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Less is more: Investigating the influence of cellular nanoparticle load on transfection outcomes in neural cells

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Running title: Magnetic nanoparticles as DNA transporters: a dose relationship

Summary: Genetic engineering of cell transplant populations offers potential for delivery of neurotherapeutic factors to modify the regenerative microenvironment of the injured spinal cord. The use of magnetic nanoparticle (MNP) based vectors has reduced the traditional reliance on viral methods and their associated obstacles in terms of scale up and safety. Studies utilising magnetic assistive platforms for MNP-mediated gene delivery have found transfection efficiency in astrocytes (a major transplant and homeostatic neural cell type) to be both frequency and amplitude-dependent. It is widely assumed that increased intracellular particle load will enhance transfection efficiency in a cell population. Therefore, we tested repeat delivery of MNP:plasmid complexes in conjunction with oscillating magnetic field parameters- a process termed 'magneto-multifection'- in astrocytes of primary origin in an attempt to enhance transfection levels. We show i) levels of transfection using magneto-multifection equal that seen with viral methods; ii) reporter protein expression using two reporter plasmids shows a diverse profile of single/dual transfected cells with implications for delivery of a 'cocktail' of neurotherapeutic proteins and, iii) contrary to expectation, an inverse relationship exists between particle load and reporter protein expression.

Keywords: amplitude; astrocytes; frequency; magneto-multifection; neurotherapeutics; transgene expression

Introduction

Astrocytes have major roles in the normal homeostatic functions of the central nervous system. While their roles in neural injury are controversial, several studies suggest that they offer promise as a cell transplant population for regenerative treatment of spinal cord injury, being of neural origin and with a key role in supporting neuronal survival (Haas & Fischer, 2013). Astrocytes also have effective cellular secretory processes meaning that genetically engineered astrocytes can play a key role as cellular delivery vehicles for gene transfer in 'combinatorial' interventions for spinal cord injury and, as such, have been effectively engineered to release neurotherapeutic proteins such as NGF and BDNF (Merienne, Le Douce, Faivre, Deglon & Bonvento, 2013). However, such studies have usually relied on viral vectors for genetic modification which, while they report high transfection efficiencies, can present significant challenges for human translational applications, in terms of scale-up and safety.

In this regard, MNPs have been proven to be highly promising gene delivery agents for genetic modification of several major neural transplant populations, offering a number of advantages over other transfection techniques (Karra & Dahm, 2010; Laurentt, Sapet, Le Gourrierec, Bertosio & Zelphati, 2011). The low transfection efficiency associated with MNP transfection has been addressed by deploying oscillating magnetic fields in conjunction with MNPs, proving highly advantageous in improving cellular transfection levels (McBain et al., 2008). Studies investigating the optimisation of magnetic field oscillation parameters for gene transfer to astrocytes have shown that transfection levels are optimal at a frequency of 1 Hz (Pickard & Chari, 2010). Additionally, the amplitude of field oscillation plays a role in influencing the *extent* of protein expression in individual cells (Tickle, Jenkins, Polyak, Pickard & Chari,

2016). A third parameter shown to further enhance MNP-mediated gene delivery is repeat administration of MNP:plasmid complexes (termed "multifection"). This approach can not only be used to increase the delivery of a single transgene to transplant populations, but also the use of different plasmids to enable release of a 'cocktail' of therapeutic molecules. Additionally, the combined use of multifection and applied magnetic field [*termed* 'magneto-multifection' (M-Mfect)] has shown significantly increased transfection efficiency in cell transplant populations (Pickard, Barraud & Chari, 2011). Therefore, we can predict that employing *the optimal frequency and amplitude for astrocytes*, M-Mfect may enhance transfection efficiency and/or the extent of transgene expression in astrocytes; however, this has not been tested previously.

A further important, but often overlooked consideration in transfection studies utilising MNPs is the correlation between the extent of nanoparticle uptake and gene expression. While many studies have focused on enhancing nanoparticle delivery to cells, no study has, so far, explored the relationship between particle load and transgene expression in neural transplant cells. To address these issues, we have utilised astrocytes of primary origin to (i) evaluate the efficacy and safety of M-Mfect for dual reporter plasmid delivery to astrocytes and, (ii) investigated the relationship between cellular nanoparticle load and transfection; both in terms of proportions of transfected astrocytes and extent of reporter protein expression per cell.

Materials & Methods

The care and use of animals was in accordance with the Animals (Scientific Procedures) Act of 1986 (UK), and approved by local ethics committee.

Following published protocols, MNP-mediated gene [red & green fluorescent protein (RFP; GFP)] transfer protocols were conducted at 24-48 h post-plating of primary rodent astrocytes (passage 2). The magnefect-nano device [42Bio, LLC (Gainesville, Florida, USA)] offers

lateral oscillation capabilities with programmable amplitude and frequency. Transfection-grade MNPs were obtained from OZ Biosciences; commercial forms (NeuroMag) and custom-synthesized forms (NeuroMag^{-Fluo}; NeuroMag conjugated to rhodamine fluorophore). Briefly, following addition of the MNP:plasmid complex, cells were exposed to the optimal magnetic field reported for astrocytes (f = 1Hz; 200 µm; 30 min); incubated in the absence of a magnetic field for 30 min, followed by 100% D10 refresh to remove any non-internalised particles (see Tickle, Jenkins, Pickard & Chari, 2015). Following a further 7 h incubation, cells were subject to a repeat gene transfer protocol. Single transfection acted as controls [Time1 (T1); Time2 (T2)] for the M-Mfect condition. RFP results in lower transfection efficiency than that seen with GFP due to the larger plasmid size of the former - 4.6 kb *vs.* 3.5 kb (Pickard, Adams, Barraud & Chari, 2015); therefore, experiments were duplicated using RFP at T1/GFP at T2 and GFP at T1/RFP at T2 (n=2; duplicates of each culture). This also enabled evaluation of RFP/GFP co-expression. Experiments were terminated 48 h following final MNP:plasmid administration. Control wells consisted of cells not exposed to MNP:plasmid complex. Safety of the M-Mfect protocol was of prime concern therefore, alongside an MTS assay as an indicator of metabolic activity (VICTOR^2 Multi-label Counter, PerkinElmer), assessment of cell viability was quantified through culture characterisation i.e. average cell count; culture purity and prevalence of pyknosis [see published protocols (Tickle, Jenkins, Pickard & Chari, 2016)].

To explore the dose relationship between MNP load and transgene expression, a subset of experiments was conducted using NeuroMag^{-Fluo}. The extent of NeuroMag^{-Fluo} cellular uptake was based on a previously described semi-quantitative method of low, medium or high particle accumulation within the cell (Jenkins, Pickard, Furness, Yiu & Chari, 2013). Fluorescence intensity was used as a representative measure of GFP expression. Briefly, fluorescence micrographs were converted to grayscale; inverted and calibrated using an optical density step

tablet, with individual cells then delineated to assess fluorescence intensity. Readings taken to correct for background fluorescence were subtracted from the fluorescence intensity cellular readings, the resulting value representing the measure of GFP expression in a transfected cell (ImageJ software). Particle accumulation *vs.* GFP expression was compared to allow for evaluation of a MNP/GFP dose-relationship.

Results

For M-Mfect, plasmids encoding RFP and GFP were used to evaluate transfection efficiency at the two time points (Fig. 1a) and to investigate the level of reporter protein co-expression in single cells (Fig. 1b). Repeat administration significantly increased overall cellular transfection efficiency compared with single MNP:plasmid dose at T1 or T2 alone [81.5 ± 1.9% (M-Mfect) *vs.* 37.9 ± 8.8% (T1) and *vs.* 43.7 ± 8.1% (T2); ***p < 0.01; *p < 0.05 respectively*] (Fig. 1c). Repeat exposure to MNP:plasmid complexes revealed that only *ca.* 22% of cells showed co-expression of RFP/GFP [Fig. 1b-image (+ inset) & Fig 1d]; with the majority of transfected cells exhibiting GFP expression (*ca.* 54%) (Fig. 1d).

Transfected cells are nanoparticle-loaded cells which, following intranuclear plasmid-DNA (pDNA) delivery, subsequently exhibit reporter protein expression. Of the cells here, approximately 80% of astrocytes exhibited NeuroMag^{-Fluo} labeling, of which *ca.* 50% exhibited reporter protein expression (Fig. 1e). Labeled cells showed low, moderate or high levels of particle accumulation as measured by a visual semi-quantitative analysis (Fig. 1f). Observation of levels of particle accumulation and transgene expression revealed an inverse relationship (Fig. 1g). High transgene expression was generally associated with low levels of particle accumulation, whereas cells that were highly labeled exhibited a low extent of transgene expression. In this regard, although low particle accumulation was always associated with reporter protein expression, the observed relationship between higher levels of particle

accumulation and GFP expression was inconsistent. Higher levels of accumulation were observed in association with high, moderate, low or no GFP expression (Fig. 1h).

The percentage of cells expressing GFAP was consistent across all conditions (98.1 ± 0.5%) (Fig. 2a), with no difference found in average cell number (Fig. 2b). Type 1 astrocytes accounted for *ca.* 92% of the GFAP+ve population *vs.* Type 2 which accounted for *ca.* 8% (Fig. 2c). The Type 1 phenotype made up the majority of transfected cells (*ca.* 89% *vs.* Type 2; *ca.* 11%) (Fig. 2d). Low levels of pyknosis were observed across all conditions (*ca.* 3% overall) (Fig. 2e), with no difference found in metabolic activity between cells exposed to plasmid only or MNP:plasmid complex across each of the experimental conditions (Fig. 2f).

Discussion

MNP-mediated gene transfer to astrocytes has previously achieved 60% transfection efficiency (Pickard & Chari, 2010). We show here that employing a M-Mfect strategy, *utilising the optimal parameters for this particular neural cell type*, significantly enhanced transfection efficiency versus our previous study (> 80% *vs.* 60%), and was associated with a high degree of cell viability. A wider population of the target cells are transfected, with a subpopulation showing dual protein expression indicative of repeat MNP:plasmid uptake. Previous studies (Pickard & Chari, 2010) have observed rapid intranuclear delivery and transgene expression in a subpopulation of astrocytes in as little as 4 h. Potentially, levels of GFP/dual protein expression could be significantly enhanced in this same subpopulation, as repeat administration of MNP:plasmid complex at 8 h intervals could increase the likelihood of pDNA exposure to i) a greater proportion of target cells of which ii) a subpopulation have the potential for dual protein expression. This has implications for therapeutic gene delivery. A subpopulation of cells exhibiting high levels of gene expression could be more beneficial for slow release applications due to the transient nature of nucleic acid, and transgene dilution through

proliferative processes. Conversely, a population containing a large proportion of low transgene-expressing cells could be advantageous for immediate 'one-step' release of a drug or growth protein. Our results on dual-transfected cells suggest however, that only a small number (*ca.* 22%) have the potential to release a 'cocktail' of proteins.

In respect of transfection efficiency, a widely accepted view is that transfection levels will directly correlate with nanoparticle load (i.e. Laurentt, Sapet, Le Gourrierec, Bertosio & Zelphati, 2011; McBain et al., 2008). Our results question this assumption. Nanoparticle uptake is dependent on a number of factors including endocytotic activity of the target cell (Treuel et al, 2015). Astrocytes possess a highly endocytotic membrane showing avid MNP uptake (Jenkins, Pickard, Furness, Yiu & Chari, 2013; Tickle, Jenkins, Pickard & Chari, 2015; Tickle, Jenkins, Polyak, Pickard & Chari, 2016). However, of the *ca.* 80% labeled cells in this present study, only *ca.* 50% were transfected. Morphological analyses revealed low intracellular particle load was associated with high protein expression. Highly labeled cells, for the most part exhibited low/no protein expression. These findings indicate an inverse relationship between particle load and gene transfer, suggesting high particle uptake is not a major determinant of successful transfection.

For non-viral gene delivery, heterogeneity between particle uptake and gene expression appears to be commonplace (Akita, Ito, Kamiya, Kogure & Harashima, 2007). A plausible explanation in relation to gene transfer could be cell cycle dynamics which reports a trafficking of the various elements – particle and pDNA - throughout the duration of the cell cycle. However, it has also clearly identified optimal timings -'hot spots'- at different stages of the cell-cycle for uptake, delivery and subsequent protein expression. The cell cycle of astrocytes in culture is *ca.* 14-20 h, with an M-phase of 2-3 h and an S-phase of *ca.* 7–10 h. Particle uptake as a delivery vector for pDNA is greatest during the G2/M-phase, with intranuclear pDNA delivery comparable throughout the cell cycle, reporting higher levels of uptake during

mitosis. Subsequent transgene expression is understood to be dependent on mitotic events although, significant increases are also reported prior to mitosis (Akita, Ito, Kamiya, Kogure & Harashima, 2007; Tseng, Haselton & Giorgio, 1999).

In this context, particle uptake and subsequent intracellular processing are not continuous but rather, a multi-stage process (Pollard et al., 1998). This implies a longer time-course than previously supposed, and that the occurrence of one event does not necessarily predict the occurrence of a subsequent event. High cellular particle loading may result in a longer trafficking time or be 'held' at one stage of the process, potentially impairing DNA release from the particle; a possible explanation for the inconsistency observed in highly labeled cells. Of importance, contrary to the belief that increased nanoparticle labeling is a precursor to enhanced protein expression, the findings from this study would suggest that **optimal transgene expression, *at least in respect of astrocytes*, may be due to lower particle uptake per cell** and in turn, more efficient intracellular processing.

For the proportion of cells showing dual protein expression, co-expression may be contingent on optimal particle loading at each time-point, or/and pDNA release at *different time-points* due to plasmid size. In this regard, electron microscopy in conjunction with cell cycle synchronisation would offer insight into the intracellular processing of the MNP:plasmid in this neural cell. Further work is needed to establish the mechanisms underlying the reasons for the division of a cell population into transfected/non-transfected cells and their capacity for dual protein expression.

Conflict of Interest Statement: The authors have no other relevant affiliations or financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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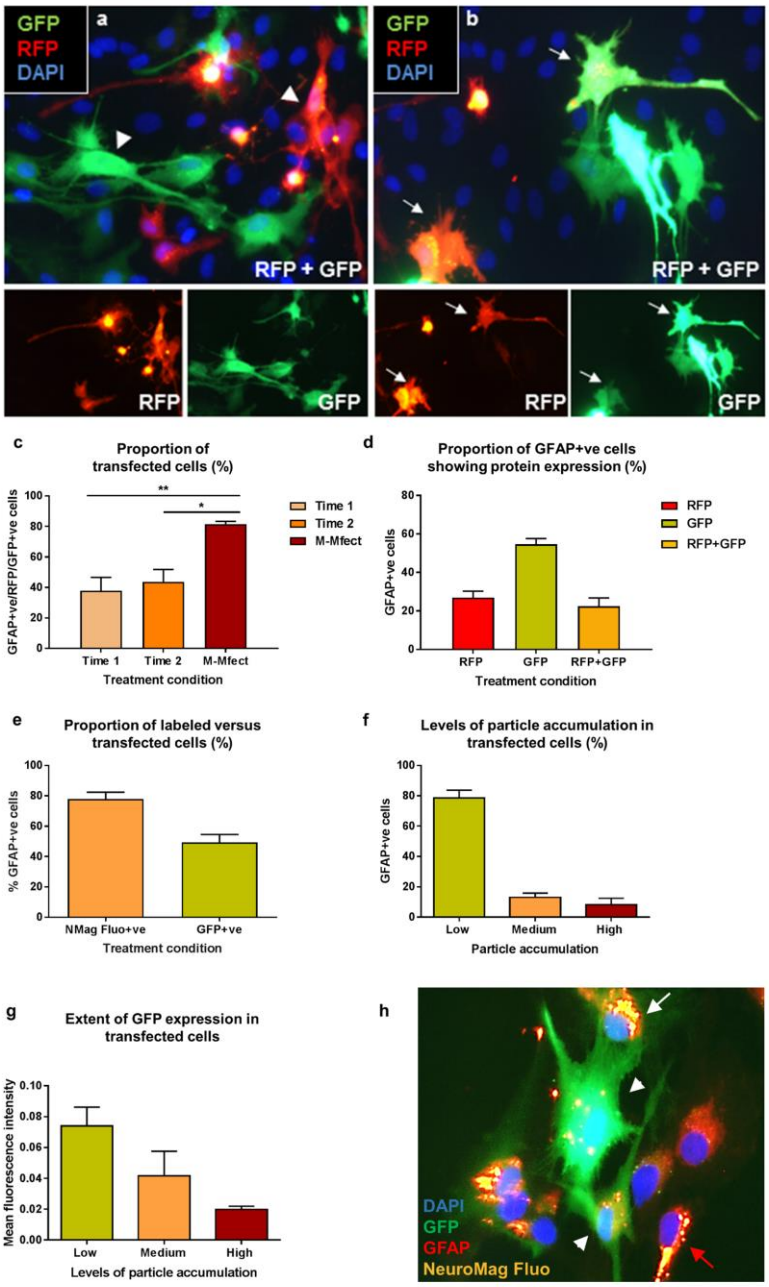

Figure 1. Magneto-multifection and transgene expression. For the M-Mfect experiments, transfection efficiency was determined as the percentage of GFAP+ve cells exhibiting reporter protein expression (RFP or GFP). Co-expressing (RFP + GFP) cells were confirmed by coincident expression of red or green fluorescent protein on unmerged images, with the merged image exhibiting a combination of both proteins. Representative fluorescence micrographs illustrating (a) two different sub-populations of cells are transfected at T1 and T2; characterised respectively by RFP and GFP expression [arrowheads; main image (corresponding un-merged images showing RFP and GFP expressing cells)]; (b) The prevalence of a subpopulation of cells co-expressing RFP/GFP expression, indicating repeat uptake of MNP:plasmid complex [white arrows; main image (corresponding un-merged images showing coincident RFP/GFP expression)]. Bar graphs showing (c) repeat administration of MNP:plasmid complex significantly increased transfection efficiency compared with a single dose at T1 or T2 (**$p < 0.01$; *$p < 0.05$ respectively), and (d) a

greater number of transfected cells exhibit GFP expression vs. RFP, with a subpopulation of cells exhibiting RFP/GFP co-expression (n = 3). Bar graphs show (e) a discrepancy between proportions of labeled cells and cells exhibiting GFP expression, with (f) transfected cells predominantly showing low levels of particle accumulation. Bar graph (g) shows an inverse relationship exists between particle accumulation and levels of GFP expression. Fluorescence micrograph (h) shows (i) high levels of GFP expression (white arrow head) in cells exhibiting low levels of particle uptake; (ii) extensive particle uptake coupled with negligible GFP expression (red arrow) and conversely, medium levels of GFP expression (white arrow) (n = 2). Scale of main image = 50 µm. Results expressed as mean ± s.e.m. (c; d). GFAP: glial fibrillary acidic protein; GFP: green fluorescent protein; MNP: magnetic nanoparticle; RFP: red fluorescent protein; T1; T2: Time 1; Time 2

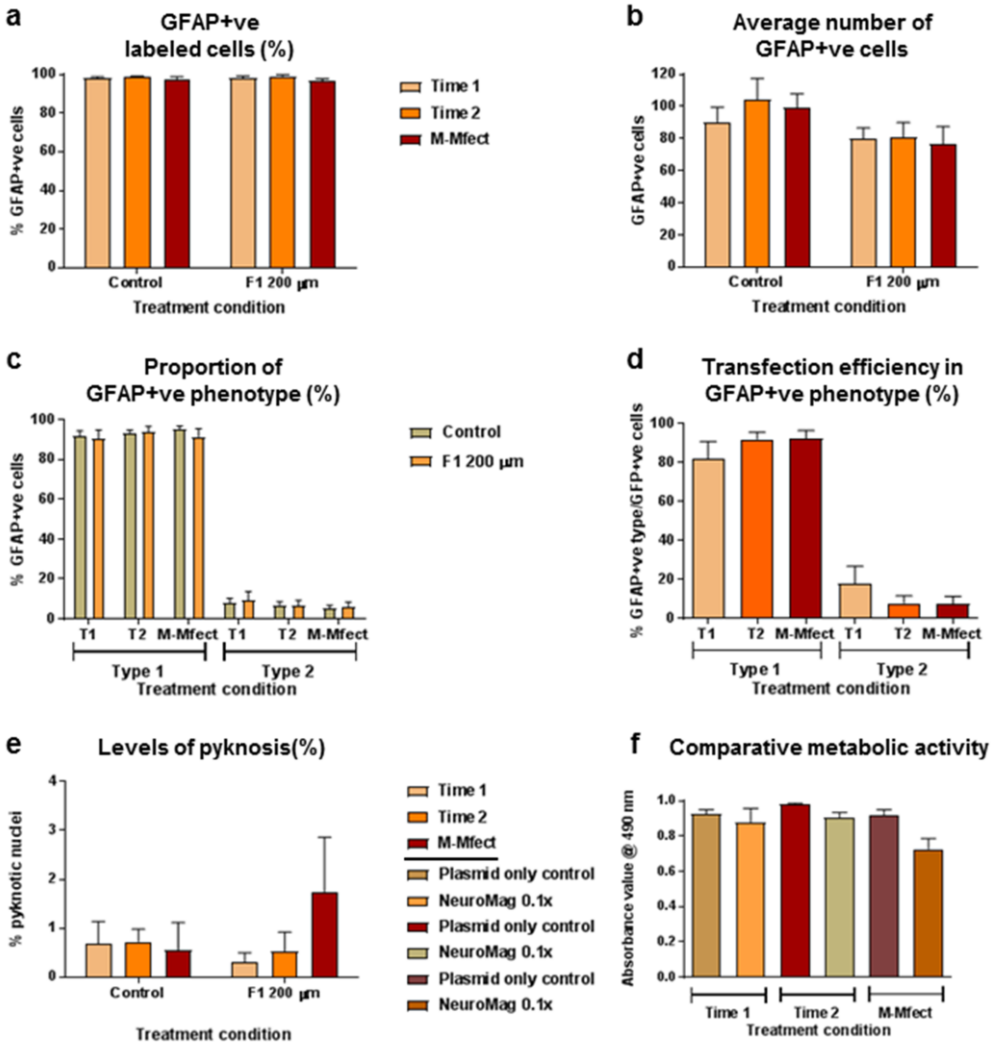

Figure 2. Safety assessment of M-Mfect protocols. Bar graphs showing (a) the percentage of GFAP+ve cells was consistent across all conditions with (b) no difference in average cell count. Bar graphs showing (c) proportion of astrocyte phenotype was predominantly Type 1 (*ca.* 92% *vs. ca.* 8% Type 2) with (d) Type 1 phenotype accounting for 89% of transfected cells *vs.* 11% Type 2 phenotype. Bar graphs showing (e) low levels of pyknosis across all conditions (*ca.* 3%) with (f) no difference seen in comparative metabolic activity in each condition. (n = 3). Results expressed as mean ± s.e.m.