Published in final edited form as:

Biomaterials. 2014 August ; 35(24): 6473–6481. doi:10.1016/j.biomaterials.2014.04.027.

Controlled Release and Gradient Formation of Human Glial-Cell Derived Neurotrophic Factor from Heparinated Poly(ethylene glycol) Microsphere-based Scaffolds

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Abstract

Introduction of spatial patterning of proteins, while retaining activity and releasability, is critical for the field of regenerative medicine. Reversible binding to heparin, which many biological molecules exhibit, is one potential pathway to achieving this goal. We have covalently bound heparin to poly(ethylene glycol) (PEG) microspheres to create useful spatial patterns of glial-cell derived human neurotrophic factor (GDNF) in scaffolds to promote peripheral nerve regeneration. Labeled GDNF was incubated with heparinated microspheres that were subsequently centrifuged into cylindrical scaffolds in distinct layers containing different concentrations of GDNF. The GDNF was then allowed to diffuse out of the scaffold, and release was tracked via fluorescent scanning confocal microscopy. The measured release profile was compared to predicted Fickian models. Solutions of reaction-diffusion equations suggested the concentrations of GDNF in each discrete layer that would result in a nearly linear concentration gradient over much of the length of the scaffold. The agreement between the predicted and measured GDNF concentration gradients was high. Multilayer scaffolds with different amounts of heparin and GDNF and different crosslinking densities allow the design of a wide variety of gradients and release kinetics. Additionally, fabrication is much simpler and more robust than typical gradient-forming systems due to the low viscosity of the microsphere solutions compared to gelating solutions, which can easily result in premature gelation or the trapping of air bubbles with a nerve guidance conduit. The microsphere-based method provides a framework for producing specific growth factor gradients in conduits designed to enhance nerve regeneration.

Keywords

microsphere; hydrogel; gradient; release; heparin; GDNF

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Competing Financial Interests The Authors have no competing financial interests.

Introduction

The importance of gradients in biological molecules is well recognized. Processes such as nerve regeneration, wound healing, embryogenesis, angiogenesis, and immunity have been found to depend significantly on biological gradients [1–12]. In chemotaxis cells follow concentration gradients in signaling molecules, with the steepness of the gradients greatly influencing cell movement more than the average concentration [5–7]. To replicate and improve upon developmental and repair processes to engineer tissues and organs, production of bioactive gradients along with spatial patterning will be essential.

In recent years an increasing number of researchers have proposed many methods to this end [13–28]. For example, Khademhosseini and colleagues have created gradients in adhesion peptides using inverse flows and photopolymerization in microfluidic channels to influence and study endothelial cell migration [25]. Shoichet, et al. have immobilized nerve growth factor in concentration gradients and observed enhanced directionality of extending dendrites [4, 6, 57]. Bellamkonda, et al. found increasing concentration gradients in laminin-1 could alter the direction of growing dorsal root ganglia and enhanced regeneration of sciatic nerves in rats with nerve growth factor [21–23].

Many of the current methods for the patterning and delivery of bioactive molecules use various forms of covalent attachment [13–18]. Irreversible coupling, however, may not be the optimal approach. Covalent attachment can potentially hinder the ability of cells to access the molecules, and chemical modification may result in a loss of activity. An alternative that our lab has explored recently is the use of heparin-decorated synthetic materials that can bind electrostatically (reversibly) many useful proteins, including proteins that promote nerve regeneration [26–34]. GDNF, a heparin binding protein, has been shown to enhance motor and sensory nerve regeneration [35, 36]. Synthetic polymer hydrogels have been extensively explored to create scaffolds for regenerative medicine, and have seen some promising results [37–39]. Functional peptides, proteins, or other biological molecules like heparin may be incorporated into these hydrogels imparting biological functions, such as cell adhesion or cell-initiated degradability [2,38,40–42]. However, bulk hydrogel scaffolds generally lack macroporosity or spatial anisotropy. To address these limitations we and others are seeking to produce heterogeneous scaffolds using modular assembly of hydrogel microparticles [28, 38, 43–48].

Gradient producing systems such as pulsatile application of picoliters of growth factor solutions, simple diffusion of molecules into a gel, gradient makers using two polymerizing solutions, and microfluidic devices have been used extensively [1, 4, 5, 10, 24–26]. However scaling issues and difficulties in pumping polymerizing solutions are only a few of the challenges faced by these methods due to the low volumes involved (e.g. about 70 μL of fluid per centimeter of conduit). The formation of gradients of growth factors, as well as addition of adhesion factors and degradability in bioactive scaffolds, is proposed to be improved by assembling microparticles in a modular manner [28,38,44,45,49,50]. To this end our lab has developed PEG hydrogel microspheres fabricated from multi-arm PEG derivatives in aqueous solution with kosmotropic salts via a thermally induced phase separation [28,38]. In this strategy, solutions are *not mixed* during microsphere formation,

with size controlled by the length of time required for gelation [58]. We have already successfully imparted different functionalities, such as cell adhesion, degradability, heparination, and protein and drug delivery to these microspheres [28,38].

In a recent study we engineered gradients into scaffolds made from these PEG microspheres, most notably decorating the microspheres with heparin and creating a gradient of *covalently coupled* GDNF [28]. However, we had not demonstrated the release of electrostatically (i.e. reversibly) bound GDNF from these scaffolds. The challenges in the previous publication that did not allow release of GDNF were overcome and the results are presented here.

Materials and Methods

Unless otherwise noted, all reagents were purchased from Sigma-Aldrich.

PEG Synthesis

PEG₈-vinylsulfone (PEG₈-VS) and PEG₈-amine was synthesized from eight-arm PEG-OH (PEG₈-OH; mol. Wt. 10,000; Shearwater Polymers, Huntsville, AL) as previously described [51]. PEG macromonomers were dissolved separately at 200 mg/mL in Dulbecco's phosphate buffered saline (PBS; 8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM sodium chloride, 10 mM potassium chloride, pH 7.4) and sterile filtered with 0.22 μ m syringe filters (Millipore).

Heparin attachment

A solution of 500 mM N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), 12.5 mM N-Hydroxy-succinimide (NHS), and 50 mg/mL heparin sodium salt (mol. wt. ~18,000, ~2.78 mM) in MES buffer (10 mM, pH 6.0) was incubated at room temperature for 30 min. The activated heparin solution was then added to a 200 mg/mL solution of PEG₈-amine at a 20:1, or 160:1 PEG₈-amine to heparin molar ratio and incubated at room temperature for another 30 min before refrigeration. For microsphere formation, heparin-conjugated PEG₈-amine was mixed with PEG₈-VS in a 1:2 ratio of the two PEG types (see Figure 1).

Microsphere Formation

PEG₈-amine (with or without bound heparin) solutions were combined with PEG₈-VS solutions at a 1:2 ratio. The PEG solutions were diluted to 20 mg/mL PEG with PBS and 1.5 M sodium sulfate (in PBS) to a final sodium sulfate concentration of 0.6 M. The PEG₈-VS/PEG₈-amine solutions were then incubated above the cloud point at 70°C for various times. Suspensions of microspheres were subsequently buffer exchanged into 8 mM sodium phosphate twice to remove the sodium sulfate by: (1) diluting the microsphere solution 3:1 with PBS and titrating, (2) centrifuging at 14,100g for 2 min, and (3) removing the supernatant.

GDNF Labeling

Dylight-488 NHS-ester (Pierce) was dissolved in dimethyl formamide at 10 mg/mL. Recombinant human GDNF (Peprotech, Rocky Hill, NJ) was dissolved in 8 mM sodium

phosphate buffer (pH 7.4). Dylight-488 was added to the solution for a final GDNF concentration of 10 µg/mL and a final Dylight-488 concentration of 50 ng/mL and incubated overnight at 2°C. The solution was then dialyzed using Slide-A-Lyzer MINI Dialysis Units (Thermo Scientific, Rockford, IL, 3500 MWCO) in 8 mM sodium phosphate buffer (pH 7.4) to remove unbound Dylight-488.

Heparin labeling

For some experiments, heparin was labeled with Dylight-488. A solution of heparin (100 mg/mL) and Dylight-488 (560 µg/mL) in PBS was incubated overnight at room temperature. The labeled heparin solution was dialyzed using Slide-A-Lyzer MINI Dialysis Units in MES buffer (10 mM, pH 6.0) to remove any unbound Dylight-488. The heparin solution was then used in the microsphere formation protocol as described above.

GDNF Loading Heparin Microspheres

Labeled or unlabeled GDNF solutions were added to buffer washed microspheres such that the GDNF concentration within the supernatant was 250 ng/mL. The microsphere/GDNF solution was well mixed, by titration and incubated overnight to allow diffusion of the GDNF into the microspheres. Immediately before scaffold formation, the microspheres were centrifuged at 14,100 g, supernatant was removed, and microspheres were resuspended in 8 mM sodium phosphate.

Gradient Formation

The glass walls of Pasteur pipettes were passivated with PLL(375)-g[7]-PEG(5) [52,53]. The pipettes were filled with a 20 mg/mL PLL-g-PEG solution, incubated for 30 seconds, and washed with DI water. After sufficient drying time, the tips of the pipettes were sealed with silicone aquarium sealant (DAP Inc., Baltimore, MD). To form scaffolds, microsphere solutions loaded or unloaded with GDNF were sequentially added to the pipettes that were placed in 15 mL conical vials. The microsphere solutions were centrifuged at 1000 g for 5 min before the next layer of microspheres was added. The supernatant was then removed once more and replaced with either 8 mM Sodium Phosphate or PBS.

Confocal microscopy

Fluorescence microscopy was performed with a Nikon Eclipse C1/80i confocal microscope. Microsphere gradients were imaged while still in the Pasteur pipettes with a 10X objective (NA=0.30, DIC L/N1, WD=16.0mm). Multiple images were taken along the length of the pipette and processed using EZ-C1 3.70 FreeViewer software (Nikon Instruments Inc.) and then combined. Fluorescence in the composite photographs was analyzed with ImageJ software.

Results and Discussion

To confirm attachment of heparin to PEG-OAm and subsequent incorporation into microspheres, heparin was labeled with Dylight-488. The mechanism to couple the NHS ester dye to heparin was conceived because of an observation that NHS-activated heparin alone will form a gel if the reaction is allowed to proceed overnight. Crosslinking was most

likely due to reactions between the NHS-esters on heparin and secondary amines on heparin, although the concentration of this chemical linkage was too low to measure by NMR or IR. Though Dylight-488 normally reacts with primary amines, adequate incubation time allowed for reaction with heparin. Unreacted Dylight-488 was removed by dialysis as well as by the washing steps after microsphere formation (by which time the NHS-esters on Dylight-488 would be hydrolyzed and unreactive). After the washes, the microspheres were photographed with a fluorescence microscope (Figure 2A). The total fluorescence was also compared to unlabeled heparin microspheres on a fluorescence plate reader to confirm the fluorescence was originating from the labeled heparin (Figure 2B). Fluorescence readings were also taken on the labeled heparin microspheres before and after the washing steps. The washed microspheres contained 46% of the fluorescence of the unwashed microspheres. This indicated that at most 46% of the heparin was successfully integrated into the microspheres.

We previously presented a method of gradient formation in one step, using density (buoyancy) differences in microspheres to form distinct layers during centrifugation [28]. However, we suspected that differences in crosslink density that resulted in differences in buoyancy may affect rates of growth factor diffusion within the microspheres. To test this, scaffolds were made from microspheres crosslinked for 11 minutes or 45 minutes. We had shown that these crosslinking times resulted in the full range of practically achievable buoyancies (less crosslinking time did not result in microsphere formation and more resulted in substantial microsphere aggregation) [28]. Single-layer scaffolds were prepared from each microsphere type in the presence of 250 ng/mL Dylight-labeled GDNF. The interface between the microsphere layer and the supernatant was imaged immediately after scaffold formation (i.e. before removing the supernatant and washing the scaffold). Representative fluorescent images are shown in Figure 3, which suggest that the scaffold made from microspheres incubated in the phase separated state for only 11 minutes had higher GDNF concentrations than the adjacent supernatant. The opposite was true for a scaffold made from microspheres incubated in the phase separated state for 45 minutes (the highest crosslinking density possible without substantial microsphere aggregation) [28]. This indicated that the more densely crosslinked microsphere had a restricted ability to absorb GDNF. Although a one-step process for gradient formation is attractive, the non-uniformity in growth factor diffusion rates for the different layers makes prediction of release kinetics extremely challenging. Thus, we subsequently used the lowest crosslinking time (11 minutes) for all microspheres to ensure high loading of growth factor in the scaffolds and predictability of release kinetics. Gradient scaffolds were thus formed by sequentially centrifuging microspheres in distinct layers, with gradients formed by incubating microspheres with different concentrations of GDNF prior to and during centrifugation. The layer-by-layer scaffold formation method served to eliminate the high sensitivity of the microsphere structure to the length of incubation time in the phase separated state during microsphere formation. Although the layer-by-layer method initially produces step gradients in GDNF, continuous gradients of soluble GDNF are rapidly generated by diffusion and dynamic interactions with heparin in the scaffold.

Figures 4 and 5 show release for single tiered scaffolds made of heparin-containing microspheres incubated in 250 ng/mL GDNF during scaffold formation. Figures 6 and 7

show scaffolds with two tiers - a lower tier with scaffold made of heparin-containing microspheres incubated in 250 nM GDNF during centrifugation, and an upper level with no GDNF present during centrifugation of heparin-containing microspheres. Figures 4A, 5A, 6A, and 7A demonstrate GDNF gradient formation within one or two tier scaffolds, with release into either physiological (Figures 4 and 6) and low salt conditions (Figures 5 and 7). The affinity of GDNF for heparin in the microspheres will be influenced by the concentration of salt in the surrounding buffer [28]. Low salt (8mM sodium phosphate) should result in slower release than physiological salt concentrations (i.e. PBS). More rapid release of GDNF into buffer at physiological salt concentration was observed, as expected, which was quantified in Figures 4B, 5B, 6B and 7B. Each of these figures also contains mathematical predictions for the GDNF concentration profile within the scaffold based on Fick's 2nd Law (Figures 4C, 5C, 6C and 7C). The prediction was obtained using a model that utilized an *effective diffusion constant* for GDNF within the scaffold:

$$D_{eff} = \frac{D_{AB}}{[H]/K_D + 1}$$

where D_{eff} = effective diffusion constant, D_{AB} = diffusion constant of GDNF in PEG scaffolds without heparin, $[H]$ = heparin concentration, K_D = equilibrium dissociation constant for the interaction of heparin with GDNF [54]. Use of an effective diffusion coefficient is justified when binding equilibrium is rapidly achieved compared to the rate of diffusion. The constants used for the predictions (D_{AB} , K_D) were initially estimated using literature values for diffusion of proteins of similar size through collagen gels ($D_{AB} = 7 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$) and interaction of GDNF with heparin at physiological salt concentration ($K_D = 1 \times 10^{-7} \text{ M}$). [55,56]. From these values, D_{eff} was predicted to be $1.52 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. However, the rate of diffusion through these PEG hydrogels may be much slower than in a collagen gel. Thus, the release data in Figures 4 through 7 were fit to solutions of Fick's second law to determine best fit effective diffusion coefficients. In physiological salt we observed a $D_{eff} = 4.84 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$, while in low salt we observed $D_{eff} = 2.52 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$. The differences may be explained by the higher affinity of GDNF for heparin in low salt conditions. All predicted curves in Figures 4 through 7 these values for the effective diffusion coefficients.

Figure 4 shows the high salt release for a single tiered scaffold made of microspheres incubated in 250 ng/mL GDNF during scaffold formation. Reasonable agreement was observed between the predicted release profile and the measured release profile. The low salt release for the same initial single-tiered profile (Figure 5) also was markedly similar to the predicted release profile. Although the predicted release profile used an effective diffusion coefficient that was partially determined from this data, subsequent results will show that these same effective diffusion coefficients are able to describe release from a variety of scaffolds. As expected, release was much slower into low salt buffer than high salt buffer.

Table 1 presents an analysis of linearity of the graphs in Figures 4 through 7. Linear regressions were performed on both experimental and predicted curves (excluding zero time

points), requiring that points at (or near) the open end(right end) be included in the regression (for examples, see Figure 8). This would correspond to the growth factor gradient that extending axons would sense. The percentage of the length of the scaffold that produced a regression with an r^2 value above a particular value is reported. For the predicted curves, we set a constraint of $r^2 \geq 0.995$ for the linear regression. Because the experimental curves contained experimental error, we set a constraint of $r^2 \geq 0.95$ for those regressions. It should be clear that the purpose of the linear regression is to characterize the morphology of the curve and not to explain the relationship between the measured and predicted curves. These values for the coefficient of determination produced similar percentages of the scaffolds with 'linear' GDNF concentration gradients (mostly below 50%) for the 1 tier scaffolds. Scaffolds with two tiers had consistently larger percentages of the scaffold with 'linear' gradients, in both the experimental and predicted curves.

For the 1 tier scaffold, the linearity varied greatly from 1 to 5 days in both experimental and both experimental and predicted cases, though the experiments have the surprising distinction of producing larger linear regions earlier and losing them over time as opposed to the model slowly growing more linear.

For the 2 tier scaffolds, the experimental profiles show larger linear regions than their predicted counterparts for all time points, though the 1 day profiles show the greatest disparity, with all or nearly all of the experimental profiles showing high linearity while the model predicts less than 25%. The presence of a linear gradient in GDNF concentration that emerges in just one day and is maintained for 12 days with only two tiers suggests that this strategy is highly promising for generating growth factor gradients within scaffolds. Furthermore, because of the high degree of predictability, more complex layer-by-layer arrangements may allow for the engineering of not only release kinetics but also gradient shape.

To demonstrate the robustness of this technique, multiple tiered scaffolds were fabricated with different amounts of GDNF in the tiers. Figure 9 shows three and four-tiered scaffolds with GDNF initially in alternating tiers. These scaffolds are the same length of their simpler counterparts and thus the tiers are smaller becoming much more homogenous in GDNF concentration after only a day. However, the initial GDNF concentration profile still strongly affects the resulting concentration profile at 1 day. These examples display the ability of this method to create more complex GDNF concentration profiles and release kinetics. The multiple tiers could also potentially be incubated with distinct concentrations of different growth factors, allowing release of multiple growth factors with different concentration profiles and release kinetics.

The heparin content in the different tiers can also be varied to affect the release kinetics and gradient-forming capabilities of the scaffolds. Figures 10 and 11 show two cases where the top tier had a lower concentration of heparin than the bottom tier. The bottom tier for both experiments had the original amount of heparin (20:1 PEG-OAm to heparin) while the second tier contained either no heparin (Figure 10) or 1/8 of the original heparin concentration (160:1 PEG-OAm to heparin; Figure 11). In both cases, the microspheres were incubated overnight with 250 ng/mL GDNF. A major caveat is that the microspheres

with less heparin were considerably less dense than their fully heparinated counterparts, and the photomicrographs clearly indicate that the microspheres with less heparin absorbed more GDNF during incubation. Thus, the top tier is initially much brighter than the bottom tier, so much so that the gain on the photodetector had to be greatly reduced below typical values. By one day, the brightness of the top tier was dramatically decreased, allowing the gain to be returned to normal values. The model predicted that with a higher initial GDNF concentration in the top layer, rapid release of GDNF from the top of the scaffold would be combined with diffusion into the bottom layer, creating a maximum in the GDNF concentration profile at one day. Although not as dramatic as predicted, this maximum in GDNF concentration was observed for both cases (Figures 10 and 11). These experiments illustrated that heparin concentration and microsphere crosslink density are both variables that can be adjusted to control the rate of release and the shape of the growth factor gradient over time. Ideally, methods would be developed in the future such that the heparin concentration did not affect the crosslink density.

A key consideration that must be addressed is whether or not the GDNF loses activity through this extensive incubation, scaffold formation, and subsequent release process. Due to significant dilution into the release medium, the concentration of GDNF was too low to test with cells, although subsequent studies will test the response of chick dorsal root ganglion cells within the scaffolds. For the current study, we asked if human GDNF retained its immuno-reactivity via ELISA measurements on the release solution. Results showed, approximately 8% of the initial activity introduced into the microspheres was released from a microsphere pellet after one day. A Matlab simulation of this process predicts a 55% release after 1 day. Adsorption to the various surfaces and the loss of activity overnight loading and one day of release may account for the difference between the model prediction and the measured activity. Further testing is required, but the retention of some GDNF immune-reactivity suggests the possibility for the retention of some biological activity in the released GDNF.

Conclusions

We devised robust methods for the creation of concentration gradients in GDNF. Through the sequential centrifugation of heparinated PEG microspheres loaded with varying amounts of GDNF, we quickly formed gradients in GDNF concentration. With relatively uncomplicated two-tiered scaffolds, linear gradients were produced and maintained for 12 days. The gradient shapes and kinetics agreed with mathematical predictions. We showed that the production of more complex gradients is possible and that microsphere characteristics, such as crosslink density and heparin content, can be tuned to alter release kinetics. This method of scaffold formation may be useful to improve nerve regeneration through engineered nerve guidance conduits.

Acknowledgments

The authors are grateful to Igor Efimov for use of the confocal microscope and funding from NIH 1R21NS0776501. We thank Casey Donahoe and Amanda Walker for technical assistance.

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Figure 1.
Heparin Attachment

Figure 2.

(A) Photomicrograph of PEG microspheres decorated with Dylight-488 labeled heparin. (20X) (B) Fluorescence of microspheres with labeled and unlabeled heparin. Excitation 488nm, Emission 530nm. (n=3, error bars shown)

Figure 3. Photomicrographs of Dylight-488 labeled GDNF on the boundaries of scaffolds made from PEG microspheres (A) incubated 11 minutes and (B) incubated 45 minutes. Scaffolds are located to the left of the red line while the supernatant lies to the right.

Figure 4.

Physiological salt (PBS) release of Dylight-488 labeled GDNF (constant initial profile) from Heparin decorated PEG microsphere (11 minute incubation) scaffold. (A) Composite photograph of fluorescence (GDNF) in scaffold at the zero time point, one day, and 5 days. (B) Graphical depiction of fluorescence (GDNF concentration) vs. the distance in the scaffold for the three time points: zero (blue), 1 day (green), and 5 days (red). $n=3$ sample error bars shown. (C) Plot of predicted release (GDNF Concentration vs. distance in the scaffold) based on Fick's 2nd law. Zero time point (blue), 1 day (green), and 5 days (red).

Figure 5.

“Low salt” (8 mM Sodium phosphate) release of Dylight-488 labeled GDNF (constant initial profile) from Heparin decorated PEG microsphere (11 minute incubation) scaffold. (A) Composite photograph of fluorescence (GDNF) in scaffold at the zero time point, one day, and 5 days. (B) Graphical depiction of fluorescence (GDNF concentration) vs. the distance in the scaffold for the three time points: zero (blue), 1 day (green), and 5 days (red). n=3 sample error bars shown. (C) Plot of predicted release (GDNF Concentration vs. distance in the scaffold) based on Fick's 2nd law. Zero time point (blue), 1 day (green), and 5 days (red).

Figure 6.

2-tier initial profile, physiological salt (PBS) release of Dylight-488 labeled GDNF from Heparin decorated PEG microsphere (11 minute incubation) scaffold. (A) Composite photograph of fluorescence (GDNF) in scaffold at the zero time point, one day, 5 days, and 12 days. (B) Graphical depiction of fluorescence (GDNF concentration) vs. the distance in the scaffold for the four time points: zero (blue), 1 day (green), 5 days (red), and 12 days (light blue). $n=3$ sample error bars shown. (C) Plot of predicted release (GDNF Concentration vs. distance in the scaffold) based on Fick's 2nd law. Zero time point (blue), 1 day (green), 5 days (red), and 12 days (light blue).

Figure 7.

2-tier initial profile, “low salt” (8 mM sodium phosphate) release of Dylight-488 labeled GDNF from Heparin decorated PEG microsphere (11 minute incubation) scaffold. (A) Composite photograph of fluorescence (GDNF) in scaffold at the zero time point, one day, 5 days, and 12 days. (B) Graphical depiction of fluorescence (GDNF concentration) vs. the distance in the scaffold for the four time points: zero (blue), 1 day (green), 5 days (red), and 12 days (light blue). $n=3$ sample error bars shown. (C) Plot of predicted release (GDNF Concentration vs. distance in the scaffold) based on Fick's 2nd law. Zero time point (blue), 1 day (green), 5 days (red), and 12 days (light blue).

Figure 8.

Sample linear regions for data presented in Table 1. Shown are linear regressions over the portion of each concentration profile which yielded a coefficient of determination above a particular threshold (exp: $R^2 \geq 0.95$, pred: $R^2 \geq 0.995$), corresponding to correlation coefficients of 0.975 and 0.9975, respectively. The percentage of length of the scaffold (x axis) that each section spanned is reported in Table 1. As in previous figures green, red, and blue lines correspond to 1, 5, and 12 days post-scaffold formation, respectively.

Figure 9. Multi-Tier Formations. The versatility of this gradient formation technique is displayed by three scaffolds with more complex patterns of GDNF. Composite photographs of fluorescence (GDNF) in the scaffolds taken at the zero time point and after one day. (A) 3-tier initial pattern: GDNF-Empty-GDNF. (B) 3-tier initial pattern: Empty-GDNF-Empty. (C) 4-tier initial pattern: GDNF-Empty-GDNF-Empty.

Figure 10.

PEG microsphere (11 minute incubation) scaffold with first half ($d < 0.5$ cm) made with 20:1 PEG-Oam to Heparin and the second half ($d > 0.5$ cm) made without heparin, releases Dylight-488 labeled GDNF (constant initial profile) under “low salt” (8 mM Sodium phosphate) conditions. (A) Composite photograph of fluorescence (GDNF) in scaffold at the zero time point, one day, and 5 days. (B) Graphical depiction of fluorescence (GDNF concentration) vs. the distance in the scaffold for the three time points: zero (blue), 1 day (green), and 5 days (red). (C) Plot of predicted release (GDNF Concentration vs. distance in the scaffold) based on Fick's 2nd law. Zero time point (blue), 1 day (green), and 5 days (red).

Figure 11.

PEG microsphere (11 minute incubation) scaffold with first half ($d < 0.5$ cm) made with 20:1 PEG-Oam to Heparin and the second half ($d > 0.5$ cm) made with 160:1 PEG-Oam to Heparin, releases Dylight-488 labeled GDNF (constant initial profile) under “low salt” (8 mM Sodium phosphate) conditions. (A) Composite photograph of fluorescence (GDNF) in scaffold at the zero time point, one day, and 5 days. (B) Graphical depiction of fluorescence (GDNF concentration) vs. the distance in the scaffold for the three time points: zero (blue), 1 day (green), and 5 days (red). (C) Plot of predicted release (GDNF Concentration vs. distance in the scaffold) based on Fick's 2nd law. Zero time point (blue), 1 day (green), and 5 days (red).

Table 1

Linearity comparisons between experimental and predicted models.

	1 Day	5 Days	12 Days
1 tier, Phys Salt (Exp)	62%	0%	-
1 tier, Phys Salt (Pred)	29%	60%	-
1 tier, Low Salt (Exp)	11%	19%	-
1 tier, Low Salt (Pred)	21%	48%	-
2 tier, Phys Salt (Exp)	92%	92%	81%
2 tier, Phys Salt (Pred)	24%	65%	63%
2 tiers, Low Salt (Exp)	100%	92%	92%
2 tiers, Low Salt (Pred)	10%	80%	62%

Shown are the percentages of the length of the scaffolds with a linear regression which yielded a coefficient of determination above a particular threshold are shown (exp: $R^2 = 0.95$, pred: $R^2 = 0.9975$), corresponding to correlation coefficients of 0.975 and 0.9975, respectively. Experimental (Exp) and predicted data (Pred) are shown for comparison. The line was required to include points at (or near) the open (right) end of the scaffolds. It should be understood that the coefficients of determination are used here to describe the morphology of the curve and not to explain the underlying relationships between the measured concentration profile and the predicted values. Sample linear regions are shown in Figure 8.