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Heparin–chitosan nanoparticle functionalization of porous poly(ethylene glycol) hydrogels for localized lentivirus delivery of angiogenic factors

Aline M. Thomas [a], Andrew J. Gomez [a], Jaime L. Palma [b], Woon Teck Yap [a], Lonnie D. Shea [b, c, d, e, f, *]

[a] Department of Biomedical Engineering, McCormick School of Engineering, Northwestern University, Evanston, IL, USA
[b] Department of Chemical and Biological Engineering, McCormick School of Engineering, Northwestern University, Evanston, IL, USA
[c] Institute for BioNanotechnology in Medicine (IBNAM), Northwestern University, Chicago, IL, USA
[d] Center for Reproductive Science (CRS), Northwestern University, Evanston, IL, USA
[e] Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL, USA
[f] Chemistry of Life Processes Institute (CLP), Northwestern University, Evanston, IL, USA

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ABSTRACT

Hydrogels have been extensively used for regenerative medicine strategies given their tailorable mechanical and chemical properties. Gene delivery represents a promising strategy by which to enhance the bioactivity of the hydrogels, though the efficiency and localization of gene transfer have been challenging. Here, we functionalized porous poly(ethylene glycol) hydrogels with heparin–chitosan nanoparticles to retain the vectors locally and enhance lentivirus delivery while minimizing changes to hydrogel architecture and mechanical properties. The immobilization of nanoparticles, as compared to homogeneous heparin and/or chitosan, is essential to lentivirus immobilization and retention of activity. Using this gene-delivering platform, we over-expressed the angiogenic factors sonic hedgehog (Shh) and vascular endothelial growth factor (Vegf) to promote blood vessel recruitment to the implant site. Shh enhanced endothelial recruitment and blood vessel formation around the hydrogel compared to both Vegf-delivering and control hydrogels. The nanoparticle-modified porous hydrogels for delivering gene therapy vectors can provide a platform for numerous regenerative medicine applications.

1. Introduction

Swift, robust vascularization of the implant is required in regenerative medicine in order to support the survival and function of endogenous or transplanted cells [1–4]. Hydrogels have traditionally supported cell infiltration by providing sites for cell-adhesion, and by their degradation can create space for cells to enter. More recently, cell infiltration has been facilitated through the creation of porous hydrogels, as cells can readily infiltrate through the pores without the requirement of degrading the hydrogel [5]. Furthermore, cell infiltration has been enhanced through the application of drug delivery technology, which has promoted processes such as vascular ingrowth [6–10]. Many technologies have delivered factors for time scales of days to weeks; however, delivering for long time scales [5] or delivering a combination of factors [11–14], which may be necessary for some applications, has been challenging.

Gene delivery provides the opportunity to obtain sustained expression for time scales consistent with the time required for tissue regeneration, as transduction of endogenous cells permits the expression of transgenes for months after delivery [15,16]. Microporous scaffolds have been extensively investigated for gene delivery for numerous applications [16–18]. Microporous poly(lactide-co-glycolide) (PLG) scaffolds have been used to provide sustained release, and upon modification, to reversibly associate with the vector and enhance vector retention [15,16,19]. However, in our laboratory, gene delivery from hydrogels has been less effective *in vivo* relative to delivery from PLG scaffolds. The inclusion of micropores within the hydrogel led to enhanced transduction through increased cell infiltration [5]. Furthermore,

* Corresponding author. Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Rd./Tech E136, Evanston, IL, 60208-3120, USA. Tel.: +1 847 491 7043; fax: +1 847 491 3728.
E-mail address: l-shea@northwestern.edu (L.D. Shea).

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the inclusion of inorganic nanoparticles that reversibly associate with the vector have increased the vector half-life and maintained greater concentrations within the material [15]. While these strategies have enhanced transgene expression, the opportunity remains to further enhance gene delivery from hydrogels.

In this report, our objective was to investigate the incorporation and properties of heparin and chitosan, separately and in combination, to immobilize lentivirus onto porous, poly(ethylene glycol) (PEG) hydrogels in order to promote localized, sustained overexpression of regenerative factors. We recently reported that surface modification of PLG scaffolds with heparin and chitosan to reversibly associate with viral vectors retained the vectors locally, enhanced the vector half-life, and increased transgene expression [16]. We hypothesized that the manner in which heparin and chitosan were included (i.e., a component of the bulk hydrogel, or as nanoparticles) would influence the interaction with lentivirus and subsequent gene transfer. Vector binding and activity were investigated *in vitro*, along with the extent and duration of transgene expression *in vivo*. Lentivirus encoding for sonic hedgehog (Shh) and vascular endothelial growth factor (Vegf) were delivered, both of which have been implicated in angiogenesis [11–14], and their ability to enhance host integration and vascularization of the hydrogel were assessed for their future use in regenerative medicine applications.

2. Materials and methods

2.1. Formation and characterization of hydrogels

Fabrication: PEG-acrylate (4 arm, 20,000 Da; Laysan biomaterials; 10% w/v) was dissolved into PBS with photoinitiator (Irgacure 2959, Ciba; 0.5% w/v). To form porous hydrogels, the solution was frozen for 16 h at −20 °C and then exposed while frozen to UV light (365 nm, 50 mW/cm^2; 2 min). To form non-porous hydrogels, UV exposure was employed to crosslink the liquid-phase PEG solution. The formed hydrogel was then immersed in PBS or distilled water until use.

Mesh Size and Swelling Ratio: PEG hydrogels were formed as before ($n = 4$ per condition), weighed, and placed in distilled water. After 24 h, the hydrogels were weighed, frozen in liquid nitrogen, lyophilized for 24 h, and weighed again. Swelling ratio and mesh size was approximated using a modified Flory-Rehner model [20–22].

2.2. Cysteine addition of heparin and chitosan

Heparin (180 USP/mg, ~16,000 Da [22]) and chitosan (8183 Da) were stirred for 30 min in varying ratios of cysteines in 1 M 2-(*N*-morpholino)ethanesulfonic acid buffer (1 mg/mL polysaccharide) in the presence of 9 mg/mL *N*-ethyl-*N*′-(3-(dimethylamino)propyl)carbodiimide (EDC; CreoSalus Inc., Louisville, KY) and 6 mg/mL *N*-hydroxysuccinimide (NHS; Research Organics, Cleveland, OH). Unbound cysteines were removed using 10,000 and 3000 molecular weight cutoff Slide-A-Lyzer Dialysis Cassettes (Pierce), respectively. Incorporation of cysteines on the polysaccharides was assessed by measuring the absorbance of the modified polysaccharides in Ellman's (Pierce) solution at 412 nm [23], and the extinction of absorbance in toluidine blue (Fisher Scientific) and orange II assay solutions at 610 nm and 480 nm [16] as described previously. For hydrogel incorporation, filtered solutions were flash frozen in nitrogen, lyophilized, and stored with desiccants until use. All materials are from Sigma unless otherwise indicated.

2.3. Heparin–chitosan nanoparticles

Heparin and chitosan solutions (0.9 mg/mL ea. in 2% v/v acetic acid) were dissolved, mixed in varying ratios, and stirred for 2 h. Aggregates were removed using a 0.22 μm filter. Size and charge density of nanoparticles were assessed using the Zetasizer Nano ZSP (Malvern Instruments). For hydrogel incorporation, filtered solutions were flash frozen in nitrogen, lyophilized, and stored with desiccants until use.

Incorporation and retention of nanoparticles were assessed using the toluidine blue assay [24]. Solutions of unbound nanoparticles were assayed by immersing the supernatant for 1 h in toluidine blue (Fisher Scientific). Assay solutions were made according to previously established methods [16]. Incorporation was defined as the percentage of nanoparticles remaining after two 5 min washes, consistent with prior reports [16,19]. Retention was defined as the percentage remaining of incorporated nanoparticles. Release rate is defined as the difference in nanoparticle retention at the specified time period divided by that time period (in days).

2.4. Lentivirus incorporation and in vitro expression

To make cell-compatible porous PEG hydrogels, precursor solution (1% w/v PEG, 0.5% w/v photoinitiator in PBS) was conjugated to GCYKNRGCYKNRCGRGD (5 mM, fabricated at the Northwestern University peptide synthesis core) by Michael-type addition at 37 °C for 10 min to promote cell-adhesion and incorporate plasmin-degradable sites, frozen at −20 °C overnight, and exposed to UV light (365 nm, 50 mW/cm^2) for 2 min. Hydrogels were washed in PBS (twice, 5 min ea.) and partially dried in air to facilitate wetting of the surface by lentivirus (1×10^7–1×10^8 particles encoding for firefly luciferase) pipetted onto the gel. To evaluate *in vitro* expression lentivirus-incorporated hydrogels were seeded with 100,000 human embryonic kidney cells and imaged as done previously [16]. In brief, the hydrogels were incubated with 50 mM of D-luciferin (Molecular Therapeutics, Inc., MI) for 4 h and imaged with two 10-s intervals for bioluminescence (integrated photon flux, p/s), which was assessed using the *In Vivo* Imaging System (IVIS; Caliper, Hopkinton, MA, USA) [16].

2.5. Subcutaneous implantation

Animals were treated according the Animal Care and use Committee guidelines at Northwestern University. Surgery was performed as previously described ($n = 4$ per hydrogel design and time-point) [5,25]. Male CD1 mice (30g, Charles River) were anesthetized using isoflurane (2% v/v). An incision was made in the upper and lower back to allow for implantation of lentivirus-incorporated hydrogels that were stored at −80 °C until use. The site was secured in place by suturing the skin together and stapling the skin. Postoperative care consisted buprenorphine (0.1 mg/kg) administered immediately after surgery. To quantify gene expression, animals implanted with hydrogels delivering firefly luciferase-encoding lentivirus were injected intraperitoneally with 150 mg/kg body weight of D-Luciferin and imaged using the IVIS at 5 min intervals until expression peaked as previously reported [16].

2.6. Immunohistochemistry: hematoxylin and eosin

Hydrogels were implanted for 8 weeks. Hydrogels extracted from mice were fixed using 4% w/v paraformaldehyde (Sigma–Aldrich), embedded in sucrose O.C.T. and frozen as done previously [5], and sectioned transversally in 18 μm thick slices and collected serially. To detect overall cell presence these sections were stained with eosin and counterstained with Mayer's hematoxylin (Surgipath Medical Industries). Images were captured at 5× magnification for light microscopy (Leica Microsystems, Wetzlar, Germany).

2.7. Immunofluorescence: vascularization

Upon retrieval of the hydrogels, mice were injected by tail vein with biotinylated lectin (175 μL at 1 mg/mL). To assess vascularization, CD31 (Abcam, ab56299; 1:400 dilution) was used with AlexaFluor 555 goat anti-rabbit IgG (1:500 dilution) and fluorescein anti-biotin IgG (1:200 dilution) secondaries to label infiltrating endothelial cells and functional blood vessels, respectively. Images were captured at 10× magnification for fluorescence microscopy (Leica Microsystems, Wetzlar, Germany) and the outer 20 μm perimeter of the hydrogel was assessed for lectin and CD31 presence.

2.8. Statistics

One- and two-way ANOVA with Bonferroni post-hoc analysis were used to assess statistical differences.

3. Results

3.1. Heparin- and chitosan-functionalized PEG hydrogels

Heparin and chitosan were investigated as potential lentivirus immobilization agents that minimally influenced the bulk properties of porous PEG hydrogels. Initially, the method of incorporation of these polysaccharides was assessed for their ability to promote gene transfer *in vitro* (Fig. 1): adsorption of unmodified polysaccharides after hydrogel formation, mixing unmodified polysaccharides before formation, or conjugating cysteine-modified polysaccharides using Michael-type addition before formation. Cysteines were added to the polysaccharides in varying proportions using EDC/NHS chemistry to facilitate their incorporation into PEG (Supp. 1a). The extent of cysteine conjugation increased linearly for heparin (6.4-fold) and chitosan (4.5-fold) as the ratio of cysteine:polysaccharide ranged from 250 to 1000. Hydrogels formed with at a ratio of cysteine:polysaccharide equal to 1000 swelled to a final volume that was larger than unmodified hydrogels (Supp. 1d and e), yet a significant difference was not observed at ratios for cysteine:polysaccharide equal to 500 (Supp. 1b and c). Expression levels were not significantly different between control hydrogels, which did not contain polysaccharides, and hydrogels that