Acta Biomaterialia 56 (2017) 141–152

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Development of multifunctional films for peripheral nerve regeneration ☆

Metin Uz [a,1], Anup D. Sharma [a,b,1], Pratish Adhikari [a,c], Donald S. Sakaguchi [b,c], Surya K. Mallapragada [a,b,*]

[a] Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011, United States
[b] Neuroscience Program, Iowa State University, Ames, IA 50011, United States
[c] Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, United States

ARTICLE INFO

Article history:
Received 8 April 2016
Received in revised form 16 September 2016
Accepted 28 September 2016
Available online 29 September 2016

Keywords:
Nerve regeneration
Poly(lactic acid) (PLLA) films
Nerve growth factor (NGF)
Surface gradient
Micropatterning
Polyanhydride microparticles
Controlled release

ABSTRACT

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/cm^2 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 6×10^{-14} and 1.67×10^{-10} cm^2/min and dissolution constants ranging from 1×10^{-4} to 1×10^{-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 total neurite length per cell 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.

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

☆ Part of the Gradients in Biomaterials Special Issue, edited by Professors Brendan Harley and Helen Lu.

* Corresponding author at: Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011, United States.
E-mail address: suryakm@iastate.edu (S.K. Mallapragada).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.actbio.2016.09.039
1742-7061/

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-L-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 allowed 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-NGF surface gradients were created on PLLA film surfaces with longitudinal micropatterns through ester-amine interactions [35] and physical self-assembly to provide initial β-NGF 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-L-lactide (L-PL-ester terminated B6002-2) was obtained from Lactel Absorbable Polymers. Beta-nerve growth factor (β-NGF, 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-NGF 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-βIII 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-NGF loaded microparticles

Random copolymers of CPTEG:CPH (1,6-bis (p-carboxy phenoxy) hexane (CPH), 1,8-bis (p-carboxyphenoxy)-3,6-dioxaoc 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-NGF encapsulation efficiency. The polymer-protein solution (200 mg of polymer and 100 μg of β-NGF 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-NGF 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-NGF release from microparticles

β-NGF 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% BSA-PBS solution. The concentration of released β-NGF collected in the sample supernatant was analyzed using a β-NGF ELISA kit following the manufacturer's procedure.

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

Dry phase inversion technique was used to prepare β-NGF/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,