SUPPORTING INFORMATION

Elucidating the Inability of Functionalised Nanoparticles to Cross the Blood Brain Barrier and Target Specific Cells In Vivo

§Priya S.R. Naidu[1], §Nikolas Gavriel[2], §Chloe G.G. Gray[2], Carole A. Bartlett[2], Lillian M. Toomey[2,3,4], Jessica A. Kretzmann[1], Diana Patalwala[5], Terence McGonigle[3], Eleanor Denham[3], Charmaine Hee[1], Diwei Ho[1], Nicolas L. Taylor[1], Marck Norret[1], Nicole Smith[1], Sarah A. Dunlop[2], §K. Swaminathan Iyer[1], *§Melinda Fitzgerald[2,3,4]

[1]*School of Molecular Sciences, The University of Western Australia, Stirling Hwy Perth 6009 Western Australia*

[2]*Experimental and Regenerative Neurosciences, School of Biological Sciences, The University of Western Australia, Stirling Hwy Perth 6009 Western Australia*

[3]*Curtin Health Innovation Research Institute, Curtin University*

[4]*Perron Institute for Neurological and Translational Science, Ralph and Patricia Sarich Neuroscience Research Institute Building, Verdun St, Nedlands 6009 Western Australia, Australia*

[5]*Centre for Microscopy, Characterization and Analysis, The University of Western Australia, Stirling Hwy Perth 6009 Western Australia*

§ indicates equal contribution

* Corresponding E-mail: lindy.fitzgerald@curtin.edu.au

A. Detailed Materials and Methods

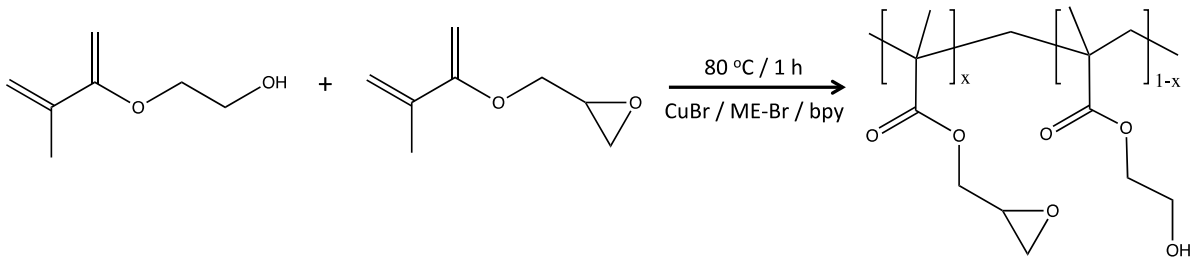
Materials

All materials and reagents used for the preparation of nanoparticles for this study were obtained from Merck© (previously Sigma-Aldrich®) unless specified otherwise. All tissue culture reagents were purchased from Gibco™ unless specified otherwise.

Nanoparticle (NP) synthesis

2-hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GMA) were copolymerized *via* atom-transfer radical-polymerization (ATRP) (refer to Schematic 1). GMA (12 mmol, 1.64 mL) and HEMA (28 mmol, 3.40 mL) were dissolved in methanol (MeOH) at monomer to solvent ratio of 1:3 and degassed 3 times *via* the freeze/pump/thaw method. An alkyl halide initiator (ME-Br, 209 µL, 280.16 g/mol, 1.34 g/mL, 1 mmol), copper (I) bromide (CuBr, 143 mg, 1 mmol) and 2,2-bipyridine (bpy, 143 mg, 2 mmol) were added to the degassed monomer solution. The reaction was heated at 80 °C under standard Schlenk conditions for 1 h and the copolymer, *p*(HEMA-*ran*-GMA), product was collected by precipitation in excess diethyl ether and dried overnight under vacuum.

Scheme S1: Atom-Transfer Radical Polymerisation (ATRP) of 2-hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GMA) to synthesize *p*(HEMA-*ran*-GMA) copolymer



The hydrophilic *p*(HEMA-*ran*-GMA) NPs were formed by a spontaneous water-in-oil (W/O) inverse nano-emulsion. The aqueous phase of the emulsion comprised of copolymer dissolved in water (25 (v/v) %) which was added to an organic phase consisting of cyclohexane (75 (v/v) %) and 0.5 M dioctyl sulfosuccinate sodium salt (AOT). Ethylene diamine (50 mol %) was added to the resulting optically clear and homogenous emulsion to enable cross-linking of the dispersed aqueous copolymer over 24 h at room temperature (RT).

To retrieve the cross-linked NPs, all organic solvent in the W/O emulsion was removed under reduced pressure, leaving behind a solid mixture of AOT and cross-linked NPs. This was re-dispersed in deionized water and ultracentrifuged at 300k × *g* for 30 min (~8 – 10 times) and the supernatant consisting of AOT was discarded after each run in order to purify the NPs.

After dialysing the NPs over 24 h against MilliQ water (Fisherbrand™ Regenerated Cellulose Dialysis Tubing MWCO 12-14 kDa) to remove any unreacted components and surfactant, aqueous ammonia (aq. 25% NH_3) was added in 5-fold excess with respect to the epoxide functional groups present in the NP suspension and reacted over 24 h at 60 °C to enable amine-functionalization of the NPs *via* epoxide ring opening. The amine-functionalized NPs were purified by dialysis overnight against MilliQ water (4 × 5 L changes over 24 h) and lyophilized for yield assessment.

Cyanine5 (Cy5)-N-hydroxysuccinimide ester (Lumiprobe) (0.27 mg; 433.6 μmol) was dissolved in 1:10 reaction volume of dimethyl sulfoxide (DMSO) and added to a 9:10 reaction volume of amine-functionalized *p*(HEMA-*ran*-GMA) NPs in amine-free buffer (50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) ; pH 8.3) as suggested by the manufacturer's protocol. The reaction mixture was briefly sonicated and stirred over 4 h at RT. Unbound fluorophore was removed to purify the Cy5-functionalized *p*(HEMA-*ran*-GMA) NPs by dialysis against MilliQ water overnight (3 × 4 L changes). Cy5-*p*(HEMA-*ran*-GMA) NPs were lyophilized for yield assessment and/or for storage at RT in a desiccator away from direct light.

Cy5-*p*(HEMA-*ran*-GMA) NPs were functionalized with αNG2 antibody and TAT using a hetero-bifunctional sulfosuccimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) cross-linker with polyethylene glycol (PEG) chain spacer, $SM(PEG)_{12}$ (Thermo Scientific™). This cross-linker consists of both N-hydroxysuccinimide and maleimide groups adjacently positioned with PEG spacers consisting of 12 ethylene glycol units. To enable sulfhydryl groups to link to the maleimide end group of the $SM(PEG)_{12}$, the α-NG2 and TAT peptide were individually exposed to 10 molar excess of Tris (2-carboxyethyl) phosphine (TCEP) in 50 mM HEPES buffer at pH 6.5 for 1 h at RT. During this time, the Cy5-*p*(HEMA-*ran*-GMA) NPs were resuspended in 50 mM HEPES buffer at pH 7 and $SM(PEG)_{12}$ in DMSO was added at 10 molar excess with respect to theoretical amine functional groups left on NPs after Cy5 conjugation. The cross-linker attachment reaction

was carried out at RT for 1 h with moderate stirring. After which, the disulphide bond reduced moieties were introduced to the NP suspension to produce respective functionalized NPs by allowing the reaction to occur at RT for 1 h. Variants with αNG2 only, TAT only and both αNG2 and TAT were synthesized. Based on the theoretical quantity of cross-linker presence on the NP surface, the α-NG2 and TAT were exposed at 10 molar excess and allowed to react for 1 h at RT. For NPs functionalized with both α-NG2 and TAT, the antibody functionalization was conducted for the first 30 min of the reaction time, followed by TAT functionalization for another 30 min. Unreacted reactants were removed from NP variants by centrifugation at 17000 × *g* for 30 min and the supernatant was assessed by Micro BCA™ analysis to allow calculation of the degree of functionalization. The functionalized NPs were resuspended in 50 mM HEPES at pH 7.4 and stored at 4 °C until required.

NP characterization

Mean hydrodynamic size and surface charges (zeta potentials) of the NP variant suspensions were characterized using dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, Malvern Panalytical®) using a 4 mW He-Ne laser operating at 633 nm with a scattering angle of 173°. Respective NP variant samples at a concentration of 0.1 mg/mL were prepared by resuspension by sonication for 30 s, immediately prior to analysis, in order to minimize incidence of particle aggregation. Measurements were taken in triplicate after an initial equilibrium of 1 min. The samples were calibrated against measurements for recorded for PGMA (refractive index 1.515; viscosity 0.05) in water at 25 °C as dispersant (refractive index 1.33; viscosity 0.887). The intensity-weighted hydrodynamic radii and zeta potentials of the NP variants were presented as mean ± standard deviation (S.D). All zeta potential measurements were taken with 1× phosphate buffered saline (PBS) at physiological pH (7.4). 10 µL NP suspensions were deposited on carbon-coated copper grids and dried overnight in preparation for transmission electron microscopy (TEM) imaging. All TEM images were obtained at 120 kV using JEOL JEM-2100.

The attachment of αNG2 antibody and TAT peptide to the NP was confirmed as follows:

- αNG2 detection

Rabbit αNG2-functionalized NPs were incubated with 1:500 AF488 goat anti-rabbit secondary antibodies for 30 min at RT with constant agitation, to allow secondary antibody binding. The NPs were then centrifuged (17000 × *g*; 10 min) to wash and remove any

unbound secondary antibodies from the NP pellet. This clean-up process was repeated 2 more times by resuspending the NP pellet with 1× PBS. After the final centrifugation, the resuspended NP pellet was assessed for fluorescence using the spectral properties of AF488 to confirm the presence of the primary antibody, αNG2.

- TAT detection

TAT-functionalized NPs were incubated with 1:500 chicken αTAT antibodies for 30 min at RT with constant agitation, to allow αTAT 1° antibody to bind to TAT peptide present on the NPs. Following this incubation, the NPs were centrifuged (17000 × *g*; 10 min) to wash and remove unbound 1° antibodies from the NP pellet. This clean-up process was repeated 2 more times by resuspending the NP pellet with 1× PBS. The resuspended αTAT-TAT-functionalized NPs were then incubated with 1:500 FITC-conjugated anti-chicken antibody for 30 min at RT with constant agitation. The FITC-αTAT-TAT-functionalized NPs were then centrifuged and washed as described before to remove any unbound FITC anti-chicken antibodies from the nanoparticle pellet. After the final centrifugation, the resuspended NP pellet was assessed for fluorescence using the spectral properties of FITC to confirm the presence of the secondary antibodies bound to the αTAT-TAT-functionalized NPs.

Preparation of mixed cortical cultures

Working under sterile conditions, brains were dissected from five humanely euthanized rat pups and placed into a petri dish containing Hanks Balanced Salt Solution (HBSS). Cortices were surgically freed from subcortical structures and meninges, using forceps and fine operating scissors, and placed into a new petri dish containing clean, ice-cold HBSS, where they were chopped into ~1 mm^3 pieces using a sterile scalpel blade (Braun). Digestion solution containing 6.8 mL HBSS, 0.4 mL DNaseI (0.2 mg/mL) and 0.3 mL trypsin (0.25 %) was added for 15 min in 5 % CO_2 at 37 °C. Digested cortices were transferred into 5 mL DMEMS20 (DMEM, 4 mM 100 × GlutaMAX, 1 mM sodium pyruvate, 20 (v/v) % fetal bovine serum (FBS), 100,000 U/mL penicillin-streptomycin). Residual tissue in the petri dish was gently washed with ~2-3 mL DMEMS20 and added to the digested cortices which were then centrifuged for 5 min at 100 × *g*, at which point, a clear tissue pellet could be distinguished. 5 mL of DMEMS20 was added to the tissue pellet, which was then triturated using a transfer pipette until almost homogenous and settled on ice for 10 min. Avoiding the loose tissue pellet, the cell suspension was passed through a 70 μm nylon strainer (VWR International) and into 5 mL DMEMS20. Cells were counted using Trypan blue exclusion

assay (0.4 % Trypan blue; Invitrogen™) with a Neubauer Improved Bright-lined haemocytometer (ProSciTech®) on an Olympus® CK30 culture microscope at 20 × magnification. Cell suspension was diluted in DMEMS20 (+ 10 ng/mL platelet derived growth factor-AA (PDGF-AA) and 10 ng/mL basic fibroblast growth factor (bFGF)) and plated onto poly-DL-ornithine (1 × concentration in DPBS overnight; Sigma-Aldrich®) coated 18 mm^2 glass coverslips in 12 well plates at a density of 1 × 10^4 cells/cm^2. Cultures were incubated at 37 °C in 5 % CO_2 for 10 days, with half media changes of DMEMS20 (containing 2× concentration of growth factors i.e. + 20 ng/mL PDGF-AA and 20 ng/mL bFGF) every 2 – 3 days.

In vitro NP treatment

After 10 days *in vitro*, stock NP suspensions (4-5 mg/mL in HEPES buffer) were added to sterilized HEPES buffer to achieve a concentration range of between 100 μg and 4 mg/mL. These NP suspensions in HEPES buffer were then further diluted in DMEMS20 (+ 20 ng/mL PDGF-AA and 20 ng/mL bFGF) at 1:10 ratio, giving a concentration range of 10 – 400 μg/mL. NPs were added to wells at the time of half media changes; final NP concentrations ranged from 5 – 200 μg/mL, whilst controls received HEPES buffer diluted in DMEMS20 (+ 20 ng/mL PDGF-AA and 20 ng/mL bFGF) at 1:10 ratio.

In separate experiments, NP variants at a final concentration of 25 μg/mL were applied to 0.17 mm thickness coverslips at 10 days post plating in 3 sets of duplicates, to allow for immunohistochemical identification of multiple cell types and NP localization therein, and cultures were incubated for a further 24 h at 37 °C in 5 % CO_2. Experiments were repeated three times using cultures derived from separate litters of rat pups. NP variants were added similarly to cultures in DMEMS20, either with or without FBS, PDGF-AA or bFGF supplementation, for 4 h at 4°C; experiments were repeated twice, using cultures derived from separate litters of rat pups.

Immunohistochemistry

Cultures were removed from the incubator and briefly washed once with 37 °C Dulbecco's phosphate buffer solution (DPBS; Thermo Fisher Scientific™). Cells were fixed by removing half of the volume of DPBS and adding an equal volume of 4 (w/v) % paraformaldehyde (Sigma-Aldrich®) in 0.1M phosphate buffer pH7.2 (PFA) for 5 min, then removing all of the solution and adding 4 (w/v) % PFA for 20 min, followed by three washes with 1 × PBS. Fixed cells on coverslips were then incubated in wells in 1 × PBS + 0.2 (v/v) % Triton™ X-

100 (PBST) + 5 (v/v) % normal donkey serum (DkS; Abcam© AB7475) for 30 min to block non-specific binding sites and permeabilize cellular membranes, then incubated at 4 °C overnight in the dark on an IKA Vibrax® VXR-VX7 orbital shaker at ~80 rpm with primary antibodies (1:500 dilution in solution of PBST + 5 (v/v) % DkS) recognizing OPC indicators NG2 (Merck©; rabbit AB5320) and Olig2 (R&D Systems®; goat AF2418); astrocyte indicator glial fibrillary acidic protein (GFAP; Abcam©; rabbit AB33922); microglia indicator ionized calcium-binding adapter molecule 1 (Iba1; Abcam©; goat AB5076); neuronal indicator beta-III tubulin (βIII-T; Abcam© rabbit AB18207) and oligodendrocyte indicator adenomatous polyposis coli clone 1 (CC1; Merck©; mouse OP80). Following overnight incubation, the fixed cells were washed 3× 5 min with 1 × PBS. Fluorescent secondary antibodies Alexa Flour® 488 and 555 (1:400; anti-rabbit, anti-goat and/or anti-mouse; Thermo Fisher Scientific™) and Hoechst 33342 (1:1000; Thermo Fisher Scientific™) were added and cells incubated at RT in the dark for 2 hours on an IKA Vibrax® VXR-VX7 orbital shaker at 100 rpm, then washed three times with 1 × PBS. Coverslips were then removed from wells using fine forceps, carefully mounted onto Superfrost Plus™ microscope slides (Thermo Fisher Scientific™) using Flouromount-G™ (Thermo Fisher Scientific™), and stored in the dark at RT until visualization.

B. Supplementary Results

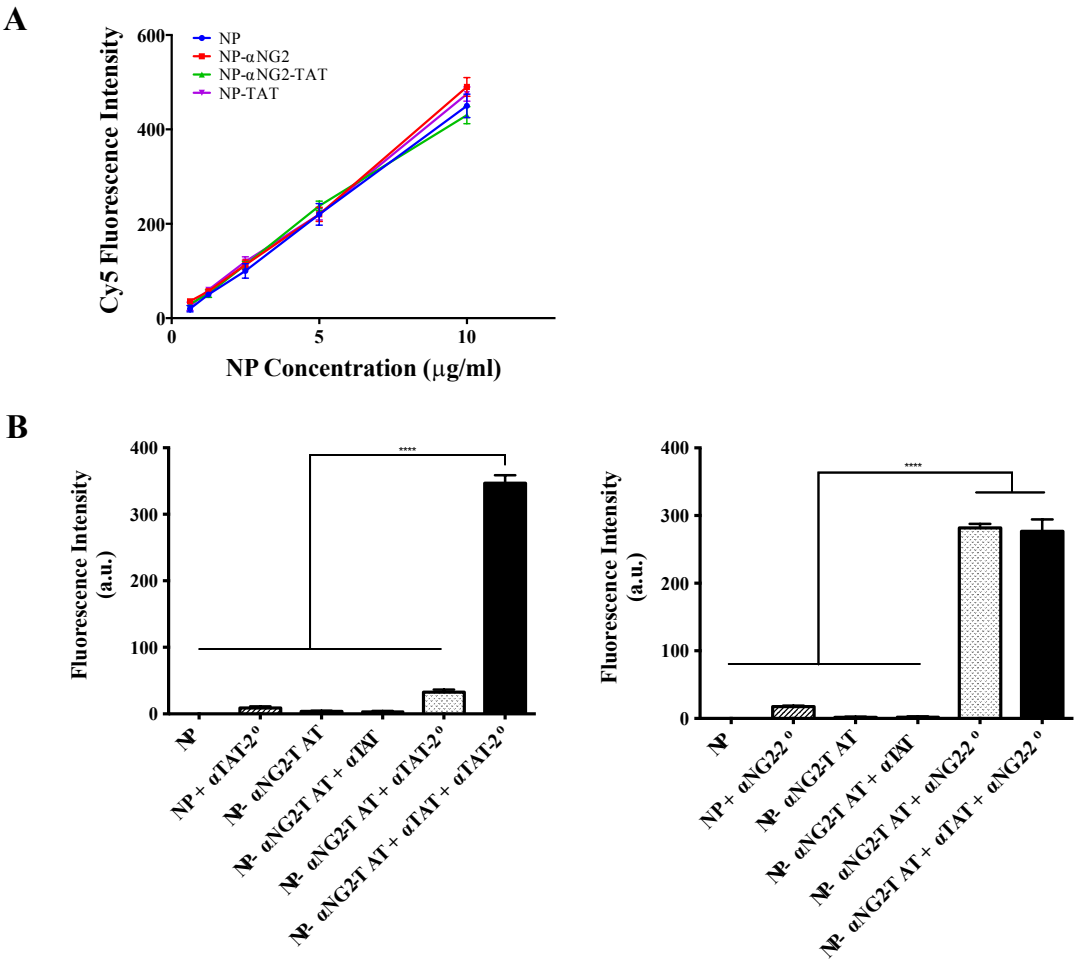


Figure S1. Additional characterization of NP variants.

(A) Assessment of Cy5 fluorescence intensity of NP variants demonstrated linear fluorescence within the tested concentration range, regardless of functionalization. (B) Fluorescence intensities of secondary (2°) antibodies bound to αNG2, or to αTAT antibodies recognizing conjugated TAT on the surface of NP-αNG2-TAT. Significant differences were indicated by ****$p \leq 0.0001$.

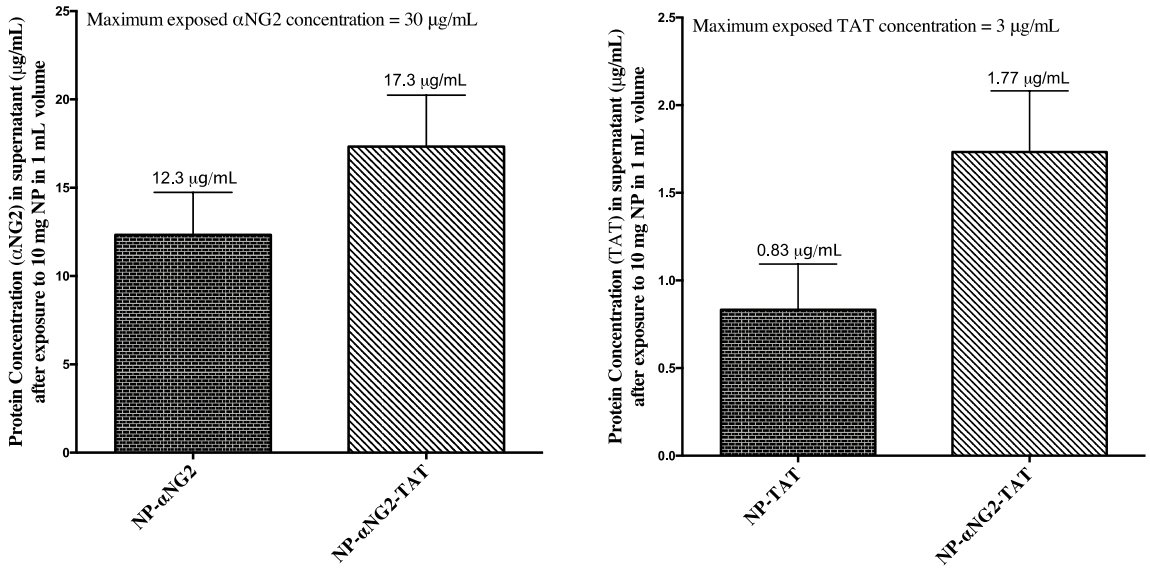


Figure S2. Degree of functionalization of targeting moieties (anti-NG2 and TAT) on respective NP variants determined by Micro BCA™ assessment of the supernatant containing unreacted antibody or peptide.

Notes:

From the known maximum concentration of exposed antibody or peptide, the amount bound to a known mass of NPs was back-calculated:

Amount of anti-NG2 per mg of NP-αNG2 = 1.8 μg
Amount of anti-NG2 per mg of NP-αNG2-TAT = 1.2 μg
Amount of TAT per mg of NP-TAT = 0.2 μg
Amount of TAT per mg of NP-αNG2-TAT = 0.09 μg

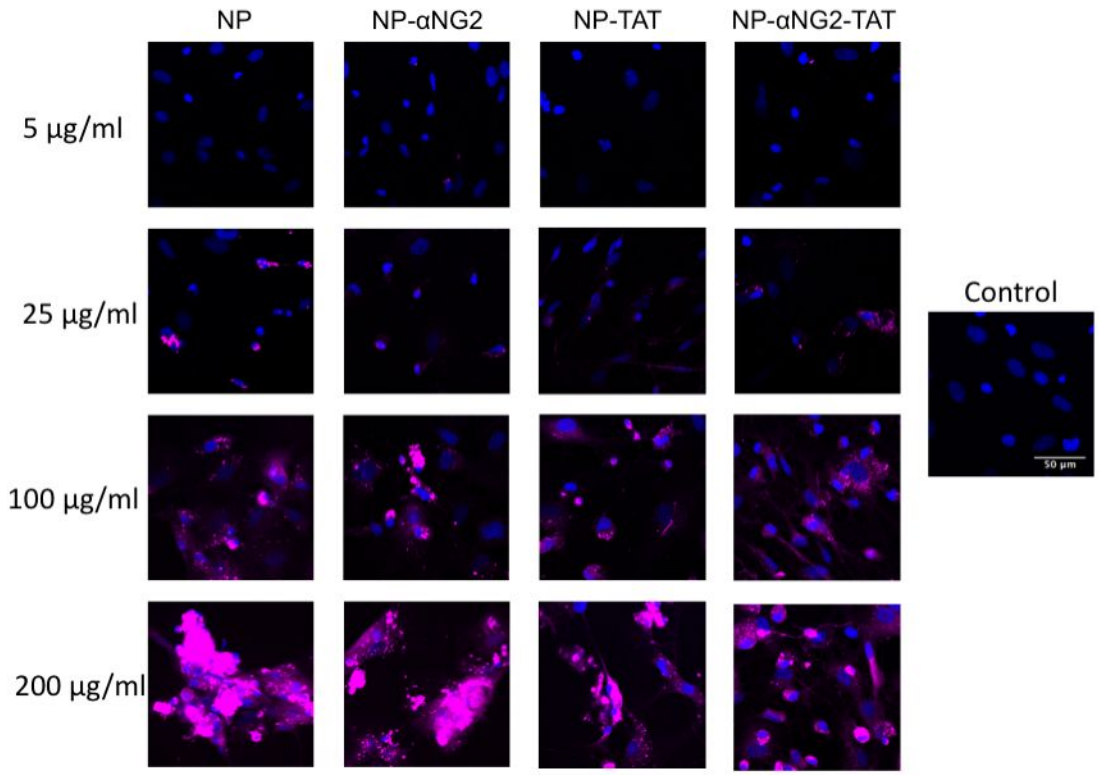


Figure S3. Representative images of mixed cortical cultures treated with Cy5-labelled *p*(HEMA-*ran*-GMA) NP variants at concentrations of either 5, 25, 100 or 200 µg/mL.

Nuclei were visualized with Hoechst (blue). Each column represents NP variant treatment, whilst each row displays variants at different dosages. Control (no NP) is on the far right. Microscope settings were consistent for all NP concentrations for comparative purposes; 25 µg/mL provided optimal discriminatory capacity between distributions of NP variants. Scale bar = 50 µm, applies to all images.

Notes:

Cultures treated with 100 and 200 µg/mL NP variants displayed high Cy5 florescence in all cells, indicating lack of discriminatory localization; 200 µg/mL of all NP variants assessed appeared to aggregate and therefore 25 µg/mL NPs was deemed optimal for analysis and used for further detailed assessments.

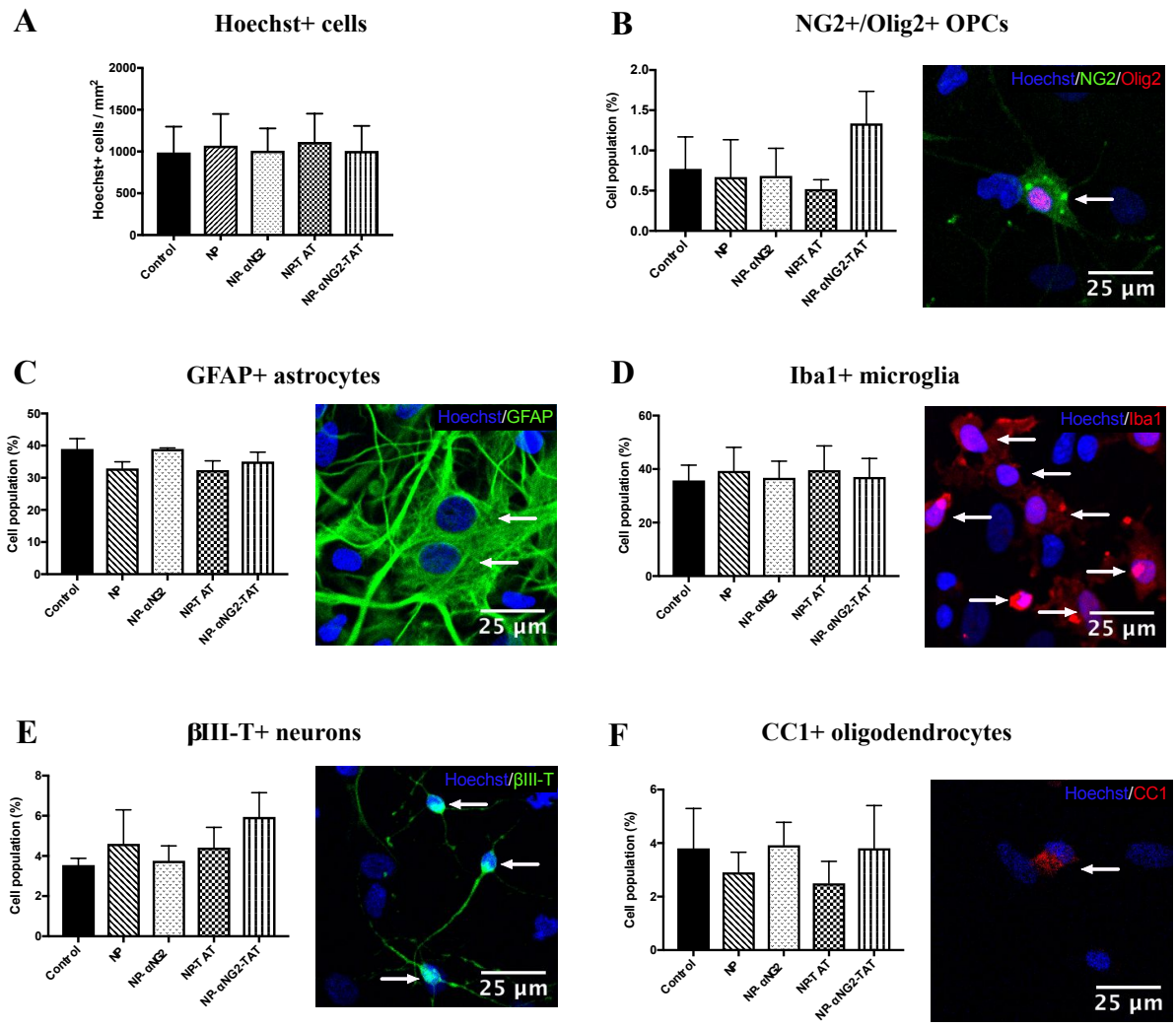


Figure S4. Effect of Cy5-labelled *p*(HEMA-*ran*-GMA) NP variants on the number of Hoechst+ cells, OPCs, astrocytes, microglia, neurons and oligodendrocytes.

Numbers of cells, expressed as mean ± standard error of measurement (SEM) for n = 2 – 3 independent experiments, following treatment with 25 µg/mL of the NP variants or HEPES buffer control did not change for (A) Hoechst+ cells ($F(4,10) = 0.027$; $p = 0.998$), (B) NG2+/Olig2+ OPCs ($F(4,10) = 0.743$; $p = 0.584$), (C) GFAP+ astrocytes ($F(4,5) = 1.607$; $p = 0.305$), (D) Iba1+ microglia ($F(4,10) = 0.051$; $p = 0.994$), (E) βIII-T+ neurons ($F(4,10 = 0.737$; $p = 0.587$) or (F) CC1+ oligodendrocytes ($F(4,10) = 0.309$; $p = 0.806$). A minimum of 150 cells per cell type was assessed. * Indicates significant differences at $p \leq 0.05$; none seen.

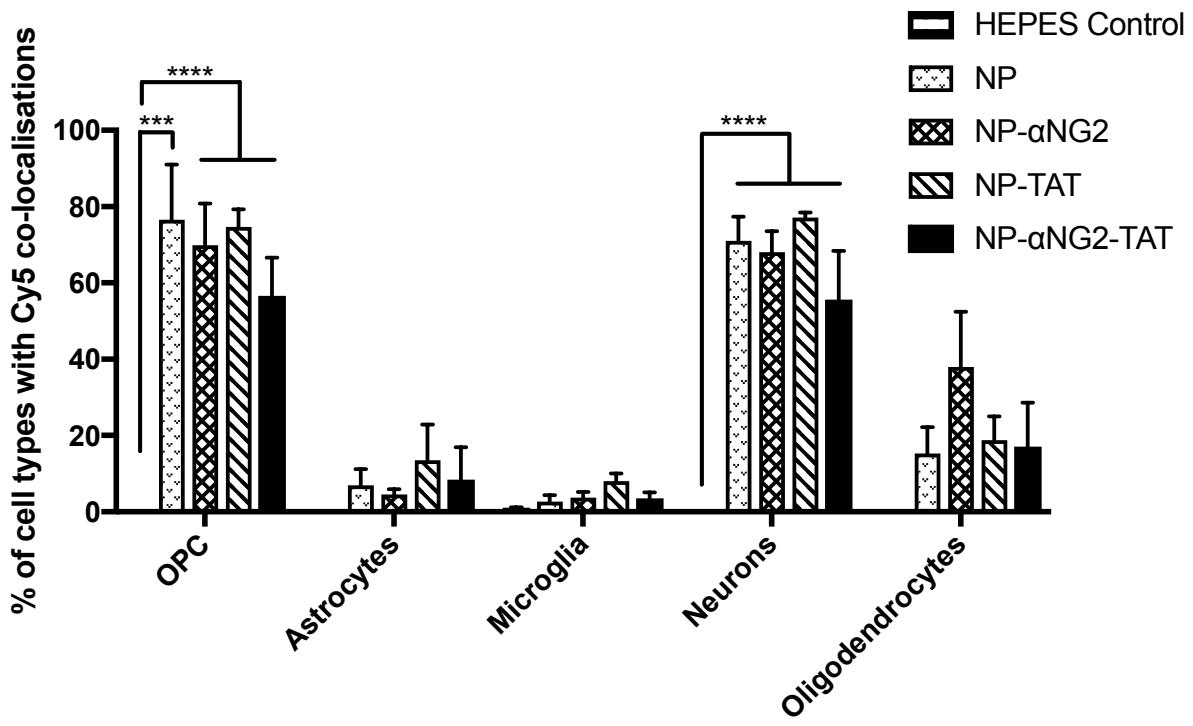


Figure S5. ***In vitro*** **assessment of cell types with Cy5 localization from Cy5-labelled** ***p*****(HEMA-*ran*-GMA) NP variants.**

Comparisons between NP variants for each of the cell types. Data presented as mean ± standard error of measurement (SEM) percentages of each cell type with Cy5 localization, for n = 2 – 3 independent experiments, following treatment with 25 μg/mL of the NP variants or

HEPES buffer control. Significant differences have been indicated by ** $p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. A minimum of 100 cells per cell type was assessed.

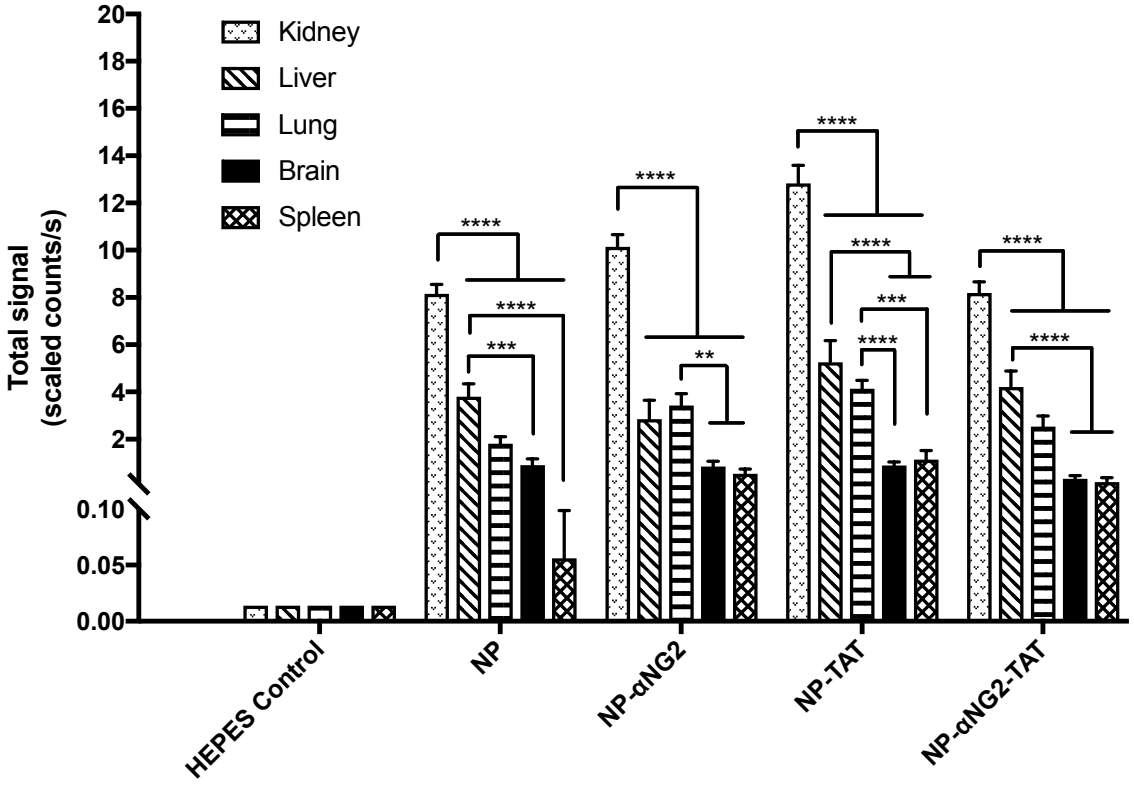


Figure S6. Comparison of the total Cy5 fluorescence signal from intravenously delivered Cy5-labelled *p*(HEMA-*ran*-GMA) NP variants, above threshold (scaled counts/s) in a range of whole organs.

Comparisons made between organ types for each of the NP variants. Histogram bars represent the mean Cy5 signal intensity above threshold (scaled counts/s) ± standard error of measurement (SEM). Higher signal intensities were indicative of a higher uptake of the respective Cy5-labelled NP variant by the given organ.

Notes:

Data was displayed to allow visualization of comparisons of NP variants between organs. In organs from rats IV-injected with each of the NP variants, the mean Cy5 fluorescence intensity was significantly higher in the kidney than in the liver, lung, brain or spleen. For unfunctionalized NPs and NP-αNG2-TAT, liver was also higher in Cy5 fluorescence than brain or spleen; for NP-αNG2, lung was higher in Cy5 fluorescence than brain or spleen; and for NP-TAT, both liver and lung were higher in Cy5 fluorescence than brain or spleen.

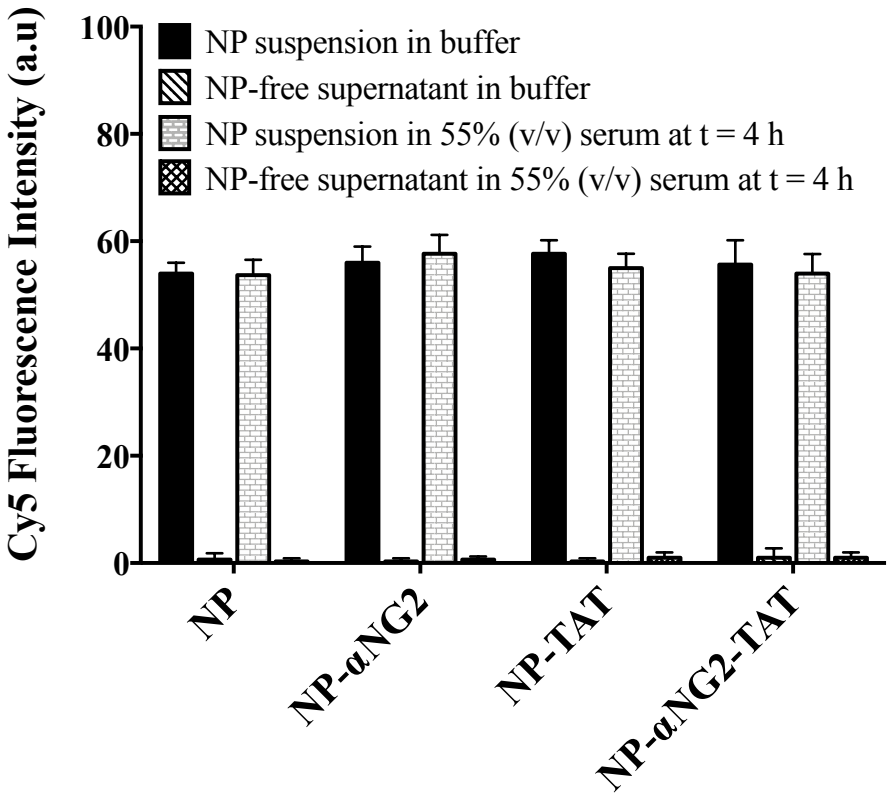


Figure S7. Stability of Cy5-labelled NPs under simulated *in vivo* conditions.

NP variants were incubated in 55 (v/v) % serum at 37 °C for 4 h to simulate the environment experienced by the NPs in the *in vivo* assessments. In order to assess the stability of the NP with respect to the Cy5 conjugation, the supernatant after the suspension was centrifuged to retain the NPs as a pellet, and assessed for free Cy5 fluorescence. Results showed that Cy5 fluorescence was not detected in the supernatant, suggesting that the NPs remained stable throughout the duration of *in vivo* circulation.

Table S1. Cy5 signal indicating the presence of Cy5-labelled NP variants in the measured organs.

	Brain	Kidney	Lung	Spleen	Liver
NP	1.36 ± 0.82	77.18 ± 4.30	3.90 ± 0.84	0.01 ± 0.01	17.56 ± 3.73
NP-αNG2	0.65 ± 0.28	81.49 ± 4.69	9.62 ± 2.47	0.31 ± 0.15	7.93 ± 4.21
NP-TAT	0.37 ± 0.08	76.88 ± 3.06	8.63 ± 1.47	0.73 ± 0.30	13.38 ± 3.53
NP-αNG2-TAT	0.20 ± 0.11	72.88 ± 6.13	7.14 ± 1.88	0.15 ± 0.15	19.64 ± 4.69

Data represented as mean percentages of the total signal per animal ± standard error of measurement (SEM).