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Hydrogel Macroporosity and the Prolongation of Transgene Expression and the Enhancement of Angiogenesis

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Abstract

The utility of hydrogels for regenerative medicine can be improved through localized gene delivery to enhance their bioactivity. However, current systems typically lead to low-level transgene expression located in host tissue surrounding the implant. Herein, we investigated the inclusion of macropores into hydrogels to facilitate cell ingrowth and enhance gene delivery within the macropores in vivo. Macropores were created within PEG hydrogels by gelation around gelatin microspheres, with gelatin subsequently dissolved by incubation at 37°C. The macropores were interconnected, as evidenced by homogeneous cell seeding in vitro and complete cell infiltration in vivo. Lentivirus loaded within hydrogels following gelation retained its activity relative to the unencapsulated control virus. In vivo, macroporous PEG demonstrated sustained, elevated levels of transgene expression for 6 weeks, while hydrogels without macropores had transient expression. Transduced cells were located throughout the macroporous structure, while non-macroporous PEG hydrogels had transduction only in the adjacent host tissue. Delivery of lentivirus encoding for VEGF increased vascularization relative to the control, with vessels throughout the macropores of the hydrogel. The inclusion of macropores within the hydrogel to enhance cell infiltration enhances transduction and influences tissue development, which has implications for multiple regenerative medicine applications.

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INTRODUCTION

In regenerative medicine, new tissue formation relies on a combination of multiple chemical, physical, and biological cues acting in concert to guide cellular processes leading to functional tissue replacements. Hydrogels have been developed to serve as scaffolds to regenerate numerous tissues such as nerve [1, 2], bone [3, 4], cartilage [5, 6], and vasculature [7, 8] since their high water content and mechanical properties are similar to native extracellular matrix (ECM) [9, 10]. Hydrogels can be readily tailored for the inclusion of biochemical cues known to guide cellular processes such as cellular adhesion peptides (e.g., RGD, YIGSR) to mimic the extracellular space or growth factors (e.g., nerve growth factor (NGF) or vascular endothelial growth factor (VEGF)) to trigger proliferation, migration, and cell survival [11, 12].

The functionality of hydrogels in regenerative medicine can be further enhanced with localized gene delivery to promote tissue morphogenesis. Gene delivery from hydrogels is a versatile approach for the sustained production of tissue inductive factors by endogenous or transplanted cells. The delivery of DNA encoding for therapeutic proteins is attractive because it enables the sustained production of inductive factors for extended, or controllable durations [13, 14]. The duration of expression is critical for many processes, such as vascularization, in which exposure of angiogenic factors must be sustained for multiple weeks to prevent apoptosis of migrating endothelial cells [15, 16]. In addition to the duration of expression, the spatial distribution of transgene expression influences tissue development and architecture [17], and obtaining expression within the implant may be necessary to fully promote functional tissue growth throughout the defect.

The mechanisms modulating gene delivery from hydrogels have begun to be elucidated. Natural and synthetic hydrogels have been employed to provide a sustained release of viral and nonviral vectors based on diffusion through the matrix or degradation of the crosslinks [18, 19]. Vector release has resulted in inefficient gene transfer to host tissue surrounding the hydrogel and produced low levels and duration of transgene expression. Synthetic hydrogels, however, can have mesh sizes comparable to or smaller than the hydrodynamic diameter of gene therapy vectors, which can serve to retain the vector [20-22]. Vector retention by physical entrapment or chemical modification can elevate levels of transgene expression by maintaining high concentrations of vector within the cellular microenvironment [23, 24]. However, retention of encapsulated vector requires that cells infiltrate the hydrogel to access the entrapped vector, and cell infiltration often depends on matrix degradation or remodeling. While cell ingrowth can elevate transgene expression, matrix degradation may compromise the matrix integrity, thereby compromising the other functions of the matrix.

In this report, we investigate the hypothesis that the incorporation of macropores, which facilitates cell ingrowth while maintaining structural support, will enable prolonged transgene expression within hydrogels. PEG hydrogels previously employed for investigating gene delivery were made macroporous using a gentle Michael-type addition chemistry to encapsulate gelatin microspheres [23-25]. Initial studies investigated the relationship between macroporosity and cell ingrowth. Lentivirus was loaded within these macroporous PEG hydrogels and the virus activity was confirmed. Subsequent studies quantified transgene expression in vivo and characterized the distribution of transduced cells within the hydrogels. The delivery of virus encoding for an angiogenic factor, VEGF, was subsequently investigated to promote vascularization throughout the hydrogel. A hydrogel system that enables prolonged localized transgene expression and induces robust angiogenesis throughout the hydrogel has numerous potential applications for guiding tissue formation.

MATERIALS AND METHODS

Virus production

Lentiviruses encoding for Gaussia luciferase (Gluc) (New England BioLabs, Ipswich, MA) firefly luciferase (Luc), or vascular endothelial growth factor (VEGF), each containing the CMV promoter, were produced in HEK 293T cells cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) in a 37°C and 5% CO₂ environment. Lentiviral packaging vectors pMDL-GagPol, pRSV-Rev, and pIVS-VSV-G along with plenti-CMV-Gluc or plenti-CMV-Luc were first complexed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) then co-transfected into HEK 293T cells. After 48 h, the culture supernatants containing lentivirus were collected, viruses were recovered using PEG-it (System Biosciences, Mountain View, CA) and centrifugation, and resuspended in sterile 1x PBS, pH 7.4. The virus titer (lentivirus particle (LP)) was determined using a HIV-1 p24 antigen ELISA kit (ZeptoMetrix, Buffalo, NY).

Gelatin microsphere fabrication and size measurement

Gelatin microspheres were fabricated using type A gelatin from porcine skin having bloom strength of 300 (Sigma-Aldrich, St. Louis, MO) using a previously established water-oil emulsification method [26]. Briefly, gelatin was added to de-ionized water heated to 80°C at a 0.1 g/mL final concentration. This gelatin solution was then added drop-wise to mineral oil heated to 80°C while stirring at 500 rpm. The water-oil emulsion was cooled with constant stirring using an ice jacket for 1.5 h. Subsequently, 200 mL acetone was added to the emulsion and stirred for another hour. Gelatin microspheres were collected by removing the oil phase washing with acetone to remove remaining mineral oil. Gelatin microspheres were stored in a dry atmosphere.

The distribution of gelatin microsphere size was measured in both dehydrated and hydrated states of three different fabricated batches. For dehydrated and hydrated size measurements, gelatin microspheres were added to mineral oil or water, respectively, and incubated for 30 mins. Each condition was subsequently imaged with phase microscopy. Ten random images were acquired per condition, and microsphere diameters were measured in ImageJ by two observers. A frequency diagram was generated indicating the percentage of microspheres at specified diameters by averaging the measurements of the three batches.

Hydrogel preparation

Degradable hydrogels encapsulating gelatin microspheres were formed based on a previously described Michael-Type addition PEG hydrogel system with modifications [23]. Briefly, four-arm poly(ethylene glycol) vinyl sulfone (PEG-VS) (20 kDa) was synthesized as previously published [27]. PEG macromer was dissolved in 0.3 M triethanolamine (TEA) pH 8.0 at a concentration of 0.5 mg/μL to yield a final PEG concentration of 10%. The plasmin-degradable trifunctional (3 cysteine groups) peptide crosslinker (Ac-GCYKNRCGYKNRCG) (custom synthesis at the Institute for BioNanotechnology in Medicine (IBNAM) at Northwestern University, Chicago, IL) was dissolved in 0.3 M TEA pH 10.0 to maintain reduction of the free thiols at a concentration that would maintain a stoichiometrically balanced molar ratio of VS:SH. Prior to gelation, gelatin microspheres were hydrated with 10 μL sterile Millipore or lentivirus solution unless noted otherwise. Subsequently, the PEG and peptide crosslinking solutions were mixed well and immediately added to the hydrated gelatin microspheres for encapsulation. Three virus loading configurations into the PEG hydrogels were investigated and consisted of (i) non-macroporous PEG (no gelatin) (PEGnp) hydrated with virus solution following gelation, (ii) PEG encapsulating gelatin microspheres (PEGmp) hydrated with virus solution following

gelation, and (iii) PEG encapsulating gelatin microspheres that have been loaded with virus prior to gelation (pPEGmp) by swelling gelatin microspheres with virus solution. Given that PEGnp and PEGmp hydrogels swell to 200% and 300% of their original weight, respectively, 15 μ L hydrogels were hydrated with 10 μ L virus solution to ensure complete loading. Gelatin microspheres hydrated with virus solution was tested as a control in the in vivo studies.

Hydrogel characterization

PEG hydrogels formed with and without gelatin microspheres were stained with Sirius red, which is commonly used for staining collagen and nonspecific staining of other proteins [28-30], to visualize the distribution of gelatin within the PEG hydrogels and to investigate degradation of gelatin over time. Hydrogels were incubated in Sirius red solution (1% in 1.3% picric acid) (Sigma-Aldrich, St. Louis, MO) for 1 h, followed by incubation in acetic acid solution for 1 h. Hydrogels were subsequently transferred to PBS and imaged 2 h and 2 days following gelation. PEG hydrogels containing no gelatin were stained as a negative control.

The effect of gelatin microsphere incorporation on hydrogel swelling and rate of dissolution was investigated through swelling studies. Hydrogels without gelatin (PEGnp) and encapsulating gelatin microspheres (PEGmp) were formed as described above and weighed immediately after gelation. Hydrogels were then incubated in PBS at 4°C to prevent gelatin dissolution and weighed at specified time points. Following the 24 h time point, four PEGmp hydrogels were transferred to 37°C to facilitate gelatin dissolution and the remaining PEGnp and PEGmp hydrogels were stored at 4°C. Hydrogels were weighed up to 7 days.

Interconnected macroporosity minimizes the barriers to cellular ingrowth into tissue engineering scaffolds in vivo [31, 32]. Hydrogels were formed containing 5 mM covalently attached RGD (Ac-GCGYGRGDSGP) (custom synthesis at IBNAM) tethers encapsulating a range of gelatin amounts (0 to 9 mass ratio of gelatin:PEG) were formed to test interconnected macroporosity in vitro. Incorporation of RGD provides cellular adhesion sites to the hydrogel matrix. Following gelation, hydrogels were incubated in PBS at 37°C for 24 h to facilitate gelatin dissolution. Subsequently, 200 μ L of 100,000 cells/mL HT-1080 cell solution, a human cell line representative of fibroblast cells which would be present in numerous regenerative medicine applications, was seeded onto hydrogels having a dry weight of 15 μ L. After 2 h, the supernatant containing unbound cells and cells loosely attached to hydrogel surfaces were removed and replaced with thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich) dissolved in PBS to label remaining viable cells for determining their location within the hydrogel structure. Initial in vitro studies investigated a range of gelatin quantities with the objective of maintaining the hydrogel shape yet allowing cell seeding throughout the structure. Subsequent studies in vivo used PEGmp hydrogels formed with an 8 to 1 mass ratio of gelatin:PEG. PEGmp and PEGnp hydrogels were implanted subcutaneously in mice to determine the extent of cell ingrowth and hydrogel mechanical stability over a time period of 6 weeks. Hydrogels were retrieved at 2, 4, and 6 weeks, sectioned and stained for histological analysis.

Assessment of virus activity

The activity of virus released and loaded within the three loading configurations described above (PEGnp, PEGmp, and pPEGmp) was assessed using the lentivirus CMV-Gluc (8×10^5 LP). Four hours after gelation, the vector supernatant containing released virus was collected for spinoculation. The control for the released virus fraction consists of virus solution incubated in 200 μ L cDMEM. Virus remaining within the hydrogel was collected

by incubating hydrogels in 200 μ L trypsin in 0.05% EDTA for 1 h at 37°C. Degraded hydrogel solution was then collected for spinoculation. The control for virus remaining within the hydrogel fraction consisted of virus solution added to 200 μ L trypsin in 0.05% EDTA for 1 h at 37°C. To spinoculate, 200 μ L of 2.5×10^5 cells/mL of HT-1080 cell solution was added to sample solutions and centrifuged at 2500 rpm at 32°C. After 45 mins, the supernatant was aspirated and the cell pellet was resuspended in 200 μ L cDMEM and plated in wells of a 96 well plate. After 48 h, the cell supernatant was collected and stored at -80°C and cells were lysed with reporter lysis buffer (Promega, Madison, WI). Gaussia luciferase activity was measured with a Gaussia luciferase assay kit (New England BioLabs, Ipswich, MA) and a luminometer (Turner Design, Sunnyvale, CA). Relative light units (RLUs) was normalized to total cellular protein using the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). Released and encapsulated virus activities were expressed as a percentage of a control virus solution containing 8×10^5 LP, the total amount delivered per hydrogel, in cDMEM or trypsin, respectively.

Bioluminescence imaging

Hydrogels loaded with 3×10^7 LP of Lenti-Luc were implanted into male CD1 28-30 g mice (Charles River, Wilmington, MA). In vivo luciferase expression was monitored using an IVIS imaging system (Caliper Life Sciences, Hopkinton, MA) for up to 6 weeks. At each time point (3 days, 1-6 weeks), the mice received an intraperitoneal injection of D-luciferin (150 mg/kg body weight) (Molecular Therapeutics, Ann Arbor, MI) using 28-gauge insulin syringes. The mice were placed within the chamber of the IVIS imaging system and bioluminescence images were acquired every 5 mins for a total of 50 mins until the peak light emission was confirmed. Bioluminescence images were superimposed over gray-scale images using the Living Image software (Caliper Life Sciences, Hopkinton, MA). The luminescence signal intensity was obtained by drawing a constant-size region of interest over the implantation site to measure the integrated light flux (photons/s). The care and use of the laboratory animals was conducted in accordance with the guidance established by the Northwestern University Institutional Animal Care and Use Committee.

Histological analysis and immunohistochemistry

Histological analysis was performed to determine the extent of cellular infiltration, distribution of transduced cells and extent of vascularization. At specified time points, hydrogels were retrieved from mice for histological analysis and immunohistochemistry. Retrieved hydrogels were fixed overnight in 4% paraformaldehyde and a previously reported adapted cryosectioning method was implemented to embed and freeze hydrogels prior to cryosectioning [33]. Briefly, hydrogels were incubated in increased sucrose solutions (5-20%) for up to 2.5 h followed by incubations of Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) to displace the sucrose solution. Samples embedded in O.C.T. were then frozen to -80°C and sections were cut at a thickness of 10 μ m using a cryostat.

PEGnp and PEGmp hydrogel sections were stained for hematoxylin and eosin (H&E) to visualize the extent of cellular infiltration into hydrogels at 2, 4, and 6 weeks post-implantation. Note that the gelatin microspheres condition tested in the subsequent gene delivery study is completely degraded by week 2, and cellular infiltration into the PEGmp condition represents ingrowth in subsequent virus delivery studies consisting of virus loading configurations: pPEGmp and PEGmp.

The distribution of transduced cells within the hydrogel at 2 and 4 weeks post-implantation was subsequently determined for both macroporous hydrogel conditions that deliver lentivirus encoding for luciferase using immunofluorescence. PEGnp hydrogels were not

analyzed for distribution of transduced cells since they did not support cellular ingrowth. Infected cells within hydrogel sections were first permeabilized with 0.5% Triton and then stained with a 1:50 dilution of polyclonal goat anti-luciferase (Promega, Madison, WI) primary antibody in buffer containing 1% normal donkey serum and 0.1% Triton and a 1:500 dilution of Dylight 549 donkey anti-goat (red) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as a secondary antibody. Cell nuclei (blue) were subsequently stained with a 1:2000 dilution of 10 mg/mL Hoechst (Invitrogen, Carlsbad, CA). Transduced cells were imaged with a cooled CCD camera (Photometrics, Tuscon, AR) on a Leica inverted fluorescence microscope.

Subsequently, the collagen content and number of endothelial cells were quantified to assess the extent of angiogenesis within macroporous hydrogels (pPEGmp and PEGmp) delivering lentivirus encoding for VEGF relative to the control lentivirus encoding for luciferase. Collagen content was assessed at 2 and 4 weeks post-implantation using Masson's trichrome stain (Polysciences, Inc., Warrington, PA) for both macroporous hydrogel conditions delivering lentivirus encoding for VEGF and macroporous hydrogels delivering lentivirus encoding for luciferase as the control. The percentage of tissue area containing collagen was measured by counting the number of collagen (blue) pixels in ImageJ and dividing by the total tissue pixel number measured. Similarly, endothelial cells were quantified at 2 and 4 weeks post-implantation by staining tissue sections with 1:100 fluorescein labeled *Lycopersicon esculentum* (tomato) lectin (Vector Laboratories, Burlingame, CA) diluted in PBS. The lectin-positive pixels were counted and normalized to the total tissue pixel number measured. For both collagen and endothelial cell quantifications, 3 images were taken per section and 3 sections within each hydrogel were quantified.

Statistics

Statistical analysis was performed using the JMP statistical software package (SAS Institute, Cary, NC). Statistical significance in the differences between groups was analyzed using a multiple comparison analysis (ANOVA) method and a post-hoc *t*-test using a 95% confidence interval. For comparison of the levels of transgene express, a Kruskal-Walls test was performed with a *p* level of 0.05. Mean values with standard deviation are reported unless stated otherwise. All experiments were tested in triplicate.

RESULTS

Size distribution and swelling characteristics of gelatin microspheres

Macropores are introduced through gelatin microspheres, and the size distributions of gelatin microspheres in the dehydrated and hydrated states were initially investigated. Dehydrated gelatin microspheres had an average diameter of $113 \pm 48 \mu\text{m}$ and hydrated gelatin microspheres had an average diameter approximately three times as large, $311 \pm 127 \mu\text{m}$ (Fig. 1a). The diameter of hydrated microspheres had a larger range with a maximum diameter reaching up to $700 \mu\text{m}$, while the maximum diameter of dehydrated microspheres was $300 \mu\text{m}$.

Macroporous hydrogel network structure and swelling characteristics

Macroporous hydrogels formed by entrapment of the gelatin microspheres were subsequently characterized by the hydrogel structure and swelling. For PEG hydrogels with entrapped gelatin that create macropores (PEGmp), the encapsulated gelatin stained dark red immediately after gelation (Fig. 1b). By day 2, minimal red staining of gelatin microspheres was observed, along with outlines of open macropores, indicating that the majority of gelatin had dissolved (Fig. 1c). PEG hydrogels with no gelatin, which has only nano-scale porosity (PEGnp), stained lightly with Sirius red both immediately after gelation (data not shown)

and on day 2 (Fig. 1e), likely due to nonspecific staining of the peptide crosslinker. The internal macroporous structure was observable with phase microscopy (Fig. 1d).

Swelling studies were performed to characterize the hydrogel stability and the rate at which the encapsulated gelatin dissolves. Initially, hydrogels were swollen at 4°C to prevent dissolution of the encapsulated gelatin. Within the first 24 h, the PEGnp hydrogels swelled to 200% of their original weight, while PEGmp swelled to 300% of their original weight (Fig. 1f). After 24 h, PEGmp hydrogels were transferred to 37°C to initiate gelatin dissolution. After 2 h, PEGmp hydrogels at 37°C decreased below 100% of their original weight, indicating the majority of gelatin was degraded. Gels that had remained at 4°C retained their original weight. All PEG hydrogels were stable and maintained their swollen weight throughout the 7-day study. The weight change of PEGmp hydrogels stored at 4°C and 37 °C were both significantly different than the PEGnp hydrogels at each time point.

Subsequent studies investigated the gelatin content to achieve interconnected macroporosity, which is necessary to support cellular infiltration in vivo [34]. PEG hydrogels encapsulating a range of gelatin amounts (0 to 9:1 mg gelatin to mg PEG) were incubated at 37°C to remove the gelatin followed by cell seeding (Fig. 2). After 2 h and removal of unbound cells, few cells were observed within the hydrogels for the conditions 0:1, 1:1, and 3:1 mg gelatin to mg PEG. For hydrogels containing more than 6:1 mg gelatin to mg PEG, cells were observed throughout the hydrogels, indicating interconnected macroporosity of the hydrogel.

Consistent with previous reports, interconnected macropores facilitated tissue ingrowth in vivo [34]. PEGmp supported cellular ingrowth throughout the hydrogel following subcutaneous implantation, while maintaining its structural support (Fig. 3a-c). PEGnp did not support cell invasion even 6 weeks post implantation (Fig. 3d). Notably, all hydrogels maintained their structural support upon retrieval.

Stability of lentivirus released and remaining within hydrogels

Virus infectivity can be affected by biomaterial encapsulation, thus the stability of lentivirus within hydrogels was subsequently investigated. We investigated three hydrogel loading approaches: i) PEGnp loaded with virus after gelation, ii) PEGmp loaded with virus after gelation, and iii) virus pre-loaded into gelatin prior to creating a macroporous PEG hydrogel (pPEGmp). Four hours after gelation, the activity of virus remaining within hydrogels ranged from 1.5 to over 5 times greater than the activity of the control virus, which consisted of virus that was not encapsulated yet incubated in trypsin in the same manner as the biomaterial. The virus remaining within pPEGmp had significantly greater activity relative to the PEGmp and PEGnp hydrogels ($p < 0.05$) (Fig. 4). The activity of lentivirus released from hydrogels was up to 5 times greater than the control virus incubated in cDMEM for the PEGnp and PEGmp conditions. However, virus released from the pPEGmp condition had significantly less activity than the control ($p < 0.05$). Together, these results indicate loading virus within the PEG hydrogels preserves its activity for all loading configurations tested.

In vivo transgene expression

Hydrogels loaded with lentivirus encoding for firefly luciferase were subsequently investigated for the extent and duration of transgene expression in vivo, and the distribution of transduced cells. For all conditions, maximal transgene expression was observed at 2 weeks (Fig. 5). For the PEGmp, maximal transgene expression persisted for 6 weeks. Expression levels for PEGnp were initially comparable to the PEGmp condition, however, expression decayed more rapidly after week 3 and was significantly less than the PEGmp

condition by week 6 ($p < 0.05$). Transgene expression levels for pPEGmp and gelatin microspheres remained an order of magnitude lower than the PEGmp for all time points.

Staining indicated that luciferase-positive cells were observed throughout both PEGmp and pPEGmp at both 2 and 4 weeks (Fig. 6). However, substantially larger numbers of luciferase-positive cells were observed within the PEGmp hydrogels (Figs. 6c, d). PEGnp did not support cell ingrowth and thus had no transduced cells within the hydrogel.

Delivery of lentivirus encoding for VEGF from macroporous hydrogels in vivo

We subsequently investigated the localized expression of angiogenic factors to promote blood vessel ingrowth throughout the macroporous hydrogel. During angiogenesis, blood vessel formation requires the synthesis and deposition of collagen to facilitate the growth of the vessel lumen [35]. Thus, initial studies employed Masson's trichrome staining of tissue sections to visualize collagen at 2 and 4 weeks, which also allowed identification of blood vessels. At week 2, few blood vessels were observed inside macroporous hydrogels, however blood vessels were apparent in the tissue surrounding the hydrogels (Fig. 7e). By week 4, collagen was observed within the macroporous hydrogels with VEGF expression (Figs. 7d, f). Hydrogels delivering luciferase-encoding vector had less collagen staining within the macropores (Fig. 7b). Collagen deposition was quantified by measuring the percentage area of collagen, which was determined as the area of collagen-rich (blue) tissue relative to the total tissue area within the hydrogel per image. At 2 weeks, no significant difference in the percentage area of collagen was observed within the macropores for VEGF expression relative to the controls (Figs. 7g). The percent collagen area within macroporous hydrogels delivering VEGF significantly increased between 2 weeks and 4 weeks, and was increased relative to the control condition ($p < 0.05$).

Angiogenesis involves the migration and proliferation of endothelial cells [36]; thus, we also assessed the number of endothelial cells. At 2 and 4 weeks, macroporous hydrogels delivering VEGF-encoding lentivirus had lectin-positive cells throughout the macropores (Figs. 8c-f). Hydrogels delivering a luciferin-encoding lentivirus similarly had positive lectin staining, though the extent of staining appeared reduced relative to the VEGF encoding lentivirus (Figs. 8a, b). Quantification of tissue sections indicated both macroporous hydrogel conditions delivering lentivirus encoding for VEGF had an increase in the percentage area that was lectin-positive relative to controls at both 2 and 4 weeks ($p < 0.05$) (Fig. 8g). Furthermore, the percentage area of lectin-positive staining significantly increased from 2 weeks to 4 weeks only for the PEGmp loaded with lentivirus encoding for VEGF ($p < 0.05$).

DISCUSSION

Hydrogels for tissue engineering function to maintain a space to support new tissue formation, and gene delivery from these hydrogels aims to promote the expression of inductive factors within this space to promote tissue formation. However, sustaining levels of transgene expression remains difficult to achieve using hydrogels due to short vector half-lives and migration of transduced cells away from the implantation site [13, 28]. Several reports have investigated the design of hydrogels to improve localized gene delivery [18, 23, 24]. Cell migration through hydrogel matrix degradation enhances gene expression levels by increasing cell exposure to the vector [23]. However, rapid matrix degradation may lead to transient transgene expression, even with strategies that aim to limit release of the vector [13, 14]. The PEG hydrogels reported herein maintain their structural stability in vivo; however, despite being enzymatically degradable, cell infiltration is limited due to the slow biodegradation rate in vivo that may impede tissue formation (Fig. 3d) [20, 37]. The incorporation of interconnected macropores within hydrogels facilitates cellular ingrowth

and have been shown to maintain structural support to promote neovascularization, fibroblast ingrowth, regeneration of bone, osteoid growth, and the growth of hepatocytes [38]. We hypothesized the incorporation of macropores within hydrogels that facilitate cell ingrowth will promote prolonged and localized transgene expression.

Macroporous PEG hydrogels that facilitate cellular ingrowth maintained elevated levels of transgene expression in vivo. Hydrogels based on PEG were made macroporous using a gentle technique based on Michael-type addition to form enzymatically degradable crosslinks around gelatin microspheres, with subsequent dissolution of the gelatin at 37°C (Fig. 1f) to create macropores (Figs. 1b-e). The amount of encapsulated gelatin required to create an interconnected pore structure was determined to be 6:1 mg gelatin per mg PEG (Fig. 2). Virus release from PEGmp resulted in maximal transgene expression in vivo that persisted for at least 6 weeks (Fig. 5). The dose initially delivered is presumed critical in determining the extent of transgene expression [39], particularly given the half-life of lentivirus to be approximately 24 h at 37°C [13]. pPEGmp had significantly decreased levels of expression, which is consistent with a decreased amount of active virus released from the gel (Fig 4). This decrease of active virus is likely due to a lower number of lentiviral particles released from the hydrogel as the entrapped lentivirus has a reported hydrodynamic diameter of 166 nm [40], which is greater than that of the reported mesh size of the nanoporous PEG, which is 25 nm [20]. Following implantation, elevated levels of expression were maintained past week 3 only for the PEGmp condition.

Macropores within the hydrogel led to transduced cells within the hydrogel and corresponded with a longer duration of expression. Mechanisms limiting sustained levels of localized expression in vivo have been identified as cell turnover and migration of transduced cells from the implantation site [13, 28, 41]. The PEGnp hydrogels had expression levels during the initial 2 weeks that were similar to PEGmp; however, the PEGmp had a longer duration of expression. In this report, we identify transduced cells within the hydrogel macropores at 2 and 4 weeks for both virus loading configurations (Fig. 6). The presence of transduced cells within the macropores likely contributes to the difference in duration of expression. In addition to facilitating the penetration of cells within hydrogels, the incorporation of macropores increases surface area to enhance cell attachment, ECM deposition, and cell proliferation [42, 43]. Macrophages and dendritic cells infiltrate implanted scaffolds and have been identified as being transduced in similar systems [28]. Thus, the incorporation of macropores within hydrogels that maintain structural stability supports tissue growth while promoting transduction within the macropores of the hydrogel.

The combination of a macroporous hydrogel and localized VEGF expression significantly enhanced vascular growth throughout the macropores. A major challenge in cell transplantation is promoting rapid vascularization that can enhance cell survival and function. Additionally, strategies based on promoting regeneration must also recruit vessels to prevent ischemia, which may subsequently recruit progenitor cells to the injury site [44]. The angiogenic factor VEGF has proven capable of promoting vascularization in regenerative medicine applications. Strategies based on protein delivery can be challenging due to an elimination half-life of less than 1 h, and the protein must be available for extended times to prevent nascent blood vessel regression [45, 46]. Macroporous hydrogels delivering lentivirus encoding for VEGF provide the opportunity for localized and extended expression to promote vascular ingrowth throughout the material. Vascularization of the macropores was observed through increased numbers of blood vessels, endothelial cell staining, and collagen deposition by week 4 for expression of VEGF relative to luciferase (Fig. 7). Interestingly, only the loading configuration that led to sustained, elevated levels of transgene expression (PEGmp) supported significantly greater amounts of endothelial cells

($p < 0.05$). Together, these results suggest angiogenesis is enhanced within macroporous hydrogels that promote prolonged transgene expression. The production of tissue inductive factors such as VEGF localized within the hydrogel macropores facilitated tissue morphogenesis, which could be applied to multiple applications simply by interchanging gene therapy vectors.

CONCLUSION

We developed a macroporous hydrogel that supports cell ingrowth, while also delivering lentiviral vectors that transduce cells within the macropores and promotes long-term transgene expression. The incorporation of macropores within hydrogels facilitates cellular ingrowth while maintaining structural stability of the scaffold over 6 weeks. The extent of transgene expression was dependent upon the quantities of active vector delivered. The duration of gene expression for PEGmp was prolonged relative to hydrogels that do not support cellular ingrowth (PEGnp). With localized gene delivery from the hydrogel, transduced cells were located within the macropores at early and late time points. Subsequent delivery of virus encoding for VEGF from macroporous hydrogels induced observable angiogenesis after 4 weeks. Together, these results demonstrate the ability to enable sustained delivery of tissue inductive factors from hydrogels in vivo using gene delivery, which has broad applicability to numerous regenerative medicine applications.

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Figure 1. Characterization of Gelatin and PEG

Size distribution of dehydrated and hydrated gelatin microspheres ($n=3$) (a). The distribution and degradation of gelatin microspheres encapsulated within PEG hydrogels was visualized with Sirius red for PEGnp hydrogels (e) and PEGmp hydrogels (b, c) immediately following gelation (b) and on day 2 (c, e). Phase microscopy images the internal architecture of the PEGmp hydrogels (d). Black scale bars represent 1000 μm and the white scale bar represents 300 μm . Subsequent swelling studies were conducted in PBS at 4°C and PEGnp and PEGmp hydrogels were weighed at specified time points for the initial 24 h (f). After 24 h, PEGmp ($n=4$) gels were incubated at 37°C to facilitate gelatin microsphere dissolution and remaining PEGnp ($n=4$) and PEGmp ($n=4$) hydrogels were maintained at 4°C. Significant difference in % weight gain at each time point relative to the PEGnp condition based on a t -test are denoted by an asterisk ($p<0.05$).

Figure 2. Interconnectedness of hydrogel macropores

PEG hydrogels encapsulating a range of gelatin amounts (0, 1, 3, 6, 9 mg gelatin to mg PEG) were imaged from a top view (**a, b, c, d, e**), respectively, and a side view (**f, g, h, i, j**). Following gelatin microsphere dissolution, HT-1080 cells were seeded on top of hydrogels. Two hours after seeding, unbound cells were removed and viable cells were stained with MTT, resulting in a purple color.

Figure 3. In vivo cellular infiltration into PEG hydrogels

PEGmp (a-c) and PEGnp hydrogels (d) were implanted subcutaneously in mice. H&E stains of hydrogels indicates cellular ingrowth at 2 (a), 4 (b), 6 (c, d) weeks post-implantation. The labels P and T denote PEG and tissue surrounding the implant, respectively. The white line denotes the hydrogel-tissue interface. Scale bars represent 100 μ m.

Figure 4. Lentivirus activity following loading within PEG hydrogels

Viral activity release from and remaining within hydrogels was assessed for PEGnp, PEGmp, and pPEGmp hydrogels. For the measurement of viral activity within hydrogels, the hydrogels were solubilized by the treatment of trypsin for 1 h. Virus in cDMEM or trypsin served as controls for the measurement of viral activity released from or remaining within hydrogels, respectively. Viral activity for each hydrogel condition is reported relative to the respective unencapsulated control (either released from hydrogel or remaining within hydrogel), which is the total amount of virus loaded per hydrogel (8×10^5 LP). Significant differences relative to the respective control based on a *t*-test are denoted by an asterisk ($p < 0.05$).

Figure 5. In vivo transgene expression from lentivirus loaded hydrogels

Representative bioluminescence images following subcutaneous implantation of hydrogels with multiple loading configurations (a). Transgene expression was measured as integrated photon flux (photons/s) using an IVIS bioluminescence imaging system (b). Asterisks represent statistical difference ($p < 0.05$) relative to the PEGmp condition at each time point based on a Kruskal-Wallis test.

Figure 6. The distribution of transduced cells within macroporous PEG hydrogels
 Macroporous hydrogels loaded with lentivirus encoding for luciferase, pPEGmp (a, b) and PEGmp (c, d), were retrieved 2 (a, c) and 4 weeks (b, d) post-implantation and analyzed by immunohistochemistry stained against luciferase antibodies. Figures show an overlay of the luciferase antibody staining (red) and Hoechst staining of cell nuclei (blue). The cells marked by the white squares are magnified in the inset to show transduced cells (red with blue cell nuclei) are located within macroporous hydrogels along with cells that do not express the transgene (blue cell nuclei only). White arrows indicate some of the transduced cells. Scale bars represent 100 μ m.

Figure 7. Collagen characterization

Representative images of macroporous PEG hydrogel sections following delivery of lentivirus encoding for VEGF. Tissues sections from pPEGmp (c, d) and PEGmp (e, f) loaded with lentivirus encoding for VEGF and PEGmp loaded with lentivirus encoding for luciferase (control) (a, b) were stained with Masson's trichrome 2 (a, c, e) and 4 (b, d, f) weeks post-implantation. Collagen (blue), erythrocytes or cytoplasm (red), and cell nuclei (black) are observed within macroporous hydrogels. Scale bars represent 100 μm. The percentage of collagen-rich tissue relative to the total tissue area was quantified using ImageJ (g). Significant difference relative to week 2 based on a t-test, are denoted by a single asterisk ($p < 0.05$). Significant difference relative to the negative control at the given time point is denoted by a double asterisk ($p < 0.05$).

Figure 8. Vascularization within macroporous hydrogels

Representative images indicate lectin-positive cells located within macroporous PEG hydrogels following the delivery of lentivirus encoding for VEGF. Tissue sections from pPEGmp (**c, d**) and PEGmp (**e, f**) loaded with lentivirus encoding for VEGF were stained with tomato lectin (green) at 2 (**a, c, e**) and 4 (**b, d, f**) weeks post-implantation, and cell nuclei were counterstained with Hoechst (blue). pPEGmp (images not shown) and PEGmp loaded with lentivirus encoding for luciferase served as controls at 2 (**a**) and 4 weeks (**b**). White arrows indicate some of the lectin-positive cells. Scale bars represent 100 μm. The percentage of lectin-positive tissue relative to the total tissue area was measured using ImageJ (**g**). Significant differences relative to week 2 based on a t-test, are denoted by a single asterisk ($p < 0.05$) and significant differences relative to the negative control is denoted by a double asterisk ($p < 0.05$).