Fundamental Studies of Hybrid Poly(2-(diisopropylamino)ethyl methacrylate)/Poly(*N*-vinyl pyrrolidone) Films and Capsules

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Abstract

Hybrid and multi-compartment carriers are of significant interest for the development of next-generation therapeutic drug carriers. Herein, fundamental investigations on layer-by-layer (LbL) capsules consisting of two different polymers are presented. The hybrid systems were designed to have pH-responsive, charge-shifting poly(2-(diisopropylamino)ethyl methacrylate) (PDPA) inner layers and low-fouling poly(*N*-vinylpyrrolidone) (PVPON) outer layers. Planar hybrid films with different

layer ratios were studied by quartz crystal microgravimetry (QCM) and atomic force microscopy (AFM). The information obtained was translated to particulate templates to prepare hybrid capsules, which were stabilized by click chemistry. The charge-shifting behavior of PDPA improved the cargo encapsulation and initial retention of a model CpG cargo, while outer layers of PVPON improved biofouling properties compared to single-component PDPA capsules. The results demonstrate the need to understand and design multifunctional systems that can successfully embody different functionalities in a single, stable construct for the fabrication of next-generation drug and gene delivery carriers aimed at overcoming the challenges encountered in biological systems.

Keywords: polymer films, capsules, layer-by-layer, click chemistry.

Introduction

The design of therapeutic polymer carriers for intracellular drug and gene delivery is integral to the advancement of next-generation therapeutics. However, such carriers need to fulfill numerous criteria to find successful application in *in vivo* therapies, which include simple and reproducible formulation, high therapeutic loading, and responsive properties tailored for the biological environment. In particular, the latter is essential for achieving triggered degradation and cargo release.^{1,2} Typically, these criteria are not satisfied in simple single-component systems, and as such, limit their biological application as therapeutic carriers. To this end, multi-component carriers with specific properties need to be designed and engineered, for example by using functional copolymers or (polymer) blends/composites.³ Additionally, post-polymerization modification of starting materials with further functional groups,⁴ and the conjugation

of carriers with poly(ethylene glycol) (PEG) to improve their circulation in blood,⁵ are widely used approaches. Matsumoto et al. reported superior properties exhibited by advanced polyion-complex micelles (PICMs), based on the combination of pH and redox responsive functionalities.⁶ Other strategies for the preparation of multi-functional carriers include merging different systems with individual properties into both hybrid systems and composites. Städler et al. demonstrated this approach by combining polymer capsules and liposomes to produce smart multi-compartment drug carriers, known as capsosomes.⁷ Therefore, the design of multi-functional systems that embody different functionalities in a single stable construct is essential for the fabrication of next generation drug and gene delivery carriers, which can surmount the specific challenges encountered in biological systems.⁸

Recently, a versatile pH-responsive polymer, poly(2-(diisopropylamino)ethyl methacrylate) (PDPA), was reported in the context of advanced therapeutic delivery systems. PDPA undergoes a hydrophilic/hydrophobic pH-induced transition within a physiological relevant pH window.⁹⁻¹³ This is a particularly useful property as the charge-shifting transition coincides with a common biological pH gradient between the bloodstream pH of 7.4, and acidic intracellular pH (endosomal pH is between 5.5 – 6.0 and lysosomal pH is between 4.5 – 5.0).^{1,14} In work reported by Armes and coworkers, the DPA monomer was used with a biocompatible 2-(methacryloyloxy)ethyl phosphorylcholine (MPC) monomer in the formulation of diblock copolymer (PMPC-*b*-PDPA) vesicles that were colloidally stable at physiological pH, whilst demonstrating complete vesicle dissociation below pH 6.⁹ These carriers show promise for intracellular cargo delivery; additionally, the assembly mechanism has been suggested to protect loaded DNA in both polymer states. At physiological pH, the DNA is

physically encapsulated within the lumen, while in an acidic environment the cationic PDPA segment forms a complex with the negatively charged phosphate groups of the DNA. Liang et al. developed PDPA-based polymer capsules that were stabilized with a redox-responsive crosslinker to obtain a dual responsive system.¹³ In contrast to other responsive delivery systems,^{15,16} such capsules can exploit the synergistic effects of both pH and redox stimuli within cells to achieve efficient cargo release. Despite having biologically-responsive properties, single-component or "naked" PDPA capsules are hydrophobic at physiological pH, and hence are expected to demonstrate poor fouling behavior when exposed to serum.^{5,17} This is problematic because just as phagocytic cells (e.g., macrophages and monocytes) remove foreign material from the body to protect it against infection, they also recognize therapeutic biomolecules and delivery systems as foreign materials, removing them from the body.⁵ Therefore, it is crucial for carrier systems to possess low-fouling properties to avoid opsonization, and therefore prolong blood circulation lifetimes for efficient delivery to target sites.¹⁷

In this work, we describe a hybrid polymer capsule system that combines the low-fouling properties of a poly(*N*-vinylpyrrolidone) (PVPON) system recently reported by our group,^{18,19} with the pH-responsive capability offered by PDPA to produce a novel multifunctional therapeutic carrier. The polymer capsules were formulated by assembling alkyne-modified PDPA (PDPA_{Alk}) and PVPON (PVPON_{Alk}) onto colloidal silica templates using the layer-by-layer (LbL) approach. The ratio of PDPA_{Alk} to PVPON_{Alk} layers in crosslinked films was systematically varied, and the resulting film properties were investigated. To understand the film properties, we first studied the single-component and hybrid films using planar techniques (i.e., QCM and AFM). This was later translated onto colloidal systems to study the shrinking/swelling

behavior of the capsules at different pHs. We also verified the charge-shifting properties of PDPA on the cargo retention ability of the capsules by loading the capsules with a biomolecule, and monitoring the retention over 48 h. Finally, protein-fouling experiments were performed on the capsules to validate the low-fouling characteristics of PVPON. This fundamental study demonstrates that the specific functional properties of individual systems can be engineered into a single entity. More importantly, it highlights the potential advancement of hybrid systems in satisfying different biological requirements to overcome limitations in existing capsule systems.

Experimental Section

Materials. Silica (SiO₂) particles (2.59 and 5.28 μ m diameter) and aminated particles (2.39 μ m diameter) were purchased from MicroParticles GmbH (Germany) as 5 wt % suspensions. Poly(methacrylic acid, sodium salt) (PMA, M_w 15 kDa) was purchased from Polysciences (USA). Alexa Fluor 488 azide (Alexa Fluor 488 5-carboxamido-(6-azidohexanyl), bis(triethylammonium salt), AF488_{Az}) and Alexa Fluor 647 azide, triethylammonium salt (AF647_{Az}) were obtained from Invitrogen (USA). 3-(*N*-morpholino)-propanesulfonic acid (MOPS) was purchased from Acros Organics (Geel, Belgium). All other chemicals, including sodium acetate (NaOAc), poly(ethyleneimine) (PEI, M_w 25 kDa), poly(*N*-vinyl pyrrolidone) (PVPON, M_w 55 kDa), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (New South Wales, Australia) and used as received, unless otherwise stated. Bovine serum albumin (BSA, minimum 98% by electrophoresis), lysozyme (human), and myoglobin (from horse skeletal muscle) were obtained from Sigma-Aldrich. Fluorescently labeled CpG (FITC-CpG) was synthesized by GeneWorks (Australia). High-purity (Milli-Q) water with a

resistivity greater than 18 M Ω cm was obtained from an in-line Millipore RiOs/Origin water purification system. All solutions were adjusted to the required pH using a pH meter (Mettler-Toledo MP220 pH meter) with 1.0 M hydrochloric acid (HCl) and 1.0 M sodium hydroxide (NaOH) solutions.

Polymer and Crosslinker Synthesis. The synthesis of the PDPA_{Alk} (See Supporting Information; Scheme S1A) has been described elsewhere,¹³ while the synthesis procedure for PVPON_{Alk} (See Supporting Information; Scheme S1B) and the disulfide reducible bisazide crosslinker can be found in previously published studies.^{18,19}

Quartz Crystal Microgravimetry with Dissipation Monitoring (QCM-D). Film buildup was analyzed using a Q-Sense E4 instrument (Q-Sense AB, Västra Frölunda, Sweden). Silicon dioxide-coated AT-cut quartz crystals with a fundamental resonant frequency of 5 MHz and a constant (*C*) of 17.7 ng cm⁻² Hz (Q-Sense AB, Västra Frölunda, Sweden) were cleaned with Piranha solution (70/30 v/v % sulfuric acid/hydrogen peroxide), followed by extensive rinsing with Milli-Q water. *Caution! Piranha solution is highly corrosive. Extreme care should be taken when handling Piranha solution, and only small quantities should be prepared.* The crystals were then blow-dried with nitrogen and treated in a UV-ozone TipCleaner (Bioforce NanoSciences, USA) for 10 min to remove any remaining contaminants. Cleaned crystals were mounted into the flow chambers of the instrument and three systems, namely PMA/(PVPON_{Alk})₇, PMA/(PDPA_{Alk})₇, and PMA/[(PDPA_{Alk})₅/(PVPON_{Alk})₂] were assembled in different chambers. The measurements were monitored at a constant temperature of 23.8 °C throughout the experiments. The crystals were first primed with a layer of PEI (1 mg mL⁻¹ in 0.5 M NaCl) for 10 min, followed by rinsing in Milli-Q water and 0.05 M NaOAc buffer. Next, a layer of PMA_{Alk} (1 mg mL⁻¹ in 0.05 M

NaOAc buffer, See Supporting Information for synthesis) was deposited onto the primer layer for 15 min, and the films were rinsed in NaOAc buffer, followed by the adsorption of unmodified PVPON (1 mg mL^{-1} in 0.05 M NaOAc buffer) and further rinsing. To construct the multilayer films, alternating layers of PMA (0.5 mg mL^{-1} in 0.05 M NaOAc buffer) and PVPON_{Alk} or PDPA_{Alk} (0.5 mg mL^{-1} in 0.05 M NaOAc buffer) were successively deposited onto the precursor layers of the crystals, to a total of seven bilayers of PMA/polymer_{Alk} (where polymer_{Alk} refers to either PVPON_{Alk} or PDPA_{Alk}). Each polymer adsorption step was for 15 min, with intermediate rinsing in 1 mL of NaOAc buffer to remove excess polymer material. All polymer solutions were flowed over the crystal at a constant flow rate of $300 \mu\text{L min}^{-1}$, and the raw data was analyzed using accompanying QTools 3.0.0.175 software. All overtones measured (1st, 3rd, 7th, 11th, and 13th) displayed the same trend; however, only the 3rd overtone frequency and dissipation values are quoted. The frequency/dissipation (f/D) ratios of the films (>10) validates the assumptions behind the Sauerbrey equation,²⁰ and hence film buildup is reported both as a frequency change and absolute mass.

Surface Structure and Morphology. A series of films were assembled in a similar way to the QCM-D experiments, by varying the inner PDPA_{Alk} to outer PVPON_{Alk} layers at ratios of 0:7, 1:6, 3:4, 5:2, and 7:0. The films were then crosslinked using 2 mL of crosslinking solution consisting of the bisazide crosslinker (0.5 mg mL^{-1}), sodium ascorbate (4.44 mg mL^{-1}), and copper (II) sulfate (1.75 mg mL^{-1}) at a ratio of 3:1:1 by volume in 0.05 M NaOAc buffer (pH 4). This crosslinking condition was scaled proportionately to that used in crosslinking the core-shell particles. The films were incubated overnight with constant agitation. Following crosslinking, the films were washed with $500 \mu\text{L}$ of 0.05 M NaOAc buffer and then washed in buffers in the

following sequence: 0.02 M MOPS, 0.05 M MOPS, 0.1 M MOPS and twice in 0.01 M PBS buffer (pH 7.4) to remove the sacrificial non-crosslinked polymer layers. This resulted in crosslinked single-component PVPON_{Alk} and PDPA_{Alk} films, and hybrid PDPA_{Alk}/PVPON_{Alk} films. The films were further washed with PBS buffer (pH 6.0) to determine the effects of solution pH on the film properties. For detailed investigation of the surface structure and morphology of the films, atomic force microscopy (AFM) images of hydrated films on freshly cleaved mica (22 mm diameter V-4, SPI Chem, USA) were acquired using a JPK Nanowizard II instrument (JPK Instruments AG, Berlin, Germany). Scans were conducted in AC (alternating current) mode with ultrasharp, high-reflectance, SiN cantilevers (CSC37/Cr-Au, MikroMasch, Tallinn, Estonia). As the AC mode images were performed in buffer, a cantilever driving frequency of ca. 10 kHz, and nominal spring constant of 0.3 N m⁻¹, was used to collect the 10 x 10 μm² images. AFM measurements were performed in PBS buffer at pH 6.0 and 7.4, and at a controlled temperature of 23.0 ± 0.3 °C, using a thermally-regulated liquid cell (BioCell, JPK Instruments AG). Processing (first-order image flattening and plane-fitting) and root-mean-square (RMS) roughness evaluation of the AFM scans were performed using accompanying JPK image processing software and algorithms.

Polymer Capsule Preparation. Polymer capsules were prepared in 0.05 M NaOAc buffer (pH 4) in a similar way to a previously reported protocol.¹⁹ Depending on the system, after depositing precursor PVPON and PMA (0.05 M NaOAc buffer at pH 4) onto SiO₂ templates (2.59 μm-diameter for PVPON and hybrid systems, 5.28 μm-diameter for PDPA capsules), subsequent layers were assembled using alternating layers of PDPA_{Alk} or PVPON_{Alk} and PMA. The assembly pH was chosen to ensure that PMA (pK_a ~6.5) was protonated and can interact with PVPON via hydrogen bonding.¹⁸

For PMA/PDPA_{Alk} assembly, this solution pH also ensures that PDPA is below its pK_a of 6.4 and is cationic, thus electrostatically interacting with negatively charged PMA.¹³ A series of capsules with a total of seven bilayers (excluding the precursor PVPON layer) was assembled by varying the inner PDPA_{Alk} layers to outer PVPON_{Alk} layers at ratios of 0:7, 1:6, 3:4, and 5:2.

Core-shell particles with a total of seven layers of alkyne-functionalized polymers (PDPA_{Alk} and/or PVPON_{Alk}) were incubated with 1 mL of crosslinking solution, as described earlier. The particles were incubated overnight with constant agitation to allow for crosslinking of the films via a “click” reaction. The particles were then washed using three wash cycles and resuspended in 300 μ L of 0.05 M NaOAc buffer (pH 4). For visualization, multilayer films were post-labeled after crosslinking, according to an established methodology.¹⁹ Hollow capsules were obtained by dissolving the SiO₂ template particles with buffered hydrofluoric acid (HF) (2 M HF/8 M NH₄F). *Caution! Hydrofluoric acid and ammonium fluoride are highly toxic and extreme care must be taken when handling.* To remove the hydrogen-bonded PMA from the crosslinked multilayers, the capsules were washed in buffers in the following sequence: 0.02 M MOPS, 0.05 M MOPS, 0.1 M MOPS, and twice in 0.01 M PBS buffer (pH 7.4) via five wash cycles.

To study the stabilizing effects of outer PVPON_{Alk} layers on increasing number of inner PDPA_{Alk} layers, a series of hybrid capsules with ratios 5:2, 10:2, 15:2 and 20:2 of inner PDPA_{Alk} to outer PVPON_{Alk} layers were assembled. Core-shell particles were incubated in crosslinking solutions that were scaled proportionately with respect to the number of polymer layers.

Differential interference contrast (DIC) and fluorescence microscopy images of the capsules were taken with an inverted Olympus IX 71 microscope equipped with a DIC slider (U-DICT, Olympus) and the corresponding filter sets, using a 60× objective lens (Olympus UPLFL20/0.5 N.A., W.D. 1.6).

Effect of Polymer Interpenetration. To investigate the interpenetration of PVPON_{Alk} in outer layers into the films, the following capsules were prepared: single-component crosslinked PDPA_{Alk} capsules consisting of (i) five and (ii) ten layers (PDPA₅ and PDPA₁₀ respectively), (iii) ten PDPA_{Alk} layers crosslinked once, before the deposition of two PVPON_{Alk} layers and a second crosslinking step, and (iv) ten PDPA_{Alk} layers, followed by two PVPON_{Alk} layers and then crosslinked once. These films were post-labeled with AF488_{Az} to aid with visualization. The templates were then removed and the capsules were washed into PBS buffer (pH 7.4). Fluorescence microscopy was used to observe these capsules and their diameters were measured. A minimum of 20 capsules was measured.

Cargo Retention. To prepare CpG-loaded capsules, 100 μ L of aminated particles ($\sim 1.8 \times 10^8$) were washed with 0.05 M NaOAc buffer using centrifugation/redispersion cycles and were then redispersed into 500 μ L 30% 0.02 M MOPS (pH 8)/70% 0.05 M NaOAc buffer (pH 4). The particles were incubated with FITC-CpG to achieve $\sim 50\%$ surface saturation (i.e., 0.2 mg m^{-2}) in an Eppendorf thermomixer at 20 °C at 1 200 rpm for 20 min. This value for achieving particle surface saturation by DNA was previously reported.²¹ After adsorption, the samples were washed with 30% 0.02 M MOPS (pH 8)/70% 0.05 M NaOAc buffer (pH 4) via three centrifugation/redispersion wash cycles, and twice more with NaOAc buffer. Two types of capsules, namely (PVPON_{Alk})₇ and (PDPA_{Alk})₅/(PVPON_{Alk})₂, were then assembled as described until a total of seven

bilayers (and a precursor PVPON layer) was deposited. Another sample of unloaded (PVPON_{Alk})₇ capsules was also assembled to serve as the control. The films were crosslinked before template removal and PBS buffer washing. The cargo retention stability was monitored by incubating the CpG-loaded capsules in PBS buffer (pH 7.4) at 37 °C, and monitoring the fluorescence intensity of FITC-CpG using flow cytometry over 48 h. Each analysis was obtained from 10 000 individual capsules, and performed in triplicate. A Partec CyFlow Space (Partec GmbH, Germany) flow cytometer, using an excitation wavelength of 488 nm, was used for all flow cytometry experiments. Data analysis was performed with Partec CyflowMax software or FlowJo analysis software to obtain the fluorescence intensities of the samples.

Protein Fouling Behavior. To study the fouling properties of the various systems, PVPON and hybrid capsule systems were assembled onto 2.59 μm -diameter SiO₂ particles, while PDPA_{Alk} capsules were assembled on larger 5.28 μm -diameter particles, as the diameter of these capsules decreases in PBS buffer (pH 7.4). These capsules were intentionally unlabeled for use with FITC-labeled BSA (FITC-BSA) protein. The proteins (BSA, lysozyme, and myoglobin) were labeled according to previously reported procedures.¹⁸ For the different systems, an equal number of capsules ($\sim 1 \times 10^6$) were incubated with 1 mg mL⁻¹ protein in PBS buffer (pH 7.4) at room temperature for 2 h, with constant mild agitation. The capsules were then washed with PBS buffer and measured using flow cytometry for any associated fluorescence. Each experiment was performed in triplicate.

Results and Discussion

Film assembly was based on the widely-reported LbL technique.^{22,23} Hybrid polymer films consisting of PDPA_{Alk}, PVPON_{Alk}, and PMA, were prepared (Scheme 1). Multilayer film assembly was performed at pH 4 to facilitate film buildup via both electrostatic interactions and hydrogen bonding between PMA and PDPA_{Alk},¹³ while PVPON_{Alk} was deposited as the capping layers via hydrogen bonding with PMA. The ratio of PDPA_{Alk} to PVPON_{Alk} layers in the films was systematically varied. The alkyne-modified layers were crosslinked via "click" chemistry using a reducible bisazide crosslinker, as described in previous work.¹⁹ For the preparation of hollow capsules, the particle support (i.e., silica particles), along with the sacrificial PMA, were removed to form hybrid PDPA_{Alk}/PVPON_{Alk} capsules. We have reported in our previous study on PDPA capsules that PMA readily diffuses out of the capsules upon increasing pH.¹³

Scheme 1. Schematic representation of hybrid PDPA_{Alk}/PVPON_{Alk} films. (a) PMA (yellow) and alkyne-modified PDPA_{Alk} (blue) were sequentially deposited onto a silica template. (b) For hybrid films, layering was performed with alkyne-modified PVPON_{Alk} (green) until the desired number of polymer layers (i.e., PDPA_{Alk}:PVPON_{Alk}) was achieved. A film of (PDPA_{Alk})₅/(PVPON_{Alk})₂ is shown here. (c) A reducible bisazide crosslinker (black) was used to crosslink PDPA_{Alk} and PVPON_{Alk} layers via "click" chemistry. (d) For the preparation of hollow capsules, colloidal templates were removed and the films were washed into PBS (pH 7.4) to produce crosslinked hybrid PDPA_{Alk}/PVPON_{Alk} films.

Planar Film Assembly and Characterization. We first characterized the hybrid polymer films on planar surfaces before translating the results and understandings to particle templates to focus on the design of multifunctional capsules.

Film buildup was first monitored using QCM-D to investigate the material deposition in single-component and hybrid films. Alternating layers of PMA/PVPON_{Alk}, PMA/PDPA_{Alk}, and hybrid PMA/[(PDPA_{Alk}/PVPON_{Alk})] were assembled to examine if altering the polymer from PDPA_{Alk} to PVPON_{Alk} had an effect on material deposition. A decrease in resonance frequency (Δf) is indicative of material deposition onto the resonator surface. As shown in Figure 1, QCM data show that PMA/PVPON_{Alk} films displayed a higher overall change in frequency compared with PMA/PDPA_{Alk} films suggesting higher material deposition in the former system. The frequency changes for the different films increases linearly with layer number, suggesting regular multilayer film growth. In all cases, the dissipation-to-frequency ratios of the films were low ($< 1 \times 10^{-6}$ per 10 Hz), and thus the Sauerbrey equation is applicable.²⁰ As such, the film buildup is reported both as a frequency change and absolute mass. The mass deposited for PVPON_{Alk} was approximately 50% more than PDPA_{Alk}. This could be attributed to the swelling of hydrophilic PVPON with associated water molecules.²⁴ The buildup also demonstrated that the amount of material deposited for each layer was equivalent (within experimental error). In the hybrid system where PMA/PDPA_{Alk} and PMA/PVPON_{Alk} were deposited

consecutively, the film buildup reflected the corresponding trends of the single-component systems with a higher increase in frequency when PVPON_{Alk} was deposited. This finding suggested that the deposition characteristics of PMA/PVPON_{Alk} was not affected when added to an existing PDPA_{Alk} film.

Figure 1. QCM-D analysis of planar PMA/PVPON_{Alk}, PMA/[(PDPA_{Alk}/PVPON_{Alk})], and PMA/PDPA_{Alk} films up to a total of seven bilayers. Change in frequency (left-axis) and mass (right-axis) for film deposition as a function of layer number. Layer 0 corresponds to the precursor layer system PEI/PMA_{Alk}/PVPON. For the subsequent layers, odd and even numbers represent PMA and alkyne-modified polymers (i.e., PVPON_{Alk} or PDPA_{Alk}), respectively.

The viscoelasticity of the films was also investigated by monitoring the dissipation of the films. An oscillatory behavior was observed for the deposition of PMA/PVPON_{Alk} compared with the deposition of PMA/PDPA_{Alk} (See Supporting Information; Figure S1). This observation could be due to the association of water molecules with hydrophilic PVPON, leading to more swollen and flexible film layers

relative to PMA.²⁴ The decrease in dissipation with every PMA layer also suggested that the PMA layers were more rigid, hence causing the release of hydration water molecules upon deposition.²⁵ The dissipation trend of PMA/PDPA_{Alk} also suggested the formation of a more rigid PDPA_{Alk} layer (Figure S1).

Film Characterization Using Atomic Force Microscopy (AFM). To study the structure and surface morphology of the films by AFM, various ratios of PDPA_{Alk}:PVPON_{Alk} layers were assembled on PEI-coated mica substrates, up to a total of seven bilayers. The films were crosslinked and washed into PBS buffer at pH 7.4 to remove the sacrificial non-crosslinked PMA layers. AFM imaging of hydrated films in PBS buffer at pH 7.4 and at pH 6.0 were acquired to observe the film properties of charge-shifting PDPA above and below its pK_a value of 6.4. The acquired images revealed a distinct difference in the surface morphology between crosslinked (PDPA_{Alk})₇ films and the other films under investigation, (PVPON_{Alk})₇ and hybrid PDPA_{Alk}/PVPON_{Alk} films (Figure 2a; see Supporting Information; Figure S2). The differences in surface roughness of these films were also confirmed by quantifying the root-mean-square (RMS) roughness (Figure 2b). (PDPA_{Alk})₇ films were rough in PBS buffer at both pH 7.4 (RMS = 4795 ± 674 nm) and pH 6.0 (RMS = 1840 ± 137 nm), while the hybrid (PVPON_{Alk})₇ films (RMS = 400 - 750 nm) were considerably smoother (pH 7.4 RMS = 596 ± 24 nm; pH 6.0 RMS = 635 ± 35 nm) at both pH values, indicating more uniform coverage of the surface with PVPON. (PDPA_{Alk})₇ films displayed sharp, distinct features at pH 7.4, and when washed into PBS buffer at pH 6.0, the surface morphology, although still rough, was more defined. This decrease in roughness for the (PDPA_{Alk})₇ films is a result of the charge-shifting transition of PDPA from hydrophobic to hydrophilic at pH values below its pK_a of 6.4. Hydrophilic

PDPA_{Alk} is charged and adopts a more stretched conformation due to repulsion along the polymer chain, resulting in a smoother morphology. Similar results have also been reported by various groups, which investigated the structure of polyelectrolyte (PE) multilayer films by AFM, where an increase in PE film roughness was correlated with decreasing charge density.^{26,27}

Figure 2. (a) Surface morphology of crosslinked PVPON_{Alk}, hybrid PDPA_{Alk}/PVPON_{Alk}, and PDPA_{Alk} multilayer films acquired using atomic force microscopy (AFM). The films consist of a total of seven crosslinked layers of PDPA_{Alk} and PVPON_{Alk} at PDPA_{Alk}:PVPON_{Alk} ratios of (i, vi) 0:7, (ii, vii) 1:6, (iii, viii) 3:4, (iv, ix) 5:2, and (v, x) 7:0. AFM images of the films were measured in (i-v) PBS buffer at

pH 7.4 and (vi-x) PBS buffer at pH 6.0. (b) Root-mean-square (RMS) roughness of the crosslinked (PVPON_{Alk})₇, hybrid PDPA_{Alk}/PVPON_{Alk}, and (PDPA_{Alk})₇ multilayer films, as measured using AFM over an area of 10 × 10 μm².

Responsive Properties of Hybrid Capsules. A series of capsules at the ratios 0:7, 1:6, 3:4 and 5:2 of PDPA_{Alk}:PVPON_{Alk} were assembled as for the planar films, and the alkyne-functionalized films were crosslinked using a bisazide crosslinker. The templates were removed and the films were washed into PBS buffer at pH 7.4 to remove the sacrificial PMA. The hybrid capsules at the different PDPA_{Alk}:PVPON_{Alk} ratios were similar in size to that of (PVPON_{Alk})₇ capsules when observed under a fluorescence microscope (Figure 3a-d). Interestingly, these hybrid capsules did not shrink, as observed for (PDPA_{Alk})₇ capsules (See Supporting Information; Figure S3),¹³ and appeared to be stabilized by the incorporated PVPON_{Alk}. Additionally, these capsules also did not undergo any visible size change when washed into PBS buffer at pH 5.5 (Figure 3e-h). From the results in the planar and capsules studies, it is clear that a minor component of PVPON was sufficient to alter both the surface properties and the responsive behavior of PDPA films and capsules.

Figure 3. Fluorescence microscopy images of crosslinked AF488-labeled PVPON_{Alk} and hybrid PDPA_{Alk}/PVPON_{Alk} capsules. The films consist of a total of seven crosslinked layers of PDPA_{Alk} and PVPON_{Alk} at PDPA_{Alk}:PVPON_{Alk} ratios of (a, e) 0:7, (b, f) 1:6, (c, g) 3:4 and (d, h) 5:2. Crosslinked capsules were washed into (a-d) PBS buffer at pH 7.4 and (e-h) PBS buffer at pH 5.5.

To determine if crosslinked hybrid capsules could regain pH-responsive behavior, a series of capsules at ratios of 5:2, 10:2, 15:2, and 20:2 PDPA_{Alk}:PVPON_{Alk} were assembled and crosslinked. Capsules were washed into PBS buffer at pH 7.4. Even at the highest ratio of PDPA_{Alk}:PVPON_{Alk} (20:2), the capsules remained stabilized by the two outer PVPON_{Alk} layers (Figure 4). This stabilizing characteristic could be attributed to chain interpenetration.²⁸ We postulate that the small molar mass PVPON_{Alk} could permeate the inner layers of PMA/PDPA_{Alk} and participate in crosslinking with the bisazide crosslinker to stabilize the bulk films. Hence, the hybrid films lose the responsive shrinking and swelling characteristics.

Figure 4. Fluorescence microscopy images of AF647-labeled hybrid PDPA_{Alk}/PVPON_{Alk} capsules. The capsules were assembled at the following ratios (a) 5:2, (b) 10:2, (c) 15:2, and (d) 20:2 PDPA_{Alk}:PVPON_{Alk} layers, crosslinked, and washed into PBS buffer at pH 7.4.

Effect of Polymer Interpenetration. To validate our hypothesis of film interpenetration and the stabilizing effects of PVPON_{Alk}, single-component PDPA_{Alk}

capsules of 5 and 10 layers were assembled and crosslinked (herein referred to as PDPA₅ and PDPA₁₀, respectively). In addition, two hybrid systems with 10 PDPA_{Alk} and two PVPON_{Alk} layers but different crosslinking sequences were prepared. The first system was crosslinked after depositing the PDPA layers and again after the deposition of the capping PVPON layers (PDPA₁₀-XL₁-PVPON₂-XL₂), while the second system was exclusively crosslinked after the deposition of all layers (PDPA₁₀-PVPON₂-XL). This variation in crosslinking procedure was used to investigate the role of PVPON_{Alk} in bulk crosslinking and its effect on the responsive nature of the capsules. When the particle templates (2.59 μm) were removed and the capsules washed into PBS buffer (pH 7.4), the capsule systems shrank to differing extents (Figure 5). PDPA₅ capsules shrank to $\sim 1.5 \mu\text{m}$, while PDPA₁₀ capsules exhibited reduced shrinkage ($\sim 2.0 \mu\text{m}$). We attribute the lower extent of shrinkage observed in PDPA₁₀ capsules to higher material deposition, leading to increased steric hindrance.

Further, it was shown that PDPA₁₀-XL₁-PVPON₂-XL₂ capsules did not shrink to the same extent as PDPA₁₀ capsules, and even less shrinkage was observed for PDPA₁₀-PVPON₂-XL capsules. The diameter of PDPA₁₀-XL₁-PVPON₂-XL₂ capsules was intermediate between that of the single-component PDPA_{Alk} (PDPA₁₀) and PDPA₁₀-PVPON₂-XL capsule systems (Figure 5b). In the hybrid systems, PVPON_{Alk} layers could have interpenetrated the films to different extents, exerting a stabilizing effect during film crosslinking. Picart et al. demonstrated that poly(L-lysine) (PLL, $\sim 30 - 50$ kDa) can readily diffuse in and out of PLL/hyaluronan (HA) PE multilayers.²⁸ Furthermore, film assembly in the presence of salt or reduced charged density is likely to enhance polymer interpenetration.^{29,30} Under such conditions, PE chains can adopt coiled conformations during deposition, leading to more swollen and porous multilayer

films that lead to enhanced interpenetration. In the PDPA₁₀-XL₁-PVPON₂-XL₂ system, PDPA_{Alk} was first crosslinked, thus consuming the majority of present alkyne groups. Hence, when the capping PVPON_{Alk} layers were subsequently deposited and the system was crosslinked a second time, less alkyne groups in the PDPA film were available for crosslinking with interpenetrating PVPON_{Alk}. Therefore, these films were stabilized to a lesser extent, and exhibited some shrinking from the template size (i.e., 2.59 μm) to $\sim 2.3 \mu\text{m}$. In the PDPA₁₀-PVPON₂-XL system, PVPON_{Alk} could readily permeate through the films and, together with the bisazide crosslinker, participate in crosslinking of the bulk film material, increasing the extent of stabilization.

Figure 5. (a) Fluorescence microscopy images of different capsule systems; (i) PDPA₅ and (ii) PDPA₁₀ are single-component crosslinked PDPA_{Alk} capsules consisting of five and ten layers, (iii) PDPA₁₀-XL₁-PVPON₂-XL₂ capsules are assembled from ten

PDPA_{Alk} layers (PDPA₁₀) which are crosslinked once (XL₁), followed by the deposition of two PVPON_{Alk} layers (PVPON₂) and a second crosslinking step, (iv) PDPA₁₀-PVPON₂-XL capsules are assembled from the deposition of ten PDPA_{Alk} layers, and two outer PVPON_{Alk} layers, before a single crosslinking step after the film assembly. (b) The diameters of each capsule type were determined using fluorescence microscopy; over twenty capsules were measured to obtain an average size.

Cargo Retention Properties of Capsules. To verify if the charge-shifting property of PDPA could still be exploited for cargo encapsulation and initial retention in the different films, single-component (PVPON_{Alk})₇ and hybrid (PDPA_{Alk})₅/(PVPON_{Alk})₂ capsules were preloaded with a model cargo, FITC-CpG, before film assembly, as reported recently.^{19,21} More recently, CpG-loading of multilayer capsules,³¹ and polypeptide replica particles, have also been reported.³² CpG (–cytosine–phosphate–guanine–) is an oligonucleotide adjuvant that enhances immune responses to an antigen. CpG dinucleotides in bacteria, viruses and parasites are typically unmethylated and thus different to mammalian CpG, which display more frequent methylation of the cytosine nucleotide; this difference is recognized by the Toll-like receptor TLR9 of dendritic cells. The CpG-loaded capsules were incubated in simulated physiological conditions using PBS buffer (pH 7.4) at 37 °C, and the fluorescence intensity of the loaded CpG was monitored over 48 h using flow cytometry. It was found that at the beginning of the experiment (i.e., t = 0 min), (PDPA_{Alk})₅/(PVPON_{Alk})₂ capsules had twice as much CpG fluorescence intensity compared with (PVPON_{Alk})₇ capsules (Figure 6), suggesting more CpG was loaded. The electrostatic interaction between positively charged PDPA_{Alk} and negatively charged CpG could have increased the retention capacity of these carriers compared with uncharged PVPON_{Alk}. After template removal, the CpG

can no longer adsorb onto the previously present positively charged particle template and given its small size, CpG can readily leach out of the semi-permeable capsule films due to the concentration gradient between the capsule interior and the solution. Hence, the higher amount of encapsulated CpG in $(PDPA_{Alk})_5/(PVPON_{Alk})_2$ capsules was attributed to the interaction between positively charged $PDPA_{Alk}$ and negatively charged CpG. At physiological pH, the hydrophobicity of PDPA in the hybrid system can further form a less permeable hydrophobic barrier around the capsule cavity, leading to improved CpG retention.^{10,11} Thus, although the hybrid capsules do not exhibit size changes with pH variation, the charge-transition property shows potential for improving cargo encapsulation and retention.

Figure 6. Cargo retention stability of capsules in simulated physiological conditions using PBS buffer (pH 7.4) at 37 °C, as monitored using flow cytometry over 48 h. $(PDPA_{Alk})_5/(PVPON_{Alk})_2$ and $(PVPON_{Alk})_7$ capsules loaded with FITC-CpG, and a control sample of unloaded $(PVPON_{Alk})_7$ capsules were measured for the FITC fluorescence intensity at various time points.

Fouling Behavior of Polymer Capsules. Effective drug and gene delivery systems need to exhibit low-fouling properties to have longer blood circulation times and to increase the bioavailability for delivery to target sites.^{5,17,33} To perform a preliminary assessment of the outer PVPON_{Alk} layers in resisting protein adsorption, and any improvement to the fouling behavior of PDPA_{Alk} capsules,¹³ (PVPON_{Alk})₇, (PDPA_{Alk})₇ and hybrid capsules at various PDPA_{Alk}:PVPON_{Alk} ratios were incubated with three different FITC-labeled proteins with different isoelectric points (pI),³⁴ namely BSA (pI ~4.7), myoglobin (pI ~7), and lysozyme (pI ~11). As (PDPA_{Alk})₇ shrinks in PBS buffer (pH 7.4), this system was assembled onto larger silica templates (i.e., 5.28 μ m) to allow for shrinking to a comparable size (i.e., surface area) as the other capsules (See Supporting Information; Figure S4).

After incubation with the different proteins, the capsules were washed into PBS buffer and the relative amount of associated protein on the capsules was measured using flow cytometry (Figure 7). The results showed that (PDPA_{Alk})₇ capsules had more associated fluorescent protein in all cases when compared to the other capsules, suggesting that PVPON_{Alk} layers decreased the fouling behavior of the capsules. There were no observable differences in the fouling properties between (PVPON_{Alk})₇ and the hybrid PDPA_{Alk}/PVPON_{Alk} capsules, suggesting a similar presence of PVPON on the outer shell of the hybrid PDPA_{Alk}/PVPON_{Alk} capsules. Noteworthy, two PVPON_{Alk} layers were sufficient to effectively improve the low-fouling properties of the capsules. These results are in accordance with previous studies of the low-fouling behavior of PVPON surfaces.^{18,35} These data also prove that the inner PDPA layers have a minor effect on the capsule biofouling properties.

Figure 7. Flow cytometry analysis of the fouling behavior of PVPON_{AIk}, PDPA_{AIk}, and hybrid PDPA_{AIk}/PVPON_{AIk} capsules using FITC-labeled BSA (a), FITC-labeled

myoglobin (b), and FITC-labeled lysozyme (c). Unlabeled capsules were assembled at various PDPA_{Alk}:PVPON_{Alk} ratios and incubated with protein (1 mg mL⁻¹) in PBS buffer for 2 h, before being measured for bound protein on the capsule surfaces.

Conclusion

We have assembled capsule systems with a pH-responsive, charge-shifting PDPA component as the inner layers and with low-fouling PVPON at the surface. While PVPON_{Alk} appeared to stabilize the pH-responsive behavior of PDPA_{Alk} in hybrid capsules, we found that the charge-shifting behavior of PDPA improved the cargo encapsulation and initial retention of a model CpG cargo, and thus this property could be exploited for biomedical application. The presence of peripheral PVPON_{Alk} in hybrid capsules improved the biofouling properties, highlighting the need to design carriers with low-fouling surfaces to avoid rapid blood clearance and increase blood circulation times. Therefore, a fundamental understanding of polymer multilayer films and capsules is paramount for the design and engineering of specific properties into polymer carriers to satisfy the criteria needed for effective therapeutic delivery.

Supporting Information

Further data, including the synthesis of PMA_{Alk}, QCM dissipation study, and additional fluorescence microscopy images, is available free of charge via the Internet at <http://pubs.acs.org>.

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