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Biostable and bioreducible polymersomes for intracellular delivery of doxorubicin

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Abstract

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. Polymersomes were prepared using poly(ethylene glycol)-b-poly(lysine)-b-poly(caprolactone), a biocompatible triblock copolymer. To chemically cross-link the polymersomes, the primary amine of the triblock copolymer was reacted with a disulfide-containing cross-linker. Doxorubicin (DOX) was chosen as a model anti-cancer drug, and was effectively encapsulated into the CLPMs. The drug-loaded polymersomes greatly retarded the release of DOX under physiological conditions (pH 7.4), whereas the release rate of DOX increased remarkably in the presence of 10 mM glutathione, mimicking an intracellular environment. Microscopic observation showed that DOX-loaded CLPMs could effectively deliver the drug into intracellular level of SCC7 cancer cells, leading to high cytotoxicity. These observations suggest that CLPMs is a promising nanocarrier for intracellular DOX delivery.

Key words: Polymersomes, cross-linking, DOX, GSH, and drug delivery
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 macromolecular amphiphiles of diblock, triblock, or graft copolymers. Owing to their unique architecture, polymersomes offer multiple advantages including tunable membrane properties, capability to encapsulate both hydrophilic and hydrophobic drugs, prolonged circulation in the blood stream, and preferential accumulation into tumor tissue via the enhanced permeation and retention (EPR) effect. However, most polymersomes have suffered from poor structural stability, which leads to disintegration upon intravenous administration. In the diluted body condition, the polymer concentration often falls below the critical aggregation level, resulting in rapid drug release at unwanted sites. Therefore, they neither hold the entrapped drug nor specifically deliver the agents to the tumor tissues. For successful in vivo applications, the polymersomes should be stable with minimal drug release during circulation, followed by enhanced drug release at the tumor site.

Recently, the cross-linking of polymersomes has been recognized as a powerful approach to hold the nanostructure in a 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 cross-linking is the selection of the cross-linker. For example, the non-degradable cross-linker may prevent the drug release from the polymersomes even at the target site, resulting in reduced therapeutic efficacy. In order to prepare biostable nanoparticles which enable the drug release at the target site, several degradable linkers have been recently investigated such as disulfide linkers and pH-sensitive or hydrolysable ester derivatives. In particular, disulfide-containing cross-linkers have been extensively used
for intracellular drug delivery, since they are rapidly reduced in the reductive environment of cytosol. Glutathione (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 blood plasma (~2 µM). This dramatic difference in the GSH concentration has encouraged researchers to develop GSH-responsive vehicles for drug delivery applications. Therefore, the introduction of GSH-responsive cross-links into the polymersomes may improve the structural stability of the polymersomes and allow for programmed drug delivery. In addition, since polymersomes have the capability to load both 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 chemically cross-linked bioreducible polymersomes (CLPMs), studies have used carriers based on non-degradable polyacrylate or polyacrylamide derivatives. Until now, there have been no reports about CLPMs based on biodegradable and biocompatible polymers. Herein, we describe the synthesis of CLPMs, composed of poly(ethylene glycol)-b-poly(lysine)-b-poly(caprolactone) (PEG-b-PLys-b-PCL), using a disulfide cross-linker to produce robust polymersomes that can preferentially release doxorubicin (DOX) inside cancer cells (Fig. 1). In contrast to the conventional cross-linked polymersomes based on non-degradable block copolymers, our system is composed of biocompatible and biodegradable copolymers. In this system, PEG can be eliminated from the body through renal excretion, whereas PLys and PCL are degraded into small molecular fragments after performing their roles as drug carriers.

The physiochemical characteristics of the CLPMs were determined using $^1$H NMR, dynamic light scattering (DLS), and transmission electron microscopy (TEM). In vitro release behavior of DOX from CLPMs was measured in the presence and absence of GSH. In addition,
the cytotoxicity and intracellular drug release behavior of polymersomes were evaluated using the SCC7 cancer cells.

**Experimental procedures**

**Materials**

- α-Methoxy-ω-amino PEG (mPEG-NH₂, \( M_n = 5000 \) g/mol), N6-carbobenzyloxy-L-lysine, ε-caprolactone, stannous octoate, 4-nitrophenyl chloroformate, doxorubicin-hydrochloride (DOX·HCl), trifluoroacetic acid (TFA), 5, 5′-dithiobis-(2-nitrobenzoic acid) (Ellman’s reagent), fluorescamine, hydrobromic acid solution in acetic acid (HBr/AcOH), sodium dodecyl sulfate (SDS), and triphosgene were purchased from Sigma-Aldrich co. (St. Louis, MO, USA). 3, 3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) was obtained from Thermo Scientific (Rockford, IL, USA). The water used in the experiments was prepared by an AquaMax-Ultra water purification system (Younglin Co., Anyang, Korea). All other chemicals were of analytical grade, and used without further purification. Nitrophenyl-activated poly(caprolactone) (PCL-ONPC) was synthesized according to a previously reported procedure.²⁵

**Synthesis of N6-carbobenzyloxy-L-lysine N-carboxyanhydride (Lysine NCA)**

Lysine NCA was synthesized by the Fuchs-Farthing method using triphosgene.²⁶ In brief, N6-carbobenzyloxy-L-lysine (5 g, 17.84 mmol) was suspended at 50 °C in THF. Triphosgene (2.1 g, 7.13 mmol) was slowly added to the solution, after which the reaction mixture was stirred for 3 h. The clear solution obtained was poured into n-hexane, cooled to -20 °C, and stayed
overnight. After the precipitate was filtered, it was washed with n-hexane and dried for one day at room temperature under vacuum.

**Synthesis of PEG-b-PLys-b-PCL triblock copolymer**

The PEG-b-PLys-b-PCL triblock copolymer was synthesized by sequential one-pot synthesis, as previously described.² Lysine NCA (0.85 g, 2.8 mmol) was added to mPEG-NH₂ (1 g, 0.2 mmol) solution in DMF under a nitrogen atmosphere and stirred at 35 °C. After 24 h, PCL-ONPC in DMF was added to the above reaction mixture, and the reaction was continued at the same temperature for an additional 24 h. PEG-b-PLys(Z)-b-PCL (0.75 g, 0.19 mmol) was isolated by precipitation with excess diethyl ether. Then, deprotection of the carbobenzyloxy group was carried out by treating the block copolymers with TFA and HBr/AcOH for 24 h at room temperature. The resulting solution was purified with a dialysis membrane against distilled water (MWCO = 1,000 Da) for two days, followed by lyophilization. The triblock copolymers obtained could form nano-sized polymersomes under physiological conditions (PBS, pH 7.4) at a concentration of 1 mg/ml. The chemical structure and molecular weight of the PEG-b-PLys-b-PCL triblock copolymer were characterized using ¹H NMR.² The molar composition ratio of EG, Lys, and CL in the copolymer was found to be 113:11:37.

**Cross-linking of polymersomes**

For chemical cross-linking, polymersomes were suspended in a PBS (1 mg/ml), DTSSP was slowly added, and the reaction mixture was stirred for 3 h at pH 9.²⁷,²⁸ The resulting solution was purified with a dialysis membrane (MWCO = 3,500 Da) against distilled water for one day to remove unreacted DTSSP and by-products. The degree of cross-linking was
controlled by varying the feed molar ratio of DTSSP to the lysine unit of the triblock copolymer ([DTSSP]: [Lys] =1:2 or 1:1). During the cross-linking process, the consumption of amine groups in the lysine units was observed using a zeta potential (ζ) measurement. Depending on the feed molar ratio of DTSSP to Lys repeating units (1:2, 1:1), the obtained CLPMs were denoted as CLPM1 and CLPM2, respectively. The non-cross-linked polymersome was denoted as NCPM. The cross-linking reaction was also monitored using fluorescamine assay.

**Stability of cross-linked polymersomes**

The stability of NCPM or CLPMs was investigated by interacting them with SDS which acts as a destabilizing agent in aqueous media. In brief, the SDS solution (5 mg/ml) was added to an aqueous solution of NCPM or CLPMs (1 mg/ml), and the solution was stirred at room temperature. The scattering light intensity of the polymersomes in the SDS solution was monitored at predetermined time intervals.

**Characterization**

The chemical structures of the polymers were characterized using $^1$H NMR (JNM-AL300, JEOL, Tokyo, Japan) operating at 300 MHz, for which the samples were dissolved in DMSO-$D_6$ or $CDCl_3$. The sizes of the polymersomes were determined at 25 °C using a FPAR-1000 fiber optics particle analyzer (Otsuka Electronics, Osaka, Japan). The morphology of the particles were observed using a TEM (JEM-2100F, JEOL, Tokyo, Japan), operated at an accelerating voltage of 200 keV. For TEM images, samples were dispersed in distilled water (1 mg/ml) and dropped onto a 200-mesh copper grid. The ζ and scattering light intensity measurement were
performed using a 90 PLUS particle size analyzer (BrookHAVEN Instruments Cooperation, New York, USA).

The critical aggregation concentration (CACs) of the copolymers were evaluated using fluorescence spectroscopy in the presence of pyrene molecules. In brief, a pyrene solution (12 × 10⁻⁷ M) was prepared in distilled water, which was then mixed with the polymersome solution to obtain a polymer concentration ranging from 1.0 × 10⁻⁴ to 2.5 mg/ml. The final concentration of pyrene in each sample was fixed at 6.0 × 10⁻⁷ M. The fluorescence spectra were recorded using an ISS K2 multi-frequency phase and modulation fluorometer (ISS, Champaign, IL, USA). The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 334 nm and 390 nm, respectively.

To observe the morphology of the polymersomes using CLSM, giant polymersomes were prepared according to a previously reported procedure. In brief, Nile Red (0.2 mg) in acetone was slowly added to the copolymer solution (10 mg/ml) in de-ionized water, and acetone was evaporated to allow the Nile Red entrapment in the hydrophobic membrane. A drop of the solution was placed on a confocal dish, prior to imaging. The fluorescent image was observed using a LSM 510 META NLO confocal laser scanning microscope equipped with a fluorescence filter set for rhodamine.

The cleavage of the disulfide bond in CLPMs was monitored using a UV-vis spectrophotometer (Optizen 3220UV, Mecasys Co., Ltd, Daejeon, Korea) in the presence of Ellman’s reagent. In brief, the CLPMs were reduced for 10 min in the presence of 10 mM GSH, followed by dialysis against excess amount of water to remove GSH. Thereafter, the sample was treated with Ellman’s reagent to determine the free thiol groups by using a UV-vis spectroscopy.
**Preparation of DOX-loaded polymersomes**

DOX-loaded polymersomes were prepared by the solvent casting method. In brief, the PEG-b-PLys-b-PCL triblock copolymer was dissolved in a chloroform/methanol (1v:1v) mixture. DOX·HCl in chloroform and 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. Then, the solution was dialyzed against distilled water (MWCO = 3,500 Da) for one day, followed by lyophilization to obtain the DOX-loaded NCPM (DOX-NCPM). For the preparation of DOX-loaded CLPMs, DOX-NCPM was further reacted with DTSSP for 3 h at pH 9.0. The feed molar ratio of DTSSP to the lysine unit of the triblock copolymer was 1:2 or 1:1. After the reaction, the solution was dialyzed against distilled water for 6 h, followed by lyophilization to obtain DOX-loaded CLPMs (DOX-CLPM1, DOX-CLPM2). The loading efficiency and content of DOX in polymersomes were determined using a UV-vis spectrophotometer at 485 nm (Optizen 3220UV, Mecasys Co., Ltd, Daejeon, Korea). For this experiment, DOX-loaded polymersomes were dissolved in a DMSO/DMF (1v/1v) mixture, and the calibration curve was obtained using DMSO/DMF (1v/1v) solutions with different DOX concentrations. The loading efficiency and loading content of DOX were calculated using the following formula:

\[
\text{Loading efficiency} (%) = \left( \frac{\text{weight of loaded drug}}{\text{weight of drug in feed}} \right) \times 100\%
\]

\[
\text{Loading content} (%) = \left( \frac{\text{weight of loaded drug}}{\text{weight of polymer}} \right) \times 100\%
\]

**In vitro release behavior of DOX from polymersomes**

DOX-loaded polymersomes (1 mg/ml) 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 30 ml of PBS (pH 7.4) or with different GSH solutions (2 µM, 1 mM, and 10 mM). Each sample was gently shaken at 100 rpm in a water bath with a temperature of 37 °C. The medium was refreshed at predetermined time intervals, and the DOX concentration was determined using UV-vis spectroscopy at 485 nm.

**Cytotoxicity and intracellular drug release**

SCC7 (squamous carcinoma) cell lines obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum and 1% (w/v) penicillin-streptomycin at 37 °C in a humidified 5% CO₂-95% air atmosphere. The cells were seeded at a density of 1×10⁴ cells/well in 96-well flat-bottomed plates. After one day of growth, the cells were washed twice with PBS (pH 7.4) and incubated for 24 h with various concentrations of polymersomes or DOX-loaded polymersomes. The cells were then washed twice with PBS to remove any remaining drug, and fresh culture medium was added. Twenty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/ml in PBS) were added to each well, and the cells were incubated for an additional 4 h at 37°C. Subsequently, the medium was removed, and the cells were dissolved in DMSO. The absorbance at 570 nm was measured using a microplate reader (BioTek, Seoul, Korea).

To observe the intracellular drug release behavior of polymersomes, the cells were incubated for 3, 6, and 12 h with DOX-CLPM2 or free DOX. The cells were then washed twice with PBS (pH 7.4) and fixed with 4% formaldehyde solution. For nuclear staining, the cells were incubated with 4,6-diamino-2-phenylindole (DAPI) for 10 min at room temperature, followed
by washing with PBS (pH 7.4). The intracellular localization of DOX was observed using a LSM 510 META NLO confocal laser scanning microscope.

**Statistical analysis**

The statistical significance of differences ($p < 0.05$) between groups tested was calculated using one-way ANOVA.

**Results and discussion**

**Synthesis of PEG-b-PLys-b-PCL**

In an attempt to prepare biostable polymersomes, an ABC-type biocompatible and biodegradable triblock copolymer was introduced. Each block in the triblock copolymers is expected to play a role in generating a robust CLPM. The PEG shell will protect the polymersomes from intermolecular bridge formation during the cross-linking process, which is a major drawback associated with AB-type block copolymers.\textsuperscript{13, 32, 33} PCL can act as a hydrophobic membrane that separates the aqueous core and enables encapsulation of hydrophobic drugs. The PLys middle block can be used for disulfide cross-linking of the polymersomes. As a consequence, it is expected that CLPMs are highly stable at the extracellular environments, whereas they can be reduced by a high concentration of GSH in the cytoplasm upon endocytosis, causing rapid release of the payloads (Fig. 1).

To prepare a polymersome nanotemplate for cross-linking, an ABC-type triblock copolymer of PEG-b-PLys-b-PCL was synthesized according to a previously reported procedure (see ESI, Fig. S1 and S2).\textsuperscript{2} Owing to its amphiphilic nature, the triblock copolymer formed
uniform polymersomes in an aqueous solution, consisting of hydrophobic PCL membrane and hydrophilic PEG-b-PLys aqueous core. From the fluorescent spectra (see ESI, Fig. S4a), it was found that as the concentration of copolymer increased, the fluorescence intensity of pyrene increased, indicating the aggregation of pyrene into hydrophobic membrane of polymersomes. The CAC of the copolymer estimated from pyrene emission spectra was found to be 0.025 mg/ml (see ESI, Fig. S4b), which was lower than that of other copolymer forming polymersomes.\textsuperscript{34}

**Preparation and characterization of CLPMs**

The cross-linking of the polymersome was performed by adding DTSSP to an aqueous solution of PEG-b-PLys-b-PCL (see ESI, Fig. S3). The cross-linking took place at the interface of the hydrophobic membrane by reaction between the primary amine of lysine and DTSSP. The degree of cross-linking was precisely controlled by varying the feed molar ratio of DTSSP to Lys repeating units. As the feed ratio of DTSSP to Lys increased, the content of primary amine in the polymersomes decreased, resulting in lower $\zeta$ values (Table 1). After cross-linking, the $\zeta$ values of CLPM1 and CLPM2 were 10 and 7 mV, respectively, which were much lower than that of NCPM ($\zeta=42$ mV).\textsuperscript{28} The extent of decrease in $\zeta$ indicated the conversion of primary amine groups into amide during the cross-linking process. In addition, decrease in the amine content also supported the cross-linking of the polymersomes. The size distribution and morphologies of NCPM and CLPMs are shown in Fig. 2. 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 cross-linking. 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 and see ESI, Fig. S5). After cross-linking, the size of the polymersomes decreased, which is consistent with the size distribution results. As shown in Fig. 3, the mean diameter of all the polymersomes did not change significantly over a period of 7 days in physiological buffer (pH 7.4).

**Stability of CLPMs**

The stability of the thermodynamically frozen CLPMs and NCPM were investigated against SDS which acts as a destabilizing agent (Fig. 4). After the preparation of polymersomes in aqueous solution (1 mg/ml), each polymersome was mixed with an aqueous solution of SDS (5 mg/ml), and the scattering intensity was measured as a function of time. As expected, NCPM showed a drastic decrease in scattering light intensity within 10 min. After 1 h, the intensity was further decreased to 65%. The autocorrelation function and polydispersity of the NCPM could not be found 30 min after the SDS treatment. This result indicated that the SDS induced disintegration of the NCPM. In contrast, the CLPMs showed minimal decreases in scattering intensity, indicating the enhancement of the stability of the polymersomes after cross-linking. In particular, greater than 95% scattering intensity was observed for CLPM2, even after 3 h of incubation in SDS. This behavior was also observed from the disulfide-crosslinked micelles. When CLPM2 was exposed to 10 mM GSH for 10 min, the disulfide bonds were rapidly cleaved, implying that CLPMs would be disintegrated at the intracellular environment (Fig. S6).

*In vitro drug release*
To investigate the drug release characteristics, DOX was chosen as a model anti-cancer drug because it is known to interact with intracellular DNA via intercalation and inhibit the growth of macromolecular biosynthesis.\textsuperscript{37} To enhance the therapeutic efficacy of DOX with reduced systemic toxicity, it should be delivered inside cancer cells. In this study, DOX was encapsulated into the polymersome by the solvent casting method.\textsuperscript{30, 31} The loading efficiency and content of the DOX-NCPM were 63\% and 6.3 wt\%, respectively. Further, the cross-linking of DOX-NCPM was carried out to obtain DOX-loaded CLPMs (DOX-CLPM1, DOX-CLPM2). Irrespective of crosslinking, loading efficiencies of DOX were always greater than 55\%, and the mean diameter of the polymersomes decreased following drug encapsulation, implying formation of compact structure by hydrophobic interactions between the PCL membrane of polymersome and DOX (Table 2).

Fig. 5 shows the release behavior of DOX from NCPM and CLPMs in the absence and presence of GSH. The release profile in the absence of GSH showed that the CLPMs more efficiently hold most of the drugs compared to NCPM (Fig. 5a). In particular, as the degree of cross-linking increased, the drug release from the polymersomes was greatly retarded. This may further support our assumption that the CLPMs would minimize the loss of encapsulated drug before reaching the target site. Since DOX-CLPM2 showed a great retardation in drug release in physiological conditions, it was chosen as a candidate for further experiments. Fig. 5b shows the drug release behavior of DOX-CLPM2 in the presence of GSH. At a GSH concentration of 2 \(\mu\)M, mimicking the extracellular GSH level, the release pattern was close to that in physiological buffer conditions. Interestingly, as the GSH concentration increased to the cytoplasmic level, the drug release was gradually facilitated. In particular, at 10 mM GSH, the DOX release of CLPM2 was similar to that of NCPM at 24 h (Fig. 5a). These results indicate that the drug release at the
extracellular environment is greatly retarded from the CLPM2, whereas rapid drug release occurs upon reaching the cytoplasm. This is primarily due to the degradation of the disulfide cross-linker disturbing the frozen state of the polymersome. Overall, it could be conclude that the CLPMs not only minimize the drug loss at unwanted sites, but also facilitate the drug release at the intracellular level for improving therapeutic efficacy.

**In vitro cell cytotoxicity and intracellular drug release**

Fig. 6 shows the cytotoxic effect of bare polymersomes and DOX-loaded polymersomes on SCC7 cells, as evaluated using an MTT assay. Owing to their biocompatibilities, all the polymersomes did not exhibit cytotoxicity to SCC7 cells. In particular, most cells were viable up to 500 µg/ml of CLPMs, indicating that chemical cross-linking with the disulfide bond did not exhibit cytotoxicity. The *in vitro* cytotoxicities of DOX, DOX-NCPM, and DOX-CLPM2 were estimated to observe the effect of cross-linking on DOX-loaded polymersomes. DOX-CLPM2 shows lower cytotoxicity compared to DOX-NCPM and DOX, which is probably due to the gradual release of DOX from stabilized polymersomes. DOX-NCPM without cross-linking starts releasing DOX immediately upon placement in the culture media. As such, the released DOX could be accumulated in the cell nucleus within a short period of time. In contrast, the DOX release rate from DOX-CLPMs was slower, because the DOX release was accelerated after entering into the intracellular level. This specific nature of the material may not only minimize the drug loss in blood, but also lead to selective accumulation in tumor tissue by the EPR effect.\(^{38,39}\) Recently, it was also demonstrated that the bioreducibly cross-linked micelles showed enhanced therapeutic efficacy *in vivo*.\(^ {27}\) Finally, intracellular drug release of the DOX-loaded polymersomes was observed using the CLSM after they were treated with the SCC7 cells (Fig.
7). As expected, for CLPMs, fluorescence of DOX in the cytosol gradually increased as a function of time. On the contrary, free DOX was directly accumulated at the nucleus within a short period of time. It is important to note that after a 12 h incubation of DOX-CLPM2, strong fluorescence was observed at the nucleus as well as cytoplasm of the cell. These results suggest that the DOX-loaded polymersomes slowly reach inside the cancer cells through endocytosis, and GSH in the intracellular level cleave the disulfide bond for the triggered drug release.

**Conclusion**

In summary, biocompatible CLPMs that could preferentially release anticancer drugs at the intracellular level of cancer cells have been demonstrated. The introduction of cross-linker into the polymersomes dramatically minimizes the initial burst release of the drug and facilitated the drug release in the presence of GSH, mimicking the intracellular reductive environment. From the cell experiment, it is evident that CLPMs could effectively deliver the DOX into the intracellular level of the SCC7 cancer cells. Overall, these bioreducible CLPMs may have potential as a useful drug carrier system for DOX.

**Acknowledgment**

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**Electronic supplementary information (ESI)**
ESI available: Reaction scheme, $^1$H NMR, CAC, and fluorescent intensity of Nile Red-loaded polymersomes.

References


### Table 1. Physicochemical characteristics of NCPM and CLPMs

<table>
<thead>
<tr>
<th>Polymersomes</th>
<th>Feed ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Size (nm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Polydispersity</th>
<th>ζ (mV)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Relative content of free amines (%)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCPM</td>
<td>-</td>
<td>208±2.11</td>
<td>0.28</td>
<td>42±0.97</td>
<td>100</td>
</tr>
<tr>
<td>CLPM1</td>
<td>0.5</td>
<td>175±3.66</td>
<td>0.26</td>
<td>10±2.86</td>
<td>49.56</td>
</tr>
<tr>
<td>CLPM2</td>
<td>1.0</td>
<td>146±1.03</td>
<td>0.29</td>
<td>7±1.76</td>
<td>23.29</td>
</tr>
</tbody>
</table>

<sup>a</sup>Molar feed ratio of DTSSP to Lysine.  
<sup>b</sup>Mean diameter measured using the particle analyzer.  
<sup>c</sup>The zeta potential of polymersomes in water (1 mg/ml).  
<sup>d</sup>Relative percentage of amine present in PLys.

### Table 2. Physicochemical characteristics of DOX-loaded polymersomes

<table>
<thead>
<tr>
<th>Polymersomes</th>
<th>Feed ratio</th>
<th>Loading efficiency (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Loading content (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Size (nm)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX-NCPM</td>
<td>10</td>
<td>63±0.62</td>
<td>6.3±0.06</td>
<td>166±3.13</td>
</tr>
<tr>
<td>DOX-CLPM1</td>
<td>10</td>
<td>60±1.23</td>
<td>6.0±0.12</td>
<td>144±2.59</td>
</tr>
<tr>
<td>DOX-CLPM2</td>
<td>10</td>
<td>58±1.52</td>
<td>5.8±0.15</td>
<td>125±3.55</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined using UV-visible spectrometer.  
<sup>b</sup>Determined using the particle analyzer.
Fig. 1. Schematic illustration of GSH-responsive release of the drug into the nucleus from DOX-loaded CLPMs.
Fig. 2. Size distributions and CLSM images of (a and b) NCPM; (c and d) CLPM1 and (e and f) CLPM2. The insets are for TEM images of each polymersomes.
Fig. 3. Particle size of the polymersomes as a function of time in a PBS (pH 7.4). The error bars in the graph represent standard deviations ($n=3$).
Fig. 4. Change in (a) light scattering intensities of NCPM and CLPMs and (b) polydispersity in the presence of SDS as a function of time.
**Fig. 5.** *In vitro* release behavior of DOX from NCPM and CLPMs (a) in the absence of GSH and (b) in the presence of GSH. Asterisks (*) indicates difference at the $p < 0.05$ significance level. The error bars in the graph represent standard deviations ($n=3$).
Fig. 6. *In vitro* cytotoxicity of (a) bare polymersomes and (b) DOX-loaded polymersomes. Asterisks (*) denote statistically significant differences ($p < 0.05$ compared with free DOX). The error bars in the graph represent standard deviations ($n=3$).
Fig. 7. CLSM images of SCC7 cells incubated with free DOX and DOX-CLPM2.
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PEG-PLys-b-PCL → Self-assembly → NCPM → DOX → Cross-linking → CLPM

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Fig. 4. T. Thambi et al
Fig. 5. T. Thambi et al
Fig. 6. T.Thambi et al

(a) Polymersome concentration (μg/ml)

![Graph showing polymersome concentration vs. cell viability.]

(b) DOX concentration (μg/ml)

![Graph showing DOX concentration vs. cell viability.]

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Fig. 7. T. Thambi et al.

Comparative analysis of DAPI, DOX, and Overlay images for Free DOX and DOX-CLPM2 over time:
- **Free DOX**
  - 3h
  - 12h
- **DOX-CLPM2**
  - 3h
  - 6h
  - 12h

The images show the distribution and fluorescence intensity of DOX within cells at different time points, highlighting the efficacy of the DOX-CLPM2 formulation compared to Free DOX.