Acid-Degradable Solid-Walled Microcapsules for Environmentally Responsive Burst-Release Drug Delivery

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Electronic Supporting Information

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General Procedures and Materials

All reagents were purchased from commercial sources and used without further purification unless otherwise specified. Water (dd-H$_2$O) for buffers and particle washing steps was purified to a resistance of 18 MΩ using a NANOpure purification system (Barnstead, USA). When used in the presence of acetal containing materials, dd-H$_2$O was rendered basic (pH 8) by the addition of triethylamine (TEA) (approximately 0.01%). Unless otherwise stated, $^1$H NMR spectra were recorded at 400 MHz and $^{13}$C spectra were recorded at 100 MHz. UV-Vis spectroscopic measurements were obtained from samples in quartz cuvettes using a Lambda 35 spectrophotometer with a cell changer (Perkin Elmer, USA) or using a Spectra Max 190 (Molecular Devices, USA) for microplate-based assays, usage courtesy of Prof. Carolyn Bertozzi. RAW 264.7 and HeLa cells were obtained from ATCC (Manassas, VA) and grown according to ATCC’s directions. All experiments with cells, unless otherwise stated, were carried out in DMEM with GlutaMAX (Invitrogen 10566-016, USA) supplemented with 10% fetal
bovine serum and 1% pen-strep. Flow cytometry was performed on a FACSCalibur Flow Cytometer, usage courtesy of Prof. Carolyn Bertozzi.

**Experimental Methods**

**Synthesis of Diethanolamine Ketal (DEAK).** A flask was charged with p-toluenesulfonic acid monohydrate (380 mg, 2 mmol), N-(2-hydroxyethyl)phthalimide (38.24 g, 220 mmol) and benzene (350 mL). A Dean-Stark apparatus was affixed and the solution was refluxed at 110 °C for 1.5 h. Reaction was cooled to 0 °C and 2-methoxypropene (21 mL, 220 mmol) was added. Reaction was capped and warmed to 40 °C for 2 hours. Dean-Stark apparatus was reattached and reaction was reheated to 110 °C. Azeotroped benzene/methanol was removed by continuously draining from Dean-Stark trap. After 5 h, reaction was added to hexanes (1 L) and precipitate was collected. This crude diphalimide ketal was deprotected by refluxing with NaOH (6M, 150 mL) at 110 °C overnight. The reaction mixture was cooled and extracted with 1:1 CHCl₃:iPrOH (3 x 200 mL). The combined organics were washed with brine and dried over MgSO₄ and concentrated in vacuo to yield a yellow-orange liquid (11.8 g, 33% yield). Spectral data was in good agreement with previous precedent.¹

**Synthesis of triamide 1.** To a stirring flask containing ethanolamine (2.3 mL, 37.7 mmol) was added dropwise a solution of trimesoyl chloride (1.00 g, 3.77 mmol) in DCM (10 mL). Reaction was stirred for 30 min then diluted with water (10 mL) and extracted with 1:1 CHCl₃:iPrOH (3 x 30 mL). The combined organics were then dried over MgSO₄ and concentrated in vacuo. The crude yellow solid was then recrystallized using hexanes and MeOH with a small amount of DCM. Crystals were collected as white fibers (199.6 mg, 16% yield). M.P. = 192-198 °C. ¹H NMR δ 8.20 (s, 1H), 3.75 (t, J = 5.5 Hz, 2H), 3.52 (t, J = 5.5 Hz, 2H). ¹³C NMR δ 42.12, 59.88, 128.81, 134.60, 168.84. Other data in line with previous report.²

**Capsule preparation (>100 µm diameter range).** To a 20 mL vial containing an aqueous solution of poly(vinyl alcohol) (PVA, Mₔ =13 000 – 23 000 g/mol, 87-89% hydrolyzed) (3 mL, 0.4 % w/w in dd-H₂O) was added toluene or capryic/caprylic triglycerides containing either TM or TR (1 mL, 6% w/v). This mixture was stirred vigorously to produce an emulsion. An aqueous solution of either DETA or DEAK (1.6 mL, 38 % v/v
in dd-H₂O) was then added dropwise. The resulting mixture was stirred gently for 3 hours then isolated by filtration, washed with acetone and ether, and allowed to dry under a stream of N₂.

**Large Microcapsule preparation (10 µm diameter range).** Microcapsules were prepared as above but using an homogenizer (Ika Ultra-Turrax with an S25GN-10G generator) at 24 000 rpm for 45 s to generate the emulsion. Instead of washing with acetone and ether, capsules were dialyzed against water (100 KDa MWCO).

**Small microcapsule preparation (300 nm diameter range).** Nanocapsules were prepared as above but the emulsion was generated by first vortexing, then sonicating (Branson Sonifier) for 45 s at an output level of 5 and a duty cycle of 80%.

**Scanning Electron Microscopy.** Microcapsules were characterized by scanning electron microscopy using a S-5000 microscope (Hitachi, Japan). Particles were suspended in dd-H₂O (pH 8) at a concentration of 1 mg/mL and the resulting dispersions were dripped onto silicon wafers. After 15 min, the remaining water was wicked away using tissue paper and the samples were allowed to air dry. The particles were then sputter coated with a 2 nm layer of a palladium/gold alloy and imaged.

**Transmission Electron Microscopy.** Microcapsules were characterized by transmission electron microscopy (TEM) using a TECNAI F20 microscope (FEI, USA) at 200 kV accelerating voltage. Particles were suspended in dd-H₂O (pH 8) at a concentration of 1 mg/mL and dropped onto a TEM grid. Excess water was wicked off 5 minutes later using a tissue paper and the samples were allowed to air dry.

**Particle Size Analysis by Dynamic Light Scattering.** Particle size distributions and average particle diameters were determined by dynamic light scattering using a Nano ZS (Malvern Instruments, United Kingdom). Particles were suspended in dd-H₂O (pH 8) at a concentration of 1 mg/mL and three measurements were taken of the resulting dispersions. Size distribution histograms are presented in Figure S1 and average sizes are reported in Table S1.
Figure S1. (a) Size distribution of capsule samples as measured by dynamic light scattering.

Table S1. Average diameters of capsule samples as measured by dynamic light scattering.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diameter (nm)</th>
<th>Width @ Half Peak Height (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>empty emulsion</td>
<td>264</td>
<td>110</td>
</tr>
<tr>
<td>empty nondegradable MCs</td>
<td>318</td>
<td>160</td>
</tr>
<tr>
<td>empty degradable MCs</td>
<td>389</td>
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</tr>
<tr>
<td>taxol emulsion</td>
<td>274</td>
<td>122</td>
</tr>
<tr>
<td>taxol nondegradable MCs</td>
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<td>144</td>
</tr>
<tr>
<td>taxol degradable MCs</td>
<td>342</td>
<td>158</td>
</tr>
</tbody>
</table>

Cytotoxicity Studies. For cell viability experiments, triamide 1 was tested using HeLa cells and RAW 264.7 macrophages (Figure S2a). Additionally, degradable and nondegradable capsules containing caprylic/capric triglycerides were each incubated with RAW macrophages (Figure S2b).

For each viability experiment, 1x10⁴ RAW macrophages or HeLa cells were seeded in a 96-well plate and allowed to grow overnight. Serial dilutions of samples were prepared and added to the cells, which were then incubated overnight. The next morning,
40 µL of 2.9 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well. Crystals were allowed to develop for 10 min for RAW macrophages and for 30 min for HeLa cells. Medium was aspirated and remaining crystals were dissolved in 200 µL DMSO and 25 µL glycine buffer (0.1M glycine, 0.1M NaCl). Absorbance was read at 570 nm. Results are presented as the mean of triplicate cultures ± 95% confidence intervals.

**Figure S2.** *In vitro* toxicity assays of capsule degradation byproducts (a) and capsules (b). 100% viability is defined as the absorbance from untreated cells. The inherent variability of living cells means that values over 100 % are often seen.

**Particle Degradation:**

**Deuterated buffer preparation.** D₂O was buffered at pH 7.5 by dissolving 100 mM Na₂DPO₄ then adjusting the pH with concentrated DCl. Because only signals between 0 and 4.5 ppm were necessary for observing degradation, pyridine was chosen as a suitable buffering agent for D₂O at pH 5. Pyridine was dissolved to 100 mM in D₂O then the pH was adjusted using concentrated DCl. All pH values were measured using a glass electrode pH meter and adjusting using the following formula: pD = 0.4 + pH reading.

**¹H NMR Study.** Empty small microcapsules (1 mg) and deuterated PBS buffer (1 mL) or pyridine buffer (1 mL) were added to an NMR tube, which was immediately sealed. After various time points additional ¹H NMR spectra were taken and the appearance of acetone,
and triamide 1 was measured as a ratio of these peaks’ integrals to the integral of the internal standard peak (3-(trimethylsilyl) propionic-2,2,3,3,4,4 acid, sodium salt). Stack plots of the NMR spectra at various time points are presented in Figure S3 and S4.

**Figure S3.** NMR of capsule degradation over time in deuterated aqueous phosphate buffer at pH 7.5.
Figure S4. NMR of capsule degradation over time in deuterated water and pyridine buffer at pH 5.0.

Figure S5. Normalized $^1$H integrations of acetone and triamide 1 from MCs shown over the course of 120 h, at which point full degradation is observed at pH 7.5.

Fluorescent sample preparation: Fluorescent samples were prepared as above, but containing 5 mg/mL of benzothiadiazole dye 2. Absorption of 2 was 464 nm and
emission was 560 nm. Preparation and use of 2 courtesy of Dr. Paul Armstrong. All spectral data matched previous report. ³

\[ \text{Dye Release experiment:} \]

Standard 3 mL quartz cuvettes were filled with CHCl₃ (2 mL) and either 30 mM pH 5 acetate buffer or 30 mM pH 7.4 phosphate buffer (1 mL). Either degradable or nondegradable fluorescent capsules (3 mg) were added. Cuvettes were gently shaken to allow capsules to settle to the interface between aqueous and organic phases. Any capsules sticking to the quartz walls were gently pushed by blowing air bubbles from a Pasteur pipette. Cuvettes were sealed absorbance was measured at 464 nm every 90 s for 150 h. Due to solvent height, this measurement was of the organic layer.

\[ \text{Figure S6.} \]

Release of an encapsulated dye from MCs over 40 h. Nondegradable capsules release less than 10% of encapsulated cargo over this time period. Degradable capsules at pH 7.4 lack a lag phase in dye release, presumably because dye begins to leach from capsules as their walls are weakened by slow degradation. Gap in data is due to an instrument failure between hours 16 and 25.
**Cell uptake studies:** RAW 264.7 cells were plated at 2 x 10⁵ cell/well in 2 mL in 24-well plates. The following day, the medium was replaced with medium containing samples at 1.0, 0.1, or 0.01 mg/mL. The following day, medium was removed and wells were gently washed once with 300 µL PBS. Trypsin (70µL) was then added to each well and the plate was incubated for 15 minutes at 37 °C. PBS (300 µL) was added and cells were washed from the plate with repeated pipetting. Combined washes were centrifuged at 3000 x g for 4 min. Buffer was removed and cells were resuspended in PBS (300 µL). Cells were centrifuged and resuspended twice more, with the final resuspension in 300 µL of 4% paraformaldehyde in PBS. Samples were put on ice and analyzed by flow cytometry.

**References**