Glucose Single-Chain Polymer Nanoparticles for Cellular Targeting

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Experimental section

Materials. Potassium ethyl xanthogenate (96%), 2-bromoethanol (95%), potassium thioacetate (98%), DL-1,2-isopropylideneglycerol (solketal, ≥97.0%), methyl α-D-glucoside (99%), methacryloyl chloride (98%), vinyl methacrylate (98%), lipase acrylic resin from *Candida antarctica* (≥5000 U/g), hydrazine monohydrate (98%), 2-(dimethylaminoethyl) acrylate (DMAEA, 98%), poly(ethylene glycol) diacrylate (PEGDA, M_n 258 g/mol), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, ≥98%), Benedict's reagent, Dulbecco modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (containing 10.000 units penicillin, 10 mg streptomycin mL^{-1}), resazurin sodium salt (Bioreagent), phosphate buffered saline (PBS, pH 7.4), Trypsin-EDTA solution (sterile filtered, BioReagent) and 4′,6-diamidino-2-phenylindole dihydrochoride (DAPI, 98%) were purchased from Sigma Aldrich. Acetone (100%), methanol (100%), tetrahydrofuran (THF, 100%) and *N,N*-dimethylformamide (DMF, ≥99.9%)) were purchased from VWR. 4,4'-azobis(4-cyanovaleric acid) (ACVA, 98%) was purchased from Fluka. Acetonitrile (99.9%, HPLC grade) was purchased from Actu-All Chemicals. 5-(4,6-Dichlorotriazinyl) aminofluorescein (5-DTAF, single isomer), Live Cell Imaging Solution (HEPES buffered physiological saline pH 7.4 by life technologies) and BacMam CellLight® reagents (early endosomes, late endosomes and lysosomes, RFP) were purchased from Thermo Fisher Scientific. Chloroform (≥99%) and EndoGro™ Basal medium were purchased from Merck. CF®405M Wheat Germ Agglutinin (WGA) was purchased from Biotium. All chemicals were used without further purification unless stated otherwise. When stated as dry, solvents were treated with molecular sieves (4 Å) 24 h before usage. SnakeSkin™ Dialysis Tubing (10K MWCO) from ThermoFisher was employed for dialysis. 4-Cyano-4-((thiobenzoyl)sulfanyl)pentanoic acid (CPADB) was prepared following a literature procedure.[1] Control XMA copolymer was synthesized as published

earlier.[2] Prior to cell studies, fluorescent-labeled materials were purified via a preparative column Superose 6 10/100 GL, GE Healthcare on an FPLC Äkta purifier 900 with a 24 mL bed volume using a low TRIS salt buffer (5.0 mM Tris, 10.0 mM NaCl, 1.0 mM KCl, 0.5 mM $MgCl_2$, pH 7.2). Subsequently, the materials were dialyzed against water and freeze-dried.

^{1}H NMR (400 MHz) and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in ppm and referenced to DMSO or water. Gel permeation chromatography (GPC) was performed on a Waters e2695 Separations Module equipped with an Agilent PLgel 5 μm MIXED-D 300 × 7.5 mm column and Waters photodiode array detector (PDA 2998), fluorescence detector (FLR 2475) and refractive index detector (RI 2414), and DMF (50 mM LiCl) was employed as eluent and molecular weights were calibrated relative to PEO/PEG. GPC samples were prepared in DMF followed by filtration using GE Healthcare Whatman SPARTAN 13/0.2 RC 0.2 μm syringe filters. Dynamic Light Scattering (DLS) measurements were carried out in DMSO or milliQ water on a Malvern Instruments Zetasizer ZS and samples were filtrated using GE Healthcare Whatman SPARTAN 13/0.2 RC 0.2 μm syringe filters prior to measurements. UV measurements were conducted on a Shimadzu 1 UV-2401PC UV-VIS Recording Spectrophotometer. Mass spectra were obtained using a Waters Micromass LCT time-of-flight (TOF) mass spectrometer featured with electrospray ionization (ESI) in positive mode. Samples were dissolved in a 1:1 water/acetonitrile solution with 0.1% formic acid and the instrument parameters were set to a capillary voltage of 3 kV, sample cone voltage of 20 V, extraction cone voltage of 5 V, RF lens 200, and desolvation and source temperatures at 150 and 80 °C respectively. Approximately 20 scans were averaged to one spectrum and measurements were calibrated against phosphoric acid. The data analysis was performed with Masslynx software. Transmission electron microscopy (TEM) images were recorded on a Philips CM300ST-FEG

Transmission Electron Microscope 300 kV equipped with GATAN Ultrascan1000 (2kx2k CCD camera) and a GATAN Tridiem energy filter (2kx2k CCD camera). The sample (5 μL, 0.5-1 mg/mL in milliQ water) was drop-cast on formvar coated 200 mesh copper grids and incubated for 30 s. Subsequently, excess solution was removed with filter paper. For staining, the grid was incubated for 60 s with 5 μL of a 1% (w/v) uranyl acetate solution.

Monomer synthesis.

2-(Ethyl xanthate) ethyl methacrylate (**XMA**) was prepared via a 2-step synthesis as described earlier by our group.[3]

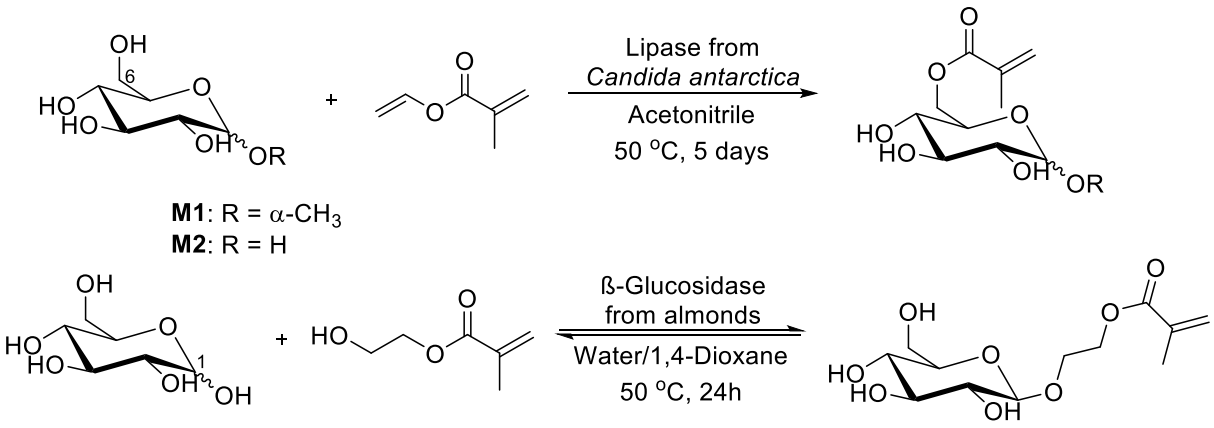

Figure S1. Reaction schemes of enzyme-catalyzed synthesis of monomers **M1-3**.

All three glucose-derivated monomers were purified via silica column chromatography and obtained in yields of 88% for **M1** and 45% for **M3**, strongly depending on the enzymatic equilibrium of the reaction. **M2** was obtained in lower yields of 24% as glucose has a lower solubility in acetonitrile and presumably, the equilibrium with the open form impedes the efficiency of the enzyme. ^{1}H NMR spectroscopy on the purified products confirmed a glucose to methacrylate ratio of 1:1 with H-6 appearing at 4.8 ppm for **M1** and at 4.4 ppm for **M2** and H-1 shifting from 4.7 to 4.5 ppm for **M3**.**Synthesis of methyl 6-O-methacryloyl-α-D-glucoside (mG$_6$MA, M1).** **mG$_6$MA**was prepared following a literature procedure.[4] Methyl α-D-glucoside (2.01 g,

10.37 mmol), lipase acrylic resin from *Candida antarctica* (0.98 g), vinyl methacrylate (VMA) (1.30 g, 1.40 mL, 11.59 mmol, 1.12 eq.) and hydroquinone (1.10 mg, 0.01 mmol) were added to a 50 mL conical flask. The flask was closed with a septum and purged with N_2. Dry acetonitrile (15 mL) was transferred to the reaction mixture and the flask was suspended into a water bath maintained at 50 °C, shaking at 150 rpm for 5 days. The reaction was terminated by filtering off the enzyme. The enzyme was washed with methanol (2 × 20 mL) and the filtrate was concentrated under reduced pressure. Following silica column chromatography (ethyl acetate, *n*-heptane and ethanol (70:20:10, R_f = 0.41) as eluent), a yellow viscous liquid (2.42 g, 88.0% yield) was obtained.

^{1}H NMR (400 MHz, D_2O) δ_H: 6.17 (m, 1H, HC=C-) 5.75 (m, 1H, HC=C-), 4.82 (d, 1H, CH), 4.50 (dd, 1H, CH_2), 4.37 (dd, 1H, CH_2), 3.91 (m, 1H, CH), 3.70 (q, 1H, CHOH), 3.60 (dd, 1H, CHOH), 3.50 (t, 1H, CHOH), 3.42 (s, 3H, CH_3), 1.95 (m, 3H, CH_3). ^{13}C NMR (400 MHz, D_2O) δ_C: 169.4, 136.6, 127.1, 99.3, 72.9, 71.1, 69.7, 69.4, 63.4, 55.1, 17.3.

Synthesis of 6-O-methacryloyl-α-D-glucose (G_6MA, M2). G_6MA was synthesized following a similar procedure as for 6-GlcMA, from D-(+)-glucose (3.00 g, 16.66 mmol), *Candida antarctica* (1.30 g), hydroquinone and vinyl methacrylate (2.24 g, 19.99 mmol, 1.20 eq.) in dry acetonitrile (25 mL). The product was purified by column chromatography (ethyl acetate, *n*-heptane and ethanol (70:20:10, R_f = 0.28) as eluent) to yield a yellow viscous liquid (1.07 g, 24.4% yield).

MS-ESI (+): m/z = 236.14 $[M+H]^+$

^{1}H NMR (400 MHz, D_2O) δ_H: 6.14 (m, 1H, HC=C-), 5.72 (m, 1H, HC=C-), 5.20 (d, 0.52H, CH-α), 4.65 (d, 0.48H, CH-ß), 4.39 (m, 2H, CH_2), 4.06 (m, 0.52H, CH-ß), 3.69 (m, 1H, CHα and CH-ß), 3.49 (m, 2H, CH-α, CH-ß and CH), 3.23 (m, 0.47H, CH-ß), 1.90 (s, 3H, CH_3).

^{13}C NMR (101 MHz, DMSO-d_6) δ 166.56, 135.90, 125.80, 96.97, 92.33, 76.39, 74.74, 73.49, 72.86, 72.23, 70.52, 70.11, 69.20, 64.26, 18.04.

Synthesis of 2-(ß-glucosyloxy)-ethyl methacrylate (G1MA, M3). G_1MA was prepared following a literature procedure.[5] D-(+)-glucose (1.68 g, 9.33 mmol) was dissolved in water (2.40 mL) in a 50 mL conical flask. 2-Hydroxyethyl methacrylate (30.34 g, 233.26 mmol, 25.00 eq.) and 1,4-dioxane (2.40 mL) was added to the flask. A solution of β-glucosidase (0.168 g, 10 wt% of D-(+)-glucose) in water (2.40 mL) was added to the flask, which was closed with a septum. The reaction was incubated at 50 °C, shaking at 80 rpm for 24 h. The product was purified by column chromatography (chloroform and methanol (80:20, R_f= 0.49) as eluent) to yield a transparent vicious liquid (1.20 g, 44.0% yield).

MS-ESI (+): m/z = 249.14 $[M+H]^+$

^{1}H NMR (400 MHz, D_2O) δ_H: 6.14 (m, 1H, HC=C-) 5.70 (m, 1H, HC=C-), 4.47 (d, 1H, CH), 4.35 (m, 2H, CH_2), 4.12 (m, 1H, CH_2), 3.97 (m, 1H, CH_2), 3.85 (dd, 1H, CH), 3.63 (m, 1H, CH), 3.50-3.14 (m, 4H, CH_2, CH, CH), 1.90 (m, 3H, CH_3). ^{13}C NMR (400 MHz, D_2O) δ_C: 169.65, 135.73, 126.95, 102.49, 75.91, 75.66, 72.99, 69.53, 67.94, 64.31, 60.67, 17.33.

Synthesis of [XMA-mG6MA], [XMA-G6MA]) and [XMA-G1MA] copolymers (P1, P2 and P3). XMA was copolymerized with monomers 1-3 at a constant molar ratio.

For example, **mG_6MA** (1.20 g, 4.58 mmol), **XMA** (0.11 g, 0.47 mmol), CPADB (3.00 mg, 0.011 mmol, stock solution in DMF, 10 mg/mL) and ACVA (1.5 mg, 0.005 mmol, stock solution in DMF, 5 mg/mL) were dissolved in DMF (4.50 mL, [GlcMA] = 1 M) in a polymerization flask. The flask was degassed by four freeze-pump-thaw cycles. The reaction was initiated by heating in an oil bath at 70 °C. After 24 h, DMF (5 mL) was added to the flask. The pink polymer was precipitated in methanol followed by centrifugation (1.18 g, 90% yield).

P1: ^{1}H NMR (400 MHz, DMSO-d_6) δ_H: 5.14 (br, s, CH), 4.94 (br, s, CH_2), 4.79 (br, s, CH_2), 4.64 (br, s, CH_2), 4.58 (br, s, CHOH), 4.15 (q), 3.79 (br), 3.55 (br), 3.32 (br), 3.17 (d), 3.02 (br), 1.77 (br, s, CH_3), 1.37 (br, s, CH_3), 0.95-0.80 (br).

P2: ^{1}H NMR (400 MHz, D_2O) δ_H: 5.25 (br, s, CH), 4.63 (br, s, CH), 4.46 (br, s, CH), 4.12-4.04 (br, m, CH and CH_2), 3.74-3.27 (br), 1.98 (br, s, CH_3), 1.58 (br, s, CH_3), 1.09-0.93 (br).

P3: ^{1}H NMR (400 MHz, D_2O) δ_H: 4.47 (br, s, CH), 4.24-4.13 (br, m, CH_2 and CH), 3.93-3.90 (br), 3.73 (br), 3.48-3.30 (br), 1.98 (br, s, CH_3), 1.59 (br, s, CH_3), 1.09-0.91 (br).

Nanoparticle formation in aqueous solution (NP1-3). Thiol aminolysis of the glucose copolymers, as well as SCNP formation, were performed as published earlier for glycol thiol copolymers.[2] In brief: a nitrogen purged copolymer solution in DMF with hydrazine was stirred for 30 min. Subsequently, the solution was filtered and slowly added to a solution of poly(ethylene glycol) diacrylate (PEGDA, 258 g/mol) and TCEP in carbonate buffer (0.1 M sodium carbonate/sodium dicarbonate, pH 9-10). DMAEA was added after 4 h and stirred overnight, after which the solution was filtered and concentrated under reduced pressure. The obtained solution was dialyzed against demi-water, filtered and freeze-dried to obtain a white lyophilisate (200 mg).

P1-deprotected: ^{1}H NMR (400 MHz, D_2O) δ_H: 4.37 (br, s, CH), 4.07 (br, s, CH), 3.83 (br), 3.67 (br), 3.58 (br), 3.46 (br), 1.94 (br, s, CH_3), 1.10-0.95 (br).

P2-deprotected: ^{1}H NMR (400 MHz, D_2O) δ_H: 5.24 (br, s, CH-α), 4.61 (br, s, CH-ß), 4.10-4.02 (br), 3.72-3.66 (br), 3.47 (br), 3.24 (br), 3.01 (br), 1.97 (br, s, CH_3), 1.06-0.91 (br).

P3-deprotected: ^{1}H NMR (400 MHz, DMSO-d_6) δ_H: 4.71 (br), 4.38-3.91 (br), 3.69 (br), 3.38 (br), 3.01 (br), 1.81 (br, s, CH_3), 0.95-0.78 (br).

NP1: ^{1}H NMR (400 MHz, D_2O) δ_H: 4.38 (br, s, CH), 4.08 (br, s, CH), 3.84 (br), 3.68 (br), 3.59 (br), 3.46 (br), 2.91 (br), 2.80 (br), 2.49 (br), 1.93 (br, s, CH_3), 0.95-0.80 (br).

NP2: ^{1}H NMR (400 MHz, D_2O) δ_H: 5.24 (br, s, CH-α), 4.61 (br, s, CH-ß), 4.46 (br), 4.12-4.04 (br), 3.74-3.68 (br), 3.49 (br), 3.28 (br), 2.95 (br), 2.55 (br), 1.97 (br, s, CH_3), 1.08-0.93 (br).

NP3: ^{1}H NMR (400 MHz, D_2O) δ_H: 4.50 (br, s, CH-β), 4.23-4.13 (br), 3.89 (br), 3.71 (br), 3.45-3.33 (br), 2.93 (br), 2.49 (br), 1.97 (br, s, CH_3), 1.11-0.91 (br).

Fluorescent labeling. For cell uptake experiments, the SCNPs were either labeled via DTAF fluorescent labels as previously described.[2] Prior to dialysis against demi-water, the solutions were filtered and labeled products were obtained through freeze-drying.

Copper reduction assay. D-(+)-glucose and sucrose were used as positive and negative controls, respectively. Polymer, nanoparticle or control (5 mg) was dissolved in water (1 mL) in a test tube. Benedict's reagent (1 mL) was added to the test tube and the solution was heated to 95 °C for 5 minutes.

Lectin studies. Concanavalin A (ConA) was dissolved in HEPES buffer (20 mM HEPES, 140 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 1.0 mM $MnCl_2$) to afford a stock solution of 60 μM (assuming ConA tetramers with a molecular weight of 104 kDa). A series of different concentrations of glycopolymers in HEPES buffer, ranging from 0.2 to 2000 μM, was prepared. Equal volumes of ConA and glycopolymers solutions were thoroughly mixed and incubated at room temperature overnight. White precipitate was centrifuged for 10 min and the supernatant was removed. The pellet was washed twice with cold buffer (200 μL) and resuspended in a 100 mM solution of methyl α-D-glucoside (600 μL).

Cytotoxicity. HeLa cells were seeded at 10 x 10^3 cells/well on 96-well plates and incubated at 37 °C in a humidified 5% CO_2-containing atmosphere. Sample solutions in DMEM medium were filtered with a 0.2 μm filter and diluted to the final concentrations (200, 100, 50, 10 and 1 μg/mL). After 24 h of incubation, 100 μL of the sample solution or medium for the references was added

per well. The medium of the negative control was aspirated 30 min prior to the assay and replaced by 70% methanol. After 24 h or 48 h of incubation, the medium was aspirated, and the cells were washed with 100 μL PBS. Subsequently, 100 μL of resazurin sodium salt (440 mM) in PBS was added to all wells and the cells were incubated for 1 h at 37 °C in a humidified 5% CO_2-containing atmosphere. The fluorescence intensity was measured on a Tecan infinite m200 plate reader at an excitation and emission of 560/590 nm.

FACS measurements. HeLa cells were seeded at 40 x 10^3 cells/well on 24-well plates and incubated at 37 °C in a humidified 5% CO_2-containing atmosphere. Sample solutions in DMEM medium were filtered with a 0.2 μm filter and diluted to a final concentration of 200 μg/mL. After 24 h of incubation, 500 μL of the sample solution or medium for the reference were added per well. The particles were incubated for 4 h. Subsequently, cells were washed with PBS and harvested with trypsin. Samples were transferred to Falcon tubes and centrifuged at 300 g for 2 min, the supernatant was removed and cells were re-suspended in 500 μL PBS. FACS analysis was performed using a BD Bioscience FACS ARIA II with excitation and emission filter of 488-530/30 nm.

Confocal laser scanning microscopy. HeLa cells were seeded at 5 x 10^3 cells/well on an FBS coated 96-well sensor plate. CellLight™ BacMam staining 2.0 (early endosome, late endosome or lysosome)-RFP (Red fluorescent protein) was added following the protocol of ThermoFisher and incubated at 37 °C in a humidified 5% CO_2-containing atmosphere. Sample solutions in DMEM medium was filtered with a 2.0 μm filter and diluted to a final concentration of 200 μg/mL. After 24 h of incubation, 100 μL of sample solution was added per well. The cells were incubated for 4 h and then washed with PBS followed by the fixation of the cells usind 4% formaldehyde. The cells were stained with CF®405M CF 408 WGA M for 20 min and DAPI (300 nM) for 3 min. Finally,

cells were washed and stored in PBS, before examination under a Nikon confocal microscope A1, equipped with lasers of the following excitation wavelengths: 405, 488 and 561 nm.

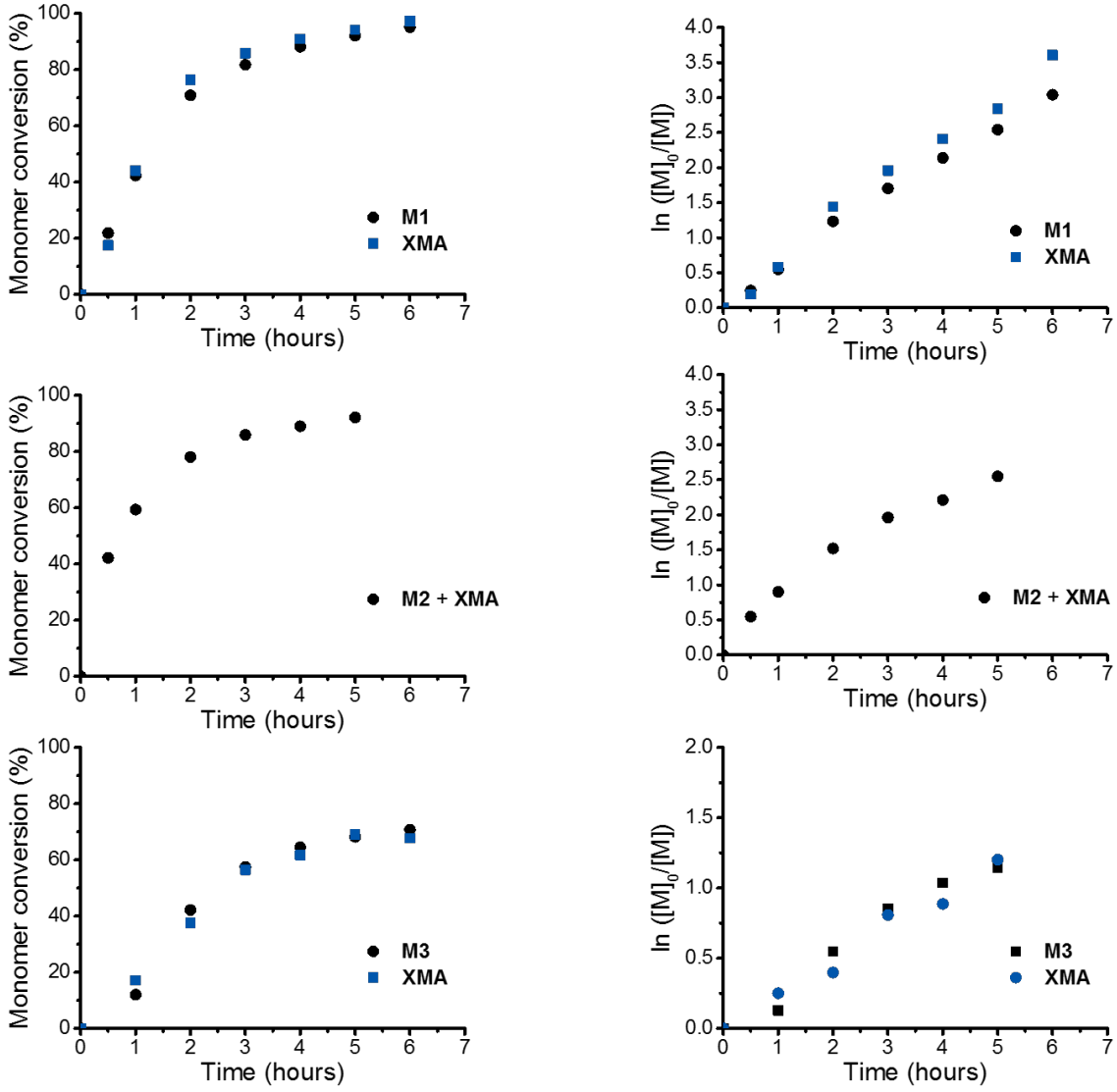

Figure S2. Kinetic plots of polymerization of **P1-3**.

Table S1. Polymerization details of polymer precursors.

	Monomer conversion (%)	Solvent	[GMA]:[XMA]:[CTA]:[I]	DP_{total}	X_{XMA} (%)	DP_{GMA}	DP_{XMA}	Ø RU_{GMA}
P1a	63	DMF	[204]:[22]:[1]:[0.2]	142	14	122	20	6.1
P1b	96	DMF	[391]:[40]:[1]:[0.4]	413	17	343	70	4.9
P2	92	DMF	[420]:[42]:[1]:[0.4]	425	15	361	64	5.6
P3	96	DMF	[338]:[34]:[1]:[0.4]	413	20	330	83	3.9

GMA = glucose/glucoside metharylate, XMA = xanthate methacrylate, DP = degree of polymerization, χ = fraction of monomer, Ø RU = average number of repeating units.

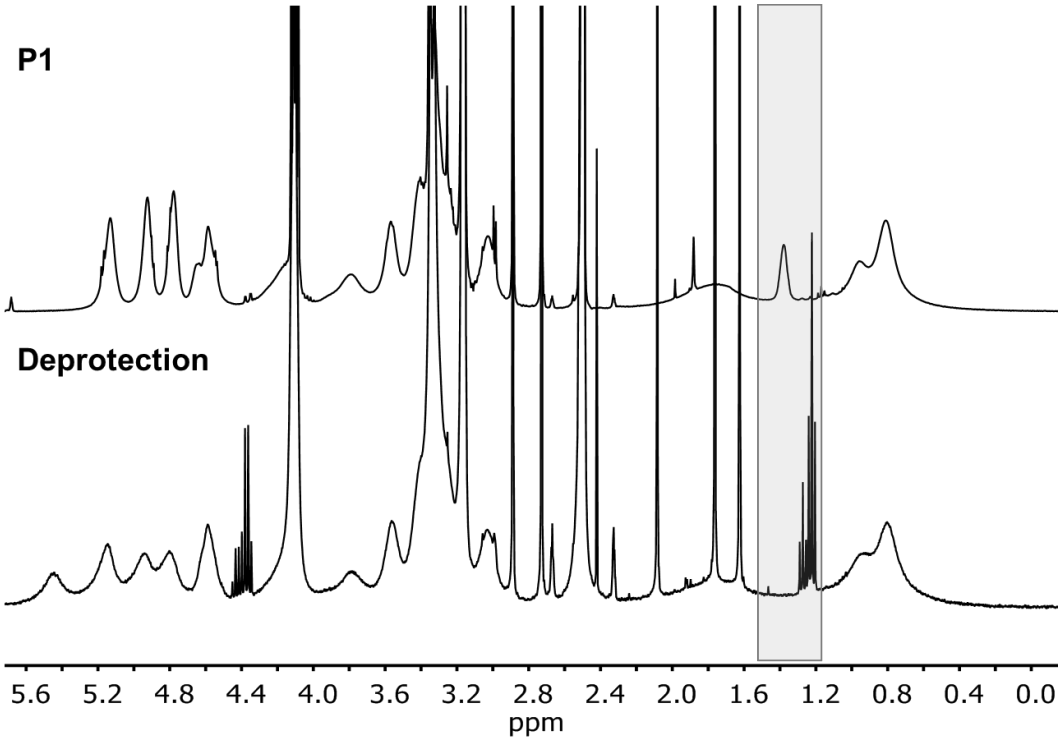

Figure S3. ^{1}H NMR spectra of **P1** before (top) and after 15 min reaction with hydrazine in DMSO-d_6.

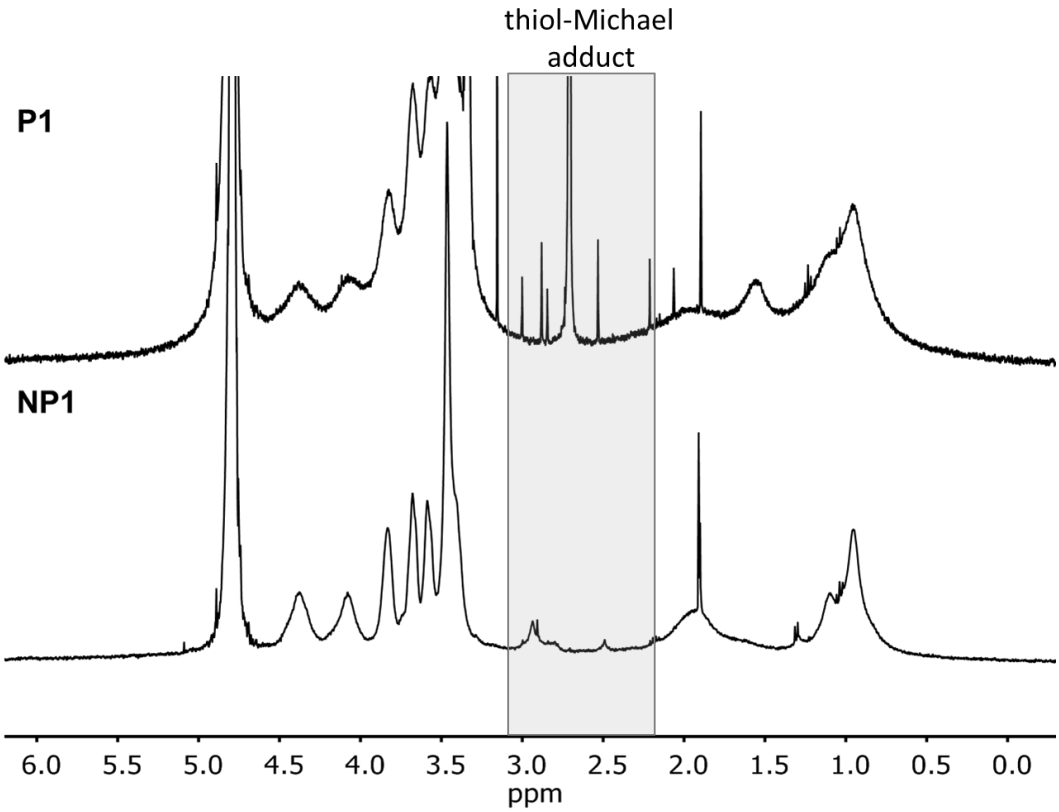

Figure S4. ^{1}H NMR spectra of **P1** and **NP1** in D_2O.

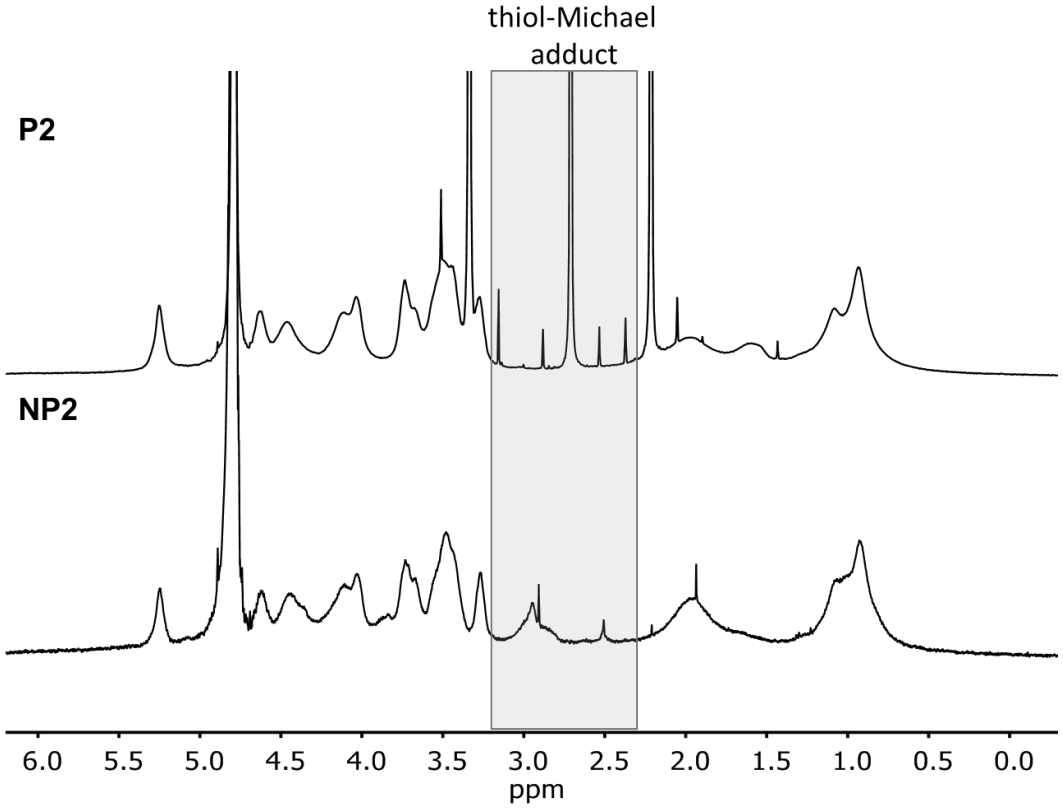

Figure S5. ^{1}H NMR spectra of **P2** and **NP2** in D_2O.

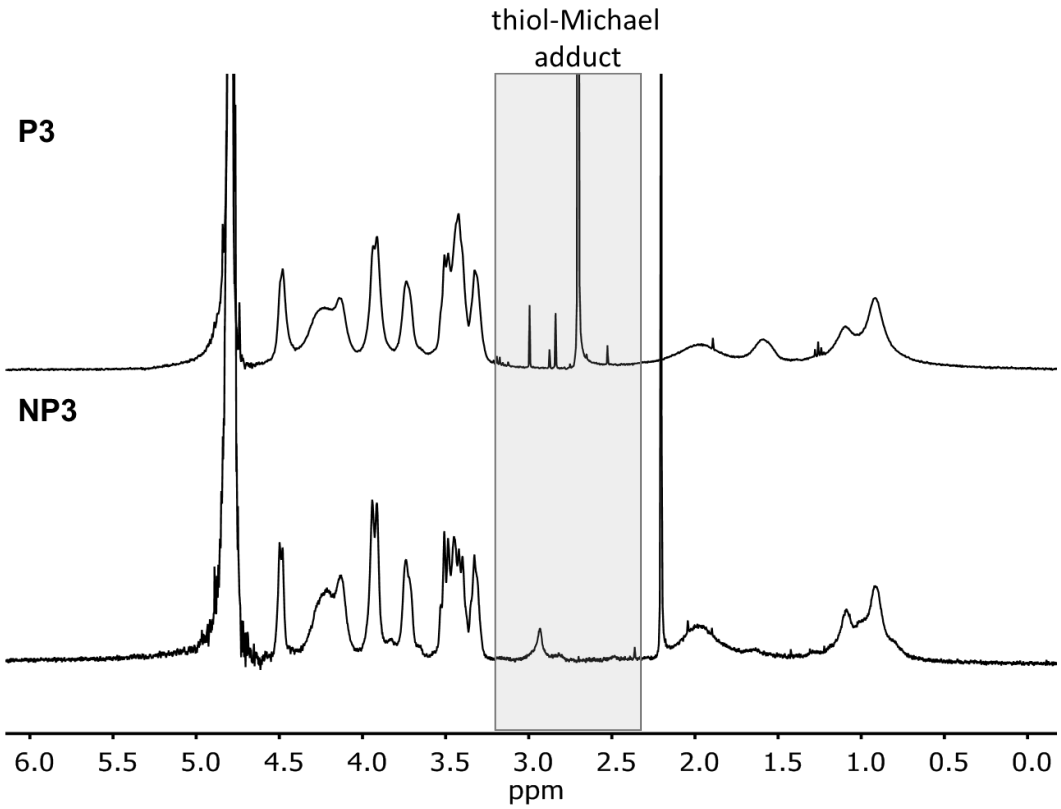

Figure S6. ^{1}H NMR spectra of **P3** and **NP3** in D_2O.

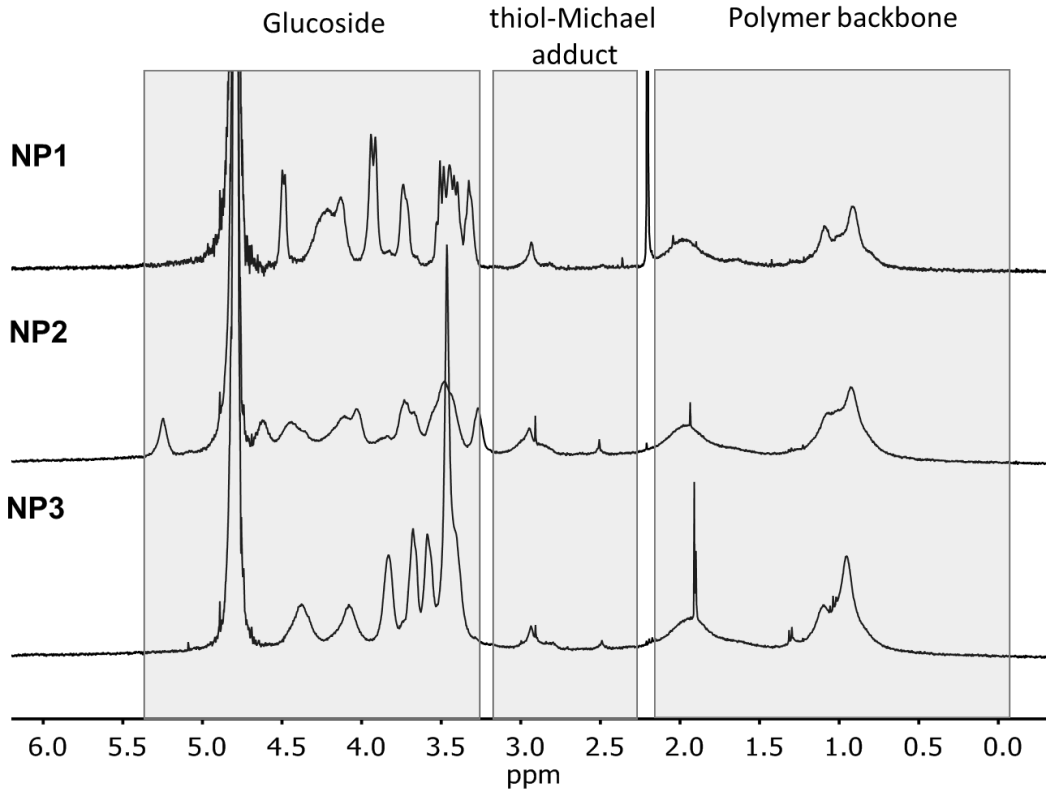

Figure S7. Overview of ^{1}H NMR spectra of **NP1-3** in D_2O.

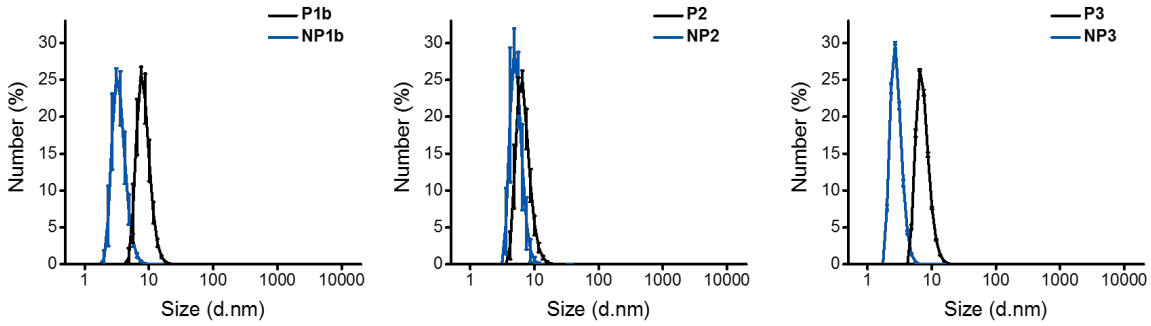

Figure S8. DLS spectra of polymers (**P1b-3**) and the corresponding nanoparticles (**NP1b-3**).

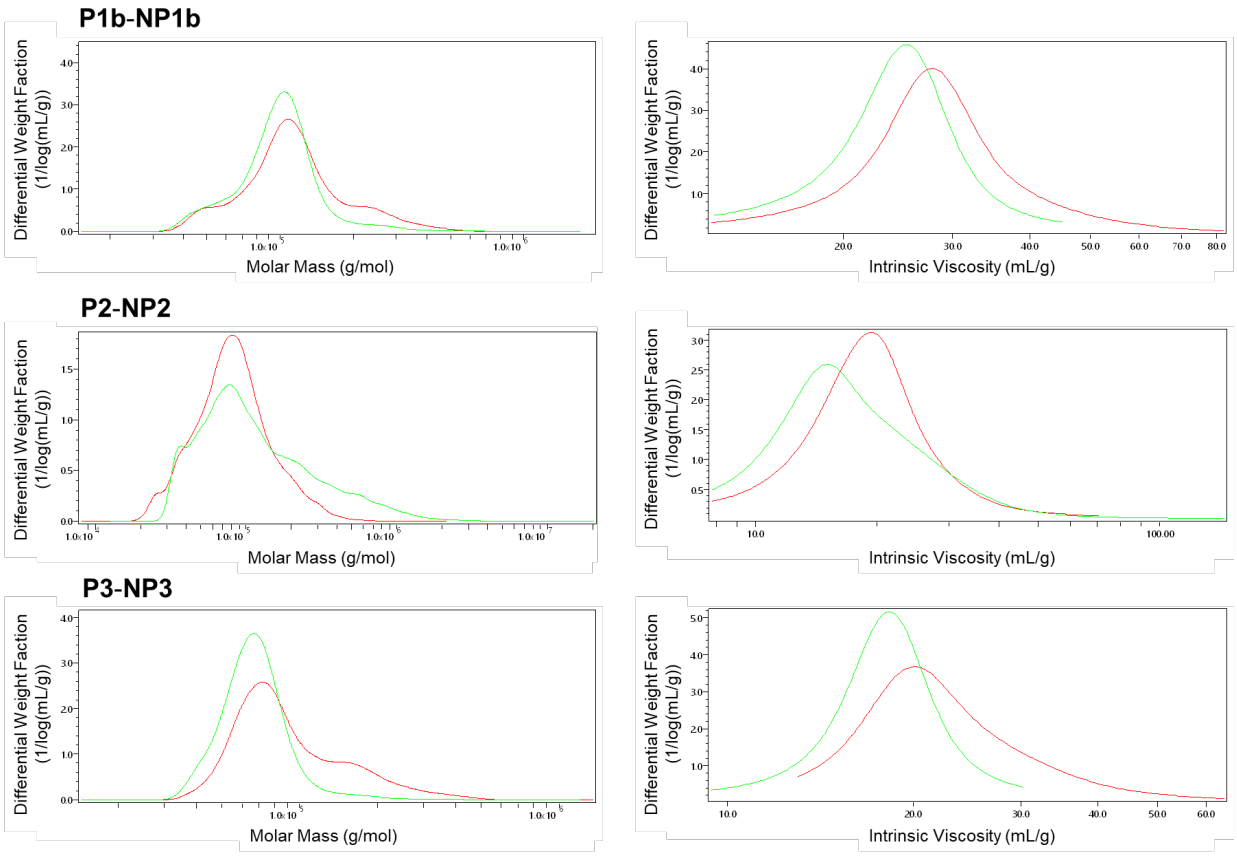

Figure S9. Differential weight distribution plots of polymers (**P1b-3**, in red) and the corresponding nanoparticles (**NP1b-3**, in green) vs. molar mass (left) and vs. intrinsic viscosity (right) as determined by GPC MALS and rheology measurements. Whereas the molar mass stays mainly the same, the intrinsic viscosity is decreasing upon cross-linking.

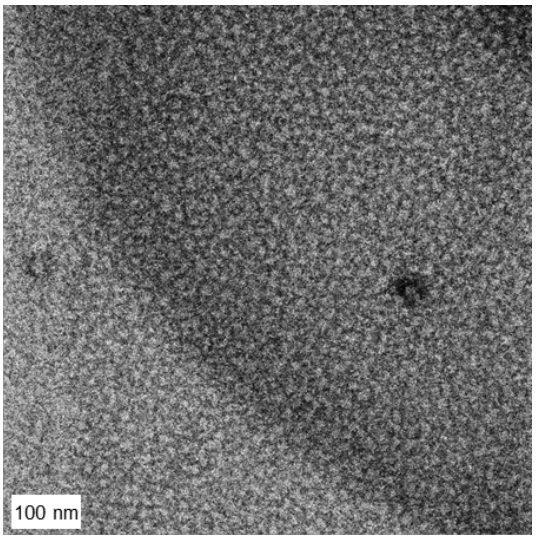

Figure S10. TEM image of stained **NP1a**.

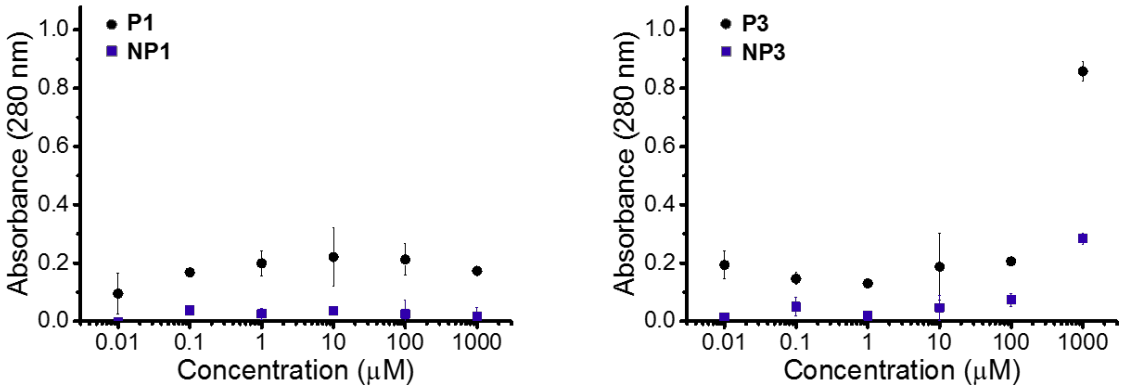

Figure S11. ConA precipitation assay for glycopolymers (**P1**, **P3**) and corresponding nanoparticles (**NP1**, **NP3**).

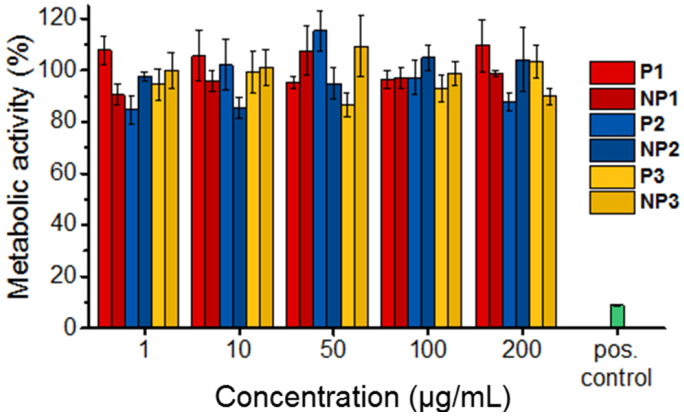

Figure S12. Metabolic activity of HeLa cells after incubation with glycopolymers (**P1**-**3**) and corresponding nanoparticles (**NP1**-**3**) for 24 h.

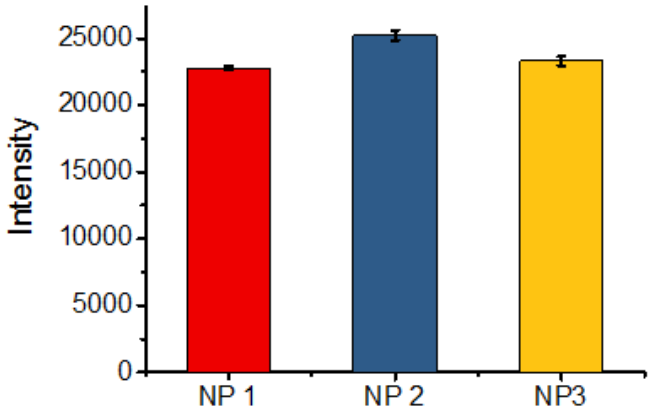

Figure S13 Comparison of the batch fluorescence intensity of nanoparticles used in cellular uptake studies (**NP1-3**).

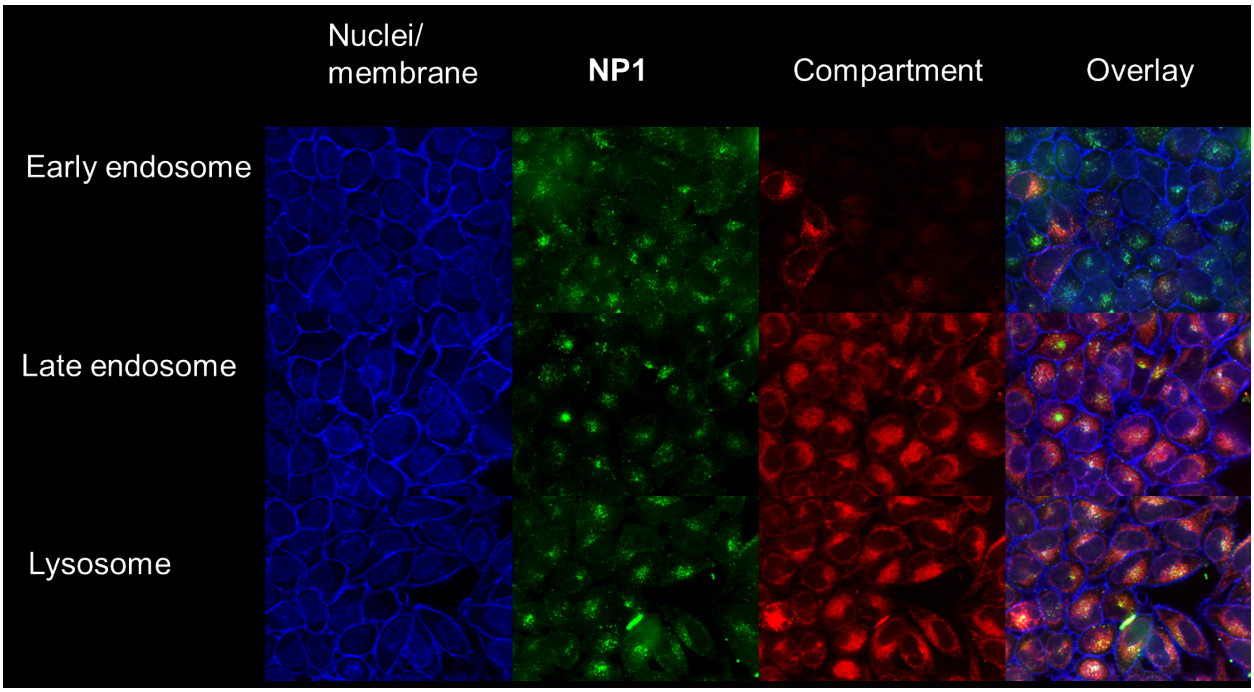

Figure S14 Confocal images of HeLa cells incubated 4 h with the glyco-SCNPs **NP1** (in green, nuclei and membrane = blue, cell compartment = red).

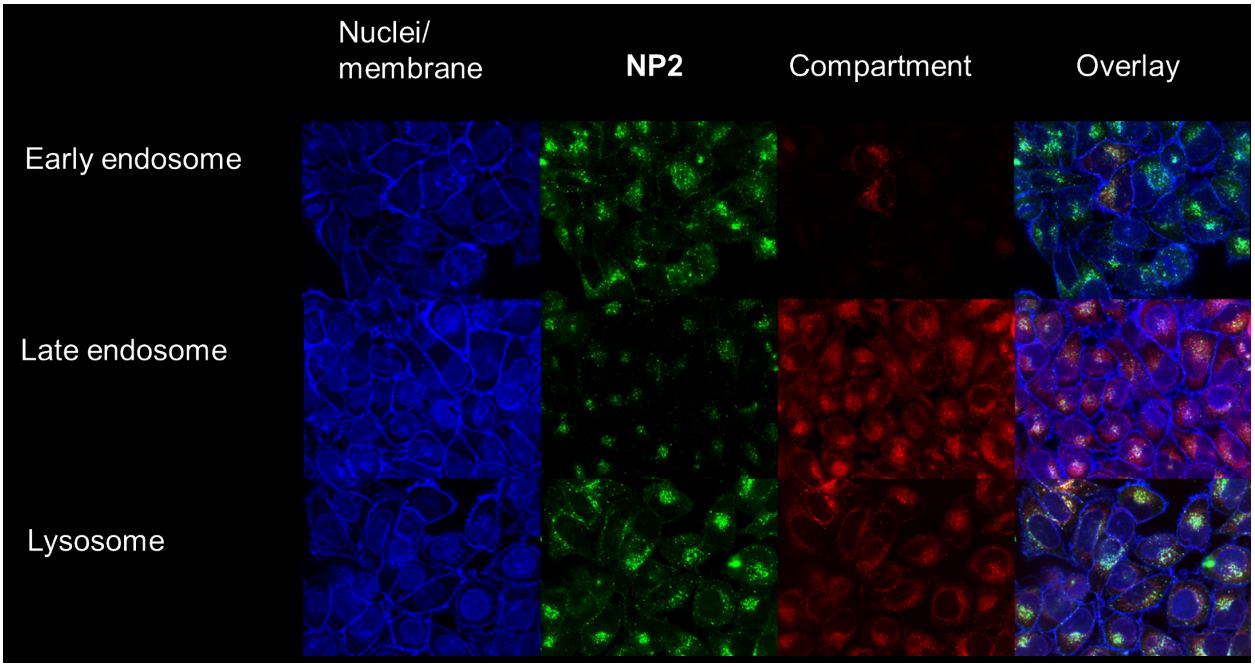

Figure S15 Confocal images of HeLa cells incubated 4 h with the glyco-SCNPs **NP2** (in green, nuclei and membrane = blue, cell compartment = red).

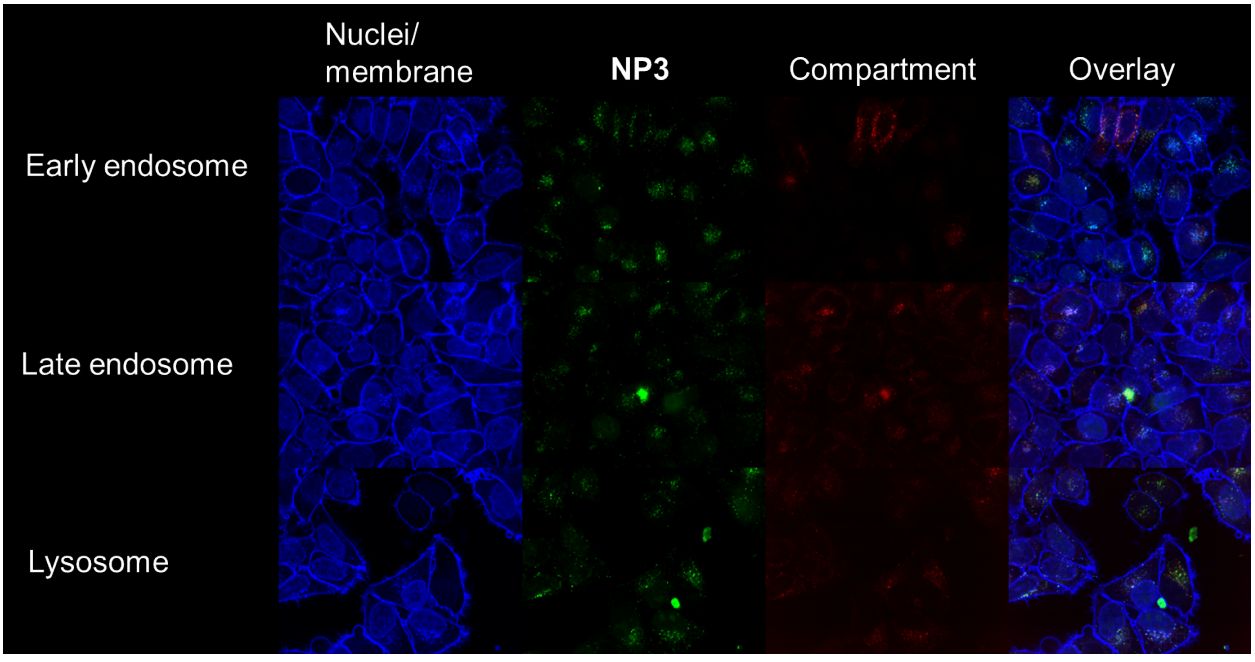

Figure S16 Confocal images of HeLa cells incubated 4 h with the glyco-SCNPs **NP3** (in green, nuclei and membrane = blue, cell compartment = red).

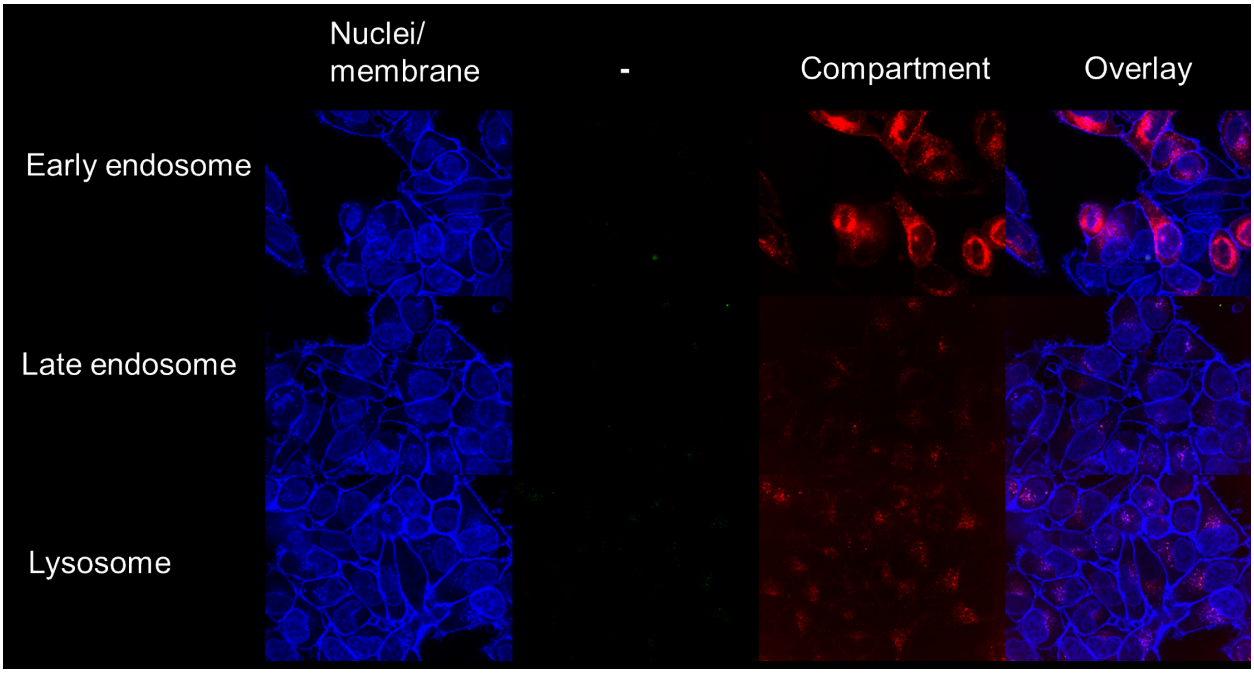

Figure S17 Confocal images of non-treated HeLa cells (nuclei and membrane = blue, cell compartment = red).

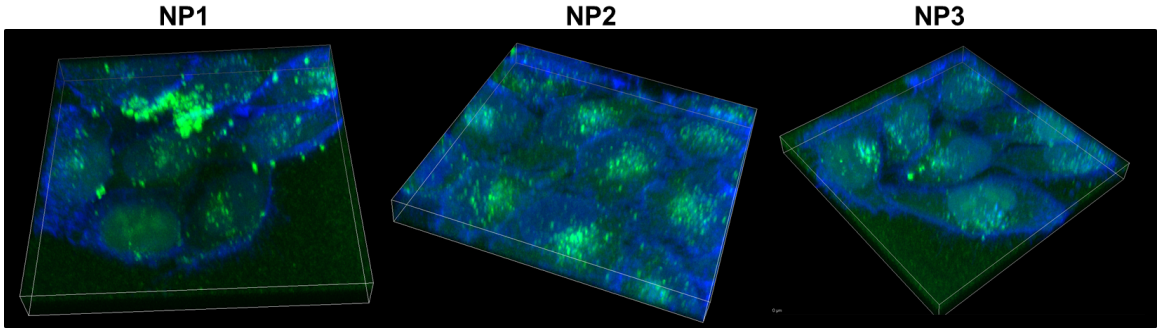

Figure S18 3D reconstruction of z-stacked confocal images of HeLa cells incubated with nanoparticles (**NP1-3**).