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Research Paper

Using Polymer Chemistry to Modulate the Delivery of Neurotrophic Factors from Degradable Microspheres: Delivery of BDNF

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Purpose. Brain-derived neurotrophic factor (BDNF) plays an important role in neuroprotection and repair, but long-term delivery from polymer systems has been challenging. We investigated the role the chemistry of the polymer played in loading and delivery of BDNF via microspheres, which are suitable for minimally invasive administration.

Methods. We synthesized polymers based on PLGA and PEG to determine what components augmented loading and delivery. We characterized microspheres fabricated from these polymers using a battery of tests, including sizing, *in vitro* release, and bioactivity of the BDNF using PC12 cells engineered to express the trkB receptor.

Results. We found that a triblock polymer of PLGA, PLL, and PEG led to the delivery of BDNF for periods of time greater than 60 days and that the BDNF delivered was bioactive. The microsphere size was amendable to injection via a 30 gauge needle, allowing minimally invasive delivery.

Conclusions. PLGA-PLL-PEG leads to greater loading and longer-term delivery of BDNF than PLGA or a blend of the polymers. We hypothesize that the introduction of an amphiphilic PLGA-based polymer increases the interaction of the BDNF with the polymer and leads to release that more closely correlates with the degradation of the polymer.

KEY WORDS: BDNF; PEG; PLGA; microparticle; microsphere.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) has shown promise for promoting axon extension and guidance (1,2), neural stem cell differentiation (3,4), and cell survival in a variety of injuries to the central nervous system (CNS) (5,6). In each of these cases, long-term delivery of BDNF is necessary for a successful response. In some cases, beneficial effects of BDNF terminate with the end of the administration of the factor (7).

Systemic delivery of BDNF has been used in clinical trials using systemic delivery via subcutaneous injections, but daily injections are required, and while positive effects are seen, there are clear and significant side effects making sustained, local delivery a more attractive option to limit the side effects and avoid repeated injections (8). In the phase III clinical trial of BDNF administration intrathecally via a pump system, 25% of the patients reported significant bowel complications in the low dose group, and 47% reported complications in the high dose group (8). High serum levels of BDNF have also been correlated with metabolic syndrome, which includes high plasma triglycerides and insulin resistance (5). Increased levels of BDNF in serum are also correlated with a number of psychiatric disorders (6–8). While it is not clear that BDNF causes these conditions, it is concerning and supports the concept of developing sustained, localized delivery of BDNF.

Previously, long-term delivery has been achieved via delivery from pumps (1,2,7), cells (9), polymer rods (6), scaffolds (10), and microspheres (11). Pumps can be cumbersome to implant. Cellular delivery raises concerns regarding the immunogenicity of the cells as well as the control over the up- and down-regulation of the factor, although the latter concern has been addressed in part by the incorporation of genes for the factor of interest. The polymeric delivery via rods, scaffolds and microspheres has been reported to be relatively short (7–14 days) (3,12). Hydrogels have been shown to deliver BDNF for slightly longer (3–4 weeks) (13,14), but longer delivery is desirable for neuroprotection.

We sought to investigate the parameters that impacted BDNF delivery via polymeric microspheres. The advantage of microspheres over other polymeric delivery systems is that they may be either injected directly (15) or combined with other polymer systems to create drug-delivery scaffolds (16,17). We found that both the double emulsion and spontaneous emulsion techniques led to an initial burst phase followed by very little subsequent release of the neurotrophin

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with a range of molecular weights of PLGA. By blending the PLGA with a graft copolymer of PLGA, polylysine and polyethylene glycol, we were able to deliver a therapeutically relevant amount of BDNF up to 65 days. This opens the possibility of delivery of BDNF for long times using minimally invasive techniques, which has applications in treating a number of neurodegenerative disorders and diseases and provides insight into the factors that impact BDNF delivery.

MATERIALS AND METHODS

Materials

Poly(lactic-*co*-glycolic acid) (PLGA; 50:50 lactic acid : glycolic acid) with carboxylic acid end groups was obtained from Boehringer Ingelheim GmbH (Ingelheim, Germany) in two molecular weights: M_n ~10 kDa and M_n ~25 kDa (502H and 503H, respectively). Polyvinyl alcohol (PVA; Mn ~25,000 Da, 88 mol% hydrolyzed) and monomethyl ether poly(ethylene glycol) (PEG; molecular weight ~1,900 Da) were obtained from Polysciences (Warrington, PA, USA). Poly(ε-carbobenzoxy-**L**-lysine) (M_w ~1,000 Da) was from Sigma-Aldrich (St. Louis, MO, USA). All cell culture media and supplements were obtained from Invitrogen (Carlsbad, California). PC12-trk B cells were a kind gift of Dr. Veronique Tran. Human recombinant brain-derived neurotrophic factor (BDNF) and the BDNF enzyme-linked immunosorbent assay (ELISA Duoset) kit were obtained from R&D Systems (Minneapolis, MN, USA). The Micro bicinchoninic (BCA) protein assay kit was from Pierce (Rockford, IL, USA). Deuterated dimethyl sulfoxide (D_6 -DMSO) was from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All other chemicals were obtained from Sigma-Aldrich and used as received.

Synthesis of PLGA-PLL-PEG

Poly(ε-carbobenzoxy-L-lysine) and PLGA were conjugated as described previously (Fig. 1) (18,19). Briefly, PLGA 503H and poly(ε-carbobenzoxy-L-lysine) (1:1 molar ratio) were dissolved in dimethyl formamide (DMF). Two molar equivalents (with respect to PLGA) of dicyclohexyl carbodiimide (DCC) and 0.1 molar equivalents of (dimethylamino-pyridine) DMAP were added, and the reaction was allowed to run for 36 h under argon. Following conjugation of PLGA and poly(ε-carbobenzoxy-L-lysine) (PLGA-PLL), the polymer solution was diluted with chloroform and filtered to remove N,N'-dicyclohexylurea (DCU), an insoluble by-product of the reaction. This PLGA-PLL copolymer was then precipitated in methanol, vacuum filtered to remove any unconjugated poly(ε-carbobenzoxy-L-lysine), and lyophilized. To expose the protected primary amines, 1.5 g of the copolymer were dissolved in hydrogen bromide (HBr), 30 wt% in acetic acid (HBr/HOAc) and allowed to stir. After 1.5 h, ether was added to the solution, and the precipitated polymer was removed. The polymer was washed with ether until an off-white brittle mass was obtained. The mass was then dissolved in chloroform, re-precipitated in ether and lyophilized for 48 h.

N,N'-Carbonyldiimidazole (CDI)-activated PEG was then conjugated to the exposed primary amines of the PLGA-PLL. The hydroxyl terminus of PEG was activated with CDI in dioxane as described previously (20). Briefly, PEG was dissolved in an excess of dioxane at 37°C. A 1:20 molar excess of CDI was added, and the resulting mixture was stirred under Argon for 2 h at 37°C. Unreacted CDI was removed by dialysis in deionized water for 12 h. The dialysate was changed every hour. The resulting solution was flash frozen in liquid nitrogen and lyophilized for 3 days. A mixture of excess activated PEG and PLGA-PLL (5:1 molar ratio PEG:PLGA-PLL) was dissolved in anhydrous DMF and allowed to stir under argon. After 48 h, the polymer solution was diluted with chloroform and precipitated in methanol. Unconjugated PEG is soluble in methanol and easily removed. Polymer dissolution and precipitation were repeated two times to ensure the removal of unconjugated PEG.

Characterization of PLGA-PLL-PEG

NMR

The successful conjugation of poly(ε-carbobenzoxy-L-lysine) and PLGA was determined as previously described (18,19). The successful conjugation of PEG to PLGA-PLL was determined using ^{1}H NMR. ^{1}H NMR spectra were recorded at room temperature in D_6 -DMSO on a 400 MHz Bruker (Germany) spectrometer and referenced to tetramethylsilane (TMS) peak ($\delta = 0.0$ ppm).

Contact Angle

Polymer wettability was characterized by measuring the water contact angle. Briefly, polymers, films were cast by dissolving the polymer in dichloromethane (DCM) (5% w/v). This polymer solution (0.2 ml) was then pipetted onto a glass coverslip and allowed to air dry. Polymer films were then stored in a desiccator until testing. Deionized water was used as the probe liquid. Contact angle measurements were taken using a video contact angle system (VCA 2000 system, AST Products, Billerica, MA, USA). Each value is the average of nine measurements conducted on different parts of the sample surface.

Fabrication of Microspheres

Microspheres were fabricated using either a double emulsion technique (21,22) or a modified version of the spontaneous emulsification technique previously described (23,24). For the double emulsion technique, 200 mg of polymer were dissolved in 7 ml of dichloromethane (DCM). 100 μl of a 100 μg/ml BDNF/0.1% bovine serum albumin (BSA) solution were added to the polymer/DCM solution dropwise, and the solution was vortexed for 30 s to create an emulsion. This was then added to 150 ml of a 1% PVA solution and homogenized at 3,000 rpm for 1 min. This was then added to 200 ml of a 5% PVA in water solution and stirred for 3 h. Microspheres were washed and collected via centrifugation, freeze-dried, and stored in a desiccator at −80°C until use. PLGA 502H and PLGA 503H were used for the double emulsion protocol.

For the spontaneous emulsion technique, a total of 200 mg of polymer was first dissolved in 5 ml of a 1:4 (v:v) DCM to trifluoroethanol solution. A 0.1 ml (0.1% w/v) BSA/ phosphate buffered saline (PBS) solution was added to 0.1 ml of a 100 μg/ml BDNF/PBS solution in an eppendorf. To this, 0.1 ml of a 250 μM Aerosol-OT, Sodium bis-2-ethylhexyl-sulfosuccinate (AOT)/PBS solution was then added to the eppendorf. The solution was vortexed for 5 s. Contents were then added dropwise to test tubes with the dissolved polymers and vortexed for 5 s. The emulsions were then added dropwise to 200 ml of chilled 5% (w/v) PVA/water solution and allowed to stir for 3 h for microsphere hardening and solvent evaporation. Microspheres were washed and collected via centrifugation, freeze-dried, and stored in a desiccator at −80°C until use. Blank microspheres were made at the same time using the same conditions, except that no BDNF was added (0.1 ml of a 0.1% (w/v) BSA/PBS solution replaced the BDNF/PBS solution). When used as an excipient, BSA has been shown to stabilize and protect the bioactivity of growth factors during encapsulation (25–27). For this reason, BSA was incorporated during microsphere fabrication for both the double and single emulsification protocols.

Three different polymer formulations were investigated for microsphere fabrication using the spontaneous emulsification protocol: 1) PLGA 502H (200 mg) alone, 2) a blend (1.8:1 molar ratio of PEG:PLGA) of PEG (molecular weight ~1,900 Da) and PLGA 502H, 48 mg and 152 mg, respectively (22), and 3) a 50:50 blend of PLGA 502H and PLGA-PLL-PEG (100 mg of each polymer).

Characterization of Microspheres

Microsphere Sizing

The volume-weighted mean diameter of the microspheres from each batch was determined using a Beckman Coulter Multisizer 3 (Fullerton, CA, USA) with a 100 μm diameter aperture based on a sample size of at least 80,000 microspheres.

Amenability of Injection via a 30 Gauge Needle

A 1 ml tuberculin syringe was loaded with 0.5 ml of a solution of microspheres in PBS. A 30 gauge needle was then placed on the end of the syringe, and pressure was applied to expel the solution. The concentration at which the solution of

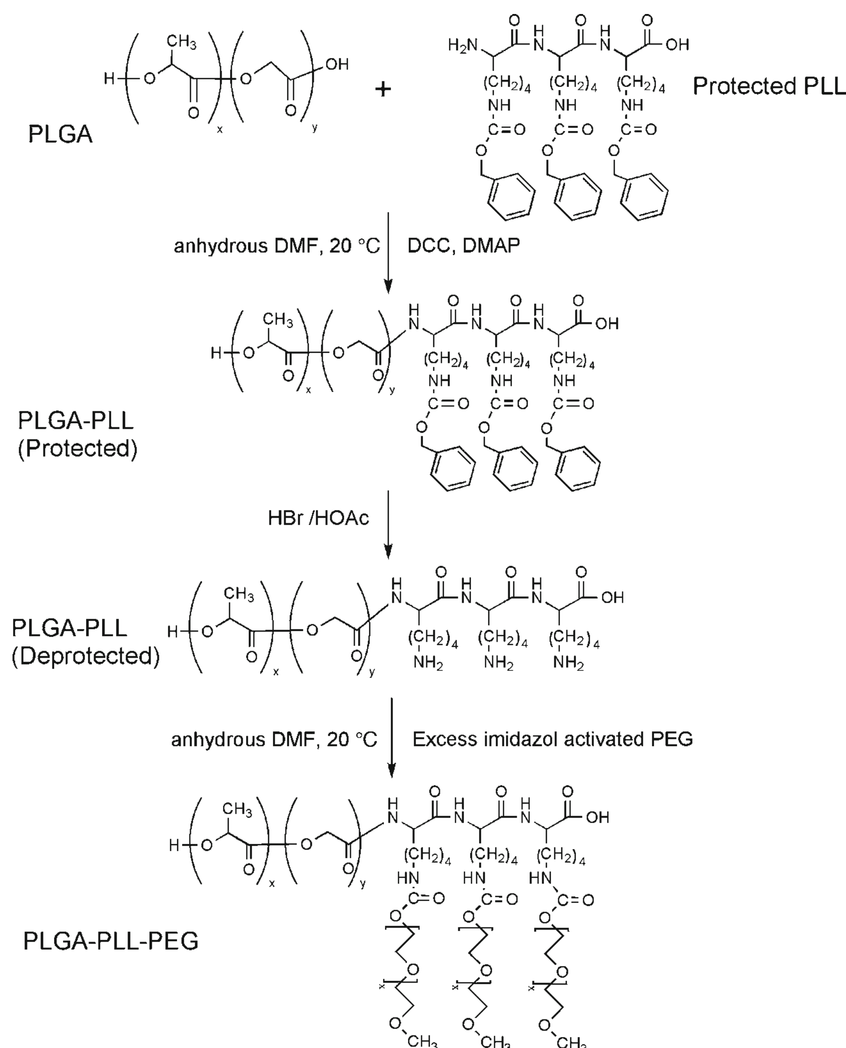

Fig. 1. Reaction scheme for the synthesis of PLGA-PLL-PEG. DMAP: 4-dimethylamino-pyridine; DMF: dimethylformamide.

spheres could be expelled with no signs of clogging, leaking, or loss was recorded.

Scanning Electron Microscopy (SEM) Micrographs

SEM analysis was used to examine microsphere morphology. Microspheres were sputter coated with gold for 30 s at 25 mA, and SEM micrographs were taken on a FEI XL-30 environmental SEM operating at 5 kV.

Microsphere Loading

Microsphere protein loading (BDNF + BSA) was determined using a Micro BCA colorimetric assay (28). Briefly, 10 mg of microspheres were added to 0.3 ml DCM and allowed to dissolve for 30 min at 37°C. Following microsphere dissolution, 1 ml of PBS was added, vortexed, and allowed to phase separate at 37°C overnight. The aqueous phase was then extracted, and protein content was determined using a Micro BCA assay (29). Each experiment was performed in triplicate.

Microsphere Release Study

In 1.5 ml eppendorf tubes, 10 mg of microspheres were suspended in 1 ml of PBS. Samples were prepared in triplicate. Mixtures were then incubated at 37°C on a Labquake Rotator (Barnstead/Thermolyne; Dubuque, Iowa USA). At specific time points (1, 3, 5, and 8 h and 1, 2, 3, 5, 7 days, and once every 7 days thereafter until no pellets were present), the mixture was centrifuged, and the supernatant was collected. One milliliter of PBS was then added to replace the withdrawn supernatant, and the microspheres were resuspended and returned to the shaker. Supernatants for each of the sets of microspheres were frozen and stored at

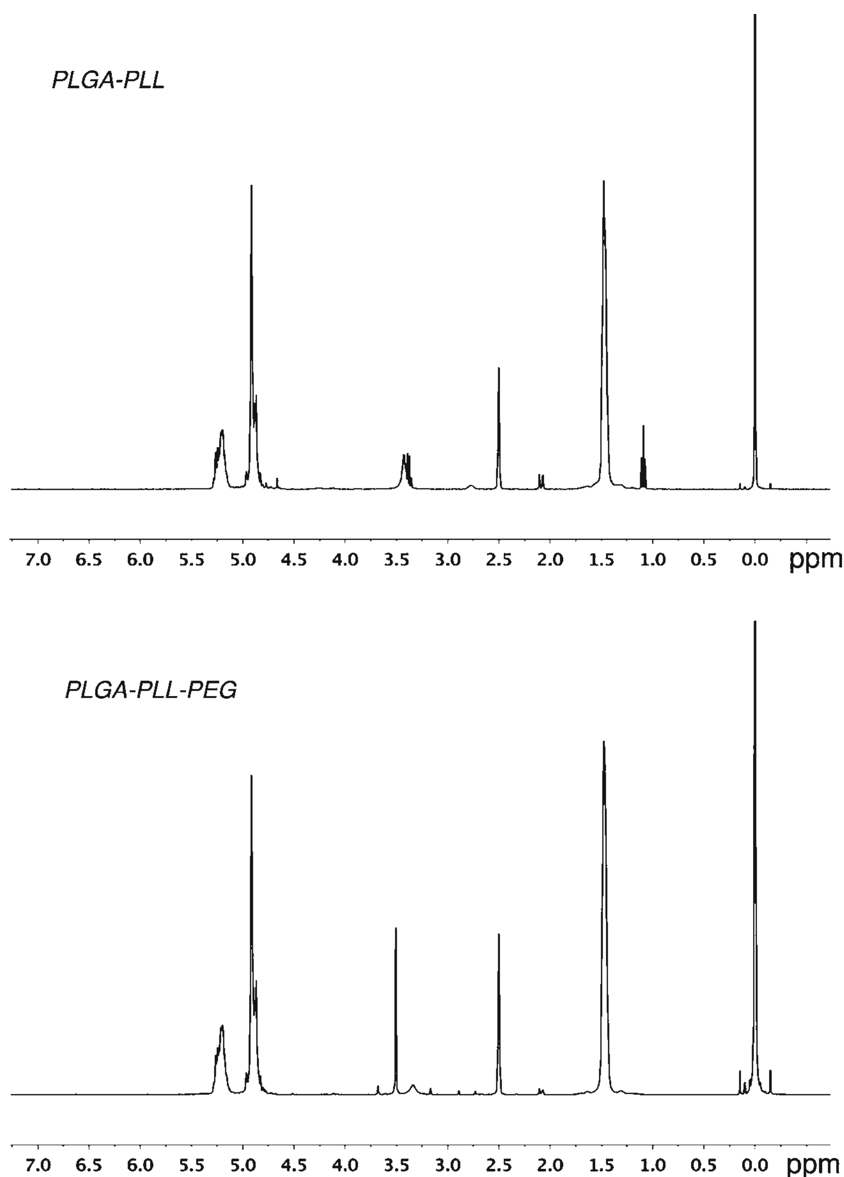

Fig. 2. ^{1}H NMR spectra for PLGA-PLL and PLGA-PLL-PEG. Peak at δ=3.51 ppm is associated with the ether bond present in the conjugated PEG. Note the absence of this peak in the PLGA-PLL copolymer prior to PEG conjugation.

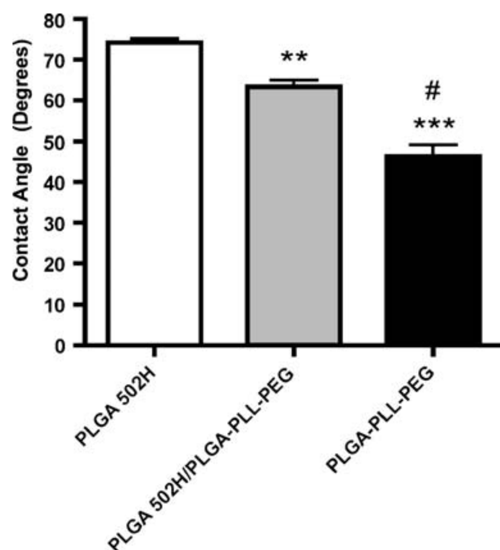

Fig. 3. The wettablility of PLGA 502H, a 50/50 PLGA 502H/PLGA-PLL-PEG blend, and PLGA-PLL-PEG was determined from water contact angle measurements. Data represents mean ± SEM (n=9). ** p<0.01 and *** p<0.001 *versus* PLGA 502H, and # p<0.001 *versus* 50/50 PLGA 502H/PLGA-PLL-PEG blend.

−80°C for subsequent BDNF quantification. Immunoactive BDNF concentrations were determined with an ELISA. Total BDNF encapsulation was defined as the total amount of BDNF released, as determined by ELISA from the release studies.

Bioactivity Assay

PLGA/PLGA-PLL-PEG microspheres (BDNF loaded or blank) were suspended in a transwell insert (0.4 μm pore size) at 2 mg/ml in 1.0 ml of media. The insert was placed in a well (24-well plate) containing 1.0 ml of media seeded with a low density (100 cells/well) of low passage (P3) PC12-trk B cells (final microsphere concentration was 1 mg/ml). Cells were cultured in DMEM, 10% Fetal bovine serum, and 1% Penicillin/streptomycin. Images were taken using a Zeiss Axiovert 200 Microscope (Zeiss, Thronwood, NY, USA). Cells were analyzed every 24 h for 1 week for the number of cells per viewing area (812 μm by 643 μm with a 10x magnification), the number of neurites per cell, and the average length of each neurite. A neurite was deemed to be any process greater than 30 μm. Neurite length was measured from the cell body to the end of the neurite, taking the shortest route. Each experiment was performed in triplicate.

Statistics

Statistical analysis is reported using a standard one-way ANOVA. When appropriate, the post-hoc Tukey test was used. Differences were accepted as statistically significant with P<0.05.

RESULTS

Characterization of PLGA-PLL-PEG

Fig. 1 shows the reaction scheme for the synthesis of PLGA-PLL-PEG. PLGA-PLL was coupled as described previously (18,19). Ultraviolet-visible (UV-Vis) spectroscopy and ^{1}H-NMR characterization confirmed successful conjugation with an average conjugation efficiency of approximately 30%. The PLGA-PLL was then deprotected using HBr/acetic acid, and imidazol-activated PEG was conjugated to the exposed primary amines. ^{1}H-NMR (Fig. 2) showed a proton shift at 3.51 ppm (30), indicating the successful coupling of PEG to the PLGA-PLL copolymer.

Following PEG conjugation, the PLGA-PLL-PEG was precipitated in methanol. Due to PEG's complete solubility in methanol, this was effective means for removing unconjugated PEG from the PLGA-PLL-PEG. To further establish that the observed ether peak in the ^{1}H-NMR spectra was due to PEG conjugation, and not simply PEG adsorption, a reaction was undertaken with unactivated PEG (without imidazol activation). Following polymer precipitation, no peak at 3.51 ppm was observed for this control reaction.

Polymer Wettability

The results of the water contact angle measurements are presented in Fig. 3. Films comprised of PLGA 502H were the most hydrophobic, resulting in the greatest contact angle of 74 ± 1° (mean ± SE). This measurement is in good agreement with previous observations (31). As expected, the 50/50 blend of PLGA with the amphiphilic PLGA-PLL-PEG, resulted in a smaller contact angle (increased wettability) of 63 ± 1.5° (mean ± SEM), with PLGA-PLL-PEG ultimately being the most hydrophilic with a contact angle of 46 ± 3° (mean ± SE). Similar trends and measurements have been reported with varying PLGA/PEG conjugates (32).

Microsphere Sizing

The volume-weighted diameter for BDNF and blank microspheres are listed in Table I. Microsphere diameters were found to be independent of both the polymer formulation as well as the incorporation of BDNF. SEM micrographs were used to verify results obtained from the Coulter Multisizer. Fig. 4 is a SEM micrograph of BDNF microspheres comprised of PLGA/PLGA-PLL-PEG. Microspheres were relatively homogenous spheres with smooth surfaces and minimal aggregation.

Amenability of Injection of Microspheres as a Function of Size

We were able to dispense the microspheres at a concentration of 25 mg/ml through the 30 gauge needle with no leaking, clogging, or apparent loss of spheres. At higher concentrations, some leakage became evident. Tuberculin syringes do not contain a luer lock. The needle is held on by

Table I. Microsphere Volume Weighted Diameters Determined by the Coulter Multisizer

	BDNF (μm)	Blank (μm)
PLGA 502H	18.8±8.3	18.6±6.1
PLGA 502H/PEG Blend	16.3±6.6	20.7±9.4
PLGA 502H/PLGA-PLL-PEG Blend	15.4±5.6	14.5±5.5

Values: (mean ± SD)

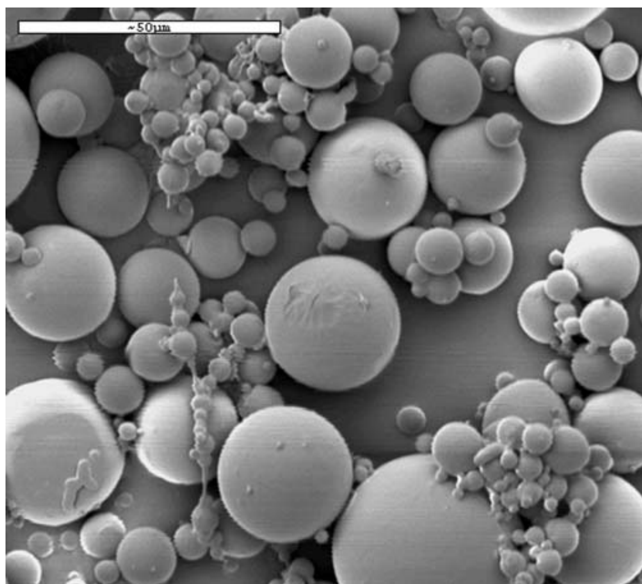

Fig. 4. SEM micrograph of BDNF microspheres composed of a 50/50 blend of PLGA 502H and PLGA-PLL-PEG. Scale bar: 50 μm.

friction, and leakage is seen readily with suspensions if the pressure is excessive.

Loading and Release from BDNF Microspheres

In our initial work, we investigated the role of encapsulation technique on the delivery of BDNF. We encapsulated BDNF using both the traditional double emulsion technique and the spontaneous emulsion technique. Each technique led to a burst of protein within the first few hours of release (24). This burst is most likely due to BDNF adsorbed to the microsphere surface. The double emulsion technique led to a substantial burst of BDNF with 95% of the growth factor being released in the first 2 h using either PLGA 502H or PLGA 503H. The spontaneous emulsification technique led to a large burst with 50% of the BDNF being released in the first 2 h, but the remaining growth factor was delivered over the next 65 days. This finding that we achieved some sustained delivery with the spontaneous emulsion technique motivated us to use that technique to study the role of the polymer in modifying BDNF delivery.

Using the BCA assay, we determined that all of the polymer formulations led to encapsulation of similar amounts of protein (Table II). However, the different polymers had significant influences on the loading and release kinetics of the BDNF (Fig. 5). Each led to a burst of BDNF with 50% of the BDNF being released from the PLGA and 70% being released from the PLGA/PEG blended spheres (Fig. 5b). However, less than 5% of the encapsulated BDNF in the PLGA/PLGA-PLL-PEG microspheres was released in this acute period. This is an important finding. Whereas the blend with PEG led to a greater burst of BDNF, the block copolymer led to a substantial reduction in the initial burst of growth factor.

Following this initial burst, minimal BDNF was released until approximately 14 days for all of the polymer formulations. After this plateau phase of release, all formulations subsequently exhibited a sustained release profile until approximately 65–75 days, when microspheres were completely degraded. The

Table II. Loading of Protein for Microspheres

Polymer	BDNF + BSA (μg/mg)
PLGA 502H	0.81±0.44
PLGA 502H/PEG Blend	1.02±0.11
PLGA 502H/PLGA-PLL-PEG Blend	0.80±0.39

Values: (mean± SD)

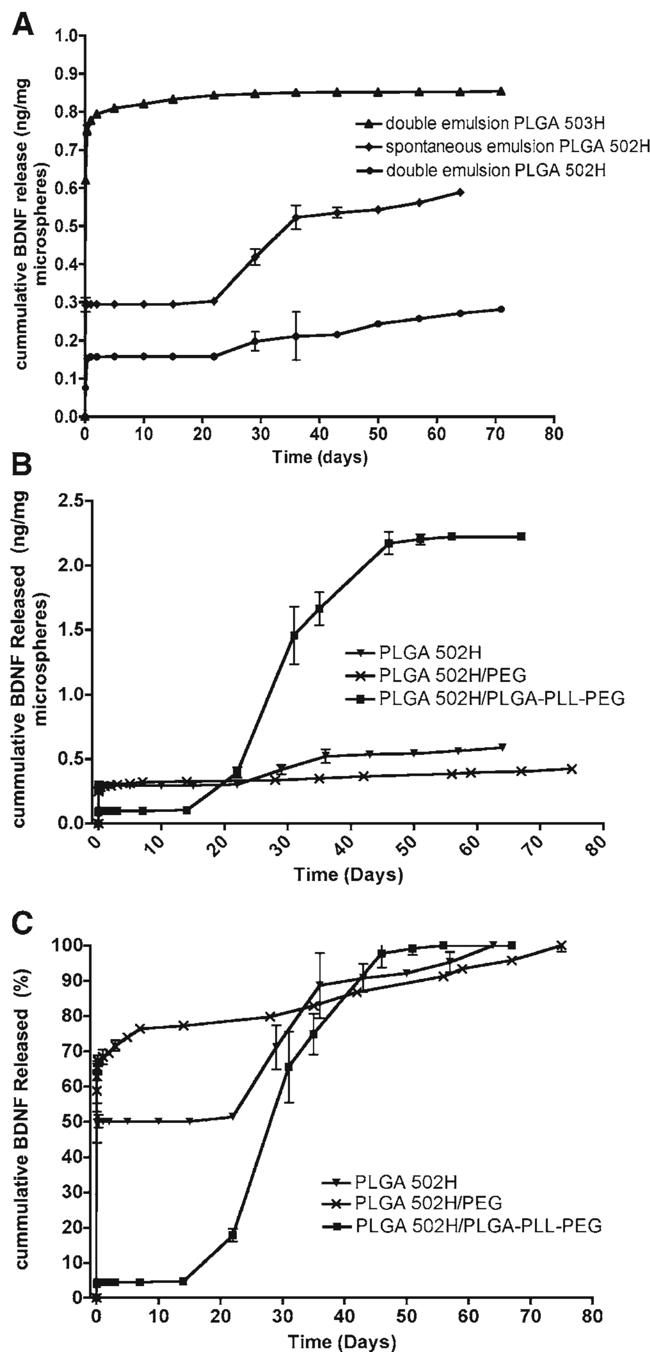

Fig. 5. Cumulative release profiles for BDNF microspheres. (**a**) BDNF delivery as a function of fabrication method. The spontaneous emulsion method led to some sustained delivery of BDNF after the first day, making it the more promising technique for subsequent encapsulation studies. (**b**) BDNF released from microspheres. (**c**) Data from (**b**) represented as % of total BDNF released. Data represent mean ± SD (n=3).

total BDNF released from PLGA/PLGA-PLL-PEG microspheres (2.2 ng/mg) was nearly 4.5-fold greater than that from PLGA or PLGA/PEG blend microspheres: 0.59 ng/mg and 0.42 ng/mg, respectively (Fig. 5b). The total BDNF released was determined by totaling the BDNF quantified in the supernatant over time using the ELISA assay. It is striking that all of the formulations led to the same total protein (BDNF + BSA) encapsulated, but the PLGA/PLGA-PLL-PEG led to the greatest amount of BDNF being encapsulated based on growth factor actually released.

The PLGA/PLGA-PLL-PEG microspheres led to delivery of greater amounts of BDNF more consistently over a longer time than either the PLGA or PLGA/PEG microspheres. While the delivery was far from linear, it more closely approximated the sustained delivery profile we sought. It also approximated the mass lost as PLGA microspheres degrade over time (43). The next question is whether the delivered BDNF is bioactive upon release from the spheres.

BDNF Bioactivity Assay

To investigate the bioactivity of encapsulated BDNF, PLGA/PLGA-PLL-PEG microspheres were incubated with PC12-TrkB cells. This cell line was chosen based on the cells' TrkB receptors' affinity for BDNF (33,34). BDNF released from PLGA/PLGA-PLL-PEG microspheres was bioactive and influenced cell morphology (Figs. 6 and 7). Released BDNF led to a significant increase in the number of neurites per cell (Fig. 7a), as well as the length of each neurite (Fig. 7b). After 7 days in culture with BDNF loaded microspheres, PC12-trkB cells had an average of 1.7 ± 0.1 neurites per cell with an average neurite length of 72.6 ± 1.4 μm. Cells cultured with blank microspheres had an average of 1.0 ± 0.1 neurites per cell with an average neurite length of 55.2 ± 2.0 μm. The number of PC12-trkB cells was also counted, and statistical differences in cell proliferation were observed after 7 days in culture (Fig. 7c).

DISCUSSION

The sustained delivery of proteins from polymeric devices enables one to administer a therapeutic level of the protein over prolonged periods (35). Moreover, the delivery of neurotrophic factors from these devices may have the potential to treat neurodegenerative diseases, where sustained administration of these factors is required (36,37). PLGA is an attractive candidate for investigating sustained

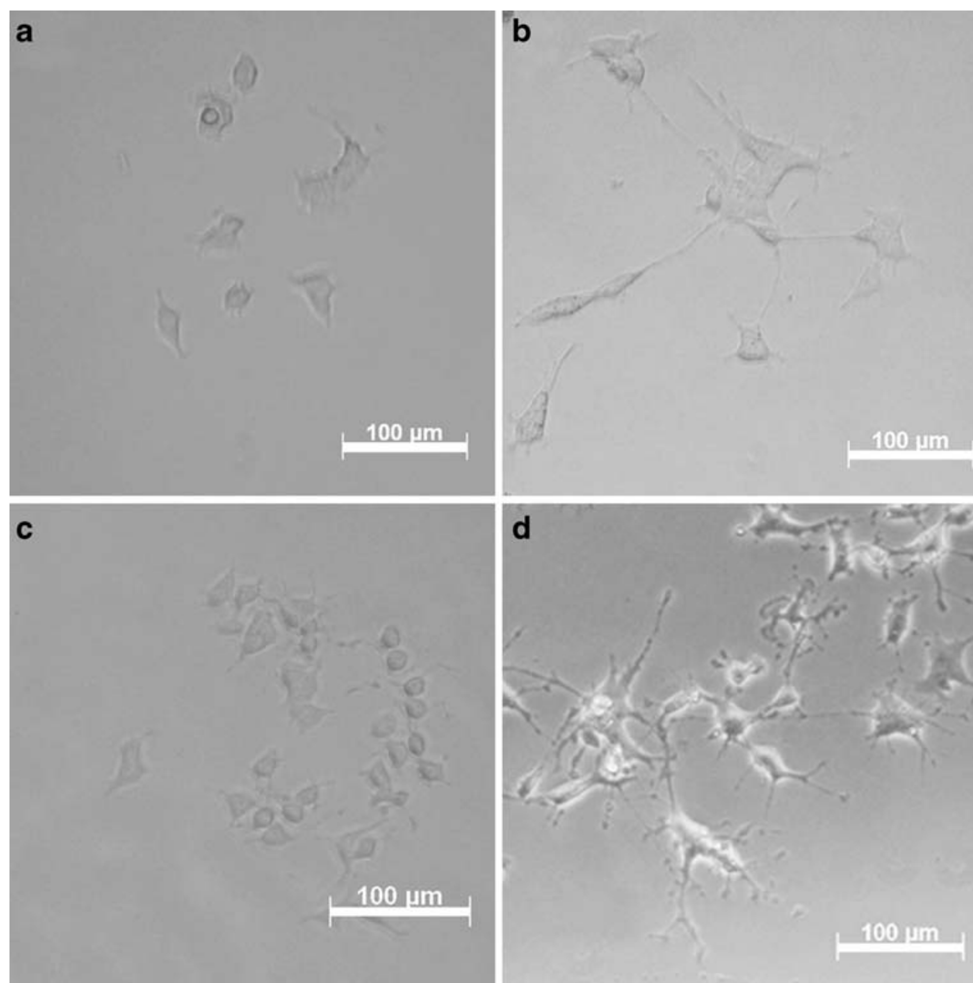

Fig. 6. PC12-trkB cells in culture with BDNF or blank PLGA/PLGA-PLL-PEG microspheres. (**a**) Blank microspheres: 1 day in culture. (**b**) BDNF microspheres: 1 day in culture. (**c**) Blank microspheres: 5 days in culture. (**d**) BDNF microspheres: 5 days in culture. Scale bar: 100 μm.

delivery because it is extremely amenable to processing, allows tailoring of degradation over a wide timescale, and can be fabricated into microspheres that are readily injected using minimally invasive approaches. To date, encapsulation of BDNF using PLGA-based systems has resulted in a delivery profile with a significant burst release and limited sustained delivery (38). Efforts to mitigate this burst and improve encapsulation (e.g. addition of salt) have often resulted in protein denaturing (39). In an effort to reduce these adverse effects, we developed a PLGA-PLL-PEG triblock polymer to facilitate the encapsulation of BDNF, preserve its bioactivity, and enable its prolonged delivery. With the incorporation of the PLGA-PLL-PEG, our microspheres sustained the release of BDNF for up to 65 days, with less than 5% of the encapsulated protein being released in the first day.

The simple addition of PEG to the aqueous phase during microsphere fabrication has proven beneficial, most notably in the preservation of bioactivity of the encapsulated protein (22). A 1.8:1 PEG:PLGA molar ratio was found to be optimal for the sustained release of nerve growth factor (NGF), with higher PEG concentrations resulting in a marked burst in the initial hours of release. Thus, it seemed reasonable to investigate the role of PEG on the encapsulation and delivery of BDNF, a member of the NGF structural family.

We found that simple incorporation of PEG was not sufficient to augment encapsulation and delivery. Both PLGA alone and PLGA/PEG blended microspheres resulted in a similar burst and plateau release profile, with approximately 50–60% of the encapsulated BDNF being released during the early time-points (Fig. 5b). The initial burst is often due to the desorption of surface-associated hydrophilic molecules on devices comprised of hydrophobic polymers, not device degradation (40). In an aqueous environment, these surface-absorbed molecules are readily dissociated. However, when the PEG was chemically coupled to the other polymers, we saw a significant improvement in sustained delivery. By replacing PEG with the PLGA-PLL-PEG triblock polymer, this initial release of BDNF was significantly decreased to approximately 5% of the total released protein. Furthermore, based on the observation that protein loading was equivalent for all formulations (Table II), but BDNF release was nearly 4.5-fold greater from PLGA/PLGA-PLL-PEG blended microspheres, the preservative attributes of PEG were still marked even after its conjugation to PLGA.

The conjugation of PEG to PLGA, and its incorporation during microsphere fabrication, did not have a dramatic influence on the size or degradation of the microspheres. All of the microspheres, PLGA, PLGA/PEG, and PLGA/PLGA-PLL-PEG spheres were similar sizes (approximately 17 μm) (Table I), suggesting that the introduction of small amounts of PEG does not change the processing behavior significantly. The PEG also did not have a dramatic impact on the degradation. The microspheres in both the PLGA and PLGA/PLGA-PLL-PEG groups were present for approximately 70 days. This suggests that the choice of PLGA molecular weight and glycolic acid to lactic acid ratio will still play a dominant role in dictating the microsphere release kinetics (41, 42). Moreover, the delivery of BDNF from the PLGA/PLGA-PLL-PEG microspheres correlates extremely well with the degradation of PLGA. Others have assessed the mass loss and molecular weight of PLGA microspheres degrading over time, and the curves follow the BDNF delivery curves in the PLGA/PLGA-PLL-PEG group very closely (43,44).

One of the controls we did not look at was the PLGA-PLL spheres. We know from other work that the PLL strongly influences interactions between growth factors and the polymer and that polymers with a low isoelectric point associate more strongly with the positively charged PLL (19). Since BDNF has an extremely high isoelectric point (45), the PLGA-PLL alone would likely to lead to lower loading than

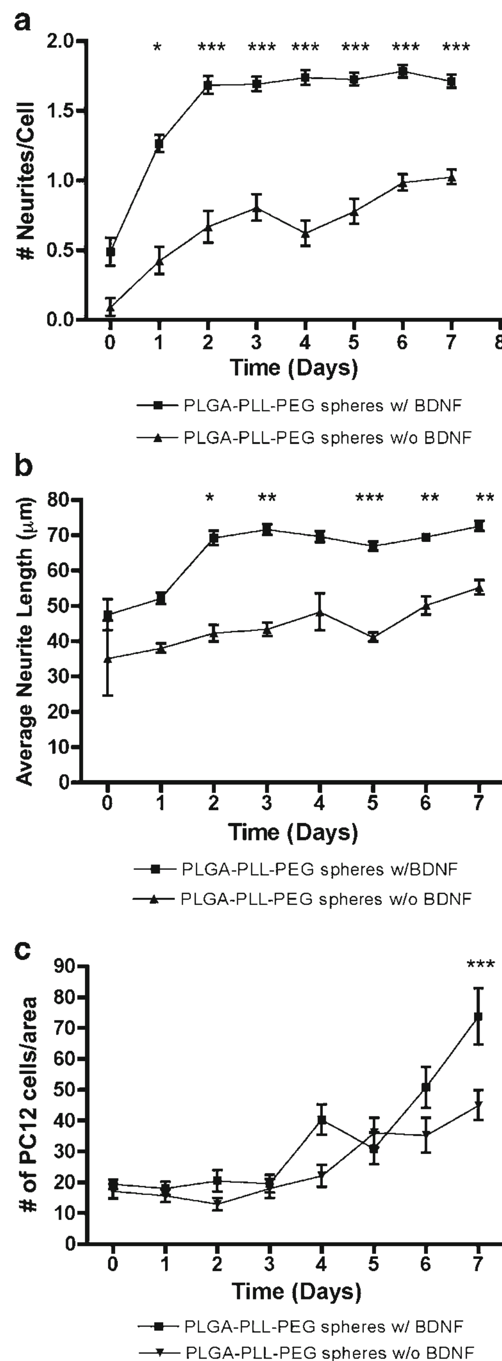

Fig. 7. Morphological and proliferative quantification of PC12-trkB cells cultured with BDNF or blank PLGA/PLGA-PLL-PEG microspheres. (**a**) Number of neuritis/cell. (**b**) Neurite length. (**c**) Number of cells. All measurements taken in a visual field (812 μm×643 μm). Data represent mean ± SEM (n=3). *p<0.05, **p<0.01, and ***p<0.001.

PLGA if it had an effect. The purpose of the PLL was to provide sites for coupling the PEG. The PLL we used was small, with only 3–4 lysine residues per chain. In other work, we have seen PLL alter release kinetics (19), but in that case, the PLL was uncoupled and, at physiological conditions, was charged. Once the PEG is coupled to the PLL, the new secondary amines will not be charged, and therefore, the PLL is likely to have minimal effect on the delivery.

Ultimately, the question arises as to what role the PEG plays. Clearly, loading is higher with the incorporation of the PLGA-PLL-PEG. The hydrophilicity of the PEG may decrease surface tension at the water/oil interface during microsphere fabrication. This may limit BDNF degradation by harsh organic solvents. Furthermore, due to the high isoelectric point, BDNF is a relatively hydrophilic neurotrophin. The hydrophilic nature of the PEG may interact preferentially with the BDNF and lead to more intimate association of the polymer with the growth factor. Thus, instead of the BDNF simply desorbing from the microspheres quickly, the release is more closely coupled to the degradation of the polymer, which occurs over the 65 day delivery period. This is an exciting finding suggesting that the coupling of the PEG enhances the interaction between the PLGA and BDNF. Thus, one may be able to further tailor the release of BDNF by the choice of PLGA in the block copolymer.

Sustained delivery of a growth factor is only meaningful if that factor is active upon delivery. It is well-established that PC12 cells (a rat pheochromocytoma tumor cell line) resemble sympathetic neurons in response to NGF exposure (34). Furthermore, tropomyosin-related kinase B expressing PC12 (PC12-trkB) cells show a similar differentiating response (neurite extension) to their ligands, BDNF or NT-4/5 (46). We found that the BDNF released from the microspheres led to morphological responses, in particular neurite extension that replicated their behavior following the addition of unencapsulated BDNF. Thus, the encapsulation of BDNF using PLGA/PLGA-PLL-PEG leads to greater loading and sustained delivery of the bioactive neurotrophin for over 65 days.

There is growing interest in the development of drug delivery systems in which the polymer interacts with the drug. These affinity-based systems have been developed using a range of structures including dextrans, peptides, and other molecules (47–50). These drug/materials interactions can fundamentally alter release kinetics and tie delivery of the drug to the degradation of the material.

We wanted to develop a simple system that leveraged materials that have been well-studied and have been approved in other devices and pharmaceutical applications by the FDA. PLGA is an obvious material to use as a basis, since it had been extremely well-studied and used in a variety of FDA-approved devices and formulations. However, developing affinity-based PLGA materials has been challenging. The intense hydrophobicity of PLGA tends to be the dominant characteristic in polymer-drug interactions. The coupling of PEG onto PLGA via the free amines on the lysine residues allows us to modify the hydrophobicity and improve BDNF loading and delivery. While new materials are often necessary to obtain desired properties, systems that use previously approved materials tend to have a shorter timeline to the clinic. By investigating polymer-drug interactions using well-studied and previously approved materials, we are able to obtain some of the benefits of affinity-based systems, including high loading and sustained delivery with readily translatable materials.

CONCLUSION

BDNF holds great promise for the treatment of a range of neurodegenerative diseases and disorders, but it is critical that it be delivered for long time periods. We sought to develop a minimally invasive microsphere formulation that delivered BDNF. After investigating fabrication techniques and seeing some sustained delivery using the spontaneous emulsion process with a substantial burst phase, we began to investigate modifying the PLGA to augment loading and promote sustained delivery. The addition of PLGA-PLL-PEG to the microsphere formulation led to a minimal burst and sustained delivery of the BDNF. We hypothesize that the PEG facilitated interaction of the BDNF with the PLGA and led to the delivery of the BDNF being more closely associated with the degradation of the microspheres as opposed to simple desorption over the first few hours.

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