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# The orientation of the neuronal growth process can be directed via magnetic nanoparticles under an applied magnetic field

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**Abstract**

There is a growing body of evidence indicating the importance of physical stimuli for neuronal growth and development. Specifically, results from published experimental studies indicate that forces, when carefully controlled, can modulate neuronal regeneration. Here, we validate a non-invasive approach for physical guidance of nerve regeneration based on the synergic use of magnetic nanoparticles (MNPs) and magnetic fields (Ms). The concept is that the application of a tensile force to a neuronal cell can stimulate neurite initiation or axon elongation in the desired direction, the MNPs being used to generate this tensile force under the effect of a static external magnetic field providing the required directional orientation. In a neuron-like cell line, we have confirmed that MNPs direct the neurite outgrowth preferentially along the direction imposed by an external magnetic field, by inducing a net angle displacement (about 30°) of neurite direction.

***From the Clinical Editor:*** This study validates that non-invasive approaches for physical guidance of nerve regeneration based on the synergic use of magnetic nanoparticles and magnetic fields are possible. The hypothesis was confirmed by observing preferential neurite outgrowth in a cell culture system along the direction imposed by an external magnetic field.


*Key words:* Nerve regeneration; Magnetic nanoparticle; Magnetic field; Neurite outgrowth orientation; Physical guidance

Nerve regeneration and recovery of nerve function have been a major issue in neuroscience in regards to the treatment of injured neurons after accidents or degenerative diseases.[1] The regeneration of peripheral nerves exemplifies the plasticity which exists within the nervous system. Following an injury to a peripheral nerve, the section distal to the injury site degenerates. Functional recovery is then totally dependent on the growth and extension of axons from the proximal end across the injured site until they reach their distal target, e.g., denervated muscle.[2] In humans, axonal regeneration occurs at a rate of about 2-5 mm/day; and thus significant complete injuries (neurotmesis) can take many months for effective return of function.[3] Extensive research in

*Abbreviation:* MNPs, Magnetic nanoparticles; M, Magnetic fields; NFs, Neurotrophic factors; NGF-β, Nerve growth factor beta; BSA, Bovine serum albumin; $NGF_{fluor}$, Fluoscently labelled NGF-β; TPF, Tetrafluorophenyl; f-MNP, MNP labelled with $NGF_{fluor}$; FBS, Fetal bovine serum; PLL, Poly-L-lysine; TEM, Transmission electron microscopy; HAADF, High angle annular dark field; EDS, Energy-dispersive X-Ray Spectroscopy; EDAX, EDS detector; SEM, Scanning electron microscopy; PI, Propidium iodide; $T_d$, Doubling time; PEI, Polyethylenimine; SPION, Superparamagnetic iron oxide nanoparticles; p-TrkA, Phosphorylated TrkA; K, Control; P, Pellet; S, Supernatant; FIB, Focused ion beam; STEM, Scanning TEM; FEM, Finite element modelling.

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bioengineering has been focused on the development of innovative strategies for reducing this prolonged recovery time. The underlying concepts of these strategies involve physical and biochemical guidance i) to direct axonal re-growth and ii) to stimulate axonal elongation across the nerve lesion site. For a long time, neuroscientists have focused on biochemical guidance cues such as molecules capable of orientating migrating and growing cells (e.g., netrins, ephrins, semaphorins) and factors influencing neuronal growth (e.g., growth factors, neurotransmitters, glial cells and extracellular matrix proteins).[4] However, exclusive focus on biochemical processes involved in neuronal regeneration has not so far resulted in significant therapeutic gains. An alternative approach is the "guidance therapy" based on the use of scaffolds (autologous tissue grafts, non-autologous tissue grafts, natural based materials, synthetic materials, etc.) working as "nerve guides" or "nerve guidance channels". They provide a conduit during the nerve regeneration process for the diffusion of growth factors secreted by the injured nerve ends and to limit the injury site infiltration by scar tissue.[5-8]

The importance of mechanical factors for the nervous system has been appreciated only recently. It has become widely accepted that cellular tension is a crucial factor in neuronal development.[9] The idea that tension is involved in the morphogenesis of the nervous system arose in the late 1970s, when experimental pioneering work revealed that neuronal processes in vitro are under tension.[10] Later, it was demonstrated that the external application of mechanical tension alone is sufficient to initiate axonal outgrowth.[11] Neurite initiation and elongation as functions of the applied tensile force were generally studied using glass microneedles but, recently, Fass et al reported the use of magnetic beads for force application.[12]

Although the role of mechanical force for neurite initiation and elongation is a well investigated topic, its influence in directing axonal re-growth is poorly studied. The control of directional movement is obviously a crucial issue in nerve regeneration as regeneration cannot occur until the proximal end of the injured nerve reaches its destination. During the regeneration process, neurons send out the leading tips of their processes called growth cones. They are highly motile structures that provide the machinery to move forward. Recent work suggests that growth cones also generate forces (through cytoskeletal dynamics, kinesin, dynein, and myosin) which induce axonal elongation, and axons lengthen by stretching.[13] They also possess detectors of guidance cues that translate environmental cues into directional movement and thus guide neuronal processes toward their destination.[14] Guidance cues are classically understood to be attractive or repulsive molecules, capable of orientating neuronal cells. Generally, a growth cone's response to a certain guidance cue depends on several factors and it is difficult to gain control on this process. An alternative approach proposed in literature is to introduce topographic cues to guide neuronal navigation.[15-18] Although several reports have demonstrated the cell culture benefit of these cues, they usually fail when tested therapeutically. One problem is that the non-invasive delivery of these cues is difficult if not impossible to achieve. Here, we validate an alternative approach for physical guidance. Specifically, we demonstrate that magnetic nanoparticles (MNPs) and magnetic fields (Ms) can be used for physical guidance of neuronal processes. We found that the application of a tensile force to a neuron or an axon can stimulate neurite initiation or axon elongation in the desired direction. In this work, MNPs are used to generate these tensile forces under the effect of an external magnetic field and to achieve directional orientation (Figure 1).

The first draft of this idea was reported by a patent submitted in 1998 (US6132360 A) which describes a methodology based on MNPs which are actively incorporated into neurons (and their axons) of a severed or interrupted nerve. This is then exposed to an external magnetic field which is moved longitudinally to the severed/interrupted nerve, thereby stretching the MNPs-loaded neurons and their axons along the desired axis for bringing the gap. To our knowledge, no practical demonstration of this concept has been provided to date and our work represents the first proof of concept.

This methodology has the potential for clinical translation as static magnetic fields are extensively used in medical imaging.[19] Additionally, magnetic nanoparticles are employed in biomedicine. Currently, many clinical diagnostic/therapeutic tools use MNPs, e.g., MRI contrast agents in magnetic resonance imaging and magnetic hyperthermia, vectors for drug delivery and magnetic cell separation, etc.[20-22]

Additionally, in combination with recent advances in functionalization chemistry MNPs can be easily functionalised with biological molecules, e.g., neural binders to enhance MNP binding to neuronal cells[23,24] or neurotrophic factors (NFs) to stimulate peripheral neurons to regenerate their axon[25] or specific antibody to track signaling endosome and control their subcellular localization in order to alter growth cone motility and to halt neurite growth.[26]

In the present work, we produced and characterised MNPs functionalised with nerve growth factor beta (NGF-β), demonstrating that these particles are able to trigger PC12 differentiation in a neuronal phenotype and, most importantly, to direct the orientation of newly formed neurites.

## Methods

### *Functionalisation of MNPs*

MNPs were synthesised by a modification of the well-established oxidative hydrolysis method (i.e., the precipitation of an iron salt in basic media with a mild oxidant).[27] Specifically, in situ polymer coating was achieved by adding polyethylenimine (PEI, 25 kDa) during the reaction, as described in a previous work.[28]

The protein (a mixture NGF-β /BSA 1:6 w/w) (Sigma, N1408) was labelled with Alexa Fluor 488 (Life Science, A10235). TFP ester of the dye efficiently reacted with primary amines of proteins. Purification through a size exclusion resin allowed to discard the unincorporated dye. The concentration of the purified labelled protein was evaluated by UV–vis analysis. The absorbance spectrum revealed the presence of two peaks: at 280 and 494 nm, typical of proteins and Alexa Fluor 488, respectively (Figures S1-2, supplementary material).

The functionalization of the particles was carried out by adding the labelled protein (concentration of $NGF_{fluor}$ 35 $\mu g ml^{-1}$) to MNPs (500 $\mu g ml^{-1}$). The resulting suspension was dispersed at