Supporting Information

Nanogel receptors for high isoelectric point protein detection: Influence of electrostatic and covalent polymer-protein interactions

*Marissa E. Wechsler,[a,b] H. K. H. Jocelyn Dang,[b] Samuel D. Dahlhauser,[c] Susana P. Simmonds,[b] James F. Reuther,[c,d] Jordyn M. Wyse,[b] Abigail N. VandeWalle,[c] Eric V. Anslyn,[*c] and Nicholas A. Peppas[*a,b,e,f,g]*

[a]Institute for Biomaterials, Drug Delivery, and Regenerative Medicine, The University of Texas at Austin, Austin, TX, USA, [b]Department of Biomedical Engineering, The University of Texas at Austin, Austin, TX, USA, [c]Department of Chemistry, The University of Texas at Austin, Austin, TX, USA, [d]Department of Chemistry, University of Massachusetts Lowell, Lowell, MA USA, [e]McKetta Department of Chemical Engineering, The University of Texas at Austin, Austin, TX, USA, [f]Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA, [g]Department of Surgery and Perioperative Care, Dell Medical School, The University of Texas at Austin, Austin, TX, USA

**peppas@che.utexas.edu*

Table of contents:

Materials and methods

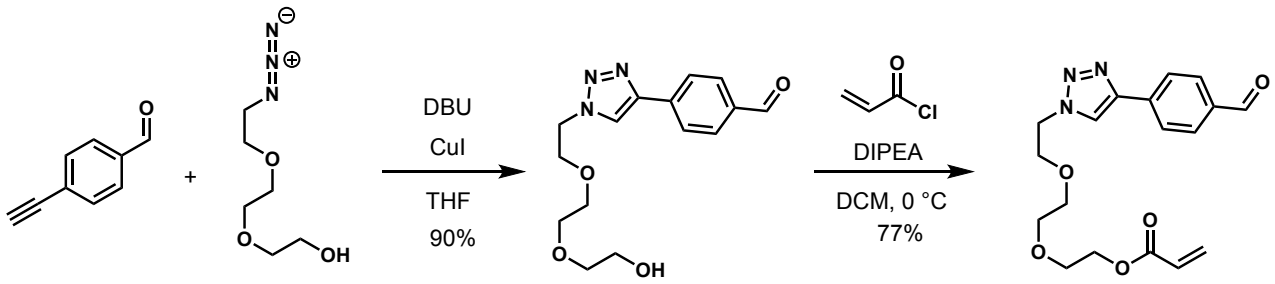
Materials

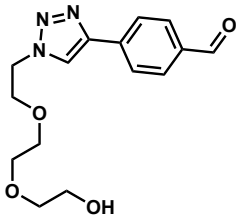
N-isopropylacrylamide, methacrylic acid, N,N'-methylenebisacrylamide, ammonium persulfate, human lysozyme expressed in rice, lactoferrin from human milk, β-lactoglobulin from bovine milk, and albumin from human serum were purchased from Sigma-Aldrich (St. Louis, MO). IgG from human plasma was purchased from Lee Biosolutions (Maryland Heights, MO). 10X phosphate buffered and all standard peptide and protein assays were purchased from Thermo Fisher Scientific (Waltham, MA). Ultrapure water (final resistance = 18.2 MΩ) was obtained from a Barnstead GenPure purification system from Thermo Fisher Scientific. Spectra/Por dialysis tubing was used for dialysis (12-14 kDa MWCO). All reagents were used as received.

Methods

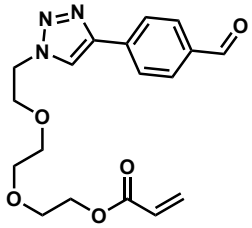
Synthesis of aldehyde-functional oligo(ethylene glycol)acrylate (Al-OEGA)

Scheme S1. Synthesis of aldehyde-functional oligo(ethylene glycol)acrylate (Al-OEGA)





4-(1-(2-(2-(2-hydroxyethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)benzaldehyde: A 500 mL 3-neck flask was charged with a stir bar, flame dried, and backfilled with argon. To the flask was added 4-ethynyl benzaldehyde (3.90 g, 29.96 mmol) followed by THF (120 mL). Next, commercially available azido-PEG_3-OH (5.0 g, 28.54 mmol) and 1,8-Diazabicyclo(5.4.0)undec-7-ene (DBU) (1.71 mL, 11.42 mmol) were added. The mixture was sparged with argon for 15 minutes and then cooled to 0°C in an ice bath. Last, copper iodide (1.35 g, 7.14 mmol) was added. The reaction stirred at 0 °C for 2 hours and was left to warm to room temperature with stirring overnight. The THF was removed under reduced pressure and the crude mixture was then diluted with EtOAc (300 mL). The organic layer was extracted with 1N HCl (3 x 100 mL), 1x with brine, dried over Na_2SO_4, and concentrated under vacuum. Product was purified by silica gel chromatography (EtOAc) and isolated as a colorless oil (7.89 g, 90% yield). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.98 (s, 1H), 8.70 (s, 1H), 8.08 – 8.03 (m, 2H), 7.98 – 7.94 (m, 2H), 4.57 (t, J = 5.7 Hz, 2H), 3.85 (t, J = 5.6 Hz, 2H), 3.55 – 3.50 (m, 2H), 3.49 – 3.45 (m, 2H), 3.43 – 3.40 (m, 2H), 3.37 – 3.33 (m, 2H). ^{13}C NMR (126 MHz, Chloroform-d) δ 191.72, 146.47 136.55, 135.75, 130.42, 126.02, 122.23, 72.42, 70.66, 70.28, 69.43, 61.72, 50.51.MS; ESI+ m/z 306.2 ($M+H^+$)



2-(2-(2-(4-(4-formylphenyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethyl acrylate: A 500 mL 3-neck flask was charged with a stir bar, flame dried, and backfilled with argon. Next, **1** (7.89 g, 25.85 mmol) was added and dissolved in anhydrous DCM (120 mL). The solution was cooled to 0 °C in an ice bath. Next, anhydrous N,N-Diisopropylethylamine (DIPEA) (5.4 mL, 31.0 mmol) was added. Last, acryloyl chloride (2.5 mL, 31.0 mmol) was added dropwise over 10 minutes. The reaction was left to stir for 2.5 hours and monitored by TLC (2:1 H:E). Once complete, the reaction was quenched with water (20 mL) followed by addition of saturated NH_4Cl (100 mL). The reaction was transferred to a

seperatory funnel, diluted with DCM (300 mL) and extracted with sat. NH_4Cl (3 x 100 mL). The organic layer was washed 1x with brine, dried over Na_2SO_4, and concentrated under vacuum. Product was purified by silica gel chromatography (gradient elution, 2:1 H:E then 1:1 H:E then EtOAc) and isolated as a colorless oil (7.14 g, 77% yield). ^{1}H NMR (400 MHz, DMSO-d6) δ 10.00 (s, 1H), 8.72 (s, 1H), 8.08 – 8.04 (m, 2H), 7.99 – 7.95 (m, 2H), 6.27 (dd, J = 17.3, 1.6 Hz, 1H), 6.11 (dd, J = 17.3, 10.3 Hz, 1H), 5.88 (dd, J = 10.3, 1.6 Hz, 1H), 4.58 (t, J = 5.6 Hz, 2H), 4.17 – 4.12 (m, 2H), 3.86 (dd, J = 5.7 Hz, 2H), 3.60 – 3.49 (m, 6H). ^{13}C NMR (126 MHz, DMSO-d6) δ 193.04, 165.93, 145.58, 136.87, 135.77, 132.15, 130.79, 128.54, 125.94, 123.78, 70.03, 70.02, 69.01, 68.64, 63.85, 50.21. MS; ESI+ m/z 382.2 ($M+Na^+$)

Nanogel fabrication

Nanogels were synthesized via free radical precipitation polymerization in water as previously described.[1] Briefly, N-isopropylacrylamide (NIPAM) was polymerized alone or co-polymerized with either a custom, tunable orthogonal reversible covalent aldehyde-functional oligo(ethylene glycol)acrylate (Al-OEGA) monomer (synthesized by the Anslyn group), and/or methacrylic acid (MAA). A final monomer concentration of 16.5 mM was obtained (**Table S1**). The synthesized nanogels were crosslinked using N,N'-methylene bisacrylamide (BIS) at 5 % relative to total monomer.

The monomer solution in water was nitrogen purged while heating in a water bath to 70°C for 30 minutes. The polymerization was then initiated with a freshly prepared solution of ammonium persulfate in water (0.88 mM). The polymerization was carried out at 70°C for 4 hours. The resulting nanogels were dialyzed against ultrapure water for several days with at least 8 water changes, dried via lyophilization, and stored at room temperature until further use. Synthesis of each nanogel formulation was repeated in triplicate.

Nanogel characterization

Lyophilized, crosslinked polymer nanogels were characterized to determine composition, hydrodynamic diameter, surface zeta potential, and particle morphology. Fourier transform infrared spectroscopy (FTIR, Thermo Fisher Scientific) with a germanium crystal was used to confirm nanogel composition. Background spectra were collected prior to each sample and used for background subtraction. Furthermore, the level of MAA incorporation into the nanogels was determined by potentiometric titration. Briefly, nanogels (7.5 mg) were suspended in 5 mM KCl at 0.125 mg/mL. The initial pH was adjusted to 10 using 1N NaOH to deprotonate all carboxylic acid moieties. A Hanna Instruments 902 Potentiometric Titrator was then used to titrate the nanogels, under dynamic dosing, to pH 3 with 0.01N HCl.

Nanogel hydrodynamic diameter and zeta potential was determined using a Zetasizer Nano ZS system (Malvern Instrument, Inc.). Dried nanogels were suspended at 1 mg/mL in 0.1X phosphate buffered saline (PBS, pH 7.4). Measurements were obtained at 25°C.

Nanogel morphology was observed by transmission electron microscopy (TEM). Lyophilized nanogels were suspended in ultrapure water at 1 mg/mL and probe sonicated. Nanogels (5 μL) were dropped onto ionized carbon coated 400 mesh copper grids and were stained with 2% uranyl acetate (pH 4) in water. TEM micrographs were obtained using a FEI Tecnai TEM operating at 80 kV. Dry nanogel diameter values were obtained from TEM images and determined using ImageJ software.

Peptide binding studies to determine aldehyde functionality

Crosslinked nanogels were suspended at 0.5 mg/mL in 0.1X PBS. A custom hydrazide containing peptide was prepared at 1 mg/mL in 0.1X PBS and combined with the nanogels in a 1:1 volume ratio, specifically, 0.125 mL of each. The nanogel and peptide mixtures were allowed to incubate at room temperature while mixing end-over-end for 24 hours. Controls were nanogels combined with 0.1X PBS, in the absence of peptide. After 24 hours, the nanogels and any bound peptide were centrifuged at 21,800 x g for 15 minutes. The supernatant containing any unbound protein was analyzed via a Pierce Quantitative Fluorometric Peptide Assay (ThermoFisher) following the manufacturer's instructions. The adsorption capacity (Q) was calculated using **Equation 1**, where C_0 and C_e are the initial and equilibrium protein concentrations,

respectively, V is the solution volume (0.3 mL), and m is the mass of the nanogels (0.075 mg). Adsorption assays were performed with three independent batches of each nanogel formulation and repeated in duplicate.

$$Q = \frac{(C_0 - C_e)V}{m} \quad \textbf{Equation 1}$$

Protein binding studies to determine nanogel use as protein receptors

All nanogel formulations were suspended at 0.5 mg/mL in 0.1X PBS. Protein solutions were prepared at a range of concentrations (0.25-1 mg/mL) in 0.1X PBS and combined with the nanogels in a 1:1 volume ratio, specifically, 0.150 mL of each. The nanogel and protein mixtures were allowed to incubate at room temperature with gentle agitation for 1 hour. Controls were nanogels mixed with 0.1X PBS alone, in the absence of protein. After 1 hour, the nanogels and any bound protein were centrifuged at 21,800 x g for 15 minutes. The supernatant containing any unbound protein was analyzed via a Pierce BCA Protein Assay Kit (ThermoFisher) following the manufacturer's instructions. The adsorption capacity (Q) was calculated using **Equation 1**. Adsorption assays were performed with three independent batches of each nanogel formulation and repeated in duplicate.

Table S1: Proteins and concentrations in human tears of healthy and dry eye patients.[2] Analysis of the solvent accessible surface area (SASA) proteins used in binding studies was performed to determine the number of surface exposed lysines which would interact with nanogel formulations containing Al-OEGA. SASA was quantified using the PDBePISA (Protein Data Bank in Europe, Proteins, Interfaces, Structures and Assemblies) SASA tool available online through the European Molecular Biology Laboratory.[3]

Protein	Isoelectric Point	Molecular Weight (kDa)	# Solvent Accessible Lysine	Concentration (healthy) (mg/mL)	Concentration (dry eye) (mg/mL)
Lysozyme	11.3	14.3	5	$2.0 \pm 1.0 \times 10^{0}$	$0.7 \pm 0.5 \times 10^{0}$
Lactoferrin	8.7	80	46	$2.0 \pm 1.1 \times 10^{0}$	$0.7 \pm 0.5 \times 10^{0}$
Lipocalin-1	5.3	19	11	$1.7 \pm 0.5 \times 10^{0}$	$1.0 \pm 0.5 \times 10^{0}$
sIgA	4.5-6.5	385	-	$1.7 \pm 0.7 \times 10^{0}$	$0.8 \pm 0.4 \times 10^{0}$
Albumin	4.7	66.5	58	$1.2 \pm 0.8 \times 10^{-2}$	$0.8 \pm 0.5 \times 10^{-2}$
IgG	6.5-9.5	153	varies	$3.6 \pm 0.6 \times 10^{-3}$	-

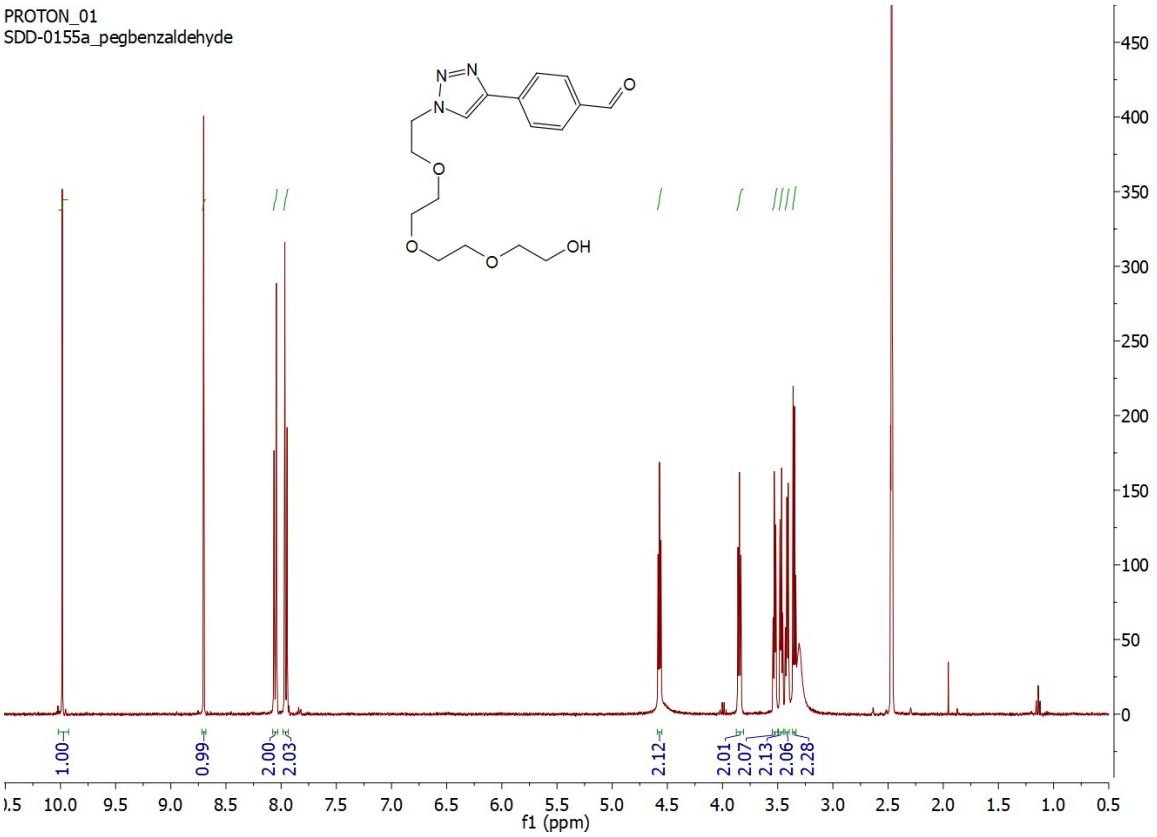


Figure S1: ^{1}H NMR spectrum of 1.

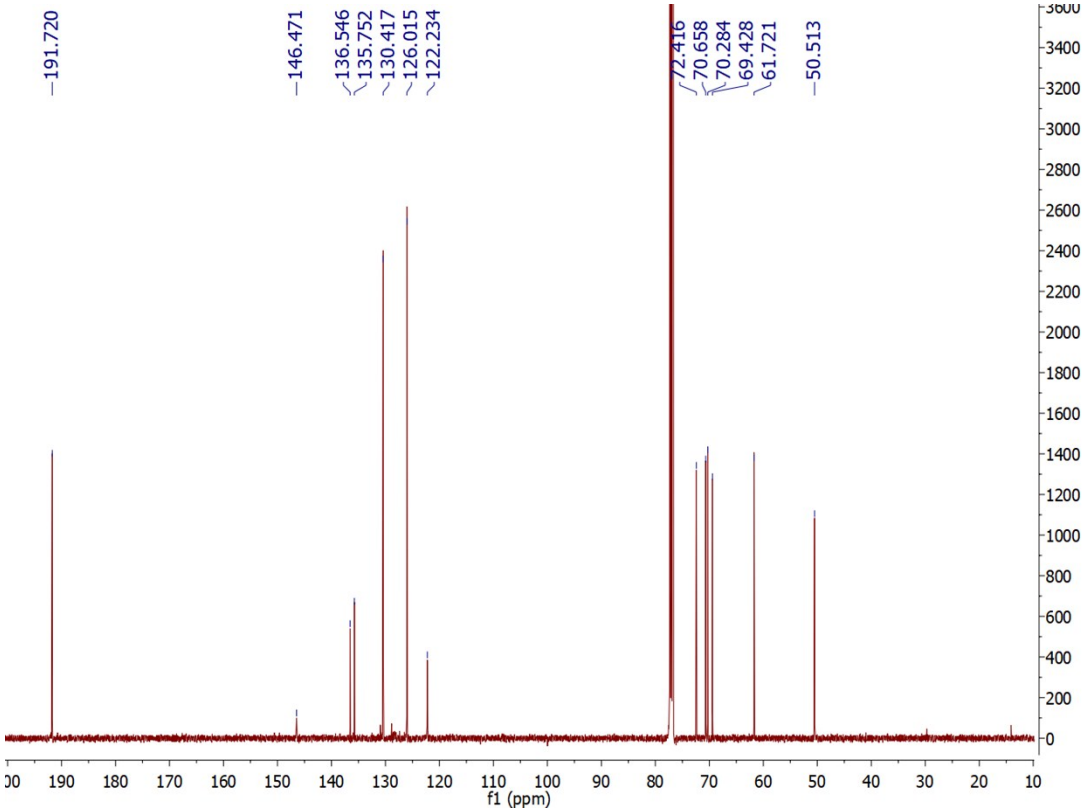


Figure S2: ^{13}C NMR spectrum of 1.

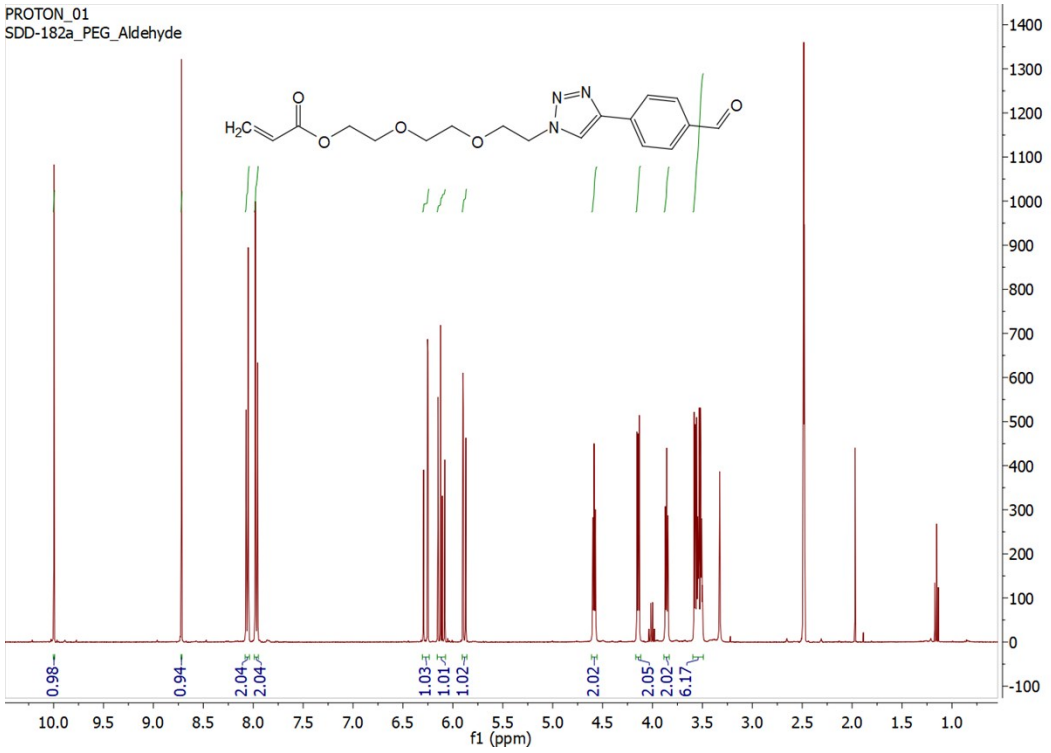


Figure S3: ^{1}H NMR spectrum of 2.

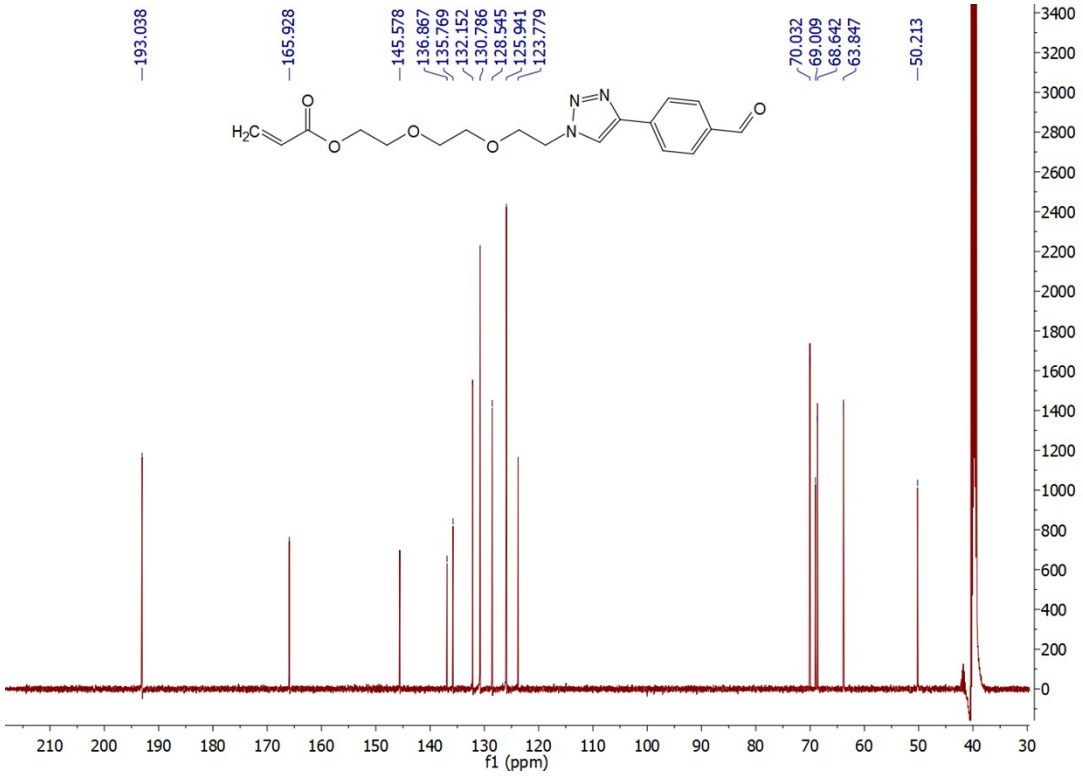


Figure S4: ^{13}C NMR spectrum of 2.

Table S2: Molar ratios of monomers used in the synthesis of P(NIPAM) nanogels. Notation subscripts indicate the ratio of MAA:Al-OEGA.

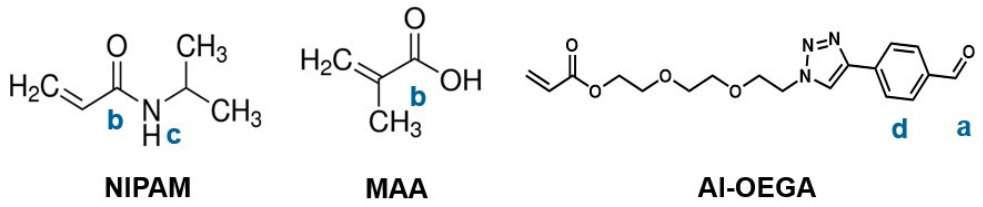
Formulation	NIPAM (mM)	MAA (mM)	PEG Aldehyde (mM)	Notation
P(NIPAM)	16.5	-	-	NN
P(NIPAM-co-Al-OEGA)	12	-	4.5	NA
P(NIPAM-co-MAA)	12	4.5	-	NM
P(NIPAM-co-MAA_2-co-Al-$OEGA_1$)	12	3.0	1.5	NM_2A_1
P(NIPAM-co-MAA_1-co-Al-$OEGA_2$)	12	1.5	3.0	NM_1A_2

Table S3: Dry mass yield of synthesized P(NIPAM) nanogels. Synthesis of each nanogel formulation was repeated in triplicate. Dry mass yield was determined after lyophilization and is reported as mean ± standard deviation. Notation subscripts indicate the ratio of MAA:Al-OEGA.

Formulation	Notation	Mass Yield (mg)

P(NIPAM)	NN	88.79 ± 11.20

a)



b)

Functional group	Wavenumber (cm^{-1})	Letter
Aldehyde, C=O stretching	1724	a
Carboxylic acid, C=O	1713	b
Amide I, C=O stretching	1635	b
Amide II, C-N stretching	1550	c
Aromatic, C-H	830	d

c)

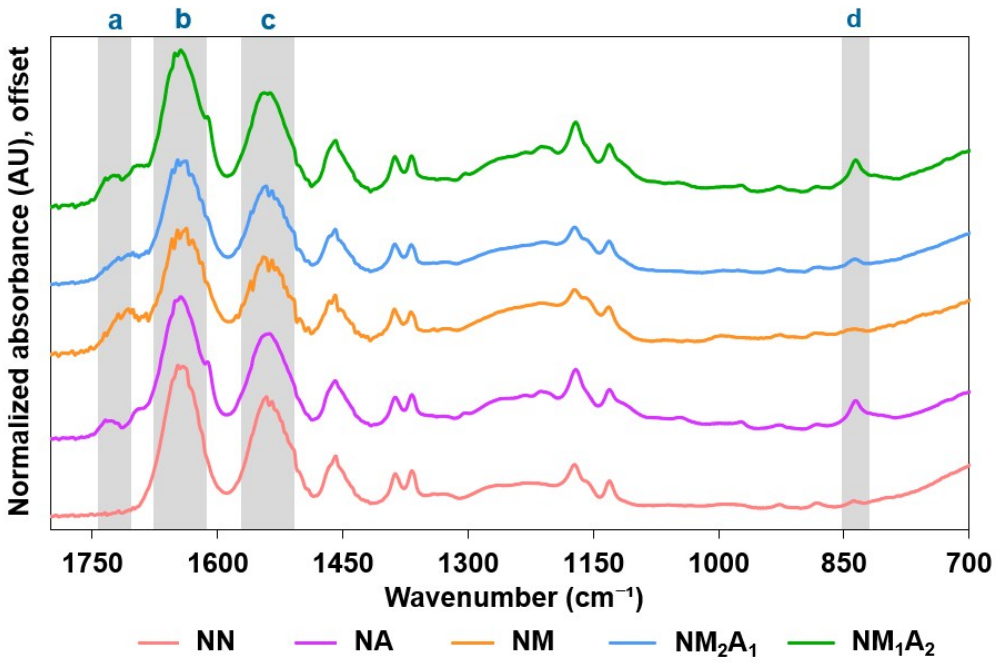


Figure S5: Functional group wavenumber associations and FTIR spectra of the synthesized P(NIPAM) nanogels. a) chemical structure of monomers used in the nanogel synthesis, b) functional group and corresponding wavenumber association, c) FTIR spectra of the synthesized nanogels. All spectra were normalized to the maximum absorbance at 1635 cm^{-1} associated with the amide I absorbance in P(NIPAM). In addition, all formulations possessed a similar IR absorbance at approximately 1550 cm^{-1} associated with the amide II absorbance also present in P(NIPAM). MAA containing

formulations revealed an absorbance peak at 1713 cm^{-1} in relation to the presence of carboxylic acids, while aldehyde and aromatic absorbance peaks were observed in the Al-OEGA containing nanogels at 1724 cm^{-1} and 830 cm^{-1}, respectively.

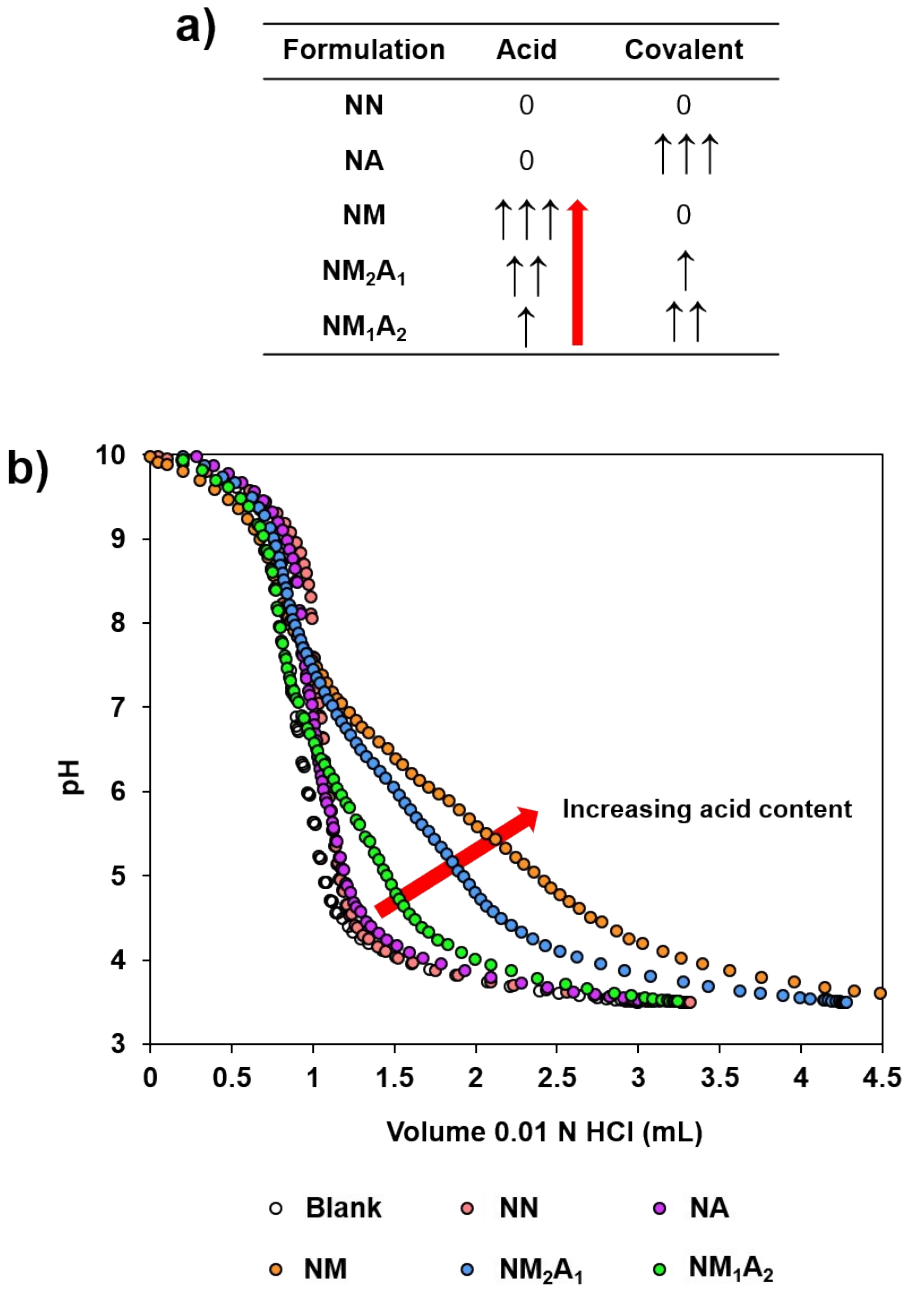
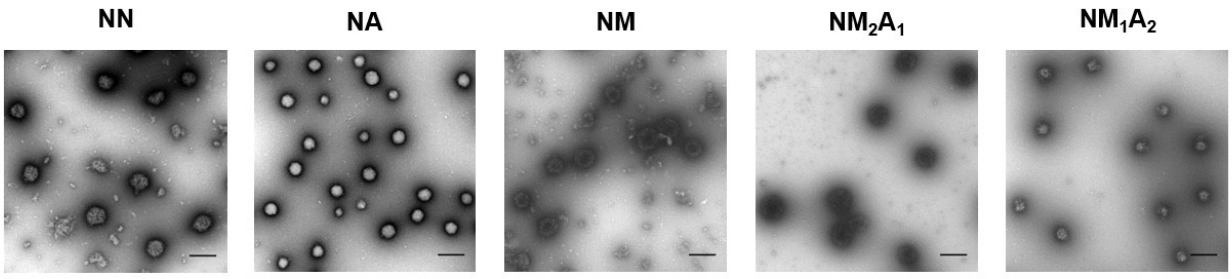


Figure S6: Potentiometric titration of P(NIPAM) nanogels. a) schematic symbolizing the qualitative acid (MAA) and covalent (Al-OEGA) moieties in the synthesize nanogels, and b) representative potentiometric titration curves of the synthesized nanogels. Nangels were suspended in 5 mM KCl at 0.125 mg/mL. The initial pH was adjusted to 10 using 1N NaOH to deprotonate all carboxylic acid moieties. Then, nanogels were titrated under dynamic dosing to pH 3 with 0.01N HCl. Blank refers to data obtained from a sample of 5 mM KCl alone.

Table S4: Hydrodynamic diameter and zeta potential of P(NIPAM) nanogels. Dried nanogels were suspended at 1 mg/mL in 0.1X phosphate buffered saline (PBS, pH 7.4) and measurements were obtained using at 25°C. Data are reported as mean ± standard deviation.

Formulation	Hydrodynamic Diameter (nm) in 0.1X PBS, pH 7.4	Zeta Potential (mV) in 0.1X PBS, pH 7.4
NN	308.2 ± 44.97	-4.019 ± 0.509
NA	178.7 ± 4.891	-7.871 ± 0.557
NM	409.0 ± 90.69	-14.86 ± 2.690
NM_2A_1	420.8 ± 150.7	-15.76 ± 0.184
NM_1A_2	240.5 ± 63.38	-20.59 ± 1.375

a)



b)

Formulation	Dry Nanogel Diameter (nm)
NN	163.2 ± 18.11
NA	147.0 ± 18.39
NM	193.2 ± 28.49
NM_2A_1	214.0 ± 38.34
NM_1A_2	126.6 ± 14.00

Figure S7: Transmission electron micrographs and dry particle diameter values of P(NIPAM) nanogels. a) representative transmission electron micrographs of synthesized nanogels. b) dry nanogel diameter values obtained from TEM images. Values are reported as mean ± standard deviation. Lyophilized nanogels were suspended in ultrapure water

at 1 mg/mL, dropped onto ionized carbon coated 400 mesh copper grids, and stained with 2% uranyl acetate (pH 4) in water. Scale bar = 300 nm.

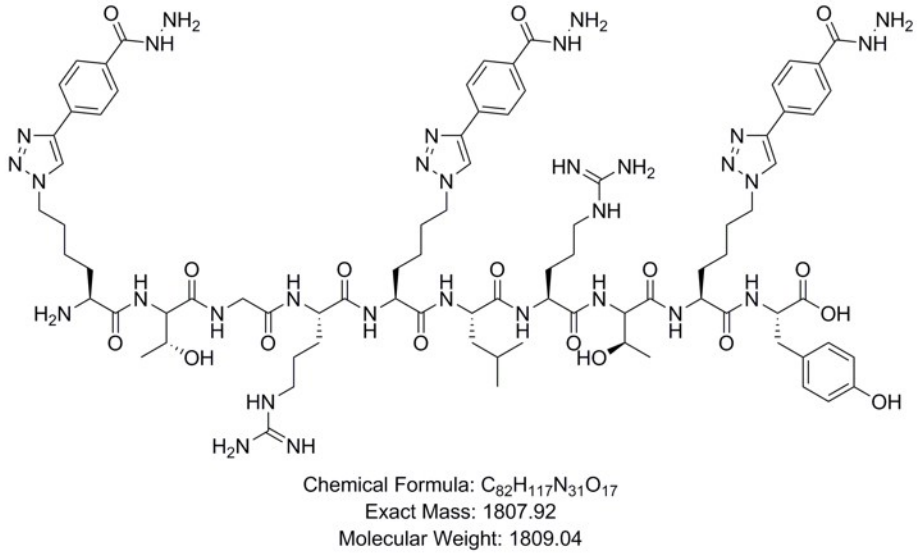


Figure S8: Custom hydrazide containing peptide used in peptide binding studies. The peptide was synthesized and characterized by the Anslyn group following previously established procedures.[4]

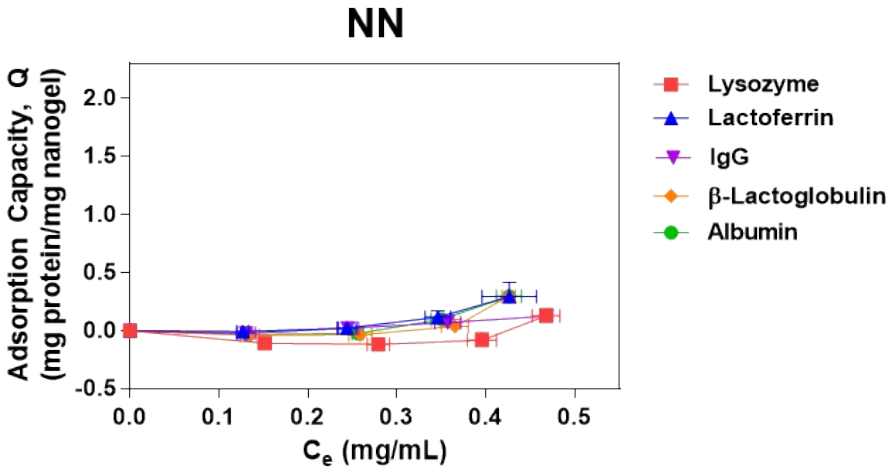


Figure S9: Adsorption capacity of select proteins to P(NIPAM) nanogels as a function of equilibrium protein concentration. Data are reported as mean ± standard deviation.

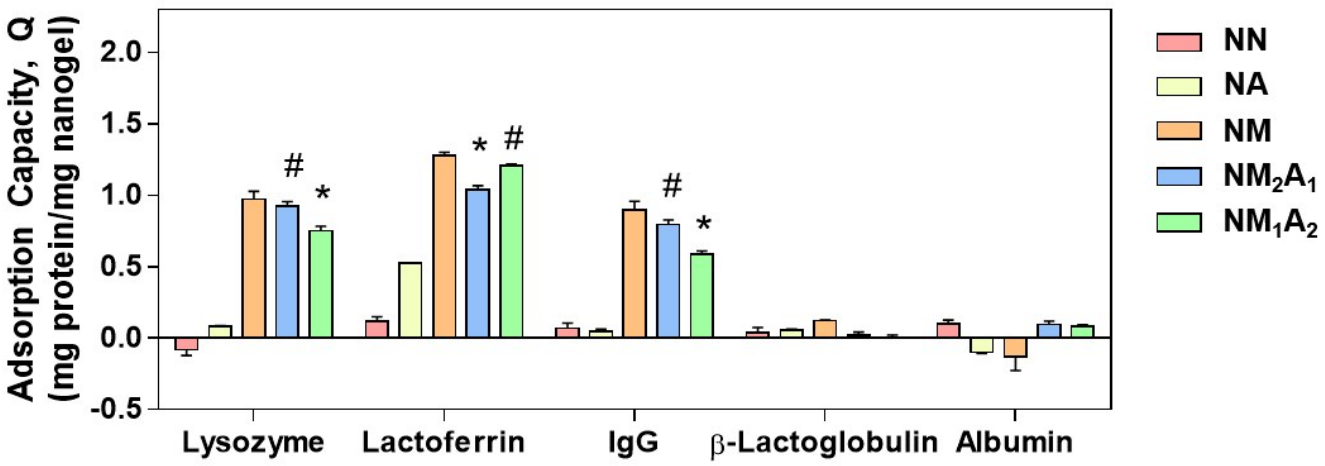


Figure S10: Adsorption capacity of select proteins to P(NIPAM) nanogels. Data are reported as mean ± standard error of the mean and represent results obtained upon incubation of proteins at an initial concentration of 0.75 mg/mL with nanogels of varying formulations. * indicates $p < 0.05$ compared to NM (orange bars). # indicates $p < 0.05$ between aldehyde formulations (blue and green bars).

References

1 H. R. Culver, I. Sharma, M. E. Wechsler, E. V. Anslyn and N. A. Peppas, *Analyst*, 2017, **263**, 111–117.
2 H. R. Culver, M. E. Wechsler and N. A. Peppas, *ACS Nano*, 2018, **12**, 9342–9354.
3 E. Krissinel and K. Henrick, *J. Mol. Biol.*, 2007, **372**, 774–797.
4 J. F. Reuther, J. L. Dees, I. V. Kolesnichenko, E. T. Hernandez, D. V. Ukraintsev, R. Guduru, M. Whiteley and E. V. Anslyn, *Nat. Chem.*, 2017, **10**, 45–50.