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***In vitro* and *In vivo* Release of Nerve Growth Factor from Biodegradable Poly-Lactic-Co-Glycolic-Acid Microspheres**

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Abstract

Regeneration of peripheral nerves after injury is suboptimal. We now report the long term delivery of nerve growth factor (NGF) by biodegradable poly-lactic-co-glycolic acid (PLGA) microspheres *in vitro* and *in vivo*. Lactic to glycolic acid ratios of 50:50 and 85:15 were fabricated using the double emulsion solvent, evaporation technique. Three different inherent viscosities (0.1dL/g: 1A, 0.4dL/g: 4A, 0.7dL/g: 7A) were analyzed. *In vitro*, release of NGF for 23 days was measured. Electron microscopy demonstrated intact spheres for at least 7 days (50:50 1A), 14 days (50:50 4A) or 35 days (50:50 7A and 85:15 7A). *In vitro* release kinetics were characterized by burst release, followed by release of NGF at a rate of 0.6%-1.6% a day. Release curves for 50:50 1A and 85:15 7A differed significantly from other compositions ($p < 0.01$). *In vivo*, release was characterized by a novel radionuclide tracking assay. Release rates varied from 0.9%-2.2% per day with linear kinetics. All but the 85:15 type of spheres showed different release profiles *in vivo* compared to *in vitro* conditions. Based on the surface morphology and release profiles we found microspheres fabricated from 50:50 4A PLGA to be best suited for the use in a rat sciatic nerve injury model.

Keywords

Nerve Growth Factor; Microspheres; Peripheral Nerve; Poly-lactic-co-glycolic-acid; Dorsal root ganglia

Introduction

Traumatic peripheral nerve injury may lead to a gap between nerve ends at a time of repair or reconstruction. When direct coaptation is not feasible, the current gold standard is the interposition of an autologous nerve graft. This has numerous disadvantages, such as graft availability, donor site morbidity, and size mismatch. The most important problem of grafting is that nerve regeneration is never complete. Over the past decades the development of artificial nerve conduits has therefore been of great interest. So far, the use of these conduits does not lead to results comparable to autologous grafting, let alone pre-injury

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levels. A variety of methods have been used to improve upon nerve regeneration. One of many options is the incorporation of growth factors within the conduit.

NGF has a potent effect on sensory and sympathetic neurons. It is readily available and relatively inexpensive. Direct injection of NGF, as well as other growth factors, into the lumen of the conduit has been shown to be beneficial to nerve regeneration.¹⁻³ Prolonged availability of NGF after injury might further enhance nerve regeneration. Barriers to the use of NGF or other neurotrophic polypeptides include the short half life and *in vivo* availability. The development and characterization of a long-term delivery system would potentially be of great value. One possibility is the introduction of microspheres as a source of growth factors into a single lumen nerve conduit. A minimum essential requirement is for release to occur for a period of 4-8 weeks during active regeneration in animal nerve injury models.

PLGA is a biodegradable polymer of which the degradation profile can be tailored.⁴ It is being used clinically for a variety of purposes; such as sutures and depot drug applications. It can be molded into many shapes, such as nerve conduits or microspheres. Microspheres have been used extensively in other studies, for delivery of proteins.⁵ When fabricated using a water-in-oil-in-water solvent evaporation technique, their release profile is characterized by an initial burst followed by a period of steady release. Microspheres fabricated as such show accurate release of biological active proteins *in vitro*.⁶ *In vivo* however, due to the alteration of micro-environment it is highly likely for release profiles to change. In addition, the biological activity of the released protein might decrease.

The aim of the current study was to define the type of microsphere best suited for use within a nerve conduit. Four types of PLGA microspheres were fabricated in order to study their *in vitro* and *in vivo* release. Radiolabeled NGF was incorporated in the spheres and the release medium was assessed weekly by gamma counting for a duration of 8 weeks. Scanning electron microscopy pictures were taken to assess the surface morphology of the spheres and thus their integrity. DRG bioassays were performed to verify biological activity of released NGF. For the *in vivo* part of our study, we subcutaneously implanted nerve conduits filled with microspheres and measured radioactivity using scintillation probes.

Methods

NGF radiolabeling

2.5S Nerve growth factor (Harlan Bioproducts for Science, Inc., Madison, WI) was labeled with iodine-125 (Perkin Elmer, Boston, MA) using the chloramine T method.⁷ Briefly, 8μl of iodine-125 (1mCi) was added to 100μl of 2mg/ml NGF solution in PBS. Next, 100μl of 1mg/ml chloramine T (Fisher Scientific, Pittsburgh, PA) was added to start the reaction. After two minutes, 100μl of 5mg/ml sodium metabisulfate (Fisher Scientific) was used to stop the labeling. The radiolabeled NGF was then transferred into a dialysis tube with a molecular weight cut off of 10kDa and dialysed for 24 hours in TBS, while refreshing the buffer every 8 hours. Last, the labeled NGF was collected for further use.

Microsphere fabrication

A water-in-oil-in-water (w-o-w) double emulsion, solvent evaporation technique was used for microsphere fabrication.⁸ Four different types of PLGA were used: PLGA with a lactic to glycolic acid ratio of 50:50 with inherent viscosities of 0.1dL/g (50:50 1A), 0.4dL/g (50:50 4A) and 0.7dL/g (50:50 7A) and PLGA with a lactic to glycolic acid ratio of 85:15, with an inherent viscosity of 0.7dL/g. For each type of sphere three different batches were created and used for subsequent characterization. Briefly, 500 mg of PLGA (Lakeshore Biomaterials, Birmingham, AL) was dissolved in 1.25 mL of dichloromethane (Fisher Scientific) in a glass test tube. While continuously vortexing (Vortex Genie, Fisher

Scientific) at 3000 rpm, 100 µl of 2mg/mL 2.5S NGF (Harlan Bioproducts for Science, Indianapolis, IN) was added to create the first emulsion. After 30 seconds, 2mL of aqueous 2% w/v PVA 87-89% hydrolyzed (Sigma, St. Louis, MO) was added to form the double emulsion. This was then poured into a 600mL beaker with 100 mL of 0.3% w/v PVA and placed on a stirplate (Fisher Scientific). After 1 min, 100mL of 2% v/v isopropylalcohol (Sigma, St. Louis, MO) was added and the mixture was continuously stirred with a magnetic stirbar for 1 hour. The microspheres were then collected by centrifugation, washed twice with distilled, deionized water and finally vacuum-dried (Virtis freeze mobile, Virtis Company, NY) at less than 500 mThor.

In vitro

Encapsulation efficiency: Prior to encapsulation in microspheres, activity of NGF-I¹²⁵ samples was measured using a Wallac Wizard 1480 gamma counter (Perkin Elmer Life and Analytical Sciences, Bridgeport, CT). Directly after microsphere fabrication, activity was measured again and the encapsulation efficiency in percentage was calculated as follows:

$$(\text{measured activity/theoretical activity}) * 100$$

In which the measured activity is the NGF-I¹²⁵ activity after sphere fabrication and the theoretical activity the NGF-I¹²⁵ sample activity prior to encapsulation.

Surface morphology and microsphere size distribution: Surface morphology was evaluated after one day and at weekly intervals for a duration of eight weeks. Before analysis, 30mg of microspheres were placed in 1.5mL microcentrifuge tubes containing 1mL of distilled water and 0.01% sodium azide (Sigma). At each timepoint, 10µl of microsphere solution was collected and placed on a metal stub. Next, this stub was palladium sputter coated using a Bio-Rad/Polaron E5400 High Resolution Sputter Coater (Biorad/Polaron, Cambridge, MA). Pictures were taken at various timepoints using a Hitachi S-4700 Cold Field-Emission Scanning Electron Microscope (Hitachi Instruments Inc., San Jose, CA). Microsphere size distribution was determined at baseline by measuring the diameter of 600 randomly selected microspheres of each type of polymer using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2000).

Release kinetics: Corning transwells (Fisher Scientific) with a pore size of 0.4µm were filled with 50mg of microspheres encapsulating radiolabeled NGF and placed in 12 well plates (Corning, Inc., Corning, NY). Each transwell contained 500µl of 0.1% BSA in PBS and each well 1.5mL of the same solution. Measurements of released radioactivity were taken at baseline, day 1, day 7 and weekly intervals thereafter. At each timepoint, transwells were transferred into a new 12-well plate with 1.5mL of 0.1% BSA in PBS. In addition, all samples were stored at -80°C for later analysis of bioactivity of released NGF.

DRG Bioassay: Bioactivity of released NGF was assessed using a rat DRG bioassay. Embryonic DRG explants were harvested from a time pregnant embryonic day 15 Sprague Dawley rat. Next, explants were placed in collagen coated 12-well plates (Corning) and cultured in DMEM with 10% FBS (Gibco, Carlsbad, CA), with the addition of a titration series of microsphere released NGF at 37°C, 5% CO₂. NGF at a concentration of 10ng/ml and 0.1ng/ml was added as a positive and negative control respectively. After 48 hours, digital pictures were acquired using a Nikon Digital Sight DS-5M camera (Nikon Corporation, Tokyo, Japan) attached to a Zeiss Axiovert 35 inverted microscope (Zeiss, Munich, Germany) and neurite extension was measured using ImageJ (National Institutes of Health, USA) software.

In vivo

Conduit fabrication: A solvent evaporation, injection molding technique was used to fabricate a conduit.⁹ First, 400mg of 85:15 DL high i.v. (Lakeshore Biomaterials, Birmingham, AL) was dissolved in 600 μ L of dichloromethane (Fisher Scientific) in a 3mL syringe (Becton Dickinson & Co., Franklin Lakes, NJ). After shaking for 3 hours (Super Mixer II, Lab-Line Instruments Inc., Melrose Park, IL), the polymer solution was injected into a teflon mold, resulting in a cylindrical conduit with an inner and outer diameter of 1.6mm and 2.2mm respectively. After overnight vacuum-drying, conduits were carefully removed from the mold and cut to the appropriate length of 12mm. Directly prior to surgery, tubes were loaded with 100mg/ml of the four different types of microspheres in PBS and both ends were sealed with bone wax (Ethicon, Inc., Somerville, NJ).

Conduit implantation: Twelve female Sprague Dawley rats weighing 200-250grams (Harlan Sprague Dawley, Indianapolis, IN) were used for this study. Procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Mayo Clinic, Rochester, Minnesota. Animals were housed in 12-hour light and dark cycles, with chow and water available *ad libitum*. Prior to surgery, rats were anesthetized using a mixture of xylazine 5 mg/kg (Lloyd laboratories, Shenandoah, IA) and ketamine 80mg/kg (Fort Dodge, IA), i.p. Procedures were performed under aseptic conditions. A 5mm skin incision was made in the distal portion of each four limbs and the skin was undermined, creating a subcutaneous pocket. Subsequently, one conduit was implanted herein. Therefore, each animal had one different conduit in every limb. The skin was closed using 6-0 ethilon sutures (Ethicon, Inc., Somerville, NJ).

In vivo release kinetics: *In vivo* radioactivity measurements were taken using three sodium iodide probes (Model 44-3, Ludlum measurements, Inc., Sweetwater, TX) connected to scalers (Model 1000, Ludlum measurements, Inc., Sweetwater, TX) (figure 1). Scalers and probes were calibrated beforehand. Small cylinders of lead tape were attached to the sodium iodide probes to prevent picking up radioactivity from samples in the other limbs. The distance of the probes to the sample were kept at 3cm, as previously described by Kempen et al.¹⁰ Right before measurements, rats were anesthetized and placed on a wooden board, with all four limbs spread out. Probes were placed over the subcutaneous tubes in the limbs and radioactivity was measured for 1 minute. Measurements were taken at baseline, day 7 and weekly intervals thereafter. One sample was measured by all three probes and values were averaged afterwards.

Statistical analysis: Statistical analysis was performed using the statistical software package Graphpad Prism version 4 (La Jolla, CA). One-way ANOVA was performed for comparison of encapsulation efficiency. A student T-test was used for comparison of neurite outgrowth in rat E15 DRG. Non linear and linear regression analyses were performed for analysis and comparison of both *in vitro* and *in vivo* release kinetics, respectively. Statistical significance was assumed at $p < 0.05$.

Results

Encapsulation efficiency

Encapsulation efficiency of NGF within the four different types of microspheres is shown in figure 4. Encapsulation efficiency was highest (74.6%) for a PLGA ratio of 50:50 1A, with an inherent viscosity of 0.1dL/g and was lower for all other types of polymer. Increasing the inherent viscosity resulted in a lowering of the encapsulation efficiency from 74.6% to 35.6%. Differences in ratio of lactic to glycolic acid did not change encapsulation efficiency substantially.

Microsphere size distribution and surface morphology

For all polymer types, 79%-98% of all spheres had a diameter of 59µm or smaller, with 57-63% being 19µm or smaller. The remaining portion was equally distributed in size and only showing a slightly higher percentage (11.6%) for microspheres from polymer type 50:50 4A for a diameter of <100µm.

Degradation profiles were assessed by SEM and are shown in figure 2. Microspheres degrade initially by pore and later dimple forming in the outer surface. This phase is followed by loss of the spherical appearance, caused by bulk degradation of the polymer. 50:50 1A microspheres showed the fastest degradation profile and were not visible after two weeks. 50:50 4A microspheres kept their integrity for over two weeks, while the other types remained present for at least 35 days.

***In vitro* release**—NGF was gradually released from all microspheres (figure 6). Release profiles were characterized by a burst release from loosely bound NGF during the first 24h. The amount of NGF released in the first day varied from 5.4% (85:15 7A) to 11.4% (50:50 4A). This phase was then followed by a period of continuous release. Depending on the microsphere type, release rates in the first 35 days varied between 0.6% and 1.6% of total per day for 85:15 7A and 50:50 1A respectively. Release curves were found to be statistically different from each other for all polymer types, except 50:50 4A and 50:50 7A. Based on these *in vitro* release kinetics as well as surface morphology, microspheres fabricated from PLGA 50:50 4A were found best suited for *in vivo* use.

DRG bioassay—Biological active NGF was released from microspheres fabricated from PLGA 50:50 4A (figure 5). Released NGF showed extension of neurites after day 8, 11, 14, 17, 20 and 23. A neurite outgrowth length of rat E15 dorsal root ganglion neurons of >900µm was seen in DRG neurons supplemented with microsphere released NGF, as compared to 440µm for negative controls ($p<0.01$).

***In vivo* release**—Two subcutaneous implanted conduits spontaneously explanted during the study and were excluded from analysis. *In vivo* release profiles showed a gradual release of NGF during a period of 5 weeks (figure 7). No initial burst release was observed in the early timepoints. Release kinetics were characterized by a more linear release profile. Release rates varied between 1% of total per day for microspheres fabricated from polymer type 85:15 7A and 2.2% of total per day for polymer type 50:50 1A.

Discussion

An important issue in peripheral nerve reconstruction and regeneration is bridging the nerve gap after nerve injury. Reconstruction using autologous nerve grafts still yields insufficient results. Various strategies are being developed to create an artificial nerve conduit which allows for regeneration superior to nerve grafting.¹¹ One of the options is to incorporate growth factors using a microsphere delivery system. Microspheres are successfully used for delivery of various proteins. The advantage of the application of a conduit with microspheres over various cell loaded conduits is the immediate off-the-shelf availability. However, prior to clinical application, the release kinetics of a sphere containing conduit should be known. It takes 2 weeks for outgrowing axons to enter the conduit and approximately 4 weeks to traverse a 1cm gap in the rat sciatic nerve. Therefore, we focused on designing a microsphere delivery system, capable of delivering active NGF for that amount of time. In addition, we know of no study investigating the *in vivo* release characteristics of NGF encapsulating PLGA microspheres. We studied the *in vitro* and *in*

vivo release characteristics of microspheres, fabricated from four different types of polymers.

PLGA was chosen for this study, considering its previous use in various forms: nanoparticles, microspheres and layered nerve conduits.¹²⁻¹⁴ Upon contact with water, microsphere degradation profiles are initially characterized by a burst release in the first 24 hours. This initial burst is mostly due to the immediate release of the loosely attached protein on the outer surface of the microspheres and to a lesser extent diffusion.¹⁵ A longer period of steady release follows. Water uptake by microspheres initiates hydrolysis of ester bonds within the microsphere matrix, resulting in degradation with time.

It is important to take fabrication parameters into account when microsphere delivery systems are designed. They allow for more accurate tailoring of the delivery system. Our study has focused on the effect of polymer monomer ratio, as well as molecular weight on encapsulation efficiency and release profiles.

We found lower molecular weight polymers to have higher encapsulation efficiencies. This could be explained by the direct interaction of the protein with the polymer. Low molecular weight polymers have smaller polymer chains. Therefore, they have a higher number of end groups compared to high molecular weight polymers, when using the same weight. In this study we used carboxylic end groups, allowing for the positively charged NGF to have an ionic interaction directly to negatively charged carboxylic groups. This is in accordance with previous findings by Blanco, in which the encapsulation efficiency of lysozyme preferentially increases when using low, compared to high molecular weight PLGA.¹⁶

A second explanation for our observations is, while using high molecular weight polymers, the viscosity of the organic phase will increase and make it more difficult for the aqueous phase to disperse evenly. On the other hand, lower molecular weight polymers cause a reduction in the viscosity of the organic phase, thereby facilitating emulsification of the W1 phase. Entrapment of more protein is the result, since less protein is lost due to diffusion to the W2 phase.⁸

Our *in vitro* study of four different types of spheres showed different degradation profiles, with protein release profiles between 0.6% and 1.6% of total per day for the first 35 days. Differences in release kinetics were shown to be dependent on polymer composition and molecular weight. Faster degradation rates were found when the glycolic acid composition of the polymer was increased. The hydrophilicity of glycolic acid causes more water uptake and thus faster degradation.¹⁷ In addition, we found the lowering of the inherent viscosity of the polymer to increase release rates. Again, lower viscosities are created by using lower molecular weight polymers. In their turn, lower molecular weight polymers need less time to degrade into smaller oligomers and monomers. This allows for more water uptake by the polymer matrix and thus for a faster release of NGF.¹⁸

Up to now, little is known about the actual *in vivo* release kinetics of NGF from PLGA microspheres. The *in vivo* part of our study demonstrated faster release (1.0% - 2.2% per day) curves for all types of microspheres compared to their *in vitro* counterparts (0.6% - 1.6% per day).

These findings indicate that extrapolation of *in vitro* kinetics to an *in vivo* situation would be inaccurate. Differences such as vascularization, the change in microenvironment, might play a role in altering the degradation profile of PLGA microspheres.

Nevertheless, subcutaneous implantation is the only approach that can mimic an *in vivo* situation with the possibility to measure accurate radioactivity counts. Though, it would be

preferable to measure counts in a situation where conduit and microspheres are used in a nerve grafting model, for instance the sciatic nerve model in the rat. We, however, did not choose this option because of potential variability in probe distance and autotomy in a rat model.¹⁹ Therefore, we were unable to account for adverse *in vivo* effects specifically related to bridging a gap with a nerve conduit, such as leakage of microspheres from coaptation sites in our study. Previously, an *in vivo* non-invasive screening method for release profiles has been validated by Kempen et al.¹⁰ This method, using scintillation probes for radioactivity counts, is similar to the one used in our study. Their findings show that the shielding of scintillation probes and maintaining a similar distance to the source is essential. The next step in the development of a growth factor releasing microsphere delivery system would be to incorporate it into a nerve conduit. We have previously investigated the effect of single and multichannel PLGA nerve conduits on peripheral nerve regeneration.⁹ Presently, the effect of NGF-microspheres incorporated into a nerve conduit is being evaluated in a rat sciatic nerve injury model.

In conclusion, we have found *in vivo* degradation profiles of microspheres to significantly differ from the *in vitro* release kinetics. In addition, we have shown release of bioactive NGF over a period of 23 days. Release kinetics can be tailored by altering polymer composition and molecular weight. Based on these findings, in combination with surface morphology data, we have selected microspheres fabricated from PLGA 50:50 4A to be best suited for use in a rat sciatic nerve injury model. Currently, the evaluation of the effect of NGF microspheres on peripheral nerve regeneration is ongoing.

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Used abbreviations

NGF	Nerve Growth Factor
PLGA	Poly-lactic- <i>co</i> -glycolic acid
PBS	Phosphate Buffered Saline
TBS	Tris Buffered Saline
BSA	bovine serum albumin
PVA	polyvinyl alcohol
DRG	dorsal root ganglia
SEM	scanning electron microscopy

Figure 1. Setup

In vivo radioactivity counting setup: four scalers are connected to sodium iodide probes. For a duration of 1 minute, counts are measured by placing the probes at a 3cm distance from the skin.

Figure 2. SEM

Surface morphology of microspheres as assessed by SEM. Degradation of microspheres showing pore forming on the outer surface first. In a later stadium, dimples are formed before the spheres loose their integrity and disintegrate into smaller particles. Scalebar is 50 µm.

Figure 3. Size distribution

The diameter of 600 randomly selected microspheres was assessed directly after fabrication. More than half of all types of microspheres have a size smaller than 19μm, with most microspheres being smaller than 59μm in diameter.

Figure 4. Encapsulation efficiency

Higher encapsulation efficiencies are yielded by the use of lower molecular weight polymers. 50:50 1A is significantly different from other types of microspheres ($p < 0.0001$)

Figure 5. Neurite outgrowth

Bioactivity evaluation of microsphere delivered NGF. A rat E15 dorsal root ganglion bioassay was used to measure neurite outgrowth. At all timepoint, neurite extension was similar to the positive control (10ng/ml NGF) and different from the negative control (0.1ng/ml NGF) ($p < 0.0001$).

Figure 6. *In vitro* release

Release curves of 4 types of microspheres *in vitro*. Spheres were placed in a transwell. Radioactivity in the release medium was assessed at day 1 and at weekly intervals. All spheres show an initial burst release at day 1 and an exponential decay afterwards. Release between different spheres differed significantly ($p < 0.0001$), with 50:50 1A release at a rate of 1.6% per day, compared to 85:15 7A releasing NGF at a rate of 0.6% per day. Regression curves of one phase exponential decay are shown.

Figure 7. In vivo release

Release curves, measured using sodium iodide probes *in vivo*. Microspheres were subcutaneously implanted in a nerve conduit sealed with bonewax. All curves show a linear release profile. Release rates vary from 1.0% - 2.2%. Error bars are omitted for clarity.