Uniform Trehalose Nanogels Improve Long Term Glucagon Stability and Solubility

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Supplementary Information

Contents:

Materials

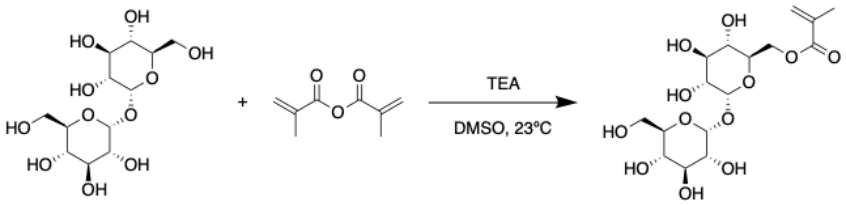
Trehalose was purchased from The Healthy Essential Management Corporation (Houston, TX) and was azeotropically dried with ethanol and kept under vacuum until use. Unless otherwise noted, all materials were of analytical grade and purchased and used as received from Fisher Scientific, Acros Organics, Oakwood Chemicals and Sigma Aldrich. Anhydrous compounds were dried over molecular sieves. Azobis(isobutyronitrile) (AIBN) was recrystallized from acetone before use. DMSO was dried using molecular sieves and kept under an inert atmosphere. Spectra/Por3 regenerated cellulose membrane (MWCO 1.0 or 3.5 kDa) was purchased from Spectrum Chemical (New Brunswick, NJ) for polymer dialysis. Thiolated glucagon (GCG-SH, sequence: HSQGTFTSDYSKYLDSRRAQDFVCWLMNT) and native GCG (sequence: HSQGTFTSDYSKYLDSRRAQDFVQWLMNT) were purchased from Biomatik at >90% purity. MilliporeSigma™ Millex™ hydrophilic PTFE filters were used for all glucagon nanogel experiments. Sheep red blood cells (RBCs) were purchased from Innovative Research (lot # 39841). Dulbecco's Phosphate buffer saline (DPBS) (pH 7.4), Dulbecco's Modified Eagle's Medium (DMEM) cell culture media, and Pen Strep (Penicillin and Streptomycin) were procured from Gibco, ThermoFisher Scientific. Fetal bovine serum (FBS) was procured from Sigma-Aldrich. Trypsin was purchased from MP Biomedicals. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was procured from RPI (Research Product International Corp.). DMSO was procured from VWR chemicals. Calcein AM and ethidium homodimer-1 were purchased from Invitrogen**.** Lactate-Glo™ assay was purchased from Promega.

Analytical techniques

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker AV 400 MHz spectrometer and the data was analyzed using MestRenova v12 software. Deuterated solvents obtained from Cambridge Isotope Laboratories were used for all NMR spectroscopic analyses. A Biotage Isolera Prime equipped with KP-SNAP Ultra Biotage columns was used for all flash column chromatography. Analytical reverse-phase high performance liquid chromatography (HPLC) was carried out on an Agilent 1260 Infinity II HPLC system equipped with an autosampler and a UV detector using a Zorbax 300SB-C18 (analytical: 3.5 μm, 3.0 × 150 mm) with monitoring at λ = 220 nm and with a flow rate of 0.8 mL/min, using a gradient 30-45% of solvent B (A: H_2O + 0.1% TFA; B: CAN + 0.1% TFA) over 18 minutes. Preparatory HPLC purification was performed on a Shimadzu HPLC system equipped with a UV detector using a Luna 5 μm C18 100 Å column (preparatory: 5 μm, 250 × 21.2 mm) with monitoring at λ = 215 and 254 nm. Gradient solvent system (H_2O:MeOH = 90:10 to 40:60 over 20 min) was used as the mobile phase at a flow rate of 20 mL/min. Gel permeation chromatography (GPC) using a size exclusion chromatography (SEC) measurements were performed in DMF on an Infinity 1260 II HPLC system from Agilent equipped with a multiangle light scattering detector MALS and a diode array detector DAD and differential refractive index detector dRI from Wyatt technology. Polymers were separated on two Plgel Mixed-D gel columns PL1110–6504 (300 × 7.5 mm) at a flow rate of 0.6 mL min^{-1}. Column temperatures were held at 40 °C in DMF with LiBr (0.1 M). Molar masses were calculated from the dn/dc. Absorbance and fluorescence readings were measured using the SpectraMax iD3Multi-Mode Microplate Reader from Molecular Devices.Electrospray ionization (ESI) mass spectra were obtained using an Agilent 6530 QTOF-ESI in tandem with a 1260 Infinity LC. Dynamic light scattering (DLS) measurements were carried out using a Malvern Zetasizer Nano at polymer concentrations of 1 mg/mL. Transmission electron microscopy (TEM) images were acquired on a FEI T12 instrument using formvar/carbon coated grids (200 mesh, Cu, Ted Pella). Grids were glow discharged for 15 seconds. 5 μL of sample (1

mg/mL) were placed on the grid and allowed to adhere for 5 minutes. After, the grids were stained with 3 µL of 2% uranyl acetate for 3 minutes. Infrared absorption spectra were recorded using a PerkinElmer FT-IR equipped with an ATR accessory. For SDS-PAGE gels, samples were loaded using 2X Laemmli sample buffer and run on Mini-Protean TGX, Any kD gels (Bio-Rad) at 195V for 35 minutes using Tris/Glycine/SDS buffer (Bio-Rad). Gels were stained with Coomassie. SDS-PAGE protein standards were obtained from Bio-Rad (Precision Plus Protein Prestained Standards). The fluorescence images were taken using Zeiss fluorescence Microscopy. Viscosity was measured using a RheoSense microVisc.

Synthesis of TrMA monomer.



Synthesis of trehalose methacrylate monomer. [1]

Anhydrous trehalose (5.02 g, 14.6 mmol, 5 eq) was dissolved in dry DMSO (60 mL) in a dry round bottom flask (250 mL) equipped with a stir bar. The solution was stirred under argon until most of the trehalose dissolved. Under argon, dry triethylamine (6.1 mL, 43.8 mmol, 15 eq) was added followed by methacrylic anhydride (0.43 mL, 2.92 mmol, 1 eq). The solution was left to stir at room temperature for 72 hours. The solution was added dropwise to ice-cold 8:2 hexanes: dichloromethane (2000 mL) with gradual stirring over 10 minutes. A sticky yellow oil was left at the bottom of the flask. The organic layer was poured off to leave the oil and some remaining solvent. The solution was transferred to a round bottom flask (1000 mL) to remove any remaining organic solvent *via* a rotary evaporator. The sticky solid was re-dissolved in deionized water. The material was purified via HPLC (C18 column, 10-60% MeOH). The pure fractions were collected and transferred to a round bottom flask containing MEHQ solution (10 µL, 4 mg/mL in water) to prevent auto-polymerization. The methanol was removed by blowing air into the round bottom for 18 hours. The material was rinsed with distilled water and transferred to a tared falcon tube and lyophilized to yield a white fluffy solid (Yield: 38 %). ^{1}H NMR (400 MHz, D_2O) δ 6.15 (p, *J* = 1.0 Hz, 1H), 5.74 (p, *J* = 1.5 Hz, 1H), 5.16 (dd, *J* = 13.5, 3.9 Hz, 2H), 4.49 (dd, *J* = 12.3, 2.2 Hz, 1H), 4.36 (dd, *J* = 12.3, 5.2 Hz, 1H), 4.07 (ddd, *J* = 10.1, 5.1, 2.1 Hz, 1H), 3.88 – 3.40 (m, 11H), 1.93 (dd, *J* = 1.6, 1.0 Hz, 3H). Spectrum matched published one.

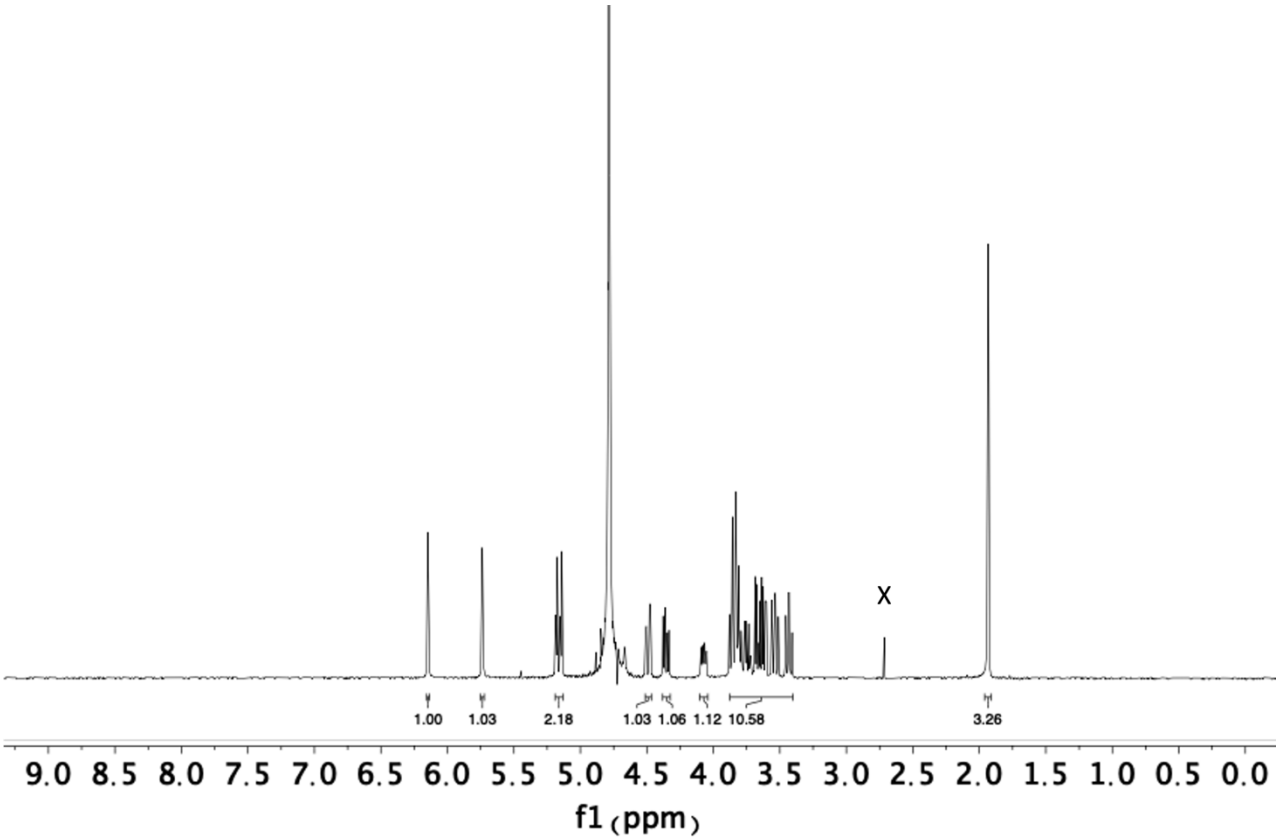
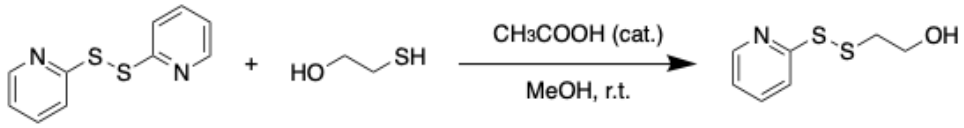


Figure S1. ^{1}H NMR spectrum of TrMA (400 MHz in D_2O).

Synthesis of PDSMA monomer.



Synthesis of pyridyl disulfide ethyl alcohol. [2]

2-2 dipyridyl disulfide, (5.052 g, 22.9 mmol) was dissolved in 25 mL of methanol. Glacial acetic acid (330 µL) was added to the mixture. 2-mercaptoethanol (800 µL, 11.4 mmol) was added to the mixture drop-wise at room temperature making a yellow solution. The reaction was stirred at 4 hours at room temperature. The solution was concentrated down to a yellow oil by removing the methanol. The oil was redissolved in dichloromethane and washed with sodium bicarbonate (30 mL), distilled water (30 mL), and brine (30 mL). The organic layer was dried over magnesium sulfate. The material was purified by flash chromatography using a 25% ethyl acetate: hexanes isocratic method to remove the 2,2-dipyridyl disulfide peak first and then increased to a gradient of 25%-40% ethyl acetate: hexanes to elute the product peak. The pure fractions were collected and rotovapped to remove the solvent. The product was concentrated to a clear oil (Yield: 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (ddd, J = 6.0, 2.0, 1.1 Hz, 1H), 7.43 (dddd, J = 8.0, 7.4, 1.8, 0.7 Hz, 1H), 7.25 (dt, J = 8.1, 1.0 Hz, 1H), 7.00 (ddt, J = 7.2, 5.0, 1.1 Hz, 1H), 3.68 – 3.61 (m, 2H), 2.82 – 2.76 (m, 2H). FT-IR (film) λ= 3307 cm^{-1}, 2853 cm^{-1}, 2842cm-1, 1573 cm^{-1}, 1406 cm^{-1}, 1042 cm^{-1}. NMR spectrum matched published one.

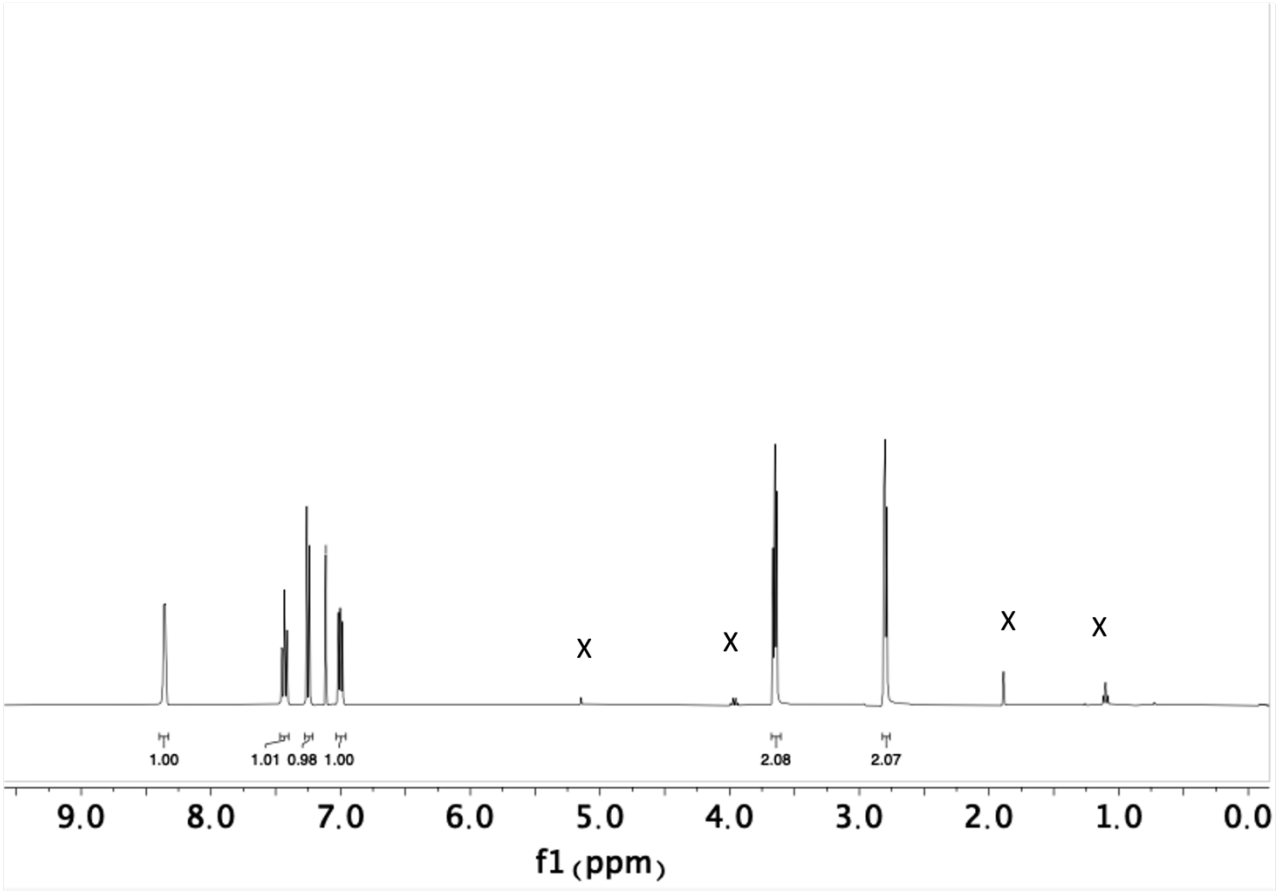


Figure S2. ^{1}H NMR spectrum of PDSOH (400 MHz in $CDCl_3$).

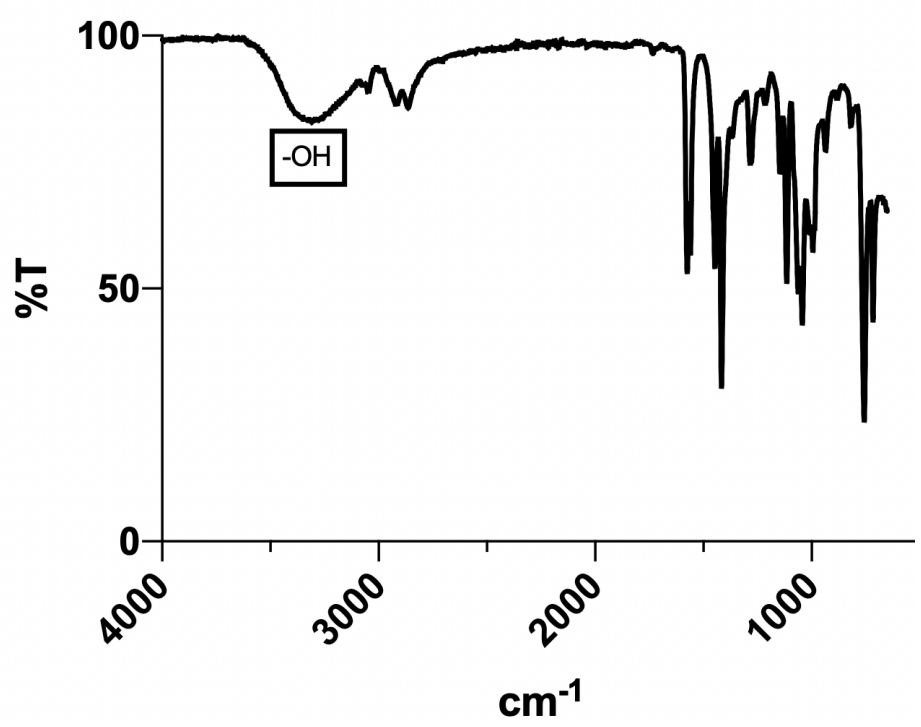


Figure S3. FT-IR spectrum of PDSOH.

Synthesis of pyridyl disulfide ethyl methacrylate monomer.[2]

Pyridyl disulfide ethyl alcohol (500 mg) was dissolved dry dichloromethane (2 mL) in a dry round bottom flask with a stir bar. Dry triethylamine (483 μL) was added and the mixture was stirred in an ice-bath under argon. Methacryloyl chloride (260 μL) was diluted in dry dichloromethane (800 μL) then added drop-wise with continuous stirring to the reaction flask. The reaction mixture was then stirred at room temperature for 6 hours. The stirring was stopped, and the reaction mixture was washed with sodium bicarbonate (3x30 mL), distilled water (3x30 mL), and brine (30 mL). The organic layer was collected and dried over anhydrous magnesium sulfate. The product was purified via flash column chromatography using a 20% isocratic method. The product eluted first and was concentrated to yield a clear oil (Yield: 56 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (ddd, J = 4.9, 1.8, 0.9 Hz, 1H), 7.70 – 7.59 (m, 2H), 7.09 (ddd, J = 7.3, 4.9, 1.2 Hz, 1H), 6.12 (dq, J = 2.0, 1.0 Hz, 1H), 5.58 (p, J = 1.6 Hz, 1H), 4.39 (t, J = 6.4 Hz, 2H), 3.09 (t, J = 6.4 Hz, 2H), 1.93 (dd, J = 1.6, 1.0 Hz, 3H). Spectrum matched literature.

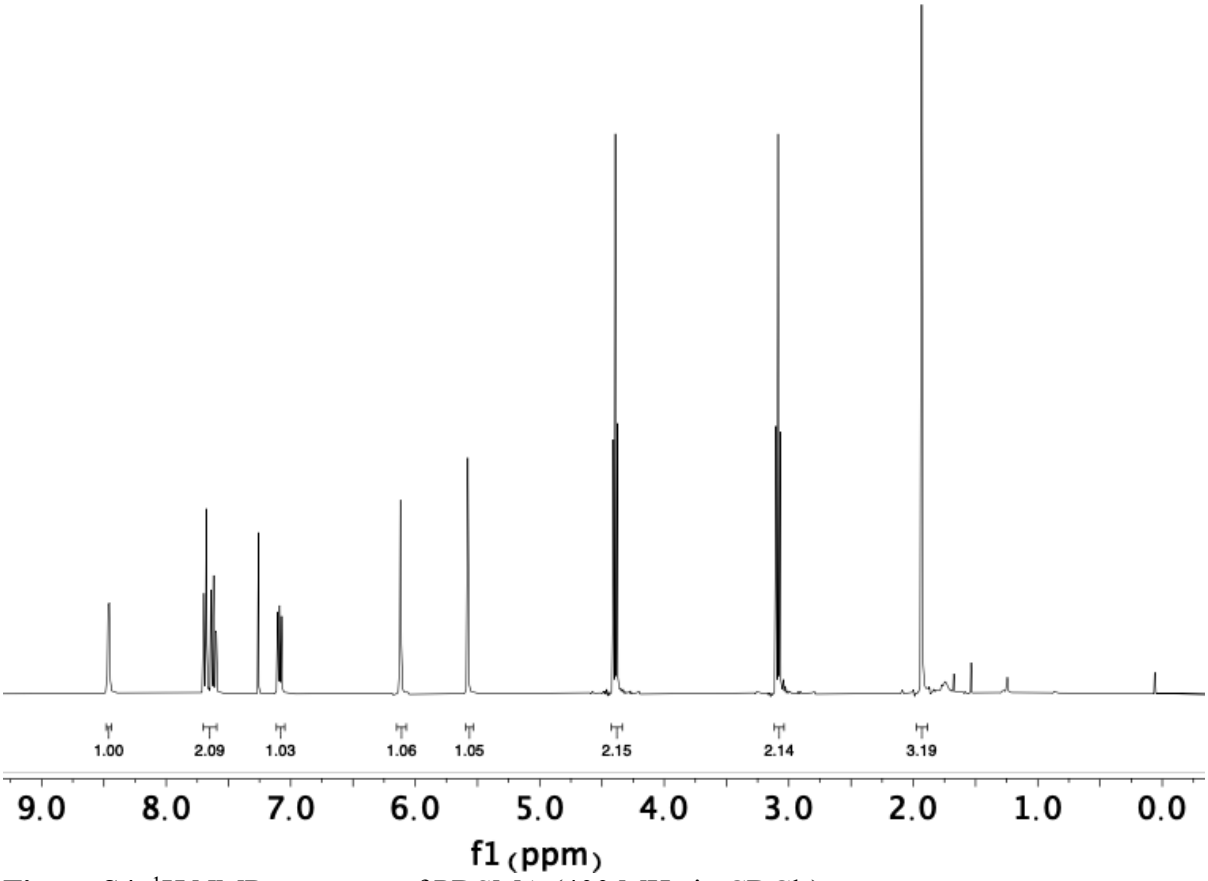
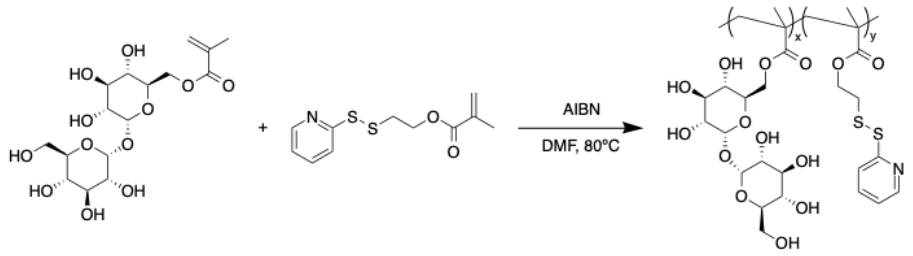


Figure S4. ^{1}H NMR spectrum of PDSMA (400 MHz in $CDCl_3$).

Synthesis of poly(PDSMA-co-TrMA).



Free radical polymerization of PDSMA and TrMA.

For a 1:1 PDSMA:TrMA feed ratio, TrMA (50 mg, 122 μmol), PDSMA (31.1 mg, 122 μmol), and azobisisobutyronitrile (AIBN) (1 mg, 6 μmol) were dissolved in dry dimethylformamide (0.80 mL) to give a [TrMA]:[PDSMA]:[initiator] ratio of 20:20:1. The solutions were degassed by freeze–pump–thawing five times. The polymerization reaction vessel was then put under argon to stir for 5 hours at 80 ºC in an oil bath. The polymerization stopped at 5 hours by exposing to air. The polymerization was purified by precipitating in ethyl acetate and dialyzing for 3 days against deionized water using 1 kDa MWCO tubing. The polymer was lyophilized for 2 days to yield a white fluffy solid (Yield: 53%). ^{1}H NMR (400 MHz, DMSO) δ 8.46 (s, 1H), 7.79 (s, 2H), 7.24 (s, 1H), 4.85 (d, J = 41.0 Hz, 10H), 1.81 (s, 4H), 0.87 (d, J = 69.0 Hz, 8H).

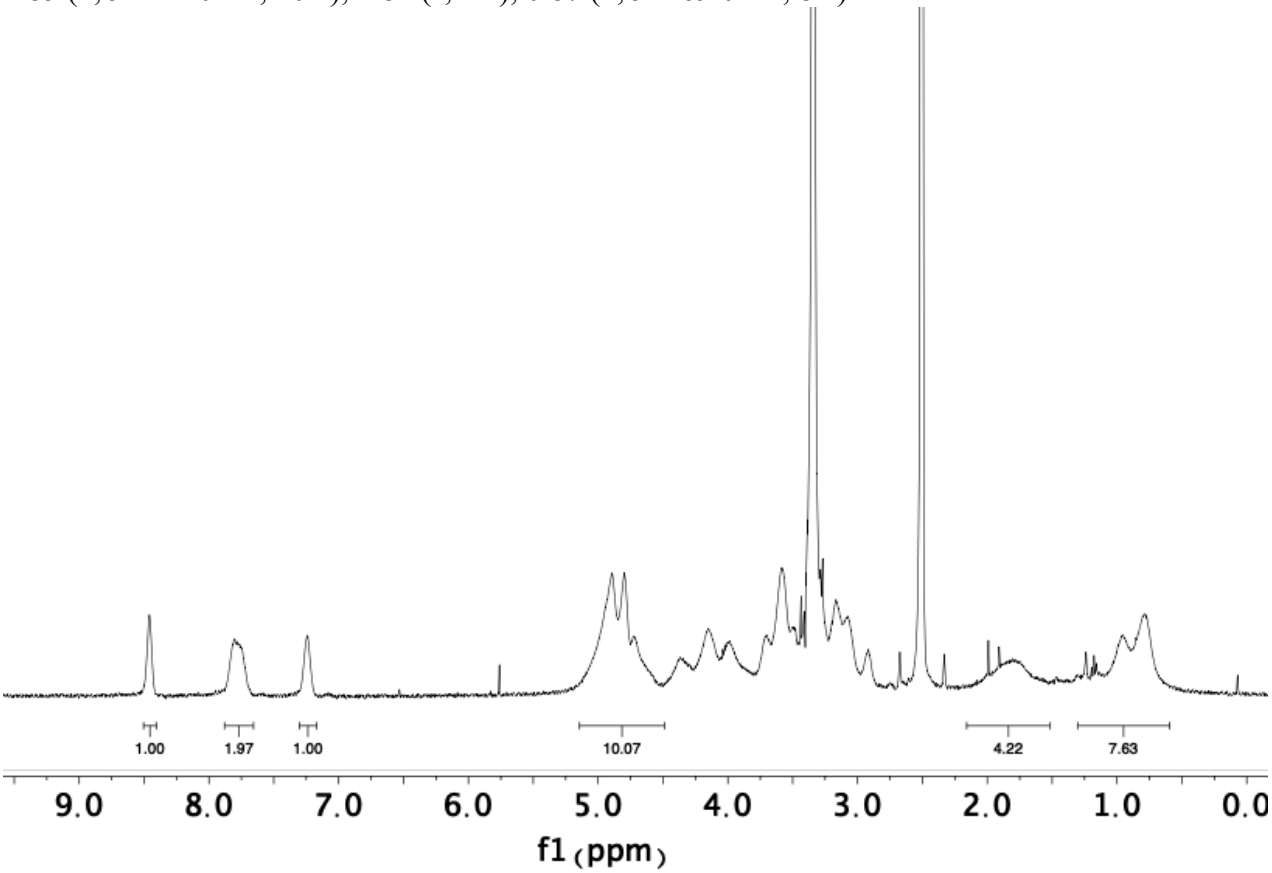


Figure S5. ^{1}H NMR spectrum of poly(PDSMA-*co*-TrMA (400 MHz in DMSO-d_6).

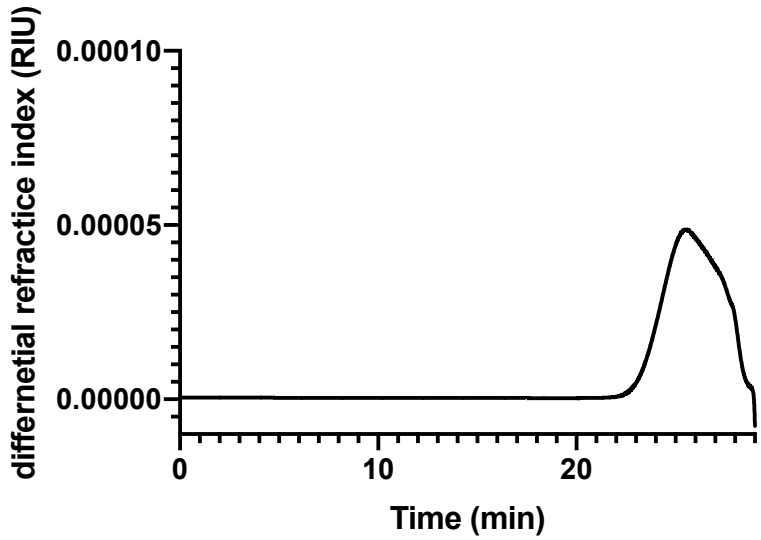


Figure S6. GPC of poly(PDSMA-*co*-TrMA) in DMF (*M*n = 9820; Đ = 1.9).

Hour	% Consumption
0	0
0.2	52
1	65
2	73
3	82

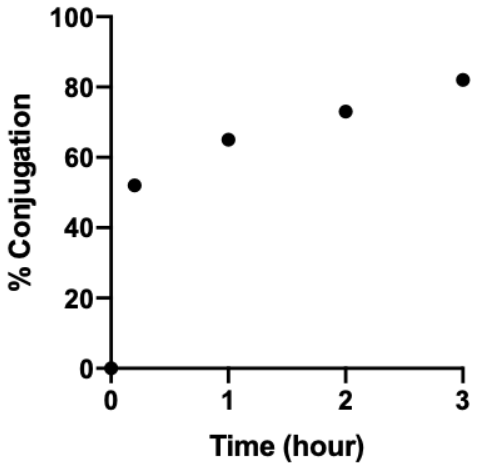


Figure S7. Glucagon-SH conjugation kinetics to poly(PDSMA-co-TrMA) was monitored via HPLC over 3 hours.

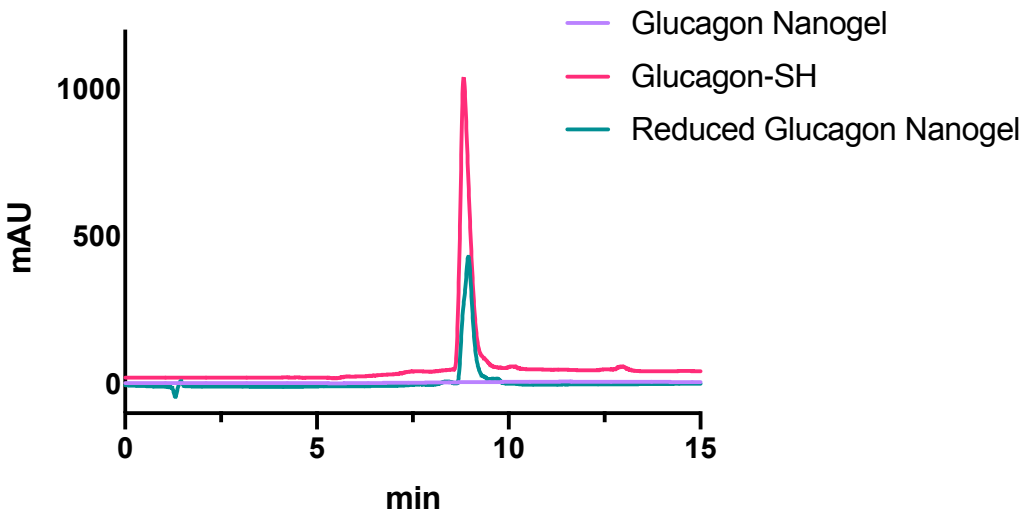


Figure S8. Representative HPLC traces of glucagon-SH conjugation and release from the glucagon nanogel.

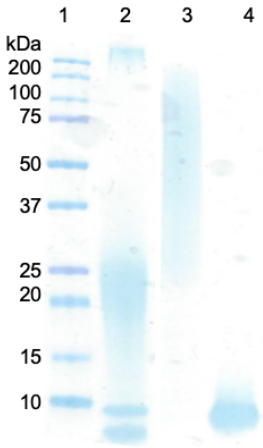


Figure S9. Representative SDS-PAGE of glucagon-SH conjugation and release from the glucagon nanogel. Lane 1: protein ladder; lane 2: crude glucagon nanogel; lane 3: purified glucagon nanogel; lane 4: glucagon nanogel from lane 3 reduced with TCEP (100 eq).

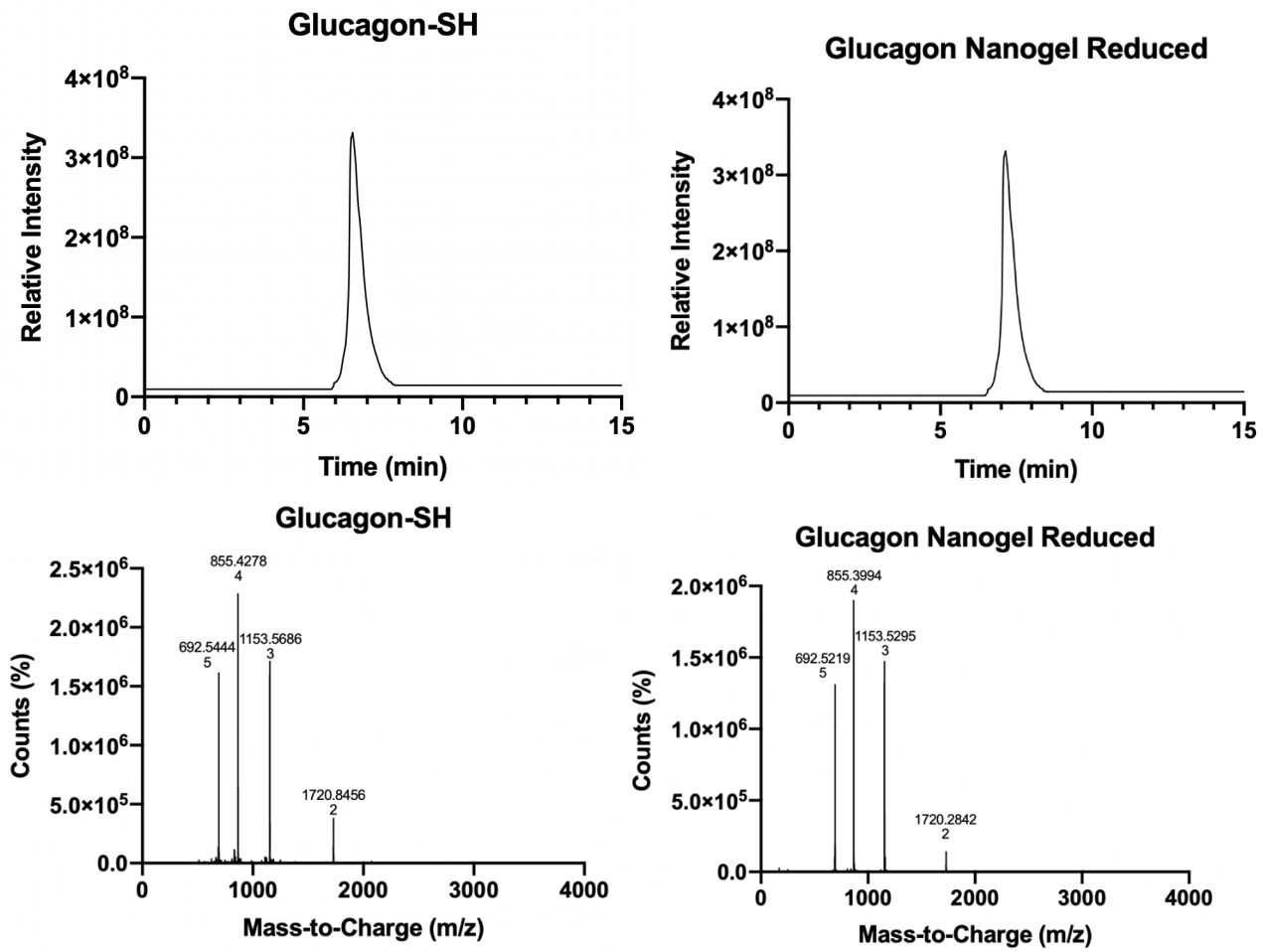


Figure S10. Representative LC-MS trace of glucagon-SH standard compared to reduced glucagon nanogels show no degradation in fresh glucagon-SH or immediately after reduction. Calculated m/z= 3460, observed m/z=3460, retention time= 6.3 minutes.

Crosslinking %	Empty Nanogel Z-average (d.nm)	Glucagon nanogel Z-average (d.nm)	Glucagon nanogel Z-average (d.nm), 24 hours 4ºC	DLS results
10	7.2	1408	1374	poor
20	7.5	196.0	2981	poor
30	7.6	1333	2326	poor
40	7.8	649.2	973.4	**good**
50	8.6	643.3	660.9	**good**

Figure S11. Varying percentages of PEG_{2000} dithiol varied the glucagon nanogel size by DLS.

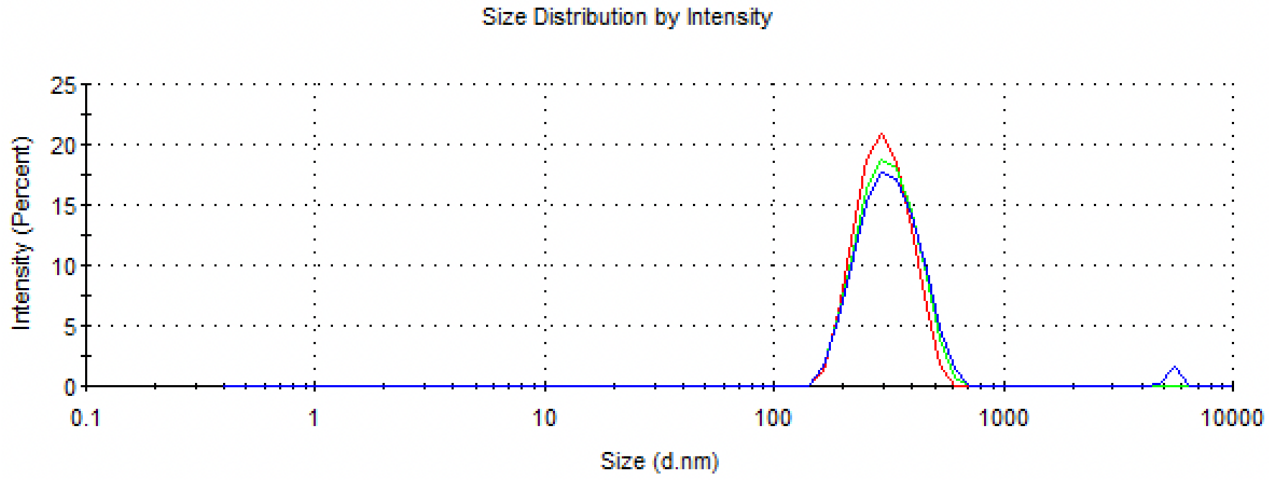


Figure S12. DLS of simultaneous crosslinking of PEG_{2000} dithiol and glucagon-SH PDI: 0.427 Z-average (d.nm): 360.0. with microdialysis purification.

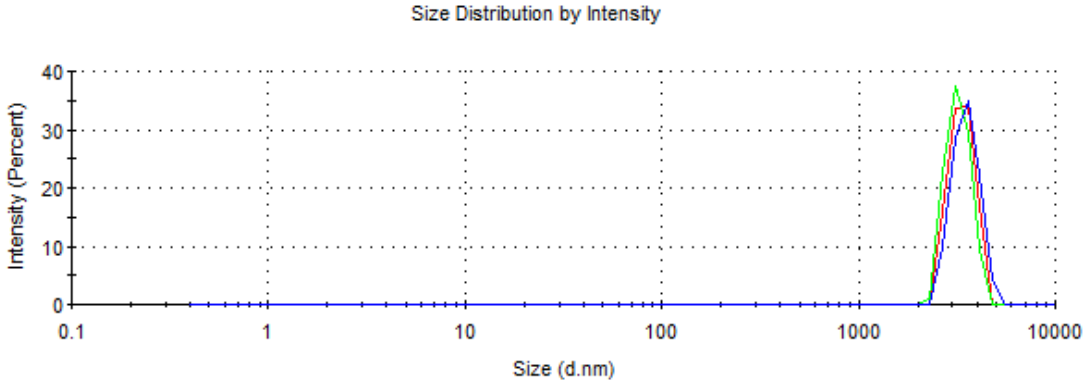


Figure S13. DLS of covalent attachment of glucagon-SH followed by crosslinking of PEG_{600} dithiol in 10 mM HCl, buffer exchanged to DPBS pH 7.4, purified by microdialysis. PDI: 0.02, Z-average (d.nm): 3189.

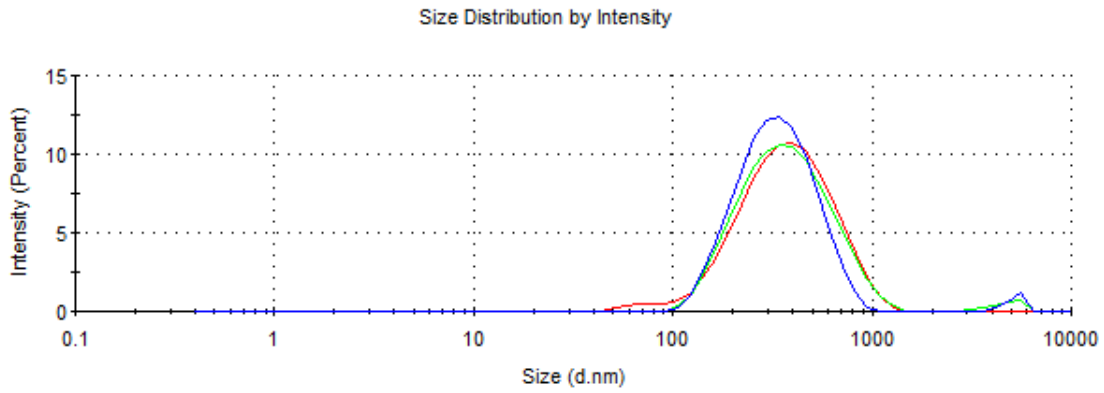


Figure S14. DLS of covalent attachment of glucagon-SH followed by crosslinking of PEG_{1000} dithiol in 10 mM HCl, buffer exchanged to DPBS pH 7.4 and purified by microdialysis. PDI: 0.23, Z-average (d.nm): 312.4.

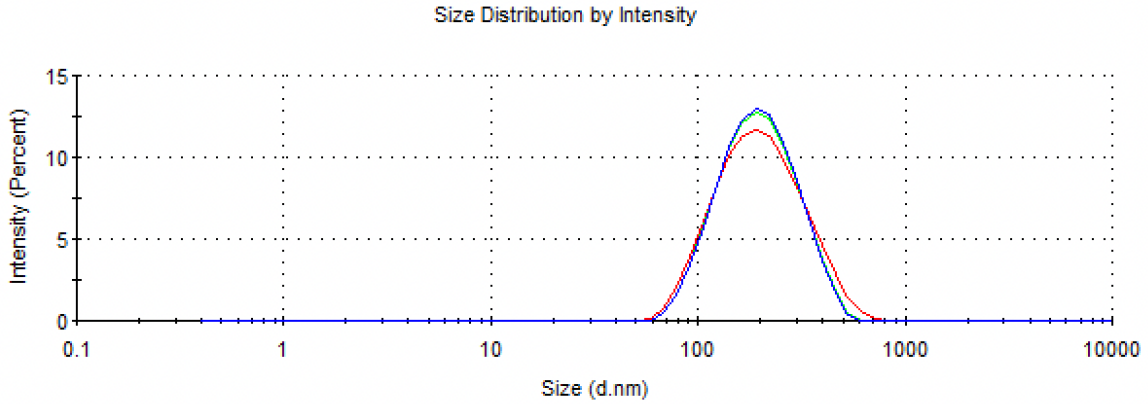


Figure S15. DLS of covalent attachment of glucagon-SH followed by crosslinking of PEG_{2000} dithiol in 10 mM HCl, buffer exchanged to DPBS pH 7.4 and purified by microdialysis. PDI: 0.18, Z-average (d.nm) 149.1.

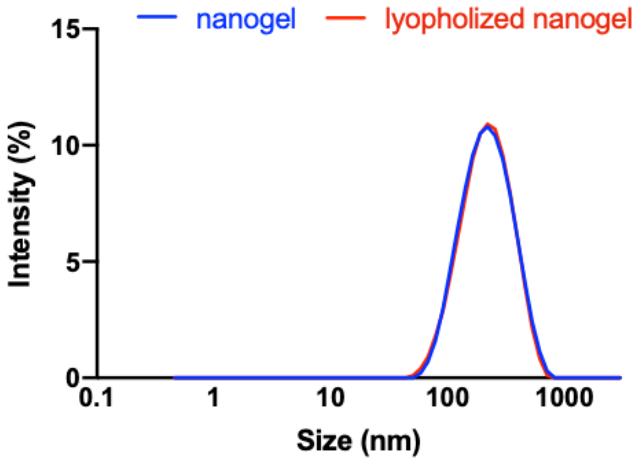


Figure S16. DLS of glucagon nanogels 1 mg/mL lyophilized versus in solution show comparable sizes of 149.1 nm (nanogel) and 155.2 nm (lyophilized nanogel).

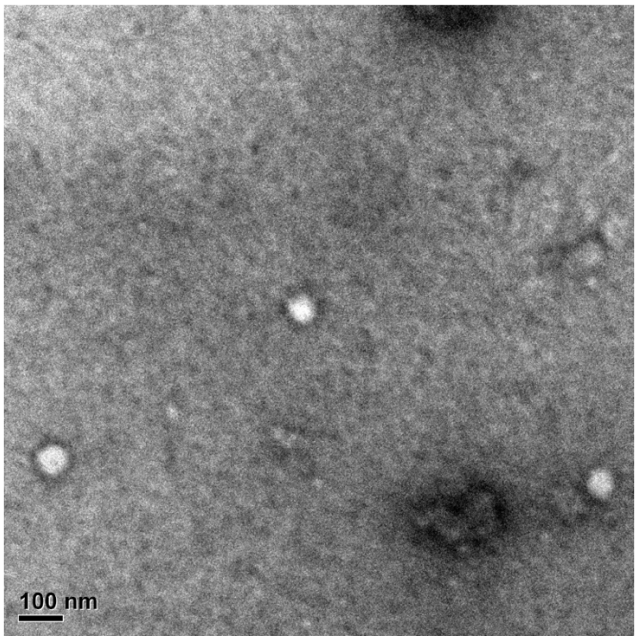


Figure S17. Transmission electron microscopy image of glucagon nanogels 1 mg/mL lyophilized.

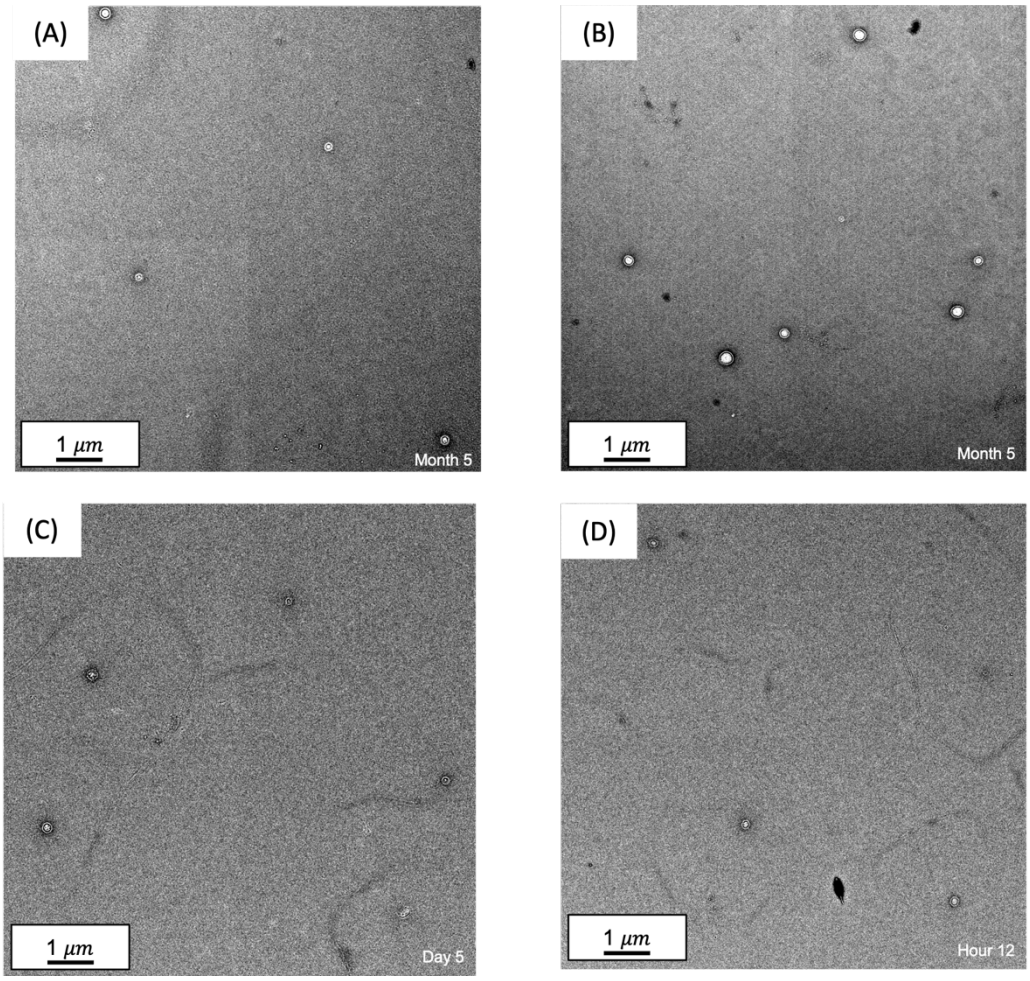


Figure S18. Zoomed out TEM of the glucagon nanogels at A) -20 ºC for 5 months, B) 4 ºC for 5 months, C) 25 ºC for 5 days, and D) 37 ºC for 12 hours.

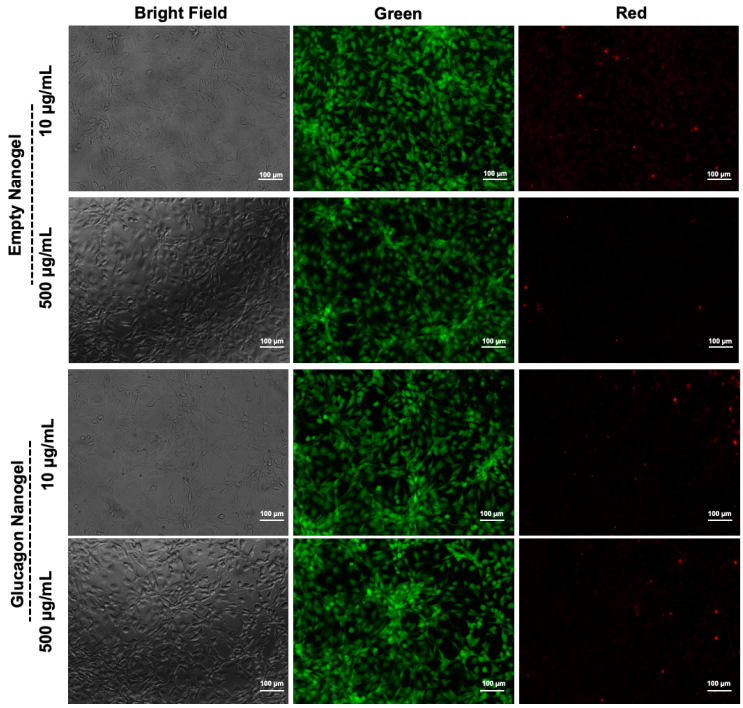


Figure S19. *In vitro* biocompatibility was evaluated after 24 hours in mouse embryonic fibroblasts, NIH3T3 by performing a live/dead assay using calcein AM and ethidium homodimer-1. The empty nanogel and glucagon nanogel are biocompatible at 500 µg/mL and 10 µg/mL.

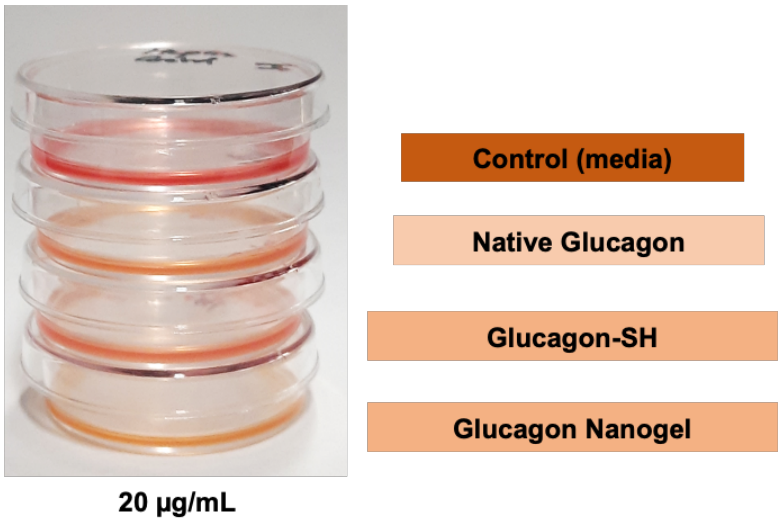
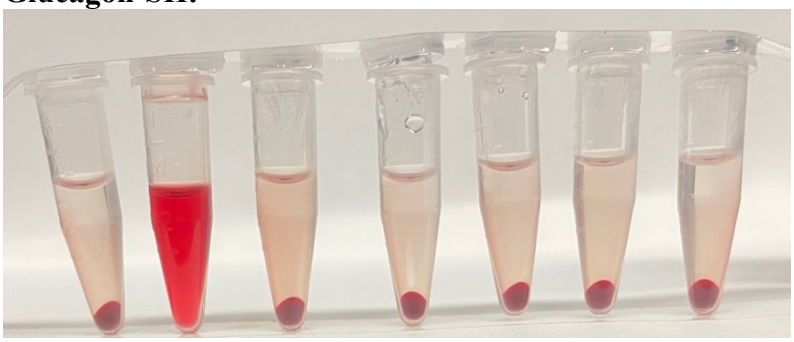
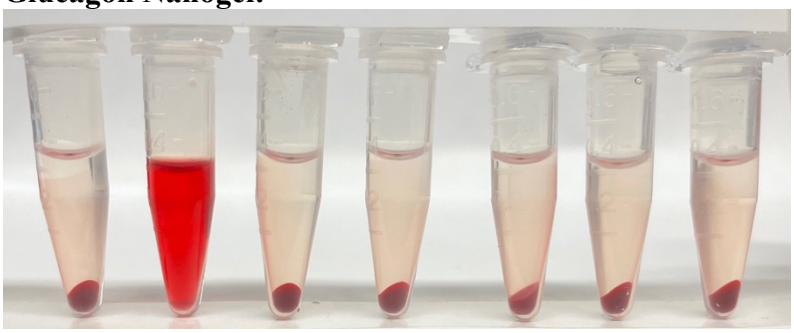


Figure S20. Metabolic activity was qualitatively evaluated after 24 hours in a liver cell model, HepG2 cells by visualizing a color change of golden yellow to phenol red of the control compared to the glucagon containing samples. $0.3x10^6$ cells/well were counted using hemocytometer

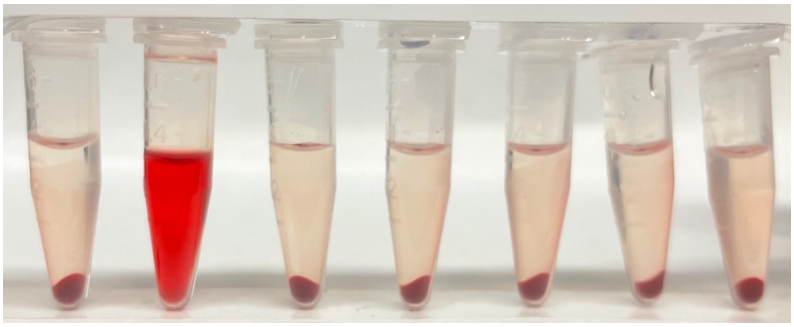
Glucagon-SH:



Glucagon Nanogel:



PEG_{2000} dithiol:



PDSMA-co-TrMA:

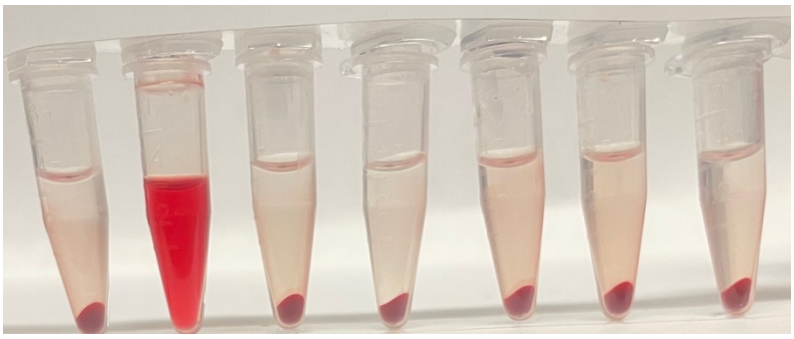


Figure S21. Qualitative hemolysis results left to right: DPBS, 20% Triton-X, 0 μM , 5 μM, 10 μM, 25 μM, 50 μM.

References:

(1) Boehnke, N.; Kammeyer, J. K.; Damoiseaux, R.; Maynard, H. D. Stabilization of Glucagon by Trehalose Glycopolymer Nanogels. *Adv. Funct. Mater.* **2018**, *28* (10), 1705475. https://doi.org/10.1002/adfm.201705475.

(2) Ghosh, S.; Basu, S.; Thayumanavan, S. Simultaneous and Reversible Functionalization of Copolymers for Biological Applications. *Macromolecules* **2006**, *39* (17), 5595–5597. https://doi.org/10.1021/ma061420x.