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The effect of glycosaminoglycan content on polyethylenimine-based gene delivery within three-dimensional collagen-GAG scaffolds

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Abstract

The design of biomaterials for increasingly complex tissue engineering applications often requires exogenous presentation of biomolecular signals. Integration of gene delivery vectors with a biomaterial scaffold offers the potential to bypass the use of expensive and relatively inefficient growth factor supplementation strategies to augment cell behavior. However, integration of cationic polymer based gene delivery vectors within three-dimensional biomaterials, particularly matrices which can carry significant surface charge, remains poorly explored. We examined the potential of polyethylenimine (PEI) as a gene delivery vector for three-dimensional collagen-glycosaminoglycan (CG) scaffolds under development for tendon repair. While acetylated versions of PEI have demonstrated improved transfection efficiency in 2D culture assays, we investigated translation of this effect to a 3D biomaterial that contains significant electrostatic charge. A reporter gene was used to examine the impact of polymer modification, polymer:DNA ratio, and the degree of sulfation of the biomaterial microenvironment on gene delivery *in vitro*. We observed highest transgene expression in acetylated and unmodified PEI at distinct polymer:DNA ratios; notably, the enhancement often seen in two-dimensional culture for acetylated PEI did not fully translate to three-dimensional scaffolds. We also found highly sulfated heparin-based CG scaffolds showed enhanced initial luciferase expression but not prolonged activity. While PEI constructs significantly reduced tenocyte metabolic health during the period of transfection, heparin-based CG scaffolds showed the greatest recovery in tenocyte metabolic health over the full 2 week culture. These results suggest that the electrostatic environment of

three-dimensional biomaterials may be an important design criterion for cationic polymer-based gene delivery.

A. Introduction

Traditionally, biomaterial design has focused on optimizing a range of biophysical properties such as structure, mechanics, and rate of degradation. Often, efforts have focused on artificial extracellular matrix (ECM) analogs that support cellular health and function by mimicking the native tissue's structure, mechanics, and composition. More recently, the impact of biomolecular supplementation and delivery methods has been incorporated into biomaterial design. While growth factors are still primarily introduced freely in solution as soluble media additives,^{1–5} efforts have turned to exploring approaches that could extend the time a growth factor is present within the biomaterial or that could spatially control factor localization. In these cases, designs have included strategies to release a biomolecule of interest from a degradable construct (*e.g.*, microspheres)^{6–8} or to covalently bind the biomolecule to the surface of the biomaterial.^{9–11} Inspired by transient immobilization of growth factors within the native ECM, recent approaches have also begun to incorporate charged moieties within the biomaterial (*e.g.*, glycosaminoglycans, heparin binding peptides) that are able to transiently sequester factors – either exogenously added or endogenously produced – to the surface of the biomaterial.^{12–16} However, growth factors still incur a high cost, require large dosages, and suffer from relatively short half-lives as well as the potential for significant diffusive loss after *in vivo* implantation.

Gene delivery approaches offer the potential to address a number of limitations associated with conventional biomolecular supplementation. Although viral-based gene vehicles are most efficient in achieving gene expression, concerns remain about their immunogenic safety, and hence regulatory approval.^{17, 18} Non-viral gene vectors, in the form of proteins, lipids and cationic polymers, provide an alternative path for transient gene delivery.^{19–27} One of the most efficient and well-studied off-the-shelf cationic polymers is polyethylenimine (PEI).^{28–31} Through electrostatic interaction, PEI binds the negatively charged phosphates on the DNA backbone, spontaneously condensing into a polymer:DNA complex (polyplex) on the order of hundreds of nanometers.³² The polymer:DNA weight-to-weight (w:w) ratio is a critical design parameter to control both transfection efficiency and cytotoxicity. If the w:w ratio is too low, there is minimal gene transfection. Alternatively, high polymer:DNA ratios often result in significant polymer-induced cytotoxicity.^{30, 33} In the context of designing a more efficient PEI-based delivery system, recent efforts have focused on selective acetylation of the primary and secondary amines of the polymer. Acetylation decreases the density of protonated amines, decreasing the cationic charge of the polymer, and allowing for a weaker polymer:DNA interaction, leading to easier release of the nucleic acid cargo and significant improvements in gene delivery efficiency.^{28, 34}

Our laboratory has recently described a series of collagen-GAG (CG) scaffolds under development for tendon regenerative medicine applications. Fabricated via lyophilization, we have described approaches to create anisotropic pore architectures to improve tenocyte bioactivity as well as influence mesenchymal stem cell differentiation down a tenogenic lineage.^{35–37} We have also explored the use of growth factor supplementation strategies to

balance tenocyte bioactivity versus proliferation.^{5, 37, 38} Recently, biomaterials such as CG scaffolds which contain charged ECM-derived matrix components have increasingly been explored in the context of bioinspired biomaterial design. For example, glycosaminoglycans (GAGs) in the native ECM are known to play an important role in sequestering biomolecules within the matrix based on their degree of sulfation and structural variations in their carbohydrate backbone.^{12, 39} Biomaterials incorporating GAGs have increasingly been explored as a means to transiently regulate growth factor availability.^{12, 15, 16, 40} Recently, we demonstrated that modifying the GAG content of collagen-GAG (CG) scaffolds imparts differential capacity for non-covalent, electrostatic growth factor binding.⁴¹ Notably, by incorporating highly sulfated heparin into the CG scaffold, we saw increased growth factor sequestration and positive impacts on cellular bioactivity compared to scaffolds containing lesser charged GAGs (hyaluronic acid, chondroitin sulfate). While modifying the degree of GAG-sulfation within three-dimensional biomaterials has been shown to impact both biomolecule sequestration and resultant cell response, its impact on the activity of cationic-polymer gene delivery vectors remains poorly understood.

The objective of this study therefore was to examine the efficiency of PEI-based gene delivery of a luciferase reporter gene to tenocytes within a collagen-GAG scaffold variant under development for tendon tissue engineering. In particular, we compared the optimal polymer:DNA ratios for transfection in two-dimensional culture and three-dimensional CG scaffolds. Further, given the known impact of scaffold GAG content on non-covalent growth factor sequestration as well as the central importance of electrostatic interactions in the context of PEI polyplexes, we explored the combined impact of polymer:DNA w:w ratio, PEI acetylation, and scaffold GAG content on polyplex efficiency. We tested the hypotheses that the electrostatic environment of the scaffold may enhance the expression or, alternatively, enhance the delivery of PEI-based gene vectors by preventing diffusion of the polyplexes out of the scaffold.

B. Materials and Methods

B.1. Collagen-glycosaminoglycan scaffold fabrication

B.1.1. CG suspension preparation—Type I collagen from bovine Achilles tendon (Sigma-Aldrich) was homogenized together with one of three glycosaminoglycans (GAGs): hyaluronic acid from *Streptococcus equi* (Sigma-Aldrich), chondroitin sulfate from shark cartilage (Sigma-Aldrich) or heparin from porcine intestinal mucosa (Sigma-Aldrich) in 0.05 M acetic acid to create a CG suspension.⁴² All experiments used suspensions with a collagen concentration of 1.5% (w/v) and a collagen:GAG ratio of 11:1. The suspensions were stored at 4°C and degassed prior to use.⁴³

B.1.2. CG scaffold fabrication via freeze drying—CG scaffolds were fabricated using a previously described approach.³⁷ Briefly, the CG suspension was added to cylindrical wells (6 mm dia., 15 mm tall) in a polytetrafluoroethylene (PTFE)-copper mold then placed onto a precooled (−10°C) freeze-dryer shelf (VirTis). The suspension was frozen at −10°C for two hours prior to the sublimation of the resulting ice crystals at 0°C and 200 mTorr.³⁷ Unidirectional heat transfer through the mold's copper bottom has

previously been shown to induce formation of anisotropic scaffolds for tendon applications.³⁷

B.1.3. Crosslinking of CG scaffold—After lyophilization, scaffolds were placed in a vacuum oven (Welch) at 105°C under vacuum for 24 hours for sterilization and dehydrothermal crosslinking.⁴² Scaffolds were cut to 5 mm length (6 mm dia.) for all experiments. All CG scaffolds were hydrated in 100% ethanol overnight and washed in phosphate-buffered saline (PBS) for 24 hours. To increase their resistance to tenocyte contraction, scaffolds were crosslinked using carbodiimide chemistry.^{44, 45} Scaffolds were covered with a 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) solution (molar ratio of 5:2:1 EDC:NHS:COOH) and agitated at room temperature for 2 hours. Following crosslinking, scaffolds were washed with PBS and stored in fresh PBS at 4°C.

B.2. Tenocyte isolation and culture

Tenocytes (tendon cells) were isolated from 2–3 year old horses that were euthanized for reasons not related to tendinopathy using previously described methods.⁴⁶ Tenocytes were expanded in standard culture flasks in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 1% Antibiotic-Antimycotic (Invitrogen), 1% L-glutamine (Invitrogen), and 50 µg/mL ascorbic acid (Wako). The media was changed every 3 days and the tenocytes were cultured to confluence at 37°C and 5% CO₂. Passage 4 cells were used for all experiments.

B.3. Polyplex fabrication

The luciferase reporter vectors pGL3 (Promega) and pGLuc (New England BioLabs) were grown in DH5α E. coli (Gibco BRL) and were purified using a commercial plasmid purification kit (BioRad). Acetylation was carried out as previously described,³⁴ with 60% acetylated PEI used throughout. Briefly, branched 25-kDa PEI (0.5 g) was transferred to a 20 mL scintillation vial in freshly distilled methanol and dissolved. Acetic anhydride was added and the reaction was carried out for 4 hours at 60°C with stirring before quenching with double distilled water, as previously described.³⁴ The solvent was removed under reduced pressure. The remaining solution was purified by gel filtration chromatography (PD-10 columns, Pharmacia) and eluted with double distilled water. The purified polymers were then concentrated and stored at –80°C until use.

B.4. Preparation of gene activated matrices

B.4.1. Preparation of polymer-DNA polyplexes—Stock polymer solutions of unmodified or 60% acetylated PEI were thawed and diluted to 1 mg/mL. DNA (2 µg per scaffold) and PEI were combined at a range of polymer:DNA weight-to-weight ratios (1:1 to 5:1) with 20 mM PIPES and distilled water to achieve a desired final volume (30 µL) to be added per scaffold. Polyplexes were allowed to equilibrate at room temperature for 20 minutes before use.

B.4.2. Scaffold seeding and culture conditions—Hydrated and crosslinked scaffolds (6 mm in diameter, 5 mm in height) were incubated in complete media at 37°C for at least

one hour prior to seeding. Excess media was removed and the scaffolds were placed in ultra-low attachment well plates. Polyplexes were added by direct loading to the scaffolds, in a manner inspired by previous efforts by Tierney *et al.*^{29, 47} Briefly, 15 μ L of polyplex suspension was added to one side of the scaffold followed by 15 minute incubation at room temperature. The scaffold was then flipped over and another 15 μ L of polyplex suspension was added. Following a second 15 minute incubation, tenocytes were trypsinized and resuspended at a concentration of 5×10^5 cells per 20 μ L of media. The cells were seeded into the polyplex containing scaffolds by directly loading 10 μ L of the cell suspension to each side of the scaffold using a previous described static seeding method.³⁷ Briefly, the scaffolds were incubated at 37°C for 15 minutes in between each seeding and then incubated without additional media for 2 hours to allow for initial cell attachment. After two hours, serum-free growth media was gently added. Four hours post-seeding, the serum-free media was replaced with full tenocyte culture media. Media was changed every 3 days for the duration of each experiment.

B.5. Quantification of luciferase expression

B.5.1. Assay for pGL3 expression—Luciferase expression was first quantified 48 hours after cell seeding as a function of polymer:DNA w:w ratio and acetylation via a destructive assay using the Promega luciferase assay system. Scaffolds were lysed with Complete Cell Lysis Reagent and 20 μ L of lysate was used for analysis. Activity was measured in relative light units (RLU) using a Lumat LB 9507 luminometer (Berthold). The results were normalized to relative light units measured from tenocyte seeded scaffolds that were treated with the pGL3 plasmid only with no PEI carrier.

B.5.2. Assay for pGLuc expression—Luciferase expression was subsequently quantified over time (through 14 days) after cell seeding using the BioLux Gaussia Luciferase Assay Kit (New England BioLabs). Aliquots of media (50 μ L) were taken at regular intervals (2, 5, 7, 14 days post transfection) and stored for analysis at either 4°C (less than 1 week storage) or –20°C (longer than 1 week storage). Activity was again reported in relative light units (RLU) versus a DNA only, no PEI carrier control using a Lumat LB 9507 luminometer (Berthold).

B.6. Quantification of metabolic health

The metabolic activity of the tenocytes within the CG scaffolds was measured using the non-destructive alamarBlue® assay (Invitrogen) as previously described.^{37, 43} Briefly, scaffolds were removed from culture at each timepoint, rinsed in PBS, then incubated in alamarBlue® solution at 37°C under shaking for 2 hours.³⁷ Using a fluorescent spectrophotometer, resorufin fluorescence was measured (excitation: 540 nm, emission: 590 nm) and compared to a standard curve created from a known number of tenocytes. Tenocyte metabolic activity has been previously shown to be tightly correlated with overall tenocyte number within the scaffold.^{5, 37} Here, metabolic activity of the tenocyte-scaffold construct was determined at each time point, then normalized to the metabolic activity of the total number of tenocytes initially seeded into the scaffold, to provide a standard comparison metric.

B.7. Statistical analysis

Two-way analysis of variance (ANOVA) was performed on all data sets followed by Tukey-HSD post-hoc test. Pair-wise comparisons were also completed as necessary. A p-value < 0.05 was used for significance. All analyses were based on a minimum of n = 3 scaffolds. In figures, error is reported as the standard error of the mean.

C. Results

C.1. The impact of polyplex polymer:DNA ratio and acetylation on gene delivery in a three dimensional CG scaffold

To screen the impact of a range of polyplex formulations on transfection efficiency within the three-dimensional CG scaffold, we quantified delivery of the pGL3 plasmid, which encodes the luciferase reporter protein, to equine tenocytes within CG scaffolds containing the conventional GAG, chondroitin sulfate (C:CS scaffold). Unmodified and acetylated PEI-containing polyplexes were tested at a range of polymer:DNA weight-to-weight ratios (1:1 to 5:1) previously validated as exhibiting high transfection activity in 2-D cultures. Luciferase expression was quantified 48 hours after seeding equine tenocytes into polyplex modified C:CS scaffolds.

Results suggested the presence of a separate optimal polymer:DNA ratio for unmodified versus acetylated PEI (Fig. 1). The acetylated PEI-mediated transfection efficiency of tenocytes in 2D culture, similar to previous studies,^{28, 34} was significantly greater at higher polymer:DNA ratios (data not shown). For three-dimensional CG scaffolds, unmodified PEI constructs showed higher efficiency at lower polymer:DNA ratios, with a 2:1 ratio showing significantly ($p < 0.014$) increased luciferase as compared to all other polymer:DNA ratios and control in the C:CS scaffold. Also like previous 2D studies,^{28, 34} acetylated PEI required higher polymer:DNA ratios, with 4:1 and 5:1 polymer:DNA polyplexes showing significantly ($p < 0.05$) increased luciferase activity. However, unlike previous studies in two-dimensional culture, acetylated PEI did not significantly outperform unmodified PEI in CG scaffolds. Unmodified PEI showed significantly increased luciferase activity compared to acetylated PEI at 1:1 ($p = 0.032$), 2:1 ($p < 0.001$), and 3:1 ($p = 0.016$) polymer:DNA ratios. While luciferase activity was greater in acetylated PEI for 4:1 and 5:1 polymer:DNA ratios compared to unmodified PEI, the increases were not statistically significant.

C.2. The impact of acetylation on the kinetics of gene expression

We next examined the time course for luciferase expression using PEI polyplexes incorporating the pGLuc plasmid encoding the *Gaussia princeps* luciferase reporter protein. This modification allowed gene expression of tenocyte seeded C:CS scaffolds to be assessed non-destructively from aliquots taken from the culture media over 14 days (Fig. 2). Here, luciferase expression was normalized against a three dimensional control that contained the plasmid DNA without a delivery vector. While the naked pGLuc gene gave very low chemiluminescent background, suggesting minimal transfection efficiency (Fig. 1), it was not possible to track cell response over time. While the pGL3 gene allowed for tracing cell response, it had not been previously optimized for gene delivery and led to significant chemiluminescent background during analysis (Fig. 2).

As observed with the pGL3 plasmid, at 2 days post-transfection the 2:1, 3:1, 4:1 and 5:1 w:w ratios of the polyplexes containing unmodified PEI showed significantly ($p < 0.001$) higher luciferase expression compared to the control and the 1:1 w:w ratio (Fig. 2A). At day 5, significantly increased luciferase expression over control was still observed for 2:1, 3:1 and 5:1 polymer:DNA ratios ($p < 0.011$) for unmodified PEI constructs (Fig. 2A). Examining the results over time, luciferase expression in tenocytes was significantly ($p < 0.001$) greater at day 2 than at later timepoints (days 5, 7 and 14) for 2:1, 3:1, 4:1 and 5:1 unmodified PEI polyplexes. By days 7 and 14, there was no significant increase in luciferase expression over control (Fig. 2A).

When considering the polyplexes based on the acetylated PEI, 4:1 and 5:1 w:w ratios again led to significantly ($p < 0.001$) greater luciferase expression than the control at both days 2 and 5 (Fig. 2B). Luciferase expression in the 4:1 and 5:1 constructs were significantly ($p < 0.001$) greater than all lower polymer:DNA ratios at day 2. Examining the results with time, the 3:1, 4:1 and 5:1 PEI:DNA w:w ratio polyplexes demonstrated significantly ($p < 0.05$) increased luciferase expression at day 2 compared to all later days (Fig. 2B). There was no statistical significance between the polyplex groups at days 5, 7 and 14 (Fig. 2B).

Comparing the top performing unmodified PEI (2:1 w:w) and acetylated PEI (4:1 w:w) groups, no significant differences were observed in the performance over time between unmodified and acetylated PEI (Fig. 2C). Both unmodified and acetylated PEI groups significantly outperformed control (bare DNA) cultures at both day 2 ($p < 0.001$) and day 5 ($p < 0.02$), following similar trends seen in earlier transfection studies using these identical systems in 2D cultures.³⁴ Contrary to those results, acetylated PEI polyplexes again did not exhibit increased gene expression versus unmodified PEI polyplexes at any time.

C.3. The impact of variable GAG incorporation on polyplex efficiency

Given the observed differences in response in three-dimensional CG scaffolds versus previously reported two-dimensional cultures, we subsequently examined the impact of sulfated scaffold GAG content on gene expression. Here, a sub-set of polyplex polymer:DNA ratios were chosen for a narrow range surrounding the identified optimal unmodified (2:1) and acetylated (4:1) PEI ratios (Fig. 2). Unmodified PEI polyplexes were tested at polymer:DNA at ratios of 1:1, 2:1 and 3:1 while acetylated PEI polyplexes were tested at w:w ratios of 3:1, 4:1 and 5:1.

The initial impact of GAG charge on tenocyte luciferase expression was examined 2 days after tenocytes were exposed to the gene activated matrices (Fig. 3). For unmodified PEI polyplexes, scaffold GAG content significantly ($p < 0.05$) impacted luciferase expression. Luciferase activity was significantly ($p < 0.03$) enhanced in the CG scaffolds containing heparin (C:HP) compared to C:CS or hyaluronic acid (C:HA) CG scaffolds for a 2:1 polymer:DNA ratio (Fig. 3A). Luciferase activity was also significantly ($p < 0.01$) enhanced in the C:HP scaffolds compared to the C:HA scaffold for the 3:1 polymer:DNA ratio, while no difference was observed between groups at 1:1 polymer:DNA ratio (Fig. 3A). A similar effect was observed with acetylated PEI polyplexes (Fig. 3B). Here, tenocytes cultured in C:HP scaffolds show significantly ($p < 0.001$) enhanced luciferase expression over C:HA and C:CS scaffolds at both the 4:1 and 5:1 w:w ratios.

The expression profiles of the most effective polymer:DNA ratios (2:1 for unmodified PEI; 5:1 for acetylated PEI) were subsequently traced over 7 days in culture to determine whether scaffold GAG content was able to mediate extended DNA delivery as well (Fig. 4). For unmodified PEI polyplexes (2:1 w:w), a significant ($p = 0.001$) decrease in expression was observed across the 7 day experiment between each time point and also within each GAG variant. The expression in the C:HP scaffolds is significantly ($p < 0.001$) higher at day 2 compared to C:HA and C:CS scaffolds (Fig. 4A). Results were similar for acetylated PEI polyplexes (Fig. 4B). Significantly ($p < 0.001$) increased luciferase expression was observed at day 2 as compared to days 5 and 7. Like with unmodified PEI, the impact of the highly charged C:HP scaffold on increased expression was limited to the first time point (day 2).

C.4. The impact of PEI gene delivery on tenocyte metabolic activity

PEI-based delivery vectors have been shown to have the potential for significant cytotoxicity.⁴⁸ However, we have previously shown that increasing the degree of glycosaminoglycan sulfation in the CG scaffold can enhance tenocyte metabolic health.⁴¹ We therefore examined the long-term (14 days) consequences of PEI-based luciferase delivery on tenocyte metabolic activity as a function of scaffold GAG content for both unmodified (2:1 polymer:DNA ratio) and acetylated (5:1 polymer:DNA ratio) PEI polyplexes (Fig. 5). Here metabolic activity is reported as normalized against the number of initially seeded tenocytes, with data from day 2 suggesting initial cell attachment efficiencies on order of 30 – 50% as previously described for CG scaffolds by *O'Brien et al.*⁴⁹ Overall, the metabolic activity of tenocytes exposed to DNA alone was significantly ($p < 0.05$) higher than tenocytes exposed to unmodified or acetylated PEI polyplexes across all time points. However, consistent with previous observations,⁴¹ scaffold GAG content impacted cell metabolic activity. For the control group that did not receive PEI, the C:HP scaffold showed significantly ($p < 0.05$) increased metabolic activity at day 7 and 14 of culture compared to day 2. Examining the overall effect of PEI type and scaffold GAG content in more detail, while the metabolic activity of tenocytes exposed to PEI (unmodified, acetylated) is reduced compared to control for each scaffold type, the reduction is not significant at day 2 (Fig. 5). However, by day 4 the metabolic activity of tenocytes in scaffolds not exposed to PEI starts to increase, with C:HA and C:HP scaffolds showing significantly ($p < 0.05$) higher metabolic activity compared to their corresponding polyplex receiving groups (Fig. 5). Identical results are also observed at day 7. By day 14 there were no significant differences in metabolic activity observed between control or either PEI groups in both the C:HA or C:CS scaffolds. Interestingly, tenocytes in the C:HP scaffolds that were exposed to PEI show significantly ($p < 0.05$) higher metabolic activity compared to all earlier time points (Fig. 5).

D. Discussion

The development of therapeutic scaffolds for tissue regeneration has largely focused on mimicking the mechanical, structural and compositional features of the extracellular matrix. Additionally, efforts are being made to increase the bioactivity of these scaffolds although much of the literature to date has focused on the presentation of recombinant proteins and growth factors.^{50–52} Alternatively, gene therapy has the potential to facilitate localized,

sustained delivery of beneficial growth factors by delivering therapeutic plasmids to the affected areas. Such approaches may eventually offer value in the context of wound healing⁵³ and for promoting lineage specification from patient-derived MSC populations.^{1, 54–56} Early work in the development of gene-activated matrices demonstrated the ability to improve bone healing in a canine defect model through administration of plasmid DNA in a collagen sponge.⁵⁴ More recent studies have shown the influence PEI and nano-hydroxyapatite, individually and in combination, have on gene (reporter and therapeutic) delivery to MSCs within collagen-based scaffolds for bone regeneration.^{26, 29, 47, 57} Further, studies focused on optimizing PEI chemistry for gene delivery have shown that increased efficiency of the PEI vector can be achieved through acetylation of the polymer. This was attributed to the lower electrostatic binding affinity of the polymer and DNA, as the cationic charge is reduced from acetylation of the polymer's amine groups.³⁴ While this previous work suggested the electrostatic environment within the cell can impact the efficiency of gene delivery, our work here suggests that the electrostatic environment surrounding the cell can also significantly impact polyplex delivery.

The work described here examines the impact of the electrostatic environment of a three-dimensional collagen-GAG scaffold on the efficiency of PEI-based polyplexes under development for a range of gene delivery applications. Glycosaminoglycans are native to the extracellular matrix and are known for their role in sequestering growth factors *in vivo*.⁵⁸ Recent work by our group has shown that the degree of sulfation (negative charge) of the GAG component of 3D CG scaffolds could be used to impact the degree in which the scaffold was able to sequester growth factors and, furthermore, the cellular activity of equine tenocytes.⁴¹ We sought to determine whether the presence of a three-dimensional CG scaffold impacted the efficiency of PEI-based polyplex gene delivery, and subsequently whether altering the degree of GAG sulfation in the CG scaffolds could impact polyplex activity or maintain their delivery over an extended period of time.

Initial experiments examined the response of the PEI polyplexes within CG scaffolds containing the moderately sulfated chondroitin sulfate (CS), our CG scaffold standard. It was found that the unmodified PEI polyplexes led to significantly higher luciferase expression at lower PEI:DNA w:w ratios when compared to acetylated PEI polyplexes 2 days after transfection (Fig. 1). While the acetylated polyplexes seemed to have higher expression at higher w:w ratios, it was not statistically significant. This is in contrast to transfection results seen in 2D.^{28, 34} In order to examine the time course of transfection and expression, luciferase expression was subsequently monitored over an extended 14 day period (Fig. 2A).

The active time of luciferase expression in the polyplex seeded C:CS scaffolds (compared to bare DNA alone) was found to be limited to 2–5 days (Fig. 2). Unmodified PEI polyplexes showed significantly improved luciferase expression at 2:1, 3:1, 4:1 and 5:1 w:w ratios at day 2 and 2:1, 3:1 and 5:1 at day 5. Consistent with initial results and those in the literature, acetylated PEI polyplexes at higher w:w ratios (4:1, 5:1) were the only variants to show significantly higher luciferase expression, again at both day 2 and 5 post-transfection (Fig. 2B). Interestingly, acetylation did not significantly improve luciferase expression, with the

top performing unmodified (2:1) and acetylated (4:1) PEI showing nearly identical performance over time (Fig. 2C).

In order to determine the effect of the electrostatic environment on gene delivery efficiency, polyplex delivery efficiency was subsequently tested across three CG scaffold variants, each with a differentially sulfated glycosaminoglycan: hyaluronic acid (HA), chondroitin sulfate (CS), or heparin (HP). A non-sulfated GAG, hyaluronic acid was not expected to have a positive impact on gene delivery efficiency or duration. Chondroitin sulfate (CS), a moderately sulfated GAG, was tested as the conventional CG scaffold formulation reported in the literature.⁴² As a highly-sulfated, negatively charged glycosaminoglycan, we hypothesized the C:HP scaffolds would more strongly interact with the positively charged PEI polyplexes, retaining more of the polyplex within the scaffold and enhancing either the efficiency or duration of gene expression.

Indeed, increasing the degree of sulfation of the CG scaffolds, through incorporating highly sulfated heparin, led to significantly improved gene delivery efficiencies in both unmodified and acetylated PEI polyplexes two days after tenocytes were exposed to the gene activated matrices (Fig. 3). The effects were observed at higher w:w ratios of the range tested for unmodified and acetylated PEI, suggesting at low cationic polymer densities the effect of scaffold GAG content was lost. Notably, significant increases in luciferase expression were noted for tenocytes in the C:HP scaffolds versus both C:HA and C:CS scaffolds for a 2:1 w:w polymer:DNA ratio using unmodified PEI (Fig. 3A). Similar results were observed at 4:1 or 5:1 w:w ratios using acetylated PEI (Fig. 3B). These results suggest that the electrostatic scaffold environment can significantly impact the short term efficiency of gene delivery. Considering recent approaches describing fabrication of CG scaffolds with regional control over composition,⁵⁹ these results also suggest a path for developing biomaterials able to spatially-enhance the efficiency of a ubiquitously added gene delivery vector. However, when examining the impact of scaffold GAG content over the course of 7 days (Fig. 4), it was apparent that scaffold GAG content may not be sufficient to enhance the duration of functional gene delivery. Here, the significant increase in luciferase expression between scaffolds only appears at day 2 (2:1 unmodified, 5:1 acetylated polyplexes), with no significant differences seen between groups at days 5 or 7 (Fig. 4).

The mechanism by which scaffold GAG content impacts PEI delivery remains undetermined. The presence of increasingly anionic GAGs within the CG scaffolds may promote the maintenance of the cationic polyplex in the scaffold, decreasing the rate of diffusive loss. Alternatively, scaffold GAG content may enhance partial dissociation of the DNA from the polymer, potentially allowing for effective transcription within the cells. As unmodified PEI constructs exhibit higher charge,³⁴ they may be sequestered in the charged C:CS and C:HP scaffolds more readily than the acetylated PEI. Further, interactions with the charged GAG, especially in the case of the more weakly associated acetylated PEI polyplexes, may also facilitate the release of the DNA from the polyplex, with acetylated PEI showing highest transfection efficiency in the C:HP scaffolds (Fig. 4). While future efforts will need to examine mechanisms by which the scaffold GAG content impacts polyplex stability or retention, it is clear that the biophysical properties of a tissue engineering scaffold can significantly impact polyplex-based gene delivery. Further, these

results highlight that polyplexes optimized in conventional two-dimensional cultures may show significantly different behavior when translated to biomaterial systems.

Moving forward, it would be important to extend these studies to examine the transfection efficiency on a per-cell basis. Experiments such as those were not performed as part of this study because established methods (e.g., normalizing to protein or DNA content) could not be performed in the presence of the collagen scaffold or the charged PEI polymer, respectively. In the future, the use of a GFP reporter and flow cytometry would be a viable way to obtain the percent of transfected cells present as a function of culture time. Further, while here we examined delivery of a reporter gene, delivery of a therapeutic gene to direct mesenchymal stem cell differentiation towards a tenogenic lineage would be an important step in the development of a functional gene delivery matrix for tendon repair. Recent efforts understanding conserved processes of tendon development and repair offer a number of therapeutic targets for incorporation into a gene activated matrix in the future.^{60–63}

Finally, a well-documented drawback of PEI-based gene delivery systems is the significant potential for PEI-induced cytotoxicity.⁴⁸ At first glance, results from this study suggest that appropriate design of the correct biomaterial platform may offer an orthogonal design parameter for enhancing the activity of a given gene delivery vector. However, here we also explored the potential for the scaffold GAG content to enhance tenocyte long-term metabolic activity as a potential strategy to help cells recover more rapidly after the cytotoxic insult associated the PEI polyplex exposure. Tenocyte metabolic activity was monitored over 2 weeks in the GAG scaffolds variants as they were exposed to different gene delivery vehicles: DNA only control, 2:1 unmodified PEI polyplexes, and 5:1 acetylated PEI polyplexes (Fig. 5). There are significant, but temporary, decreases in the metabolic activity at early time points (4, 7 days) for cells exposed to the DNA polyplexes, corresponding to the active period of time for polyplex transfection. Notably, we observed significantly enhanced recovery of the metabolic health of tenocytes cultures in the heparin-based CG scaffolds by day 14. These results are encouraging as they suggest no long term damage may have been done to the cells as a result of the transfection. Further, these results mimic previous findings showing that C:HP scaffolds can enhance cell activity in metabolically taxing environments.

Conclusions

It has been previously shown that the GAG content of CG scaffolds can impact the transient sequestration of growth factors and subsequent cellular responses. Here we described the effect that sulfated GAG content of CG scaffolds has on gene delivery via cationic polymeric polyplexes. Using the cationic polymer polyethylenimine (PEI) to condense DNA into polyplexes, we were able to also examine the impact of polymer acetylation and polymer:DNA weight-to-weight ratio on delivery of a luciferase reporter gene within a three-dimensional biomaterial. We found that increasing the weight-to-weight ratio and employing the highly sulfated heparin glycosaminoglycan in the scaffold design increased the initial luciferase expression in tenocytes treated for acetylated PEI polyplexes. Notably, heparin-based CG scaffolds also enhanced metabolic activity over 14 days. These results suggest that the electrostatic environment of a gene activated scaffold may prove to be an

important design parameter to consider in order to enhance the efficiency and perhaps to moderate cytotoxic side-effects of cationic polymer based gene therapy for tissue engineering applications.

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Figure 1.

Luciferase expression (pGL3 vector) 48 hours post-transfection from tenocytes cultured in 3D C:CS scaffolds. (*) significant ($p < 0.05$) up-regulation compared to all other unmodified PEI groups. (^) significant ($p < 0.05$) up-regulation compared to control, 1:1, and 2:1 acetylated PEI groups. (#) significant ($p < 0.05$) up-regulation between unmodified and acetylated PEI groups at given polymer:DNA ratio.

Figure 2.

Luciferase expression (pGLuc vector) in tenocytes over two weeks of culture in C:CS scaffolds. Expression of polyplex variant with unmodified PEI (**A**) or acetylated PEI (**B**) over the full range of weight-to-weight ratios. Relative light units (RLUs) are normalized to a 3D control of DNA without a polymer vector. (**C**) Top performing ratios (w:w) for unmodified and acetylated PEI over 14 days. (*) significance ($p < 0.05$) when compared to control. (^) significance ($p < 0.05$) between 1:1 ratio at same day. (†) significance ($p < 0.05$) between groups.

Figure 3.

Day 2 luciferase expression (pGLuc) of a focused range of weight-to-weight ratios for each type of PEI within scaffolds of varying charge. (*) significance ($p < 0.05$) between groups.

Figure 4. Luciferase expression (pGLuc) from the most effective weight-to-weight ratios in scaffolds with variant glycosaminoglycan content over 7 days. (*) significant ($p < 0.05$) increase relative to other timepoints within the same scaffold group.

Figure 5.

Tenocyte metabolic activity as a function of scaffold GAG content as well as polyplex group. (*) significance ($p < 0.05$) over corresponding experimental groups at same timepoint. (^) significance ($p < 0.05$) between earlier timepoints. (†) significance ($p < 0.05$) to day 2 groups.