Bioreducible Robust Polymersomes for Anticancer Drug Delivery
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ABSTRACT SUMMARY
To minimize the premature drug release of nanocarriers, we have developed chemically cross-linked bioreducible polymersomes (CLPMs) that can specifically release the drug inside cancer cells. The polymersomes were prepared using the biocompatible triblock copolymer, poly(ethylene glycol)-b-poly(lysine)-b-poly(caprolactone) (PEG-b-PLys-b-PCL). For chemical crosslinking of polymersomes, the primary amine of the triblock copolymer was reacted with the disulfide-containing crosslinker. Doxorubicin (DOX), chosen as a model anticancer drug, was effectively encapsulated into the CLPMs. The drug-loaded polymersomes greatly retarded DOX release under physiological condition (pH 7.4), whereas the release rate of DOX increased remarkably in the presence of 10 mM GSH, similar to the intracellular environment.

INTRODUCTION
Polymersomes, composed of biocompatible polymers, have emerged as a promising nanocarrier for drug delivery. Unlike liposomes, prepared from low molecular weight lipids, polymersomes are constructed by amphiphiles of diblock, triblock, and graft copolymers. Owing to their unique architecture, polymersomes offer many advantages including tunable membrane properties, capability to encapsulate large quantity of hydrophilic as well as hydrophobic drugs, prolonged circulation in the blood stream, and preferential accumulation into the tumor tissue via the enhanced permeation and retention (EPR) effect. However, most of polymersomes have suffered from poor structural stability, leading to disintegration upon intravenous administration. In the diluted body condition, the polymer concentration often falls below the critical aggregation level, which leads to the drug release at unwanted sites. Therefore, they neither hold the entrapped drug nor specifically deliver the agents into the tumor tissues. For successful in vivo applications, the polymersomes should be stable with minimal drug release during circulation in the body, followed by enhanced drug release at the tumor site.

Recently, crosslinking the polymersomes has been recognized as a powerful approach to hold the nanostructure in frozen state. In general, cross-linking not only improves the structural stability of the polymersomes but also decreases the release rate of the encapsulated drugs. The most challenging aspect in crosslinking is selection of the cross-linker. For example, the non-degradable cross-linker may prevent the drug release from the polymersomes at the target site, resulting in the reduced therapeutic efficacy. On the other hand, in order to prepare biostable polymeric nanoparticles, several degradable linkers have been used such as disulfide linker and pH-sensitive or hydrolysable ester derivatives. In particular, disulfide-containing crosslinkers have been extensively used for intracellular drug delivery, since they were rapidly reduced under reductive environment of the cytosol. GSH, a thiol containing tripeptide capable of reducing disulfide bonds, is abundant in the cytoplasm of the cell (1-10 mM), whereas it is rarely present in the blood plasma (~ 2 μM).1 This dramatic change in the GSH concentration has encouraged the researchers to develop GSH-responsive vehicles for drug delivery applications. Therefore, the introduction of GSH-responsive crosslinks into the polymersomes may improve the structural stability of the polymersomes and allow programmed drug delivery. In addition, since polymersomes have the capability to load both of hydrophilic and hydrophobic agents, GSH-responsive biostable polymersomes may be useful for combination therapy to enhance antitumor efficacy. Although there have been many reports on CLPMs, most studies involve carriers based on non-degradable polyacrylate or polyacrylamide derivatives. Until now, no literatures are available for bioreducible CLPMs. Herein, we describe synthesis and physicochemical characterization of CLPMs using a disulfide crosslinker to produce robust polymersomes that can preferentially release DOX in response to the intracellular GSH.

EXPERIMENTAL METHODS
PEG-b-PLys-b-PCL triblock copolymers were synthesized according to the previously reported procedure.2 In brief, lysine N-carboxy anhydride (NCA) (0.85 g, 2.8 mmol) was added to the PEG-NH2 (1 g, 0.2 mmol) solution in DMF under a nitrogen atmosphere and stirred at 35 °C. After 24 h, nitrophenyl-activated poly(caprolactone) (PCL-ONPC) was dissolved in DMF and added to the reaction mixture. The reaction was continued for a further 24 h. PEG-b-PLys(Z)-b-PCL (0.75 g, 0.19 mmol) was isolated by precipitation with excess diethyl ether. Then, the copolymers were treated for 24h with trifluoroacetic acid and HBr/acetic acid to remove carbobenzyloxy group. The solution was then purified with a dialysis membrane against distilled water (MWCO=1000) for two days, followed by lyophilization to obtain triblock copolymer. For chemical crosslinking, the polymersome was suspended in a PBS at a concentration of 1 mg/ml, followed by addition of 3, 3′-dithiobis (sulfosuccinimidylpropionate) (DTSSP) solution. The resulting solution was stirred for 3 h at pH 9. Thereafter, the solution was purified with dialysis membrane (MWCO=3500) against distilled water for 1 day to remove unreacted DTSSP. Degree of cross-linking
was controlled by varying the feed molar ratio of DTSSP to Lys.

DOX-loaded polymersomes were prepared by the solvent casting method. In brief, PEG-\(b\)-PLys-\(b\)-PCL triblock copolymer was dissolved in a chloroform/methanol (1v:1v) mixture. DOX-HCl in chloroform/triethylamine (1v:1v) was added to the polymer solution and stirred for 3 h. Then, the solvent was completely removed using a rotary evaporator to form a thin film. PBS (pH 7.4) was added and the solution was stirred for an additional 1 h, followed by filtration through a 0.45 µm filter to remove unloaded DOX. The solution was dialyzed against distilled water (MWCO = 3,500 Da) for one day and lyophilized to obtain the DOX-loaded polymersomes (DOX-NCPM). For the preparation of DOX-loaded cross-linked polymersomes, DOX-NCPM was reacted with the DTSSP for 3 h at pH 9.0, in which a feed molar ratio of DTSSP to Lys was varied from 1:2 to 1:1. Then, the solution was dialyzed against distilled water for 6 h followed by lyophilization to obtain DOX-loaded CLPMs (DOX-CLPM1 and DOX-CLPM2). For the release experiments, DOX-loaded polymersomes were dispersed in a PBS (pH 7.4), and the solutions were transferred to cellulose membrane tubes (MWCO = 3,500 Da). The dialysis tubes were then immersed in a PBS (pH 7.4) with or without GSH (2 µM, 1 mM, or 10 mM). Each sample was gently shaken in a 37 °C water bath at 100 rpm. The medium was refreshed at predetermined time intervals, and the DOX concentration was determined using UV-vis spectroscopy at 485 nm.

RESULTS AND DISCUSSION

GSH-sensitive CLPMs were synthesized to deliver the active agents into the cytosol of the cancer cell. To prepare a polymersome nanotemplate for crosslinking, PEG-\(b\)-PLys-\(b\)-PCL triblock copolymer was prepared. Each block in the copolymer plays a significant role in CLPMs. PEG was chosen as hydrophilic part to protect nanocarrier from proteolytic digestion and offers the stealth character in vivo. The PLys block with primary amine group was used for crosslinking, and PCL was selected as a biodegradable hydrophobic block to encapsulate the hydrophobic drugs. The synthesized copolymer was characterized using \(^1\)H NMR. From the result, the composition ratio of ethylene glycol, lysine, and caprolactone was found to be 113:11:37. Based on the molecular composition, the triblock copolymer was denoted as PEG_{113}-\(b\) PLys_{11}-\(b\) PCL_{37}. Crosslinking of the polymersome was performed by adding DTSSP to an aqueous solution of PEG_{113}-\(b\) PLys_{11}-\(b\) PCL_{37}. The crosslinking took place at the interface of the hydrophobic membrane by the reaction between the primary amine of lysine and DTSSP. The degree of crosslinking was precisely controlled by varying the feed molar ratio of DTSSP and Lys, and the obtained CLPMs were denoted as CLPM1 and CLPM2. The non-crosslinked polymersome was labeled as NCPM. As the feed ratio of DTSSP to Lys increased, the content of primary amine in the polymersomes decreased, which was observed from the \(\zeta\) values. The size distribution of NCPM and CLPMs are shown in Fig. 1. As expected, all the copolymers formed uniform polymersomes with unimodal size distributions. TEM images indicated that the morphological structure of polymersomes was not significantly changed by chemical crosslinking. Confocal laser scanning microscopy (CLSM) images of large polymersomes containing nile red revealed the clear location of the dye in the hydrophobic membrane, thus forming a fluorescent ring (Fig. 2). From the drug release tests, it was observed that CLPMs could release DOX in a sustained manner in a PBS (pH 7.4), whereas DOX was rapidly released at the intracellular environments containing GSH.

![Figure 1](image1.png)

**Figure 1.** Size distributions of NCPM and CLPMs. The insets are for TEM images of each polymersomes.

![Figure 2](image2.png)

**Figure 2.** CLSM images of NCPM and CLPM2 encapsulated with nile red. Graph represents the fluorescent intensity profiles.

CONCLUSION

A triblock copolymer (PEG-\(b\)-PLys-\(b\)-PCL), forming uniform polymersomes in an aqueous solution, was prepared as the potential carrier for anticancer drugs. To improve the stability of polymersomes and control the drug release pattern, they were chemically modified using the GSH-cleavable crosslinker. The results have demonstrated that CLPMs were highly stable and release DOX specifically at the intracellular environments.

REFERENCES

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