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The Development of Non-Viral Gene-Activated Matrices for Bone Regeneration Using Polyethyleneimine (PEI) and Collagen-Based Scaffolds

Erica G. Tierney ^a, Garry P. Duffy ^{a, b}, Alan J. Hibbitts ^c, Sally-Ann Cryan ^c,
Fergal J. O'Brien ^{a, b*}

^aDepartment of Anatomy, Royal College of Surgeons in Ireland, Dublin

^bTrinity Centre for Bioengineering, Trinity College, Dublin

^cSchool of Pharmacy, Royal College of Surgeons in Ireland, Dublin

*Corresponding author. Tel.: +353 1 4022149; fax +353 1 4022355.

Email address: fjobrien@rcsi.ie

Abstract

The healing potential of scaffolds for tissue engineering can be enhanced by combining them with genes to produce gene-activated matrices (GAMs) for tissue regeneration. We examined the potential of using polyethyleneimine (PEI) as a vector for transfection of mesenchymal stem cells (MSCs) in monolayer culture and in 3D collagen-based GAMs. PEI-pDNA polyplexes were fabricated at a range of N/P ratios and their optimal transfection parameters (N/P 7 ratio, 2µg dose) and transfection efficiencies ($45 \pm 3\%$) determined in monolayer culture. The polyplexes were then loaded onto collagen, collagen-glycosaminoglycan and collagen-nanohydroxyapatite scaffolds where gene expression was observed up to 21 days with a polyplex dose as low as 2µg. Transient expression profiles indicated that the GAMs act as a polyplex depot system whereby infiltrating cells become transfected over time as they migrate throughout the scaffold. The collagen-nHa GAM exhibited the most prolonged and elevated levels of transgene expression. This research has thus demonstrated that PEI is a highly efficient pDNA transfection agent for both MSC monolayer cultures and in the 3D GAM environment. By combining therapeutic gene therapy with highly engineered scaffolds, it is proposed that these GAMs might have immense capability to promote tissue regeneration.

Keywords

Non-viral gene therapy; polyethyleneimine; vector; mesenchymal stem cell; gene-activated matrix; bone regeneration

Introduction

The ultimate goal of tissue engineering and regenerative medicine is to develop substitutes to help guide the growth of new functional tissue using biological, mechanical and structural cues. Scaffolds developed from biomaterials act as templates for tissue formation where the interaction between the scaffold, infiltrating cells and the aforementioned cues is intrinsic to its success. Biomaterial scaffolds for tissue engineering applications have primarily been modified to contain growth factors in the form of recombinant proteins to boost the therapeutic response [1-5]. However, using proteins in this environment is associated with a number of distinct disadvantages such as the large dose required, the need for repeated applications, poor distribution, expense, short half-life and protein instability [6]. Essentially, the main downfall is in attempting to achieve a long-term effect using proteins with a short half-life [7]. Gene therapy can be a valuable tool to avoid such limitations by providing gene expression and protein production over an extended time frame to ultimately ensure an enhanced therapeutic response. The idea of a gene delivery vector contained within a biodegradable scaffold, although not new, is a recent development in the field of regenerative

medicine and the system has been termed a ‘gene activated matrix’ (GAM). The scaffold essentially acts as a depot for the gene while simultaneously offering structural support and a matrix for new tissue deposition.

The lack of a widely accepted gene vector coupled with difficulty in transfecting primary cells such as stem cells is a significant hurdle in the advancement of gene therapy [8]. An ideal vector must possess a number of key qualities including efficient transfection with minimal associated cytotoxicity and safety concerns. In addition, it should also allow for cell or spatial specificity and have a controllable timeframe of expression [6, 9-11]. One of the first and most successful non-viral gene vectors to date [6], polyethyleneimine (PEI) was first introduced as a non-viral vector both *in vitro* and *in vivo* in a landmark paper by Boussif et al. in 1995 [12]. As a non-viral vector, PEI exhibits a number of key advantages over viral vectors in that it is less immunogenic, less cytotoxic, there are no carcinogenic concerns, it is safe for clinical use and it induces transient gene expression [6]. Moreover, the vector is associated with a relatively high transfer efficiency [13-15] due to a phenomenon known as the ‘proton sponge effect’ [16] and is regarded as providing transfection efficiencies comparable with viral gene delivery agents [17]. Across the literature the branched 25kDa PEI polymer is favoured as a gene transfer agent [18-20] and as a common positive control often used as a gold standard in *in vitro* studies [21]. Concisely, PEI-pDNA polyplex properties can be changed by simply altering the N/P ratio (PEI amines/DNA phosphates). Higher ratios of PEI to pDNA usually result in for higher transfection efficiencies but using these higher ratios is associated with proportional increases in cytotoxicity [12, 22]. In order to optimise PEI for MSC gene transfer, a balance must be reached between these attributes.

Mesenchymal stem cells (MSCs) are non-haematopoietic stromal cells which can differentiate into or become involved in the regeneration of mesenchymal tissues such as bone [23]. Their use in therapeutic applications is particularly appealing due to their rapid proliferation, ease of isolation, ability to self-renew and more imperatively for their inherent ability to differentiate into desired cell types given the right cues [23, 24]. MSCs have specific scope in the field of bone tissue engineering where they are well known and employed for their role in fracture repair. MSCs are the subject of countless cell-based repair strategies either as a standalone treatment or in conjunction with scaffolds for bone regeneration [25]. PEI has been successfully used in a variety of adherent cell lines such as HeLa, HepG2 and NIH3T3 [26, 27], and a marrow derived stem cell line [28]. Furthermore, Saraf et al. have reported transfection efficiencies of just 12 % using branched PEI to transfect human MSCs [29]. Similarly, PEI has also been used to transfect adipose derived stem cells [26] and has even been the basis of an *in vivo* gene activated matrix (GAM) for bone repair [20]. It is for this reason that it was sought to determine whether PEI could provide the same transfection efficiency for pDNA in rMSCs as the targeted *in vivo* cell type and to also establish if PEI polyplexes could be successfully merged with our scaffolds which are specifically engineered for bone regeneration.

Seminal work by Bonadio et al. [9] saw the development of the first GAM, whereby gene therapy was combined with scaffolds for enhanced tissue regeneration. However, the development of such materials is still in its infancy in the field. Gene transfer from a GAM guides cells towards a selected lineage without the need for repeat administration as the *in vivo* response induced by sustained transgene expression over time governs effective healing [30]. Moreover, when the complexes are distributed throughout the scaffold rather than solely applying gene therapy to the defect, the scaffold also confers a level of protection to the complexes. Clearance of the complexes is thereby delayed from the site of injury and they are

shielded against physiological degradation which results in enhanced complex efficacy, increased availability to cells and more critically, maintains effective long term concentrations of the transgene [31].

Novel lyophilisation techniques developed in our laboratory have enabled the fabrication of a series of collagen-based scaffolds with pore size, porosity and mechanical properties tailored specifically for bone tissue regeneration [32-38]. The composition of these scaffolds has been adapted to contain various constituents such as, for example, glycosaminoglycans (GAG) which effectively increases cellular attachment, migration and infiltration throughout the matrix. Bone is comprised of approximately 70% nano-hydroxyapatite (nHa) which makes nHa another logical inclusion in a scaffold designed for bone tissue engineering. The beneficial effects of nHa incorporation include improved osteointegration, increased mechanical properties and enhanced cell function [39-41]. In addition, the excellent capacity of these scaffolds to repair and regenerate bone defects has been exhibited in a number of *in vivo* models [41-43]. However, in order to heal very large defects such as those which typically require bone grafting in humans, an extra agent, i.e. a therapeutic protein or gene therapy technology, is a warranted addition to these scaffolds. By applying gene therapy to scaffolds which already possess proven healing potential we hypothesise that the amalgamation of gene therapy with these scaffolds could result in a matrix with immense capability for healing. It has been shown that although GAGs increase biocompatibility they may have a negative or inhibitory effect on the transfection process [44, 45] and that an osteoinductive biomineral phase such as nHa can also augment gene transfection. Therefore, we sought to examine which of three collagen-based scaffolds - collagen, collagen-GAG or collagen-nHa, provides the most suitable platform for gene transfer [46, 47]. The aim of this work was to thus merge gene therapy with these collagen-based scaffolds to produce a series of GAMs. Specifically, this involved (i) the development of the PEI polyplexes as a gene transfer tool in rMSCs, (ii) incorporating the optimised polyplexes into the scaffolds and (iii) determining whether various gene expression profiles could be attained by altering the baseline scaffold composition.

Materials and Methods

Stem cell isolation and expansion

MSCs were isolated from 8 week old female Fischer 344 rats [37] with the approval of the Research Ethics Committee of the Royal College of Surgeons in Ireland as outlined in application (REC 237). After euthanasia, marrow was flushed from the tibiae and femora with phosphate buffered saline (PBS) (Sigma-Aldrich, Ireland) and a single cell suspension was recovered. After centrifugation (600x g, 10 minutes) cells were plated at 120×10^6 cells/cm², in complete rat MSC growth medium [10 % foetal bovine serum (FBS; Hyclone), 45 % F12-Ham and 45 % α -MEM (Biosciences, Ireland) supplemented with antibiotics (100 U/ml penicillin G and 100 μ g/ml streptomycin sulphate; Gibco)]. Flasks were incubated at 37°C in 5 % CO₂/90 % humidity. After 8 days colonies became compact and cells were detached with 0.25 % trypsin/EDTA and re-plated at 2000 cells/cm². Subsequently, cultures were passaged at 5-7 day intervals and expanded to passage 5 for experimentation. The MSCs were cultured in Dulbecco's Modified Eagles Medium (Sigma-Aldrich, Ireland) supplemented with 2 % penicillin/streptomycin (Sigma-Aldrich, Ireland), 0.6 % L-glutamine (Sigma-Aldrich, Ireland), 10 % FBS (BioSera, East Sussex, UK), 0.6 % glutamax (Biosciences, Ireland) and 1.2 % non essential amino acids (Biosciences, Ireland).

Optimisation of rMSC monolayer transfection using PEI-pDNA polyplexes

Branched 25kDa PEI (Sigma-Aldrich, Ireland) was condensed with pDNA, (Green Fluorescent Protein plasmid DNA, (pGFP), Amaxa, Lonza, Cologne AG, Germany) or luciferase (luc) (pGaussia-Luciferase, New England Biolabs, Massachusetts, USA). These two reporter genes are routinely used in gene therapy to visualise (GFP) and quantify transgene expression (luc). Cells were cultured until 90% confluency and seeded at a density of 5×10^4 cells per well in adherent 6 well plates 24 hours prior to transfection. Media was removed an hour before transfection and the monolayer of cells was washed with 1.5ml of PBS per well. 1.5 ml of OptiMEM serum-free media (Gibco, Ireland) was then added to each well and incubated at 37°C for one hour. PEI-pDNA complexes were produced in OptiMEM at varying N/P ratios (5, 7, 10) and doses (1µg, 2µg, 4µg) of pDNA and incubated at room temperature for 30 minutes. The N/P ratio can be defined as moles of amines in PEI (N)/moles of phosphate in pDNA (P) and the term dose refers to the quantity of pDNA contained within the polyplex. Furthermore, polyplexes of increasing dose therefore contain increased levels of PEI to preserve a consistent N/P ratio. The OptiMEM was removed from the wells and the polyplex solutions was added to the monolayer in 500 µl suspensions and incubated at 37°C for 15 minutes. After 15 minutes a further 1ml of OptiMEM was added and the cells were incubated for approximately 4 hours. Following this transfection period the transfection medium was removed and a PBS wash was performed. Complete culture media was then added and the cells were incubated at 37°C to allow expression of the transgene. Imaging and flow cytometry analysis were used to illustrate PEI-pGFP transfection and a LumiFlex GLuc assay (New England Biolabs, Isis Ltd, Ireland) was used to estimate luciferase expression.

Flow cytometry analysis to assess transfection efficiency

To demonstrate transfection efficiency, flow cytometry was performed on PEI-pGFP transfected cells. Cells were transfected with PEI-pGFP polyplexes and cultured for 72hrs. Following this incubation period the media was aspirated from the wells and they were rinsed with PBS. 500µl of PBS was added to each well and cells were lifted using cell scrapers (Sarstedt, Ireland). Briefly, cells were formalin (5%) fixed for 20 minutes and centrifuged at 500g for 5 minutes. The pellet was resuspended in 500µl of PBS and passed through a FACS filter (Partec UK) into a FACS cartridge (Partec UK). The suspension was then analysed for GFP fluorescence and a percentage of fluorescent over non fluorescent cells gauged the transfection efficiency of the complex using FACS Canto 11, DIVA software.

Luciferase assay to analyse transgene expression in monolayer and 3D culture

A LumiFlex GLuc assay (New England Biolabs, Isis Ltd, Ireland) was used to quantify the expression of luciferase from MSCs in monolayer and 3D culture. The GLuc assay solution was prepared by adding 50 µl of the 100x substrate into 800 µl of the GLuc assay buffer. This solution was vortexed to mix, and stored in a tightly sealed, light protected container. Aliquots (20 µl) of culture media were plated in triplicate in a 96 well plate and 50µl of GLuc assay solution was subsequently added. Luciferase expression was determined using luminescence measurements (Wallac Victor2™ 1420 multilabel counter, Perkin Elmer Life Sciences, Finland).

Cell viability

An evaluation of cell viability 24hr and 72hr post transfection using a range of polyplex N/P ratios and pDNA doses was performed with an MTT Cell Growth Assay (Millipore™, Ireland). Briefly, 10µl of MTT reagent was added to the cells in 90µl of media and incubated for an additional 4hr at 37°C. The supernatant was then removed and 50µl of DMSO was

added to dissolve the formazan crystals formed as a result of MTT cleavage by viable cells. Absorbance was measured at 570nm with a reference wavelength of 630nm with non-transfected cells serving as a 100% viability control. Cell viability (%) was estimated according to the following equation: $\text{abs}_{[\text{transfected}]} / \text{abs}_{[\text{control}]} \times 100$.

Scaffold fabrication

To produce highly porous homogeneous scaffolds, collagen, collagen GAG and collagen nHa scaffolds were fabricated by a lyophilisation technique using a procedure developed by O'Brien et al. [32]. Collagen slurries were made by adding 3.6g of collagen (Integra Life Sciences) to 720ml of 0.05M glacial acetic acid (HOAc) and blending at 15,000rpm for 90 minutes using an overhead blender maintained at 4°C. (Ultra Turrax T18 Overhead Blended, IKA Works Inc.). For collagen GAG scaffolds, 3.6g of collagen was added to 600ml of HOAc and 0.32g of chondroitin-6-sulfate was added to 120ml of HOAc. The chondroitin-6-sulfate solution was subsequently added dropwise to the collagen solution using a peristaltic pump. The collagen nanohydroxyapatite (nHa) scaffold was fabricated by the addition 3.6g nHa to the standard collagen scaffold. nHa was synthesised using a novel dispersant-aided precipitation technique [48] which was incorporated into collagen slurries using a suspension method [40]. Scaffolds were cut to 9.5mm diameter using circular biopsy punches post-lyophilisation. All scaffolds were dehydrothermally crosslinked using and subsequently chemically crosslinked with a mixture of 14 mM N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) (Sigma Aldrich, Ireland) and 5.5 mM N-Hydroxysuccinimide (NHS) (Sigma Aldrich, Ireland) [38] to both sterilize and structurally reinforce the scaffold.

GAM development

Scaffolds were manufactured as above and cut to 9.5mm diameter. PEI-pDNA polyplexes were prepared at a ratio of N/P 10 as detailed above using 2µg-20µg of pDNA in 50µl solutions. 25 µl of the polyplex solution was added per side of the scaffold by direct loading, incubating for 15 minutes at room temperature after loading. Subsequently, 2.5×10^5 MSCs were seeded per side of the scaffold in a 25µl suspension with 15 minutes incubation between seeding each side. 2ml of OptiMEM was then added and the plates were incubated at 37°C for four hours after which the media was removed and replaced with 2ml of normal rMSC media. Media was changed every 3 days and spent media was stored at -80°C for analysis. 3D transfections were performed with pGFP and luc plasmids whereby imaging indicated the success of PEI-pGFP transfection a luciferase assay was employed to demonstrate PEI-pluc transgene expression. Scaffolds were imaged at intervals using a Leica DMIL microscope (Leica Microsystems, Switzerland).

Statistical analysis

Results are expressed as mean $\pm$ standard deviation. Statistical significance in Figure 1 was assessed using One-way ANOVA followed by Tukey post-hoc analysis. In the case of Figures 2d-e, 3 and 4c, Two-way ANOVA analysis was carried out followed by Tukey post-hoc analysis. The sample size was n=3 and $P < 0.05$ values were considered statistically significant.

Results

Figure 1 PEI-pDNA polyplex transfection in rMSCs. Fluorescent images depicting GFP expression in rMSCs in monolayer culture 3 days post transfection using 2 μ g doses of N/P ratio 5 (a), 7 (b) and 10 (c) PEI-pGFP polyplexes. Flow cytometry was performed on the MSCs 3 days post transfection with PEI-pGFP polyplexes to determine transfection efficiency (d). Luciferase gene expression was observed over time in rMSCs transfected with N/P 7 PEI-pluc polyplexes in monolayer culture (e). Data plotted shows mean standard deviation (n=3) and $p>0.05$.

Optimisation of transfection of rMSCs in monolayer culture using PEI-pDNA

To optimise gene transfer in monolayer culture, rMSCs were transfected with polyplexes with a range of PEI-pDNA ratios using PEI-pGFP and PEI-pluc plasmids. Since the use of N/P 1 was discontinued, N/P 5 was co-analysed alongside N/P 7 and 10 to determine transfection efficiency across a range of viable polyplex ratios. rMSCs were transfected in monolayer culture with 2 μ g PEI-pGFP polyplexes and gene expression was imaged 3 days post transfection (**Figure 1a-c**). The fluorescent green colour of the cultures is indicative of successful transfection with the level of GFP expression dependent on the N/P ratio used. This was further confirmed using flow cytometry to determine the % transfection efficiency of each polyplex in the rMSCs as displayed in **Figure 1d**. Flow cytometry analysis revealed N/P 10 polyplexes result in 45 ± 2.99 % of cells being transfected, N/P 7 results in 30 ± 8.42 % whereas only 7 ± 1.21 % of wells are transfected when N/P 5 is used (**Figure 1d**). Despite the apparent increase in transfection using N/P 10 polyplexes no statistically significant difference existed between the transfection efficiencies of N/P 7 and N/P 10 polyplexes. Consequently, a charge ratio of 7 was utilized to transfect rMSCs in all subsequent monolayer experiments. In order to quantitatively determine this gene expression over time, rMSCs were transfected in monolayer culture with N/P 7 PEI-pluc and luciferase expression was analysed over a 9 day period by assaying spent media from the cultures (**Figure 1e**). Luciferase expression was shown to peak at day 6 at approximately 5×10^5 Relative

Luciferase Units (RLU) suggesting that the PEI transfection technique is a transient gene expression system.

Figure 2a-c

Figure 2d

Figure 2e

Figure 2 Viability of rMSCs transfected with PEI-pDNA. Phase images of PEI-pDNA transfected rMSCs at N/P ratio 5 (a), N/P ratio 7 (b) and N/P ratio 10 (c). The cell viability of rMSCs 3 days post transfection with various polyplex N/P ratios (d) and the effect of various N/P 7 pDNA polyplex doses on rMSCs (e). Data plotted shows mean standard deviation (n=3) and $p < 0.05$.

Effect of PEI-pDNA polyplex transfection on rMSC viability

The viability of cells post transfection is paramount for any pDNA/vector system. An MTT assay was utilised as a viability assay by measuring the metabolic activity of transfected cells versus a non-transfected control. The effect of different PEI-pGFP polyplex ratios on rMSC viability is displayed in **Figure 2**. N/P 5 polyplexes appear to have little effect on cell viability as demonstrated in **Figure 2a**. However, this is in tandem with an inability to induce high levels of transfection (**Figure 1**) so N/P 5 polyplexes were eliminated from subsequent experiments. In **Figure 2a-c** the N/P ratio 10 group does not appear as confluent as the other two groups which may indicate a decline in cell number either through a lack of proliferation or cytotoxicity. This visual decrease in cell number was confirmed with using an MTT assay (**Figure 2d**). In the supplementary information Figures I and II, it was conveyed that low N/P ratios are unsuitable for transfection due to poor physical characteristics. Similarly, the use of relatively high N/P ratios are not recommended as they have the potential to cause cytotoxicity. In order to establish a definitive negative effect of N/P ratio on viability, N/P 15 polyplexes were co-analysed alongside N/P 5, N/P 7 and 10 polyplexes. rMSCs were

transfected with 2 μ g doses of PEI-pDNA at various N/P ratios and the MTT assay was performed 24 and 72 hours post transfection. Interestingly, PEI alone has a significantly toxic effect on cells 72hrs post transfection but it is expected that uncomplexed free PEI can have this effect on cells. There is a significant decrease in cell viability proportional to increasing N/P ratio used. 70 % of N/P 15 transfected cells remained viable 24hr post transfection and only 36% of cells after 72hr of culture. There was also a marked decline in viability in the N/P 10 treated group but this was only seen after 72hr of culture. Taken together, the combination of physical characteristics (**Supplementary information**), transfection efficiency (**Figure 1**) and cell viability analysis (**Figure 2**), this data demonstrates that N/P 7 is the optimal N/P ratio for rMSC transfection in monolayer culture. N/P 7 facilitates efficient gene expression while minimizing cytotoxicity. N/P 10 polyplexes do induce more transfection but this also slightly compromises cell viability. However, in applications whereby higher transfection efficiencies are preferential, N/P 10 is still a suitable vector for consideration as there was less than a 35% reduction in cell viability.

In order to ascertain the correct pDNA dose within a polyplex to apply to rMSCs in monolayer culture an MTT assay was performed on rMSCs transfected with N/P 7 PEI polyplexes containing varying doses of pDNA and analysed 24 and 72hr post transfection (**Figure2**). A sharp decline in cell number was witnessed in the 4 μ g group after 24hr which further declined to approximately 22% remaining viable relative to the cells alone control after 72hr. With little effect on cell viability in applying 1 μ g or 2 μ g polyplex doses to the rMSCs and previous experiments demonstrating that 2 μ g polyplexes resulted in enhanced transfection in comparison to 1 μ g polyplexes (Data not shown), 2 μ g is the chosen polyplex dose to apply to rMSCs in monolayer culture.

Figure 3

Figure 3 Dose optimisation for PEI-pDNA (N/P10) polyplex incorporation into collagen-based scaffolds to produce GAMs. Relative luciferase expression from collagen-GAG scaffolds containing various doses of PEI-pluc polyplexes was monitored over 7 days. Data plotted shows mean standard deviation (n=3).

Incorporation of PEI-pDNA polyplexes into collagen-GAG scaffolds

pDNA dose and N/P ratio were both found to have a significant effect on the transfection efficiency and viability of monolayer rMSCs therefore the initial step in the fabrication of a GAM was to investigate the appropriate polyplex N/P ratio and dose for the 3D environment (**Figure 3**). Initially, N/P 7 polyplexes were used but it was decided to revert to N/P 10

polyplexes as they exhibited significantly higher levels of transfection in the GAM (Data not shown). It is thought that excess PEI is required to necessitate transfection in the 3D environment. Using an N/P 10 2 μ g polyplex dose, a gene-activated matrix was fabricated using PEI, pGFP and collagen-based scaffolds. A luciferase assay of media samples demonstrating the luciferase protein expression from the GAMs containing N/P 10 PEI-pluc polyplexes over 7 days was performed. **Figure 3** shows that there was no significant difference in protein expression between any of the doses used which implied that the 2 μ g dose optimised for monolayer use could also be used in the GAM. During this 7 day time course, gene expression did not yet decline, irrespective of the polyplex dose applied to the cells. This is in contrast to monolayer transfections (**Figure 1e**) where it transpired that transfection peaks at day 6 with a loss of gene expression thereafter. It is speculated that the cells are being continuously transfected over time as they become exposed to the polyplexes upon migration throughout the scaffold. Despite the variability of the data, there appears to be a trend of increasing protein expression over 7 days which is desirable for tissue engineering applications.

Figure 4a-b

Figure 4c

Figure 4 Effect of scaffold composition on gene expression. Images depict rMSC transfection in PEI-pGFP gene-activated collagen-GAG matrices soak loaded with 2 μ g N/P 10 PEI-pGFP polyplexes 3 days (a) and 21 days (b) post cell seeding. Relative luciferase expression in collagen alone, collagen-GAG and collagen-nHa gene activated matrices was determined by assaying media samples over time (c). Data plotted shows mean standard deviation (n=3) and $p < 0.05$.

Comparative analysis of a series of gene-activated matrices by incorporating PEI-pDNA with collagen, collagen-GAG and collagen-nHa scaffolds.

Collagen, collagen-GAG and collagen-nHa scaffolds exhibited successful transfection with continuous transfection and gene expression up to 21 days *in vitro* using N/P 10 2 μ g doses. Collagen-GAG GAMs were produced using 2 μ g PEI-pGFP polyplexes loaded onto collagen-GAG scaffolds and subsequently seeded with rMSCs (**Figure 4a-b**). These matrices facilitated gene expression up to 21 days as depicted. Importantly, this expression has begun to notably decline at this 21 day timepoint but since these GAMs are designed for tissue

regeneration applications whereby transient expression is required only for the initiation of tissue formation this is a welcome decline in gene expression. In addition, when the inclusion of nHa and GAG in the scaffold were investigated and compared to the collagen only scaffold, the composition of the baseline scaffold was seen to affect the performance of a GAM. Luciferase expression was comparatively analysed over 14 days in each GAM (**Figure 4c**) to ascertain the length of gene expression. It was shown that the composition of the scaffold can indeed have an effect on transfection efficiency or gene expression within the matrix. Collagen and collagen-GAG based matrices provided similar expression profiles throughout the time frame implying that the addition of GAG did not have any effect on gene expression. In contrast, the collagen-nHa scaffold facilitated increased gene expression which did not peak until day 7 and had significantly higher expression at Day 10 and Day 14 in comparison to both collagen and collagen-GAG scaffolds.

Discussion

In order to heal large defects, biomaterial scaffolds for tissue engineering applications have primarily been modified to contain growth factors in the form of recombinant proteins for greater therapeutic potential [1-5]. Recently, due to the limitations associated with protein delivery [6, 49], gene therapy has alternatively been applied to provide sustained protein production by transfected cells over an extended time frame to ultimately render an enhanced therapeutic response. In this study, we developed and evaluated a gene activated matrix using PEI polyplexes optimized within for MSC transfection. MSCs were the target cell type for their exciting ability to differentiate into mesenchymal lineages when provided with the right cues, i.e. rMSCs can be instructed to differentiate by inducing the expression of certain genes. Since there exists no ideal vector for MSC gene transfer, a gene delivery system using PEI was successfully optimised for MSC transfection in monolayer culture and then incorporated into three collagen-based scaffolds designed for bone regeneration.

A major obstacle in stem cell transfection, gene therapy and the development of gene-activated matrices for regenerative medicine is the lack of an ideal vector for non-viral transfection. Although the capability of PEI as a non-viral vector has been previously explored we sought to optimise charge ratio and dose specifically for MSCs to aid in the subsequent development of a GAM for bone repair. Several groups [13, 15, 21] have previously demonstrated the high transfection efficiency of PEI in various cell types and cell lines. Although PEI has been reported as an efficient transfection tool in human adipose derived stem cells [25], human bone marrow MSCs [50] and a bone marrow derived stem cell line [27], collectively very little work has been reported using PEI as an efficient MSC transfection agent, despite its widespread availability as a vector. The majority of research conducted using gene delivery technology is carried out using readily available cells lines. By optimising the polyplexes specifically for MSCs this research has greater clinical relevance. The suitability of PEI mediated transfection of rMSCs has been investigated and verified here, both in monolayer culture and in the 3D environment.

As predicted, the N/P ratio of the polyplex affects the transfection efficiency in rMSCs as portrayed in **Figure 1a-c**. These results also imply that the PEI polyplexes can be tailored to give the desired transfection efficiency for various applications by simply altering the N/P ratio. Several groups [13, 15, 21] have discussed the high transfection efficiency of PEI with other cell types which has now been verified here with a transfection efficiency in rMSCs of up to $45 \pm 2.99\%$. Viral vectors are often applied in place of non-viral vectors due to their ability to achieve higher transfection efficiencies. However, most viral vectors differ to non-viral vectors in that they usually cause stable transgene expression by integrating with the

host genome. This is detrimental to the tissue engineering application as gene expression is only desired for a therapeutic window after which a decline in gene expression is essential. Hamm et al.[8] reported a nucleofection transfection efficiency in rMSCs of 28.9-45.3% using nucleofection, a gene transfer technique regarded as one of the most efficient of the physical and non-viral transfection methods. Since nucleofection is restricted to *ex-vivo* use, the equally high transfection efficiencies realised using PEI holds great promise for stem cell transfection *in vivo*. PEI-pluc transgene expression peaked at Day 6 with a subsequent downturn in expression indicating that the vector induces transient gene expression which is desirable for tissue regeneration applications.

Recent work has begun to utilize gene therapy in combination with scaffolds for tissue regeneration [9, 20, 51, 52]. In the original GAM developed by Bonadio et al. [9] it was necessary to use plasmid DNA in the order of milligrams to elicit a therapeutic effect. Huang et al. [19, 27] found it necessary to use a 200 µg polyplex dose per scaffold. Endo et al. [51] improved upon these doses by developing a GAM which achieved increased bone healing using 12µg calcium phosphate BMP-2 polyplexes. However, the current study has developed a GAM which requires a lower dose than that used in these other studies. **Figure 3** shows no significant difference in transgene expression in GAMs containing either 2µg, 5µg, 10µg or 20µg polyplex doses and gene expression has been witnessed up to 21 days within our GAMs across a range of polyplex doses. These data thus suggest that this system has the potential to provide a therapeutic response using a dose as low as just 2µg of polyplex within the scaffold. The polyplexes do not have a significant effect on cell viability in monolayer culture when applied using N/P ratios of 7 or lower and with doses below 4µg. Cell viability is slightly compromised with the N/P 10 ratio 72hr post transfection but still holds a respectable level of cell viability of above 65%. Since the GAMs were developed using these ratios and doses there should be no cytotoxic concerns in 3D as incorporation into a 3D matrix increases the surface area available to the cells and therefore decreases cell contact with the polyplexes. N/P 7 was found to be the optimal charge ratio to use in monolayer culture but it was necessary to use an N/P 10 ratio within the 3D scaffold. PEI has been shown to be fully condensed at N/P 2 [27], we can therefore deduce that excess PEI must be binding to or being sequestered by the scaffold, making it necessary to use higher ratios to attain transfection. The subtle difference between the monolayer and 3D environment is unsurprising; transfection in a 3D environment is a very different scenario for transfecting adherent cells. When polyplexes are applied to a monolayer of cells, the decreased surface area means that all cells present will be exposed to the polyplexes. Conversely, when the larger surface area and intricacies of the interconnected scaffold pore structure are taken into account, we propose that transfection is very much reliant on cellular migration throughout the GAM. This theory is based on previous work within the group which showed cellular migration throughout the scaffold [33, 53].

Although PEI-pluc transfections in monolayer verified that PEI-pDNA is a transient expression system in rMSCs, transgene expression was seen in PEI GAMs at 21 days post cell seeding (**Figure 4a-b**). This supports the theory that the GAM may act as a polyplex depot system whereby infiltrating and migrating cells become transfected as they come in contact with the GAM. Our data corroborates with findings by Huang et al. [27] where they showed that 3D transfection on a PLGA sponge lasts for a longer duration even though higher levels of transgene expression were seen in adherent cells in monolayer.

There was no difference in collagen and collagen-GAG expression profiles. However, the collagen nHa GAM was found to be the best performing scaffold considering the more

significant sustained release profile it exhibits over a 14 day period as shown in **Figure 4c**. From a bone repair perspective, the addition of a nHa phase to the matrix serves to theoretically increase the osteogenic potential of the scaffold. Hydroxyapatite is an osteoconductive material that encourages osteoblast differentiation [54] and when hydroxyapatite is combined with collagen in a tissue engineering scaffold, it leads to increased matrix deposition by seeded cells [55]. Its use in scaffolds for bone tissue engineering therefore has obvious advantages, especially if further enhanced by combining nHa scaffolds with gene therapy. Using biominerals in biomaterials has been previously shown by other research groups as another way to induce more transgene expression within a matrix. Enhanced osteogenesis observed by implementing a biomineral phase has been documented previously [36, 40, 52] and the ability of a collagen-calcium phosphate scaffold to act as its own vector for transgene expression has also been shown [52]. The presence of a biomineralised or calcium phosphate phase can also augment gene transfer [47, 52] so perhaps the presence of nHa in this PEI-GAM is testament to that effect. Within this work, the presence of the nHa mineral phase in the scaffolds correlated with a more sustained expression of the transgene, implying that transgene expression and the release profile of the transgene can be customised by changing the composition of the collagen-based scaffold.

In the race to provide increased gene delivery using non-viral vectors in GAMs, research has begun to focus on incorporating minerals in matrices to not only boost mechanical strength and cellular responses but also to augment transfection efficiency. Kong et al. reported that stiffer substrates have a direct impact on gene expression whereby cells cultured on stiffer biomaterials yielded enhanced gene expression [56]. Furthermore, Shen et al. have shown that the extent of gene transfer in a matrix can even be an adjustable system simply by altering the mineral composition of the material [57]. Polyplexes may be attached to a nHa scaffold in one of two possible ways, either by adsorption to the collagen or to the nHa mineral itself. If the cells preferentially attach to the nHa rather than to the collagen it could explain why cells exhibit higher transfection levels in the nHa GAMs. Cells directly interact with their surrounding matrix via focal adhesions [33, 47, 56] and cells respond to mechanical cues such as substrate stiffness via these focal adhesions. Leonova et al. showed that the mineralisation of substrates stimulates focal adhesion and motility of MSCs [58]. It is suspected that the addition of nHa is resulting in the adherence of more cells than in the collagen alone and collagen-GAG scaffolds due to increased scope for focal adhesion because of the presence of nHa. In summary, the sustained and elevated levels of gene expression seen in the collagen-nHa scaffolds is likely attributable to increased cellular attachment, proliferation and motility throughout the GAM meaning that the cells are exposed to more PEI polyplexes than their mineral-free counterparts which results in more transfection.

This study has made a considerable contribution to the field of gene therapy and tissue engineering. Much progress has been reported in the application of non-viral vectors to 3D scaffolds [10, 51, 59, 60] but by specifically optimising a vector for the target cell type and using the refined polyplex doses and compositions in a 3D scaffold, previously optimised for tissue repair, this offers a superior bioactive scaffold for tissue engineering applications. Engineering a GAM using merely 2µg polyplex doses to effectively cause considerable levels of transfection in 3D is beneficial in terms of reducing the amount of product required which decreases cost and lessens the presence of exogenous materials in a defect site. Furthermore, while the system developed in this study might have therapeutic applications in a number of areas, from a bone repair perspective, the performance of the nHa GAM is perhaps one of the most interesting findings. The combination of an efficient gene transfer system residing in a

GAM which contains nHa, a material which has dual benefits in that it boosts both gene transfer and osteoinductivity, is a considerable advancement in the field. Ultimately, the application of this GAM might involve replacing the reporter genes with therapeutic genes for bone repair, perhaps most obviously BMP2, to produce efficient, sustained protein expression within a defect without the side effects of the current recombinant protein-based technologies.

Conclusion

The non-viral vector PEI has been optimised within this research to attain a transfection efficiency of up to 45% with MSCs, a cell type typically regarded as difficult to transfect. Upon replacing the reporter genes used within with therapeutic genes, this gene transfer system has enormous scope in genetically directing MSCs towards a particular lineage for regeneration. PEI polyplex GAMs developed using merely 2µg of pDNA per scaffold were capable of producing a sustained release profile of gene expression up to 21 days when seeded with rMSCs. In addition, the nHa mineral phase had a significant effect on the GAMs expression profile by contributing to prolonged elevated levels of gene expression over 14 days. This suggests that this PEI nHa GAM may have significant potential for bone regeneration applications. By converging gene therapy (using therapeutic genes) with scaffolds with a proven track record *in vitro* and *in vivo*, it is proposed that the resultant GAMs might have immense capability to promote tissue regeneration and holds great promise for the field of tissue engineering.

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References

- [1] B. De la Riva, E. Sánchez, A. Hernández, R. Reyes, F. Tamimi, E. López-Cabarcos, A. Delgado, C. Évora, Local controlled release of VEGF and PDGF from a combined brushite-chitosan system enhances bone regeneration, *Journal of Controlled Release*, (2010) 143–45-52.
- [2] Z.S. Patel, S. Young, Y. Tabata, J.A. Jansen, M.E.K. Wong, A.G. Mikos, Dual delivery of an angiogenic and an osteogenic growth factor for bone regeneration in a critical size defect model, *Bone*, 43 (2008) 931-940.
- [3] F.-M. Chen, M. Zhang, Z.-F. Wu, Toward delivery of multiple growth factors in tissue engineering, *Biomaterials*, 31 (2010) 6279-6308.
- [4] C.A. Simmons, E. Alsberg, S. Hsiong, W.J. Kim, D.J. Mooney, Dual growth factor delivery and controlled scaffold degradation enhance *in vivo* bone formation by transplanted bone marrow stromal cells, *Bone*, 35 (2004) 562-569.
- [5] R. Visser, P.M. Arrabal, J. Becerra, U. Rinas, M. Cifuentes, The effect of an rhBMP-2 absorbable collagen sponge-targeted system on bone formation *in vivo*, *Biomaterials*, 30 (2009) 2032-2037.
- [6] D.W. Pack, A.S. Hoffman, S. Pun, P.S. Stayton, Design and development of polymers for gene delivery, *Nat Rev Drug Discov*, 4 (2005) 581-593.
- [7] S. Orsi, A. De Capua, D. Guarnieri, D. Marasco, P.A. Netti, Cell recruitment and transfection in gene activated collagen matrix, *Biomaterials*, 31 (2010) 570-576.
- [8] A. Hamm, N. Krott, I. Breibach, R. Blindt, A.K. Bosserhoff, Efficient transfection method for primary cells, *Tissue Engineering*, 8 (2002) 235-245.
- [9] J. Bonadio, E. Smiley, P. Patil, S. Goldstein, Localized, direct plasmid gene delivery *in vivo*: prolonged therapy results in reproducible tissue regeneration, *Nat Med*, 5 (1999) 753-759.
- [10] C. Holladay, M. Keeney, U. Greiser, M. Murphy, T. O'Brien, A. Pandit, A matrix reservoir for improved control of non-viral gene delivery, *Journal of Controlled Release*, 136 (2009) 220-225.
- [11] H. Hosseinkhani, T. Azzam, H. Kobayashi, Y. Hiraoka, H. Shimokawa, A.J. Domb, Y. Tabata, Combination of 3D tissue engineered scaffold and non-viral gene carrier enhance *in vitro* DNA expression of mesenchymal stem cells, *Biomaterials*, 27 (2006) 4269-4278.
- [12] O. Boussif, F. Lezoual'ch, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine, *PNAS*, 92 (1995) 7297-7301.
- [13] A. Akinc, M. Thomas, A. Klibanov, M. , R. Langer, Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis, *The Journal of Gene Medicine*, 7 (2005) 657-663.
- [14] J. Schäfer, S. Höbel, U. Bakowsky, A. Aigner, Liposome-polyethylenimine complexes for enhanced DNA and siRNA delivery, *Biomaterials*, 31 (2010) 6892-6900.
- [15] R. Deng, Y. Yue, F. Jin, Y. Chen, H.-F. Kung, M.C.M. Lin, C. Wu, Revisit the complexation of PEI and DNA -- How to make low cytotoxic and highly efficient PEI gene transfection non-viral vectors with a controllable chain length and structure?, *Journal of Controlled Release*, 140 (2009) 40-46.
- [16] J.P. Behr, The proton sponge: a trick to enter cells the viruses did not exploit, *Chimia*, 51 (1997) 34-36.

- [17] B. Abdallah, A. Hassan, C. Benoist, A powerful nonviral vector for *in vivo* gene transfer into the adult mammalian brain, *Human Gene Therapy*, (1996) 1947-1954.
- [18] O.A. Ali, D.J. Mooney, Sustained GM-CSF and PEI condensed pDNA presentation increases the level and duration of gene expression in dendritic cells, *Journal of Controlled Release*, 132 (2008) 273-278.
- [19] Y.C. Huang, K. Riddle, K.G. Rice, D.J. Mooney, Long-term *in vivo* gene expression via delivery of PEI-DNA condensates from porous polymer scaffolds, *Human Gene Therapy*, 16 (2005) 609-617.
- [20] Y.C. Huang, C. Simmons, D. Kaigler, K.G. Rice, D.J. Mooney, Bone regeneration in a rat cranial defect with delivery of PEI-condensed plasmid DNA encoding for bone morphogenetic protein-4 (BMP-4), *Gene Therapy*, 12 (2005) 418-426.
- [21] K. Park, PEI-DNA complexes with higher transfection efficiency and lower cytotoxicity, *Journal of Controlled Release*, 140 (2009) 1-11.
- [22] W.T. Godbey, K.K. Wu, A.G. Mikos, Poly(ethylenimine) and its role in gene delivery, *Journal of Controlled Release*, 60 (1999) 149-160.
- [23] G. Chamberlain, J. Fox, B. Ashton, J. Middleton, Concise Review: Mesenchymal Stem Cells: Their Phenotype, Differentiation Capacity, Immunological Features, and Potential for Homing, *STEM CELLS*, 25 (2007) 2739-2749.
- [24] D.J. Mooney, H. Vandenburgh, Cell Delivery Mechanisms for Tissue Repair, *Cell Stem Cell*, 2 (2008) 205-213.
- [25] A.H. Undale, J.J. Westendorf, M.J. Yaszemski, S. Khosla, Mesenchymal Stem Cells for Bone Repair and Metabolic Bone Diseases, *Mayo Clin Proc*, 84 (2009) 893-902.
- [26] H.H. Ahn, J.H. Lee, K.S. Kim, J.Y. Lee, M.S. Kim, G. Khang, I.W. Lee, H.B. Lee, Polyethyleneimine-mediated gene delivery into human adipose derived stem cells, *Biomaterials*, 29 (2008) 2415-2422.
- [27] Y.C. Huang, M. Connell, Y. Park, D.J. Mooney, K.G. Rice, Fabrication and *in vitro* testing of polymeric delivery system for condensed DNA, *Journal of Biomedical Materials Research Part A*, 67A (2003) 1384-1392.
- [28] Y.T. Nguyen, H.K. Kim, J.S. Kwon, Y.S. Kim, T.R. Yoon, Y. Ahn, M.H. Jeong, I.K. Park, Efficient transfer of reporter gene-loaded nanoparticles to bone marrow stromal cells (D1) by reverse transfection, *J Nanosci Nanotech*, 10 (2010) 3170-3174.
- [29] A. Saraf, M.C. Hacker, B. Sitharaman, K.J. Grande-Allen, M.A. Barry, A.G. Mikos, Synthesis and Conformational Evaluation of a Novel Gene Delivery Vector for Human Mesenchymal Stem Cells, *Biomacromolecules*, 9 (2008) 818-827.
- [30] M.O. Avilés, C.-H. Lin, M. Zelivyanskaya, J.G. Graham, R.M. Boehler, P.B. Messersmith, L.D. Shea, The contribution of plasmid design and release to *in vivo* gene expression following delivery from cationic polymer modified scaffolds, *Biomaterials*, 31 (2010) 1140-1147.
- [31] X. Yang, X.F. Walboomers, J. van den Dolder, F. Yang, Z. Bian, M. Fan, J.A. Jansen, Non-Viral Bone Morphogenetic Protein 2 Transfection of Rat Dental Pulp Stem Cells Using Calcium Phosphate Nanoparticles as Carriers, *Tissue Engineering: Part A*, 14 (2008) 71-81.
- [32] F.J. O'Brien, B.A. Harley, I.V. Yannas, L. Gibson, Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds, *Biomaterials*, 25 (2004) 1077-1086.
- [33] C.M. Murphy, M.G. Haugh, F.J. O'Brien, The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering, *Biomaterials*, 31 (2010) 461-466.
- [34] M.G. Haugh, C.M. Murphy, F. O' Brien, Novel freeze-drying methods to produce a range of collagen-glycosaminoglycan scaffolds with tailored mean pore sizes, *Tissue Engineering Part C*, 16 (2010) 887-894.
- [35] A.A. Al-Munajjed, F.J. O'Brien, Influence of a novel calcium-phosphate coating on the mechanical properties of highly porous collagen scaffolds for bone repair, *Journal of the Mechanical Behavior of Biomedical Materials*, 2 (2009) 138-146.
- [36] A.A. Al-Munajjed, N.A. Plunkett, J.P. Gleeson, T. Weber, C. Jungreuthmayer, T. Levingstone, J. Hammer, F.J. O'Brien, Development of a biomimetic collagen-hydroxyapatite scaffold for bone tissue engineering using a SBF immersion technique, *J Biomed Mater Res B Appl Biomater*, 90 (2009) 584-591.
- [37] E. Farrell, F.J. O'Brien, P. Doyle, J. Fischer, I. Yannas, B.A. Harley, B. O'Connell, P.J. Prendergast, V.A. Campbell, A Collagen-glycosaminoglycan Scaffold Supports Adult Rat Mesenchymal Stem Cell Differentiation Along Osteogenic and Chondrogenic Routes, *Tissue Engineering*, 12 (2006) 459-468.
- [38] C.M. Tierney, M.G. Haugh, J. Liedl, F. Mulcahy, B. Hayes, F.J. O'Brien, The effects of collagen concentration and crosslink density on the biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering, *Journal of the Mechanical Behavior of Biomedical Materials*, 2 (2009) 202-209.
- [39] N. Tran, T.J. Webster, Nanotechnology for bone materials, *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, 1 (2009) 336-351.
- [40] G.M. Cunniffe, G.R. Dickson, S. Partap, K.T. Stanton, F.J. O'Brien, Development and characterisation of a collagen nano-hydroxyapatite composite scaffold for bone tissue engineering, *J Mater Sci Mater Med*, 21 (2010) 2293-2298.
- [41] J.P. Gleeson, N.A. Plunkett, F. O' Brien, Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration, *Eur Cell Mater*, 4 (2010) 218-230.
- [42] F.G. Lyons, A.A. Al-Munajjed, S.M. Kieran, M.E. Toner, C.M. Murphy, G.P. Duffy, F.J. O'Brien, The healing of bony defects by cell-free collagen-based scaffolds compared to stem cell-seeded tissue engineered constructs, *Biomaterials*, 31 (2010) 9232-9243.
- [43] M. Alhag, E. Farrell, M.E. Toner, N. Claffey, T.C. Lee, F. O' Brien, Evaluation of early healing events around mesenchymal stem cell-seeded collagen-glycosaminoglycan scaffold. An experimental study in Wistar rats., *Oral Maxillofac Surg*, 15 (2011) 31-39.
- [44] M. Ruoponen, S. Yla-Herttuala, A. Urtti, Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies, *Biochimica et Biophysica Acta*, 1415 (1999) 331-341.
- [45] P. Lampela, P. Soininen, K.A. Puttonen, M. Ruoponen, A. Urtti, P.T. Männistö, A. Raasmaja, Effect of cell-surface glycosaminoglycans on cationic carrier combined with low-MW PEI-mediated gene transfection, *International Journal of Pharmaceutics*, 284 (2004) 43-52.
- [46] H. Nie, C.-H. Wang, Fabrication and characterization of PLGA/HAp composite scaffolds for delivery of BMP-2 plasmid DNA, *Journal of Controlled Release*, 120 (2007) 111-121.
- [47] R. Rao, R., J. He, J.K. Leach, Biomaterialized composite substrates increase gene expression with nonviral delivery, *Journal of Biomedical Materials Research Part A*, 94A (2010) 344-354.
- [48] G.M. Cunniffe, F. O' Brien, S. Partap, T. Levingstone, K.T. Stanton, G.R. Dickson, The synthesis and characterization of nanophase hydroxyapatite using a novel dispersant-aided precipitation method, *Journal of Biomedical Materials Research Part A*, 95 (2010) 1142-1149.
- [49] H. Storrer, D.J. Mooney, Sustained delivery of plasmid DNA from polymeric scaffolds for tissue engineering, *Advanced Drug Delivery Reviews*, 58 (2006) 500-514.
- [50] W. Wang, W. Li, L. Ou, E. Flick, P. Mark, C. Nesselmann, C.A. Lux, H.H. Gatzert, A. Kaminski, A. Liebold, K. Lutzkow, A. Lendlein, R.K. Li, G. Steinhoff, N. Ma, Polyethyleneimine-mediated gene delivery into human bone marrow mesenchymal stem cells from patients, *J Cell Mol Med*, (2010).
- [51] M. Endo, S. Kuroda, H. Kondo, Y. Maruoka, K. Ohya, S. Kasugai, Bone Regeneration by Modified Gene-Activated Matrix: Effectiveness in Segmental Tibial Defects in Rats, *Tissue Engineering*, 12 (2006) 489-497.

- [52] M. Keeney, J.J.J.P. van den Beucken, P.M. van der Kraan, J.A. Jansen, A. Pandit, The ability of a collagen/calcium phosphate scaffold to act as its own vector for gene delivery and to promote bone formation via transfection with VEGF165, *Biomaterials*, 31 (2010) 2893-2902.
- [53] M.G. Haugh, C.M. Murphy, R.C. McKiernan, C. Altenbuchner, F.J. O'Brien, Crosslinking and mechanical properties significantly influence cell attachment, proliferation and migration within collagen glycosaminoglycan scaffolds, *Tissue Eng Part A*, 17 (2011) 1201-1208.
- [54] J. Xie, M.J. Baumann, L.R. McCabe, Osteoblasts respond to hydroxyapatite surfaces with immediate changes in gene expression, *Journal of Biomedical Materials Research Part A*, 71A (2004) 108-117.
- [55] C.M. Serre, M. Papillard, P. Chavassieux, G. Boivin, In vitro induction of a calcifying matrix by biomaterials constituted of collagen and/or hydroxyapatite: an ultrastructural comparison of three types of biomaterials, *Biomaterials*, 14 (1993) 97-106.
- [56] H.J. Kong, J. Liu, K. Riddle, T. Matsumoto, K. Leach, D.J. Mooney, Non-viral gene delivery regulated by stiffness of cell adhesion substrates, *Nat Mater*, 4 (2005) 460-464.
- [57] H. Shen, J. Tan, W.M. Saltzman, Surface-mediated gene transfer from nanocomposites of controlled texture, *Nat Mater*, 3 (2004) 569-574.
- [58] E.V. Leonova, K.E. Pennington, P.H. Krebsbach, D.H. Kohn, Substrate mineralization stimulates focal adhesion contact redistribution and cell motility of bone marrow stromal cells, *J Biomed Mater Res B Appl Biomater*, 79 (2006) 263-270.
- [59] L. De Laporte, J. Cruz Rea, L.D. Shea, Design of modular non-viral gene therapy vectors, *Biomaterials*, 27 (2006) 947-954.
- [60] S. O'Rourke, M. Keeney, A. Pandit, Non-viral polyplexes: Scaffold mediated delivery for gene therapy, *Progress in Polymer Science*, 35 (2010) 441-458.

Graphical abstract

Collagen-based matrices support optimised PEI-DNA polyplexes delivery to MSCs. Collagen nano-hydroxyapatite matrices induce more elevated and sustained transgene expression in comparison to collagen and collagen-glycosaminoglycan scaffolds over the culture period.

Figure 1.

Figure 2.

Figure 3.

Figure 4.