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Endocytic pH-Triggered Degradation of Nanoengineered Multilayer Capsules

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The design of stimuli-responsive polymeric drug carriers is a burgeoning research area because such carriers can selectively degrade and release therapeutic agents inside target cells, thereby improving therapeutic outcomes.[1] A range of stimuli-responsive mechanisms have been integrated into responsive carriers for achieving selective and site-specific degradation through both biological and non-biological triggers.[2] Among them, carriers with biological triggers (e.g., pH, redox, and enzyme) are attracting particular interest because they can respond to naturally occurring conditions and variations within cells. Specifically, the pH drop experienced during endocytosis is a desirable trigger because it is common across cell lines and extensively studied.[3] Furthermore, polymer carriers are typically internalized via endocytic pathways, and therefore the pH shift between the extracellular environment (pH 7.4) and endosomal vesicles (pH 6.5–4.5) is an attractive biological trigger for degradation and drug release.[3] To harness this trigger, several pH-degradable liposomes/polymersomes and polymer nanoparticles have been reported.[4] However, for layer-by-layer (LbL) assembled capsules, which are an emerging class of polymer drug carriers, cellular pH-triggered degradation has not been demonstrated.

LbL assembly has proven to be a robust and versatile technique for synthesizing polymer capsules, largely due to the ability to fine-tune the physicochemical properties and functionality of the obtained capsules.[5] However, the narrow pH shift experienced during endocytosis[6] has made it difficult to assemble capsules with the relevant pH degradability. As a result, LbL capsules have been designed to be sensitive to other cellular triggers (e.g., redox reactions and enzymes) to cause capsule degradation and subsequent cargo release. Unlike pH reductions experienced during endocytosis, the concentrations of "redox chemicals" (e.g., glutathione) and enzymes in the endosomal compartments vary for different cells.[7] Therefore, it is desirable to design LbL capsules that can be degraded by the well-characterized reduction in pH experienced during the endocytic process. Such a degradation mechanism is broadly applicable to a range of cells. Herein, we report the synthesis of LbL capsules that degrade solely in response to endocytic pH changes. We also demonstrate their use for encapsulating and delivering various model drugs and therapeutics.

The capsules prepared are based on the charge-shifting polymer, poly(2-diisopropylaminoethyl methacrylate) (PDPA).[8] To achieve endocytic pH-induced degradation of the PDPA-based capsules, we engineered PDPA with a minor component of lauryl methacrylate ($PDPA_{C12}$), and used $PDPA_{C12}$ as the building block for capsule assembly. The use of the modified PDPA permits the formation of $PDPA_{C12}$ multilayers through non-covalent stabilization, allowing for the assembly of highly pH-responsive, single-component multilayer capsules. This represents a significant difference from our previous work on PDPA capsules stabilized with a disulfide-containing cross-linker, where a combination of intracellular reducing conditions and pH changes was required to degrade the capsules.[8] Further, in the absence of C_{12} groups on PDPA, stable capsules could not be formed without chemically cross-linking the PDPA capsules.[8] The addition of C_{12} groups in the PDPA is expected to increase the overall hydrophobicity (Supporting Information, Figure S1), which could non-covalently stabilize the multilayers at pH 7.4 (**Scheme 1**). The merging of pH-degradable and non-covalently stabilized polymer multilayer capsules, as reported in the current study, offers a number of important advantages. Firstly, the synthesized capsules are stable at physiological pH (pH 7.4) and respond to narrow cellular pH shifts to rapidly degrade under endosomal pH. Secondly, the assembled capsules demonstrate pH-dependent encapsulation and release of hydrophilic molecules (e.g., therapeutic agents) with a wide range of molecular weights (ca. 500 Da to ca. 70 kDa), improving the capacity to encapsulate small (less than 10 kDa) molecules in polymer hydrogel capsules.[9] Thirdly, this encapsulation strategy overcomes the limitations of conjugating small therapeutics to the carrier, which involves chemical reactions, can be time consuming, and may alter the properties of the molecules. Fourthly, the prepared capsules are stabilized via hydrophobic interactions, avoiding the use of potentially toxic substances such as Cu (I), which is involved in some chemical cross-linking processes.[10] This non-covalent stabilization method is expected to have application in a wide range of responsive multilayer drug carriers.

$PDPA_{C12}$ was synthesized with a minor component of lauryl methacrylate and a short poly(ethylene glycol) methacrylate side group via atom transfer radical polymerization (ATRP) using an azide-containing initiator (see Supporting Information). The azide groups at the polymer end can be used for post-functionalization of the capsules via click chemistry. This could involve

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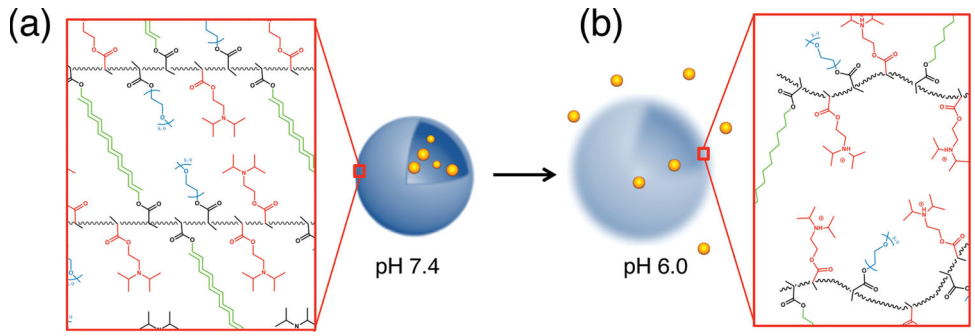

Scheme 1. a) Multilayered PDPA capsules containing hydrophobic C_{12} groups (green) and DPA groups (red) at pH 7.4. b) Reducing the pH to below 6.4, to mimic endosomal pH conditions, results in protonation of DPA, causing charge repulsion between polymer layers, leading to rapid deconstruction of the capsules and release of encapsulated therapeutics.

dyes for imaging or antibodies for targeting.[11] NMR characterization showed that the C_{12} and PEG contents from the $PDPA_{C12}$ were ca. 10% and ca. 8%, respectively (see Supporting Information). LbL assembly between $PDPA_{C12}$ and PMA at pH 4 was investigated firstly on planar gold substrates using a quartz crystal microbalance (QCM). Film assembly is likely to be a combination of hydrogen bonding between protonated carboxyl groups of PMA and the PEG moieties in the $PDPA_{C12}$, as well as electrostatic interactions between charged PMA and $PDPA_{C12}$ tertiary amine groups (Supporting Information, Figure S2).[8] Uniform buildup of five $PDPA_{C12}$/PMA bilayers was observed (Supporting Information, Figure S3). Using the Sauerbrey equation, the mass deposited for each $PDPA_{C12}$ and PMA layer was calculated to be 350 and 400 ng cm^{-2}, respectively, which are similar to those observed for electrostatically-associated LbL films.[12] Upon increasing the pH to above the pKa (ca. 6.4) of the tertiary amine groups in the PDPA, more than 99% of the PMA was removed from the multilayers (Supporting Information, Figure S3).

$PDPA_{C12}$/PMA multilayer growth was then investigated on ca. 2.6 µm-diameter spherical silica particles using flow cytometry. A linear increase in the intensity of Alexa Flour 488-labeled PMA was observed for $PDPA_{C12}$/PMA multilayer assembly, suggesting regular growth of the multilayers (Supporting Information, Figure S4a). Following deposition of five bilayers, the silica particles were removed, leaving hollow multilayer capsules (**Figure 1**). Flow cytometry confirmed removal of PMA upon raising the pH to 7.4 for 5 min (Supporting Information, Figure S4b). Furthermore, controlled reversible shrinking and swelling over three cycles was observed upon varying the pH from 7.4 to 6.0. The swollen capsules were stable for 24 h without shaking. Fluorescence microscopy and TEM images of these capsules prepared on silica particles showed reversible size changes from 1.2 µm to 3.5 µm at pH 7.4 and 6.0, respectively (Figure 1). Compared with the covalently cross-linked PDPA capsules reported earlier,[8] these non-covalently stabilized PDPA capsules showed more significant pH-induced size responses (tripling size versus doubling size).

Due to the semipermeable nature of polymeric hydrogel capsules, it is often challenging to retain small water-soluble therapeutic molecules (below 10 kDa).[9] One of the advantages of $PDPA_{C12}$ capsules is the significant size change due to the hydrophilic to hydrophobic shift, which can be explored to achieve pH-selective permeability of small water-soluble molecules. To demonstrate the feasibility of pH-controlled permeation of $PDPA_{C12}$ capsules, fluorescein isothiocyanate (FITC)-labeled dextran of different molecular weights was used as a fluorescent probe (Supporting Information, Figure S5). At pH 7.4, the capsules were found to be impermeable to the FITC-dextran of different molecular weights (4–500 kDa) due to the hydrophobic nature of the capsule shell. However, at pH 6.0, the capsules swelled and became permeable to FITC-dextran of molecular weight up to 500 kDa (**Figure 2**; Supporting Information, Figure S5). The pH-dependent capsule permeability to a water-soluble model drug, rhodamine 6G (R6G) (479 Da) was also investigated. (The R6G-loaded capsules were used to demonstrate intracellular cargo release-see later.) The results indicate that $PDPA_{C12}$ capsules at pH 7.4 remained impermeable to R6G (Figure 2). These results suggest that the hydrophobic shell of $PDPA_{C12}$ capsules at physiological pH prevents the trafficking of small molecules, whereas at endosomal pH, these capsules swell, causing free diffusion of these molecules across the capsule walls.

To demonstrate the effective encapsulation and release of small hydrophilic therapeutic agents, an oligonucleotide adjuvant[13] (FITC-labeled CpG, negatively charged, ca. 6.5 kDa) and a cell penetrating peptide (FITC-labeled peptide with the sequence of R4H4, positively charged, ca. 1.7 kDa) were used as model therapeutics. $PDPA_{C12}$ capsules were incubated in pH 6.0 PBS containing the small hydrophilic molecules for one minute without shaking. This allows for diffusion of the molecules into the capsules. The capsules were then transferred into pH 7.4 PBS to lock the molecules inside the capsules, followed by three wash cycles to remove excess molecules. At pH 7.4, successful encapsulation of both DNA and peptide was demonstrated. The average amount of DNA or peptide loaded inside each capsule was calculated to be 2.1 pg and 4.7 pg, respectively, as determined from fluorescence spectroscopy according to predetermined calibration curves (data not shown). The DNA appeared mostly on the capsule wall due to the electrostatic interaction between the negative charged DNA and slightly positively charged multilayers (**Figure 3**a). On the other hand, the positively charged peptides were distributed evenly in the capsules (Figure 3c). Upon reduction of pH to 6.0 to simulate endosomal conditions, successful release of the cargo was observed within 10 s due to the capsule swelling and

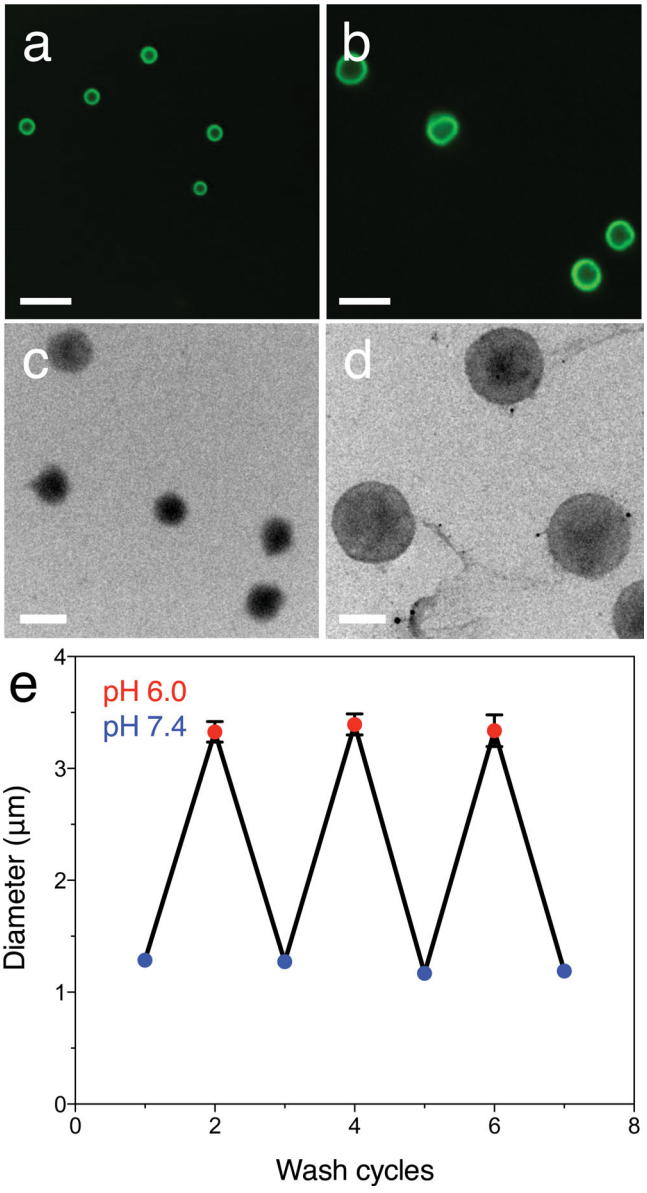

Figure 1. Non-covalently stabilized $PDPA_{C12}$ capsules obtained from 2.6 μm-diameter silica templates, as observed: by a) and b) fluorescence microscopy ($PDPA_{C12}$ capsules labeled with AF488), and c) and d) TEM at pH 7.4 (left column) and pH 6.0 (right column). a, b) Scale bars are 5 μm; d, e) scale bars are 2 μm. e) Diameters of $PDPA_{C12}$ capsules in response to alternate pH wash cycles. The diameters of 20 capsules were measured using a fluorescence microscope.

subsequent deconstruction, as seen by the fluorescence evenly distributed in the solution (Figure 3b,d).

The $PDPA_{C12}$ capsules were expected to rapidly respond the physiological pH changes during endocytosis, causing rapid degradation specifically in the acidic cellular compartments. To examine the pH-selective degradability of $PDPA_{C12}$ capsules, they were incubated in both pH 7.4 and 6.0 at 37 °C (with constant shaking) to simulate the extracellular and endosomal conditions, respectively. The total number of capsules in each sample was monitored by flow cytometry over time.

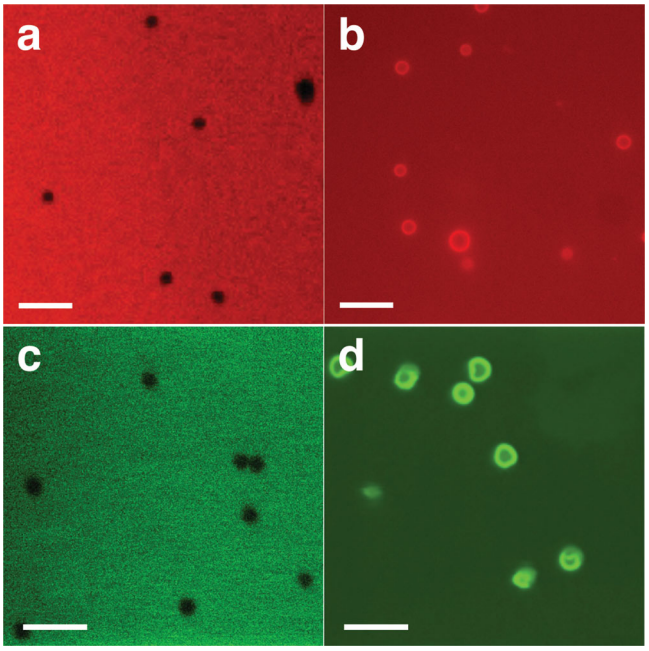

Figure 2. Confocal fluorescence microscopy images of $PDPA_{C12}$ capsules in R6G solution (in PBS) at a) pH 7.4 and b) pH 6.0, and capsules in 500 kDa FITC-dextran solution (in PBS) at c) pH 7.4 and d) pH 6.0. Scale bars are 10 μm.

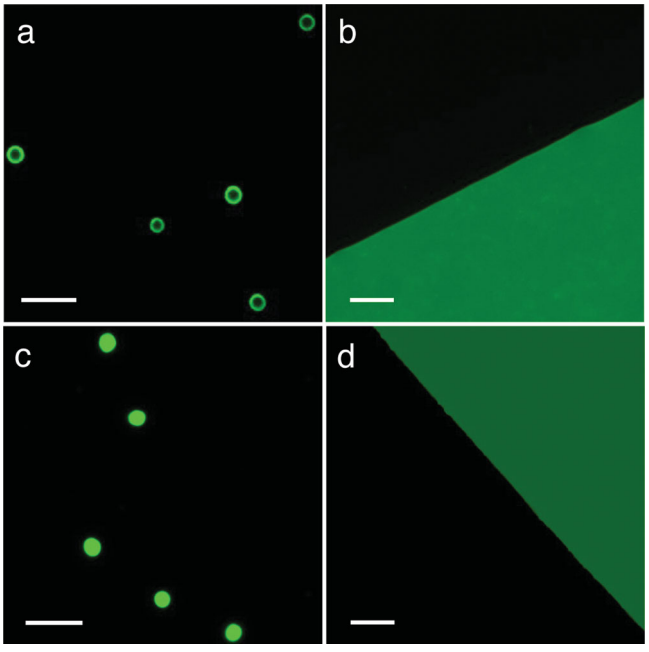

Figure 3. Confocal fluorescence microscopy images of DNA-loaded $PDPA_{C12}$ capsules in a) pH 7.4 and b) pH 6.0 PBS, and peptide-loaded $PDPA_{C12}$ capsules in c) pH 7.4 and d) pH 6.0 PBS. Scale bars are 5 μm.

At pH 7.4, >90% of the capsules remained at the end of the assay (**Figure 4**a), and these capsules showed excellent colloidal stability for months (data not shown). In contrast, at pH 6.0, about 80% of the capsules were degraded within 10 min, demonstrating their rapid and pH-selective degradation.

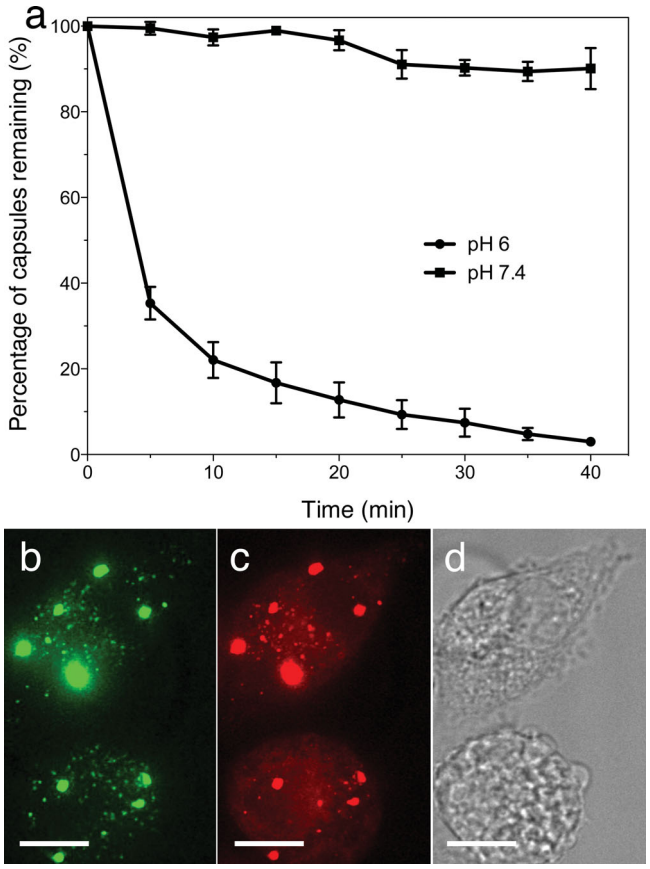

Figure 4. a) Degradation profiles of $PDPA_{C12}$ capsules in pH 7.4 (squares) and pH 6.0 (circles) PBS, as monitored by flow cytometry. Experiments were performed in triplicate. b,c) Deconvolution optical microscopy images (maximum intensity projection) and d) DIC images of JAWS II cells incubated with R6G loaded (red) $PDPA_{C12}$ capsules (fluorescently labeled with AF488, green) for 30 min at 37 °C. Capsule:cell ratio of 20:1. Scale bars are 10 µm.

The cellular interactions between $PDPA_{C12}$ capsules and JAWS II cells were studied to examine their intracellular degradation and cargo release. R6G was loaded in the capsules as a small model drug. JAWS II cells are immortalized immature dendritic cells (DCs) harvested from mouse bone marrow. DCs play a critical role in activating both T and B cells during an immune response, and are widely studied as a target cell line for immunotherapy against cancers and infectious diseases.[14] AF488-labeled $PDPA_{C12}$ capsules were incubated with JAWS II cells for 2 h at 4 °C to associate the capsules on the cell membrane (without active uptake), and the temperature was then raised to 37 °C prior to live cell imaging. Evidence of capsule degradation within 30 min was observed, as seen by the transformation of their shape from intact capsules to smaller fluorescent fragments and diffused fluorescence (Figure 4b). In contrast, the R6G signal showed diffused fluorescence in the cells, suggesting the cargo was successfully released in the cell cytosol. Furthermore, 3-(4,5-dimethylthiaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to evaluate the cytotoxicity of $PDPA_{C12}$ capsules against HeLa cells (Supporting Information, Figure S6). The results showed negligible influence on cell viability even at high capsule dosages (capsule-to-cell ratio up to 200:1).

In conclusion, we have reported the synthesis of cross-linker free, endocytic pH-degradable multilayer capsules through non-covalent stabilization. These capsules can successfully load and release hydrophilic molecules within a wide range of molecular weights (from 0.5 to 500 kDa). Further, physiological pH variations from extracellular to intracellular acidic compartments trigger capsule degradation and cargo release within 30 min, highlighting the potential of these capsules as intracellular delivery vehicles.

Experimental Section

Capsule Preparation: 100 µL of a SiO_2 particle suspension (5 wt%, 2.59 µm-diameter; Microparticles GmbH, Berlin, Germany) was centrifuged and washed three times in sodium acetate buffer (pH 4, 50 mM). This procedure was used as the standard washing procedure. Assembly of the polymers was achieved by consecutive incubation of the silica templates with 200 µL of PMA (1 mg mL^{-1}) at pH 4 in Milli Q water and 200 µL of $PDPA_{C12}$ (1 mg mL^{-1}) at pH 4 in 50 mM sodium acetate buffer with constant shaking at 23 °C. A 15 min adsorption time was allowed for all layers, and particles underwent three centrifugation/wash cycles before the addition of the following layer. This procedure was repeated until five bilayers of $PDPA_{C12}$/PMA were adsorbed. Removal of the SiO_2 particles to form hollow capsules was achieved by mixing the particle suspension with ammonium fluoride (13.3 M) buffered hydrofluoric acid (HF) (5 M) at a volumetric ratio of 1:1.5. [*Caution! HF solution is highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared.*] The capsules were then washed three times through centrifugation/wash cycles in 50 mM sodium acetate buffer (pH 4). The removal of electrostatically bound PMA layers from the $PDPA_{C12}$ multilayers was achieved by incubating capsules in borate buffer (50 mM, pH 7.4) followed by three centrifugation/wash cycles in PBS.

Fluorescent Labeling of Capsules: For a starting volume of 100 µL of particles, 1.5 µL of Alexa Fluor 488 alkyne (1 mg mL^{-1} in DMSO), 50 µL of sodium ascorbate (4.4 mg mL^{-1}), and 50 µL of copper sulfate (1.8 mg mL^{-1}) were mixed with 150 µL of sodium acetate buffer (pH 4, 50 mM). This solution was added to the $PDPA_{C12}$/PMA particles followed by constant shaking overnight. The particles were washed three times before removal of the core templates. At each pH an average of 20 capsules were sized using fluorescence microscopy.

Degradation of the $PDPA_{C12}$ Capsules: Fluorescently labeled $PDPA_{C12}$ capsules were washed into PBS and counted using flow cytometry. Then, a set of samples with a total capsule population of 1×10^7 was suspended in PBS buffer, the pH of which was adjusted to 6.0 and 7.4. The samples were incubated at 37 °C with constant shaking at 200 rpm in a thermomixer (Eppendorf, Germany). At each measurement, approximately 5×10^4 capsules were assessed. The mean fluorescence intensity and the number of capsules were measured to determine the capsule population at each time point.

Live Cell Imaging: JAWS II cells (ATCC, VA, USA) were seeded in an 8-well chambered cover glass (Thermo Scientific) at 37 °C (5% CO_2) overnight at a population of 4.0×10^5 cells per well. The cells were then incubated at 4 °C. Capsules were added at a capsule:cell ratio of 20:1 in each well. After 2 h, the cells were washed with warm media (37 °C) twice to remove free-floating capsules. The cells were finally covered with 500 µL of media in each well. The cover glass was then fitted to a DeltaVision (Applied Precision) microscope. Cells were kept alive in the incubation chamber, equipped with a CO_2 inlet, at 37 °C and mounted on the microscope stage, for imaging. Deconvolution images were taken on a series of *z*-sections within the top and bottom of the cells.

Supporting Information

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

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