Title: **Development of a Nanomaterial Bio-Screening Platform for Neurological Applications**

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Abstract: Nanoparticle platforms are being intensively investigated for neurological applications. Current biological models used to identify clinically relevant materials have major limitations, *e.g.* technical/ethical issues with live animal experimentation, failure to replicate neural cell diversity, limited control over cellular stoichiometries and poor reproducibility. High-throughput neuro-mimetic screening systems are required to address these challenges. We describe an advanced multicellular neural model comprising the major non-neuronal/glial cells of the central nervous system (CNS), shown to account for ~99.5% of CNS nanoparticle uptake. This model offers critical advantages for neuro-nanomaterials testing whilst reducing animal use: one primary source and culture medium for all cell types, standardized biomolecular corona formation and defined/reproducible cellular stoichiometry. Using dynamic time-lapse imaging, we demonstrate in real-time that microglia (neural immune cells) dramatically limit particle uptake in other neural subtypes (paralleling post-mortem observations after nanoparticle injection *in vivo*), highlighting the utility of the system in predicting neural handling of biomaterials.

Keywords: biomaterials screening; multicellular models; neural cells; glia; protein corona

Introduction

Advanced functional material design has led to a global increase in clinical nanomaterial use for regenerative medicine, particularly platforms such as magnetic particles (MPs), in applications including imaging and biomolecule delivery, with several therapeutic nanoparticles under clinical trials or in pre-clinical development.[1, 2] Identification and optimization of such medical biomaterials requires dedicated design and realization of surface functionalization with appropriate materials characterization tools, and parallel biomedical testing using relevant biomimetic screening models. Neurological applications represent a unique challenge in this regard, given the complex, multicellular composition of the brain and spinal cord (termed the central nervous system or CNS).[3] Neural cells are classed into neurons (transmitters of electrical information) or glia (the supporting cells). Glia outnumber neurons by about 10-fold[4–7] and comprise several subtypes that regulate the neural environment including, critically, clearance of nanomaterials.[8] One study recently proved that glial uptake of nanoparticles accounts for *ca.* 99.5% of nanoparticle clearance in the CNS, with neurons accounting for the small balance[9] - identifying the former as the overwhelmingly dominant population governing CNS nanoparticle uptake. Consequently, the overall response of the glial population to introduced nanomaterials is *the most critical predictor* of the CNS characteristic response as a whole.

We recently reported major differences in MP uptake/handling between glia.[10] The immune components (microglia) showed rapid and avid particle uptake with extensive degradation. In contrast, other glial subtypes (the astrocytes, oligodendrocytes and their precursors) showed significantly lower but stable particle accumulation. Based on these observations, we predicted that the rapid and high particle accumulation by a dominant cell population, such as microglia, would constitute a critical ‘extracellular barrier’ to particle uptake in mixed neural

cell populations, such as the intact nervous system. This is pertinent as high numbers of activated microglia are typically present in neurological pathology.[11] Accordingly, the development and testing of neuro-compatible materials for clinical use must account for *both intercellular dynamics and constituent glial cell numbers*, using appropriate multicellular neural models.

Despite this major need there is a substantial lack of sophisticated and accessible neural models for high-throughput screening of neuro-nanomaterials.[12] In terms of widely used current approaches, live animal models are biologically relevant but involve significant ethical issues, technical complexity and expense, whilst being low-throughput. 'Reductionist' models addressing the 3Rs principles (Reduction, Replacement and Refinement of animal experimentation[13,14] for which there is a current global drive) have several drawbacks, chiefly pertaining to their biological relevance. These include use of inappropriate sources/combinations of cells/tissue, *e.g.* cell lines of unknown age/provenance combined with primary cells, adult plus immature cells or peripheral nervous system (PNS) and CNS cells,[15,16] significantly limiting their neuro-mimetic capacity. Large variability is also inherent in these models, making reproducibility and robust analyses problematic. Tissue explants are technically challenging, showing uneven cellular distribution and stoichiometry, limiting robust quantification of material uptake,[16] and reducing their predictive utility.

Another major point of note is that biomolecule interactions with materials at the nanoscale – the same length scale as proteins – underpin the affinity between the surface and the biomolecule upon adsorption. In biological media, with ~30,000 different proteins likely present at varying abundance,[17] there is competition for adsorption sites on nanoparticles.[18] The so-called 'protein corona' formed around nanoparticles is highly dependent on their

characteristics (surface chemical functionality and nano-topography).[19–21] Most studies investigate cell-material interactions in isolated, purified mono-cultures, propagated in *cell-specific* media wherein differentially modified materials are presented to cells, even when the same defined starting nanoparticles are used.[10] This would substantially impact the readouts of intercellular comparisons of materials' handling (as the cells encounter this corona rather than the material surface).[19] Given this important confounding variable, it is important that the *same biological medium* be used with all cell types under study, to standardize experiments and elucidate true cellular responses to nanomaterials. This is especially relevant for neural cells, which co-exist in the same extracellular fluid in the intact nervous system, but with individual subtypes typically requiring biochemically distinctive media for survival and propagation *in vitro*.

To address these challenges, we developed a multi-glial cell screening model for nanomaterials with the following key features: (i) a standardized culture medium developed in-house for the model, which permits survival of all cell types; (ii) derivation of all cells from a single primary source; (iii) reproducible experimenter control over cellular stoichiometry; (iv) ease of nanomaterial delivery and (v) compatibility with a range of analytical/microscopic techniques. To evaluate the biological utility of the new model in predicting neural responses to introduced materials, we have challenged the system with well-characterized MPs, to test the hypothesis that a ‘microglial barrier’ exists, limiting particle uptake by other neural subtypes (a phenomenon previously only inferred from post-mortem observations following nanoparticle introduction into the intact CNS).[22]

Materials and Methods

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.

Materials

Tissue culture-grade plastics, media, and media supplements were from Fisher Scientific (Loughborough, UK) and Sigma-Aldrich (Poole, UK). DAPI mounting medium was from Vector Laboratories (Peterborough, UK). TrypLE (trypsin replacement) and monoclonal anti-biotin-FITC (fluorescein isothiocyanate) secondary antibody (clone BN-34) were from Sigma-Aldrich (Poole, UK). All other secondary antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

Sphero MPs and coronal protein characterization

Sphero MPs (mean diameter 360 nm, range 200 – 390 nm, 15 – 20% Fe w/v; Spherotech Inc., Illinois, USA) have previously been characterized in detail[10] and detection of Sphero-labelled cells using MRI illustrates their biological utility.[23] MPs were incubated in media (3 h; 20 μg mL^{-1}), magnetically separated, washed and air dried onto aluminium discs. FTIR data was collected on a Bruker Alpha system using a DRIFT attachment, with 512 scans being averaged at a resolution of 4 cm^{-1}. Amide I band component peak fitting was performed using previously defined parameters,[24] and an in-house program built using Omnic Macros Basic (Thermofisher Scientific). Eigen Vector Solo was used for PCA analysis, with all data being mean centered. The hydrodynamic diameter and zeta-potential of Sphero particles in cellular media were determined using a Zetasizer Nano ZS (Malvern, UK). All media contain carbonate buffer to maintain a pH of ~7.4 while incubated (37 °C, 5% CO_2/95% humidified air). As pH can influence particle-media interactions, cell culture conditions were replicated:

media were incubated for 24 h prior to particle addition (50 µg mL^{-1}) with the 5% CO_2 headspace being sealed between removal and measurement. Measurements were made at 37 °C, 5 min and 24 h following particle addition.

Development of the co-culture model

'Staggered Culture' approach for simultaneous cell derivation and stoichiometrically defined co-cultures

The McCarthy and de Vellis mixed glial culture method[25] (with modifications by Chen *et al.*)[26] was used to derive all glial cell types. Parallel seeding of flasks with dissociated tissue at different densities [poly-D-lysine (PDL)-coated 75 cm^2 flasks; D10; 37 °C, 5% CO_2/95% humidified air], ensured cells reached confluence at different times, enabling simultaneous derivation of high purity cellular fractions **(Figure 1)**. Cells were plated on PDL-coated glass coverslips in 24 well plates and subjected to 50% medium change (D10-CM 'gliosupportive' medium) every 2 – 3 d. For an initial assessment of competitive MP uptake dynamics, 50:50 co-cultures were used to ensure comparable cell numbers were present for head-to head analyses. A density of 6 x 10^4 cells per cm^2 was selected to avoid the adverse effects of confluence, whilst permitting survival of all cell types **(Supplementary Table S1)**.

Development of the standardized 'gliosupportive' medium

All cells were plated on PDL coated 24 well plates (astrocytes at 4 x 10^4 cells cm^{-2}; microglia at 9 x 10^4 cells cm^{-2}; OPCs at 6 x 10^4 cells cm^{-2}). In pilot experiments, mono-cultures of each cell type were tested for 48 h in various cell specific media (**Supplementary Table S2**). To develop a gliosupportive medium, D10 medium supplemented with conditioned D10 medium

from parent mixed glial cultures was tested (D10-CM; conditioned medium derived 48 h after last medium change, sterile-filtered and stored at 4 °C).

Competitive uptake studies

To test our proposed '*extracellular barrier*' hypothesis using our multicellular model, Sphero particles (20 µg mL^{-1}) were added to glial mono-cultures or 50:50 co-cultures, 24 h after plating in the gliosupportive medium. Mono-cultures served as internal controls, demonstrating intrinsic particle uptake by each cell type. After 24 h, all cultures were washed and fixed (4% paraformaldehyde) for immunocytochemistry.

Immunocytochemistry

Fixed cells were incubated with blocking solution (RT; 30 min), then primary antibody or lectin in blocking solution (**Supplementary Table S3**; 4 °C; overnight), washed with PBS, and incubated with the appropriate FITC-conjugated secondary antibody (1:200; RT; 2 h) and mounted with nuclear stain DAPI.

Fluorescence microscopy for toxicity and uptake analyses

Samples were photographed on an Axio Scope A1 fluorescence microscope (Carl Zeiss MicroImaging, Germany) and images merged using Photoshop CS3. A minimum of three microscopic fields and 100 nuclei per culture were assessed for all conditions. Toxicity was assessed by morphological observations and by comparing proportions of pyknotic nuclei (pyknotic/ healthy plus pyknotic), identified as small, intensely stained and often fragmenting. Culture purity and stoichiometry were determined by assessing the percentage of cells expressing cell-specific markers. Extent of MP-loading was assessed using a semi-quantitative technique by comparison with the average cross-sectional area of an OPC, as described previously.[10] Briefly, uptake was scored as low (<10% of the area of an average nucleus), medium (10 – 50%) or high (>50%). Elsewhere, we have discussed the benefits of this technique versus techniques deriving an average value for fluorescence or iron per cell.[10]

Further, measurements of 'intracellular' iron content (using colorimetric absorbance assays) include substantial proportions of extracellular (membrane-bound) particles: 20% of the iron per cell value for microglia,[27] and up to 50% for astrocytes.[28] Such techniques also assume an even distribution between cells and we have shown that considerable heterogeneity exists within glial subtypes in terms of extent of uptake.[10]

Statistical analysis

Data were analyzed using Prism software (GraphPad, CA, USA) and are expressed as mean ± standard error of the mean unless stated otherwise. '*n*' refers to the number of primary cultures from which mixed glial fractions were derived, each established from a different litter. Unpaired two-tailed *t*-tests were performed to compare the following between mono- and co-cultures: (i) the percentage of MP-labelled cells, (ii) proportions of pyknotic nuclei, (iii) proportions of cells showing 'low', 'medium' or 'high' levels of MP-loading.

Live cell dynamic time-lapse imaging

To study microglial behavior (specifically membrane activity and survival), mono-cultures were plated in PDL-coated 24 well plates (6×10^4 cells cm^{-2}, D10). After 24 h, cultures were imaged using time-lapse phase contrast microscopy (Nikon Eclipse Ti fluorescence microscope with Nikon DS-U2/L2 camera and NIS Elements BR 3.22.14 software), then Sphero MPs were added (20 µg mL^{-1}) and cultures imaged using time-lapse microscopy. To assess if microglial behavior was similar in mono versus co-cultures, a mixed glial culture was subjected to time-lapse imaging before and after Sphero addition (20 µg mL^{-1}, D10-CM). In separate experiments, cells were fixed and stained for transmission electron microscopy and scanning electron microcopy to visualize the ultrastructure of cells **(Supplementary methods)**.

Results

Differences in MP protein coronas in different cell media highlight the need to develop a single gliosupportive medium

Astrocytes, microglia, OPCs and oligodendrocytes are typically cultured in distinct media (D10, OPC-MM and Sato; see supplementary methods). Sphero particles incubated in each medium showed differences in MP-associated coronas **(Figure 2)** highlighting the importance of employing a single cell medium for intercellular comparisons of nanoparticle uptake. In pilot experiments, *no single* cell-specific medium could support all cell types without adversely affecting survival, proliferation or increasing the proportion of undesirable cell phenotypes[29] **(Supplementary table S2)**. Often, '*conditioned*' media are used for cell culture, wherein proteinaceous materials secreted by cells better support cell populations compared to standard media - offering a potential solution to this problem. In our cultures, multiple factors are secreted by the astrocyte bedlayer into base medium which becomes conditioned, so it was rationalized that D10 conditioned medium from parent cultures could provide an enhanced chemical medium to sustain multiple glial cell types in our co-cultures. Indeed, we found that a 20% supplement successfully supported the attachment and survival of all glial cell types whilst limiting cell differentiation and genesis of undesirable cell phenotypes **(Supplementary table S2)**, identifying this as an appropriate gliosupportive medium.

Analysis of particle characteristics between cell specific glial culture media versus the new gliosupportive medium

Detailed analyses of corona formation were performed in the standard media and gliosupportive medium. To assess if particles exhibited different size/charge characteristics in

different media, dynamic light scattering (DLS) and zeta potential measurements were performed **(Figure 3A)**. Particles exhibited similar hydrodynamic diameters in different media, and after differing incubation periods (5 min versus 24 h). Zeta-potential measurements demonstrated a similar negative charge for particles across media. These measurements may be expected to show similarity due to generalization of proteinaceous adsorption with similar adsorbed protein layer thickness (and similar hydrodynamic diameters) and surface charge states. By contrast, FTIR analysis of the amide I band, 1600-1700 cm^{-1}, is well-documented to be highly sensitive to changes in protein secondary structure.[18,24] Although also a global measure of protein structure, this technique discriminated between the nature of the adsorbed layer compositions formed from various media **(Figure 3B)**. Variation in amide II and III was also observed (data not shown). Component amide I band fitting of each of the particle coronas formed in different media highlights significant differences between global corona secondary structures **(Figure 3A)**.

Principal component analysis (PCA) of FTIR spectra was carried out to determine the variation patterns of the amide I band (1710-1590 cm^{-1}) and whole mid-infrared region (4000-400 cm^{-1}, data not shown). PCA is a statistical approach for the examination of complex variance between samples; when applied to spectroscopic data it is often referred to as a reverse Beer-Lambert law, with loadings representing the origin of the variability and scores highlighting the relative amount (or concentration) of this change between samples. The analysis highlights variances matching well with protein secondary structure components: α-helix (~1655 cm^{-1}), extended chain or β-sheet (~1636 & 1628 cm^{-1}) and side chain (~1614 cm^{-1}; **Figure 3B)**. A component of PC1 includes a peak at 1601 cm^{-1}, a highly indicative band in the styrene coating of the MPs used here,[10] possibly indicating a change in the presentation of this coating after protein adsorption. PCA scores show excellent

discrimination between all four samples using only PC1 and PC2 **(Figure 3C)**. TEM analyses of particles revealed electron dense rings, indicating the presence of iron around a polystyrene core, consistent with the reported physical diameter range: 200 – 390 nm **(Figure 3D)**.

Co-cultures of defined stoichiometry could be propagated in the gliosupportive medium

High purity glial fractions were derived from mixed glial parent preparations - astrocytes (97.8 ± 1.0% $GFAP^+$), microglia (98.0 ± 0.9% lectin-reactive) and OPCs (98.1 ± 0.4% $A2B5^+$ or $NG2^+$; **Supplementary figure F1A**). Individual cell types were successfully combined to produce co-cultures with approximately 1:1 cellular stoichiometry in the gliosupportive medium **(Supplementary figure F1A)**. In co-cultures, each cell type was evenly distributed, ensuring a reliable head-to-head comparison of competitive particle uptake dynamics. In some experiments, mature and highly branched oligodendrocytes were identified **(Supplementary figure F1B)**, and stained with the late-stage marker MBP (data not shown) indicating that this medium can support all stages of the oligodendrocyte lineage. This was confirmed in further pilot experiments, in which astrocytes, microglia and OPCs were added to an oligodendrocyte culture (at 8 DIV) and all four cell types could be successfully co-cultured in D10-CM for 48 h.

Microglia dramatically reduce MP uptake by other cell types proving the 'extracellular barrier' hypothesis in our model

For all cell types, the particle dose used here has previously been tested in monocultures, without evidence of toxicity at 24 h.[10,23,30] To rule out Sphero-induced toxicity in multicellular cultures, cell viability assays were conducted. No toxicity was observed in mono- or co-cultures: no differences in cellular adherence or cellular/nuclear morphology

were apparent by phase/fluorescence microscopy following immunostaining. No significant differences in numbers of pyknotic nuclei were found between mono- and co-cultures (less than 5% of cells, consistent with our previous reports).[10,23,30]

Glial Monocultures: MP-labelled cells were readily identified in all cultures. In mono-cultures, the cellular hierarchy in percentage of cells labelled and extent of loading was consistent with our previous report:[10] microglia > astrocytes > OPCs, with ~100% of microglia and astrocytes being labelled **(Figure 4A, B, E)**. The extent of loading varied between cell types with more microglia exhibiting 'high' loading than astrocytes (~55% versus ~35%). OPCs showed lower proportions of labelled cells (~75%), and lower extent of accumulation [~5% showing 'high', with ~20% showing 'low' loading **(Figure 4C, D, F)**].

50:50 co-cultures with microglia: Particle uptake features in astrocytes and OPCs were dramatically altered in the presence of microglia, both in proportions of cells labelled and extent of loading. Microglia mainly exhibited 'high' loading in co-cultures **(Figure 4A, B, E)**. Percentages of labelled astrocytes and OPCs were markedly reduced (*ca.* 100% to 70% and 75% to 35% respectively, **Figure 4A, C-F**) along with a reduced extent of loading.

Ultrastructural and dynamic live cell imaging to understanding the basis for the 'microglial barrier' effect

Ultrastructural analyses of microglia revealed extensive membrane ruffling and infoldings **(Figure 5A, B)** versus OPCs **(Figure 5C)** and astrocytes (not shown). This suggests high levels of microglial endocytotic/phagocytic activity versus other cell types. The rounded morphologies observed using electron microscopy resembled those of activated microglia under light/fluorescence microscopy **(Figures 4 and 5)**. Supporting the

ultrastructural observations, live microglia under time-lapse microscopy showed rounded, ruffled morphologies and sweeping projections of membrane rapidly extruded and retracted (**Video 1**: individual frames 20 seconds apart; **Figure 5D**). Membranes of astrocytes and OPCs showed motility, but with a lesser rate/extent of activity than microglial membranes **(Video 1; Figure 5D)**. Comparable microglial morphologies and membrane activity were observed using time-lapse imaging of monocultures **(Video 2; Figure 5E)**, demonstrating that these microglial behaviors are not dependent on the presence of other glial cells. Microglia remained within a region of approximately 80 µm diameter, appearing to explore their immediate microenvironment with membrane projections, an observation consistent with the proposed surveillance role of the microglia in the CNS.

Following MP addition, microglia became MP-loaded within 1 h, with large intracellular accumulations apparent within 90 min **(Figure 5F, G)**. In mixed cultures (in gliosupportive medium), labelled microglia were identified within 15 min and heavily MP-loaded cells, with increasingly spherical morphologies, were apparent by 2 h **(Video 3)**. Notably, *no* MP-loading was apparent in astrocytes or OPCs in mixed cultures over the same period. Some microglia extended processes over and around neighboring cells, a behavior that may be expected to limit the latter cells' access to particles.

Discussion

Here, we have successfully developed a multicellular (multi-glial) model for the developmental testing of medical biomaterials. Representation of all the major glial subtypes in the model ensures mimicry of the *in vivo* situation where the glia account for *ca.* 99.5% of nanoparticle uptake from the extracellular environment.[9] We utilized our approach to demonstrate for the first time, the existence of a competitive 'microglial barrier' to particle uptake in other neural cells in real-time. Parallel derivation of all glial types for the model from a single primary source, as achieved here, avoids problems with cell lines which are often of unknown provenance (origin and treatment history)[31] and altered physiology,[32] potentially leading to dramatically different nanoparticle uptake dynamics and toxicity profiles compared with primary cells.[16] Our method also ensures that constituent cells possess identical ages/anatomical origins, with culture under identical conditions. Further, by achieving defined cellular stoichiometry with high reproducibility, direct intercellular comparisons can be reliably drawn. The even cellular distribution in monolayers facilitated light and fluorescence microscopical analysis, obviating the need for confocal or z-stack identification of labelled cells. This system was also analyzed using time-lapse light and fluorescence microscopy, highlighting the potential to provide dynamic detail about particle uptake and particle-induced changes in cellular behavior (*e.g.* altered motility). As such, we consider that our model offers significant advantages over alternative neural co-culture systems currently used within the nanomedicine community.

As far as we are aware, we are also the first to demonstrate that different biomolecular coronas are formed in different neural media, identifying this as a critical confounding variable in cross-cellular comparisons of materials handling. Competitive protein binding to interfaces is a highly dynamic process, with distinct variability in the composition of the

formed biolayer in different biochemical media.[19,21,33,34] Changes in the protein corona presented at the particle surface lead to variance in how cells 'perceive' particles, through non-specific interaction or specific receptor mediated responses. Therefore, the cell-material interactions can be expected to vary between media. Secondary structure changes within the protein corona are indicative of a global change within the adsorbed protein layer.[18,35] Here we clearly highlight this variability. Differences in global secondary structure were observed from component amide I band fitting, particularly with respect to the α-helical component. PCA analysis of spectra further supported this finding, with discrimination between coronas formed from the four different media being highly resolved depending upon secondary structure component bands. Consequently, development of a *single* medium to support all cell types was a major outcome, to overcome issues associated with medium-specific corona formation. Therefore, we consider this model can provide a true reflection of multiple glial responses to nanomaterials, as pertains in complex neural tissue. Further analysis of the protein corona in future studies would allow for a more detailed insight into the mechanisms underpinning particle-neural cell interactions, where a major knowledge gap currently exists. Techniques such as 2D PAGE and mass spectrometry can be used to characterize the protein components of the corona, for various particle-medium combinations. This may reveal correlations between the presence of particular proteins and specific cell-particle interactions in those media; the predictive value of such data would greatly aid particle design for optimized cell interaction and internalization.

Astrocytes and OPCs showed dramatic reductions in MP uptake upon culture with microglia, confirming that extensive and avid microglial uptake is a major extracellular barrier limiting particle uptake in other cells. These results indicate that MP-loading observed in neural mono-cultures *cannot* be extrapolated to mixed populations (such as the intact

CNS). Indeed, mono-culture data, as is widely-reported within the nanomedicine community, will likely provide significant over-estimates of the extent of MP-loading possible for neural cells within mixed cell populations, thereby providing insufficient insight into responses within the intact nervous system. Further, our *in vivo* studies have shown that delivery of the Sphero particles employed here into the spinal cord parenchyma results in extensive particle localization within microglia/macrophages, with negligible uptake in other neural cells (unpublished data). Similarly, post-mortem studies of glioblastoma patients who received thermotherapy with MPs have shown that particles are predominantly localized within macrophage like cells.[36] By contrast, isolated neural tumor cells do have the capacity to take up particles *in vitro*[22] which would have predicted uptake in the intact CNS, highlighting the critical importance of *developing multicellular models that incorporate the CNS immune component* in order to make reliable predictions about the neural handling of introduced materials. Direct delivery of other MPs to the CNS also results in competitive uptake dynamics between glial cell types, with reported microglial dominance of this uptake[22] (as reported here). The striking similarity of these findings to ours, highlights the neuro-mimetic and predictive value of our advanced model. Complementary ultrastructural and dynamic (live cell) imaging applied to the model have provided insight into the basis for the 'microglial barrier' effect[10] by confirming the highly phagocytic and active nature of microglia in terms of cellular motility, membrane re-organization and surveillance behaviors, all of which will limit particle uptake by other neural cells in the vicinity.

We consider that the versatility of the model allows for diverse screening applications in regenerative neurology. For example, biomaterials intended to evade microglial clearance and/or target specific neural cell types could be tested, and the effects of drugs on competitive uptake dynamics could be assessed. There is also considerable scope to increase the

sophistication of the model in terms of cellular complexity (including addition of neurons) and tailored stoichiometry **(Figure 6)**. Consequently, this facile system can be employed to conduct head-to-head comparisons of biomaterials handling by glial cells, and provides a foundation by which screening approaches can be standardized. We predict that such neuro-mimetic models have the potential to accelerate the rate of discovery of neurocompatible and efficacious materials for neuroregenerative applications, whilst taking a major step towards reducing live animal experimentation.

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Figure Legends

Figure 1. Schematic diagram showing 'Stoichiometrically Defined' co-culture method.

Figure 2. A pan-gliosupportive medium is necessary to standardize the protein corona. (A) Schematic of protein corona formation in biological medium. (B) Amide I region of protein corona formed from saline and different culture media.

Figure 3. Magnetic particles developed measurably different coronas in different neural culture media. Analyses of MPs incubated in different neural media: (A) Zetasizer and FTIR analyses (comparative amide I component bands), (B) PCA loadings, (C) PC1 and PC2 score plot and (D) transmission electron micrograph of Sphero particles showing electron dense iron ring around polystyrene core.

Figure 4. Astrocytes and OPCs show marked reduction in proportions of MP-labelled cells and extent of loading in co-culture with microglia. Fluorescence micrographs of (A) astrocyte: microglia co-culture showing extensive microglial loading (white arrows). Contrast unlabelled $GFAP^+$ astrocytes (yellow arrows) with several labelled astrocytes in mono-cultures (inset; arrows show 'high' loading). (B) Microglial mono-culture exhibiting extensive loading (arrows; $GFAP^-$; phase contrast counterpart inset). (C) OPC: microglia co-culture showing extensive microglial loading (arrows; $DAPI^+/A2B5^-$) - note lack of labelled OPCs. Inset, OPC mono-culture with multiple labelled OPCs, arrows show 'high' loading. (D) OPC: microglia co-culture showing extensive loading in microglia - note lack of OPC

labelling ($DAPI^+$/lectin-unreactive; phase contrast counterpart inset). (E) Bar graph showing proportions of MP-labelled cells/extent of loading in microglia:astrocyte cultures. Proportions of MP-labelled astrocytes were significantly reduced versus mono-cultures ($^{+++}p < 0.001$) with more astrocytes exhibiting 'medium' ($^{*}p < 0.05$) or 'high' ($^{*}p < 0.05$) loading, and fewer exhibiting 'low' loading ($^{***}p < 0.001$); $n = 3$. (F) Bar graph showing proportions of MP-labelled cells/extent of loading in microglia: OPC cultures. When co-cultured with microglia, proportions of MP-labelled OPCs were significantly reduced ($^{+++}p < 0.001$). More OPCs exhibited 'medium' ($^{***}p < 0.001$) loading in mono-cultures than in co-cultures ($n = 4$).

Figure 5. Microglia possess highly active membrane projections and exhibit rapid and extensive uptake of MPs. (A) Transmission electron micrograph of an MP-labelled microglial cell (red arrows) showing extensive membrane ruffles/folds (black arrows). SEM reveals highly ruffled microglial membrane (B), compared with relatively quiescent OPC membranes (C). Note similarities in (A) and (B), in morphologies and membrane folds. (D) Time-lapse micrographs of a mixed culture without MPs (stills from Video 1) show astrocytes with flattened phenotypes in the bedlayer, while OPCs exhibit relatively small, dark cell bodies with fine processes. Microglia display rounded morphologies with membrane being rapidly extruded and retracted (arrow). Astrocytes and OPCs show limited membrane motility (also see Video 1), compared to the microglial cell (arrow) over the same period. (E) Time-lapse series from a microglial mono-culture in the absence of MPs (still from Video 2). Arrows indicate the same cell in each frame, showing extensive and rapid membrane remodeling. (F) Representative phase-contrast image of a live microglial mono-culture after

90 min MP incubation, with counterpart fluorescence micrograph showing high MP-loading (G).

Video 1: Mixed glial culture, no MPs, 20 s between frames, 79 min 20 s length. The sequence shows high levels of microglial activity, relative to other cell types.

Video 2: Microglial mono-culture, no MPs, 2 min between frames, 2 h 58 min length. The sequence confirms that microglial activity is similar in mono- and co-cultures.

Video 3: Mixed glial culture, 20 min post-MP addition, 2 min between frames, 12 h 8 m length. The sequence demonstrates the microglial dominance of particle uptake versus other cell types present.

Figure 6. Schematic showing potential enhancements and applications of the stoichiometrically defined neural co-culture in vitro screening platform.