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Gene Activated Titanium Surfaces Promote *In Vitro* Osteogenesis

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Abstract

Commercially pure titanium (CpTi) and its alloys possess favorable mechanical and biological properties for use as implants in orthopedics and dentistry. However, failures in osseointegration still exist and are common in select individuals with risk factors such as smoking. Therefore, in this study, a proposal was made to enhance the potential of CpTi discs for osseointegration by coating their surfaces with nanoplexes comprising polyethyleneimine (PEI) and plasmid DNA encoding bone morphogenetic protein-2 (*pBMP-2*). The nanoplexes were characterized for size and surface charge at a range of N/P ratios. CpTi discs were surface characterized for morphology and composition before and after nanoplex coating using scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and X-ray powder diffraction (XRD). The cytotoxicity and transfection ability of CpTi discs coated with nanoplexes of varying N/P ratios in human bone marrow derived mesenchymal stem cells (BMSCs) was measured via MTS assays and flow cytometry, respectively. The CpTi discs coated with nanoplexes prepared at an N/P ratio of 10 (N/P-10) were considered optimal, resulting in 75% cell viability and 14% transfection efficiency. ELISA results demonstrated a significant enhancement in BMP-2 protein secretion by BMSCs 7 days post-treatment with CpTi discs coated with PEI/*pBMP-2* nanoplexes (N/P-10), compared to the controls. Real time PCR data demonstrated that the BMSCs treated with PEI/*pBMP-2* nanoplex coated CpTi discs resulted in an enhancement of *runx-2*, *alkaline phosphatase* and *osteocalcin* gene expressions on day 7, post-treatment. In addition, these BMSCs demonstrated enhanced calcium deposition on day 30 post-treatment as determined by qualitative (alizarin red staining) and quantitative (atomic absorption spectroscopy)

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assays. Thus, from all the above data it can be concluded that PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs have the potential to induce osteogenesis and enhance osseointegration.

Keywords

Titanium; implant surface; gene therapy; non-viral; BMP-2

Introduction

The osseointegration of titanium implants is dependent on the migratory response of cells to titanium implants which is influenced by implant characteristics such as surface topography, surface roughness, hydrophilicity and biomimetic coatings.(6) In order to enhance the osseointegrative or bone regenerative properties of the implant surface, a variety of techniques have been developed such as coating the surface with recombinant bone regenerative proteins(7–10), bisphosphonates(11) or hydroxyapatite.(12)

Bone morphogenetic protein-2 (BMP-2) is a member of the transforming growth factor- β superfamily and is considered to be a potent morphogen that plays a pivotal role in human development and is extensively involved in bone formation and regeneration.(1) BMP-2 is synthesized by osteoblastic cells and it exerts its osteogenic effect through the Smad signaling pathway by binding to type I and II serine/threonine kinase receptors and also via the p38/44/42 MAPK signaling pathway.(2) Several studies have demonstrated the potential of BMP-2 in regenerating bone and, as a result, BMP-2 has been clinically utilized for bone regeneration applications(3) In the United States, recombinant human BMP-2 (rhBMP-2) is cleared for use in dentistry in sinus augmentation as well as in ridge preservation procedures. However, due to its rapid degradation by proteases, the half-life of rhBMP-2 in animals was 7–16 min and 8 days, when given systemically and locally (through an implantable collagen sponge), respectively.(4–6) In attempts to achieve improved clinical outcomes, rhBMP-2 protein has to be administered to patients in larger doses. The reduced bioavailability of these recombinant proteins and the resulting supraphysiological dosage has resulted in side effects such as ectopic bone formation, vertebral osteolysis, cervical soft tissue swelling and radiculitis.(7)

Gene therapy is considered a logical alternative to protein therapy as it can address many of the drawbacks attributed to protein therapy that were mentioned earlier.(8) In this study, in order to overcome the high dosage requirement of recombinant BMP-2 protein delivery, plasmid DNA (*pDNA*) encoding BMP-2 (*pBMP-2*) was utilized. The bioactivity of CpTi discs was proposed to enhance by coating them with bone morphogenetic protein-2 (BMP-2) encoded plasmids complexed with polyethyleneimine (PEI). Exogenous *pDNA* delivered to cells without a vector has less chance to be taken up by the target cells and can be more readily degraded by extracellular or cytoplasmic nucleases.(9) Polyethyleneimine (PEI), a cationic polymer was utilized as a vector for *pDNA* delivery. PEI (25 kDa) can complex with *pDNA* electrostatically and has proven to be less cytotoxic than other vectors (10, 11) and have efficient transfection ability both *in vitro*(12) and *in vivo*(13). Thus, in this study the CpTi disc surfaces were coated with PEI/*pBMP-2* nanoplexes (N/P-10) and were

further investigated for osteogenic differentiation potential on human bone marrow derived mesenchymal stem cells (BMSCs).

Materials and Methods

Culture of BMSCs

Human bone marrow-derived mesenchymal stem cells (BMSCs) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The passage number of BMSCs used in this study was between 5 and 10. Cells were cultured as a monolayer in DMEM containing 10% fetal bovine serum (FBS) and antibiotics (penicillin-100 U/mL and streptomycin sulfate-100 mg/mL) in a humidified incubator at 37 °C and 5% CO₂ (Sanyo Scientific, Wood Dale, IL) and were passaged using 0.25% trypsin-EDTA (Life Technologies, Madison, WI).

Isolation of plasmid DNA (*pDNA*) encoding EGFP-N1, and BMP-2

The plasmids used in this study encoded for either enhanced green fluorescent protein (EGFP-N1 - 4.7 Kb) (Elim Biopharmaceuticals, Inc. Hayward, CA) or bone morphogenetic protein-2 (BMP-2 – 5 Kb) (Origene Technologies, Inc. Rockville, MD, USA) and were both driven by the CMV promoter/enhancer. Plasmids were independently transformed into chemically competent DH5α *Escherichia coli* and purified after bacterial culture using a GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich; St-Louis, MO) according to the manufacturer's protocol. The concentration and the quality of the isolated *pDNA* was determined by measuring absorbances at A260 nm and A280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA).

Fabrication of PEI/*pDNA* nanoplexes

Different molar ratios of PEI (N-amine) to *pDNA* (P-phosphate) were used to make the complexes (N/P ratios of 1, 5, 10, 15 and 20). Here, the concentration of branched PEI (Mol wt. 25 kDa; Sigma-Aldrich, St-Louis, MO) was altered while maintaining a constant *pDNA* concentration to get the required N/P ratios. Required amounts of *pDNA* (*pEGFP-N1* or *pBMP-2*) and PEI were diluted separately in DNase/RNase free water to a final volume of 500 µL and then the PEI solution was added to the *pDNA* solution resulting in a 50 µg final *pDNA* concentration with the desired N/P ratio. The final 1 mL solution was then vortexed immediately for 30 sec and the mixture was then set aside for 30 min at room temperature before utilization.

Characterization of the size and surface charge of PEI/*pDNA* nanoplexes

Immediately after preparation, the PEI/*pBMP-2* nanoplexes were analyzed for size and zeta potential using a Zetasizer Nano-ZS (Malvern Instruments, Westborough, MA). PEI/*pBMP-2* nanoplexes of 1 mL volumes were taken into polystyrene cuvettes and polystyrene folded capillary cells to measure particle size and zeta potential, respectively. Particle size and size distribution were analyzed by dynamic light scattering using a 4 mW He–Ne laser at a fixed wavelength of 633 nm and 173° backscatter at 25 °C in 10 mm diameter cells. The principle of electrophoresis was employed to determine the zeta potential.

Coating CpTi discs with PEI/*pDNA* nanoplexes

CpTi discs were polished with a variable speed grinder-polisher (Buehler-Ecomet 3) using grinding paper (Buehler Carbimet special silicon carbide for metallography) starting from grit number 120, and then progressing to 240, 400 and 600. Then the CpTi discs were sandblasted with a sandblaster (EWL Type 5423) using 50 μm white aluminium oxide blasting compound (Ivoclar Vivadent). The sandblasted CpTi discs were then sonicated in tap water for 5 min, then sonicated in milli Q water for 30 min to remove any remnants of aluminium oxide particles. The CpTi discs were cleaned with methyl ethyl ketone for 15 min and acid passivated using 30% HNO_3 for another 30 min. They were then rinsed using ultrapure water for 20 min and dried in a vacuum dessicator. After drying, the CpTi discs were sterilized using UV light at 300 uW/cm^2 on both sides for 10 min. The sterilized CpTi discs were then attached to 24 well plate lid (using glue and tape) at appropriate locations of the wells. These constructs (CpTi discs attached to 24 well plate lid) were left in a sterile hood for 24 h and sterilized again with UV light for 2 h. After sterilization, the CpTi discs were coated uniformly by pipetting 50 μl of previously prepared nanoplexes (either PEI/*pEGFP* or PEI/*pBMP-2*) dropwise and these nanoplex coated CpTi discs were allowed to air dry for 30 min under sterile hood.

Surface topographical analysis of CpTi discs

The CpTi discs, after coating with PEI/*pBMP-2* (N/P-10) nanoplexes, were dried in a biosafety cabinet for 24 h. The CpTi discs were then sputter coated with gold-palladium using an argon beam K550 sputter coater (Emitech Ltd., Kent, England) under reduced pressure to make them electrically conductive prior to examination by scanning electron microscopy (SEM) (Hitachi S-4800). SEM images were captured at 4 kV accelerating voltage in an argon atmosphere. Atomic force microscopy (AFM) images were obtained using a molecular force probe 3D AFM (Asylum Research, Santa Barbara, CA) equipped with a high intensity illuminator (Fiber-Lite MI-150 R). Images were collected using tapping mode (AC mode) and silicon probes (MikroMasch, Model CSC37) with a spring constant of 0.3 N/m and a 10 nm tip radius of curvature. Three samples of each surface condition were analyzed and all images were collected at room temperature.

Surface chemistry analysis using XPS

XPS analysis was carried out using a Kratos Axis Ultra x-ray photoelectron spectrometer with concentric hemispherical electron energy analyzers combined with the established delay-line detector (DLD). The incident monochromatic Al $\text{K}\alpha$ X-ray (1486.6 eV) radiation at 150 W (accelerating voltage 15 kV, emission current 10 mA) was projected 45° to the sample surface and the photoelectron data was collected at a takeoff angle of $\theta = 90^\circ$. The absolute energy scale was calibrated to Copper (Cu) $2p_{3/2}$ peak binding energy at 932.6 eV using sputter-etched copper foil. The base pressure in the analysis chamber was maintained at 1.0×10^{-9} torr. Low energy electrons were used for charge compensation to neutralize the sample. Survey scans were taken at pass energy of 160 eV, and carried out over 1200 eV $\sim$ 5 eV binding energy range with 1.0 eV steps and a dwell time of 200 ms. High resolution scans of Ti 2p, O 1s, C 1s, N 1s and P 2p were taken at pass energy of 20 eV with 0.1 eV steps and a dwell time of 1000 ms. The spectra analysis was carried out using CasaXPS software

(version 2.3.17dev6.4k). Shirley type background was routinely used to account for inelastically scattered electrons that contribute to the broad background. Transmission corrected RFS/Kratos library relative sensitivity factors (RSFs) was used for elemental quantification. The spectra were calibrated using adventitious carbon C 1s peak at 285.0 eV.

Microstructural characterization of CpTi discs using X-ray diffraction (XRD)

X-ray powder diffraction is a well-established technique used to measure the degree of crystallinity of samples. The surface crystallinity and composition of PEI/*pBMP-2* nanoplex (N/P-10) coated and uncoated CpTi discs were analyzed using a Bruker D-5000 q - q diffractometer equipped with a Kevex energy-sensitive detector. Coupled 2 θ / θ scan type in the 2 θ range of 5° to 40° with a scanning step width of 0.01° was used to carry out all the measurements. The incidence and receiving slits were set at 1 mm and 0.013°, respectively. Copper (Cu) radiation of 1.5418 Å was used to record the data with the anode voltage and current set at 40 kV and 30 mA respectively. Diffrac suite software was used to analyze the data obtained.

Evaluation of cytotoxicity of CpTi discs coated with PEI/*pEGFP* nanoplexes

MTS cell growth assay reagent (Cell Titer 96 AQueous One Solution cell proliferation assay, Promega Corporation, Chicago, IL) was used to determine the cytotoxicity of CpTi discs coated with PEI/*pEGFP* nanoplexes prepared at various N/P ratios. BMSCs were plated at a seeding density of 80,000 cells/well 24 h before the assay. After 24 h, the medium was changed to serum free DMEM and the PEI/*pEGFP* nanoplexes (N/P-1, 5, 10, 15 and 20) coated CpTi discs were suspended into the 24 well plate as described earlier. The well plate was shaken using an orbital shaker at a speed of 60 rpm for 30 min at room temperature to force the release of nanoplexes from the CpTi disc surfaces. The well plate was further incubated for 3.5 h in a humidified incubator set at 37 °C and 5% CO₂ (Sanyo Scientific, Wood Dale, IL), after which the BMSCs were washed with 1X PBS and then 500 μ L of DMEM containing 10% FBS was added. After 20 h, the BMSCs were treated with 100 μ L of MTS assay reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). The plates were then incubated for 4 h at 37 °C and 5% CO₂. After 4 h, 120 μ L of the solution from each well was transferred to the wells of a 96 well plate and the cell viability was determined by assessing the relative levels of soluble formazan formed using SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA, USA) microplate reader at an absorbance of 490 nm. The following equation was used to calculate cell viability:

$$\% \text{ Cell Viability} = (\text{absorbance of assay sample at 490 nm}) / (\text{absorbance of untreated (control) sample at 490 nm}) \times 100$$

Determination of percent transfection using flow cytometry

Transfection efficiencies of PEI/*pEGFP* nanoplexes coated on CpTi discs were determined using flow cytometry. BMSCs were treated in a similar way as mentioned earlier. After incubating the BMSCs with CpTi discs for 48 h, DMEM was removed and the BMSCs were washed with 1X PBS and trypsinized using 0.25% trypsin. The BMSCs were then resuspended in DMEM containing 10% FBS to neutralize the trypsin. Cell suspensions were

then subjected to a FACScan (Becton Dickinson, Franklin, WI) flow cytometer equipped with a 15 mW, 488 nm argon ion laser. Forward scatter (FSC), side scatter (SSC), and green fluorescence (FL1) parameters were measured and the data from 5000 events was obtained. Dot-plots of FSC versus SSC were generated and by using the FlowJo software the percent of BMSCs that were positive for green fluorescence (FL1) compared to the controls was obtained. Percent of BMSCs expressing detectable green fluorescence were scored and compared to the total cell number. Positive events obtained from the BMSCs treated with uncoated CpTi disc controls were subtracted from the test samples to eliminate background noise.

Quantification and characterization of PEI/*pBMP-2* nanoplex release from the surface of CpTi discs

The CpTi discs were coated with PEI/*pBMP-2* nanoplexes at N/P-10 and attached to a 24 well plate lid as described earlier. CpTi discs coated with *pBMP-2* was used as a control. The coated CpTi discs were then suspended in the wells of a 24 well plate containing 500 μ L of 1X PBS/well in such a way that the CpTi disc surfaces containing the PEI/*pBMP-2* nanoplexes (N/P-10) and *pBMP-2* were submerged in PBS. At 1, 2, 3, 4, 8, 12 and 24 h, 100 μ L of the sample supernatants were removed and replaced with 100 μ L of fresh 1X PBS. To the 100 μ L of sample supernatants, heparin sodium salt (from porcine intestinal mucosa, Alfa Aesar A16198) was added to a final concentration of 5% w/v and incubated for 15 min. The solution was then centrifuged at $12,800 \times g$ for 2 min and the supernatant was analyzed using picogreen assay (Quant-iTTM PicoGreen[®] dsDNA Assay Kit – molecular probes by Life Technologies, Madison, WI). To further support the data obtained from the picogreen assay, gel electrophoresis was performed on the collected supernatant samples to determine if the presence of heparin had enhanced the separation of *pBMP-2* from PEI. The collected samples were loaded in 1% agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) with blue juice loading buffer for easy loading and tracking of *pBMP-2* samples in agarose in the presence of 1 $\times$ TAE buffer with 5 μ g/mL ethidium bromide. Electrophoresis was carried out at 80 mA and 150 V. After 2 h, the gel was analyzed using a UV transilluminator.

Determination of BMP-2 protein secretion using ELISA

BMP-2 protein secretion by BMSCs transfected with PEI/*pBMP-2* nanoplexes (N/P-10) (delivered through CpTi discs) was analyzed using BMP-2 ELISA kits (Quantkine, R&D Systems, Minneapolis, MN). BMSCs were treated in a similar manner as described earlier. On day 7 post-treatment with CpTi discs, 500 μ L of cell supernatant was collected and centrifuged at $230 \times g$ for 5 min to remove any cell debris prior to performing an ELISA. ELISAs were performed in accordance with the manufacturer's protocol (R&D systems, Minneapolis, MN, USA).

Determination of osteoblastic gene expression using real time PCR

Real time PCR was employed to determine osteoblastic gene (*Runx-2*, *alkaline phosphatase-ALP* and *osteocalcin*) expression in transfected cells.(12) BMSCs were treated in a similar manner as described earlier. On day 7 post-treatment with CpTi discs, the BMSCs were washed with 1X PBS and total cell RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) by following the manufacturer's protocol. Total RNA was eluted out

from homogenized BMSCs (QIAshredder column, Qiagen) using an RNeasy column. The amount and the quality of RNA isolated was measured using a NanoDrop 2000 UV–Vis spectrophotometer (Thermoscientific, Wilmington, DE) by assessing the absorbance at A260 nm and the ratio of A260/A280, respectively. The extracted RNA was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) and the process of reverse transcription was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, BioRad, Waltham, MA, USA). The mixture was subjected to varying temperatures of 25 °C (10 min), 48 °C (30 min), 95 °C (5 min) and finally cooled to 4 °C. 18S rRNA was used as an endogenous control and TaqMan Ribosomal RNA Control Reagent Kit (Applied Biosystems) was used for its detection. TaqMan Universal PCR Master Mix (Applied Biosystems), primers and probes for *runx-2*, *alkaline phosphatase* (*ALP*), *osteocalcin*, the endogenous 18S rRNA control, and the cDNA were all mixed in 96-well Optical Reaction Plates (Applied Biosystems). The real time PCR analysis was carried out in an Applied Biosystems 7300 Real Time PCR System, with thermal cycling parameters set at 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.(12) The mRNA levels were normalized with respect to 18S rRNA levels and measured relative to untreated controls by “the relative quantitation using comparative CT” in Multiplex Reactions (PerkinElmer).(12) One way ANOVA was used to analyze the fold change of comparative threshold values (2^{-CT}) values followed by Tukey’s post-hoc test.

Demonstration of mineralization by alizarin red staining and atomic absorption spectroscopy

Qualitative calcium detection using alizarin red staining—On day 30 post-treatment with CpTi discs, the BMSCs were washed twice with 1X PBS and then fixed with 10% formalin for 10 min. Then, 500 µl of 2% w/v alizarin red S solution (pH 4.2) was added to the wells and incubated for 10 min. The wells were then carefully washed five to six times with distilled water and observed under a light microscope and images were captured. The alizarin red stain percent was quantified using ImageJ software using Analyze-Measure tool. Percent surface area was calculated by dividing the approximate surface area covered by the stain to the total surface area multiplied by 100.

Quantitative determination of calcium content—The deposition of extracellular calcium was quantitatively measured using atomic absorption spectroscopy (PerkinElmer model 2380). The BMSCs on day 30 post-treatment with CpTi discs were hydrolyzed for 24 h using 1 mL of 0.6 N HCl in PBS per well. After 24 h, the solution was mixed well by pipetting several times so that the calcium present in the extracellular matrix (ECM) of BMSCs was released into the supernatant. Then, 450 µL of this supernatant was mixed with 550 µL of 2.5% lanthanum oxide in 0.6 N HCl. The atomic absorption spectrophotometer was operated at 422.7 nm wavelength, 0.7 nm slit width, with air–acetylene (55:15) as a recommended flame. Interference from the phosphates was removed by using lanthanum oxide. Commercial calcium standards were used to calibrate the instrument. To prepare the calcium standards, required volumes of 20 ppm calcium stock were dissolved in 0.6 N HCl in 1X PBS and 2% lanthanum oxide. The calcium standards and cell supernatants were directly injected into the instrument and calcium concentration readings were recorded.

Statistical Analysis

All the measurements were made in triplicates (n=3). Treatments and controls were compared using one way analysis of variance (ANOVA) and the statistical significance was determined using Tukey's post-hoc test by using statistical and graphing software GraphPad PRISM (GraphPad, San Diego, CA). Values are expressed as mean $\pm$ SEM, and *p*-values less than 0.05 were considered statistically significant.

Results and Discussion

Biocompatibility and osteoconductivity are generally considered key factors for the long term successful performance of an implant which conventional dental implants lack in select individuals with risk factors such as smoking.(14, 15) In order to overcome this problem, many implant topographical modifications such as grit blasting, acid etching, coating with ceramics such as calcium phosphates and bioglasses were explored to make the implant surface more osseointegrative.(16–18) However, ceramics cannot sustain high amounts of tensile strain and can undergo debonding, cracking and fragmentation from the titanium surface resulting in adverse tissue reactions *in vivo*.(19) In order to overcome these drawbacks, bioinspired organic bone implant coatings with organic compounds derived from ECM biomolecules such as fibronectin, vitronectin, type I collagen, osteopontin, sialoprotein and osteogenic proteins such as BMP, FGF, PDGF, TGF- β etc., are extensively studied.(20–24) However, these proteins upon adsorption to the implant surface can undergo conformational changes which can reduce their stability, resulting in reduced efficacy.(21) In addition and as mentioned previously, BMP as proteins are short lived and require high doses for their action. Thus, in this study PEI complexed BMP-2 encoded *pDNA* was employed as an alternative to direct BMP-2 protein delivery. The procedure used to coat the CpTi disc surfaces with PEI/*pBMP-2* nanoplexes in this study is novel and at the same time straight forward when compared to many other coating techniques. These complexes when freeze dried will be stable for at least 8 months, making it more clinically viable and translatable.(25)

Synthesis and characterization of PEI/*pBMP-2* nanoplexes

Nanoplexes of various N/P ratios of 1, 5, 10, 15 and 20 were prepared by maintaining the amount of *pDNA* constant at 50 μ g and varying the amount of PEI. Based on the mobility of *pDNA* in gel electrophoresis (figure not shown) it was determined that the amount of PEI used at N/P-5 ratios or greater has the ability to form stable nanoplexes with *pDNA*.(12) Size and surface charge of particles have a significant impact on cell viability and cellular uptake. Particles lower than 150 nm in diameter and with a positive surface charge are considered highly amenable to endocytosis.(26) The size (fig 1-I) of PEI/*pBMP-2* nanoplexes with N/P ratios of 1 to 20 ranged from 155 nm to 80 nm with a polydispersity index (PDI) ranging between 0.1 and 0.3. The zeta potential of PEI/*pBMP-2* nanoplexes prepared at N/P ratios of 1 to 20 ranged between –40 mV and +45 mV. It was found that by increasing the amount of PEI, the size of nanoplexes decreased and the zeta potential became more positive.

Surface topographical analysis of CpTi discs

Several studies demonstrated the importance of surface roughness of CpTi discs on cellular attachment and proliferation.(27, 28) In this study, the difference in the surface topography of sand blasted CpTi discs before and after coating with PEI/*pBMP-2* nanoplexes (N/P-10) were analyzed using SEM (Fig 1-II) and AFM (Fig 2). SEM images showed that, although the PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi disc surfaces had a glazed appearance, no significant morphological differences in the nanoplex coated CpTi disc surface compared to the uncoated CpTi disc surface were evident. The deposited nanoplex coatings were found to be crack free and exhibited a uniform homogenous distribution over the CpTi disc surface. AFM measurements were carried out to confirm the results obtained from SEM. The two-dimensional amplitude retrace AFM images, shown in Fig 2-Ia and IIa, indicate that there was no significant change in the surface morphology of PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi disc surfaces compared to the uncoated CpTi disc surfaces. The average roughness (R_a) was found to be $\sim 0.450 \mu\text{m}$ for both PEI/*pBMP-2* nanoplex (N/P-10) coated and uncoated CpTi disc surfaces. Thus, the CpTi surfaces (both nanoplex coated and uncoated) used in this study can be considered as minimally rough.(29) Furthermore, the height distributions (Fig 2-Ib and IIb) of both the PEI/*pBMP-2* nanoplex (N/P-10) coated and uncoated CpTi disc surfaces were found to be symmetric and followed gaussian distribution, in addition to root mean square roughness (RMS-Rq) being similar between coated and uncoated discs (Fig 2-III) and an Rq/Ra of 1.22 indicating that the surfaces were homogeneous.(30) The surface uniformity, homogeneity and platykurtic nature are the essential parameters which can ultimately affect the cellular attachment, proliferation and differentiation *in vitro* and osseointegration *in vivo*. The surface coating seemed to have very little effect on these above parameters.

Surface chemistry analysis using X-ray photoelectron spectroscopy

Analysis of the atoms present on the surface of PEI/*pBMP-2* nanoplex (N/P-10) coated and uncoated CpTi discs was performed using XPS. The detection of the presence of titanium, aluminum, oxygen, nitrogen, phosphorus and their relative levels on the uncoated CpTi discs, PEI, *pBMP-2* and PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi disc surfaces was the key purpose of using XPS. Figure 3-IA shows the XPS survey level spectra of uncoated CpTi discs and demonstrated the presence of oxygen, carbon, aluminum and titanium with atomic ratios of 60.95, 11.83, 18.21 and 8.02%, respectively. Aluminum was present due to the residual Al_2O_3 particles left on the CpTi disc surfaces after standard sand blasting procedure. Adventitious carbon, which was used as a reference to calibrate the XPS spectra, and minor contamination of fluorine were also detected. No nitrogen or phosphorus was detected on the uncoated CpTi discs. Ti 2p core level spectrum (Fig 3-IA inset) of the uncoated CpTi disc shows the binding energy of Ti 2p_{3/2} at 458.9eV shifted from 454.1eV (metal Ti) due to oxygen binding. This Ti 2p orbital doublet separation by 5.7 eV is characteristic of TiO_2 . Survey spectrum (Fig 3-IB) of the CpTi disc coated with *pBMP-2* showed the presence of oxygen, carbon, nitrogen, phosphorus, aluminum and titanium in atomic ratios of 47.78, 27.51, 2.56, 3.22, 12.26, and 4.94%, respectively. Nitrogen and phosphorus were detected at a ratio of 0.8, which was the result of the chemical structure of *pBMP-2*. Survey spectrum (Fig 3-IC) of the CpTi disc coated with PEI demonstrated the presence of oxygen, carbon, nitrogen, phosphorous, aluminum and titanium in the atomic

ratios of 31.57, 43.85, 10.78, 1.94, 7.83, and 2.52%, respectively. The atomic ratio of nitrogen to phosphorus got substantially increased to 5.5 on the PEI coated CpTi disc, when compared to the ratio of 0.8 on the CpTi disc coated with *pBMP-2*, which was due to the presence of a large amount of primary, secondary and tertiary amines in PEI and this occurrence of abundant nitrogen has contributed to the increase in the nitrogen to phosphorus atomic ratio. CpTi discs coated with PEI/*pBMP-2* (N/P-10) nanoplexes (Fig 3-ID) demonstrated the presence of oxygen, carbon, nitrogen, phosphorus, aluminum and titanium in the atomic ratios of 44.83, 26.72, 4.95, 2.82, 13.71, and 5.82%, respectively. The ratio of nitrogen to phosphorus for PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi disc was 1.8 and this value was in between the atomic ratio of *pBMP-2* coated CpTi disc (0.8) and PEI coated CpTi disc (5.5). Minor contaminants such as fluorine, calcium, zinc and sodium were also detected. Figure 3-II A, B and C demonstrates the core level spectra of N 1s on CpTi discs coated with *pBMP-2*, PEI and PEI/*pBMP-2* nanoplexes (N/P-10), respectively. Five nitrogen peaks, N₁, N₂, N₃, N₄ and N₅ were detected with the binding energies of 401.5, 400.3, 399.4, 398.3 and 397.2 eV, corresponding to -NH₃⁺, -NH₂, -NH-, -N= and TiN, respectively. Appearance of N₅ in PEI indicates its reaction with Ti forming TiN. Along with TiN nitrogen, the protonated amine group was also detected on the PEI coated CpTi disc. Results from the CpTi disc coated with PEI/*pBMP-2* nanoplexes (N/P-10) showed five peaks with particularly intense N₃ signals confirming the presence of *pBMP-2* and PEI.

Microstructural characterization of CpTi discs using X-ray diffraction (XRD)

The X-ray diffraction patterns of both the PEI/*pBMP-2* nanoplex (N/P-10) coated and uncoated CpTi discs (Fig 3-III) showed the presence of Ti element as indicated by the 2θ values at 35°, 38.5°, 40.2° and 53°. The 2θ values at 25.5°, 35°, 43.5°, and 57.5° represents aluminium oxide (corundum). The presence of Al₂O₃ was the result of its deposition during the sand blasting process. There were no detectable differences in the peak intensities and the peak position patterns of the uncoated CpTi discs and the PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs. Thus, it can be concluded that there were no significant differences in the elemental composition and crystalline nature of the PEI/*pBMP-2* nanoplex (N/P-10) coated and uncoated CpTi discs.

N/P ratio of nanoplexes can significantly affect cell survival

In order to obtain optimal protein expression from a transfected cell population, a balance between percent uptake (of nanoplexes) and percent cell viability is desired.(31) The cytotoxicity of PEI/*pEGFP* nanoplexes coated on the surface of CpTi discs at N/P ratios of 1, 5, 10, 15 and 20 was tested using an MTS assay (Fig 4-I). BMSCs treated with uncoated CpTi discs, PEI coated and *pEGFP* coated CpTi discs served as controls. Previous data reported (with nanoplexes alone) that 4 h is an optimal time point for achieving high cell viabilities and effective transfections. Therefore in this study, BMSCs were treated with both the treatment and control CpTi discs for 4 h. After 4 h, the BMSCs were washed to remove any extra cellular nanoplexes and the CpTi discs were resuspended in respective wells.(32) The results demonstrate that the BMSCs treated with *pEGFP* coated CpTi discs and PEI/*pEGFP* nanoplex coated CpTi discs (N/P-1 and N/P-5) had cell viabilities ranging between 90 and 95%. Whereas, the BMSCs treated with PEI/*pEGFP* nanoplex (N/P-10) coated CpTi discs had ~75% cell viability and the BMSCs treated with PEI/*pEGFP* nanoplex (N/P-15

and N/P-20) coated CpTi discs had a cell viability ranging between 50 and 60%. From the above data, it is clear that the CpTi discs coated with PEI/*pEGFP* nanoplexes prepared at N/P ratios of 15 and 20 demonstrated high cytotoxicity which is probably due to the increased toxicity of free uncomplexed PEI.(33) These results are consistent with previous studies which reported that increasing the N/P ratio of nanoplexes increases cytotoxicity.(34)

Transfection efficiency depends on the N/P ratio of nanoplexes

Transfection efficiencies of PEI/*pEGFP* nanoplexes at N/P ratios of 5, 10 and 15 coated on the surface of CpTi discs were analyzed using flow cytometry (Fig 4-II). Transfection efficiency was measured via EGFP expression. It was found that the BMSCs treated with PEI/*pEGFP* nanoplex (N/P-5, N/P-10 and N/P-15) coated CpTi discs resulted in a 2%, 14% and 11% EGFP expression, respectively. Treatment of BMSCs with PEI/*pEGFP* nanoplex (N/P-10) coated CpTi discs resulted in an almost 10-fold ($*p < 0.05$) and a 7-fold ($*p < 0.05$) enhancement in the EGFP expression compared to the BMSCs treated with uncoated CpTi discs and PEI/*pEGFP* nanoplex (N/P-5) coated CpTi discs, respectively. These results were consistent with the size and surface charge of nanoplexes prepared at these different N/P ratios. The nanoplexes with higher surface charge and smaller size resulted in greater transfection compared to the nanoplexes with lower charge and larger size.

Quantification and characterization of PEI/*pBMP-2* nanoplex (N/P-10) release from the surface of CpTi discs

Quantification of PEI/*pBMP-2* nanoplex (N/P-10) release from the CpTi discs was performed using a PicoGreen assay. Several studies reported that the fluorescence produced by PicoGreen probes can get drastically reduced due to the interference caused by PEI complexation with *pDNA*.(35) Therefore, in this study heparin was used to separate *pBMP-2* from PEI. Gel electrophoresis (Fig 5-I) demonstrated that the addition of heparin to the nanoplexes (N+H) resulted in the release of *pBMP-2* into the gel, whereas the nanoplexes without heparin addition (N-H) resulted in no detectable release of *pBMP-2*. *pBMP-2* with heparin (P+H) and *pBMP-2* without heparin (P-H) served as controls. Following the collection of nanoplex samples, as detailed in the methods section, *pBMP-2* was separated from PEI using heparin and the amount of *pBMP-2* released was quantified using a PicoGreen assay at various time points. Fig 5-II demonstrates that, during the first 4 h, there was an almost 68% and 88% release of *pBMP-2* complexed and uncomplexed with PEI, respectively. This behavior clearly indicates that the release of PEI complexed *pBMP-2* from the CpTi disc surfaces was more sustained compared to the uncomplexed *pBMP-2*. This sustained release of nanoplexes can be speculated to be due to the electrostatic interactions between negatively charged hydroxyl groups present on the CpTi disc surfaces and the positively charged PEI. This is further supported by the XPS data which showed the presence of oxygen on CpTi discs indicating the formation of titanium oxide.

Treatment with PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs resulted in a significant enhancement of BMP-2 protein expression

ELISA was employed to analyze the amount of BMP-2 produced by the BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs in comparison with the BMSCs treated with *pBMP-2* coated, PEI coated, and uncoated CpTi discs, which served as controls.

Fig 6-I demonstrates that the BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs resulted in a significant 1.9-fold ($**p < 0.01$), 1.6-fold ($*p < 0.05$) and 3-fold ($***p < 0.001$) enhancement in the BMP-2 expression compared to the BMSCs treated with uncoated CpTi discs, *pBMP-2* coated and PEI coated CpTi discs, respectively. These results suggest that the BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs resulted in a significant internalization of *pBMP-2* when compared to the BMSCs treated with *pBMP-2* coated CpTi discs (as is also evident from EGFP expression data).

Enhancement in the osteogenic marker expression due to PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi disc treatment

Runx-2 is known to regulate the expression of many osteoblast-specific genes and is also considered to be an inhibitor of muscle tissue formation or myogenesis.(36, 37) In this study, the level of *Runx-2* gene expression was determined using real time PCR on day 7 post-treatment with CpTi discs. Here, BMSCs treated with uncoated CpTi discs, *pBMP-2* coated and PEI coated CpTi discs served as controls. Fig 6-IIa demonstrates that, although there were no significant differences in *Runx-2* gene expressions between the treatments and controls, there was a 1.8, 1.5 and 4 fold enhancement in *Runx-2* gene expression in BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs compared to the BMSCs treated with uncoated CpTi discs, PEI coated and *pBMP-2* coated CpTi discs respectively. ALP is a metalloenzyme and high levels of ALP expression in bone were reported almost a century ago by Robison et al.(38) and is known to be among the first few osteogenic markers expressed in the calcification process.(39) Fig 6-IIb demonstrates the expression of the *ALP* gene in BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated, *pBMP-2* coated, PEI coated and uncoated CpTi discs serving as controls. BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs showed a significant enhancement in *ALP* gene expression ($*p < 0.05$), compared to the controls. *Runx-2* and *ALP* are known to be expressed in the early stages of bone development, whereas in the later stages, the expression of osteocalcin is upregulated.(40) *Osteocalcin* is one among the few bone specific genes which is extensively studied in the context of osteogenic differentiation of mesenchymal stem cells (MSCs).(41–44) It is also known to upregulate bone matrix deposition and is considered to be a true osteoblast-specific gene.(12, 45) In this study, the *osteocalcin* gene expression was analyzed using real time PCR on day 7 post-treatment with CpTi discs. It was shown (Fig 6-IIc) that although there were no significant differences between the treatments and controls, there was an almost 2-fold enhancement in *osteocalcin* expression in the BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs compared to the BMSCs treated with uncoated CpTi discs. This data suggests that, although insignificant, there was a trend toward upregulation of the osteogenic genes in the BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs compared to the controls which indicates that the PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs have osteogenic potential.

Demonstration of mineralization by alizarin red staining and atomic absorption spectroscopy

In this experiment the effect of PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs on calcium deposition and mineralization of BMSCs was investigated both qualitatively and

quantitatively using alizarin red staining and atomic absorption spectroscopy, respectively, on day 30 post-treatment with CpTi discs. Alizarin red staining (Fig 7) showed that the BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs resulted in a more intense alizarin red staining (Fig 7-Id) compared to the BMSCs treated with uncoated CpTi discs (Fig 7-Ia), *pBMP-2* coated (Fig 7-Ib), and PEI coated CpTi discs (Fig 7-Ic). In addition, the amount of stain obtained was quantified using ImageJ software (Fig 7-II). This data demonstrated a significant enhancement ($*p < 0.05$; $**p < 0.01$) in the alizarin red stain in BMSCs treated with PEI/*pBMP-2* nanoplex (N/P-10) coated titanium disc compared to the other controls. The amount of calcium produced was also quantified using atomic absorption spectroscopy on day 30 post-treatment with CpTi discs. Results (Fig 7-III) show that the BMSCs treated with the PEI/*pBMP-2* nanoplex (N/P-10) coated titanium disc resulted in an almost 2-fold ($*p < 0.05$) and 3-fold ($*p < 0.05$) enhancement in calcium ion deposition compared to the BMSCs treated with uncoated CpTi discs and *pBMP-2* coated CpTi discs, respectively.

Conclusions

Within the limits of this *in vitro* proof of concept study, the following conclusions were made:

1. CpTi disc surfaces modified with PEI/*pBMP-2* nanoplex coating can enhance the local production of BMP-2 protein which further enhances osteogenesis as determined by the increased expression of *Runx-2*, *alkaline phosphatase* and *osteocalcin* activity.
2. This study demonstrates a new approach to modifying CpTi disc surfaces with the goal of promoting osteogenesis and osseointegration.
3. In future studies, *in vivo* studies evaluating the osseointegrative attributes of this coating strategy in comparison with BMP-2 protein coating, would be valuable to move this approach closer to clinical applications.

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Fig 1.
I) Chart showing the particle size and zeta potential of PEI/*pBMP-2* nanoplexes formed at N/P ratios of 1, 5, 10, 15 & 20. Values are expressed as mean $\pm$ SEM with $n = 3$. II) SEM images showing the surface topography of (a) Uncoated and (b) PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs.

Fig 2. AFM images showing the surface topography of (I) Uncoated and (II) PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs. a) Amplitude retrace and b) Histograms. III) Graph representing the root mean square roughness (RMS-R_q) of uncoated (red) and PEI/*pBMP-2* nanoplex (N/P-10) coated (blue) CpTi discs. Values are expressed as mean $\pm$ SEM with n = 3.

Fig 3. Plots representing (I) XPS survey level scan spectra of: A) Uncoated B) *pBMP*-2 coated C) PEI coated and D) PEI/*pBMP*-2 nanoplex (N/P-10) coated CpTi discs. (II) XPS core level spectra of A) *pBMP*-2 coated B) PEI coated and C) PEI/*pBMP*-2 nanoplex (N/P-10) coated CpTi discs. (III) X-ray diffraction patterns of uncoated (red) and PEI/*pBMP*-2 nanoplex (N/P-10) coated CpTi discs (blue).

Fig 4.

I) Chart demonstrating the MTS cell viability analysis of BMSCs treated with PEI/*pEGFP* nanoplex coated CpTi discs at various N/P ratios (indicated). II) Histogram demonstrating the percent EGFP expression by BMSCs treated with PEI/*pEGFP* nanoplex coated CpTi discs at various N/P ratios (indicated) obtained by flow cytometry. BMSCs treated with uncoated, *pEGFP* and PEI coated CpTi discs served as controls. One-way ANOVA was employed to assess the significant differences between treatments and controls followed by Tukey's post-test ($*p < 0.05$). Values are expressed as mean $\pm$ SEM with $n = 3$.

Fig 5.

I) Gel electrophoresis image demonstrating the effect of heparin sodium salt on separating *pBMP-2* from PEI. In the figure, N+H represents PEI/*pBMP-2* nanoplex (N/P-10) with heparin, N-H indicates PEI/*pBMP-2* nanoplex (N/P-10) without heparin or nanoplex alone, P+H represents *pBMP-2* with heparin and P-H represents *pBMP-2* without heparin or *pBMP-2* alone. *pBMP-2* with (P+H) and without (P-H) heparin. II) Graph demonstrating the cumulative release profile of PEI/*pBMP-2* nanoplexes (N/P-10) from the CpTi discs with respect to time obtained using PicoGreen assay. *pBMP-2* coated CpTi discs served as controls. Values are expressed as mean $\pm$ SEM with n = 3.

Fig 6.

I) ELISA data demonstrating the amount of BMP-2 produced by BMSCs on day 7 post-treatment. II) Osteogenic gene expression by BMSCs on day 7 post-treatment obtained using real time PCR analysis (a) *Runx-2* expression (b) *Alkaline phosphatase (ALP)* expression and (c) *Osteocalcin* expression. Here PEI/pBMP-2 nanoplex (N/P-10) coated CpTi discs served as treatments and uncoated CpTi discs, pBMP-2 and PEI coated CpTi discs served as controls. One-way ANOVA was employed to assess the significant differences between treatments and controls followed by Tukey's post-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Values are expressed as mean $\pm$ SEM with $n = 3$.

Fig 7.

I) Light microscopic images of alizarin red stained BMSCs on day 30 post-treatment as an indication of mineralization. a) BMSCs treated with uncoated; b) *pBMP-2* coated; c) PEI coated; and d) PEI/*pBMP-2* nanoplex (N/P-10) coated CpTi discs; BMSCs treated with uncoated, *pBMP-2* and PEI coated CpTi discs served as controls. II) Chart representing the alizarin red stain quantification data of light microscopic images for indicated treatments obtained using ImageJ software. III) Atomic absorption spectrophotometric results of calcium levels produced by BMSCs on day 30 post-treatment. One-way ANOVA was employed to assess the significant differences between treatments and controls followed by Tukey's post-test (**p* < 0.05; ***p* < 0.01). Values are expressed as mean ± SEM with n = 3.