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Tissue Engineering Applied to the Retinal Prosthesis: Neurotrophin-Eluting Polymeric Hydrogel Coatings

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Abstract

Several groups are developing visual prostheses to aid patients with vision loss. While these devices have shown some success in the clinic, they are severely limited by poor resolution, and in many cases have as few as 15 electrodes. Pixel density is poor because high stimulation thresholds require large electrodes to minimize charge density that would otherwise damage the electrode and tissue. A significant contributor to high stimulation threshold requirements is poor biocompatibility. We investigated the application of one system popular in tissue engineering, drug-releasing hydrogels, as a mechanism to improve the tissue-electrode interface. Hydrogels studied (i.e., PEGPLA photocrosslinkable polymers) released neurotrophic factors (i.e., BDNF) known to promote neuron survival and neurite extension in the retina. Hydrogels were examined in co-culture with retinal explants for 7 and 14 days, at which time neurite extension and neurite density was measured. Neurite extension was enhanced in samples exposed to BDNF-releasing hydrogels at 7 days; however, these increases were absent by day 14 suggesting declining drug release. Thus, PEGPLA hydrogels are excellent candidates for short-term (< 14 day) acute release of therapeutic factors in the retina, but will require additional modifications for application with neural prostheses. Additionally, these results suggest that the effects of neurotrophic factors are short-lived in the absence of additional support cues, and tissue engineering systems employing such factors may only produce transient benefits to the patient.

Introduction

Millions of individuals suffer from vision loss or visual impairment, and treatment options are extremely limited [1]. One potential therapy is the development of prosthetic devices to restore lost visual function. Our group (Figure 1) [2] and others [3-5] are engaged in ongoing research to develop a retinal prosthesis that restores vision to patients with degeneration of the outer retina (i.e. the photoreceptors). The electrodes used by all groups that are delivering current to the electrodes from outside sources have been relatively large,

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on the order of 400 μm or greater. The large size of the electrodes has limited the density of electrodes that can be placed over a given area. The choice of electrode size has been dictated mostly by the potential for damage to the electrode and surrounding tissue posed by high charge densities [6,7]. Perceptual threshold measurements obtained in blind humans have varied widely, although 0.5 $\mu\text{C}/\text{phase}$ is reasonably good representation of the collective results [8]. However, charge densities of $>2000 \mu\text{C}/\text{cm}^2$ can result in substantial damage to the electrode and tissue [9,10]. Thus, large electrodes ($>175 \mu\text{m}$ diameter) are required to supply the needed charge to minimize charge density and the risk of electrode or tissue damage.

High stimulation thresholds are at least partially the result of a poor interface between the electrode array and the target tissue [8,11,12]. Chronically implanted devices may become enveloped by fibrotic tissue, which likely increases thresholds by increasing the electrical resistance of the tissue and also by creating a greater physical separation between the electrodes and the neurons. [13] Several approaches are being studied to reduce stimulation thresholds, including the use of penetrating electrode materials [14], modification with cell adhesion molecules [15] and cell transplantation [16].

The field of tissue engineering has the potential to improve the biocompatibility of prosthetic devices by minimizing inflammatory reactions and by enticing neurons closer to the stimulation electrodes, which should reduce stimulation thresholds. In this manuscript, we examine the use of biodegradable drug-releasing hydrogels, which are popular neural tissue engineering constructs [17], to improve the electrode-tissue interface. The hydrogels employed (i.e., poly(ethylene glycol)-poly(lactic acid) (PEGPLA)) are composed of polymer elements that have been shown to dramatically increase biocompatibility of implanted devices [18], potentially reducing scar formation at the implant site. Additionally, these hydrogels can be designed to release a variety of bioactive compounds including neurotrophins, which are biomolecules that can influence neuronal survival and neurite extension in the retina [19-21]. Neurotrophins should promote growth of neuronal extensions toward the array, and thus decrease electrode-target separation distance and therefore thresholds.

Previously, we developed PEGPLA hydrogels capable of releasing biomolecules for 1-3 weeks [22]. We demonstrated that these hydrogels produce neurite extension in PC12 cells, a model neuron class, in response to elution of nerve growth factor (NGF) [22]. Here, we demonstrate the ability of PEGPLA hydrogels: 1) to emit neurotrophins that have been shown by others to attract retinal neurons (e.g. brain derived neurotrophic factor (BDNF)); and 2) to explore the response of retinal explants to their release.

Materials and Methods

Creation of Polymer Boluses

Hydrogel boluses were composed of acrylated poly(ethylene glycol)-poly(lactic acid) (PEGPLA) copolymers synthesized as described previously [22,23]. Two polymers were investigated 1540LA2, containing 1540 MW PEG with 2 lactide groups on each PEG terminus, and 1540LA4 containing 1540 MW PEG with 4 lactide groups on each PEG terminus. Previous investigations of hydrogels composed using these polymers yielded bovine serum albumin (BSA) release durations of 1-3 weeks [22].

To create hydrogel boluses, acryl-PEGPLA was mixed with distilled deionized water at 22% (wt/v) with 0.5% (wt/v) 1-Vinyl-2-pyrrolidone co-monomer (Sigma), 0.1% (wt/v) Irgacure 2959 (Ciba) photoinitiator, and 5% (wt/v) BSA (Jackson Immunochem) excipient. For brain derived neurotrophic factor (BDNF) release experiments, this solution was filtered using a

0.22 μm sterile filter and added to lyophilized BDNF (Peprotech) at 100 ng/ μL solution. Boluses were created by depositing the final solution in 2 μL drops onto a Teflon substrate and exposing to UV light (UV III Systems) for 30 seconds. Gelation was confirmed by comparing to a diacryl-PEG positive control (400 MW, Polysciences).

Animals

Adult New Zealand White rabbits, weighing 2.5-3 kg, were obtained from Millbrook (Amherst, MA). The rabbits were maintained in a 12 h light (<300 lux)/12 h dark environment with high fiber rabbit chow (Purina 5326) and water available *ad libitum*. All experimental methods and animal care procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Boston VA Institutional Animal Care and Use Committee.

Retinal Preparation

General anesthesia was induced with ketamine (35-50 mg/kg, i.m.) and xylazine (3-5 mg/kg, i.m.) and maintained with 2-3% isoflurane, administered through a nose mask. Lidocaine hydrochloride (20 mg/ml) was applied locally to the eyelids and surrounding tissue prior to enucleation. Following enucleation, the anterior portion of the eye was surgically removed and discarded. Following removal of the vitreous humor with gentle suction from a Pasteur pipette, the retina was dissected from the choroid. Retinal explants were isolated from gross retinal sections using a 2 mm diameter trephine (Millennium Surgical). Only retina from the inferior portion of the eye was used.

Explant/Bolus Co-culture

For explant/bolus co-culture, five boluses were removed from the Teflon substrate and placed on a transwell insert membrane (Corning, 6 well size). This insert was submerged in cell culture medium consisting of Dulbecco's modified essential medium (DMEM, Sigma) supplemented with 1.5 g/L sodium bicarbonate (Sigma), 10% fetal bovine serum (FBS, Sigma) and 1% penicillin-streptomycin (Invitrogen). The explants were cultured on separate transwell insert membranes (12 well size) at the level of the culture medium nested in the bolus-containing inserts. The use of nested inserts prevented direct contact of the PEGPLA boluses with explants and facilitated medium exchange, which occurred every 2-3 days. Explants were exposed to six experimental conditions: 1540LA2 and 1540LA4 BDNF-releasing boluses, 1540LA2 and 1540LA4 BSA-releasing shams, a positive control receiving 100 ng/ml of BDNF (Peprotech) added to cell culture medium immediately before medium exchange, and a negative control receiving unsupplemented cell culture medium.

Explant Neurofilament Staining

To observe neurite extension, explants were fixed and stained for neurofilament at 7 and 14 days. Briefly, explants (3 per experimental condition) were washed 2 $\times$ with phosphate buffered saline (PBS, without Ca^{2+} and Mg^{2+} , pH = 7.4, Sigma) and exposed to 4% (wt/v) formaldehyde solution (prepared freshly from p-formaldehyde (Sigma) in PBS with 4% (wt/v) sucrose (Sigma)) for 15 minutes. Samples were then washed 2 $\times$ with PBS and exposed to blocking medium containing 1% (wt/v) BSA and 0.2% (v/v) Triton X-100 in PBS for 30 minutes. Next, samples were incubated with mouse anti-neurofilament 200 kDa monoclonal antibody (Chemicon) in blocking buffer [1:500] overnight. Then, samples were washed 3 $\times$ with PBS and exposed to goat anti-mouse IgG (H+L) FITC conjugate (Jackson Immunochem) in blocking buffer [1:100] for 1 hour. Samples were rinsed 3 $\times$ with PBS and sealed with slow fade gold mounting medium (Invitrogen) for observation.

Neurite Counting and Analysis

Sequential explant segments were photographed using an Olympus CKX41 phase contrast/fluorescence optical microscope equipped with 4X, 10X, 20X, and 40X objectives and a Sony iCY-shot DXC-S500 color digital camera. Composite explant images were reconstructed using Adobe Photoshop. Neurite lengths and numbers were assessed using Image J image processing software (National Institutes of Health) and normalized versus the explant perimeter. Neurites length was measured from the edge of the explant to the extended tip. Very little branching was observed, but when branching occurred, the length from the neurite base to the tip of each terminus was measured. Neurites that did not extend outside of the border of the explant were not measured.

The length and number of neurites/mm explant were analyzed using the Sigmaplot (SYSTAT) statistical software package. All tests were evaluated using a p value < 0.05 to establish significance. The resulting distributions for neurite length data were non-Gaussian and either bimodal or skewed; therefore, comparisons of the average and standard deviation were not appropriate. Instead, data from 3 individual samples for each experimental set were pooled and analyzed using the Kruskal-Wallis test with Dunn's modification, which permits comparisons between non-Gaussian data sets with unequal numbers of data points (N , pooled data set > 58). Data for neurite number were analyzed using one way Anova with the Holm-Sidak modification. The primary objective of this study was to identify the effect of BDNF releasing polymer boluses on neurite *length*; therefore, the sample size for neurite number ($N = 3$) was limited, and the statistical power of this test was low (0.769 vs. 0.80 required). Additional experiments would be needed to fully capture statistical differences amongst sample groups.

Results

We examined the potential of biodegradable, neurotrophin-eluting PEGPLA hydrogels to promote neurite extension using a retinal explant model. Six experimental conditions were examined: 1540LA2 (3 week release polymer loaded with BDNF), 1540LA4 (1.5 week release polymer loaded with BDNF), Sham LA2 (unloaded 3 week release polymer), Sham LA4 (unloaded 1.5 week release polymer, BDNF⁺ control (receiving 100 ng/ml of BDNF added to culture medium), and control (negative control receiving no supplement and no boluses).

After 7 days (Figures 2-3), neurite length was significantly increased for samples exposed to BDNF, including the BDNF-releasing boluses (1540LA2 and 1540LA4) and BDNF⁺ control (Figure 3A). Explants exposed to the 1540LA2 (3 week releasing) polymer displayed the longest neurites, which were statistically longer than those from any other group ($p < 0.05$). The sham and control samples displayed statistically insignificant differences from each other, indicating that the polymer alone does not account for increases in neurite length.

In most cases, the neurite density (number/mm explant perimeter) was substantially unaffected by BDNF application (Figure 3B). The 1540LA4 group did show a comparatively large increase in neurite number ($p < 0.10$ for all groups except BDNF⁺ control); however, the sample size explored ($N = 3$) was insufficient to establish the power necessary for statistical analysis (0.769 vs. 0.80 required). Additional experiment beyond the scope of this publication would be required to substantiate this result. These data seem to indicate that slower BDNF release rates (e.g., 3 week-releasing 1540LA2) promote increased neurite length, whereas higher release rates (e.g., 1.5 week-releasing 1540LA4) promote increased neurite density. This is consistent with previous data demonstrating differential concentration-dependant effects of neurotrophin application [20, 24].

After 14 days, neurite extension increased only for the BDNF⁺ samples (Figure 4). A qualitative difference can be seen between the BDNF⁺ samples and all others. In samples exposed to BDNF-releasing boluses (Figure 4A,B) and BSA-releasing sham boluses (Figure 4D,E), neurite extension proceeds tangential to the edge of the explant; however, the BDNF⁺ control samples exhibit radial extension (Figure 4C). Based on subsequent experiments on differentially treated culture surfaces (e.g., collagen, laminin, or polylysine coated, manuscript in preparation), the most logical explanation for this behavior is differential adhesion of the explant to the cell culture substratum. This suggests that the presence of BDNF in the cell culture medium could influence adhesion; however, the mechanism of this process is unclear, although previous research has shown a synergistic response between neurotrophins and ECM components [25].

Another possible explanation is that a gradient of BDNF in the medium has been established. Researchers have shown that concentration gradients influence neurite extension patterns [26,27], with extension usually favoring the direction of the gradient maximum. It is expected that as the amount of BDNF released by boluses declines, a concentration gradient with a maximum near the explant surface would be created as a result of endogenous BDNF secretion. In either case, these effects are not fully realized until > 1 week in culture (compare Figure 2 vs. 4).

Quantitative analysis confirms reduced neurite extension for all samples excluding the BDNF⁺ control (Figure 5). Only the BDNF⁺ control sample shows an increase in neurite length from day 7 to day 14 ($p < 0.05$). The BDNF-releasing bolus samples exhibit a statistically significant decrease ($p < 0.05$) in neurite length from day 7 to 14. The control sample also exhibits a significant decrease in neurite length, whereas the BSA-releasing sham boluses do not. Additionally, the BDNF-releasing 1540LA4, BSA-releasing sham 1540LA4, and control samples are statistically indistinguishable from each other.

Although the sample size ($N=3$) was limited, neurite density appears to increase when comparing values for day 7 to 14 for the BDNF⁺ control sample (14 ± 0.55 neurites/mm vs. 27 ± 7.8 neurites/mm). The 1540LA2 BDNF-releasing bolus sample shows a slight increase in neurite density (10 ± 3.0 neurites/mm on Day 7 vs. 14 ± 4.1 neurites/mm on Day 14), whereas the 1540LA4 BDNF-releasing bolus samples demonstrate a dramatic decrease in average neurite number, dropping from 26 ± 9.0 neurites/mm on Day 7 to 16 ± 0.25 neurites/mm on Day 14. The negative control and BSA-releasing sham bolus samples demonstrate small changes in neurite density.

Discussion

Our results indicate that PEGPLA hydrogel boluses can be used for short-term (<14 days) drug delivery to the retina to promote neurite extension. Initial data (Day 7) demonstrate that BDNF release from PEGPLA boluses produces neurite extension equal to or better than a BDNF⁺ control that received BDNF directly in the cell culture medium. This is significant because the extremely short half-life of neurotrophic factors [28,29] requires some method of chronic delivery to produce therapeutic effects *in vivo*. PEGPLA hydrogel boluses have the advantage of providing slow, controlled neurotrophin release directly to the target site, whereas chronic systemic delivery would be extremely difficult in the retina.

However, the impact of BDNF factor delivery on retinal neurons using PEGPLA boluses *in vitro* is limited in time. By day 14, neurites extension in response to BDNF elution either stopped or reversed. Based on previous studies[22], it was anticipated that neurotrophin release from polymer boluses would proceed for approximately 1.5-3 weeks. However, these experiments were performed in the absence of cells. It is likely that cells metabolize

some BDNF, which would thus reduce the concentration of this factor in solution. As release is primarily diffusion driven [30], a decrease in the external concentration will accelerate release rates. Release duration can be increased by altering the composition of PEGPLA boluses [23] or by combining PEGPLA boluses with poly(lactic-co-glycolic) acid (PLGA) microspheres [31]. These changes should produce a hydrogel drug release system that can deliver neurotrophic factors for many weeks or longer, which could potentially make this method of drug delivery relevant for incorporation into neural prosthesis applications.

Perhaps, the most significant finding of this study is that neurotrophin withdrawal, in this case through declining drug release, results in neurite retraction. Several researchers have proposed the use of neurotrophin as a therapeutic treatment for peripheral nerve and spinal cord injuries [32,33]. However, our results suggest that any extension obtained would be short-lived in the absence of other cues. Similarly, application of this approach to a neural prosthesis will require additional modifications. Neurotrophins may provide acute increases in neurite proximity to neural prosthesis electrodes, but these effects must be maintained by other mechanisms.

One possible method to sustain the effect of elutable neurotrophic factors is the use of cell adhesion molecules. In preliminary unpublished work in our laboratory, we have shown that adding cell adhesion molecules to PEGPLA boluses prevents neurite retraction following BDNF withdrawal and increases neurite extension over that seen with BDNF application alone in a 14 day period. A multi-faceted system containing both soluble and adherent guidance factors could address both acute and chronic biocompatibility of neural prosthesis materials. It is hoped that increased biocompatibility will lower required thresholds for stimulation, thereby permitting smaller electrodes to be employed and increasing device resolution.

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Figure 1.
Multi-electrode polyimide array for retinal prosthesis studies. The array has 15 electrodes each 400 μ m in diameter arranged in a 3×5 matrix.

Figure 2. Neurite extension in retinal explants exposed to (A) 1540LA2 and (B) 1540LA4 BDNF-releasing boluses, (C) BDNF⁺ control, (D) 1540LA2 and (E) 1540LA4 BSA-releasing shams, and (F) negative control receiving no BDNF for 7 days *in vitro*.

Figure 3.

Comparison of neurite lengths and number/mm after 7 days of culture. (A) Average neurite length. (Error bars = S.D., $N > 16$ * = Statistically insignificant difference $p > 0.05$.) (B) Average number of neurites normalized to explant perimeter (mm). LA4 sample is statistically different from all samples other than BDNF⁺ control. However, statistical power was low (0.769 vs. 0.80 required). Additional tests should be performed to confirm results. (Error bars represent S.D., $N = 3$).

Figure 4. Neurite extension in retinal explants exposed to (A) 1540LA2 and (B) 1540LA4 BDNF-releasing boluses, (C) BDNF⁺ control, (D) 1540LA2 and (E) 1540LA4 BSA-releasing shams, and (F) negative control receiving no BDNF for 14 days *in vitro*.

Figure 5.

Comparison of neurite lengths and number/mm after 14 days of culture. (A) Average neurite length. (Error bars = S.D., $N > 15$ * = Statistically insignificant difference $p > 0.05$.) (B) Average number of neurites normalized to explant perimeter (mm). BDNF⁺ sample is statistically different from all others. LA4 is statistically different from control and sham samples. LA2 is statistically different from control and Sham LA4. However given small sample size, additional tests should be performed to confirm results. (Error bars represent S.D., $N = 3$).