Polyethylenimine-coated Albumin Nanoparticles for BMP-2 Delivery

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DOI 10.1021/bp.12
Published online in Wiley InterScience (www.interscience.wiley.com).

Nanoparticle (NP)-based delivery has gained importance for improving the potency of therapeutic agents. The bovine serum albumin (BSA) NPs, obtained by a coacervation process, was modified by electrostatic adsorption of cationic polyethylenimine (PEI) to NP surfaces for delivery of bone-inducing growth factor, bone morphogenetic protein-2 (BMP-2). Different concentrations of PEI were utilized for coating BSA NPs to stabilize the colloidal system and to control the release of BMP-2. The NPs were characterized by size and zeta potential measurements, as well as by Scanning Electron Microscopy and Atomic Force Microscopy. The encapsulation efficiency was typically >90% in all NP preparations. In vitro release kinetics showed that the PEI concentration used for coating the NPs efficiently controlled the release of BMP-2, demonstrating a gradual slowing, sustained release pattern during a 10-day study period. The bioactivity of the encapsulated BMP-2 and the toxicity of the NPs were examined by the alkaline phosphatase (ALP) induction assay and the MTT assay, respectively, using C2C12 cells. The results indicated that PEI was the primary determinant of NP toxicities, and BSA NPs coated with 0.1 mg/mL PEI demonstrated tolerable toxicity, retained the bioactivity of BMP-2, and efficiently slowed the release rate of BMP-2. We conclude that BMP-2 encapsulated in BSA NPs might be an efficient way to deliver the protein for in vivo bone induction.
Keywords: albumin nanoparticles, polyethylenimine, bone morphogenetic protein-2, controlled release

Introduction

Bone-inducing growth factor, bone morphogenetic protein-2 (BMP-2), plays a critical role in bone healing by means of its ability to stimulate differentiation of mesenchymal cells to an osteo-chondroblastic lineage.[1] After Wozney et al. reported the nucleotide sequence of first bone morphogenetic proteins in 1988,[2] various BMPs were produced in abundance with the use of recombinant gene technology. The therapeutic applications involving the recombinant BMPs demonstrated robust efficacy in the treatment of bone diseases. For example, BMP-2 showed good ability to heal critical size defects in rat, rabbit, sheep, dog, and primate models,[3–7] and it has become part of a new treatment modality in orthopaedic practice.[8] Unlike the traditional surgical repair that necessitates the use of autografts, stimulation of local bone healing via the delivery of BMPs can fulfill the aim of bone repair without morbidity associated with the surgical techniques. However, BMP-2 is a low-molecular-weight peptide[9] and it exhibits a short in vivo half-life. The clinical BMP-2 administration was focused on local delivery by incorporating the protein into a carrier matrix to provide a slow delivery formulation.[10] The delivery mechanism has relied on adsorption of the protein in collagenous biomaterials; slow protein desorption from the implanted biomaterial has provided the necessary concentration at the local site. The collagen sponge appeared to be the most effective matrix in animal studies, and it remains the only FDA-approved recombinant human BMP-2 carrier in clinical practice.[11,12]

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A better way to control the BMP-2 delivery is to encapsulate the protein in nanoparticles (NPs) so that the physicochemical properties of the particles can control the BMP-2 release more precisely. Nanoparticulate drug delivery system may provide a promising mechanism, not only for local application, but also for targeted bone stimulation via intravascular injection. The colloidal micro/nanoparticles were originally explored for delivery of other cytokines. For example, erythropoietin, NGF, and IL-2 were formulated in poly(lactic acid)/poly(D,L-lactic-*co*-glycolic acid) (PLA/PLGA) particles for delivery.[13] Drawbacks of these materials include low encapsulation efficiency and rapid initial release of proteins, as well as the need for special formulations to maintain protein stability.[14] Current techniques often produce NPs by employing harsh organic solvents, synthetic polymers, or surfactants having high toxicity or immunological activity.[15] More recently, BMP-2 was encapsulated in 20–40 μm particles of dextran-glycidylmethacrylate/poly(ethylene glycol).[16] BMP-2 was shown to remain bioactive after in vitro release, which displayed a burst release for all particles prepared. Another study investigated the BMP-2 nanospheres prepared by a coacervation-co-precipitation method,[17] with the use of polysaccharide/*N*,*N*-diethylaminoethyl dextran/BSA to form particles stabilized by phosphatidylcholine. The size of the particles ranged from 160 to 250 nm, but BMP-2 release from these particles was not investigated in that study.

NPs based on natural biopolymers will be more advantageous over particles made of synthetic materials. Bovine serum albumin (BSA) is a physiological protein that was widely used for NP preparation by different techniques.[18–21] BSA NPs are considered suitable for drug delivery because they are naturally biodegradable, nontoxic, and nonantigenic. The BSA NPs can be easily prepared under mild conditions by coacervation, or controlled desolvation processes.[22–24] To improve NP stability, BSA NPs is typically crosslinked by glutaraldehyde.[25–27] The glutaraldehyde reacts with free amines on BSA, and possibly with any amines on the encapsulated drug, which could adversely affect the drug integrity. The potential toxicity of glutaraldehyde is also a concern for in vivo delivery. As an alternative to glutaraldehyde, we considered the possibility of stabilizing BSA NPs with polyethylenimine (PEI). Branched PEI, because of its high density of positive charges, was expected to readily adsorb to the surface of BSA particles. The PEI coating could provide a means of controlling release rate of the encapsulated BMP-2. In this study, we explored the feasibility of such an approach by entrapping BMP-2 in BSA NPs and coating the particles with PEI. The results indicated that BMP-2 encapsulated in BSA NPs retained its bioactivity, and was released from the NPs as a function of PEI coating on the particles.

Materials and Methods

Materials

Bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the ALP substrate *p*-nitrophenol phosphate (*p*-NPP), 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (TCDG), and branched PEI ($M_w \sim$ 25,000 by LS, $M_n \sim$ 10,000 by GPC) were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human bone morphogenetic protein-2 (BMP-2, from *E.coli*.) was prepared as described earlier.[28] Fluorescein isothiocyanate (FITC) was obtained from Pierce (Rockford, IL). $Na^{125}I$ (in 0.1 M NaOH) was obtained from GE Healthcare (Piscataway, NJ). Dulbecco's modified eagle medium (DMEM), Hank's balanced salt solution (HBSS), penicillin (10,000 U/mL solution), and streptomycin (10,000 μg/mL solution) were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Atlanta Biologics (Atlanta, GA). All tissue culture plasticware was from Corning (Corning, NY). The Spectra/Por dialysis tubings with 12–14 kDa and 100 kDa cut-off were acquired from Spectrum Laboratories (Rancho Dominguez, CA). Distilled/deionized water (ddH_2O) used for buffer preparations were derived from a Milli-Q purification system.

Preparation of BMP-2 encapsulated, PEI-coated BSA NPs

BSA NPs were prepared by a coacervation method, or desolvation, as described previously,[29–31] except the crosslinker glutaraldehyde was eliminated in this process. Briefly, 250 μL of 10 mg/mL BSA solution (50 mg of BSA dissolved in 5 mL ddH_2O) was added to 250 μL of 10 mM NaCl solution (pH = 7.0) in a glass vessel under constant stirring (600 rpm) at room temperature. The stirring was allowed to proceed for 15 min. Then, 72 μL of 0.5 mg/mL BMP-2 in ddH_2O was added into the above solution. This aqueous phase was then desolvated with dropwise addition of 3.0 mL of ethanol after 2 h of incubation. The mixture was stirred (600 rpm) under room temperature for 3 h. The BMP-2 encapsulated BSA NPs so formed were coated with different PEI concentrations (see Legends for exact concentrations). Specially, different concentrations of PEI in 0.5 mM NaCl solution were added to the above NPs solution by a volume ratio of 1.25 to 1. The coating was allowed to proceed for 1 or 4 h under shaking. The coated NPs were extensively dialyzed (MWCO: 12–14 kDa) against phosphate buffered saline (pH = 7.3, 3×) and, for the bioactivity studies, then against DMEM with 1% penicillin/streptomycin (1×). The dialyzed buffer was exchanged every 3 h. All solutions used for NP preparation were sterilized by passing through 0.20 μm sterile filter (SARSTEDT, Nümbrecht, Germany) before use, and the manufacture process was carried out under sterile conditions.

For the determination of the percentage of BSA transformed into NPs before coating, the NPs were separated from the supernatant by centrifugation at 10,000 rpm for 10 min at room temperature. The amount of BSA in the supernatant was determined by the Bradford protein assay.[32] To 50 μL of the supernatant, 1 mL of the protein reagent was added, and the samples were analyzed spectrophotometrically at 595nm. A calibration curve was based on known concentrations of BSA standards. The yield of BSA in the NPs was calculated as: 100% × {(initial amount of BSA – BSA amount in the supernatant)/(initial amount of BSA)}.

Particle size and zeta potential

The mean particle size and polydispersity index of the PEI-coated and uncoated BSA NPs were determined by dynamic light scattering at 25°C with a Zetasizer 3000 HS (Malvern Instruments, UK) using a 633 nm He-Ne laser at a scattering angle of 90°. The surface charge of the coated and

uncoated BSA NPs were investigated by measuring the electrophoretic mobility of the particles using the zeta potential modus of the same instrument at 25°C. The samples for measurement were prepared after appropriate dilution and suspended in 1 mM NaCl solution. Both particle size and zeta potential measurements were measured for three batches of particles, each measurement being the average of 3 runs.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to study the morphology of the NPs. The samples were first dialyzed (MWCO: 100 kDa) against ddH_2O (3×), then centrifuged at 8,000 rpm for 10 min (3×) to remove any free BSA and PEI. The pellet was then redispersed in ddH_2O. Three microliter of the sample was added onto a clean silica surface, and dried under room temperature with natural convection. The prepared samples were, then, scanned on a JOEL Jamp-9500F SEM.

Atomic force microscopy

MFP-3D atomic force microscopy (AFM) (Asylum Research, Santa Barbara, CA) was used for the AFM studies of PEI-coated BSA NPs. AC240TS cantilever was used throughout all AFM measurements. The oscillation amplitude of the scanning tip was registered at 0.6 V and the frequency of the oscillation was in the range of 60–70 kHz. All AFM imaging was under conventional ambient tapping mode. The scan rate was typically 0.5–1.0 Hz, and the sample size was 512 × 512 pixels. Images were processed and analyzed by the Igor Pro imaging software (version 5.04B). The NP samples were sonificated for 5 min and then 1.5 μL of the sample was dropped onto the mica surface (PELCO^R Mica Discs; TED PELLA, Redding, CA), and imaged after drying under room temperature.

Coating efficiency with FITC-PEI

In one study, the amount of FITC-PEI coated on BSA NPs was determined by fluorescence measurements. To obtain the labeled polymer, a stock solution of FITC (10 mM in DMSO) was added to PEI solution (10 mg/mL in ddH_2O) for 2.5 h to give a final concentration of 0.1 mM FITC, after which the samples were dialyzed (MWCO: 12–14 kDa) against ddH_2O (3×) to remove the unreacted FITC.[33] Different concentrations of FITC-PEI were used to coat BSA NPs. The PEI-coated NPs (1 h and 4 h coating) were first dialyzed (MWCO: 100 kDa) against ddH_2O (3×), centrifuged (3×) for 10 min at 8,000 rpm to remove ethanol, free BSA, and uncoated PEI, and then redispersed in ddH_2O. A 200 μL of the aliquot in the duplicate was then added to black 96-well plates (NUNC, Rochester, NY) and the fluorescence (λ_{ex}: 485 nm; λ_{em}: 527 nm) was determined with a multiwell plate reader (Thermo Labsystems, Franklin, MA). The amount of FITC-PEI coated on the BSA NPs was calculated based on a calibration curve generated by using the FITC-PEI in ddH_2O. The calibration curve was also generated in a mixture of FITC-PEI and BSA solution (0.5 and 1.0 mg/mL of BSA, respectively), and the results showed that they were comparable to the calibration curve generated in ddH_2O (data not shown). The coating efficiency of FITC-PEI was calculated as: 100% × {(final FITC-PEI of the pellet)/(initial FITC-PEI for coating)}.

Encapsulation efficiency and in vitro release of BMP-2

To determine the encapsulation efficiency and in vitro release of BMP-2, BMP-2 was labeled with ^{125}I by using the Iodo-Gen procedure.[34] Microcentrifuge tubes were coated with TCDG (200 μL of 20 μg/mL TCDG in chloroform), and 20 μL of BMP-2 solution (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 $Na^{125}I$. After reacting for 25 min, free ^{125}I was separated from the radiolabeled BMP-2 using a Sephadex G-25 column. After precipitating an aliquot of the purified samples with 20% trichloroacetic acid (TCA), the counts in the supernatant and the pellet was determined with a γ-counter (Wizard 1470; Wallac, Turku, Finland), and it was confirmed that the iodinated samples contained <4% free ^{125}I. To determine the encapsulation efficiency, ^{125}I-labeled BMP-2 was first diluted by ddH_2O (1:5), and then mixed with BSA solution as described earlier to prepare the NPs. Five hundred microliter of ^{125}I-labeled BMP-2 encapsulated BSA NPs, uncoated and coated with different concentrations of PEI, were then added into the microcentrifuge tubes in triplicate. The samples were centrifuged at 5,500 rpm for 10 min. The counts in the supernatant and pellet were determined by a γ-counter, and the encapsulation efficiency (EE) was calculated as: EE = 100% × {(counts in the pellet)/(counts in the pellet + counts in the supernatant)}.

The NPs containing ^{125}I-labeled BMP-2 was used to measure the release rate of BMP-2 in vitro. The NPs were coated with PEI concentrations of 0.6, 0.3, 0.1, and 0 mg/mL for 1 h for this study. The release experiment was performed by incubating the NPs (in triplicate) in 1 mL DMEM with 1% penicillin/streptomycin at 37°C ± 1°C and under constant shaking. At indicated time points, the samples were centrifuged at 5,500 rpm for 10 min. The counts in the supernatant and the pellet were determined separately by a γ-counter. One milliliter of fresh release medium was added back to the pellet to maintain a constant volume during release period. To test for free iodine in the supernatant, an aliquot of the purified samples was precipitated with 20% TCA after each centrifugation. The counts in the TCA supernatant and the pellet were determined by a γ-counter, and ^{125}I-labeled BMP-2 released to the supernatant was calculated accordingly.

Toxicity of BSA NPs by MTT assay

The MTT dye reduction assay[35] was used to assess the cytotoxicity of PEI-coated NPs. Various concentrations of PEI were coated on BMP-2 encapsulated BSA NPs for 1 h. After coating, the samples were first dialyzed (MWCO: 12–14 kDa) against phosphate buffered saline (pH = 7.3; 3×), followed by dialysis against DMEM with 1% penicillin/streptomycin (1×). The samples were then centrifuged at 8,000 rpm for 10 min, and the supernatant was removed. The same volume of fresh DMEM with 1% penicillin/streptomycin was added to the pellet, which was sonicated for 20 min. An aliquot of the supernatant and the suspended pellet was incubated with human C2C12 cells grown on 48-well plates (in triplicate). After 48 h incubation, 100 μL of sterile filtered 5.0 mg/mL MTT solution in HBSS were added to 0.5 mL cell culture medium in each well. The plates were incubated in the dark for 2 h, the supernatant was aspirated, and 0.5 mL DMSO was added to each well. Then the plates were incubated for 5 min and mixed to dissolve the crystals.

The optical density of the crystals was measured by an ELISA reader at 570 nm. The untreated cells served as the reference. The mean optical density was used as a measurement of cell number/well.

BMP-2 bioactivity by kinetic ALP assay

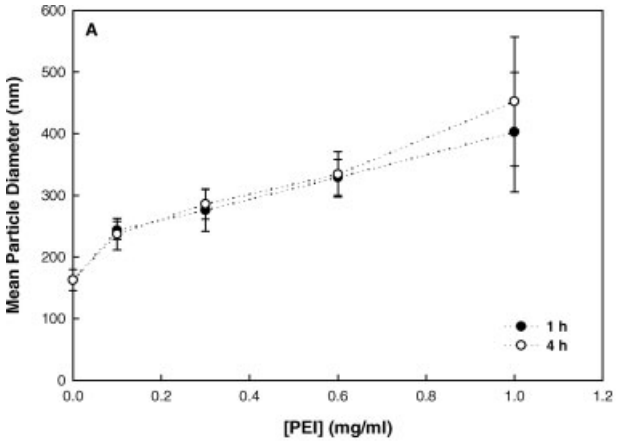
A kinetic ALP assay was performed to determine the bioactivity of encapsulated BMP-2.[36] Human C2C12 cells, grown in 48-well plates, were first incubated with the NPs (supernatant and suspended pellet), which were processed as described in the MTT assay. After 72 h of incubation, the C2C12 cells were washed with HBSS (2×) and lysed with 200 μL ALP buffer (0.5 M 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton-X; pH 10.5). After 1 h of lysis, 200 μL of 1.0 mg/mL ALP substrate (*p*-nitrophenol phosphate) was added to the lysed cells. The changes in optical density ($\lambda_{absorbance}$: 405 nm) were determined in a multiwell plate reader at an intervals of 1.5 min for eight cycles. The kinetic ALP activity was expressed as the change in optical density of the wells per unit time (mAbs/min). All results were expressed as mean ± standard deviation (SD) of triplicate wells.

Data analysis

All data shown in the figures are summarized as mean ± SD of the specified number of replicates. The number of independent experiments (n) was specified for each figure in the legends. Where indicated, statistical differences between group means were analyzed by the two-sided Student's t-test or single factor analysis of variance (ANOVA, Microsoft Office Excel 2003).

Results and Discussion

A new formulation of BMP-2 in NPs was pursued by first eliminating the glutaraldehyde used in a typical BSA NP fabrication process. The reason for this was the possibility of glutaraldehyde crosslinking the protein amines to the BSA matrix, producing a protein-conjugate, which could affect the protein release and bioactivity. This was noted for the encapsulation of recombinant IFN-γ, a cytokine of the Th1-type cellular immune response, with albumin NPs. The IFN-γ inside the BSA matrix completely abrogated its bioactivity, whereas the adsorbed IFN-γ on the glutaraldehyde crosslinked NPs retained its bioactivity.[26] A previous study performed by the same group,[27] in which no release of IFN-γ was observed from the glutaraldehyde crosslinked BSA NPs, also highlighted the need to eliminate glutaraldehyde in the BSA NP fabrication process. In addition to proteins, glutaraldehyde crosslinking was also problematic for the small molecular drug doxorubicin, which gave only ~8% free drug release in a 3-day study period due to drug conjugation to the BSA matrix.[25] This incomplete release of drug was also reported by another group studying the glutaraldehyde crosslinked albumin microspheres for the release of adriamycin.[37] We, therefore, turned to branched PEI, which is known to display exceptional affinity to anionic surfaces. This physical interaction between PEI and BSA matrix should not affect the BMP-2 integrity during encapsulation and at the same time control the release of BMP-2 through the network of BSA-PEI matrix formed on the NP surface. The properties of NPs formed from such a process were first investigated, followed by the BMP-2 release kinetics and bioactivity in vitro.



B. Polydispersity

1 h	Mean ± SD	4 h	Mean ± SD
1	0.42 ± 0.21	1	0.26 ± 0.23
0.6	0.47 ± 0.12	0.6	0.44 ± 0.06
0.3	0.20 ± 0.05	0.3	0.24 ± 0.17
0.1	0.32 ± 0.17	0.1	0.27 ± 0.16
0	0.44 ± 0.16	0	0.44 ± 0.16

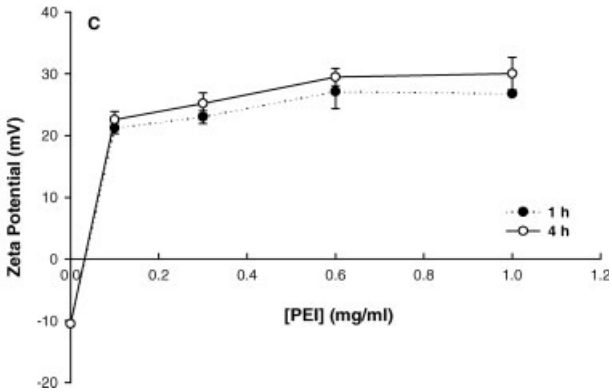


Figure 1. The mean particle diameter (A), polydispersity index (B), and zeta potential (C) of BSA NPs coated with different PEI concentrations (n = 3).

Three independent batches for each coating concentration were used for measurements, and each measurement was performed in 3 runs. The mean particle diameter increased from ~150 nm for uncoated BSA NPs to ~400 nm for the highest PEI coating concentration (1.0 mg/mL). The zeta potential of the coated NPs increased dramatically as compared to uncoated NPs, and slightly increased as PEI concentration was increased from 0.1 to 1.0 mg/mL for both 1 and 4 h coating times.

Characterization of PEI-coated BSA NPs

To examine the influence of PEI coating, BSA NPs were prepared by changing the PEI concentration during the coating process as well as the contact time between the NPs and the PEI. Using PEI concentrations of 0.1, 0.3, 0.6, and 1.0 mg/mL, the size of the NPs was observed to gradually increase as the PEI coating concentration was increased; from 150 nm for the uncoated NPs, to ~230, ~280, ~330, and ~400 nm for 0.1, 0.3, 0.6 and 1.0 mg/mL PEI, respectively (Figure 1A). A coating time of 1 or 4 h did not seem to make a difference in the size of the particles (Figure 1A).

With higher PEI concentration, the availability of more PEI presumably facilitated the formation of a thicker and more intensive layer of a PEI-BSA network on NP surfaces.[38] The coating appeared to be rapid, because it seemed to be complete within 1 h of contact between the NPs and the PEI. The extent of PEI coating controlling the size of NPs was also reported for a preparation of PLA/PLGA NPs, where PEI was used to reduce the interfacial surface energy between the particle surface and the aqueous media.[39] Coating by using 1.0 mg/mL PEI led to relatively significant variations in the NP size (see large SDs in Figure 1A), probably because of higher aggregation of the BSA NPs with the availability of abundant PEI capable of forming linkages among the particles. The polydispersities of the NPs are summarized in Figure 1B for three independent batches of NPs. The relatively high polydispersity of NPs was attributed to two possible reasons: (1) the manual performance of the desolvation process by adding the desolvating agent, ethanol, to the protein solution drop by drop as mentioned by other groups before[29]; (2) some aggregation of particles formed for PEI coated NPs, or unstable particles disassociated for uncoated NPs during the dialysis process. There was no significant difference in the polydispersity of NPs from different PEI concentrations used for coating (ANOVA).

The zeta-potential of the particles increased significantly as a result of PEI coating (Figure 1C), from −10 mV for uncoated NPs to +21–29 mV for the PEI coated NPs. A slight increase from +21 to +29 mV was exhibited for coated NPs as the PEI concentration was increased from 0.1 to 1.0 mg/mL. This was indicative of increased PEI adsorption with increased concentration, an observation consistent with increasing particle size in this concentration range. There was a small increase of the zeta potential as the coating time was increased from 1 to 4 h, but this was not significant and it paralleled the effect of coating time on particle size.

Using the Bradford protein assay, we obtained >90% BSA transformation into the NPs after ethanol desolvation. This was in agreement with earlier results of Langer and Weber et al.[29,40] The amount of PEI adsorption on BSA NPs was directly quantified by using a FITC-labeled PEI polymer and measuring the extent of adsorbed fluorescence on NPs. The results were summarized as PEI amount (μg) adsorbed on NPs (Figure 2A). As the PEI concentration was increased, the amount of adsorbed PEI was increased as expected. There was no difference in the adsorbed PEI between 1 and 4 h for PEI coating concentration of 0.1 mg/mL, but a difference was noted for other PEI concentrations. Between the lowest and highest PEI concentrations used in this study, ~2-fold and ~3-fold increase in PEI coating were seen for 1 h and 4 h coating time, respectively. The effect of such an increase did not have a corresponding consequence in size and/or charge of the NPs. The PEI coating efficiency (Figure 2B) was dependent on the PEI concentration; as expected, coating with 0.1 mg/mL PEI demonstrated the highest coating efficiency (~45%) among all PEI concentrations. The coating efficiency was increased by 4–6% as the coating time was increased from 1 to 4 h for other PEI concentrations. The highest coating efficiency obtained with the lowest PEI concentration was consistent with our previous results of PEI adsorption to hydroxyapatite: the percentage of hydroxyapatite adsorption was decreased as the PEI concentration increased; the total amount of binding, however, increased with the increasing PEI concentration in the medium.[33] It

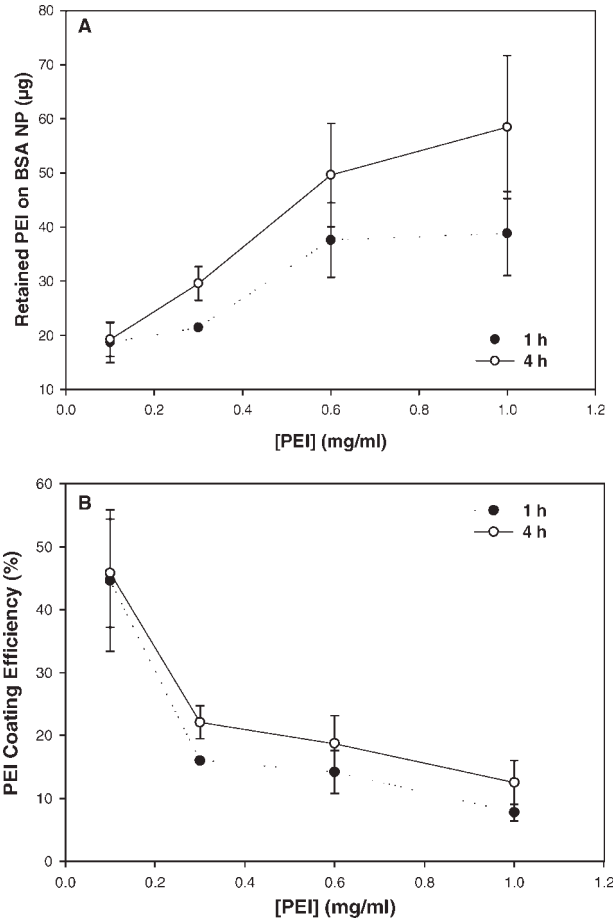


Figure 2. The quantification of PEI coating on BSA NPs, as measured by adsorption of FITC-labeled PEI (n = 3).

The fluorescence of the PEI adsorbed on BSA NPs was measured as a function of PEI coating concentration, and converted to mass (μg) of PEI adsorbed (A) based on FITC-PEI calibration curve. The amount of PEI adsorbed on the NPs was increased with the PEI concentration and the coating time. The coating efficiency on BSA NPs was calculated from the adsorbed PEI and the initial PEI in solution (B). Note that coating concentration of 0.1 mg/mL PEI gave the highest coating efficiency among the PEI concentrations used.

was interesting to note that FITC-labeled PEI continued to adsorb to BSA NPs from 1 to 4 h, even though this was not immediately clear from size and charge measurements. It is likely the adsorbed PEI was penetrating into the interior of the NPs during this time without affecting the size or surface properties.

As shown in Figure 3, the NPs generally possessed spherical shapes and smooth surface characteristics after analysis by SEM and AFM. Figures 3A,B are SEM images for particles coated with 1.0 and 0.1 mg/mL PEI, respectively. Although more uniform particles were evident for coating with the lower PEI concentration, coating with higher PEI concentration resulted in some nonspherical particles, typically appearing as elongated particles. Presumably, this resulted from fusion of two separate spherical particles. This might also be the underlying mechanism for increased sizes of the particles measured by the dynamic light scattering. Figures 3C,D are height-mode AFM images for 1.0 and 0.1 mg/mL PEI coating, respectively. Larger particles for coating with 1.0 mg/mL PEI were also evident in comparing these two images. Figure 3E is the amplitude-mode AFM

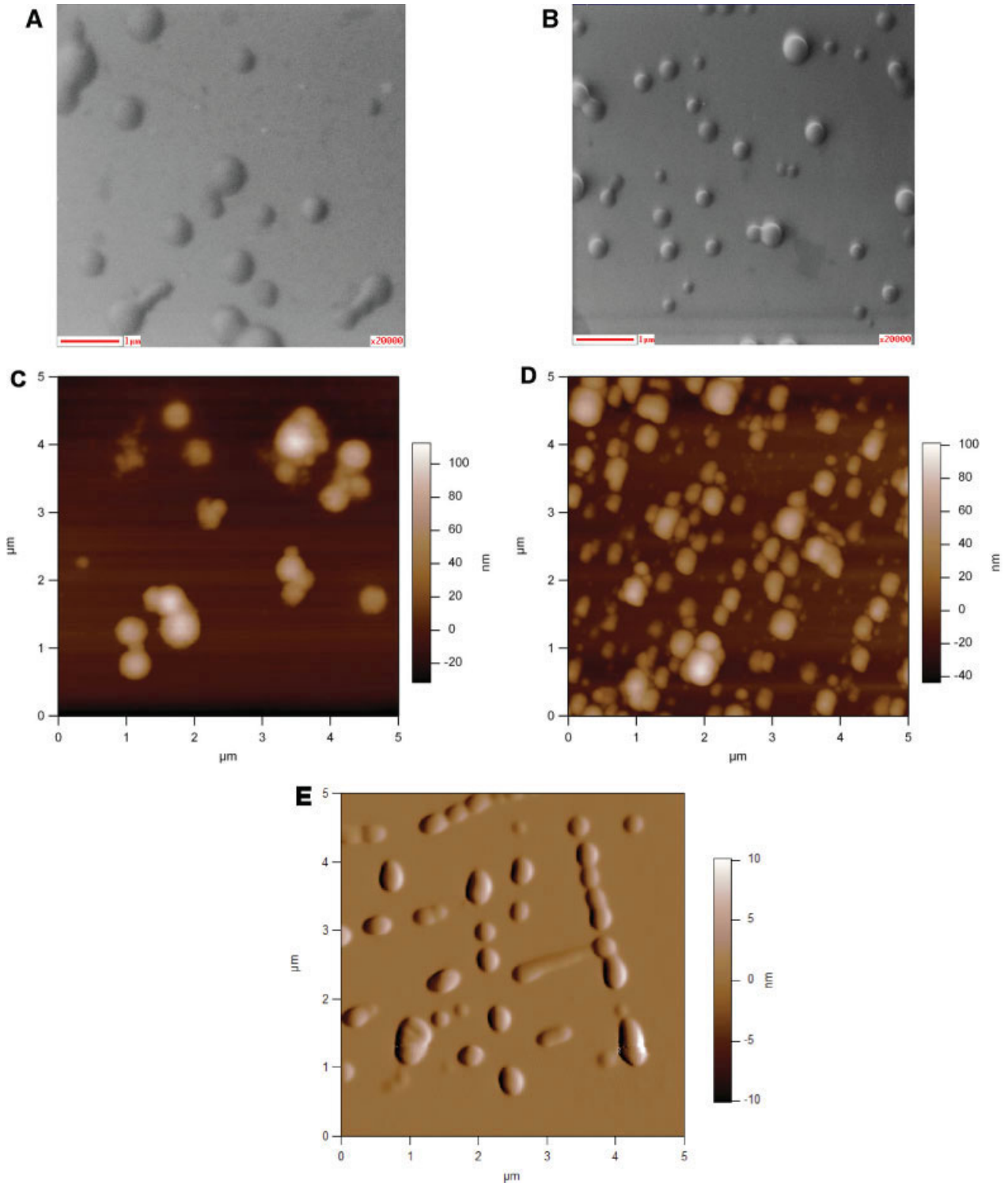


Figure 3. SEM images of BSA NPs coated with 1.0 mg/mL PEI (A) and 0.1 mg/mL PEI (B); AFM images (height-mode) of BSA NPs coated with 1.0 mg/mL PEI (C), 0.1 mg/mL PEI (D) and an amplitude-mode image of PEI (0.1 mg/mL)-coated BSA NPs with BMP-2 encapsulated (E).

Larger diameter NPs were evident in the samples coated with the higher PEI concentration.

image for 0.1 mg/mL PEI coated BSA NPs with BMP-2 encapsulated (14.4 μg/mg bulk BSA). Encapsulating BMP-2 in the NPs did not increase the size of the particles (Figure 3E).

The particle size and surface charge are two main properties that can influence biodistribution of colloidal carriers upon administration.[41] This is not likely to be a critical issue when NPs are used for local delivery of proteins after implantation, but it will be a significant issue when NPs are injected systemically. A positive surface charge of the NPs is regarded beneficial for penetrating plasma membrane[42]; however, it is a major disadvantage after systemic administration because cationic NPs can bind nonspecifically to cell

Table 1. Encapsulation Efficiency Obtained by Using^{125} I-labeled BMP-2 (in Triplicate)

	Encapsulation Efficiency	
PEI concentration (mg/mL)	Before Coating	After Coating
0.6	–	96.53 ± 0.08
0.3	–	96.88 ± 0.15
0.1	–	96.20 ± 0.27
0	90.10 ± 0.38	–

The coating time for all preparations was 1 h ($n = 2$).

surfaces and activate the complement system.[43] In addition, particulates are rapidly opsonized with serum proteins,[44] leading to preferential uptake of particles by the cells of the reticuloendothelial system and rapid clearance. These adverse effects associated with the use of cationic NPs in vivo can be reduced with smaller NPs[45,46] and lower surface charges.[44] It was reported that the extent of opsonization decreased as the size of the particles decreased from 800 to 200 nm, and no enhancement of phagocytic uptake was recorded at particle sizes below 200 nm.[41] However, particles below 100 nm were able to cross the fenestration in the hepatic sinusoidal endothelium, leading to a hepatic accumulation instead of long circulation times. Though deformity of particles was considered to be a factor for crossing endothelial fenestration,[47] the albumin-based particles are regarded as a solid particle matrix. One study investigated in vivo distribution of surface-modified albumin NPs in rats, and observed no differences in blood circulation times and organ accumulation among positive, negative, and neutral surface charged NPs.[48] Evaluating in vivo biodistribution of the PEI-coated BSA NPs was beyond the scope of this study, but it appears that BSA NPs coated with lower PEI concentrations might be more desirable for systemic administration due to reduced size, reducing the chances of opsonization. It remains to be seen whether in vivo biodistribution will be unaffected by the extent of PEI coating, as suggested in Ref. *48*, or whether PEI will impart a unique biodistribution pattern to the BSA NPs.

Encapsulation efficiency and BMP-2 release

The encapsulation efficiency and in vitro release of BMP-2 was assessed by using ^{125}I-labeled BMP-2 in BSA NPs. The BMP-2 encapsulation efficiency was typically >90% for all NPs prepared (Table 1), and it was even higher after PEI coating of the NPs. A significant effect of PEI coating was readily observed on the BMP-2 release kinetics, analyzed by assessing the BMP-2 amount in the tissue culture release medium (Figure 4A) or the BMP-2 amount retained in the NPs (Figure 4B). A burst release of BMP-2 was evident for uncoated NPs, where >70% of the BMP-2 content was released into the medium within the first day. When NPs were treated with PEI, a pronounced decrease in the BMP-2 burst release (<15% in 1 day) was seen, while the release period was extended over the 10-day study period (Figure 4). Increasing the PEI concentration used for coating NPs decreased the extent of BMP-2 release; the cumulative release at the end of the 10 days was 51, 42, and 29% for NPs coated with 0.1, 0.3, and 0.6 mg/mL PEI, respectively (Figure 4A). The differences in accumulative release between 0.6 and 0.3 mg/mL, and 0.6 and 0.1 mg/mL PEI

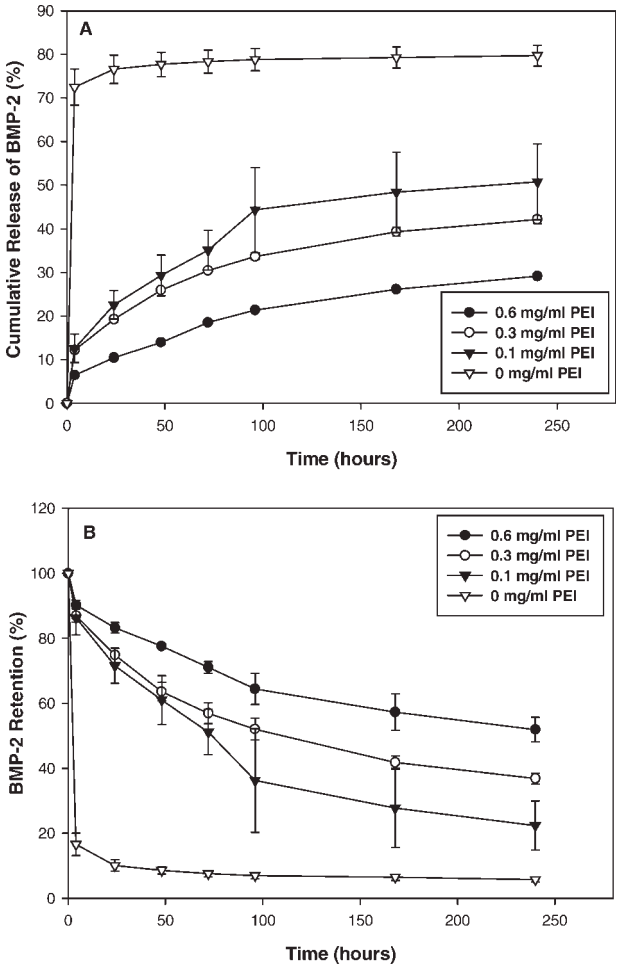


Figure 4. The release profile of BMP-2 from the PEI-coated BSA NPs with different PEI coating concentrations.

The release was performed after encapsulating ^{125}I-labeled BMP-2 in BSA NPs and using a release medium of DMEM containing 1% penicillin/streptomycin at 37°C ± 1°C (A). PEI coating time was 1 h and the results were expressed as the mean ± SD (triplicate) percentage of BMP-2 loaded in NPs. At each time point, the amount of BMP-2 in the supernatant (A) and the pellet (B) was determined after separating the supernatant from the NPs by centrifugation. There was a burst release of BMP-2 for uncoated BSA NPs within hours of incubating the NPs in the release medium. The burst release was effectively suppressed by PEI coating. Note that the gradual reduction of BMP-2 release as the PEI coating concentration was increased from 0.1 to 0.6 mg/mL. In case where SDs are not seen, they are smaller than the symbols and this study was repeated once more with similar results ($n = 2$).

coating were significant ($P < 0.05$), whereas the difference between 0.3 and 0.1 mg/mL PEI coating was not. Taken together, an effective layer of PEI appeared to be formed around the NP surfaces to control the BMP-2 release, and treatment with higher PEI concentration allowed slower release of BMP-2 with a minimal burst release. It is likely that the effect of PEI coating was mediated by two mechanisms, one in which the PEI coating physically reduced the BMP-2 diffusion through the NP surface, and one in which the PEI stabilized the particles, preventing premature disintegration of the BSA NPs. The relative contribution of each mechanism remains to be determined.

The 10-day, 29–51% cumulative BMP-2 release obtained with the current PEI-coated BSA NPs was less than the VEGF release obtained from NPs formed by VEGF-bound dextran and PEI (~50% encapsulation efficiency with >10

days of ~75% cumulative release).[49] The ionic interaction between anionic dextran sulfate and cationic PEI built up the NPs in that system and effectively controlled the release of VEGF. Chitosan and poly-L-lysine were also investigated in that study as alternative polycations, but they all showed similar VEGF release trends. However, the influence of PEI concentration on particles formation and VEGF release was not examined in that study. Similar approach for protein delivery was also employed for the delivery of insulin and amphotericin B,[50,51] where 40–100% release were observed in the study period depending on the ionic strength and the nature of the release medium. BMP-2 is not readily soluble at the physiological pH, and this may explain the lower amount of BMP-2 release in our system. A lower release, however, may be advantageous for in vivo application where increased retention at implant site may give a more robust bone induction.[52]

Toxicity of PEI-coated BSA NPs

The MTT assay was employed to determine the cytotoxicity of the PEI-coated BSA NPs. Among the components of the prepared NPs, only PEI was expected to display cytotoxicity because cationic polymers strongly interact with anionic cellular surfaces and might compromise the integrity of cellular membranes.[53] PEI is known to induce cytotoxicity because of its unique architecture and an increased toxicity was noted with high molecular weight and branching.[54] Initial studies indeed showed a strong toxicity of the prepared NPs on C2C12 cells, especially for particles coated with high PEI concentrations (not shown). To elucidate the source of the toxicity, BSA NPs were separated into two fractions by centrifugation; a supernatant containing soluble BSA and PEI molecules, and a pellet containing the NPs, and the cytotoxicity of these two fractions was evaluated separately. The supernatant of the BSA NPs displayed differential cytotoxicity as a function of PEI concentration used for coating (Figure 5A). A clear toxicity was evident for the two higher PEI concentrations (1.0 and 0.6 mg/mL) while the toxicity of 0.3 mg/mL PEI coating was dependent on the volume of the supernatant added to the cells. The lowest concentration of the PEI (0.1 mg/mL) did not indicate any additional toxicity as compared to the uncoated NPs. In contrast, the pellet containing the NPs from different preparations did not reduce the viability of the cells, based on the relatively similar MTT absorbance for all PEI concentrations used for coating (Figure 5B). Two additional batches of NP preparations were evaluated in the similar manners, and both batches gave similar results as earlier (not shown).

These results were indicative of free PEI being present in the NP preparations. The dialysis procedure used for removing free PEI apparently did not lead to complete removal of the PEI, but a centrifugation process was successful to prepare the NPs with no/little cytotoxicity. This was important in further bioactivity testing of the NP preparations, because any toxicity of NPs might lead to underestimation of the BMP-2 bioactivity retained in the NPs. The PEI immobilized on the BSA NPs was apparently not toxic, either because of its low concentration or its immobilized state which prevented its association with cellular surfaces. An independent study on PEI based DNA-polyplexes reported a significant proportion of the polymer utilized remained in free form; this was in part responsible for cell dysfunction and cytotoxicity. After purification of the PEI/DNA complex, the particles demonstrated reduced toxicity,[55] which suggested that the purification of the NPs could reduce the toxicity of the PEI coating. It might be necessary to replace PEI in future studies with more biocompatible cationic polymers, but for the purposes of this study, PEI-coated NPs was further tested to investigate whether BMP-2 activity was retained in the NP formulations.

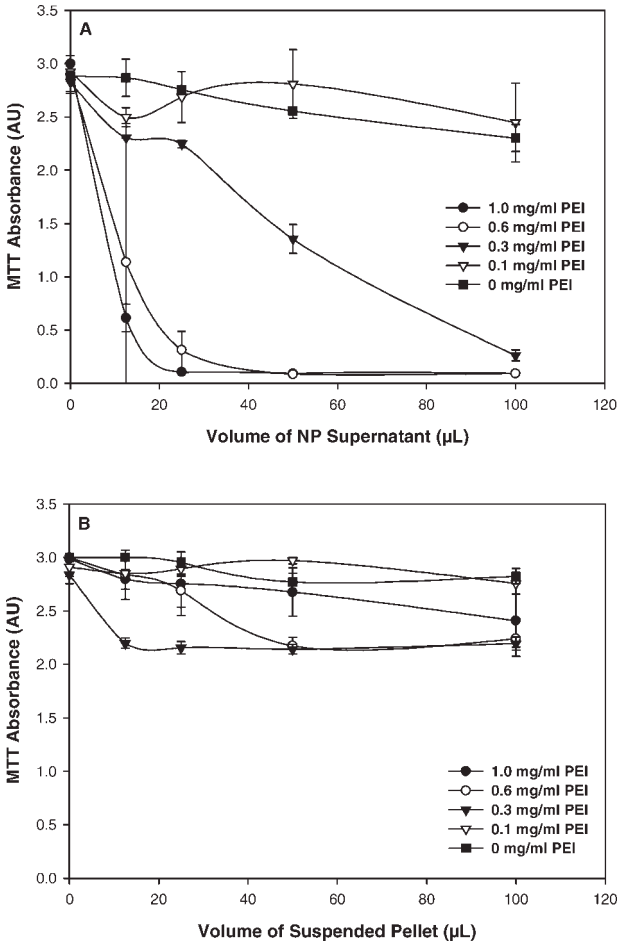


Figure 5. Cytotoxicity of BSA NPs coated with different PEI concentrations.

The cell viability by the MTT assay was determined by using C2C12 cells incubated with the supernatant (A) and the suspended pellet (B) after separating the two fractions by centrifugation. Cells were grown in 48-well plates (in triplicate) and incubated with the samples for 48 h. Note that the observed toxicity was dependent on the PEI concentrations. In the supernatant, no toxicity was observed with uncoated BSA NPs but significant toxicity was evident for PEI coating concentration >0.3 mg/mL. The pellet separated from the supernatant, on the other hand, gave no clear toxicity for all PEI concentrations. Two additional batches of NP preparations were evaluated in the similar manners, and both batches gave similar results as above ($n = 3$).

BMP-2 activity in PEI-coated BSA NPs

ALP is an early marker of osteoblast differentiation.[56] We used the BMP-2 induced ALP induction in C2C12 cells in vitro as a measure of BMP-2 activity. We first measured the ALP induction by four groups: (i) BSA NPs without BMP-2, (ii) BSA/BMP-2 solution that was subjected to NP formation process without the addition of ethanol so that no NPs was formed, (iii) BSA NPs with encapsulated BMP-2, and (iv) PEI (1.0 mg/mL)-coated BSA NPs with encapsulated BMP-2 (Figure 6). The BMP-2 loading in the above samples was

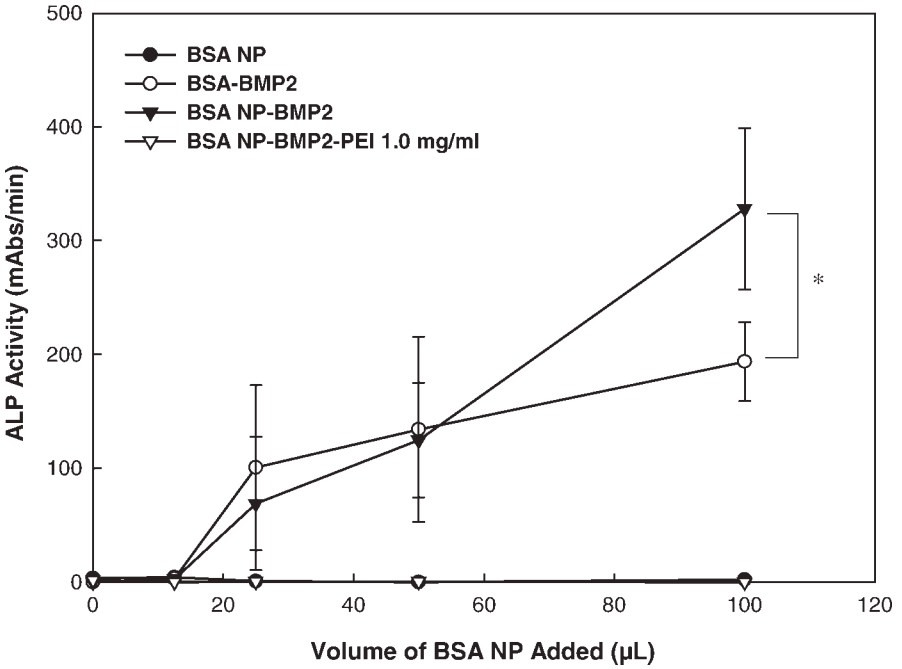


Figure 6. The BMP-2 activity in different NP formulations, as measured by ALP induction assay with C2C12 cells (in triplicate).

The formulations used were a mixture of BSA and BMP-2 solution without NP formation (i.e., no ethanol addition), BSA NPs without any BMP-2, BMP-2 encapsulated in uncoated BSA NPs, and BMP-2 encapsulated in BSA NPs coated with 1.0 mg/mL PEI. The bioactivities of BMP-2 in BSA solution or in BSA NPs were comparable. No activity was seen in BSA NPs in the absence of BMP-2, and BMP-2 in NPs coated with 1.0 mg/mL PEI gave no activity, presumably due to toxicity of this formulation. *$P < 0.05$ at this volume added.

14.4 μg BMP-2/mg bulk BSA. The samples were analyzed without centrifugal purification. An equivalent BMP-2 activity was evident in two samples, the BMP-2/BSA mixture and the BMP-2 entrapped in BSA NPs (Figure 6). For the other two groups, BSA NPs without BMP-2 and PEI-coated BSA NPs with encapsulated BMP-2, there was no detectable activity. This was expected for the former group in the absence of BMP-2, as well as the latter due to excessive toxicity of PEI at this coating concentration (see Figure 5A). This study confirmed the retention of BMP-2 activity as a result of entrapment in BSA NPs. The fact that a similar activity was seen with and without NP formation suggests an equivalent level of BMP-2 retention in these samples. We expected almost complete entrapment of the BMP-2 in NPs based on the obtained encapsulation efficiencies (see Table 1); we also expected to retain a high amount of BMP-2 in the BSA solution after processing, because (i) the dialysis tubing used for NP purification had a MW cut-off (12–14 kDa) smaller than the MW of BMP-2 (~32 kDa), and (ii) the presence of abundant BSA (~100-fold higher) should stabilize BMP-2 in solution and prevent its loss due to adsorption to the dialysis apparatus.

A subsequent study was performed by using BMP-2 containing NPs that were coated with lower PEI concentrations. The NP preparations were additionally fractionated into a supernatant containing the soluble molecules and a pellet containing the NPs before the bioactivity testing. The ALP induction is shown in Figure 7A for the NPs before fractionation, in Figure 7B for the pellet after fractionation and in Figure 7C for the supernatant after fractionation. For the unfractionated NPs, the ALP activity was induced as a function of NP volume added for uncoated BSA NPs and BSA NPs coated with 0.1 mg/mL PEI. No significant difference was evident between these two groups, indicating no adverse effects of the 0.1 mg/mL PEI coating. For BSA NPs coated with 0.6 and 0.3 mg/mL PEI, however, the induced ALP activity decreased as the volume of NPs increased, presumably due to the toxicity of the higher amount of PEI in the medium. Upon fractionation, free PEI was expected to be removed from the preparation, so that bioactivity could be tested in the absence of toxicity issues associated with the PEI. In this case (Figure 7B), all PEI-coated NPs containing BMP-2 gave significant activity in the pellet and the level of ALP induction of BSA NPs coated with 0.1 mg/mL PEI was significantly higher than 0.6 and 0.3 mg/mL PEI coating ($P < 0.05$) at the highest volume (100 μL) of suspended pellet added. For the samples coated with the higher PEI concentration (0.6 and 0.3 mg/mL), there was still a decline in ALP activity at the higher volumes of samples added, indicating continued toxicity even for the pellet fraction. It is possible that a high concentration of PEI adsorbed onto the NPs might directly damage the cells or be released into the medium to manifest its toxic effect. The NPs with the lowest PEI coating (0.1 mg/mL) gave a dose-response curve in the ALP induction assay without any sign of toxicity. BMP-2 entrapped in uncoated BSA NPs gave a little activity in the pellet fraction. This was presumably due to rapid release of BMP-2 into the supernatant of NP preparations, which suggested that the uncoated NPs are stable in ethanol, but relatively unstable during the dialysis procedure. The ALP activity was demonstrated when the supernatants of the same samples were analyzed in the bioassay; the uncoated BSA NPs gave a significant BMP-2 activity whereas the 0.6 and 0.3 mg/mL PEI-coated NPs gave nearly no BMP-2 activity and 0.1 mg/mL PEI-coated NPs gave some activity. Presumably, there was little release of BMP-2 for the 0.6 and 0.3 mg/mL PEI-coated NPs samples. It is possible that the lack of BMP-2 activity is obscured by the toxicity of 0.3 and 0.6 mg/mL PEI samples (from Figure 5A), but this was not valid for the 0.1 mg/mL PEI-coated sample because toxicity

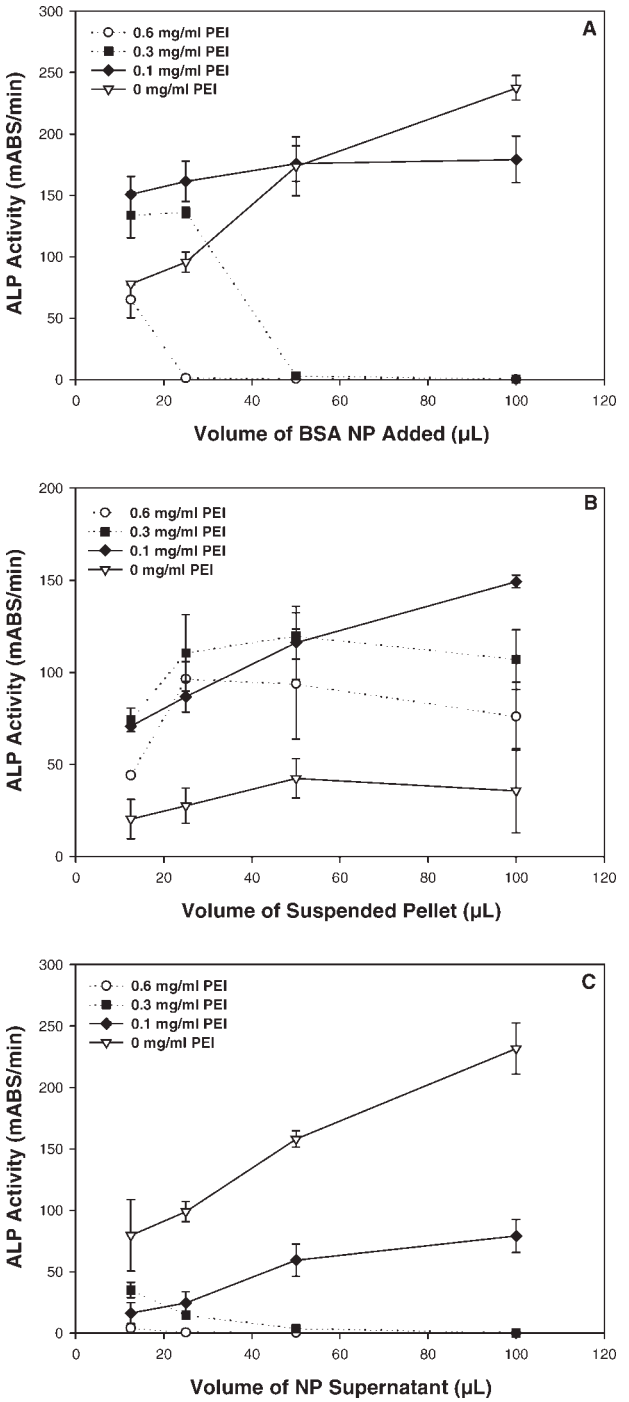


Figure 7. The BMP-2 activity in different NP formulations, as measured by ALP induction assay with C2C12 cells (in triplicate).

The formulations were tested without purification (A), or after centrifugation to separate the pellet containing the NPs (B) and the supernatant containing the soluble molecules (C). The BMP-2 activity observed was dependent on the PEI concentration used to coat the NPs. In the original NP formulation (A), uncoated and 0.1 mg/mL PEI coated BSA NPs retained the BMP-2 activity to an equivalent degree, while the NPs coated with 0.6 and 0.3 mg/mL PEI gave a decreasing BMP-2 activity as higher volumes added. In the pellet with NPs (B), NPs coated with all three PEI concentrations displayed an ALP induction activity higher than the uncoated BSA NPs, which showed no activity. In the supernatant (C), the uncoated BSA NPs induced ALP activity, while the BSA NPs coated with 0.1 mg/mL PEI induced some ALP activity. The supernatant from the 0.3 and 0.6 mg/mL PEI coated NPs did not yield any BMP-2 activity. This study was repeated twice more with similar results ($n = 3$).

of the original preparation was minimal, if any, with this sample. In this case, some release of BMP-2 might have been achieved during the 3-day C2C12 cell incubation study, so that the BMP-2 was available during this time for the ALP induction.

The results from the bioactivity studies indicated that BSA NPs coated with 0.1 mg/mL PEI was the preferred formulation. Such NPs provided a release formulation that was compatible with the mammalian cells in vitro. The BMP-2 entrapped in such a NP formulation was bioactive based on the ALP induction assay with C2C12 cells. The bioactivity studies also provided supportive evidence for the slower release of the BMP-2 from these formulations, unlike uncoated BSA NPs, which displayed the most BMP-2 activity in the supernatant and not in the pellet containing the NPs. The fact that little BMP-2 was recovered from the supernatant of NP formulations is encouraging for developing an aqueous-based process for NP fabrication, because protein loss due to release from NPs will be minimized during the fabrication process. It remains to be seen whether this is true for other bioactive proteins; BMP-2 is usually not soluble under physiological conditions (i.e., ionic strength and pH) and this might have helped with improved encapsulation efficiency and a more-sustained release profile. More-readily soluble proteins might behave differently in this respect. We are aware that C2C12 cells are myogenic origin and more studies will be needed to assess the bioactivity of the proposed BMP-2 formulations with more relevant cellular phenotypes. For example, cells derived from bone marrow cavity[36] or osteoblastic cell lines such as mouse calvarial MC3T3-E1 cells[28] will need to be further explored to better extrapolate the bioactivity results to physiological responses.

Conclusions

The bone-inducing growth factor BMP-2 was encapsulated in albumin NPs, which were coated with the cationic polymer, PEI, for better control of BMP-2 delivery kinetics. The electrostatic interaction between the anionic albumin and cationic PEI was sufficient to create an effective PEI coating on the NPs. The size of spherical NPs ranged from ~150 nm for uncoated BSA NPs, and significantly increased up to 400 nm as a function of PEI concentration used for coating the particles. The zeta-potential of the particles similarly increased significantly as a result of PEI coating. The encapsulation efficiency was typically >90% in all NP preparations. In vitro release of encapsulated BMP-2 was controlled by the PEI coating of the NPs, providing a gradual reduction of BMP-2 release as the PEI concentration for coating was increased. The cytotoxicity was a significant issue for NPs coated with high concentrations of PEI, but it was possible to minimize the toxic effect of PEI by using lower PEI concentration during coating process. The overall results indicated that BMP-2 encapsulated BSA NPs coated with 0.1 mg/mL PEI gave tolerable toxicity, retained a robust ALP induction activity in C2C12 cells, and efficiently slowed the release of BMP-2 from the BSA NPs. These studies established the foundation for testing NP formulations for BMP-2 in animal models, and in particular evaluating the effect of sustained-release formulations on BMP-2 induced bone formation.

Acknowledgments

We thank Sara Elhasi (Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta) for the help with Zetasizer

3000 HS (Malvern Instruments, UK). This project was financially supported by an operating grant from the Canadian Institutes of Health Research (CIHR). Infrastructure support was provided by Canadian Foundation for Innovation (CFI) and Alberta Heritage Foundation for Medical Research (AHFMR).

Literature Cited

1. Lieberman JR, Daluiski A, Einhorn TA. The role of growth factors in the repair of bone: biology and clinical applications. *J Bone Joint Surg Am*. 2002;84:1032–1044.
2. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. Novel regulators of bone formation: molecular clones and activities. *Science*. 1988;242: 1528–1534.
3. Yasko AW, Lane JM, Fellinger EJ, Rosen V, Wozney JM, Wang EA. The healing of segmental bone defects, induced by recombinant human bone morphogenetic protein rhBMP-2. A radiographic, histological, and biomechanical study in rats. *J Bone Joint Surg Am*. 1992;74:659–670.
4. Bostrom M, Lane JM, Tomin E, Browne M, Berberian W, Turek T, Smith J, Wozney J, Schildhauer T. Use of bone morphogenetic protein-2 in the rabbit ulnar nonunion model. *Clin Orthop*. 1996;327:272–282.
5. Gerhart TN, Kirker-Head CA, Kriz, MJ, Holtrop ME, Hennig GE, Hipp J, Schelling SH, Wang E. Healing segmental femoral defects in sheep using recombinant human bone morphogenetic protein. *Clin Orthop*. 1993;293:317–326.
6. Sciadini MF, Johnson KD. Evaluation of recombinant human bone morphogenetic protein-2 as a bone-graft substitute in a canine segmental defect model. *J Orthop Res*. 2000;18:289–302.
7. Reddi AH. Bone morphogenetic proteins: an unconventional approach to isolation of first mammalian morphogens. *Cytokine Growth Factor Rev*. 1997;8:11–20.
8. Jones AL, Bucholz RW, Bosse MJ, Mirza SK, Lyon TR, Webb LX, Pollak AN, Golden JD, Valentin-Opran A. Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects. A randomized, controlled trial. *J Bone Joint Surg*. 2006;88:1431–1441.
9. Khan SN, Bostrom MPG, Lane JM. Bone growth factors. *Orthop Clin North Am*. 2000;31:1–14.
10. Luginbuehl V, Meinel L, Merkle HP, Gander B. Localized delivery of growth factors for bone repair. *Eur J Pharm Biopharm*. 2004;58:197–208.
11. Maeda H, Sano A, Fujioka K. Controlled release of rhBMP-2 from collagen minipellet and the relationship between release profile and ectopic bone formation. *Int J Pharm*. 2004;275:109–122.
12. Geiger M, Li RH, Friess W. Collagen sponges for bone regeneration with rhBMP-2. *Adv Drug Delivery Rev*. 2003;55:1613–1629.
13. Putney SC, Burke P. Improving protein therapeutics with sustained-release formulations. *Nature Biotech*. 1998;16:153–157.
14. Cleland JL, Duenas ET, Park A, Daugherty A, Kahn J, Kowalski J, Cuthbertson A. Development of poly-D,L-lactide-*co*-glycolide microsphere formulations containing recombinant human vascular endothelial growth factor to promote local angiogenesis. *J Control Release*. 2001;72:13–24.
15. Bala I, Hariharan S, Kumar MN. PLGA nanoparticles in drug delivery: the state of the art. *Crit Rev Ther Drug Carrier Syst*. 2004;21:387–422.
16. Chen F, Wu Z, Sun H, Wu H, Xin S, Wang Q, Dong G, Ma Z, Huang S, Zhang Y, Jin Y. Release of bioactive BMP from dextran-derived microspheres: a novel delivery concept. *Int J Pharm*. 2006;307:23–32.
17. Jiang B, Gao C, Hu L, Shen J. Water-dispersed bone morphogenetic protein nanospheres prepared by co-precipitation method. *Zhejiang Univ SCI*. 2004;5:936–940.
18. Arshady R. Albumin microspheres and microcapsules: methodology of manufacturing techniques. *J Control Release*. 1990;14: 111–131.
19. Morimoto Y, Fujimoto S. Albumin microspheres as drug carriers. *Crit Rev Ther Drug Carrier Syst*. 1985;2:19–63.
20. Rhodes BA, Zolle I, Buchanan JW, Wagner HN Jr. Radioactive albumin microspheres for studies of the pulmonary circulation. *Radiology*. 1969;92:1453–1460.
21. Praveen Reddy B, Dorle AK, Krishna DR. Albumin microspheres: effect of process variables on size distribution and in-vitro release. *Drug Dev Ind Pharm*. 1990;16:1791–1803.
22. Das S, Banerjee R, Jayesh B. Aspirin loaded albumin nanoparticles by coacervation: implications in drug delivery. *Trends Biomater Artif Organs*. 2005;18:203–212.
23. Lin W, Coombes AGA, Davies MC, Davis SS, Illum L. Preparation of sub-100 nm human serum albumin nanoshperes using a pH-coacervation method. *J Drug Targeting*. 1993;1:237–243.
24. Merodio M, Arnedo A, Renedo MJ, Irache JM. Ganciclovir-loaded albumin nanoparticles: characterization and in vitro release properties. *Eur J Pharm Sci*. 2001;12:251–259.
25. Leo E, Vandelli MA, Cameroni R, Forni F. Doxorubicin-loaded gelatin nanoparticles stabilized by glutaraldehyde: involvement of the drug in the cross-linking process. *Int J Pharm*. 1997;155: 75–82.
26. Segura S, Gamazo C, Irache JM, Espuelas E. Gamma interferon loaded onto albumin nanoparticles: in vitro and in vivo activities against *Brucella abortus*. *Antimicrob Agents Chemother*; 2007;51:1310–1314.
27. Segura S, Espuelas S, Renedo MJ, Irache JM. Potential of albumin nanoparticles as carriers for interferon gamma. *Drug Dev Ind Pharm*. 2005;31:271–280.
28. Chatzinikolaidou M, Zumbrink T, Jennissen HP. Stability of surface-enhanced ultrahydrophilic metals as a basis for bioactive rhBMP-2 surfaces. *Materialwiss Werkstofftech*. 2003;34:1106–1112.
29. Langer K, Balthasar S, Vogel V, Dinauer N, Von Briesen H, Schubert D. Optimization of the preparation process for human serum albumin nanoparticles. *Int J Pharm*. 2003;257:169–180.
30. Lin W, Garnett M, Davies MC, Bignotti F, Ferruti P, Davis SS, Illum L. Preparation of surface-modified albumin nanospheres. *Biomaterials*. 1997;18:559–565.
31. Rahimnejad M, Jahanshahi M, Najafpour GD. Production of biological nanoparticles from bovine serum albumin for drug delivery. *Afr J Biotechnol*. 2006;5:1918–1923.
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–254.
33. Zhang S, Wright JEI, Özber N, Uludağ H. The interaction of cationic polymers and their bisphosphonate derivatives with hydroxyapatite. *Macromol Biosci*. 2007;7:656–670.
34. Gittens SA, Bansal G, Kucharski C, Borden M, Uludağ H. Imparting mineral affinity to fetuin by bisphosphonate conjugation: a comparison of three bisphosphonate conjugation schemes. *Mol Pharm*. 2005;2:392–406.
35. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65:55–63.
36. Varkey M, Kucharski C, Takrima H, Sebald W, Uludağ H. In vitro osteogenic response of rat bone marrow cells to bFGF and BMP-2 treatments. *Clin Orthop Relat Res*. 2006;443:113–123.
37. Willmott N, Cummings J, Florence AT. In vitro release of adriamycin from drug-loaded albumin and haemoglobin microspheres. *J Microencapsulation*. 1985;2:293–304.
38. Park TG, Cohen S, Langer R. Controlled protein release from polyethyleneimine-coated poly L-lactic acid/pluronic blend matrices. *Pharm Res*. 1992;9:37–39.
39. Kim IS, Lee SK, Park YM, Lee YB, Shin SC, Lee KC, Oh IJ. Physico-chemical characterization of poly-L-lactic acid and poly-D,L-lactide-*co*-glycolide nanoparticles with polyethylenimine as gene delivery carrier. *Int J Pharm*. 2005;298:255–262.
40. Weber C, Coester C, Kreuter J, Langer K. Desolvation process and surface characterisation of protein nanoparticles. *Int J Pharm*. 2000;194:91–102.
41. Moghimi SM, Hunter AC, Murray JC. Long-circulating and target-specific nano-particles: theory to practice. *Pharmacol Rev*. 2001;53:283–318.

42. Thassu D, Deleers M, Pathak Y. *Nanoparticulate Drug Delivery Systems*. New York, USA: Informa Healthcare; 2007:51–99.
43. Plank C, Mechtler K, Szoka FC Jr., Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum Gene Ther*. 1996;7:1437–1446.
44. Owens DE III, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *Int J Pharm*. 2006;307:93–102.
45. Devine DV, Wong K, Serrano K, Chonn A, Cullis, PR. Liposome complement interactions in rat serum: implications for liposome survival studies. *Biochim Biophys Acta*. 1994;1191:43–51.
46. Harashima H, Sakata K, Funato K, Kiwada H. Enhanced hepatic uptake of liposomes through complement activation depending on the size of liposomes. *Pharm Res*. 1994;11:402–406.
47. Romero EL, Morilla MJ, Regts J, Koning GA, Scherphof GL. On the mechanism of hepatic transendothelial passage of large liposomes. *FEBS Lett*. 1999;448:193–196.
48. Roser M, Fidher D, Kissel T. Surface-modified biodegradable albumin nano- and microspheres. II. Effect of surface charges on in vitro phagocytosis and biodistribution in rats. *Eur J Pharm Biopharm*. 1998;46:255–263.
49. Huang M, Vitharana SN, Peek LJ, Coop T, Berkland C. Polyelectrolyte complexes stabilize and controllably release vascular endothelial growth factor. *Biomacromolecules*. 2007;8:1607–1614.
50. Tiyaboonchai W, Woiszwillo J, Sims RC, Middaugh CR. Insulin containing polyethylenimine-dextran sulfate nanoparticles. *Int J Pharm*. 2003;25:139–151.
51. Tiyaboonchai W, Woiszwillo J, Middaugh CR. Formulation and characterization of amphotericin B-polyethylenimine-dextran sulfate nanoparticles. *J Pharm Sci*. 2001;90:902–914.
52. Uludag H, D'Augusta D, Golden J, Li J, Timony G, Riedel R, Wozney JM. Implantation of recombinant human bone morphogenetic proteins with biomaterial carriers: a correlation between protein pharmacokinetics and osteoinduction in the rat ectopic model. *J Biomed Mater Res*. 2000;50:227–238.
53. Rhaese S, Von Briesen H, Rübsamen-Waigmann H, Kreuter J, Langer K. Human serum albumin–polyethylenimine nanoparticles for gene delivery. *J Control Release*. 2003;92:199–208.
54. Hunter AC. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. *Adv Drug Delivery Rev*. 2006;58:1523–1531.
55. Boeckle S, Von Gersdorff K, Van der Piepen S, Culmsee C, Wanger E, Ogris M. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. *J Gene Med*. 2004;6:1102–1111.
56. Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I. Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Dev Biol*. 1994;161:218–228.

Manuscript received Oct. 16, 2007, and revision received Mar. 11, 2008.

BTPR0703836