**Supporting Information**

# Exploring the potential of polypeptide-polypeptoide hybrid nanogels for mucosal delivery

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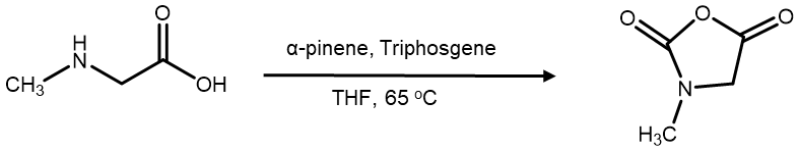
## 1 Experimental Section

**Materials.** N, N'-Dicarbobenzoxy-L-cystine ((Z-dCys-OH)$_2$) and allylamine were purchased from Bachem. L-Phenylalanine (H-Phe-OH) and bis(trichloromethyl) carbonate (triphosgene) were purchased from Fluorochem. Sarcosine, α-pinene, tetrahydrofuran (THF, anhydrous), thionyl chloride ($SOCl_2$) (distilled before use), n-hexane, ethyl acetate (anhydrous), dichloromethane (DCM, anhydrous), diethyl ether (anhydrous), dimethyformamide (DMF, anhydrous), 1,1,1,3,3,3,-hexafluoro-2-propanol (HFiP), IR780 idoide (IR780, dye content ≥95 %), RPMI 1640 amino acid solution (50x), type II mucin from porcine stomach, egg yolk emulsion, diethylenetriaminepentaacetic acid, deoxyrybonucleic acid sodium salt from salmon testes, sodium chloride, potassium chloride and Dulbecco's Modified Eagle's Medium (high glucose) were purchased from Sigma–Aldrich (Merck, UK). Deuterated dimethyl sulfoxide (DMSO)-$d_6$ and deuterated chloroform ($CDCl_3$) were purchased from Apollo Scientific. Nuclease-free water was purchased from Integrated DNA Technologies (Ireland). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) were obtained from Thermo Fisher Scientific (Ireland).

**Methods and Instrumentation.** Proton nuclear magnetic resonance ($^1$H NMR) and Diffusion ordered spectroscopy (DOSY) spectra were recorded on a Bruker Avance 400 (400 MHz) spectrometer. All chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal reference. Attenuated total reflection (ATR) FTIR measurements were performed on a ThermoFisher Nicolet iS10 instrument. Spectra were obtained from 16 scans with a resolution of 2 $cm^{-1}$ in the spectral region of 500–4000 $cm^{-1}$. Samples were analyzed without prior preparation both in the liquid and solid form. Size exclusion chromatography (SEC) was conducted in 1,1,1,3,3,3-Hexafluoro-2-propanol HFiP using an PSS SECurity GPC system equipped with a PFG 7 µm 8 × 50 mm pre-column, a PSS 100 Å, 7µm 8 × 300 mm and a PSS 1000 Å, 7µm 8 × 300 mm column in series and a differential refractive index (RI) detector at a flow rate of 1.0 mL·min$^{-1}$. The systems were calibrated against Agilent Easi-Vial linear poly(methyl methacrylate) (PMMA) standards and analyzed by the software package PSS winGPC UniChrom. Concentration of the SEC samples was 3mg/1mL HFiP. The samples were filtered with 0.2 µm membranes prior to injection into the instrument. The dialysis purifications were performed using Snakeskin dialysis tubing (ThermoFisher) with molecular weight cut off (MWCO) at 3500 Da against 2 L of deionized water. Dynamic light scattering (DLS) was performed using a Malvern Zetasizer Nano ZSP instrument to determine the size and size distribution of the particles at a laser wavelength of 633 nm at a scattering angle of 173°. Measurements were carried out at 25°C with triplicates for each sample. Transmission electron microscopy (TEM) images were

recorded on a Hitachi H-7650 instrument at 100 kV. The samples (1 mg/mL) dilute with water were dropped on a Copper grid, coated with Formvar 200 mesh and wiped off after 10 minutes. The grids were left drying at room temperature before analysis. Nanoparticle tracking analysis (NTA) was performed on the NanoSight NS3000 (Malvern Instruments). Samples were injected slowly using a 1mL syringe until the nanogels could be seen on the display. Measurements were taken when approximately 30 particles were visible on screen. A screen gain of 1 and camera level of 9 -15 were used. A measurement of 60 seconds duration was taken for each sample. Ultraviolet–visible (UV-vis) spectra were recorded on Libra-S22 UV-vis spectroscopy (Biochrom). The concentration of IR780 were quantified by recording the absorbance at 780 nm using ultraviolet–visible spectroscopy (UV-vis). High performance liquid chromatography (HPLC) was performed on Agilent 1120 (Isocratic, variable wavelength detector) at the wavelength of 240 nm. The control panel for Open LAB software (Agilent) was used to process the data obtained.

**Synthesis of sarcosine NCA (Sar NCA).** The synthesis of Sar NCA was conducted as previously described in literature (Scheme S1, Figures S1 andS2).[1]




Scheme S1. Synthesis of sarcosine NCA (Sar[1] NCA).

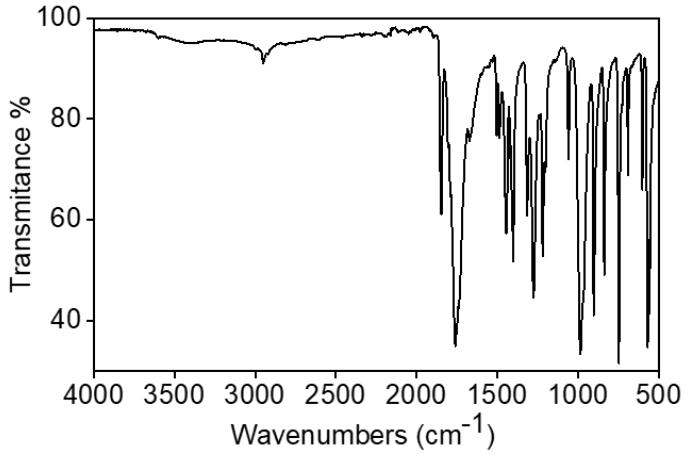



Figure S1. FTIR spectrum of Sar NCA.

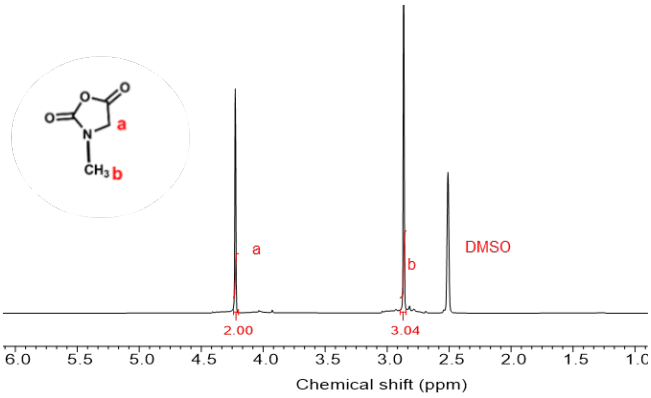
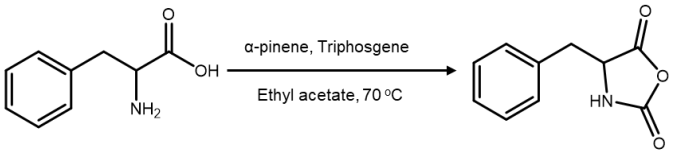



Figure S2. $^{1}$H-NMR spectrum of Sar NCA.

**Synthesis of phenylalanine NCA (Phe NCA).** The synthesis of Phe NCA was conducted as previously described in literature (Scheme S2, Figures S3 and S4).[2]




Scheme S2. Synthesis of L-Phenylalanine NCA (Phe NCA).

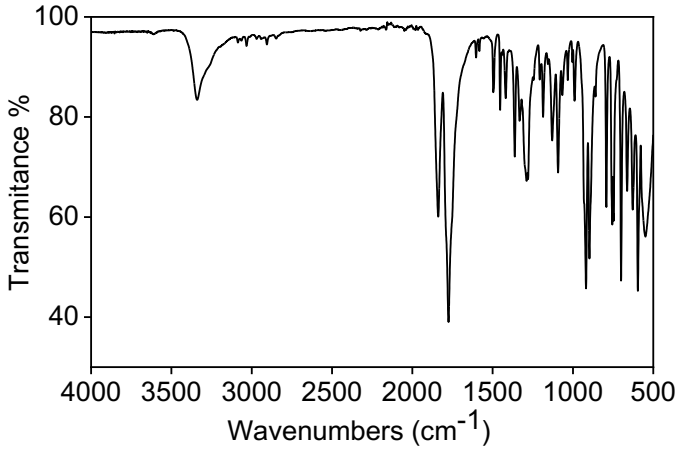



Figure S3. FTIR spectrum of Phe NCA.

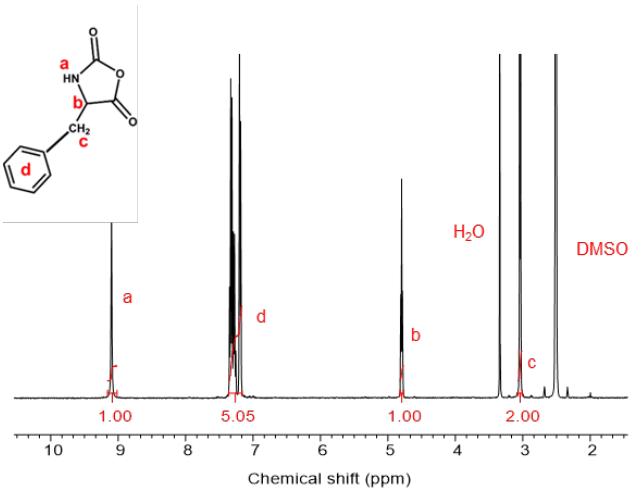
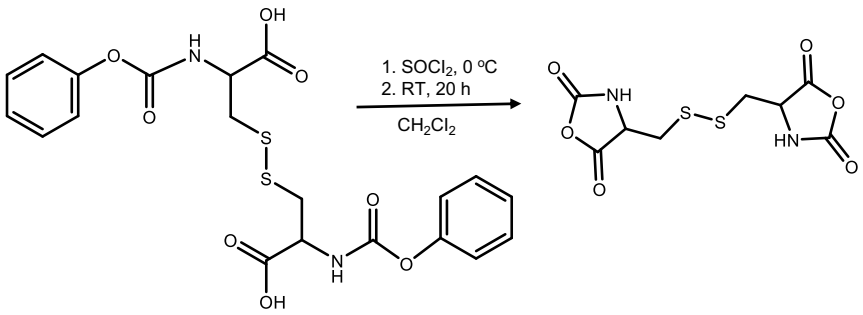



Figure S4. $^{1}$H-NMR spectrum of Phe NCA.

**Synthesis of cystine NCA (dCys NCA).** N, N'-dicarbobenzoxy-L-cystine (3.0 g, 5.90 mmol, 1 eq.) was added to 60 mL of dry Dichloromethane (DCM) leading to a suspension. Then, at 0°C and under continuous nitrogen flow, thionyl chloride (1.8 g, 15.16 mmol, 2.6 eq.) diluted in 20 mL of DCM were added dropwise to the reaction medium. The resulting yellowish turbid mixture was vigorously stirred at this temperature for 3 h and afterwards was left at room temperature for 20 more hours for the completion of the reaction. The obtained white precipitate was filtered in vacuum and the solids were washed repeatedly using dichloromethane and diethyl ether for the complete removal of side products. The white powder was then dried under vacuum at room temperature overnight (yield 45%, Scheme S3, Figures S5 and S6).




Scheme S3. Synthesis of cystine NCA (dCys NCA).

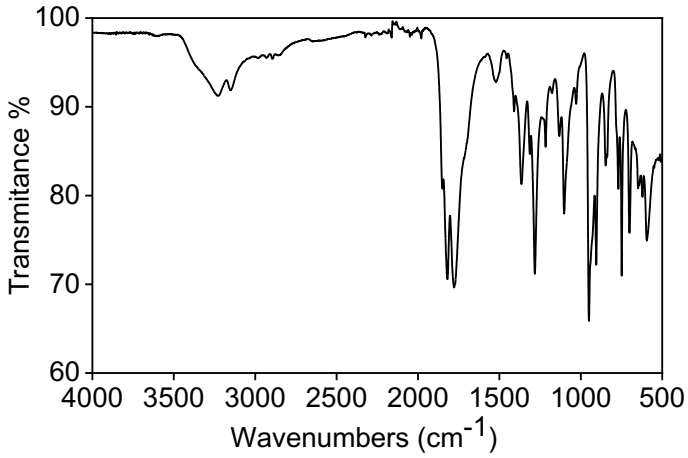



Figure S5. FTIR spectrum of dCys NCA.

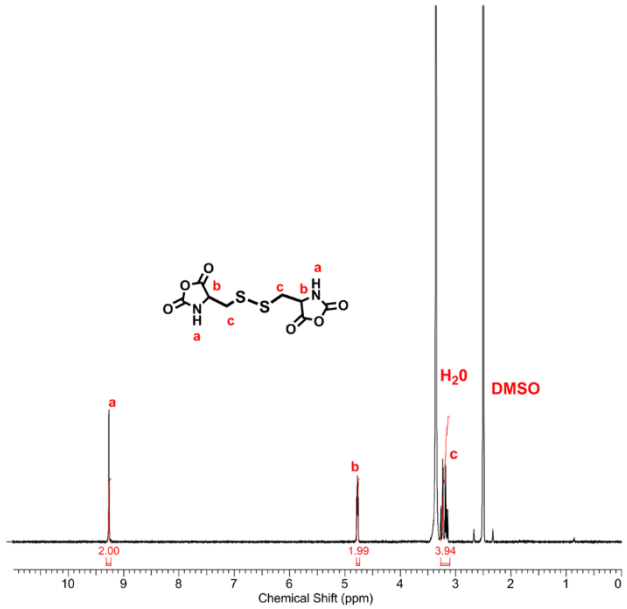



Figure S6. $^{1}$H-NMR spectrum of dCys NCA.

**Synthesis of PSar-*b*-P(Phe-*co*-dCys) nanogels.** The following is a representative procedure. To a pre-dried Schlenk tube equipped with a magnetic stirrer under a nitrogen atmosphere, a solution of the Sar NCA (465.7 mg, 4.05 mmol) in DMF (5 mL) was prepared. A solution of allylamine initiator (3.0 mg, 0.053 mmol) in DMF (1 mL) was also prepared and charged to the reaction solution *via* syringe. The solution was stirred at room temperature overnight and it was periodically degassed under vacuum. Fourier-transform infrared (FTIR) spectroscopy was used to monitor the complete consumption of the monomer. Then, a mixture solution of the Phe NCA (607.3 mg, 3.18 mmol) and dCys NCA (163.9 mg, 0.56 mmol) in DMF (7 mL) was prepared and added to the reaction solution *via* syringe. The solution was allowed to stir until full monomer consumption was confirmed by FTIR and it was periodically degassed under vacuum. After the termination of polymerization indicated by FTIR, the polymer was precipitated into an excess of cold diethyl ether and centrifuged for 5 minutes at 7000 revolutions per minute (rpm). The supernatant was decanted and the solid polymer was dried in a vacuum oven at 37°C overnight. Next day, the polymer was dissolved in deionized water and dialyzed against 2 liters

of deionized water, which was refreshed twice per day for 3 days. Finally, the aqueous polymer solution was freeze-dried to achieve the corresponding polymer (743 mg, 60% yield).

The nanogels were prepared by directly dispersing the lyophilized polymer powder in 10 mM PBS at pH 7.4. The resulted nanogels were then characterized by DLS, NTA and TEM for size and morphology. Before measurement, 0.45µm membranes were utilized to filter the samples.

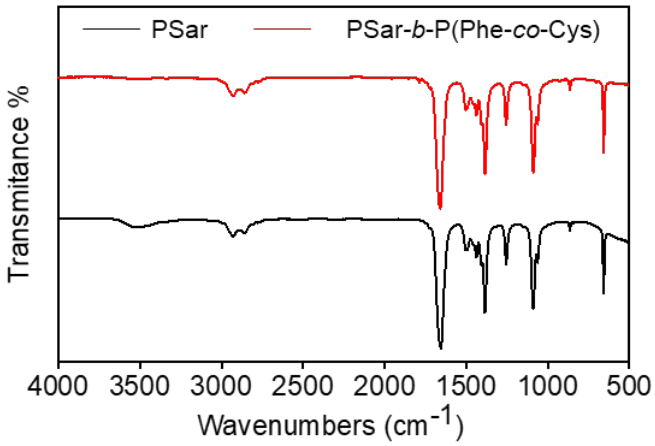



Figure S7. FTIR spectrum of PSar and PSar-*b*-P(Phe-*co*-dCys).

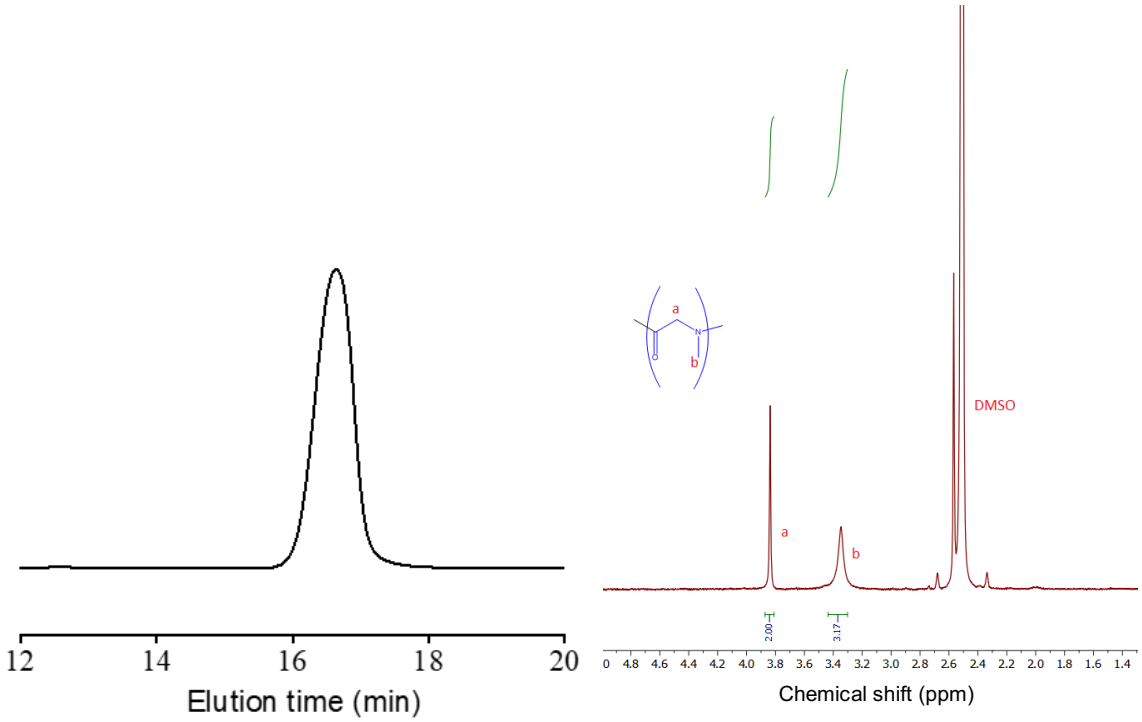



Figure S8. SEC chromatogram ($M_n$ = 12700 g/mol and $Đ$ = 1.05) and $^1$H-NMR spectrum of PSar.

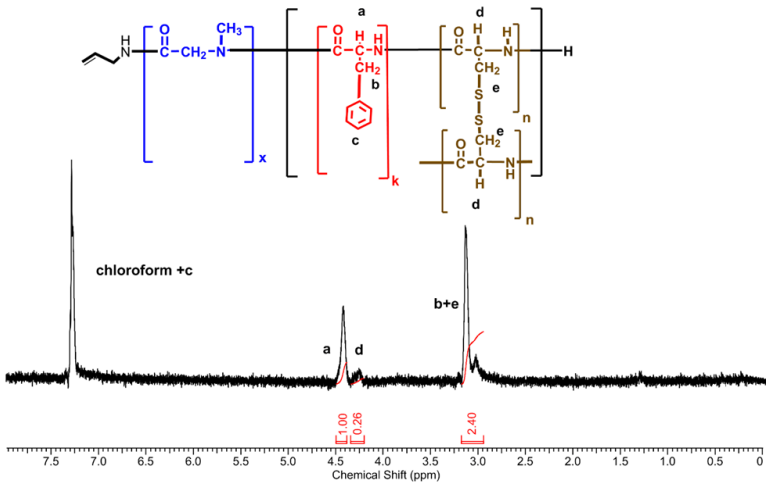



Figure S9. $^{1}$H-NMR spectrum of NG1 in $CDCl_3$.

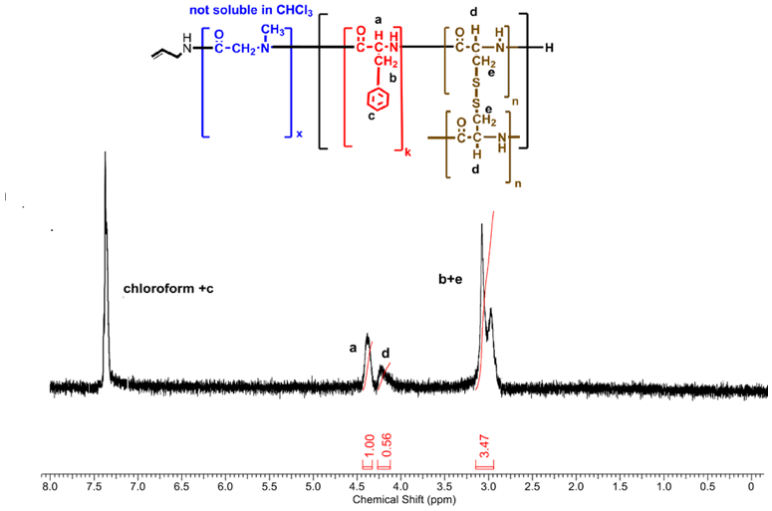



Figure S10. $^{1}$H-NMR spectrum of NG2 in $CDCl_3$.

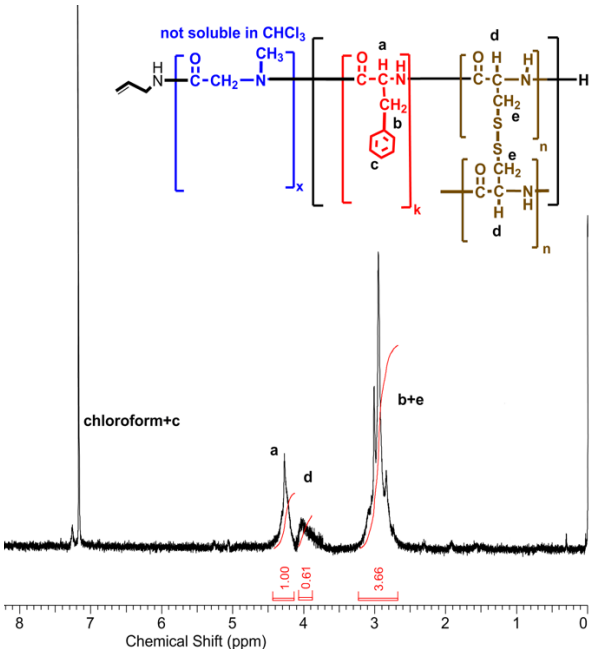



Figure S11. $^{1}$H-NMR spectrum of NG3 in $CDCl_3$.

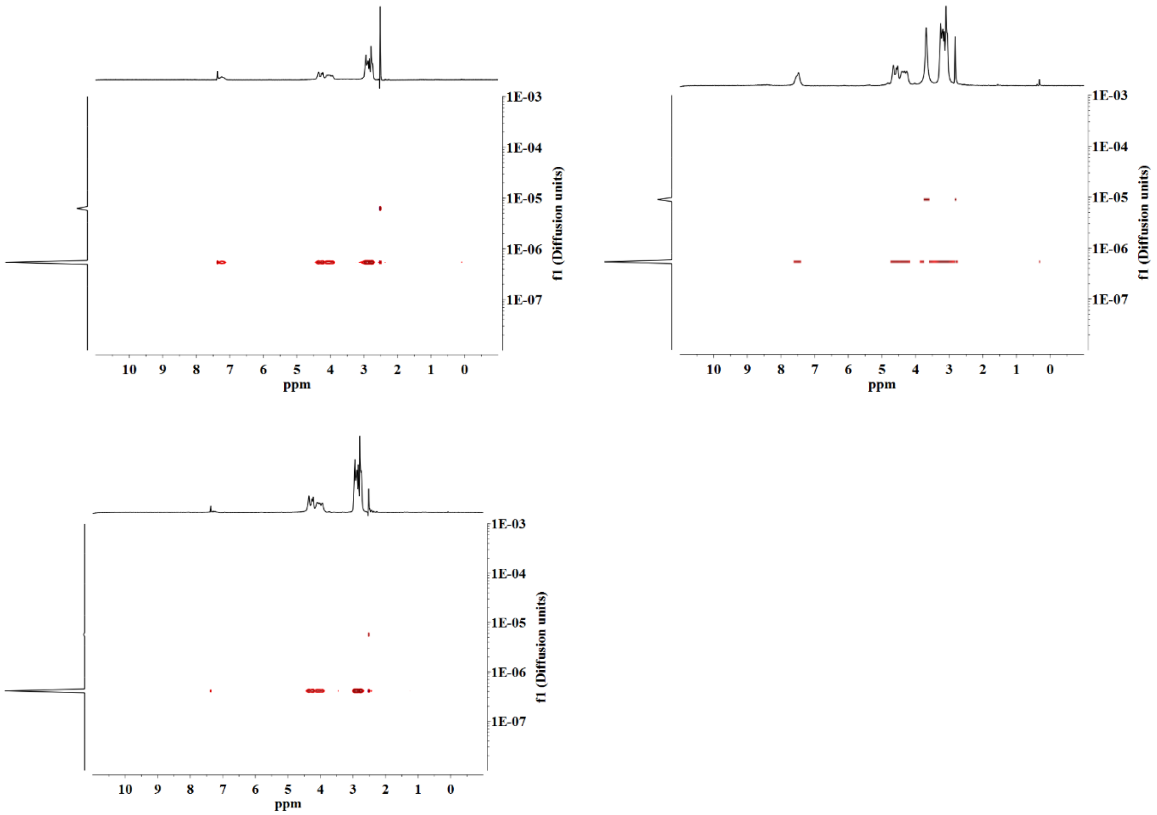



Figure S12. DOSY spectra of NG1 (top left), NG2 (top right), NG3 (bottom left).

**Redox-Sensitivity of Nanogels.** The reduction sensitivity of nanogels was studied by monitoring the size change of nanogels over time in response to GSH at a concentration equivalent to intracellular

GSH (10mM). Briefly, the polymer was dissolved in PBS (1 mg/mL) and it was degassed with $N_2$ for 20 min. Then GSH was added to yield a final concentration of 10 mM (3.073 mg/mL). The vial was sealed and incubated at 37°C. The size of the nanogels was determined by DLS at different time intervals. Nanogels without GSH were used as reference. After 24 h of incubation, GSH-treated nanogel was also observed by TEM. The reaction of GSH and disulfide bond was also studied by GPC analysis. The polymer was mixed with GSH in PBS and incubated at 37°C for 24 h before freeze-dried.

**Dye Encapsulation and Characterization.** The nanoprecipitation method was employed to prepare NG-IR780 nanoparticles. Briefly, specific amount of polymer and IR780 were mixed in 1 mL of DMF. After stirring at room temperature for 30 minutes, 3 mL of deionized water was added dropwise to the solution and stirred for 2 h. The mixture was then transferred to the dialysis bag (MWCO: 3500 Da) and was dialyzed against deionized water for 24 h to remove the organic solvents and free dye. The obtained loaded NG solution was filtered and freeze dried. The content of IR780 in the obtained nanoformulations were quantified by the absorbance at 780 nm using UV-vis. Then, the dye loading content (DL%) and dye encapsulation efficiency (EE%) of IR780 was calculated by the following equations:

DL% = amount of dye entrapped in NG / amount of dye-loaded NG × 100%

EE% = amount of dye entrapped in NG / amount of dye added initially × 100%

Size distribution and morphology were measured by DLS, NTA and TEM. Briefly, the dye-loaded NG dispersion was prepared in a concentration of 1 mg/mL. DLS was used to measure the Z-average size and PDI, NTA to observe the distribution and movement of nanoparticles and TEM to observe the morphology and size without hydrate layer.

**In Vitro Release of NG-IR780.** The lead polymer nanogel (NG4) was used to load IR780 for further use. The in vitro release experiment of IR780 was conducted at reduction insensitive (without GSH) and reduction-sensitive (with GSH) conditions using the dialysis method. Release study was performed at 37°C, 100 rmp in a water containing incubator shaker (OLS200, Focus Scientific, Ireland). For the reduction insensitive experiment, 1 mL of predetermined amount of the nanogels loaded with IR780 was transferred into dialysis kits (MWCO: 3.5 kDa) and dialyzed against 30 mL of PBS (pH 7.4, 10 mM). At predetermined time intervals, 1 mL of the dialysis solution was removed to quantify the released dye with UV−vis spectroscopy and same volume of fresh medium was replenished. The same method was employed for the reduction-sensitive experiment with the exception of the replacement of the pure PBS by PBS solution with 10 mM GSH. The cumulative amount of released dye percentages were calculated and plotted against time. The in vitro release studies were conducted in triplicate, and the results are presented as average data with standard deviations.

**Mucus Penetration.** The mucus penetration was performed using a Transwell-Snapwell diffusion chamber. Artificial mucus was prepared as previously described.[1] Briefly, 500 mg of deoxyribonucleic acid (DNA), 250 mg of mucin, 0.295 mg of diethylenetriaminepentaacetic acid (DTPA), 1 mL of RPMI 1640 amino acid solution, 250 µL of egg yolk emulsion, 250 mg of NaCl, and 110 mg of KCl were mixed together in a final volume of 50 mL of DNase-free water. This dispersion was allowed to equilibrate at 25°C overnight. The assay was performed in 12 well cell culture insert plates (VWR), with a 12 mm polycarbonate membrane and 3 µm pores. The basolateral compartment was filled with 1.0 mL of PBS while 200 µL sample solution containing 20 µg of IR780 were added to the apical side of the inserts in 400 µL of PBS or a 1:1 mixture of artificial mucus and PBS. 200 µL of the nanogels loaded with the same total amount of IR780 (20 µg) were added to the apical compartment of the Transwells with or

without mucus. Samples (300 µL) were withdrawn from the basolateral compartment every 60 min for 240 min. The basolateral compartment was replaced with 300 µL of fresh PBS each time. The content of the withdrawn samples was measured at 780 nm using UV-vis. The cumulative transport of IR780 across the membrane was calculated from a calibration curve generated at the same time.

**Cytotoxicity of the Nanogels.** Cytotoxicity of the main nanogel was tested by MTT assay.[3, 4] Briefly, B16F10 cells and 293T cells were seeded in a 96-well plate (n = 3 technical replicates x 3 biological replicates) at a seeding density of $4 \times 10^3$ cells per well and left to adhere and grow overnight in a $CO_2$ incubator (5%) at 37°C. After overnight incubation, medium was collected and cells were treated with various concentrations of NGs in complete medium (0.1 to 2 mg/mL). A negative control (NC), consisting of cells incubated only with medium, and a positive control (PC), consisting on cells incubated with 5% of DMSO in medium, were also prepared and treated similarly to the sample wells. After 24 h of incubation, the MTT assay was performed according to the manufacturer's protocol to determine the biocompatibility of NG polymer. Cellular viability percentage is expressed based on the following Equation:

Cellular viability (%) = (OD of NG treated cells or PC / OD of NC) × 100%.

**2 Additional characterisation of nanogels**

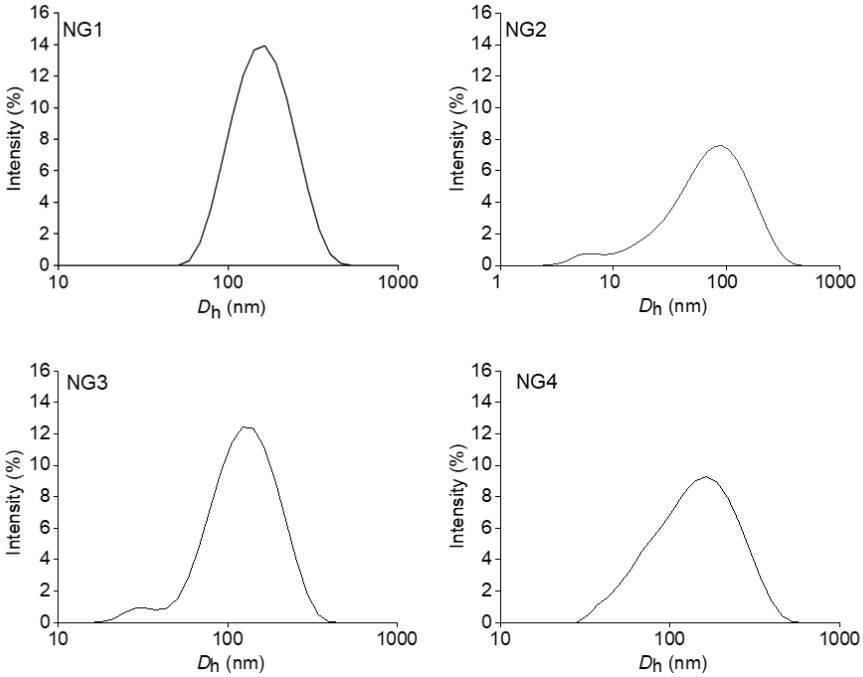



Figure S13. DLS traces of size distribution of NG1-4.

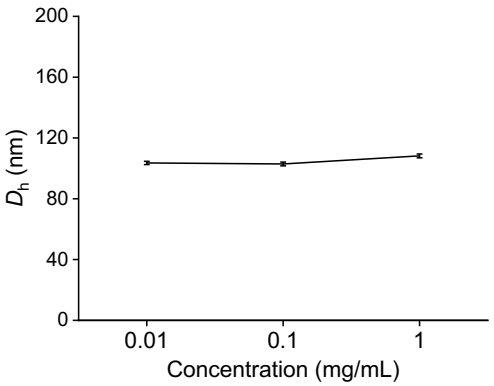



Figure S14. $D_h$ values at 10 and 100 times dilutions for NG4 in PBS buffer monitored by DLS.

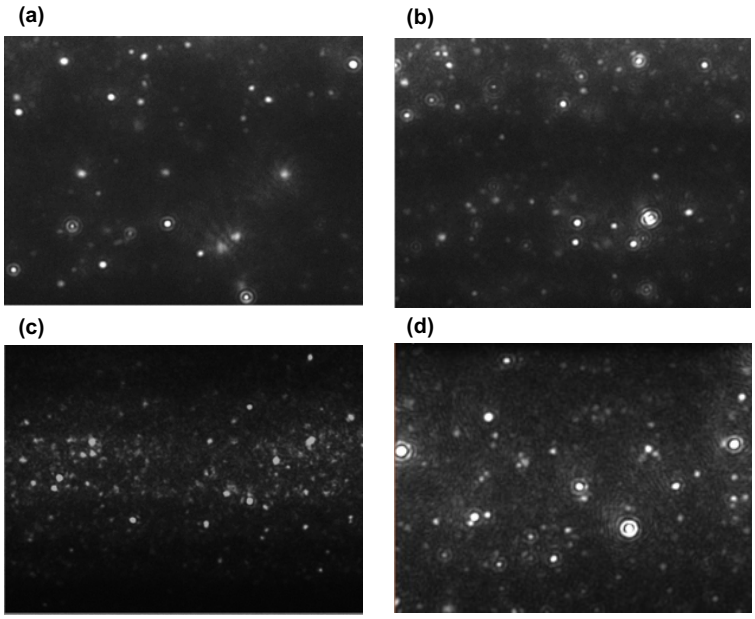



Figure S15. NTA camera images of (a) NG1, (b) NG2, (c) NG3 and (d) NG4.

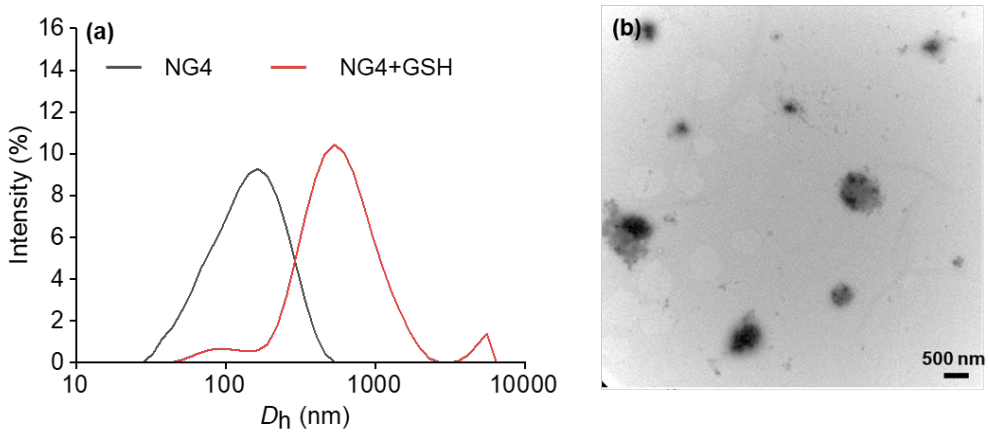



Figure S16. (a) Size distribution of NG4 after 24 h with and without 10.0 mM GSH; (b) TEM image of NG4 after incubation with 10 mM GSH for 24 h.

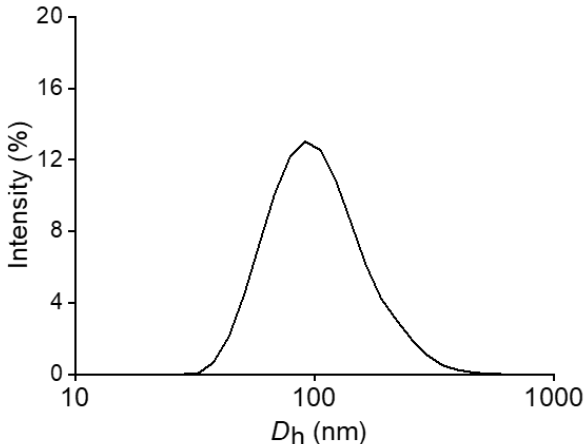


Figure S17: DLS trace of NG4 loaded with IR780.

Table S1: Summary of DLS/TEM/NTA characterization and dye loading content (DL%) /dye encapsulation efficiency (EE%) of IR780 loaded NG4.

| NG/IR780 feeding ratio (mg) | EE of IR780 (% w/w) | DL of IR780 (% ) | Z-average size (nm) | TEM size (nm) | NTA size (nm) |
|---|---|---|---|---|---|
| 10:1 | 74.15±2.84 | 4.52±1.62 | 121.7±5.5 | 69.7±5.6 | 104.5±25.3 |

[1] Skoulas, D.; Stuettgen, V.; Gaul, R.; Cryan, S. A.; Brayden, D. J.; Heise, A., Amphiphilic Star Polypept(o)ides as Nanomeric Vectors in Mucosal Drug Delivery. *Biomacromolecules* **2020,** 21, (6), 2455-2462.
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[3] Bernard, M.; Jubeli, E.; Pungente, M.D.; Yagoubi, N., Biocompatibility of polymer-based biomaterials and medical devices – regulations, in vitro screening and risk-management. *Biomater. Sci* **2018**, 6, 2025-2053.
[4] Kejlová, K.; Labský, J.; Jírová, D.; Bendová, H., Hydrophilic polymers--biocompatibility testing in vitro. *Toxicol In Vitro* **2005,** 19, (7), 957-962.