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Polymersome-Loaded Capsules for Controlled Release of DNA**

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We report the formation of a novel drug delivery carrier that comprises layer-by-layer polymer capsules subcompartmentalized with pH-sensitive nanometer-sized polymersomes, for the controlled release of plasmid DNA. The amphiphilic diblock copolymer poly(oligoethylene glycol methacrylate)-*block*-poly(2-(diisopropylamino)ethyl methacrylate) forms polymersomes at physiological pH, but transitions to unimeric polymer chains upon acidification to cellular endocytic pH. These polymersomes can thus release an encapsulated payload in response to a change in pH from physiological to endocytic conditions. Multi-component layer-by-layer capsules were formed by exploiting the ability of tannic acid to act as an efficient hydrogen bond donor for both the polymersomes and poly(*N*-vinyl pyrrolidone) at physiological pH. These capsules showed release of a plasmid DNA payload encapsulated within the polymersome subcompartments in response to changes in pH within the physiological range.

1. Introduction

The efficient delivery of therapeutic agents such as DNA, short interfering RNA (siRNA) and anti-cancer drugs to target sites in the body is highly important for the effective treatment of diseases such as genetic disorders and cancer. In their free state, these therapeutic agents are rapidly eliminated following their intravenous administration into the body, for example via interaction with blood plasma proteins or renal clearance. To prolong their blood circulation half-life and enhance their uptake by target cells, it is generally accepted that therapeutic agents need to be incorporated or loaded into a suitable carrier, which satisfies the size and surface character requirements for a long circulation time. A diverse range of micrometer- and nanometer-sized carriers, for example polymersomes,[1] micelles,[2] lipid-DNA complexes,[3] and polyethylenimine-DNA complexes,[4] have been explored for their potential in therapeutic delivery, and have shown efficient cellular uptake by the process of endocytosis. This typically involves invagination of the cell plasma membrane around the particle to be internalized, followed by membrane fission, resulting in the particle being contained within a membrane-enclosed cellular organelle known as an endosome.

Another class of delivery vectors, layer-by-layer (LbL) polymer capsules, have been shown to be internalized by cells, even when the size of the capsules is in the 2-3 μm size range,[5] and one particular study has shown that polymer capsules can be efficiently internalized via the endocytic pathway of macropinocytosis.[6] LbL capsules are generated via the sequential deposition of two interacting polymers upon a sacrificial core template. The two polymers interact, for example, by electrostatic

interactions,[7] or hydrogen[8] or covalent bonding.[9] Removal of the core template results in the isolation of hollow capsules, the size of which can be tuned through the choice of template diameter. Various techniques have been used to load therapeutic agents in LbL capsules, including: adsorption onto functionally modified templates prior to multilayered film assembly;[10] use of polymer-drug conjugates;[11] or use of the therapeutic agent[12] as a component in the multilayered film. The advantages of LbL polymer capsules in drug delivery include the ability to load and deliver a high dose of the drug using a single carrier, and the potential to readily decorate the capsule surface with cell-specific targeting ligands.[13] However, it remains a challenge to generate LbL capsules that remain stable and non-permeable at physiological pH, but disassemble and release an encapsulated cargo at endocytic pH.

Recently, several methods to subcompartmentalize LbL capsules have been developed. Capsosomes, which comprise LbL capsules containing nanometer-sized liposomes,[14-17] have demonstrated their effectiveness in microreactor[17] and drug delivery applications.[18] Encapsulation of multiple liposomal layers[17] allows tuning of the payload dose and can facilitate confinement of different therapeutic components to specific subcompartments. Another approach is to incorporate nanometer-sized LbL capsules within micrometer-sized LbL capsules, in which triggered release of a therapeutic cargo can be achieved by the selective degradation of the various subunits in response to different chemical stimuli.[19] Multilayered polyelectrolyte capsules have also been subcompartmentalized with liquid crystalline cubic phase structures, termed cubosomes™, for application in drug delivery.[20]

Polymer micelles have also been used extensively to allow subcompartmentalization of LbL films for drug delivery applications.[21]

Polymersomes are a further widely investigated example of an effective drug delivery vector. These are highly organized 'soft' structures formed via the self-assembly of amphiphilic block copolymers in the presence of a solvent that is selective for one polymer block.[22] Polymersomes have several benefits over their lipid counterparts.[23] The higher molecular weight of block copolymers compared to phospholipids results in the generation of thicker membranes, and interdigitation (caused by entanglement) of the hydrophobic chains,[24,25] yielding greater membrane mechanical strength.[26] Importantly, the enhanced toughness of the polymer membrane compared to lipid membranes does not compromise its elastic- or fluid-like nature.[24] Furthermore, block copolymers display very slow membrane dynamics, leading to the formation of kinetically stable, non-ergodic (i.e., the exchange of block copolymer chains between locally isolated structures occurs extremely slowly) structures.[27] Polymersomes can thus retain encapsulated cargo for long time periods (i.e., at least 3 months).[28] Compared to polymer micelles, polymersomes have the added advantage of being able to encapsulate and deliver both hydrophilic (within their hollow aqueous lumen) and hydrophobic (within their membrane core) cargo simultaneously.[29] Micrometer-sized polymersome assemblies comprising multiple confined polymersome subcompartments have been generated from double emulsions using a microfluidic device.[30] The size of each individual polymersome within the multi-compartment assembly can be precisely controlled "without cross-contamination"[31] between cargo loaded within different polymersomes.[30]

Herein, we present a novel drug delivery carrier that incorporates nanometer-sized stimuli-responsive polymersomes into LbL polymer-based planar films and hollow capsules. These polymersomes are formed from a diblock copolymer (POEGMA-PDPA) composed of hydrophilic oligoethylene glycol methacrylate (OEGMA) and pH-sensitive[32-34] 2-(diisopropylamino)-ethyl methacrylate (DPA) units. At physiological pH, the DPA units are deprotonated, rendering the diblock copolymer amphiphilic, thereby causing self-assembly into polymersomes. Acidification to endocytic pH causes protonation of the DPA units and dissociation of the polymersomes,[28,35] allowing release of an encapsulated cargo. The polymersomes are used to load a model therapeutic molecule, plasmid DNA (pDNA), which can be readily incorporated in polymersomes comprising PDPA.[33] Nanostructures using PDPA have been used extensively for cell tracking[36,37] and cytosolic delivery applications,[28,33,37,38] and have shown minimal effect on cell viability.[28,33,34,39,40]

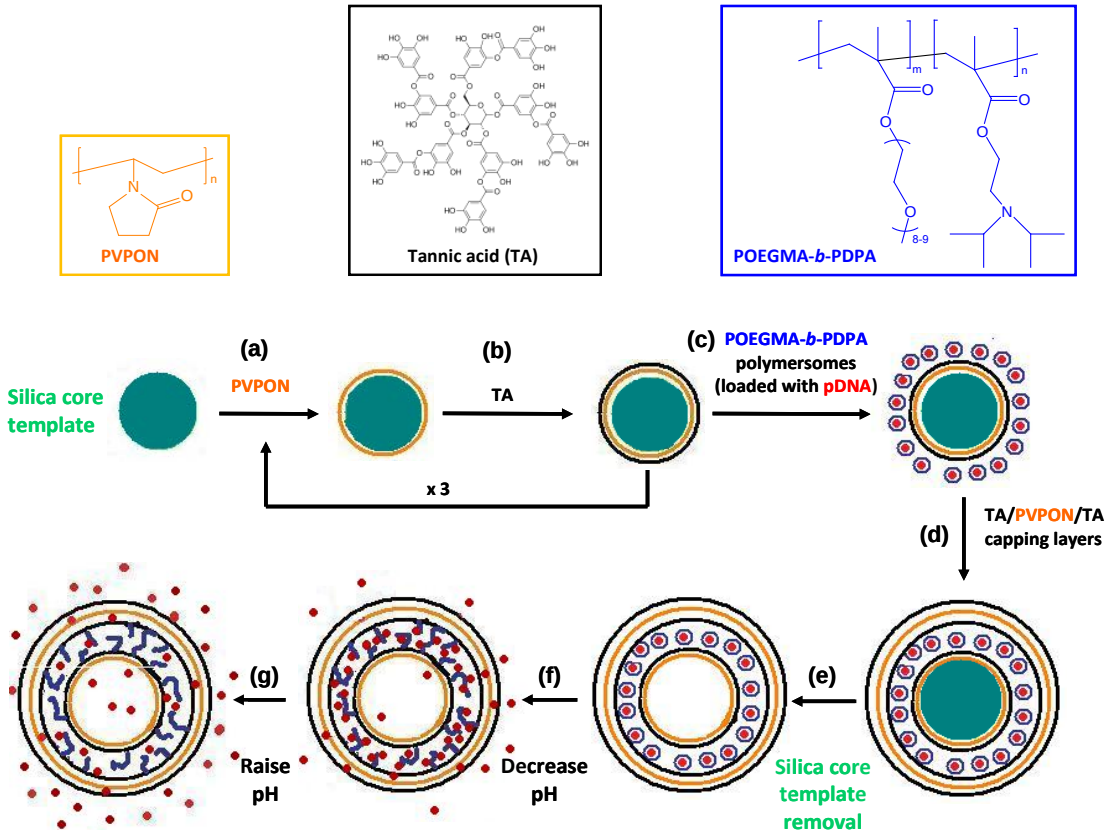
LbL films of poly(*N*-vinylpyrrolidone) (PVPON) and tannic acid (TA), incorporating POEGMA-PDPA polymersomes were assembled on both planar and particle substrates using the ability of TA to act as an effective hydrogen-bond donor at physiological pH,[41] to both PVPON and the poly(ethylene glycol) (PEG) chains in the polymersome corona. TA has previously demonstrated anti-cancer and anti-bacterial behavior,[42] while PVPON[43] and PEG[44] are biocompatible and low fouling. Furthermore, TA has previously been used for: (i) the formation of micrometer-sized capsules,[45] exploiting its ability as an efficient hydrogen bond donor for a variety of non-ionic polymers, and (ii) for the formation of a pH-

responsive subcompartmentalized LbL system through the incorporation of block copolymer micelles into a multilayered film.[46] The novel subcompartmentalized capsule system described herein combines the benefits of both LbL polymer capsules (i.e., efficient cellular uptake via macropinocytosis; the ability to incorporate multiple drugs within a single carrier; and the ability to be readily functionalized with antibodies) and stimuli-responsive polymersomes (i.e., the ability to retain an encapsulated payload for long time periods and demonstrate controlled payload release).

We first investigated whether POEGMA-PDPA polymersomes could be loaded into a LbL film comprising PVPON and TA assembled on a planar substrate, via quartz crystal microgravimetry (QCM). Next, polymersome-loaded capsules were prepared and probed for their ability to release an encapsulated cargo in response to changes in environmental pH. pDNA was exploited for this purpose, due to its high loading efficiency in polymersomes comprising PDPA,[28,33,47] which is the result of its electrostatic interaction with PDPA, forming 'polyplexes', at weakly acidic pH conditions. Herein, we show not only the novel incorporation of pDNA-loaded POEGMA-PDPA polymersomes into a LbL capsule carrier, but also the ability of the carrier to release pDNA in response to physiological pH changes, demonstrating its potential to effectively protect the pDNA in both physiological and endocytic pH conditions via its physical encapsulation and polyplex formation, respectively.

2. Results and Discussion

The structure of the block copolymer, poly(oligoethylene glycol)-*block*-poly(2-(diisopropylamino)-ethyl methacrylate) ($POEGMA_{26}$-$PDPA_{50}$), where subscripts denote mean degree of polymerization, is shown in **Scheme 1**. POEGMA was selected for the hydrophilic block due to the higher density of hydrogen-bond accepting groups compared to linear PEG, thus a stronger interaction with TA was expected. At pH values below the pK_a of 6.4, the amine groups in the PDPA block are protonated, initiating dissolution of the block copolymer chains in aqueous media due to their net hydrophilicity.[33] At pH 7.3, the PDPA block is deprotonated, rendering it hydrophobic. This results in the self-assembly of the block copolymer chains into polymersomes, due to the hydrophobic effect.[48] The assembly is a rapid, spontaneous and reversible process[28,35] forming colloidally stable, locally isolated structures below a certain maximum block copolymer concentration, which depends on the block copolymer molecular weight.[49] The formation of polymersomes was confirmed by the increase in turbidity of the solution. The particle size distribution of GPC-purified polymersomes was assessed by dynamic light scattering (DLS) (see **Supporting Information Figure S1)**. DLS displays the typical distribution of diameters for these polymersomes, which ranges from 50-300 nm.



Scheme 1. Schematic for the preparation of the polymersome-loaded LbL capsules and pDNA payload release. (a) The first layer of PVPON is deposited onto a silica core template with a diameter of approximately 3 μm. (b) A layer of TA is then deposited at pH 7.3. These steps are repeated until three precursor bilayers of PVPON and TA have been deposited. (c) A layer of $POEGMA_{26}$-$PDPA_{50}$ polymersomes is deposited (the chemical structure of $POEGMA_{26}$-$PDPA_{50}$ is shown with the RAFT end groups omitted); optionally, these polymersomes are loaded with pDNA (labeled with Cy^{TM}5). (d) Capping layers of TA, PVPON, and finally TA are adsorbed. (e) The silica core template is removed via the addition of hydrofluoric acid buffered to pH 7.3 (which does not affect the activity of the DNA[46]), resulting in the isolation of hollow capsules. (f) To release the pDNA payload, the pH is first lowered to 5 to dissociate the polymersomes and trigger the formation of electrostatic interactions between the now protonated PDPA chains and the pDNA phosphate

backbone. (g) The pH is raised to 7.3 to simulate cytoplasmic pH conditions and trigger loss of the pDNA-PDPA electrostatic interaction and subsequent gradual pDNA release from the capsules.

As illustrated in Scheme 1, polymersome-loaded samples were prepared by the deposition of three bilayers of PVPON and TA onto silica core templates. Alternatively, a planar substrate, e.g., a poly(ethylenimine) (PEI)-coated silicon wafer, was used to assemble and act as a support for the multilayered film. Subsequently, a layer of polymersomes was adsorbed onto the TA surface of the core-shell particles, followed by capping layers of TA, PVPON, and finally TA, to prevent the polymersomes from readily disassembling from the core-shell particles. Control samples (i.e., without polymersomes) comprised four bilayers of PVPON and TA adsorbed onto planar or particulate surfaces. TA solutions were prepared fresh and were protected from light to prevent oxidation.[41] Particles were found to display greater colloidal stability/lower levels of aggregation when TA was present on the surface rather than PVPON (data not shown).

The ability to incorporate $POEGMA_{26}$-$PDPA_{50}$ polymersomes into a multilayered film comprising TA and PVPON was investigated by quartz crystal microgravimetry with dissipation monitoring (QCM-D). Gold-coated quartz sensor crystals were first coated with a layer of PEI. Suitable conditions for preparing a LbL film comprising TA and PVPON were investigated. The frequency decrease upon the deposition of each layer, corresponding to deposited mass,[50] was higher when the layering was carried out in sodium acetate buffer rather than phosphate buffered saline (PBS);

therefore the former was used in subsequent multilayered film assembly. Furthermore, the addition of an extra 100 mM sodium chloride to 50 mM sodium acetate was found to enhance the mass deposited at each layer (data not shown). A multilayered film comprising seven layers of TA and PVPON (with TA on the surface) was found to be stable overnight, with no significant change in the relative frequency. A frequency decrease of approximately 175 Hz (see **Figure 1**) was observed for the subsequent deposition of a layer of $POEGMA_{26}$-$PDPA_{50}$ polymersomes. This frequency change is comparable to the adsorption of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes to a precursor layer of poly(allylamine hydrochloride) (PAH) (~ 200 Hz).[14]

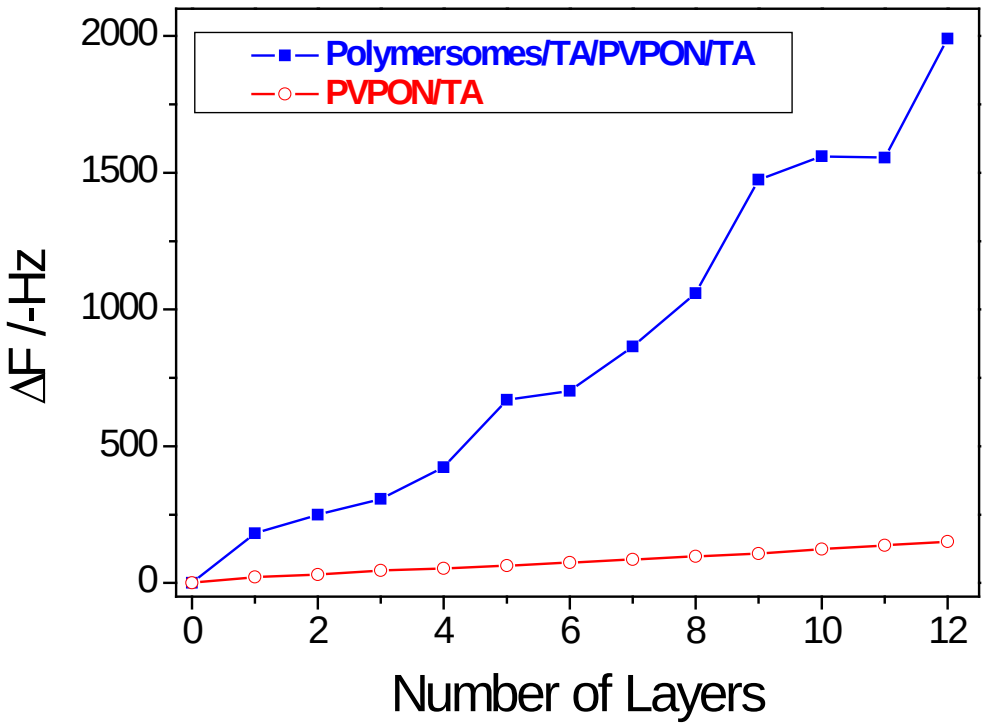


Figure 1. QCM-D frequency changes for LbL film deposition as a function of the number of layers. The squares (blue) show the deposition of $POEGMA_{26}$-$PDPA_{50}$

polymersomes/TA/PVPON/TA between layers 0-4, 4-8 and 8-12. (Note that 0-1 refers to the deposition of polymersomes onto a PEI/TA/$(PVPON/TA)_3$ precursor film). The circles (red) show the alternate deposition of PVPON/TA as a 'control' LbL film.

The potential for this LbL system to incorporate multiple layers of $POEGMA_{26}$-$PDPA_{50}$ polymersomes was studied. Figure 1 shows that three layers of these polymersomes can be loaded into a LbL film, with layers of TA/PVPON/TA assembled between each polymersome layer and at the film surface. The change in dissipation was found to be slightly less than 1×10^{-6} per frequency change of 10 Hz, thus the film can be described as having low levels of water infiltration and being relatively rigid. The greater deposition of the TA and PVPON layers onto a LbL film comprising polymersomes is presumably due to the greater surface roughness of this film compared to the 'control' film prepared without polymersomes.

Although the QCM-D data clearly demonstrates the incorporation of the $POEGMA_{26}$-$PDPA_{50}$ polymersomes into the multilayered film, in order to prove that the polymersomes were still intact upon their incorporation and adsorption of capping layers, atomic force microscopy (AFM) analysis of the film was performed. TA and PVPON were alternately deposited on PEI-coated silicon wafers until seven layers had been assembled. After depositing a layer of $POEGMA_{26}$-$PDPA_{50}$ polymersomes, capping layers of TA, PVPON and finally TA were deposited. **Figure 2** shows an AFM micrograph of the planar LbL film incorporating intact $POEGMA_{26}$-$PDPA_{50}$ polymersomes. The cross-sectional profile indicates that the polymersomes are

flattened upon their loading into the film, with a height ranging from 15-35 nm, and their diameters (50-300 nm) correspond to their size distribution measured by DLS (see Supporting Information Figure S1). The AFM data suggest that the polymersomes are still intact. Previous work by Discher et al. has demonstrated that polymersomes have highly flexible membranes[26] that can tolerate deformation. Furthermore, due to their slow membrane dynamics,[27] polymersomes display very high levels of payload retention. Despite deformation upon loading into the LbL system, the polymersomes should remain non-leaky to encapsulated payloads for relatively long time periods.

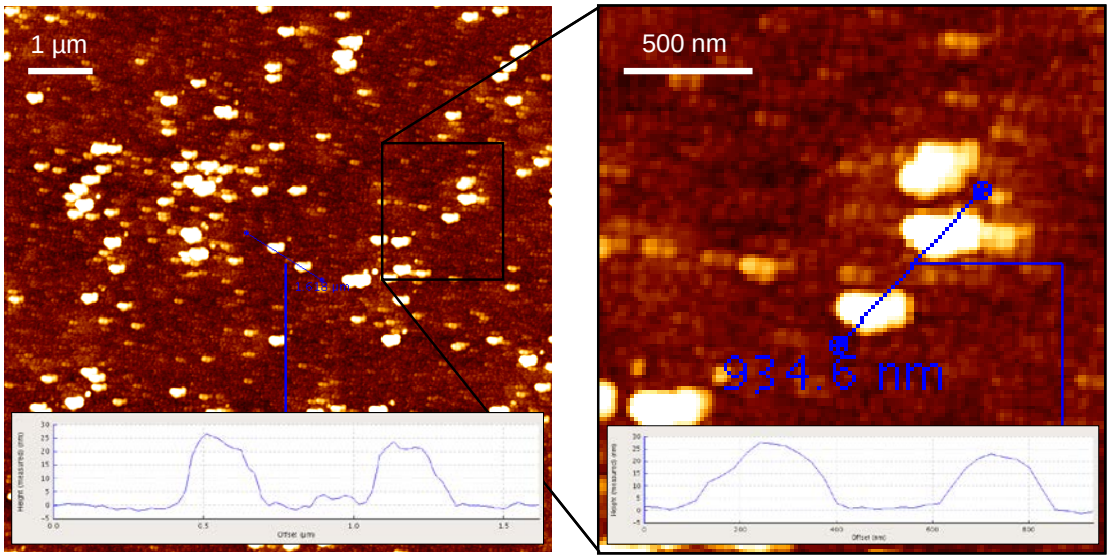


Figure 2. AFM image of a planar LbL film comprising $POEGMA_{26}$-$PDPA_{50}$ polymersomes deposited on a PEI-coated silicon wafer. The film comprises $TA/(PVPON/TA)_3/POEGMA_{26}$-$PDPA_{50}$ polymersomes/TA/PVPON/TA. The insets show cross-sectional profiles of the film to assess polymersome incorporation and film roughness.

We next investigated whether isolated, hollow, micrometer-sized capsules incorporating polymersome subcompartments could be prepared. To confirm polymersome incorporation into multilayered capsules, the amphiphilic fluorescent dye Oregon Green® 488 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Oregon Green® 488 DHPE) was loaded into the polymersome membrane. Fluorescence microscopy performed on the silica core-multilayered shell particles indicated embedding of the polymersomes within multilayered shells comprising TA and PVPON (data not shown). Furthermore, fluorescence microscopy carried out after adsorption of the capping layers indicated that this process did not cause polymersome rupture and subsequent loss of the amphiphilic dye from the particle shells.

To isolate micrometer-sized hollow polymersome-loaded capsules, the silica cores were removed via the addition of hydrofluoric acid (HF) buffered to pH 7.3 (see Experimental Section 4.2.9. for details). Fluorescence microscopy (**Figure 3a)** showed the successful isolation of hollow multilayered capsules incorporating polymersomes loaded with Oregon Green® 488 DHPE. The capsules displayed good morphological integrity and colloidal stability. To further demonstrate the incorporation of intact nanometer-sized polymersomes within a ~ 3 μm-sized carrier capsule, AFM and transmission electron microscopy (TEM) analyses of the capsules were performed (Figure 3b and **Supporting Information Figure S2**, respectively). As well as demonstrating the loading of intact polymersomes, the AFM image indicates that the surface of the capsules is rough compared to the 'control' capsules prepared without loaded polymersomes, as shown by taking cross-sectional profiles of

the capsules (**Supporting Information Figures S3** and **S4**). Closer examination of the TEM image in Supporting Information Figure S2 indicates the incorporation of hundreds of intact polymersomes within a larger carrier capsule. The surface is clearly rough, and is a striking contrast to the capsules prepared without polymersomes, shown in **Supporting Information Figure S5**, providing further evidence for successful polymersome loading. In contrast to the capsosome system, whereby it is often necessary to use polymers modified with cholesterol in order to anchor the liposomes into multilayered films,[15] these polymersomes are incorporated into the LbL system simply through hydrogen bonding interactions, and neither the polymersomes nor the polymers used to form the multilayered films require further modification or the use of cross-linkers in order to form a stable system.

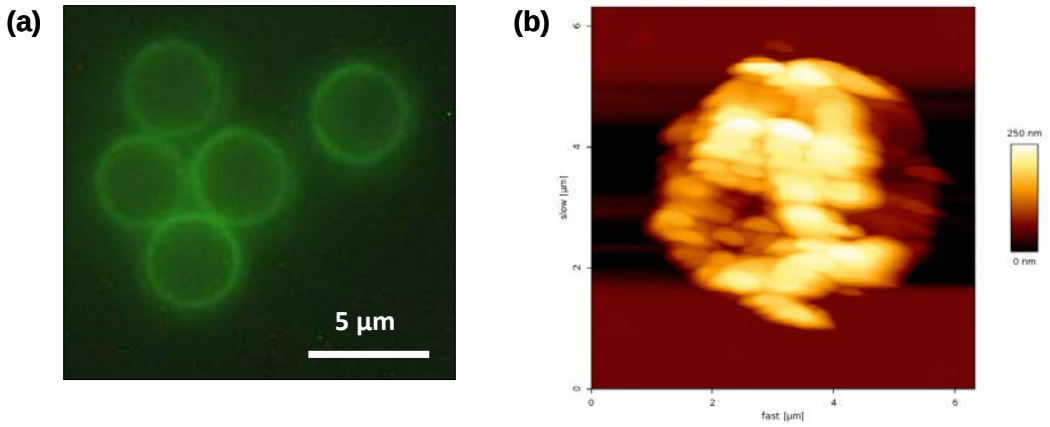


Figure 3. LbL polymer capsules comprising $(PVPON/TA)_3$/$POEGMA_{26}$-$PDPA_{50}$ polymersomes/TA/PVPON/TA. (a) Fluorescence microscopy image of capsules loaded with $POEGMA_{26}$-$PDPA_{50}$ polymersomes incorporating Oregon Green® 488 DHPE in the polymersome membrane. (b) AFM image of a $POEGMA_{26}$-$PDPA_{50}$ polymersome-loaded capsule.

Next, the ability of these polymersome-loaded capsules to be loaded with and release a therapeutic cargo in response to changes in pH was investigated. pDNA was used for this purpose, since it has previously shown high loading efficiencies within polymersomes comprising PDPA.[28,33,47] (Labeling of the pDNA with a fluorescent dye ($Cy^{TM}5$) facilitates the tracking of loading into and release from the polymersome-loaded capsules.) Loading efficiencies of up to 60% were obtained for the $POEGMA_{26}$-$PDPA_{50}$ polymersomes (see Experimental Section 4.2.6. for details). The obtained encapsulation efficiencies are in agreement with those previously recorded for PDPA-containing polymersomes.[47]

The high polymersome loading efficiency of pDNA is a result of adding a solution of the pDNA to the block copolymer just below its pK_a of 6.4.[33] Within this pH range, the PDPA block is protonated, permitting electrostatic interaction between the cationic PDPA amine groups and the anionic phosphate groups on the pDNA. The formation of block copolymer-pDNA complexes (termed 'polyplexes') has been shown to improve the pDNA encapsulation efficiency within polymersomes.[33] As the pDNA is already in close proximity to block copolymer chains at weakly acidic pH, loss of the electrostatic interaction at pH 7.3, due to deprotonation of the PDPA block, allows encapsulation of a significant proportion of the pDNA upon polymersome self-assembly. Further processing of the polymersomes by sonication and/or extrusion can further enhance the loading efficiency.[28]

Fluorescence microscopy (**Figure 4a**) shows successful incorporation of pDNA-loaded polymersomes into LbL capsules. To investigate the release rate of the pDNA,

the pH of the solution was then lowered to mildly acidic conditions (pH 5) to mimic the environment within late endosomes, since it is assumed that these LbL capsules are internalized by cells by endocytosis. It is hypothesized that these pH conditions cause the polymersomes within the film to dissociate; however, the block copolymer chains are presumably still strongly bound to the TA through hydrogen bonding between the TA phenol H-donor groups and the POEGMA ester and ethylene glycol H-acceptor groups. Furthermore, the PDPA block of the copolymer is protonated within this pH range, permitting electrostatic interaction with pDNA and thereby protecting it in endosomal conditions.[33] Raising the pH back to physiological conditions leads to loss of this electrostatic interaction, allowing the pDNA to be released from the multilayered capsule walls. By fluorescence microscopy analysis, the release was found to be relatively slow rather than a burst release mechanism since the multilayered film comprising the TA, PVPON and POEGMA-PDPA block copolymer is still stable under these conditions. pDNA is a relatively large macromolecule, and is thus unable to readily penetrate the polymer capping layers comprising the capsules.

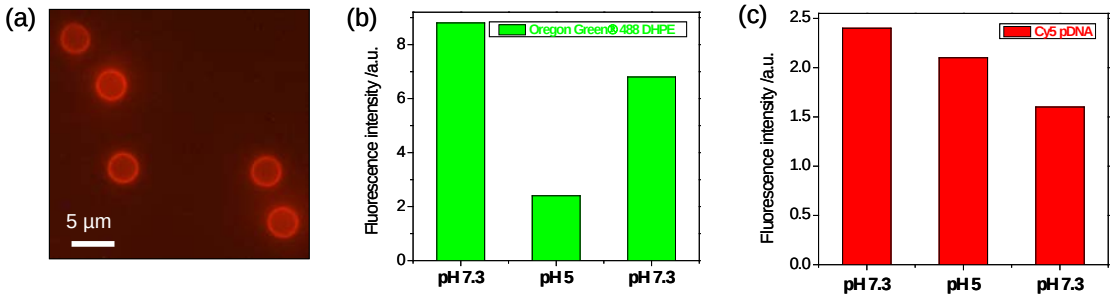


Figure 4. (a) Fluorescence microscopy image of LbL capsules incorporating $POEGMA_{26}$-$PDPA_{50}$ polymersomes loaded with Cy™5-labeled pDNA. (b) Mean fluorescence intensity of the capsules measured by flow cytometry, using an

excitation wavelength of 488 nm, corresponding to the amount of Oregon Green® 488 DHPE loaded within the capsules. (c) Mean fluorescence intensity of the capsules using an excitation wavelength of 647 nm, corresponding to the amount of Cy^{TM}5-DNA loaded within the capsules. All flow cytometry measurements are relative to the 'control' unlabeled polymersome-loaded capsules.

To quantify pDNA release, flow cytometry analysis of the capsules was performed two weeks after storing the capsules in PBS at pH 7.3, pH 5, and pH 7.3 (for this latter sample, the pH was lowered to 5 for 1 h, raised again to pH 7.3, and then stored at pH 7.3 for 2 weeks). The mean fluorescence intensity of the capsules using both the green and far red channels in the flow cytometer, for detection of Oregon Green® 488 DHPE and Cy^{TM}5-DNA fluorescence, respectively, was measured for each sample (Figures 4b and 4c, and **Supporting Information Figure S6**). The drop in green fluorescence at pH 5 is due to the pH-sensitivity of Oregon Green® 488 DHPE. There is a slight reduction in the green relative fluorescence intensity from 8.8 to 6.8 a.u. (23% reduction) following the pH drop and increase, indicating some loss of Oregon Green® 488 DHPE from the capsules as a result of disruption of the polymersome structure. The Cy^{TM}5-pDNA relative fluorescence intensity is reduced firstly from 2.4 to 2.1 a.u. (13% reduction) after lowering the pH to 5, and is reduced even further to 1.6 a.u. (33% reduction from the initial fluorescence intensity) after the pH is raised back to 7.3, indicating pDNA release upon disruption of the polymersome structure, and more enhanced pDNA release upon breakage of the pDNA-PDPA electrostatic interactions at physiological pH. Complete release of the encapsulated pDNA was not observed due its macromolecular size and inability to

readily penetrate the capping layers comprising the capsules; however, a reduction in Cy^{TM}5-pDNA fluorescence intensity of the capsules was clearly observed following the pH modifications.

These data show the potential of this system to control the amount of pDNA that is released in response to changes in pH within the physiological range. In future work, it would be useful to quantify the release as a function of time. Further, it would be worthwhile investigating the ability of POEGMA$_{26}$-PDPA$_{50}$ polymersome-loaded LbL capsules to load and release a low molecular weight payload such as siRNA, since the release rate is expected to be significantly faster than for pDNA.

The work presented here has confirmed not only that these polymersomes can efficiently load pDNA within their aqueous interiors, but also that multiple layers of polymersomes can be embedded within multilayered polymer films and capsules, providing the potential for multiple protection strategies for the pDNA as the carrier capsule is transported through the bloodstream. Furthermore, under endosomal pH conditions, the pDNA would be protected via the formation of copolymer-pDNA complexes, which would subsequently disassemble upon entry into the cell cytosol, facilitating pDNA entry into its target organelle, the nucleus. Polymersomes promote greater control over membrane permeability than liposomes,[52] rendering polymersome-loaded multilayered capsules excellent candidates for the efficient cellular delivery of a variety of therapeutic agents.

3. Conclusions

We have demonstrated the successful incorporation of pH-sensitive $POEGMA_{26}$-$PDPA_{50}$ polymersomes into multilayered polymer films and capsules. This system is based on hydrogen-bonding interactions, whereby TA acts as an efficient hydrogen bond donor for both PVPON and the POEGMA corona of the polymersomes at physiological pH and salt concentration. pDNA can be effectively loaded within $POEGMA_{26}$-$PDPA_{50}$ polymersomes and subsequently incorporated within multilayered capsules. We have demonstrated that pDNA can be released from the capsule carrier in response to pH changes within the physiological range.

This work is significant as it demonstrates the formation of a novel multi-component drug carrier that has the potential to simultaneously load and deliver multiple drugs with tailored degradation kinetics. This system combines the advantageous properties of both polymersomes and polymer capsules; the polymersomes have high payload retention and are pH-responsive within the physiological range, whilst micrometer-sized LbL polymer capsules are known to be internalized by cells by endocytosis. The fact that the carrier is synthetically-made creates endless possibilities for the incorporation of different functionalities and properties, leading to the ability to tailor the vector for a required purpose. One can envisage these polymersome-loaded capsules finding application in the delivery of therapeutics.

Analysis of the loading and release of a low molecular weight payload such as siRNA would provide a more effective ‘proof of concept’ of the ability of these polymersome-loaded capsules to release an encapsulated drug in response to pH

modifications in the physiological range. Furthermore, the use of degradable polymers[53]/chemical cross-linkers,[54] mixed polymer systems with tailored degradation kinetics,[55] or polymer capsule shells with tunable permeability,[56] to trigger faster/more controlled release of the nucleic acid payload, would be desirable for *in vivo* applications.

4. Experimental Section

4.1. Materials

Two batches of SiO_2-particles were used for the experiments: (i) diameter 3.03 µm, standard deviation 0.17 µm, solids content 50 mg/mL (Lot SiO_2-R-L1195), and (ii) diameter 3.59 µm, standard deviation 0.1 µm, solids content 50 mg/mL (Lot SiO_2-R-11610) (Microparticles GmbH, Berlin, Germany). Oligo(ethylene glycol) methyl ether methacrylate (OEGMA, M_n = 475, mean degree of polymerization (DP) = 8-9) (Sigma-Aldrich) and 2-diisopropylaminoethyl methacrylate (DPA) (Polysciences, Inc.) were passed over basic alumina (Scharlau) twice to remove any inhibitors present and stored below 4 °C prior to use. The RAFT agent 2-cyanoprop-2-yl dithiobenzoate (CPDB) was prepared according to the literature.[57] 2,2′-Azobis(2-methylpropionitrile) (AIBN, 98%) (Acros), dioxane, tetrahydrofuran (THF), diethyl ether, and *n*-hexane were used without further purification.

The pEGFP DNA construct pME18s, comprising 3.3 kilo base pairs, was used to prepare EGFP-encoding plasmid DNA. The Plasmid Maxi Kit used to isolate and purify the DNA was purchased from Qiagen (Australia). The One Shot Top10 Chemically Competent *Escherichia coli* (*E. coli*) used for transformation with the

pME18s, and ampicillin were purchased from Invitrogen (Australia). Luria-Bertani (LB) broth tablets, glycerol (for molecular biology, > 99%), 2-propanol (for molecular biology, > 99%), ethanol (molecular biology grade) and ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) were purchased from Sigma-Aldrich (Australia). Tris(hydroxymethyl) methylamine (Tris) was purchased from Chem Supply (Australia). The Label IT® Nucleic Acid Labeling Kits, Cy™3 and Cy™5 were brought from Mirus (US).

Tannic acid (TA), poly(*N*-vinyl pyrrolidone) (PVPON) (M_n 55 000), Sepharose 4B and polyethylenimine (PEI) (high molecular weight, water free, typical M_n 10 000 (GPC)) were purchased from Sigma-Aldrich (Australia). Disodium hydrogen phosphate heptahydrate (PBS), sodium acetate tetrahydrate (NaOAc) and sodium hydroxide (NaOH) were purchased from Merck (Germany). Hydrochloric acid (HCl) (37%) was sourced from AnalaR. Sodium chloride (NaCl), chloroform, methanol, ethanol and 2-propanol were purchased from Chem Supply (Australia). Oregon Green® 488 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Oregon Green® 488 DHPE) was sourced from Invitrogen (Australia). High purity and resistivity (>18 MΩ.cm) de-ionized water (MilliQ water) was obtained from an inline Millipore RiOs/Origin water purification system. The pH of all solutions was measured with a Mettler-Toledo MP220 pH meter. For QCM measurements, gold-coated quartz sensor crystals were purchased from ATA Scientific Pty Ltd (Australia). Silicon wafers were obtained from MMRC Pty Ltd (Melbourne, Australia).

4.2. Methods

4.2.1. Block Copolymer Characterization

Polymer molecular weight characterization was carried out on THF-eluting GPC: Shimadzu size-exclusion chromatogram equipped with a Wyatt DAWN EOS MALLS detector (690 nm, 30 mW) and Wyatt OPTILAB DSP interferometric refractometer (690 nm) using three Phenomenex Phenogel columns in series (500, 10^4 and 10^6 Å porosity; 5 µm bead size) operating at 30 °C. THF was used as the eluent at a flow rate of 1 mL/min. The samples were dissolved in eluent at the designed concentration and passed through a 0.45 µm filter and injected into the GPC for analysis. Astra software (Wyatt Technology Corp.) was used to determine the molecular weight characteristics.

^{1}H NMR measurements were conducted on a Varian Unity 400 MHz spectrometer using the deuterated solvent as the reference and a sample concentration of around 20 mg/ml.

4.2.2. Synthesis of Poly(oligoethylene glycol methacrylate)-block-poly(2-diisopropyl aminoethyl methacrylate) (POEGMA-PDPA)

The POEGMA macroRAFT agent was prepared by RAFT polymerization. OEGMA (4.75 g, 10 mmol), CPDB (90.7 mg, 0.33 mmol), and AIBN (6.8 mg, 0.04 mmol) were dissolved in dioxane (5 mL). The mixture was degassed through freeze-pump-thaw cycles (× 3) and then reacted at 70 ^{o}C for 16 h. The mixture was quenched in liquid nitrogen and diluted with THF before it was precipitated into excess diethyl ether. This precipitation procedure was repeated three times. The resulting product was dried *in vacuo* to yield POEGMA as a red viscous solid, 3.9 g (82%). GPC-MALLS (THF): M_w = 11.5 kDa, M_w/M_n = 1.16. The degree of polymerization (DP) of the homopolymer was determined to be 26 from ^{1}H NMR.

The block copolymer (POEGMA-PDPA) was prepared by RAFT polymerization of DPA using POEGMA as the macroRAFT agent. In a typical run, POEGMA (0.6 g, 0.049 mmol), DPA (0.62 g, 2.9 mmol), and AIBN (1.0 mg, 0.006 mmol) were dissolved in dioxane (2 mL). The mixture was degassed through freeze-pump-thaw cycles (× 3) and then reacted at 70 ^{o}C for 20 h. The mixture was quenched in liquid nitrogen and diluted with THF before it was precipitated into cold *n*-hexane. This precipitation procedure was repeated two times. The resulting product was dried *in vacuo* to yield the block copolymer as a red viscous solid, 1.0 g (80%). GPC-MALLS (THF): M_w = 21.2 kDa, M_w/M_n = 1.18. The degree of polymerization (DP) of DPA was determined to be 50 from ^{1}H NMR. The obtained diblock copolymer was denoted as $POEGMA_{26}$-$PDPA_{50}$.

4.2.3. Plasmid DNA Preparation

The EGFP-encoding plasmid DNA (pDNA) was prepared by first transforming the Top10 Chemically Competent *E. coli* with the DNA construct. Selection for the transformed bacteria was carried out by growing the *E. coli* culture at 37 °C in LB media that contained 100 μg/mL ampicillin. The pDNA was then isolated and purified from the bacteria using a Plasmid Maxi Prep kit, following the procedure provided by the manufacturer. pDNA purity was assessed by running a 1% agarose gel stained with ethidium bromide and calculating the absorbance ratio at 260 and 280 nm, using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Australia). The absorbance at 260 nm and the molar extinction coefficient for double-stranded DNA were used to calculate the pDNA concentration.

4.2.4. Fluorescent Labeling of Plasmid DNA

Both Cy™3- and Cy™5- labeling reagents were used to fluorescently label the pDNA, using the protocol provided by the manufacturer. Briefly, pDNA (5 µg) was mixed with the labeling reagent (5 µL) in 3-(*N*-morpholino)propanesulfonic acid) (MOPS) buffer (50 µL, 20 mM). The mixture was then placed in an Eppendorf tube in an Eppendorf Thermomixer Comfort at 37 °C, 1200 rpm for 1 h. Excess labeling reagent was then separated off from the pDNA using a filter centrifuge with a molecular weight cut-off of 100 000 (Pall Life Sciences). Eight washes in Tris-EDTA (TE, 10 mM Tris and 1 mM EDTA) buffer (pH 8.0) were performed using a centrifugation speed of 2000 *g* (3 min per spin). The purified pDNA was redissolved in TE buffer and stored at -18 °C until use.

4.2.5. Preparation of $POEGMA_{26}$-$PDPA_{50}$ Polymersomes

Two different methods were used to prepare nanometer-sized $POEGMA_{26}$-$PDPA_{50}$ polymersomes. For polymersomes which were not loaded with amphiphilic fluorescent dyes, the direct hydration method was adopted. The block copolymer was dissolved at a concentration of 3 mg/mL in 50 mM NaOAc and 100 mM NaCl at approximately pH 2.5. The pH was then increased slowly under vigorous stirring to pH 7.3 via the dropwise addition of 1 M NaOH. The solution was then sonicated for 30 min to narrow the particle size distribution of the formed structures. $POEGMA_{26}$-$PDPA_{50}$ forms ~ 10% micelles in addition to polymersomes (data not shown); thus the polymersomes were purified by preparative GPC using a column comprising Sepharose 4B, with a column length of 14 cm and a diameter of 1.5 cm. The dilution factor was found to be a factor of 3 (upon adding 1 mL solution to the column).

Fractions containing pure polymersomes were identified by DLS analysis of the samples (the procedure for which is described in section 4.2.14). Transmission electron microscopy analysis of polymersomes formed from diblock copolymers comprising PDPA has been extensively conducted previously by the research groups of Armes and Battaglia.[28,32,33,36,47]

To prepare polymersomes loaded with Oregon Green® 488 DHPE, the film rehydration technique was adopted. In a typical experiment, 4 mg $POEGMA_{26}$-$PDPA_{50}$ was dissolved at a concentration of 2 mg/mL in a solution of 2:1 chloroform:methanol, adapting a previously reported protocol.[43] 0.1 mg Oregon Green® 488 DHPE dissolved in methanol was added to this solution, and the solvent of the mixture was removed via rotary evaporation, resulting in a thin film of the block copolymer and the amphiphilic dye on the walls of the round bottom flask. This film was hydrated at ~ pH 2.5 and a solution of pure polymersomes was obtained using the method described above for the direct hydration of the block copolymer.

4.2.6. Preparation of $POEGMA_{26}$-$PDPA_{50}$ Polymersomes Loaded with Plasmid DNA

A solution of $POEGMA_{26}$-$PDPA_{50}$ (4 mg/mL) was prepared using the direct hydration method, as described above, and the pH was adjusted to ~ pH 5.9. 20 µg of $Cy^{TM}5$-labeled pDNA was dissolved in 333 µL TE buffer (pH 8.0) and the resulting solution was added dropwise to 1 mL of the block copolymer solution under stirring, taking care to ensure that the pH of the block copolymer solution did not rise above pH 6.1. The concentration of the block copolymer solution at this stage was 3 mg/mL. The pH was slowly raised to pH 7.3 under stirring to allow the formation of polymersomes loaded with pDNA. The resulting mixture was sonicated for 30 min,

and the polymersomes were separated off from micelles and free pDNA by preparative GPC. Fractions containing pure polymersomes loaded with pDNA were identified by DLS and UV-vis analysis. For the UV-vis measurements to determine DNA concentration, the pH of a small aliquot of the fraction was lowered to pH 5.5 to dissociate the polymersomes and prevent the polymersome absorbance close to 200 nm masking the absorbance at 260 nm due to the presence of DNA. The encapsulation efficiency was calculated by measuring the pDNA absorbance at 260 nm both before and after preparative GPC purification of the polymersomes, and using the Beer-Lambert law to convert the absorbance readings to pDNA concentration.

4.2.7. Preparation of LbL Planar Films Incorporating Polymersomes

Solutions of TA and PVPON were prepared at a concentration of 1 mg/mL in 50 mM NaOAc and 100 mM NaCl and the pH adjusted to 7.1. Silicon wafers were cleaned by immersing them in Piranha solution (7:3 v/v sulfuric acid:hydrogen peroxide) for 20 min. (*Caution! Piranha solution is highly corrosive. Extreme care should be taken when handling Piranha solution and only small quantities should be prepared*.) The wafers were then washed with MilliQ water and dried under a stream of nitrogen. First, a layer of PEI (1 mg/mL in 500 mM NaCl) was deposited onto the wafers for 20 min. The wafers were then washed three times with MilliQ water before adding TA for 15 min. After washing three times with buffer (50mM NaOAc and 100 mM NaCl) this cycle was repeated to deposit a further three bilayers of PVPON and TA. Next, a layer of $POEGMA_{26}$-$PDPA_{50}$ polymersomes (1 mg/mL in 50 mM NaOAc and 100 mM NaCl) was deposited, allowing 1.5 h for the polymersomes to adsorb. After

washing in MilliQ water (pH 7.1), capping layers of TA, PVPON and TA were deposited, and the following day the multilayered film was analyzed by AFM (stored dry overnight).

4.2.8. Preparation of LbL Capsules Incorporating Polymersomes

Solutions of tannic acid (TA) and PVPON were prepared at a concentration of 1 mg/mL in 50 mM NaOAc and 100 mM NaCl. The pH of these solutions was adjusted to pH 7.1, and the solutions were filtered (0.2 μm, Pall Corporation) prior to use. A 1 mg/mL solution of PVPON in MilliQ water was also prepared. Silica particles (50 μL of a 50 mg/mL solution) were centrifuged (1000 *g* for 30 s), and the supernatant was removed. For the initial washing step, 300 μL of MilliQ water was added, the particles redispersed and centrifuged again; this procedure was repeated twice. Subsequently, 50 μL PVPON (1 mg/mL in MilliQ water) was added to the particles and allowed to adsorb for 15 min (to deposit the first layer of the multilayered shell). The particles were then washed using the aforementioned protocol using MilliQ water (1x) then pH 7.1 50 mM NaOAc and 100 mM NaCl (2x). All subsequent washing and layering steps were conducted in pH 7.1 50 mM NaOAc and 100 mM NaCl. TA (50 μL, 1 mg/mL) was then adsorbed to the particles for 15 min which were subsequently washed. This procedure was then repeated to deposit two further bilayers of PVPON and TA, followed by a layer of $POEGMA_{26}$-$PDPA_{50}$ polymersomes (100-500 μL), allowing an adsorption time of 2.5 h for the polymersomes. After washing three times in buffer, capping layers of TA, PVPON and finally TA were added. Polymersome-loaded capsules were left overnight prior to removing the silica core templates using buffered HF, as described below.

'Control' samples of capsules without polymersomes were prepared using a similar protocol, depositing a total of 4 bilayers of PVPON and TA.

4.2.9. Isolation of Hollow Capsules via Silica Core Template Removal

Particle suspensions were mixed well by vortexing and transferred to 500 µL Eppendorf tubes. The particles were washed once with pH 7.3 MilliQ water and resuspended in 50 µL pH 7.3 MilliQ water. The template silica cores were then removed to isolate hollow capsules by the addition of hydrogen fluoride (HF) buffered to pH 7.3 with ammonium fluoride (NH_4F) (250 µL, 1:13 5 M HF:13.3 M NH_4F). (*Caution! Note that hydrogen fluoride and ammonium fluoride are highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared.)* The samples were tapped gently to dissolve the silica cores and after ~ 15 minutes, the samples were spun at 1500 *g* for 20 min. The supernatant was removed, the pellet gently tapped, and the resulting capsules were resuspended in 300 µL MilliQ water and spun again at 1500 *g* for 20 min. Three such washing steps were performed before the capsules were resuspended in a suitable volume (typically 120 µL) of pH 7.3 150 mM PBS or MilliQ water.

4.2.10. Measuring the Release of Plasmid DNA from Polymersome-Loaded Capsules

The pH of isolated hollow capsules in 150 mM PBS buffer was lowered to 5 via the addition of 0.1 M HCl (to a small aliquot of capsules). Capsules were analyzed by fluorescence microscopy and flow cytometry, then spun at 1500 *g* for 20 min. The supernatant was removed and the capsule pellet was resuspended in pH 7.3 PBS.

4.2.11. TEM Sample Preparation and Analysis

5 µL of the sample was allowed to adsorb for 5 min onto a carbon-coated Formvar film mounted on 300 mesh copper grids (ProSciTech, Australia). The grids were UV-treated for 10 min prior to sample loading. After the sample had adsorbed onto the grid, the grid was blotted dry using filter paper. The grid was then passed into a drop of pH 7.4 MilliQ water for 5 s, blotted dry, and allowed to air dry overnight. Samples were analyzed using a transmission electron microscope (FEI Tecnai F20 (FEI Company, Eindhoven, The Netherlands)). The microscope was operating at 200 kV and was equipped with a charge-coupled device (CCD) camera. Images were processed using DigitalMicrograph Demo software (Gatan).

4.2.12. AFM Sample Preparation and Analysis

AFM images were acquired of air-dried LbL capsules on microscope glass slides with a JPK NanoWizard BioAFM. A total of 1.5 µL of a capsule suspension was allowed to dry on a PEI-coated clean microscope glass slide. Typical scans were conducted in intermittent contact mode with MikroMasch silicon cantilevers (NSC/CSC). Capsule thicknesses and roughnesses were determined using the accompanying JPK image processing (version V.3.3.32) software.

4.2.13. Phase Contrast and Fluorescence Microscopy

Samples were analyzed by differential interference contrast (DIC) and fluorescence microscopy using an inverted Olympus IX71 microscope equipped with a DIC slider (U-DICT, Olympus) and a CCD camera. A tungsten lamp was used to visualize samples in DIC mode. For fluorescence images, samples were illuminated with an

Hg arc lamp, using a UF1032 filter cube. A 60× objective lens (Olympus UPFL20/0.5 NA, W.D. 1.6) was used to record images.

4.2.14. Dynamic Light Scattering

For dynamic light scattering (DLS) measurements, 20-50 µL of sample was diluted to 500 µL with a filtered buffer solution comprising 50 mM NaOAc and 100 mM NaCl, transferred to a low volume disposable cuvette, and analyzed at a 90° angle to the incident laser beam using a particle sizer (Malvern HPPS). The diameter by intensity method was used to determine the particle size distribution.

4.2.15. Quartz Crystal Microgravimetry

Quartz crystal microgravimetry (QCM) measurements were performed using a QCM-D E4 device with four flow cells (Q-Sense AB, Västra Frölunda, Sweden). All frequency values quoted are for the third overtone. The fifth, seventh, ninth and eleventh overtones followed the same general trend as the third. The temperature was set at 24 °C for all experiments. Gold-coated quartz sensor crystals were cleaned with Piranha solution (7:3 v/v sulfuric acid:hydrogen peroxide) for 5 min prior to use. (*Caution! Piranha solution is highly corrosive. Extreme care should be taken when handling Piranha solution and only small quantities should be prepared*.) They were then washed thoroughly with MilliQ water, 2-propanol and then again with MilliQ water prior to drying under a stream of nitrogen. The crystals were then placed in a UV-cleaner for 15 min. The crystals were set up in the QCM instrument in MilliQ water and a layer of PEI (1 mg/mL in 500 mM NaCl) was deposited. The conditions were then switched to acetate buffer (50 mM NaOAc and 100 mM NaCl) at pH 7.3

before depositing layers of TA and PVPON. A flow rate of 250 µL/min was applied for the deposition of each layer onto the crystals, with an incubation time of 15 min applied for TA and PVPON to saturate the crystal surface. An adsorption time of 1.5 h was applied for the polymersomes. 250 µL of each polymer solution was added to the crystals, and washing steps between each layer were carried out by flowing 500 µL buffer solution over the crystals.

4.1.16. Flow Cytometry

For flow cytometry measurements, 2 µL of capsules was diluted to 800 µL in PBS buffer (pH 7.3, 150 mM). The relative fluorescence intensity of the capsules was assessed using an excitation wavelength of 488 nm for observation of the Oregon Green® 488 DHPE signal and 647 nm for analysis of the Cy™5 label on the DNA. Measurements were performed on a Partec CyFlow Space (Partec GmbH) flow cytometer.

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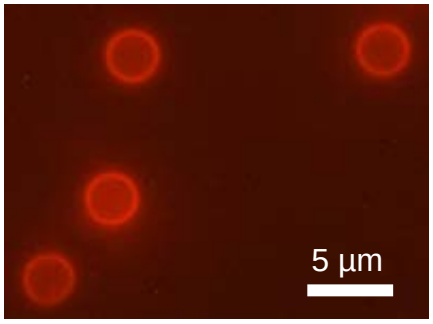
Table of Contents

Polymersome-Loaded Capsules

H. Lomas, A. P. R. Johnston, G. K. Such, Z. Zhu, K. Liang, M. P. van Koeverden, S. Alongkornchotikul, F. Caruso*

Polymersome-Loaded Capsules for Controlled Release of DNA



We report the formation of a novel drug delivery carrier for the controlled release of DNA. This carrier comprises a layer-by-layer capsule subcompartmentalized with pH-responsive polymersomes loaded with Cy™5-labeled plasmid DNA. The DNA is released from the capsules in response to pH changes within the physiological range.

Supporting Information

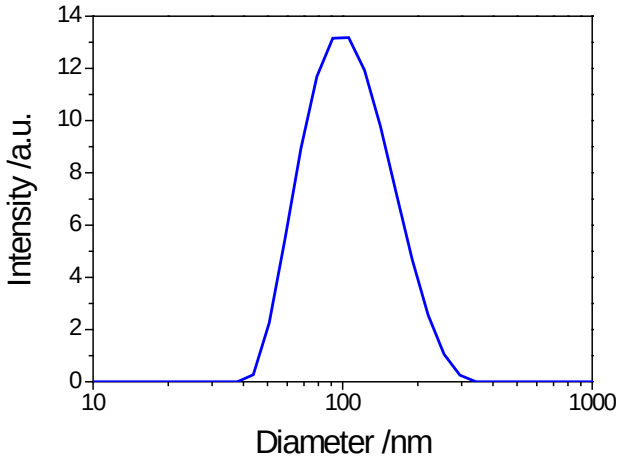


Figure S1. Typical particle size distribution of GPC-purified $POEGMA_{26}$-$PDPA_{50}$ polymersomes, as measured by DLS.

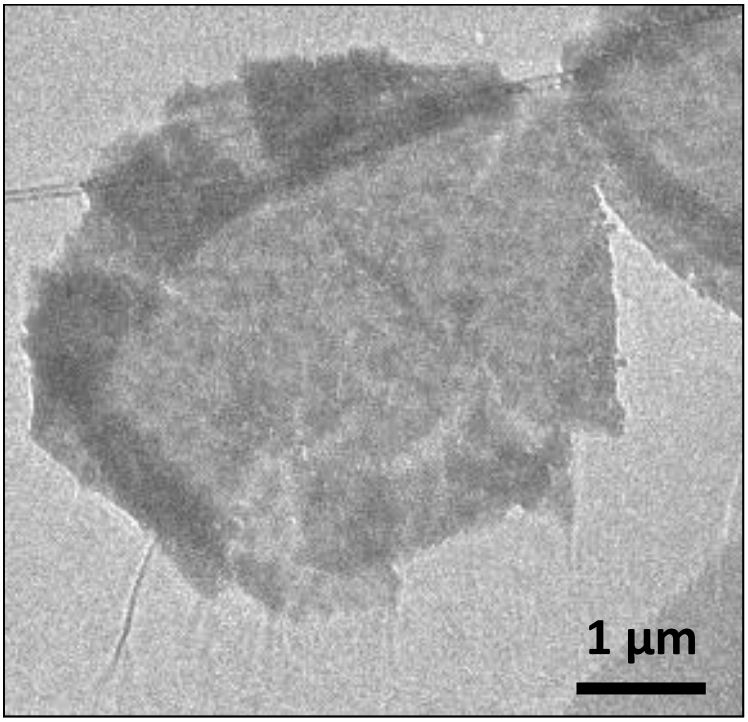


Figure S2. TEM image of a LbL capsule comprising $(PVPON/TA)_3$/$POEGMA_{26}$-$PDPA_{50}$ polymersomes/TA/PVPON/TA.

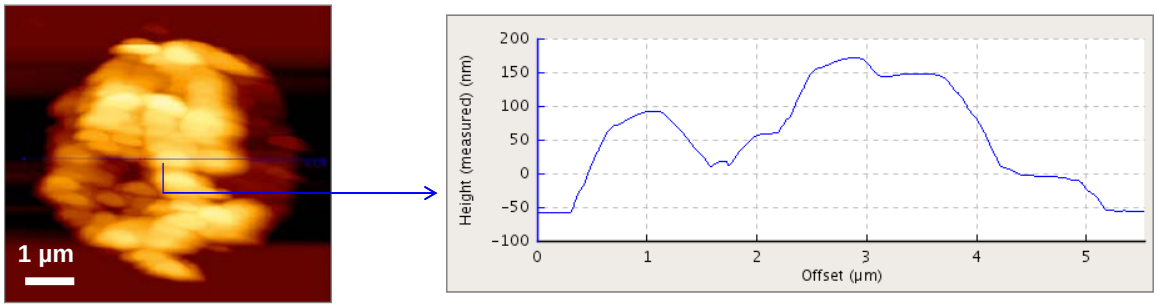


Figure S3. AFM image of a LbL capsule comprising $(PVPON/TA)_3/POEGMA_{26}$-$PDPA_{50}$ polymersomes/TA/PVPON/TA, and the cross-sectional profile to assess capsule roughness.

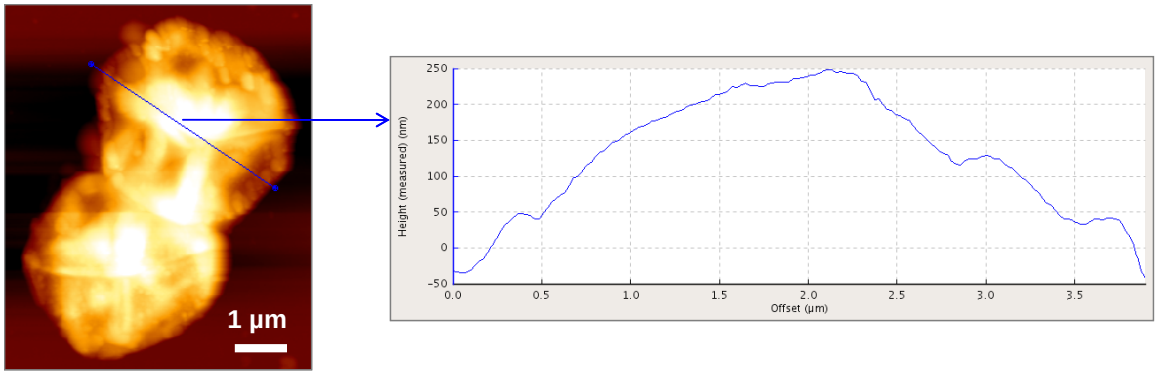


Figure S4. AFM image of LbL 'control' capsules comprising $(PVPON/TA)_4$, and the cross-sectional profile to assess capsule roughness.

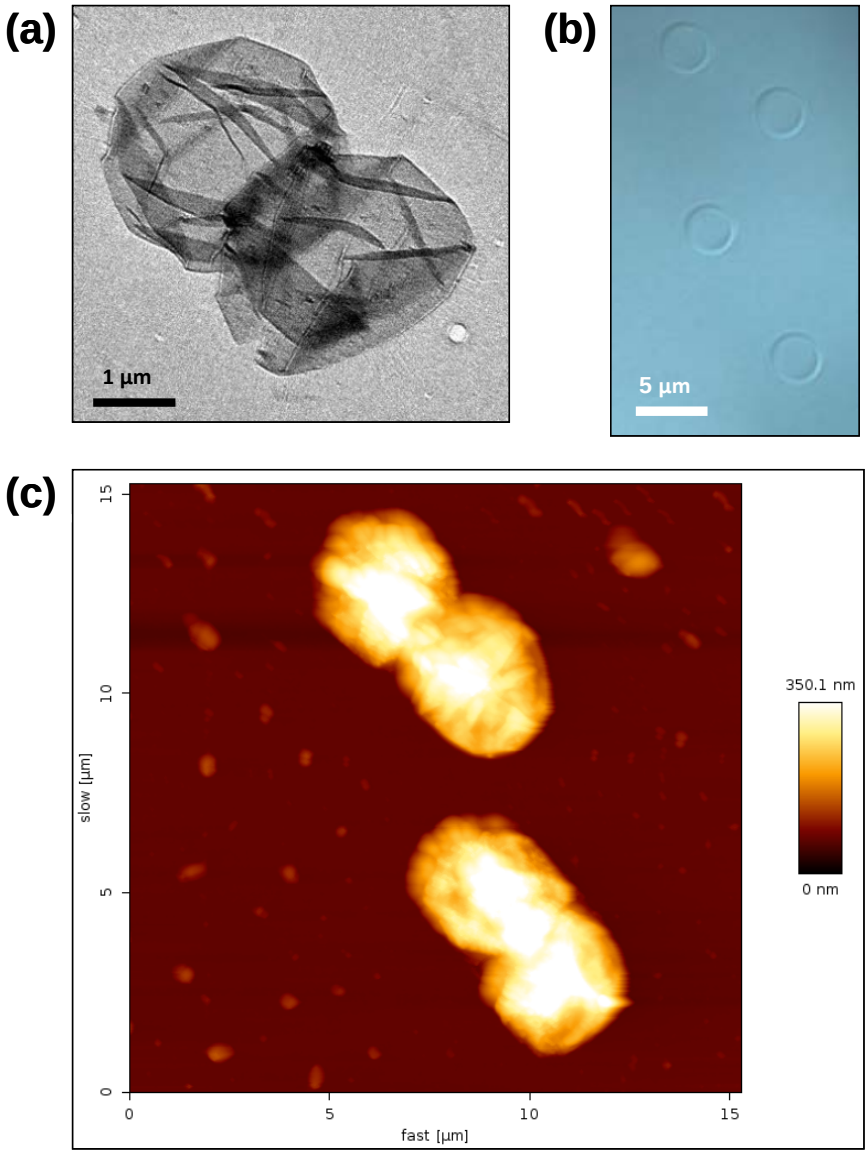


Figure S5. (a) TEM micrograph, (b) phase contrast micrograph, and (c) AFM image of LbL 'control' capsules comprising $(PVPON/TA)_4$.

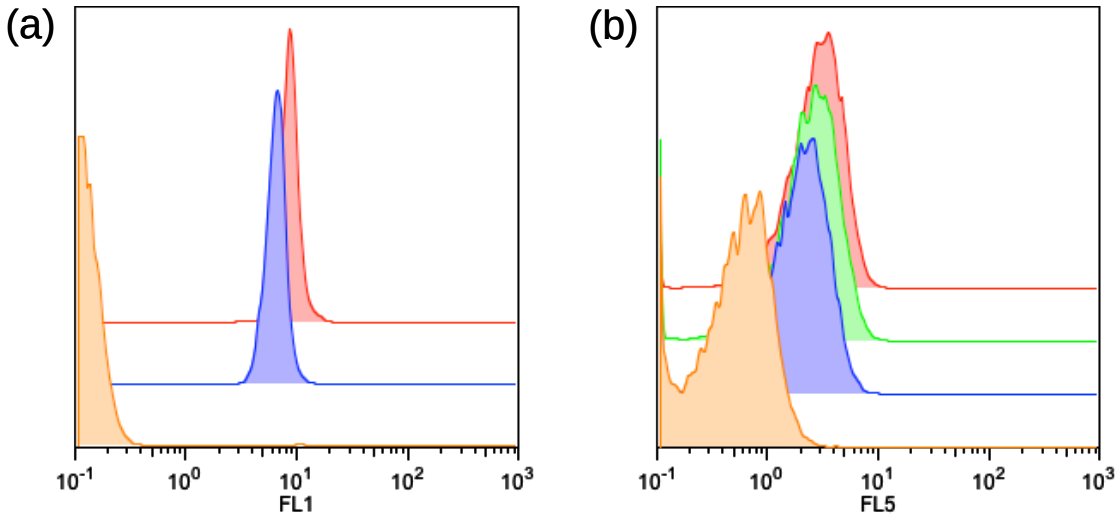


Figure S6. Flow cytometry data for capsules incorporating $POEGMA_{26}$-$PDPA_{50}$ polymersomes loaded with Oregon Green® 488 DHPE and Cy™5-pDNA, measured at pH 7.3 (red), pH 5 (green), and pH 7.3 following a reduction in pH to 5 (blue). As a control, unlabeled capsules incorporating $POEGMA_{26}$-$PDPA_{50}$ polymersomes were analyzed at pH 7.3 (orange). All measurements were performed in 150 mM PBS. (a) Shows the fluorescence intensity of the capsules using an excitation wavelength of 488 nm for Oregon Green® 488 DHPE detection and (b) shows that at an excitation wavelength of 647 nm for Cy™5-pDNA detection.