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

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Keywords: *p*(HEMA-*ran*-GMA) nanoparticles; functionalization; protein corona; blood brain barrier; oligodendrocyte precursor cells

Abstract

The adsorption of serum proteins on the surface of nanoparticles (NPs) delivered into a biological environment has been known to alter NP surface properties and consequently their targeting efficiency. In this present article we use random copolymer (*p*(HEMA-*ran*-GMA)) - based NPs synthesized using 2-hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GMA). We show that serum proteins bind to the NP and that functionalization with antibodies and peptides designed to facilitate NP passage across the blood brain barrier (BBB) to bind specific cell types is ineffective. In particular, we use systematic *in vitro* and *in vivo* analyses to demonstrate that *p*(HEMA-*ran*-GMA) NPs functionalized with HIV-1 transactivating transcriptor (TAT) peptide (known to cross the BBB) and α neural/glial antigen 2 (NG2) (known for targeting oligodendrocyte precursor cells (OPCs)), individually and in combination, do not specifically target OPCs and are unable to cross the BBB, likely due to the serum protein binding to the NPs.

TEXT

Introduction

Neurological disease and traumatic brain injury represents a substantial threat to global health primarily due to a lack of drugs that can significantly modify suitable molecular targets, and enter the central nervous system (CNS) through the protective blood brain barrier (BBB).¹ While the BBB can open following severe injury, the duration for which it remains open is relatively unknown and can vary depending on the type and severity of injury.^{2,3} Following neurotrauma, the damage spreads into surrounding tissue in the form of chronic secondary degeneration⁴, and therefore delivery of protective treatments through the BBB will still be required after the BBB has closed. Hydrophilic, random copolymer -

based nanoparticles (NPs) synthesized from 2-hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GMA) monomers, given as *p*(HEMA-*ran*-GMA) NPs, have potential to be attractive candidates that may enable hydrophilic drug delivery across the BBB into the damaged CNS, due to the capacity of the material to retain high water content and inherent low toxicity.⁵ Highly reactive epoxide functional groups present on *p*(HEMA-*ran*-GMA) add to its appeal for use in the development of therapeutic NPs as functionalization can be achieved with ease.⁶ *p*(HEMA-*ran*-GMA) NPs consist of a polymer network structure between which are water filled permeation channels that can enable water soluble drugs to diffuse out to the external environment from within in a controlled fashion.⁷ However, therapeutic agents and/or the carrier's hydrophobicity are important determinants of passive BBB permeation, and a positive correlation exists between a solute's hydrophobicity and its BBB permeability.^{8,9} High, non-specific CNS uptake rates have also been associated with positive charge, due to negatively charged cell membranes¹⁰, and shape and size are further important factors that influence a therapeutic agent's penetrance through the BBB. The cell-penetrating HIV-1 trans-activating transcriptor peptide (TAT) can translocate across the BBB *via* protein transduction independent of transporters and receptor-mediated endocytosis.¹¹ Therefore, the major benefit of TAT is that it may be conjugated to NPs and facilitate their transport across the BBB, independent of the physico-chemical properties of the NPs. The size of the NP to which TAT is conjugated is not a rate-limiting factor for transport¹² and this peptide has been widely used for NP delivery across the BBB in a number of species.^{13,14} TAT is also neuroprotective following excitotoxic insult and oxygen deprivation^{15,16}, possibly by inhibition of the activation of NMDA-mediated calpain, a calcium ion (Ca²⁺) cysteine protease.¹⁵

Myelin is particularly susceptible to neurotrauma, leading to cognitive dysfunction.^{17, 18} In the CNS, myelin is formed by oligodendrocytes, which wrap axons in insulating protective sheaths to enhance the efficacy of nerve signaling.¹⁹ Oligodendrocyte precursor cells (OPCs), which express the chondroitin sulphate proteoglycan, neural/glial antigen 2 (NG2)^{20, 21}, are able to respond to myelin damage by rapidly proliferating and differentiating into new myelinating oligodendrocytes.²² However, OPCs are particularly susceptible to the Ca^{2+} associated glutamate excitotoxicity that occurs after neurotrauma due to the high concentration of Ca^{2+} permeable receptors on OPCs.²³⁻²⁵ Furthermore, compared to other glia, OPCs also have a reduced capacity to cope with reactive oxygen species (ROS) that are in excess following neurotrauma due to relatively low concentrations of antioxidants.²⁶ OPC vulnerability and their subsequent limited differentiation into myelinating oligodendrocytes is associated with demyelination²⁷ and loss of function following neurotrauma.⁴ Preservation of OPCs by targeted delivery of therapeutic agents to these cells can improve myelination *in vitro* and *in vivo*²⁸; however, in that study, targeted delivery of NPs to OPCs was not confirmed and injury site delivery obviated the need for crossing the BBB.

In biological environments, spontaneous adsorption of biomolecules from the serum and interstitial fluid on the surface of NPs due to high surface free energy²⁹, may alter the effects of surface functionalizations, with variations in functionalization altering the amount and types of biomolecular species (chiefly proteins) adsorbed.^{30, 31} This “protein corona” on the NP surface, predominantly made up by serum proteins and lipids, alters the interfacial composition of NPs upon exposure to biological conditions and subsequently their interactions with cells.^{32, 33} It is now widely accepted that it is necessary to consider formation of the protein corona when

characterizing NP preparations for therapeutic use as well as the effects of that corona on efficacy of functionalizations.³⁴ This current proof-of-principle study utilizes novel *p*(HEMA-*ran*-GMA) NPs designed to specifically target OPCs *via* conjugation with anti-NG2 antibody (α NG2). The NPs were further conjugated with TAT to facilitate active crossing of the closed BBB. The concentration of proteins surrounding each NP variant was quantified and the presence of functionalization moieties confirmed. Targeting efficacy of the α NG2 functionalization was assessed using mixed cortical cultures *in vitro*, and the efficacy with which the NPs cross the BBB was assessed using whole organ imaging *ex vivo* following intravenous (IV) administration.

Materials and Methods

Materials

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

NP synthesis and characterization

p(HEMA-*ran*-GMA) copolymer used for the NP preparation was synthesized by atom-transfer radical polymerisation (ATRP) according to previously established protocols.³⁵ *p*(HEMA-*ran*-GMA) NPs were synthesized via spontaneous ‘water-in-oil’ (W/O) inverse nano-emulsion. In brief, the copolymer was completely dissolved in water and allowed to disperse as spontaneous aqueous micelles in a continuous organic phase made up by n-hexane and the surfactant, sodium dioctyl sulfosuccinate,

also known as Aerosol OT (AOT). Ethylene diamine was added to the W/O emulsion to cross-link the solvated polymer chains within the micelles *via* the epoxide groups present, to form discrete *p*(HEMA-*ran*-GMA) NPs. The NPs were retrieved from the emulsion after the cross-linking reaction was allowed to occur overnight, by solvent removal and ultracentrifugation. The cross-linked *p*(HEMA-*ran*-GMA) NPs were resuspended in water and amine-functionalized *via* epoxide ring-opening by reacting with excess aqueous ammonia. The amine-functionalized *p*(HEMA-*ran*-GMA) NPs were lyophilized and then further reacted with Cyanine5-N-hydroxysuccinimide (Cy5-NHS) ester (Lumiprobe) to form fluorescent Cy5-*p*(HEMA-*ran*-GMA) NPs. Functionalization of the Cy5-labelled NPs with α -NG2 antibodies (NP- α NG2) and/or TAT (NP- α NG2-TAT, NP-TAT) was done using a hetero-bifunctional sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) cross-linker with polyethylene glycol (PEG) chain spacer, SM(PEG)₁₂ (Thermo Scientific™). Full details of NP synthesis, functionalization and methods for characterization are provided in the Supporting Information. Demonstration of linear Cy5 fluorescence intensity with increasing concentrations of each of the NP variants is shown in Figure S1A in the Supporting Information.

Determination of the concentration of protein adsorbed on NP variants

Known masses of each NP variant were resuspended and exposed to 55 (v/v) % rat serum in 1× phosphate buffered saline (PBS), pH = 7.2, or in PBS only, and incubated with gentle agitation for 1 h at 37 °C. 1 mL of the serum solution or PBS incubated NP suspensions were loaded onto 1 mL of 0.7 M sucrose cushion and centrifuged at 20000 × *g* for 30 min at 4 °C and the NP pellet was washed three times with 1× PBS by centrifugation (20000 × *g* for 30 min at 4 °C) to remove unbound serum proteins.

The recovered NPs were resuspended by sonication in 100 μ L solution consisting of 8 M urea and 2 (w/v) % 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) on ice, centrifuged ($20000 \times g$ for 30 min) and the eluted protein concentration in the supernatant was determined using a Micro BCA™ Protein Assay Kit (Thermo Scientific™).

Animals and study design

Piebald Virol Glaxo (PVG) rat pups (postnatal age 0 – 2 days) or adult females (160 – 180 g) were obtained from the Animal Resource Centre in Murdoch, Western Australia. All procedures were approved by the Animal Ethics Committee of The University of Western Australia (RA3/100/1485) and were conducted in accordance with the National Health and Medical Research Council of Australia Guidelines on the Use of Animals in Research. For cell culture, rat pups were euthanized with intraperitoneal injection of pentobarbital sodium (Provet®, Western Australia). Cortices from five animals were pooled together for each independent experiment. For *in vivo* imaging, adult rats ($n = 5/\text{group}$) were IV – administered a 5 mg/kg dose of their allocated NPs or vehicle control (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer (50 mM, pH 7.4) *via* tail vein injection, and left in their home enclosures for 4 h to allow NP circulation.³⁶ Rats were euthanized with pentobarbital sodium at 160 mg/kg dosage, transcardially perfused with 100 mL of 0.9 % saline, and dissected to remove the brain and major reticuloendothelial organs including the spleen, one kidney, and one liver and lung lobe. Lobes of organs were unilaterally dissected from the right of the animal, lateral to the mid-line and were stored at -20 °C in the dark for a maximum of 24 h prior to *ex vivo* fluorescence imaging.

Tissue culture procedures

Mixed cortical cultures were prepared as described ³⁷, and as detailed in full in the Supporting Information. Due to the fact that OPC cultures prepared using the method described by Y. Chen *et al* did not contain sufficient numbers of relevant cell types such as microglia to assess NP localization, an abridged method employing fewer purification steps was employed so as to maintain a range of cell populations including astrocytes and microglia. NPs diluted in Dulbecco's Modification of Eagles Medium (DMEM) supplemented as detailed in ESI and containing 10 ng/mL platelet derived growth factor-AA (PDGF-AA) and 10 ng/mL basic fibroblast growth factor (bFGF), were administered to cultures. In initial dose response studies where cells were counted, NPs were administered at a final concentration range of 5 – 200 µg/mL (as noted in Figure legends), in triplicate wells. NPs were administered at a final concentration of 25 µg/mL to triplicate wells for all subsequent experiments; all experiments were conducted 2 – 3 times using separate cultures. Cultures were incubated for 24 h at 37 °C, in 5 % CO₂, or 4 h at 4 °C, prior to fixation in 4 (w/v) % paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Immunohistochemical procedures, used primary antibodies 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 β-III tubulin (βIII-T; Abcam© rabbit AB18207) and oligodendrocyte indicator adenomatous polyposis coli clone 1 (CC1; Merck©; mouse OP80). Fluorescent secondary antibodies were Alexa Fluor® 488 and 555 (1:400; anti-rabbit,

anti-goat and/or anti-mouse; Thermo Fisher Scientific™); Hoechst 33342 nuclear stain (1:1000; Thermo Fisher Scientific™) was also added at this time.

Imaging and analysis

In vitro: Fixed cultures on 0.17 mm coverslips were visualized using a Nikon® C2plus camera attached to a Nikon® Ni-E confocal fluorescence microscope. Nikon® NIS Elements Advanced Research software was used for acquisition and device control. For characterization of cultures and assessing the effects of NP variants on cell numbers, 4 randomly sampled, non-overlapping Z-stack images were taken per well with a 20× NA0.75 objective lens, encompassing a 4 µm range, with 0.75 µm steps. To determine the proportion of each cell type containing NPs, 1 – 2 randomly sampled, non-overlapping images were taken per well, with a 40× NA0.95 objective lens encompassing a 6 µm range, with 0.75 µm steps. Using the open-sourced Fiji/ImageJ software, a consistently sized and located region of interest (ROI) was defined within each image for quantification of total cell count *via* Hoechst+ staining, quantification of numbers of specific cell types by immunohistochemical identification and quantification of the number of these cells that contained Cy5 fluorescence, indicative of NP presence. Data from 43,767 Hoechst+ cells were used for characterization of cultures and assessments of the effects of NP variants on cell numbers, whilst data collected for the assessment of cell types containing NPs came from a total of 10,613 Hoechst+ cells, with a minimum of 100 cells per cell type counted.

In vivo: A CRi Maestro 2™ *in vivo* imaging system was used to auto-expose at 5000 ms and 1×1 binning and imaged using a yellow excitation filter set with custom 600 – 800 nm emission wavelength with a 10 nm step size. Using manual spectral

unmixing, the auto-fluorescence of tissue IV injected with HEPES buffer was subtracted from tissue IV injected with Cy5 labelled NPs to produce unmixed Cy5 fluorescence component images for each organ type. A consistent threshold (0.00019 scaled counts/second) was set to capture definitive regions of Cy5 signal, excluding areas of weak, diffuse signal for all organs. Organs with no signal above this threshold, such as controls, were assigned a signal value equal to the threshold minimum value.

Statistical Analyses

All data were analyzed using IBM SPSS software and graphed using GraphPad Prism® 7 software.

In vitro data: Comparisons of numbers of each cell type expressed as a percentage of the total number of Hoechst+ cells following treatment with the NP variants were analyzed using one-way ANOVA with Tukey or Games-Howell post-hoc tests as applicable ($\alpha = 0.05$). Comparisons were confined to between cell types for each NP variant and between NP variants for each cell type and were analyzed using two-way ANOVA with Tukey post-hoc tests. Levene's test revealed unequal variance, with log10 and Arcsine transformations unable to normalize variations. As such, the confidence threshold for NP targeting analyses was lowered to 0.01 to reduce the chance of type I errors.³⁸

In vivo data: Similarly, Levene's test revealed unequal variance ($p \leq 0.0001$), with log10 and Arcsine transformations unable to normalize variations. Two-way ANOVA were used to assess differences in the data sets and Tukey's post-hoc tests at $p \leq 0.01$ used to define differences between experimental groups, confining comparisons to within NP types or within organs.

Results and Discussion

Functionalization and Characterization of *p*(HEMA-*ran*-GMA)-based NPs

Synthesis of Cy5-labelled *p*(HEMA-*ran*-GMA) NPs has been detailed in the Supporting Information. Mean hydrodynamic sizes of the various NPs used in this study were characterized using Dynamic Light Scattering (DLS). The NP variants were approximately similar in size as observed by their hydrodynamic size distributions (Figure 1A). Transmission electron microscopy (TEM) images were obtained from a dried sample of an aqueous suspension of Cy5-labelled *p*(HEMA-*ran*-GMA) NPs placed on a Formvar®-coated copper grid used for TEM sample preparation (Figure 1B). While the images indicated that the NPs were monodispersed in size, the intensity profile across the NP images showed that they had not retained their spherical shape when dried onto the TEM grid. Instead, the NPs had collapsed, becoming flatter and wider when viewed on the TEM. The projected NP diameters viewed on the TEM were therefore augmented when compared to the undistorted mean spherical hydrodynamic sizes obtained from the DLS data. The attachment of α NG2 antibody and TAT peptide to generate the appropriate NP variants was confirmed by detection of fluorescent secondary antibodies recognizing the α NG2 antibody, and α TAT antibodies recognized by fluorescent secondary antibodies to detect the TAT peptide (Figure 1C). Attachment of both α NG2 and TAT to NP- α NG2-TAT is demonstrated in Figure S1B in the Supporting Information. The conjugation technique utilized in this work to functionalize the NPs with the TAT peptide has been adapted from literature,³⁹ which have indicated the conservation of

the peptide's function by means of increased cell transfection and low cytotoxicity of the various TAT-functionalized therapeutic vehicles.

Figure 1. Characterization of Cy5-labelled *p*(HEMA-*ran*-GMA) NP variants. “NP” in figure labels denotes unfunctionalized, Cy5-*p*(HEMA-*ran*-GMA) NPs. (A) Dynamic Light Scattering (DLS) displaying hydrodynamic size distributions for each of the NP variants represented as the percentage of light scattering intensity (normalized to 1). (B) Transmission electron microscopy (TEM) image of dried Cy5-*p*(HEMA-*ran*-GMA) NPs on copper grid with carbon support film (Scale bar = 0.5 μ m). (C) Confirmation of functionalization on NP variants by fluorescence intensities of secondary (2°) antibodies bound to α NG2 (left panel), or to α TAT (right panel) antibodies recognizing conjugated TAT. Significant differences are indicated by * $p \leq 0.05$, **** $p \leq 0.0001$. (a.u. stands for arbitrary units.)

Protein corona quantification on *p*(HEMA-*ran*-GMA)-based NP variants

Once placed into a biological environment containing plasma, serum or blood, exogenous NPs are rapidly modified by the adsorption of biomolecules.⁴⁰ This highly complex formation of biomolecules, known as a protein corona that is modulated based on the physico-chemical properties of any given NP, imposes a ‘biological identity’ on the synthetic surfaces, and may influence NP biodistribution.⁴¹ Some studies however have deemed that the presence of a protein corona had no impact on the fate of NPs in biological conditions^{42, 43}, or that the protein corona may in fact benefit therapy by reducing NP cytotoxicity.⁴⁴ Given the differing conclusions arising from the inevitable protein corona formation on various NP formulations, it is necessary to evaluate the design of any therapeutic NP not just based on their synthetic properties, but also in the presence of any protein corona that they may attract, so as to be able to better predict or understand NP behaviour in biological conditions. To determine whether the hydrophilic *p*(HEMA-*ran*-GMA)-based NPs

attracted a significant amount of serum proteins to their surfaces, each of the NP variants were incubated with rat serum to mimic the experimental conditions of *in vivo* assessments of functionalization to follow, and the total amount of tightly adsorbed serum proteins (commonly referred to as the ‘hard corona’) on the respective NP variants’ surfaces was measured.

Figure 2. Assessment of protein corona on Cy5-labelled *p*(HEMA-*ran*-GMA)-based NP variants. “NP” in figure labels denotes unfunctionalized, Cy5-*p*(HEMA-*ran*-

GMA) NPs. (A) Quantification of the mean total protein amount from the protein corona adsorbed on the NP variants ($\mu\text{g protein/mg of NP}$) $\pm$ standard error of measurement (SEM). Significant differences are indicated by * $p \leq 0.05$, **** $p \leq 0.0001$. (B) Hydrodynamic size (d.nm) and NP polydispersity index (PDI) of each of the NP variants, with and without incubation with 55 (v/v) % rat serum assessed by Dynamic Light Scattering (DLS). Surface charges of NP variants with and without incubation with 55 (v/v) % rat serum given by zeta potential measurements ($\pm$ standard deviation (S.D.)). (C) Colocalization of NP variants, including unfunctionalized control NP (purple), within cell bodies in mixed cortical culture, immunohistochemically recognized for NG2 (green). (Scale bar = 20 μm .)

The amount of serum proteins adsorbed on each of the functionalized NP variants, i.e. NP- αNG2 , TAT-NP and $\alpha\text{NG2-TAT-NP}$, was significantly higher than that surrounding unfunctionalized NPs ($F(3,16) = 299.5$, $p < 0.05$). Post hoc analysis indicated that more proteins bound to NP-TAT and to NP- $\alpha\text{NG2-TAT}$ than to NP- αNG2 or unfunctionalized NPs (Figure 2A). Protein concentrations eluted from NP variants incubated in serum-free conditions were low, indicating negligible contributions to protein concentration by the targeting moieties. DLS measurements reflected the adsorbed protein amount with increased hydrodynamic size of the NP variants, particularly of $\alpha\text{NG2-TAT-NP}$ following incubation with serum (Figure 2B). The surface charge of the various NP suspensions were further characterized from zeta potential measurements, indicating that the surface charges of the NP variants changed from negative to neutral following TAT functionalization in the absence of serum. Subtle changes to zeta potential measures following incubation with rat serum (Figure 2B) may also be indicative of surface modification of the NP variants by serum protein adsorption. Taken together, it appeared that the presence of TAT

increased the protein corona formation on these NPs, likely due to protein-protein interactions between TAT and serum proteins, despite the fact that NP-TAT were neutral and NP- α NG2-TAT were negatively charged (Figure 1A). Assessment of whether the NP- α NG2 entered NG2⁺ cells within a mixed cortical culture preferentially, when compared to unfunctionalized NPs, indicated that each of the NP variants entered NG2⁺ cells regardless of functionalization (Figure 2C).

Targeting efficacy of the α NG2 functionalization *in vitro*

A significant amount of hard corona proteins were adsorbed to NP- α NG2 incubated in serum when compared to NP- α NG2 incubated without serum (Figure 2A). To further assess whether the formation of the corona on the nanoparticle variants hampered the α NG2 functionalization, mixed cortical cultures were employed to quantitatively assess NP targeting *in vitro*. The cultures comprised of 39 % GFAP⁺ astrocytes, 36 % Iba1⁺ microglia, 4 % CC1⁺ oligodendrocytes, 3.5 % β III-T⁺ neurons and 0.8 % NG2⁺/Olig2⁺ OPCs; remaining cells (16.7 %) were not identifiable by the immunohistochemical markers employed. Cultures treated with an optimized concentration of 25 μ g/mL of the NP variants showed Cy5 within and around cells at a suitable fluorescence for visualization, with no evidence of cytotoxicity when viewing Hoechst⁺ cell nuclei. Representative confocal images of the cultures treated with varying concentrations of the NP variants have been provided in the Supporting Information. (Refer to Figure S2.) Treatment of the mixed cortical cells with each of the NP variants resulted in no changes to the numbers of Hoechst⁺ cells, or NG2⁺/Olig2⁺ OPCs, GFAP⁺ astrocytes, Iba1⁺ microglia, β III-T⁺ neurons or CC1⁺ oligodendrocytes, expressed as a proportion of Hoechst⁺ cells ($p > 0.05$) (Refer to Figure S3 in the Supporting Information for quantification,

representative images and full statistical information). The data demonstrated that at the tested NP concentration, the Cy5-labelled *p*(HEMA-*ran*-GMA) NP variants did not reduce cell numbers of a range of different neuronal and glial cell subtypes, regardless of α NG2 and/or TAT functionalization.

To assess the localization of the NP variants within specific cell types and determine whether the α NG2 functionalized NPs were located more in NG2+/Olig2+ OPCs than in other cell types within the mixed cortical cultures, the proportions of each cell type that contained each NP variant, as indicated by internalized Cy5 fluorescence, were counted. Two-way ANOVA revealed a statistically significant effect of treatment with the NP variants on the percentages of the analyzed cell types containing Cy5 fluorescence ($F(24,43) = 16.658$; $p \leq 0.0001$). The percentages of each cell type with NPs were compared separately for each NP variant (Figure 3A). In cultures treated with unfunctionalized NP or NP-TAT, both OPCs and neurons contained significantly more NPs than astrocytes, microglia and oligodendrocytes ($p \leq 0.01$), with no significant difference between the proportions of neurons and OPCs with NPs regardless of functionalization (Figure 3A). Preferential presence of NP- α NG2 and NP- α NG2-TAT in OPCs and neurons was similar but less pronounced (Figure 3A). There were no significant differences between the uptake of NP variants for any of the tested cell types, indicating a lack of targeting efficacy of NP- α NG2 towards OPCs (Representative confocal images: Figure 3B; Refer to Figure S4 in the Supporting Information.)

Figure 3. *In vitro* assessment of cell types with Cy5 localization from Cy5-labelled *p*(HEMA-*ran*-GMA) NP variants. “NP” in figure labels denotes unfunctionalized, Cy5-*p*(HEMA-*ran*-GMA) NPs. (A) Comparisons between cell types for each of the

NP variants. Data presented as mean $\pm$ standard error of measurement (SEM) percentages of each cell type with Cy5 localization, for $n = 2 - 3$ independent experiments, following treatment with 25 $\mu\text{g/mL}$ 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. (B) Representative confocal microscopy images were taken from a single optical slice within the Z stack in the middle of Hoechst+ nuclei in order to demonstrate intracellular localization.

It has been previously demonstrated that an adsorbed corona of proteins from the biological environment may cause functionalized NPs to lose the ability to specifically target cells by masking the targeting ligands on the NP surface, preventing them from interacting specifically with receptors/ligands on target cells.⁴⁵
⁴⁶ Thus, biomolecules from tissue culture supplements and secreted cellular components may have adsorbed to the NP surface and limited targeting efficacy of NP- αNG2 towards OPCs. An additional experiment assessing the uptake of the NP variants in either the presence or absence of serum and media growth factors was conducted, to assess the contribution of the protein corona to the lack of efficacy of functionalization. Cells were incubated with NP at 4°C for 4 hours, to ensure cell viability was maintained. Two way ANOVA demonstrated an effect of serum across the NP variants of $F(1,72) = 9.439$, $p = 0.003$, with a trend to a reduction in the uptake of control and αNG2 functionalized NP by NG2+ cells in the presence of serum (Figure 4A, representative images Figure 4B). However Sidak's *post-hoc* tests correcting for multiple comparisons did not indicate a significant effect of serum for

any individual NP variant (Figure 4A). As expected, there was no effect of serum on uptake of NP-TAT into NG2+ cells.

Figure 4. *In vitro* assessment of the effect of serum on NP uptake by NG2+ cells at 4°C over 4 h. (A) Percentage of cells in mixed cortical culture that were NG2+, colocalized with Cy5-labelled NP variants. Data are presented as mean $\pm$ standard error of the mean (SEM). Two separate experiments were conducted, showing similar outcomes; in each experiment duplicate wells were assessed for each NP variant and

quantification based on 5 images/ well. (B) Representative confocal microscopy images of NP- α NG2 in NG2+ cells were taken from a single optical slice within the Z stack in the middle of Hoechst+ nuclei in order to demonstrate intracellular localization $\pm$ addition of serum at 4°C at the 4 h time point. (Scale bar = 10 μ m).

Similar uptake of the Cy5-labelled NP variants by neurons and OPCs may be due to the surface charges of neurons, OPCs and the NPs. Negatively charged NPs can be significantly and selectively internalized by neurons compared to astrocytes, microglia and oligodendrocytes⁴⁷, attributed to neuronal electrical spiking activity.⁴⁸ In addition, it has been previously shown that positively charged NPs display little neuronal uptake, regardless of spiking activity.⁴⁸ The Cy5-labelled *p*(HEMA-*ran*-GMA)-based NPs employed in the current study were negatively charged or neutral, perhaps explaining their internalization by neurons. Recently, a subset of OPCs had been discovered to initiate action potentials after depolarization, dependent on the expression of voltage-gated sodium channels,⁴⁹ and it is feasible that an attractive relationship may also occur with OPCs. It should be noted that NG2 is not OPC-specific, and within the CNS is also present on pericytes and some smooth muscle cells of the vascular system⁵⁰, which this study did not assess. However, α NG2-conjugated polymeric NPs have been previously shown to effectively target OPCs both *in vitro* and *in vivo*.²⁸

It was interesting to note the apparent lack of active phagocytosis of the NP variants, with little Cy5 fluorescence detected within microglia after 24 h exposure. Microglia numbers were not reduced following incubation with the NPs, indicating no toxic effect of phagocytosis of the NPs. The degree of opsonization of NPs can be influenced by their surface charge and hydrophobicity/hydrophilicity⁵¹, which in turn

are a direct result of the types of proteins adsorbed onto the NP surfaces. Hydrophobic NPs are generally thought to be opsonized more quickly and readily than hydrophilic NPs, due to their enhanced absorbability.⁵¹ Negatively charged NPs, like the ones used in this study, also often have a lower macrophage uptake than positively charged NPs.⁵² However, the possibility remains that the *p*(HEMA-*ran*-GMA)-based NPs were internalized by microglia and degraded within the 24 h incubation period, or eliminated from the cells by secretion *via* exosomes. Future time course studies addressing microglial and macrophage activation in the presence of the NPs would be useful. Taken together, it was clear that the NPs did not preferentially target OPCs *in vitro*, as they were also found in neurons, perhaps due to the electrical spiking activity of these cells.⁴⁸ However, there did not appear to be non-specific internalization into any cell where the NPs settled in the culture, nor active phagocytosis.

Efficacy of the TAT and α NG2 functionalization of NPs *in vivo*

Although the α NG2 functionalization did not result in selective uptake by OPCs *in vitro*, the release of therapeutics diffusely into brain tissue remains clinically relevant following neurotrauma, where myelin abnormalities and diffuse axonal injury is associated with ionic imbalances, apoptosis and inflammation.⁵³ To determine whether the TAT functionalization could enable *p*(HEMA-*ran*-GMA)-based NPs to cross the BBB, it was necessary to conduct *in vivo* assessments. All NP variants were compared, to assess the effect of multiple surface functionalizations on the efficacy of the TAT functionalization. Uninjured adult rats, where the BBB is known to be intact, were used as a model system, and the organ distribution of the NP variants was determined 4 h following their IV administration *via* tail vein injection. The distribution of Cy5-labelled *p*(HEMA-*ran*-GMA) NP variants in the brain and non-

target organs kidney, lung, liver and spleen was assessed by imaging whole excised organs using the Maestro™ 2.6 *in vivo* imaging system (Representative images: Figure 5A). Comparative two-way ANOVA of the mean total fluorescence signal above threshold showed that for each NP variant, the amount of fluorescence was different, depending upon the organ being assessed ($F(16,122) = 13.418$, $p \leq 0.0001$). Data were analyzed and displayed to show significant differences within each organ, relative to NP variants (Figure 5B), to illustrate any increases in NP-TAT relative to other NP variants, in brain or other organs. Complementing this analysis, it was found that the Cy5-labelled NP variants remained stable throughout a similarly timed incubation in serum *in vitro*, with no free Cy5 released, indicating that free Cy5 is unlikely to be contributing to the *in vivo* signal (Refer to Figure S7 in the Supporting Information). Post hoc tests showed that the mean Cy5 fluorescence intensity was significantly higher in the kidneys of animals receiving each of the NP variants than the animals IV injected with the control vehicle, HEPES buffer (50 mM, pH 7.4), showing that the detected fluorescence was above background (Figure 4B). Within the kidney, Cy5 fluorescence was higher in animals injected with NP-TAT, than any of the other variants, including NP- α NG2-TAT. Similarly, in liver, Cy5 fluorescence intensity was significantly higher in animals receiving each of the NP variants than HEPES buffer ($p \leq 0.05$), but there were no significant differences between the NP variants ($p > 0.05$). In lungs, animals IV-injected with NP- α NG2 or NP-TAT had higher fluorescence than animals receiving HEPES buffer (Figure 5B). In brain and spleen, fluorescence was not higher following injection with any of the NP variants, than following injection with buffer control, highlighting the low percentage of NP, functionalized or otherwise, that reached the target organ of the brain (Refer to Table S1 in the Supporting Information). Confocal microscopy of multiple brain sections

from an additional cohort of animals, searching the choroid plexus and ventricles of the brain and particularly examining NG2+ pericytes, failed to detect Cy5 fluorescence above background following injection with any of the NP variants, including those that were TAT-functionalized (data not shown).

Taken together, the *in vivo* analysis failed to show TAT functionalized *p*(HEMA-*ran*-GMA)-based NPs could cross the BBB and access the brain. It was also clearly indicated that there was a lack of targeted delivery of NPs across the BBB into the brain, and a predominance of NPs in kidney and to a lesser extent, liver and lung. (Refer to Supplementary Figure 5 in the ESI.) Further, in the kidney, fluorescence following administration of the NP-TAT variant was particularly prominent, possibly due to its prolonged circulation when compared to the other NP variants due to its relatively neutral charge (Figure 2B).⁵⁴ Additional pilot analysis using sliced organs gave a similar distribution pattern to that observed using the whole organs. However, it is important to note that fluorescent labels in the far infra-red range such as Cyanine7 (Cy7) would likely allow for deeper light penetration, reduced light scattering and minimal background auto-fluorescence as compared to Cy5,⁵⁵ and could allow for greater sensitivity of detection in future studies.

Figure 5. (A) Images representing the distribution of Cy5-labelled *p*(HEMA-*ran*-GMA) NP variants in whole organs obtained by *ex vivo* fluorescence imaging. Cy5 fluorescence of NP variants (colours) was superimposed over autofluorescence (grey).

Cy5 fluorescence localized to pineal gland in brains (black arrows). Representative images for each organ from $n = 5$ animals / group shown; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. (B) Assessment of 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 the NP variants within whole organ types. Histogram bars represent the mean Cy5 signal intensity above threshold (scaled counts/s) $\pm$ 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.

Cell penetrating peptide functionalizations like TAT, can lack specificity and result in uptake of NPs by non-target organs.^{11, 56} NP size can also influence non-specific internalization. Previously it had been found that large poly-methoxypolyethyleneglycol NPs of 240 nm diameter were taken up to a greater extent by macrophages than smaller NPs of the same composition, despite their hydrophilicity.⁵⁷ While our observed lack of uptake of the Cy5-labelled *p*(HEMA-*ran*-GMA)-based NPs by microglia *in vitro* indicated that phagocytosis of these NPs was minimal, their relatively large hydrodynamic size may favour non-specific internalization. A relationship also exists between the mean hydrodynamic diameter of NPs and renal filtration, and any molecule above 10 nm cannot be excreted into urine.⁵⁸ NPs larger than 10 nm can be taken up by mesangial cells, which are found surrounding and supporting the glomerular apparatus.⁵⁹ However, NPs larger than 75 nm do not penetrate mesangium significantly⁶⁰, with clearance by Kupffer cells in the liver.^{60, 61} Although it was demonstrated that the NPs were stable in serum over a 4 h period, it may be possible that the *p*(HEMA-*ran*-GMA)-based NPs were actively

dissembled into smaller polymer chains during the 4 h by Kupffer cells in the liver before being renally excreted. This hypothesis is in accordance with the rapid renal excretion of 100 nm large cyclodextrin polymer based NPs, where the fraction of NPs renally cleared was attributed to their disruption.⁶² Future *in vivo* time-course studies may demonstrate a predominance of NPs in hepatic cells at earlier timepoints.

There is precedent in literature for the presence of a protein corona masking NP functionalizations designed to facilitate crossing of the BBB when assessed *in vitro*⁴⁵, although other *in vitro* assessments have indicated that targeting can be maintained.⁴² It is becoming increasingly clear that the nature of the protein corona depends heavily upon the features of the NP and the nature of the proteins it encounters.^{31, 63} The resultant rapid clearance of drugs from blood circulation to non-target organs is a major hindrance to CNS targeted drug delivery.⁶⁴ Pre-coating of NPs with specific serum proteins such as apolipoprotein E (ApoE) could facilitate successful crossing of the BBB.⁶⁵ Further functionalization with stealth moieties as with the use of PEG chains to limit non-specific internalization and protein corona formation by steric hindrance, may assist in enhancing the usefulness of α NG2 functionalization of NPs.⁶⁶ Future assessments of the composition of the protein corona surrounding each NP variant and the effect of each protein on any functionalizations will be important to understand how choice of functionalizations can be optimised. If successful, functionalization of NPs with α NG2 antibodies has potential for wide-ranging application beyond targeting of OPCs in the brain, as NG2 is expressed on tumour cells, particularly those associated with metastasis⁶⁷, and could be used to deliver chemotherapies. While it is important to consider the immunogenicity of surface-conjugated antibodies, NPs can be conjugated with humanized antibodies, in which

all but the binding sites are human-derived (~95 % human, ~5 % rodent).⁶⁸ However, this can come at the expense of targeting affinity.⁶⁹

Conclusions

The presence of TAT on Cy5-labelled *p*(HEMA-*ran*-GMA) NPs was associated with an increased protein corona relative to the other functionalized NP and a lack of efficacy at crossing the BBB to the brain following IV injection *in vivo*. If the NPs had accessed the brain, *in vitro* assessments indicated that it is unlikely that the α NG2-TAT-NP would have targeted OPCs. Despite the fact that TAT functionalized NPs have been demonstrated to cross the BBB, our study indicated that functionalization with antibodies and peptides designed to facilitate NP passage across the BBB to bind OPCs is not necessarily effective and should be specifically assessed on a case by case basis.

ASSOCIATED CONTENT

Supporting Information. Complete list of materials used in the study, along with detailed experimental procedures are provided in the electronic supporting information (ESI). Supplementary results, detailing additional NP characterization, representative confocal images of mixed cortical cultures with NP treatment and supporting *in vitro* and *in vivo* assessments, are also provided in the ESI.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Authors labeled with § indicate equal contribution. There are no conflicts of interest to declare.

Funding Sources

We acknowledge financial support from the National Health and Medical Research Council (NHMRC), Australia (APP1082403). MF is supported by an NHMRC Career Development Fellowship (APP1087114).

ACKNOWLEDGMENT

The authors acknowledge the facilities and scientific and technical assistance of the National Imaging Facility, a National Collaborative Research Infrastructure Strategy (NCRIS) capability, at the Centre for Microscopy, Characterization and Analysis (CMCA), University of Western Australia. JAK would like to acknowledge Cancer Council Western Australia for a PhD Top Up scholarship.

ABBREVIATIONS

NP, nanoparticle; HEMA, hydroxyethyl methacrylate; GMA, glycidyl methacrylate; BBB, blood-brain barrier; TAT, trans-activating transcriptor; NG2, alpha neural/glial antigen 2; OPC, oligodendrocyte progenitor cell; CNS, central nervous system; DLS, dynamic light scattering; TEM, transmission electron microscopy; Cy5, Cyanine 5; NHS, N-hydroxysuccinimide; SMCC, sulfosuccinidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate; ATRP, atom-transfer radical polymerization; W/O, water-in-oil; AOT, sodium dioctyl sulfosuccinate; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate; ApoE,

apolipoprotein E; IV, intravenous; PEG, polyethylene glycol. PBS, phosphate buffered saline; DPBS, Dulbecco's phosphate buffered saline; PDGF-AA, platelet derived growth factor-AA; bFGF, basic fibroblast growth factor; PFA, paraformaldehyde.

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