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SUPPLEMENTARY INFORMATION

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Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity

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Methods:

Materials: α-chymotrypsin from bovine pancreas type II, lyophilized powder, ≥40 units/mg protein (sigma), copper(I) bromide (CuBr, 99.999%, Aldrich), copper(II) bromide ($CuBr_2$, 99.999%, aldrich), 1,1,4,7,10,10-Hexamethyltriethylenetetramine (HMTETA 97%, aldrich), N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (Sigma), and methoxy PEG succinimidyl carboxymethyl ester, MW 5,000 (Jenkem Technology, China).

***N*-Hydroxysuccinimide terminated poly(carboxybetaine methacrylate) polymer synthesis:** Synthesis of the *N*-Hydroxysuccinimide (NHS) ATRP initiator[1] and poly(carboxybetaine) methacrylate with t-butyl protected ester (CBMA-1-tBut)[2] were performed as published. Atom transfer radical polymerization was carried out in anhydrous dimethylformamide (DMF) using a Cu(I)Br/HMTETA catalyst (Supplementary Fig. 1a). In a typical polymerization, DMF and HMTETA were separately purged of oxygen by bubbling with nitrogen. 1g (3.67 mmol) of CBMA-1-tBut monomer and 125 mg (0.5 mmol) of NHS-initiator were added to a Schlenk tube. To a second Schlenk tube was added 71.7 mg (0.5 mmol) of Cu(1)Br. Both tubes were deoxygenated by cycling between nitrogen and vacuum three times. 8 and 2 mL of deoxygenated DMF were added to the monomer/initiator and Cu(I)Br tubes respectively. 136 uL (0.5 mmol) of deoxygenated HMTETA was added to the Cu(I)Br containing solution and was stirred for 30 min under nitrogen protection. The catalyst solution (Cu(1)/HMTETA) was then all added to the monomer/initiator solution to start the reaction. The reaction was run to completion at room temperature with monitoring by NMR. After

polymerization, the reaction was fully precipitated in ethyl ether. The precipitate was then dried under vacuum and redissolved in minimal DMF (3-5 mL). The solution was reprecipitated in anhydrous acetone to remove the soluble catalyst and trace monomer. This was repeated for a total of 3 times to fully remove the catalyst. The remaining ester polymer was dried overnight under vacuum and analyzed by NMR.To hydrolyze the tert-butal group, 500 mg NHS-pCBMA-1-tBut was dissolved in 5 mL trifluoroacetic acid (Supplementary Fig. 1b). This was allowed to sit for 2 hours. The solution was then precipitated in ethyl ether, dried overnight under vacuum and subsequently analyzed by NMR (MW = 4.2 kDa) and GPC (MW = 2.5 kDa, PDI = 1.05).

α-chymotrypsin polymer conjugation and purification (Supplementary Fig. 1c): α-chymotrypsin conjugates were prepared in 200 mM HEPES buffer, pH 8.5. The α-chymotrypsin concentration was fixed at 4 mg/mL, while the polymer solutions varied at 4 mg/mL, 8 mg/mL and 16 mg/mL. This was done for all three polymers: PEG, pCB M_n and pCB R_h. Solutions were mixed with a stir bar for 30 min, then incubated at 4°C for 3 hours. The conjugates were purified and concentrated by ultrafiltration using a 30kDa MWCO membrane. The pCB R_h conjugates were further purified using size exclusion column with a Superdex 200 10/300 column. These samples were then again concentrated using ultrafiltration. The number of polymers per protein for each sample was determined using a trinitrobenzene sulfonate (TNBS) assay to determine the number of unreacted surface amines, along with AUC of SEC chromatograms from size exclusion chromatography.

Urea stability: Conjugates were incubated in 0.1 M Tris at pH 8.0 for 8 hours at 0.5 ug/mL with and without 5M Urea. Measurements were performed in triplicate. Solutions were also incubated with 0.1 mg/mL BSA as a blocking agent to prevent depletion of enzyme from surface adsorption. After 8.0 hours, activities were measured of all samples by adding N-

Succinyl-Ala-Ala-Pro-Phe p-nitroanilide at 667 ug/mL. Activities were measured as the increase in absorbance at 412nm with time. Values were recorded as percentage of activity with urea, relative to without urea.

Thermal stability: Conjugates were incubated in 0.1 M Tris, pH 8.0, at temperatures of 40, 45, 50 and 55°C at a concentration of 5.0 ug/mL. After 10 min at each temperature, each conjugate was diluted into refrigerated buffer to a concentration of 0.5 ug/mL. Solutions were also incubated with 0.1 mg/mL BSA as a blocking agent. Activities were measured of all samples by adding by adding N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide to a final concentration of 667 ug/mL. Activities were measured as the increase in absorbance at 412nm with time. Values were recorded as percentage of each conjugates activity at 40°C.

Conjugate kinetics with N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide: Conjugates were incubated at 20 pM in 0.1 M Tris, pH 8.0 with 0.1 mg/mL BSA as a blocking agent. Low enzyme concentrations were required to achieve accurate activity measurements at low substrate concentrations. N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide was added at varying concentrations from 0 to 667 ug/mL (0-1,070 μM). Concentrations of each substrate were prepared in triplicate and activity measured as an increase in absorbance with time at 412nm.

Conjugate kinetics with resorufin bromoacetate: Conjugates were incubated at 20 pM in 0.1 M MES, pH 6.0 with 0.1 mg/mL BSA as a blocking agent. A lower pH was required to retain the stability of the base liable substrate. Resorufin bromoacetate was added at varying concentrations from 0 to 50 ug/mL (0-150 μM). Concentration of each substrate were prepared in triplicate and activity measured as an increase in absorbance with time at 571nm.

Evaluation of polymer-substrate solubility: Dialysis containers containing either buffer (control), 5 mg/mL PEG or 5mg/mL pCB were exposed to a large bath in excess volume

containing 2.5 ug/mL of peptide substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide). A dialysis membrane separated the polymers from the substrate, which allowed the substrate to pass freely, but not to the polymers. After 3 days, the sample solutions were measured by HPLC for peptide concentration.

Supplementary References:

1. Lecolley, F. et al. A new approach to bioconjugates for proteins and peptides ("pegylation") utilising living radical polymerisation. *Chemical Communications*, 2026-2027 (2004).
2. Cao, Z.Q., Yu, Q.M., Xue, H., Cheng, G. & Jiang, S.Y. Nanoparticles for Drug Delivery Prepared from Amphiphilic PLGA Zwitterionic Block Copolymers with Sharp Contrast in Polarity between Two Blocks. *Angewandte Chemie-International Edition* **49**, 3771-3776 (2010).

Supporting Figures:

Table S1 - Molecular weights determined by NMR and GPC including PDI's for all polymers.

	NMR	**GPC**	
Name	M_n (kDa)	M_n (kDa)	M_w/M_n
PEG	5.0	4.9	1.01
pCB M_n Eq (PEG molecular weight equivalent)	4.2	2.5	1.04
pCB R_h Eq (PEG hydrodynamic size equivalent)	12.0	6.7	1.17

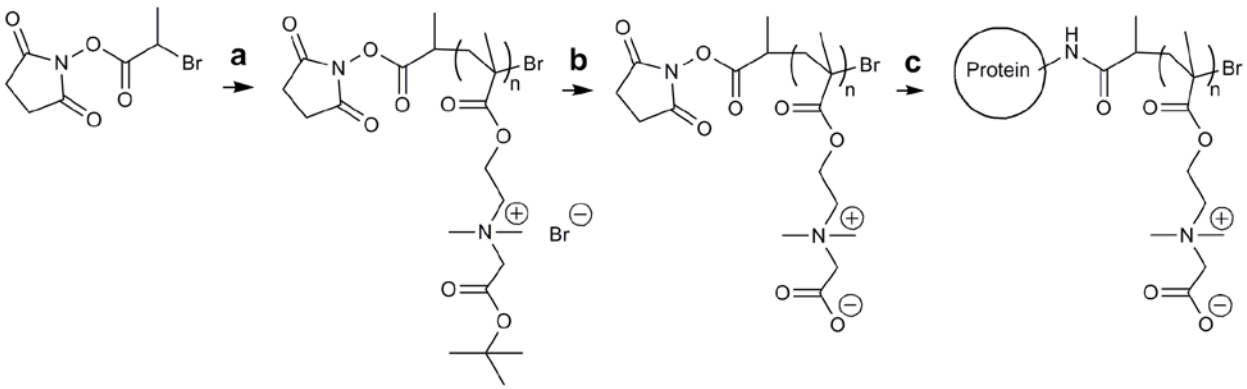

Figure S1 - Synthesis and formation of carboxybetaine-protein conjugates.

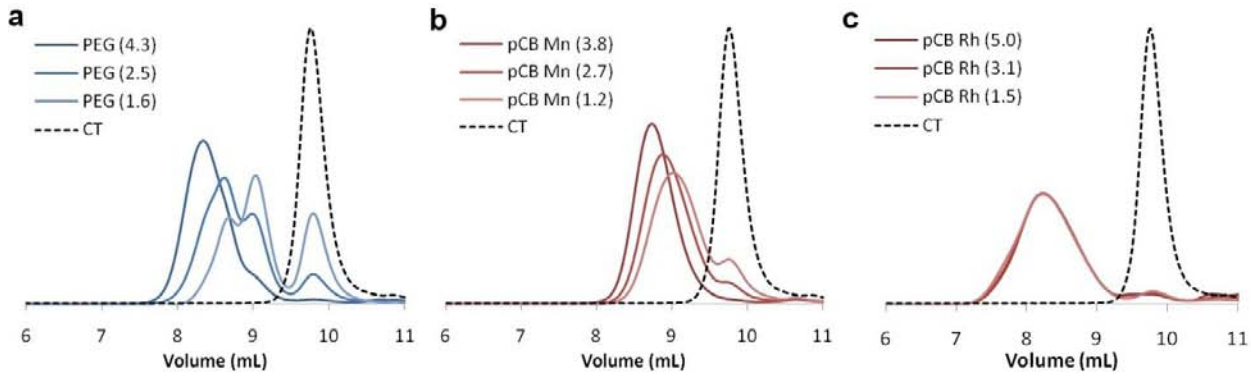

Figure S2 – Size exclusion chromatographs of **a,** PEG, **b,** pCB M_n, and **c,** pCB R_h chymotrypsin conjugates respectively.

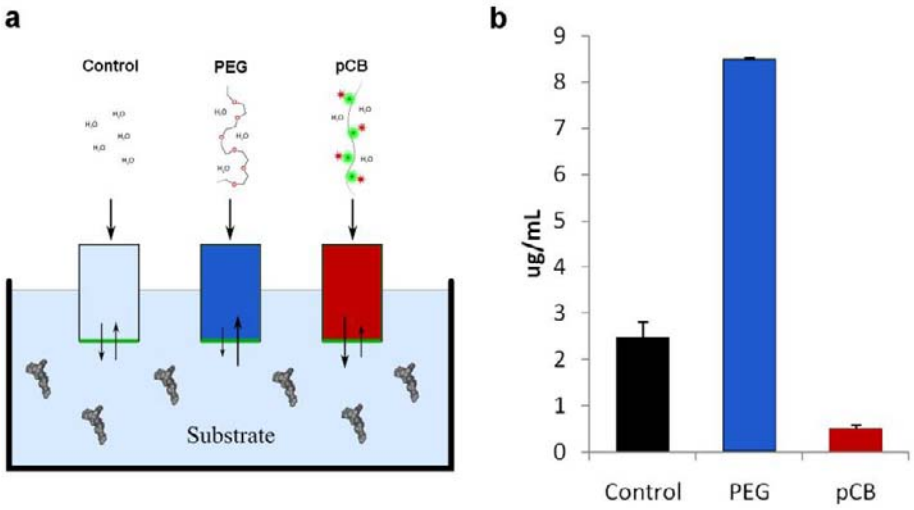

Figure S3 – Evaluation of polymer-substrate solubility. **a**, Dialysis setup with buffer (control), PEG or pCB, exposed to a large bath in excess volume containing peptide substrate. **b**, Substrate concentration in each container after 3 days.

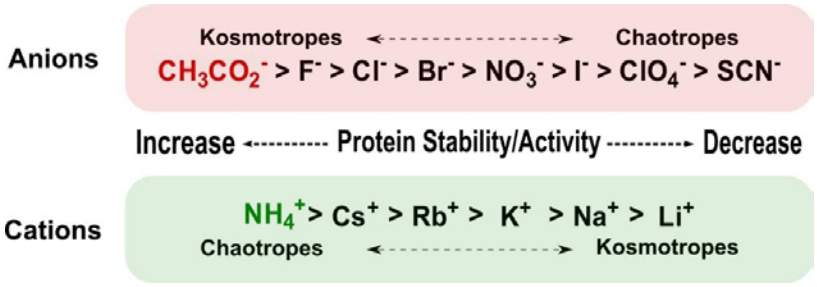

Figure S4 – The Hofmeister Series for monovalent ions.

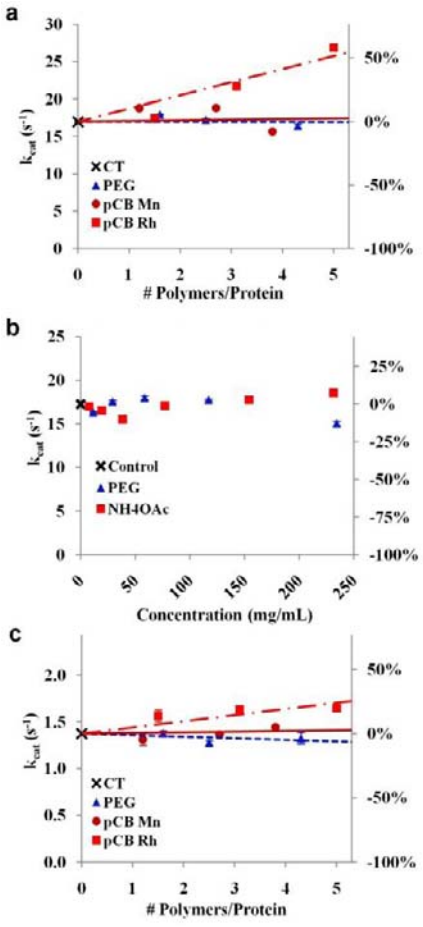

Figure S5 – Values of k_{cat} for **a**, conjugates with the N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide, **b**, bare α-chymotrypsin with the peptide molecule substrate in solutions of ammonium acetate (NH_4OAc) and 650 M_n PEG, and **c**, the conjugates with resorufin bromoacetate.