Controlled release of glial cell line-derived neurotrophic factor from poly (ε-caprolactone) microspheres

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2014

The final published version of this article can be found at:

http://dx.doi.org/10.1007/s13346-013-0189-0

Controlled Release of Glial Cell Line-Derived Neurotrophic Factor from Poly (Ɛ-Caprolactone) Microspheres

Glial cell line-derived neurotrophic factor (GDNF), a growth factor expressed in the central nervous system, promotes the survival of both dopaminergic and motor neurons, making it a promising candidate for neurodegenerative disease therapy. Although GDNF is currently being evaluated in clinical trials for the treatment of Parkinson's disease (PD), the current delivery method using catheter implantation has certain limitations in terms of delivering GDNF safely and effectively. As a proof of concept, we encapsulated GDNF into poly (ε-caprolactone) (PCL) microspheres to enable controlled drug release for 25 days. First, microspheres were loaded with bovine serum albumin (BSA) to determine the optimal fabrication conditions necessary to achieve the desired release rates of protein. BSA was then used as a carrier protein to preserve GDNF activity during the fabrication process in the presence of organic solvents. GDNF encapsulated microspheres were created and characterized using scanning electron microscopy. Next, the in vitro release of GDNF along with microsphere morphology was tracked over 25 days. Finally, the bioactivity of the released GDNF was confirmed using PC12 cells. This work demonstrates the potential of such microspheres for the delivery of bioactive GDNF with the end goal of developing a suitable, clinically relevant formulation for injection to appropriate regions of the brain in PD patients.

Keywords: Glial Cell Line-Derived Neurotrophic Factor (GDNF), Parkinson’s disease (PD), Microspheres, Controlled Release

1. Introduction

An estimated seven to ten million people worldwide currently suffer from Parkinson's disease (PD) with the majority of the affected being over the age of 65 [1]. In Canada, approximately 100,000 people suffer from PD with around 5,500 new cases being diagnosed annually [2]. PD results from the death of dopaminergic neurons in the substantia nigra region of the brain, resulting in impaired motor control. In the healthy brain, these neurons produce the neurotransmitter dopamine to ensure proper motor function and coordination [3]. As the adult brain does not regenerate these lost cells, treating PD remains challenging [4, 5]. Current pharmacological approaches attempt to restore these decreased dopamine levels through oral administration of L-dihydroxyphenylalanine (L-DOPA), a precursor molecule, that is converted into dopamine through an enzymatic decarboxylation reaction [6]. L-DOPA is taken orally and absorbed by the intestine before entering the bloodstream where it can then cross the blood-brain barrier (BBB) into the brain. As a result, approximately 10% of the initial dose of L-DOPA reaches the brain and the L-DOPA that metabolizes before reaching the brain can cause serious side effects such as motor fluctuations and dyskinesias [7]. Thus, major challenges include how to increase the amount of L-DOPA delivered to the brain and that L-DOPA has a short half-life, requiring constant delivery to be effective [8]. Long term treatment with L-DOPA leads to dyskinesias and thus more effective, long term therapies for PD are being investigated.

Several research groups have investigated the delivery of glial cell line-derived neurotrophic factor (GDNF) for the treatment of PD as an alternative to the use of L-DOPA. [9, 10]. Due to its neuroprotective properties, GDNF treatment promotes the survival and differentiation of embryonic dopaminergic neurons grown in culture, making it a desirable therapeutic target [11]. Following injury, neurons and related cells elevate their GDNF expression to preserve the remaining cells [12]. Recent studies suggest that long term delivery of glial cell line-derived neurotrophic factor (GDNF) promotes the survival of dopaminergic neurons in animal models of PD [13]. After using 6-hydroxydopamine injections to simulate the effects of PD in rats, Sullivan *et al.* administered GDNF weekly into the substantia nigra, showing that GDNF significantly inhibited the death of dopaminergic neurons at 13 weeks post surgery [14]. Administration of GDNF directly into the right substantia nigra pars compacta of the adult rat brain showed significant protection of midbrain dopamine neurons against 6-hydroxydopamine (6-OHDA) toxicity [15]. These pre-clinical studies demonstrate the potential for GDNF to serve as a long term therapy for PD by promoting the survival of dopaminergic neurons.

Similar to the delivery issues associated with L-DOPA, the major challenge with GDNF-based therapies is how to successfully deliver appropriate concentrations to the correct region of the brain over extended time periods. Thus, a delivery method must address how to maintain bioactivity while avoiding unwanted receptor binding as well as to address how to cross the BBB and establish an appropriate diffusion profile in the brain [16]. Several delivery methods have been evaluated, including the use of pump-catheter systems for the direct infusion of the protein, cell transplants with genetically modified GDNF-expressing cells, and injection of viral strains modified with the GDNF gene [17-19] . The first open-label clinical trial to evaluate the efficacy of GDNF involved continuous, yearlong delivery into the putamen region of the forebrain of five Parkinson patients, and it showed an increase in dopamine

storage [17]. However, this study raised toxicity concerns due to accumulation of GDNF, resulting from poor drug distribution in the brain [20, 21]. To address this issues, a second clinical trial delivered GDNF using microcatheters combined with a process known as convection enhanced delivery (CED), where a pressure gradient drives drug transport through bulk flow, leading to greater homogenous drug delivery [22]. This technique resulted in a clinically effective drug distribution, but the bioactivity of the GDNF was low (2-9%) [23]. Additionally, challenges still remain in minimizing the toxicity and optimizing the doses of delivered GDNF. For example, loss of infusion pressure in the system could result in GDNF back flow into other regions of the brain, causing serious side effects. Thus, finding an alternative method of GDNF delivery would be desirable.

Controlled release of GDNF from biomaterials can be used as an alternative delivery method to using microcatheter based techniques. In particular, microspheres, porous particles which have been used since 1996, can serve as drug delivery systems when they are loaded with growth factors to be then released over extended time periods [24-26]. For instance, in a pioneer work by Jameela *et al.* encapsulation of bovine serum albumin (BSA) into poly(ε-caprolactone) (PCL) microspheres has been carried out successfully and the *in vitro* release over 16 days has been assayed [25]. Properties such as particle size, degradation rate, and polymer concentration can be manipulated during the fabrication process, influencing the resulting drug release profile [27]. Their relatively small size allows for injection into the specific locations for drug release, which is desirable for the treatment of PD as it afflicts a localized region of the brain. Previous studies have demonstrated successful drug release from microspheres fabricated from a number of biodegradable polymers, including poly(lactide) (PLA), poly(glycolide) (PGA), poly(l-lactic acid) (PLLA), poly(lactic-co-glycolic acid) (PLGA), and PCL [29-32]. These microspheres have been used to deliver neurotransmitters, neurohormones, neuromodulators and neurotrophic factors [28]. In terms of GDNF delivery, controlled release from PLGA microspheres has been characterized in *vitro* [33] and *in vivo* in a rat nerve injury model [34, 35]. Andrieu-Soler et al. have observed a burst release of GDNF from PLGA mircospheres of 35 % within the first day and then continuous release was reported reaching 60 % of the total GDNF at 56 days [36] .In another work, Checa-Casalengua et al. reported an initial burst release of 16.0±0.9 ng GDNF/mg microsphere in the first 24 h. Controlled release of GDNF for 41 days with the release rate of 117.3±11.1 pg GDNF/mg microsphere/day was reported [37]. Wu *et al.* have used different concentrations of chitosan in their chitosan-PCL copolymer system to control the release of GDNF from PCL-chitosan microspheres [38]. Additionally, PLGA microspheres that released GDNF can improve motor function in a short term PD rat model [39, 40].

The aim of this study was to develop PCL microspheres that provide controlled release of clinical grade human GDNF at low concentrations for extended time periods. Compared to the more commonly used polymers like PLA and PLGA, PCL has a much slower degradation rate with the potential to generate sustained drug delivery for up to a year [41, 42]. In terms of clinically relevant delivery, the longer that these microspheres can release biological active GDNF reduces the need for additional treatments via injection [43]. In this study, we fabricated PCL microspheres for the delivery of GDNF produced using good manufacturing practices provided by MedGenesis Therapeutix, which is the same protein currently being used in their on-going clinical trial. In order to control the release of GDNF, we varied the concentration of PCL used (6 and 9 % (w/v)) to encapsulate BSA as a stabilizer protein for GDNF, using a water/oil/water (w/o/w) emulsion method. BSA is encapsulated inside the microsphere

due to its protective role to preserve drug activity during the fabrication of drug delivery systems in the presence of organic solvents [44, 45]. The resulting microspheres were characterized using scanning electron microscopy (SEM) and their release profiles determined using a protein quantification assay. We then repeated this process by encapsulating GDNF at the same two PCL concentrations. The resulting microspheres were characterized and the release profile was determined using a GDNF enzyme-linked immunosorbent assay (ELISA), showing that at the end of a 25 day release study that ~5-10% of the total GDNF loaded had been released depending on the concentration of PCL used during encapsulation. Finally, the bioactivity of GDNF after being release from microspheres was confirmed using pheochromocytoma (PC12) cells, which differentiate into a neural phenotype upon exposure to GDNF [46]. Using this assay, the released GDNF was confirmed to be biologically active GDNF after 25 days. Overall, our microsphere based approach serves as a clinical relevant method for achieving extended delivery of GDNF for the treatment of PD.

2. Materials and methods

2.1. Materials

Poly (ε-caprolactone) (PCL) (M_n ~ 45000), polyvinyl alcohol (PVA) (M_w ~ 13,000–23,000, 87–89% hydrolyzed), bovine serum albumin (BSA) (M_w 66 kDA, lyophilized powder), laminin derived from the basement membrane of Engelbreth-Holm-Swarm murine sarcoma cells, and 0.01% poly-L-ornithine solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dichloromethane (DCM) and phosphoric acid (FW 98.00) were purchased from Fisher Scientific (Ottawa, ON, Canada). Methanol (MeOH) was purchased from VWR International (Edmonton, AB, Canada). Phosphate buffer solution (PBS) and Dulbecco's modified eagle medium (DMEM) (1X, 4.5g/L D-Glucose, L-Glutamine) were purchased from Invitrogen (Burlington, ON, Canada). Good manufacturing practice (GMP) Liatermin (r-metHuGDNF), which will be subsequently referred to as GDNF in this paper, was provided by MedGenesis Therapeutix Inc. (Victoria, BC, Canada). Polyclonal goat IgG human GDNF biotinylated antibody, monoclonal rat IgG anti-GDNF antibody, and wash buffer were purchased from R&D Systems (Minneapolis, MN, USA). ImmunoPure® streptavidin horseradish peroxidase conjugated and fetal bovine serum (FBS) were purchased from Thermo Scientific (Rockford, IL, USA). Enzyme-linked immunosorbent assay (ELISA) plates and 24-well tissue culture plates were purchased from Grenier (Monroe, NC). Milk diluent/blocking solution concentrate and SureBlue Reserve™ TMB microwell peroxidase substrate (1-component) were purchased from KPL (Gaithersburg, MD, USA). The adrenal rat PC12 cell line was generously provided by the Burke lab (Department of Biochemistry and Microbiology, University of Victoria).

2.2. Preparation of encapsulated microspheres

The microspheres were fabricated using a well-known water-in-oil-in-water (w/o/w) double emulsion technique [25, 26, 42]. . In order to prepare BSA-encapsulated microspheres, the first emulsion (w/o) was obtained by mixing the first water phase with the oil phase by sonication (Fisher Scientific Model 100) for 30 seconds at the lowest setting (level 1), followed by vortex mixing (Fisher Scientific) for 15 seconds. This (w/o) emulsion was

immediately added to the second water phase and held at 35°C at a mixing speed of 400 rpm for 1.5 hours to achieve evaporation of the organic solvent. After mixing, the microspheres were isolated by vacuum filtration using Whatman filter paper, washed with de-ionized water, and dried at 4°C for at least 24 hours. The microsphere protocol used for the BSA encapsulated microspheres was scaled down for the encapsulation of GDNF into PCL 9% (w/v) microspheres by reducing the volumes used by 1/2. Since GDNF is quite expensive compared to BSA, scaling down the procedure reduced the amount of GDNF necessary for the fabrication process. BSA serves as a carrier protein to maintain the bioactivity of the GDNF [47]. The first water phase included GDNF and BSA at a ratio of 1:11.5. The GDNF-BSA mixture was added to diluted PVA to make 0.25 Ml of 0.5% BSA and GDNF in 1% PVA (w/v). The 1/2 oil phase was made by dissolving PCL in 4 mL of DCM and 1 mL of MeOH for 6% and 9% (w/v) concentrations to make PCL 6% (w/v) and PCL 9% (w/v) microspheres. The rest of the procedure remained unchanged from BSA encapsulated microsphere preparation.

2.3. Characterization of microspheres

2.3.1. Surface morphology and particle size analysis

Morphological characterization was performed using a Hitachi S-4800 FE scanning electron microscopy (SEM) machine to image the microspheres at different time intervals during the release studies. Dry microspheres were transferred to loading stubs and coated twice with carbon using a Cressington 208 high vacuum carbon coater for duration of 6 seconds at 10^{-4} *m*bar to enhance surface conductivity for morphological analysis by SEM. Images were captured using accelerated voltages of 1.0 and 1.5 kV with working distances of 7.8 mm and 7.0 mm. The average diameter of the microspheres was determined by using Quartz-PCI Image Management Systems® software.

2.4. *In-vitro* release studies

2.4.1. Drug encapsulation efficiency

To calculate encapsulation efficiency, the ratio of the actual encapsulated protein to the amount of protein originally added was used. The BSA encapsulated per unit weight of microspheres was determined by an extraction method patented by Tice and Gilley [48]. A measured amount of dried microspheres was placed in a 45 mL propylene conical tube. DCM (8 mL) was added and vortexed for 1 min to dissolve PCL. Next, PBS (2 mL) was added and vortexed for 15 seconds to allow extraction of BSA into the PBS. The solution was then centrifuged at 22°C and 5000 rpm for 20 min using an Eppendorf 5804 R Centrifuge. The aqueous portion was then transferred to a 15 mL conical tube. 200 µL of this aqueous layer was then transferred to a 1.5 mL microfuge tube and diluted with 800 µL of PBS. The concentration of protein in the sample was determined by using the Microtitre Plate Protocols per manufacturer's instructions. This assay detects the total amount of protein in the sample. Sample and standard absorbance were read by a microplate reader (Molecular Devices Versa max) and Softmax Pro version 5.2 software (595 nm). GDNF encapsulated per unit weight of microspheres was also determined by extraction into 2 mL PBS as previously described. The concentration of the sample was determined by a solid phase sandwich ELISA specific to GDNF (Amgen procedure #A0195r04). 96-well ELISA plates were coated with monoclonal anti-GDNF antibodies and then incubated with samples and standards. After washing, subsequent incubation and wash cycles were

performed with polyclonal anti-GDNF antibodies (biotin conjugated), Streptavidin (HRP conjugated), substrate, and H_3PO_4 stop solution. The absorbance of samples and standards were read by a microplate reader (Molecular Devices Versa max) and Softmax Pro version 5.2 software (450 nm and 650 nm).

2.4.2. BSA release study

In vitro release studies were carried out in triplicate for PCL 6% (w/v) and PCL 9% (w/v) microspheres. For each replicate, 200 mg of microspheres were placed in 1.5 mL microfuge tubes and suspended in 1 mL PBS. These tubes were then loaded onto a Sarstedt Sarmix mr-1 tube rotator and incubated at 37°C. Samples were taken at 24 hour intervals for 25 days. For sampling, the tubes were centrifuged (Revolutionary Science microcentrifuge) at 6000 rpm for 10 minutes. After centrifuging, 100 µL of the supernatant was removed from each tube, replaced with fresh PBS, and put back in the incubator. The 100 µL supernatant sample was diluted with 400 µL of PBS and assayed for BSA release according to the Bio-Rad Protein Assay Microassay protocol. Absorbances of the samples and standards were determined as previously described.

2.4.3. GDNF release study

In vitro release studies were carried out in triplicate for PCL 6% (w/v) and PCL 9% (w/v) microspheres. For each replicate, 50 mg of PCL 6% (w/v) microspheres or 60 mg of PCL 9% (w/v) microspheres were placed in 1.5 mL microfuge tubes and suspended in 1 mL PBS. These tubes were then loaded onto a Sarstedt Sarmix mr-1 tube rotator and incubated at 37°C. Samples were taken at 24 hour intervals for 25 days. For sampling, the tubes were centrifuged as previously described. After centrifuging, 1 mL of the supernatant was removed from each tube, replaced with fresh PBS, and put back in the incubator. The 1 mL supernatant sample was then assayed for GDNF release by ELISA. Absorbances of the samples and standards were read by a microplate reader (Molecular Devices Versa max) with Softmax Pro version 5.2 software at 450 nm and 650 nm.

2.5. *In vitro* bioactivity assay

2.5.1. PC12 cell culture

PC12 cells were grown in suspension in untreated T-25 cell culture flasks (Grenier Bio One, Monroe, NC) in DMEM supplemented with 15% FBS at 37°C and 5% CO_2. Cells were passaged once a week using a 22 gauge needle to separate cell clumps.

2.5.2. Bioactivity of released GDNF

For neurite outgrowth experiments, PC12 cells were seeded into 24-well plates coated with laminin at a concentration of $2.15x10^4$ cells/mL for the test experiment and $1.12\ x10^4$ cells/mL for the positive control. Cells were then exposed to GDNF released from microspheres during the 25 day release study at concentrations ranging from 0 to 100 ng/mL. After 10 days following administration, the cultures were imaged using a Leica DMI3000 B microscope. Neurite outgrowth was assessed visually by counting the number of cells with one or more neurites at a length of more than twice the width of its body. Untreated cells served as a negative control and cells were treated with stock GDNF at varying concentrations as a positive control.

2.6. Statistical analysis

Data are presented as mean values ± standard deviation of the mean. Statistical analysis using STATISTICA 9 applying a standard *t*-test was carried out for comparisons between 6% and 9% PCL concentration release data. Two independent tests were carried out for both sets of release studies (BSA and GDNF). Significance was considered at the $p < 0.05$ level.

3. Results

3.1. Morphological studies

Three different PCL concentrations of BSA-loaded microspheres were produced and microsphere size was analyzed with SEM imaging. Size affects the versatility of microspheres in a given system. In an effort to decrease the average diameter of microsphere size and reduce the amount of GDNF required for loading, the oil phase volume was downscaled by 1/2 for the GDNF-BSA loaded microspheres. The average diameter of microsphere batches varied among different concentrations and degree of downscale ($p < 0.05$) (Table 1). Large variations in the microsphere size distribution were noted. Microsphere diameter generally increased correspondingly with PCL concentration with PCL 3% (w/v) being the smallest. The only sample contrary to this pattern was the PCL 6% (w/v) BSA microspheres having an average diameter larger than PCL 9% (w/v) BSA microspheres. PCL 3% (w/v) microspheres did not release detectable levels of BSA and thus were not considered for further characterization. As expected, the GDNF-BSA downscale analogues of PCL 6% (w/v) and PCL 9% (w/v) were found to be smaller in diameter than their BSA counterparts. The concentration of polymer hadan effect on the diameter of the microspheres during fabrication. Along with diameter measurements, the surface structure of microspheres was also characterized by SEM. Fabricated microspheres were observed to be spherical in shape and exhibited a smooth surface with micro-pores interspersed.

During the 25 day study, images taken on day 7 through 25 show swelling of microsphere structure over time in both BSA and BSA-GDNF microspheres (Figures 1 and 2). The microspheres exhibited morphological changes (Figures. 1f, 1d, 2c, and 2g), increased deformation (Figures. 1c, 1h, 2c, and 2e), and appearances of possible cleavage planes (Figures. 2d and 2h) as time went on. These morphological changes were more prominent in the latter portion of the 25 day study. In summary, the swelling and morphological changes of the microspheres were observed to increase over time, independent of polymer concentration.

3.2. Drug encapsulation efficiency

The efficiency of encapsulation reflects the amount of protein encapsulated in the microspheres compared to how much was added in the fabrication process. Encapsulation efficiency determines the amount of protein loaded into the microspheres and therefore, ultimately affects their drug release profiles. This important property influences the release characteristics of the microspheres. When using BSA to load the microspheres, PCL 3% (w/v) microspheres had an encapsulation efficiency of 3.33 ± 2.4%, PCL 6% (w/v) microspheres had an encapsulation

efficiency of 15.8 ± 3.1%, and PCL 9% (w/v) microspheres had an encapsulation efficiency of 34.1 ± 7.6% of 6 mg BSA added in each concentration (Table 2). PCL 9% (w/v) was observed to have the highest encapsulation efficiency of BSA while PCL 3% (w/v) had the lowest. Accordingly, increasing PCL concentration increased encapsulation efficiency. For GDNF-BSA PCL 6% (w/v) and GDNF-BSA PCL 9% (w/v) microspheres, 0.2 mg of GDNF was added with encapsulation efficiencies of 18.5 ± 6.6% for PCL 6% (w/v) and 47.4 ± 8.9% for PCL 9% (w/v), respectively. Again, encapsulation efficiency increased with PCL concentration.

3.3. BSA and GNDF release profiles

The release kinetics of protein loaded PCL microspheres was first examined using BSA to model differential release from different polymer concentrations and to confirm sustained release from the microspheres. PCL 6% (w/v) microspheres released a maximum BSA amount of 9.7 μg on day 3 of release studies while PCL 9% (w/v) microspheres released a maximum of 3.8 μg on day 2. The percent cumulative release of BSA over time from total encapsulated BSA is shown in Figure 3A for both PCL 6% (w/v) and PCL 9% (w/v). For PCL 6% (w/v), the maximum cumulative release of BSA at the end of 25 days was 88% of the 176.93 μg BSA encapsulated. The maximum cumulative release of BSA for PCL 9% (w/v) was 19% of 239.47 μg BSA encapsulated. Following the analysis of BSA release profiles, GDNF release was tracked similarly over 25 days from GDNF-BSA microspheres. The microspheres demonstrated a sustained release of GDNF at low concentrations over 25 days. PCL 6% (w/v) microspheres released a maximum GDNF amount of 178 ng on day 1 of release studies while PCL 9% (w/v) microspheres released a maximum of 186 ng on day 1 as well. Again, the amount of protein released by the microspheres decreased each subsequent day following maximum release. Figure 3B shows the normalized release data for both sets of microspheres. PCL 6% (w/v) exhibited a maximum cumulative release of 11.3 % of its total GDNF while PCL 9% (w/v) showed a cumulative release of 5.3% of its total. The release data at each day between the PCL 6% (w/v) and PCL 9% (w/v) microspheres was significantly different ($p<0.05$).

3.4 Bioactivity of released GDNF

GDNF bioactivity was assessed to determine if the drug was still viable after microsphere release. Cells were treated with PCL 6% (w/v) and PCL 9% (w/v) GDNF washes from day 1, 3, 12, 19, and 25 of the release study, corresponding to 89 ng/mL, 42 ng/mL, 10 ng/mL, 5 ng/mL, and 3 ng/mL of GDNF. Qualitatively, it was evident that PC12 cells exposed to release study wash GDNF experienced greater neurite growth than the control (Figure 4). By counting neurite extensions, GDNF wash tests at each day for the PCL 9% (w/v) sample yielded a greater amount of positive PC12 cells observed than the control. There was no significant difference at days 12, 19 and 25 for the PCL 6 % (w/v) compared to the control. Neurite outgrowth in test concentrations of 42 ng/mL, 10 ng/mL, 5 ng/mL, and 3 ng/mL decreased successively from the 89 ng/mL test. All tests except PCL 6% (w/v) at 3 ng/mL were statistically greater than the negative control (Figure 5). There was no statistical difference between PCL 6% (w/v) and PCL 9% (w/v) samples at each time point. The positive control test yielded similar results. Cells were treated with stock GDNF at concentrations of 100 ng/mL, 50 ng/mL, 10 ng/mL, 5 ng/mL, and 2.5 ng/mL. Again, it was evident that PC12 cells exposed to stock GDNF experienced greater neurite growth than the negative control. Neurite outgrowth in test concentrations of 50 ng/mL, 10 ng/mL, and 2.5 ng/mL decreased successively

from the 100 ng/mL test with the only test concentration of 5 ng/mL having a similar extent of neurite outgrowth to 10 ng/mL. There was no significant difference between stock GDNF and released GDNF compared for both sets of microspheres at 2.5 ng/mL, 10 ng/mL and 100 ng/mL.

4. Discussion

We were able to successfully encapsulate clinical grade GDNF into PCL microspheres that could release these proteins over 25 days. When fabricating the microspheres, increasing PCL concentration increased microsphere diameter and encapsulation efficiency of the target molecule. The microspheres with the largest diameter and highest encapsulation efficiency were 9% (w/v) polymer microspheres. This pattern is consistent with previous work demonstrating that an increase polymer concentration increases both microsphere diameter and encapsulation efficiency [24, 49, 50]. As polymer concentration increases, the solution becomes more viscous with a higher degree of polymer chain structure. During the fabrication process, these properties may allow conservation of larger microspheres in the high shear stress environment caused by the magnetic mixer and prevent protein loss to the outer solution. The size distribution of microspheres in each batch was very broad. Although microsphere size and its distribution are reproducible, standard deviations up to 50 % of the average diameter are not unusual. Control of sphere size and size distribution has several important implications for controlled-release drug delivery. There are different methods to fabricate polymer microspheres with controlled and uniform size, such as spraying [51]. For clinical applications, filtration could be also used to select a homogenous size distribution of microspheres. We would strongly suggest sieving microspheres to fabricate more homogenously microspheres for the clinical applications of such drug delivery systems.

Morphology deformation over time was observed and coincided with the release of encapsulated drug over time. As the microsphere structure changes, drug diffuses from the microsphere matrix into the surrounding outer solution by simple diffusion. The corresponding morphological change observed using SEM imaging and its correlation with our quantitative protein release data demonstrates long-term drug delivery from the microspheres.

When encapsulating BSA, the release profile of PCL 6% (w/v) microspheres displays more diffusion based kinetics as they release almost all (88%) of the encapsulated protein over 25 days. In contrast, we observed that PCL 9% (w/v) retained more protein over time, releasing only 19% of encapsulated protein. Here we observed that PCL 9% (w/v) has a slower release rate than PCL 6% (w/v). This was expected as a higher concentration of PCL used in the fabrication process would create denser polymer structure more resistant to swelling and consequently changing in morphology. A possible theory to explain this is that a thicker shell fabricated by PCL 9% (w/v) solution could provide more resistance to diffusion of theproteins. It is probable that if the release study was continued beyond 25 days, additional morphological changes of PCL 9% (w/v) microspheres would occur with the subsequent release of the remaining encapsulated protein . With this observation, PCL 9% (w/v) could be used for the encapsulation of GDNF where a slow, prolonged release of the drug is desired such as the application to PD. Conducting a longer release study of up to one year would further determine the relevance of these microspheres for clinical applications

The fabrication volumes were downscaled to make PCL microspheres encapsulating GDNF. The release profile of PCL 6% (w/v) GDNF-BSA microspheres display a slower rate of release of GDNF (total release of 11.3%) compared to BSA in PCL 6% (w/v) BSA microspheres (total release of 88%) over 25 days. Similarly, we observed that PCL 9% (w/v) GDNF-BSA microspheres had a slower GDNF release rate (total release of 5.3%) compared to the BSA counterpart (total release of 19%). Our results showed that GDNF release was slower than BSA release. This effect could be due to the differing concentration gradients of BSA and GDNF having an effect on diffusion rates and thus release rates. BSA release alongside GDNF from GDNF-BSA microspheres supports this as there was a larger accumulative release of BSA compared to GDNF (data not shown). In the BSA study a maximum of 1.7 mg of BSA was encapsulated in PCL 9% (w/v) microspheres, meanwhile in the GDNF study, only a maximum of 0.087 mg of GDNF was encapsulated inside PCL 9% (w/v) microspheres. One of the drawbacks of using double emulsion technique is low encapsulation efficiency of proteins [52]. To overcome this issue, using other techniques such as spray-drying and single phase oil in oil solvent evaporation have been reported [52, 53] . Our GDNF microsphere batches released a maximum of 178 ng/mL and 186 ng/mL on day 1 for PCL 6% (w/v) and PCL 9% (w/v) microspheres respectively. The concentration decreased each subsequent day reaching between 5 and 25 ng/mL from days 8-25. These concentrations are therapeutically effective based on the literature data [33].Previous studies on GDNF delivery from PLGA microspheres show faster rates of release at around 45% and 55% cumulative release at 25 days [54]. Our results confirm that fabricating microspheres using PCL results in slower protein release. One possible theory for explaining why the GDNF release is slower than BSA is GNDF increased affinity to the PCL matrix than BSA, since the pI value of GDNF (~9.4 - 10) is greater than BSA (~4.7-5). The relatively low release of GDNF, around 5% and 11% over 25 days for both types of microspheres, shows that additional assays would be recommended as future work to study the release GDNF for a longer time of up to one year. Possible methods for improving GDNF release from PCL microspheres could be the use of plasma modification in order to change the PLC surface properties such as increasing its hydrophilicity. Moreover, synthesis of amphiphilic copolymers based on PCL polymer to add hydrophilic polymeric chains into PCL backbone to increase the level of swelling and consequently increasing the release of loaded drug. Finally, future *in vivo* release studies in animal models would help to evaluate the potential of such microspheres for neural tissue repair

In reservoir-based delivery systems, such as microspheres, a polymer structure surrounds the drug reservoir and the release is modulated through different mechanisms [28]. In microspheres, drugs are non-covalently embedded in the porous polymer structure. Generally, delivery of drug is firstly controlled by diffusion of the drug through the biodegradable polymer structure. The release mechanism then can be governed by the surface or either bulk degradation of microspheres. The type of release kinetic can be distinguished by several empirical well-known models including Peppas, Hixon-Crowell, Higuchi models and etc. [55]. For example, if the release mechanism is mainly governed by diffusion mechanism, the kinetics of drug release from can be estimated using the Peppas model:

$$Q = Kt^n \quad or \quad Log(Q) = Log\ k + nLog(t)$$

Where, Q (%) is the accumulated amount of the released drug (BSA or GDNF), *k* (%/t^n) is the rate constant of release which depends on the structural characteristics, and *t* (day) is the release time, *n* is the release exponent which indicates the release mechanism. Figure 6a and 6b illustrate the linear regression of the BSA and GDFN release data from PCL 6% (w/v) and PCL 9% (w/v), and PCL 6% (w/v) and PCL 9% (w/v) microspheres respectively. Table 3 summarizes the Peppas model parameters for the controlled release of BSA and GDNF from both sets of microspheres.

Here, the *n*-values of the PCL BSA microspheres were 0.97 and 0.79 for PCL 6% (w/v) and PLC 9 respectively. The R^2 correlation coefficients were around 0.96 for both sets of microspheres. Since the n values were reported higher than 0.5, we assume that the release kinetics is controlled by diffusion. In the case of PCL-GDNF microspheres *n*-values were reported around 0.40 which is a good approximation for the *n*-value of 0.5 which indicates that the release mechanism is controlled by diffusion. Compared to PCL-BSA microspheres, these results are consistent since we have very low release rate for the PCL-GDNF microspheres which proved that the release is controlled by diffusion mechanism. As shown in Table 3, the *k*-values of the PCL-BSA microspheres were decreased as the PCL concentration of PCL solution is increased. These results confirmed the slower rate of BSA release from PCL 9% (w/v) compared to PCL 6% (w/v) microspheres. Similarly, in the case of PCL-GDNF microspheres, faster release rate is related to PCL 6% (w/v) samples.

GDNF washes from the release study were observed to induce PC12 neurite outgrowth. Since all wash GDNF samples yielded more cells with neurite extensions than the negative controls at significance, the highest neurite outgrowth was induced from the highest concentration of GDNF subsequently decreasing to the lowest concentration, and neurite extensions compared to stock GDNF (positive control) were not statistically different at 2.5 ng/mL, 10 ng/mL and 100 ng/mL, it can be inferred that the bioactivity of GDNF was retained through the fabrication and release study process. Since GDNF was released from a biocompatible and biodegradable polymer, there would be no change in the GDNF bioactivity. Consequently, this method of delivery would be introduced as a more efficient alternative compared to the CED technique. In continuing the study, it would be preferable to use a human derived protein as a carrier alongside GDNF to replace BSA, an animal product. Such an adjustment would provide a more clinically relevant formula for studies with PD patients.

5. Conclusions

This study investigated a GDNF delivery system composed of PCL degradable microspheres for long term drug release. We successfully fabricated GDNF-loaded PCL microspheres using a double emulsion technique. The polymer concentration used during microsphere fabrication affected drug release rates and these microspheres were shown to exhibit a slower GDNF release profile over 25 days compared to previous studies using PLGA microspheres. PC12 cell neurite outgrowth confirmed the bioactivity of released GDNF from the microspheres. On-going work is aimed at incorporating these novel microspheres into a composite biomaterial scaffold as a potential therapy for PD.

Acknowledgments

The authors would like to acknowledge support from an NSERC Discovery Grant (S.M.W.) and an NSERC Engage Grant with MedGenesis Therapeutix. They would also like to acknowledge the Advanced Microscopy Facility at the University of Victoria and MedGenesis Therapeutix for their on-going support of this project.

Potential Conflicts of Interest

The corresponding author previously held an Engage Grant with MedGenesis Therapeutix and MedGenesis Therapeutix supported this project via donation of GDNF.

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Table 1. Average diameter of encapsulated microspheres (± standard deviation).

Microsphere	Microsphere diameter (µm)
PCL 3% (w/v) - BSA	57.86 ± 21.32 (n=43)
PCL 6% (w/v) - BSA	123.22 ± 77.16 (n=145)
PCL 9% (w/v) - BSA	100.73 ± 45.72 (n=369)
PCL 6% (w/v) GDNF-BSA	82.75 ± 49.87 (n=458)
PCL 9% (w/v) GDNF-BSA	155.22 ± 64.61 (n=85)

Table 2. Average encapsulation efficiency of loaded microspheres (± standard deviation).

Microsphere	Average encapsulation efficiency (%)
PCL 3% (w/v) - BSA	3.33 ± 2.4%
PCL 6% (w/v) - BSA	15.8 ± 3.1%
PCL 9% (w/v) - BSA	34.1 ± 7.6%
PCL 6% (w/v) GDNF-BSA	34.6 ± 15.6%*
PCL 9% (w/v) GDNF-BSA	43.7 ± 10.3%*

*refers to the percentage of GDNF encapsulated as opposed to total protein

Table 3. The Peppas model kinetic parameters of BSA and GDFN released from microspheres

Peppas Model Parameters		**k**	**n**	**R^2**
BSA	PCL 6% (w/v)	5.04	0.97	0.967
	PCL 9% (w/v)	1.82	0.79	0.9619
GDNF	PCL 6% (w/v)	1.81	0.4	0.931
	PCL 9% (w/v)	1.56	0.42	0.9626

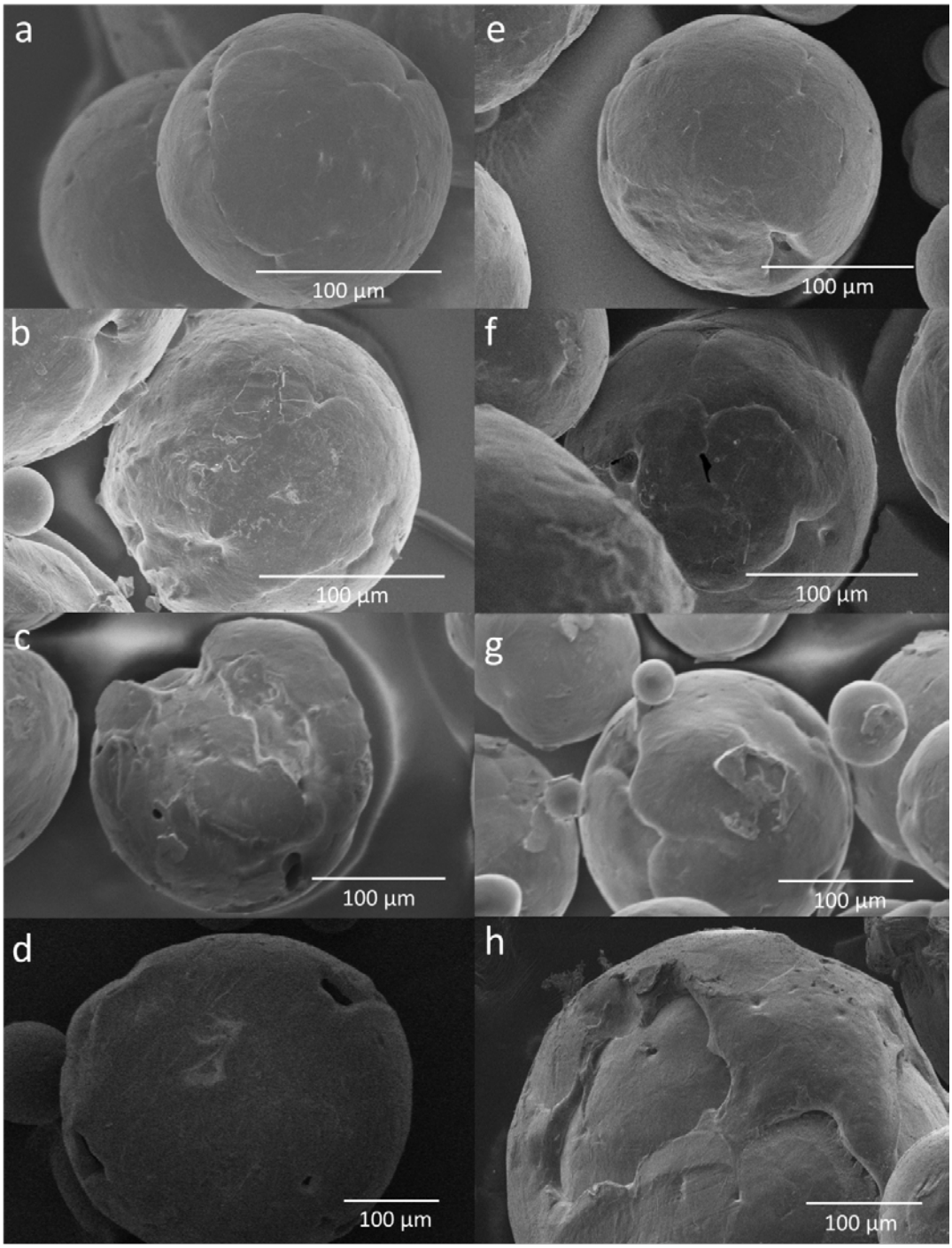

Fig. 1. Scanning electron microscopy images of (a, b, c, d) PCL 6% (w/v) and (e, f, g, h) PCL 9% (w/v) BSA microspheres taken on day 1 (a, e), day 7 (b, f), day 14 (c, g), and day 25 (d, h) of the release studies.

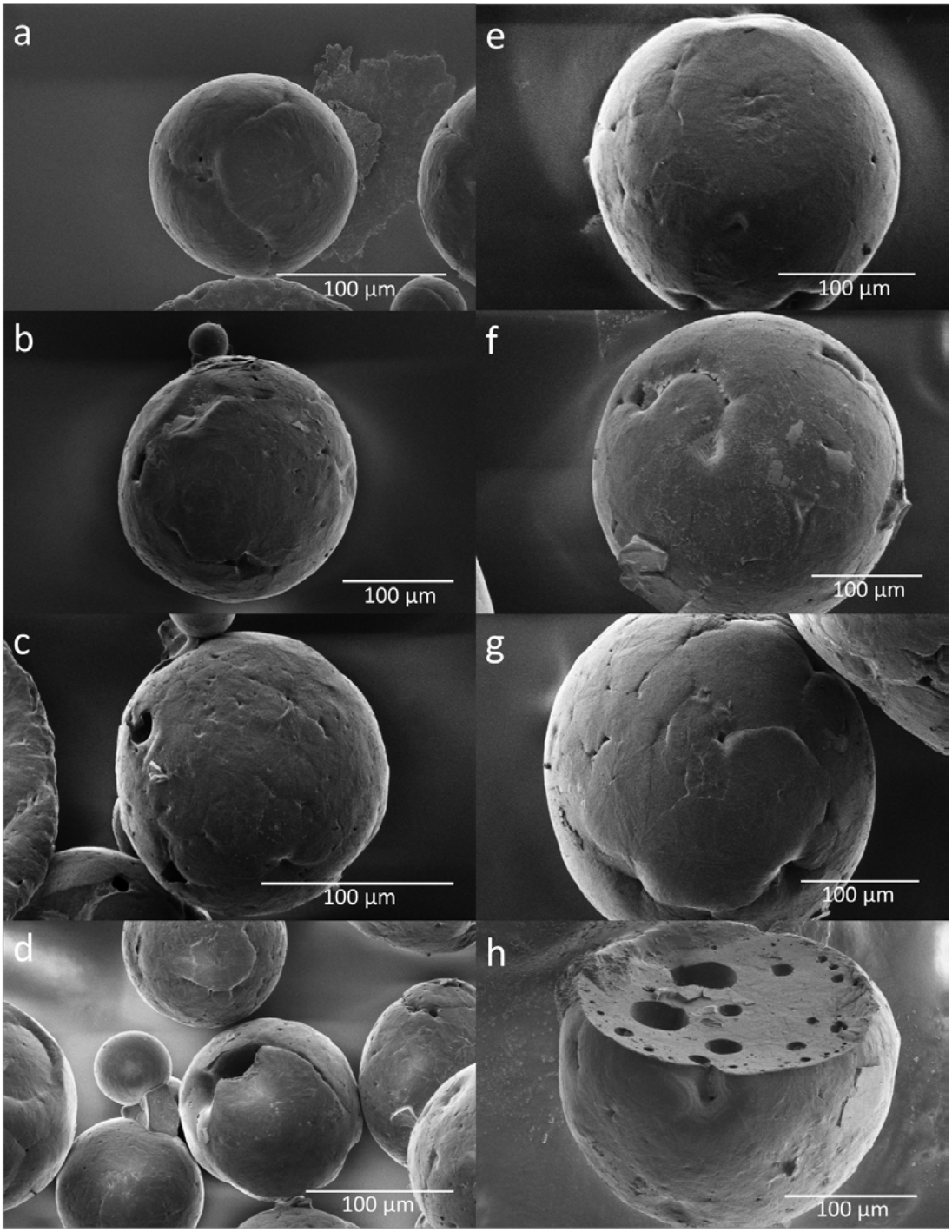

Fig. 2. Scanning electron microscopy images of (a, b, c, d) PCL 6% (w/v) and (e, f, g, h) PCL 9% (w/v) GDNF-BSA microspheres on day 1 (a, e), day 7 (b, f), day 14 (c, g), and day 25 (d, h) of the release studies.

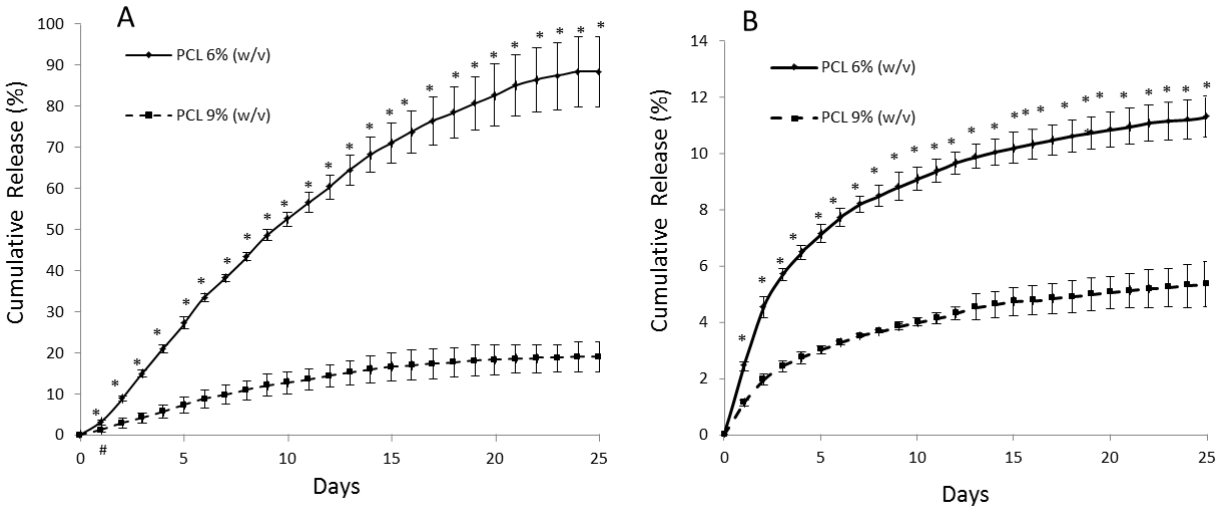

Fig. 3. Release Profiles: (A) *In vitro* cumulative BSA release from PCL 6% (w/v) and PCL 9% (w/v) microspheres. Standard deviations are shown. The release data at each day between the PCL 6% (w/v) and PCL 9% (w/v) microspheres was significantly different ($p<0.05$). (B) *In vitro* cumulative GDNF release from PCL 6% (w/v) and PCL 9% (w/v) GDNF-BSA encapsulated microspheres with BSA as a carrier protein. Release was calculated from total theoretical encapsulated GDNF in each microsphere sample (13600 ng in 6% and 15200 ng in 9%). Standard deviations are shown. The release data at each day between the PCL 6% (w/v) and PCL 9% (w/v) microspheres was significantly different ($p<0.05$).

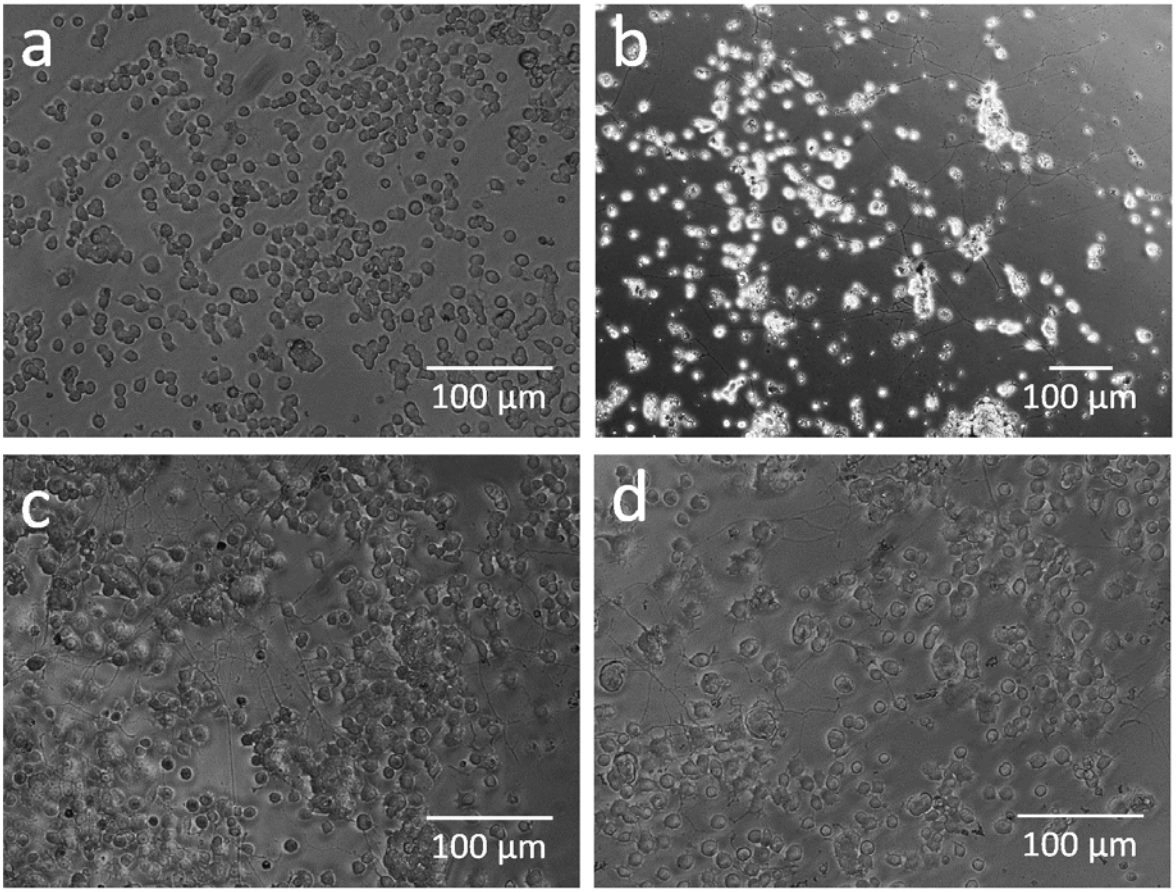

Fig. 4. Neurite extensions observed at 10X magnification of PC12 cells. (a) Control (no GDNF present), (b) Positive Control (100 ng/mL), (c) Day 1 Wash (89 ng/mL GDNF), and (d) Day 12 Wash (20 ng/mL GDNF) (c). Cells were plated on laminin in a 96 well plate, incubated at 37˚C and 5% CO2, and photographed in phase contrast 10 days after treatment with varying concentrations of GDNF released during a 25 day release study from PCL-based microspheres.

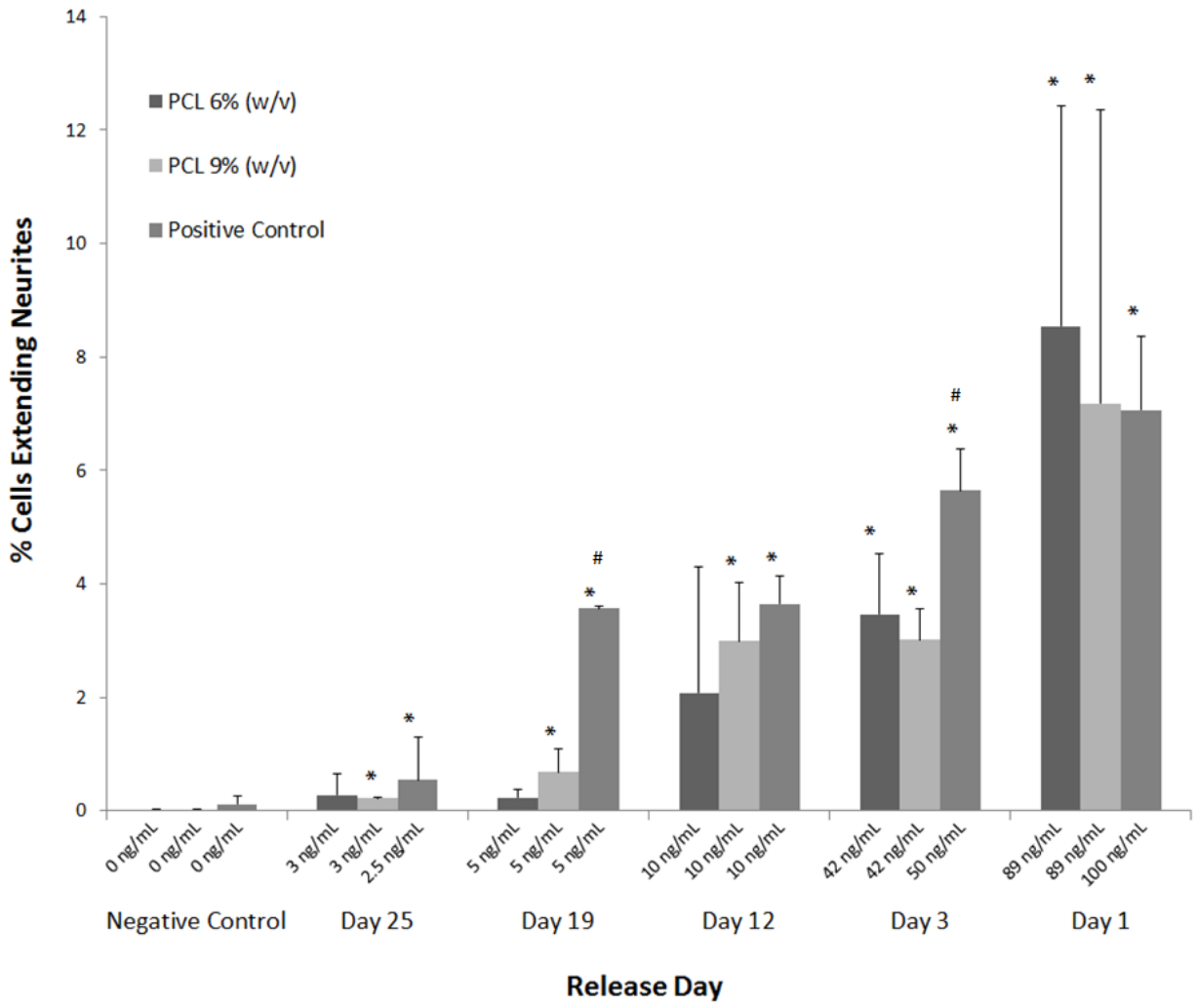

Fig. 5. (A) Percentage of cells extending neurites in total cells observed at 10X magnification of PC12 cell replicates. Cells were plated on laminin in a 96 well plate, incubated at 37˚C and 5% CO_2, and photographed 10 days after treatment with varying concentrations of stock GDNF and GDNF released during a 25 day release study. There is no significant difference reported between the GDNF released from the two microspheres for each wash concentration. * indicates $p<0.05$ versus control. # indicates $p< 0.05$ versus the two microspheres.

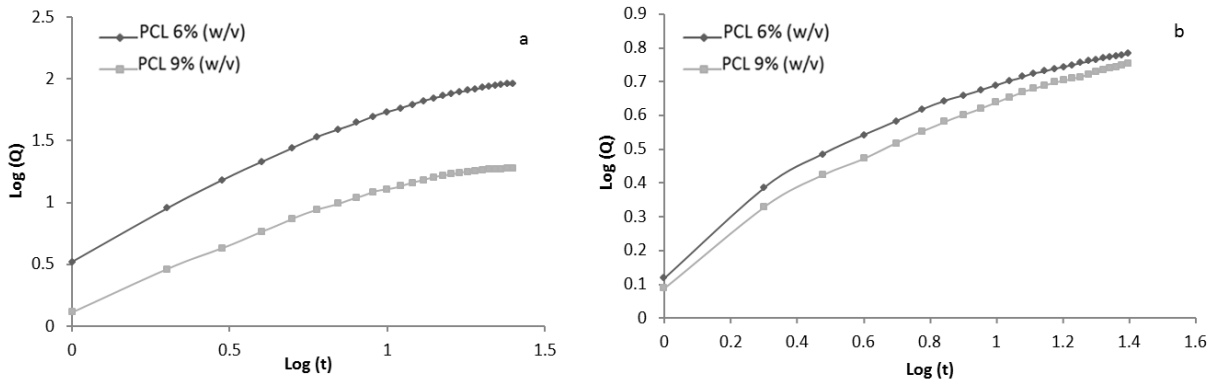

Fig.6. The linear regression of (a) BSA and (b) GDNF release data based on the Peppas Model.