Supporting Information

Templated Self-Assembly of a Covalent Polymer Network for Intracellular Protein Delivery and Traceless Release

Kingshuk Dutta,[1] Ding Hu,[1] Bo Zhao,[1] Alexander E. Ribbe,[2] Jiaming Zhuang*,[1] and S. Thayumanavan*[1,3,4]

[1]Department of Chemistry, [2]Department of Polymer Science and Engineering, [3]The Center for Bioactive Delivery, Institute for Applied Life Sciences, [4]Molecular and Celluar Biology Program, University of Massachusetts, Amherst, Massachusetts 01003-9336, United States

Contents **Page No.**

Materials S2
Methods S2
Synthesis of p(PEGMA-*co*-PDSMA), P_{PcP}. S2
Modification of synthesized p(PEGMA-*co*-EDSMA) polymer, P_{PcE} S2
Synthesis of p(PEGMA-*co*-NPC) (*NPC: p-nitrophenylcarbonate*), P1 S3
^{1}H-NMR spectra for polymer samples P_{PcP}, P_{PcE} and P1 S3
Gel permeation chromatography (GPC) for P_{PcP}, P_{PcE} and P1 S5
Synthesis of polymer−protein nanoassemblies S5
NA-Empty^{ED} and NA-Empty^{PEG} S5
NA-Lys^{ED}, NA-Lys^{PEG}, NA-RNaseA^{ED}, NA-RNaseA^{PEG}, NA-CytC^{ED} and NA-CytC^{PEG} S5
Monitoring protein-polymer conjugation, degree of NPC group modification by protein and crosslinking density S6
Encapsulation efficacy and loading capacity S8
DLS and zeta potential plots S9
Cryo-TEM sample preparation and instrumentation for Figure 2 in the manuscript S11
Estimation of approximate number of proteins per particle S13
Enzymatic degradation (trypsin digest) study S14
Assessment of serum stability of NA-Lys^{PEG}, NA-RNaseA^{PEG} and NA-CytC^{PEG} nanoassemblies S16
Lysine residues in proteins S16
SDS-PAGE for protein-polymer conjugation and release studies S17
Release kinetics of proteins from the protein-polymer nanoassemblies S18
Activity assays S20
Circular dichroism (CD) spectra S23
MALDI-MS spectra for the released proteins S24
Labeling of Proteins with Rhodamine B S25
Cell culture S25
Cellular uptake studies for protein delivery and endosomal escape S26
Cell viability with alamarBlue® assay S28
Study of apoptosis with NA-CytC^{PEG} nanoassembly S29
Encapsulation, release and cellular internalization studies with eGFP S30
References S31

Materials:

All chemicals, polyethylene glycol monomethyl ether methacrylate (PEGMA; MW 500), 2,2′-dithiodipyridine, 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (chain transfer agent), D,L-dithiothreitol (DTT), lysozyme, RNase A, cytochrome C and rhodamine B isothiocyanate were obtained from Sigma-Aldrich and were used without further purification unless otherwise mentioned. 2,2′-azobis-(2-methylpropionitrile) (AIBN) was procured from Sigma-Aldrich and purified by recrystallization before usage. Pyridyl disulfide ethyl methacrylate (PDSMA) was synthesized using previously reported procedure.[1]

Methods:

Synthesis procedure

Synthesis of p(PEGMA-*co*-PDSMA), P_{PcP}

PDSMA (0.511 g, 2 mmol), PEGMA (1 g, 2 mmol) and 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (55.7 mg, 0.2 mmol) were weighed and dissolved with 2 mL THF in a 25 mL Schlenk flask. To the reaction mixture, 1 mL AIBN (6.7 mg, 0.0408 mmol) solution in THF was added and mixed for 5 min. The flask was purged with argon and performed three freeze-pump-thaw cycles. After that the reaction vessel was sealed and transferred to an oil bath preheated at 70 °C. The polymerization was quenched after 24 h by cooling down the reaction flask with cold water and the solvent was evaporated. The viscous reaction product was purified by repeated washing with cold diethyl ether and finally dried in high vacuum at room temperature for 24 h. Yield: 96%, GPC (THF) M_n: 27 K. Đ: 1.1. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.47, 7.70, 7.13, 4.21−4.07, 3.64−3.37, 3.03, 1.93-1.82, 1.03−0.87. From ^{1}H NMR, integration of the methoxy proton (in PEG unit) and the aromatic proton (in pyridine unit) provided the molar ratio of two monomers to be 1:1 (PEG/PDS).

Modification of synthesized p(PEGMA-*co*-EDSMA) polymer, P_{PcE}

PDS polymer (1 g, 1.32 mmol PDS repeat unit) was weighed in a 20 mL glass vial and dissolved in 8 mL DCM. Catalytic amount (100 μL) of glacial AcOH was added to it and stirred for 5 min. Afterwards, 2-mercaptoethanol (0.9 mL, 13 mmol) was added dropwise to the reaction mixture and the solution was stirred for 24 h at room temperature. After that, the modified polymer was

purified by dialyzing against methanol using a membrane of MWCO: 3.5 kDa. After dialysis, the solvent was evaporated and the polymer was dried under vacuum for 24 h. Yield: 90%, GPC (THF) M_n: 26 K. Đ: 1.2. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.24−4.09, 3.87, 3.65−3.53, 3.37, 2.95-2.90, 1.93-1.84, 1.04-0.89.

Synthesis of p(PEGMA-*co*-NPC) (*NPC: p-nitrophenylcarbonate*), P1

Modified polymer P2 (1 g, 1.39 mmol) and 4-Nitrophenyl chloroformate (325 mg, 1.61 mmol) were dissolved in 5 mL DCM taken in a 20 mL glass vial. The reaction mixture was cooled in ice bath for 10 min. To the cold mixture, pyridine (130 µL, 1.61 mmol) was added dropwise under vigorous stirring. Finally, the reaction mixture was stirred at room temperature for 24 h and the self-immolative polymer was purified by dialyzing against DCM/MeOH 1:1 mixture using a MWCO 3.5 kDa membrane. Yield: 98%, GPC (THF) M_n: 32 K. Đ: 1.2. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.29-8.27, 7.41-7.42, 4.54, 4.23-4.08, 3.63-3.37, 3.04-2.94, 1.89-1.80, 1.03−0.88.

^{1}H-NMR spectra for polymer samples P_{PcP}, P_{PcE} and P1

^{1}H-NMR spectra of the samples were recorded on a 400 MHz Bruker NMR spectrometer using residual proton resonance of the solvent as the internal standard and chemical shifts were reported in parts per million (ppm).

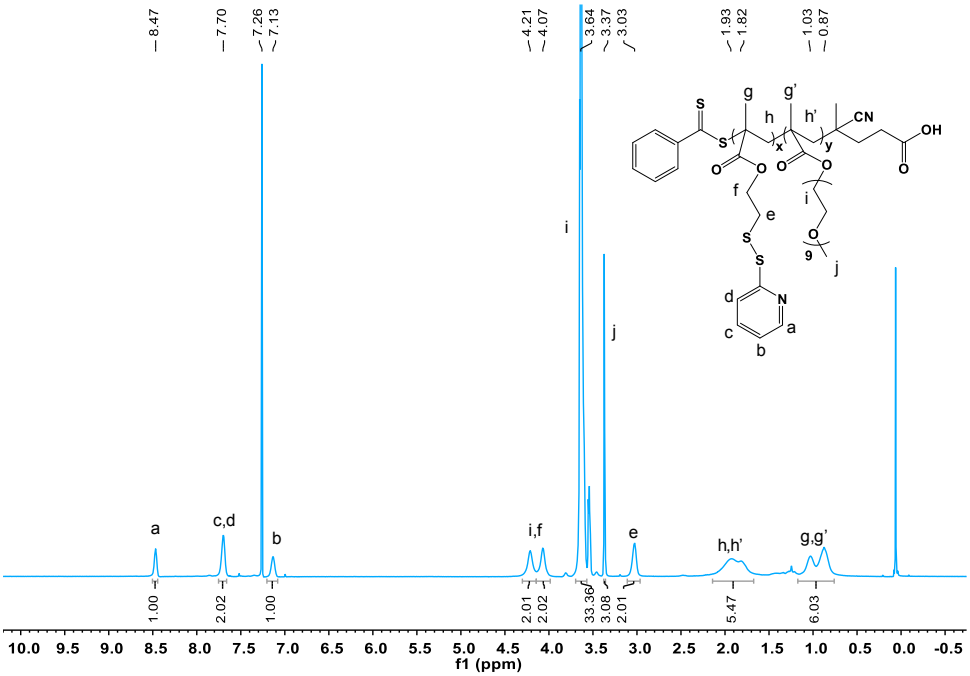


Figure S1. ^{1}H-NMR spectra of p(PEGMA-co-PDSMA), $\mathbf{P_{PcP}}$

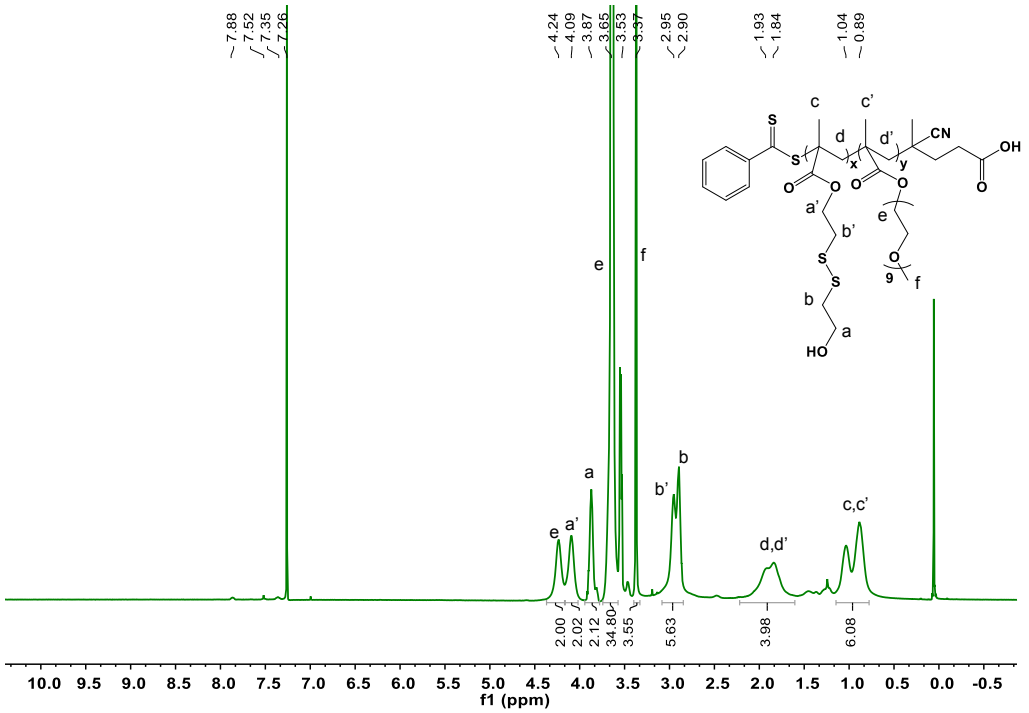


Figure S2. [1]H-NMR spectra of p(PEGMA-co-EDSMA), $\mathbf{P_{PcE}}$

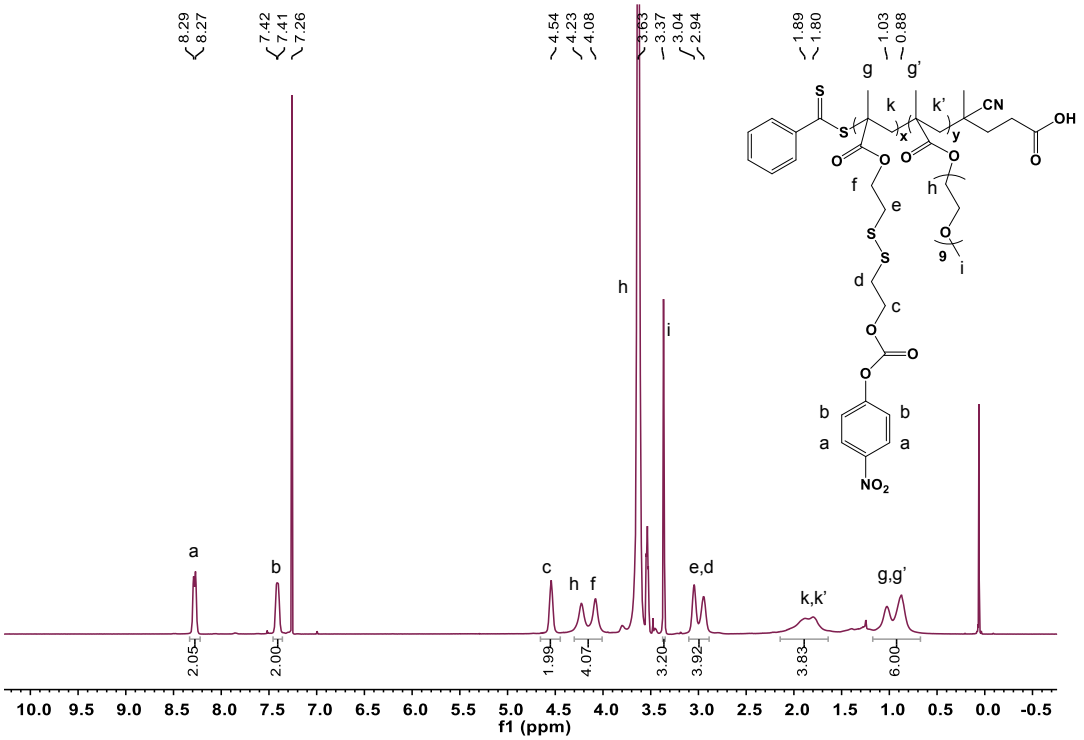


Figure S3. [1]H-NMR spectra of p(PEGMA-co-NPC), P1

Gel permeation chromatography (GPC) for P_{PcP}, P_{PcE} and P1

Molecular weights of all synthesized polymers were estimated by GPC in THF using poly(methyl methacrylate) (PMMA) standards with a refractive index detector.

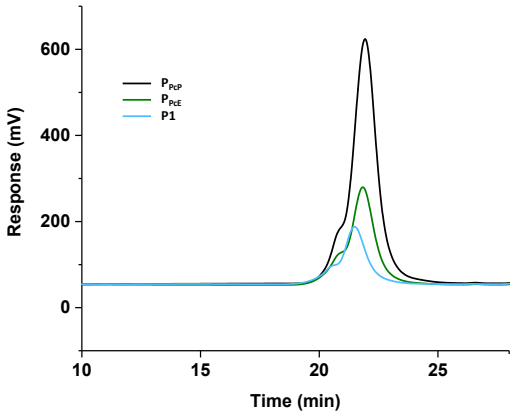


Figure S4. GPC(THF) for polymers **P_{PcP}, P_{PcE} and P1**

Synthesis of polymer-protein nanoassemblies

NA-Empty^{ED} and NA-Empty^{PEG}

The self-immolative polymer P1 (10 mg) was dissolved in 2.5 mL phosphate buffer (adjusted to pH 8.5) at 20 ºC. A calculated amount (~0.005 mmol) of ethylenediamine (for NA-Empty^{ED}) or $(PEO)_4$-bis-amine (for NA-Empty^{PEG}) was added to the solution and kept stirring for 24 h for cross-linking. The resulting nanoassembly was purified by repeated washing (five times) with phosphate buffer pH 7.4 Amicon Ultra Centrifugal Filters MWCO 30 kDa. The final volume of empty nanoassembly was adjusted to 500 µL with phosphate buffer of pH 7.4.

NA-Lys^{ED}, NA-Lys^{PEG}, NA-RNaseA^{ED}, NA-RNaseA^{PEG}, NA-CytC^{ED} and NA-CytC^{PEG}

Initially, polymer P1 (10 mg) was dissolved in 1.5 mL phosphate buffer (adjusted to pH 8.5). To this solution, 1 mL solution of a specific protein (1 mg lysozyme or RNase A or cytochrome C in phosphate buffer, pH 8.5) was added dropwise and stirred for 24 h at 20 ºC to generate P2. Then, calculated amount (~0.005 mmol) of ethylenediamine (for NA-Protein^{ED}) or $(PEO)_4$-bis-amine (for NA-Protein^{PEG}) was added to each solution for cross-linking and mixed for another 24 h at 20 ºC. Finally, the reaction mixture was washed (five times) with phosphate buffer (pH 7.4)

using Amicon Ultra Centrifugal Filters MWCO 30 kDa to get purified nanoassemblies (NA) encapsulated with proteins. The final volume of all conjugates was adjusted to 500 µL with phosphate buffer of pH 7.4.

Monitoring protein-polymer conjugation, degree of NPC group modification by protein and crosslinking density

The conjugation process and cross-linking density for the protein-polymer conjugates can be evaluated by UV-Vis spectroscopy. The amount of released 4-nitrophenol was monitored at its λ_{max} 400 nm (measured molar extinction co-efficient $\approx 12.14*10^3$ $LM^{-1}cm^{-1}$ at 400 nm). Absorbance of each solution was measured after the conjugation and cross-linking processes. For each absorbance measurement, 25 µL of sample was withdrawn and diluted to 1 mL with distilled water. Cross-linking density was calculated by assuming that the formation of a single chain crosslinking bond would require cleavage of two NPC units and produce two 4-nitrophenol molecules.

The molar ratio of NPC:PEG in the polymer, P1= 50:50 (x mol : y mol, from NMR)

NPC molecular weight = 387 g/mol, PEG molecular weight = 500 g/mol

So, x mol * 387 g/mol + y mol * 500 g/mol= 0.1 mg

x mol = 50/50 y mol

Amount of polymer used = 0.1 mg/mL for each absorbance measurement
Moles of NPC-unit (x mol) in the solution= (0.1/887)/1000= $1.13 * 10^{-7}$ mol

Example for NA-Lys^{ED}:

From Beer-lamber's law: A = ε.c.l and path-length=1 cm
So, concentration of 4-nitrophenol, [c]= 0.3692/ $(12.14 * 10^3) = 3.04 * 10^{-5}$ M
Thus, moles of 4-nitrophenol in 1 mL solution= $3.04 * 10^{-8}$ mol
This represents 27 mol% of total NPC unit. As we assume that two 4-nitro phenol are released per crosslinking bond formation and NPC unit is 50 mol% of total polymer.
Therefore, crosslinking density =27/2 *0.5 = 6.7 %

Hydrolysis from control polymer sample contributes ~1% towards release of 4-nitrophenol and adjusted for all calculations.

Table 1: NPC modification and crosslinking density of nanoassemblies:

Sample	Degree of NPC modification by protein*, %	Max. available Lysine per NPC group#, %	Crosslinking density, %
NA-Lys^{ED}	0.38	2.0	5.7
NA-Lys^{PEG}	0.61	2.1	7.0
NA-RNaseA^{ED}	0.21	3.6	6.0
NA-RNaseA^{PEG}	0.43	3.6	6.0
NA-CytC^{ED}	3.3	8.7	5.2
NA-CytC^{PEG}	3.5	9.2	6.0

* Amount of reacted NPC group, calculated based on amount of 4-nitrophenol released and original content of the same in the reactant polymer.

moles of lysine/moles of NPC * 100; calculated based on initial polymer dosage (10 mg) and encapsulated proteins (see encapsulation efficiency)

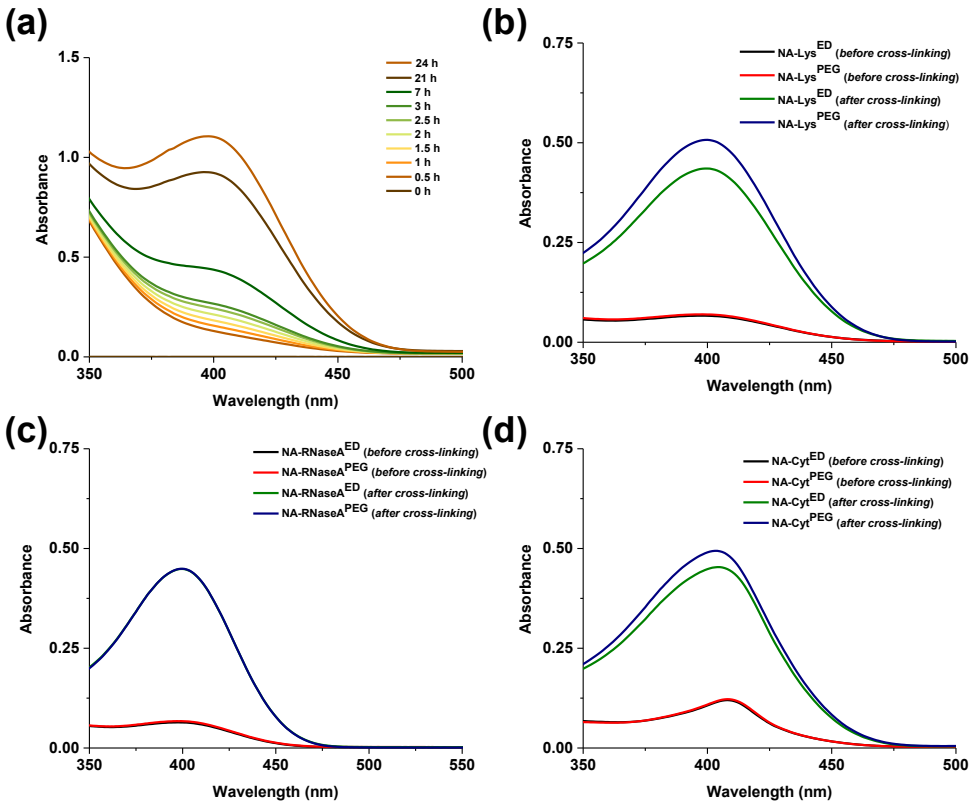


Figure S5. (a) Time-course of absorbance profile for released 4-nitrophenol as a fate of conjugation of lysozyme with polymer P1; Absorbance spectra of polymer-protein conjugates- before and after crosslinking for (b) lysozyme; (c) RNase A and (d) cytochrome C. UV−visible absorption spectra were recorded on a PerkinElmer Lambda 35 spectrophotometer.

Encapsulation efficacy and loading capacity:

All nanoassemblies were evaluated for amount of protein encapsulation after conjugation and cross-linking process. Protein concentration in each sample was measured from the filtrate after crosslinking reaction and the amount of protein was back-calculated in the conjugate. An absorbance based assay (with Pierce^{TM} 660 nm Protein Assay Reagent) was utilized to quantify the protein amount. The encapsulation efficiency (EE) and loading capacity (LC) were calculated based on the following formulas:

$$\text{EE, \%} = \left[\frac{\text{initial protein loaded} - \text{free “unencapsulated” protein}}{\text{initial protein loaded}}\right] \times 100$$

$$\text{LC, \%} = \left[\frac{\text{amount of “encapsulated” protein}}{\text{amount of polymer}}\right] \times 100$$

Table 2: Encapsulation efficiency and loading capacity of nanoassemblies:

Sample	Amount of protein encapsulated, µg (Initial dose= 1mg)	Encapsulation efficiency (EE), %	Loading capacity (LC), %
NA-Lys^{ED}	550	55	5.5
NA-Lys^{PEG}	560	56	5.6
NA-RNaseA^{ED}	558	56	5.6
NA-RNaseA^{PEG}	553	55	5.5
NA-CytC^{ED}	637	64	6.4
NA-CytC^{PEG}	673	67	6.7

DLS and zeta potential plots:

Dynamic light scattering (DLS) and zeta potential measurements were performed using a Malvern Nanozetasizer-ZS. All samples were diluted with phosphate buffer pH 7.4 to adjust final concentration to 1 mg/mL.

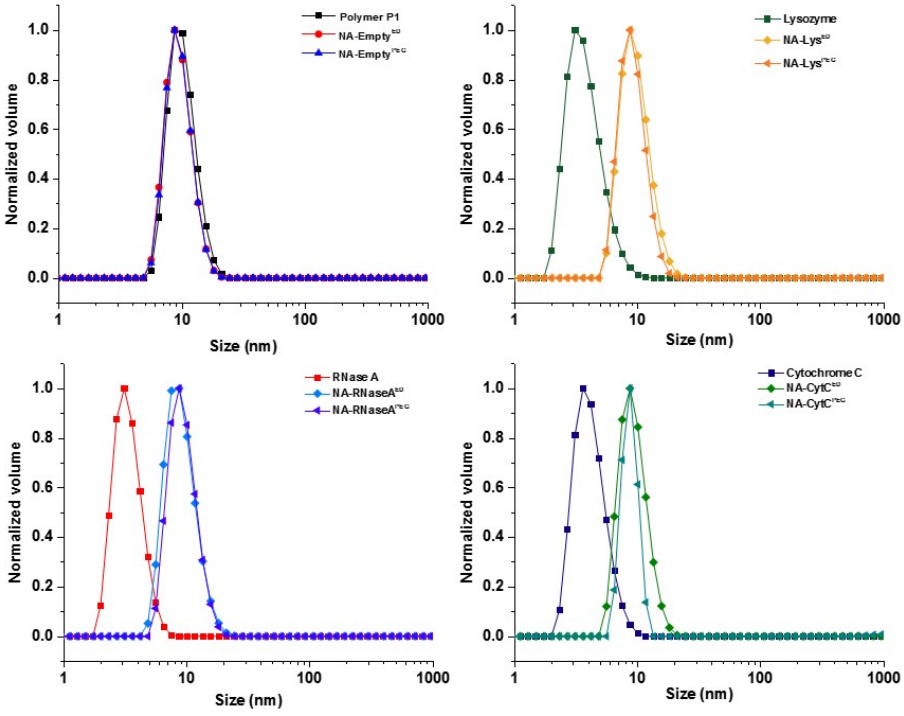


Figure S6. Particle size analysis of protein-polymer nanoassemblies from DLS measurements

Table 3: Particle size values for the nanoassemblies:

Sample	Size, nm
Lysozyme	3.6 ± 0.4
RNase A	3.3 ± 0.2
Cytochrome C	4.3 ± 0.1
NA-Empty^{ED}	9.7 ± 0.1
NA-Empty^{PEG}	9.5 ± 0.1
NA-Lys^{ED}	9.7 ± 0.2
NA-Lys^{PEG}	9.4 ± 0.3
NA-RNaseA^{ED}	9.1 ± 0.1
NA-RNaseA^{PEG}	9.5 ± 0.1
NA-CytC^{ED}	9.2 ± 0.2
NA-CytC^{PEG}	8.6 ± 0.1

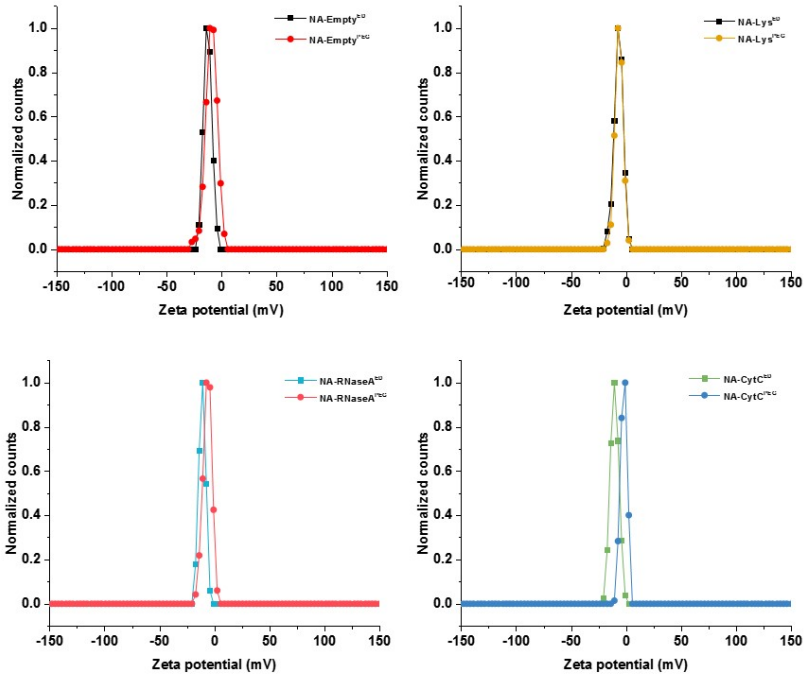


Figure S7. Zeta potential plots for protein-polymer nanoassemblies

Table 4: Particle size and zeta potential values for the nanoassemblies:

Sample	Zeta potential, mV
NA-Empty^{ED}	-7.3 ± 1.3
NA-Empty^{PEG}	-9.2 ± 1.5
NA-Lys^{ED}	-7.6 ± 0.7
NA-Lys^{PEG}	-7.1 ± 0.4
NA-RNaseA^{ED}	-8.2 ± 0.7
NA-RNaseA^{PEG}	-8.7 ± 0.7
NA-CytC^{ED}	-6.4 ± 0.5
NA-CytC^{PEG}	-2.1 ± 0.5

Cryo-TEM sample preparation and instrumentation for Figure 2 in the manuscript:

(a top) Cryo-EM sample preparation was done using a FEI Vitrobot MKII applying purified NA-Protein sample solution (20-21 mg/ml) to a c-flat holey carbon grid (hole size 2 µm) after washing the grid in chloroform. The samples were then transferred using a Gatan cryo-transfer holder to a FEI TecnaiT12 TEM operating at 120 kV acceleration voltage. Images were acquired using standard low dose methods at 5 µm under focus. (a bottom) Sample preparation and HAADF imaging conditions are described under Figure S10.

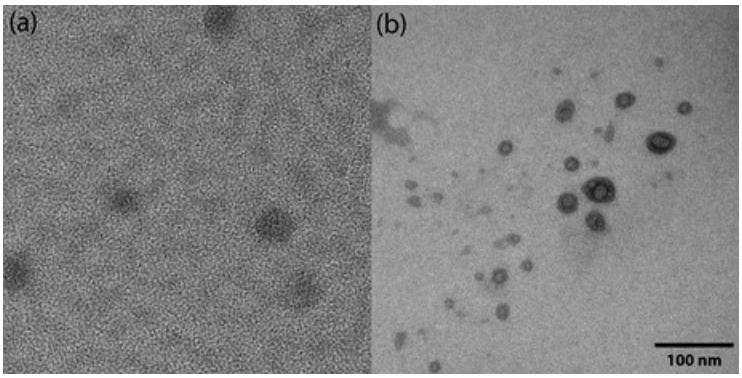


Figure S8. (a) Bright Field image of NA-CytC^{PEG} was obtained after allowing a drop of sample solution to dry on a carbon coated grid and subsequent transfer into the TEM at room temperature mounted on a cryo-TEM holder. The sample was then cooled to -182 °C for observation. The bright field image shows particles in the size of 20-30 nm, which corresponds well to the size observed after cryo-transfer sample preparation. The contrast is naturally not as good as after the cryo-EM sample

preparation (see main text Figure 2a) due to the carbon support film and most likely particles have undergone some deformation due to drying. This sample was also used for the HAADF and EDS measurements at cryo-temperature (main Text Figure 2a bottom and SI Figure S10). Due to relatively high beam currents and long acquisition times necessary for EDS measurements it was only possible to perform these measurements on these samples since samples prepared through cryo-transfer are quickly destroyed during the measurements. The bright field image here, all HAADF images (Main text and SI) and all EDS measurement (SI) were done using a JEOL FEM-2200FS field emission TEM equipped with an Oxford 80mm^2 X-Max EDS-Spectrometer. Acceleration Voltage was 200 kV and camera length for HAADF was 100 cm. Samples were mounted on a Gatan 636 Double Tilt Cryo-Holder with Beryllium cradle to avoid Fe signal typical for standard stainless steel holders. **(b)** Bright Field image of NA-CytC^{PEG} obtained after allowing a drop of sample solution to dry on a carbon coated grid and subsequently observed in a TEM at RT. No additional staining was performed. Compared to the sample in part (a) observed at cryo temperatures the sample quickly degrades under electron beam exposure and forms ring like structures where the average diameter of the inner dark ring is in the range of 10-30 nm. Interestingly, individual NA-CytC^{PEG} particles in aggregates each form a single ring allowing to identify the number of NA-CytC^{PEG} particles within an aggregate (see calculation and Figure S11 for # of proteins per particle). The imaging was done using a JEOL JEM-2000FX TEM operating at 200kV acceleration voltage.

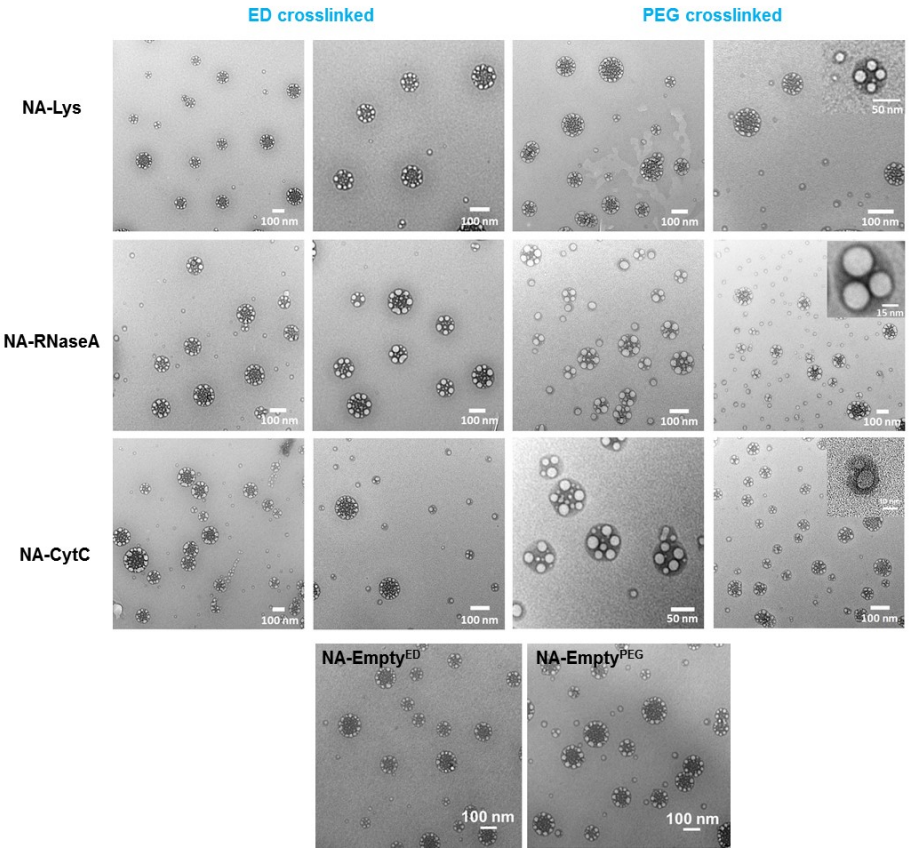


Figure S9. Room temperature TEM images for NA-Protein samples (samples were drop-casted on a carbon coated grid and dried at room temperature before subjecting room temperature TEM study. For all

samples, a mixture of individual as well as clustered particles were observed because of drying on TEM grids. This is also in agreement with the cryo-EM images provided in Figure 2a in manuscript and Figure S8 in SI.

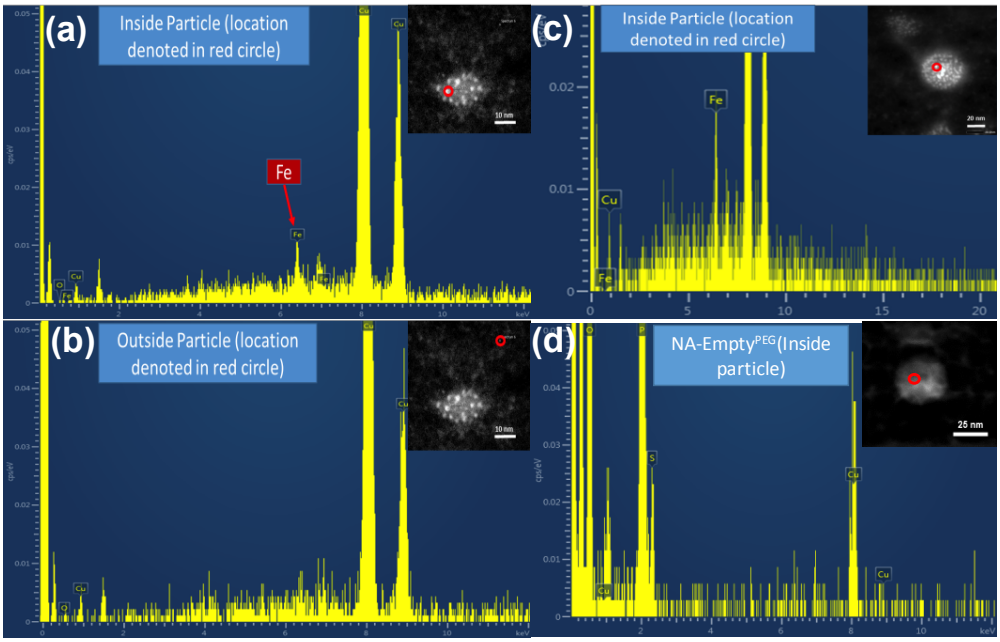


Figure S10. Energy Dispersive X-Ray Spectroscopy (EDS) analyses and High Angle Annular Dark Field Microscopy (HAADF, inset) images at cryo temperature (-178 °C) for NA-CytC^{PEG}. Figure S10 insets shows HAADF images of individual nano-assemblies. For HAADF and EDS analysis, samples were transferred using a Gatan 636 Double Tilt Cryo-Holder with Beryllium cradle to avoid any contamination from external matal sources. The whole assembly has a diameter of ~20 nm and the bright spots in the range of ~2-3 nm are caused by the Fe-content of discrete CytC protein molecules.[2] EDS analysis confirmed the presence of Fe inside (a, c) the nanoassemblies and none in the outer space (c). As expected, we did not observe any bright spots of Fe in NA-Empty^{PEG} particle (d, inset) and EDS confirms the absence of Fe in it(d).

Estimation of approximate number of proteins per particle:

Example for NA-Lys^{ED}:

Diameter of nanoassembly, D = 9.7 nm, radius, R = 4.85 nm

Volume of each particle, $V_{PPC} = (4/3)\pi R^3 = (4/3)\,\pi(4.85)^3$ nm^3 = 477.6×10^{-21} cm^3

Again, for lysozyme, diameter of protein, d = 3.6 nm, radius, r = 1.8 nm

Volume of each protein, $V_{Ly} = (4/3)\pi r^3 = (4/3)\,\pi(1.8)^3$ nm^3 = 24.4×10^{-21} cm^3

Assuming that maximum sphere packing efficiency to be ~74%,[3] number of lysozyme per nanoassembly to be = $(477.6 \times 10^{-21} / 24.4 \times 10^{-21}) \times (0.74) = 14$

The results for all other nanoassemblies are summarized below:

Table 5: Theoretically calculated number of proteins per nanoassembly based on size:

Sample	# of Protein per PPC particle
NA-Lys^{ED}	14
NA-Lys^{PEG}	14
NA-RNaseA^{ED}	16
NA-RNaseA^{PEG}	18
NA-CytC^{ED}	7
NA-CytC^{PEG}	6

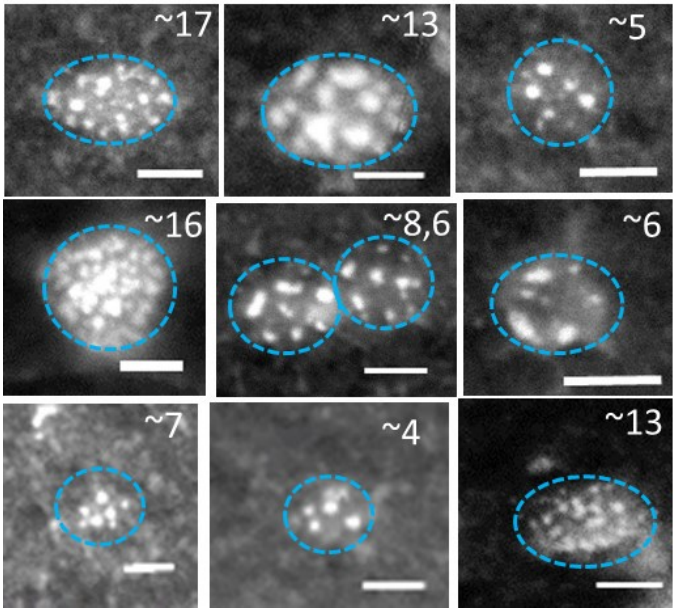


Figure S11. HAADF images of different NA-CytC^{PEG} particles. The bright spots with a diameter of 2-3 nm are caused by the Fe-content of the Cyt C. This allows to identify and approximately count number of CytC proteins per particle through Image J software (see discussions in Figure S8b), approx. # of proteins are reported in the inset, scale bar: 20 nm.

Enzymatic degradation (trypsin digest) study:

Polymer-protein conjugate solutions and native proteins (lysozyme, RNase A and cytochrome C) were subjected to enzymatic degradation study to evaluate the stealth power of polymeric nanoassemblies to encapsulate and protect the sensitive cargos from protease mediated cleavage. Sample solutions were prepared with polymer-protein complexes (with final protein

concentration of 0.39 mg/mL based on previous protein analysis) in $NaHCO_3$ buffer (pH=8.0). The concentrations of native proteins in each control sample were also kept identical for comparison purpose. After that 10% acetonitrile was added to each sample to denature the protein and incubated at 50 ºC for 45 minutes. For RNase A, samples were treated with 15% AcOH and incubated at 90 ºC for 4 h. After hydrolysis, samples were freeze-dried and finally added 10% acetonitrile and 90% $NaHCO_3$ buffer of pH 8.0. Finally, all samples were digested with trypsin from porcine pancreas at a ratio of 1:25 (trypsin:protein) at 37 ºC for 17 h. After digestion samples were collected by centrifugation and subjected to MALDI-MS analysis. The matrix was prepared with a solvent mixture of acetonitrile, water and trifluoroacetic acid (with a ratio 50:47.5:2.5) containing 10 mg/mL α-cyano-hydroxycinnamic acid. The matrix and digested samples were mixed at 1:1 ratio and spotted on the MALDI target for fragmental analysis.

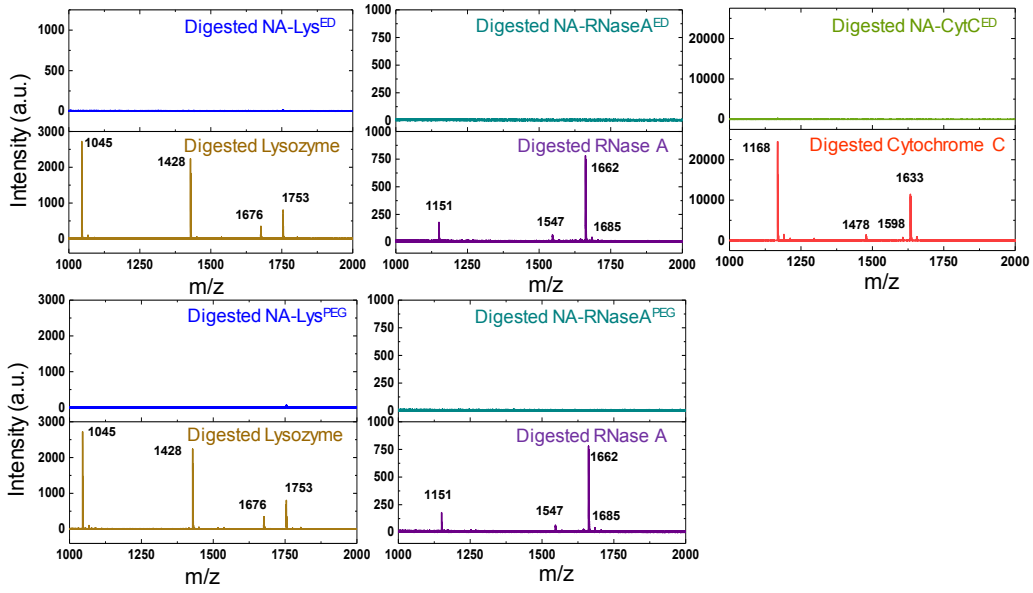


Figure S12. Trypsin digest for ED and PEG-crosslinked polymer-protein nanoassemblies

Table 6. Major MS-Digest fragments for Lysozyme, RNase A and Cytochrome C from MALDI-MS analysis

Lysozyme

m/z	Start	End	Sequence
1045	135	143	(K)GTDVQAWIR(G)
1428	52	63	(K)FESNFNTQATNR(N)
1676	116	130	(K)IVSDGNGMNAWVAWR(N)
1753	64	79	(R)NTDGSTDYGILQINSR(W)

RNase A

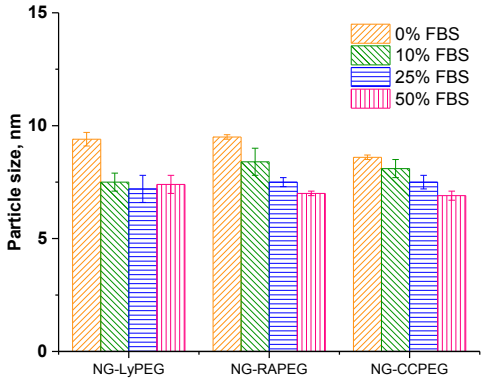
m/z	Start	End	Sequence
1151	1	10	(-)MPAPATTYER(I)
1547	85	98	(K)LWSSLTLLGSYKGK(N)
1662	1	14	(-)MPAPATTYERIVYK(N)
1685	26	41	(R)LEFQDGGVGLTAAQFK(Q)

Cytochrome C

m/z	Start	End	Sequence
1168	29	39	(K)TGPNLHGLFGR(K)
1478	89	100	(K)KTEREDLIAYLK(K)
1478	90	101	(K)TEREDLIAYLKK(A)
1598	40	54	(R)KTGQAPGFTYTDANK(N)
1633	10	23	(K)IFVQKCAQCHTVEK(G)

Assessment of serum stability of NA-Lys^{PEG}, NA-RNaseA^{PEG} and NA-CytC^{PEG} nanoassemblies

To the stability of nanoassemblies in serum is considered to be an important criterion to perform as an efficient delivery vehicle. The serum stability of the PPCs were performed by monitoring the changes in particle size through DLS.[4] All samples were incubated with differential amounts of serum (0%, 10%, 25% and 50%) for 6 h at 37 ºC before subjecting to DLS measurements. Conjugates were found to quite stable with negligible shifts in the particle sizes confirming no protein adsorption leading to aggregation and biofouling.



Sample	0% FBS, nm	10% FBS, nm	25% FBS, nm	50% FBS, nm
NA-Lys^{PEG}	9.4 ± 0.3	7.5 ± 0.4	7.2 ± 0.6	7.4 ± 0.4
NA-RNaseA^{PEG}	9.5 ± 0.1	8.4 ± 0.6	7.5 ± 0.2	7.0 ± 0.1
NA-CytC^{PEG}	8.6 ± 0.1	8.1 ± 0.4	7.5 ± 0.3	6.9 ± 0.2

Figure S13. Particle size analysis of nanoassemblies in presence of serum

Lysine residues in proteins

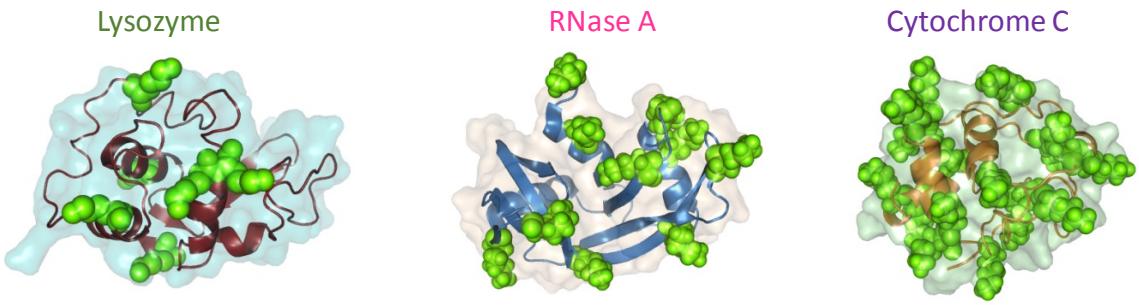


Figure S14. Lysine residues in Lysozyme (#6), RNase A (#10) and Cytochrome C (#19)

SDS-PAGE for protein-polymer conjugation and release studies:

30 µL of different samples containing NA-Empty^{ED}, NA-Empty^{PEG}, NA-Lys^{ED}, NA-Lys^{PEG}, NA-RNaseA^{ED}, NA-RNaseA^{PEG}, NA-CytC^{ED} and NA-CytC^{PEG} were mixed with 10 µL of loading buffer (DTT free) and 25 µL of each sample was loaded on acrylamide gel. For release experiment, identical protein-polymer conjugate samples were treated with 10 mM DTT and incubated at 37 ºC for 4 h before subjecting to acrylamide gel electrophoresis. To calculate the amount of released protein from each sample, standard curves were generated from the known concentrations of pure protein samples loaded into the gel lanes. The gel image analysis and quantification were performed with Bio-Rad Image Lab^{TM} software.

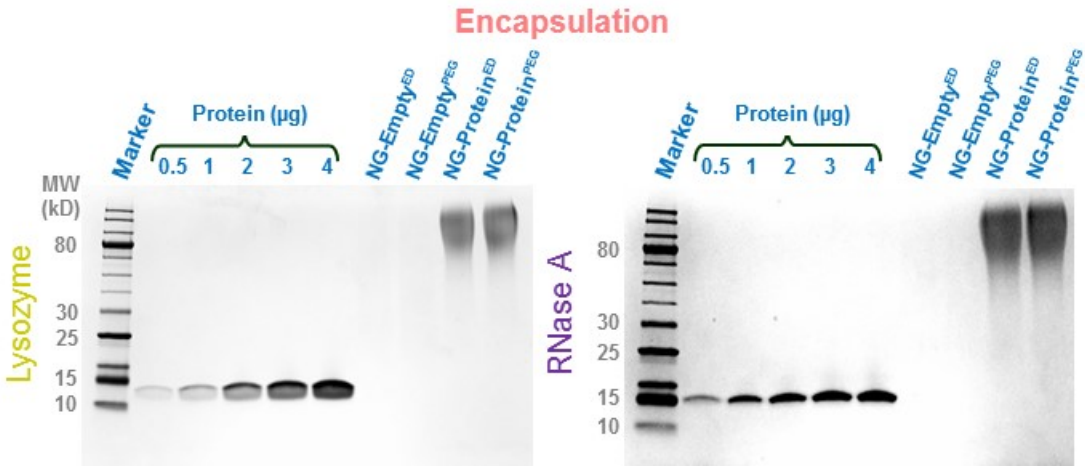


Figure S15. SDS-PAGE for encapsulation analysis with nanoassemblies containing Lys and RNaseA

Release kinetics of proteins from the protein-polymer nanoassemblies:

To monitor the release kinetics of proteins (lysozyme, RNase A and cytochrome C), 30 µL of NA-Lys^{ED}, NA-Lys^{PEG}, NA-RNaseA^{ED}, NA-RNaseA^{PEG}, NA-CytC^{ED} and NA-CytC^{PEG} samples were incubated at 37 ºC with requisite amounts of 10 mM DTT for different time intervals. After each incubation time, samples were collected and immediately frozen at -20 ºC. Finally, all samples were subjected to SDS-PAGE analysis to quantify the amount of released proteins.

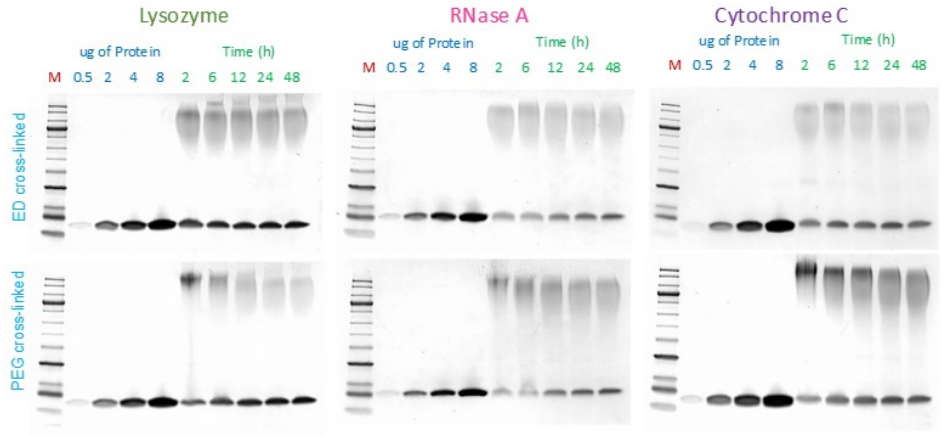


Figure S16. Release kinetics of proteins from the nanoassemblies by SDS-PAGE at disulfide of polymer to DTT ratio 1:1

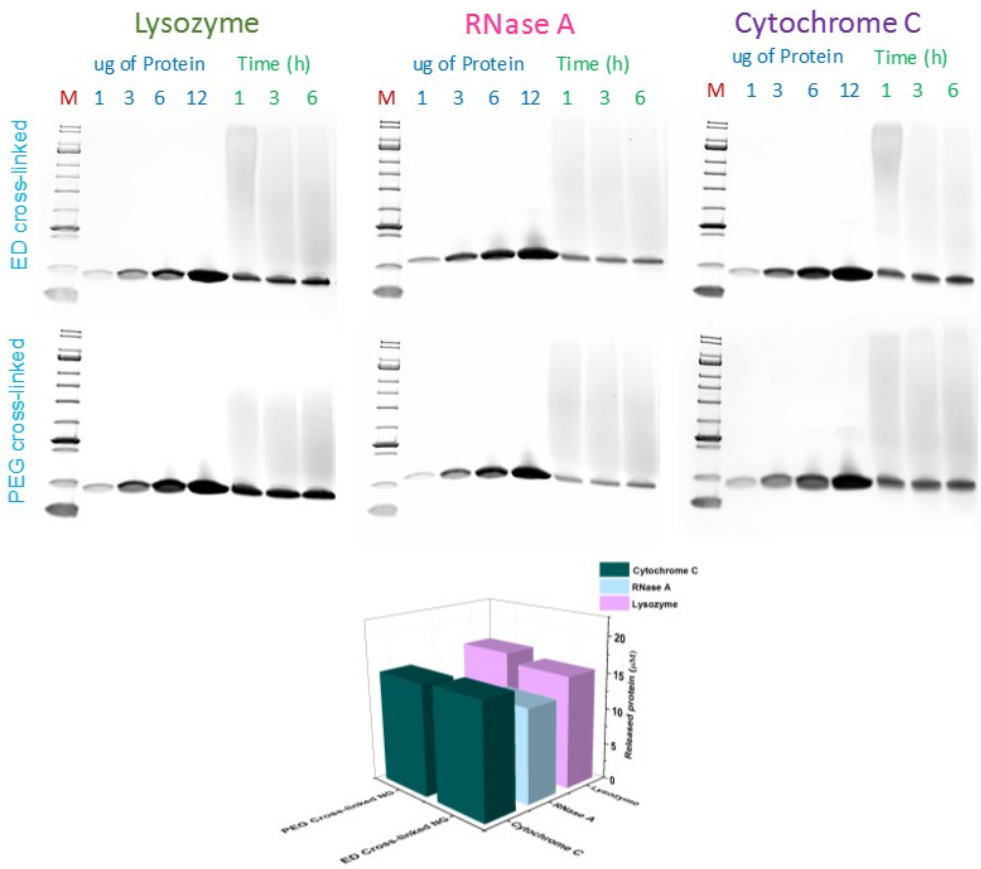


Figure S17. Release kinetics of proteins from the PPCs by SDS-PAGE at disulfide of polymer to DTT ratio 1:10, quantification data provided in the 3D bar graph after 6 h of release.

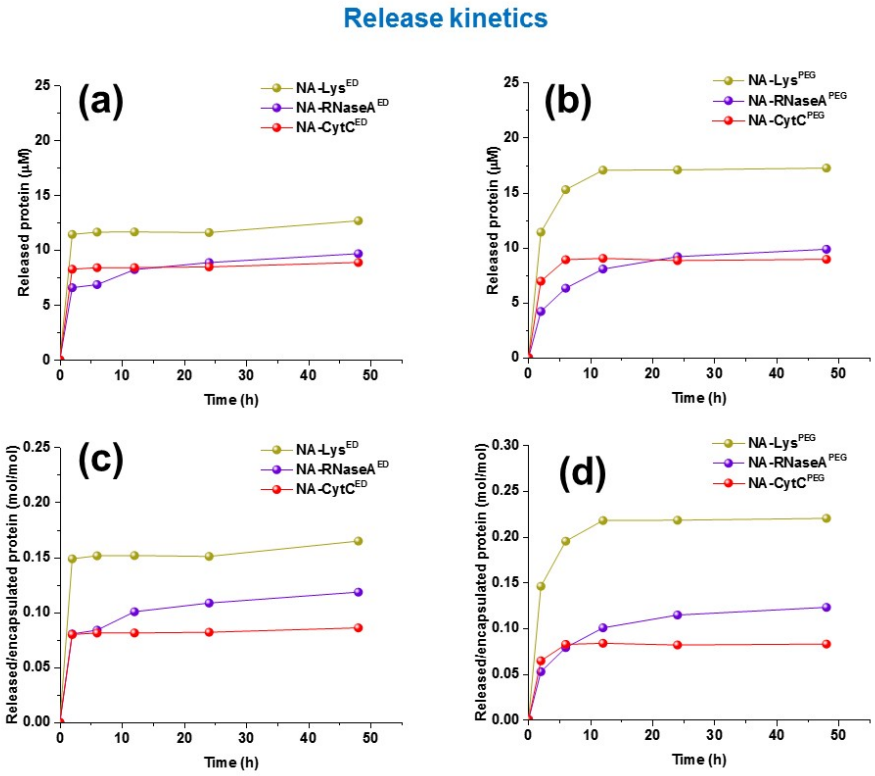


Figure S18. Release kinetics of proteins from the nanoassemblies: (a, b) Plot of concentration of protein released (μM) vs. time; (c, d) Normalized plot of released/encapsulated protein vs. time. Although the concentration/amount of released proteins are approximately same for both RNaseA and Cyt C, the normalized plots (moles of released/encapsulated protein vs. time) differentiates the protein release behavior more clearly and the released protein amounts for each can be correlated with their lysine content.

Activity assays:

To measure the activity of released proteins from different polymer-protein conjugates, first samples were treated with 10 mM DTT and incubated at 37 °C for 4 h. Identical samples were subjected to 50 μM DTT mimicking extra-cellular reducing environment and incubated under similar condition.

SpectraMax® M5 spectrophotometer (Molecular Devices) was utilized for evaluating all activities through absorbance and fluorescence measurements.

a. For lysozyme:

The EnzChek® Lysozyme Assay Kit (Thermo-Fisher Scientific) was used to check the lysozyme activity on a substrate based on *Micrococcus lysodeikticus* cell walls which was labeled with

fluorescein to such an extent that the fluorescence is quenched. Due to lysozyme's enzymatic activity, the mucopolysaccharide cell walls of the labelled microorganism containing β-(1-4)-glucosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues were hydrolysed releasing proportional amount of fluorescein. To perform the assay, 20 µL sample was mixed with 20 µL prepared substrate solution and subjected to fluorescence measurement (Ex/Em: 494/518 nm) over 1h with SpectraMax® M5 spectrophotometer.

b. For RNase A:

RNaseAlert® activity kit (Thermo-Fisher Scientific) was used to check the activity of released RNase A for all samples. RNase A cleaves the oligonucleotide substrate of the assay consisting a fluorophore and a quencher present at two extreme ends, thus releasing the fluorophore which can be detected and quantified with a fluorometer. For a typical kinetic experiment, the substrate was mixed with 5 µL test buffer, 35 µL nuclease free water and 10 µL sample (diluted X10,000 from DTT experiment). 40 µL of the prepared sample mixture was transferred to a black 96-well plate and immediately measured for fluorescence (Ex/Em: 490/520 nm) with SpectraMax® M5 spectrophotometer over a 30 min time course.

b. For cytochrome C:

The peroxidase activity of cytochrome C was determined by examining the catalytic conversion of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Cytochrome C catalyzes the reduction of H_2O_2 to water which is coupled with one-electron oxidation of chromogenic ABTS forming a brilliant blue-green ABTS radical cation. The assay kinetics can be monitored by observing the changes in absorbance spectra of the radical cation at 418 nm. Before subjecting to activity measurement, DTT treated samples were washed thoroughly with PBS buffer of pH 7.4 to remove DTT and other byproducts. The test solution was prepared by mixing 100 µL sample solution with 400 µL H_2O_2 (25 mM) and 500 µL ABTS (1 mg/mL). Absorbance spectra were recorded for all samples at 418 nm for a time course of 5 min using SpectraMax® M5 spectrophotometer.

Table 7. Abbreviations used in activity assay plots

Sample	Details	Sample	Details
Protein_DTT 0.05 mM	Native protein treated with 0.05 mM DTT	NA-Protein^{PEG} 0.05 mM	Protein encapsulated PEG-crosslinked nanoassemblies treated with 0.05 mM DTT
Protein_DTT 10 mM	Native protein treated with 10 mM DTT	NA-Protein^{ED} 10 mM	Protein encapsulated ED-crosslinked nanoassemblies treated with 10 mM DTT
NA-Empty^{ED}	Empty ED-crosslinked nanoassemblies treated with 10 mM DTT	NA-Protein^{PEG} 10 mM	Protein encapsulated PEG-crosslinked nanoassemblies treated with 10 mM DTT
NA-Empty^{PEG}	Empty PEG-crosslinked nanoassemblies treated with 10 mM DTT	Blank	Only phosphate buffer, pH 7.4
NA-Protein^{ED} 0.05 mM	Protein encapsulated ED-crosslinked nanoassemblies treated with 0.05 mM DTT		

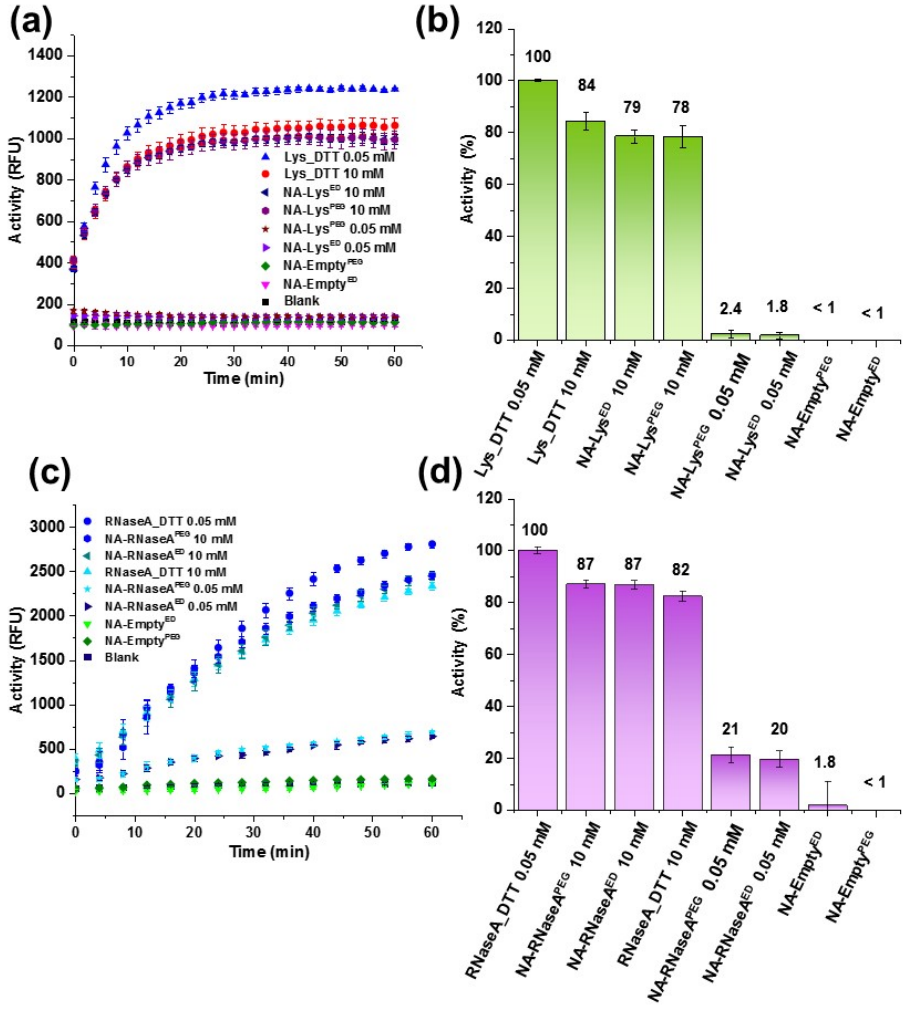


Figure S19. Activity of released proteins from nanoassemblies containing (a-b) Lysozyme; (c-d) RNase A.

Circular dichroism (CD) spectra:

CD spectra of the released and native protein samples were recorded on JASCO J-1500 spectrophotometer. In a typical experiment, NA-protein sample was incubated with requisite amount of DTT for 24 h. After that, the sample was dialyzed against PBS buffer pH 7.4 with a membrane MWCO 20 kDa for 2 days to separate the polymer. Finally, the purified sample was concentrated with Amicon Ultra Centrifugal Filters MWCO 3K and the concentration was measured with Pierce 660 nm Protein Assay Reagent. For recording the spectra, 200 µL protein solution was injected into a quartz cuvette of 1-mm path length, equilibrated at 25 ºC for 10 min and scanned from 190 to 250 nm (scan rate: 20 nm/min, interval: 0.2 nm, average of three spectra).

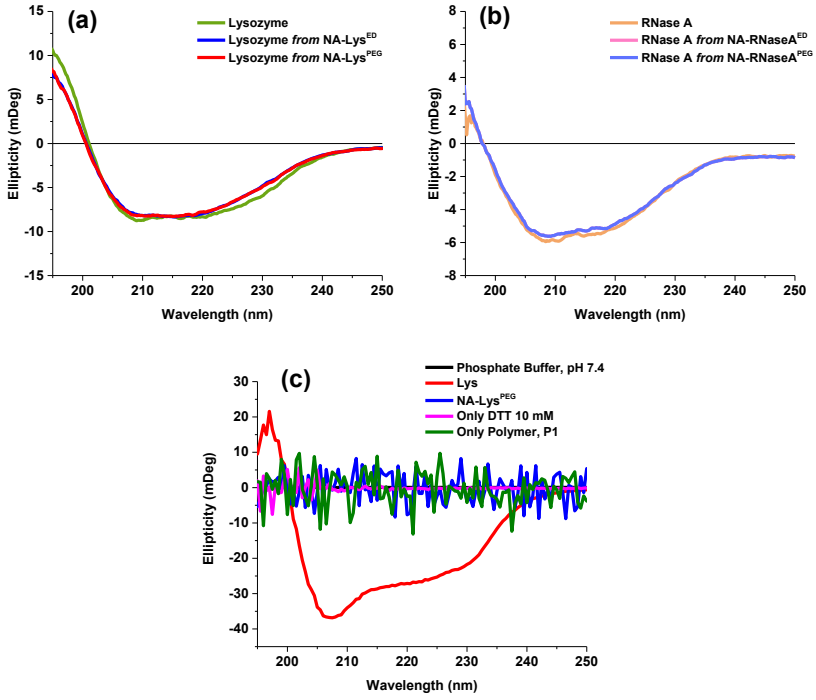


Figure S20. CD spectra of native proteins and polymer-protein nanoassemblies

MALDI-MS spectra for the released proteins:

MALDI-MS analyses were performed with Bruker Autoflex III time-of-flight mass spectrometer. All mass spectra were acquired in the reflectron mode with an average of 500 laser shots at ~60% optimized power.

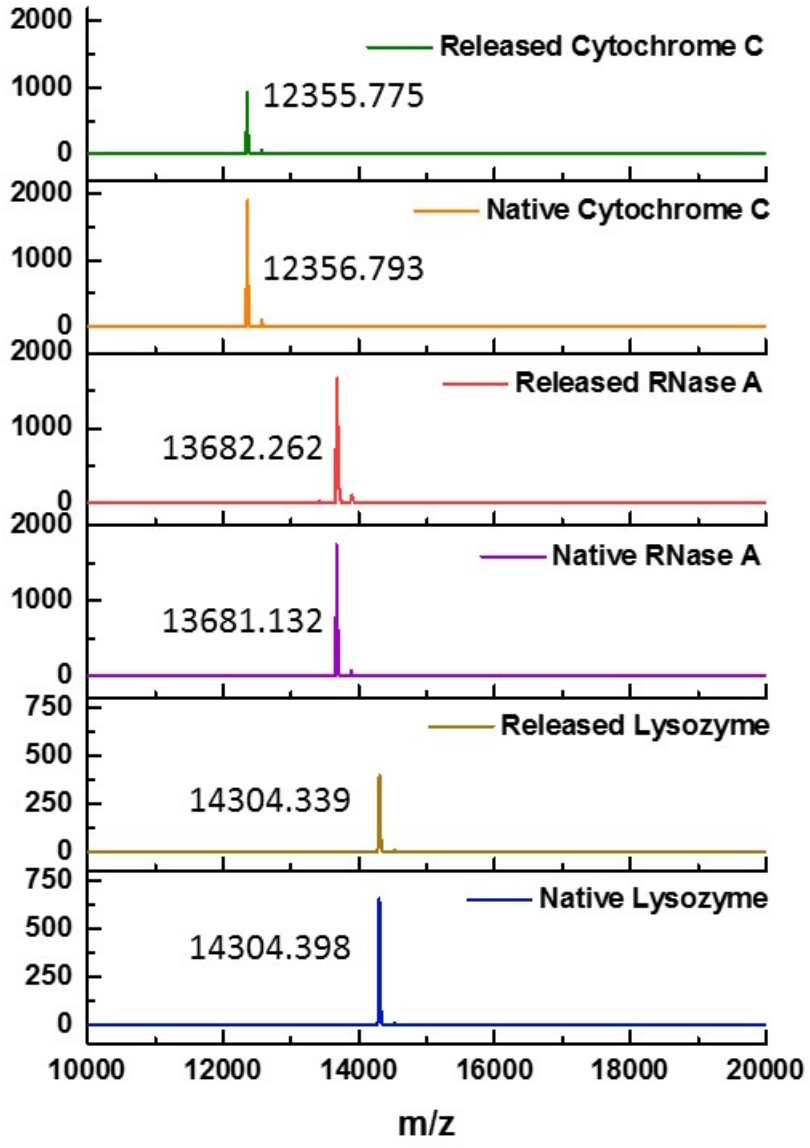


Figure S21. Comparison of MALDI-MS spectra of the native proteins and released proteins from nanoassemblies.

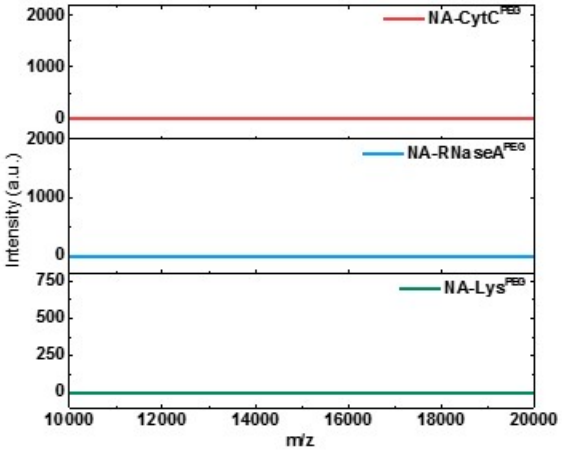


Figure S22. MALDI-MS spectra of the NA-Protein^{PEG} nanoassemblies.

Labeling of Proteins with Rhodamine B:

To perform the cell-uptake studies, fluorescence-labelled proteins (lysozyme, RNase A and cytochrome C) were prepared with Rhodamine B isothiocyanate (RB). In a typical labelling procedure, proteins (4 mg) were dissolved separately in 2 mL of 0.1 M $NaHCO_3$ buffer (pH 8.5) and stirred for 15 min at 4 ºC. RB (5 eq. of each protein, 10 mg/mL in DMSO) was added dropwise to each protein solution and stirred at 4 ºC for 2 h protected from light. The RB-labelled-proteins were purified by extensive dialysis with 50 mM Tris pH 7.4 and 50 mM NaCl mixture to remove excess RB and concentrated using 3 kDa Amicon Ultra Centrifugal Filters. Protein concentrations in each labelled conjugate were calculated using UV-Vis spectroscopy. The molar ratio of RB and labelled lysozyme, RNase A and cytochrome C were estimated to be 0.62, 0.43 and 0.63, respectively.

All labelled polymer-protein conjugates were prepared with the RB-labelled proteins following the method described under ‘Synthesis of polymer−protein nanoassemblies’.

Cell culture:

Human cervical carcinoma (HeLa) cells were cultured in T75 cell culture flask containing Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) in a humidified

incubator with 5% CO_2 at 37 °C. Culture media was supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic-antimycotic (100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Amphotericin B).

Cellular uptake studies for protein delivery and endosomal escape:

Cell internalization studies were performed with HeLa cells seeded at 100,000 cells/mL in glass-bottomed petri-dishes and cultured for 1 day at 37°C in a 5% CO_2 incubator. Prior to delivery cells were washed three times with PBS buffer and incubated with 1 mL media containing 6 µL polymer-Rhodamine B-protein conjugate or Rhodamine B-protein conjugate (protein concentration 1 mg/mL) at 37 °C for 4 h. After that, cell nucleus was stained with Hoechst 33342 (8 µM) and finally the media was replaced with fresh stock and incubated for another 1 h before subjecting to CLSM analysis. In addition, to study the endosomal escape of the labelled proteins, HeLa cells were incubated with labelled nanoassemblies for 4 and 24 h. After that it was stained with LysoTracker® Green to label endosomes/lysosomes and studied the co-localization of red and green fluorescence channels. Live cell imaging was performed using Nikon Spectral $A1^+$ confocal microscope.

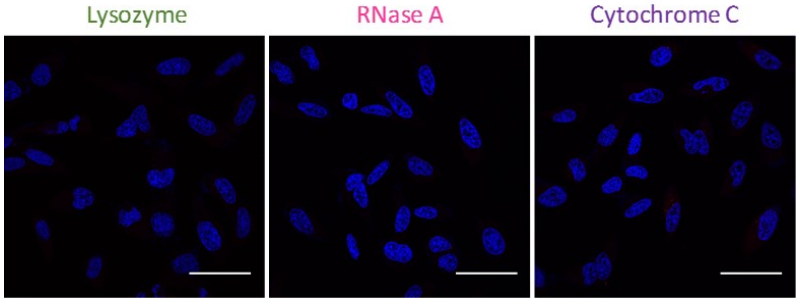


Figure S23. Negligible uptake for HeLa cells incubated with only proteins, cell nucleus was stained with Hoechst 33342, scale bar: 50 µm.

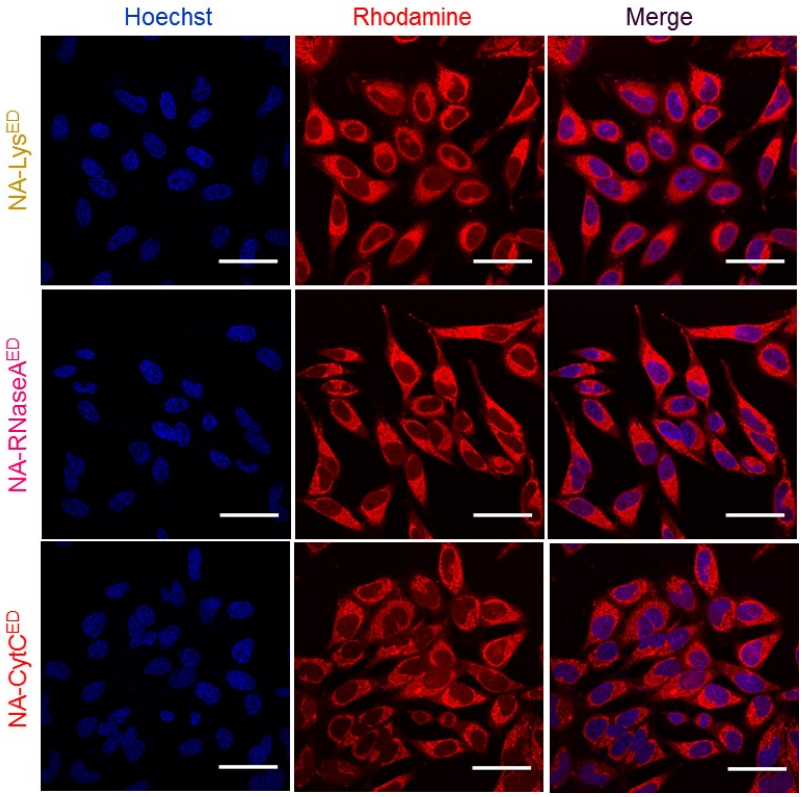


Figure S24. Cellular internalization with ED-crosslinkied nanoassemblies, cell nucleus was stained with Hoechst 33342, scale bar: 50 µm.

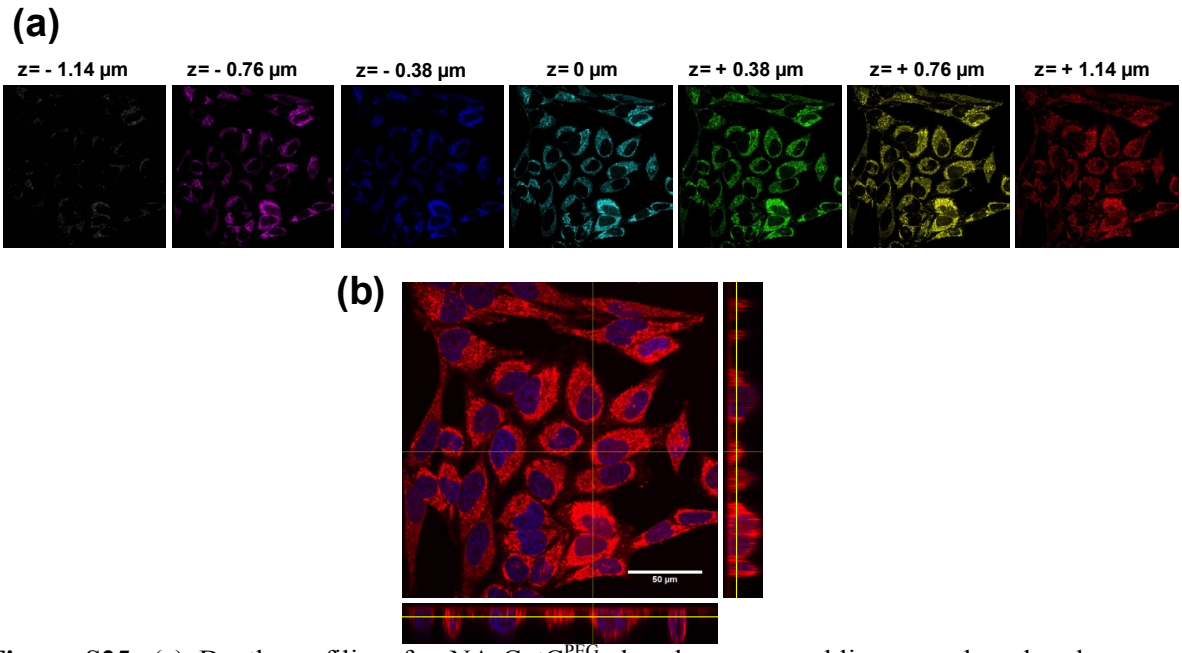


Figure S25. (a) Depth profiling for NA-CytC^{PEG} dosed nanoassemblies: pseudo-colored consecutive slices through z-axis and (b) z-stack orthogonal projection from CLSM experiment showing localization of cytochrome C inside HeLa cells.

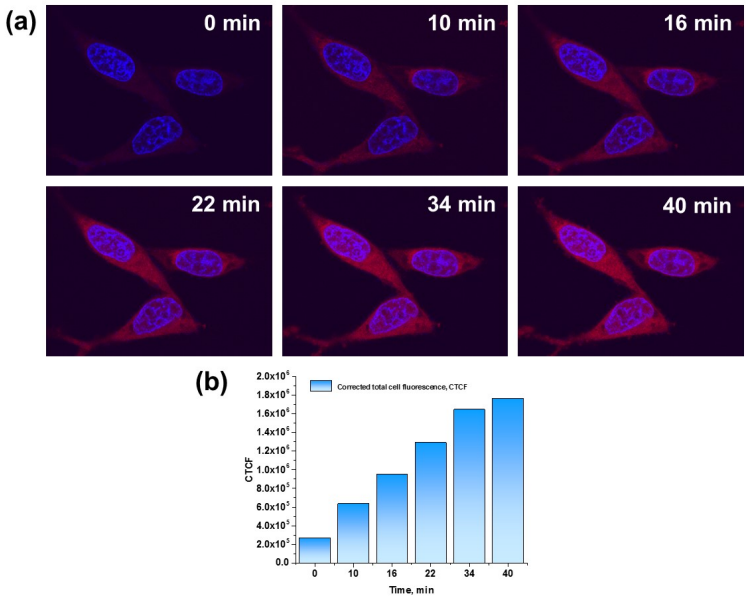


Figure S26. (a) Time course of uptake for NA-CytC^{PEG}, (b) Fluorescence intensity measurement from the red channel by Image J software[5] at different time points of uptake experiment, expressed as Corrected total cell fluorescence, CTCF = Integrated Density - (Area of selected cell × Mean fluorescence of background).[6]

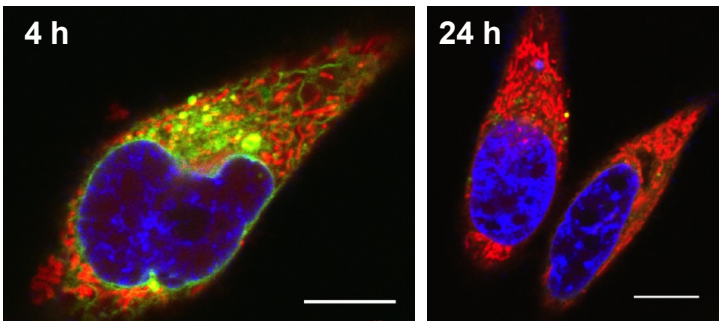


Figure S27. Endosomal escape studies by co-localization of LysoTracker Green and Rhodamine B tagged protein; co-localization of dyes after 4 h incubation confirms existence in the endosomes and after 24 h distinct red fluorescence confirms release of proteins into the cytosol from endosomes. Scale bar: 10 µm.

Cell viability with alamarBlue® assay:

HeLa cells were seeded into 96-well tissue culture plates at a density of 5000 cells/well/100 µL sample and incubated at 37 °C. After 24 h, culture media was replaced and cells were treated with different concentrations of protein-polymer conjugates and control protein samples (0.1 mg/mL to 2 mg/mL NA-Protein^{PEG} concentration; for naked protein concentration is matched with the encapsulated one in the NA-Protein sample and can be calculated from encapsulation efficacies, 2 mg/mL of NA-CytC^{PEG} refers to 126 µg/mL of cytochrome C) in 100 µL media. All

samples were incubated for 6 h at 37 °C, then the media was replaced and incubated for another 66 h at 37 °C. Afterwards media was replaced, washed with PBS buffer for three times and each well was treated with 100 µL 10% alamarBlue in media with serum. Finally, samples were incubated for 1 h and subjected to fluorescence measurement with SpectraMax® M5 at 560 nm excitation/590 nm emission wavelength in a black 96-well flat bottomed plate.

Study of apoptosis with NA-CytC^{PEG} nanoassembly:

HeLa cells were seeded at 40,000 cells/mL density in glass-bottomed petri-dishes and cultured for 1 day at 37°C in a 5% CO_2 incubator. Cells were washed three times with PBS buffer and incubated with 1 mL media containing NA-CytC^{PEG} conjugate (2 mg/mL) at 37 °C for 4 h. After that, the media was replaced and cells were incubated for another 68 h. To detect the apoptotic cells, each sample was treated with CellEvent™ Caspase-3/7 Red Detection Reagent (10 µM) and hoechst 33342 (8 µM) to stain the nucleus by incubating for 30 min before subjecting to CLSM analysis. The apoptosis assay reagent consists of a DEVD peptide attached to a nucleic acid-binding cy5-dye. When bound with the peptide, the dye becomes intrinsically non-fluorescent as the DEVD peptide retards the DNA-binding ability of the dye. Once caspase-3/7 enzymes are activated in apoptotic cells by the delivery of cytochrome C, the DEVD peptide is cleaved by those and enable the dye to bind to DNA to produce a bright, fluorogenic response. Co-localization of blue (hoechst) and red (cy5) channels was studied to check the nuclei of the apoptotic cells.

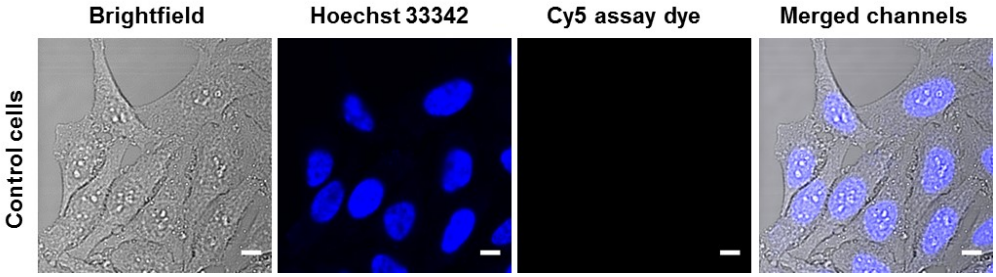


Figure S28. Detection of activated caspase-3/7 after 72 h in control HeLa cells treated with NA-Empty^{PEG} sample; scale bar: 10 µm, no co-localization of hoechst and cy5-tagged assay reagent was observed in the nucleus.

Encapsulation, release and cellular internalization studies with eGFP

To check the robustness of the polymeric delivery system, we investigated the encapsulation and release properties of eGFP that has higher MW and lower pI (MW ~27 kDa, pI 5.5[7]) compared to lysozyme, RNase A and cytochrome C (MW between 12-15 kDa and pI >8.5[8]). eGFP was encapsulated within polymer matrix by the reactive coupling method described in "synthesis of polymer-protein nanoassemblies" section, and was subsequently released with the aid of redox-stimuli DTT (see section 'SDS-PAGE for protein-polymer conjugation and release studies', Figure S26). NA-eGFP^{PEG} assemblies were subjected to cellular uptake studies with HeLa cells (dosage 2 mg/mL, see previous 'cellular uptake studies for protein delivery' section) and imaged using confocal microscope. Evenly distributed green fluorescence of delivered eGFP (Figure S30) demonstrates successful delivery of the protein into HeLa cells.[9,10]

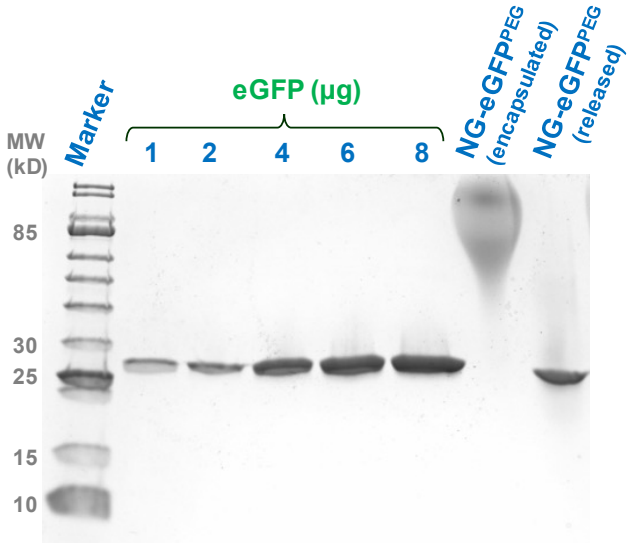


Figure S29. SDS-PAGE for encapsulation and release analysis with nanoassembly containing eGFP.

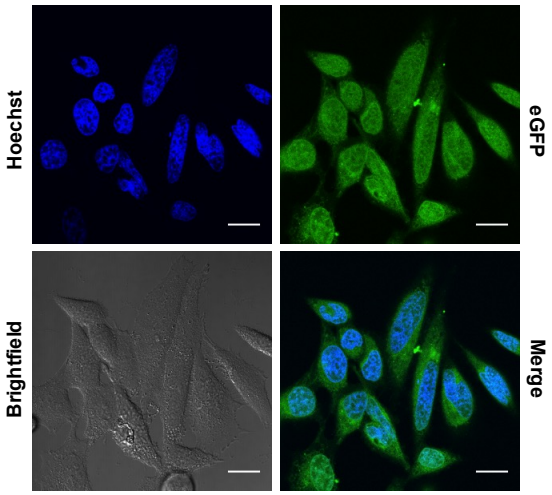


Figure S30. Cellular internalization with NA-eGFP^{PEG} nanoassemblies, cell nucleus was stained with Hoechst 33342, scale bar: 20 µm.

Acknowledgement

We thank Francesca Anson for providing eGFP.

References

[1] Ghosh, S.; Basu, S.; Thayumanavan, S. *Macromolecules* **2006**, *39*, 5595.
[2] Plascencia-Villa, G.; Ponce, A.; Collingwood, J. F.; Arellano-Jiménez, M. J.; Zhu, X.; Rogers, J. T.; Betancourt, I.; José-Yacamán, M.; Perry, G. *Sci. Rep.* **2016**, *6*, 24873.
[3] Based on *Carl Friedrich Gauss* theory on close packing of sphere in space: highest average packing density is given by $\pi/3\sqrt{2} \approx 0.74$
[4] Mohr, K.; Müller, S. S.; Müller, L. K.; Rusitzka, K.; Gietzen, S.; Frey, H.; Schmidt, M. *Langmuir* **2014**, *30*, 14954.
[5] Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. *Nat. Methods* **2012**, *9*, 671.
[6] Burgess. A.; Vigneron, S.; Brioudes, E.; Labbé, J-C.; Lorca, T.; Castro, A. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 12564.
[7] Protein identification and analysis tools on the ExPASy server.
[8] Chiti, F. Relative Importance of Hydrophobicity, Net Charge, and Secondary Structure Propensities in Protein Aggregation. In *Protein Misfolding, Aggregation and Conformational Diseases: Part A: Protein Aggregation and Conformational Diseases* [Online]; Uversky, V. N.; Fink, A., Eds; ProteinReviews; Atassi, M. Z., Series Ed.; Springer Science & Business Media, 2007; Chapter 3, Volume 4, pp 43-59. http://www.springer.com/us/book/9780387259185.
[9] Seibel, N. M., Eljouni, J., Nalaskowski, M. M., Hampe, W. *Anal. Biochem.* **2007**, *368*, 95.
[10] Dingwall, C.; Laskey, R. A. *Annu. Rev. Cell Biol.* **1986**, *2*, 367.