Developing Super-Paramagnetic Nanoparticles for Central Nervous System Axon Regeneration

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ABSTRACT

Neurons in the central nervous system (CNS) fail to regenerate their axons after injury or in degenerative disease, and stimulating regenerative growth remains a major goal of neuroscience. Mechanical tension plays a key role in stimulating axon growth *in vitro* and *in vivo* and might be used to enhance regeneration. Simply applying a tensile force to a neuron or an axon can stimulate neurite initiation or axon elongation. We are exploring novel nanotechnology-based approaches by which magnetic nanoparticles (MNPs) could be used to generate tensile forces and manipulate axons to elongate, to overcome inhibitory substrates, and to enhance the trophic signaling of axon growth. We have investigated how MNPs interact with optic nerve and spinal cord tissue *in vivo* and CNS neurons *in vitro*. We asked whether these nanoparticles can be incorporated into axons and cells. *In vivo*, MNPs were localized to the site of injection with little to no particle-specific toxicity detected. *In vitro*, we found MNP endocytosis by embryonic and postnatal retinal ganglion cells and embryonic hippocampal cells. Ongoing experiments are directed at identifying the subcellular localization of the MNPs, functionalizing MNPs for optimized binding to axons, and then, using magnetic fields to exert forces on neuronal growth cones *in vitro* and *in vivo*.

Keywords: central nervous system, axon regeneration, magnetic nanoparticles

1. BACKGROUND

Neurons in the central nervous system (CNS), which includes the eye, brain and spinal cord, fail to regenerate their axons after injury. The failure of retinal ganglion cells' (RGCs') axons to traverse an injured optic nerve leads to permanent loss of vision. In the spinal cord, a similar failure leads to permanent loss of motor and sensory function. This inability of CNS axons to regenerate is apparent not only after trauma, but also in neurodegenerative diseases. Thus, the failure of CNS regeneration underlies a major clinical need.

Little is known about how axons grow. There are probably two different modes of axon growth *in vivo (1)*. During the early developmental wiring of the nervous system, axons extend towards their synaptic targets mainly by elongating at the growth cone, the motile tip of the axon. Later, however, after neurons form synaptic connections and no longer have motile growth cones, they elongate along the length of the axon, not just at the tip. During normal body growth, this allows the axon length to keep up with the increase in distance between its cell body and synaptic target.

Research addressing both of these modes of growth have supported a role for tension in stimulating axon elongation (*1, 2*). Over 20 years ago, Dennis Bray demonstrated that tension plays a key role in stimulating neurite growth. In these experiments, pulled glass pipette tips were coated with collagen and polylysine (*3*). Then, the pipette tip surface was adhered to the cell membrane of cultured neurons and pulled from the cell body at velocities in the order of 1μm/min. This application of mechanical tension elicited neurite-like process outgrowth.

Subsequent studies used glass microneedles with known mechanical compliances to measure the force necessary to initiate and elongate neurites (*4-7*). These studies contributed new insight into the role of tension in stimulating neurite outgrowth. However, it was difficult to apply forces accurately below 100pN (*8*). Furthermore, long, thin, stiff needles may have random thermal fluctuations larger than the needle deflections used to estimate applied forces, which may confound force measurements.

An alternate method employing superparamagnetic microparticles in a high-gradient magnetic field, called "magnetic bead force application" (MBFA), may provide a precise and reliable approach to apply well-controlled forces to neurons and axons (*8*). This approach was adapted from previous studies in which it was used to measure mechanical properties of cells. The microparticles (4.5μm) were coated with anti-β1-integrin antibody to allow attachment to embryonic chick forebrain cells. Magnetic field application generated a dynamic range of forces from 15 to 2000 pN, and a typical stiffness of ~10 pN/μm. This reduced stiffness in the manipulator greatly reduced the fluctuations in the applied force. Later, MBFA was used to mechanically elicit neurites from synapse-competent neuron cultures that ranged in age from 7-22 embryonic equivalent days (*9*). These results show that simply applying a force to an axon, for example, by pulling on the growth cone with a glass microneedle, or with a magnetic particle in an applied high gradient magnetic field, can stimulate neurite initiation and elongation from the cell body, and that direct application of mechanical tension to growth cones causes elongation independent of extrinsically

applied trophic factors. This suggests that trophic factors signal growth cones to create tension on the axon. Furthermore, recent *in vitro* studies have shown that mechanical tension can be used to elongate fasciculated bundles of axons (*10, 11*).

Magnetic nanoparticles (MNPs) have been used extensively in biological and medical research as well as in potential therapeutic approaches. MNPs are used as a contrasting agent in MRI (*12*), as targeted drug delivery systems (*13*), as a cancer therapy system (*14*), and as a system that can apply mechanical forces to cells (*15*). If the particle size is optimized, these particles may be endocytosed by cells, and the location of the MNPs may be manipulated using an external magnetic field. For example, Won et al reported that when streptavidin-conjugated MNPs approximately 50nm in diameter were labeled with transducible fusogenic TAT-HA2 peptide, they were endocytosed into HeLa cells, and furthermore, they could be translocated with an external magnetic field by using a ~1.1T permanent magnet (*16*).

Furthermore, maghemite (γFe_2O_3), a ferromagnetic crystal, can be synthesized with 10-80nm diameters. Such MNPs were endocytosed by HeLa cells and contained in vesicles (*17, 18*). These studies also quantified MNP endocytosis by these cells using magnetophoresis. It was shown that the cellular in take of these particles were in the range of 2-10 millions of nanoparticles per cell. Furthermore, it was shown that MNPs with negatively charged surfaces had higher affinity for cell membranes than dextran-coated MNPs, and that this affinity can be reduced by coating anionic MNPs with albumin. These results show the importance of surface specificity for endocytosis of MNPs *in vitro*.

As a step towards understanding the interplay between trophic signaling and mechanical tension in axon growth, we are investigating how commercially available magnetic and non-magnetic nanoparticles interact with optic nerve and spinal cord tissue. Specifically, we asked whether these nanoparticles can be incorporated into neurons and axons *in vivo* and *in vitro*.

2. EXPERIMENTAL METHODS

2.1 Nanoparticles

We used MACS® MNPs (Miltenyi Biotec Inc., CA), carboxyl and tosyl activated MNPs (Dynal/Invitrogen, CA; Micromod, Germany) and RetroBeads™ (Lumafluor Inc., FL). The nanoparticle sizes varied from 50-1000nm. Surfaces were modified with fluorescently labeled molecules for microscopy.

2.2 Optic nerve and spinal cord MNP injections

All animal pre-operative and post-operative care was carried out according to University of Miami Animal Care Use Committee guidelines. Adult Sprague-Dawley (SD) rats were anesthetized by an intraperitoneal injection of Ketamin and Xylazine, (80-100mg + 10mg)/Kg. For optic nerve injections, the optic nerve was surgically exposed via an orbital approach. 1µl of MNPs was injected approximately 2-4mm retrobulbar from the nerve head; the contralateral optic nerve was injected with 1µl of vehicle as a control. Skin was sutured with 6-0 vicryl (Ethicon, INC., New Jersey). Then, 1µl of fluorescently labeled cholera toxin-subunit B (CTB) was injected intravitreally into both eyes. After 24hrs, rats were euthanized by CO_2 inhalation and cervical dislocation. For spinal cord injections, postnatal day 2 SD rats were deeply anesthetized by hypothermia. The dorsal surface of the spinal cord was exposed by laminectomy, and 1µl of MNP solution was injected on each side of the spinal cord, lateral to the medial dorsal artery. As a control, same procedure was followed with 1µl of vehicle. Then the skin was sutured and rats were euthanized by decapitation 24 hours later.

For both injection sites, after euthanasia, the relevant tissues were removed and immediately fixed using 4% paraformaldehyde, and stored in sucrose overnight. The tissues were frozen and embedded in Optimal Cutting Temperature (OCT) Compound (Sakura, California). Sections 20-50 µm thick of the eye and optic nerve or the brain and spinal cord were made using a cryostat (Leyca CM 1850 Germany).

2.3 Purification and culture of retinal ganglion cells (RGCs) and hipppocampal neurons

RGCs from E20 through P7 Sprague-Dawley rats were purified by sequential immunopanning to 99.5% purity and cultured on poly-D-lysine (PDL; 70 kDa, 10 µg/ml; Sigma) and laminin (2 mg/ml; Telios/Gibco) in serum-free defined medium containing BDNF (50 ng/ml), CNTF (10 ng/ml), insulin (5 mg/ml) and forskolin (5 mM) (*19*).

Hippocampal neurons were prepared from embryonic day 18 rat hippocampi (Brain Bits LLC, IL) according to Goslin et al. (*20*) Cells were cultured in Hibernate-E medium with B27 and glutamax supplement (Invitrogen).

2.4 Immunostaining and confocal imaging

Tissue sections were fixed with 4% PFA for 30 min, blocked with 20% serum with 0.4% Triton-X for ~2 hours, and incubated with primary anti-GFAP (Sigma, 1:500) at 4°C overnight. Incubation in secondary, goat anti-Mouse Alexa 647 (Mol. Probes, 1:500) for ~2 hours and DAPI for nuclei staining was followed by mounting and microscopy.

Confocal laser scanning microscopy was performed on a Leica TCS SP with a 40X oil immersion objective (Leica HCX PL APO with 1.26NA), and a 63X oil immersion objective (HCX PL APO lambda blue with 1.4NA). For fluorescent microscopy, 405 Diode, Argon, and HeNe 543 lasers were used. Digital images were collected and analyzed with Leica Application Suite Advanced Fluorescent (LAS AF) version 1.5.1 image processing software.

2.5 Transmission Electron Microscopy (TEM)

Tissues were fixed with 2% glutaraldehyde in sodium cacodylate buffer, post-fixed with 1% osmium tetroxide, and dehydrated with a series of ethanols before Spurr™ resin embedding. Tissue samples were then ultra microtome sectioned and stained with lead citrate. Sections were observed with a Philips 300 TEM. MNPs were characterized using energy dispersive spectroscopy function.

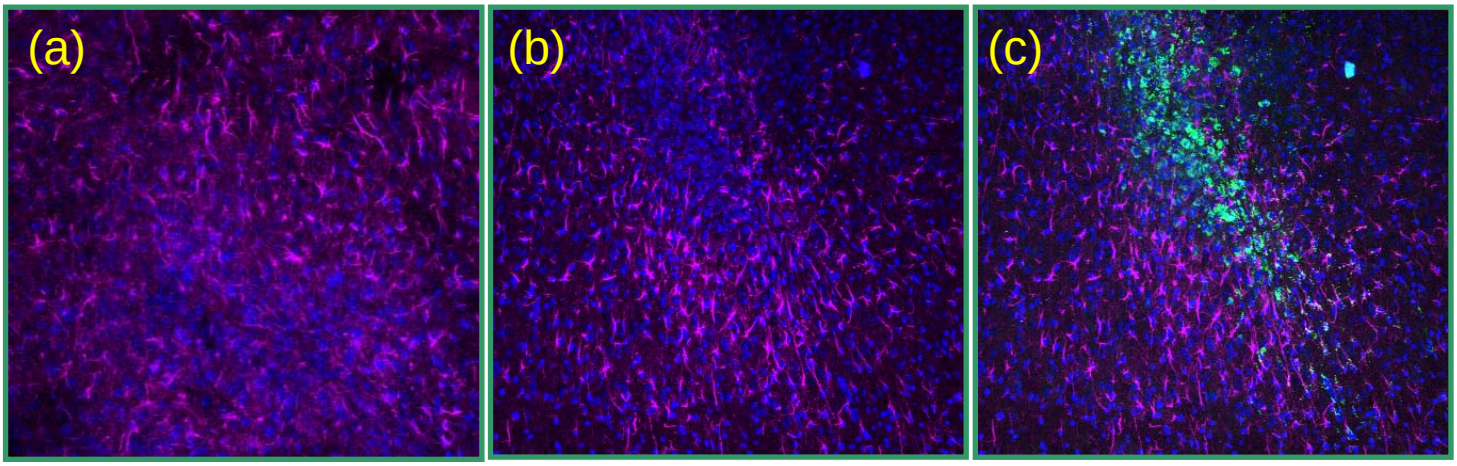

Figure 1: Comparison of (a) control versus (b) and (c) MNP injections into rat spinal cord. Green – nanoparticles including MNPs, Blue – DAPI staining of cell nuclei, Magenta – GFAP staining showing activated astrocytes. There are similar levels of astrocyte activation in saline- and MNP-injected spinal cords, suggesting MNPs confer no additional toxicity over the injection itself.

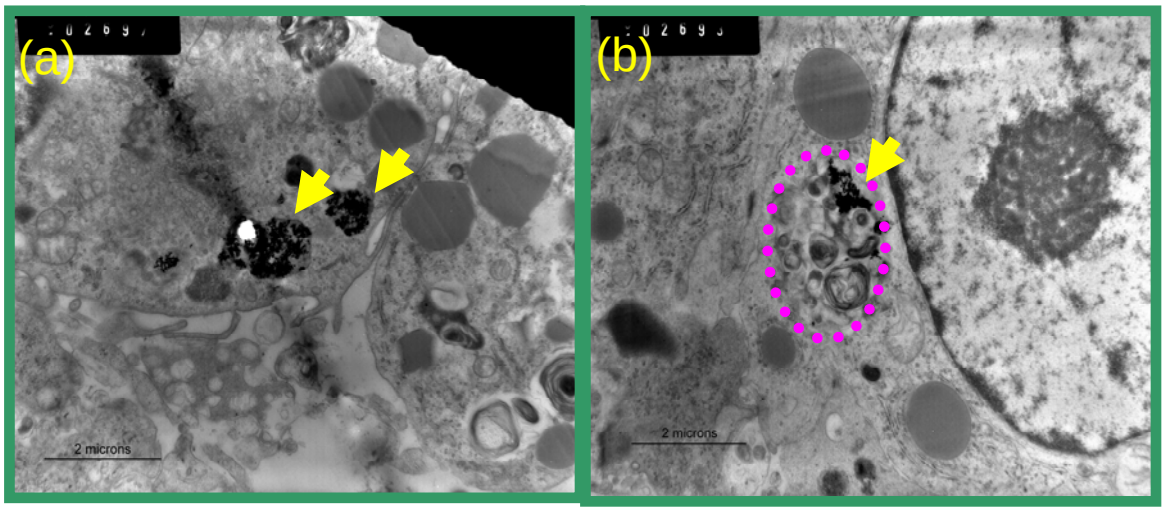

Figure 2: TEM micrographs showing endocytosis of magnetic nanoparticles by cells in spinal cord tissue. The endocytosed particles are contained in vesicle (arrows in a and b), and some sections showed magnetic particles in vesicles with degenerated myelin (b).

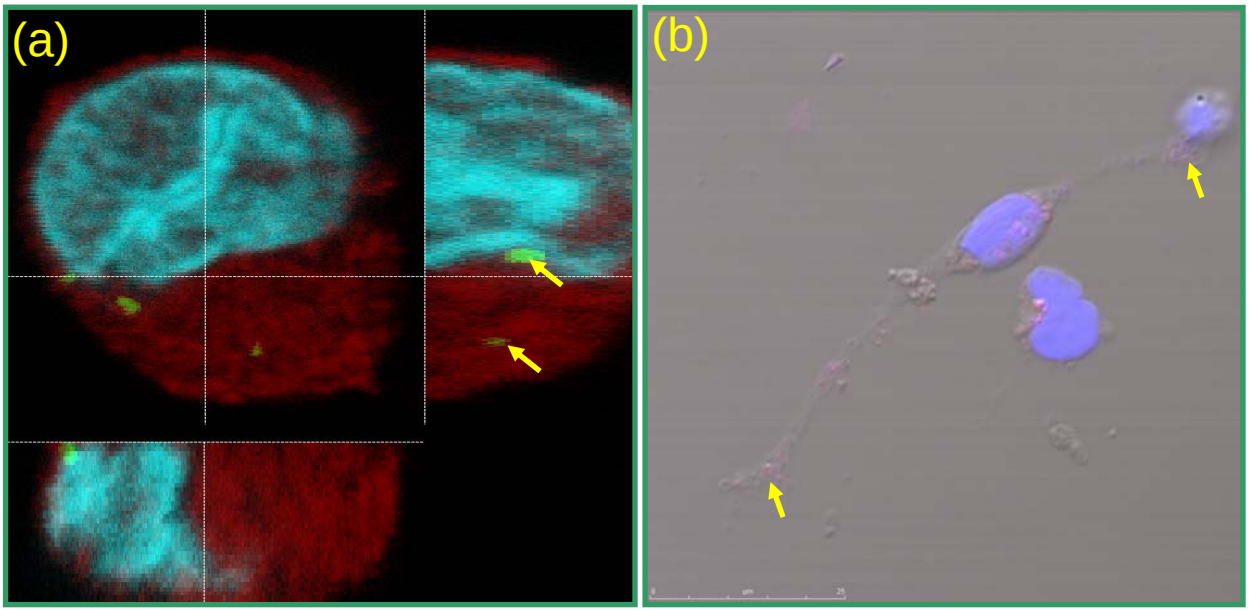

Figure 3: Confocal images of (a) E20 RGC and (b) E18 rat hippocampal neurons showing endocytosis of nanoparticles. Yellow arrows in the z-stacks in (a) show nanoparticles are in the cytoplasm. In (b), red staining demonstrates MNPs (arrows).

3. RESULTS

3.1 Incorporation of nanoparticles into cells and axons *in vivo*

We first asked whether MNPs are toxic *in vivo*. We compared astrocyte activation as measured by glial fibrillary acidic protein (GFAP) upregulation in control (saline) versus nanoparticle injections in spinal cord. In both cases, the injection sites were similarly surrounded by activated astrocytes, suggesting MNPs confer no additional toxicity (Fig. 1). Also, we observed an increase in the cell nuclei density around the injection sites compared to an uninjected tissue site. Cell nuclei near injection sites were co-localized with nanoparticles. Transmission electron microscopy (TEM) showed endocytosis of the magnetic nanoparticles (Fig 2).

3.2 Incorporation of MNPs into cells and axons *in vitro*

Confocal images of *in vitro* cell cultures showed endocytosis of particles in embryonic RGCs and hippocampal neuron cell bodies and in their processes (Fig. 3). The average size of fluorescent puncta ranged from 500-1200nm. No gross toxicity of MNPs was noted *in vitro* (data not shown).

4. DISCUSSION

These data demonstrate that MNPs can be delivered to cells *in vivo* and endocytosed in the spinal cord. *In vitro* experiments showed similar results where embryonic RGCs and hippocampal neurons endocytosed MNPs. Preliminary experiments directed at identifying potential toxicity have not detected MNP-specific toxicity *in vitro* or *in vivo*. Ongoing experiments are directed at identifying the subcellular localization of the MNPs, functionalizing MNPs for optimized binding to axons, and using magnetic fields to exert forces on neuronal growth cones *in vitro* and *in vivo*.

Our ultimate goal is to apply the use of MNPs to the study of trophic signaling and mechanical tension as mediators of axon regenerative ability. Such investigation into the use of magnetic fields and MNPs may ultimately allow for external control of axon growth and pathfinding. Our hope is that these studies will illuminate the basic biology of trophic signaling at the nanometer scale, as well as lead to potential "nanotherapeutics" that could ultimately be developed to treat human disease. Our research will also lay a foundation for future projects to investigate the possibility of translating tension based neurite regeneration into a novel approach in axon regeneration for clinical use.

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