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Magnetic NGF-Releasing PLLA/Iron Oxide Nanoparticles Direct Extending Neurites and Preferentially Guide Neurites along Aligned Electrospun Microfibers

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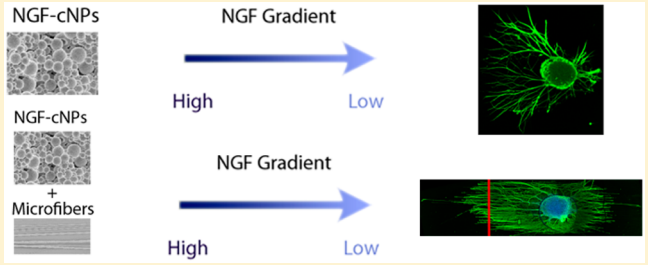
Supporting Information

ABSTRACT: Nerve growth factor releasing composite nanoparticles (NGF-cNPs) were developed to direct the extension of neurite outgrowth from dorsal root ganglia (DRG). Iron oxide magnetic nanoparticles were incorporated into poly-L-lactic acid (PLLA) nanoparticles in order to position the NGF-cNPs in a culture dish. Neurites growing from DRG extended toward the NGF released from the NGF-cNPs. DRG were then cultured on aligned PLLA microfibers in the presence of NGF-cNPs, and these biomaterials combined to align DRG neurite extension along one axis and preferentially toward the NGF-cNPs. This combinatorial biomaterial approach shows promise as a strategy to direct the extension of regenerating neurites.



KEYWORDS: *Poly-L-lactic acid, magnetic nanoparticle, electrospun fiber, nerve growth factor, neurite extension*

Spinal cord injury (SCI) results in debilitating loss of function, caused by the disruption of axonal tracts in the spinal cord that are necessary for sensory and motor functions.[1] Damaged neurons adjacent to the lesion site experience axonal dieback, and when these axons recover and attempt to traverse the lesion site they are stalled by several inhibitory mechanisms.[2−5] These mechanisms include, but are not limited to, interactions with immune cells and their excreted factors,[5] inhibitory changes to the extracellular matrix (ECM), such as an upregulation of proteoglycans,[2−5] formation of a glial scar,[2−4] and a lack of guidance for regenerating axons.[2] The complexity of the injury environment has led to the development of several approaches to counteract the above mechanisms including an enzyme to digest glycosaminoglycans within the inhibitory ECM,[6] therapeutics that block neural receptors to inhibitory ECM glycosaminoglycans,[7] administration of growth factors in the injury site,[8,9] microtubule stabilization using taxol,[10] and peripheral nerve grafts that traverse the lesion site.[11,12] While the above studies demonstrate the capability of different approaches to enhance axonal regeneration and foster functional recovery, there is no approach to guide axonal regeneration to appropriate synaptic targets. Such a strategy has the potential to fully reinnervate the injured spinal cord and improve functional outcomes following SCI.

Chemotropic guidance of neurite extension was originally proposed by Ramón y Cajal nearly a century ago,[13] and has been investigated to discern the molecular mechanisms responsible for axonal guidance. Gundersen and Barrett first demonstrated the ability of nerve growth factor (NGF) gradients to chemoattract neurites extending from dorsal root ganglion neurons in 1979,[14] and since then the responsiveness of neurite extension to different chemicals including neurotransmitters,[15,16] cyclic AMP,[17,18] and calcium[19,20] has been studied. Chemotropic guidance also supports directed axonal extension in vivo, specifically when neurotrophin-3 gradients have been established using lentiviruses.[21,22] This regeneration strategy has enhanced extension of axons through a site of SCI[21] and has been used to form synapses to reinnervate neurons in the brain stem.[22]

Topographical guidance of neurite extension is being studied extensively due to recent advances in the development of tissue-engineering biomaterials.[23−25] It is well-known that nano- and microtopographies have the ability to direct the growth of extending neurites,[26−36] and aligned biomaterial scaffolds can induce directed axonal regeneration following both SCI[37−43] and sciatic nerve injury.[41−43] These studies demonstrate that chemotropic gradients and aligned top-

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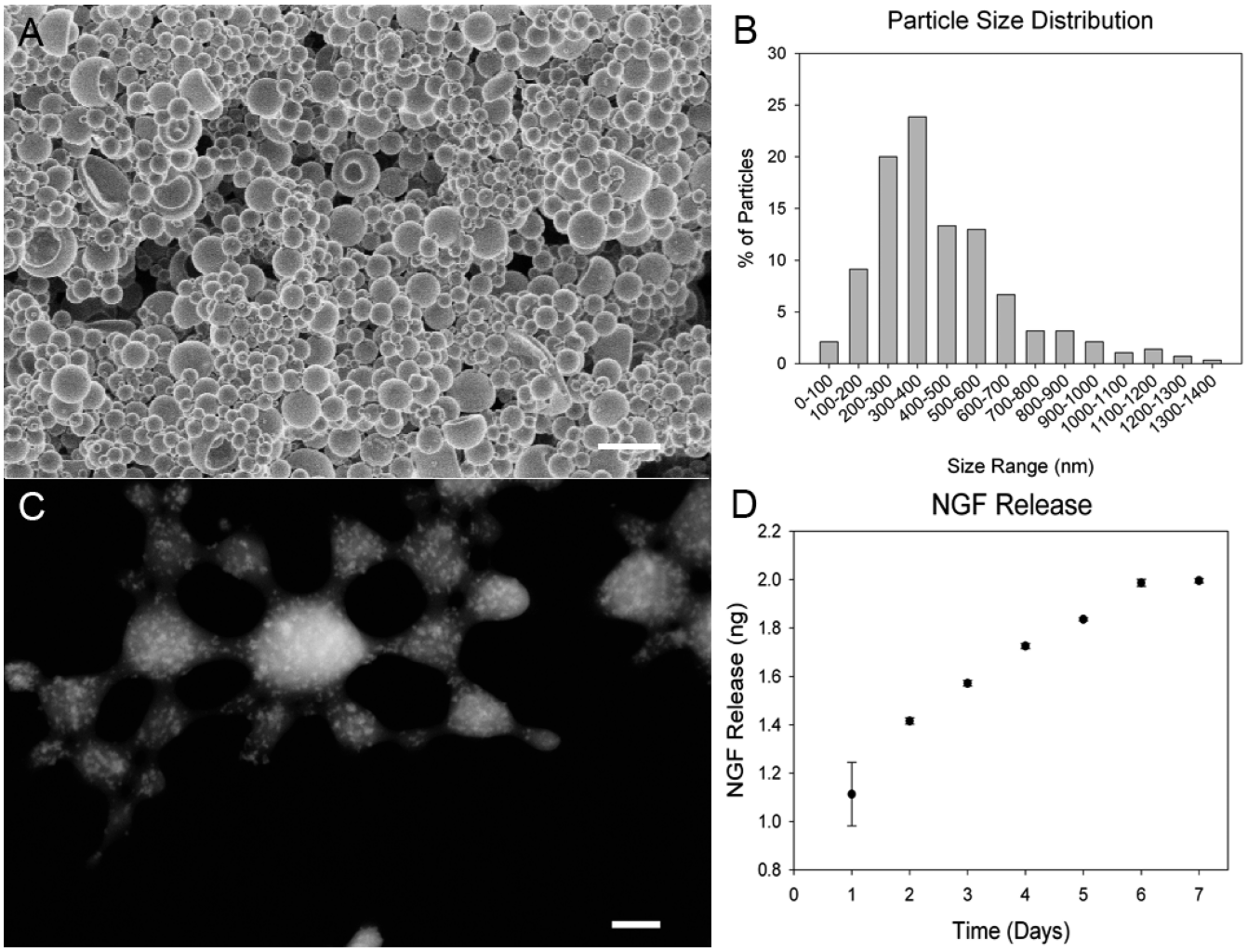

Figure 1. cNP characterization. (A) cNPs were fabricated by a water/oil/water double emulsion method, and the scanning electron micrograph depicts the spherical morphology and size distribution of the cNPs. (B) The cNPs had a large size distribution; however, nearly 80% of the cNPs fell within 200−600 nm. (C) Oleic acid coated MNPs were incorporated into the PLLA NPs, and the focused ion beam scanning electron micrograph image shows the incorporated MNPs. The MNPs are conductive, so they appear brighter than the PLLA in the micrograph. (D) cNPs loaded with NGF show release profiles over 6 days. There is a burst release over the first 24 h (1.1 ng), followed by less NGF released over the second 24 h (0.3 ng), and then a linear release curve over days 3−6 (~0.15 ng/day). (A) Scale = 1 μm; (C) scale = 200 nm.

ography are potent mechanisms that can be utilized both in vitro to direct extending neurites and in vivo to encourage directed axonal regeneration. Therefore, we created a combinatorial biomaterial approach to exploit the ability of both soluble chemotropic factors and aligned microtopography to direct neurite outgrowth.

We developed a movable composite nanoparticle (cNP) to create NGF gradients at specific locations within a tissue culture dish to direct neurite extension. The cNPs were fabricated by combining NGF-releasing poly-L-lactic acid (PLLA) nanoparticles with magnetic iron oxide nanoparticles (MNPs) through the modification of water/oil/water double emulsion techniques.[44,45] The particle diameter distribution of the composite particles ranged from >100 to 1400 nm, but the diameter of nearly 80% of the particles was between 200 and 600 nm (Figure 1A,B). Focused ion beam scanning electron microscopy was used to verify the presence of MNPs (10−15 nm) within the bulk of the PLLA nanoparticles. The bright areas within the cNPs (Figure 1C) reveal the location of the MNPs in the bulk PLLA nanoparticles. The ability of external magnetic fields to move the cNPs was then determined. The cNPs responded almost instantaneously to an external magnetic field (BH_{max} = 48 MGOe) when the cNPs were suspended in saline (Supporting Information Movie 1). To demonstrate that the cNPs could move in an environment with mechanical properties similar to tissue of the central nervous system (CNS) (modulus ranges from 0.2 to 1 kPa[46]), the cNPs were suspended in a 2% (wt/wt) agarose hydrogel (a shear modulus of 1.2 kPa[47]), which closely mimics the CNS mechanical environment. The cNPs were directed by the same external magnetic field through the 2% agarose hydrogel, but at a much slower rate (~3.2 mm/min) than their movement in saline (Supporting Information Movie 2). There are particles in both experiments that appear to lag behind the leading particles, and this is assumed to be due to both cNP size and the density of iron oxide in the PLLA nanoparticle bulk (more iron oxide will increase the response of the cNPs). This experiment suggests that the cNPs have the potential to be manipulated in vivo through administration of an external magnetic field gradient. The particles can therefore be moved over the course of the therapy, providing an approach to alter the location of the NGF gradient externally.

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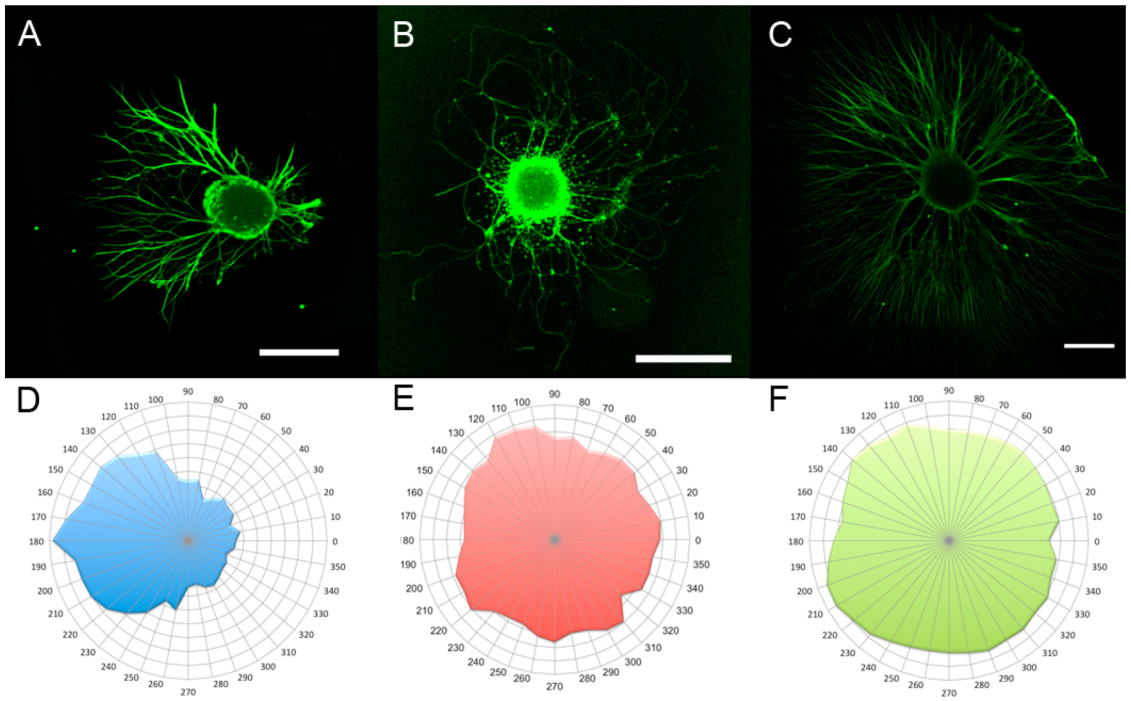

Figure 2. NGF gradients generated by cNPs direct the growth of extending neurites from DRG. (A–C) E9 chick DRG were subjected to (A) NGF-cNPs, (B) cNPs, and (C) NGF containing medium. (A) NGF-cNPs directed the extension of neurites from DRG. (B) Control cNPs had no effect on the directional outgrowth of extending neurites, and (C) NGF containing medium did not impart a growth preference on neurites extending from DRG. (D–F) Polar histograms of neurites extending from DRG cultured with (d) NGF-cNPs, (e) cNPs, and (f) NGF containing medium depict the normalized directional growth of neurites. (d) Polar histogram of neurites cultured with NGF-cNPs shows substantially longer neurite outgrowth toward the particles (130–230°) compared to neurite extension in all other directions. However, (E, F) DRG cultured with (E) cNPs and (F) NGF containg medium show no preferential growth in any direction around the polar histograms. (A–C) Green = RT-97; scale = 500 μm.

Following diameter characterization and verification of cNP responsiveness to an external magnetic field gradient, the release of NGF from 3 mg of cNPs in 0.5 mL of PBS (to ensure adequate NGF release for detection) was measured using an NGF ELISA. There was an initial burst release of NGF, with 1.1 ng NGF released in the first 24 h of the study (Figure 1D), followed by 0.3 ng released from 24 to 48 h. The release then became linear, with about 0.15 ng of NGF released in each of the next 4 days. The release levels off at day 7, suggesting that release from the cNPs lasts for 6 days. The EC_{50} of NGF to initiate neurite outgrowth is in the range of 0.1–30 ng/mL.[48] Thus, the amount of released NGF was within the range necessary to promote directed neurite outgrowth. Conditions such as NGF loading concentration could be further optimized if extended release or higher concentrations of NGF are desired. For this study, the necessary concentrations and time of release were suitable to induce a response from DRG neurons. We then determined the ability of the cNPs to direct neurite outgrowth from E9 chick dorsal root ganglia (DRG). DRG were cultured with NGF-cNPs held 5 mm away from the body of the DRG for 48 h. The 5 mm distance was chosen because it was the shortest distance that could be measured with accuracy in our culture system. cNPs without NGF were used as the negative control, and NGF containing (3 ng/mL) serum free Neurobasal media was used as the positive control. The concentration of 3 ng/mL was chosen because the cNPs release ~1.5 ng of NGF over the 48 h culture period, and the DRG were cultured in 500 μL of media (1.5 ng/0.5 mL = 3 ng/mL, a concentration known to promote a response in neurons[48]). Polar histograms of DRG neurite outgrowth were created using a modified protocol from previous studies,[49,50] and the images of the DRG cultured with cNPs were all positioned so that the center of the cNPs was to the left of the DRG (@ 180° on the polar histograms). DRG demonstrated robust neurite extension preferentially in the direction of the NGF-cNPs (Figure 2A,D). DRG in negative control cultures (+cNPs, −NGF) extended neurites without any preferential alignment (Figure 2B,E), and the outgrowth was not nearly as robust as that of the neurites in the experimental or positive control cultures (Figure 2A–C). The lack of NGF in the culture media restricts the number of extending neurites and the length of those extending neurites. The positive control cultures (+3 ng/mL NGF, −cNPs) show DRG with robust neurite extension without any preferential alignment (Figure 2C,F). This is the first time to our knowledge that DRG neurite extension has been directed by a soluble gradient of NGF released from magnetic cNPs.

NGF-cNPs were then combined with aligned PLLA microfibers to create a biomaterial approach that utilizes both topography and chemotropic gradients to direct extending neurites. We have previously shown that aligned PLLA microfibers direct neurite outgrowth and align astrocytes, endothelial cells, and macrophages.[26,51−54] Neurites from DRG extending on aligned fibers are not only directed parallel to fiber alignment, but they extend farther than if they are cultured on flat or unaligned surface topographies.[37] Therefore, we combined the NGF-cNPs with aligned PLLA microfibers in an attempt to induce directed neurite outgrowth preferentially on one side of the DRG explant to demonstrate the ability of a combinatorial biomaterial technology to alter neurite extension. Electrospun PLLA microfibers were fabricated as previously described.[26] The fibers used in these experiments had an

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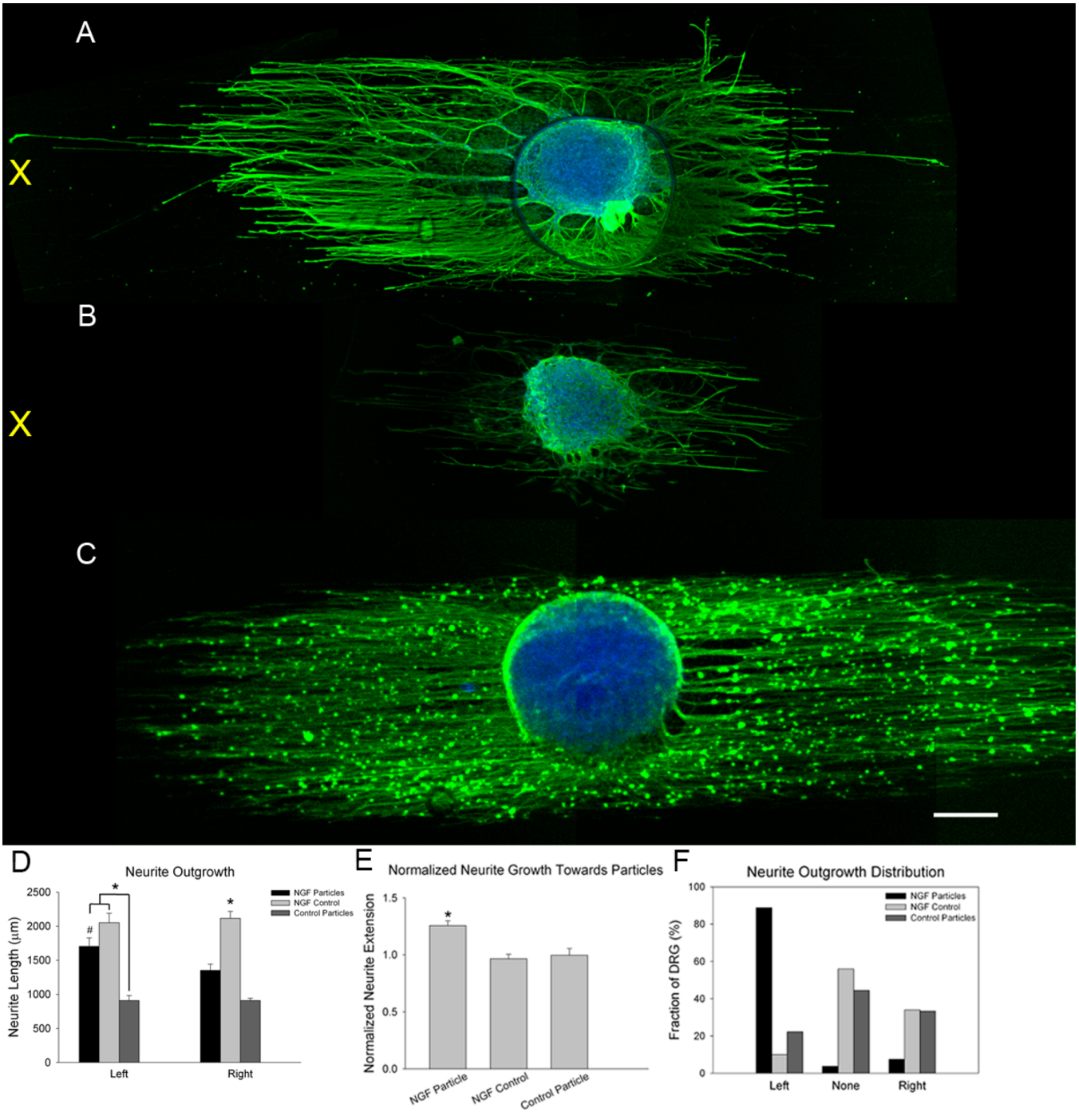

Figure 3. Aligned neurite extension from DRG cultured on PLLA microfibers is preferentially directed by NGF-cNPs. (A–C) E9 chick DRG cultured on PLLA microfibers were subjected to (A) NGF-cNPs, (B) cNPs, and (C) NGF containing medium. (A) Neurites extending from DRG cultured on the aligned microfibers preferentially grew longer in the direction of the NGF-cNPs than away from the NGF-cNPs, while neurites extending from DRG cultured on aligned microfibers in the presence of (B) cNPs and (C) NGF containing medium. (D–F) Analysis of neurite outgrowth determined that neurites extend significantly farther toward NGF-cNPs than they away from NGF-cNPs. This was shown by determining (D) total neurite outgrowth, (E) normalized neurite outgrowth, and (F) the fraction of DRG that exhibit significantly longer neurites growing toward the NGF-cNP side of the DRG. (A,B) X denotes direction of cNPs. (A–C) Green = RT-97; blue = DAPI; scale = 500 μm. (D–F) *Significant differences between experimental groups; #significant differences within experimental groups ($p < 0.05$).

average diameter of 2.42 ± 0.41 μm (previously shown to direct extending neurites from chick DRG[52]), had a density of 127 fibers/mm, and were preferentially aligned, with no fibers being more than 12° away from the median angle of alignment (Supporting Information Figure 1). E9 chick DRG were then cultured on the aligned fiber scaffolds for 48 h, with the same groups that were used previously (NGF-cNPs, cNPs, NGF control). The PLLA fibers direct the outgrowth of extending neurites, aligning them in the direction of fiber orientation (Figure 3A–C). When NGF-cNPs are placed 5 mm away from the body of the DRG, the neurites extending toward the NGF gradient grow farther (1703 ± 125 μm) than the neurites

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extending away from the NGF-cNPs (1353 ± 90 μm) (Figure 3A,D). Extending neurites show no preferential growth direction when cultured with control cNPs (toward, 910 ± 72 μm; away, 909 ± 34 μm) (Figure 3B,D) or in the NGF control cultures (toward, 2051 ± 140 μm; away, 2116 ± 102 μm) (Figure 3C,D). Again, NGF inclusion in the cultures promotes qualitatively more robust neurite extension (Figure 3C), while the DRG in the cNP control cultures show limited neurite outgrowth (Figure 3B,D). Neurites extending to the left (toward the particles) in both the NGF-cNP and the NGF control cultures were significantly longer than the neurites extending from the cNP control DRG (Figure 3D). DRG cultured in NGF control medium showed similar outgrowth to the left of the DRG with the NGF-cNPs since the control DRG were activated at the onset of the experiment. Even though NGF gradients direct neurite outgrowth, the stimulation for the duration of the experiment was enough to induce neurite extension similar to the gradient produced by the NGF-cNPs. Neurite extension to the right of the DRG (away from the particles) is significantly longer in the NGF control cultures than both the NGF cNP and control cNP cultures (Figure 3D). The NGF control and cNP control groups show no preferential outgrowth in either direction, while the DRG cultured with NGF-cNPs are the only DRG explants that demonstrate preferential growth in one direction (toward the cNPs) (Figure 3D). This was further characterized by analyzing normalized neurite extension, where the NGF-cNP group showed significantly more neurite extension toward the particles (1.26×) while neither of the control groups had a significant difference in normalized neurite extension (Figure 3E). The distribution of DRG that showed preferential growth was also characterized, and in the NGF-cNP cultures 90% of DRG extended significantly longer neurites to the left, 3% showed no significant differences, and 7% had significantly longer neurite extension to the right (Figure 3F). The DRG cultured with control cNPs had 22% of DRG with significantly longer neurites to the left, 44% showed no preferences, and 34% had significantly longer neurites extending to the right (Figure 3F). The DRG cultured with NGF and no cNPs had 11% of DRG with significantly longer neurite extension to the left, 56% showed no significant difference, and 34% had significantly longer neurite extension to the right (Figure 3f). Taken together, these results demonstrate that neurites extending along aligned PLLA microfibers can be preferentially directed toward an NGF gradient using magnetic, NGF-releasing PLLA/iron oxide cNPs.

It has been previously shown that neurites from DRG extend farther when cultured on PLLA fibers than compared to DRG cultured on flat surface controls.[37] This study also demonstrated this, where the longest neurites seen for NGF-cNP, cNP, and NGF cultures were 831, 625, and 1274 μm, respectively. Comparing these numbers to the average longest neurites of the DRG cultured on the microfibers shows that the fibers themselves play a larger role in the distance extended than the NGF gradient. The longest neurite in the NGF-cNP cultures was 831 μm (which was toward the NGF gradient), while neurites extending toward the NGF gradients on PLLA fibers were seen to extend up to 3000 μm. However, the NGF gradient created by the NGF-cNPs could be used on its own to direct neurite extension in instances where the use of PLLA fibers may be unwarranted (mild trauma to CNS), and the combinatorial approach provides two growth mechanisms to induce neurons through areas that are known to restrict growth (such as the glial scar).

This study is the first to develop magnetic NGF-cNPs to direct the extension of neurites growing from DRG explants. Several other studies have demonstrated release of growth factors from nano- and microparticles,[55−58] but only one utilized magnetic fields to manipulate particles.[59] Alginate/magnetite microparticles loaded with NGF have previously been used to examine the importance of distance the NGF gradient is from PC12 cells.[59] This study demonstrates the potential for magnetic microparticles releasing NGF to affect neuron-like cells. The current study expands on these findings by incorporating NGF into magnetic nanoparticles while also directing primary neuron neurite outgrowth. Development of NGF-conjugated iron oxide nanoparticles has previously been studied, and these materials promote differentiation and outgrowth of PC12 cells,[60] as well as enhancing neural fiber sprouting.[61] NGF-conjugated iron oxide nanoparticles that are incorporated into the cell through the TrkA receptor can be used to mechanically stretch neurites using an applied magnetic field, inducing neurite extension in a particular direction.[62] Facilitating neurite extension through the use of internalized magnetic particles is different from the chemotropic mechanism presented here, but again demonstrates the potential of nanoparticles in directing neurite extension.

The NGF-cNPs were combined with aligned PLLA microfibers in order to determine if these two biomaterials could work in unison to direct extending neurites from DRG explants. This combinatorial biomaterial was developed to entice neurites extending from one side of the DRG to grow longer than the neurites growing in the opposite direction. Several impactful studies have utilized both chemical and topographical guidance cues to determine cellular responses to the presentation of multiple guidance mechanisms. Neurite extension in hydrogels engineered with differing RGD ligand densities has been studied in the presence of NGF gradients, showing that the density of the binding sites for neurons is important for the sensitivity of neurite turning toward NGF gradients.[63] Surface attached poly-L-lysine patterns and topographic guidance channels were combined to study cytoarchitectural polarization in primary hippocampal neurons.[64] Microfluidic-created vascular endothelial growth factor gradients were combined with aligned electrospun fibers, and these experiments showed that gradients created parallel to fiber alignment increased the persistence time of the migration of human umbilical vein endothelial cells.[65] These studies used innovative techniques to demonstrate the importance of both topography and chemical factors in directing cellular migration. Our results expand on these previous studies by combining chemical gradients with topological structures to guide neurite extension along one axis and preferentially in one direction.

In conclusion, we have demonstrated that magnetic, NGF-releasing PLLA/iron oxide cNPs can direct neurite outgrowth. The cNPs have diameters in the range of hundreds of nanometers, can be positioned by external magnetic field gradients, and release NGF for up to 6 days. When cNPS are held in position with an external magnetic field, the NGF released from the cNPs can direct the extension of growing neurites from DRG explants. Furthermore, neurites extending from DRG explants cultured on aligned PLLA microfibers can be directed to preferentially grow in one direction along the oriented fibers. These two technologies have the potential to be combined in the future, where they could be used in an in vivo

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strategy to entice directed neurite regeneration following nervous system injury.

METHODS

Fabrication of NGF-Releasing PLLA/Iron Oxide cNPs. The procedure to fabricate NGF-releasing PLLA/iron oxide cNPs was adapted from two previous water/oil/water (w/o/w) NP fabrication methods.[44,45] PLLA (Natureworks; grade 6201D) was dissolved in dichloromethane at a concentration of 12.5 mg/mL. Next, 10 mg of oleic acid coated iron oxide nanoparticles (10−15 nm) (fabricated as previously described[66,67]) and 100 μL of SPAN-80 were then added to 3 mL of the PLLA solution and vortexed to disperse the particles. In a new vial, 4.26 mL of dH_2O, 5 mL of glycerin, and 740 μL of Tween-80 were mixed thoroughly. Then 100 nanograms of NGF (2.5S NGF, Mouse maxillary Glands, EMD Millipore) in 300 μL of dH_2O was added to the polymer solution to form the first w/o emulsion, and the solution was sonicated for 20 s using an ultrasonic homogenizer (model 300 V/T, BioLogics, Inc.). The glycerine/dH_2O solution was then added to the emulsion and again sonicated for 20 s to finish the w/o/w emulsion. This emulsion was then stirred at room temperature for 12 h to evaporate the solvent, and the particles were collected by centrifugation and washed three times. Following the washes, the particles were resuspended in dH_2O and placed at −80 °C overnight (~16 h). The particles were then lyophilized and stored at −20 °C until use.

In Vitro NGF Release. The release of NGF from PLLA/iron oxide cNPs was determined according to the manufacturer's instructions using a mouse NGF/NGF beta ELISA kit (Boster Immunoleader). An amount of 3 mg of NGF releasing PLLA/iron oxide NPs was suspended in 500 μL of phosphate buffered saline (PBS) and placed in an incubator at 37 °C. Following 24 h of incubation, the particles were spun down using a centrifuge and the supernatant was removed for analysis. This procedure was carried out everyday for 7 days. The data represents the average of three independent batches of NGF releasing PLLA/iron oxide cNPs for each time point.

Isolation and Culture of Chick DRG. E9 chick DRG were isolated from the dorsal roots of the lumbar spinal cord of chicks and placed in Hank's balanced salt solution as previously described.[26] Next 12-well culture dishes were coated with 1 μg/mL poly-D-lysine and 20 ng/mL laminin (Sigma-Aldrich) for 2 h. The coating solution was removed, and the wells were then washed twice with dH_2O. A volume of 500 μL of neurobasal (NB) medium (Gibco) containing 2% (v/v) B-27 supplement, 1% (v/v) penicillin/streptomycin, and 0.5 mM L-glutamine was then added to the wells. The DRG were cleaned of all tissue debris and added to the culture wells. DRG adhered for 12 h, and then three different experimental conditions were applied. DRG were subjected to a 3 ng/mL concentration of NGF for the positive control group, 3 mg of PLLA/iron oxide cNPs without NGF for the negative control group, and 3 mg of NGF-releasing PLLA/iron oxide cNPs for the experimental group. The NPs were held in place 5 mm away from the DRG using a 1/2 in. neodymium magnet (BH_{max} = 48 MGOe) (Applied Magnets). DRG were cultured for 48 h following particle or NGF incorporation after which they were fixed, stained, and imaged. For PLLA microfiber cultures, the fibers were coated with 1 μg/mL poly-D-lysine for 2 h and then washed twice with dH_2O. Next 30 μL of NB was placed on the fibers and the DRG were allowed to adhere for 12 h in this small volume of media.

Immunocytochemistry. DRG were fixed in 4% paraformaldehyde and then blocked and permeabilized with 0.4% Triton-X and 5% bovine serum albumin in PBS. Neurons were stained against RT97 neurofilament primary antibody (Developmental Studies Hybridoma Bank) at a concentration of 1:100. DAPI (Sigma-Aldrich) was used at a concentration of 1 μg/mL to stain nuclei. The secondary antibodies used were Alexa-Fluor anti-mouse 488 (1:1000, Invitrogen).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00189.

Additional methods; characterization of aligned PLLA microfiber scaffolds (PDF)

Composite PLLA/Fe_3O_4 nanoparticle movement in saline buffer (AVI)

Composite PLLA/Fe_3O_4 nanoparticle movement through 2% agarose hydrogel (AVI)

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Notes

The authors declare no competing financial interest.

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