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Gene Therapy Vectors with Enhanced Transfection Based on Hydrogels Modified with Affinity Peptides

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Abstract

Regenerative strategies for damaged tissue aim to present biochemical cues that recruit and direct progenitor cell migration and differentiation. Hydrogels capable of localized gene delivery are being developed to provide a support for tissue growth, and as a versatile method to induce the expression of inductive proteins; however, the duration, level, and localization of expression is often insufficient for regeneration. We thus investigated the modification of hydrogels with affinity peptides to enhance vector retention and increase transfection within the matrix. PEG hydrogels were modified with lysine-based repeats (K4, K8), which retained approximately 25% more vector than control peptides. Transfection increased 5- to 15-fold with K8 and K4 respectively, over the RDG control peptide. K8- and K4-modified hydrogels bound similar quantities of vector, yet the vector dissociation rate was reduced for K8, suggesting excessive binding that limited transfection. These hydrogels were subsequently applied to an *in vitro* co-culture model to induce NGF expression and promote neurite outgrowth. K4-modified hydrogels promoted maximal neurite outgrowth, likely due to retention of both the vector and the NGF. Thus, hydrogels modified with affinity peptides enhanced vector retention and increased gene delivery, and these hydrogels may provide a versatile scaffold for numerous regenerative medicine applications.

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Keywords

gene delivery; hydrogel; affinity peptide; vector retention; three-dimensional

Introduction

In regenerative medicine, tissue engineering scaffolds aim to create an instructional milieu for guiding tissue formation. Tissue formation is complex and directed by a range of stimuli, requiring multifunctional systems that can present biomolecular cues such as growth factors or cell adhesion molecules in the local environment [1]. As an example, the regeneration of the spinal cord will require trophic factors to promote neuronal survival and neurite outgrowth, while also blocking or degrading inhibitory factors that limit regeneration. However, the development of tissue engineering scaffolds that efficiently deliver one or more biomolecular cues locally to facilitate neurite outgrowth has been difficult due to loss of these factors from the injury site.

Hydrogels that deliver gene therapy vectors provide a system to present multiple biomolecular cues locally, which can direct the response of either endogenous or transplanted cells. Hydrogels serve to create and maintain space for tissue growth and are attractive tissue engineering scaffolds as their mechanical properties are similar to many soft tissues. Additionally, the mechanics, degradation rates, and the amounts and identities of functional groups can be tailored for specific applications [2, 3]. Gene delivery from hydrogels provides a versatile approach to induce the expression of tissue inductive factors locally; yet can be limited by insufficient transfection [4]. In past studies, gene therapy vectors have been entrapped within hydrogels, which often provide a sustained release of the vector by diffusion through the hydrogel mesh [5, 6]. Cell infiltration into the hydrogel is often accompanied by degradation of the matrix that increases the mesh size and enhances release of the vector [7–9]. This enhanced vector release with matrix degradation contributes to mass transport limitations that limit gene delivery.

Strategies to circumvent mass-transport limitations in gene delivery from biomaterials have investigated the interactions of the vector with the material, such as covalent tethering or non-specific interactions such as van der Waals, electrostatic, or hydrophobic [10, 11]. A significant challenge for obtaining transfection throughout the hydrogel is vector retention. For 2D cell culture, immobilization of the vector onto biomaterial surfaces has co-localized the vector with adherent cells to reduce mass transport limitations and localize delivery [12–14]. Immobilization must adequately retain the vector, while permitting cellular internalization. These strategies have included specific interactions between the vector and material, such as biotin-neutravidin binding, which requires the functionalization of both the biomaterial and the vector [10]. Alternatively, non-specific interactions have been employed to slow the release of vectors through electrostatic interactions with the matrix, as exemplified by the use of oligo(poly(ethylene glycol) fumarate) [15], cationized gelatin [16], and poly-L-lysine (PLL) in collagen [17]. Adapting these strategies to hydrogels in order to retain vectors may enhance transfection locally in order to promote regeneration.

In this report, we investigate the hypothesis that the efficacy of localized gene delivery within hydrogels can be enhanced by incorporating functional groups that increase vector retention within the hydrogel, yet dissociate adequately to improve transfection. PEG-based hydrogels that support cell adhesion and are crosslinked with plasmin-sensitive peptides were functionalized with lysine-based peptides (K4, K8) that can reversibly associate with lipoplexes [18, 19]. Hydrogels functionalized with these peptides were studied for their ability to retain lipoplexes. Binding and release studies were conducted to measure the

association and dissociation of the vector to the peptides, with dissociation necessary to allow for cellular association of the vector. Transfection studies subsequently measured the extent and duration of transgene expression within hydrogels modified with affinity peptides. The utility of these hydrogels in regenerative medicine was assessed in a model of neurite outgrowth, in which a nerve growth factor (NGF) construct was delivered to accessory cells that were co-cultured with neurons. NGF binding and release to the affinity peptides was assessed, as previous studies suggest that lysine-containing hydrogels are capable of binding NGF [20]. This report identifies lysine-containing affinity peptides for enhancing the retention of lipoplexes that can increase transfection locally. PEG hydrogels modified with these peptides may be a versatile scaffold that can be readily tailored for delivery of gene therapy vectors for numerous regenerative medicine applications.

Materials and methods

PEG-vinyl sulfone(PEG-VS) synthesis

Multiarm PEG-VS was synthesized as previously described [21]. Briefly, 4-arm PEG-OH (5 g) was dried by azeotropic distillation in toluene to remove traces of water and then was dissolved directly in 300 mL of anhydrous dichloromethane. NaH was added under nitrogen at a 10-fold molar excess over OH groups. After hydrogen evolution, divinylsulfone was added very quickly at 100-fold molar excess over OH groups. The reaction was refluxed for 3 days under nitrogen atmosphere with constant stirring. Afterwards, the reaction solution was neutralized with concentrated acetic acid, filtered through paper until clear, and reduced to a small volume (ca. 10 mL) by rotary evaporation. PEG was precipitated by adding the remaining solution dropwise into ice-cold diethyl ether. The precipitated polymer was recovered by filtration, washed with diethyl ether, and dried under vacuum. Derivatization was confirmed with ^1H NMR (D_2O , 50°C): 3.6 ppm (PEG backbone), 6.2 ppm (d, 1H, dCH_2), 6.3 ppm (d, 1H, dCH_2), and 6.8 ppm (dd, 1H, $-\text{SO}_2\text{CHd}$). The degree of end group conversion, as shown by NMR, was found to range from 95 to 98%.

Plasmids

Plasmids containing a cytomegalovirus promoter were produced with a QiagenMaxiprep kit (Valencia, CA) and stored in Tris-EDTA buffer at -20°C . Reporter plasmid encoding for *Gaussia*Luciferase (pGLuc) (New England BioLabs, Ipswich, MA) was used to quantify transgene expression. Plasmid encoding for firefly luciferase/enhanced green fluorescent fusion protein (pEGFP-Luc) was used in retention studies. Plasmid encoding for full-length mouse NGF in the RK5 vector backbone (pRK5-NGF) (gifted by Dr. H. Nomoto, Gifu Pharmaceutical University, Japan) was used in DRG outgrowth studies.

Hydrogel preparation

HT-1080 cells (gifted by Dr. J. Jones of Northwestern University, Chicago, IL) were maintained in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA) supplemented with 10% FBS and 1% streptomycin/penicillin. Cells were cultured at 37°C and 5% CO_2 and split using trypsin containing 0.05% EDTA. Hydrogels of 15 μL encapsulating cells and DNA were formed with Michael-type addition by reacting the free thiols of cysteine residues of mono-functional or tri-functional peptides with unsaturated PEG moieties as described previously [19]. All peptides were custom synthesized at the Institute for Bionanotechnology in Medicine Core Facility at Northwestern University (Chicago, IL). PEG monomers were functionalized with 5 mM GCGYGRGDSPG (RGD) cellular adhesion sites per previous studies [9, 19] and 5 mM of candidate vector affinity peptides: GCGKKKK (K4) or GCGYGKKKKKKKK (K8). Additionally, two control peptides were employed: a peptide with charged amino acids yet overall neutral charge GCGKDKG (KDKG) or a peptide lacking lysines that does not support cell adhesion but is

employed to achieve the same degree of substitution without supporting cell adhesion GCGYGRDGSPG (RDG). The kinetics of conjugation of each peptide to PEG-VS at pH 8 was tested by tracking the concentration of free thiols using an Ellman's test. These studies indicated that all conditions had the same degree of functionalization with the tested peptide. To encapsulate lipoplexes, PEG-VS was dissolved in 0.3 M triethanolamine (TEOA), pH 8.0 at a concentration of 100 mg/mL. RGD and affinity peptides were added to the PEG solutions at a known final concentration and allowed to react for 15 mins at 37 °C. Meanwhile, lipoplexes were formed following the manufacturer's protocol by adding 1.5 µL TransFast transfecting reagent (Promega, Madison, WI) to 1 µg pGLuc and incubating 15 mins at room temperature. Cell solutions were prepared to yield a final cell concentration of 1.8×10^6 cells/mL. Cell and vector solutions were added to functionalized PEG solutions and encapsulated upon gelation by addition of a molar ratio of 1:1 vinyl sulfone to free thiol of the crosslinking peptide to maintain the same crosslink density (i.e., mechanical properties) across all the conditions tested. The tri-functional, plasmin degradable peptide sequence (GCKYKNCRGYKNCRG) was dissolved in 0.3 M TEOA, pH 10.0 to maintain the reduction of free thiols in accordance to previous reports encapsulating cells and lipoplexes [7, 8]. This tri-functional peptide sequence results in more efficient and homogenous crosslinking, while still degrading locally by cells within the matrix, than previously used bi-functional crosslinking peptide sequences [8, 22]. Gelation was permitted to occur for 15 mins at 37 °C. Following gelation, hydrogels were transferred to wells of a 96 well plate and washed immediately and after swelling 1–2 h with 200 µL supplemented DMEM (cDMEM).

DNA retention

Plasmid pEGFP-Luc was radiolabeled with α - ^{32}P -dATP (PerkinElmer, Waltham, MA) using a nick translation kit (Amersham Pharmacia Biotech, Piscataway, NY) according to the manufacturer's protocol with minor modifications [23]. Radiolabeled plasmid was complexed with TransFast as described above. The retention of the lipoplexes within 10% PEG hydrogels containing 5 mM RGD and 5 mM RDG, KDKG, K4, or K8 peptide was measured. HT-1080 cells (1.8×10^6 cells/mL) and radiolabeled lipoplexes (3 µg/gel) were co-encapsulated within hydrogels. Following gelation, hydrogels were incubated in cDMEM and washes were collected immediately and at 2 and 4 h followed by periodic sampling with replacement for 15 days. Results obtained were converted to DNA amount using a standard curve, and the amount of DNA retained in the presence of cells was normalized to the measured total amount of DNA encapsulated per hydrogel.

Assessment of DNA association and dissociation

Binding and release of lipoplexes to hydrogel surfaces was investigated to determine the kinetics of association and dissociation of the vectors to the affinity peptide without the complication due to physical entrapment [24]. Hydrogels containing 5 mM RDG, KDKG, K4, or K8 peptide were formed as described above in the absence of cells and DNA. Hydrogels were washed immediately and after 1 h of swelling in unsupplemented DMEM (uDMEM) to remove unreacted monomers. Radiolabeled lipoplexes suspended in uDMEM were added to wells at a concentration of 2 µg DNA/well and incubated for 2 h at room temperature. Following incubation, the DNA solution was removed and added to 5 mL Biosafe II scintillation cocktail for subsequent counting with a scintillation counter. Hydrogels were washed twice with uDMEM immediately and after 2, 4 and 6 h followed by periodic sampling with replacement for 6 days. The acquired counts were correlated to DNA amount using a standard curve, and results are recorded in amount DNA bound to the hydrogel and released over the measured time points.

Quantification of expression levels

Transfection studies were carried out to quantify the expression level profiles of cells cultured within hydrogels containing 10% PEG with 5 mM RGD and 5 mM RDG, K4, or K8. Hydrogels were formed as described above encapsulating HT-1080 cells and 2 μ g pGLucilipoplexes. Hydrogels were cultured in a 96 well plate containing 200 μ L cDMEM at 37°C and 5% CO₂ for 15 days based on previously published results. Media was collected and replaced once per day and collected media was stored at -80 °C for subsequent luciferase measurements. Following one freeze/thaw cycle, cell-secreted luciferase levels were measured using the Gaussia luciferase assay kit (New England BioLabs, Ipswich, MA) and a luminometer (Turner Design, Sunnyvale, CA). The luminometer was set for a 3 s delay with 10 s signal integration time. Luciferase levels are reported in relative light units (RLUs) per well.

In vitroneurite outgrowth and quantification

Hydrogel precursor solutions containing 10% PEG, 5 mM RGD, 5 mM RDG, KDKG, K4 or K8, HT-1080 cells, and lipoplexes encoding for NGF were formed as described above. Dorsal root ganglia (DRG) explants were isolated from day 8 white leghorn chicken embryos (Phil's Fresh Eggs, Forrester, IL) and maintained in Hank's buffered salt solution (HBSS) supplemented with 6g/l dextrose until ready for use. Before encapsulation, DRG explants were washed in precursor solutions by mouth pipeting with fire-polished glass Pasteur pipettes to remove HBSS. Explants were transferred to precursor solutions and encapsulated upon addition of the crosslinking peptide. After gelation, hydrogels were transferred to wells of a 96 well plate and cultured in cDMEM. DRG explants were imaged with phase microscopy and the radius of the explants upon encapsulation was determined using the program ImageJ. After the 4 day co-culture, the hydrogels were washed with PBS and fixed with 4% paraformaldehyde. The neurofilament of the DRG explants were stained using anti-neurofilament 200 antibody (Sigma, N-4142) diluted 1:300 in 1% normal goat serum (Vector Labs, S-1000) in PBS with a 2 h incubation period. After PBS washes, explants were incubated with Alexa 546 goat anti-rabbit secondary antibody (Invitrogen, A-11010) diluted 1:500 in 1% normal goat serum in PBS for 1 h. Cell nuclei were stained for using Hoechst 33258 (Invitrogen, H3569). The neurofilament of DRG explants was captured with fluorescence microscopy, and the radius of neurite outgrowth was determined using ImageJ. The radial length of neurite outgrowth was calculated by subtracting the radius of the explants upon encapsulation from the radial neurite outgrowth on day 4.

Assessment of NGF binding and release

The association and dissociation of NGF protein to affinity peptides was measured with hydrogels containing 10% PEG and 5 mM RDG, K4, or K8 peptides in the absence of cells. Hydrogels were formed as described above, transferred to a 96 well plate, and washed with uDMEM. Recombinant rat β -NGF (R&D Systems, Minneapolis, Minnesota) was diluted with uDMEM to yield a final concentration of 0.05 μ g/mL and 200 μ L of this solution was added to wells containing hydrogels. After 2 h, media was collected with replacement and at 4, 6, 24 and 48 h, and solutions were stored at -80 °C. At the end of the study, the amount of NGF per sample was analyzed using a ChemoKine NGF sandwich enzyme-linked immunosorbent assay (ELISA) (Millipore, Billerica, MA). Results are reported as the amount of NGF bound to the hydrogels and the subsequent cumulative release.

Mathematical modeling of NGF concentration profiles

Mathematical modeling of the NGF concentration profile within the hydrogel was employed to provide mechanistic insights regarding the impact of affinity peptides on transfection and

retaining the NGF protein. The binding and release of NGF to affinity peptides are described by the following chemical reactions:

where N , AP , and NAP stand for NGF, affinity peptide, and NGF bound to affinity peptide, respectively. NGF association and dissociation rate constants for binding to affinity peptides were calculated from NGF ELISA data collected for the binding and release studies (Table 1).

Reaction-diffusion (RD) equations 3–7 describe changes in the concentration of all species involved. In these equations, the terms involving Laplacian operators, ∇^2 , account for the diffusion of mobile species (N , AP -unbound, and NAP -unbound), while the remaining terms describe reaction kinetics as prescribed by Equations 1,2 and also the production of NGF by transfected cells and its degradation in solution

$$\frac{\partial C_N}{\partial t} = D_N \nabla^2 C_N - k_f C_N (C_{AP, bound} + C_{AP, unbound}) + k_r (C_{NAP, bound} + C_{NAP, unbound}) - k_{deg} C_N + k_{prod} C_{transfected\ cells} \quad (3)$$

$$\frac{\partial C_{AP, unbound}}{\partial t} = D_{AP, unbound} \nabla^2 C_{AP, unbound} + \frac{V_m C_{AP, bound}}{K_m + C_{AP, bound}} - k_f C_N C_{AP, unbound} + k_r C_{NAP, unbound} \quad (4)$$

$$\frac{\partial C_{AP, bound}}{\partial t} = -\frac{V_m C_{AP, bound}}{K_m + C_{AP, bound}} - k_f C_N C_{AP, bound} + k_r C_{NAP, bound} \quad (5)$$

$$\frac{\partial C_{NAP, unbound}}{\partial t} = D_{NAP, unbound} \nabla^2 C_{NAP, unbound} + \frac{V_m C_{NAP, bound}}{K_m + C_{NAP, bound}} + k_f C_N C_{AP, unbound} - k_r C_{NAP, unbound} \quad (6)$$

$$\frac{\partial C_{NAP, bound}}{\partial t} = -\frac{V_m C_{NAP, bound}}{K_m + C_{NAP, bound}} + k_f C_N C_{NAP, bound} - k_r C_{NAP, bound} \quad (7)$$

The degradation of the hydrogel matrix by plasmin is described using Michaelis-Menten kinetics. The maximum rate of crosslink degradation, V_m , was estimated as 7.41×10^{-8} M/min based on the rate of hydrogel degradation. The Michaelis-Menten constant, K_m , was obtained from the literature (640×10^{-6} M) [19]. Values for the diffusivity of NGF, D_N , and the rate constant for NGF degradation, k_{deg} , in buffered solution were determined from published reports to be 12×10^{-7} cm²/s and 0.0029 min⁻¹, respectively [25, 26]. NGF

association and dissociation, k_f and k_r , respectively, were determined from data describing vector binding and release from the hydrogel surfaces (Fig. 4). Constant protein production rate, $k_{prod} = 7.1 \times 10^{-5}$ pg/cell/min was assumed for all conditions in initial simulations based on previously published reports [27]. In subsequent simulations, protein production rates were correlated to transfection data for hydrogels containing K4, K8, or KDKG peptide, and were determined to be 9.5×10^{-5} , 3.8×10^{-5} , and 4.7×10^{-6} pg/cell/min, respectively.

The diffusion coefficient of the remaining species in the gel was approximated with the molecular weight of each species using a power-law expression and correlation for diffusion of protein through porous hydrogels (Equation 8) [28–30].

$$D_{species,hydrogel} = 0.9 A M_w^{-1/3} \times 10^{-7} \quad (8)$$

where M_w is the molecular weight of each species and the constant $A = 260 \text{ cm}^2/(\text{s} \cdot \text{Dalton})$.

The gel was represented as a rectangular slab of dimensions 2.75 mm in length and width. The locations of transfected cells within the gel were randomly distributed throughout the matrix. The initial condition of the bound affinity peptide, $C_{AP,bound}$, was 5 mM, and the concentration of remaining components was zero throughout the culture (Equation 9).

$$C_i(x, y, z, t=0) = 0 \quad \text{for } i = N, AP - \text{unbound}, NAP - \text{bound}, \text{ or } NAP - \text{unbound} \quad (9)$$

The boundary conditions were imposed to account for the diffusive flux of the mobile species out of the gel and into the media, in the z -direction (Equations 10–12).

$$\frac{\partial C_N}{\partial z}(x, y, z=z_{\max}, t) = D_N \nabla^2 C_N - k_f C_N C_{AP,unbound} + k_r C_{NAP,unbound} \quad (10)$$

$$\frac{\partial C_{AP,unbound}}{\partial z}(x, y, z=z_{\max}, t) = D_{AP,unbound} \nabla^2 C_{AP,unbound} - k_f C_N C_{AP,unbound} + k_r C_{NAP,unbound} \quad (11)$$

$$\frac{\partial C_{NAP,unbound}}{\partial z}(x, y, z=z_{\max}, t) = D_{NAP,unbound} \nabla^2 C_{NAP,unbound} + k_f C_N C_{AP,unbound} - k_r C_{NAP,unbound} \quad (12)$$

No-flux boundary conditions were applied at these walls for all species present (Equation 13).

$$\begin{aligned}
& \frac{\partial C_i}{\partial x}(x=0, y, z, t) \\
&= \frac{\partial C_i}{\partial x}(x=x_{\max}, y, z, t) \\
&= \frac{\partial C_i}{\partial y}(x, y=0, z, t) \\
&= \frac{\partial C_i}{\partial y}(x, y=y_{\max}, z, t) \\
&= 0 \quad \text{for } i \\
&= N, AP - bound, AP - unbound, NAP - bound, \text{ or } NAP - unbound
\end{aligned} \tag{13}$$

The concentration of NGF within the gel was averaged over the three-dimensional domain for comparison with the concentrations reported in the literature [27]. With these preliminaries, the RD equations were solved numerically using the forward Euler integration method [31] with a time step of 1 s and node spacing of 55 μm .

Statistics

Results were analyzed using ANOVA with post hoc *t*-test for multiple comparisons with a 95% confidence level using JMP software (SAS Institute, Cary, NC). All experiments were performed in quadruplicate and mean values with standard deviations are reported.

Results

Vector retention

Initial studies investigated the retention of the lipoplexes within hydrogels that were modified with affinity peptides. Lipoplexes, which had a zeta potential of -41.3 ± 7.21 mV consistent with previous reports [32, 33], were encapsulated within the hydrogel, and were expected to interact with the cationic lysine based peptides. Previously, we reported that DNA retention was reduced from 86% after 16 days in the absence of cells, to 10% after 16 days in the presence of cells [9]. Thus, vector retention was determined in the presence of HT-1080 cells that were homogeneously distributed throughout the hydrogel, as these cells secrete proteases that degrade the crosslinks to increase the mesh size, potentially enhancing release. Hydrogels containing K8 peptide retained a statistically greater amount of DNA throughout the 15-day study relative to the RDG condition ($p < 0.05$) (Fig. 1). For the K4 peptide, a statistically greater amount of DNA was retained relative to RDG only at the later time points ($p < 0.05$) (days 5–15). Hydrogels containing KDKG retained similar DNA quantities as the RDG condition. Interestingly, vector retention relative to the RDG condition was enhanced only for the K8 condition during the initial hydrogel swelling. After 15 days, the amounts of vector retained within the hydrogels was greatest for K8, while K4 resulted in intermediate retention, and KDKG and RDG resulted in the lowest retention.

DNA binding and release

Vector association and dissociation with the affinity peptides incorporated into the matrix was subsequently assessed. To isolate the contribution of the affinity peptides to vector retention, vectors were incubated with peptide-functionalized hydrogels for 2 h to allow binding. After replacing the media to remove the unbound lipoplexes, the hydrogels were incubated for an additional 2 h and media was collected to quantify the release of bound vectors. Following the initial incubation, hydrogels containing K4 and K8 bound significantly greater amounts of DNA ($p < 0.05$) (96 and 94 ng, respectively) than hydrogels

containing RDG or KDKG peptide (54 and 53 ng, respectively) (Fig. 2a). The release studies indicate that the amount of DNA retained was greatest for K8-containing hydrogels (41% by day 6), which was the only condition that was significantly different than the RDG condition (20% by day 6) over the 6-day study (Fig. 2b). The mean quantity of vector bound to hydrogels containing K4 peptides was approximately 5% greater than the KDKG and RDG conditions throughout the study.

Gene expression

Transfection levels were subsequently measured to determine the impact of enhanced vector retention on transgene expression. Expression was observed throughout the 14-day culture for all conditions; however, expression levels for K4 and K8 conditions were significantly increased relative to the RDG condition (Fig. 3). Interestingly, maximal transfection occurred within hydrogels containing K4 peptides (15-fold increase relative to RDG between days 2 and 6, $p < 0.05$). The K4 condition had maximal DNA association and an intermediate dissociation rate relative to the other peptides. Cells encapsulated in hydrogels containing the K8 peptide, which had DNA association similar to the K4 peptide yet a decreased dissociation, produced transfection levels intermediate between the K4 and RDG peptide conditions (5-fold increase relative to RDG between days 2 and 6). At later culture times (days 12 and 14), both the K4 and K8 peptide conditions resulted in transfection levels significantly greater than the RDG ($p < 0.05$). These results indicate that increasing DNA retention increases transgene expression levels, and suggests that excessive binding of the vector limited the extent to which gene expression was enhanced.

Neurite extension

The utility of enhanced vector retention and gene expression was subsequently demonstrated using a co-culture model targeted to promote neurite outgrowth. Affinity peptide modified hydrogels were formed that entrapped three components: lipoplexes encoding for NGF, HT-1080 cells, a cell line representing accessory cells that infiltrate into the injury site, and a DRG explant. Interestingly, neurite outgrowth was greatest for the K4-containing hydrogels and was statistically greater than that of hydrogels containing either KDKG or RDG peptide (Fig. 4). These results indicate that enhancing vector retention to the matrix and gene expression correlates with enhanced neurite extension.

NGF binding and release

The mechanism by which the affinity peptides enhance neurite outgrowth was subsequently investigated. The NGF protein has previously been reported to associate with lysine-containing peptides [18], which could also function to retain NGF to enhance neurite outgrowth. Thus, the association and dissociation of NGF protein to hydrogels containing affinity peptides was subsequently investigated. Hydrogels containing RDG, K4, or K8 affinity peptides were incubated with 10 ng/well NGF in uDMEM. After the incubation, the amount of NGF remaining in solution measured with an ELISA, and was greatest for the RDG condition (3.8 ng), which was statistically greater than the K8 condition (3.2 ng, $p < 0.05$) (Fig. 5a). The results for the K4 condition were intermediate between the RDG and K8 conditions with 3.4 ng NGF measured in the media. After the initial binding, the rate of NGF dissociation from the hydrogels was monitored over 48 h. Release of NGF remained lowest for the K8-containing hydrogels and was statistically lower than that of the RDG negative control ($p < 0.05$) (Fig. 5b). The K4-containing hydrogels released NGF intermediate between the RDG and K8 conditions. These results are consistent with previous reports have that shown that lysine-containing hydrogels are able to retain NGF protein [20].

Mathematical modeling to verify the contribution of DNA and NGF retention

A mathematical model was developed to quantify the relative contributions of the affinity peptides that retain either the vector for enhanced gene delivery or NGF protein to enhance the local concentration of NGF and neurite outgrowth. A set of partial differential equations was employed to determine the concentration of NGF, both temporally and spatially, throughout the hydrogel and the adjacent media. According to the model, production of the protein by cells within the hydrogel leads to NGF concentrations in the hydrogel that exceed that in the bulk media. The contribution of NGF binding to affinity peptides on the concentration in the hydrogel was subsequently investigated by varying the binding affinity of the protein to the affinity peptides (Fig. 6a), with the relative affinities based on the measured binding of NGF to the peptides. In these simulations, the NGF concentration inside the gels increased over time for all conditions. Hydrogels with K4 peptide had the maximal predicted NGF concentration (1.18-fold greater than KDKG), while hydrogels with K8 had an intermediate NGF concentration (1.17-fold greater than KDKG). The KDKG control peptide had the lowest NGF concentration.

Subsequently, the contribution of the lipoplex binding to affinity peptides was assessed by applying protein production rates that correlate with the transfection data[34]. NGF concentrations were greatest for the K4 condition, which were 24-fold greater than KDKG, while K8 had an intermediate NGF concentration, 9.5-fold greater than KDKG. KDKG had the lowest predicted NGF concentration (Fig. 6b). These results suggest affinity peptides have the greatest contribution to the local NGF concentration through retaining the vector and enhancing transfection rather than by limiting release of the produced protein.

Discussion

Currently, the localization, duration, and level of expression hinders the utility of vector-loaded hydrogels for regenerative medicine. This report investigates the development of PEG-hydrogels modified with affinity peptides to retain the vectors within the hydrogel, thereby maintaining an elevated concentration locally that can enhance gene delivery. Hydrogels were modified with lysine containing peptides of varying lengths that reversibly associate with the vector. Vector retention is expected to be particularly critical for *in vivo* translation, as targeting the infiltrating cells will require that the vector be retained until the cells infiltrate the gel, which occurs on the time course of days. Modifying hydrogels with these affinity peptides could provide a versatile tool for the localized delivery of various gene therapy vectors, including naked plasmid, lipoplexes, and polyplexes,

The cationic peptides of the hydrogel interact with the anionic lipoplexes to limit release from the hydrogel. We had previously reported that physical entrapment of the lipoplexes within the PEG hydrogel limited release since the mesh size of the hydrogels (~25 nm) is less than the particle diameter (~486 nm) [8, 9]; however, this retention was substantially reduced in the presence of cells [9]. Cells secrete proteases that degraded the peptide crosslinkers, which enabled migration through the hydrogel [19]. HT-1080 cells produce and secrete urokinase-type plasminogen activator that converts the inactive zymogen plasminogen to the active plasmin proteinase, which can degrade the peptides crosslinking these hydrogels [34]. In the absence of affinity peptides, this local degradation of the matrix led to the release of the entrapped vector, with diffusion from the gel occurring preferentially over cell association. For the report herein, the cationic lysine-containing peptides interact with lipoplexes that have a negative zeta potential (-41.3 ± 7.21 mV), which is within the range previously reported for lipoplexes[32]. The peptides do not impact cell viability nor activity of the vector over time (data not shown). Vectors encapsulated within the hydrogels may be stabilized by the incorporated cationic peptides, which has been suggested to enhance vector activity [10]. Cationic peptides possessing lysine repeats have been previously shown to

bind DNA and produce condensates that are more easily internalized by the cell, leading to increases in gene expression [18]. Since hydrogel degradation could provide lysine peptides that interact with DNA, we tested the ability of cleaved affinity peptides to enhance transfection, and observed no increase in transgene expression compared to the control (data not shown). Based on these controls, increased transfection within hydrogels was attributed to an increased retention of the lipoplexes.

Vector retention was significantly enhanced with K4 and K8 peptides, while transgene expression was maximal with the K4 peptide. Maximal expression with K4 likely reflects enhanced retention of the vector, while also providing sufficient dissociation from the matrix to enable cellular association. The overall neutral peptide KDKG produced the same amount of vector retention as the control peptide RDG (Fig. 1), which was less than that of the polylysine peptides. The K4 and K8 peptides bound similar quantities of the vector, indicating a similar association; however, the amount of bound vector that was released was significantly greater for K4 relative to K8 (Fig. 2), which is consistent with previous reports regarding the affinity of polylysine peptides for DNA [18, 35]. Expression levels within hydrogels containing the K8 peptide were less than expression levels for hydrogels containing K4 (Fig. 3), suggesting transfection is related to the binding strength between DNA and the material [35]. Therefore, both the K4 and K8 affinity peptides effectively retain the vector as the matrix degrades; however, the more rapid dissociation of the vector from the K4 peptide relative to K8 may have facilitated cellular internalization and enabled greater expression.

The increased localized transgene expression provided by the affinity peptides was applied to promote tissue development using neurite outgrowth as a model. In nerve regeneration, neurotrophic factors, such as NGF, serve as chemotactic agents for supporting neuron survival and inducing and directing axonal elongation of neurons [36]. Delivery of these factors as gene therapy vectors that will induce sustained expression can extend their availability over long periods of time, which will be important for *in vivo* translation. *In vivo*, localized transfection will be essential to induce axonal growth into and across the injury. This transfection should likely be transient to encourage re-entry into the host tissue [37, 38]. In the co-culture model, maximum neurite outgrowth occurred in hydrogels containing K4 (Fig. 4), the condition that induced the highest levels of gene expression (Fig. 3). Although previous studies indicate that lysine-conjugated hydrogels improved NGF protein retention [20], this contribution of the affinity peptide to maintaining localized NGF concentration was less of a factor than the enhanced gene delivery (Fig 6). The contributions of the affinity peptides binding NGF and lipoplexes were assessed using a mathematical model, which indicated that, for the same protein production (i.e., level of expression), the NGF concentration within the hydrogels increased only 17% or 18% for K4 or K8 relative to KDKG, respectively. However, for protein production rates that correlate with the transfection data, the NGF concentration within the hydrogels was 24- or 9.5-fold greater for K4 or K8 relative to KDKG, respectively (Fig. 6). Therefore, the affinity peptides within hydrogels offered dual functionality; increasing the extent of transgene expression, and retaining the expressed protein, both of which contribute to elevating the local concentration of neurotrophic factors that enhance neurite outgrowth.

Conclusion

Regeneration of lost or damaged tissue will likely require the delivery of multiple signals to facilitate repair, particularly for complex tissues such as in the nervous system. The versatility of gene delivery and the mechanics and tailorability of hydrogels makes gene delivery from hydrogels an attractive approach to enable soft tissue regeneration. To investigate the hydrogel design parameters that modulate localized gene delivery, we have

modified PEG-based hydrogels with cationic, lysine-based peptides, which can reversibly associate with the lipoplexes, thereby maintaining their concentration within the hydrogel that leads to enhanced transfection of cells throughout the hydrogel. Affinity peptides were identified that reversibly bound the gene therapy vector; however, peptides with the slowest vector dissociation had reduced transfection relative to those with a more rapid release. Interestingly, the affinity peptides functioned in a dual role in associating with both the vector and the expressed protein to increase neurite extension within hydrogels. These findings illustrate an opportunity for identifying specific peptides that bind both the vector and the protein product. This affinity peptide strategy could be adapted to a wide range of hydrogels to enhance their functionality in numerous regenerative medicine applications.

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Figure 1.

Vector retention within hydrogels. Lipoplex retention in the presence of HT-1080 cells for 10% PEG hydrogels containing 5 mM RDG, KDKG, K4, or K8 peptide demonstrate enhanced vector retention. Significant differences in retention at each time point of each condition relative to 5 mM RDG based on ANOVA are denoted by an asterisk ($p < 0.05$).

Figure 2.

DNA binding (a) and subsequent release (b) from 10% PEG hydrogels containing 5 mM RDG, KDKG, K4, or K8. Significant differences in binding relative to the RDG condition based on ANOVA are denoted by asterisks with $p < 0.05$ (a). Significant differences in release at each time point relative to 5 mM RDG based on ANOVA are denoted by asterisks with $p < 0.05$ (b).

Figure 3.

Expression levels within 10% PEG hydrogels containing 5 mM RDG, K4, or K8 peptide. Significant differences in expression level at each time point relative to 5 mM RDG based on ANOVA are denoted by an asterisk ($p < 0.05$).

Figure 4. NGF binding (a) and subsequent release (b) from 10% PEG hydrogels containing 5 mM RDG, K4, or K8 peptide. Significant differences in binding relative to the RDG condition based on ANOVA are denoted by an asterisk ($p < 0.05$) (a). Significant differences in release at each time point relative to 5 mM RDG based on ANOVA are denoted by an asterisk ($p < 0.05$) (b).

Figure 5. DRG neurite outgrowth within 10% PEG hydrogels containing 5 mM RDG, KDKG, K4, or K8 peptide. DNA lipoplexes encoding for NGF were encapsulated with HT-1080 cells for transfection and DRG explants within hydrogels. The radial distance of neurite outgrowth was measured for each condition (a). NGF production from transfection of HT-1080 cells led to varying extents of neurite outgrowth depending on the type of peptide incorporated. Representative images demonstrating neurite outgrowth in hydrogels containing KDKG (b) was decreased relative to hydrogels containing K4 (c). Significant differences in neurite outgrowth based on ANOVA are denoted by an asterisk ($p < 0.05$). Scale bars denote 200 μm.

Figure 6.

Predicted NGF concentration profiles within 10% PEG hydrogels containing 5 mM RDG, KDKG, K4, or K8 peptide. Concentration profiles were modeled with the same transfection profile across conditions on days 1, 2, and 4 (a) and with protein production rates that correlate to transfection profiles experimentally measured for each condition at 4, 8, 12, 24, 48, and 96 hrs (b). Note that NGF concentration within hydrogels containing K4 and hydrogels containing K8 peptides are overlapping in panel (a).

Table 1

NGF association (k_f) and dissociation (k_r) rate constants for binding to the affinity peptides KDKG, K4, and K8 based on experimental measure.

Affinity peptide	k_f (1/M·min)	k_r (1/min)
KDKG	1.313×10^3	1.393×10^{-2}
K4	1.437×10^3	8.22×10^{-3}
K8	1.574×10^3	9.208×10^{-3}