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Engineering Enzyme-Cleavable Hybrid Click Capsules with a pH-Sheddable Coating for Intracellular Degradation

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The ability for nanoengineered carriers to intelligently respond to biological variations is fundamental for their application as effective therapeutic delivery systems.[1] Extensive research has been performed on carriers that respond to intracellular triggers such as pH gradients or changes in redox potential.[2] A related emerging area is the use of specific peptide sequences for biologically-inspired degradation of therapeutic carriers.[3] Such peptide sequences can be engineered to degrade specifically at certain sites, based on the presence of a specific enzyme. Cathepsin B (CtsB) is one of the earliest identified lysosomal cysteine proteases in the papain family.[4] It is a ubiquitous enzyme, which predominantly localizes in the acidic cellular compartments (e.g., lysosomes).[4a,5] The common internalization pathway

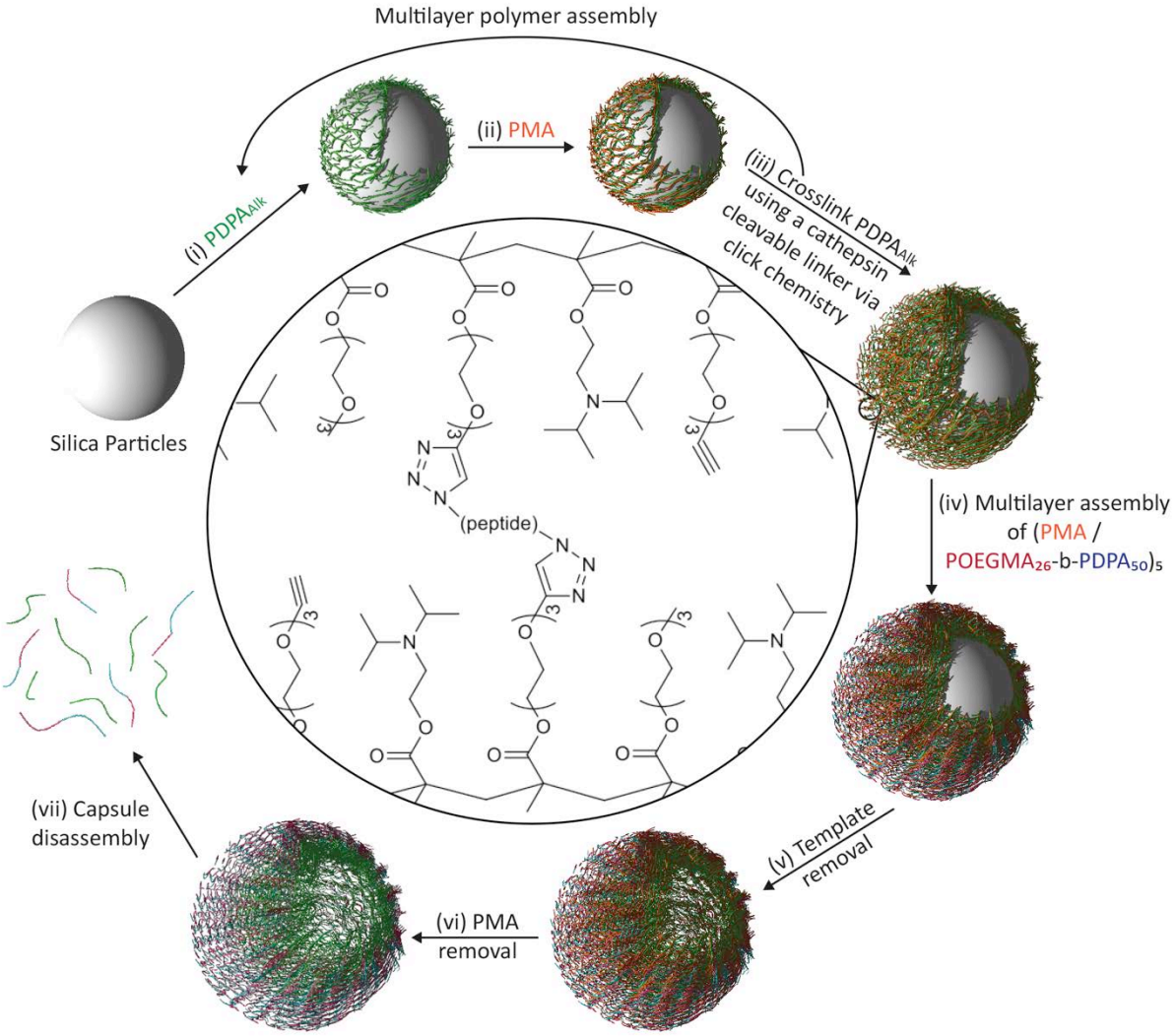
for particulate carriers is endocytosis, in which the carrier is dynamically transported through early endosomes (pH 6.8–5.9), late endosomes, and then lysosomes (pH 6.0–4.9).[6] Hence, the use of CtsB as an enzyme-specific trigger for engineered carriers[7] provides possibilities for precise spatial control over cargo release and carrier degradation (e.g., in lysosomes). To achieve this, a peptide sequence with a CtsB recognition sequence (GFQGVQFAGF) can be incorporated into the carrier.[8] While various degradable polypeptide carriers have been developed,[9] there are few studies that combine polymeric carriers with an enzyme-specific peptide sequence for enhanced cellular degradation.

Recently, we reported a layer-by-layer (LbL)-assembled polymer capsule system based on poly(2-diisopropylaminoethyl methacrylate) (PDPA) that responds to changes in pH and redox potential.[10] These dual-responsive PDPA capsules showed tailored degradation due to capsule swelling (at pH below 6.4)[11] and cleavage of the redox-responsive cross-linker in reducing conditions.[10] However, the use of redox potential has limitations in such delivery systems, as the concentration and activity of reducing agents can be depleted in the endosomes/lysosomes upon usage.[10b] In contrast, the catalytic properties of enzymes allows for continuous regeneration and hence retention of high concentrations and activities of the enzymes within such compartments.

Herein, we report the synthesis of hybrid capsules, combining the pH-responsive behavior of PDPA and an enzyme-specific degradable cross-linker, for enhanced cellular degradation. The hybrid capsules consist of CtsB-cleavable cross-linked inner layers and a pH-responsive outer coating. The outer coating confers colloidal stability and provides protection for the enzyme-sensitive components from premature hydrolysis. This carrier exhibits almost instantaneous carrier degradation (within 10 min) *in vitro*. The significance of this work lies in several important areas: firstly, in the design of a novel hybrid capsule with a non cross-

linked outer coating, which is sheddable upon changes in intracellular pH; secondly, to our knowledge, this is the first system that integrates specific enzyme cleavage sites for lysosomal hydrolysis within a LbL polymer capsule system; and thirdly, this bio-responsive cleavage mechanism enables extremely rapid carrier degradation, which is desirable for instantaneous burst release of cargo.[12] This represents a highly modular approach, combining the advantages of an engineered peptide and a pH response to enhance *in vitro* polymer carrier degradation. Exploiting the nature of biological conditions and the innate responsive properties of materials in the design of carriers is highly promising for effective and specific therapeutic delivery in targeted cellular compartments.

Hybrid capsules, comprising an inner CtsB-cleavable cross-linked polymer capsule with an outer pH-sheddable coating, were prepared by LbL assembly. Assembly of the inner layers of alkyne-functionalized PDPA ($PDPA_{Alk}$), which was obtained by copolymerization of DPA and an alkyne-containing oligo(ethylene glycol) methyl ether methacrylate (OEGMA), and poly(methacrylic acid) (PMA) on silica (SiO_2) particles, was performed at pH 4. Film buildup is based on hydrogen bonding between poly(ethylene glycol) (PEG) moieties in $PDPA_{Alk}$ and protonated PMA carboxyl groups as well as electrostatic interactions between slightly charged PMA and protonated tertiary amine groups of $PDPA_{Alk}$. Uniform film buildup of $PDPA_{Alk}$/PMA bilayers, on planar substrates and on particles, has been reported earlier.[10a] Subsequent to the deposition of five $PDPA_{Alk}$/PMA bilayers, the alkyne groups in PDPA were cross-linked by a bisazide-functionalized CtsB-cleavable (GFQGVQFAGF) linker in the presence of copper (II) sulfate and sodium ascorbate via the copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry reaction (**Scheme 1**). The remaining alkyne moieties in PDPA were used for post-labeling of the inner layers using fluorescence dyes.



Scheme 1. Layer-by-layer (LbL) fabrication of hybrid PDPA click capsules with a pH-sheddable coating: (i) $PDPA_{Alk}$ deposition onto non-porous SiO_2 particle templates; (ii) PMA deposition onto polymer-coated particles; alternate layers of $PDPA_{Alk}$ and PMA were deposited onto polymer-coated particles until the desired number of layers was achieved; (iii) $PDPA_{Alk}$ multilayers were cross-linked between their alkyne moieties using a CtsB-cleavable linker (GFQGVQFAGF) functionalized with a bisazide via click chemistry (the structure of the peptide linker is provided in Scheme S1); (iv) LbL assembly of PMA and $POEGMA_{26}$-b-$PDPA_{50}$ onto the polymer-coated particles until the desired number of layers was achieved; (v) Sacrificial template removal via HF; (vi) Sacrificial PMA layer removal via high pH washing; and (vii) Capsule disassembly upon exposure to endosomal pH and CtsB.

To protect the functionality of the peptide, an outer coating of non cross-linked, pH-sheddable multilayers based on poly[oligo(ethylene glycol) methyl ether methacrylate]-block-poly(2-diisopropylaminoethyl methacrylate) ($POEGMA_{26}$-b-$PDPA_{50}$) was deposited. Previous studies have shown the importance of an outer coating serving as a protective barrier for the inner layers.[13] Four layers were found to be the minimum coating thickness to hinder protease accessibility to the inner layers and to prevent unwanted degradation.[13a] Thus, in this system, five bilayers of rhodamine B isothiocyanate (RITC)-labeled $POEGMA_{26}$-b-$PDPA_{50}$ and PMA were assembled at pH 4 via LbL assembly, utilizing similar interactions to the inner layers for film buildup. Film assembly on planar and particle templates suggest the uniform deposition of PMA/$POEGMA_{26}$-b-$PDPA_{50}$ bilayers, as analyzed by ellipsometry and flow cytometry (Figure 1). Ellipsometry measurements on air-dried, ten bilayer-thick films (including cross-linking) yielded a total film thickness of 34 ± 1 nm (Figure 1).

To obtain hybrid PDPA click capsules, the silica templates were dissolved using hydrofluoric acid (HF). The sacrificial PMA layers in the inner layers and outer coating were then removed by exposing the polymer capsules to high pH (Scheme 1). At pH > pK_a of PDPA (> 6.4), the tertiary amine groups are deprotonated and switch the PDPA from being hydrophilic to hydrophobic, hence disrupting both hydrogen bonding and electrostatic interactions with PMA. Fluorescence microscopy images showed uniform green (Alexa Fluor (AF) 488-labeled inner capsules) and red (RITC-labeled outer coating) fluorescence around the polymer capsules (Figure 2a, b). This implies the successful formation of an enzyme-cleavable PDPA click capsule with a non cross-linked pH-sheddable coating. Capsule formation was further confirmed by differential interference contrast (DIC) microscopy and transmission electron microscopy (TEM) images (Figure 2c, d).

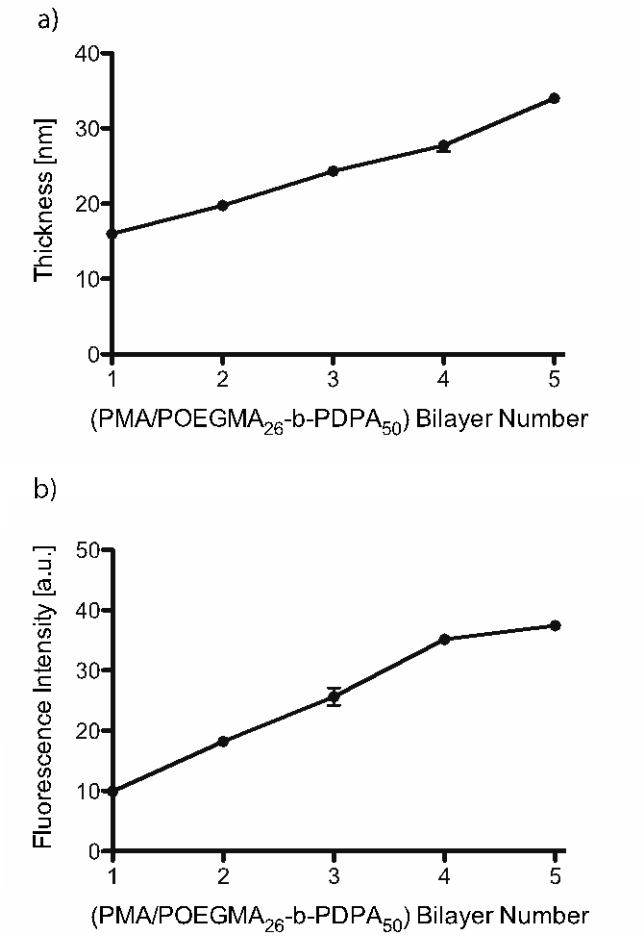


Figure 1. LbL assembly of the pH-sheddable coating on a $(PDPA_{Alk}/PMA)_5$ film cross-linked by a CtsB-cleavable linker on (a) planar substrates, as measured by ellipsometry, and (b) on colloidal templates, as followed by flow cytometry.

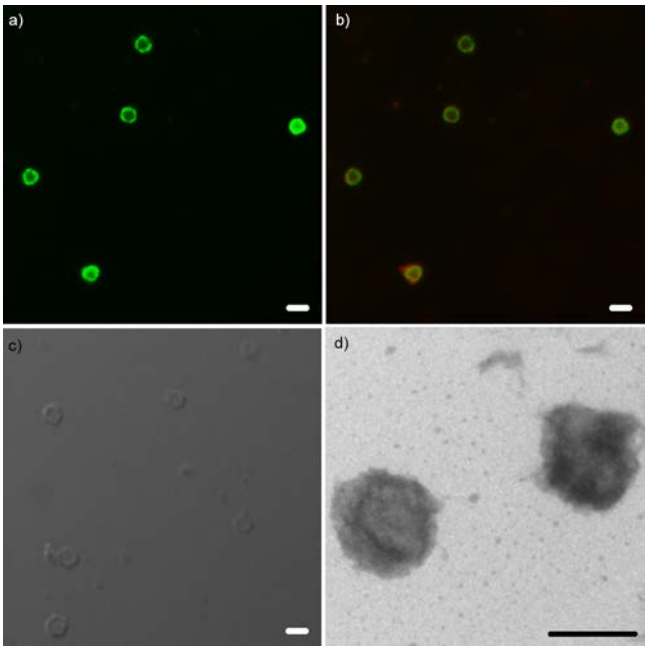


Figure 2. Microscopy images of hybrid PDPA click capsules prepared from 2.59 μm-diameter SiO_2 template particles. (a, b) Fluorescence and (c) DIC images of capsules dispersed in PBS (pH 7.4); (d) TEM images of the air-dried capsules. Capsules were dual

fluorescently labeled with AF488 (green) for the inner layers and RITC (red) for the outer coating. Scale bars are 2 μm.

Although the outer coating was not cross-linked, it was found to be stably attached to the cross-linked inner layers. This is likely to be due to the non-covalent hydrophobic interactions between the outer coating, peptide linker, and inner layers at physiological pH (pH 7.4). The peptide linker mostly comprises hydrophobic amino acids with an overall isoelectric point of ~6.0,[14] resulting in close to zero net charge within the pH range 5.0–7.5. Hence, the peptide linker is slightly positively charged during the assembly at pH 4 and subsequently switches to being hydrophobic at physiological pH. The hydrophilic to hydrophobic switch of the peptide linker and PDPA was exploited for integrating $POEGMA_{26}$-b-$PDPA_{50}$ as an outer coating via hydrophobic interactions. The significance of these interactions was confirmed in both particle and planar systems. The stability of the pH-sheddable coating on the inner layers was characterized by monitoring the fluorescence intensity of the RITC-labeled outer coating. The sample was incubated at physiological conditions, at 37 °C suspended in phosphate buffered saline (PBS) pH 7.4 or cell media, with gentle shaking, and analyzed by flow cytometry. The negligible change in the fluorescence intensity indicates retention of the pH-sheddable coating dispersed in PBS buffer. A reduction of ~30% in fluorescence intensity was observed in the samples suspended in cell media, which is likely due to the protein-capsule interactions during early incubation. Despite this decrease, the fluorescence intensity of the outer coating remains constant at ~70% in cell media, even after 48 h incubation (Figure S1). This implies successful and stable attachment of the pH-sheddable coating on the polymer capsules through non-covalent hydrophobic interactions. This finding was also verified by planar film analysis using a quartz crystal microbalance (QCM). Attachment of the outer coating was found to be stable upon washing at high pH. In contrast, a 60% reduction in the outer coating was observed upon exposure to simulated endosomal pH conditions (PBS, pH 5.9) (Figure

S2). The acidic pH converts PDPA from hydrophobic to hydrophilic and thus disrupts the stabilizing effect of the outer coating. The sensitivity to endosomal pH is of potential use, as it can induce release of the outer coating upon carrier internalization. Subsequently, enzymatic degradation can occur once the carriers are dynamically transported from endosomal to lysosomal compartments where CtsB is present.[4a,5]

Despite the hydrophobic nature of these hybrid PDPA click capsules, the 50% size reduction that occurs upon a decrease in pH, as demonstrated in our previous work with disulfide-stabilized PDPA click capsules, was not observed.[10a] It is speculated that the peptide linker also acts as a structural framework that prevents capsule shrinkage. To verify peptide attachment on the inner layer surface, the zeta (ζ)-potential of non cross-linked and peptide cross-linked $(PDPA_{Alk}/PMA)_5$ layers was measured in pH 4 buffer. The measurements demonstrated charge alteration from negative (due to PMA) to neutral upon cross-linking (due to the peptide linker) (Figure S3). Therefore, it is likely that the peptide linker not only penetrated into the PDPA inner layers for cross-linking but also attached onto the inner layer surface, which is possibly because of its coiled peptide conformation.[15] This argument is supported by the findings from analysis of CtsB-cleavable cross-linked PDPA capsules assembled without an outer coating. At simulated physiological conditions (PBS, pH 7.4), where the peptide has a close to zero net charge (pH range 5.0–7.5),[14] the capsules were found to heavily aggregate (Figure S4), suggesting that the peptide coverage on the capsule surface induces inter-capsule hydrophobic interactions. Further verification was obtained from AFM analysis of planar films in pH 4 buffer (Figure S5), where it was found that domains were present in the cross-linked films (Figure S5b), but not in the non-cross-linked films (Figure S5a). Evidence of polymer domains was also observed on the surface coated with pH-sheddable layers, showing the influence of the peptide linker and the $POEGMA_{26}$-b-$PDPA_{50}$ coating on the overall surface morphology (Figure S5c).

To examine enzyme-specific degradation of the hybrid PDPA click capsules *in vitro*, the capsules were exposed to simulated endosomal conditions (PBS, pH 5.9) in the presence of CtsB. However, upon exposure to an endosomal pH, the capsules were found to aggregate, as the outer coating is released from the capsule surface (Figure S6). No significant capsule degradation was observed (data not shown), most probably due to the restricted access of the enzyme into the network of aggregated capsules.

Consequently, the enzymatic degradation of the hybrid PDPA click capsules was investigated intracellularly, employing JAWS II, an immortalized dendritic cell (DC) line extracted from primary mouse bone marrow.[16] DCs are vital in regulating the immune system due to: (i) their ability as antigen presenting cells to initiate immune responses; (ii) their ability to migrate tumor cells; and (iii) their capacity to activate resting T cell lymphocytes, resulting in anti-tumor responses.[17] Degradable capsules were dual fluorescently labeled using AF488 (green) for the inner layers and RITC (red) for the pH-sheddable coating. Non-degradable capsules were labeled using AF647 as negative controls (false-colored blue) and fabricated using an identical method to that used for the degradable capsules, hence yielding the same multilayer structures. These capsules were cross-linked using a bisazide-functionalized peptide linker with a scrambled sequence (GGQGVFQAFF), and thus the linker has similar hydrophobicity and is non-degradable by CtsB. Both degradable and non-degradable capsules were added to the cells at approximately a 10:1 capsule-to-cell ratio and incubated for six different time periods (10 min, 30 min, 1 h, 2 h, 4 h, and 6 h). Afterwards, cells were fixed using paraformaldehyde (PFA, 3%) for 10 min at 37 °C and washed by using Dulbecco's PBS (DPBS) prior to analysis. Figure 3 shows representative deconvolution microscopy images of fixed cells after incubation with capsules at 10 min and 6 h. Images for all six time points are given in the Supporting Information (Figure S7). The fluorescence microscopy images for

degradation of the capsules at 10 min are also given as insets (Figure 3a, c, e). These images highlight the fragments of degraded capsules. *In vitro* cell degradation analysis suggests limited capsule internalization after 10 min. However, rapid capsule degradation was observed upon cellular uptake (at 10 min), demonstrated by green fluorescence fragments throughout the cellular compartments. Fluorescence fragmentation indicates a high degree of capsule degradation, as the fragmentation extent is sufficient to spread out inside the cellular compartments. Red fluorescence (due to the outer coating) was found to both fragment and spread throughout the cells, suggesting partial release of the outer coating prior to capsule degradation. On the other hand, capsules with the non-degradable cross-linker (blue) were found to swell, but to maintain their shape and structural integrity, highlighting the high specificity of the enzyme degradation mechanism.

The intracellular fate of the hybrid PDPA click capsules was investigated by performing lysosomal staining of the cells. Subsequent to overnight incubation of the degradable capsules with JAWS II cells, at approximately a 10:1 capsule-to-cell ratio, the cells were fixed (see above). Degradable capsules were dual fluorescently-labeled using AF647 for the inner layers and RITC for the outer coating, whereas lysosomes were stained using LAMP-1 and AF568-goat anti-rat IgG. Figure 4 demonstrates partial disassociation of the outer coating from the inner layers prior to capsule disassembly, as some lysosomal compartments were found to contain only the outer coating (and some the outer coating and inner layers). As a large number of cellular lysosomes were formed within the time course of these experiments, we were unable to ascertain if endosomal escape occurred; however, the intracellular fate of the capsules is the lysosomal compartments.

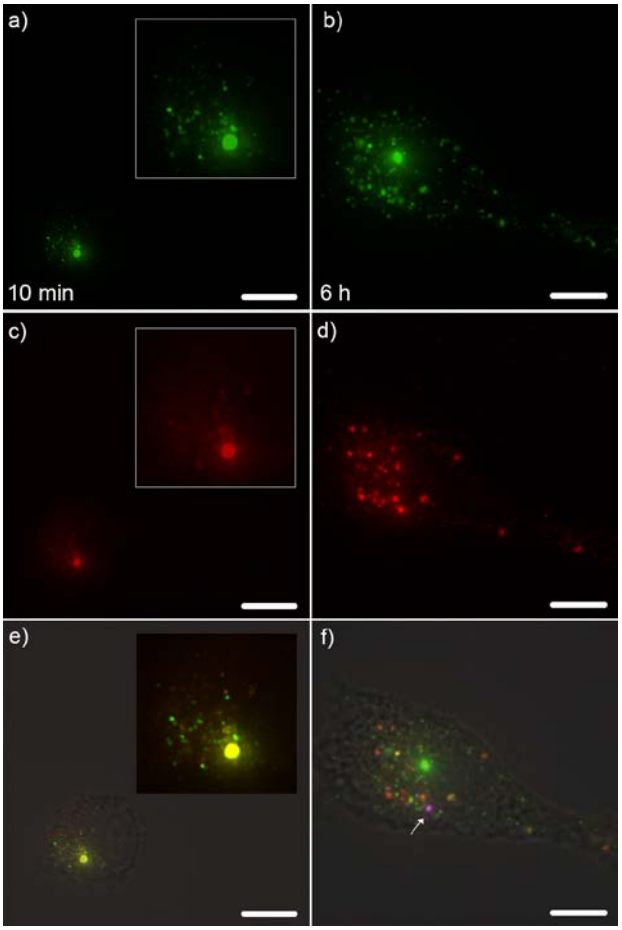


Figure 3. Deconvolution optical microscopy images of *in vitro* cell degradation of hybrid PDPA click capsules incubated with JAWS II cells for 10 min and 6 h. Fluorescence images (with maximum intensity projection) after (a, c) 10 min and (b, d) 6 h incubation. Overlay of bright field and fluorescence images after (e) 10 min and (f) 6 h incubation. The insets are provided to highlight capsule degradation at 10 min. CtsB-cleavable capsules were dual fluorescently labeled, with AF488 (green) for the inner layers and RITC (red) for the outer coating. Non-degradable capsules were dual fluorescently labeled, with AF647 (blue) for the inner layers and RITC (red) for the outer coating. White arrow indicates intact non-degradable capsules. Scale bars are 5 μm.

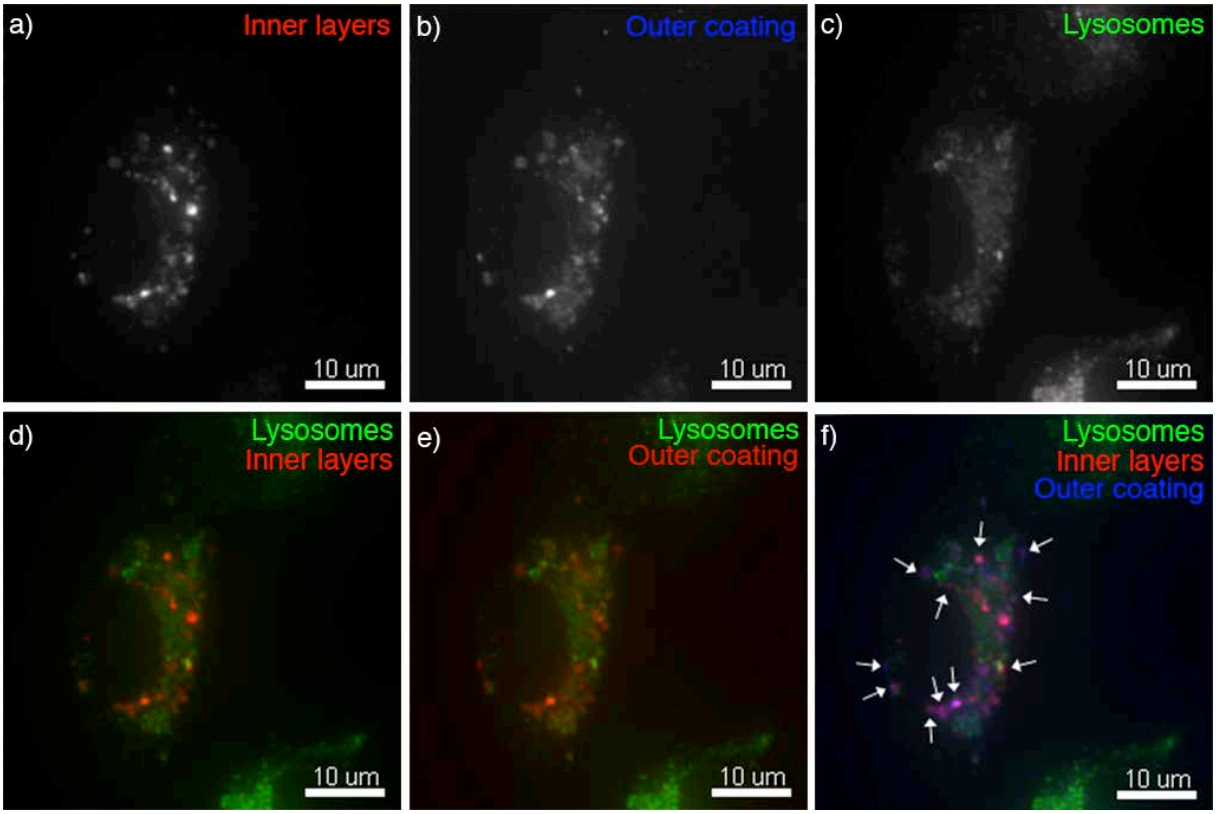


Figure 4. Deconvolution optical microscopy images of the colocalization of hybrid PDPA click capsules with lysosomes after overnight incubation in JAWS II cells. Fluorescence images of the (a) inner layers, (b) outer coating, (c) lysosomes, (d) overlay of inner layers (red) and lysosomes (green), (e) overlay of outer coating (red) and lysosomes (green), and (f) overlay of inner layers (red), outer coating (blue), and lysosomes (green). CtsB-cleavable capsules were dual fluorescently labeled, with AF647 for the inner layers and RITC for the outer coating. Lysosomal compartments were stained using LAMP-1 and AF568-goat anti-rat IgG. White arrows indicate colocalization of both the inner layers and the outer coating with the lysosomal compartments.

In conclusion, we have demonstrated the fabrication of bioresponsive hybrid PDPA click capsules, stabilized by CtsB-cleavable cross-linking of the inner layers and non-covalent hydrophobic interactions of a pH-sheddable outer coating. The stability of this outer coating implies that non-covalent hydrophobic interactions provide stabilization. These carriers were engineered to be inherently responsive in intracellular conditions, specifically to endosomal pH and CtsB. The mechanism of carrier degradation consists of two virtually simultaneous stages. Exposure to endosomal pH was shown to release the outer coating, based on charge shifting of PDPA, and subsequently inducing polymer capsule degradation based on an enzyme-specific hydrolysis. The synergistic combination of enzyme-induced cross-linker

degradation and pH-response of PDPA constituents results in rapid carrier degradation upon cellular internalization. Despite the short incubation period (10 min), a high degree of carrier degradation occurred, as observed *in vitro* inside the cells. The colocalization study demonstrated that the outer coating and inner layers end up in different lysosomal compartments. The simple and versatile technique of exploiting the innate properties of materials to induce carrier assembly and disassembly is promising for developing biologically intelligent carriers. This feature is of importance for therapeutic applications, as it allows for control over carrier degradation in specific cellular compartments.

Experimental Section

Materials: Poly(methacrylic acid) (PMA,15 kDa) and 2-diisopropylaminoethyl methacrylate (DPA) were purchased from Polysciences (Pennsylvania, USA). The CtsB-cleavable (GFQGVQFAGF) and non-degradable peptide (GGQGVFQAFF) linkers with bisazide-functionalization were purchased from Mimotopes (Victoria, Australia). Boric acid, phosphate buffered saline (PBS), bovine serum albumin (BSA), and Triton X-100 were obtained from Sigma-Aldrich (Sydney, Australia). Non-porous colloidal silica (SiO_2) particles (2.59 μm) were purchased from MicroParticles GmbH (Berlin, Germany). LAMP-1 (CD107a clone 1D4B) was obtained from BD Biosciences (Australia). Alexa Fluor 568 (AF568)-goat anti-rat IgG (H+L) was purchased from Life Technologies (Victoria, Australia). High purity and resistivity water (Milli-Q gradient A 10 system, resistivity >18 MΩ·cm, TOC < 4 ppb, Millipore Corporation, Massachusetts, USA) was obtained from an inline Millipore RiOs/Origin water purification system. All other materials were obtained from Sigma-Aldrich and used as received.

Polymer Synthesis: The detailed synthesis of $PDPA_{Alk}$ was described previously.[10a] The detailed synthesis of $POEGMA_{26}$-b-$PDPA_{50}$ can be found in a previous publication.[11b]

Capsule Preparation: SiO_2 particles (2.59 μm average diameter) were washed by three standard centrifugation/re-dispersion cycles in sodium acetate buffer (50 mM, pH 4). LbL capsules were fabricated through the alternate deposition of $PDPA_{Alk}$ (1 mg mL^{-1}) and PMA (1 mg mL^{-1}) on SiO_2 particles. The polymer was allowed to interact for 15 min with constant shaking for adsorption onto the particles. The polymer-coated particles were then washed three times to remove the excess polymer. LbL assembly was repeated until five bilayers of $PDPA_{Alk}$/PMA were deposited. Non-functionalized polymer (PMA) was used as the final layer to prevent polymer bridging between the polymer-coated particles upon cross-linking. Afterwards, the multilayers underwent cross-linking through overnight incubation in a solution comprising peptide linker (1 mg mL^{-1}), sodium ascorbate (4.4 mg mL^{-1}), and copper (II) sulfate (1.8 mg mL^{-1}) in sodium acetate buffer at a volumetric ratio of 3:1:1 with constant shaking. Subsequently, the outer coating was assembled via five alternate depositions of PMA (1 mg mL^{-1}) and $POEGMA_{26}$-b-$PDPA_{50}$ (1 mg mL^{-1}) with 15 min incubation and followed by three centrifugation/re-dispersion cycles in sodium acetate buffer to remove the excess polymer. To form hollow polymer capsules, the core SiO_2 particles were removed by suspending polymer-coated particles into HF solution (5 M), followed by three centrifugation/re-dispersion cycles in sodium acetate buffer. The sacrificial PMA layers were then removed via multiple washes in borate buffer and finally PBS.

Cellular Degradation: CtsB-cleavable (AF488- and RITC-labeled) and non-degradable (AF647- and RITC-labeled) capsules were added to JAWS II cells, with approximately a 10:1 capsule-to-cell ratio for each time point (10 min, 30 min, 1 h, 2 h, 4 h, and 6 h). The cells were then fixed by 10 min incubation in paraformaldehyde (3%, 37 °C) and washed multiple times using DPBS.

Lysosomal Staining: CtsB-cleavable (AF488- and RITC-labeled) capsules were added to JAWS II cells, with approximately a 10:1 capsule-to-cell ratio, and incubated overnight. The cells were fixed by 10 min incubation in paraformaldehyde (3%, 37 °C) and permeabilized with a blocking buffer (3% BSA, 0.2% Triton X-100) for 2 h at room temperature (RT, 23 °C). The cells were then stained using LAMP-1 antibody (1 μg mL^{-1} in blocking buffer) for 2 h at RT and AF568-goat anti-rat IgG (10 μg mL^{-1} in blocking buffer) for 30 min at RT. A washing procedure was performed after antibody incubation: three washes in a washing buffer (0.2% BSA, 0.05% Triton X-100) for 10 min at RT were performed. The cells were finally immersed in DPBS for imaging.

Supporting Information
Supporting Information is available online from the Wiley Online Library or from the author.

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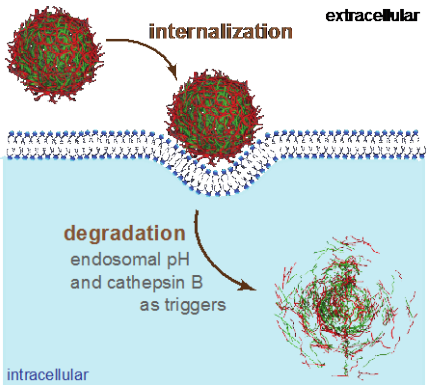
The table of contents entry should be 50−60 words long (max. 400 characters), and the first phrase should be bold.

Keyword click chemistry, polymer capsules, LbL, enzyme-responsive, nanotechnology

S. T. Gunawan, K. Liang, G. K. Such, A. P. R. Johnston, M. K. M. Leung, J. Cui, F. Caruso*

Title Engineering Enzyme-Cleavable Hybrid Click Capsules with a pH-Sheddable Coating for Intracellular Degradation

The engineering of layer-by-layer (LbL) hybrid click capsules that are responsive to biological stimuli is reported. The capsules comprise a pH-sheddable non cross-linked outer coating that protects enzyme-cleavable inner layers. Upon cellular uptake, the outer coating is released and the capsules are enzymatically degraded. *In vitro* cell degradation results in rapid capsule degradation (10 min) upon cellular internalization.



Supporting Information

Engineering Enzyme-Cleavable Hybrid Click Capsules with a pH-Sheddable Coating for Intracellular Degradation

*Sylvia T. Gunawan, Kang Liang, Georgina K. Such, Angus P. R. Johnston, Melissa K. M. Leung, Jiwei Cui, and Frank Caruso**

Materials: Alexa Fluor 488 (AF488) azide and Alexa Fluor 647 (AF647) azide, Dulbecco's phosphate buffered saline (DPBS), Recombinant Mouse Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) recombinant mouse protein and MEM alpha were purchased from Life Technologies (Victoria, Australia). Poly(ethylene imine) (PEI, 25 kDa), sodium acetate (NaOAc), sodium-L-ascorbate, copper (II) sulfate ($CuSO_4$) anhydrous, hydrofluoric acid (HF), boric acid, phosphate buffered saline (PBS), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DTMM), sodium phosphate dibasic (Na_2HPO_4), and propargylamine hydrochloride (95%) were purchased from Sigma-Aldrich (Sydney, Australia). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Kansas, USA). All other materials were obtained from Sigma-Aldrich and used as received.

Three types of buffer were used in this experiment: (i) NaOAc buffer consisting of 50 mM NaOAc (pH 4); (ii) PBS buffer consisting of 10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl (pH 7.4); (iii) Borate buffer consisting of 50 mM boric acid (pH 7.5). The buffer solutions used in all experiments were prepared with high purity and resistivity water (Milli-Q gradient A 10 system, resistivity >18 MΩ·cm, TOC < 4 ppb, Millipore Corporation, Massachusetts, USA).

Synthesis of PMA_{Alk}: The alkyne-functionalized PMA was prepared using a coupling reaction catalyzed by DMTMM. PMA (120.77 mg, 0.462 mol) and DMTMM (34.55 mg, 0.125 mol) were dissolved and incubated in Na_2HPO_4 buffer (100 mM, pH 7.2) for 15 min. Propargylamine hydrochloride (5.71 mg, 0.0624 mol) was then added into the solution and the reaction was left to proceed overnight with constant stirring, followed by polymer dialysis and freeze drying. The resulting polymer contains 9% alkyne functional groups, characterized by ^{1}H NMR.

Multilayer assembly on planar supports: Gold-coated quartz crystals (5 MHz, AT cut) were cleaned using Piranha solution (7:3 v/v sulfuric acid/hydrogen peroxide) *(Caution! Piranha solution is highly corrosive. Extreme care should be taken when handling Piranha solution and only small quantities should be prepared)* for 5 min, followed by extensive rinsing with water, 2-propanol, and water. The crystals were then dried with a stream of nitrogen and exposed to UV (Bioforce NanoScience, USA) for 20 min. Film assembly on planar surfaces was characterized using a quartz crystal microbalance with dissipation monitoring (QCM-D, Q-sense E4, Frolunda, Sweden). Water was run through all the chambers for setup measurements after mounting the cleaned QCM crystals inside the flow chambers. This is important to ensure data reliability throughout the experiment. Unless otherwise stated, the mass and dissipation measurements were conducted at 24.0 ± 0.1 °C and the quoted frequency values correspond to the third overtone, with equivalent trends for the fifth, seventh, ninth, eleventh, and thirteenth overtones. The experiment was initialized by obtaining a stable NaOAc baseline. After a NaOAc baseline was obtained, precursor layers containing PEI (1 mg mL^{-1}) and PMA_{Alk} (1 mg mL^{-1}) were sequentially introduced. This was then followed by the alternate deposition of $PDPA_{Alk}$ (1 mg mL^{-1}) and PMA (1 mg mL^{-1}) until five $PDPA_{Alk}$/PMA bilayers were deposited. For each layer assembly, the polymer solution was allowed to interact for 15 min and the excess polymer was removed through extensive NaOAc

washing. The multilayer films were then cross-linked overnight by a solution of peptide linker (1 mg mL^{-1}), sodium ascorbate (4.4 mg mL^{-1}), and copper (II) sulfate (1.8 mg mL^{-1}) in NaOAc buffer. The QSoft software was turned off during the cross-linking process to prevent the influence of copper on the gold-coated QCM crystals. Subsequent to the cross-linking process and prior to switching on the QSoft software, the polymer-coated surfaces were rinsed by NaOAc to remove the unreacted copper. The frequency and dissipation values were again measured to ensure data accuracy. Upon stabilization of the NaOAc baseline, five bilayers of PMA and $POEGMA_{26}$-b-$PDPA_{50}$ (1 mg mL^{-1}) were alternately deposited, employing an identical procedure as described above. The sacrificial PMA layers were then removed by sequential washing in borate and PBS buffer.

For the outer coating release experiments, the films were exposed to a lower pH (PBS, pH 5.9). The frequency was then compared in order to quantify the degree of film release.

Multilayer films on planar supports for AFM and ellipsometry characterization were prepared following the method described above. Cleaning of silicon wafers was performed by immersing the slides in Piranha solution for 10 min, followed by extensive rinsing with water. The slides were then sonicated in 50 v/v isopropyl alcohol/water for 15 min, heated to 60 °C in RCA solution (5:1:1 water/hydrogen peroxide/ammonia) for 20 min, extensively rinsed with water, and dried under a stream of nitrogen.

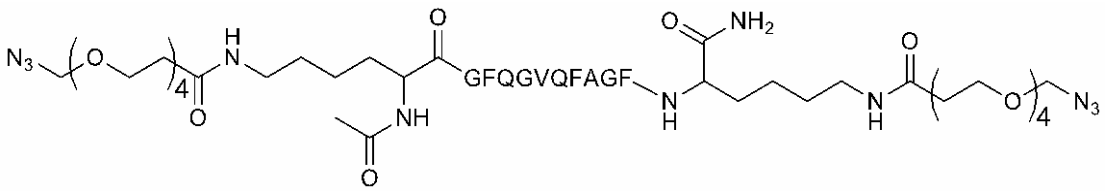
Fluorescence-labeling of capsules: The remaining alkyne moieties of $PDPA_{Alk}$ in 100 μL polymer-coated SiO_2 particles were fluorescently labeled using a mixture of 2.5 μL AF488 or AF647 azide, 300 μL sodium acetate buffer, 100 μL sodium ascorbate (4.4 mg mL^{-1}), and 100

μL copper (II) sulfate (1.8 mg mL^{-1}) via click chemistry. Prior to the deposition of the outer coating, these polymer-coated particles were washed three times to remove the unreacted dyes.

Stability of the non cross-linked outer coating: Samples containing at least 1×10^9 capsules were suspended in PBS or cell media (80 mL MEM Alpha, 20 mL FBS and 5 μL GM-CSF) and incubated at 37 °C under constant and gentle shaking. The fluorescence intensity of RITC-$POEGMA_{26}$-b-$PDPA_{50}$ layers in the polymer capsules were monitored by flow cytometry (Partec Cyflow Space, Partec GmbH, Germany), utilizing an excitation wavelength of 510 nm. At least 2×10^4 capsules were analyzed in each sample run. Experiments were performed in triplicates and the mean fluorescence intensity was evaluated to analyze the stability of the non cross-linked outer coating at physiological conditions (PBS and cell media).

Characterization: The LbL films on silicon wafers were dried and measured in air using an AutoSE spectroscopic ellipsometer (HORIBA Jobin Yvon). Film thicknesses were acquired between 5 nm and 50 nm with a 1 nm increment and the data were then extracted using the integrated DeltaPsi2 software by data fitting to Tauc Lorentz 2 spectroscopic model. AFM characterization (NanoWizard II BioScience AFM) in solution was performed on LbL films assembled on silicon wafers utilizing the deflection of sharp force-sensing tips attached to cantilevers. Flow cytometry experiments were performed using a Partec CyFlow Space with Partec CyflowMax software for data analysis. The thresholds for FSC and SSC were adjusted to obtain the optimum signal-to-noise ratio. Sample solutions were inserted and passed through the laser beam at an excitation length of 510 nm for RITC. The emission in FSC, SSC and FL2 was observed and plotted on 2D histograms. Fluorescence and DIC microscopy images of the hollow capsules were obtained using an inverted Olympus IX71 microscope.

The 60× oil objective lenses for 488, 510 and 647 excitation filters were used. Microscopy images were captured by a black and white camera mounted on the left port of the fluorescence microscope. For transmission electron microscopy (TEM) measurements, 3 μL of capsules was allowed to adsorb onto a carbon-coated Formvar film mounted on 300 mesh UV-treated copper grids (ProSciTech, Australia). The grids were blotted dry using filter paper and placed in a drop of water (of the appropriate pH) for 5 s. After blotting dry, the grids were left to air-dry overnight and then analyzed using TEM (Philips CM120 BioTWIN, operated at 120 kV). The *in vitro* cell degradation analysis was performed using a deconvolution microscope (DeltaVision, Applied Precision, 60× 1.42 NA oil objective). The filter sets used were FITC, TRITC and CY5 to observe AF488, RITC, and AF647, respectively. The resulting images were deconvolved and then analyzed using Imaris (Bitplane) with maximum intensity projection. Zeta-potential experiments of the $(PDPA_{Alk}/PMA)_5$ multilayer films assembled on SiO_2 particles, before and after cross-linking, were measured using a Zetasizer (Nano ZS, Malvern). Each measurement was performed eight times.



Scheme S1. Chemical structure of the CtsB-cleavable (GFQGVQFAGF) linker functionalized with a bisazide.

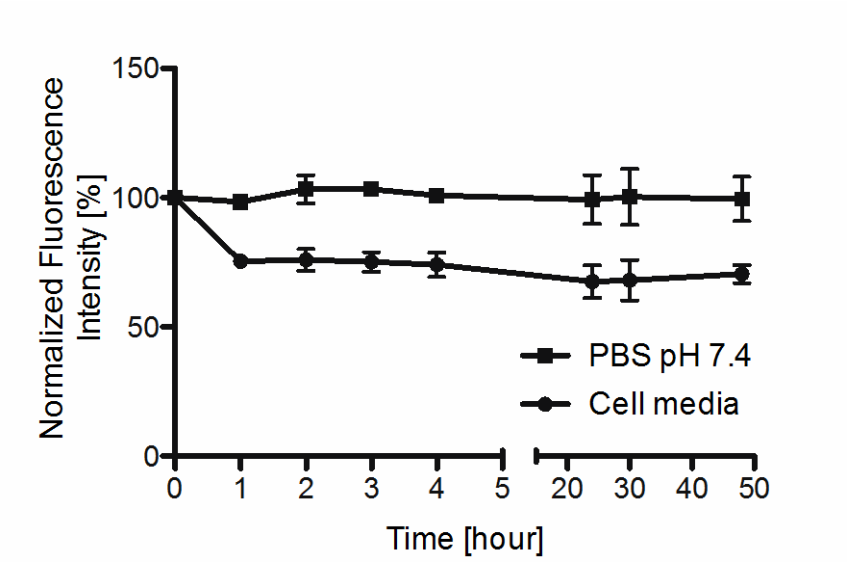


Figure S1. Stability of the outer (pH-sheddable) coating attached to CtsB cross-linked inner PDPA layers in the hybrid PDPA click capsule system. The sample was incubated in pH 7.4 PBS or cell media at 37 °C, with gentle shaking.

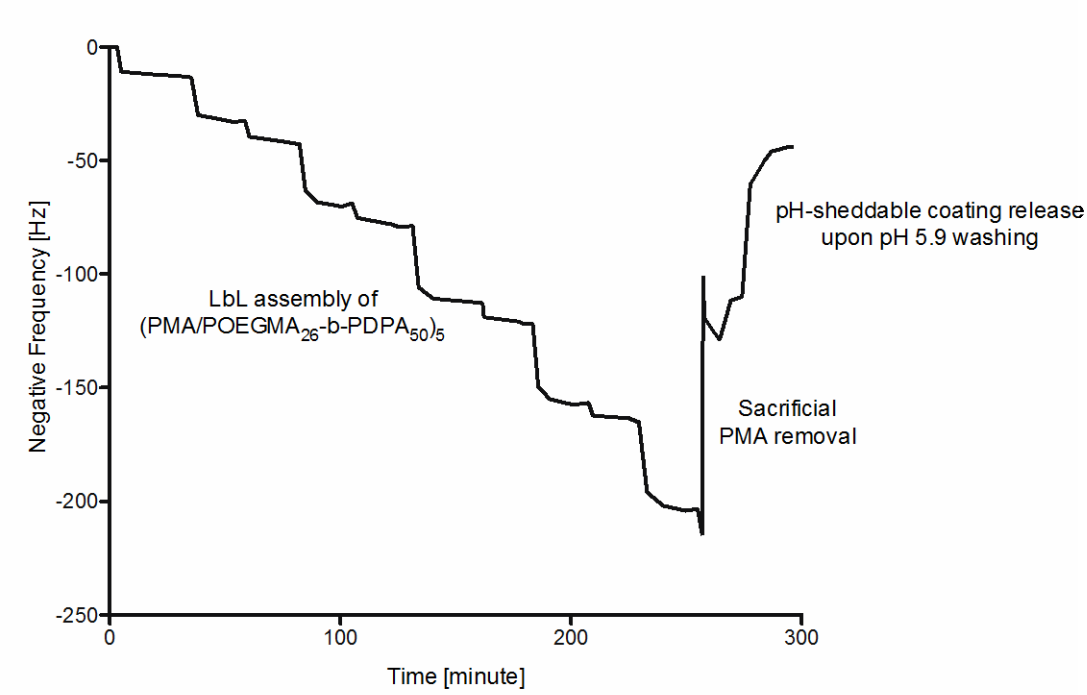


Figure S2. LbL assembly of the outer (pH-sheddable coating) on CtsB cross-linked inner PDPA multilayers, followed by sacrificial PMA removal (washing at pH > 6.4), and outer (pH-sheddable) coating release upon exposure to simulated endosomal pH (5.9).

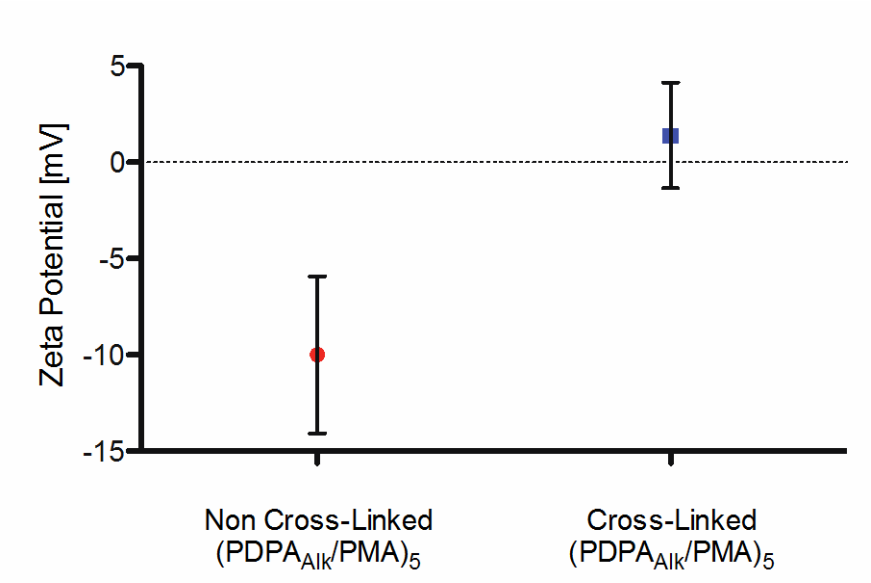


Figure S3. Zeta (ζ)-potential values for the $(PDPA_{Alk}/PMA)_5$ multilayer films assembled on SiO_2 particles before and after CtsB-cleavable cross-linking, measured in pH 4 buffer.

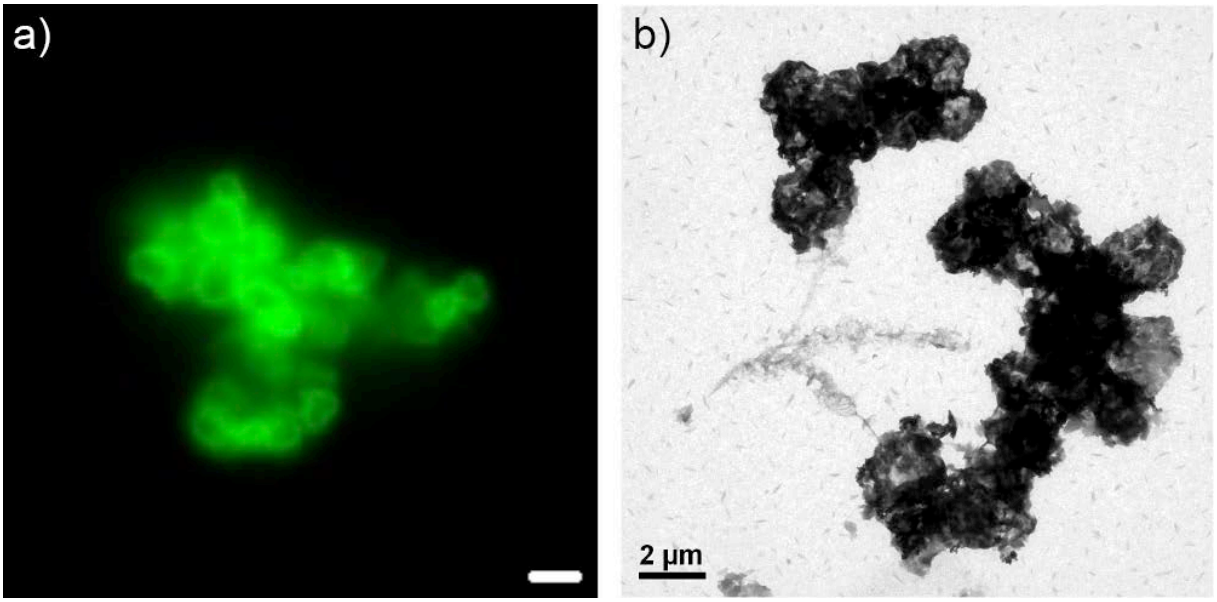


Figure S4. Microscopy images of CtsB-cleavable PDPA capsules without an outer coating, prepared from 2.59 µm-diameter SiO_2 template particles. (a) Fluorescence image of capsules dispersed in PBS (pH 7.4) and (b) TEM image of air-dried capsules. Capsules were fluorescently-labeled with AF488 (green). Scale bars are 2 µm.

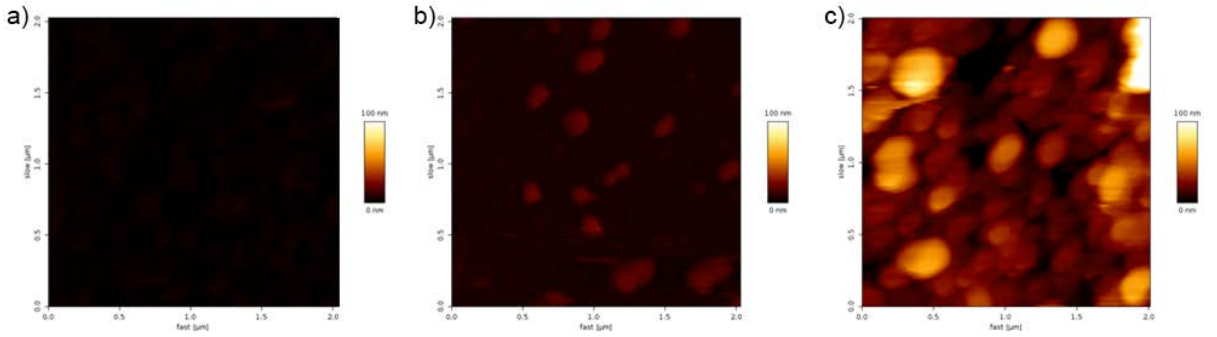


Figure S5. AFM images of (a) the inner PDPA multilayers, (b) CtsB-cleavable cross-linked PDPA multilayers, and (c) pH-sheddable-coated PDPA multilayers. The multilayers were separately assembled on silicon planar substrates and dispersed in pH 4 for characterization. The scale ranges from 0 nm to 100 nm.

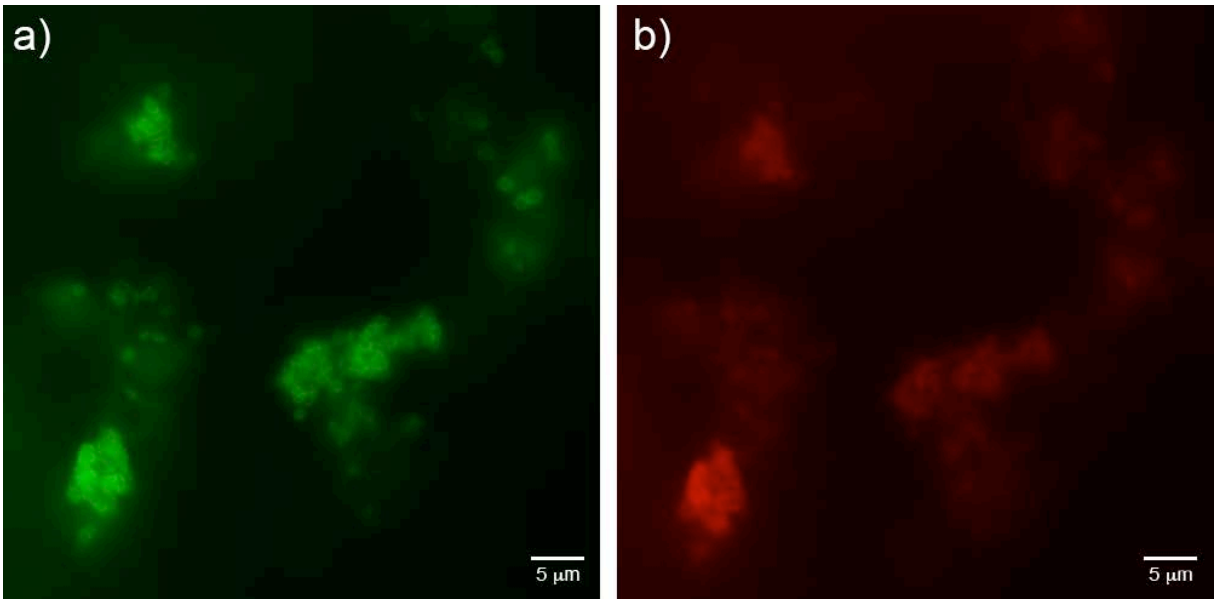
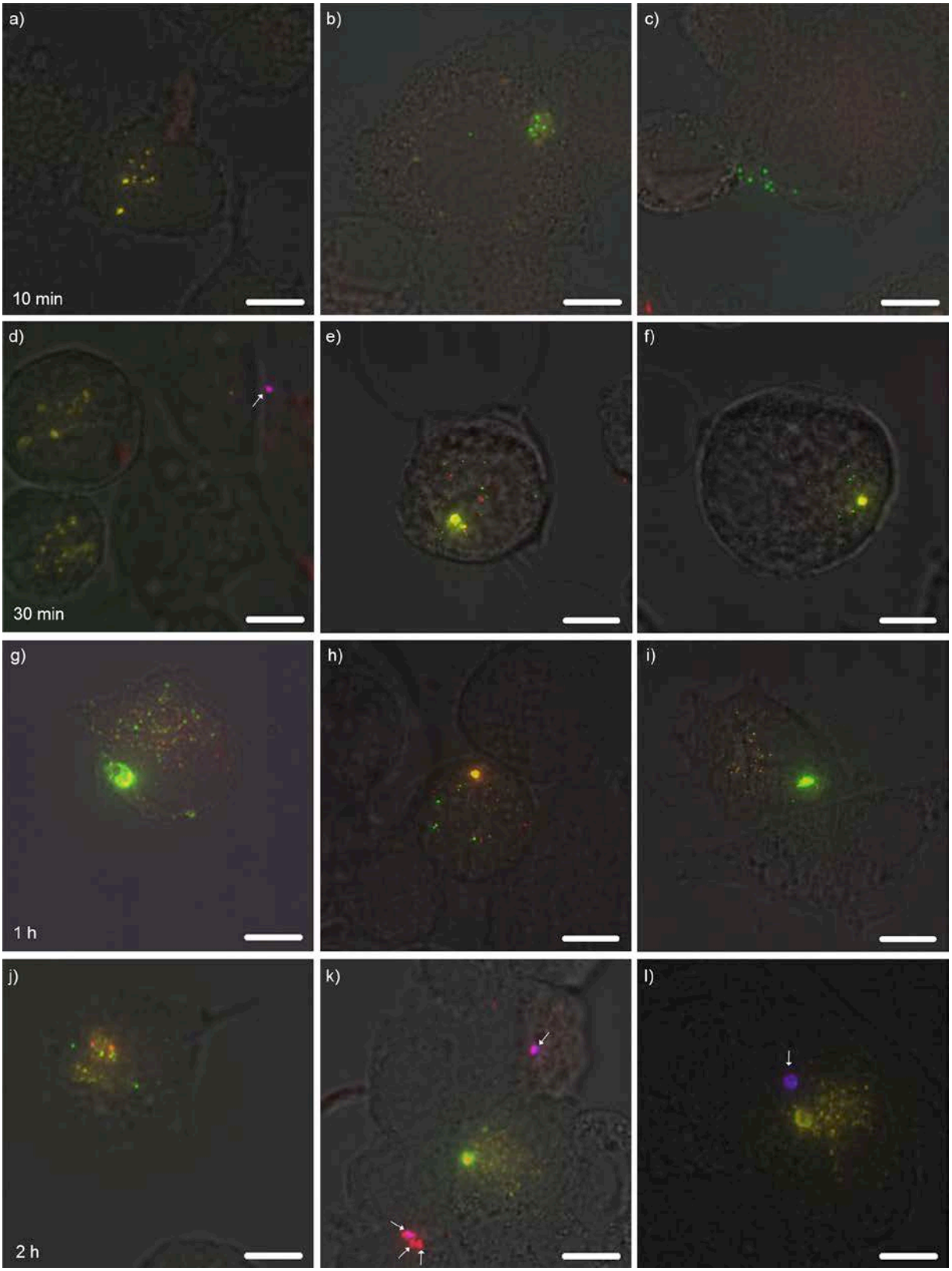


Figure S6. Fluorescence microscopy images of hybrid PDPA click capsules, prepared from 2.59 µm-diameter SiO_2 template particles, dispersed in simulated endosomal conditions (PBS, pH 5.9). The capsules were dual fluorescently labeled using (a) AF488 for the inner layers and (b) RITC for the outer coating. Scale bars are 5 µm.


a)
b)
c)
10 min
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30 min
g)
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1 h
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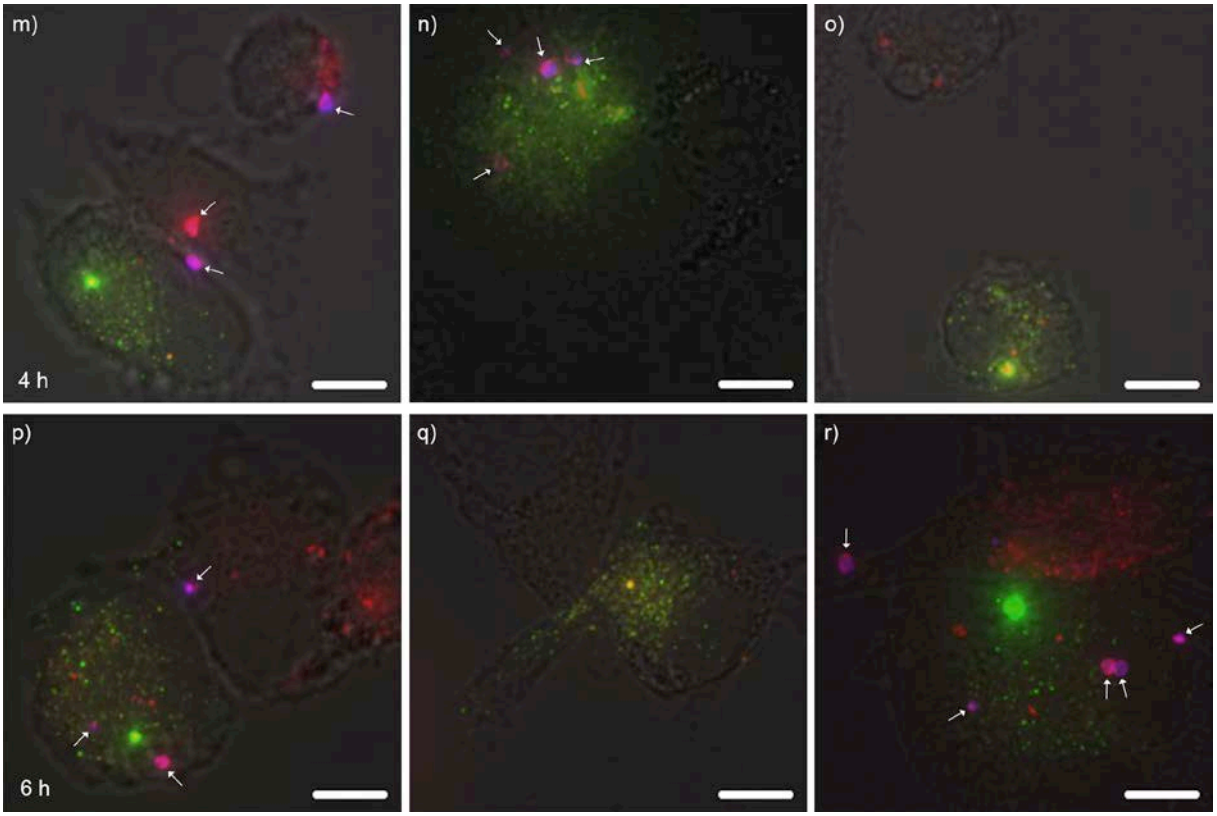

Figure S7. Deconvolution optical microscopy images (maximum intensity projection) of *in vitro* cell degradation of hybrid PDPA click capsules incubated in JAWS II cells for 10 min, 30 min, 1 h, 2 h, 4 h, and 6 h. Overlay of bright field and fluorescence images after (a, b, c) 10 min, (d, e, f) 30 min, (g, h, i) 1 h, (j, k, l) 2 h, (m, n, o) 4 h, and (p, q, r) 6 h incubation. CtsB-cleavable capsules were dual fluorescently labeled, with AF488 (green) for the inner layers and RITC (red) for the outer coating. Non-degradable capsules were dual fluorescently labeled, with AF647 (blue) for the inner layers and RITC (red) for the outer coating. White arrows indicate intact non-degradable capsules. Scale bars are 5 μm.

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