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2 # Supporting Information
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5 **Chitosan-PEG Hydrogel with Sol-Gel Transition Triggerable by Multiple External**
6 **Stimuli**
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8 *Ching Ting Tsao, Meng Hsuan Hsiao, Mengying Zhang, Sheeny Lan Levengood, Miqin*
9 *Zhang**
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12 ## Supplementary Experimental Section

13 *Reagents*

14 All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.
15 Chitosan (85% de-acetylated, medium molecular weight), methoxy-poly(ethylene glycol)
16 (mPEG, 750 Da), succinic anhydride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
17 (EDC) and N-hydroxysuccinimide (NHS) were used as received.

18

19 *Synthesis of carboxylic acid-terminated methoxy-poly(ethylene glycol) (mPEG-acid)*

20 The carboxylic acid-terminated methoxy-poly(ethylene glycol) (mPEG-acid) was prepared
21 following reported methods[1,2] with slight modifications. Specifically, mPEG (9.75 g) was
22 dehydrated at 50ºC under vacuum for 8 hr before initiating the reaction. Succinic anhydride
23 (1.35 g) was added to dehydrated mPEG, and the mixture heated at 100ºC for 2 hr to allow
24 the succinic anhydride to fully dissolve. The molar ratio of succinic anhydride to mPEG is
25 1.038. The reaction then proceeded under reflux at 120ºC for 24 hr. During the reaction, it
26 was observed that some succinic anhydride sublimed inside the reaction vessel. Therefore a
27 small excess of succinic anhydride was used to ensure that the terminal hydroxyl groups of
28 mPEG were completely reacted. The mixture was then cooled to room temperature, and the
29 mPEG-acid was collected by filtration through filter paper to eliminate unreacted succinic

30 anhydride. The yield of mPEG-acid is 70 wt%, and the conversion is 99 mol%. The mPEG-
31 acid was stored at −20ºC for future use.
32
33 *Synthesis of methoxy-poly(ethylene glycol)-g-chitosan (mPEG-g-chitosan)*
34 Chitosan (0.3 g) was dissolved in 30 mL of 1.0 w/v % acetic acid. To this mPEG-acid (0.43 g)
35 was added, and the two components were mixed with constant stirring until homogeneous. A
36 catalyst solution was prepared by adding EDC (0.2 g) and NHS (0.12 g) in 20 mL of DI water.
37 Subsequently, the catalyst solution was added dropwise into the mixture of chitosan and
38 mPEG-acid. The resulting solution was stirred for 4 hr at room temperature allowing for
39 amide linkage formation between chitosan and mPEG-acid.[3,4] A 0.5 M NaOH solution was
40 then added dropwise into the mixture until a pH value of 7 was reached. The resultant mixture
41 was dialyzed with a dialysis membrane (MW 12,000–14,000 cutoff) against DI water to
42 ensure unreacted chemicals and salts were removed. The dialysis step was repeated three
43 times, and the resulting solution after dialysis was snap frozen with liquid nitorgen. mPEG-g-
44 chitosan was obtained by removal of water using lyophilization.
45
46 *FTIR*
47 Chitosan, mPEG, succinic anhydride, mPEG-acid, and mPEG-g-chitosan were analyzed by
48 Fourier transform infrared spectroscopy (FTIR, Perkin-Elmer Spectrum RX1 System, MA) to
49 examine the peak intensity variation of the hydroxyl groups, carboxylic acid groups and the
50 amide linkages. A 0.5 w/v % sample solution of each was prepared in acetone, and a droplet
51 of sample solution was placed onto a transparent KBr disk for FTIR analysis.
52
53 *NMR*
54 The chemical structures of chitosan and mPEG-g-chitosan were confirmed using ^{1}H nuclear
55 magnetic resonance spectroscopy (^{1}H NMR, Bruker AV-301 spectrometer, MA), and the

56 spectra were acquired at 500 MHz and 50ºC. mPEG-g-chitosan samples of 10–20 mg each
57 were dissolved in D_2O (0.7 mL). The degree of PEG-grafting (PEGylation) was defined by
58 the molar ratio of H1 to H7 as shown in **Figure 1e** using the integral function in Topspin
59 (Bruker, MA).
60
61 *Rheology*
62 Thermal sensitive gelation behavior of mPEG-g-chitosan was further studied by rheological
63 analysis. Briefly, water-soluble mPEG-g-chitosan was reconstituted with DI water, 1× PBS,
64 or 10× PBS to make solutions of different solute concentrations and salt concentrations. The
65 solutions were maintained on ice for 4 hr with periodic vortexing to ensure full dissolution of
66 mPEG-g-chitosan. The rheological properties of samples were measured using a stress-
67 controlled rheometer (MCR 301, Anton Paar, Germany) with a cone and plate configuration
68 of 24.982 mm diameter and 0.994º cone angle. A layer of silicon oil was carefully applied to
69 prevent water evaporation during the experiment.[5] All dynamic frequency experiments were
70 conducted in the linear viscoelastic regime of tested samples, as determined by dynamic
71 strainsweep trials over a strian range of 0.05–50%.
72 The viscoelastic measurement was taken in the dynamic oscillatory mode with a constant
73 frequency of 1 Hz and strain of 10% (no effect on gel formation) with temperature ramping at
74 a rate of 1°C/min. The values of the storage and loss moduli (G' and G", respectively) and
75 phase angle (Θ) were obtained accordingly.[6] The incipient of gel network formation, which
76 is defined by the gelation temperature, is given by the crossover of G' and G". The
77 measurement of the gelation temperature showed good reproducibility.
78 For the pH dependence of rheological properties, the solution was adjusted with 1N NaOH
79 and 1 N CH_3COOH to the targeted pH value with constant ionic strength of 1N. The samples
80 were completely sealed to prevent solution exposure to CO_2 in the air. The samples were also

81 analyzed for zeta potential using a DTS Zetasizer Nano (Malvern Instruments, Worcestershire,
82 UK).
83
84 *In vitro protein release*
85 Bovine serum albumin (BSA) was dissolved in 1× PBS to yield BSA solutions of 0, 100, 500,
86 800 and 1000 μg/mL. The BSA solutions were filtered through a 0.22 μm syringe filter for
87 sterilization. Ethylene oxide (EtO)-sterilized mPEG-g-chitosan was dissolved in previously
88 prepared BSA solutions to yield a final mPEG-g-chitosan concentration of 1.5 wt%. The
89 mixtures were ice-chilled overnight to ensure full dissolution. After light vortexing of the
90 polymer/protein mixtures, air bubbles were removed by centrifugation. The 0.5 mL solutions
91 containing mPEG-g-chitosan and BSA were incubated at 37ºC for 10 minutes to form gels.
92 Following gelation, 0.5 mL of sterile PBS (37ºC) was added on top of the gel. The BSA-
93 containing gels and PBS were maintained at 37ºC without mixing. At specified sample
94 collection times, 0.5 mL of PBS was collected and transferred to a siliconized 1.5 mL
95 microcentrifuge tube, and 0.5 mL of fresh PBS was added. The protein content of each
96 sample was analyzed with modified Coomassie blue protein assay (BioradR) in a 96-well
97 plate using UV spectroscopy at 590 nm. A calibration curve was generated at each time
98 interval using a non-loaded gel in order to correct for the intrinsic absorbance of the polymer.
99
100 *Cell compatibility*
101 1.5 wt% EtO-sterilized mPEG-g-chitosan was fuly dissolved in Dulbecco's Modified Eagle
102 Media (DMEM). Matrigel (Corning, Tewksbury, MA) was thawed at 4°C overnight to obtain
103 liquidized solution. Pre-chilled pipette tips and 24-well tissue culture polystyrene (TCPS)
104 plates were utilized. 200 μL of mPEG-g-chitosan gel or Matrigel was pipetted into TCPS
105 wells, and maintained at 37°C for 2 hours to achieve gelation. Murine mammary carcinoma
106 (MMC) cells (1×10^5) were seeded in uncoated, mPEG-g-chitosan gel pre-coated (200 μL),

107 and Matrigel pre-coated (200 μL) wells. The fully supplemented DMEM medium (800 μL)
108 was added 2 hours after seeding. MMC cell proliferation was assessed using an Alamar blue
109 assay (Alamar BioSciences, Sacramento, CA) one and four days after cells seeding. Briefly,
110 media were gently aspirated and replaced with the Alamar blue solution (10× dilution with
111 DMEM). After incubation for 2 hrs, the Alamar blue solution was collected and the
112 fluorescence of the solution was measured on a SpectraMax M2 microplate reader (Molecular
113 Device, Sunnyvale, CA) at 550 nm excitation and 590 nm emission. The cell number was
114 determined from calibration curves generated with known numbers of MMC cells.
115
116 *Microstructural characterization*
117 Scanning electron microscopy (SEM) was utilized to investigate the mophology of mPEG-g-
118 chitosan. Gels were frozen in liquid nitrogen and lyophilized for dehydration. The samples
119 were mounted, sputter-coated with platinum, and imaged using a JSM-7000F SEM (JEOL,
120 Tokyo, Japan) operated at 10 kV and 5 nA.
121
122 *Statistical Analysis*
123 The results were presented as mean values ± standard deviation (mean ± s.d.). The
124 statistical difference was determined by one-way analysis of variance (ANOVA) and unpaired,
125 two-tailed Student's t-test. Values were considered to be statistically significant at $p < 0.05$
126 (*).
127

128 **Supplementary Results and Discussion**

129 *Chemical structure analysis*

130 mPEG-acid was prepared via the ring opening reaction of succinic anhydride with mPEG.
131 The characteristic assignments of mPEG-acid were: δ = 4.2 (H-4), 3.5–3.7 (H-2, H-3), 3.4 (H-
132 1), 2.6 ppm (H-5).[7] No remaining unreacted succinic anhydride (δ = 3 ppm) was found in
133 the mPEG-acid.

134

135 *Controlled release study*

136 mPEG-g-chitosan gel was investigated for controlled release of a model protein. **Figure S3a**
137 shows the cumulative release of bovine serum albumin (BSA) from 1.5 wt% mPEG-g-
138 chitosan prepared in 1× PBS. The hydrogels were loaded with BSA of different
139 concentrations ranging from 0, 100, 500, 800, to 1000 μg/mL, and the BSA release was
140 quantified at 1, 5, 30 and 93 hours. mPEG-g-chitosan gel loaded with 100 μg/mL or 500 μg
141 /mL BSA showed minimal burst release within the first hour and a controlled release profile
142 over the course of 90 hours. When high concentrations of BSA were loaded into mPEG-g-
143 chitosan gels (800 and 1000 μg/mL), more than 60% of the BSA was released during the first
144 30 hours. This might result from disturbance of optimal hydrogel chain packing by the
145 presence of too much protein within the hydrogel network. The weight loss of mPEG-g-
146 chitosan gel is around 10% at the end of this controlled release study (data not shown).

147

148 *Biocomaptibility*

149 Cell viability is the most fundamental feature of biocompatibility for biomedical materials.
150 The viability of murine mammary carcinoma (MMC) cells cultured on mPEG-g-chitosan gel
151 was assessed with the Alamar blue assay. Tissue culture polystyrene (TCPS) and Matrigel
152 were utilized as control materials. **Figure S3b** shows the number of MMC cells on all three
153 substrates over a culture period of 4 days. Cell proliferation was observed on all three

154 substrates. The initial attachment of MMC cells on TCPS was higher than on Matrigel and on
155 mPEG-g-chitosan gel given the same seeding density. However, the number of MMC cells
156 incresed more than twice on Matrigel and mPEG-g-chitosan gel than on TCPS. The difference
157 in initial cell attachment and subsequent proliferation might result from topological
158 differences between 3D (Matrigel and mPEG-g-chitosan) and 2D (TCP) culture substrates.
159 Though MMC cells showed higher initial attachment on TCPS, rigid, two-dimensional
160 substrates have been reported as poorly representive of biological environments.[8,9] Cells
161 cultured on 2D are forced to adhere to a rigid surface and are geometrically constrained. The
162 adopted flat morphology alters cytoskeletal regulation that is important to celluar signaling
163 thus affecting proliferation, migration and apotosis.[10] Therefore the cell attachment and
164 proliferation on TCPS may not accurately reflect *in vivo* cellular activity. On the other hand,
165 3D matrices have been reported as more biomimetic.[9,10] Overall, the cell number was
166 significantly higher on mPEG-g-chitosan gel than on Matrigel, which is a more comparable
167 and biologically-relevant culture substrate than TCPS. This result confirms that mPEG-g-
168 chitosan gel is biocompatible and a suitable material for culture of MMC cells.
169
170 Microstructure
171 The microstructure of mPEG-g-chitosan is visible via scanning electron microscopy as shown
172 in **Figure S3c**. The hydrogel possesses interconnected pores on the order of 1–10 μm in
173 diameter. A uniform microstructure was observed throughout a hydrogel sample.
174

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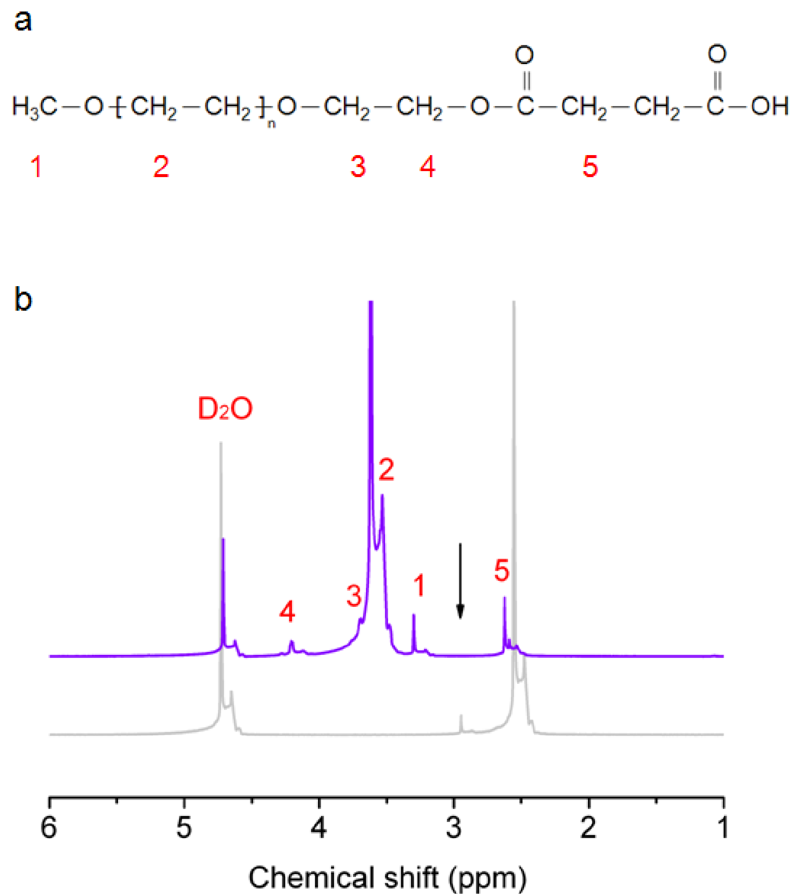

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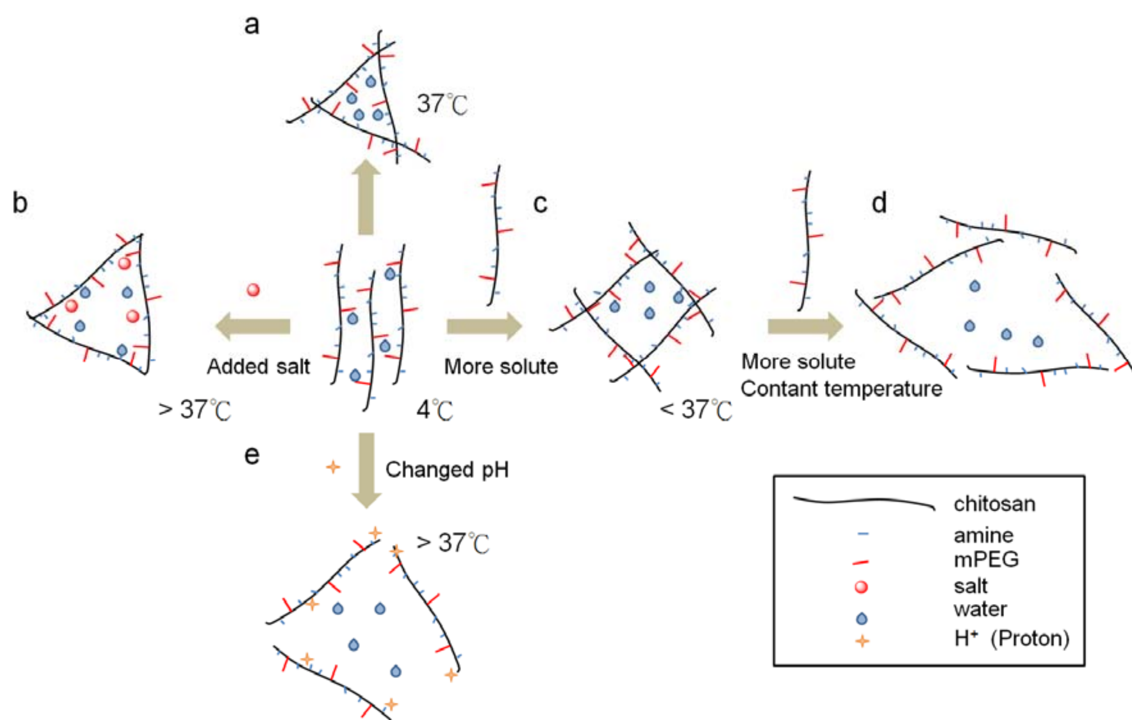
177 **Figure S1.** Chemical structure analysis: (a) Chemical structure of mPEG-acid, (b) ^{1}H NMR
178 analysis of mPEG-acid (purple) and succinic anhydride (gray).
179



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181 **Figure S2.** Schematic illustration of mechanisms for sol–gel transition of mPEG-g-chitosan
182 in response to multiple stimuli: temperature, salt concentration, pH and solute concentration.
183 (**a**) Rearrangement of mPEG-g-chitosan chains occurs at elevated temperatures allowing for
184 the dominance of hydrophobic interactions and resulting in gel network formation. (**b**) The
185 addition of salt interrupts gel network formation causing an increase in sol-gel transition
186 temperature. (**c**) Gel network formation occurs more easily with an increase in mPEG-g-
187 chitosan concentration, causing a decrease in temperature associated with sol-gel transition,
188 but (**d**) further increase in mPEG-g-chitosan concentration disrupts network formation by
189 disrupting optimal polymer packing required for gelation. (**e**) The protonation of the chitosan
190 amines prevents mPEG-g-chitosan packing and thereby gel formation due to increased
191 electrostatic repulsions among polymer chains.
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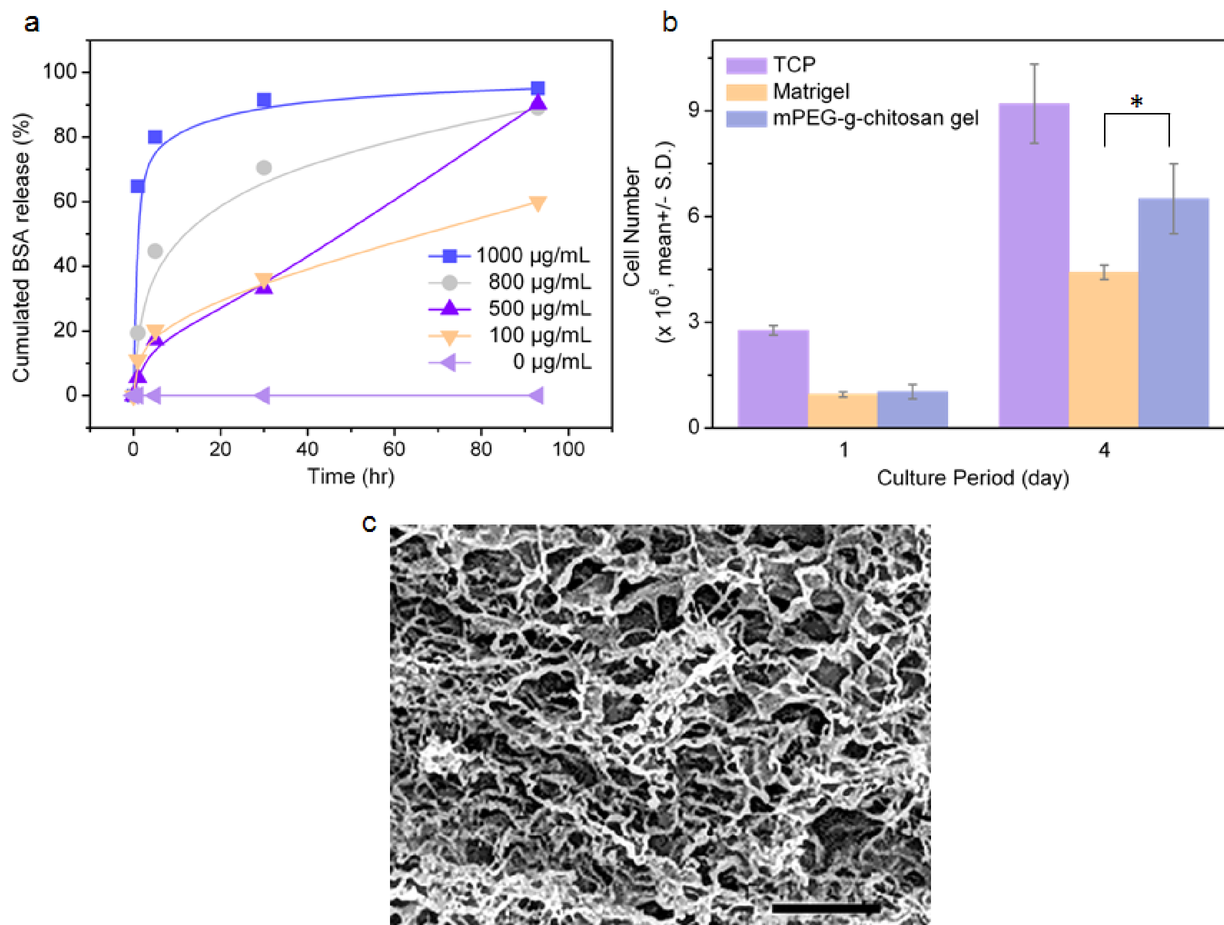

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197 **Figure S3.** Model protein release profile, biocompatibility and microstructure of mPEG-g-
198 chitosan: (**a**) *in vitro* cumulative percent release of bovine serum albumin (BSA) from mPEG-
199 g-chitosan. Solutions of 1.5 wt% mPEG-g-chitosan in 1× PBS were loaded with different
200 BSA concentrations (0, 100, 500, 800, and 1000 μg/mL) and incubated at 37° to induce
201 gelation. (**b**) the growth of murine mammary carcinoma (MMC) cells on TCPS (tissue culture
202 polystyrene), Matrigel, and mPEG-g-chitosan gel over a 4 day culture period as determined
203 by Alamar blue assay. Stastistic analysis was conducted between Matrigel and mPEG-g-
204 chitosan gel. Results are presented as mean ± s.d., and * indicates statistical significance as
205 determined by unpaired, two-tailed Student's t-test, $p < 0.05$, $n = 3$. (**c**) the SEM image of the
206 porous strucutre of mPEG-g-chitosan. The scale bar is 10 μm.
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