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Pharmacokinetics and bone formation by BMP-2 entrapped in polyethylenimine-coated albumin nanoparticles

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ABSTRACT

The osteoinductive growth factor, bone morphogenetic protein-2 (BMP-2), is capable of inducing *de novo* bone formation after implantation. A nanoparticulate (NP) system was developed for BMP-2 delivery based on NPs fabricated from bovine serum albumin (BSA) and stabilized by polyethylenimine (PEI) coating. In this study, the pharmacokinetics and osteoinductivity of BMP-2 delivered with different BSA NP formulations were determined by subcutaneous implantation in rats. A 7-day pharmacokinetics study showed that PEI coating on NPs effectively reduced the initial burst release of BMP-2 and prolonged the BMP-2 retention at implantation site. However, the uncoated BMP-2 NPs (BMP-2 loading of 1.44% w/w) were able to induce a robust ectopic bone formation, while no bone formation was found by the BMP-2 NPs coated with PEI. The toxicity of the PEI used for NP coating was determined to be the reason for lack of osteoinduction. Increasing BMP-2 loading (up to 5.76% w/w) was then employed to formulate NPs with lower PEI content; the higher BMP-2 loading was found to better promote induction of *de novo* bone. Our findings indicated that PEI coating on BSA NPs was effective for controlling BMP-2 release from NPs, but the toxicity of cationic PEI was a concern for the osteoinductive activity, which should be alleviated by further optimization of NP formulations.

1. Introduction

The bone morphogenetic proteins (BMPs) are a group of secreted proteins that belong to the transforming growth factor-β (TGF-β) superfamily [1]. BMPs exert an osteoinductive effect by acting on non-committed progenitor cells and causing cell differentiation into the bone-depositing osteoblast phenotype. Recombinant human BMP-2 has been shown to induce osteogenesis at various sites in many animal species [2–7]. The high potency of BMP-2 to successfully stimulate bone formation at implantation sites makes it an alternative for autogenous bone grafting in bone healing, obviating the need for graft harvest, and eliminating the donor-site morbidity in patients [8–10]. Currently, BMP-2 is clinically utilized for treatment of bone fractures and in spinal fusion procedures [11,12]. The clinical administration of BMP-2 was focused on local BMP-2 delivery. Studies showed that BMP induces bone induction only locally at the site of implantation, and the bone induction is limited temporally only to the time when the BMP is present [13]. Because BMP-2 diffuses rapidly from the site of administration when applied without a carrier, it has been widely accepted that BMP-2 needs a biomaterial carrier in order to maximize the induced osteogenic effect. The carrier maintains BMP-2 at the treatment site, optimizing the release profile of BMP-2, and restricts BMP-2 to the site of application to prevent extraneous bone formation [14,15].

The BMP-2 has been delivered with different carriers for ectopic and orthotopic bone regeneration, including inorganic materials (e.g. hydroxyapatite [16], tricalcium phosphate [17] and calcium phosphate-based cements [18]), synthetic polymers (e.g. polylactide [19], polyglycolide [20], and poly (D,L-lactide)-*co*-glycolide [21]), natural polymers (e.g. collagen [22] and hyaluronan [23]) and the composites of the above three materials [24–27]. The disadvantages associated with inorganic and synthetic carriers for BMP-2 are the poor biodegradability and the possible inflammatory response, respectively [28]. Currently, one of the most effective carriers for BMP-2 delivery is the Type I bovine absorbable collagen sponge (ACS) [15]. As a natural polymer, it can be transformed into a porous structure with excellent operational properties, and good biocompatibility with tissues [29,30]. The carrier properties of ACS have been extensively studied [31–33] and it

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was found acceptable for clinical delivery of BMP-2. The ACS/BMP-2 combination, however, has some inherent problems in controlling the release rate of BMP-2, often resulting in a high initial burst release, and consequently unable to provide a long-term BMP-2 release [26,34].

Polymer-based nanoparticle (NP) systems have been developed since the early 1980s for drug delivery and targeting purposes [35,36]. The NPs are a colloidal system in the sub-micron size range. NPs possess the potential of eliminating problems associated with non-specific drug distribution, and rapid breakdown and/or clearance of drugs *in vivo*. By encapsulating the drug in a protective environment, NPs increase the drug's bioavailability in circulation and may provide a sustained release that may be beneficial for local application [35]. NPs made from natural or synthetic materials have been explored for protein delivery [37]. Previously, we reported a coacervation method to fabricate BMP-2-containing bovine serum albumin (BSA) NPs with a polyethylenimine (PEI) coating [38]. These NPs were prepared without any chemical modification (e.g., glutaraldehyde cross-linking) of the ingredients, so that the integrity of the BMP-2 was not compromised after the process of NP entrapment. The encapsulation efficiency of BMP-2 in BSA NPs was typically greater than 90%, making it suitable for pharmaceutical development. The surface coating with cationic polymer PEI effectively controlled the release of BMP-2 from the NPs under *in vitro* culture conditions [38]. The released BMP-2 was capable of inducing the alkaline phosphatase (ALP) activity in human C2C12 cells *in vitro* with equivalent potency to that of the free BMP-2 [38]. However, the NP formulation of BMP-2 was not previously evaluated in an animal model, and it was not known if the prepared formulations were osteoinductive *in vivo*.

In this study, PEI-coated BMP-2-containing BSA NPs were evaluated in a subcutaneous implant model with two main goals: i) to examine the pharmacokinetics of BMP-2 release; and ii) to assess the osteoinductive activity of the NP formulations of BMP-2. The BMP-2-containing NPs were soaked into ACS implants, and then subcutaneously implanted into rats. In the pharmacokinetics study, the local BMP-2 concentration was determined by using ^{125}I-labeled BMP-2 and it was our intent to determine if the PEI coating influenced the BMP-2 release *in vivo*. The rat ectopic assay was chosen for assessing osteoinductive activity of BMP-2 formulations, due to its robustness and its long-history of use to investigate bioactivity of BMP delivery systems for bone formation.

2. Materials and methods

2.1. Materials

BSA, branched PEI (M_w w25,000 by light scattering, M_n w10,000 by gel permeation chromatography), ALP substrate *p*-nitrophenol phosphate (*p*-NPP), 2-amino-2-methyl-1-propanol (AMP), 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (TCDG), *o*-cresolphthalein, 8-hydroxyquinoline, trichloroacetic acid (TCA) and calcium assay standards were obtained from Sigma–Aldrich (St. Louis, MO, USA). Recombinant Human Bone Morphogenetic Protein-2 (BMP-2, from *Escherichia coli*) was prepared as described in Ref. [39]. Na^{125}I (in 0.1 M NaOH) was obtained from GE Healthcare (Piscataway, NJ, USA). Metofane™ (methoxyflurane) was obtained from Janssen Inc. (Toronto, ON, Canada). Sterile saline used for implantation (0.9% NaCl, non-pyrogenic) was obtained from Baxter Corporation (Toronto, ON, Canada). Hemostatic Absorbable Collagen Sponge (ACS, Helistat®) was obtained from Integra Life Sciences Corporation (Plainsboro, NJ, USA). Triton X-100 was obtained from Feinbiochemica (Heidelberg/NY, USA). Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), penicillin (10,000 U/mL) and streptomycin (10,000 μg/mL) were from Invitrogen (Carlsbad, CA, USA). Rat osteocalcin Enzyme Immunoassay (EIA) Kit was from Biomedical Technologies Inc. (Stoughton, MA, USA). All tissue culture plasticware was from Corning (Corning, NY, USA). Where indicated, in-house prepared distilled/de-ionized water (ddH_2O) was used for buffer preparations and for dialysis, and it was derived from a Milli-Q purification system (Millipore; Billerica, MA, USA).

2.2. NP preparation

The NPs were fabricated by the same procedure described previously [38]. The BMP-2:BSA mass ratios were controlled during the preparation of individual batches of NPs (see Section 3). The BMP-2 encapsulation efficiency was assumed to be 100%. For the coating, 300 μL of NPs was typically mixed with 375 μL of 0.6 or 0.1 mg/mL PEI solution for 1 h, respectively. The resultant 675 μL of NP solution was centrifuged at 8000 rpm for 10 min to remove the supernatant containing ethanol, free polymer and NaCl. For *in vitro* ALP studies, the obtained pellet was re-dispersed in 675 μL of DMEM containing 1% of penicillin/streptomycin; while for implantation preparations, such a pellet was re-dispersed in 50 μL of saline (concentrated by 13.5-fold), to achieve 3 μg of BMP-2 per 50 μL NP re-dispersion.

To investigate the effect of BMP-2 loading on osteoinductive activity, 200 μL of 10 mg/mL BSA in ddH_2O was mixed with 28.8, 57.6 and 115.2 μL of 1.0 μg/μL of BMP-2 solution to give 1.44%, 2.88% and 5.76% w/w BMP-2 loaded NPs. After 15 min of mixing (600 rpm), NaCl solution was added to each NP formulation to give 10 mM NaCl solution. After 2 h of mixing, 2.4 mL of ethanol was added dropwise to each formulation. The desolvation was allowed to proceed for 3 h, and 300 μL of NP solution was then added to 375 μL of 0.1 mg/mL PEI solution for coating. After 1 h of coating, the NPs were centrifuged at 8000 rpm for 10 min. The pellets were concentrated and re-suspended in sterilized phosphate buffered saline (PBS × 1, pH = 7.3) to obtain 3 μg of BMP-2 per 50 μL of NP re-dispersion for each formulation.

2.3. Assessment of ALP induction by NP formulation of BMP-2

The activity of BMP-2 in NP formulations was assessed *in vitro* by determining its ability to induce ALP activity in human C2C12 cells. Five groups were examined in the first experiment: (1) BSA NPs without any BMP-2; (2) free BMP-2 solution; (3) uncoated BMP-2/BSA NPs; (4) 0.1 mg/mL PEI-coated BMP-2/BSA NPs, and (5) 0.6 mg/mL PEI-coated BMP-2/BSA NPs. The free BMP-2 group was prepared from a stock solution of 0.5 μg/μL BMP-2 by diluting it to1.0 μg/mL, with the use of BSA and NaCl mixture that of the same ratio for making NPs. Six aliquots were prepared for each group for the 6 time points to be examined. These aliquots were placed on an orbital shaker, and incubated at 37 °C with 5% of CO_2 under gentle shaking. At designated time point (1, 5, 8, 14, 22 and 28 days), one aliquot from each group was taken out, centrifuged (10 min at 8000 rpm) to separate the supernatant and the pellet. The pellets were re-dissolved in 675 μL of DMEM/1% penicillin–streptomycin. Human C2C12 cells were seeded in 48-well plates, and used after w24 h of seeding. Each sample from the five groups was added to the cells at volumes of 100, 50, 25 and 0 μL, with the final culture medium of 0.5 mL/well. The final BMP-2 concentration in the 0.5 mL culture medium was estimated to be 1.0 μg/mL for the 100 μL sample adding in all groups except the blank NPs. A kinetic ALP assay was performed to determine the bioactivity of encapsulated BMP-2 as described before [38].

In order to determine the *in vitro* bioactivity of the implant formulation with different BMP-2 loadings, the NP samples were produced by the same procedure as in the implant preparations, except that the BMP-2 loading was scaled down (e.g., 1.44% w/w BMP-2/BSA was reduced to 0.144% w/w BMP-2/BSA) to adjust the BMP-2 concentrations to the working range of the bioassay. The NPs were prepared with three different BMP-2 loadings (0.14%, 0.29% and 0.58%), and coated with 0.01, 0.03 and 0.1 mg/mL PEI, respectively, with the uncoated BMP-2/BSA NPs serving as control. Immediately following the fabrication, NPs from different groups were separated into the supernatant and pellet fractions by centrifugation at 8000 rpm for 10 min. The supernatant was discarded, and the pellet was re-dispersed into DMEM/1% penicillin–streptomycin. The re-dispersion was centrifuged again to separate the supernatant and pellet for ALP assay. There were two volumes of pellet dispersion that were incubated with C2C12 cells for each BMP-2 dosage, giving estimated concentrations of 1.0 and 0.5 μg/mL of BMP-2 in the culture medium. For the supernatant fraction, the same volumes as for the pellet dispersion were added to cells and the kinetic ALP assay was performed as described before [38].

2.4. Pharmacokinetics study

2.4.1. Preparation of BSA NPs containing ^{125}I-labeled BMP-2 [38]

The BMP-2 was labeled with ^{125}I [40] to determine the retention of BMP-2 encapsulated in NPs. Microcentrifuge tubes were coated with TCDG (200 μL of 20 μg/mL TCDG in chloroform), and 20 μL of BMP-2 solution (containing 10 μg of BMP-2) was added to the coated tubes, along with 50 μL of 0.1 M phosphate buffer (pH = 4.5) and 20 μL of 0.2 mCi of Na^{125}I in 0.1 M NaOH. After reacting for 25 min, free ^{125}I was separated from the radiolabeled BMP-2 by using a Sephadex G-25 column. After precipitating an aliquot of the purified samples with 20% (w/v) TCA, the counts in the supernatant and the pellet were determined with a γ-counter (Wizard 1470; Wallac, Turku, Finland), and it was confirmed that the iodinated sample contained < 5% free ^{125}I. After iodination, 10 μL of ^{125}I-labeled BMP-2 was first diluted with 40 μL of ddH_2O, and then mixed with BSA solution for NP preparation. 250 μL of 10 mg/mL BSA solution was added to 250 μL of 10 mM NaCl solution (pH = 7.0) in a glass vessel under constant stirring at room temperature (600 rpm for 15 min). Then, 50 μL of ^{125}I-labeled BMP-2 in ddH_2O was added to this solution. This solution was desolvated with dropwise addition of 3.0 mL of ethanol and mixed (600 rpm) at room temperature for 3 h. The NPs so formed

Table 1
Experimental groups for the three osteoinduction studies conducted in the rat subcutaneous implant model.

Study	Implant set	Group	BMP-2 dose per implant (μg)	Number of animals (number of implants)	Harvest (days)	Analysis
I[a]	G1	ACS	0	3 (6), 3 (6)	10, 16	Weight, Ca^{2+}, ALP
	G2	ACS + BMP-2	3	3 (6), 3 (6)	10, 16	Weight, Ca^{2+}, ALP
	G3	ACS + BMP-2/BSA NP	3	3 (6), 3 (6)	10, 16	Weight, Ca^{2+}, ALP
	G4	ACS + 0.6 mg/mL PEI-coated BMP-2/BSA NP	3	3 (6), 3 (6)	10, 16	Weight, Ca^{2+}, ALP
II[a]	G1	ACS	0	3 (6), 3 (6)	14, 21	Weight, Ca^{2+}, ALP, Micro-CT
	G21	ACS + BMP-2/BSA NP	3 + 0	3 (6), 3 (6)	14, 21	Weight, Ca^{2+}, ALP, Micro-CT
	G22	ACS + BMP-2/BSA NP	3 + 3	3 (6), 3 (6)	14, 21	Weight, Ca^{2+}, ALP, Micro-CT
	G31	ACS + 0.6 mg/mL PEI-coated BMP-2/BSA NP	3 + 0	3 (6), 3 (6)	14, 21	Weight, Ca^{2+}, ALP, Micro-CT
	G32	ACS + 0.6 mg/mL PEI-coated BMP-2/BSA NP	3 + 3	3 (6), 3 (6)	14, 21	Weight, Ca^{2+}, ALP, Micro-CT
	G41	ACS + 0.1 mg/mL PEI-coated BMP-2/BSA NP	3 + 0	3 (6), 3 (6)	14, 21	Weight, Ca^{2+}, ALP, Micro-CT
	G42	ACS + 0.1 mg/mL PEI-coated BMP-2/BSA NP	3 + 3	3 (6), 3 (6)	14, 21	Weight, Ca^{2+}, ALP, Micro-CT
III	G1	ACS	0	3 (6), 3 (6)	17, 23	Weight, Ca^{2+}, ALP, Micro-CT
	G2	ACS + BMP-2/BSA NPs (5.76%)	3	3 (6), 3 (6)	17, 23	Weight, Ca^{2+}, ALP, Micro-CT
	G3-5.76	ACS + 0.1 mg/mL PEI-coated BMP-2/BSA NP (5.76%)	3	3 (6), 3 (6)	17, 23	Weight, Ca^{2+}, ALP, Micro-CT
	G4-2.88	ACS + 0.1 mg/mL PEI-coated BMP-2/BSA NP (2.88%)	3	3 (6), 3 (6)	17, 23	Weight, Ca^{2+}, ALP, Micro-CT
	G5-1.44	ACS + 0.1 mg/mL PEI-coated BMP-2/BSA NP (1.44%)	3	3 (6), 3 (6)	17, 23	Weight, Ca^{2+}, ALP, Micro-CT

[a] In Studies I and II, the weight ratio of BMP-2/BSA was 1.44% in NPs.

were coated with 0.1 and 0.6 mg/mL PEI, respectively. PEI dissolved in 0.5 mM NaCl solution was added to the above NP solution by a volume ratio of 1.25:1. The coating was allowed to proceed for 1 h under stirring and then the samples were centrifuged for 5 min to remove the supernatant. The pellet was re-dispersed into 675 μL of saline for implantation.

2.4.2. Implantation and assessment of BMP-2 retention in implants

6–8-week-old female Sprague–Dawley rats were purchased from Biosciences (Edmonton, Alberta). The rats were acclimated for 1 week under standard laboratory conditions (23 °C, 12 h of light/dark cycle) prior to the beginning of the study. While maintained in pairs in sterilized cages, rats were provided with standard commercial rat chow, and tap water *ad libitum* for the duration of the study. All procedures involving the rats were approved by the Animal Welfare Committee at the University of Alberta (Edmonton, Alberta). The ACS used for implantation was 1 cm × 1 cm square cut from a 7.5 cm × 10 cm ACS (5.0 mm thickness). The dry sponges were then soaked for 10 min with 50 μL of ^{125}I-labeled test samples: (1) BMP-2 in BSA NPs, (2) BMP-2 in BSA NPs coated with 0.1 mg/mL PEI, and (3) BMP-2 in BSA NPs coated with 0.6 mg/mL PEI. The exact counts in the added 50 μL sample were determined by a γ-counter prior to implantation, and used as the total implanted BMP-2 dose. Once rats were anesthetized with inhalational Metofane™, two implants (duplicates of the same type) were implanted subcutaneously into bilateral ventral pouches in each rat. A total of twenty-four rats were utilized for the three study groups; 2, 3 and 3 rats from each group were sacrificed at 1, 4 and 7 days post-implantation, respectively. The rats were euthanized with CO_2, the implants were recovered, and the counts associated with the excised implants were quantified by a γ-counter. The amount of BMP-2 retention, expressed as a percentage of implanted dose, was calculated as: 100% × [(recovered counts in implants)/(initial counts in implants)]. The results were summarized as mean ± standard deviation (SD) of %implant retention of BMP-2 at each time point.

2.5. Osteoinduction in rat subcutaneous implant model

The three series of implantations conducted for this study are summarized in Table 1. Four groups of implants were investigated in the first study: (1) saline (i.e., negative control), (2) free BMP-2 (i.e., positive control), (3) uncoated BMP-2/BSA NPs and (4) 0.6 mg/mL PEI-coated BMP-2/BSA NPs (BMP-2 loading of 1.44%). The NPs were centrifuged, and then the pellet was concentrated in saline to obtain 50 μL of NP re-dispersion containing 3 μg of BMP-2 [41]. Free BMP-2 solution was prepared by using 50 μL of 0.06 μg/μL BMP-2 solutions for each implant. 50 μL of sample from each group was soaked into a piece of ACS (1 cm × 1 cm) for 10 min prior to implantation. Once rats were anesthetized with inhalational Metofane™, two implants (duplicates of the same type) were implanted subcutaneously into bilateral ventral pouches in each rat. The implants were recovered on days 10 and 16 after rat euthanasia with CO_2, weighed, and processed for ALP and calcium assays as described below.

In a second study, free BMP-2 was added to each group to initiate bone formation in addition to the NPs. There were seven groups: (1) saline; (2) uncoated BMP-2/BSA NPs; (3) uncoated BMP-2/BSA NPs with 3 μg of free BMP-2; (4) 0.6 mg/mL PEI-coated BMP-2/BSA NPs; (5) 0.6 mg/mL PEI-coated BMP-2/BSA NPs with 3 μg of free BMP-2; (6) 0.1 mg/mL PEI-coated BMP-2/BSA NPs; and (7) 0.1 mg/mL PEI-coated BMP-2/BSA NPs with 3 μg of free BMP-2. The implants were prepared with the NPs as described above, and 3 μg of free BMP-2 solution was added to appropriate groups before implantation. Forty-two rats were used in this study, and the implants were recovered on days 14 and 21 for ALP activity assay, calcium content and mineralization by micro-CT as described below.

In a third study, five groups of implants were investigated: (1) sterilized phosphate buffered saline, (2) uncoated BMP-2/BSA NPs, (3) 0.1 mg/mL PEI-coated NPs with 5.76% BMP-2 loading, (4) 0.1 mg/mL PEI-coated NPs with 2.88% BMP-2 loading, and (5) 0.1 mg/mL PEI-coated NPs with 1.44% BMP-2 loading. Thirty rats were used in this study, and the implants were recovered on days 17 and 23 for ALP activity assay, calcium content and mineralization by micro-CT.

2.6. ALP activity and osteocalcin deposition in implants

The explants were incubated in 2.0 mL phosphate buffered saline (PBS × 1, pH = 7.3) at 4 °C in 24-well plates overnight with gentle shaking to remove any serum contaminants. The explants were then transferred into 1.0 mL of 25 mM of $NaHCO_3$ (pH = 7.4) containing 0.01% Triton X-100 for a 72 h incubation at 4 °C with gentle shaking. After 72 h of incubation, 200 μL of the sample solution (in duplicate) from each well was placed into a 48-well plate, and then 200 μL of *p*-NPP in ALP buffer (pH = 10.5, containing 0.1% of Triton X-100) was added to each extraction solution [42]. The changes in optical density ($l_{absorbance}$: 405 nm) were determined in a multi-well plate reader at intervals of 1.5 min for 8 cycles. The kinetic ALP activity was expressed as the change in optical density of the wells per minute (mABS/min). All results were expressed as mean ± SD of duplicate wells for each recovered implant.

To determine the osteocalcin content in explants, the remaining cell lysates were analyzed with a sandwich EIA Kit specific for rat osteocalcin. Briefly, a monoclonal antibody directed against the N-terminal region of osteocalcin was first bound to 96-well polystyrene wells for sample capture. Manufacturer supplied standards of highly purified rat osteocalcin were used to generate a standard curve along with the explant solutions. 100 μL of sample buffer (blank), standards, controls and tested samples were added into designated duplicate wells. The plate was incubated at 37 °C for 2.5 h, and then the wells were aspirated completely and washed three times with 0.3 mL PBS. 100 μL of the osteocalcin detection antiserum was added to each well and incubated at 37 °C for 1 h. The plate was washed again as described in last step. 100 μL of the diluted Donkey anti-Goat IgG Peroxidase was added to each well and incubated at room temperature for 1 h. Following another wash, 100 μL of the mixture of Peroxidase substrate TMB (3,3′,5,5′-tetramethylbenzidine) solution and hydrogen peroxide solution (volume ratio = 1:1) was added to all wells and incubated at room temperature in the dark for 30 min. 100 μL of stop solution was added to all wells, the plate was swirled and the absorbance was measured at 450 nm. Based on the obtained calibration curve, the concentration of osteocalcin in explants was summarized as mean ± SD of duplicate wells.

2.7. Micro-computed tomography (micro-CT) imaging of explants

After the implants were evaluated using the ALP assay (but prior to decalcification), the recovered implants were imaged non-invasively at high resolution using a micro-CT imager (Skyscan-1076, Skyscan NV, Belgium). Briefly, the recovered implants were removed from the ALP buffer, placed into a 1.5 mL microcentrifuge tube, and loaded into the imager gantry along with volumetric micro-CT calibration phantoms of known calcium phosphate density. Samples were scanned at 18 μm resolution, using a tube voltage of 48 kVp and a current of 100 μA. The X-ray beam was hardened using a 0.025 mm titanium filter in order to remove low energy

photons and reduce beam hardening and edge artifacts. An imaging step of 0.35° (through 180° of rotation) was chosen in order to adequately sample the low density mineralized explants, with an image acquisition frame averaging of 3, and with a scan duration of 23 min per batch of 3 samples. The raw image data were Gaussian filtered and reconstructed using a modified Feldkamp back-projection algorithm, thresholded at an image to cross-section of 0.0004 to 0.0414 using vendor supplied NRecon reconstruction software (version 1.5.1). Reconstructed images for each sample were quantified for percent bone volume using vendor supplied histomorphometric image analysis software (CT-An, Skyscan NV, Belgium). Reconstructed images were rendered into 3D representations using vendor supplied CT-Vol software.

2.8. Assessment of calcium deposition in implants

After ALP assay and micro-CT evaluation, the explants were washed with 2.0 mL of PBS (1×, pH = 7.3), and transferred to a new 24-well plate with 1.0 mL of

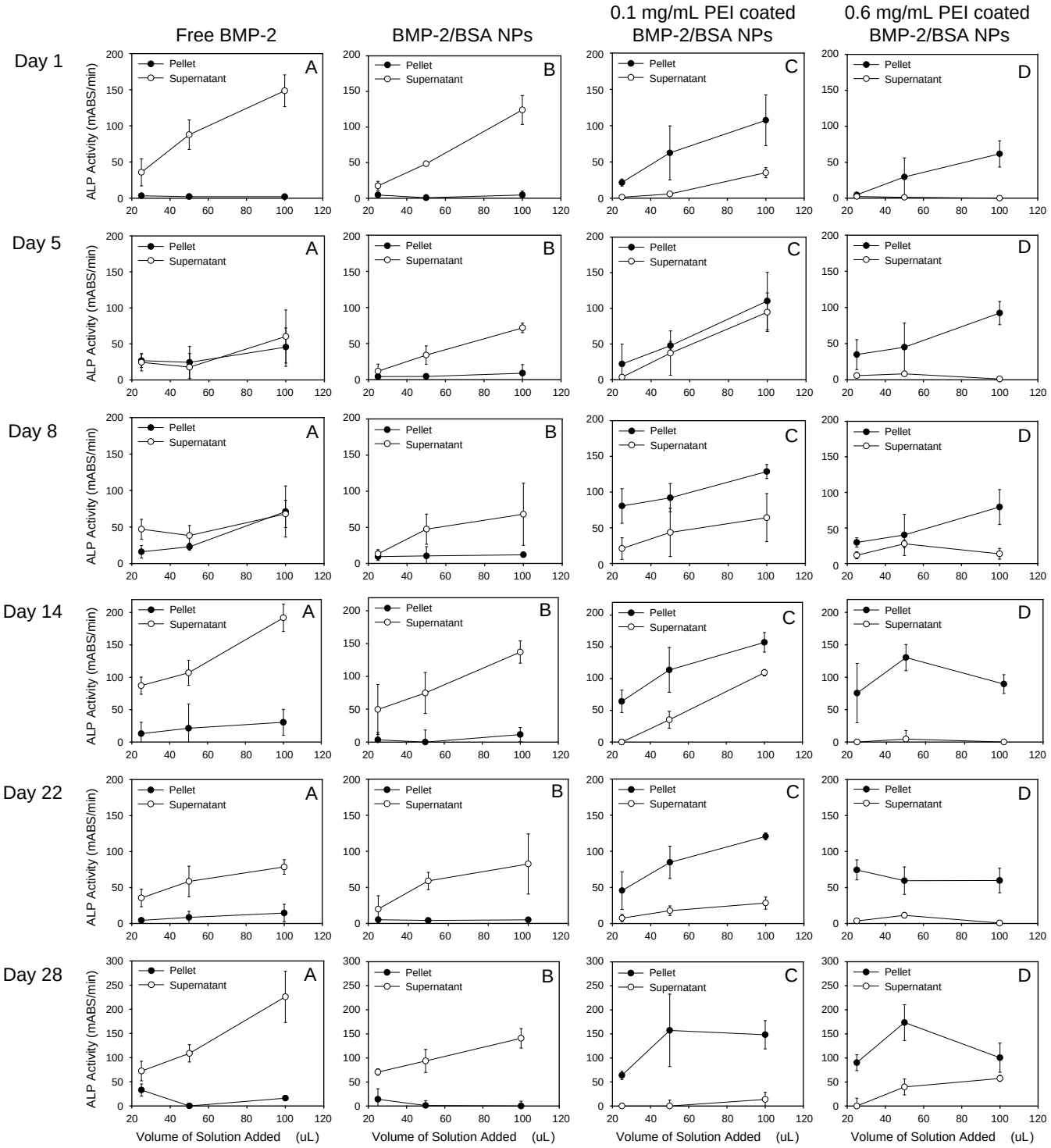

Fig. 1. *In vitro* ALP induction in C2C12 cells for BMP-2 formulations. The study groups included free BMP-2 (**A**), BMP-2 in BSA NPs (**B**), and BMP-2 in BSA NPs that were coated with 0.1 (**C**) and 0.6 mg/mL PEI (**D**). The formulations were maintained at 37 °C and processed to obtain a supernatant and a pellet fraction for BMP-2 activity assessment. For free BMP-2 and BMP-2 in BSA NPs, most activity was recovered in the supernatant fraction, whereas the BMP-2 activity was retained in the pellet for the PEI-coated NPs. Note that the BMP-2 retained its bioactivity for at least 28 days in the NP formulations.

0.5 N HCl per well. The explants were gently shaken for 24 h to extract the mineralized calcium. 20 μL of the dissolved calcium solution was added to 50 μL of 28 mM of 8-hydroxyquinoline in 0.5% (v/v) sulfuric acid, as well as 0.5 mL of 0.37 mM of *o*-cresolphthalein in 1.5% (v/v) of AMP. The absorbance was determined with a multi-well plate reader at 570 nm. A standard curve based on known concentration of calcium standards was used to convert the obtained absorbance values into calcium concentrations. The level of calcification was summarized as the mean concentration of calcium (mg/dL) ± SD of duplicate wells for each recovered implant.

2.9. Statistical analysis

All quantitative data were expressed as the mean ± SD. In the pharmacokinetics study, statistical analysis was performed by unpaired Student's *t*-test. In the ectopic bone formation study, statistical analysis was performed by the non-parametric Mann–Whitney–Wilcoxon test. Where indicated, statistical differences between group means were analyzed by single factor analysis of variance (ANOVA) or Kruskal–Wallis one-way analysis of variance (for non-parametric test). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. In vitro bioactivity of BMP-2 in NPs

The bioactivity of BMP-2 in NPs was assessed by ALP induction in the C2C12 cells (Fig. 1). The NP formulations were loaded with BMP-2 at 1.44% (w/w) and incubated at 37 °C for 28 days, with the BMP-2 activity in NP supernatants (i.e., released BMP-2) and pellets (i.e., entrapped BMP-2) being assessed at periodic intervals. The blank NPs containing no BMP-2 did not induce any ALP activity in either the pellet or supernatant fractions (not shown). Free BMP-2 demonstrated the most ALP induction activity in supernatants at all times as expected, except day 5 and day 8 where some ALP induction activity appeared in the pellet. This might be due to precipitation of a fraction of the BMP-2 protein. With uncoated NPs, ALP induction was obtained mainly in the supernatant, likely due to the rapid release of BMP-2 into the supernatant during the study period. For 0.1 mg/mL PEI-coated NPs, the ALP induction activity was mainly localized in the pellet during the study period. The ALP induction activity in the supernatant initially increased (day 1 to day 5) and later decreased (day 14 to day 28) for these NPs. For the 0.6 mg/mL PEI-coated NPs, the ALP induction activity in the pellet was lower than that seen for the 0.1 mg/mL PEI-coated NPs until day 8, after which they were equivalent. The supernatant of 0.6 mg/mL PEI-coated NPs was not capable of inducing ALP activity as much as the 0.1 mg/mL PEI-coated NPs. These results indicated that BMP-2 entrapped in PEI-coated BSA NPs was released over a prolonged period, and the released BMP-2 in the supernatant as well as the entrapped BMP-2 in the NP pellets both remained in a bioactive form.

3.2. Pharmacokinetics of BMP-2 in NPs

The BMP-2 retention in ACS implants soaked with PEI-coated and uncoated NPs is shown in Fig. 2. After 1 day, a burst release was evident for the uncoated NPs, with only 22% of the implanted BMP-2 remaining in the ACS. A reduced BMP-2 burst release was observed for both 0.6 and 0.1 mg/mL PEI-coated NPs, with 83% and 67% of the BMP-2 remaining in the implants, respectively. At 4 and 7 days post-implantation, the BMP-2 retentions for 0.6 and 0.1 mg/mL PEI-coated BMP-2/BSA NPs were 51% and 33%, and 47% and 30%, respectively. For the uncoated BMP-2/BSA NPs, the BMP-2 retention was 12% and 10% at day 4 and day 7, respectively. There were no significant differences in the BMP-2 retention between 0.6 and 0.1 mg/mL PEI-coated NPs; on the other hand, the differences between the uncoated NPs and PEI-coated NPs were significant at all time points ($p < 0.03$ by ANOVA).

3.3. Ectopic bone formation

The osteoinductive activity of NP formulations of BMP-2 was assessed by subcutaneous implantation in rats. We used an implant dose of 3 μg BMP-2 per 50 μL of NP solution that had a BMP-2 loading of 1.44% (w/w). The NPs were concentrated 13.5-fold by centrifugation to obtain 3 μg BMP-2/50 μL NP volume. The weights of the explants on days 10 and 16 are summarized in Fig. 3A, and to compensate for variations in explant weights, the ALP activity and calcium deposition were normalized with the weights. A higher ALP activity was observed in the uncoated BMP-2/BSA NPs and free BMP-2 solution, compared with the negative control (no BMP-2) and 0.6 mg/mL PEI-coated BMP-2/BSA NPs, which did not demonstrate any significant ALP activity at both time points (Fig. 3B). The relatively large error bars are typical of this *in vivo* bioassay. Calcium deposition in implants was also evident on day 16 in both the uncoated BMP-2/BSA NPs and free BMP-2, but not detected in the control and 0.6 mg/mL PEI-coated BMP-2/BSA NPs (Fig. 3C). There was an increased ALP activity and calcification at day 16 for both the uncoated BMP-2/BSA NPs and free BMP-2. Correlations between the ALP activity and calcium deposition for all explants are shown in Fig. 3D. A significant correlation was observed between the ALP activity and calcification on day 10 ($p < 0.0001$), and 16 ($p < 0.0001$ by 2-tailed Pearson Correlation).

The osteocalcin levels in the explants are summarized in Fig. 4. The free BMP-2 and uncoated BMP-2/BSA NP groups yielded significantly higher osteocalcin levels compared with the control group and 0.6 mg/mL PEI-coated BMP-2/BSA NPs at both day 10 and day 16 ($p < 0.05$). The osteocalcin deposition in free BMP-2 and uncoated BMP-2/BSA NP groups was significantly increased from day 10 to day 16 ($p < 0.02$ for both groups). There was no osteocalcin deposition in PEI-coated BMP-2/BSA NPs, since there was no significant difference between the control and PEI-coated BMP-2/BSA NPs for both time points ($p > 0.70$).

Given the lack of osteoinductive activity with PEI-coated NPs, a follow-up study was conducted with two changes: (1) a lower concentration of PEI (0.1 mg/mL) was employed for NP coating, and (2) free BMP-2 was added to implants to assess osteoinduction independent of NP formulations. The explants were recovered on

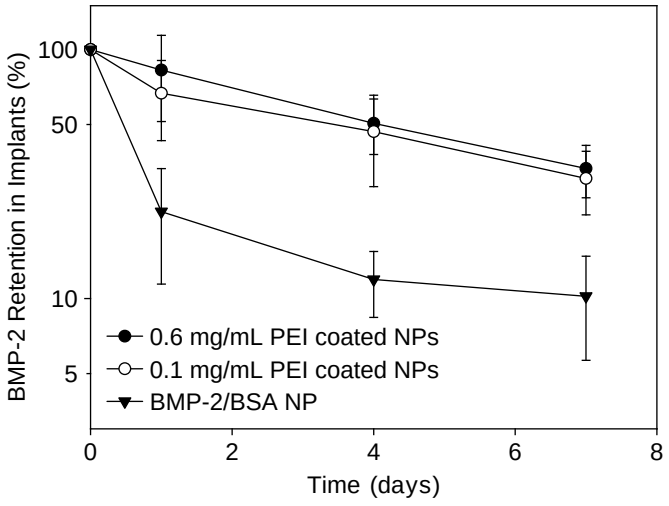

Fig. 2. Pharmacokinetics of BMP-2 in BSA NPs in the rat ectopic implant model. The results were summarized as the percent retention of the implanted BMP-2 dose as a function of time. The study groups were ACS implants soaked with BMP-2 containing NPs (uncoated) and BMP-2 containing NPs coated with 0.1 and 0.6 mg/mL PEI. The PEI-coated BMP-2/BSA NPs gave significantly higher BMP-2 retention at all time points as compared with the uncoated BMP-2/BSA NPs ($p < 0.03$ by ANOVA).

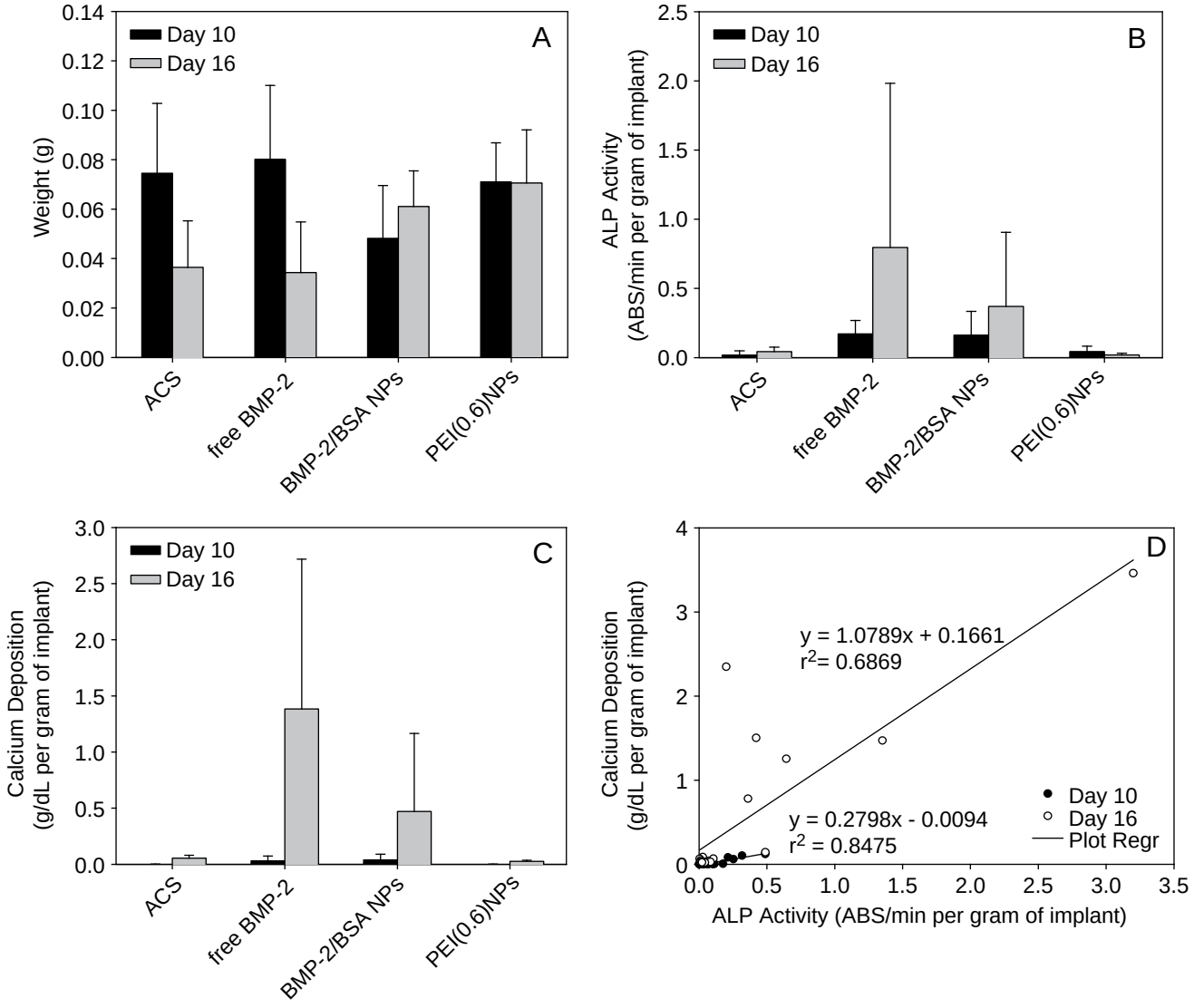

Fig. 3. The osteoinductive activity of different NP formulations in the rat ectopic model. The study groups were ACS implants soaked with saline, free BMP-2 and BMP-2 in uncoated BSA NPs and PEI-coated (0.6 mg/mL) BSA NPs. The amount of BMP-2 was 3 μg in the appropriate groups. **A.** Wet weight of recovered implants at day 10 and day 16. **B.** Normalized ALP activity for the study groups at day 10 and day 16. **C.** Normalized calcium deposition for the study groups at day 10 and day 16. **D.** Correlation between the calcium deposition and ALP activity of the recovered implants at day 10 and day 16. Note the significant ALP activity and calcium deposition in free BMP-2 implants and implants soaked with BMP-2 in uncoated BSA NPs.

days 14 and 21 and the explant weights are summarized in Fig. 5A. There was no ALP activity or calcification in the negative control group at both time points. The uncoated BMP-2/BSA NPs, with and without free BMP-2, showed higher ALP activity than all other groups on days 14 and 21 (Fig. 5B). On the other hand, PEI-coated NPs (both 0.6 and 0.1 mg/mL), with or without free BMP-2 addition, did not demonstrate any significant ALP activity even at day 21 ($p > 0.31$ vs. control). The calcification results exhibited similar trends to that of the ALP activity, except that the uncoated BMP-2/BSA NPs with free BMP-2 resulted in more calcium deposition than the NPs without free BMP-2 at day 21 (Fig. 5C), but this difference was not significant ($p > 0.14$). The PEI-coated NPs, with and without free BMP-2 addition, did not give significant calcium deposition compared with control at both time points. The correlation between ALP activity and calcification (Fig. 5D) was significant for day 14 ($p < 0.0001$), and day 21 ($p < 0.01$). The micro-CT images for recovered implants at day 14 visually demonstrated mineralization inside the implants (Fig. 6), which were in accordance with the calcification results obtained independently. Based on the micro-CT analysis, only the uncoated BMP-2/BSA NP + free BMP-2 group gave strong mineralization and adding free BMP-2 to PEI-coated NPs did not give an increase in calcification. These results suggested an undesired effect of PEI on the osteoinductive activity of BMP-2, even for free BMP-2 implanted along with the PEI-coated NPs.

3.4. NPs with different BMP-2 loading ratio: in vitro BMP-2 bioactivity

Higher BMP-2 loading in NPs were attempted to reduce the need to concentrate NP formulations, thereby reducing the PEI content in implanted formulations. NPs were prepared at BMP-2 loading levels of 0.14%, 0.29%, and 0.58%, and coated with PEI concentrations between 0.1 and 0.01 mg/mL. The final BMP-2 concentrations in the assay medium were 1.0 and 0.5 μg/mL after concentrating the NPs 13.5 (for 0.14% NPs), 6.75 (for 0.29% NPs) and 3.38 (for 0.58% NPs) fold. The pellet fraction of 0.14% NPs did not

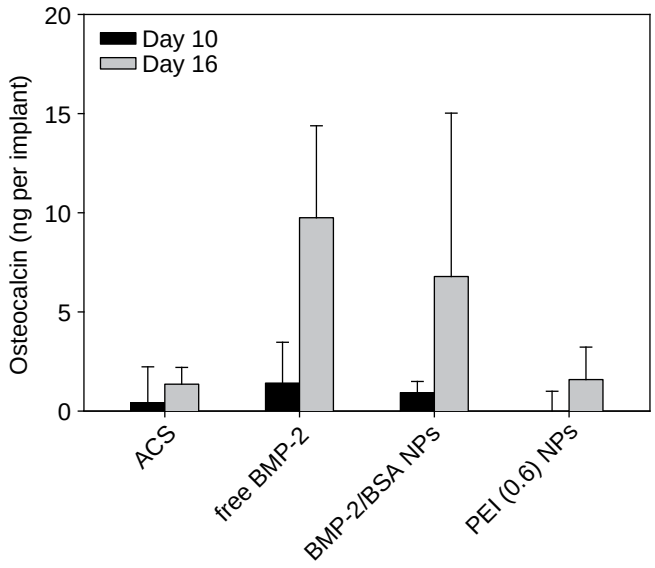

Fig. 4. Osteocalcin deposition in implants at day 10 and day 16. The osteocalcin level in free BMP-2 and the uncoated BMP-2/BSA NPs was significantly higher than the control and 0.6 mg/mL PEI-coated BMP-2/BSA NPs at both day 10 and day 16 ($p < 0.05$). PEI-coated BMP-2/BSA NPs did not yield osteocalcin deposition, with no significant difference at both time points from the control ACS ($p > 0.70$).

induce any ALP activity when coated with 0.1 mg/mL PEI; however, 0.29% NPs induced some ALP activity, and 0.58% NPs exhibited a dose-dependent ALP induction at the PEI coating concentration of 0.1 mg/mL. For 0.03 and 0.01 mg/mL PEI-coated NPs, the pellet fraction of all three types of NPs induced ALP activity in a dose-respondent manner (Fig. 7A, B). In the supernatant, no significant ALP activity was observed for all formulations tested, except the 0.01 mg/mL PEI-coated NPs, which showed the highest ALP activity with 0.14% NPs (Fig. 7C, D). The overall results indicated that the induced ALP activity was increased as the PEI coating decreased from 0.1 to 0.01 mg/mL.

3.5. NPs with different BMP-2 loading ratio: ectopic bone formation

The NP formulations with different BMP-2 loading were tested for osteoinduction. The implant dose was 3 μg BMP-2/50 μL of NP solution. The BMP-2 loading in NPs was 1.44%, 2.88% and 5.76%, with 0.1 mg/mL PEI coating, and the NPs were concentrated 13.5, 6.75 and 3.38-fold (same concentration regimen used for the *in vitro* BMP-2 bioassay in Section 3.4) to achieve the desired 3 μg BMP-2/50 μL dose. The implants were recovered at days 17 and 23. As before, the uncoated NPs showed the highest ALP activity that was significantly different from other groups at both time points ($p < 0.006$ by Kruskal–Wallis one-way ANOVA; Fig. 8B). The 5.76% NPs showed a higher ALP activity than the negative control on day 23, but the difference remained statistically insignificant ($p < 0.055$). This was the case for 1.44% NPs as well, where the higher ALP activity on day 23 did not reach a significantly higher level than the control ($p < 0.109$).

The calcification results showed a similar trend to the ALP activity, with the uncoated NPs giving the highest calcium deposition at both time points ($p < 0.002$; Fig. 8C). The calcification in 5.76% NPs was higher than the control and the remaining coated NP groups, with significant difference on day 23 ($p < 0.011$), but not on day 17 ($p > 0.16$). Little calcium deposition was obtained with other NPs. The correlation between ALP activity and calcification was significant for both day 17 and day 23 recovered implants ($p < 0.0001$) (Fig. 8D).

The micro-CT analyses for day 17 (Fig. 9A) and day 23 (Fig. 9B) explants were in line with the ALP activity and calcification results; extensive calcification was observed with the uncoated NPs, followed by less calcification in 5.76% NPs, and sporadic or no calcium deposition in other groups. Based on the estimated bone volume (Fig. 9C), significant bone volumes were observed on days 17 and 23 in uncoated NPs, but not in other groups. The decreased bone volume in this case from day 17 to day 23 was likely due to the progressive resorption of the induced bone at the mechanically unloaded ectopic site.

4. Discussion

BMP-2 is a potent stimulant of bone formation as it induces differentiation of mesenchymal stem cells into bone-depositing osteoblasts [43]. The necessity of a carrier for BMP-2 delivery is due to the short biological half-life of BMP-2 at implant sites; moreover, safety issues arising from uncontrolled diffusion of free BMP-2 after implantation make the use of a carrier necessary. In order to develop an effective and safe approach for *in situ* bone formation, we previously reported a BMP-2 delivery system based on PEI-coated BSA NPs [38]. *In vitro* release studies suggested that the release of BMP-2 from the NPs can be controlled by the extent of PEI coating on the NPs [38]. In this study, we first evaluate the native activity of BMP-2 in NP formulations, since it is imperative to maintain protein bioactivity in NPs over a period of time suitable for *de novo* bone induction. Our 28-day *in vitro* ALP induction study suggested that BMP-2 remained bioactive over the entire time period for both the uncoated and PEI-coated NPs (0.1 and 0.6 mg/mL PEI for coating). Compared with the uncoated NPs, PEI-coated NPs exhibited enhanced ALP activity in the pellet, indicating better BMP-2 retention by the PEI coating that was consistent with our previous study [38]. A similar controllable release pattern was also observed for other PEI coatings in drug release formulations [44–46]. We then continued to investigate the proposed BMP-2 delivery system by evaluating BMP-2 pharmacokinetics and osteoinductive activity in a rat ectopic model.

4.1. Pharmacokinetics of BMP-2

The pharmacokinetics of BMP-2 in different carriers, such as ACS, demineralized bone matrix and bone mineral, has been studied thoroughly [31,32,41]. Previous studies suggested that BMP loss from the implanted scaffolds occurred in two phases: an initial burst release and a gradual protein loss afterwards [41]. The concentration of implanted BMP did not affect the *in vivo* pharmacokinetics [31]. The BMP-2 retention in the rat ectopic model was w 10% in ACS after 7 days [41]. In this study, with the same methodology [41], a similar retention was achieved for the uncoated BMP-2/BSA NPs. A high burst release from uncoated BSA NPs was expected in early time periods since the NPs without a PEI coating will be unstable after implantation [38]. The release from the uncoated BSA NPs was similar to the release of free BMP-2 in ACS (not shown). This study also showed that > 30% of BMP-2 was retained in the ACS after PEI stabilization of the NPs at 7 days post-implantation. The PEI coating on BSA NPs could effectively reduce the initial burst release of BMP-2 at > 0.1 mg/mL PEI coating concentrations. The PEI coating was expected to delay BSA degradation and/or dissolution from the NPs, hence slowing the release of BMP-2 from the BSA matrix. Although this study did not investigate the degradation pattern of BSA NPs, Lin et al. studied the degradation of albumin NPs in rat serum *in vitro* [47]. Approximately 17% of albumin NPs was degraded after 4 days at 37 °C, so that significant degradation of our BSA NPs should also occur at the ectopic site. Other factors are also likely to affect BMP-2 retention in

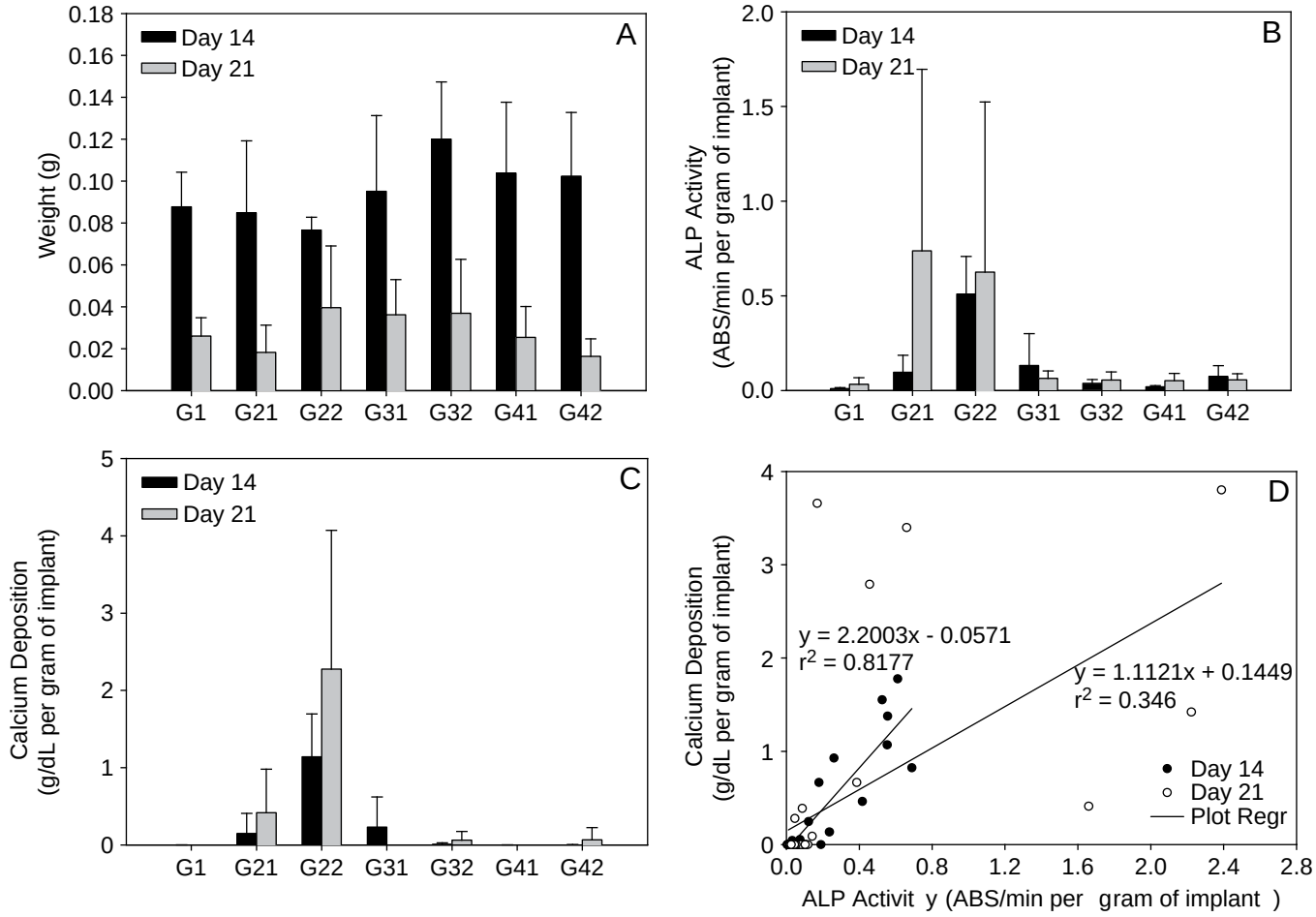

Fig. 5. The osteoinductive activity of different NP formulations in the presence of BMP-2. The study groups were G1: ACS soaked with saline; G21: ACS soaked with uncoated BMP-2/BSA NPs; G22: ACS soaked with uncoated BMP-2/BSA NPs + 3 μg of BMP-2; G31: ACS soaked with BMP-2/BSA NPs coated with 0.6 mg/mL PEI; G32: ACS soaked with BMP-2/BSA NPs coated with 0.6 mg/mL PEI + 3 μg of BMP-2; G41: ACS soaked with BMP-2/BSA NPs coated with 0.1 mg/mL PEI; G42: ACS soaked with BMP-2/BSA NPs coated with 0.1 mg/mL PEI + 3 μg of BMP-2. **A.** Wet weight of recovered implants at day 10 and day 16; **B.** Normalized ALP activity for different groups at day 14 and day 21; **C.** Normalized calcium deposition for different groups at day 14 and day 21; **D.** Correlation between calcium deposition and ALP activity at day 14 and day 21. Note that the presence of free BMP-2 enhanced ALP activity and calcium deposition for BMP-2/BSA NPs, but did not display an additional osteoinductive effect for the PEI-coated NPs.

implants, including diffusion of BMP-2 and/or NPs, the degradation rate of ACS sponge, and the interactions among the BMP-2, BSA, PEI and ACS. The NP size should play a significant role in BMP-2 release, but this issue was not explored in this study.

Although BMP-2 retention in carriers with diverse matrix materials and geometries, as well as at different surgical sites and animal models were investigated, only a single study investigated BMP-2 pharmacokinetics with an NP system. Hosseinkhani et al. investigated BMP-2 release from nanofibers constructed from a self-assembled peptide amphiphile (PA; $CH_3(CH_2)_{14}CO(A_4G_3ERGD)$) after subcutaneous injection in rats [48]. The BMP-2 solution was injected simultaneously with the PAs to form a nanofiber scaffold at the injected site. There was no burst release of BMP-2 on the first day, and 30–40% of BMP-2 remained in the scaffold after 7 days. This level of retention was comparable to our results with the PEI-coated BSA NPs. The degradation of the nanofibers paralleled BMP-2 release (30–40% degradation within 7 days), indicating a close relationship between the scaffold degradation and BMP-2 release rate. Other BMP-2 pharmacokinetics studies with microparticles made from poly(D,L-lactic-*co*-glycolic acid) (PLGA) [49] and gelatin hydrogels [50] also suggested a correlation between the local BMP-2 retention and the carrier degradation. We anticipate a similar correlation to be valid for the BSA NPs described here, but experimental evidence for such a relationship will be the focus of future studies. We also expect the BMP-2 loss to be faster in an orthotopic site, based on the evidence reported with other BMP-2 delivery systems (i.e., PLGA microparticles entrapped in a poly(propylene fumarate) (PPF) scaffold surrounded by a gelatin hydrogel [51]).

4.2. Ectopic bone formation

Implantation of BMP-2 typically results in new bone formation at ectopic sites and stimulation of bone deposition at osseous sites. The BMP-2 concentration required for effective bone formation depends on the features of the wound, and the state of the organism in the evolutionary scale, with larger amount of BMP-2 needed for higher organisms [52]. It is thought that large animals may have a smaller pool of responsive stem cells from the bone and soft tissue envelopes than the small animals [14]. Two micrograms of BMP-2 was considered minimal for osteoinduction in the rat ectopic model [41], but this is expected to depend on the choice of carrier. Our NP formulation was originally designed to provide 3 μg of BMP-2 in 675 μL of NP solution. With an intention to implant 3 μg of BMP-2 per implant, the NP formulations had to be significantly "concentrated" to obtain 3 μg BMP-2 in 50 μL NP volume.

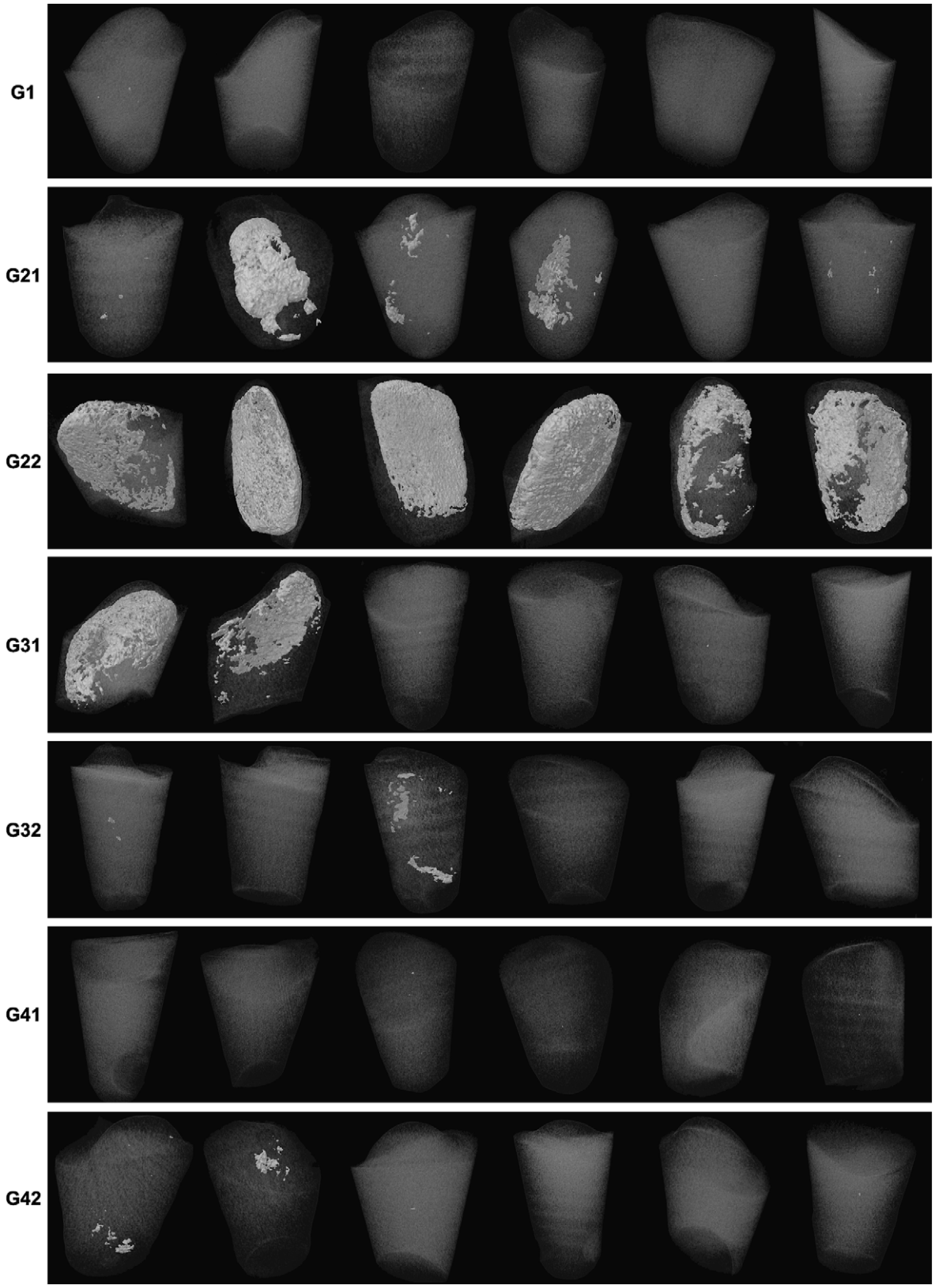

Fig. 6. Micro-CT images of the implants corresponding to the study groups described in Fig. 5. The implants were recovered at day 14 post-implantation. Note that mineralization was evident for only the study groups where BMP-2 was delivered with uncoated BSA NPs. There was no significant mineralization in the case of BMP-2 delivery with the PEI-coated NPs.

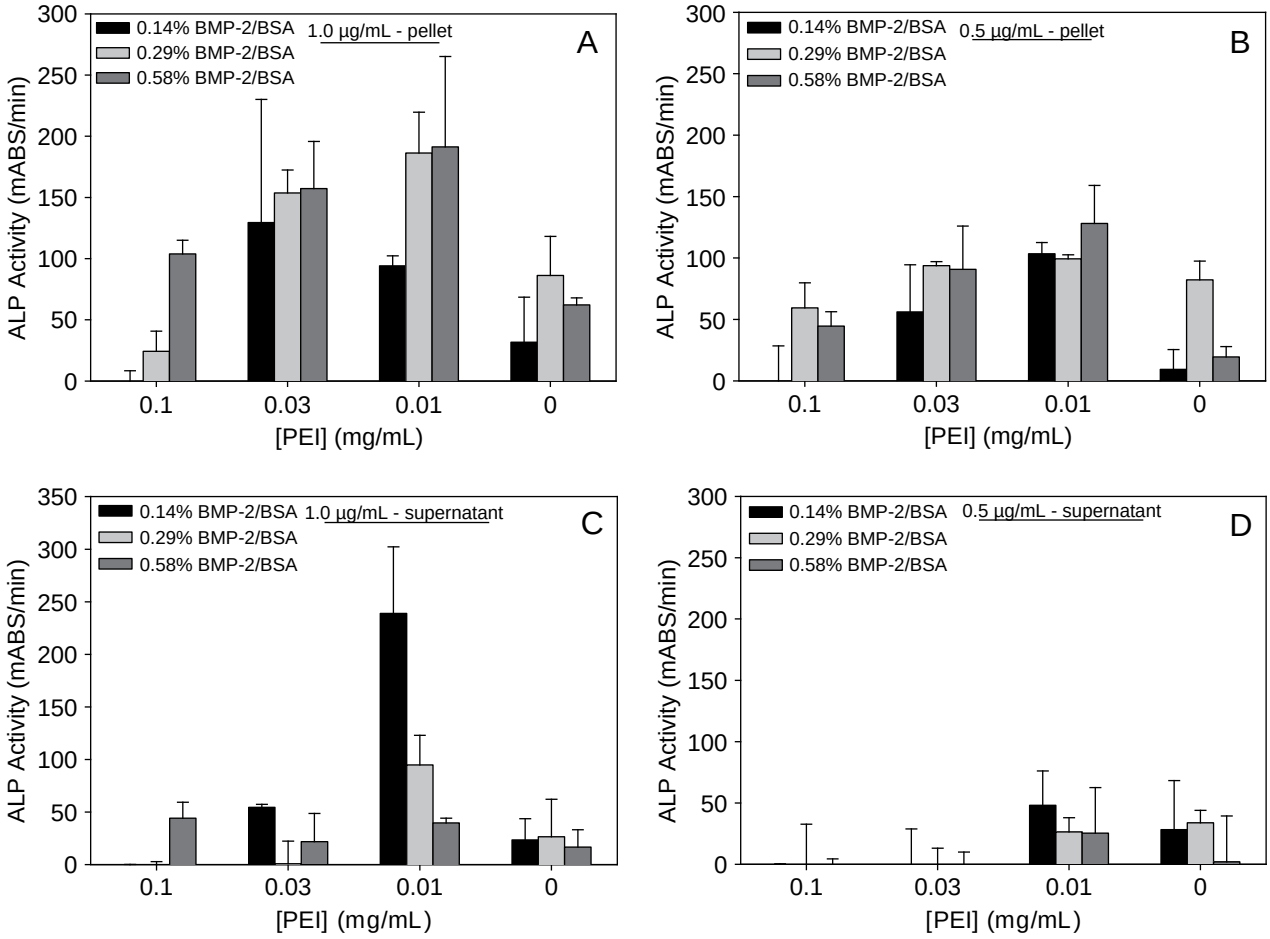

Fig. 7. *In vitro* ALP activity for NPs processed by the 'concentration' procedure reminiscent of implant preparations. The NPs were prepared with different BMP-2 loadings (0.14%, 0.29% and 0.58% BMP-2/BSA) and PEI coatings, and centrifuged for 13.5, 6.75 and 3.38-fold concentration, respectively. The ALP induction assay was performed with both the pellet (**A** and **B**) and supernatant (**C** and **D**) fractions. Both fractions were added to the C2C12 cells to give an estimated BMP-2 concentration of 1.0 (**A** and **C**) and 0.5 μg/mL (**B** and **D**). Most significant ALP induction was observed for 0.01 mg/mL PEI-coated NPs (for both pellet and supernatant fractions). The ALP induction was significantly reduced for the 0.1 mg/mL PEI-coated NPs.

The BMP-2 induced bone formation is initiated within 5–10 days post-implantations in the ectopic model [53–55] and, hence, we evaluated bone formation between 10 and 16 days for the first study. Free BMP-2 loaded ACS implants and the uncoated BMP-2/BSA NPs loaded in ACS induced robust bone formation, based on all the investigated markers including osteocalcin deposition. Osteocalcin is synthesized by mature osteoblasts, which is not present at early calcification stages, but is expressed in concert with calcium deposits [48]. It appears that the NP fabrication process did not adversely affect the osteoinductive activity of BMP-2. In contrast, the NPs coated with 0.6 mg/mL PEI did not demonstrate any osteoinduction. This was not consistent with the BMP-2 pharmacokinetics, since higher amount of BMP-2 was retained in PEI-coated NPs than the uncoated NPs. BMP-2 functions only locally and higher retention of BMP-2 in the carrier should have resulted in more bone formation. Takita et al. reported that the slow release of BMP-2 from a carrier can give a late response in bone formation [56]. There was no evidence of a delayed bone formation by these NPs since our collective studies evaluated bone formation up to 23 days. Therefore, two possible reasons were attributed to the lack of bone formation: (i) the lack of BMP-2 burst release could not recruit responsive cells from the subcutaneous tissues to initiate bone deposition, and relatively slow release afterwards did not allow the BMP-2 to reach a threshold concentration to induce the bone formation [50,57]; and (ii) the cytotoxicity of PEI associated with the NP surfaces was detrimental to the BMP-2 bioactivity.

We first tested the necessity of initial BMP-2 release by delivering free BMP-2 along with the NP formulations. With the uncoated NPs, the addition of free BMP-2 further increased bone formation as compared with NPs alone. The bioassay showed that the ALP activity initiated by BMP-2 increased and saturated at 3 weeks while the calcium deposition augmented steadily with time. Such a kinetics was also observed in other studies [48,58], and was considered typical for the bone induction at the subcutaneous tissue. On the other hand, there was little sign of bone formation with PEI-coated NPs, even with the addition of free BMP-2. Two recovered implants in the 0.6 mg/mL PEI-coated groups were exceptions (both from the same rat), which showed good osteoinduction. This might be due to uneven dispersion of the concentrated pellet, which might have contained relatively less PEI or more BMP-2 for these two implants. Therefore, the collective observations pointed out the possibility of PEI cytotoxicity abolishing the BMP-2 activity. Our initial studies had identified the PEI to be the most toxic component in our system [38]. Since previous [38] and current studies (Fig. 1) showed that the unconcentrated PEI-coated NPs (0.1 and 0.6 mg/mL PEI) induced the expected ALP activity *in vitro* [38], the 'concentration' process was considered the likely reason for abolishing the BMP-2 activity. The cytotoxicity of PEI is well documented, since cationic

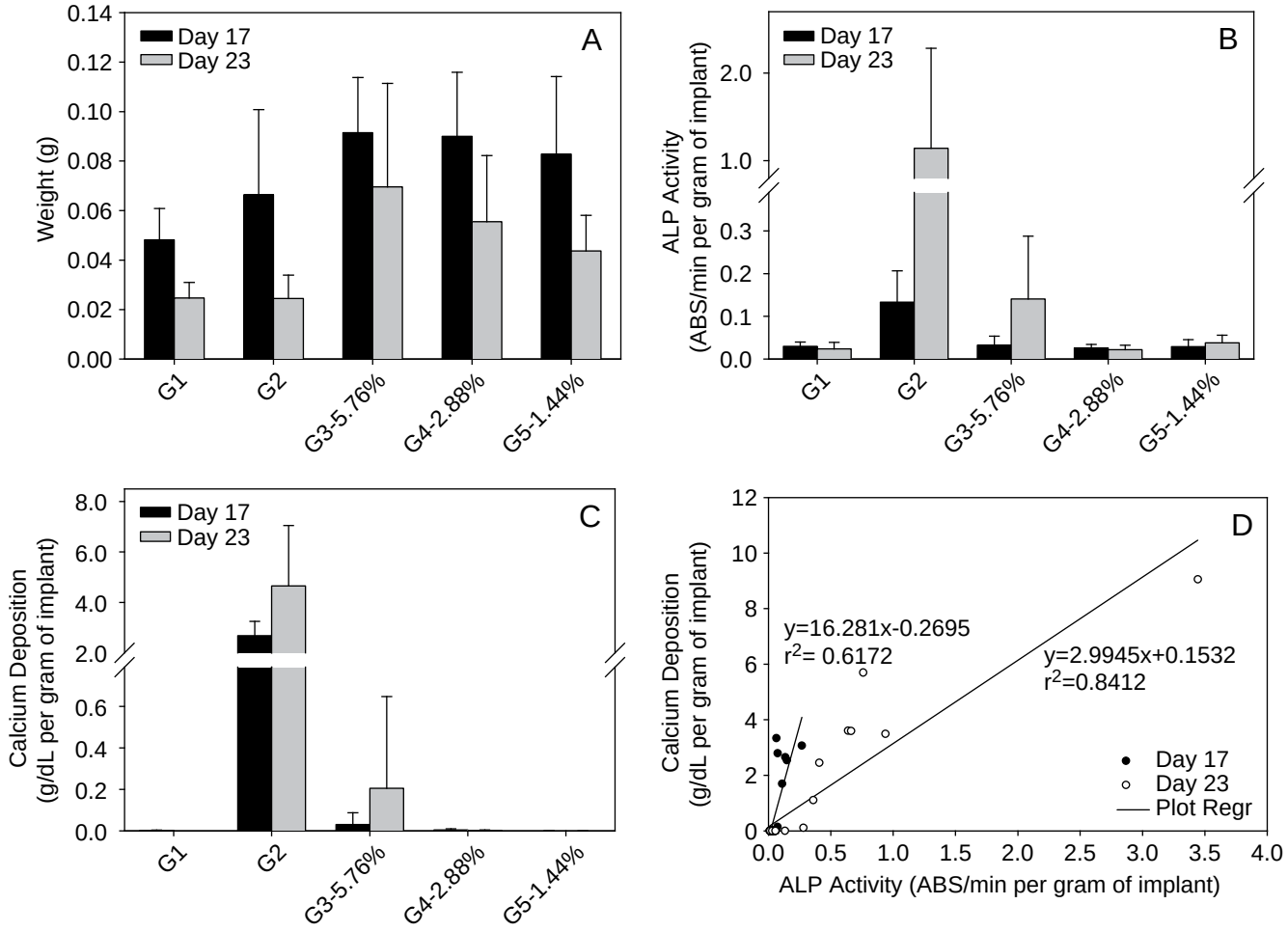

Fig. 8. The osteoinductive activity of different NP formulations with different BMP-2 loadings. The study groups were G1: ACS soaked with sterilized phosphate buffered saline; G2: ACS soaked with uncoated BMP-2/BSA NPs (BMP-2 loading: 5.76%); G3: ACS soaked with BMP-2/BSA NPs coated with 0.1 mg/mL PEI (BMP-2 loading: 5.76%); G4: ACS soaked with BMP-2/BSA NPs coated with 0.1 mg/mL PEI (BMP-2 loading: 2.88%); G5: ACS soaked with BMP-2/BSA NPs coated with 0.1 mg/mL PEI (BMP-2 loading: 1.44%). **A.** Wet weight of recovered implants at day 17 and day 23; **B.** Normalized ALP activity for different groups at day 17 and day 23; **C.** Normalized calcium deposition for different groups at day 17 and day 23; **D.** Correlation between the calcium deposition and ALP activity at day 17 and day 23. Note that PEI-coated BSA NPs displayed some osteoinductive activity at the highest BMP-2 loading.

polymers strongly interact with anionic cell surfaces and compromise the integrity of cellular membranes and normal cellular process [59,60]. Another effect of this 'concentration' process was an increase in the BSA amount in the formulations, but this change seemed to be compatible with the osteoinductive process (since uncoated BMP-2/BSA NPs subjected to the same process robustly induced bone).

The *in vitro* bioassay designed to evaluate the 'concentration' process indicated a detrimental effect of PEI used for particle coating (Fig. 7), unless the PEI concentration was reduced to 0.01 mg/mL. Cell death was visually observed for samples that did not give ALP induction. It appears that *in vitro* bioassay required a maximal concentration of 0.01 mg/mL (w 0.14 mg/mL diluted 13.5-fold in tissue culture medium) PEI for induction of BMP-2 induced ALP activity. Although we can estimate the initial PEI concentration in implants (w 32 and 16.7 μg in 50 μL for 0.6 and 0.1 mg/mL PEI coating, respectively, taking into account the coating efficiencies at these two concentrations [38]), it is not easy to convert this into an effective PEI concentration in implants, since (i) PEI is expected to complex with ACS sponges (likely reducing its toxicity); (ii) PEI will diffuse away from the implants either freely or with NPs; and (iii) cell flux into the ACS implant will take several days and the exact PEI exposure concentration will be variable during this time. Nevertheless, the fact that even free BMP-2 did not sustain an osteoinductive effect in ACS sponges was the clearest indication of the undesirable effect of PEI-coated NPs.

One way to reduce PEI toxicity is to increase BMP-2 loading in NPs. The *in vitro* ALP induction study with three BMP-2 loadings (0.14%, 0.29% and 0.58% necessitating 13.5, 6.75 and 3.38-fold NP 'concentration') showed ALP activity that clearly favored the high BMP-2 loading. These results pointed out to an approach for improving the NP formulations, which will be stabilized by PEI or other cationic polymer coating. This approach was also tested in the ectopic model with the BMP-2 loadings of 1.44%, 2.88% and 5.76% in NPs, corresponding to 13.5, 6.75 and 3.38-fold PEI 'concentration', respectively. The BMP-2 amount contained in NPs was in the practical range for implantation (3 μg of BMP-2 per 50 μL NP volume) while the amount of PEI was reduced significantly. The results from the implant study validated the better performance of higher BMP-2 loading. The osteoinductive activity of the highly loaded NPs, however, was still significantly lower than the uncoated NP formulations, suggesting that the PEI in the former samples was still an impediment to the osteoinductive effect of BMP-2. We will explore other, more-biocompatible polymers to produce effective coatings for the designed NPs. Alternatively, chemical modification of PEI with such reagents as poly(ethylene

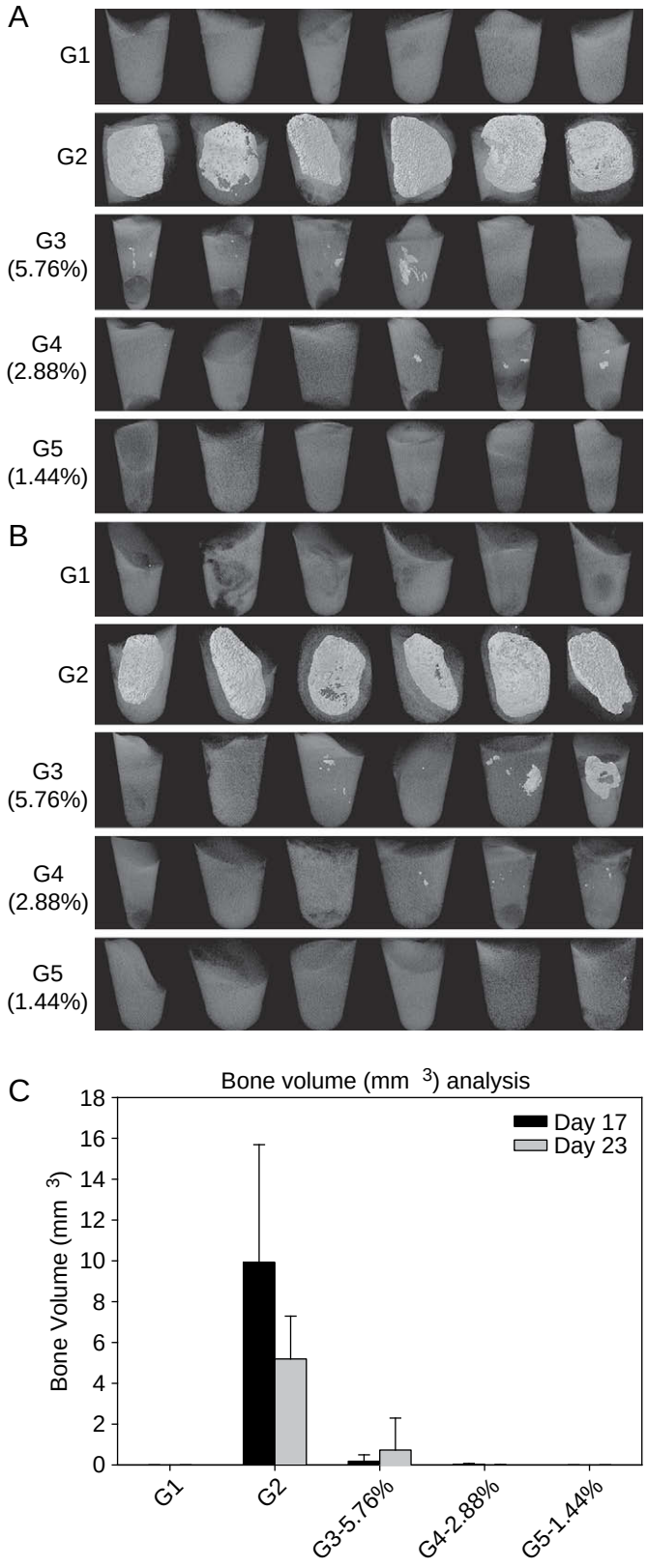

Fig. 9. Micro-CT images of the implants corresponding to the study groups described in Fig. 8. The implants were recovered at days 17 (**A**) and 23 (**B**) post-implantation. The calculated bone volumes (mean ± SD) for the scanned images are summarized in **C**. Note that robust mineralization was evident for the BMP-2 in uncoated BSA NPs, and only highest BMP-2 loaded (5.76%) PEI-coated NPs gave significant mineralization and bone volume as compared with the less BMP-2 loaded NPs.

glycol) (PEG) could be explored for improved biocompatibility of the NPs and this will be the focus of our future studies.

The concentration and retention time of BMP-2 at implant sites play an important role in inducing new bone formation [50]. The optimal conditions are not clear at present and cellular and biochemical studies are required to evaluate the effect of BMP-2 release kinetics on proliferation and differentiation of osteoprogenitor cells. With the use of NP-based delivery systems, it may be necessary to additionally characterize the NP transport kinetics to better understand the local pharmacokinetics of BMP-2. In consideration of clinical application of BMP-2, reducing the dose of BMP-2 for successful bone regeneration and diminishing the potential side effects elicited by BMP-2 at extraskeletal sites is a critical issue. A controlled and localized delivery system to deliver the appropriate amount of BMP-2 and efficiently retain BMP-2 at the site remains a worthy goal for further investigation. NPs could be important in that respect, since they have the potential to be engineered for precise release profiles.

5. Conclusions

We investigated the pharmacokinetics and osteoinductive activity of a BMP-2 delivery system based on PEI-coated BSA NPs by using a rat subcutaneous implant model. The results of pharmacokinetics study indicated that PEI coating on BSA NPs effectively reduced the initial burst release of BMP-2 from the NPs. The uncoated BMP-2/BSA NPs were capable of inducing new bone formation, supporting the possibility of formulating BMP-2 with NPs without losing its osteoinductive activity. However, the PEI-coated BMP-2/BSA NPs did not yield new bone formation. Presumably, the 'concentration' process used to prepare sufficient BMP-2 necessary for osteoinduction, also increased the toxicity of the NPs. In order to reduce the extent of PEI 'concentration', NPs loaded with increased BMP-2 amounts for osteoinductive activity were explored, and the results showed that relatively higher amounts of bone formation were obtained with increased BMP-2 loading. Our studies indicated that by optimizing the NP formulations, the toxicity of PEI coating on NPs could be reduced in order to retain the osteoinductive activity of BMP-2.

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