Differences in Magnetic Particle (MP) Uptake by Central Nervous System (CNS) Neuroglial Subclasses: Implications for Neural Tissue Engineering

Stuart I Jenkins[a], *Mark R Pickard*[a], *David N Furness*[a], *Humphrey HP Yiu*[b], *Divya M Chari*[a]*

[a]Cellular and Neural Engineering Group,

Institute for Science and Technology in Medicine,

Keele University,

Staffordshire, ST5 5BG (UK)

E-mail: d.chari@hfac.keele.ac.uk

[b]Chemical Engineering,

School of Engineering and Physical Sciences,

Heriot-Watt University,

Edinburgh, EH14 4AS (UK)

Abstract

Aims: To analyze MP uptake and intracellular processing by the four main non-neuronal subclasses of the CNS [viz. oligodendrocyte precursor cells (OPCs), oligodendrocytes, astrocytes and microglia].

Materials and Methods: MP uptake and processing were studied in rat OPCs and oligodendrocytes, using fluorescence and transmission electron microscopy, and results collated with previous data from microglia and astrocytes.

Results: Significant intercellular differences were observed between glial subtypes, with microglia demonstrating the most rapid/extensive particle uptake, followed by astrocytes, with OPCs and oligodendrocytes showing significantly lower uptake. Ultrastructural analyses suggest that MPs are extensively degraded in microglia but are relatively stable in other cells.

Conclusions: Our findings have implications for use of the MP platform in a range of neural tissue engineering applications such as transfection, cell labeling and direct CNS biomolecule delivery.

Keywords: Magnetic particles, endocytosis, neuroglia, uptake, oligodendrocyte precursor cell

Introduction

Magnetic particles (MPs) have emerged in recent years as an important platform for advanced biomedical technology, due to innovations in their large-scale synthesis and complex surface functionalization [1,2]. These advanced materials are used in key applications such as drug/gene delivery, magnetic drug targeting, thermotherapies, stem cell targeting, and in diagnostic imaging (as contrast agents) [2–6]. The size and surface chemistry of MPs can be tailored for molecules with which they are 'functionalized', enabling the construction of multimodal particles that can mediate combinations of cellular applications, whilst retaining nanoscale dimensions [7]. The unique versatility achievable with their structural design therefore confers on MPs the ability to serve as a 'theragnostic platform' to integrate therapeutic strategies with diagnostic methods such as MRI [8].

Despite their proven therapeutic potential, the use of MPs for central nervous system or CNS (*ie.* brain and spinal cord) applications has been a relatively underexploited area to date [2]. Their utility is being increasingly demonstrated for a range of regenerative applications including neural progenitor/stem cell transplant imaging [9,10], gene delivery deploying novel 'magnetofection' methods [11–15] and diagnostic imaging in neurological injury [16]. However, there are critical gaps in our knowledge of fundamental parameters governing the utility of this emergent technology for CNS applications, especially the factors that determine MP uptake and intracellular processing in neural cells. The influence of parameters such as MP size, coating and charge on particle uptake have been studied in non-neural cell types [17-20] but the influence of neural cell *subtype* on particle uptake has never been assessed. This is especially pertinent when considering MP applications for CNS tissue; the latter is uniquely complex and contains several, specialist, interacting cell types meaning that data obtained from other physiological systems cannot be extrapolated to the CNS. The neurons mediate electrical conduction but a diverse population of *non-neuronal* cells, collectively termed the 'neuroglia' undertake distinct and critical functions in

such as immune surveillance, blood-brain barrier maintenance, genesis of myelin (the insulating sheath around neurons) and regulation of electrochemical signalling [21-23]. Neuroglial subclasses consist of the oligodendrocytes [that co-exist with their parent population- the oligodendrocyte precursor cells (OPCs)], microglia, and astrocytes. These subclasses have unique structural, functional and molecular properties and can therefore be predicted to show important differences in their uptake and handling of synthetic materials. Indeed, our recent studies point to significant inter-cellular differences in toxicity and transfection levels between neuroglia, the reasons for which are unknown [12,14,24]. The neurogliaform an extensive cellular network and outnumber neurons by a factor of approximately ten. Consequently, understanding particle handling by this major class of cells, is a critical component in understanding interactions between synthetic biomaterials and the CNS as a whole.

A small number of studies have reported MP uptake by neuroglial cells [25,26] but no study to date has systematically compared particle uptake and handling by individual neuroglial subclasses. Here, we have assessed the uptake and intracellular processing of a single type of MP (of specific size and formulation) by oligodendrocyte lineage cells *ie.* oligodendrocytes and OPCs derived from primary cell cultures. These findings were collated with data from our previous studies in astrocytes [27] and microglia [24] (also derived from primary cultures) to provide, for the first time, comparative data on the uptake characteristics and particle handling by these major subclasses of CNS cells.

Materials and Methods

Reagents and equipment: Tissue culture-grade plastics, media, and media supplements were from Fisher Scientific (Loughborough, UK) and Sigma-Aldrich (Poole, UK). Recombinant human platelet-derived growth factor (PDGF-AA) and basic fibroblast growth factor (FGF2) were from Peprotech (London, UK). Monoclonal rat anti-MBP was from Serotech (Kidlington, UK), monoclonal mouse anti-A2B5 was from Sigma-Aldrich (Poole, UK), and secondary antibodies (FITC-conjugated) were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Mounting medium with DAPI (4',6-diamidino-2-phenylindole) was from Vector Laboratories (Peterborough, UK). Paramagnetic, carboxyl-modified Sphero^{TM} Nile Red fluorescent MPs (diameter 0.20 – 0.39 μm, iron content 15 – 20% w/v) were obtained from Spherotech Inc. (Lake Forest, Illinois, USA). We have observed that their fluorophore does not leach, and their emission spectra remain stable following long periods of storage, making these an optimal choice for this study.

Particle characterization: FTIR spectroscopic analysis was carried out using a Perkin Elmer Spectrum 100 spectrometer fitted with an ATR (attenuated total reflection) sampling unit. For the sample measurement, 32 scans in the region from 650 to 4000 cm^{-1} were accumulated with a resolution of 4 cm^{-1}. Powder XRD analysis on the iron oxide component of the particles was carried out using a Bruker D8 Advance diffractometer with Cu $K\alpha_1$ radiation (λ = 1.542 Å). The diffract pattern was collected from 2θ = 5° to 80°, at a step size of 0.009° and a step time of 120 s. The particle size of iron oxide was estimated using Scherrer analysis on the most intense peak (311).

OPC and oligodendrocyte cultures for uptake experiments: The care and use of animals was in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom) with approval by the local ethics committee. Primary mixed neuroglial cultures were prepared from cerebral

cortices of Sprague-Dawley rats at postnatal day 1-3, based on an established protocol [28]. Cultures were maintained in D10 medium (DMEM supplemented with 10% fetal bovine serum, 2 mM glutaMAX-I, 1 mM sodium pyruvate, 50 U mL^{-1} penicillin, and 50 µg mL^{-1} streptomycin) at 37°C in 5% CO_2/95% humidified air for 8-10 days, then shaken for 2 h on a rotary shaker at 200 rpm. This medium, containing largely microglia was discarded, fresh D10 medium added, and flasks shaken overnight at 200 rpm. This medium, containing largely OPCs, was transferred to non-tissue-culture grade petri dishes, to which microglia, but not OPCs readily attach, reducing microglial contamination. After 30 min, unattached cells were resuspended in either OPC maintenance medium (OPC-MM: DMEM supplemented with 2 mM glutaMAX-I, 1 mM sodium pyruvate, 10 nM biotin, 10 nM hydrocortisone, 30 nM sodium selenite, 50 µg mL^{-1} transferrin, 5 µg mL^{-1} insulin, 0.1% bovine serum albumin, 50 U mL^{-1} penicillin, 50 µg mL^{-1} streptomycin, 10 ng mL^{-1} PDGF-AA, and 10 ng mL^{-1} FGF2) for OPC cultures, or in Sato medium (DMEM supplemented with 2 mM glutaMAX-I, 1 mM sodium pyruvate, 1X N2 supplement, 30 nM thyroxine, 30 nM triiodothyronine, 50 U mL^{-1} penicillin, and 50 µg mL^{-1} streptomycin) to generate oligodendrocyte cultures. Cells were plated onto PDL-coated glass coverslips in 24-well plates (0.3 mL/well, at 3 x 10^4 cells/cm^2). OPC cultures were maintained for 24 h before incubation with MPs, to allow cell adherence and re-growth of processes. Oligodendrocyte cultures were maintained for 7 days (50% medium changes every 2-3 days) to allow cell differentiation, before incubation with MPs. Cells were then incubated with 2-50 µg mL^{-1} MPs for 1 – 24 h. For long-term studies, OPCs were pulse-labeled with MPs for 24 h, then switched to Sato medium and maintained for 1 month. Control cultures were treated with equal volumes of fresh medium, without MPs. Samples were washed with PBS, then fixed and either immunostained, or processed for Perl's Prussian blue histochemical staining.

Methodological considerations - extracellular particle adherence: OPC and oligodendrocyte cultures are not as strongly adherent to PDL-coated coverslips as microglial and astrocyte cultures.

This presented a methodological problem for particle uptake experiments with regard to the removal of extracellular particles when terminating incubations. In previous work, extensive washing with PBS was necessary to reduce non-specific binding of MPs to minimal levels, and this was well tolerated by microglial and astrocyte cultures, resulting in minimal cell detachment. In the present study a similar washing protocol resulted in the loss of many OPCs and oligodendrocytes, even without the prior addition of MPs. To minimize the loss of cells, several washes were performed by gently applying PBS to the walls of culture wells.

Toxicity assessment: Initial experiments used a 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTS) assay to assess MP toxicity in OPCs and oligodendrocytes, as described earlier, for comparison with astrocyte and microglia data [24,27]. However, in our hands, typically low levels of MTS reduction to formazan were consistently found in both control and MP-treated cultures, which was not deemed sufficiently sensitive to accurately assess toxicity. Therefore, epifluorescence microscopy was used to count the pyknotic and total (healthy plus pyknotic) nuclei per microscopic field, as measures of MP-related toxicity. A minimum of five fields were counted for every condition.

Immunocytochemistry: Washed cells were fixed with 4% paraformaldehyde [PFA; room temperature (RT); 25 min] then washed again. For staining, cells were incubated with blocking solution (5% serum in PBS, with 0.3% Triton X-100 for MBP; RT; 30 min), then with primary antibody in blocking solution (A2B5 1:200; MBP 1:200; 4°C; overnight). A2B5 and MBP are widely-used markers of the oligodendroglial lineage: the A2B5 antibody recognizes cell surface ganglioside epitopes; MBP is one of the major protein constituents of myelin, and is used to label late-stage oligodendrocytes. Cells were then washed with PBS, incubated with blocking solution (RT; 30 min), and incubated with the appropriate FITC-conjugated secondary antibody in blocking

solution (1:200; RT; 2 h). Finally, coverslips were washed with PBS and mounted with the nuclear stain DAPI.

Fluorescence microscopy & image analysis: Samples were imaged using fixed exposure settings on an Axio Scope A1 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Goettingen, Germany), and the images merged using Adobe Photoshop CS3 (version 10.0.1). The purity of each culture was determined by scoring at least 100 DAPI-stained nuclei for coincidence with A2B5 or MBP staining. Z-stack fluorescence images of samples were created using fixed exposure settings on a Nikon Eclipse 80i microscope fitted with a CA742-95 camera (Hamamatsu Photonics, Hamamatsu, Japan), with manual focus stepping at 0.5 or 1.0 µm, and the image manipulations performed using Nikon NIS Elements (version 3.00). The proportion of $A2B5^+$ OPCs or MBP^+ oligodendrocytes with coincident Nile Red fluorescence was assessed (minimum 100 DAPI-stained nuclei for each concentration and timepoint), with the proportion of MP-labeled cells exhibiting any perinuclear MPs also being recorded. Some samples were also imaged using a BioRad MRC1024 confocal laser scanning microscope.

Semi-quantitative assessment of MP uptake: It was deemed unsuitable to assess levels of uptake by means of a fluorescence plate reader, or in terms of incorporated iron per cell, as these techniques quantify the total fluorescence or iron present in a culture, and therefore assume an even distribution between cells. Such techniques would not enable the determination of uptake heterogeneity within the cell population, as observed in our cultures. A flow cytometry approach was also considered and rejected, as it is likely that extracellular MPs adherent to the plasma membrane would lead to a number of ‘false positives'. Microscopic cell counting analysis was therefore considered the most appropriate method to assess whether MPs were intracellular, or merely extracellularly attached to plasma membrane. To ensure that cellular uptake was judged correctly, z-stack analyses were performed for both OPC and oligodendrocyte cultures to confirm that MPs were intracellular, rather

than adherent to the plasma membrane (minimum of 100 cells for all concentrations and timepoints). Oligodendrocyte cultures were particularly problematic, as MPs were often observed amongst their extensive, lipid-rich processes however, from the z-stack analyses it was apparent that MPs were external to the oligodendrocyte processes. Therefore, for these analyses, only MPs that were clearly within the oligodendrocyte soma, the main cell body, were considered to be intracellular, and all other particles were deemed to be extracellular. The level of MP uptake in individual cells was assessed in a semi-quantitative manner by comparison with the average cross-sectional area of an OPC/oligodendrocyte nucleus, and scored as either 'low' (<10% of the area of an average nucleus), 'medium' (10 – 50%), or 'high' (>50%). We consider that such detailed microscopic assessment of MP uptake more accurately reflects the heterogeneity that is typically found in primary cell cultures.

Perls' Prussian blue staining: Washed samples were fixed with 4% PFA (RT; 25 min), then washed again. Samples were incubated with 2% potassium ferricyanide in 2% HCl for 30 min, then washed three times with distilled water and mounted without DAPI. Images were then taken using light microscopy, to visualize intracellular iron, and fluorescence microscopy, to visualize Nile Red particle fluorescence. Images were merged to assess colocalization of iron deposits with fluorescence.

Scanning electron microscopy: MPs in OPC-MM were air-dried onto aluminium stubs and visualized uncoated using a high resolution field emission SEM (Hitachi S4500) operated at an accelerating voltage of 5 kV.

Transmission electron microscopy: Microglial, astrocyte and OPC cultures were established on PDL- or polyornithine-coated aclar sheet (microglial [24] and astrocyte [27] cultures were prepared

as described previously), cut to fit a 24-well plate (0.3 mL/well, at 6 x 10^4 cells/cm^2), then incubated with 20 µg mL^{-1} MPs for 4 or 24 h. Samples were fixed with 2.5% glutaraldehyde [in sodium cacodylate buffer (SCB); RT; 2 h], then washed with SCB. Samples were postfixed with 1% osmium tetroxide in SCB for 1 h, washed with SCB, dehydrated in a graded series of ethanol, then infiltrated with Spurr resin, before polymerization at 60°C for 16 h. To obtain sections, the block was trimmed to expose the aclar sheet which was peeled off, leaving the cells in the resin. Ultrathin sections were then cut parallel to the original plane of the sheet on a Reichert Ultracut E ultramicrotome, mounted on 200 mesh thin bar copper grids, and stained with 2% uranyl acetate in 70% ethanol (RT; 20 min) and 2% Reynolds lead citrate (RT; <5 min). A minimum of three sections were examined for each cell type using a JEOL 100-CX transmission electron microscope operated at 100 kV. Images were acquired using a SIS systems Megaview III digital camera (Olympus).

Statistical analysis: Data were analyzed using GraphPad Prism statistical analysis software. Data are expressed as mean ± SEM. The number of experiments (n) refers to the number of mixed glial cultures from which OPC or oligodendrocyte cultures were derived, with each primary culture being established from a different rat litter. Data were analyzed by two-way analysis of variance (ANOVA), with Bonferroni's multiple comparison post-tests for *post hoc* analysis.

Results

Characterization of MPs

The MPs used here have a multilayered design **(Figure 1a)**, comprising a polystyrene core (stained with the fluorophore nile red), coated with a polystyrene/iron oxide composite layer; we have found that these are stable in physiological media and consistently perform robustly under our experimental conditions, justifying their use in this study. These 'test' MPs are relatively large (200 – 390 nm diameter; mean = 360 nm); we have previously justified the use of particles of this diameter for *in vivo* biological applications [24]. Indeed, particles of up to 1 μm diameter have been used to image cells transplanted into the CNS without demonstrable effects on even complex biological functions such as myelination [29] and other particles, including functionalized transfection grade ones are of comparable diameter [29–32].

Batch-to-batch variability can occur during MP synthesis, and particle size is an important factor in cellular uptake and processing [19]. Therefore, MPs were analyzed by scanning electron microscopy (SEM) for size and shape assessments; our data were consistent with our previous findings, showing that the particles used here were regular in shape, and within the size range reported by the manufacturer **(Figure 1b)**. Perls' Prussian blue staining of cells which had been pulse-labeled with these MPs for 24 h revealed the presence of iron coincident with Nile Red fluorescence at 24 h **(Figures 1c & d)**, and at 30 days post-treatment (data not shown). This demonstrates the reliability of fluorescence as an indicator of MP presence, and suggests the stability of MPs in the culture media and the cells. No blue staining was observed after Perls' staining of control cultures. The zeta potential of the particles was measured as -23.02 mV with *ca.* 1.63×10^6 carboxyl groups per particle (data provided by supplier, Spherofluor Inc.). The FTIR spectrum of these Spherotech particles was dominated by the polystyrene component (**Figure 1 e**) with a small number of caroboxylic groups, shown as $\nu_{C=O}$ (C=O stretching) at 1707 cm^{-1}. The powder XRD pattern (**Figure 1f**) revealed that the iron oxide crystallites in the particles are of an

inverse spinel structure, *eg.* Fe_3O_4 (magnetite) or gamma-Fe_2O_3 (maghemite). However, due to the small crystal size or low crystallinity of the iron oxide, the diffraction peaks are broad and of low intensity. This is also partly due to the predominant amount of polystyrene (*ca.* 80%) present in the particles. The average crystal size was also estimated to be around 18.5 nm in diameter using Scherrer analysis on the diffraction peak (311).

OPC Culture Characteristics

Phase contrast microscopy of untreated and MP-treated cultures revealed phase-bright cells with bipolar morphologies characteristic of OPCs **(Figure 2a)**. High purity OPC cultures were routinely derived, as assessed by immunostaining for the OPC marker A2B5 (95.4 ± 0.9%; n = 4), and DAPI-staining showed typical round or oval nuclei (Figure 2a, inset).

Concentration- and Time-Dependence of MP Uptake and Perinuclear Localization in OPCs

Fluorescence microscopy, including z-stack and confocal analyses, confirmed the intracellular MPs in OPCs (**Figure 2b**; confocal data not shown) with particles typically observed in small clusters. MP uptake was time- and concentration-dependent, with the highest dose and longest exposure tested resulting in labeling of *ca.* 60% of $A2B5^+$ cells **(Figure 2c)**. Concentrations of 20 and 50 µg mL^{-1} labeled a significantly greater percentage of cells than 2 µg mL^{-1}, at all timepoints. The extent of MP-uptake by individual cells was heterogenous, therefore a semi-quantitative approach was employed to classify categories of uptake as described previously [24,27]. Under all conditions, the majority of MP-labeled OPCs exhibited a 'low' level of particle accumulation. At 4 and 24 h, a small percentage of MP-labeled cells exhibited 'medium' levels of uptake at all concentrations with 'high' levels of uptake rarely observed (**Table 1**). The percentage of labeled OPCs with MPs in a perinuclear localization was time- and concentration-dependent **(Figure 2d)**. Intranuclear particles were not observed using confocal or z-stack analyses.

Oligodendrocyte Culture Characteristics

Oligodendrocytes were derived from high purity parent OPC cultures, with 70.3 ± 1.3% of cells staining positive for the late-stage oligodendrocyte marker myelin basic protein (MBP; **Figure 3a**). All stages of the oligodendroglial lineage are typically present in such cultures, and our own analyses show that a further ~25% stain for earlier markers such as NG2 and O4, with the remainder (<5%) being microglial contamination (data not shown) [33]. Cells were phase-bright, with multipolar morphologies, including the complex highly-branched, membrane elaborating morphologies typical of mature oligodendrocytes.

Concentration- and Time-Dependence of MP Uptake and Perinuclear Localization in Oligodendrocytes

Z-stack and confocal fluorescence analyses, confirmed intracellular MPs in oligodendrocytes, including perinuclear accumulations (**Figure 3b**; confocal data not shown). The percentage of labeled oligodendrocytes was time- and concentration-dependent, with the greatest dose and exposure tested resulting in MP-labeling of *ca.* 45% of MBP^+ cells **(Figure 3c)**. MP concentrations of 20 and 50 μg mL^{-1} labeled a significantly greater percentage of MBP^+ cells than 2 μg mL^{-1}, at all timepoints. Heterogeneity in uptake was apparent and this was assessed semi-quantitatively using the same criteria used for scoring OPCs **(Table 2)**. Under all conditions, the majority of MP-labeled cells exhibited a 'low' level of particle accumulation. At 4 and 24 h, oligodendrocytes with 'medium' levels of MP accumulation were observed with 'high' levels of accumulation rarely observed (at the greatest MP concentration and incubation time) (Table 2). By 24 h, cultures exposed to 2 and 5 μg mL^{-1} MPs exhibited a time-dependent increase in the percentage of MP-labeled MBP^+ oligodendrocytes with perinuclear particles **(Figure 3d)**.

Comparison of MP Uptake in OPCs and Oligodendrocytes

When data for OPCs and oligodendrocytes were compared, the proportions of labeled cells were similar at 1 h post-MP addition (Figures 2c & 3c). At subsequent time points, a greater percentage of OPCs than oligodendrocytes were MP-labeled with the exception of the 24 h incubation with 20 $\mu g\ mL^{-1}$ MP (Tables 1 and 2). With respect to cells scored as having 'low' levels of uptake, the accumulations of MPs present within OPCs were typically larger than those within oligodendrocytes (compare Figures 2b and 3b, which show typical levels of MP accumulation). Further, cells at more advanced stages of oligodendrocyte differentiation (judged by number/complexity of processes), contained smaller particle accumulations than cells with relatively immature and less branching morphologies (data not shown). This suggests an inverse relationship between cellular maturity and capacity for MP uptake. For both cell types, the percentage of cells with 'medium-high' levels of MP uptake was time- ($p<0.001$; two-way ANOVA; $n = 4$) and concentration-dependent ($p<0.001$; two-way ANOVA; $n = 4$), suggesting that the uptake mechanism is non-saturable in the time-frame examined.

Assessment of MP toxicity in OPCs and oligodendrocytes

No significant effects of MPs were observed in OPC or oligodendrocyte cultures with respect to (a) cell adherence, judged by number of DAPI-labeled nuclei (either healthy or pyknotic) per microscopic field **(Figures 4a & b)**, and (b) cell death, judged by the percentage of nuclei exhibiting pyknotic features **(***ie.* shrunken or fragmenting morphologies; **Figures 4c & d**). OPC and oligodendrocyte cultures appeared morphologically similar to controls at 24 h, and this was also true for oligodendrocytes at one month post- pulse labeling with MPs (data not shown). No effects of MPs were noted on the time taken to reach confluence or extent of membrane elaboration in OPC or oligodendrocyte cultures.

Intercellular differences in extent of MP uptake

Data from OPCs and oligodendrocytes were combined with data obtained from rat astrocytes [27] and microglia [24] using identical particle concentrations and timings of exposure. From these analyses, there are clear differences between individual neuroglial classes in the rate and extent of MP accumulation, with the relationship with respect of these parameters being: microglia > astrocytes > OPCs > oligodendrocytes (Figures 5a-d). Comparing the extent of MP-uptake at 4 h highlights the rapid labeling of >90% of microglia, even with 'low' MP concentrations, compared to the gradual time- and concentration-dependent uptake observed in the other cell types (Figure 5e). Intercellular differences in perinuclear particle localization were also apparent, with microglia showing the most rapid and extensive accumulation (Figure 5f).

Intercellular differences in MP processing in cells

Transmission electron microscopy (TEM) analyses were used to study the intracellular disposition of MPs. This is a time consuming and technically demanding method compared with other histological approaches, but we consider that this is the most robust ultrastructural approach to obtain morphological data on modes of particle uptake, evidence of vacuolar internalization, perinuclear trafficking, and lysosomal degradation/end products of MP breakdown, with high resolution. Electron dense MPs were easily detected in within cells. Astrocytes and OPCs contained morphologically intact MPs, generally observed to be free in the cytosol, including the perinuclear accumulations (Figures 6a & b) [27]. MP clusters were strikingly smaller in OPCs compared with astrocytes, supporting our observations using fluorescence microscopy (Figures 6a & b). By contrast, microglia revealed extensive MP accumulations seen associated with multilamellar bodies, likely to be lysosomes (Figure 6c). In contrast to astrocytes and OPCs, MPs appeared to be undergoing extensive degradation in microglia (Figure 6c, inset). Following 24 h MP exposure toxicity was not observed in OPCs, oligodendrocytes, or astrocytes [27], but was marked in microglial cultures [24] (Figure 6d).

Discussion

To the best of our knowledge, this is the first investigation into inter-cellular differences in MP uptake and handling by the major neuroglial subtypes. Our data reveal that these subpopulations exhibit key differences in the uptake and intracellular processing of MPs. We consider that such variations could have important implications for the biological utility of MP platforms for neural tissue engineering applications. As such, we consider that the findings have broader implications for the design and development of novel neurocompatible materials for regenerative medicine, and associated protocols for their use.

Most work to date evaluating cellular MP uptake and handling has utilized cell lines [34-38]. This has yielded valuable data but warnings about cell line identity have been made since the 1950s, with many instances of published work carried out in misidentified cell lines [39-41]. Cell lines possess an altered physiology compared with the corresponding primary cells and behave in a relatively homogenous clonal manner. Consequently, their properties may not represent biological variations that exist *in vivo* that reflect cell proliferation, differentiation and varying states of biological activation (particularly relevant in neurological injury), all of which will influence the outcomes of MP use. Cell lines also show relatively high survival/proliferation rates and resistance to adverse stimuli such as cell death signals; continuous passage of cells without robust quality control also risks chronic contamination by mycoplasma - such 'cryptic' contamination can alter cell structure, metabolism and growth, all of which can impact the interpretation of data [42]. These considerations, combined with the risk of cellular aneuploidy, can make cell lines a relatively poor model for toxicity testing [42,43]. This is an issue of high relevance to nanotechnology, where the neurotoxicity of nano- and micro- sized particles is currently an issue of major public and scientific concern. As such, we contend that cells derived from primary cultures, as used here, are of higher biological relevance than cell lines, particularly when developing protocols for translational applications.

MP uptake is dependent on endocytosis (macropinocytosis, clathrin-, and caveolin-mediated uptake) in mammalian cells [2,19,44] but little data exists on MP uptake mechanisms in primary neuroglia. The cells studied here display a range of endocytotic mechanisms overall, and differences in MP accumulation likely relate to variations in basal levels of endocytosis. We can predict that the specific endocytotic pathways utilized by each cell type, and their levels of activity, closely correlate with the *functional* role for each cell type. For example, microglia are phagocytic cells and continuously survey their microenvironment using highly motile processes, removing debris and foreign materials; they utilize all endocytotic mechanisms, including receptor-mediated endocytosis, macropinocytosis and phagocytosis [24,45] The astrocytes, in turn, have a major role in uptake of extracellular biomolecules, and possess well-developed endocytotic machinery; we recently showed that astrocytes take up the MPs studied here, by macropinocytosis and coated pit mechanisms [27]. In line with their broad regulatory and defence roles, microglia and astrocytes showed extensive and rapid particle uptake. By contrast, cells of the oligodendrocyte lineage have specialist roles in generating oligodendrocytes and the myelin sheath [23]. As such, we can predict that endocytotic cycling in these cells is lower, an expectation strikingly reflected in the finding that percentages of labeled oligodendroglial cells are about half that observed in astrocytes and microglia, along with substantially lower levels of MP accumulation. We are not aware of studies quantifying endocytotic mechanisms across neuroglial cells, and such a comparison will be a pre-requisite to providing formal proof of this idea.

A further factor influencing uptake could be the relative extent of cell membrane available to undertake endocytosis. Astrocytes display characteristically flattened morphologies and large amounts of membrane. By contrast, OPCs and oligodendrocytes have small oval or round cell bodies, and, as far as we are aware, their processes are not involved in particle uptake - observations supported by our histological analyses. Differences in cell surface area may account for the

differences in particle uptake, and it is not clear whether using particles of altered dimensions may result in greater MP accumulation [19,46]. Also, in line with their phagocytic role, microglia cells appear to inactivate particles by sequestration within vesicles and active lysosomal degradation. Extensive particle accumulation in conjunction with iron toxicity post-degradation is the likely pathological correlate of MP toxicity in these cells [47]. By contrast, in the other cell types, particles appeared to be relatively stable morphologically with negligible associated toxicity.

We consider that the overall profiles of MP uptake and handling exhibited by neuroglia, as reported here, have broader predictive value with biological implications regarding MP use in neural tissue engineering (see Figure 7 for a schematic overview). First, if using MPs for cell labeling and imaging of neural transplant populations, then protocols will need to be individually tailored by cell type, with OPCs requiring higher particle concentrations and incubation times, to achieve high efficiency labeling. A previous study in an OPC cell line suggests that sub-10 nm magnetic nanoparticles show extensive accumulation in these cells [9] indicating that MP physicochemical properties (such as size, shape, coating, geometry and charge) can be tailored to achieve optimal labeling [8].

Second, while MPs are effective transfection agents, the specific mode of intracellular trafficking of particles will exert an important influence on transfection levels achieved. Efficient perinuclear accumulation of MPs (a pre-requisite for DNA delivery [48]) in microglia, suggests that MPs could act as effective transfection agents in these cells, but we recently reported that microglial transfection was rarely achieved using transfection grade MPs [24]; particle sequestration and breakdown provides a reasonable explanation for our observations and indeed, there are few reports of non-viral, plasmid based transfection of microglia. Microglial transfection is a desirable goal, allowing immunomodulation in neurological injury, but our results indicate that there are significant 'intracellular barriers' to MP-mediated transfection in microglia that will need to be overcome, using

strategies such as MP targeting to particular intracellular compartments to evade degradation mechanisms. By contrast, the relative stability and non-sequestration of MPs in astrocytes and OPCs, is in line with our findings that both cell types can be transfected (with the same transfection grade MPs tested in microglia), albeit with major differences in maximal transfection levels (*ca.* 55% for astrocytes versus *ca.* 23% for OPCs) achieved [12,14]. This trend correlates with the relative extent of basal MP uptake in astrocytes versus OPCs. There is a sharp drop in MP mediated transfection of oligodendrocytes (up to 4%; unpublished observations) despite perinuclear MP accumulation. This could be related to lower particle uptake but successful transfection may also depend on other factors. For example, nuclear breakdown during mitosis may facilitate entry of nucleic acids, so proliferative populations such as astrocytes/OPCs may be relatively amenable to MP-mediated transfection compared to non-dividing cells such as oligodendrocytes [17].

Third, robust microglial uptake of MPs is beneficial for imaging sites of CNS injury/disease [49]. However, rapid and extensive particle uptake by endogenous microglia may limit particle uptake by other cell types where mixed cell populations of neural cells exist, for example, neurological injury sites [24]. Such considerations suggest that microglial MP uptake is a significant ‘extracellular barrier' when considering applications such as direct MP mediated drug/gene delivery to the CNS, that may potentially require strategies such as immunosuppression, cell targeting approaches or variations in particle physiochemical properties, to reduce microglial uptake. Further, MP related toxicity in microglia could become a cause of secondary CNS pathology, therefore particle doses and formulations must be selected with caution.

Conclusions

CNS neuroglial subclasses show considerable variability in MP uptake and intracellular handling. Such variations are likely related to differences in (a) their functional roles; (b) basal endocytotic activity; and (c) extent of membrane elaboration. We predict that the differences have implications for the use of the MP platform in neural tissue engineering and translational applications such as imaging, cell labeling and gene/drug delivery. It is therefore essential for future work to take account of the complexity of neuroglial responses to synthetic materials, in order to optimize and refine the use of MPs for neural regenerative applications. Such work will need to be informed by a detailed understanding of the relationship between the physicochemical properties of MPs and neuroglial uptake, processing and toxicity - parameters that are poorly documented at present.

Future Perspective

There is a critical knowledge gap in nanotechnology and neurotoxicology, regarding the influence of the chemistry of materials platforms on the biology of CNS cells [50]. Such information is vital to robustly evaluate the utility of novel materials for the development and optimization of new therapeutics. Further studies will need to characterize neuroglial cell interactions with MPs of a range of physicochemical properties to identify those with optimal features for specific biomedical applications. For each cell type, such investigations should include ultrastructural analyses of particle fate; endocytotic blocker studies to establish specific uptake mechanisms; microarray analyses for detailed toxicity analysis; evaluation of vesicular trafficking of MPs, to develop particles with the potential for endosomal escape or to suggest the specific endocytotic mechanisms to which MPs should be preferentially targeted.

Summary points

- Non-neuronal cells of the CNS, termed the neuroglia, derived from primary cultures, show significant differences in MP uptake.
- Microglia show the most rapid and extensive MP uptake followed by astrocytes; oligodendrocyte precursor cells and oligodendrocytes show comparatively less uptake.
- MPs undergo extensive degradation in microglia whereas particles appear relatively intact in the other cell types. Significant microglial toxicity was observed at concentrations that were non-toxic in the other cell types.
- The differences in the extent of MP uptake and processing in the neuroglia could have significant implications for MP use in neural tissue engineering applications.
- Rapid uptake and degradation of MPs in microglia could represent a significant 'extracellular barrier' for direct MP use (in gene/drug delivery) in the CNS. Such degradation is also a major 'intracellular barrier' in applications such as transfection.
- MP stability in astrocytes and oligodendroglial cells indicates that MPs are compatible with these cells, but the relative levels of cellular uptake will determine the efficacy of MP use.
- Utilising cells derived from primary cultures, as in the current study, offers several advantages over cell lines that are widely employed in bio-nanotechnology research.
- Future studies will need to characterize relationships between MP physicochemical properties and cellular uptake, processing and toxicity in CNS cells, to identify optimal neurocompatible materials.

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