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A PLG/HAp composite scaffold for lentivirus delivery

RM Boehler^{1,^}, S Shin^{1,^}, AG Fast¹, RM Gower¹, and LD Shea^{1,2,3,4,5,*}

¹Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL, USA

²Chemistry of Life Processes Institute (CLP), Northwestern University, Evanston, IL, USA

³Institute for BioNanotechnology in Medicine (IBNAM), Northwestern University, Chicago, IL, USA

⁴Department of Biomedical Engineering, Northwestern University, Evanston, IL, USA

⁵The Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, IL, USA

Abstract

Gene delivery from tissue engineering scaffolds provides the opportunity to control the microenvironment by inducing expression of regenerative factors. Hydroxyapatite (HAp) nanoparticles can bind lentivirus, and we investigated the incorporation of HAp into poly(lactide-co-glycolide) (PLG) scaffolds in order to retain lentivirus added to the scaffold. PLG/HAp scaffolds loaded with lentivirus enhanced transgene expression over 10-fold *in vitro* relative to scaffolds without HAp. Following *in vivo* implantation, PLG/HAp scaffolds promoted transgene expression for more than 100 days, with the level and duration enhanced relative to control scaffolds with lentivirus/HAp complexes added to PLG scaffolds. The extent of HAp incorporated into the scaffold influenced transgene expression, in part through its impact on porous architecture. Expression *in vivo* was localized to PLG/HAp scaffolds, with macrophages the primary cell type transduced at day 3, yet transduction of neutrophils and dendritic cells was also observed. At day 21 in PLG/HAp scaffolds, non-immune cells were transduced to a greater extent than immune cells, a trend that was opposite results from PLG scaffolds. Thus, in addition to retaining the virus, PLG/HAp influenced cell infiltration and preferentially transduced non-immune cells.

INTRODUCTION

The ability to modulate the biological cues within the local environment has emerged as an essential step in creating a controlled microenvironment to promote tissue regeneration. Tissue engineering scaffolds are a central tool that can serve as a platform to provide a multitude of signals essential to regeneration. The surface properties can be designed to present adhesive signals and can be manipulated to enable controlled delivery of tissue inductive factors, such as growth factors or hormones, which can influence the phenotype of endogenous or transplanted cells. However, direct delivery of these factors can be complicated by controlling the release and maintaining the bioactivity of the factor. Gene

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*Address correspondence to: Lonnie D. Shea, Northwestern University, Department of Chemical and Biological Engineering, 2145 Sheridan Rd./E156, Evanston, IL 60208-3120, Phone: 847-491-7043, Fax: 847-491-3728, l-shea@northwestern.edu.

[^]Authors contributed equally to this work

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delivery, in contrast, targets the infiltrating cells to serve as bioreactors for sustained, local secretion of active factors into the microenvironment. Delivery of viral vectors from biomaterials is a promising platform for tissue regeneration due to their high transduction efficiency and the ability to induce the expression of one or more factors.

Strategies for delivery of viral vectors from biomaterials are currently being developed to enhance localization and magnitude of gene expression, as reviewed by Jang et. al. [1]. The reversible association of gene therapy vectors to biomaterials can enhance gene delivery, through creation of elevated local concentration, co-localization of the vector with the cell surface, and stabilization of the vector against inactivation [1, 2]. Viral vectors may interact non-specifically with the biomaterial and have been immobilized on the polymer surface by direct application to scaffolds [3]. Immobilization of virus onto fibronectin-coated surfaces has shown increased transduction efficiency *in vitro* [4]. Biotinylated virus has been tethered to avidin-conjugated materials [5]. Biomaterial scaffolds have also been modified with phosphatidylserine (PS) to promote specific interaction between the scaffold and virus, which increased loading and enhanced virus activity [6]. More recently, hydroxyapatite (HAp) has been used as a nanoparticle complexing agent for maintaining activity of viral vectors for gene delivery [7]. These nanoparticles complexed with lentivirus have been incorporated into hydrogels [7] or loaded onto microporous scaffolds [8].

In this report, we investigated the surface modification of microporous poly(lactide)-co-glycolide (PLG) scaffolds with HAp nanoparticles in order to provide binding sites that retain and stabilize the vectors as a means to facilitate gene transfer. PLG scaffolds create and maintain a space for tissue growth, and cells infiltrating the pores are targeted for delivery. PLG/HAp composite scaffolds are fabricated by mixing HAp particles with PLG and salt using the gas foaming/particulate leaching method, with lentivirus added after scaffold fabrication. The activity and binding capacity of lentivirus to HAp was initially investigated, as was the impact of HAp on the architecture. The extent and duration of transgene expression was analyzed by bioluminescence imaging. Furthermore, the identity of infiltrating and transduced cells in virus-loaded PLG/HAp scaffolds was quantified. Scaffolds capable of inducing localized transgene expression could have applications for a multitude of applications in regenerative medicine.

MATERIALS AND METHODS

Virus production

Lentivirus was produced by co-transfecting HEK-293T cells with lentiviral packaging vectors (pMDL-GagPol, pRSV-Rev, pIVS-VSV-G), previously described by Dull et. al. [9], and the gene of interest (pLenti-CMV-Luciferase, pLenti-CMV- β -galactosidase, or pLenti-CMV-GFP) using Lipofectamine 2000 (Roche Biosciences). After 48 hours, supernatants were collected, and cell debris was spun down and removed. Virus particles were concentrated using PEG-it (Systems Biosciences) and re-suspended in PBS. Lentivirus titers were determined by HIV-1 p24 Antigen ELISA Kit (ZeptoMetrix Co.).

Dynamic Light Scattering

The mean particle size of HAp nanoparticles (Sigma) was measured in water at a concentration of 0.1 mg/ml after 5 minutes of pulsed ultrasonication using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK)

Assay of active lentivirus binding to HAp

HAp (Sigma-Aldrich) nanoparticles were suspended in PBS at a concentration of (10 mg/ml) and sonicated for 1 min to dissociate aggregates. Increasing amounts of lentivirus (LV)

encoding for luciferase (5×10^6 , 1×10^7 , 5×10^7 , 1×10^8 , and 5×10^8 lentivirus particles (LP)) were mixed with HAp-PBS (1 mg/ml of final concentration of HA) for 10 min. The uncomplexed LV control and HAp/LV complex were then mixed with HEK-293T cells seeded on a 24 well plate. At 2 days following infection, cells were lysed with reporter lysis buffer (Promega) and assayed for enzymatic activity. The luminometer was set for a 3 second delay with signal integration for 10 seconds. Luciferase activity was normalized to total cellular protein using the bicinchoninic acid (BCA) assay kit (Pierce). For the measurement of the amount of lentivirus bound to HAp, HAp/LV complexes were centrifuged to remove unbound lentivirus. The lentivirus was then eluted from HAp particles with 0.5 M KPO_4 buffer (pH7.4) and quantified by HIV-1 p24 Antigen ELISA Kit (ZeptoMetrix Co.).

Scaffold formation and characterization

PLG microspheres were created for fabrication into micro-porous scaffolds as previously described [10]. PLG (75:25 lactide: glycolide, i.v. = .76 dL/g) (Lakeshore Biomaterials) was dissolved in dichloromethane to make a 6% (w/w) solution, which was emulsified in 1% poly(vinyl alcohol) (PVA) at 7000 rpm to create microspheres. Microspheres were washed three times to remove PVA and lyophilized overnight. Scaffolds were fabricated by mixing HAp (Sigma-Aldrich), PLG, and NaCl ($250 \mu\text{m} < d < 425 \mu\text{m}$) in varying amounts (0:1.6:48.4 mg, 1.6:1.6:46.9 mg, 2.3:2.3:45.5 mg, or 4.2:4.2:41.7 mg HAp:PLG:NaCl), which will be referred to as (0:1:30, 1:1:30, 1:1:20, and 1:1:10) ratios respectively. Mixtures were pressed to 1,000 psi in a 5-mm KBr die using a Carver press. The scaffolds were then equilibrated with high-pressure CO_2 gas (800 psi) for 16 h in a custom-made pressure vessel and fused during a 25 min pressure release. Scaffolds were leached in water for 1 hour while rocking to remove salt. The scaffolds were then disinfected in 70% ethanol and washed with water. Scanning electron microscopy (Hitachi 3500N, Dallas, TX, USA) was used to image the scaffolds.

In vitro transduction of lentivirus-loaded scaffolds

In vitro cell transduction was analyzed by seeding and culturing cells on scaffolds with immobilized virus. Lentivirus (10 μL) encoding β -galactosidase (LV- β -gal, 5×10^8 LP) or luciferase (LV-Luc, 5×10^8 LP) were incubated with scaffolds for 10 min. Scaffolds were washed with PBS and placed into 12 well plates. Scaffolds were then seeded with 1×10^6 HEK-293T cells. The expression of β -galactosidase in the scaffolds was visualized at 3 days after transduction by staining with X-gal. The expression of luciferase was quantified by lysing the cells 3 days after transduction and assaying for enzymatic activity.

Bioluminescence imaging of lentivirus-loaded scaffolds

The level of gene expression from lentivirus-loaded scaffolds was measured over time using live animal imaging to determine the differences in gene expression profile among the different conditions. LV-Luc (8×10^9 LP; $n=4$) was loaded onto 0:1:30, 1:1:30, 1:1:20, or 1:1:10 PLG/HAp composite scaffolds. As an additional control, LV-Luc (8×10^9 LP; $n=4$) was complexed with HAp nanoparticles (1 mg/ml) and loaded onto PLG scaffolds as previously described [8]. Scaffolds were implanted into the right epididymal fat pad (EFP) of CD1 male mice (Charles River) as previously described [11]. For imaging, the animals were injected i.p. with d-luciferin (150 mg/kg body weight; Molecular Therapeutics). Luciferase expression was monitored (every 5 minutes for a total of 30 minutes) until peak light emission was confirmed using an IVIS imaging system (Xenogen Corp., Alameda, CA, USA) with a cooled CCD camera. Signal intensity in the region of interest was reported as an integrated light flux (photons/second) as determined by IGOR software (WaveMetrics). Care and use of the laboratory animals followed the guidelines established by the Northwestern University Institutional Animal Care and Use Committee (IACUC).

Transgene expression localization in the scaffold

Immunohistochemistry was performed on tissue sections to analyze the distribution of transduced cells in the scaffold. LV-GFP (5×10^9 LP; $n=3$) was loaded onto 1:1:20 PLG/HAp scaffolds and implanted into the right EFP of CD1 mice. LV-Luc (5×10^9 LP) was used as a control. After 3 or 7 days, EFPs containing scaffolds were retrieved, snap-frozen in isopentane (Fisher Scientific), and stored in siliconized tubes at -80°C . Tissue samples were cryopreserved in optimum cutting temperature (OCT) compound and sections of the scaffold and surrounding tissue were sliced in $14\text{-}\mu\text{m}$ thick sections using a cryostat. Sections were stained with polyclonal rabbit anti-EGFP (1:100; Invitrogen) as a primary antibody and counter-stained with hematoxylin to visualize nuclei.

Antibodies for flow cytometry

The following antibodies were purchased from BD Biosciences: v500-conjugated RM4-5 against CD4 and APC-conjugated 30-F11 against CD45. PE-Cy7-conjugated N418 against CD11c was purchased from eBioscience. The following antibodies were purchased from Biolegend: FITC-conjugated 53-6.7 against CD8, Percp-conjugated RB6-8C5 against Gr1, and APC-Cy7-conjugated BM8 against F4/80. Flow cytometry antibodies were used at predetermined saturating concentrations.

Flow cytometry for identification of infiltrating and transduced cells

Flow cytometry was performed to determine the identity of both infiltrating and transduced cells within the scaffolds. LV-td-tomato (8×10^9 LP; $n=3$) was loaded onto 0:1:30 and 1:1:20 PLG/HAp composite scaffolds and implanted into the right EFP of CD1 mice. After 3 or 21 days, EFPs containing scaffolds and spleens (for flow cytometry compensation) were removed, minced with microscissors, and digested in liberase (0.2 Wünsch Units; Roche Applied Science) for 20 minutes at 37°C . After incubation, $750\text{ }\mu\text{L}$ of 0.5M EDTA (Life Technologies) was immediately added to inhibit digestion. Digested tissues were pushed through $70\text{ }\mu\text{m}$ strainers and washed with HBSS (VWR). Red blood cells were then lysed with ACK lysing buffer (Invitrogen) and blocked with a solution containing 1% of normal mouse and rat serum (Sigma-Aldrich) and anti-mouse CD16/32 (eBioscience). Cells were then stained for viability using fixable violet dead cell stain (Invitrogen) and stained with the antibodies listed above. Data were acquired on a BD LSR II cytometer (San Jose, CA, USA), and analyzed using FloJo software. Fluorescence minus one staining was used as a negative control.

Statistical analysis

For multiple comparisons, statistical significance between groups was determined by ANOVA with post-hoc testing. For single comparisons, the statistical significance between pairs was determined by unpaired t test. All statistics test significance using a p value of 0.05 unless noted. Error bars represent standard error in all figures. Prism (GraphPad) software was used for all data analysis.

RESULTS

Lentivirus binding to HAp nanoparticles

The lentivirus binding to HAp was investigated to confirm binding of lentivirus and retention of virus activity. After pulsed ultrasonication, the average HAp nanoparticle diameter was 203.8 nm (Fig. 1A). Lentivirus binding to HAp was quantified at multiple ratios of lentivirus to HAp. Lentivirus binding increased as the number of lentiviral particles per mg of HA was increased from 1×10^8 to 2×10^9 . Maximal binding (B_{max}) of lentivirus was determined to be 1.6×10^9 particles per mg of HAp (Fig. 1B). HAp/virus complexes

produced between 2 and 3-fold greater viral activity at all HAp/LV ratios relative to virus in PBS (Fig. 1C, $p < 0.0001$), consistent with previous results [7].

Incorporation of HAp into PLG scaffolds

The impact of HAp on the architecture of PLG scaffolds was subsequently investigated. HAp was added directly to the PLG:NaCl mixture as a powder at three ratios of HAp:PLG:NaCl, 1:1:30, 1:1:20, and 1:1:10 prior to the gas foaming process. Polymer scaffolds without HAp (0:1:30) served as a control. The total weight of the mixture was fixed, which resulted in decreasing NaCl amount as HAp:PLG content increased. Consistent with previous reports, scaffolds without HAp (0:1:30) have a highly porous architecture (Fig. 2A). At the lowest level of HAp incorporation (1:1:30), the scaffold had an architecture similar to the PLG scaffolds (0:1:30) (Fig. 2B). As the amount of HAp was increased, the salt content decreased, leading to the presence of pores that were less open (Fig. 2C,D). The 1:1:10 scaffolds had the most constricted pores, consistent with their having the least amount of porogen (Fig. 2D).

***In vitro* transduction of virus-loaded PLG/HAp composite scaffold**

Lentivirus binding to PLG/HAp composite scaffolds and transduction of cells cultured on the scaffolds was subsequently investigated. Transgene expression on scaffolds with HAp did not differ significantly from each other (Fig. 3A); however, the presence of HAp increased transgene expression by approximately an order of magnitude relative to the control scaffold ($p < 0.05$). The reduced expression on the control scaffolds was correlated with the presence of few transduced cells (Fig. 3B), consistent with prior reports [3]. In contrast, PLG/HAp composite scaffold had large numbers of transduced cells (Fig. 3C–E). As the concentration of HAp was increased, an increasing number of transduced cells was observed in the scaffold.

***In vivo* gene expression of virus-loaded PLG/HAp composite scaffolds**

Lentivirus-loaded PLG/HAp composite scaffolds were subsequently implanted *in vivo* for quantification of transgene expression. PLG scaffolds loaded with lentivirus or HAp/LV complexes served as control conditions. Transgene expression for the three PLG/HAp composite scaffold formulations was significantly increased above the controls at day 3 (Fig. 4). The three PLG/HAp composite scaffolds sustained levels of transgene expression for 105 days, while the scaffold controls did not have expression above background beyond 21 days. Among the three PLG/HAp conditions, only the 1:1:20 composite had significantly greater expression than control conditions at day 21. However, at day 28 and 42, both the 1:1:20 and 1:1:30 scaffolds had significantly greater expression than both control conditions. These studies demonstrated that incorporation of HAp into the scaffold can both increase initial transgene expression and sustain expression for long times.

Distribution of transduced cells

The distribution of transduced cells within the scaffold was determined using histology. Lentivirus encoding for GFP or luciferase (control) was loaded onto 1:1:20 PLG/HAp scaffolds and implanted into the right EFP to investigate the localization of transduced cells in the scaffold at early time points (day 3 and day 7). Cells are present throughout the scaffold at days 3 and 7 (Fig. 5). Large numbers of GFP positive cells were observed in the scaffold at both time points (Fig. 5B,C). Most transduced cells were located at or near the surface of the polymer. These results indicate that the lentivirus-loaded PLG/HAp composite scaffolds supported cell infiltration and transduced infiltrating cells.

Identity of infiltrating and transduced cells

The identity of infiltrating cells and the percentage of cells transduced were characterized in order to investigate the mechanisms underlying the enhanced transgene expression. Immune cells (i.e., CD45+) were the focus of the studies, as they are present in a scaffold following implantation as part of a typical foreign body response [12]. PLG/HAp (1:1:20) scaffolds and PLG (0:1:30) scaffolds were compared as these conditions had significant differences in gene expression at both 3 and 21 days. Immune cell infiltration (as a percentage of total cells) was similar at day 3, and decreased at least two-fold for both PLG scaffolds and PLG/HAp scaffolds between day 3 and day 21 (Fig. 6A). At day 21, the percentage of immune cells in the PLG/HAp scaffolds was increased relative to PLG alone. At day 3, the two scaffolds had similar numbers of immune cells, though a significantly greater proportion of the immune cells in PLG/HAp scaffolds were F4/80+ cells (macrophages) relative to PLG alone, while the proportion of Gr-1+ (neutrophils) and CD11c+ (dendritic cells) cells were consistent between scaffold types (Fig. 6B). At day 21, the small number of immune cells proved insufficient to resolve the various cell types (data not shown). Additionally, the number of cells in the scaffold that were not immune cells (i.e., CD45-) did not differ significantly between scaffold types or time point (data not shown).

Next, the identity of transduced cells was compared between PLG/HAp and PLG scaffolds. At day 3, greater than 60% of transduced cells were CD45+ immune cells for both PLG/HAp and PLG scaffolds (Fig. 7A). A significantly greater percentage of F4/80+ macrophages were transduced at day 3, consistent with their relatively high infiltration into PLG/HAp scaffolds (Fig. 7B). Gr-1+ neutrophils were the only other cells type above 10% of transduced immune cells. At day 21, the majority of transduced cells in PLG scaffolds were also CD45+ immune cells. However, for PLG/HAp scaffolds at day 21, the majority of transduced cells in the scaffold were CD45- (Fig. 7C). These results demonstrated that PLG scaffolds were populated with mostly transduced immune cells at both 3 and 21 days. However, PLG/HAp scaffolds had greater levels of macrophage infiltration and transduction at day 3, and transduction of persisting CD45- (non-immune) cells at day 21.

DISCUSSION

In this manuscript, we demonstrate immobilization of lentivirus to PLG/HAp scaffolds to significantly enhance transgene expression *in vitro* and *in vivo*. PLG/HAp composite scaffolds have been produced for enhanced function in bone regeneration [13–15]. However, HAp has also been implicated in the binding of gene therapy vectors [7, 8, 16, 17], and could provide a versatile tool to functionalize scaffolds in order to retain and stabilize the vectors for enhanced gene delivery. HAp contains calcium, phosphate, and hydroxyl groups, which provides a complex combination of cationic and anionic residues for interaction with lentiviral vectors that have become widely used gene transfer tools due to their relative ease of production, the availability of vector libraries, and their capacity to stably transduce both dividing and non-dividing cells with high efficiency. Additionally, HAp has previously been employed for the association of adenoviral vectors [17]. Interestingly, binding of lentivirus to HAp nanoparticles resulted in an extension of virus half-life from 29 hours to 34 hours [7]. HAp nanoparticles have been applied to a collagen gel to retain entrapped lentivirus [7, 11, 18]. Incorporation of HAp into microporous scaffolds may similarly function to enhance retention of the virus, and can co-localize the vector with cells that attach to the scaffold surface to promote localized gene expression (Fig. 5). Additionally, a balance of HAp content for virus binding and open pore structure of these scaffolds can support rapid cell infiltration and uptake of bound virus, which has previously been reported to enhance gene transfer [19]. The presence of HAp in the PLG scaffold increased the extent of transgene expression relative to PLG alone, and supported transgene expression for more than 100 days, whereas PLG scaffolds alone supported expression for less than 28 days.

Identifying cell types within the scaffold revealed differences in cell infiltration and types of cells transduced, which may underlie the impact on transgene expression. Previous reports suggested that scaffolds containing HAp induce a similar level of host immune response when compared to unmodified scaffolds *in vivo* [20]. Herein, both scaffolds had similar numbers of immune cells at day 3, with a dramatic decline in immune cells through day 21. PLG/HAp scaffolds had more immune cells relative to PLG scaffolds at day 21. Macrophages, which had increased numbers in the PLG/HAp scaffolds at day 3 relative to PLG alone, are a significant component of the foreign-body response that can critically impact regeneration [12] and are frequent targets of lentiviral transduction *in vivo* [8, 11], which may explain the greater levels of transgene expression at day 3. This transduction of macrophages could support approaches aimed at modulating the immune response using scaffolds [21]. Interestingly, at day 21, approximately 25% of the transduced cells in the PLG/HAp scaffolds were immune cells, whereas on PLG scaffolds, approximately 90% of the transduced cells were immune cells. For PLG scaffolds, the decline in immune cell numbers between days 3 and 21 is consistent with the decrease in expression levels. The large number of persisting non-immune cells transduced in the HA/PLG scaffolds may underlie the sustained expression for over 100 days. The total number of CD45⁺ cells was similar at days 3 and 21 (data not shown); thus, the high percentage of transduced CD45⁺ cells at day 21 suggests that this cell population is preferentially transduced in PLG/HAp scaffolds relative to PLG scaffolds. The virus binding ability and/or protection of virus activity provided by the PLG/HAp scaffolds may result in higher concentrations of virus in the scaffold for longer times, which would enable transduction of the non-immune cells that typically require longer times to infiltrate the scaffold.

Reversible association of the lentivirus directly to the HAp embedded in the PLG/HAp scaffold allows for virus retention and preservation of activity. A number of approaches have been investigated for the immobilization of virus, which include vector biotinylation for binding to avidin-modified surface [5], immobilization of antibodies specific for the virus [22, 23], phosphatidylserine (PS) functionalization for binding VSV-G pseudotyped lentivirus [6], and extracellular matrix protein [4] or self-assembled monolayer [24] sequestration. These approaches have identified the challenge of providing specific virus immobilization without reduction of activity due to virus destabilization [1]. Previous studies with HAp have used nanoparticles added to the microporous scaffolds; however, the nanoparticles may be actively cleared from the scaffold by the immune system [25] or removed from the scaffold similar to the way ECM proteins are displaced (i.e., Vroman effect), which could contribute to the reduced duration of transgene expression. Fortunately, the HAp nanoparticles are relatively stable which allows their facile incorporation into the scaffold. Incorporation within the scaffold limits their ability to be displaced and cleared. The incorporation of HAp into the structure also allows for the virus to be added following scaffold fabrication, which avoids exposing the virus to the processing conditions of the polymer. Interestingly, relative to the other immobilization strategies, HAp seemed to influence cell infiltration and the cell types transduced.

CONCLUSIONS

We have developed a PLG scaffold with incorporated HAp nanoparticles to immobilize lentivirus. These PLG/HAp composite scaffolds bind active lentivirus, and promote transgene expression at elevated levels for extended time periods *in vitro* and *in vivo*. Expression levels were significantly increased at day 3 *in vivo* in all PLG/HAp scaffold formulations relative to PLG alone, and PLG/HAp scaffold formulations had expression that persisted in excess of 100 days in the epididymal fat pad. PLG/HAp scaffold binding of the lentivirus was also shown to induce local transduction of cells within the scaffold. Finally, we determined that increase macrophage infiltration and transduction was responsible for

increased gene expression at day 3, and that transduction of persisting non-immune cells is responsible for sustaining the gene expression past day 21. Thus, PLG/HAp retained and stabilized the virus, while also influencing cell infiltration and preferentially transducing non-immune cells.

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References

1. Jang JH, Schaffer DV, Shea LD. Engineering biomaterial systems to enhance viral vector gene delivery. *Mol Ther.* 2011; 19:1407–15. [PubMed: 21629221]
2. Jang JH, Koerber JT, Gujrati K, Bethi SR, Kane RS, Schaffer DV. Surface immobilization of hexahistidine-tagged adeno-associated viral vectors for localized gene delivery. *Gene Ther.* 2010; 17:1384–9. [PubMed: 20508598]
3. Shin S, Salvay DM, Shea LD. Lentivirus delivery by adsorption to tissue engineering scaffolds. *J Biomed Mater Res A.* 2010; 93:1252–9. [PubMed: 19827108]
4. Bajaj B, Lei P, Andreadis ST. High efficiencies of gene transfer with immobilized recombinant retrovirus: kinetics and optimization. *Biotechnol Prog.* 2001; 17:587–96. [PubMed: 11485416]
5. Hu WW, Lang MW, Krebsbach PH. Development of adenovirus immobilization strategies for in situ gene therapy. *J Gene Med.* 2008; 10:1102–12. [PubMed: 18618417]
6. Shin S, Tuinstra HM, Salvay DM, Shea LD. Phosphatidylserine immobilization of lentivirus for localized gene transfer. *Biomaterials.* 2010; 31:4353–9. [PubMed: 20206382]
7. Shin S, Shea LD. Lentivirus immobilization to nanoparticles for enhanced and localized delivery from hydrogels. *Mol Ther.* 2010; 18:700–6. [PubMed: 20051940]
8. Tuinstra HM, Aviles MO, Shin S, Holland SJ, Zelivyanskaya ML, Fast AG, et al. Multifunctional, multichannel bridges that deliver neurotrophin encoding lentivirus for regeneration following spinal cord injury. *Biomaterials.* 2012; 33:1618–26. [PubMed: 22130565]
9. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. *J Virol.* 1998; 72:8463–71. [PubMed: 9765382]
10. Jang JH, Rives CB, Shea LD. Plasmid delivery in vivo from porous tissue-engineering scaffolds: transgene expression and cellular transfection. *Mol Ther.* 2005; 12:475–83. [PubMed: 15950542]
11. Aviles MO, Shea LD. Hydrogels to modulate lentivirus delivery in vivo from microporous tissue engineering scaffolds. *Drug Deliv Transl Res.* 2011; 1:91–101. [PubMed: 22229129]
12. Boehler RM, Graham JG, Shea LD. Tissue engineering tools for modulation of the immune response. *Bio Techniques.* 2011; 51:239–40. 42, 44. passim.
13. Jongpaiboonkit L, Franklin-Ford T, Murphy WL. Growth of hydroxyapatite coatings on biodegradable polymer microspheres. *ACS Appl Mater Interfaces.* 2009; 1:1504–11. [PubMed: 20161578]
14. Kim SS, Ahn KM, Park MS, Lee JH, Choi CY, Kim BS. A poly(lactide-co-glycolide)/hydroxyapatite composite scaffold with enhanced osteoconductivity. *J Biomed Mater Res A.* 2007; 80:206–15. [PubMed: 17072849]
15. Son JS, Appleford M, Ong JL, Wenke JC, Kim JM, Choi SH, et al. Porous hydroxyapatite scaffold with three-dimensional localized drug delivery system using biodegradable microspheres. *J Control Release.* 2011; 153:133–40. [PubMed: 21420453]
16. Nie H, Ho ML, Wang CK, Wang CH, Fu YC. BMP-2 plasmid loaded PLGA/HAp composite scaffolds for treatment of bone defects in nude mice. *Biomaterials.* 2009; 30:892–901. [PubMed: 19010530]
17. Hu WW, Wang Z, Hollister SJ, Krebsbach PH. Localized viral vector delivery to enhance in situ regenerative gene therapy. *Gene Ther.* 2007; 14:891–901. [PubMed: 17344901]

18. Kidd ME, Shin S, Shea LD. Fibrin hydrogels for lentiviral gene delivery in vitro and in vivo. *J Control Release*. 2012; 157:80–5. [PubMed: 21907251]
19. Shepard JA, Virani FR, Goodman AG, Gossett TD, Shin S, Shea LD. Hydrogel macroporosity and the prolongation of transgene expression and the enhancement of angiogenesis. *Biomaterials*. 2012; 33:7412–21. [PubMed: 22800542]
20. Kim JH, Kim TH, Jin GZ, Park JH, Yun YR, Jang JH, et al. Mineralized poly(lactic acid) scaffolds loading vascular endothelial growth factor and the in vivo performance in rat subcutaneous model. *J Biomed Mater Res A*. 2012; 101:1447–55. [PubMed: 23114998]
21. Ali OA, Huebsch N, Cao L, Dranoff G, Mooney DJ. Infection-mimicking materials to program dendritic cells in situ. *Nat Mater*. 2009; 8:151–8. [PubMed: 19136947]
22. Mei L, Jin X, Song C, Wang M, Levy RJ. Immobilization of gene vectors on polyurethane surfaces using a monoclonal antibody for localized gene delivery. *J Gene Med*. 2006; 8:690–8. [PubMed: 16634113]
23. Levy RJ, Song C, Tallapragada S, DeFelice S, Hinson JT, Vyavahare N, et al. Localized adenovirus gene delivery using antiviral IgG complexation. *Gene Ther*. 2001; 8:659–67. [PubMed: 11406760]
24. Gersbach CA, Coyer SR, Le Doux JM, Garcia AJ. Biomaterial-mediated retroviral gene transfer using self-assembled monolayers. *Biomaterials*. 2007; 28:5121–7. [PubMed: 17698189]
25. Zolnik BS, Gonzalez-Fernandez A, Sadrieh N, Dobrovolskaia MA. Nanoparticles and the immune system. *Endocrinology*. 2010; 151:458–65. [PubMed: 20016026]

Figure 1. Binding of lentivirus to HAp

(a) Average HAp nanoparticle diameter. (b) Saturated lentivirus binding capacity of HAp nanoparticles. (c) Virus activity for different concentrations of lentivirus (5×10^6 , 1×10^7 , 5×10^7 , and 1×10^8 LP) when mixed with either PBS or 0.05 mg of HAp nanoparticles ($n = 3$). Statistical analysis completed using a two-way ANOVA with Bonferroni's post-hoc test following log transformation of the data. *, significant difference compared to uncomplexed LV at the same dose ($p < 0.0001$).

Figure 2. Scanning electron microscopy (SEM) imaging of PLG/HAp scaffolds
Images of fabricated (a) 0:1:30 (PLG:NaCl, w/w) PLG, (b) 1:1:30 (HAp:PLG:NaCl, w/w/w) PLG/HAp, (c) 1:1:20 PLG/HAp, and (d) 1:1:10 HAPLG scaffolds.

Figure 3. *In vitro* cell transduction of virus-loaded PLG/HAp composite scaffolds
 Lentivirus (5×10^8 LP, LV- β gal or LV-Luc) was mixed with scaffolds and assayed for (a) luciferase expression ($n = 3$) or (b–e) β -gal expression for (b) 0:1:30 PLG, (c) 1:1:30 PLG/HAp, (d) 1:1:20 PLG/HAp, or (e) 1:1:30 PLG/HAp. Statistical analysis completed using a one-way ANOVA with Tukey's post-hoc test following log transformation of the data. *, significant difference compared to the 0:1:30 PLG control ($p < 0.05$).

Figure 4. *In vivo* cell transduction of virus-loaded PLG/HAp composite scaffold

Lentivirus encoding luciferase (LV-Luc, 3×10^8 LP) was added to 0:1:30 PLG, 1:1:30 PLG/HAp, 1:1:20 PLG/HAp, and 1:1:10 PLG/HAp scaffolds. HA/LV complexes were also added to 0:1:30 PLG scaffolds. Integrated light flux (photons/second) was measured using constant-size regions of interest over the implant site ($n = 4$). Statistical analysis completed using a one-way ANOVA with Tukey's post-hoc test at each time point following log transformation of the data. Significant differences compared to both 0:1:30 PLG+HAp/LV and 1:30 PLG+LV are indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Figure 5. *In vivo* cell transduction of virus-loaded PLG/HAp composite scaffold

Lentivirus encoding (a) Luc (control) or (b,c) GFP (5×10^8 LP) was mixed with 1:1:20 PLG/HAp. Virus-loaded scaffolds were implanted into the EFP pad of mice. (a,b) 3 or (c) 7 days after implantation, scaffolds were stained with anti-GFP antibody. Hematoxylin was used as counterstaining. Arrows indicate the position of cells that stained positive for GFP. Scale bar indicates 100 μ m.

Figure 6. Identification of immune cell types infiltrating into scaffolds

(a) Quantification of infiltrating immune cells in PLG/HAp and PLG scaffolds by flow cytometry at day 3 and 21. Statistical analysis completed using an unpaired t test. * indicates significant difference compared to the day 21 ($p < 0.05$). (b) Quantification of immune cells types in PLG/HAp and PLG scaffolds at day 3 by flow cytometry. Statistical analysis completed using a two-way ANOVA with Bonferroni's post-hoc test. ** indicates significant difference compared to PLG scaffold ($p < 0.05$).

Figure 7. Identification of transduced cells in scaffolds

(a,c) Quantification of transduced immune and non-immune cells types in PLG/HAp and PLG scaffolds at (a) day 3 and (c) day 21 by flow cytometry. Statistical analysis completed using an unpaired t test. *, significant difference compared to the CD45- cells ($p < 0.01$). (b) Quantification of transduced immune cells in PLG/HAp and PLG scaffolds by flow cytometry at day 3. Statistical analysis completed using a two-way ANOVA with Bonferroni's post-hoc test. *, significant difference compared to PLG control ($p < 0.05$).