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Membrane Stabilization of Biodegradable Polymersomes

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Biodegradable polymersomes are promising vehicles for a range of applications. Their stabilization would improve many properties, including the retention and controlled release of polymersome contents, yet this has not been previously accomplished. Here, we present the first example of stabilizing fully biodegradable polymersomes through acrylation of the hydrophobic terminal end of polymersome-forming poly(caprolactone-*b*-ethylene glycol). Exposure of the resulting polymersomes loaded with a hydrophobic photoinitiator to ultraviolet light polymerized the acrylates, without affecting polymersome morphology or cell cytotoxicity. These stabilized polymersomes were more resistant to surfactant disruption and degradation. As an example of stabilized polymersome utility, the unintended release of doxorubicin (DOX) due to leakage from polymersomes decreased with membrane stabilization and slower sustained release was observed. Finally, DOX-loaded polymersomes retained their cytotoxicity following stabilization.

Introduction

Recent advances in polymer technology offer much promise for the development of novel, smart materials for use in medical diagnostics and disease treatment. One class of materials that has received significant attention for drug delivery and tissue imaging is synthetic polymer vesicles or polymersomes.[1−3] Polymersomes are advantageous for *in vivo* applications because of their long circulation times and high payload capacity of both hydrophilic and hydrophobic agents. They have been fabricated from a vast range of di- and triblock copolymers, and have recently been designed to incorporate functionalities such as surface peptides, biodegradability, and stimulus-responsiveness.[4−8]

Many drugs, especially those showing promise for cancer therapies, are severely limited in their clinical use because of a narrow therapeutic index.[9] A range of strategies have been suggested to improve drug efficacy while reducing side effects, including active targeting through peptide conjugation, PEGylation, and encapsulation to take advantage of the enhanced permeability and retention (EPR) effect observed in solid tumors.[10,11] Polymersomes have been suggested as useful vehicles for the encapsulation of potent cancer drugs with narrow therapeutic indices. Their PEG-coated surfaces make them invisible to the immune system, and they can be designed to be several hundred nanometers in diameter and accumulate in tumors due to the EPR effect. Additionally, because they can encapsulate both hydrophilic and hydrophobic molecules, polymersomes are ideal candidates as carrier vehicles for combination therapies.[12] However, it may be advantageous to have enhanced control over polymersome structure and degradation to better control delivery rates.

In recent work, we characterized drug release from poly(caprolactone-*b*-ethylene glycol) (PCL-PEG) polymersomes loaded with the potent anticancer drug, doxorubicin (DOX).[6] In this study, DOX was released from polymersomes following pH-dependent, first order kinetics. At endosomal pH, a single release rate was observed, hypothesized to be caused by membrane hydrolysis, while at physiological pH there was a burst phase of release hypothesized to be caused by transmembrane diffusion, followed by extended release at a rate matching that seen at lower pH and therefore thought to be caused by membrane hydrolysis.

To minimize the release of a drug from polymersomes prior to membrane hydrolysis, we sought to decrease the release rate of DOX by stabilizing the membrane structure. Specifically, the terminal hydroxyl end group of the PCL block of the diblock polymer was acrylated prior to polymersome formation. This strategy has been employed previously to increase the stability of lipid vesicles.[13] With the addition of an initiator and light source, the acrylates underwent free-radical

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polymerization and stabilized the polymersome membrane. While not affecting overall polymersome morphology, the polymersomes became more resistant to surfactant disruption and exhibited a decreased release rate of encapsulated DOX at physiological pH.

Materials and Methods

Materials. Except where noted, all materials were used as received. Acryloyl chloride, triethylamine (TEA, anhydrous grade), poly(ethylene glycol) methyl ether (PEG, M_n = 2000 Da), stannous octoate, ε-caprolactone (CL), 2,2-dimethoxy-2-phenylacetophenone (DMPA), TritonX-100, and doxorubicin hydrochloride (DOX) were purchased from Sigma Aldrich. CL was dried for 48 h over calcium hydride and distilled under reduced pressure immediately prior to use. PEG was dried under vacuum at 90 °C overnight before use. All solvents were reagent grade or better.

Polymer Synthesis. The functionalized polymer was produced in two steps (Scheme 1). In the first, the diblock copolymer was synthesized as previously reported utilizing monomethoxy PEG as a macroinitiator for the ring-opening polymerization of caprolactone with stannous octoate as the catalyst.[6] Briefly, 12 g of CL (11.65 mL) was added to a 100 mL round-bottomed flask containing 2.0 g of the previously dried PEG under an argon atmosphere. Twelve drops of stannous octoate were added, and the flask was sealed. The reaction was heated to 90 °C for 30 min to fully dissolve the PEG in the CL and then heated to 130 °C for 1.5 h while under vacuum. The crude polymer was dissolved in tetrahydrofuran (THF), precipitated into hexanes, and dried overnight. The resulting polymer contained an inert methoxy cap on the PEG and a reactive hydroxyl at the PCL terminus. The hydroxyl terminus of the PCL was acrylated using acryloyl chloride and TEA. A total of 5.2 g of PCL-PEG was dissolved in 200 mL of dichloromethane (DCM) under a stream of argon in a 500 mL three-neck round-bottomed flask equipped with an addition funnel. A total of 500 μL (~10×) of TEA was injected into the flask, and the temperature was reduced to 0 °C. The addition funnel was charged with 300 μL (~10×) of acryloyl chloride and 30 mL of DCM. The acryloyl chloride solution was added dropwise to the reaction vessel over 1 h. The reaction was allowed to proceed for 4 h at 0 °C and overnight at room temperature. Pure polymer was recovered by concentration, dissolution in benzene (to precipitate triethylammonium salts), filtration, reconcentration in DCM, precipitation into hexanes, and drying. Upon purification, the polymer was characterized by NMR and GPC. NMR spectra were recorded on a Bruker Avance 360 MHz spectrometer in deuterated chloroform. GPC spectra were obtained on a Waters 1525 Binary HPLC equipped with an autosampler, refractive index detector, and Styragel HR 4E and 5E columns in series utilizing THF as the mobile phase.

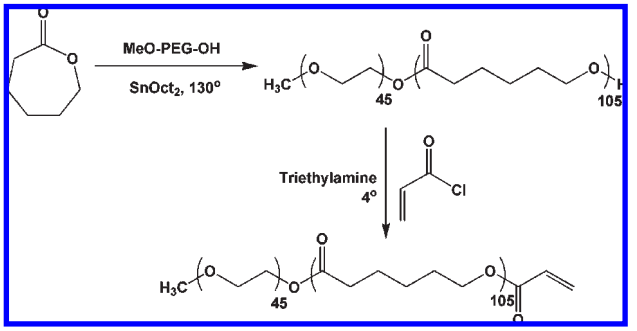
Polymersome Formation and Characterization. Polymersomes were fabricated by the self-assembly of polymer thin films on roughened Teflon into aqueous medium (70−100 mg/mL solution of polymer in DCM, drying, immersion in aqueous solution), followed by sonication at 65 °C, freeze−thaw cycling (five cycles liquid nitrogen to 65 °C), and heated, automated extrusion (400 and 200 nm membranes). The photoinitiator DMPA (18 μg/mg polymer for 1/1 mol/mol) was co-cast for inclusion into the membrane, and UV light exposure was completed with an OmniCure Series 1000 spot-curing lamp with a collimating lens (Exfo, Ontario, Canada; 365 nm, 55 mW/cm^2). DOX was encapsulated utilizing an ammonium sulfate gradient (280 μL of 10 mg/mL DOX in water was added to 2 mL of a 14 mg/mL polymersome suspension and incubated at 65 °C for 7 h),[14] and free DOX was removed on two HiTrap desalting columns in series (GE Healthcare). Release into phosphate-buffered saline (PBS) was monitored by recording the fluorescence of polymersome suspensions over time (SPEX Fluorolog-3 fluorimeter, λ_{ex} = 480 nm, λ_{em} = 590 nm). The amount of DOX encapsulated was determined by polymersome dissociation with addition of 100 μL of 30% TritonX-100 and incubation for 60 min at 37 °C. Polymersomes were analyzed for acrylate conversion via NMR and GPC of dehydrated samples reconstituted in organic solvents.

Dynamic light scattering (DLS) measurements were made in PBS or acetate buffer (pH = 5, 290 milliosmolal) on a Malvern Zetasizer NanoS (Malvern Instruments, Southboro, MA) instrument. Samples were prepared by a 20× dilution of the polymersome suspension into the appropriate buffer. TritonX-100 solutions were prepared prior to the addition of the polymersomes, such that the addition of the polymersomes brought the Triton to a final concentration of 0.5 vol %. These samples were stirred at 37 °C for 1 h before transfer to cuvettes. Samples that were kept for long-term degradation studies (> 1 day) were capped and sealed in their cuvettes to prevent evaporation.

Cryo-transmission electron microscopy (TEM) images were obtained on a JEOL 1210 transmission electron microscope. All grids for cryo-TEM were prepared within a controlled environment vitrification system (CEVS)[15] in a saturated water vapor environment at 25 °C. A droplet (~10 μL) of sample was placed on a carbon-coated copper TEM grid (Ted Pella) held by nonmagnetic tweezers. Filter paper was used to blot excess sample away, resulting in a thin film of solution spanning the grid. The sample was allowed to relax for approximately 30 s to remove any residual stresses imparted by blotting and then quickly plunged into liquefied ethane (~90 K) cooled by a reservoir of liquid nitrogen to ensure the vitrification of water. Prepared grids were stored under liquid nitrogen until imaging. The imaging of the grids was executed at −178 °C using a Gatan 626 cryogenic sample holder in a JEOL 1210 transmission electron microscope operating at 120 kV. A cooled Gatan 724 multiscan CCD camera was used to record the images. Image processing, including background subtraction, was completed with Gatan Digital Micrograph software version 3.9.1.

Cytotoxicity Analysis. NIH 3T3 fibroblasts (ATCC, cultured in DMEM with 10% fetal bovine serum, 1% sodium bicarbonate, 1% pen/strep) were seeded at a density of 10 000 cells/well in a 24-well plate. After 24 h, the cells were washed and incubated with a suspension of polymersomes in 9:1 media/PBS. The suspensions were sterilized with 20 min exposure to a germicidal lamp prior to addition to cells. After 24 or 72 h of polymersome exposure, the cells were washed and incubated for 4 h with a 10% solution of Alamar Blue (Biosource, Camarillo, CA) in media. The media was removed from the cells, and a 100 μL aliquot was read for fluorescence on a plate reader (Bio-Tek Synergy HT). For cytotoxicity studies with

Scheme 1. Synthesis of Acrylate-Terminated PCL-PEG Copolymer



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DOX-loaded polymersomes, the polymersomes were prepared as described above. To prevent DOX leakage, these polymersomes were kept on ice until immediately before dilution into media and feeding to cells.

Statistics. Student's *t* test was used to determine statistical significance between cell viability groups. A *p* value of <0.05 was considered to be significant.

Results and Discussion

While previous work has demonstrated the stabilization of polymersome membranes,[16−21] we aimed to design stabilized polymersomes that are also biodegradable. To that end, a functional group (i.e., acrylate) was incorporated at the PCL terminal end of PCL-PEG diblock polymers. Once assembled into polymersomes and in the presence of a photoinitiator, UV light exposure induces a radical polymerization through the functional groups (Scheme 2). This approach does not hinder hydrolysis of the PCL chain and yields oligo-caprolactone units, PEG, and kinetic chains of poly(acrylic acid) as the degradation products.[22]

During self-assembly into polymersomes, it is expected that the individual polymer backbones align with each other, forming the membrane.[2] We postulated that, despite the semicrystalline nature of the PCL block within the membrane which could hinder molecular movement,[23] the acrylate groups would be aligned with a close enough proximity to undergo free-radical polymerization upon initiation. To test this hypothesis, we loaded a hydrophobic radical photoinitiator (DMPA) into the bilayer of acrylated PCL-PEG (AcPCL-PEG) polymersomes. Only in the case where DMPA was loaded into the bilayer and the polymersomes were exposed to UV irradiation was polymerization of the acrylate groups observed (i.e., disappearance of acrylate peaks in NMR spectra, Figure 1A). Additionally, significant peak broadening can be seen in the NMR spectrum of the UV-exposed polymersomes containing DMPA, indicative of an increase in molecular weight that would be expected to accompany acrylate polymerization. UV light alone or simply the presence of DMPA were both insufficient to induce polymerization.

The amount of DMPA necessary for complete conversion of the acrylate groups was also investigated (Figures 1B and 2). A 1:1 mol/mol ratio of DMPA to polymer was necessary for complete conversion of acrylates, while partial conversion was observed in the NMR spectra for lower amounts of DMPA. Significant peak broadening was observed in the GPC traces of dehydrated polymersomes reconstituted in THF that had been loaded with between 50 and 100 mol % DMPA. Furthermore, in all of these cases, approximately half of the polymer remained insoluble in THF for GPC analysis, due to an excessively large molecular weight

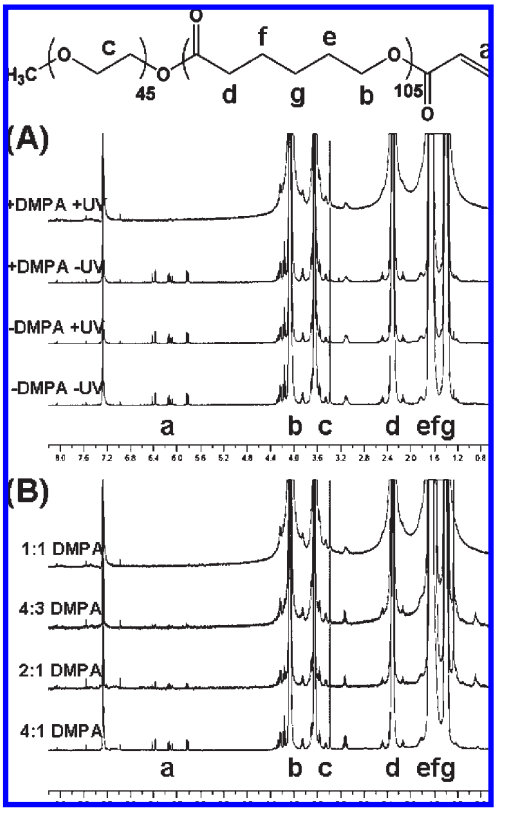
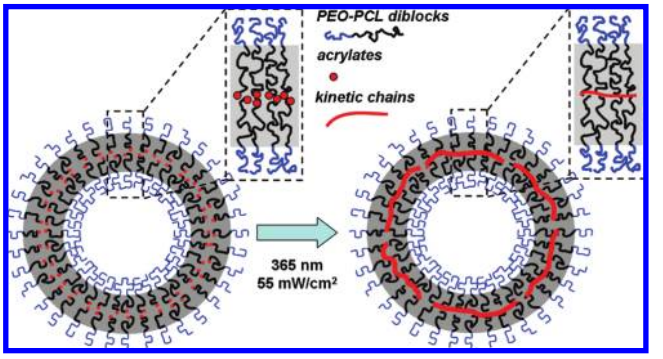


Figure 1. (A) NMR spectra of dehydrated polymersomes of AcPCL-PEG with or without DMPA loaded into the membrane before and after UV light exposure as indicated. The −DMPA + UV sample received a 30 min dose of UV light, while the + DMPA + UV sample received a 5 min dose. (B) NMR spectra of AcPCL-PEG polymersomes with varying amounts of DMPA loaded into the membrane (reported as molar ratio of polymer: DMPA). All samples received a 10 min dose of UV light. Lowercase letters indicate assignment of peaks to the chemical structure shown.



Scheme 2. Schematic of Hydrophobic End Group Polymerization for Stabilization of Polymersome Membranes

resulting from chain linkage, indicative of polymerization within the membrane. While the concentration of DMPA does seem high to achieve complete conversion, the DMPA would be fairly immobile within the membrane, as the PCL is well below its melting temperature of ~60 °C. As a result, though such a high dose of DMPA is required, only those DMPA molecules that have assembled near the acrylates would actually initiate polymerization.

To ensure complete acrylate conversion, we used a 1:1 ratio of DMPA to polymer in subsequent studies. However, it was first important to confirm that the high loading of DMPA did not significantly affect the structure of the membrane or

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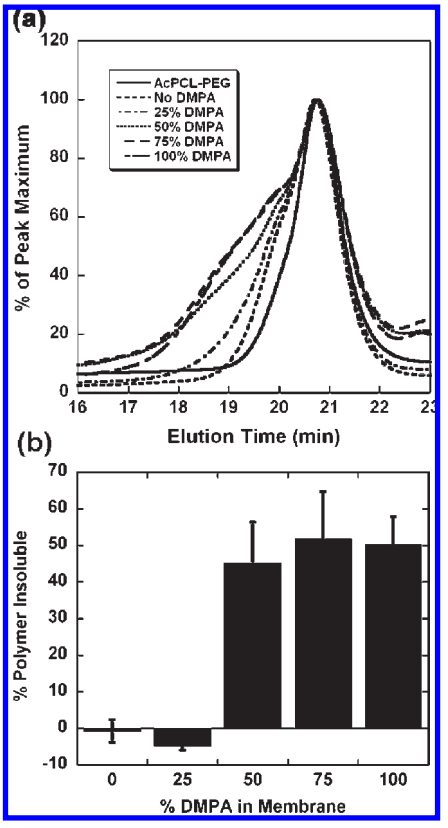

Figure 2. (a) GPC traces of reconstituted polymersomes containing varying amounts of DMPA in the membrane and exposed to UV light. GPC traces include only the soluble portions of the THF samples. (b) Percentage of polymer that remained insoluble during reconstitution in THF.

resulting macromolecular assembly. We fabricated polymersomes of AcPCL-PEG on both the nanoscale and the microscale (results not shown) with and without DMPA loaded in the membrane. A robust yield of polymersomes was observed via cryo-TEM (Figure 3a) with no significant change in size or shape. The polymersomes appear to be multilamellar at this point, but this phenomenon could be allayed by increasing the number of freeze−thaw cycles or passes through the extrusion membrane during processing. Furthermore, to alleviate concerns that a high dose of free radicals in the membrane could lead to polymer cleavage and result in micelle formation, DLS was performed on DMPA-loaded polymersomes before and after UV irradiation (Figure 3b). The peak for samples before and after light exposure is centered at ~200 nm, and there is no change with light exposure, confirming that micelles do not form. Therefore, while the 1:1 ratio of DMPA to polymer does appear to be quite high, such loading did not affect the resulting macromolecular structure.

To eliminate any concerns about the biocompatibility of the new polymer chemistry or inclusion of photoinitiator, the potential cytotoxicity was investigated with fibroblasts in standard cultures for up to 72 h (Figure 4). A slight drop in viability (~5−10%) was observed with the AcPCL-PEG + DMPA formulation prior to stabilization, and then only at the highest concentration compared to PBS controls. This decrease in viability was eliminated when the same polymersomes were stabilized by exposure to UV light, suggesting that the toxicity is caused by the high amount of DMPA present. Thus, there are no concerns with the toxicity of UV-stabilized polymersomes, which ultimately

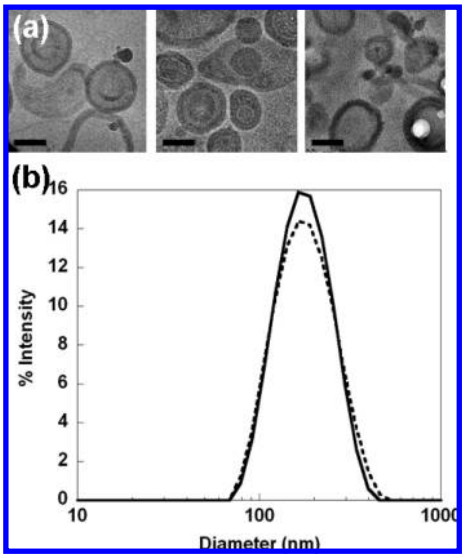

Figure 3. (a) Cryo-TEM images of AcPCL-PEG polymersomes without initiator or light (left), with initiator alone (center), and with both initiator and UV light exposure (right), indicating that the polymersome morphology is not affected by initiator or light. Scale bars = 100 nm. (b) DLS intensity distributions of polymersomes loaded with DMPA before (solid line) and after (dashed line) UV exposure.

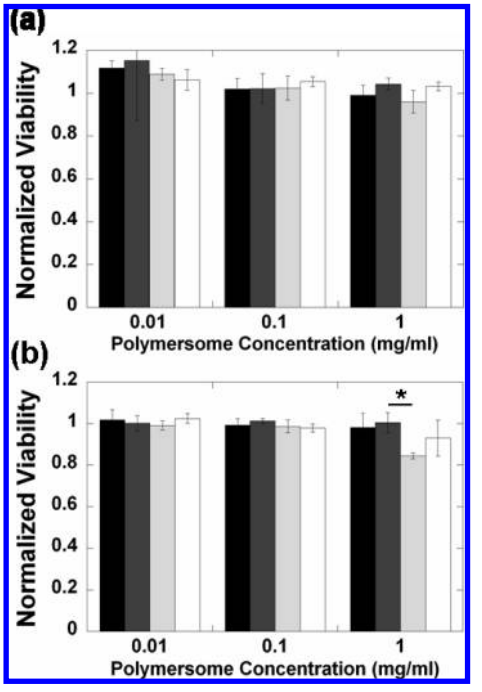

Figure 4. Fibroblast viability when cultured in the presence of polymersomes consisting of PCL-PEG (black), AcPCL-PEG (dark gray), AcPCL-PEG + DMPA (light gray), or AcPCL-PEG + DMPA + UV (white) for (a) 24 and (b) 72 h. All samples (n = 3) are normalized to cultures without polymersomes present. (*) $p < 0.05$

degrade into components commonly used in the biomaterials field.[22]

In order to confirm that a physical stabilization had occurred within the polymersome membranes, samples were treated with the detergent TritonX-100 and examined by DLS to check for morphological changes. The changes in size distributions are shown in Figure 5. For DMPA-loaded

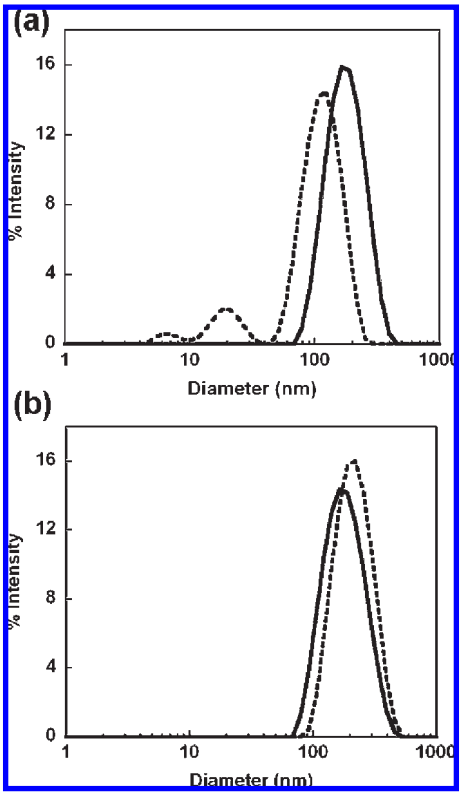

Figure 5. Self-assembled polymer morphologies before (solid lines) and after (dashed lines) treatment with TritonX-100 for DMPA-loaded polymersomes (a) before and (b) after UV exposure.

samples that were not exposed to UV light (Figure 5a), a second peak appears around 20 nm and there is a shift of the main peak from ~200 to ~100 nm after exposure to the detergent (the peak around 8 nm is from free Triton). However, for polymersomes exposed to UV light (Figure 5b), no micelle peak appears in the DLS and there is a slight increase in the peak diameter of the polymersomes, suggesting that the Triton is partitioning into the membrane but is unable to cause a complete disruption. Thus, there appears to be enhanced stabilization in polymersomes with polymerization of the acrylates within the membrane.

While we have aimed to produce polymersomes with stabilized membranes, for biomedical applications it is also important to ensure that these polymersomes remain biodegradable. Figure 6 demonstrates the decrease in polymersome size following incubation for 12 days in acetate buffer at 37 °C. During this period of time, the size and dispersity of the polymersomes decreased, indicative of degradation of the hydrophobic (PCL) backbone in the membrane. While a shift is seen for samples both before and after UV exposure, it is clearly more pronounced for the nonexposed sample. As PCL is known to degrade slowly, it is not surprising that, over this period of time, only small changes in the polymersome size are observed. However, this data still indicate that, as time progresses, the polymersomes degrade.

As a proof of concept that the stabilization of polymersome membranes is useful, a clinically relevant anticancer drug (DOX) was encapsulated in AcPCL-PEG polymersomes loaded with DMPA in the membrane and the release was monitored via fluorescence dequenching of DOX. We compared formulations with and without 15 min exposure to UV light (Figure 7a). As DOX releases from the polymersome and is diluted into the surrounding solution, its fluorescence

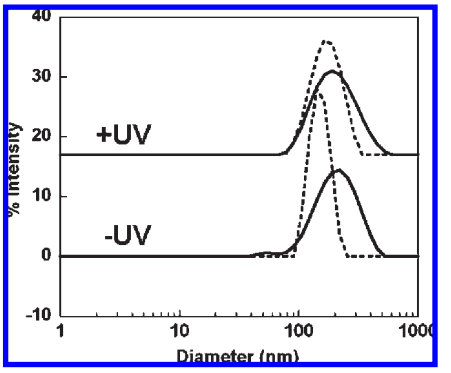

Figure 6. DLS of polymersomes before (−UV) and after (+UV) exposure initially (solid line) or when incubated in acetate buffer for 12 days (dashed line).

increases over a baseline level,[24] enabling tracking of the release from the polymersomes. There is specific interest in improved formulations of DOX that slow and target its release, as it is well-known for causing serious and fatal cardiac and hepatic side effects, as well as exhibiting fast metabolism and degradation at physiological pH.[25−29] Results are normalized to the initial amount of DOX encapsulated (determined by membrane disruption through Triton exposure to an additional sample for each group) less the baseline fluorescence. Formulations were also highly stable, exhibiting negligible release (< 1%) when stored at 4 °C over the same period of time.

For both stabilized and nonstabilized polymersomes, a large initial burst of release was observed; this release was slightly larger for the nonstabilized polymersomes. This burst could be either from the large osmotic gradient or by DOX that partitioned into the membrane prior to stabilization (DOX is amphiphilic). However, following the initial burst, the rate of release was much lower for stabilized polymersomes compared to the nonstabilized polymersomes (Figure 7b). By 7 days, only ~5% more of the DOX was observed to be released following the burst, compared to ~25% more being released for the nonstabilized samples, similar to what was observed previously.[6] It should be noted that, because of the degradation of DOX released into solution,[25,27] exact release profiles cannot be elucidated by this method. However, it is clear from the two observed profiles that release is significantly hindered by stabilization of the membrane.

As a final proof of concept, fibroblasts were cultured in the presence of DOX-loaded polymersomes (1.22 or 12.2 μg/mL) and their viability was measured for up to 72 h (Figure 8). The concentration of DOX loading in the polymersomes was calculated to be 25% based on the UV absorbance of a diluted sample exposed to Triton after separation on the HiTrap columns (ε = 23 cm^2/mg).[30] As seen in Figure 8, the viability

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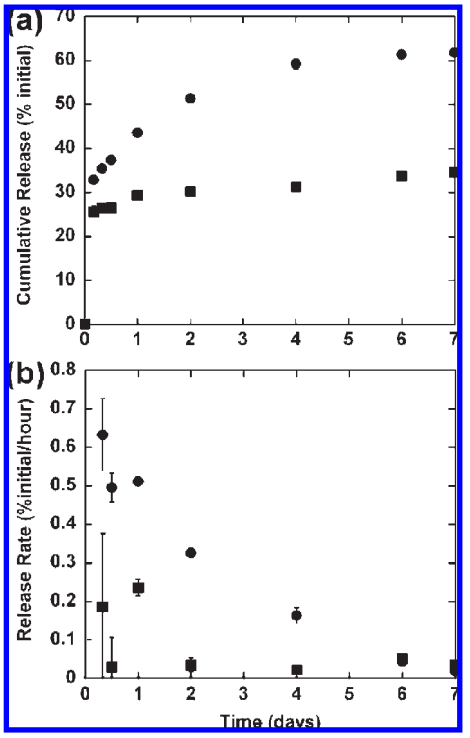

Figure 7. (a) Cumulative percent released and (b) release rates of DOX encapsulated in AcPCL-PEG polymersomes with 1:1 DMPA either without exposure (circles) or with exposure to 15 min UV light (squares). The amount released was normalized to the initial amount encapsulated and is reported as means ($n = 3$) and standard deviations.

dropped significantly for cells treated with DOX-loaded polymersomes in both a time- and dose-dependent manner. After 24 h, viability ranged from ~55% for the high dose to over 95% for the lower dose. However, by 72 h, viability dropped to ~10−20% for all doses. Importantly, this *in vitro* study confirms that the UV-stabilization did not affect the ability of the DOX to adversely affect the cells.

There are several other systems that have demonstrated stabilization of self-assembled membranes by combinations of radical polymerization and polymer blending.[13,16−21] However, to the best of our knowledge, this is the first demonstration of stabilizing a polymersome that still remains entirely biodegradable, degrading into PEG, oligo-caprolactone units, and poly(acrylic acid). Additionally, while the membrane is stabilized, it is not cross-linked, a feature that could be incorporated by doping a small diacrylate into the membrane. While we were not able to fully block the burst release of DOX after stabilization, the observed decrease demonstrates that this system is a step toward the development of smarter therapeutic delivery vehicles that could allow for better accumulation of drug at the target site and lower systemic doses.

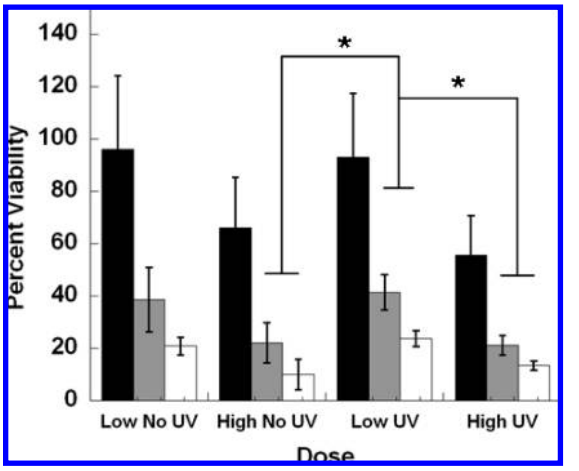

Figure 8. Cellular response to treatment with DOX-loaded polymersomes. Total DOX dose was 12.2 μg/mL (high) or 1.22 μg/mL (low) with or without UV-stabilization, and viability was measured in comparison to PBS controls at 24 (black), 48 (gray), and 72 (white) h. Data are reported as $n = 3$ and standard deviations. (∗) $p < 0.05$ for the indicated groups. Additionally, all groups saw significant ($p < 0.05$) changes between the 24, 48, and 72 h time points.

Conclusions

We report here the first example of a stabilized polymersome that remains fully biodegradable following polymerization. The ease of preparation and low cytotoxicity make this system a promising candidate for use *in vitro* and *in vivo*. The high payload capacity of polymersomes further makes them ideal candidates for clinical use. The decreased release rate observed for stabilized vesicles is significant for situations in which a high, site-specific dose is required for treatment. Future modifications could involve designing multiple degradable linkages at the PCL terminus, allowing for a stronger stabilization, or incorporating another polymer functionality into the acrylated polymer, such as PEG-surface modification, to enable active targeting *in vivo*.[31]

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