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The Enhancement of Bone Regeneration by Gene Activated Matrix Encoding for Platelet Derived Growth Factor

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Abstract

Gene therapy using non-viral vectors that are safe and efficient in transfecting target cells is an effective approach to overcome the shortcomings of protein delivery of growth factors. The objective of this study was to develop and test a non-viral gene delivery system for bone regeneration utilizing a collagen scaffold to deliver polyethylenimine (PEI)-plasmid DNA (pDNA) [encoding platelet derived growth factor-B (PDGF-B)] complexes. The PEI-pPDGF-B complexes were fabricated at amine (N) to phosphate (P) ratio of 10 and characterized for size, surface charge, and *in vitro* cytotoxicity and transfection efficacy in human bone marrow stromal cells (BMSCs). The influence of the complex-loaded collagen scaffold on cellular attachment and recruitment was evaluated *in vitro* using microscopic techniques. The *in vivo* regenerative capacity of the gene delivery system was assessed in 5 mm diameter critical-sized calvarial defects in Fisher 344 rats. The complexes were ~100 nm in size with a positive surface charge. Complexes prepared at an N/P ratio of 10 displayed low cytotoxicity as assessed by a cell viability assay. Confocal microscopy revealed significant proliferation of BMSCs on complex-loaded collagen scaffolds compared to empty scaffolds. *In vivo* studies showed significantly higher new bone volume/total volume (BV/TV) % in calvarial defects treated with the complex-activated scaffolds following 4 weeks of implantation (14- and 44-fold higher) when compared to empty defects or empty scaffolds, respectively. Together, these findings suggest that non-viral *PDGF-B* gene-activated scaffolds are effective for bone regeneration and are an attractive gene delivery system with significant potential for clinical translation.

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Keywords

Plasmid DNA; Polyethylenimine; Gene Delivery; Scaffold; Platelet Derived Growth Factor; Collagen; Bone regeneration

1. Introduction

Identification of key molecules involved in bone formation and fracture healing has led to the development of biomimetic materials for clinical applications [1, 2]. One such development in dentistry is the introduction and usage of recombinant growth factors and morphogenetic proteins [3]. Major barriers with protein therapy are cost, low bioavailability and supraphysiological dosage for therapeutic efficacy [4]. One strategy to overcome these drawbacks is gene therapy [5, 6]. There are two primary methods of gene therapy for bone regeneration: 1) transfection of cells *in vitro* and subsequent transplantation into the site of the bone defect [7], and 2) direct delivery of osteogenic plasmid genes immobilized in a scaffold matrix [8]. The latter approach has been shown to be more advantageous in generating a persistent expression of the growth factors by the transfected wound repair cells, more cost-effective, and may be more clinically safe for use [8–11].

The first set of *in vivo* studies involving non-viral gene activated matrices for bone regeneration utilized plasmids encoding bone morphogenetic protein-2 (BMP-2) and/or human parathyroid hormone peptide [8, 9]. Non-viral gene delivery vectors are relatively safe compared to viral vectors but have lower transfection efficiencies that can often limit their potential [12]. One non-viral gene delivery system showing strong transfection capabilities is cationic polymer, polyethylenimine (PEI). In previous studies, the branched form of PEI has shown significantly higher gene transfer efficiency than the linear form of PEI [13]. Branched PEI exhibits considerable buffering capacity over a wide pH range due to its protonability, has the highest cationic-charge potential, and condenses plasmid DNA (pDNA) to a greater extent than the linear PEI. This protects the DNA from serum DNases, cytosolic nuclease digestion, facilitates endocytosis and promotes the 'proton sponge effect' [14–17]. Different molecular weights of branched PEI have been investigated *in vivo* for their transfection efficiencies with 25 kDa PEI yielding the highest transfection efficiency. Low molecular weight PEIs resulted in weak PEI-pDNA complexes that readily dissociated, thus reducing the transfection efficiency relative to 25 kDa PEI [18–21].

Platelet derived growth factor (PDGF) is a potent mitogen and chemoattractant for mesenchymal and osteogenic cells and a stimulant for the expression of angiogenic molecules that play a pivotal role in bone healing [22]. There are several preclinical and clinical reports that have shown the safety and efficacy of PDGF in achieving bone regeneration [23–25]. Past studies on the use of PDGF have been through viral vector delivery or as a recombinant protein [23–25]. The objective of this study was to develop, optimize and test a non-viral based gene delivery system for bone regeneration utilizing a collagen scaffold loaded with cationic PEI-pDNA [encoding PDGF-B] complexes.

2. Materials and methods

2.1. Materials

Branched PEI (mol. wt. 25 kDa) was purchased from Sigma-Aldrich® (St. Louis, MO). The GenElute™ HP endotoxin-free plasmid maxiprep kit was obtained from Sigma-Aldrich®. The luciferase assay system was purchased from Promega Corporation (Madison, WI). The microBCA™ protein assay kit was purchased from Pierce (Rockford, IL). The PDGF-BB ELISA kit was purchased from Quantikine® (R & D Systems®, Minneapolis, MN). Plasmid

DNA (6.4 Kb) encoding for firefly luciferase reporter protein (pLUC) driven by cytomegalovirus (CMV) promoter/enhancer (VR1255 pDNA) was obtained from Vical®, Inc. (San Diego, CA). Plasmid DNA (4.7 Kb) coding for the enhanced green fluorescent protein (pEGFP-N1) driven by CMV promoter/enhancer was obtained from Elim Biopharmaceuticals, Inc. (Hayward, CA). Plasmid DNA (4.9 Kb) encoding PDGF-B protein (pPDGF-B) driven by CMV promoter/enhancer was obtained from Origene Technologies, Inc. (Rockville, MD). Absorbable type-I bovine collagen was obtained from Zimmer Dental Inc. (Carlsbad, CA). All other chemicals and solvents used were of reagent grade. Human bone marrow stromal cells (BMSCs) and Dulbecco's modified eagle medium (DMEM) were purchased from American Type Culture Collection (ATCC®, Manassas, VA). Trypsin-EDTA (0.25%, 1X solution) and Dulbecco's phosphate buffered saline (PBS) was purchased from Gibco® (Invitrogen™, Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals® (Lawrenceville, GA). Gentamycin sulfate (50 mg/ml) was purchased from Mediatech Inc. (Manassas, VA). MTS cell growth assay reagent (Cell Titer 96® AQueous One Solution cell proliferation assay) was purchased from Promega Corporation. Alexa Fluor® 568 phalloidin was purchased from Invitrogen. Triton X-100 was obtained from Sigma-Aldrich®. Vectashield®, Hardset™ mounting medium with 4',6-diamidino-2-phenylindole (DAPI, H-1500) was obtained from Vector Labs Inc. (Burlingame, CA).

2.2. Preparation of pDNA encoding different proteins: pLUC, pEGFP-N1 or pPDGF-B

The chemically competent DH5α™ bacterial strain (*Escherichia coli* species) was transformed with pDNA to amplify the plasmid. The pDNA in the transformed cultures was then expanded in *E. coli* in Lennox L Broth (LB Broth) overnight at 37 °C in an incubator shaker at 300 rpm. Plasmid DNA was extracted using GenElute™ HP endotoxin-free plasmid maxiprep kit and was analyzed for purity using a NanoDrop 2000 UV-Vis Spectrophotometer (ThermoScientific, Wilmington, DE) by measuring the ratio of absorbance (A260 nm/A280 nm). The concentration of pDNA solution was determined by absorbance at 260 nm.

2.3. Fabrication of PEI-pDNA complexes

Complexes were prepared by adding 500 µl PEI solution drop wise to 500 µl pDNA (pLUC/pEGFP-N1/pPDGF-B) solution containing 50 µg pDNA and mixed by vortexing for 20 s. The mixture was incubated at room temperature for 30 min to allow complex formation between the positively charged PEI (amine groups) and the negatively charged pDNA (phosphate groups) [16, 26]. Complexes were fabricated using different N (nitrogen) to P (phosphate) ratios (molar ratio of amine groups of PEI to phosphate groups in pDNA backbone) by varying the PEI amounts and maintaining the amount of pDNA constant (N/P ratios of 5, 10, 15 and 20, Table 1). Final volume of the complexes used in the transfection and cytotoxicity experiments was 20 µl containing 1µg of pDNA.

2.4. Size and surface charge of the PEI-pPDGF-B complexes

Measurements were carried out using a Zetasizer Nano-ZS (Malvern Instruments, Westborough, MA). The particle size and size distribution by intensity was determined by dynamic laser light scattering (4mW He-Ne laser with a fixed wavelength of 633 nm, 173° backscatter at 25 °C) in 10 mm diameter cells. Surface charge (zeta potential) was measured electrophoretically by the laser scattering technique using folded capillary cells. All measurements were done in triplicates. The mean value was recorded as the average of three different measurements.

2.5. Cell culture

Human BMSCs were maintained in DMEM supplemented with 10 % FBS and 50 µg/ml gentamycin in a humidified incubator at 37 °C containing 95 % air and 5 % CO₂ (Sanyo Scientific, Wood Dale, IL). Cells were grown as a monolayer on 75 cm² polystyrene cell culture flasks (Corning Incorporated, Corning, NY) and subcultured (subcultivation ratio of 1:9) after 80–90 % confluence. Cell lines were started from frozen stocks and the medium was changed every 2–3 days. Cell passage numbers used in the experiments were between 4 and 10.

2.6. In vitro evaluation of the transfection efficiency of PEI-pLUC complexes in BMSCs

The PEI-pLUC complexes were prepared using N/P ratios of 1, 5, 10, 15 and 20. Cells were seeded at a density of 80,000 cells/well in 24-well plates (Costar®, Corning Inc, NY). The next day, at ~ 80 % cell confluence, the cell culture medium was changed to serum-free medium and the treatments were gently vortexed and added drop wise into the wells. Each well was treated with 20 µl complexes containing 1 µg pLUC. Untreated cells were the controls while cells treated with PEI alone were the negative controls. Cells treated with uncomplexed pDNA served as a control comparison with complex-treated cells. Complexes were incubated with cells for 4 h or 24 h. At the end of each treatment period, cells were washed with 1X phosphate buffered saline (PBS) followed by addition of fresh complete medium. After a total incubation time of 48 h, cells were washed with 1X PBS, and treated with 1X lysis buffer and subjected to two freeze-thaw cycles whereupon cells were scraped and centrifuged at 14,000 rpm for 5 min. Luciferase expression was detected by a standard luciferase assay system. The relative light units (RLU) values per mg of the total cell protein, indicative of the transfection efficiency, were normalized against the protein concentration in cell extracts using a microBCA protein assay kit. The values are expressed as mean ± SD for each treatment (n = 3).

2.7. In vitro evaluation of toxicity of PEI- pLUC complexes in BMSCs

Cell survival assays were conducted to demonstrate the effect of N/P ratio of the PEI-pLUC complexes on the biocompatibility of complexes in BMSCs. Cells were seeded in clear polystyrene, flat bottom, 96-well plates (Costar®, Corning Inc.) at a density of 10,000 cells/well and allowed to attach overnight and further processed as described in section 2.6. Untreated BMSCs were used as controls. Cells treated with PEI alone or uncomplexed pLUC alone served as additional controls. The complexes were incubated with the cells for 4 h or 24 h to mimic the conditions used in the transfection experiments. At the end of the incubation period, the cells were washed with 1X PBS and fresh complete medium was added to the cells followed by addition of 20 µL MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell growth assay reagent. The plates were then incubated at 37°C in a humidified 5 % CO₂ atmosphere for 4 hours. The amount of soluble formazan produced by reduction of MTS reagent by viable cells was measured spectrophotometrically using SpectraMax® Plus³⁸⁴ (Molecular Devices, Sunnyvale, CA) at 490 nm. The cell viability was expressed by the following equation: cell viability (%) = (absorbance intensity of treated cells / absorbance intensity of untreated cells (control)) × 100. Values are expressed as mean ± SD for each treatment performed in triplicate.

2.8. In vitro visualization of transfection of BMSCs with PEI-pEGFP-N1 complexes

To determine the *in vitro* qualitative fluorescence expressed by EGFP-N1, BMSCs were plated at a density of 50,000 cells/well in clear, flat-bottom, 8-chambered glass slides with cover (Lab-Tek, Nunc™, NY) previously coated with 0.1 % poly-L-lysine and allowed to attach overnight. The next day, cell culture medium was removed and cells were incubated with complexes fabricated at a N/P ratio of 10 containing 1 µg pEGFP-N1 in serum-free

medium for 4 h or 24 h and processed as described in section 2.6. Untreated cells, cells treated with uncomplexed pEGFP-N1 and cells treated with PEI alone were used as controls. After a total incubation time of 48 h, cells were fixed with 4 % paraformaldehyde (Hatfield, PA), followed by permeabilization of cells with 0.2 % Triton[®] X-100. Cellular actin was fluorescently tagged by treating the cells with Alexa Fluor[®] 568 phalloidin. The specimen was mounted with Vectashield[®] Hardset[™] medium containing DAPI. Cells were washed with PBS during every step in the process. The cellular fluorescence was observed using confocal laser scanning microscopy (60X, Carl Zeiss 710, Germany) equipped with Zen 2009 imaging software. The images were processed using ImageJ[®] open access software (National Institutes of Health, MD).

2.9. In vitro evaluation of transfection of BMSCs with PEI-pPDGF-B complexes

The transfection in BMSCs was further evaluated by using pDNA encoding for the PDGF-B protein. Cells were plated at a seeding density of 80,000 cells/well in 24-well plates. The PEI-pPDGF-B complexes containing 1 μ g pPDGF-B were prepared at a N/P ratio of 10, incubated with cells for 4 h and the experiment processed as in section 2.6. After a total incubation time of 48 h, the cell culture supernatants were then harvested by centrifugation after 4 h of incubation with medium supplemented with 10 μ g/ml heparin to prevent retention of PDGF-BB on the cell surface. The amounts of PDGF-BB in cell culture supernatants were quantified using a PDGF-BB ELISA kit. Untreated cells and cells treated with naked, uncomplexed plasmids were employed as controls. The data is represented as mean $\pm$ SD for each treatment carried out in triplicate.

2.10. Fabrication and characterization of PEI-pPDGF-B complex-loaded scaffolds

Polyethylenimine was mixed with pPDGF-B to prepare complexes at an N/P ratio of 10 according to the method described above. Complexes were then injected into the collagen scaffolds (cut into 5 mm x 2 mm) and were freeze-dried for subsequent use. The surface characteristics of the scaffolds were studied using a scanning electron microscope (SEM, Hitachi Model S-4800, Japan). The scaffolds were either empty or loaded with the PEI-pPDGF-B complexes. The scaffolds were mounted on aluminum specimen stubs using adhesive carbon tape and coated by ion sputtering with conductive gold set at 10 mA for 2.5 min (K550 Emitech Sputter Coater, TX). The surface morphology was examined using the microscope operated at 3 kV accelerating voltage.

2.11. Attachment and proliferation of BMSCs on collagen scaffolds

The interaction and proliferation of BMSCs within the scaffolds loaded with PEI-pPDGF-B complexes prepared at a N/P ratio of 10 was evaluated *in vitro* using SEM and confocal microscopy, respectively. Empty scaffolds and complex-loaded scaffolds incorporating 50 μ g pPDGF-B were placed into the wells of a 48-well plate. For the SEM analysis of cell attachment, 100 μ l of cell culture medium containing 90,000 cells were seeded onto a single scaffold per well. After 3 to 4.5 h, 400 μ l of culture medium was added to cover the scaffold completely and kept in culture for 6 days. For cell proliferation analysis, 390,000 cells were seeded onto each scaffold in a single well and cultured for 3 days. At the end of the experiment, the scaffolds were washed and fixed overnight in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for SEM and in zinc formalin for confocal microscopy.

2.12. SEM sample preparations

Standard methods for SEM were employed. Briefly, after aldehyde fixation, the scaffolds were post-fixed for 1 h at room temperature with a 1 % solution of osmium tetroxide in 0.1 M sodium cacodylate buffer. The samples were dehydrated through a series of ethanol washes up to 100 % ethanol before to be submitted to the critical point dry of CO₂ for 2 h.

The samples were then mounted onto aluminum stubs, sputter-coated and examined with a Hitachi S-4800.

2.13. Immunocytochemical staining

The fixed samples were cryo-embedded and cryo-sectioned at 10 μm thickness along the vertical plane of the scaffolds. Cryo-sections were collected on Superfrost Plus Slides (Fisher Scientific®, Fairlawn, NJ) and were post-fixed before incubating for 10 min under a 0.1 % solution of Triton X-100. After endogenous biotin block, the background staining was blocked by treating the samples with 5 % normal goat sera diluted in PBS (Sigma) for 1 h. This was followed by an incubation of the samples for 30 min with 5 $\mu\text{g}/\text{ml}$ mouse anti-proliferating cell nuclear antigen (PCNA) primary antibody (Invitrogen) against PCNA. The samples were then treated with biotinylated anti-mouse IgG secondary antibody (Vector Laboratories, Inc.) for 10 min. Finally, the sections were incubated for 45 min with Streptavidin-Alexa Fluor 488 conjugate (Invitrogen). Negative controls were treated as described above but the specific primary antibody was replaced by a normal mouse IgG match at the same final concentration (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). The washes between each step were done with 1X PBS. The sections then mounted under coverglasses with Vectashield® containing DAPI and observed for the fluorescently-labeled antigen using a Zeiss 710 laser scanning microscope.

2.14. In vivo implantation of complex-loaded collagen scaffolds

Inbred 14 week-old male Fisher (CDF®) white rats (F344/DuCrI, ~ 250 g) were obtained from Charles River Laboratories International, Inc (Wilmington, MA) and housed and cared in the animal facilities. The surgical procedures were approved by and performed according to guidelines established by the University of Iowa Institutional Animal Care and Use Committee, Iowa. The animals were anaesthetized by intra-peritoneal injection of ketamine (80 mg/kg)-xylazine (8mg/kg) mixture (provided by the Office of Animal Resources, University of Iowa). A sagittal incision, ~ 1.5–2 cm, was made on the scalp of each rat, and the calvaria was exposed by blunt dissection. Two 5 mm diameter x 2 mm thickness critical-sized defects were generated using a round carbide bur on the parietal bone, on both sides of the sagittal suture. The defects were randomly allocated into the following study groups: (1) empty defect (n = 3); (2) empty scaffold (n = 5); and (3) PEI-pPDGF-B complex-loaded scaffold (n = 5). Where applicable, the scaffold was cut into cylinders with a diameter of 5 mm and a thickness of 2 mm and implanted into the rats. The incision was closed in layers using sterile silk sutures. Buprenorphine (0.15 mg, intramuscular), as an analgesic, was administered to each rat thereafter and the animals were carefully monitored during post-operative recovery. The rats were able to function normally after this procedure. After 4 weeks, all the animals were euthanized and the bony segments containing the regions of interest were cut from the calvarial bone and fixed in 10 % neutral buffered formalin.

2.15. micro-CT measurement

Three-dimensional microfocus x-ray microcomputed tomography (μCT) imaging was performed on the specimens using a cone-beam μCT system ($\mu\text{CT}40$, Scanco Medical AG, Switzerland). Specimens were scanned in 70% ethanol at 55 kVp and 145 μA with a voxel size of 10 μm and an integration time of 300 ms. Analysis was performed using a constant 3.5 mm diameter circular region of interest that was placed in the center of the machined defect and spanned a total of 50 reconstructed slices, such that a total cylindrical volume of interest of ~3.8 mm^3 oriented perpendicular to the outer table of the calvarium was analyzed for each specimen using the manufacturer's software (sigma = 0.8, support = 1.0, and threshold = 250). Bone volume (BV) per total volume (TV) and connectivity density (Conn.D.) in the bone defect were obtained.

2.16. Histological observation of rat bone samples

After treatment, the bone samples underwent a decalcification (Surgipath, Decalcifier II) procedure. When the decalcification end point test returned negative for the presence of calcium, the rat bone specimens ($n = 3$ for empty defect, $n = 5$ for empty scaffolds, $n = 5$ for complex-loaded scaffolds) were introduced into a paraffin processor for paraffin processing, paraffin embedded and the blocks were sectioned in the sagittal plane for each specimen. Histological analysis was performed on the $5\ \mu\text{m}$ sections in the central portion of the wound. The sections were collected on Superfrost Plus Slides (Fisher Scientific®), deparaffinized and stained with Harris hematoxylin and eosin (H & E staining) according to standard protocols. Five to six sections, representing the central area of each defect, were used to observe the presence of collagen, new bone formation, and cells in order to evaluate bone regeneration after 4 weeks *in vivo* implantation. The brightfield examination of the slides was done with an Olympus Stereoscope SZX12 and an Olympus BX61 microscope, both equipped with a digital camera.

2.17. Data presentation and statistical analysis

Nonparametric methods were emphasized to avoid inappropriate distributional assumptions, and exact tests were used whenever feasible. The Kruskal-Wallis procedure was used to assess differences in the outcome of interest among groups; the Wilcoxon Rank Sum test is the equivalent procedure for comparisons of two groups. A Type I error level of 0.05 was utilized throughout, and adjustment for multiple pairwise group comparisons was primarily made using an adaptation of the Tukey method due to Conover in conjunction with an overall 5% level of significance [27]. This asymptotic approach was used for all but the *in vivo* studies, where it was feasible to adjust for multiple pairwise comparisons using exact Wilcoxon Rank Sum tests and a standard Bonferroni correction, again specifying an experiment-wise Type I error of 0.05. Spearman rank correlations were used to evaluate the relationship between N/P ratio and cell viability. Statistical analyses were carried out using SAS® software, version 9.3 (SAS Institute Inc., Cary, NC). Graphs were generated using Prism 5.0 (GraphPad Software Inc., San Diego, CA); numerical data were represented as means with bars representing standard deviations.

3. Results and discussion

This report investigates the effects of delivery of PEI-complexed pDNA encoding for PDGF-B from porous 3-D collagen scaffolds on bone tissue regeneration. This form of gene-activated matrix (GAM) provides localized transient gene therapy since the *PDGF-B* gene will not be integrated into the host chromosome [28, 29]. The transfection efficiencies of PEI-condensed pDNA complexes are significantly affected by the type of cells being transfected, therefore making it necessary to optimize the gene delivery method for every cell line used. As a result, we first optimized the amine to phosphate (N/P) ratio of the PEI-pPDGF-B complexes so as to generate the maximum transfection in BMSCs with minimal cytotoxicity. We prepared complexes at different N/P ratios and evaluated the influence of the ratio stoichiometry on transfection efficiency and toxicity in BMSCs. It is critical for gene therapy applications that clinical amounts of proteins are produced by the transfected cells and that the gene expression levels are tightly regulated. To address the feasibility and potency concerns, GAMs containing physically entrapped *PDGF-B* plasmid genes were implanted in a rat calvarial defect model and the bone regenerative capacity was assessed. In this study, it is also important to note that the amounts of PEI and pDNA utilized were significantly lower than the amounts used in other studies evaluating GAMs [30, 31].

3.1. Generation of pDNA encoding LUC, EGFP-N1 or PDGF-B proteins

The purity of extracted pDNA as determined by the ratio of absorbance ($A_{260\text{ nm}}/A_{280\text{ nm}}$) was within the range of 1.8 to 2.0 (recommended by the manufacturer's protocol). The concentration of pDNA solution was determined by UV absorbance at 260nm and was further concentrated as needed. Agarose gel electrophoresis confirmed the size and quality of pDNA without any signs of degradation.

3.2. Size and surface charge of PEI-pPDGF-B complexes

The PEI-pPDGF-B complexes at a N/P ratio of 10 were prepared as described above. The complexes were $102 \pm 2\text{ nm}$ in size with a net surface charge of $+37 \pm 1\text{ mV}$. The polydispersity index (PDI) was approximately 0.1, thus indicating narrow size distribution, high uniformity in particle size distribution and overall general homogeneity of the sample. The size and surface charge of the complexes are both important parameters for their interaction and entry into cells [32, 33]. The small size of the polycation-condensed pDNA complexes is critical for both, efficient *in vitro* cellular uptake by clathrin-coated endocytosis [34] as well as *in vivo* distribution and diffusion in the tissues [35]. With regards to the surface charge of the complexes, there has to be a balance between the maximal transfection efficiency and the amount of cell death associated with transfection [14, 36]. In this study, we focused on localized gene therapy, and therefore the effects of the net positive charge on binding and inactivation of the cationic polymer-pDNA complexes by the circulating proteins and the subsequent complement activation, along with the induced recognition by cells of the reticuloendothelial system [37], are of minimal concern.

3.3. In vitro gene expression by PEI-pLUC complexes

One microgram of pLUC was combined with different amounts of PEI (Table 1) to generate complexes with varying N/P ratios. We quantified the luciferase protein formation due to gene expression after incubating the complexes with BMSCs for 4 h or 24 h (Fig. 1). The levels of *LUC* gene expression were significantly affected by the transfection efficiencies of the different N/P ratios of the complexes. Kruskal-Wallis procedure indicated that the distribution of transfection outcomes differed among the treatment groups at both 4 h ($p = 0.0019$) or 24 h ($p = 0.0024$). The transfection efficiency of the PEI-pLUC complexes increased as the N/P ratio at which PEI-pLUC complexes were prepared increased from 1 ($0.13\text{ }\mu\text{g PEI}$) to 10 ($1.30\text{ }\mu\text{g PEI}$). The transfection efficiency then dropped in cells treated with PEI-pLUC complexes prepared at a N/P ratio of 15 ($1.95\text{ }\mu\text{g PEI}$) and dropped further in cells treated with PEI-pLUC complexes prepared at a N/P ratio of 20 ($2.6\text{ }\mu\text{g PEI}$). At 24 h of incubation with the cells, complexes prepared at a N/P ratio of 5 showed an increase in the amount of transgene expression obtained compared to only 4 h incubation. This may be due to higher uptake and entry of the complexes into the cells over a period of time. The factors contributing to low transfection efficiencies of complexes prepared at N/P ratios < 10 include size and surface charge, pDNA binding and condensation capacity, and stability of the complexes. The mean response for complexes prepared at a N/P ratio of 10 at 4 h of treatment was significantly greater (more efficient transfection of BMSCs) than that for complexes prepared at all other N/P ratios considered after adjustment for all multiple pairwise comparisons. However, the LUC protein expression obtained in cells decreased at 24 h of incubation with complexes prepared at a N/P ratio of 10 as a result of cytotoxicity induced by PEI (see section below). The toxicity of PEI in BMSCs also led to a decline in the levels of protein expression achieved when the cells were treated with complexes prepared at higher N/P ratios of 15 and 20 at 4 h or 24 h of treatment. The gene expression generated by complexes prepared at a N/P ratio of 15 was found to be lower than the transfection resulting from complexes prepared at a N/P ratio of 10 at 4 h of treatment which reduced further at 24 h of treatment. At 4 h of incubation with BMSCs, the transgene

expression generated by complexes prepared at a N/P ratio of 20 was lower when compared to complexes prepared at a N/P ratio of 15, and in a manner similar to complexes at N/P ratios of 10 and 15, the transfection efficiency decreased at 24 h of incubation time.

3.4. In vitro cell viability assay for PEI-pLUC complexes

The toxicity of PEI-pLUC complexes prepared at various N/P ratios containing 1 μ g of pLUC (Table 1) was assessed in BMSCs treated with the complexes for 4 h or 24 h. The amount of PEI in the complexes prepared at different N/P ratios was found to have a significant effect on cell viability (Fig. 2). Results of the Kruskal-Wallis test provided evidence that the distribution of cell viability outcomes differed significantly among the treatment groups assayed at both 4 h ($p = 0.0057$) or 24 h ($p = 0.0024$). A strong and highly significant negative correlation between N/P carrier ratio and % cell viability was detected using the Spearman rank correlation at both 4 h ($r = -0.88$, $p < 0.0001$) or 24 h ($r = -0.97$, $p < 0.0001$). Approximately 82 % of BMSCs were viable at 4 h when treated with PEI-pLUC complexes prepared at a N/P ratio of 10. However, cell viability decreased to 34 % when complexes were incubated with cells for 24 h, leading to a corresponding decrease in the transgene expression (Figs. 1 and 2). Complexes of PEI-pLUC prepared at N/P ratios higher than 10 resulted in elevated cytotoxicity and therefore lower transgene expressions following incubation in cells at both 4 h or 24 h. This data clearly suggests that high amounts of PEI and prolonged cell-PEI exposure times are cytotoxic. These findings are in agreement with previously reported results showing successful non-viral gene delivery with PEI-pDNA complexes as a critical balance between sufficient PEI to ensure high transfection efficiency without causing high cytotoxicity [19, 38]. The PEI-pLUC complexes prepared at N/P ratios of 1 and 5 were relatively non-toxic, but demonstrated low transfection efficiencies in BMSCs. In our study, only the PEI-pLUC complexes prepared at N/P ratio of 10 displayed a balance between relatively high transgene expression and low cytotoxicity. Accordingly, the PEI-pDNA complexes used in the subsequent *in vitro* and *in vivo* experiments were fabricated at a N/P ratio of 10.

3.5. In vitro gene expression by PEI-pEGFP-N1 complexes

The transfection in BMSCs was further evaluated using confocal microscopy with fluorescent probes. Cells were transfected with complexes prepared at N/P ratio of 10 containing 1 μ g of pEGFP-N1 at the treatment time points of 4 h or 24 h (Fig. 3). Confocal images (Z-series, 63 X) showed the characteristic green fluorescence in the transfected cells at both 4 h or 24 h due to expression of the gene and formation of the EGFP-N1 protein. In these fixed cells, phalloidin permeated the plasma membrane to stain the cytoplasmic F-actin in red. The cell nuclei were stained blue by DAPI. The cells in the control groups (untreated cells, cells treated with uncomplexed pEGFP-N1 and PEI-treated cells) did not show any green fluorescence (data not shown). Confocal microscopy imaging, along with the quantitative results obtained earlier, thus confirmed the capability of the PEI-pDNA complexes to efficiently transfect BMSCs.

3.6. In vitro investigation of gene expression by PEI-pPDGF-B complexes

Since this study is targeted towards bone regeneration in a defect, we evaluated the gene delivery efficacy of the PEI-pPDGF-B complexes through expression of PDGF-BB. Platelet-derived growth factor is a required element in angiogenesis, is a potent mitogen for mesenchymal and progenitor cells and drives the chemotaxis of osteoblast and vascular endothelial cells [39, 40]. It also stimulates osteoblast type-1 collagen synthesis and extracellular matrix secretion. Based on the N/P ratio optimization experiments performed previously, we assessed the transfection efficiency of PEI-pPDGF-B complexes prepared at a N/P ratio of 10 containing 1 μ g pPDGF-B in BMSCs for 4 h. The PDGF-BB ELISA

quantified PDGF-BB protein formation further confirming the transfection potential of the PEI-pDNA complexes in our target cells. After the transfection of cells with the PEI-pPDGF-B complexes, PDGF-BB levels in cell culture medium supernatants (83 pg/ml) were approximately 6-fold higher than in cells treated with the naked, uncomplexed pPDGF-B (14 pg/ml) (Fig. 4). The highest PDGF-BB protein expression levels (83 pg/ml) were obtained by treating the cells with 1 μ g pPDGF-B for 4 h, followed by further incubation for 44 h. Initial attempts at detecting PDGF-BB using ELISA resulted in low levels of detectable PDGF-BB. Heparin can prevent the reuptake of secreted proteins, thereby allowing for a more accurate estimation of its secretion but has never been evaluated for its potential to improve detection of proteins following PEI-pDNA complex transfections [41]. We showed that heparin, when added to the cells 4 h prior to the analysis, resulted in an increased detection in the protein levels of PDGF-BB (132 pg/ml). The data provided strong evidence that the distribution of PDGF-BB concentrations differed among the treatment groups ($p = 6.5 \times 10^{-5}$, Kruskal-Wallis test). After adjustment for multiple pairwise comparisons, significantly more PDGF-BB was found to be produced by the cells transfected with PEI-pPDGF-B complexes or PEI-pPDGF-B complexes followed by heparin treatment relative to that produced by control or naked pDNA groups. Furthermore, significantly more PDGF-BB was secreted by the cells transfected with PEI-pPDGF-B complexes followed by heparin treatment versus without heparin treatment. These results also verified the ability of PEI-pDNA complexes to efficiently transfect BMSCs with therapeutically relevant genes.

3.7. SEM analysis of collagen scaffolds

The collagen scaffold was characterized using SEM. The resorbable collagen scaffold showed a highly interconnecting porous structure, with pore diameters ranging from 100–200 μ m (Fig. 5a). The incorporation of complexes within the scaffolds and the subsequent lyophilization procedure did not appear to have any significant effect on the morphology or the microarchitecture of the final scaffold biomaterial (Fig. 5b). Three-dimensional porous polymer scaffolds such as the collagen scaffold utilized here can create and maintain a space within the defect *in vivo*. This helps recruit the healthy pre-osteoblasts and osteoblasts to the wound site, enhances their proliferation and differentiation and forms a space-filling tissue [42]. These important processes ultimately help control the size and shape of the regenerated bone tissue within the defect.

3.8. SEM and confocal analysis of attachment and proliferation of BMSCs on collagen scaffolds

In order to provide a favorable environment for bone regeneration, the scaffold must provide sites for cellular attachment and support cell survival and growth. It is desirable that the implanted scaffold is inert and biodegradable as the new tissue is regenerated by the osteogenic cells [43, 44]. Type I collagen constitutes the main protein component of natural extracellular matrices and plays an important role in the process of repair of damaged tissue [45, 46]. It is particularly important for bone regeneration involving this guided bone-growth approach that the collagen scaffold has sufficient physical and mechanical properties to provide physical support, and retain its original geometry following *in vivo* implantation which is necessary for filling-in specific critical-sized defects. Type I collagen matrices serve as a platform for cell adhesion and migration, and direct the growth of cells [8, 45, 47, 48]. After 6 days in cell culture, we found that the scaffold surface was vastly different from the images obtained earlier in this study prior to incubating it with cells and the cell culture medium (Figs. 5a–c). This may possibly be due to the degradation of collagen. Morphological changes were observed on the surface of the porous scaffold material and inner walls of the pores after cell culture treatment. High magnification SEM imaging demonstrated the recruitment and attachment of BMSCs into the scaffold containing PEI-

pPDGF-B complexes (Fig. 5d). We observed BMSCs adhering to the scaffolds via various cell processes. The cell morphology was found to be spindle-shaped with branched cytoplasm. These highly porous scaffolds supported cell anchorage and with pore sizes greater than the size of cells, provided adequate space within the scaffold to allow migration of cells into the scaffold through the pores. The complex-loaded scaffolds would therefore be expected to allow cells from the surrounding tissues into the wound site, and stimulate their growth and differentiation, thus promoting tissue development [49].

It has been determined from the experiment above that PEI-pPDGF-B complexes are able to transfect BMSCs efficiently, which then allow for expression of the PDGF-BB protein. Platelet derived growth factor is a cytokine that regulates cell growth and cellular division for bone-forming cells and functions as a mitogenic and chemotactic agent [40, 50, 51]. The complex-loaded scaffold matrix was therefore evaluated for its ability to promote *in vitro* cell proliferation next, by detecting and quantifying the presence of dividing cells in culture using indirect immunocytochemistry and immunofluorescence assays [52]. Immunofluorescence staining was utilized to investigate cellular proliferation by the indirect fluorescent labeling of the nuclear protein, PCNA. This was accomplished using mouse anti-PCNA primary antibody expressed against PCNA, to detect the levels of expression of PCNA in the proliferating cells. PCNA plays an integral role in the eukaryotic cell cycle and is essential for cellular DNA synthesis [53]. This unconjugated, monoclonal, IgG2a antibody is specific to multiple PCNA, which is expressed during DNA synthesis, and hence is a useful tool for studying the proliferating healthy cells. As a control for the experiment, negative staining done using normal IgG in place of the specific primary antibody did not show any green fluorescence (data not shown). After incubating the complex-loaded scaffolds for 3 days in culture medium with BMSCs, the scaffolds were harvested, stained and observed under a confocal microscope to detect proliferating cells (green fluorescence). The nuclei of cells were stained by DAPI (blue fluorescence). Cells were counted under the same magnification of microscope (20X). At day 3, significantly higher number of proliferating BMSCs was observed with scaffolds containing PEI-pPDGF-B complexes compared to empty scaffolds ($p = 0.0079$, Wilcoxon Rank Sum test) (Figs. 6a–b). The number of proliferating cells in the scaffolds was 3.4-fold higher with the complex-treated group than that obtained with the untreated group (Fig. 6c). Confocal imaging confirmed the recruitment of BMSCs into the scaffold containing PEI-pPDGF-B complexes and their subsequent proliferation compared to empty scaffolds, therefore validating the important role of PDGF-BB in chemotaxis and growth of cells potentially capable of osteogenesis.

3.9. In vivo bone regeneration

The collagen scaffold matrix containing PEI-pPDGF-B complexes was evaluated *in vivo* for its efficacy as a bone regenerative biomaterial unit. Critical-sized calvarial defects were created in rats and were utilized as a model to test the *in vivo* efficacy of three different treatment groups: (1) empty defect (untreated) as a control, (2) defect filled with empty collagen scaffold, and (3) defect filled with PEI-pPDGF-B complexes entrapped in collagen scaffold. The rats were sacrificed after 4 weeks and newly-formed bone tissue was evaluated for its volume and connectivity density using μ CT scans. The μ CT scan imaged the circular bone defects induced and the regenerated bone tissue in the defects as a result of various treatments (Figs. 7a–f). The defect site was most significantly bridged with bone when treated with the scaffolds containing PEI-pPDGF-B complexes compared to other groups tested. The amount of bone tissue regenerated was quantified by analyzing the bone formation parameters, trabecular bone volume fraction of the total tissue volume of interest (BV/TV) and the degree of trabecular connectivity (connectivity density or thickness). The BV/TV was 44-fold and 14-fold higher in defects treated with complex-embedded scaffolds when compared to the empty scaffold and empty defect control groups, respectively (Fig.

7g). The data provided evidence that the distribution of BV/TV differed significantly among the three comparison groups ($p = 0.0025$, Kruskal-Wallis test). When pairwise group comparisons were made using exact Wilcoxon Rank Sum tests, a significant difference was found in the distribution of BV/TV between the complex-embedded scaffold and the empty scaffold groups ($p = 0.0079$); this remained significant after Bonferroni adjustment for multiple comparisons. The difference between the complex-embedded scaffold and the empty defect groups, after adjustment for multiple comparisons, gave a p value that was equal to 0.0357. The connectivity density of the regenerated bone was 36-fold and 52-fold greater for the complex-loaded scaffold group than for the empty scaffold group and empty defect control group, respectively (Fig. 7h). The distribution of connectivity density differed significantly among the three comparison groups ($p = 0.0016$, Kruskal-Wallis test). Pairwise comparisons via exact Wilcoxon Rank Sum test provided strong evidence of a difference in connectivity density between the complex-embedded scaffold and the empty scaffold groups ($p = 0.0079$) which was significant after Bonferroni adjustment for multiple comparisons; a difference was also seen between the complex-embedded scaffold and the empty defect control groups ($p = 0.0357$). Histology images with H & E staining further validated the μ CT results. The empty defect images showed that the gap between the healthy native bone edges was unfilled, while the empty scaffold group showed only loose, soft tissue formation with a thin rim of new bone forming at the edges of the defect (Figs. 8a–b). For the complex-loaded scaffold group, we observed complete bridging of the defect by the mature, mineralized bone tissue indicated by the arrows (Fig. 8c). We hypothesize that the complexes may have released from the degrading matrix, which then will transfect the surrounding cells. It is also possible that the cells could have migrated into the porous matrix containing the complexes followed, by their transfection by the complexes. These transfected cells produce PDGF-BB which stimulates cellular proliferation, cell growth and division, and angiogenesis [39]. Platelet derived growth factor signaling is also involved in cell migration, tissue remodeling and cellular differentiation of pre-osteoblasts into osteoblasts that initiate bone formation by secreting the osteoid matrix that mineralizes to form mature bone tissue [54]. The chemotactic action of PDGF further augments these processes. Ultimately, new bone material is laid down by the osteogenic cells by communication through cytokine PDGF signaling.

4. Conclusions

In summary, BMSCs were efficiently transfected with a variety of reporter or therapeutic genes when using PEI-pDNA complexes prepared at an optimal N/P ratio of 10. These PEI-pDNA complexes were stable and nanometer-sized, with a net positive surface charge. The PEI-pPDGF-B complex-activated collagen scaffolds favored cellular attachment and promoted cellular proliferation *in vitro*. The complex-loaded scaffolds promoted osteogenesis and demonstrated superior tissue regeneration efficacy in calvarial defects in rats when compared to the empty defect and empty scaffold groups. This system efficaciously delivered pDNA into the cells without any apparent adverse effects. Gene activated matrices encoding for PDGF-B protein therefore have a strong potential for clinical applications that require enhanced bone regeneration.

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Fig. 1. Luciferase assay assessing the effect of N/P ratio on the transfection capability of PEI-pLUC complexes in BMSCs at 4 h or 24 h (n = 3).

Fig. 2. MTS assay assessing the effect of N/P ratio on the biocompatibility of PEI-pLUC complexes in BMSCs at 4 h or 24 h (n = 3).

Fig. 3. Confocal microscopy demonstrating transfection in BMSCs after 4 h or 24 h of treatment with PEI-pEGFP complexes fabricated at a N/P ratio of 10. Scale bar, 20 μ m.

Fig. 4. ELISA assay demonstrating the expression of PDGF-BB protein as a result of transfection of BMSCs with PEI-pPDGF-B complexes for 4 h (n = 3).

Fig. 5. SEM images of empty collagen scaffolds (a) and collagen scaffolds embedded with PEI-pPDGF-B complexes (b). SEM images showing complex-loaded scaffolds seeded with BMSCs, low magnification (200X) (c) and adhesion of BMSCs to the complex-loaded scaffolds, high magnification (3500X) (d) at day 6 of incubation.

Fig. 6. Influence of the complex-loaded scaffolds on proliferation of BMSCs: confocal image demonstrating proliferating cells (DAPI- and PCNA-positive cells) on empty scaffolds (20X) (a) and on PEI-pPDGF-B complex-loaded scaffolds (20X) (b), and measurement of proliferation of BMSCs seeded on empty scaffolds compared to complex-loaded scaffolds (c) at day 3 of culture (n = 6). Scale bar, 20 μ m.

Fig. 7.

Evaluation of *in vivo* bone formation: representative μ CT scans showing the level of regenerated bone tissue after 4 weeks in empty defects (a, d, $n = 3$), empty scaffolds (b, e, $n = 5$) and PEI-pPDGF-B complex-loaded scaffolds (c, f, $n = 5$), assessment of regenerated bone volume fraction (g), bone connectivity density (h) in different groups.

Fig. 8. Representative histology sections demonstrating the extent of new bone formation in the defects at 4 weeks due to various treatments: empty defects (a, n = 3), empty scaffolds (b, n = 5) and PEI-pPDGF-B complex-loaded scaffolds (c, n = 5). OB = old bone and NB = new bone. Note the complete bridging of new bone in the PEI-pPDGF-B complex-loaded test group indicated by the arrows. Scale bar, 50 μm.

Table 1

PEI amount in different N/P ratio used for formulating PEI-pDNA complexes

N/P ratio	PEI amount (µg)
1	0.13
5	0.65
10	1.30
15	1.95
20	2.6