Supporting Information for:

Concurrent Binding and Delivery of Proteins and Lipophilic Small Molecules Using Polymeric Nanogels

Daniella C. González-Toro,[‡] Ja-Hyoung Ryu,[‡,†] Reuben T. Chacko, Jiaming Zhuang and S. Thayumanavan*

Department of Chemistry, University of Massachusetts Amherst, Massachusetts 01003

Email: thai@chem.umass.edu

Materials and Methods:

All chemicals, 2,2′-Dithiodipyridine, polyethylene glycol monomethyl ether methacrylate (MW 450), D,L-dithiothreitol (DTT), 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI), β-galactosidase (β-gal) from *E. coli,* 2-nitrophenyl-β-D-galactopyranosidase, UltraPure Agarose and solvents were purchased from commercial sources and were used as received, unless otherwise mentioned. Pyridyl disulfide ethyl methacrylate (PDSEMA) was prepared using a previously reported procedure (*Macromolecules* **2006**, *39*, 5595-5597). ^{1}H-NMR spectra were recorded on a 400mHz Bruker NMR spectrometer using the residual proton resonance of the solvent as the internal standard. Chemical shifts are reported in parts per million (ppm). Molecular weights of the polymers were estimated by gel permeation chromatography (GPC) using PMMA standard with a refractive index detector. Dynamic light scattering (DLS) measurements were performed using a Malvern Nanozetasizer. UV-vis absorption spectra were recorded on a Varian (model EL 01125047) spectrophotometer. The fluorescence spectra were obtained from a JASCO FP-6500 spectrofluorometer.

Experimental Section:

Synthesis of Random Copolymer: A mixture of 2-cyano-2-propyl benzodithioate (21 mg, 0.0949 mmol), PDSEMA (860 mg, 3.37 mmol), polyethylene glycol monomethyl ether methacrylate (620 mg, 1.31 mmol) and AIBN (5.0 mg, 0.0305 mmol) was dissolved in THF (3 mL) and degassed by performing three freeze-pump-thaw cycles. This reaction vessel was sealed and then placed in a pre-heated oil bath at 60 ^{o}C for 12 h. To remove unreactive monomers and purify the polymer, the resultant mixture was precipitated in cold ethyl ether (20 mL) to yield the random copolymer as a waxy solid. GPC (THF) M_n: 23 K. PDI: 1.38. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.45, 7.66, 7.09, 4.20-4.06, 3.90-3.36, 3.01, 2.15-1.62, 1.43-0.86. The molar ratio between two blocks was determined by integrating the methoxy proton in the polyethylene glycol unit and the aromatic proton in the pyridine and found to be 29%:71% (PEO:PDSEMA)

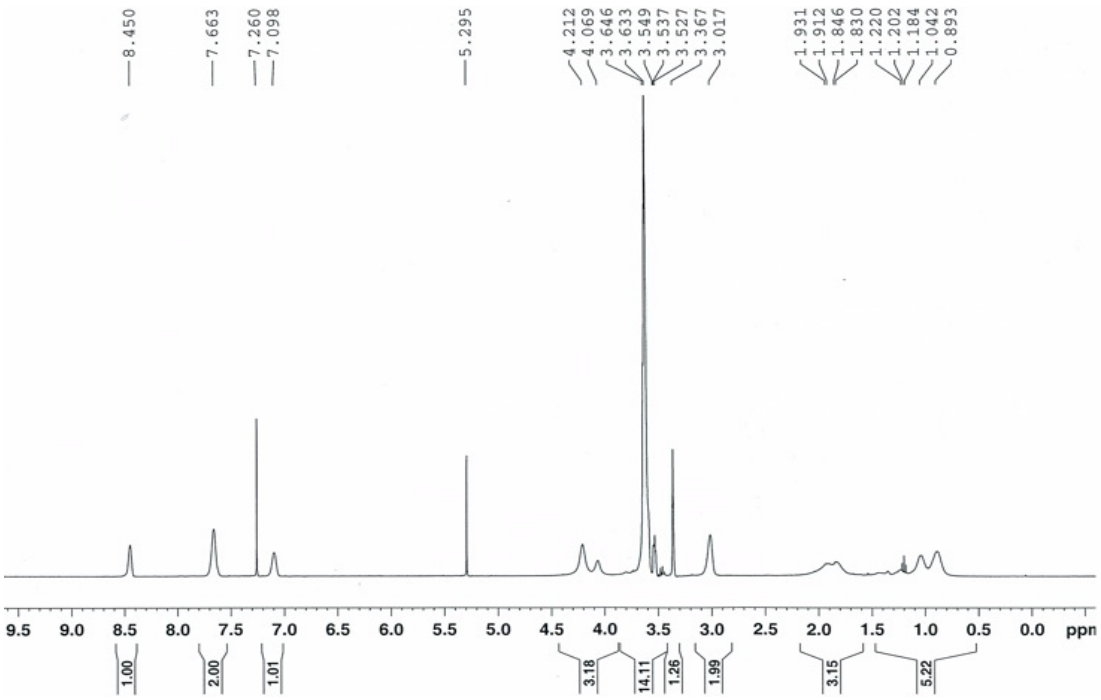

Figure S1. NMR spectra of PEO:PDSEMA polymer

Synthesis of CRRR peptide: 2-chloro-trityl chloride resin was selected as the solid support to prepare the peptide using solid phase synthesis. Coupling reaction was carried out in the presence of 3 equiv. of Fmoc protected amino acid, 3 equiv. of HATU and 3 equiv. of DIPEA and was tested by Kaiser Test. Deprotection of Fmoc group was obtained by treating the resin with 20% piperidine in DMF. The peptide was finally cleaved from resin by reacting with TFA/TIS/H_2O/EDT mixture. Precipitating the mixture in cold ether 5 times affords the crude peptide. Peptide was used without further purification. Yield: 80%. Peptide was characterized by ^{1}H NMR and MS. ^{1}H NMR (400 MHz, D_2O) δ:4.45-4.52, 4.30-4.41, 4.03-4.11, 3.15-3.26, 2.92-3.01, 1.73-1.96, 1.55-1.96. MS (FAB): exact mass calculated: 590.7. Found: 590.0.

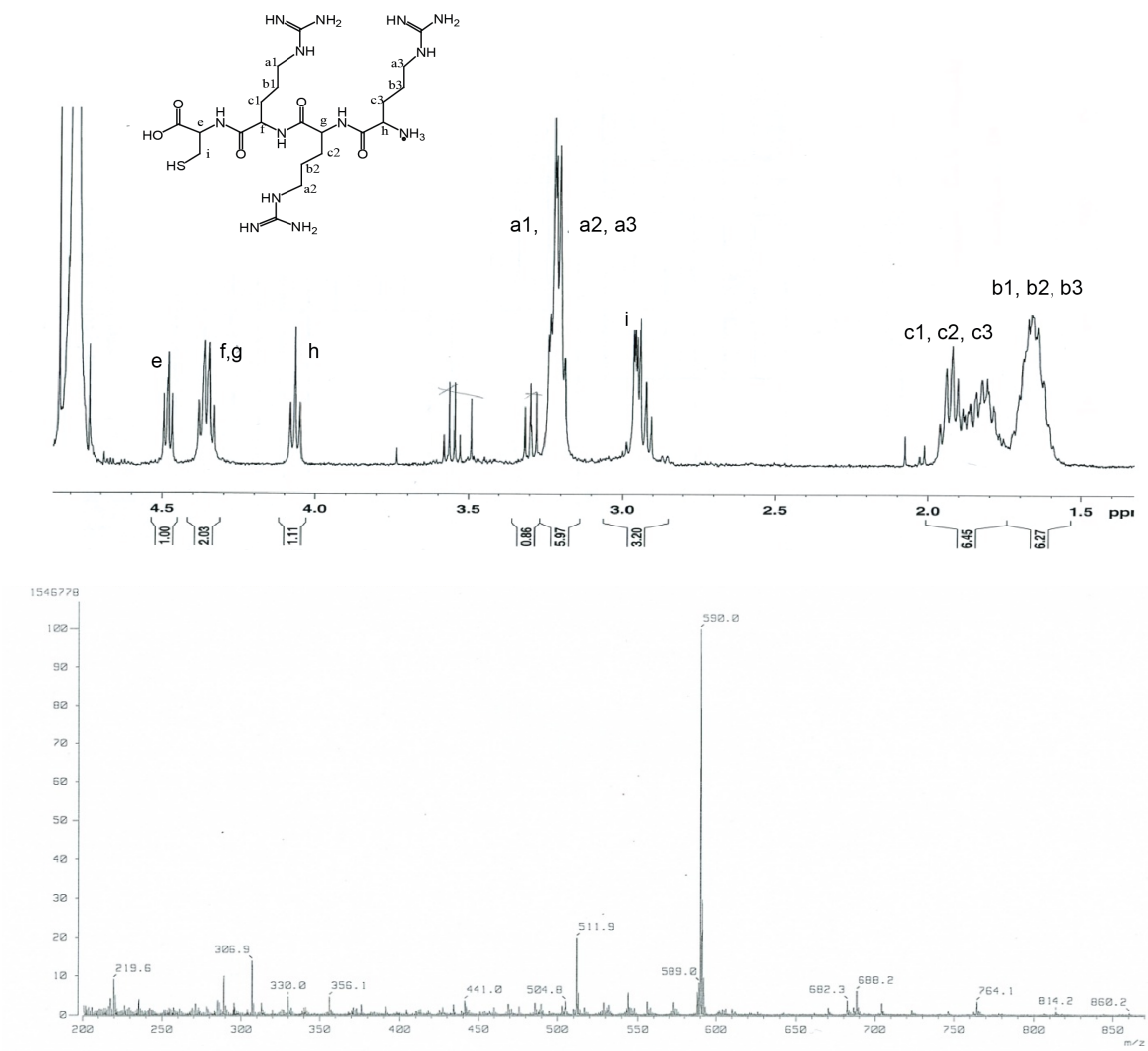

Figure S2: NMR and Mass spectra of triarginine peptide.

<u>Nanogel synthesis and surface modification:</u> The polymer (10 mg) was dissolved in water (1 mL) and the hydrophobic dye (0.2 mg of DiI) in acetone (100 µL) was added. The mixture was stirred for 6 h at room temperature, open to the atmosphere allowing the organic solvent to evaporate. To this micellar aggregate solution, was added a measured amount of DTT and then the mixture was stirred for 12 h at room temperature to allow for crosslinking. To modify the nanogel's surface, the ligand (5 mg), CRRR, was added and then stirred for another 12 h. The resulting nanogels were purified by filtration and unattached excess ligand was removed from the nanogel solution by ultrafiltration (triplicate) using a membrane with a molecular weight cutoff of 10,000 g/mol (Amicon Ultra cell-10K).

Crosslinking density and peptide modification: In order to determine the crosslinking density, UV-vis measurements were performed with samples of this solution diluted ten times. Once this was measured, we calculated the amount of pyridothione based on its known molar extinction coefficient (8.08 x 10^3 $M^{-1}cm^{-1}$ at 343 nm) (*Bioconjugate Chem.* **2006**, *17*, 1376-1384). The percentage of cross-linking was calculated by assuming that formation of a single, crosslinking disulfide bond would require cleavage of two PDS units and produce two pyridothione molecules. The attachment of the ligand was evaluated by further increase in pyridothione absorption (Figure S3).

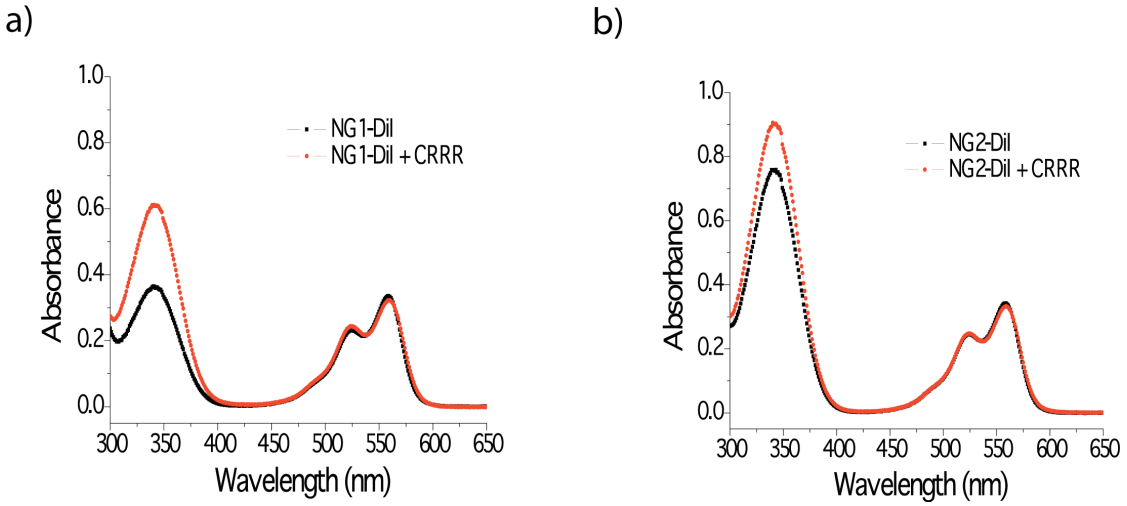

Figure S3. Absorbance of: (a) NG1 (10 mol % DTT) and (b) NG2 (20 mol% DTT) before and after modification with CRRR peptide.

Calculation of crosslinking density:

- Sample concentration in UV: 0.1mg/ml of polymer.
- The molar ratio of each unit: PDS:PEG= *x* mol : *y* mol=71:29 (from repeating unit by NMR)
- PDS molecular weight = 255 g/mol, PEG molecular weight = 475 g/mol

So, *x* mol * 255 g/mol + *y* mol * 275 g/mol= 0.1mg
x mol = 71/29 *y* mol

Therefore, *x mole* (PDS) is 2.23 * 10^{-7} mol in this solution

NG1

Absorbance is 0.31 at 343 nm.
By Beer's law,
A=εbc
0.31= 8.08*10^3 $M^{-1}cm^{-1}$ * 1 cm * c
c = 3.84 * 10^{-5} M
Therefore, 1ml of resulting nanogel solution contains 3.84 *10^{-8} mol pyridothione (byproduct).

It is 17 mol% of total PDS unit ($2.23*10^{-7}$ mol). We assume that two pyridothione are from one disulfide formation and PDS unit is 71 mol% of total polymer.
Therefore, 17 % /2 *0.71 = 6 % crosslinking density.

NG2
Absorbance is 0.703 at 343 nm.
By Beer's law,
$A=\varepsilon bc$
$0.703=8.08*10^{3}\ M^{-1}cm^{-1} * 1\ cm * c$
$c = 8.70 * 10^{-5}$ M
Therefore, 1ml of resulting nanogel solution contains $8.70 *10^{-8}$ mol pyridothione (byproduct).

It is 39 mol% of total PDS unit ($2.23*10^{-7}$ mol). We assume two pyridothiones are from one disulfide formation and PDS unit is 71 mol% of total polymer.
Therefore, 39 % /2 *0.71 = 14 % crosslinking density.

Loading Degree of Hydrophobic Dye (DiI):
We added 2 wt% DiI (0.2 mg/mL) into polymer solution (10 mg/mL). The final loading amount was calculated based on molar extinction coefficient of DiI (104 x 10^3 $M^{-1}cm^{-1}$) at 555 nm.
- The polymer concentration: 0.1mg/mL solution (diluted from 5 mg/mL stock from the nanogels used for UV measurement to 0.1mg/ml to final volume of 0.6mL)

NG1:
absorbance at 555nm= 0.310

So:
C= 0.310/ ($104x10^3$ $M^{-1}cm^{-1}$ x 1 cm) = $2.98 x 10^{-6}$ M

Conversion to grams of DiI:
2.98 µM X 961.32 g/mol x 0.6mL= 1.7µg DiI

wt% loading= DiI in nanogel/ weight total nanogel= 1.7µg/0.1mg x 100= 1.7wt%
Loading efficiency: loaded amount/feed amount= 1.7 µg / 2 µg x 100 = 85%

NG2:
absorbance at 555nm= 0.323

So:
C= 0.323/ ($104x10^3$ $M^{-1}cm^{-1}$ x 1 cm) = $3.11 x 10^{-6}$ M

Conversion to grams of DiI:
3.11 µM X 961.32 g/mol x 0.6mL= 1.8µg DiI

wt% loading= DiI in nanogel/ weight total nanogel= 1.8µg/0.1mg x 100= 1.8 wt%
Loading efficiency: loaded amount/feed amount= 1.8 µg / 2 µg x100 = 90%

Labeling of β-gal with FITC: Fluorescein isothiocyanate isomer I (FITC) was dissolved in dimethyl sulfoxide at a concentration of 4 mg/mL. The β-gal (2.5mg) was dissolved in 900µL of 0.1 M sodium bicarbonate solution (pH 9.0) and mixed with 250 µL freshly prepared FITC solution. The mixture was protected from light and stirred gently at room temperature for 2 h. The resulting FITC labeled β-gal was purified by size exclusion chromatography with Sephadex G-25 as stationary phase and phosphate buffer (5mM, pH 7.4) as mobile phase. Finally, the β-gal concentration and labeling efficiency were measured by UV-vis absorption spectroscopy.

Nanogel-protein complex optimization: NG-CRRR with encapsulated DiI (2 mg/mL) and FITC labeled β-gal (1 mg/mL) stock solutions were prepared in 5mM Sodium phosphate buffer. β-gal-NG-CRRR complex solutions were prepared from 1:1 to 1:6 ratios (β-gal:NG-CRRR) and incubated for 1h before spectroscopic measurements. The change of the intensity of FITC, which occurs by FRET between FITC in β-gal and DiI inside nanogels, was recorded by fluorescence spectroscopy with increasing nanogel concentration at same β-gal concentration in the solution. The intensity of FITC was decreasing as increasing the DiI-containing nanogels and stopped changing for NG1-CRRR- β-gal complex at 2:1 ratio and at 4:1 ratio for NG2-CRRR- β-gal, indicating that the stable complexation is formed above this concentration.

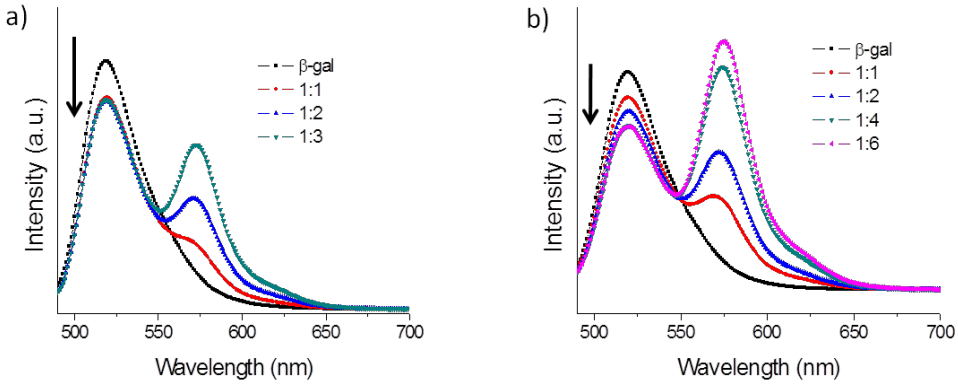

Figure S4: Fluorescence emission change of FITC labeled β-gal and encapsulated DiI in (a) NG1-CRRR and (b) NG2-CRRR varying nanogel ratios.

Estimation of number of nanogels per protein in complex:

Diameter Values(DLS):
β-gal= 10 nm
NG1-CRRR= 20 nm *NG1-CRRR- β-gal= 40 nm*
NG2-CRRR= 12 nm *NG2-CRRR- β-gal= 30 nm*

Calculation for NG1:

NG1-CRRR- β-gal complex radius= 20 nm
Volume = $4/3\ \pi\ (20\ nm)^3 = 3.35 \times 10^4\ nm^3$

NG1-CRRR radius= 10 nm
Volume = $4/3\ \pi\ (10\ nm)^3 = 4.19 \times 10^3\ nm^3$

β-gal radius= 5 nm
Volume = $4/3\ \pi\ (5\ nm)^3 = 5.23 \times 10^2\ nm^3$

Note: The ratio is 2:1 of NG1 and β-gal.

Assuming that number of NG1-CRRR is *2p* and number of β-gal is *p* in complex.

The summation of volume of *2p* NG1-CRRR and *p* β-gal = the volume of complex:
$2p \times 4.19 \times 10^3\ nm^3 + p \times 5.23 \times 10^2\ nm^3 = 3.35 \times 10^4\ nm^3$

$p = 3.35 \times 10^4\ nm^3 / (2 \times 4.19 \times 10^3\ nm^3 + 5.23 \times 10^2\ nm^{3)} = 3.76$

Thus, the average numbers of NG1-CRRR and β-gal per complex are 7.6 and 3.8 respectively.

Calculation for NG2:

NG2-CRRR-β-gal complex radius= 15nm
Volume = $4/3\ \pi\ (15\ nm)^3 = 1.41 \times 10^4\ nm^3$

NG2-CRRR radius= 6 nm.
Volume = $4/3\ \pi\ (6\ nm)^3 = 9.04 \times 10^2\ nm^3$

β-gal radius= 5 nm
Volume = $4/3\ \pi\ (5\ nm)^3 = 5.24 \times 10^2\ nm^3$

Note: The ratio is 4:1 of NG2 and β-gal.

Assuming that number of NG2 is *4q* and number of β-gal is *q* in complex.

The summation of volume of *4q* NG2 and *q* β-gal = the volume of complex:
$4q \times 9.04 \times 10^2\ nm^3 + q \times 5.24 \times 10^2\ nm^3 = 1.41 \times 10^4\ nm^3$

$q = 1.41 \times 10^4\ nm^3 / (4 \times 9.04 \times 10^2\ nm^3 + 5.24 \times 10^2\ nm^{3)} = 3.41$

Result:
Thus, the average numbers of NG2-CRRR and β-gal per complex are 13.6 and 3.4 respectively.

Agarose Gel Electrophoresis Studies: We performed agarose gel electrophoresis to observe nanogel and protein complexation taking advantage of the nanogel (positive) and β-gal (negative) charges differences. 10 µL samples were prepared in Milli Q water: (1) β-gal control (100µg), (2) NG1-CRRR control (200µg), (3) NG1-CRRR: β-gal complex (200µg: 100µg:, (4) NG2-CRRR control (400 µg), and (5) NG2-CRRR: β-gal complex (400 µg: 100 µg: with the addition of 1 µL Bromophenol blue (10mg/mL). The nanogels were incubated with the protein for 1 hour at room temperature. To prepare the gel, 270 mg of Ultra Pure Agarose was diluted in 1x TAE buffer pH 8.0 and the solution was microwaved until getting a homogeneous solution. The gel was poured in a FisherBiotech Electrophoresis System (FB-SB-710) and let stand until the gel solidified with an 8 well comb. The gel was run with 1x TAE buffer at 100 mV for 1 hour and 10 minutes at room temperature. The gel was washed with Milli Q water for 10 minutes and stained with Gel Code Blue for 1 hour.

As shown in Figure S2 complexation of NG1-CRRR with β-gal is evident in Lane 3. Free protein in lane 3 almost disappeared and there is some extension of the nanogel mark towards the cathode showing interaction between the nanogel and protein charges meaning that the positive charges of the nanogel are shielded by negative charges of β-gal. Some complexation between NG2-CRRR and β-gal is observed as well, however this interaction is weaker than that with NG1.

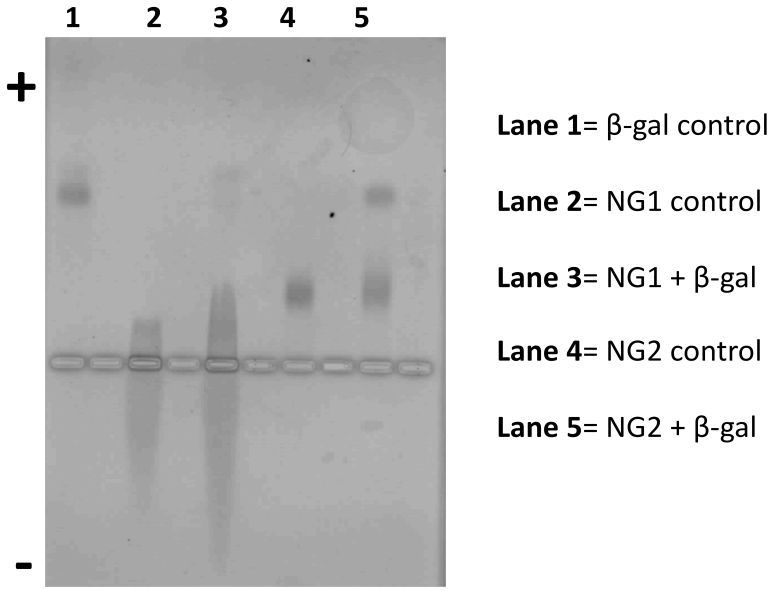

Figure S5: Agarose Gel Electrophoresis for β-gal complexed to NG1-CRRR or NG2-CRRR.

β-gal activity assay: Solutions of native β-gal (MW=116, 300 g/mol monomeric form), β-gal-NG1-CRRR (1:2 ratio) and β-gal-N2-CRRR (1:4 ratio) were prepared in PBS

buffer pH 7.4 and incubated for 1 hour. β-gal activity was assessed in a 96 well plate using SpectraMax M5 plate reader. By adding the colorimetric substrate 2-nitrophenyl-β-D-galactopyranoside (2.5 mM) activity of β-gal was recorded by the absorbance increase over time of pNP at 405 nm excitation wavelength.

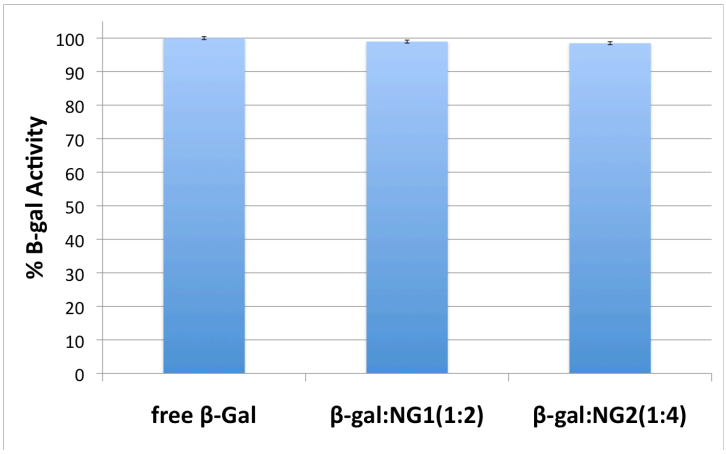

Figure S6: Activity assay of β-gal complexed to NG1-CRRR and NG2-CRRR.

Laser Scanning Confocal Microscopy: The laser confocal experiment was performed with HeLa cells which were cultured in T75 flasks containing DMEM/F12 with 10% FBS supplement. The cells were seeded at 10,000 cells/100 µL in cover slip-bottomed Petri dishes and allowed to grow for 1 day at 37 °C in a 5% CO_2 incubator. The cells in 2 mL of culture medium were treated with nanogels (0.1 mg/mL) containing dye and bound to protein; incubated for 3 hours at 37 °C before monitoring the cells by confocal microscopy (excitation of both dye molecules at 488 nm and 543 nm for FITC and DiI, respectively)

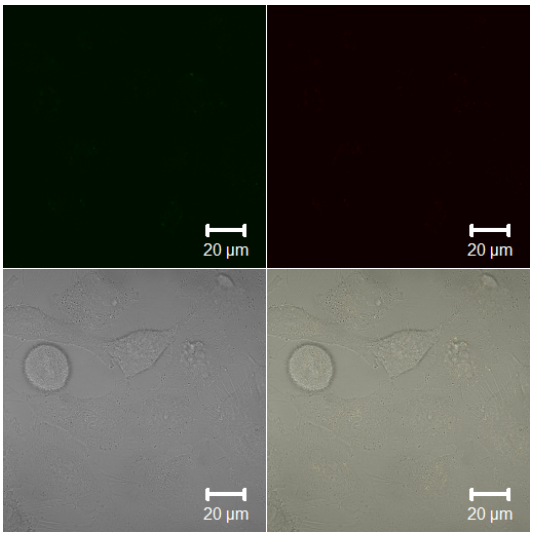

Figure S7: Intracellular delivery of native β-gal is not efficient without nanogel.

X-gal Studies:

HeLa cells were seeded in a 96 well plate at 10,000 cells/well. After 24 hours cells were washed with PBS. NG1 and NG2 complexes with β-gal were prepared at ratios of 2:1 and 4:1 respectively and added to cells in serum free DMEM/F12 media. Cells were incubated with the complexes for 3 hours. This was followed by washing cells with PBS twice and addition of DMEM/F12 with 10% FBS supplement. After another 3 hours of incubation, X-gal staining studies were carried out according to the manufacturers' protocol (Genlantis). Briefly, after fixing cells for 15 minutes they were washed twice with 1x PBS followed by addition of 80 µL 1x staining buffer. After overnight incubation cells were washed again and images were taken with an inverted microscope at 40x magnification using the Micron software.