Supporting Information

Hybrid Vesicles Enable Mechano-Responsive Hydrogel Degradation

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Experimental Details

Materials: 18:1 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonil (18:1 Liss Rhod PE), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Poly (ethylene oxide)-b-polybutadiene (PEO_{18}-b-PBD_{22}, M_n = 1800 g/mol, hereafter referred to as PEO-PBD) was purchased from Polymer Source Inc. (Montreal, Quebec, Canada). Sulfo-Cyanine5 carboxylic acid (Cy-5, MW = 681 g/mol) was purchased from Lumiprobe (Hallandale Beach, FL, USA). Nalgin™ HG medium viscosity sodium alginate was a gift from Prof. Ariella Shikanov (University of Michigan), purchased from Ingredients Solutions Inc (Waldo, ME, USA) (Lot# DYS201109010). FluoSpheres™ 0.1 μm yellow-green fluorescent microspheres were purchased from Invitrogen (Carlsbad, CA, USA). EGTA and nylon net filter (#NY2004700, 20 µm pore size) were purchased from Millipore Corp. (Billerica, MA, USA). Thermo Scientific™ Nunc 96-Well Optical-Bottom Plate (#165305) and Tris base were purchased from Thermo Fisher Scientific Co. (Waltham, MA, USA). 24-well glass bottom plate (#P24-0-N) was purchased from Cellvis (Mountain View, CA, USA). Silicone oil, mineral oil, HEPES, OptiPrep™ density gradient medium, fluorescein isothiocyanate (FITC, MW = 389 g/mol), Triton™ X-100, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Confocal imaging: Images were taken with an oil immersion UplanFL N 40 x/1.30 (Olympus, Tokyo, Japan) objective on an inverted microscope (IX-81 Olympus) equipped with a spinning-disk confocal (CSU-X1, Yokogawa, Tokyo, Japan), an iXON3 EMCCD camera (Andor Technology, Belfast, United Kingdom), and a National Instrument DAQ-MX controlled laser (Solamere Technology, Santa Barbara, CA, USA). MetaMorph (Molecular Devices, San Jose, CA, USA) software was used to acquire images and videos. Images of Cy-5 and fluorescent beads were taken with 640-nm and 488-nm excitation lasers, respectively, with 200 ms exposure time. To acquire videos for Brownian motion of fluorescent beads, "stream acquisition" function in the MetaMorph was used for 50 or 80 frames at 286 ms intervals (~23 sec), with 200 ms exposure using 488-nm laser excitation.

Preparation of vesicles: Vesicles were generated using the modified continuous droplet interface crossing encapsulation (cDICE) technique, which is an emulsion-based method. Three different solutions were prepared: an inner encapsulation solution, a lipid-in-oil dispersion, and an outer aqueous solution. The inner encapsulating solution was prepared with 5 μM Cy-5 or FITC fluorescent dye, 880 mM EGTA, and 10 v/v% OptiPrep (1.320 g/mL density gradient solution). For the non-EGTA inner solution, a diluted solution from the HEPES-Tris buffer stock solution (1.1 M HEPES, 1 M Tris, pH 8) was used instead. For lipid-in-oil dispersion, lipids and block copolymer were added to a 20 mL glass vial from respective chloroform stock solutions to obtain a final concentration of 0.2 mg/mL and then dried with a gentle argon flow. The composition used for most of the experiments consisted of 50/50 mol% of PEO-PBD block copolymer and DOPC, with a trace amount of rhodamine-PE (< 0.05 mol%) for fluorescent vesicle labeling unless specified otherwise. The dried lipid-polymer film was resuspended with 300 μL of chloroform, and while vortexing, 4.7 mL of oil mix (4:1 of silicone and mineral oil) was added dropwise. The vial was then tightly closed and vortexed for 2 min and bath sonicated for 15 min. The outer solution was dilute from a HEPES-Tris buffer stock solution to achieve an osmolarity 5–10% higher than that of the inner solution.

Next, a 3D-printed cylindrical chamber, which is opened at the top, was mounted on a tabletop
stirring motor that rotates at 1,200 rpm. The chamber was filled with 390 μL of the outer solution and then with 3.2 mL of lipid-in-oil dispersion to form an oil-water interface. In a separate tube, 800 μL of lipid-in-oil dispersion was added to 70 μL of the inner solution, and the mixture was thoroughly pipetted up and down to produce water-in-oil monolayer emulsion droplets, which were then added dropwise to a cDICE chamber. As droplets cross through the oil-water interface because of the centrifugal force, they acquire a second layer of lipid to form a bilayer structure of vesicles and are suspended in the outer solution. Collected vesicles (~ 340 μL) were washed five times using centrifugation at 100 × g with fresh outer solution (250 μL) to remove any un-encapsulated inner solution contained in the outer solution.

**Preparation of vesicle-embedded alginate hydrogels:** Alginate solution (3 w/v% in DI), 0.1 μm fluorescent beads, vesicle pellet, and HEPES-Tris stock solution (1.1 M HEPES, 1 M Tris, pH 8) were gently mixed in a 1.5 mL microtube to achieve 0.8% final alginate concentration, with the osmolarity that is 5–10% higher than that of the internal solution of vesicles. The purpose was to make vesicles be in a hyper-osmotic condition to prevent them from rupturing during mixing with the viscous alginate solution. However, it is possible that some vesicles may have ruptured while mixing, and thus the actual volume of vesicles included can be lower. Fluorescent beads were added to 1:500 dilution from stock solution. The volume of the vesicles included in the alginate solution mix was found to be 18.6%. This was determined by multiplying the added volume of the vesicle pellet (23.15%) with the vesicle volume percentage in the pellet (80.5%) based on image analysis (Figure S2A). 10 μL of the mixed solution was then transferred to a 1.5 mL microtube using a cut pipet tip. 80 μL of 50 mM CaCl₂ solution was then gently placed on top of the alginate solution with cut tip without disturbing the surface, and the hydrogel was incubated for 20 min at room temperature to be crosslinked. The crosslinked hydrogel was then washed twice with HEPES-Tris solution before use.

**Monitoring of vesicles under compressive stress:** To examine the ability of vesicles to release compounds in response to applied compressive stress, we wanted to monitor the same vesicles while applying different stress conditions. Thus, we used a compression setup designed to minimize potential hydrogel position changes during compression. We made 100 μL of alginate hydrogel solution that contains FITC-loaded vesicles and stiffened it in between slide glasses separated by 1 mm glass spacers by adding 50 mM CaCl₂ solution around the hydrogel. The stiffened hydrogel was then transferred to a clear glass bottom 24-well plate (15.5 mm diameter) in the center, and an additional 100 μL alginate hydrogel solution was added around the stiffened hydrogel to fill out the rest of the bottom area of the well. 50 mM CaCl₂ solution was added to stiffen the alginate, and the hydrogel was washed twice with HEPES-Tris solution. 14 mm diameter cover glass, which is smaller than the bottom area but larger than the vesicle-containing hydrogel area, was placed on the hydrogel, and various amounts of cylindrical weight were placed on top of the cover glass to apply compression (Figure S4A). The applied stress was calculated by the weight divided by the cover glass area (154 mm²). Applied weights of 45, 90, 150, and 200 g corresponded to the compressive stress of 2.9, 5.8, 9.6, and 12.8 kPa. Images were taken immediately after the compression. As we increased the stress stepwise from 2.9 to 12.8 kPa, the shape of the vesicles within the hydrogel gradually deformed, and 33.3% of the monitored vesicles ruptured and lost their FITC fluorescence signal (n = 12, Figure S4B). Each of the last images in Figure S4B shows a flattened vesicle membrane due to rupture.

**Required EGTA concentration for hydrogel degradation:** To produce a hydrogel that degrades
rapidly within a short period of compression time (~20 min), we carried out an experiment to determine the required EGTA concentration to degrade 10 μL of 0.8% alginate hydrogel in 20 min. 40 μL of various EGTA concentration solutions (0, 25, 50, 100, 125, 150 mM) were added to 10 μL stiffened hydrogel, which corresponds to the final EGTA concentrations of 0, 20, 40, 80, 100, 120 mM, considering the hydrogel volume. After 20 min of incubation at room temperature, we determined the degradation percentage by measuring the volume of the solution that could be pipetted up. The result in Figure S5 shows that a concentration of at least 100 mM of EGTA is needed to achieve 100% degradation under the designed settings. However, since EGTA will be encapsulated in a confined volume of vesicles, we calculated a concentration required to achieve a final 100 mM concentration when EGTA is released from the vesicles under compression. We took into account the dilution due to hydrogel volume (100/18.6 times, considering 18.6% of the vesicle volume in hydrogel) and added liquid volume (1.5 times, considering 5 μL was added which accounts for 50% of the hydrogel volume) before the compression. This calculation resulted in a final required concentration of 806.45 mM (100 mM × 100/18.6 × 15/10 = 806.45 mM). However, to account for a potential loss (~10%) of EGTA during the vesicle embedment process or EGTA encapsulation process, the EGTA concentration was set to 880 mM.

**EGTA encapsulation efficiency analysis:** As it is common that the inner solution of the droplets can be diluted by the outer solution while the bilayer is formed by emulsion-based methods, the following experiment was conducted to estimate the final EGTA concentration inside the vesicles when starting inner solution is 880 mM EGTA. First, generated vesicles were collected in a pellet through centrifugation (100 × g), and the internal solution was released by adding surfactant Triton™ X-100 to 2% to degrade vesicle membranes. To determine the EGTA concentration of the released inner solution, we used a simple assay that measures the alginate degrading ability of EGTA (Figure S6A). We generated 1.5% alginate hydrogel containing Cy-5 fluorescent dye (10 μM) in a 10 μL volume and crosslinked with 50 mM CaCl₂ solution as previously described in a microtube. The crosslinked hydrogel was then immersed in 40 μL of the released inner solution. After 100 sec of incubation, we removed the undissolved hydrogel and measured the Cy-5 fluorescence intensity of the solution using a microplate reader (Synergy H1, BioTek Instruments) at λex-em 646–670 nm. The higher the EGTA concentration in the solution, the faster the alginate hydrogel degrades and accelerates the dye release into the solution. Using a standard curve obtained from testing known EGTA concentrations (0, 440, and 880 mM), we determined the EGTA concentration of the released inner solution based on the measured Cy-5 fluorescence intensity (Figure S6B). The EGTA concentration contained in the DOPC/PEO-PBD hybrid vesicle was found to be 843.8 mM, indicating 95.9% encapsulation efficiency (Figure S6C). This is a 16.4% increased efficiency compared to that of DOPC phospholipid vesicles, which showed 699.8 mM inner EGTA concentration (79.5% encapsulation efficiency) (Figure S6C).

**Hydrogel compression setup:** Hydrogel (diameter ~3.4 mm) was transferred to a 6.3 mm diameter glass bottom 96-well plate. 5 μL of HEPES-Tris solution was added to the hydrogel prior to compression application to prevent drying of the hydrogel. A cylindrical support (6 mm diameter, 18 mm height) was 3D-printed by Form 3 3D printer (Formlabs, Somerville, MA, USA) using clear resin and placed on top of the hydrogel. Then, 30 or 50 g weight was placed on top of 3D-printed support to apply compressive stress for 20 min. The stress applied to the hydrogel was calculated by the weight (30 or 50 g) plus the 3D support weight (0.6 g) divided by the contact area (9.08 mm²) of the hydrogel. Placing 30 or 50 g of weight corresponds to a stress of 33 or 55 kPa. The
frictional force between the 3D support and the wall was assumed to be negligible. Note that the calculation was based on the initial contact area of the gel and did not consider the area expansion during the compression.

**Brownian motion analysis:** Trajectories of fluorescent beads from the videos were acquired using an ImageJ software (NIH) plugin TrackMate.\[^{[4]}\] Individual bead trajectory was plotted as mean square displacement (MSD) as a function of time interval (Δt up to 10 sec) using the following equation in MATLAB (Mathworks, Natick, MA, USA):

\[
MSD = \frac{1}{N} \sum_{i=1}^{N} |r_i(t) - r_i(t_0)|^2
\]

where \(N\) is the number of points, \(r_i(t)\) is the position of the bead at \(i\)th position at time \(t\). Diffusion coefficient \(D\) was determined from the slope of the MSD plot, using the equation,

\[
MSD = 4Dt
\]

Five beads were analyzed from three independent samples for each condition (\(n = 15\)).

**Hydrogel weight measurement:** To measure the remaining weight of the hydrogel after the compression application, we used a glass vacuum filtration apparatus with a hydrophilic nylon filter membrane (20.0 µm pore size) to separate the solid hydrogel from the dissolved portion. The filter membrane was placed on the filter holder, wetted with 3 mL of deionized water and applied with a vacuum for 30 sec, and the filter membrane was removed from the holder to measure the weight (wet filter membrane weight, \(W_f\)) using a scale (AB104-S, Mettler Toledo, Greifensee, Switzerland). The measured filter membrane was then placed back to the holder, and the hydrogel sample was placed on the top of the filter membrane. Again, 3 mL of deionized water was added to the sample and applied with the vacuum for 30 sec to remove soluble portion of the hydrogel. The weight of the wet filter membrane and remaining hydrogel sample (\(W_{f+s}\)) was measured. The residual sample weight (\(W_s\)) was determined using the equation, \(W_s = W_{f+s} - W_f\). (\(n = 5\) for non-compressed control tests, \(n = 3\) for the compressed tests).

**Hydrogel mechanical testing:** The mechanical properties of the hydrogels were measured using the test instrument Hysitron TI-950 TribolIndenter (Bruker, Billerica, MA, USA). A displacement of 100 nm was applied to all specimens. The experimental data was analyzed using the Oliver-Pharr method,\[^{[5,6]}\] using TI-950 TribolIndenter software to determine the reduced modulus. The reduced modulus of elasticity \(E^*\) is related to Young’s modulus \(E\) and Poisson’s ratio \(\nu\) by the ratio:

\[
\frac{1}{E^*} = \frac{1-\nu^2}{E} + \frac{1-\nu_i^2}{E_i}
\]

Where \(E_i\) and \(\nu_i\) are the elastic modulus and the Poisson’s ratio of the probe, respectively.

The Young’s modulus of the probe used for the measurement is 116 GPa which significantly exceeds the estimated value of the hydrogel \(E_i >> E\). Therefore, it is further assumed without loss of accuracy, the formula can be represented as the following equation:
\[ \frac{1}{E^*} = \frac{1 - \nu^2}{E} \tag{4} \]

A Poisson’s ratio (\(\nu\)) of 0.5 was used, assuming hydrogel is incompressible,\(^7\) and the elastic modulus of the sample can be calculated by the following equation:

\[ E = (1 - 0.5^2) \times E^* \tag{5} \]
Supporting Figures

Figure S1. Retention of encapsulated molecules comparison. (A) Images of Cy-5 encapsulated vesicles made with two different membrane compositions (DOPC and 50/50 DOPC/PEO-PBD) monitored for 48 hours in a buffer. (B) Quantified result shows DOPC/PEO-PBD hybrid vesicles present a better ability to retain internal Cy-5 fluorescence signal over time compared to DOPC phospholipid vesicles. Internal fluorescence and external fluorescence signal values were obtained using a “Plot profile” function in ImageJ (n = 5).
Figure S2. Size distribution and volume of vesicles embedded in hydrogel. (A) Photograph of the hybrid vesicle pellet obtained after centrifugation (left) and an image of the vesicle pellet (right). (B) Vesicle size distribution. The diameters of the vesicles were measured using ImageJ software by analyzing vesicle pellet images (n = 198). The average diameter was 21.03 ± 8.46 μm.
Figure S3. Ability of vesicles to retain small molecule compounds while embedded in a hydrogel matrix. 5 μM Cy-5 fluorescent dye (MW = 681 g/mol) loaded in vesicles were stable for one month. Fixed bead positions are shown with white arrows. The images in the top row are the same as in Figure 2B.
Figure S4. Ability of vesicles to release compounds in response to applied compressive stress. (A) Schematic showing how hydrogel was formed between glass slides using spacers (top) and how the compressive stress was applied to the hydrogel in the 24-well glass bottom plate (bottom). (B) Representative images showing vesicle shape deformation and release of fluorescent dye (FITC) from vesicles due to compression.
Figure S5. Required EGTA concentration test results. At least 100 mM of EGTA concentration is required to entirely (i.e., 100%) degrade the 10 μL of 0.8% calcium-crosslinked alginate hydrogels in 20 min (n = 3).
Figure S6. Investigation of final EGTA concentration in vesicles. (A) Schematic of an assay procedure for determining EGTA concentration by measuring the alginate degradation in EGTA-containing solutions. (B) A standard curve was obtained by using 0, 440, 880 mM EGTA concentrations (n = 3–4). (C) EGTA encapsulation efficiency of the vesicles made using DOPC or 50/50 DOPC/PEO-PBD. DOPC/PEO-PBD hybrid vesicles showed 16.4% higher encapsulation efficiency compared to DOPC phospholipid vesicles.
**Figure S7.** Brownian motion of fluorescent beads within unstiffened/stiffened alginate hydrogel. Trajectories of the fluorescent bead movement within uncrosslinked 0.8% alginate solution (left) and calcium crosslinked (right) 0.8% alginate hydrogel. Videos were taken for 50 frames at 286 ms intervals using 200 ms exposure at 488 nm laser excitation.
Figure S8. Mean square displacement (MSD) plots of fluorescent beads embedded in hydrogels. Trajectories of fluorescent beads plotted as MSD as a function of time interval (Δt). Slopes of each graph were used to calculate the diffusion coefficient values shown in Figure 3B using Equation 2. Only the beads in the hydrogel containing EGTA-loaded vesicles exhibit increased diffusion after the compression application of 55 kPa for 20 min (bottom rightmost plot).
References


