In this study, a poly(lactic acid) (PLLA) porous film with longitudinal surface micropatterns was fabricated by a dry phase inversion technique to be used as potential conduit material for peripheral nerve regeneration applications. The presence of a nerve growth factor (NGF) gradient on the patterned film surface and protein loaded, surface-eroding, biodegradable, and amphiphilic polyanhydride (PA) microparticles within the film matrix, enabled co-delivery of neurotrophic factors with controlled release properties and enhanced neurite outgrowth from PC12 cells. The protein loading capacity of PA particles was increased up to 80% using the spray drying technique, while the surface loading of NGF reached 300 ng/cm² through ester-amine interactions. The NGF surface gradient provided initial fast release from the film surface and facilitated directional neurite outgrowth along with the longitudinal micropatterns. Furthermore, the variable backbone chemistry and surface eroding nature of protein-loaded PA microparticles within the film matrix ensured protein stability and enabled controlled protein release. This novel co-delivery strategy yielded tunable diffusion coefficients varying between $10^{14}$ to $10^{10}$ cm²/min and dissolution constants ranging from $10^{-4}$ to $10^{-3}$ min⁻¹ with released amounts of 100–300 ng/mL. This strategy promoted guided neurite extension from PC12 cells of up to 10 μm in 2 days. Overall, this unique strategy can potentially be extended for individually programmed delivery of multiple growth factors through the use of PA microparticle cocktails and can further be investigated for in vivo performance as potential conduit material for peripheral nerve regeneration applications.

**Statement of Significance**

This manuscript focuses on the development of multifunctional degradable polymer films that provide topographic cues for guided growth, surface gradients of growth factors as well as nanoparticles in the films for tunable release of growth factors to enable peripheral nerve regeneration. The combination of cues was designed to overcome limitations of current strategies to facilitate peripheral nerve regeneration. These multifunctional films successfully provided high protein loading capacities while persevering activity, protein gradients on the surface, and tunable release of bioactive nerve growth factor that promoted directional and guided neurite extension of PC12 cells of up to 10 μm in 2 days. These multifunctional films can be made into conduits for peripheral nerve regeneration.

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**1. Introduction**

Bioengineered artificial nerve conduits, based on synthetic, degradable and functional polymeric biomaterials, have been considered as a promising tool to facilitate peripheral nerve regeneration [1–6]. The current artificial conduit systems with various functionalities have aimed to circumvent the limitations of autologous nerve grafting, such as biological complexity, donor site
availability, morbidity and requirement of multiple surgeries [4,7–10]. However, these conduits systems have not yet reached the regeneration potential of autologous nerve grafts or cell-based regenerative therapies [11,12]. Different conduit development strategies based on various functional biomaterials have been investigated in the literature. Most have used degradable polyester- (such as, poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA)) based conduits or particle systems that provide an environment mimicking the extracellular matrix (ECM) for peripheral nerve regeneration and enable efficient controlled release of nerve growth factors through cross-linking, changes in backbone chemistry, molecular weight or hydrophobic surface coatings [13–18]. However, these polymers potentially suffer from the bulk polymer erosion [19,20] and formation of acidic metabolites [21]. A porous structure, enabling nutrient permeability along with available space for proper cellular network formation, is a desirable conduit property [22–24]. However, the use of pore forming agents prevents the possible distribution of neurotrophic factors or microparticles through the conduit matrix, limiting the loading capacity and controlled release properties [24]. Recently, porous conduits were modified through surface nano/micropatterning [9,10,25–27] and neurotrophic factor attachment with surface gradients [14,28,29] to promote guidance along with the controlled release properties. However, the neurotrophic factor loading capacity and controlled release are limited by the conduit surface area, conjugation efficiency and crosslinking strategy, which can potentially lead to protein denaturation and activity loss as well as prominent burst release.

In this study, the combination of multiple properties, including porosity, micropatterns that provide physical guidance, surface growth factor gradients and controlled growth factor release that aid peripheral nerve regeneration have been incorporated into a single film. Such biodegradable films can facilitate potential conduit design, using approaches that we have developed in our past work [27]. For this work, ester terminated poly-ε-lactic acid (PLLA) was selected as the film material due to biodegradability, low toxicity and functional end groups [3]. The porous film structure was created through a dry-phase inversion technique [30]. This approach not only allows growth factor/microparticle distribution within the film matrix but also enabled establishment of an efficient growth factor surface gradient, resulting in a combination that provided simultaneous controlled release of multiple growth factors. There have been many different studies focusing on the controlled release of multiple growth factors using various strategies [31–34]. However, using both the film surface with gradients, and the matrix embedded with microparticles at the same time, while maintaining porosity, is a novel strategy to provide enhanced protein loading and controlled release properties. β-NFG surface gradients were created on PLLA film surfaces with longitudinal micropatterns through ester-amine interactions [35] and physical self-assembly to provide initial β-NFG release and facilitate guided neurite outgrowth in PC12 cells. A model protein, ovalbumin (OVA), encapsulated in spray dried polyanhydride (PA) microparticles with various chemistries, was incorporated into the porous film matrix to demonstrate sustained/controlled release properties. Biocompatible, biodegradable and amphiphilic polyanhydrides were utilized as a promising alternative to their polyester counterparts due to high protein loading and stability, surface erosion controlled release features, and less acidic degradation products [36–38]. The characterization of the developed films, controlled release properties of multiple proteins along with their diffusion coefficients, and in vitro neurite extension and outgrowth performance testing using PC12 cells were used to gauge the efficacy of these multifunctional films as potential conduit materials. This novel strategy can be further aimed for simultaneous delivery of multiple neurotrophic factors to enhance peripheral nerve regeneration both in vitro and in vivo.

2. Materials and methods

2.1. Materials

Poly-ε-lactide (ε-PL-ester terminated B6002-2) was obtained from Lactel Absorbable Polymers. Beta-nerve growth factor (β-NFG, 556-NG/CF) was purchased from R&D Systems. Ovalbumin (OVA) was purchased from InvivoGen. Methylene chloride and pentane were supplied from Fisher Scientific. β-NFG ELISA kit (ab100757) and OVA ELISA kit (EKU06441) was ordered from Abcam and Biomatik, respectively. RPMI-1640 cell culture media and supplies, fetal bovine serum (FBS) and heat inactivated horse serum (HS), and phosphate-buffered saline (PBS) were obtained from Invitrogen. Bovine serum albumin (BSA), paraformaldehyde (PFA) and triton-x100 were obtained from Fisher Scientific. Cultrex® Mouse Laminin I was purchased from Trevigen. Anti-ßIII tubulin antibody conjugated with Cy3 (AB15708C3) was obtained from EMD Millipore while DAPI (4',6-diamidino-2-phenylindole) dye was ordered from Invitrogen. All the other required buffers were prepared by using ultrapure water according to standard laboratory procedures.

2.2. Synthesis of copolymers and fabrication of bare and β-NFG loaded microparticles

Random copolymers of CPTEG:CHP (1.6-bis (p-carboxy phenoxy) hexane (CHP), 1.8-bis (p-carboxyphenoxy)-3,6-dioxao tane (CPTEG)) were synthesized from the corresponding monomers with different copolymer ratios via a melt polycondensation reaction as described previously [36,39]. Nuclear magnetic resonance spectroscopy (NMR) was used for resolving the structure and determining the molecular weight of the copolymer. Polymeric microparticles (bare or NGF loaded) were fabricated using a benchtop spray-dryer (Buechi, Switzerland) to increase the β-NFG encapsulation efficiency. The polymer-protein solution (200 mg of polymer and 100 µg of β-NFG in 20 mL methylene chloride) was fed to the spray-dryer through a nozzle with argon as the feed gas at the feed temperature of 24 °C, vacuum of −50 bar. The particles with 0.05% β-NFG loading were collected using a high efficiency cyclone. Scanning electron microscopy (SEM, Quant FEG 250) imaging was performed to determine the dimensions and structure of nanoparticles obtained.

2.3. β-NFG release from microparticles

β-NFG release from the microparticles (5 mg) was conducted in 200 µL PBS, supplemented with 0.5 wt% BSA as stabilizer, at 37 °C with continuous shaking. Sample supernatants were collected at predetermined time points up on centrifugation (10 min at 15,000 rpm) and replaced by fresh 200 µL of 0.5 w/o BSA-PBS solution. The concentration of released β-NFG collected in the sample supernatant was analyzed using a β-NFG ELISA kit following the manufacturer’s procedure.

2.4. Preparation and characterization of β-NFG/microparticle incorporated, porous and surface micropatterned PLLA films

Dry phase inversion technique was used to prepare β-NFG/microparticle-incorporated, porous and surface micropatterned PLLA films. For this purpose, PLLA (1 g) was dissolved in chloroform (10 mL) to create a 10 wt% polymer solution. At the same time,
β-NGF-encapsulated polyanhydride particles (20 mg) were dispersed in 400 μL pentane (volatile non-solvent), which was used to provide a protective layer around particles preventing their dissolution in chloroform and facilitating their uniform distribution in the PLLA film matrix. Then, 1 mL of 10 wt% PLLA solution was added on top of particles in pentane and shortly vortexed. This 1.4 mL casting solution was poured on a silicon wafer with longitudinal micropatterns (4 x 4 cm² area) and spin coated for 10 min. Then, the films were left to dry at room temperature for 6 h, where the different evaporation rates of pentane (non-solvent) and chloroform (solvent) resulted in phase separation and porous structure formation. At the end of drying, the films were peeled off the wafer surface and their structure was characterized by SEM.

2.5. Formation of β-NGF concentration gradient on the film surface

For the formation of β-NGF concentration gradient on the film surface, the method described elsewhere was applied with modifications [14]. The PLLA films with dimensions of 1 x 3 cm² were placed upright in a tube as shown in Supplementary Information, Fig. S.1. The β-NGF solution (10 μg/mL) was pumped into the tube at a velocity of 5 μL/min using a syringe pump in a cold room (at 4 °C) during 6 h. Then, the films were stored at 4 °C and rinsed with sterile PBS three times before use. The differential β-NGF exposure along the length of the films parallel to the tube walls created concentration gradients on the film surface (Fig. S.1.). It was anticipated that the β-NGF molecules were attached to the film surface through the interaction between the ester ends of PLLA and amine groups of β-NGF along with hydrophobic self-assembly and protein–protein interactions [35]. The presence of a β-NGF gradient was determined by X-ray photoelectron spectroscopy (XPS). Analyses were performed on the film surface starting from section-1 to section-3 by taking at least 3 random measurements in each section (Fig. S.1.). Throughout the analysis a monochromatic Al K α X-ray source (1486.6 eV) with an Omnic Focus III small area lens and multichannel detector was used. Measurements were taken at an electron take-off angle 45° from a normal sampling surface depth of ~50 Å. Survey scans were collected from 10 eV to 1100 eV with a pass energy of 187.85 eV. All spectra were referenced by setting the C 1s peak to 285.0 eV to compensate for residual charging effects.

2.6. β-NGF and OVA release kinetics from PLLA films

Polyanhydride particles loaded with different amounts of model protein OVA (1, 3 and 5%) were prepared as described in Section 2.2 for the release experiments. PLLA films containing OVA-loaded PA microparticles within the matrix and with an NGF gradient on the surface were fabricated as described in Sections 2.4 and 2.5. OVA-loaded PA particles incorporated into the PLLA films with surface NGF gradients were used to distinguish the controlled release characteristics from the film matrix and surface. Prepared PLLA films (1 x 3 cm²) were cut into 3 sections (1 x 1 cm²) each section representing the higher, middle and lower β-NGF surface concentration gradients (Fig. S.1.). The sections were placed in 1 mL PBS buffer at pH 7.4 and 37 °C. The simultaneous release of OVA from the PLLA film matrix and β-NGF from the film surface were observed. The samples were collected at predetermined times and the released amounts of OVA and β-NGF were detected using respective ELISA kits following the manufacturer’s procedure.

2.7. Determination of transport properties of PLLA films

Following the experimental data obtained through the release kinetics tests, the diffusion coefficient of OVA, released from the microparticles distributed in PLLA matrix, and dissolution constants of β-NGF, released from the PLLA film surface, were calculated using related mathematical models. The release rate of OVA was assumed to be controlled only by its diffusion through the microparticles distributed in the PLLA film matrix, where Fick’s second law was used as the main transport equation. It was assumed that there was no chemical reaction between OVA and the film, the mass transfer is by diffusion only, the diffusivity of OVA is constant, and the thickness of the film does not change due to desorption of OVA. According to a solution of Fick’s second law with one initial and two boundary conditions applied [40], the total amount of active compound desorbed from the film at any time t, due to Fickian diffusion, M₀ is given by the following expression.

\[ M_t = M_\infty \left[ 1 - \sum_{n=1}^{\infty} \frac{2\alpha(1 + \alpha)}{1 + \alpha' q_n} \exp\left(-Dq_n^2t/L^2\right) \right] \]  \( (1) \)

where \( M_\infty \) is the amount of protein released at equilibrium, and the \( q_n \)’s are the non-zero positive roots of \( \alpha = \frac{\ln q_n}{\ln \frac{C}{C_0}} \).

\[ \tan q_n = -\alpha q_n \]  \( (2) \)

The partition coefficient, K, used in the definition of \( \alpha \) was defined as the ratio of OVA concentration in the film to that in the solution at equilibrium and it was calculated from the difference in the equilibrium and the initial concentrations of OVA measured in the solution, assuming that OVA does not degrade during the test.

\[ \text{Eq. (1)} \] clearly indicates that in cases where the release is controlled only by Fickian diffusion, the diffusivity of OVA in the film is the main transport parameter and it was determined by minimizing the difference between the experimental data and the model predictions from Eq. (1).

\[ \beta-NGF \] directly released from the film surface is based on the dissolution as described in the following equation adopted from previous work [41].

\[ M_{\text{Dissolution}} = M_\infty \left[ 1 - \exp\left(-kt\right) \right] \]  \( (3) \)

\( M_{\text{Dissolution}} \) represents the released \( \beta-NGF \) amount at time, t, and \( M_\infty \) is the amount of \( \beta-NGF \) released at equilibrium. ‘k’ is the dissolution constant.

2.8. Cell culture of PC12 cells

PC12 cells (CRL-1721, ATCC), which are known to extend their neurites in the presence of \( \beta-NGF \), were grown in complete cell culture media containing 85% RPMI-1640 Medium (30-2001, ATCC) supplemented with 10% (v/v) heat-inactivated horse serum and 5% (v/v) fetal bovine serum at 37 °C under a humidified atmosphere containing 5% CO₂. Cells were subcultured approximately every 2–3 days.

2.9. Bioactivity of \( \beta-NGF \) encapsulated microparticles on PC12 cells

Neurite extension assay of PC12 cells to assess the bioactivity of \( \beta-NGF \) released from 50:50 CPTEG:CPH microparticles was performed. Previously reported nontoxic concentration of particles (125 μg/mL or less) was used to assess the bioactivity of the released \( \beta-NGF \) [42,43]. Before the experiment, the 6 well plates were coated with mouse laminin (10 μg/mL) and incubated at 37 °C overnight to facilitate the attachment of PC12 cells. PC12 cells were then plated to the wells (2 x 10⁴ cells per cm²) in 3 mL of complete cell culture media while the \( \beta-NGF \) encapsulated particles were placed onto the transwell permeable membrane support (BD Falcon) to perform a non-contact bioactivity assay. \( \beta-NGF \) at a concentration of 100 ng/mL was used as a positive control while bare microparticles (without \( \beta-NGF \) encapsulated)
and PC12 cells were used as negative controls. After 2 days of incubation at 37 °C under a humidified 5% CO₂ atmosphere, cells were immunostained with anti-βIII tubulin antibody conjugated with Cy3 and DAPI to visualize neurite extension and nuclei of PC12 cells, respectively. Imaging of the cells was conducted using an ImageXpress Micro high content imaging system (Molecular Devices) and the length of neurites quantified using the Neurite outgrowth module of MetaXpress software (Molecular Devices). At least 36 random fields per condition were imaged using a 10× objective in the ImageXpress Micro high content screening system. After imaging, the Neurite outgrowth module of the MetaXpress software was used to quantify the total number of cells using DAPI counts and also neurite length using the corresponding βIII-tubulin-Cy3 images. All the cells present in all the fields were used for the quantification. This software module is capable of segmenting the nuclei even in clusters by defining the minimum and maximum diameter of the objects and intensity above the background. This software also permits the quantification of cell body size and can be set to quantify the length of processes emanating from the cell bodies depending upon the signal to background intensity ratios above a particular threshold. Because all the cells were used for quantification in this assay, including the cells which had neurites as well as the cells which did not have neurites, the average value of neurite outgrowth per cell is expected to be smaller than those reported in the literature.

2.10. Assessment of bioactivity of β-NGF released from PLLA films on PC12 cells

The PLLA films, incorporated with 0.5% β-NGF encapsulated in 50:50 CPTEG:CPH microparticles, and possessing surface β-NGF gradient were prepared, glued on a glass coverslip (micropatterned surface is upward) and sterilized. The micropatterned films were placed into a 6 well plate and coated with mouse laminin (10 μg/mL) at 37 °C for 6 h to facilitate the attachment of PC12 cells. PC12 cells (2 × 10⁴ cells/cm²) were seeded on section-2 (Fig. S.1.), which contains the medium β-NGF surface concentration, to observe both the effect of micropatterning and surface gradient on neurite alignment and extension. Following 2 days of incubation at 37 °C under a humidified atmosphere containing 5% CO₂, the cells on the films were immunostained, imaged by ImageXpress Micro high content imaging system and length of neurites was quantified using Neurite outgrowth module of MetaXpress software as mentioned in Section 2.9.

2.11. Neurite alignment of PC12 cells on β-NGF gradients and micropatterned PLLA films

PLLA films (1 × 3 cm²), incorporated with 0.5% β-NGF encapsulated 50:50 CPTEG:CPH microparticles and possessing surface β-NGF gradient were prepared, glued on a glass coverslip (micropatterned surface is upward) and sterilized. The micropatterned films were placed into a 6 well plate and coated with mouse laminin (10 μg/mL) at 37 °C for 6 h to facilitate the attachment of PC12 cells. PC12 cells (2 × 10⁴ cells/cm²) were seeded on section-2 (Fig. S.1.), which contains the medium β-NGF surface concentration, to observe both the effect of micropatterning and surface gradient on neurite alignment and extension. Following 2 days of incubation at 37 °C under a humidified atmosphere containing 5% CO₂, the cells on the films were immunostained, imaged by ImageXpress Micro high content imaging system and length of neurites was quantified using Neurite outgrowth module of MetaXpress software as mentioned in Section 2.9.

2.12. Statistical analysis

Throughout this study, significant differences between groups were evaluated using ANOVA analysis by Tukey's method with 95% confidence interval. The results are presented as average ± standard deviation calculated from at least three independent experiments.

3. Results

3.1. Characterization of polymers, particles and films

3.1.1. Polymer and particle characterization

The NMR characterization shown in Supplementary Information Fig. S.2 indicated that polymer molecular weight, structure,
and composition were as intended and in agreement with previous studies [44–46]. SEM images indicated that the microparticles were found to be of spherical shape and polydisperse in nature possessing sizes ranging from a few nanometers to \( \approx 4 \, \mu m \) (Fig. 1). It was also observed that \( \beta \)-NGF or OVA encapsulation did not affect the particles in terms of shape, size and structure regardless of the loaded amount (Fig. 1).

3.1.2. Film characterization

Porous PLLA films with longitudinal micropatterns, containing 50:50 and 20:80 CPTEG:CPH microparticles loaded with different amounts of OVA or \( \beta \)-NGF, were prepared by the dry phase inversion technique. The type and compositions of the prepared films used in the release tests is shown in Table 1.

The SEM images of the prepared films represented in Fig. 2A and B show the micropatterns and surface porosity on both patterned and flat surfaces. The average pore size of films increased from \( \approx 1 \, \mu m \) to \( \approx 2 \, \mu m \) following the use of films for release experiments for 15 days (Fig. 2C and D). However, the pattern structure and shape were maintained. The cross-sectional images of the films in Figs. 2E and E' indicate the formation of interconnected pore structures.

Upon the preparation of the films with protein-loaded polyanhydride particles, the film surfaces were modified by \( \beta \)-NGF surface gradient through ester amine interactions [35] and physical self-assembly. The peaks at 288.8 and 288.1 eV of the high-resolution C1s spectra are attributed to N–O–C\(^\ominus\) and N–C\(^\ominus\), respectively, which resulted from the ester end groups of PLLA (Supplementary Information Fig. S3A) [35]. Similarly, the XPS spectra of N1s indicate distinguishable increases at 402 eV of N–O as the portion of primary amines in the structure of \( \beta \)-NGF attached on the film surface (Supplementary Information Fig. S3B) [35]. The presence of N1s band on the \( \beta \)-NGF modified film (Supplementary Information Fig. S3D) as compared to the plain film (Supplementary Information Fig. S3C) indicates the presence of \( \beta \)-NGF on the surface. Fig. 3 indicates the changes in surface \( \beta \)-NGF concentration along the film sections. As the exposure time of \( \beta \)-NGF solution increases, the attached \( \beta \)-NGF amount increases along the film sections (Sections-1: lowest, Section-3: highest) leading to the formation of concentration gradient as depicted in Fig. 3.

3.2. \( \beta \)-NGF release from microparticles

The preliminary \( \beta \)-NGF release data obtained from different particles indicated that only 50:50 and 20:80 CPTEG:CPH copolymer chemistries showed reliable \( \beta \)-NGF release in picogram levels in PBS. The released \( \beta \)-NGF amount was subsequently increased to nanogram levels by using 0.5 w\% BSA in PBS as stabilizer. It was

Table 1

<table>
<thead>
<tr>
<th>Film Type</th>
<th>PLLA</th>
<th>DCM</th>
<th>Pentane</th>
<th>Particle type</th>
<th>OVA loading</th>
<th>( \beta )-NGF coating</th>
<th>Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA-1</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 50:50 CPTEG:CPH</td>
<td>1%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-2</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 50:50 CPTEG:CPH</td>
<td>3%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-3</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 50:50 CPTEG:CPH</td>
<td>5%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-4</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 20:80 CPTEG:CPH</td>
<td>1%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-5</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 20:80 CPTEG:CPH</td>
<td>3%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PLLA-6</td>
<td>10%</td>
<td>64%</td>
<td>25%</td>
<td>1% 20:80 CPTEG:CPH</td>
<td>5%</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*, represents the presence of \( \beta \)-NGF coating and micropattern.

Fig. 2. SEM images of porous, OVA encapsulated 50–50:CPTEG-CPH particles incorporated PLLA films. (A) Patterned and (B) flat surfaces of unused films. (C) Patterned and (D) flat surfaces of used films. Films were maintained in PBS at 37 °C for 15 days. Magnification: 2000x. (E) and (E') cross-section of films at magnifications of 500x and 1500x, respectively.
observed that 50:50 and 20:80 CPTEG:CPH particles were able to release β-NFG for up to 8 weeks (Fig. 4). The 50:50 CPTEG:CPH showed a slight burst release with higher amounts of β-NFG at the start (~150 ng/mL), while the 20:80 CPTEG:CPH provided slower release with lower amounts of β-NFG (~40 ng/mL). This was mainly due to the increased hydrophobic CPH content (from 50 to 80) in the polymer chemistry leading to the slow surface erosion of the particles and thus, slow β-NFG release [36,37,39,44,47]. Therefore, controlled NGF release could be achieved through the manipulation of particle chemistry.

3.3. β-NFG and OVA release from films

OVA was used to observe the release characteristics of microparticles distributed in the PLLA film matrix, while β-NFG was used to distinguish the release from the film surface. Diffusion coefficients of OVA and dissolution constants of β-NFG were calculated for each film represented in Table 1 by using the equations mentioned in Section 2.7. The selected release plots, showing good fit of the experimental results with the theoretical calculations, are shown in Supplementary Information Fig. S4. This analysis was conducted for all of the cases and tabulated in Table 2.

The effect of the surface gradient, which was previously confirmed by XPS analysis (Fig. S3 and Fig. 3), was further supported by the release curves of OVA and β-NFG (Fig. 5). An initial fast β-NFG release from the film surface was observed for all cases represented in Fig. 5. It was noted that the cumulative amount of released β-NFG increased as the film sections changed from Section-1 (with the lowest β-NFG concentration) to Section-3 (with the highest β-NFG concentration) for both of the films PLLA-1 (Fig. 5A) and PLLA-4 (Fig. 5C) regardless of the type of the particles used. It can be supposed that higher cumulative amount of released β-NFG implies a higher β-NFG-loading on the film surface.

The mass transfer resistance on the film surface is low and the surface β-NFG release rate is dissolution dependent. It was noticed that the dissolution constant of β-NFG changes with respect to particle type and hydrophobic content (Table 2). The dissolution constant of β-NFG was found to be low in the PLLA films containing 20:80 CPTEG:CPH particles with more hydrophobic content than those with 50:50 CPTEG:CPH particles for all sections and all OVA loadings (Table 2). On the other hand, the increase in the OVA loading (from 1 to 5%) induced the β-NFG release (Table 2) particularly for the films with 50:50 CPTEG:CPH particles having relatively less hydrophobic content. However, an irregular trend was noted for the case of films with 20:80 CPTEG:CPH particles (Table 2). This could be attributed to the higher hydrophobic content of the 20:80 CPTEG:CPH particles enhancing the strong surface β-NFG attachment while suppressing the weak self-assemblies [48-50].

An increasing trend in the β-NFG dissolution constant was also observed in Table 2 from Section-1 to Section-3 for all OVA loading percentages and particle types (except 50:50 CPTEG:CPH particles with 5% OVA loading). This change was thought to be independent of surface NGF concentration and related to the internal particle gradient along the film matrix, affecting the hydrophobic content in different sections.

The additional mass transfer resistance throughout the film matrix resulted in much slower OVA release rates than that of the β-NFG from the film surface for all cases investigated (Fig. 5 and Table 2). Contrary to the case of β-NFG released from the surface, the amount of OVA released from the PLLA film matrix was highest in Section-1 (the lowest β-NFG surface concentration) and lowest at Section-3 (the highest β-NFG surface concentration) due to the internal particle gradient along the film matrix (Fig. 5B and D). Although the amount of OVA loss was small during the surface modification (~15 ng/mL OVA was lost from Section-3 for 50:50 particles while ~5 ng/mL was lost from 20:80 particles), the release curves in Fig. 5B and D indicated that it was enough to

Table 2

<table>
<thead>
<tr>
<th>Film type</th>
<th>Diffusion Coefficient of OVA (cm²/min)</th>
<th>Dissolution Constant of β-NFG (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Section-1</td>
<td>Section-2</td>
</tr>
<tr>
<td>PLLA-1 (50:50 CPTEG:CPH 1% OVA)</td>
<td>$3.10 \times 10^{-10}$</td>
<td>$2.57 \times 10^{-10}$</td>
</tr>
<tr>
<td>PLLA-2 (50:50 CPTEG:CPH 3% OVA)</td>
<td>$3.57 \times 10^{-10}$</td>
<td>$2.76 \times 10^{-10}$</td>
</tr>
<tr>
<td>PLLA-3 (50:50 CPTEG:CPH 5% OVA)</td>
<td>$7.51 \times 10^{-10}$</td>
<td>$6.51 \times 10^{-10}$</td>
</tr>
<tr>
<td>PLLA-4 (20:80 CPTEG:CPH 1% OVA)</td>
<td>$6.00 \times 10^{-14}$</td>
<td>$5.59 \times 10^{-13}$</td>
</tr>
<tr>
<td>PLLA-5 (20:80 CPTEG:CPH 3% OVA)</td>
<td>$2.54 \times 10^{-12}$</td>
<td>$2.00 \times 10^{-12}$</td>
</tr>
<tr>
<td>PLLA-6 (20:80 CPTEG:CPH 5% OVA)</td>
<td>$6.70 \times 10^{-10}$</td>
<td>$1.23 \times 10^{-10}$</td>
</tr>
</tbody>
</table>
create backward internal OVA concentration gradient through the film matrix. During the β-NGF surface modification, it was likely that the loosely self-assembled OVA molecules on the particle surface also leached out. It was expected that not all the OVA molecules were encapsulated within the particle matrix, but some of the OVA loosely attached to the surface, particularly at higher OVA loading percentages. Therefore, the loosely assembled OVA on the particles surface was expected to be high, causing faster OVA release and higher diffusion coefficients from Section-1 to Section-3 (Table 2). This situation was true for all of the films regardless the type of the particle or OVA loading percentage.

The OVA loading percentage also had a significant effect on the release kinetics and diffusion coefficients. As shown in Fig. 6, the increase in OVA content from 1 to 5% resulted in higher amount of OVA release for all sections and particle types as expected. However, higher OVA loadings led to the presence of loosely assembled OVA molecules around particle surfaces with low mass transfer resistance, promoting faster release and higher diffusion coefficients (Table 2).

Besides the OVA loading and film sectioning, the type of particle used played a dominant role in controlling the OVA release rate and diffusion coefficient. The presence of 20:80 CPTEG:CPH particles with higher hydrophobic CPH polymer content in films resulted in retarded surface erosion, slower OVA release and 1–4 orders of magnitude smaller diffusion coefficient values compared to the films with 50:50 CPTEG:CPH particles (Figs. 5, 6 and Table 2) for all sections and OVA loading percentages.

3.4. Bioactivity of β-NGF released from microparticles

The bioactivity of released β-NGF from 50:50 CPTEG:CPH particles was tested against PC12 cells by observing neurite outgrowth for 2 days. The 20:80 CPTEG:CPH particles were not tested since the released β-NGF amount was found to be lower than the
A threshold necessary to trigger neurite outgrowth in PC12 cells within 2 days of incubation (Fig. 4) [51]. It was observed that both β-NGF released from microparticles and soluble β-NGF, directly added to PC12 cells in culture media, caused significant neurite extension in PC12 cells, whereas the PC12 cells growing in media alone and in contact with blank particles (without β-NGF) had little to no neurites (Fig. 7A-C). Similarly, the quantitative measurement of neurite length per cell indicated that β-NGF released from microparticles caused ~2 μm long neurite extension per cell which was close to the effect of soluble β-NGF, directly added to PC12 cells in culture media (Fig. 7D). This assay showed that β-NGF released from 50:50 CPTEG:CPH microparticles caused stable and bioactive and showed a neurite extension pattern similar to that of free recombinant β-NGF in PC12 cell culture media. Furthermore, the process of β-NGF loading and storage time (one to two weeks) did not cause a significant decrease in bioactivity.

3.5. Bioactivity of β-NGF released from films

The bioactivity of released β-NGF from films containing 0.05% β-NGF loaded 50:50 CPTEG:CPH particles and β-NGF surface concentration gradients was tested using PC12 cells. The quantitative neurite extension analysis shown in Fig. 8A indicated that the increased β-NGF surface concentration along the film surface section (Sections 1–3) resulted in higher neurite outgrowth length per cell. The films containing only β-NGF loaded 50:50 CPTEG:CPH particles showed similar neurite extension effect on PC12 cells with the films possessing only β-NGF surface modification of Sections 2 and 3. However, a larger neurite extension was noted as compared to Section-1 of the film possessing only β-NGF surface modification. The films with both β-NGF surface modification and β-NGF loaded 50:50 CPTEG:CPH particles produced the highest neurite extension in PC12 cells (Fig. 8). It was also noted that the presence of β-NGF loaded 50:50 CPTEG:CPH particles in PLLA film matrix did not alter the overall β-NGF surface gradient effect. It can be anticipated that following the initial burst β-NGF release from the film surface, the 50:50 CPTEG:CPH particles provided sustained release, leading to higher and longer NGF activity. The images presented in Fig. 8B-G also showed neurite extensions upon exposure of released β-NGF. Therefore, the β-NGF loaded in particles and PLLA film matrix retained its stability and activity during the production steps. The small neurite lengths calculated by MetaXpress software may not be visible in the original images shown in Figs. 7 and 8, even at high resolutions. The cell body size, neurite length/width along with intensity threshold are parameters used for optimizing the neurite outgrowth measurement analysis by the MetaXpress software. The resolution of original images were enhanced by the software after all parameters were set during the analysis as shown in Fig. S6. Accurate high throughput MetaXpress analysis was conducted with these enhanced images.

The alignment and guided neurite extension was also achieved by synergic effect of surface micropatterning and NGF gradient. Fig. S5 in Supplementary Information clearly indicates the alignment of the extended neurites parallel to the direction of the micropattern and highest NGF surface concentration. However, the fast β-NGF release likely resulted in the rapid formation of a homogeneous β-NGF concentration in the cell culture media suppressing the surface gradient effect.

**Fig. 7.** Bioactivity of β-NGF released from 50:50 CPTEG:CPH microparticles (125 μg/ml) on the neurite extension of PC12 cells. PC12 cells density: 2 x 10⁴ cells per cm².

Incubation time: 2 days. Neurites of the cells in every condition were immunostained with βIII-tubulin along with Cy3 secondary (shown in red), DAPI for nuclei (shown in blue). (A) Differentiated PC12 cells by the influence of β-NGF released from microparticles. (B) Soluble β-NGF, directly added to PC12 cells in culture media (100 ng/mL). (C) PC12 cells only. (D) Quantification of neurite extension using ImageXpress Micro high content screening system and MetaXpress software. (N = 3 independent experiments and Scale bar = 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
4. Discussion

In this study, a porous PLLA film with longitudinal micropatterns, possessing NGF gradients on the surface and protein loaded polyanhydride microparticles with controlled release properties within the matrix, was designed as potential conduit material.

In contrast with the other studies [22–24], the porous structure of the PLLA based films was achieved by using dry phase inversion technique, which avoids the use of any pore forming agent and allows the uniform distribution of growth factor loaded PA particles within porous film matrix [30]. The average pore size of films was reported as ~1 μm which was small enough to prevent fibrous tissue infiltration and provide nutrition permeation and retain neurotrophic factor loaded particles [3].

Fig. 8. (A) Bioactivity of β-NGF released from PLLA films possessing β-NGF surface gradient alone, containing 0.05% β-NGF encapsulated 50:50 CPTEG:CPH microparticles alone and combination of the two, both containing 0.05% β-NGF loaded 50:50 CPTEG:CPH particles and possessing β-NGF surface gradient, on the neurite extension of PC12 cells. PC12 cells density: 2 × 10^4 cells per cm². Incubation time: 2 days. Same letters and * represents the statistically significant difference (p < 0.05). Bioactivity of β-NGF released from PLLA films. PLLA films possessing β-NGF surface gradient alone (B) Section-1 (C) Section-2 (D) Section-3. PLLA films possessing both 0.05% β-NGF encapsulated 50:50 CPTEG:CPH microparticles and β-NGF surface gradient (E) Section-1 (F) Section-2 (G) Section-3. PC12 cells density: 2 × 10^4 cells per cm². Incubation time: 2 days.

The porous films developed in this study possess longitudinal micropatterns and surface β-NGF concentration gradients in order to provide guided and directed neurite outgrowth from PC12 cells. The β-NGF coating on the surface was achieved through ester-amine interaction [35] and physical self-assembly reducing multiple steps and risk of growth factor denaturation during the coating processes involving various cross-linking chemistries or layer-by-layer coating techniques [13,14,34,52–54]. In most of the past studies, porous structures have been achieved by pore forming agents, leading to limited growth factor loading. For instance, in the work of Chen [53], the maximum β-NGF coating on the conduit surface through carbodiimide crosslinking was reported as ~80 ng/cm² whereas our results indicated ~300 ng/cm² surface loadings without using specific cross linkers. More importantly, in our strategy, we used both the film surface and matrix not only to provide high loadings of multiple growth factors, but also to enable controlled release properties by taking advantage of the dry phase inversion technique allowing distribution of various polyanhydride particle chemistries. The presence of high amounts of neurotrophic factors may adversely affect the axonal growth and neural regeneration [55,56]; however, a proper controlled release strategy can circumvent the adverse effect of high loadings.

As a promising alternative to the commonly used polyester-based particles, spray dried polyanhydride microparticles with
surface erosion properties, tuned by the polymer backbone chemistry and copolymer ratios, led to competitive encapsulation efficiency, protein stability and wide control over the release properties [37,38,44,57–61]. Previous reports [15,62,63], based on polyester particles, reported growth factor encapsulation efficiencies ranging between ~15 and 90%. In addition, they managed to achieve sustained growth factor release over 30 days showing efficient bioactivity. In our work, almost ~80% loading was achieved at various loading percentages by using spray dried polyanhydride microparticles with various chemistries.

Our previous [36–38,47] and current results indicated that the particle chemistry and ratio of hydrophilic to hydrophobic polyanhydride copolymers were the determining factors in controlling the release rate of growth factors from the particles. With the strategy of using both film matrix incorporated with growth factor loaded polyanhydride particles and β-NGF coating on the film surface, we showed that the growth factor diffusion coefficient and dissolution constant can be controlled over a wide range. The dissolution constant of β-NGF was found to change depending on the particle hydrophobicity. It was hypothesized that the presence of more hydrophobic polymers enabled strong β-NGF attachment to the PLLA film surface, resulting in slower β-NGF release and lower dissolution constant values [48–50]. On the other hand, partial encapsulation of OVA within the particle matrix led to the presence of loosely assembled OVA on the particle surface. This formation on the particle surface enhanced the chance of weak self-assembly between OVA and β-NGF causing fast β-NGF release from the surface and high dissolution constant values [15,64]. Similarly, the internal particle gradient along the film matrix also affected the hydrophobic content in different sections, leading to weak or strong β-NGF attachment to the film surface. For instance, in Section-3, where the amount of particles is the least, relatively high dissolution constants and fast β-NGF release were reported. However, for the case of more hydrophobic 20:80 CPTEG:CPH particles, the strong surface β-NGF attachment is promoted while suppressing the weak self-assemblies [48–50].

In the work of Rosner [65], the diffusion coefficient of β-NGF released from Schwann Cells (SCs) was reported to be between 3.94 × 10⁻⁹ and 4.6 × 10⁻¹¹ cm²/s on various substrates mimicking the real conditions. In addition, Tse [66] reported β-NGF diffusion coefficients ranging from 1.95 × 10⁻⁷ to 14 × 10⁻⁷ cm²/s for the efficient neurite outgrowth in PC12 cells caused by the β-NGF gradient release. They showed that the change in the diffusion coefficient affected the neurite outgrowth time response of the PC12 cells, pointing out that at higher diffusion coefficients, β-NGF releases faster resulting in the loss of control over the directional guidance of neurite outgrowth, whereas, at lower diffusion coefficients, β-NGF releases slowly and does not exceed the threshold during the lag time to show significant neurite outgrowth. Therefore, they proposed 1.95 × 10⁻⁷ cm²/s as the optimum diffusion coefficient value facilitating the directional guidance of neurite outgrowth. In another study, Sierra-Fronseca [51] reported that ~50 ng/mL β-NGF is enough to trigger neurite outgrowth in PC12 cells. Considering the previously published diffusion coefficients and released β-NGF amounts, our design provides wide control over the diffusion coefficient, ranging from 1 × 10⁻¹⁵ and 0.28 × 10⁻¹¹ cm²/s and dissolution constants of 0.16 × 10⁻⁵ to 0.16 × 10⁻⁴ s⁻¹ with released amounts of ~100–300 ng/mL, combinations of which resulted in wide adjustment of release rate and neurite extension. More importantly, this strategy has a potential for the incorporation of multiple growth factor loaded polyanhydride particle cocktails within the film matrix enabling simultaneous neurotrophic factor release with individually tunable diffusion coefficients. Therefore, different growth factor specific signaling pathways can be individually triggered and the amount of required growth factors can be maintained in the desired levels for longer times.

Our results indicate that the released β-NGF showed good bioactivity by inducing neurite extension from PC12 cells. The applied spray drying technique in particle preparation and dry phase inversion technique in conduit preparation played an important role in maintaining the stability and biological activity of the loaded growth factors. In addition, the presence of OVA also enhanced the stability and bioactivity of β-NGF [15,64]. The initial β-NGF release from the surface promoted β-NGF accumulation during the lag time and sustained release from the particles in the conduit matrix maintained the β-NGF concentration constant at the minimum desired level (50 ng/mL) over time to observe neurite growth from PC12 cells. For instance, Zeng [67] achieved ~0.8 μm per cell neurite length at the end of 28 days incubation while Sierra-Fronseca [51] reported ~0.5 μm per cell neurite length in PC12 cells. On the other hand, our work showed around 10 μm per cell neurite length in 2 days of incubation. In addition to considerable neurite extension, our design also promoted directed outgrowth as a result of surface micropatterning and β-NGF gradients.

5. Conclusions
In this study, multifunctional PLLA porous films with longitudinal micropatterns possessing β-NGF surface gradients and β-NGF -encapsulated surface eroding biodegradable and amphiphilic polyanhydride microparticles distributed within the film matrix were successfully produced by dry phase inversion technique. This novel strategy using both film surface and matrix achieved significant control over the simultaneous controlled release of neurotrophic factors as well as the guided and directed neurite outgrowth in PC12 cells. This unique strategy can potentially be extended for the design of conduit systems possessing individually programmed controlled release of multiple growth factors through the use of PA microparticle cocktails and can further be investigated in vivo efficacy.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.09.039.

References


