslinked micelles was observed during 30 h at pH 7.4 (Figure 6.5B and 6.6B). Interestingly, a small increase in the size of reversibly crosslinked micelles (RCM) from 58 nm to 120 nm was observed after incubation of these micelles for 4 h at pH 7.4 in the presence of 10 mM GSH due to the cleavage of the disulfide bonds (Figure 6.5B). However, no further significant changes were observed when the RCM were kept in this medium for prolonged times (*i.e.* 30 h).

The results can be explained by the fact that although the micelles were de-crosslinked, no acetal hydrolysis in the core of the micelles took place. Furthermore, the micelle concentration (40 $\mu\text{g/mL}$) was higher than the CMC (1.46 $\mu\text{g/mL}$) of the uncrosslinked RCM. In comparison, no obvious size change was discerned for irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles in 30 h under otherwise the same conditions (Figure 6.6B).

Figure 6.5. pH and redox-induced size change of reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles in time followed by DLS. (A) pH 5.0; (B) pH 7.4. The micelle concentration was set as 40 $\mu\text{g/mL}$.

Figure 6.6. pH and redox-induced size change of irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles in time followed by DLS. (A) pH 5.0; (B) pH 7.4. The micelle concentration was set as 40 $\mu\text{g/mL}$.

It should be noted that a pronounced size change of reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles was observed at pH 5.0 in the presence of 10 mM GSH, in which large aggregates with a diameter over 1000 nm were formed in 20 h (Figure 6.5A). The combination of de-crosslinking under reductive conditions and acetal hydrolysis at acidic pH for the RCMs will likely induce synergistic effects for intracellular drug release. In contrast, the size change of the irreversible crosslinked micelles (IRCMs) under the same conditions (pH 5.0 in the presence of 10 mM GSH) was similar to that observed when the

micelles were only exposed to pH 5.0 (Figure 6.6A) and this can be explained by the presence of non-degradable polyacrylate core-crosslinks. Therefore, reversibly core-crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles while possessing superior colloidal stability under extracellular conditions are prone to rapid dissociation under acidic pH and redox dual-stimuli mimicking the intracellular conditions.

6.3.4. Loading and *in vitro* release of DOX

DOX was loaded into non-crosslinked and crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles at theoretical DLC's of 5, 10 and 20 wt.%. Drug loading levels for PEG-P(TMBPEC-*co*-PDSC) crosslinked and non-crosslinked micelles were similar, with drug loading efficiencies (DLE) in the range of 59.8 to 66.8 % (Table 6.1). The average diameters of non-crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles increased from 62.4 to 167.2 nm with DOX loading contents increasing from 3.4 to 12.1 wt.% (Table 6.1). However, the average size of DOX-loaded crosslinked micelles was smaller than that of DOX-loaded non-crosslinked micelles and increased from 63.6 to 84.6 nm with the same increasing DOX loading contents. It should be noted that only small differences in the size of DOX-loaded crosslinked micelles with different DLC have been observed, which is probably due to the disulfide crosslinked core-structure. Analogous trends were also observed for DOX-loaded irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles.

Table 6.1. Characteristics of DOX-loaded non-crosslinked and reversibly core-crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles.

Micelle	DLC (wt %)		DLE (%)	Size (nm)	PDI
	Theory	Determined			
Non-crosslinked	5	3.4	66.8	62.4 ± 0.8	0.08
	10	6.5	65.2	90.3 ± 1.6	0.12
	20	12.1	60.5	167.2 ± 2.1	0.15
Crosslinked	5	3.3	65.3	63.6 ± 2.6	0.16
	10	6.3	62.6	77.4 ± 1.6	0.13
	20	12.0	59.8	84.6 ± 2.3	0.13

^a Determined by UV measurement; ^b Determined by DLS.

The *in vitro* release of DOX from reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles was investigated at 37 °C under different conditions, *i.e.* (i) pH 7.4, (ii) pH 5.0, (iii) pH 7.4 and 10 mM GSH, (iv) pH 5.0 and 10 mM GSH, and (v) 4 h at pH 5.0 followed by pH 7.4 and 10 mM GSH (mimicking the intracellular trafficking pathway). The results showed that the release of DOX from both crosslinked micelles was largely inhibited, even at a low micelle concentration of 40 µg/mL, in which only ca. 19.8 % and 17.2 % DOX was released at physiological pH in 24 h from reversibly (Figure 6.7A) and irreversibly crosslinked micelles (Figure 6.8), respectively. It is interesting that DOX release from the RCMs was boosted in the first two hours (22.4 % of DOX) at pH 7.4 in the presence of 10 mM GSH, which may be

attributed to the S-S cleavage of the crosslinked micelles triggered by the reduction-stimulus. DOX release from the de-crosslinked micelles was still inhibited due to the high hydrophobic interaction between DOX and TMBPEC units, in which only 44.1 % of drug was released in 24 h. The release results are in accordance with the size changes observed for RCMs at pH 7.4 in the presence of 10 mM GSH (Figure 6.5B). The release of DOX was significantly accelerated at pH 5.0 mainly due to pH-induced acetal hydrolysis. For example, 64.2 % of DOX was released from the RCMs in 24 h (Figure 6.7A), which was also observed for the IRCMs under otherwise the same conditions (Figure 6.8). Notably, the fastest and almost complete drug release was observed at pH 5.0 in the presence of 10 mM GSH wherein 98.7 % of DOX was released in 12 h (Figure 6.7A). This is in line with the observation that RCMs were rapidly destabilized at pH 5.0 in the presence of 10 mM GSH (Figure 6.6A). It was found that the addition of 10 mM GSH had no effect on DOX release from irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles both at pH 5.0 and pH 7.4 (Figure 6.8). In order to simulate the intracellular trafficking process, drug release studies were first performed at pH 5.0 (mimicking the acidic endosomal compartments) for 4 h and subsequently at pH 7.4 and 10 mM GSH (mimicking the reductive environment of the cytosol), which has interestingly resulted in faster and more thorough DOX release from PEG-P(TMBPEC-*co*-PDSC) micelles (ca. 95.0 % of DOX released in a total time of 24 h) than at either pH 5.0 or pH 7.4 and 10 mM GSH (Figure 6.7B). DOX release from the IRCMs at pH 5.0 was similar as that for the RCMs during the first 4 h, but was significantly inhibited at pH 7.4 in the presence of 10 mM GSH (Figure 6.7B). It appears, therefore, that reversibly crosslinked pH-sensitive degradable micelles on one hand possess superior stability at physiological conditions due to the core-crosslinked structure with S-S bonds and on the other hand are able to rapidly and thoroughly release drug by the pH and reduction dual-stimuli simulating the intracellular trafficking process.

Figure 6.7. pH and/or redox-triggered release of DOX from crosslinked micelles at 37 °C. (A) DOX release from reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles at pH 7.4 or pH 5.0 in the presence or absence of 10 mM GSH; and (B) DOX release from reversibly and irreversibly crosslinked micelles at pH 5.0 for 4 h followed by pH 7.4 and 10 mM GSH (mimicking the intracellular trafficking pathway). The micelle concentration was set as 40 µg/mL.

Figure 6.8. *In vitro* release of DOX from irreversibly crosslinked PEG-P(TMBPEC-co-AC) micelles under different conditions at 37 °C. The micelle concentration was set as 40 µg/mL.

6.3.5 Intracellular DOX release and antitumor activity

The cellular uptake and intracellular drug release profiles of DOX-loaded crosslinked micelles were investigated with CLSM using RAW 264.7 cells. Notably, comparable DOX fluorescence was observed in the cytoplasm of RAW 264.7 cells following 6 h incubation with both of the DOX-loaded crosslinked micelles (Figure 6.9A and 6.9C), in which the micelles are encountering the endosomal pH, inducing a similar DOX release. After incubation of the cells with DOX-loaded RCMs for an incubation time of 12 h, the DOX fluorescence in the cell nuclei became even stronger (Figure 6.9B) and similar to the DOX fluorescence in the nuclei observed for RAW 264.7 cells following 6 h incubation with free DOX (control experiments) (Figure 6.9E). The uptake of DOX by the cell nuclei is crucial because DOX has to intercalate with DNA to induce cell death [46]. In contrast after 12 h incubation with DOX-loaded IRCMs, the fluorescence was not increased and still mainly present in the cytosol as well as in the perinuclei regions of the cells (Figure 6.9D). These different intracellular drug release behaviors of RCM's and IRCM's are most likely due to fast DOX release from RCMs after their escape from the endosomes as a result of reduction-triggered de-crosslinking in the cytosol. In comparison, drug release from IRCMs would be much slower in the cytosol (neutral pH), as indicated in Figure 6.8. These results have demonstrated that reversibly crosslinked pH-sensitive micelles could mediate more efficient intracellular anticancer drug release than the IRCMs.

Figure 6.9. CLSM images of RAW 264.7 cells following 6 or 12 h incubation with DOX-loaded micelles and free DOX (10 $\mu\text{g/mL}$). For each panel, the images from left to right show cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlays of both images. The scale bars correspond to 25 μm in all the images. A and B: DOX-loaded reversibly crosslinked PEG-P(TMBPEC-co-PDSC) micelles, incubation for and 6 and 12 h, respectively; C and D: DOX-loaded irreversibly crosslinked PEG-P(TMBPEC-co-AC) micelles, incubation for and 6 and 12 h, respectively; and (E) free DOX, 6 h.

Figure 6.10. Cytotoxicity of reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles and irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles. (A) HeLa cells; (B) RAW 264.7 cells. The cells were incubated with micelles for 48 h. Data are presented as the average $\pm$ standard deviation ($n = 4$).

MTT assays using HeLa and RAW 264.7 cells revealed that RCMs and IRCMs were practically non-toxic (cell viabilities $\geq 94\%$) up to a tested concentration of 0.8 mg/mL (Figure 6.10), confirming that these degradable micellar particles have good biocompatibility. DOX-loaded RCMs and DOX-loaded IRCMs, however, displayed significant anti-tumor activity towards HeLa and RAW 264.7 cells following 48 h incubation (Figure 6.11). It should be noted that DOX-loaded reversibly crosslinked micelles had low IC_{50} (half inhibitory concentration) values of 1.65 and 1.14 $\mu\text{g DOX equiv./mL}$ for HeLa and RAW 264.7 cells, respectively, which were close to those obtained with the pH and reduction dual-responsive non-crosslinked PEG-SS-PTMBPEC micelles ($IC_{50} = 0.75$ and $0.60 \mu\text{g DOX equiv./mL}$ for HeLa and RAW 264.7 cells, respectively) [44]. IRCMs displayed inferior antitumor activity under otherwise the same conditions with IC_{50} values of 19.8 and 12.6 $\mu\text{g DOX equiv./mL}$ for HeLa and RAW 264.7 cells, respectively, which was mainly due to the incomplete and slow drug release from the irreversibly crosslinked structure. The higher anti-tumor activity of DOX loaded RCMs as compared to DOX-loaded IRCMs is in accordance with the results from confocal microscopy that RCMs mediate efficient intracellular drug release as compared to the corresponding IRCMs. Interestingly, the anti-tumor activity of DOX-loaded RCMs approached that of free DOX ($IC_{50} = 0.41$ and $0.38 \mu\text{g/mL}$ for HeLa and RAW 264.7 cells, respectively). It should further be noted that the anti-tumor activity of DOX-loaded reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles might be further enhanced by using targeting ligands such as folic acid, aptamer, peptide, antibody or antibody fragments that facilitate efficient and specific cellular uptake of micelles. It is concluded that these reversibly crosslinked pH-sensitive micelles have superior stability at extracellular conditions and can rapidly and completely deliver anticancer drugs intracellularly by pH and reduction dual-stimuli.

Figure 6.11. Tumor cell killing activity of DOX-loaded reversibly crosslinked PEG-P(TMBPEC-*co*-PDSC) micelles, DOX-loaded irreversibly crosslinked PEG-P(TMBPEC-*co*-AC) micelles and free DOX as a function of DOX dosage. (A) HeLa cells; (B) RAW 264.7 cells. The cells were incubated with DOX-loaded micelles or free DOX for 48 h. Data are presented as the average $\pm$ standard deviation ($n = 4$).

6.4 Conclusions

We have demonstrated that reduction-responsive reversibly crosslinked pH-sensitive micelles based on PEG-P(TMBPEC-*co*-PDSC) copolymers while possessing superior stability in the extracellular environment undergo rapid de-crosslinking and disassembly at endosomal pH followed by the exposure to cytoplasmic glutathione, resulting in enhanced intracellular anticancer drug release. In these intelligent micelles several features have been integrated: (i) they have low cytotoxicity and are degradable; (ii) they show good drug loading levels; (iii) at low concentrations they show superior stability with inhibited (premature) extracellular drug release; and (iv) they promote fast and maximum drug release inside the cancer cells in which drug release is activated during the whole intracellular trafficking process, *i.e.* not only in the mildly acidic endosomal compartments but also in the highly reducing cytoplasm and cell nuclei. These reduction-responsive reversibly crosslinked pH-sensitive biodegradable micelles provide an interesting platform for targeted and controlled intracellular release of potent chemotherapeutics.

6.5 References

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Chapter 7

Biodegradable Glycopolymer-Poly(ϵ -caprolactone) Block Copolymer Micelles: Versatile Construction, Tailored Galactose Functionality, and Hepatoma-Targeted Drug Delivery*

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Abstract

Biodegradable glycopolymer-poly(ϵ -caprolactone) (GP-PCL) block copolymer micelles ('glycomicelles') with tailored galactose functionalities were developed and investigated for hepatoma-targeted drug delivery. Amphiphilic GP-PCL copolymers were readily prepared with controlled degrees of post-polymerization functionalization of 20 %, 40 %, 80 %, and 100 % of poly(acryloyl cyclic carbonate)-b-poly(ϵ -caprolactone) (PAC-b-PCL, 11.6-6.4 kg/mol) block copolymer (denoted as GP20-PCL, GP40-PCL, GP80-PCL, and GP100-PCL, respectively) with thiol-containing galactose (Gal-SH) and 2-(2-methoxyethoxy)ethanethiol ((EO)₂-SH) via Michael-type addition reaction. All GP-PCL copolymers exhibited a low critical micelle concentration (CMC = 1.35-3.72 mg/L) and self-assembled into monodisperse nano-sized micelles with mean hydrodynamic diameters ranging from 31.9 to 76.8 nm, of which the diameter decreased with increasing Gal density of the block copolymers. The glycomicelles had excellent doxorubicin (DOX) loading capacities, in which high drug loading efficiencies of 83.0-89.2 % were obtained at a theoretical drug loading content of 20 wt.%. *In vitro* release studies showed that DOX release was faster at pH 5.0 than at pH 7.4 and increased when using micelles with higher Gal contents. Flow cytometric analyses using asialoglycoprotein receptor (ASGP-R) positive HepG2 liver cancer cells revealed that the DOX concentration in the cells after cellular uptake of DOX-loaded glycomicelles after 4 h incubation was 6.6-17.1 fold higher for micelles with Gal densities increasing from 20 % to 80 %, as compared to the free DOX concentration in the cells after uptake of the corresponding DOX loaded non-glycomicelles (100 % substitution with (EO)₂-SH). MTT assays using HepG2 cells as well as ASGP-R negative MCF-7 cells demonstrated that after 72 h incubation DOX-loaded glycomicelles were taken up more effectively by HepG2 cells than by ASGP-R negative MCF-7 cells. MTT assays also showed that free HepG2 cells were much more efficiently killed by DOX-loaded glycomicelles after incubation for 4 h and subsequent culture for 72 h than HepG2 cells blocked by lactobionic acid, emphasizing the role of ASGP-R mediated endocytosis. DOX loaded GP20-PCL, GP40-PCL, GP80-PCL and GP100-PCL micelles had lower IC₅₀ (half maximal inhibitory concentration) values of 2.05, 0.75, 0.45 and 0.43 μ g DOX equiv./mL, respectively when incubated with HepG2 cells than DOX-loaded non-glycomicelles (IC₅₀: 6.55 μ g/mL DOX equiv./mL). Under the same conditions, glycomicelles and non-glycomicelles without DOX were non-toxic for HepG2 cells up to a tested concentration of 0.5 mg/mL. Glycomicelles with tailored galactose functionalities, high drug loading capacity, and high uptake by ASGP-R positive cells are promising for liver cancer chemotherapy.

7.1 Introduction

Biodegradable polymeric micelles have appeared as one of the most promising nanosystems for targeted cancer chemotherapy [1-5]. Micelles based on amphiphilic copolymers of poly(ethylene glycol) (PEG) and aliphatic polyesters/polycarbonates such as polylactide (PLA), poly(ϵ -caprolactone) (PCL), poly(lactide-*co*-glycolide) (PLGA), and poly(trimethylene carbonate) (PTMC) are among the most studied due to their excellent biocompatibility, *in vivo* biodegradability and easy synthesis [6, 7]. *In vitro* and *in vivo* studies have demonstrated that these biodegradable polymeric micelles have great potential for passive targeting and controlled delivery of hydrophobic anti-cancer drugs such as paclitaxel (PTX) and doxorubicin (DOX), because they possess several physico- and bio-chemical advantages, including enhancing the water solubility of lipophilic drugs, prolonging circulation time, passive targeting to the tumor tissues via the enhanced permeability and retention (EPR) effect, decreasing side effects, and improving drug bioavailability [8-10]. It should be noted that PEG shielded micelles, although they are nanosized and relatively stable, display inferior cellular uptake leading to low intracellular drug concentrations and concomitantly low antitumor activity. In recent years, many micellar nanosystems containing PEG end-capped with specific ligands, such as folic acid (FA), β -D-galactose and cyclic RGD peptide, have been designed for the development of tumor-targeted drug delivery. It was shown that the antitumor efficiency of these ligand containing micellar nanosystems was significantly improved due to an enhanced cellular uptake [11, 12].

Liver cancer, especially hepatocellular carcinoma (hepatoma), is one of the most common deadly cancers all over the world and may reach epidemic levels fast because of increased viral-induced hepatitis [13]. In recent years, many reports have demonstrated that micellar nanoparticles decorated with β -D-galactose, *N*-acetylgalactosamine, and lactose ligands can be used for the targeting of liver cancer, since on the mammalian hepatocyte surface, there are asialoglycoprotein receptors (ASGP-R), which can well recognize and bind these sugars [14-16]. It is interesting that copolymers with the appropriate density and relative spatial arrangement of the carbohydrate residues can improve the binding affinity for specific proteins due to polyvalent interactions, an effect known as the “glycoside cluster effect” [17-20]. Well-defined synthetic polymers with carbohydrate-containing pendants (glycopolymers) have recently received much attention in sugar-related drug discovery, molecular diagnosis, and targeted drug/gene delivery because of these multivalent sugar-protein interactions [21-23]. Biodegradable glycopolymer-based micelles are particularly useful for tumor-targeted drug delivery applications and other receptor-mediated targeting of genes and drugs to specific tissues/cells that require the recognition of carbohydrate-binding proteins known as lectins [24-26]. A number of glycopolymer architectures have been developed for drug delivery over recent years including linear polymers [27-30], dendrimers [31-34], and micellar nanoparticles [35-37]. For example, Jain *et al.* reported that galactose coated drug dendrimer formulations revealed almost 5 times higher phagocytosis and drastically reduced toxicity compared to uncoated drug formations as well as plain drug against lung and liver macrophages of male albino rats [38]. Yang and coworkers prepared biodegradable amphiphilic glycopolymer-PTMC block copolymers by a

multi-step procedure, starting with the synthesis of protected sugar-containing (D-glucose, D-galactose and D-mannose) cyclic carbonate monomers, followed by sequential ring-opening copolymerization of sugar-containing carbonate monomers and trimethylene carbonate (TMC) and deprotection using formic acid [39]. They found that galactose-based glycomicelles exhibited significant cellular uptake and anti-tumor efficacy using ASGP-R positive HepG2 cells.

In this study, we aimed to develop non-toxic and biodegradable glycomicelles based on block glycopolymers with tunable galactose density for active hepatocyte-targeted drug delivery (Scheme 7.1). Galactose-bearing glycopolymers provide a platform for which multiple copies of a saccharide can be presented simultaneously, thus enhancing their affinity and selectivity for lectins. We synthesized the biodegradable PAC-*b*-PCL block copolymer efficiently by one-pot sequential ring-opening polymerization of (acryloyl carbonate) AC and ϵ -CL, in which the acryloyl functionalized copolymers could provide versatile access to diverse functional groups at mild conditions via the Michael-type addition reaction [40-42]. Synthesis and self-assembly of biodegradable block glycopolymers, loading and *in vitro* release of DOX, as well as the uptake of DOX-loaded glycomicelles by ASGP-R positive HepG2 liver cancer cells and their toxicity towards these cells have been investigated.

Scheme 7.1. Illustration of versatile construction of biodegradable glycopolymer-PCL micelles with tailored galactose functionality for hepatoma-targeted drug delivery.

7.2 Materials and methods

Materials. 2-(2-Methoxyethoxy)ethanethiol ((EO)₂-SH, 97 %, Aldrich), lactobionic acid (LBA, 97%, Acros), dithiothreitol (DTT, 99%, Merck), N-hydroxysuccinimide (NHS, 98%, J&K), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 98%, J&K), zinc bis[bis(trimethylsilyl)amide] (97 %, Aldrich), triethylamine (Et₃N, 99%, Alfa Aesar), and doxorubicin

hydrochloride (DOX·HCl, 99%, Beijing Zhong Shuo Pharmaceutical Technology Development Co.Ltd.) were used as received. ϵ -Caprolactone (ϵ -CL, 99%, Alfa Aesar) was dried over CaH₂ and distilled under reduced pressure prior to use. Acryloyl carbonate monomer (AC) was synthesized according to our previous report [40]. Isopropanol and dichloromethane (DCM) were dried by refluxing over CaH₂ under an argon atmosphere.

Synthesis of PAC-*b*-PCL block copolymer. The PAC-*b*-PCL block copolymer was synthesized by one-pot sequential ring-opening polymerization of AC and ϵ -CL in DCM using isopropanol as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst [43]. Briefly, in a glove-box under a nitrogen atmosphere, to a stirred solution of AC (0.520 g, 2.60 mmol) in DCM (2.6 mL) was quickly added isopropanol stock solution (0.26 mL, 200 mM) and zinc bis[bis(trimethylsilyl)amide] stock solution (0.20 mL, 100 mM). The reaction vessel was sealed and placed in a glove box and the reaction mixture was magnetically stirred for 4 h at room temperature. A sample was taken for determination of the monomer conversion and polydispersity using ¹H NMR and GPC, respectively. Then the second monomer ϵ -CL (0.296 g, 2.6 mmol) in 2.6 mL of DCM was added. The reaction was allowed to proceed at 40 °C for another 20 h. Also, one sample was taken for determination of ϵ -CL conversion using ¹H NMR. The copolymer was precipitated into cold diethyl ether and dried *in vacuo* at room temperature. ¹H NMR (400 MHz, CDCl₃) for PAC-*b*-PCL copolymer: δ 1.06 (-C(CH₂)CH₃, PAC), 1.38 (-CH₂CH₂CH₂-, PCL), 1.65 (-CH₂CH₂CH₂-, PCL), 2.30(-C(O)CH₂-, PCL), 4.05 (-CH₂-O-C(O)-, PCL), 4.11 (-CH₂-O-C(O)-O-CH₂-, PAC), 5.85-6.43 (double bond, PAC).

Synthesis of thiol-containing galactose (Gal-SH). To a stirred solution of lactobionic acid (LBA, 3 g, 8.37 mmol) in deionized water (20 mL) was added EDC (3.2 g, 16.74 mmol) and NHS (1.9 g, 16.74 mmol). The pH of the solution was adjusted to 5 and then the solution was stirred for 4 h at room temperature. Under N₂ protection, 2-mercaptoethylamine hydrochloride (2.4 g, 20.93 mmol) was added into the mixture and the pH of the solution was adjusted to 7.4 by an aqueous solution of sodium hydroxide (1 M). The reaction was allowed to proceed for another 24 h. The solution was then treated with DTT (2.0 g, 12.98 mmol) for 5 h. The crude product was isolated by precipitation in isopropanol and washed with methanol to yield Gal-SH. The thiol functionality was determined via the Ellman assay (functionality of SH = 80%).

Synthesis of glycopolymers. The synthesis of glycopolymers by Michael-type addition was carried out in DMSO/DMF at room temperature under a nitrogen atmosphere according to our previous report [40]. Glycopolymers with different amounts of galactose pending groups were synthesized by controlling the feed ratio of (EO)₂-SH and Gal-SH. As an example of the synthesis of GP20-PCL, to a solution of PAC-*b*-PCL copolymer (50 mg, 16 μ mol of AC units) and Gal-SH (1.8 mg, 3.5 μ mol of SH groups) in DMSO/DMF (v/v = 5/1) under a nitrogen atmosphere was added a catalytic amount of Et₃N. The reaction was allowed to proceed with stirring for 8 h at room temperature. One sample was taken for determination of the functionality of the PAC segment using ¹H NMR. To the rest of the reaction solution, (EO)₂-SH (2.6 mg, 19 μ mol of SH groups) dissolved in 0.5 mL of DMSO was added. The reaction was allowed to proceed at room temperature for another 8 h. The resulting glycopolymer was isolated by dialysis against deionized

water to remove excess thiol-containing molecules with a molecular weight cut-off (MWCO) of 3500 at room temperature for 48 h, and freeze-dried.

Characterization. ^1H NMR spectra were recorded on the Unity Inova 400 operating at 400 MHz. CDCl_3 and $\text{DMSO}-d_6$ were used as solvents and the chemical shifts were calibrated against residual solvent signals. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using CHCl_3 as the eluent at a flow rate of 0.5 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns.

Micelle formation and critical micelle concentration. Micelles were typically prepared under stirring by dropwise addition of 200 μL of copolymer solution in DMSO (2.0 mg/mL) to 2 mL of ultrapure water at room temperature, followed by dialysis to remove the organic solvent. The size, size distribution and surface charge of the micelles were determined by dynamic light scattering (DLS) and zeta potential measurements. Measurements were carried out at 25 °C with three independent replicates using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He-Ne laser using back-scattering detection. The data are presented as mean value $\pm$ standard deviation.

The critical micelle concentration (CMC) was determined using pyrene as a fluorescence probe. The concentration of functional copolymer was varied from 1.0×10^{-5} to 0.5 mg/mL and the concentration of pyrene was fixed at 1.0 μM . Fluorescence spectra were recorded using a FLS920 fluorescence spectrometer and an excitation wavelength of 330 nm. Fluorescence emissions at 372 and 383 nm were monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions. The stability of the micelles in PB (pH 7.4, 10 mM) containing 10% (v/v) fetal bovine serum (FBS) was studied at 37 °C by monitoring the particle size change as a function of time using DLS.

Loading of DOX into micelles and *in vitro* release. DOX was loaded into micelles by dropwise addition of a mixture of 0.2 mL of copolymer solution in DMSO (2.0 mg/mL) and 20 μL of DOX solution in DMSO (5 mg/mL, theoretical drug loading content was set as 20%) to 2 mL of ultrapure water under stirring at room temperature, followed by stirring for 0.5 h and dialysis against deionized water with MWCO of 3500 at room temperature in the dark for 6 h.

The release profiles of DOX from the micelles were studied using a dialysis tube (MWCO 12 000) in PB (10 mM, pH 7.4) or acetate buffer (10 mM, pH 5.0) at 37 °C. In order to acquire sink conditions, drug release studies were performed with 0.6 mL of micelle suspension with a DLC of 5 wt.%, which were dialyzed against 20 mL of the same medium. At desired time intervals, 5.0 mL release media was taken out and replenished with an equal volume of fresh media. The amount of DOX released was determined by using fluorescence (FLS920) measurements (excitation at 480 nm). For determination of the drug loading content (DLC), DOX-loaded micellar suspensions were freeze-dried, then dissolved in DMSO and analyzed with fluorescence spectroscopy. A calibration curve was obtained using DOX/DMSO solutions with different DOX concentrations. To determine the amount of DOX released, calibration curves were run

with DOX/phosphate buffer solution (pH 7.4, 10 mM) with different DOX concentrations. The emission at 600 nm was recorded. Release experiments were conducted in triplicate. The results presented are the average data $\pm$ standard deviation.

DLC and drug loading efficiency (DLE) were calculated according to the following formulas:

$$\text{DLC (wt.\%)} = (\text{weight of loaded drug} / \text{total weight of polymer and loaded drug}) \times 100\%$$

$$\text{DLE (\%)} = (\text{weight of loaded drug} / \text{weight of drug in feed}) \times 100\%$$

Analysis of the cellular uptake of DOX-loaded micelles by flow cytometry. HepG2 cells were seeded onto 6-well plates at 1×10^5 cells per well for 24 h using DMEM medium containing 10% FBS. After 24 h incubation, the medium was replaced by 0.9 mL of fresh DMEM and 0.1 mL of prescribed amounts of DOX-loaded micelles. After incubation at 37 °C for 4 h, the cells were digested by 0.25 w/v% trypsin/0.03 w/v% EDTA. The suspensions were centrifuged at 1500 rpm for 5 min at 25 °C, pelleted in eppendorf tubes, washed twice with cold PBS, and then resuspended in 500 μ L of PBS. Fluorescence histograms were recorded with a BD FACSCalibur (Beckton Dickinson) flow cytometer and analyzed using Cell Quest software. We analyzed 10 000 gated events to generate each histogram. The gate was arbitrarily set for the detection of DOX fluorescence.

MTT assays. The cytotoxicity of micelles and DOX-loaded micelles was studied by MTT assays using HepG2 and MCF-7 cells. Cells were seeded onto a 96-well plate at a density of 1×10^4 cells per well in 100 μ L of Dulbecco's Modified Eagle medium (DMEM) containing 10% FBS and incubated for 72 h (37 °C, 5% CO₂). The medium was replaced by 90 μ L of fresh DMEM medium containing 10% FBS, and then 10 μ L samples of various concentrations of the micelle suspensions were added. The cells were incubated for another 72 h, the medium was aspirated and replaced by 100 μ L of fresh medium, and 10 μ L of MTT solution (5 mg/mL) was added. The cells were incubated for 4 h, and then the medium was aspirated and replaced by 150 μ L of DMSO to dissolve the resulting purple crystals. The optical densities at 570 nm were measured using a BioTek microplate reader. Cells cultured in DMEM medium containing 10% FBS (without exposure to micelles) were used as controls.

To evaluate whether the high drug efficacy was specially caused by ASGP-R mediated endocytosis, HepG2 cells seeded in a 96-well plate at a density of 1×10^4 cells per well for 24 h were incubated with (+ LBA) or without (-LBA) free LBA (2 mg/mL) 4 h before the addition of DOX-loaded micelles or free DOX. The ASGP-R on HepG2 cell surface can be blocked in DMEM medium containing free LBA. The cytotoxicity studies for DOX-loaded glycomicelles, DOX-loaded Gal-free micelles and free DOX (drug dosage of 10 μ g/mL) were carried out in a similar way. The medium was replaced by 90 μ L of fresh DMEM medium and 10 μ L of DOX-loaded micelles or free DOX. After additional 4 h incubation, the medium was replaced by 100 μ L of fresh DMEM medium and the cells were cultured for another 72 h. After that, the same procedure was carried out as above-mentioned. 10 μ L of MTT solution (5 mg/mL) was added and the cells were incubated for 4 h. The culture medium was aspirated and replaced by 150 μ L of DMSO to dissolve the resulting purple crystals. The optical densities at 570 nm were measured using a BioTek microplate reader. Cells cultured in DMEM medium containing 10% FBS (without exposure to micelles) were used as controls.

7.3 Results and discussion

7.3.1 Synthesis of PAC-*b*-PCL block copolymer

The biodegradable PAC-*b*-PCL block copolymer was prepared by one-pot sequential ring-opening polymerization of AC and ϵ -CL in DCM (Scheme 7.2). Firstly, the polymerization of AC using isopropanol as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst was carried out in DCM for 4 h at r.t. In our previous reports, we found that AC could be controlled copolymerized with ϵ -CL or LA to provide a versatile approach to functionalizing biodegradable copolymers [16, 40, 42, 44]. ^1H NMR showed that the AC monomer conversion was close to 100% (Fig. 1A), which indicated that the synthesis of the PAC segment could be performed smoothly and efficiently. Next, the second monomer, ϵ -CL, was added and the polymerization was continued for another 20 h at 40 °C. ^1H NMR showed complete ϵ -CL monomer conversion after 20 h polymerization. The resulting PAC-*b*-PCL block copolymers were isolated by precipitation in diethyl ether. ^1H NMR showed signals characteristic of both AC units (δ 5.85-6.43, 4.11 and 1.06) and CL units (δ 4.05, 2.30, 1.65 and 1.38), (Fig. 1B). Importantly, signals due to double bond protons at δ 5.85-6.43 were maintained, indicating that AC functional groups remained intact during sequential polymerization and subsequent work-up procedures. The number-average molecular weights (M_n) of PAC and PCL segments estimated from ^1H NMR end group analysis by comparing the integrals of peaks at 1.06 (methyl protons in PAC block) and 2.30 (methylene protons next to carbonyl in PCL block) with δ 1.25 (methyl protons of isopropyl ester end group) were 11.6 kg/mol and 6.4 kg/mol, respectively, which were close to the theoretical values (PAC: 10 kg/mol, PCL: 5.7 kg/mol). Gel permeation chromatography (GPC) measurements using polystyrene as standards showed that the PAC segment had a narrow polydispersity (PDI) of 1.23 after the first step. After the following sequential polymerization the PAC-*b*-PCL block copolymer had a moderate PDI of 1.78. It is therefore evident that the synthesis of PAC-*b*-PCL block copolymer by one-pot sequential ring-opening polymerization of AC and ϵ -CL can be well controlled with respect to functionality and molecular weights.

Figure 7.1. ^1H NMR spectra (400 MHz, CDCl_3) of PAC prepolymer (A) and PAC-*b*-PCL block copolymer (B).

Scheme 7.2. Synthesis of amphiphilic block glycopolymer by one-pot sequential ring-opening polymerization of AC and ϵ -CL in DCM using isopropanol as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst, followed by sequential post-polymerization modification with thiol-containing molecules through Michael-type conjugate addition reaction. Conditions: (i) r.t., 4 h; (ii) 40 °C, 20 h; (iii) r.t., 8 h; (iv) r.t. 8 h..

7.3.2. Synthesis of GP-PCL block copolymer

The conjugation of Gal to PAC-*b*-PCL copolymer was performed in DMSO/DMF at room temperature by Michael-type addition reaction, to afford amphiphilic block glycopolymers (Scheme 7.2). Gal-SH was synthesized from lactobionic acid with 2-mercaptoethylamine using carbodiimide chemistry. The thiol-functionality of Gal-SH was over 80% as determined via the Ellman assay. ^1H NMR spectra (Fig. 2A) displayed new signals at δ 3.2-3.7 (attributed to the Gal pendants), and 2.6-2.8 (the methylene protons from the converted double bonds of AC units). It should be noted that 100% functionalization with Gal-SH was performed based on the complete disappearance of peaks assignable to acryloyl groups. The ratio of integrals of resonances at 1.06 (methyl protons in PAC block) and 2.30 (methylene protons next to the carbonyl in the PCL block) did not change, which suggests that the block copolymer (denoted as GP100-PCL) does not degrade during the modification procedure. Sequential modification of PAC-*b*-PCL copolymer with Gal-SH and $(\text{EO})_2$ -SH could be carried out with high efficiency, in which quantitative modification with Gal-SH was obtained with different molar ratios (1, 0.8, 0.4 and 0.2) of Gal-SH/AC units followed by the complete functionalization of residual AC groups with $(\text{EO})_2$ -SH yielding the corresponding glycopolymers denoted as GP100-CL, GP80-PCL, GP40-PCL and GP20-PCL, respectively.. The amounts of Gal present in the copolymers were determined by comparing the integrals of resonances at 1.06 (methyl protons in PAC block) with those of remaining acryloyl groups of the sample solution, from which it was found that the Gal-functionality of all the copolymers was close to what was anticipated. After $(\text{EO})_2$ -SH addition, the rest of the double bonds were quantitatively functionalized. ^1H

NMR spectra (Fig. 2B) showed new signals at δ 3.2-3.7 (attributed to the Gal and oligo(EO) pendants), and 2.6-2.8 (the methylene protons from conversion of double bonds of AC units). At these mild modification conditions no degradation of the biodegradable copolymers occurred. The copolymer modified with only oligo(EO) pendants was prepared as non-Gal control (denoted as P(EO)-PCL). ^1H NMR spectra also showed the successful modification (Fig. 2C). We hypothesized that different amounts of Gal on the glycomicelle surface may induce different rates of uptake by hepatoma cells.

Figure 7.2. ^1H NMR spectra (400 MHz) of PAC-*b*-PCL copolymer modified with Gal-SH and (EO)₂-SH. (A) GP100-PCL; (B) GP40-PCL; (C) P(EO)-PCL.

7.3.3 Formation of glycomicelles and non-glycomicelles

Micelles of Gal-functionalized copolymers were prepared by dropping polymer solutions into water, followed by dialysis to remove the organic solvent. The sizes of the micelles determined by dynamic light scattering (DLS) measurements are shown in Table 7.1. The functional copolymers formed micelles with average sizes of 31.9-88.2 nm and low polydispersities (PDIs) of 0.07-0.18 (Figure 3). It was interesting that the size of the micelles decreased with increasing amounts of Gal on the micelle surface, which was probably due to the higher molecular weights of the hydrophilic segments functionalized with Gal. The critical micelle concentration (CMC) determined using pyrene as a probe showed that all the functionalized copolymers had low CMCs of 3.72~1.03 mg/L, respectively (Table 7.1). The CMC of the copolymers decreased with increasing amounts of oligo(EO) on the micelle surface, indicating that the stability of the micelles increased with increased oligo(EO)-functionalization. Zeta potential measurements showed that the completely Gal-functionalized micelles had positive surface charges (5.3 mV) in water (Table 7.1), while micelles, shielded with various molar percentages of oligo(EO) had close to neutral or even a negative surface charge (-0.1 ~ -16.5 mV), indicating that the surface potential of the micelles could be well regulated by varying the amounts of Gal in the micelles.

Table 7.1. Characteristics of biodegradable glycopolymer-PCL micelles with tailored galactose functionality.

Entry	Functional copolymer ^a	Size (nm) ^b	PDI ^b	ξ (mV) ^b	CMC (mg/L) ^c
1	GP100-PCL	31.9 $\pm$ 1.6	0.18	5.3	3.72
2	GP80-PCL	38.5 $\pm$ 2.3	0.12	-0.1	1.87
3	GP40-PCL	57.3 $\pm$ 1.5	0.13	-3.2	1.43
4	GP20-PCL	76.8 $\pm$ 1.2	0.10	-8.2	1.35
5	P(EO)-PCL	88.2 $\pm$ 0.9	0.07	-16.5	1.03

^a Synthesized by sequential post-polymerization modification of PAC-*b*-PCL block copolymer by Michael-type addition reaction with Gal-SH and (EO)₂-SH; ^b The average particle size (nm), size distribution and ξ -potentials measured by DLS at a micelle concentration of 0.2 mg/mL in water; ^c Critical micelle concentration determined using pyrene as a fluorescent probe.

Figure 7.3. Size distribution of biodegradable glycopolymer-PCL micelles with different contents of galactose determined by DLS.

The stability of the micelles in PB (pH 7.4, 10mM) containing 10% FBS at 37 °C was studied by monitoring the particle size in time. The stability of the micelles was confirmed by the absence of large aggregates and preservation of particle size. The size of the micelles remained unchanged for 12 h, indicating that the presence of serum did not affect the stability of the micelles.

7.3.4 Loading and *in vitro* release of DOX

Table 7.2. Characteristics of DOX-loaded biodegradable glycopolymer-PCL micelles with different amounts of galactose at a theoretical DLC of 20 wt.%.

Entry	Micelle	DLC (wt.%) ^a	DLE (%)	Size (nm) ^b	PDI ^b
1	GP100-PCL	17.8	89.2	42.6 ± 2.1	0.20
2	GP80-PCL	17.1	85.5	50.3 ± 1.8	0.17
3	GP40-PCL	16.8	86.2	61.2 ± 2.6	0.15
4	GP20-PCL	16.6	83.0	75.1 ± 1.4	0.17
5	P(EO)-PCL	16.5	82.3	81.5 ± 1.7	0.16

^a DOX loading content determined by fluorescence measurements; ^b Determined by DLS.

DOX was loaded into micelles at theoretical drug loading contents (DLC) of 20 wt.% using a dialysis method. Interestingly, these micelles exhibited similar DOX loading levels (Table 7.2, DLE: 82.3~89.2%),

indicating that the molecular weights and character of the hydrophilic segments had little influence on the drug loading behavior, which preferentially depended on the hydrophobic PCL core. Moreover, the size and size distribution of the micelles were not affected significantly after loading DOX. The diameters of the micelles increased somewhat compared to the blank micelles possibly because of an increase in the inner core volume after loading DOX [45].

The *in vitro* release of DOX from the different micelles (DLC of 5 wt.%) was investigated using a dialysis tube (MWCO 12000) in PB (10 mM, pH 7.4) or acetate buffer (10 mM, pH 5.0) at 37 °C. No burst drug release was observed at both pH's. It is interesting to note that the release of DOX from all the micelles was significantly faster at pH 5.0 than at pH 7.4. For instance, approximately 16.4% and 36.4% DOX was released after 12 h, while 40.3% and 73.1% of DOX was released after 72 h from DOX-loaded GP80-PCL micelles at pH 7.4 and 5.0, respectively (Figure 7.4). This pH-dependent release behavior of DOX was also observed for PEG-*b*-PCL micelles, although with a much lower drug release rate (approximately 30% release in 3 d and 65% in 35 d) [46]. The faster release of DOX from the micelles under acidic conditions, similarly to our previously reported PCL-g-PHEMA graft copolymer micelles, is likely due to improved water solubility of DOX following protonation[47]. It should further be noted that the drug release rate increased with increasing galactose density, in which 80.9%, 73.1%, 57.7% and 50.8% of DOX was released in 72 h at pH 5.0 from GP100-PCL, GP80-PCL, GP40-PCL, and P(EO)-PCL micelles, respectively (Figure 7.4). This is likely because micelles with more galactose decoration have a less densely packed core and are somewhat less stable based on their higher CMC values.

Figure 7.4. *In vitro* release profiles of DOX from DOX-loaded glycopolymer-PCL micelles in PB (10 mM, pH 7.4) or acetate buffer (10 mM, pH 5.0) at 37 °C.

7.3.5 Cellular uptake and anti-tumor activity of DOX-loaded micelles

To evaluate the uptake of micelles by hepatoma cells, we studied the fluorescence of HepG2 cells over-expressing ASGP-R after incubation with DOX-loaded micelles for a period of 4 h. The ASGP-R

receptor of the HepG2 cells can strongly bind galactose residues and induces ASGP-R mediated endocytosis of glycomicelles [48]. The presence of free DOX in the cells after 4 h incubation with micelles was analyzed quantitatively via flow cytometry. After uptake of the DOX-loaded micelles by the HepG2 cells DOX will be released from the core of the micelles and this will induce fluorescence in the cells. The rate of release of DOX from the micelles in the cells will be dependent on the type of micelle (higher Gal decoration, higher release rate) and the pH of the cell compartment, in which the micelles will reside (see also Figure 7.4 for comparison). The contribution of DOX still present in the core of the micelles to the fluorescence of the cells will be negligible due to quenching. In principle, DOX released by the micelles in the medium can also enter the cells by diffusion and can contribute to the cell associated fluorescence. In order to compare the uptake of the different micelles by the HepG2 cells using the fluorescence of free DOX released in the cells it is necessary to compare the fluorescence data with the results of the release of DOX from the micelles in buffer at pH 7.4. From Figure 7.4 it can be seen that there is a built up of the concentration of free DOX during the first 6 h after release. After 6 hours the concentration of free DOX in the medium varies from 12.7 % to 6.4 % of the original overall DOX concentration (DOX incorporated in micelles) and decreases for micelles with decreasing Gal decoration. It has to be realized further that the effective free DOX concentration in the medium used for incubation of the DOX loaded micelles with the cells will be lower than that based on the percentages given above because of the shorter incubation time of 4 h and also because the uptake of DOX loaded micelles by the cells will take place at the same time. It is expected that the uptake of DOX loaded micelles will be more rapid with higher percentages of Gal decoration. At the other hand the release of DOX from micelles with higher percentages of Gal will be faster (compare for Figure 7.4).

Figure 7.5. Flow cytometric analysis of HepG2 cells after 4h incubation with DOX-loaded glycopolymer-PCL micelles or non-glycomicelles (DOX concentration of 5 $\mu\text{g/mL}$, cell counts 10000). (A) Histogram of HepG2 cells after incubation with DOX-loaded GP100-PCL or P(EO)-PCL micelles, respectively; (B) Maximal fluorescence intensities of HepG2 cells after incubation with different DOX-loaded glycomicelles and non-glycomicelles.

The results showed that the cellular content of DOX was much higher (17.1-6.6 fold increase) for glycomicelles as compared to that for Gal-free micelles (Figure 7.5A). It should also be noted that the

cellular concentration of DOX was enhanced significantly when the micelles had a higher Gal-functionality. This is in line with a higher uptake rate of glycomicelles with a higher Gal surface concentration by the cells and also a more rapid release of DOX from glycomicelles with a higher Gal content. For example, the cellular concentration of free DOX was more than 2 times higher for GP80-PCL micelles than that for GP40-PCL micelles (Figure 7.5B), although the release rate of DOX from these micelles in medium at pH 7.4 was not very much different. However, there was no obvious difference between the cellular concentration of DOX for GP80-PCL micelles and GP100-PCL micelles (Figure 7.5B), which indicated that receptor-mediated endocytosis could not be improved any more beyond a certain level of sugar-ligands present in the micelles. These findings further demonstrate the high affinity of glycomicelles for HepG2 cells.

Figure 7.6. Cytotoxicity of glycopolymer-PCL micelles using HepG2 cells (A) and MCF-7 cells (B). The cells were incubated with micelles for 72 h. Data are presented as the average $\pm$ standard deviation ($n = 4$).

MTT assays revealed that micelles were non-toxic to HepG2 and MCF-7 cells with cell viabilities of more than 92 % up to a tested concentration of 0.50 mg/mL (Figure 7.6). The cytotoxicity of free DOX and DOX-loaded micelles was tested using ASGP-R positive HepG2 and ASGP-R negative MCF-7 cells. As shown in Figure 7.7A, at the same DOX concentration, the viability of HepG2 cells was much more efficiently suppressed when micelles with higher Gal-functionality were used. The IC_{50} values (the concentration of DOX that causes 50% inhibition of maximal cell growth) for GP100-PCL, GP80-PCL, GP40-PCL, GP20-PCL, and P(EO)-PCL micelles were estimated to be 0.43, 0.45, 0.75, 2.05 and 6.55 μ g/mL, respectively. The improved drug efficacy of DOX achieved with the glycomicelles was due to the enhanced cellular uptake of DOX containing micelles (Figure 7.5). The IC_{50} value of Gal-decorated micelles with high Gal-density was somewhat higher than that of free DOX ($IC_{50} = 0.20$ μ g/mL), which could be possibly attributed to the attenuated DOX release from the Gal-decorated micelles. However, for ASGP-R negative MCF-7 cells (Figure 7.7B), the cytotoxicity of DOX loaded glycomicelles and DOX

loaded Gal-free micelles at the same DOX concentration was similar, because their cellular uptake rate was also similar. For example, the IC_{50} values for DOX-loaded GP100-PCL, GP80-PCL, GP40-PCL, GP20-PCL glycomicelles were 3.6, 5.0, 5.2, 6.5 and 7.0 $\mu\text{g/mL}$, respectively, which were much higher than those for HepG2 cells.

Figure 7.7. Viabilities of HepG2 cells (A) and MCF-7 cells (B) following 72 h incubation with DOX-loaded glycopolymer-PCL micelles and free DOX as a function of DOX concentration. . All the data are presented as the average $\pm$ standard deviation ($n = 4$).

The anti-tumor activity of DOX-loaded micelles was further evaluated by endocytosis of the micelles by HepG2 cells for 4 h, followed by incubation with fresh culture medium for another 72 h. The cellular uptake of DOX-loaded glycomicelles can take place efficiently by HepG2 cells via receptor-mediated endocytosis. The cell killing efficacy of DOX-loaded glycomicelles using free HepG2 cells was much better than that of DOX-loaded Gal-free micelles, which is in agreement with the results from flow cytometry and intracellular release of DOX. For instance, the cell viabilities of free HepG2 cells (- LBA) incubated with DOX-loaded GP100-PCL, GP80-PCL, GP40-PCL, and GP20-PCL micelles were 38.2, 29.2, 53.4 and 65.2%, respectively (Figure 7.8), which were lower than the cell viability for DOX loaded Gal-free micelles (86.3%). It should be noted that a low cell killing activity was observed for the lactose-blocked HepG2 cells (+ LBA) incubated with either DOX-loaded glycomicelles or with DOX-loaded Gal-free micelles after 72 h, in which the cell viabilities for all the micelles ranged from ca. 76.4-88.7%. It is concluded that the biodegradable glycomicelles have excellent ASGP-R targetability and are highly promising for liver tumor-targeted cancer therapy.

Figure 7.8. Cytotoxicity of DOX-loaded micelles by MTT assay using HepG2 cells. The cells were incubated with free LBA (+ LBA) or without free LBA (- LBA) for 4 h, then the medium was replaced by 90 μ L of fresh DMEM medium and 10 μ L of DOX-loaded micelles or free DOX. DOX dosage was 10 μ g/mL. After 4 h incubation, the medium was replaced by 100 μ L of fresh DMEM medium and the cells were cultured for another 72 h. Data are presented as the average $\pm$ standard deviation ($n = 4$).

7.4 Conclusion

We have demonstrated the successful synthesis of amphiphilic biodegradable glycopolymers with controlled galactose-density by sequential ring-opening polymerization of AC and ϵ -CL, followed by efficient post-polymerization modification with Gal-SH and (EO)₂-SH via a Michael-type addition reaction. These functionalized biodegradable glycopolymers can be self-assembled into glycomicelles in aqueous solutions, which have a mean size below 100 nm with low size polydispersities. These micelles do not show significant cytotoxicity against HepG2 and MCF-7 cells tested in this study. Interestingly, the glycomicelles exhibit excellent cellular uptake by ASGP-R positive HepG2 liver cancer cells with an increase in uptake with increasing Gal-functionality of the micelles. Due to the receptor-mediated uptake DOX-loaded glycomicelles have a significantly higher cytotoxicity towards HepG2 cells, as compared to ASGP-R negative MCF-7 cells and as compared to HepG2 cells blocked by lactobionic acid. Biodegradable glycomicelles provide an interesting platform for targeted and controlled intracellular release of potent chemotherapeutics.

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Chapter 8

In Situ Forming Reduction-Sensitive Degradable Nanogels for Facile Loading and Triggered Intracellular Release of Proteins*

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Abstract

In situ forming reduction-sensitive degradable nanogels were designed and developed based on poly(ethylene glycol)-*b*-poly(2-(hydroxyethyl) methacrylate-*co*-acryloyl carbonate) (PEG-P(HEMA-*co*-AC)) block copolymers for efficient loading as well as triggered intracellular release of proteins. PEG-P(HEMA-*co*-AC) copolymers were prepared with controlled M_n of 9.1, 9.5 and 9.9 kg/mol and varying numbers of AC units per molecule of 7, 9 and 11, respectively (denoted as copolymers **1**, **2** and **3**) by reversible addition-fragmentation chain transfer (RAFT) copolymerization. These copolymers were freely soluble in phosphate buffer but formed disulfide-crosslinked nanogels with defined sizes ranging from 72.5 to 124.1 nm in the presence of cystamine (Cys) via ring-opening reaction with cyclic carbonate groups. The sizes of nanogels decreased with increasing AC units as a result of increased crosslinking density. Dynamic light scattering (DLS) studies showed that these nanogels though stable at physiological conditions were rapidly dissociated in response to 10 mM dithiothreitol (DTT). Interestingly, FITC-labeled cytochrome C (FITC-CC) could be readily loaded into nanogels with remarkable loading efficiencies (up to 98.2 %) and loading contents (up to 48.2 wt.%). The *in vitro* release studies showed that release of FITC-CC was minimal under physiological conditions but significantly enhanced under reductive conditions in the presence of 10 mM DTT with about 96.8 % of FITC-CC released in 22 h from nanogel **1**. In contrast, protein release from 1,4-butanediamine (BDA) crosslinked nanogels (reduction-insensitive control) remained low under otherwise the same conditions. MTT assays showed that these nanogels were non-toxic to HeLa cells up to a tested concentration of 2 mg/mL. Confocal microscopy results showed that nanogel **1** delivered and released FITC-CC into the perinuclei region of HeLa cells following 8 h incubation. CC-loaded reductively degradable nanogels demonstrated apparently better apoptotic activity than free CC as well as reduction-insensitive controls. These *in situ* forming, surfactant- and oil-free, and reduction-sensitive degradable nanogels are highly promising for targeted protein therapy.

8.1 Introduction

In the past decades, protein drugs have emerged as effective treatments for severe human diseases including cancers [1-3]. As compared to chemotherapeutics, protein drugs have merits of high specificity, high therapeutic activity, and low cytotoxicity. The clinical applications of protein drugs, however, encounter several challenges such as swift degradation *in vivo*, possible immune responses, inferior cellular uptake, and poor intracellular trafficking [4]. The clinical success of protein therapy, therefore, relies on the development of safe and efficient delivery systems.

In recent years, various types of polymeric nanocarriers including polyion complex micelles [5-7], polymersomes [8-10], nanocapsules [11-13], and nanogels [14-16] have been developed for protein delivery. Notably, nanogels with advantages of relatively uncomplicated fabrication, high protein loading capacity, high stability, and controlled protein release profile have appeared as one of the most ideal protein nanocarriers [17, 18]. For example, Akiyoshi *et al.* reported that cationic cholesteryl-modified pullulan nanogels mediated effective intracellular delivery of various proteins including β -galactosidase [19], cytokines [20], and cancer vaccines [21]. De Smedt *et al.* reported the controlled release of bovine serum albumin (BSA) and lysozyme from degradable dextran nanogels [22]. Park *et al.* found that heparin-Pluronic nanogels were able to deliver RNase A into HeLa cells giving significant anticancer effects [23]. The co-delivery of paclitaxel and DNase using heparin-Pluronic nanogels exhibited a dose-dependent synergistic cytotoxicity [24]. In particular, bio-responsive nanogels that release cargos in response to endosomal pH or cytoplasmic glutathione (GSH) have received recent interests [25-29]. For instance, Akiyoshi *et al.* reported that pH-sensitive pullulan nanogels released proteins much faster under acidic conditions than at physiological pH [30]. Kataoka and Matyjaszewski developed reduction-sensitive degradable nanogels by inverse miniemulsion atom transfer radical polymerization (ATRP) [31]. Park *et al.* reported that disulfide-crosslinked heparin-Pluronic nanogels with enhanced colloidal stability effectively released encapsulated RNase A inside cells [32]. Chen *et al.* reported a one-step preparation of disulfide-crosslinked PEG-polypeptide nanogels by NCA copolymerization using L-cystine N-carboxyanhydride as a crosslinker [33]. Thayumanavan *et al.* developed reduction-sensitive self-crosslinked nanogels based on a random copolymer containing pendant oligoethyleneglycol and pyridyl disulfide side chains [34, 35]. The disulfide-crosslinked nanogels are of particular interest for intracellular protein release in that they on one hand possess superior colloidal stability and on the other hand are prone to rapid de-crosslinking and dissociation inside cells due to the presence of a high reduction potential in the cytoplasm and nuclei of cells [36, 37]. However, most of the reported nanogels require the use of oil, surfactants, and/or toxic reagents that might lead to protein denaturation/deactivation as well as cytotoxicity concerns. It should further be noted that preformed nanogels also often exhibit inferior drug loading and significant burst release.

In this paper, we report *in situ* forming reduction-sensitive degradable nanogels from PEG-P(HEMA-*co*-AC) block copolymers using cystamine (Cys) as a crosslinker for facile loading and triggered intracellular release of proteins (Scheme 8.1). To the best of our knowledge, this represents a first report on *in situ* forming, surfactant and oil-free, catalyst-free, chemically crosslinked and stimuli-sensitive degradable nanogels. Poly((2-hydroxyethyl) methacrylate) (PHEMA) possesses excellent biocompatibility and is used widely for biomedical applications [38]. The pendant cyclic carbonate units would facilitate

crosslinking of PEG-P(HEMA-co-AC) with Cys in aqueous conditions via ring-opening reaction. Notably, our crosslinking reaction does not involve any catalysts and yield any by-products. These *in situ* forming degradable nanogels could efficiently be loaded with proteins under mild conditions while quickly releasing proteins inside cells. Here, the preparation of *in situ* forming reduction-responsive degradable nanogels, loading and reduction-triggered release of CC, intracellular protein release as well as anti-tumor activity of CC-loaded nanogels were investigated.

Scheme 8.1. Illustration of *in situ* forming reduction-sensitive nanogels for facile loading and triggered release of proteins.

8.2 Experimental section

Materials. Methoxy poly(ethylene glycol) (PEG, $M_n = 5.0$ kg/mol, PDI = 1.03) was purchased from Fluka. PEG was dried by dissolution in anhydrous toluene followed by azeotropic distillation. 5-Methyl-5-prop-2-enoyloxymethyl-1,3-dioxan-2-one (acryloyl carbonate, AC) was synthesized according to our previous report [39]. 4-Cyanopentanoic acid dithionaphthalenoate (CPADN) was synthesized according to the described procedure for 4-cyanopentanoic acid dithiobenzoate [40]. PEG-CPADN was synthesized by an esterification reaction of PEG and CPADN, similar to the synthesis of CPADN-PCL-CPADN macro-RAFT agent [41]. 2-Hydroxyethyl methacrylate (HEMA, 97%, Fluka) was purified by passing through a basic alumina column before use. Azobisisobutyronitrile (AIBN, 98%, J&K) was recrystallized from methanol. Cystamine dihydrochloride (Cys-2HCl, >98%, Alfa Aesar), dithiothreitol (DTT, 99%, Merck), fluorescein isothiocyanate (FITC, 95%, Fluka), cytochrome C from equine heart (CC, Sigma), 1,4-butanediamine (BDA, 99%, J&K) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Amresco) were used as received.

Characterization. ^1H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at

400 MHz using deuterated dimethylsulfoxide (DMSO- d_6) as the solvent. The chemical shifts were calibrated against residual solvent signals of DMSO- d_6 . The molecular weight and polydispersity of copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using THF as the eluent at a flow rate of 1.0 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The size distribution of nanogels was determined using dynamic light scattering (DLS). Measurements were carried out at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He-Ne laser using back-scattering detection. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 µL of nanogel dispersion (0.2 mg/mL) on the copper grid followed by staining with phosphotungstic acid.

Synthesis of PEG-P(HEMA-co-AC) copolymers. PEG-P(HEMA-co-AC) diblock copolymer was synthesized by reversible addition-fragmentation chain transfer (RAFT) copolymerization of HEMA and AC using PEG-CPADN ($M_n = 5.0$ kg/mol) as a macro-RAFT agent and AIBN as an initiator (Scheme 8.2). In a typical example, under a nitrogen atmosphere, HEMA (64 mg, 490 µmol), AC (36 mg, 180 µmol), PEG-CPADN (100 mg, 20 µmol), AIBN (0.164 mg, 1.0 µmol) and 3 mL DMF were added into a 10 mL Schlenk flask. The flask was sealed and placed in an oil bath thermostated at 65 °C. The reaction proceeded with magnetic stirring for 24 h. One sample was taken for the determination of monomer conversions. The resulting copolymers were isolated by precipitation in diethyl ether, filtration and drying *in vacuo* at room temperature. Yield: 65–70 %. ^1H NMR (400 MHz, DMSO- d_6): PEG: δ 3.50, 3.38; HEMA moieties: δ 4.81, 3.90, 3.58, 1.89, 1.01; AC moieties: δ 4.27, 3.90, 1.89, 0.79.

***In situ* formation of reduction-sensitive nanogels.** To a solution of PEG-P(HEMA-co-AC) copolymer (2.0 mg) in phosphate buffer (PB, pH 7.4, 10 mM) at a concentration of 1.0 mg/mL was added Cys in PB (1.0 mg/mL). The molar ratio between amino groups in Cys and cyclic carbonate groups in PEG-P(HEMA-co-AC) copolymer was set at 1/1. The combined solution following adjusting its pH to 8.0 was stirred at 50 °C for 4 h. The size distribution of formed nanogels was determined by DLS and TEM.

Size change of nanogels triggered by DTT. Reduction-sensitive PEG-P(HEMA-co-AC) nanogels were prepared at a concentration of 1.0 mg/mL. The size change of nanogels in response to 10 mM DTT was monitored by DLS. In a typical experiment, to 1.0 mL of nanogel dispersion in PB was added 10 µL of 1.0 M DTT (final DTT concentration is 10 mM). At different time intervals, the size distribution of nanogels was determined. BDA-crosslinked nanogels were used as a reduction-insensitive control.

Encapsulation and triggered release of FITC-CC. To 200 µL of PEG-P(HEMA-co-AC) copolymer solution (5.0 mg/mL) was added varying volumes of CC or FITC-labeled cytochrome C (FITC-CC) solution in PB (pH 7.4, 10 mM) to obtain protein/polymer ratios of 20 wt.%, 50 wt.% and 100 wt.%, respectively. Cys-crosslinked nanogels were formed as described above. Free protein was removed by dialysis (MWCO 500 kDa) against PB (pH 7.4, 10 mM) at room temperature for 10 h. The dialysis medium was refreshed each hour.

In order to determine protein loading contents (PLC), thus prepared protein-loaded nanogels were

treated for 1 h with 100 mM DTT, diluted 1000 times, and analyzed using fluorescence measurements (FLS920, excitation at 492 nm, emission from 510 to 690 nm). The amount of loaded protein was obtained by comparing the fluorescence of the above samples to a calibration curve of known FITC-CC concentrations. PLC and protein loading efficiency (PLE) were calculated according to the following formula:

$$\text{PLC (wt. \%)} = (\text{weight of loaded protein/weight of copolymer}) \times 100 \%$$

$$\text{PLE (\%)} = (\text{weight of loaded protein/weight of protein in the feed}) \times 100 \%$$

Triggered protein release from reduction-sensitive PEG-P(HEMA-*co*-AC) nanogels was investigated using a dialysis method (MWCO 500 kDa) at 37 °C in two different media, *i.e.* PB (100 mM, pH 7.4) and PB (100 mM, pH 7.4) with 10 mM DTT. The protein release from BDA-crosslinked nanogels was used as reduction-insensitive control. At desired time intervals, 5 mL of release media was taken out and replenished with an equal volume of corresponding fresh media. The amount of released protein was determined by fluorescence measurements (FLS920, excitation at 492 nm, emission from 510 to 690 nm). The release experiments were conducted in triplicate.

Activity of released CC by ABTS assay. The activity of CC released from CC-loaded PEG-P(HEMA-*co*-AC) nanogels was examined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. CC was loaded into Cys-crosslinked nanogels as mentioned above and released in PB (pH 7.4, 100 mM) for 2 days. The released CC was collected, quantified by bicinchoninic acid (BCA) method, and diluted to a final concentration of 10 µg/mL using PB (pH 7.4, 100 mM). To CC solution in quartz cuvettes were simultaneously added 20 µL of hydrogen peroxide solution (45 mM) and 200 µL of ABTS solution (1.0 mg/mL) in PB. The absorbance at 410 nm of the oxidized product was monitored for 10 min. Native CC at a concentration of 10 µg/mL in PB (pH 7.4, 100 mM) was used as a control.

MTT assay. The cytotoxicity of PEG-P(HEMA-*co*-AC) nanogels was studied by MTT assays using HeLa cells. The cells were seeded onto a 96-well plate at a density of 1×10^4 cells per well in 100 µL of Dulbecco's Modified Eagle medium (DMEM) containing 10 % FBS and incubated for 24 h (37 °C, 5 % CO₂). The medium was replaced by 80 µL of fresh DMEM medium containing 10 % FBS and 20 µL of various concentrations of nanogel dispersion. The cells were incubated for another 24 h, the medium was aspirated and replaced by 100 µL of fresh medium, and 20 µL of MTT solution (5.0 mg/mL) was added. The cells were incubated for 4 h, and then 100 µL of DMSO was added to dissolve the resulting purple crystals. The optical densities at 570 nm were measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 570 nm with control wells containing only cell culture medium. Data are presented as average $\pm$ standard deviation ($n = 4$).

Confocal microscopy observation of HeLa cells incubated with FITC-CC loaded nanogels. HeLa cells were plated on microscope slides in a 24-well plate (5×10^4 cells/well) using DMEM medium containing 10% FBS. The cells were incubated with predetermined amounts of FITC-CC loaded nanogels or free FITC-CC at 37 °C under 5 % CO₂ atmosphere. After 4 h incubation, the culture medium was removed and the cells on microscope plates were washed three times with PBS. The cells were fixed with 4% paraformaldehyde and the cell nuclei were stained with Hoechst 33342. Fluorescence images of cells

were observed with a Nikon Digital Eclipse C1si Confocal Laser Scanning Microscope (Nikon, CLSM).

Apoptotic activity of CC-loaded nanogels. HeLa cells were plated in a 24-well plate (1×10^5 cells/well) under 5 % CO₂ atmosphere at 37 °C using DMEM medium supplemented with 10% FBS, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 µg/mL) for 24 h. The cells were treated with CC-loaded Cys-crosslinked reduction-sensitive nanogels, CC-loaded BDA-crosslinked reduction-insensitive nanogels or free CC (CC dosage: 80 µg/mL) for 24 h under 5 % CO₂ atmosphere at 37 °C. To quantify the percentage of apoptotic cells, the Annexin V-FITC kit was used as described by the manufacturer (KenGEN, China). Briefly, HeLa cells were detached from the surface with EDTA-free trypsin, washed twice with cold PBS and re-suspended in binding buffer. Then, the cells were stained with 5 µL of Annexin V-FITC solution and 5 µL of PI solution for 15 min at room temperature in the dark. The cells were analyzed immediately by flow cytometry (BD FACSCalibur, Mountain View, CA).

8.3 Results and discussion

8.3.1 Synthesis of PEG-P(HEMA-co-AC) copolymers

PEG-P(HEMA-co-AC) diblock copolymers were prepared by RAFT copolymerization of HEMA and AC in DMF at 65 °C using PEG-CPADN ($M_n = 5.0$ kg/mol, CPADN: 4-cyanopentanoic acid dithionaphthalenoate) as a macro-RAFT agent (Scheme 8.2). CPADN is a particularly versatile RAFT agent, through which we have obtained well-defined diblock [42, 43], triblock [41, 44, 45], and graft [46] copolymers for gene, protein or drug delivery. The conversions of both monomers in each polymerization were over 80% as determined by ¹H NMR. The results of copolymerization are summarized in Table 8.1. ¹H NMR shows clearly besides peaks characteristic of PEG (δ 3.50 and 3.38) also signals attributable to the hydroxyl protons of HEMA units and methylene protons on the cyclic carbonate group of AC units at δ 4.81 and 4.27, respectively (Figure 8.1). The chemical shift of methylene protons on the cyclic carbonate group remained unchanged, indicating that the cyclic carbonate remained intact during copolymerization and subsequent work-up procedures. The average numbers of HEMA and AC units per P(HEMA-co-AC) block could be determined by comparing the intensities of signals at δ 4.81 and δ 4.27 with 3.38 (methoxyl protons of PEG), respectively. Notably, the results demonstrated that the number of AC units per polymer chain increased from 7, 9 to 11 (denoted as copolymer **1**, **2** and **3**) with increasing the amount of AC monomer in the feed (Table 8.1). GPC results showed that these diblock copolymers had unimodal distributions with moderate polydispersities of 1.09-1.30 (Table 8.1), further supporting the successful synthesis of PEG-P(HEMA-co-AC) copolymers.

Scheme 8.2. Synthesis of PEG-P(HEMA-*co*-AC) Diblock Copolymer Using RAFT Polymerization.

Table 8.1. Synthesis of PEG-P(HEMA-*co*-AC) Diblock Copolymers and Nanogels.

Copolymer	HEMA/AC/CPADN ^a		M_n (kg/mol)		PDI	Size (nm)	
	Design	¹ H NMR	¹ H NMR ^b	GPC ^c		Polymer ^d	Nanogel ^e
1	24.6/9/1	20.8/7/1	9.1	7.8	1.09	5.2±0.8	124.1±1.3
2	24.6/12/1	20.8/9/1	9.5	8.3	1.30	8.3±1.1	95.8±2.1
3	24.6/15/1	20.8/11/1	9.9	9.5	1.10	10.5±0.6	72.5±1.8

^a Polymerization was carried out in DMF at 65 °C for 24 h using PEG-CPADN (M_n = 5.0 kg/mol) as a macro-RAFT agent and AIBN as an initiator at three different HEMA/AC/CPADN molar ratios; ^b Estimated by ¹H NMR end-group analysis; ^c GPC was performed using THF as an eluent at a flow rate of 1.0 mL/min at 35 °C and a series of narrow polystyrene standards for the calibration of the columns; ^d Hydrodynamic size of copolymer in PB (pH 7.4, 10 mM) determined by DLS; ^e Hydrodynamic size of *in situ* forming reduction-sensitive nanogels in PB (pH 7.4, 10 mM) determined by DLS.

Figure 8.1. ^1H NMR Spectrum (400 MHz, $\text{DMSO}-d_6$) of Copolymer **2** (Table 8.1, Entry 2).

8.3.2 Formation of reduction-sensitive crosslinked nanogels

PEG-P(HEMA-*co*-AC) copolymers were well-soluble in PB (pH 7.4, 10 mM). DLS studies showed that PEG-P(HEMA-*co*-AC) copolymers had average hydrodynamic diameters ranging from 5.2 to 10.5 nm at a polymer concentration of 1.0 mg/mL (Figure 8.2A), indicating that they exist as a unimer in PB. Disulfide-crosslinked nanogels were prepared by adding equivalent amounts of Cys relative to the AC units into the copolymer solution in PB followed by adjusting the pH of the solution pH to 8.0 and stirring at 50 °C for 4 h. Interestingly, Figure 8.2A shows that nanogels with average sizes varying from 72.5 to 124.1 nm, depending on the amounts of AC units in the copolymers, and moderate polydispersity indices (PDI) of 0.16 - 0.21 were formed. The sizes of nanogels decreased with increasing AC contents, likely due to increased crosslinking densities. TEM micrographs showed that these nanogels had a spherical morphology with particle sizes in accordance with those determined by DLS (Figure 8.2B). We hypothesized that nanogels were formed through nucleophilic ring-opening reaction of cyclic carbonates with the amino groups in Cys. To confirm our hypothesis, a model reaction was carried out by dropwise adding a solution of copolymer **1** in PB into BDA solution (20-fold excess, to prevent crosslinking) at 50 °C for 4 h. The product was isolated and analyzed by ^1H NMR. The results revealed that signals at δ 4.27 due to methylene protons on the cyclic carbonate of AC units vanished (Figure 8.3), indicating complete ring-opening of cyclic carbonate groups. This model reaction confirms that the cyclic carbonates in PEG-P(HEMA-*co*-AC) copolymers undergo rapid ring-opening reaction in the presence of diamines under mild conditions, in line

with a previous report by Endo *et al.* for ring-opening of six-membered cyclic carbonates with diamines [47].

Figure 8.2. Size distributions of PEG-P(HEMA-*co*-AC) copolymers and *in situ* forming reduction-sensitive nanogels in PB (pH 7.4, 10 mM) determined by DLS (A) and TEM image of nanogel **1** stained with phosphotungstic acid (B).

Figure 8.3. ^1H NMR spectrum (400 MHz, $\text{DMSO-}d_6$) of the model reaction product obtained from copolymer **1** and 20-fold BDA.

The reduction sensitivity of Cys-crosslinked PEG-P(HEMA-*co*-AC) nanogels was studied by monitoring the change of nanogel sizes over time in response to 10 mM DTT using DLS. The results showed that nanogel **1** was quickly dissociated in the presence of 10 mM DTT in which a large population

with reduced sizes of about 30 nm and 15 nm was observed in 2 h and 8 h, respectively (Figure 8.4A). In contrast, little size change was detected for BDA-crosslinked nanogels (reduction-insensitive control) in 24 h under the same reducing conditions (Figure 8.4B). Moreover, no size change was observed for nanogel **1** in 12 h in the absence of DTT (Figure 8.4A). It is evident, therefore, that Cys-crosslinked PEG-P(HEMA-*co*-AC) nanogels can be rapidly disrupted under intracellular-mimicking reduction conditions.

Figure 8.4. Size change of reduction-sensitive nanogel **1** (A) and BDA-crosslinked copolymer **1** nanogels (reduction-insensitive control, B) in response to 10 mM DTT measured by DLS.

8.3.3 Loading and Triggered Release of Proteins.

Proteins could be readily loaded into PEG-P(HEMA-*co*-AC) nanogels by mixing PB solutions of copolymer and protein prior to crosslinking with Cys. The unloaded protein was removed by extensive dialysis. It should be noted that lysine amino groups in protein are mostly protonated at pH 8.0 due to their high pK_a of approximately 10. The much higher pK_a of amino groups in protein than Cys ($pK_a = 8.2$) would render little, if present, involvement of protein amino groups in the crosslinking reaction with cyclic carbonate. To confirm this, we performed a control experiment on a mixture of CC and PEG-P(HEMA-*co*-AC) (*i.e.* without Cys) under otherwise the same conditions (pH 8.0, 50 °C, 4 h). DLS showed that nanogels were not formed. Furthermore, trinitrobenzene sulfonic acid (TNBSA) assays revealed that there was essentially no change in the amount of amino groups in protein, supporting absence of reaction between protein and cyclic carbonates under nanogel preparation conditions. It should further be noted that thiol groups of cysteine in CC have formed thioether groups with the heme group and reactions between the disulfide bonds of Cys and thioether groups in CC are not anticipated. The results showed that all PEG-P(HEMA-*co*-AC) nanogels had high loading capacity for FITC-CC, in which AC contents appeared to have little influences (Table 8.2). For example, high PLE ranging from 96.3 to 98.2 % were obtained at a theoretical PLC of 20 wt.%. PLE decreased with increasing theoretical PLC. Notably, a high protein loading content of 48.2 wt.% was accomplished at a theoretical PLC of 100 wt.%. The size of nanogels increased from 134.2 to 182.3 nm with increasing PLC from 19.6 to 47.0 wt.%. The high protein

loading capability of nanogels might be attributed to the hydrophobic and hydrogen bonding interactions between P(HEMA-*co*-AC) blocks with CC, as well as the initial charge interaction between the protonated amino groups in Cys and negatively charged groups in CC. The hydrophobic and charge interactions would, however, decrease during nanogel formation as amino groups in Cys react with cyclic carbonate units. Hence, interactions between protein and “core-shell” nanogels following complete crosslinking would become weak. The proteins could be effectively released from nanogels upon de-crosslinking under reductive conditions. It should further be noted that protein-loaded nanogels could also be prepared at 40 °C for 8 h.

Table 8.2. Characteristics of Protein-Loaded Nanogels. ^a

Nanogel	PLC (wt.%)		PLE (%)	Size ^c (nm)	PDI ^c
	Theory	Determined ^b			
1	20	19.6	98.2	134.2±1.5	0.15
	50	37.3	74.6	145.6± 2.3	0.20
	100	47.0	47.0	182.3±1.3	0.24
2	20	19.2	96.3	102.5±2.4	0.16
	50	36.2	72.4	150.2±2.6	0.15
	100	48.2	48.2	176.6±1.6	0.20
3	20	19.6	98.1	128.4±2.5	0.21
	50	34.8	69.6	156.3±1.6	0.18
	100	45.1	45.1	170.2±2.1	0.24

^a Nanogels were prepared in PB (pH 7.4, 10 mM) at a final concentration of 1 mg/mL; ^b Determined by fluorescence measurements; ^c Determined in PB (pH 7.4, 10 mM) by DLS.

The *in vitro* release of proteins from nanogels was investigated using PB (100 mM, pH 7.4) at 37 °C in the presence or absence of 10 mM DTT. As shown in Figure 8.5A, FITC-CC was rapidly released from nanogel **1** in the presence of 10 mM DTT, in which 50.6 % and 96.9 % of FITC-CC was released in 5 h and 22 h, respectively. The fast protein release is likely due to de-crosslinking and disruption of nanogels under reductive conditions (Figure 8.4A). In contrast, low FITC-CC release (~ 30 %) was observed in 22 h for reduction-insensitive BDA-crosslinked nanogels under otherwise the same conditions as well as for nanogel **1** in the absence of DTT (Figure 8.5A). Nanogels **2** and **3** exhibited similar protein release profiles, in which much faster release of FITC-CC was observed in the presence of 10 mM DTT (Figure 8.5B). Notably, the protein release rate decreased with increasing AC contents wherein 96.9 %, 80.0 % and 55.6 % of FITC-CC was released in 22 h from nanogels **1**, **2**, and **3**, respectively (Figure 8.5B), indicating that the protein release rate from these nanogels could be tuned by crosslinking densities. Similar results were

reported by Thayumanavan *et al.* for the release of Nile red from disulfide-crosslinked nanogels with different crosslinking densities [48]. It should be noted that in all cases no burst release of protein was observed. These results indicate that protein release from disulfide-crosslinked PEG-P(HEMA-*co*-AC) nanogels proceeds in a controlled manner and can be triggered by a reductive environment analogous to that of the intracellular compartments such as the cytosol and the cell nucleus.

Figure 8.5. *In vitro* release of FITC-CC from *in situ* forming reduction-sensitive PEG-P(HEMA-*co*-AC) nanogels in PB (pH 7.4, 100 mM) at 37 °C. (A) Release of FITC-CC from nanogel 1 in the presence or absence of 10 mM DTT. BDA-crosslinked copolymer 1 nanogel was used as a reduction-insensitive control; (B) Release of FITC-CC from nanogels 1, 2 and 3 in the presence or absence of 10 mM DTT.

8.3.4 Intracellular Release of Proteins.

The cellular uptake and intracellular protein release behavior of protein-loaded nanogels was studied using CLSM in HeLa cells. Interestingly, strong fluorescence was observed in the perinuclear region of HeLa cells following 4 h incubation with FITC-CC loaded reduction-sensitive nanogel 1 (Figure 8.6A). In contrast, weak fluorescence was detected in HeLa cells incubated with FITC-CC loaded reduction-insensitive control under otherwise the same conditions (Figure 8.6B), signifying the important role of cleavable disulfide crosslinks for intracellular protein release. As expected, little FITC-CC fluorescence was observed in HeLa cells treated with free FITC-CC, likely due to poor cellular uptake (Figure 8.6C). These confocal observations support that reduction-sensitive PEG-P(HEMA-*co*-AC) nanogels are able to deliver and release proteins into cancer cells.

Figure 8.6. CLSM images of HeLa cells incubated with FITC-CC loaded nanogels and free FITC-CC (60 $\mu\text{g/mL}$) for 4 h. For each panel, the images from left to right showed FITC-CC fluorescence in cells (green), cell nuclei stained by Hoechst 33342 (blue), and overlays of the two images. The scale bars correspond to 30 μm in all the images. A: nanogel **1**; B: BDA-crosslinked copolymer **1** nanogel (reduction-insensitive control); C: free FITC-CC.

8.3.5 Apoptotic Activity of CC-Loaded Nanogels.

It is of great importance for protein delivery systems that released proteins maintain their biological activity. PEG and PHEMA are among the few polymers approved by the U.S. FDA for biomedical uses. Our previous studies have shown that various functional degradable polymers based on AC monomer have low cytotoxicities [39, 49, 50]. MTT assays demonstrated that all reduction-sensitive PEG-P(HEMA-*co*-AC) nanogels were essentially non-toxic to HeLa cells (cell viabilities $\geq 95\%$) up to a tested concentration of 2 mg/mL (Figure 8.7). It should be noted that protein-loaded nanogels were prepared under mild conditions, which unlike most reported nanogels does not involve any oil, surfactant, and catalyst that possibly lead to protein denaturation/deactivation. The enzymatic activity of horse heart cytochrome C (CC) released from nanogel **1** was examined using the ABTS assay [45]. The results showed that there are no significant differences in oxidation of ABTS between CC released from nanogels and native CC, implying that released CC maintains its bioactivity (Figure 8.8).

Figure 8.7. MTT assays of *in situ* forming reduction-sensitive PEG-P(HEMA-*co*-AC) nanogels. HeLa cells were incubated with nanogels for 24 h. Data are presented as average $\pm$ standard deviation ($n = 4$).

Figure 8.8. Oxidation of ABTS catalyzed by native CC and CC released from nanogel 1.

CC is known to play a role in programmed cell death and intracellularly injected CC has shown to induce effective cell apoptosis [51-53]. We reported that reduction and temperature dual-responsive crosslinked polymersomes could deliver CC into MCF-7 cells resulting in enhanced apoptosis. The apoptotic activity of CC-loaded Cys-crosslinked nanogels in HeLa cells was studied using flow cytometry. The results showed that CC-loaded reduction-sensitive nanogels induced obviously more significant cell death than CC-loaded reduction-insensitive control as well as free CC (Figure 8.9). Moreover, the apoptotic activity of CC-loaded Cys-crosslinked nanogels decreased with increasing crosslinking densities, in which about 15 %, 11 %, and 8 % cell death was observed for nanogels 1, 2, and 3, respectively. In comparison, both reduction-insensitive nanogels and free CC caused little apoptosis of HeLa cells under the same conditions (Figure 8.9D, E), in accordance with poor cellular uptake of free CC and minimal intracellular release of CC from reduction-insensitive nanogels. These results confirm that released CC maintains its

apoptotic activity. It should be noted, nevertheless, that anti-tumor activity of CC-loaded PEG-P(HEMA-*co*-AC) nanogels is modest, likely due to inefficient cellular uptake as a result of PEG shielding. The cellular uptake of nanogels could be greatly enhanced by installing a targeting ligand such as folic acid, aptamer, peptide, and antibody fragment [54-56]. These *in situ* forming, surfactant-free, and reduction-sensitive nanogels form an innovative and promising platform for tumor-targeting protein therapy.

Figure 8.9. Contour diagram of Annexin V-FITC/PI flow cytometry of HeLa cells following 24 h incubation with CC-loaded nanogel **1** (A), CC-loaded nanogel **2** (B), CC-loaded nanogel **3** (C), CC-loaded BDA-crosslinked copolymer **1** nanogel (reduction-insensitive control) (D), and free CC (E). CC dosage was set at 80 $\mu\text{g/mL}$.

8.4 Conclusions

We have demonstrated for the first time that reduction-sensitive degradable nanogels are readily formed *in situ* from water soluble PEG-P(HEMA-*co*-AC) diblock copolymers and cystamine in phosphate buffer via nucleophilic ring-opening of cyclic carbonate with amino groups of cystamine. These *in situ* forming nanogels are particularly interesting for intracellular protein delivery in that they offer several unique advantages: (i) they are formed *in situ* under mild conditions, in which no oil, surfactant, and catalyst is required and no byproducts are generated; (ii) they homogeneously encapsulate proteins in a friendly environment and furthermore possess high protein loading capacity; (iii) they exhibit excellent stability with limited protein release under physiological conditions; (iv) they are prone to rapid de-crosslinking and disassociation in an intracellular-mimicking reductive environment, resulting in efficient intracellular protein release; and (v) importantly, released proteins preserve their bioactivity. These

in situ forming reduction-sensitive degradable nanogels provide a novel and promising approach for targeted intracellular protein delivery.

8.5 REFERENCES

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Summary

Biodegradable nanosystems based on functional polycarbonate-based polymers have propelled the development of targeted and controlled drug and gene delivery. Functional polycarbonate-based polymers have overcome many drawbacks which limited the application of the common polyester materials in this area. It is apparent that recently introduced novel functionalities in polycarbonate-based polymers allowed the preparation of novel nanocarriers, which are bioresponsive (*i.e.* pH, temperature, and redox) and which have improved stability (*i.e.* crosslinking and hydrogen bonding) and the ability to conjugate therapeutic agents, targeting ligands and fluorescence probes. Furthermore, functionalized polycarbonate-based polymers could be successfully applied as gene delivery systems.

This thesis focuses on the design of functional cyclic carbonate monomers, which are used to create functional degradable polymers containing versatile pendants, providing interesting platforms for targeted and triggered intracellular release of potent chemotherapeutics.

In **Chapter 1** a general background with regard to the work in this thesis is presented. The current literature on advanced biodegradable nanosystems used for the preparation of intelligent nanocarriers for controlled drug and gene delivery is presented in **Chapter 2**. In this chapter, biodegradable functional polycarbonate-based polymers bearing diverse functionalities such as hydroxyl, carboxyl, amine/urea, alkene, alkyne, halogen, azido and sugars, are discussed with respect to their use for controlled drug and gene delivery. In **Chapter 3** reduction-sensitive polycaprolactone-*graft*-SS-poly(ethylene glycol) (PCL-*g*-SS-PEG) graft copolymers were prepared via the ring-opening copolymerization of ϵ -caprolactone (ϵ -CL) with pyridyl disulfide cyclic carbonate (PDSC), followed by an exchange reaction between PDS-functionalized PCL and thiolated PEG (PEG-SH). Micelles self-assembled by PCL-*g*-SS-PEG copolymer with a diameter of 110~120 nm in phosphate buffer (PB, pH 7.4, 10 mM), while sufficiently stable under physiological conditions were prone to rapid shell-shedding and aggregation under reductive conditions. *In vitro* release studies showed that ca. 82.1 % doxorubicin (DOX) was released in 12 h in a reductive environment mimicking intracellular compartments such as the cytosol and the cell nucleus whereas only ca. 17.5 % DOX was released in 24 h under non-reductive conditions. Confocal microscopy revealed that DOX was delivered into the nuclei of HeLa cells following 8 h incubation with DOX-loaded PCL-*g*-SS-PEG micelles. MTT assays using HeLa cells demonstrated that blank PCL-*g*-SS-PEG micelles were not cytotoxic up to a tested concentration of 1.0 mg/mL while DOX-loaded PCL-*g*-SS-PEG micelles retained high anti-tumor activity with low IC₅₀ (half maximal inhibitory concentration) of 0.82-0.95 μ g DOX equiv./mL. In **Chapter 4** the thiol-disulfide exchange reaction between thiolated galactose (Gal-SH) and PDS-functionalized PCL was used to obtain biodegradable polycaprolactone-*graft*-SS-galactose (PCL-*g*-SS-Gal) copolymer, which was self-assembled to Gal-shielded redox-sensitive nanoparticles for hepatoma-targeted intracellular delivery of DOX. PCL-*g*-SS-Gal nanoparticles while relatively stable under physiological conditions were prone to rapid shell-shedding and aggregation under reductive conditions. *In vitro* release studies revealed that ca. 80.3% DOX was released in 24 h in a reductive environment whereas only ca. 20.7 % DOX was released in 24 h under non-reductive conditions, as well as minimal DOX was

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released from polycaprolactone-*graft*-galactose (PCL-g-Gal) reduction-insensitive nanoparticles under otherwise the same conditions. Flow cytometry and confocal microscopic analyses revealed that cellular uptake and intracellular release of DOX into HepG2 cells *via* asialoglycoprotein receptor (ASGP-R) mediated endocytosis were taken place efficiently using PCL-g-SS-Gal nanoparticles. Enhanced cytotoxicity of DOX-loaded PCL-g-SS-Gal nanoparticles further confirmed that the sensitive micelles are more potent for intracellular drug delivery as compared to the reduction insensitive control.

Redox and pH dual-responsive biodegradable micelles were developed based on PEG-SS-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PEG-SS-PTMBPEC) copolymer and investigated for dually-activated intracellular DOX release (**Chapter 5**). The *in vitro* release studies showed that under physiological conditions DOX release from the micelles was significantly inhibited with only 24.5 % release in 21 h, while the release rate was significantly accelerated at pH 5.0 or in the presence of 10 mM glutathione (GSH) at pH 7.4. The drug release was further boosted under 10 mM GSH and pH 5.0 conditions, with 94.2 % of DOX released in 10 h. DOX release was also facilitated by 2 or 4 h incubation at pH 5.0 and then at pH 7.4 with 10 mM GSH, which mimics the intracellular pathway of endocytosed micellar drugs. MTT assays revealed that DOX-loaded PEG-SS-PTMBPEC micelles had higher anti-tumor activity, with low IC₅₀ values of 0.75 and 0.60 µg/mL for HeLa and RAW 264.7 cells, respectively, following 48 h incubation while blank PEG-SS-PTMBPEC micelles displayed low cytotoxicity up to a concentration of 1.0 mg/mL. In **Chapter 6** reduction-sensitive reversibly core-crosslinked pH-responsive biodegradable micelles were developed based on PEG-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate-*co*-pyridyl disulfide carbonate) (PEG-P(TMBPEC-*co*-PDSC)) copolymers and investigated for intracellular DOX release. PEG-P(TMBPEC-*co*-PDSC) micelles with an average diameter of 58.6 nm were readily crosslinked by disulfide bond formation via the PDSC units after treatment with half the stoichiometrical amount of dithiothreitol (DTT). *In vitro* release studies showed that under physiological conditions only ca. 19.9 % DOX was released from the reversibly crosslinked micelles in 24 h at a relatively low micelle concentration of 40 µg/mL. The drug release was boosted at pH 5.0 and 10 mM GSH, with 98.8 % of DOX released in 12 h. Confocal microscopy indicated that DOX was delivered and released into the nuclei of HeLa cells following 12 h incubation with DOX-loaded reversibly crosslinked micelles. MTT assays revealed that DOX-loaded reversibly crosslinked micelles had significant tumor cell killing activity, with low IC₅₀ values of 1.65 and 1.14 µg/mL for HeLa and RAW 264.7 cells, respectively, following 48 h incubation. The reversibly crosslinked pH-sensitive biodegradable micelles with superior extracellular stability and dually-activated intracellular drug release provide a novel platform for tumor-targeting drug delivery.

Biodegradable glycopolymer-PCL (GP-PCL) block copolymer micelles ('glycomicelles') with tailored galactose functionalities were developed and investigated for hepatoma-targeted drug delivery as described in **Chapter 7**. The glycomicelles had excellent DOX loading capacities, in which high drug loading efficiencies of 83.0-89.2 % were obtained at a theoretical drug loading content of 20 wt.%. Flow cytometry using ASGP-R positive HepG2 liver cancer cells revealed that the DOX concentration in the cells after cellular uptake of DOX-loaded glycomicelles after 4 h incubation was 6.6-17.1 fold higher for micelles

with Gal densities increasing from 20 % to 80 %, as compared to that of non-glycomicelles (100 % substitution with thiolated oligo(ethylene glycol)). MTT assays using HepG2 cells as well as ASGP-R negative MCF-7 cells demonstrated that after 72 h incubation DOX-loaded glycomicelles were taken up more effectively by HepG2 cells than by ASGP-R negative MCF-7 cells. It was also shown that free HepG2 cells were much more efficiently killed by DOX-loaded glycomicelles after incubation for 4 h and subsequent culture for 72 h than HepG2 cells blocked by lactobionic acid, emphasizing the role of ASGP-R mediated endocytosis. In **Chapter 8** *in situ* forming reduction-sensitive degradable PEG-*b*-poly(2-(hydroxyethyl) methacrylate-*co*-acryloyl carbonate) (PEG-P(HEMA-*co*-AC)) nanogels were developed for efficient loading as well as triggered intracellular release of proteins. PEG-P(HEMA-*co*-AC) copolymers were freely soluble in PB but formed disulfide-crosslinked nanogels with defined sizes ranging from 72.5 to 124.1 nm in the presence of cystamine (Cys) via ring-opening reaction with cyclic carbonate groups. DLS studies showed that these nanogels though stable at physiological conditions were rapidly dissociated in response to 10 mM dithiothreitol (DTT). FITC-labeled cytochrome C (FITC-CC) could be readily loaded into nanogels with remarkable loading efficiencies up to 98.2 %. The *in vitro* release studies showed that release of FITC-CC was minimal under physiological conditions but significantly enhanced under reductive conditions in the presence of 10 mM DTT. Confocal microscopy results showed that nanogels delivered and released FITC-CC into the perinuclei region of HeLa cells following 8 h incubation. CC-loaded reductively degradable nanogels demonstrated apparently better apoptotic activity than free CC as well as CC loaded 1,4-butanediamine (BDA) crosslinked reduction-insensitive controls.

Summary

Samenvatting

Biodegradeerbare nanosystemen gemaakt van functionele op polycarbonaat gebaseerde polymeren hebben de ontwikkeling van de doelgerichte en gecontroleerde afgifte van geneesmiddelen en genen enorm gestimuleerd. Met het gebruik van functionele op polycarbonaat gebaseerde polymeren zijn veel nadelen overwonnen, die de toepassing van bestaande en gebruikelijke polyesters in dit gebied hebben beperkt. Het is duidelijk dat recent geïntroduceerde nieuwe functionaliteiten in polycarbonaten het mogelijk gemaakt hebben om nieuwe nanodeeltjes te ontwikkelen, die reageren op biologische signalen, dat wil zeggen reageren op veranderingen in lokale pH, temperatuur en reducerende omstandigheden. De nieuwe functionaliteiten in de polycarbonaten maken het ook mogelijk om nanodeeltjes te vervaardigen met een verhoogde stabiliteit door de vorming van verknopingen en/of waterstofbruggen en verder wordt de mogelijkheid geboden voor het binden van geneesmiddelen, liganden voor het transport van het nanosysteem naar het gewenste doel en/of fluorescente labels, waarmee zowel het transport als de locatie van de nanosystemen kunnen worden gevolgd. Functionele op polycarbonaat gebaseerde polymeren konden ook met succes worden toegepast voor de afgifte van genen.

Dit proefschrift is gericht op het ontwerp van functionele monomeren met een cyclische carbonaatgroep die vervolgens gebruikt kunnen worden voor de vervaardiging van functionele degradeerbare polymeren met verschillende types zijgroepen, waarmee een interessant platform wordt verkregen voor de doelgerichte en gestimuleerde intracellulaire afgifte van cytostatica.

In **Hoofdstuk 1** wordt de algemene achtergrond van het werk beschreven in dit proefschrift gepresenteerd. Een overzicht van de huidige literatuur op het gebied van geavanceerde biodegradeerbare nanosystemen voor de vervaardiging van zogenaamde intelligente nanodeeltjes voor de afgifte van geneesmiddelen en genen wordt gegeven in **Hoofdstuk 2**. In dit hoofdstuk worden functionele biodegradeerbare op polycarbonaat gebaseerde polymeren met verschillende functionele groepen, zoals hydroxyl, carboxyl, amine/ureum, alkeen, alkyn, azido, halogeen en suikers bediscussieerd voor hun mogelijkheden voor gecontroleerde geneesmiddel- en genafgifte.

In **Hoofdstuk 3** worden reductiegevoelige poly(ϵ -caprolacton)-*graft*-SS-poly(ethyleenglycol), (PCL-g-SS-PEG) copolymeren beschreven, die werden verkregen via een ringopening copolymerisatie van ϵ -caprolacton (ϵ -CL) en pyridyl disulfide cyclisch carbonaat (PDSC) gevolgd door een uitwisselingsreactie tussen PCL voorzien van PDS groepen en PEG met een eindstandige thiol groep (PEG-SH). Micellen verkregen via zelfassemblage van PCL-g-SS-PEG copolymeren in fosfaat buffer (PB, pH 7.4, 10 mM) met diameters tussen 110-120 nanometer waren voldoende stabiel onder fysiologische omstandigheden, terwijl zij onder reducerende condities snel hun buitenste schil verloren wat vervolgens aanleiding gaf tot aggregatie. *In vitro* afgiftestudies toonden aan dat over een periode van 12 uren ongeveer 81.2 % doxorubicine (DOX) werd afgegeven vanuit micellen, die zich in een reducerende omgeving bevonden, vergelijkbaar met die van celcompartimenten zoals de cytosol en de celkernen, terwijl over een periode van 24 uren ongeveer 17.5 % DOX werd afgegeven vanuit dezelfde micellen onder niet reducerende condities. Met behulp van confocale microscopie werd aangetoond dat DOX werd afgegeven naar de kernen van HeLa

cellen na 8 uren incubatie met DOX geladen PCL-g-SS-PEG micellen. MTT bepalingen met HeLa cellen toonden aan dat niet geladen PCL-g-SS-PEG micellen niet cytotoxisch waren tot een concentratie van 1.0 mg/ml, terwijl de DOX geladen PCL-g-SS-PEG micellen een sterke antitumor werking lieten zien met IC₅₀ waarden (helft van de maximaal inhiberende concentratie) van 0.82-0.95 µg DOX equiv./mL. In **Hoofdstuk 4** wordt beschreven hoe de thiol-disulfide uitwisselingsreactie tussen gethioleerd galactose (Gal-SH) en PDS gefunctionaliseerd PCL gebruikt kan worden om biodegradeerbare PCL-g-SS-Gal copolymeren te maken. Deze copolymeren konden vervolgens worden gebruikt voor de vervaardiging via zelfassemblage van reductiegevoelige nanodeeltjes, voorzien van galactose moleculen in de buitenschil, waarmee doelgerichte intracellulaire afgifte van DOX in hepatoom cellen tot stand kon worden gebracht. De PCL-g-SS-Gal nanodeeltjes waren relatief stabiel onder fysiologische condities, maar vertoonden een snel verlies van de buitenste schil en aggregatie onder reducerende omstandigheden. *In vitro* afgifte studies lieten zien dat in een reducerende omgeving ongeveer 80.3 % DOX werd afgegeven in 24 uren, terwijl maar ongeveer 20.7 % DOX werd afgegeven onder niet reducerende omstandigheden. Er werd een minimale hoeveelheid DOX afgegeven vanuit DOX beladen niet reductiegevoelige PCL-g-Gal nanodeeltjes onder overigens dezelfde omstandigheden. Flowcytometrie en confocale microscopie werden gebruikt om aan te tonen dat de opname door en intracellulaire afgifte van DOX in HepG2 cellen plaats vond via asialoglycoprotein receptor (ASGP-R) gefaciliteerde endocytose van PCL-g-SS-Gal nanodeeltjes. De relatief hoge cytotoxiciteit van met DOX beladen PCL-g-SS-Gal nanodeeltjes in vergelijking met DOX beladen niet reductiegevoelige PCL-g-Gal nanodeeltjes bevestigde de belangrijke rol van de reductiegevoeligheid van de deeltjes voor de intracellulaire afgifte van cytostatica. Gecombineerde reductie en pH gevoelige biodegradeerbare micellen gebaseerd op PEG-SS-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonaat) (PEG-SS-PTMBPEC) copolymeren werden vervaardigd en geëvalueerd voor duale responsieve intracellulaire afgifte van DOX (**Hoofdstuk 5**).

In vitro studies toonden aan dat de afgifte van DOX onder fysiologische omstandigheden vrij traag verliep met een afgifte van 24.5 % DOX in 21 uren, terwijl de afgifte aanzienlijk werd versneld bij pH 5 of in de aanwezigheid van 10 mM glutathion (GSH) bij pH 7.4. De DOX afgiftesnelheid werd verder verhoogd tot 94.2 % DOX afgifte na 10 uren bij pH 5.0 in de aanwezigheid van 10 mM GSH. De afgifte van DOX werd ook goed gefaciliteerd door eerst de micellen gedurende 2 of 4 uur in een omgeving te brengen van pH 5.0, gevolgd door afgifte bij pH 7.4 in de aanwezigheid van 10 mM GSH. De omstandigheden en het tijdpad in dit traject simuleren het intracellulaire traject dat wordt afgelegd door micellen, die door de cel worden opgenomen via endocytose. Met behulp van MTT bepalingen werd aangetoond dat met DOX beladen PEG-SS-PTMBPEC micellen na 48 uur incubatie met cellen een sterke antitumor werking hadden, met lage IC₅₀ waarden van 0.75 en 0.60 µg/ml voor respectievelijk HeLa en RAW 264.7 cellen, terwijl de niet met DOX beladen micellen tot aan een concentratie van 1.0 mg/mL slechts een zeer lage toxiciteit hadden. In **Hoofdstuk 6** worden pH gevoelige micellen gebaseerd op PEG-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonaat-co-pyridyl disulfide carbonaat) (PEG-P(TMBPEC-co-PDSC)) copolymeren besproken. De micellen hadden een gemiddelde diameter van 58.6 nm. De kern van de micellen kon op een omkeerbare wijze worden vernet door vorming van

disulfide bruggen tussen de PDSC eenheden na behandeling met de helft van de stoichiometrische hoeveelheid dithiothreitol (DTT). Hiermee werden de micellen ook reductiegevoelig. De micellen werden verder onderzocht voor de mogelijkheid van intracellulaire afgifte van DOX. *In vitro* afgifte studies toonden aan dat onder fysiologische condities maar ongeveer 19.9 % DOX werd afgegeven vanuit de omkeerbaar vernette micellen in een tijdsbestek van 24 uren, waarbij een relatief lage micelconcentratie van 40 µg/ml werd gebruikt. De afgiftesnelheid van DOX werd sterk verhoogd bij pH 5.0 en in de aanwezigheid van 10 mM GSH, zodat onder deze condities in 12 uren 98.8 % van de beladen hoeveelheid DOX werd afgegeven. Met behulp van confocale microscopie werd aangetoond dat DOX werd afgegeven in HeLa cellen na 12 uur incubatie met DOX beladen omkeerbaar vernette micellen en dat DOX werd getransporteerd naar de celkernen. Met behulp van MTT testen werd aangetoond dat na 48 uur incubatie van HeLa of RAW 264.7 cellen met DOX beladen omkeerbaar vernette micellen, deze een vrij hoge tumorcel dodende werking hadden, met lage IC₅₀ waarden van 1.65 en 1.14 µg/ml voor respectievelijk HeLa en RAW 264.7 cellen.

Er wordt geconcludeerd dat met deze pH gevoelige, biodegradeerbare, omkeerbaar vernette micellen, die zeer stabiel zijn buiten de cel, maar waarmee binnen de cel via de duale respons van pH en reductie een snelle afgifte van geneesmiddelen kan worden bewerkstelligd, een nieuw platform is gecreëerd voor tumor-gerichte geneesmiddelaafgifte.

In **Hoofdstuk 7** worden biodegradeerbare glycopolymeer-PCL(GP-PCL) blok-copolymeer micellen ('glycomicellen') met verschillende graden van galactose functionaliteit beschreven. De glycomicellen werden onderzocht voor doelgerichte afgifte van cytostatica in hepatoom-cellen. De glycomicellen konden zeer goed beladen worden met DOX met een efficiency van 83.0-89.2 % bij een theoretische belading van 20 gewichtsprocent. Met behulp van flowcytometrie kon worden vastgesteld dat de DOX concentratie in ASGP-R positieve HepG2 leverkanker cellen na vier uur incubatie met DOX beladen glycomicellen aanzienlijk hoger was dan wanneer met DOX beladen niet galactose bevattende micellen (non-glycomicellen) werden gebruikt. De cellulaire DOX concentratie in vergelijking met die na incubatie met DOX beladen non-glycomicellen was 6.6 tot 17.1 keer hoger wanneer DOX beladen glycomicellen met een toenemend galactose dichtheid van 20% - 80% werden gebruikt. Met behulp van MTT testen, waarbij zowel HepG2 cellen als ASGP-R negatieve MCF-7 cellen werden gebruikt kon worden vastgesteld dat na 72 uur incubatie met DOX beladen glycomicellen, deze effectiever door de HepG2 cellen werden opgenomen dan door de ASGP-R negatieve MCF-7 cellen. Verder werd aangetoond dat na incubatie gedurende 4 uren gevolgd door kweken gedurende 72 uren, vrije HepG2 cellen effectiever gedood werden door de met DOX beladen glycomicellen dan HepG2 cellen, waarvan de receptor van te voren geblokkeerd werd met behulp van lactobionzuur. Hiermee wordt nog eens aangetoond dat de ASGP-R receptor een essentiële rol speelt bij de endocytose van de glycomicellen.

In **Hoofdstuk 8** wordt de ontwikkeling van *in situ* vormende reductiegevoelige degradeerbare PEG-*b*-poly(2-hydroxyethylmethacrylaat-*co*-acryloyl carbonaat), (PEG-P(HEMA-*co*-AC)) nanogelen beschreven. Deze nanogelen kunnen op een efficiënte wijze worden beladen met eiwitten, die daarna intracellulair via een stimulus kunnen worden afgegeven. De PEG-P(HEMA-*co*-AC) copolymeren waren

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zeer goed oplosbaar in fosfaat buffer (PB) en vormden disulfide vernette nanogelen met goed gedefinieerde afmetingen variërend van 72.5 to 124.1 nm in de aanwezigheid van cystamine (Cys). Hierbij reageerde Cys met de cyclische carbonaatgroepen van AC. DLS studies toonden aan dat de nanogelen stabiel waren bij fysiologische condities, maar snel uit elkaar vielen in de aanwezigheid van 10 mM DDT. De nanogelen konden met een hoge efficiëntie (98.2 %) worden beladen met FITC-gelabeld cytochroom C (FITC-CC). Hoewel de afgifte van FITC-CC vanuit de nanogelen onder fysiologische condities minimaal was, nam deze zeer sterk toe wanneer de beladen nanogelen geplaatst werden in een reducerende omgeving (10 mM DTT). Met behulp van confocale microscopie werd aangetoond dat na 8 uur incubatie van de HeLa cellen met de beladen nanogelen, FITC-CC werd afgegeven in de “perinuclei” van de cellen. Behandeling van HeLa cellen met CC beladen degradeerbare reductiegevoelige nanogelen leidde tot een hogere apoptose, dan behandeling met vrij CC of met CC beladen 1,4-butaandiamine (BDA) vernette, reductieongevoelige nanogelen.

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Enschede

Curriculum Vitae

Wei Chen was born on 16th April, 1984 in Yixing, P. R. China. In September 2003, he entered the Department of Chemical Engineering and Technology in Huaihai Institute of Technology (Lianyungang, P. R. China). In June 2007, he obtained his Bachelor degree in Chemical Engineering and Technology with the award of Excellent Graduate. In the same year, he started his Master studies in Soochow University majoring in Applied Chemistry (Suzhou, P. R. China). During his Master education, he joined the groups of Polymer Chemistry and Biomaterials (PBM), University of Twente (Enschede, The Netherlands), as an exchange student for a period of three months under the supervision of Professor Jan Feijen and Professor Dirk W. Grijpma. In June 2010, he obtained his Master degree in Applied Chemistry in Soochow University under the supervision of Professor Zhiyuan Zhong with the award of Excellent Graduate and Excellent Master Thesis in Jiangsu Province. From July 2010, he started his Ph.D studies under the supervision of Professor Jan Feijen and Professor Zhiyuan Zhong. During the period from July 2010 to April 2013, he did his research work in the Biomedical Polymers Laboratory, College of Chemistry, Chemical Engineering and Materials Science, Soochow University (Suzhou, P. R. China). From then on, he has taken a research position in the Department of Polymer Chemistry and Biomaterials, Faculty of Science and Technology, University of Twente (Enschede, The Netherlands). His major research interests are: design and synthesis of smart biodegradable polymers, preparation of multifunctional biodegradable polymeric drug nanocarriers, and construction of tissue engineering scaffolds based on functional biodegradable polymers.