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Bisphosphonate-decorated lipid nanoparticles designed as drug carriers for bone diseases

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Abstract: A conjugate of distearoylphosphoethanolamine-polyethylene glycol with 2-(3-mercaptopropylsulfanyl)-ethyl-1,1-bisphosphonic acid (thiolBP) was synthesized and incorporated into micelles and liposomes to create mineral-binding nanocarriers for therapeutic agents. The micelles and liposomes were used to encapsulate the anticancer drug doxorubicin (DOX) and a model protein lysozyme (LYZ) by using lipid film hydration (LFH) and reverse-phase evaporation vesicle (REV) methods. The results indicated that the micelles and LFH-derived liposomes were better at DOX loading than the REV-derived liposomes, while the REV method was preferable for encapsulating LYZ. The affinity of the micellar and liposomal formulations to hydroxyapatite (HA) was assessed *in vitro*, and the results indicated that all the thiolBP-incorporated nanocarriers had stronger HA affinity than their counterparts without thiolBP. The thiolBP-decorated liposomes also displayed a strong binding to a collagen/HA composite scaffold *in vitro*. More importantly, thiolBP-decorated liposomes gave increased retention in the collagen/HA scaffolds after subcutaneously implantation in rats. The designed liposomes were able to entrap the bone morphogenetic protein-2 in a bioactive form, indicating that the proposed nanocarriers could deliver bioactive factors locally in mineralized scaffolds for bone tissue engineering. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 100A: 684–693, 2012.

Key Words: micelle, liposome, bisphosphonate, bone mineral affinity, drug delivery

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INTRODUCTION

Development of nanocarriers for delivering therapeutic agents specifically to bone is urgently needed for the treatment of a wide range of bone diseases. The carriers are expected to retain the therapeutic agents in an active form, enhance the efficacy of therapeutic agents by restricting their delivery to bone tissue (i.e., increase *in situ* concentration of therapeutic agents) and reduce the undesired side effects by minimizing nonspecific distribution to other organs.[1–3] Micellar and liposomal systems have been used for the delivery of wide array of therapeutic agents, in particular anticancer therapeutics, but their utility in bone diseases have been limited due to lack of bone affinity. These versatile nanocarriers can be used for bone targeting of therapeutic agents as long as they can be engineered for bone affinity. This may be possible by incorporating a ligand with strong affinity to the unique component of the bone tissue, namely the hydroxyapatite (HA). HA does not exist at other sites in the body under normal conditions. Such mineral-binding carriers could be used for systemic administration of drugs where the drugs in circulation are targeted for bone deposition.[3] Alternatively, the mineral-binding carriers could also be used for local administration of drugs with mineralized scaffolds that are actively pursued for bone tissue regeneration. In the latter case, the mineral-binding nanocarriers will help localize therapeutic agents in the scaffolds and sustain a more robust pharmacological effect due to improved retention of the drugs at the local repair and regeneration site.

Bone targeting of simple molecules by conjugation with bone-seeking ligands bisphosphonates (BPs) has been achieved *in vivo* for various prodrug candidates such as estradiol,[4] cisplatin,[5] prostaglandin E_2,[6] and several model proteins.[7–9] Anada et al.[10] synthesized an amphipathic molecule containing a BP head group, 4-*N*-(3,5-ditetradecyloxybenzoyl)-aminobutane-1-hydroxy-1,1-bisphosphonic acid disodium salt, which was subsequently formulated into liposomes along with distearoylphosphotidylcholine (DSPC)

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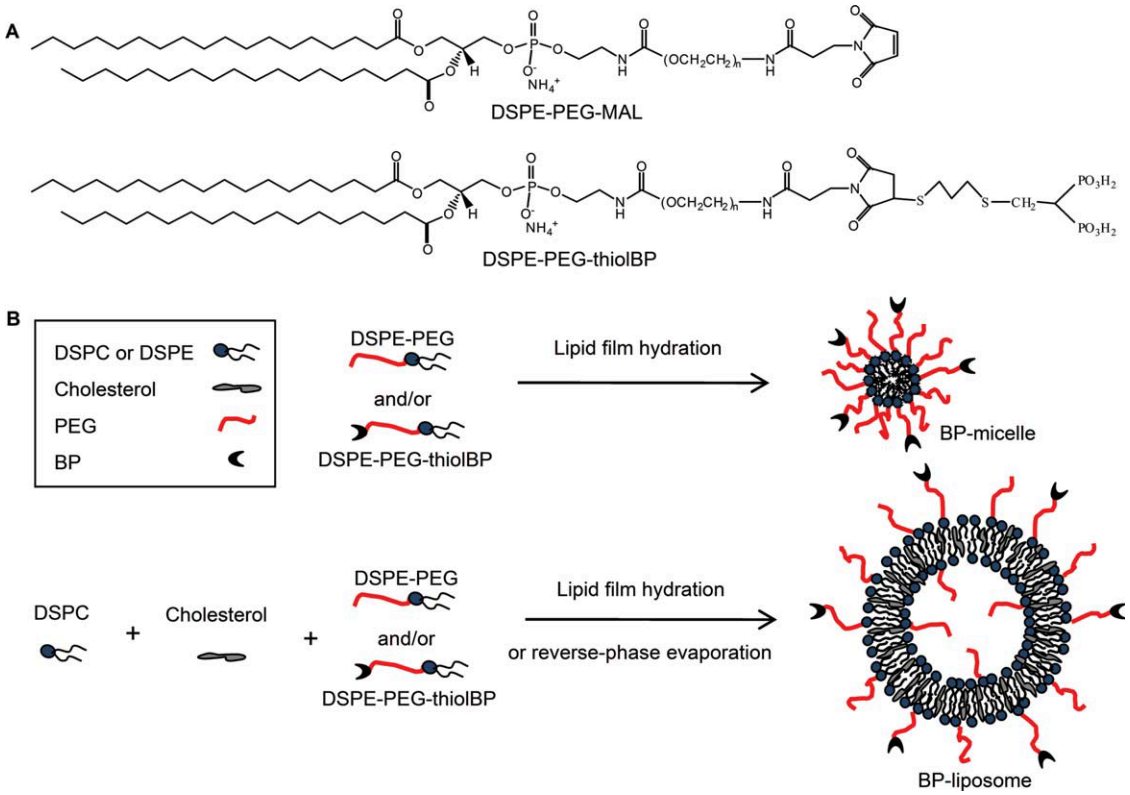

FIGURE 1. Chemical structures of DSPE-PEG-MAL and DSPE-PEG-thiIBP (A) and schematic for the preparation of the bisphosphonate-decorated micelles and liposomes (B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and cholesterol (CH). The liposomes decorated with BP moieties were shown to display high affinity for pure HA particles *in vitro*. Henst et al.[11] successfully reported liposomes incorporating with cholesteyl-trisoxyethylene-bisphosphonic acid (CH-TOE-BP) designed for mineral affinity as well. However, the desired HA affinity of these liposomes has been shown in *in vitro* tests with HA particles, and no *in vivo* studies were reported on their capability for mineral-binding. This is important considering that the protein-rich serum could compete for mineral binding under the physiological conditions. Unlike larger liposomes, micellar nanocarriers could be also advantageous for certain applications requiring smaller drug carriers; however, no micellar delivery system with bone affinity was reported to-date.

This study explored the feasibility of creating of micellar and liposomal nanocarriers from building blocks that display bone mineral (HA) affinity. Toward this goal, we conjugated a thiol-containing BP, 2-(3-mercaptopropyl-sulfanyl)-ethyl-1,1-bisphosphonic acid (thiolBP), with distearoylphosphoethanolamine-polyethylene glycol-maleimide (DSPE-PEG-MAL) to form a DSPE-PEG-thiolBP conjugate. The preparation and *in vitro* characterization of bone-targeted micelles and liposomes derived from DSPE-PEG-thiolBP (Fig. 1) was described in this report. The capacity of the prepared vehicles to encapsulate anticancer drug doxorubicin (DOX) and a model protein lysozyme (LYZ) was assessed. The mineral affinity of the vehicles was investigated using HA particles as well as a HA-embedded collagen scaffold that contains the major components of bone tissue. We imparted an effective mineral affinity to the prepared micelles and liposomes both *in vitro* and in an *in vivo* implant model, indicating that the described HA-binding nanocarriers will facilitate a novel approach for drug delivery in treatment of bone diseases.

MATERIALS AND METHODS

Materials

CH, LYZ from chicken egg white, 1,1′-dioctadecyl-3,3,3′,3′-tetra-methylindocarbocyanine (DiI), and doxorubicin hydrochloride (DOX) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC) was obtained from Pierce (Rockford, IL, USA) and used to label LYZ as described elsewhere.[12] 1,2-Distearoylglycero-3-phosphatidylcholine (DSPC) and 1,2-distearoylglycero-3-phosphoethanolamine-*N*-[poly(ethyleneglycol) 2000] (DSPE-PEG) were provided by Lipoid GmbH (Ludwigshafen, Germany). DSPE-PEG_{3400}-maleimide (DSPE-PEG-MAL) was purchased from Creative PEGWorks (Winston-Salem, NC, USA). ThiolBP was synthesized and characterized as previously described.[13] Recombinant human bone morphogenetic protein-2 (BMP-2) was expressed in *E. coli* and purified as described elsewhere.[14] Synthetic HA was prepared according to the method described by Bernardi.[15]

Conjugation of ThiolBP to DSPE-PEG

A total of 100 mg DSPE-PEG-MAL was slowly dissolved in 3 mL of 0.1*M* phosphate buffer (pH = 7.0) and mixed with 1 mL of thiolBP (molar ratio of DSPE-PEG-MAL: thiolBP =

1:4) dissolved in the same buffer. The mixture was incubated for 1 h and then transferred into dialysis tubing (MWCO 100 kDa) for dialysis against 50 m*M* sodium chloride (2 × 1 L; 3–5 h per period), followed by ddH_2O (3 × 1 L). The final solution was lyophilized to yield the DSPE-PEG-thiolBP as white solid, and analyzed by ^{1}H NMR using H_2O as the solvent (Appendix). Major peaks correspond to lipid —C$\underline{H}_3$ at 0.85 ppm (peak 6), lipid —C$\underline{H}_2$— at 1.25 ppm (peak 5), lipid —C$\underline{H}_2$—C(O) — at 2.5 ppm (peak 4a), thiolBP —C$\underline{H}_2$—s at 2.8 ppm (peak 4d) 2.5 ppm (peak 4b) and 1.9 ppm (peak 4c), and PEG —C$\underline{H}_2$—C$\underline{H}_2$—O— at 3.5–3.8 ppm (peak 2).

Micelle formation

DSPE-PEG or DSPE-PEG-thiolBP (2 μmol) and trace amount of DOX (10 μg) or LYZ (0.2 mg) (labeled with FITC) were dissolved in chloroform (1 mL). The organic solvent was removed by evaporator to form thin films integrated with DOX or LYZ. The lipid film was then hydrated with 1 mL of 10 m*M* HEPES-buffered saline (HBS; 10 m*M* HEPES with 150 m*M* NaCl, pH = 7.4) at room temperature for 30 min to form the micelles spontaneously. The micelles were purified by dialysis (MWCO 14 kDa for DOX, 100 kDa for LYZ) against at least 300-fold excess of HBS (× 3) for 24 h to remove the unloaded DOX or LYZ. In one set of preparation, micelles with different ratios of DSPE-PEG and DSPE-PEG-thiolBP (0:100, 20:80, 50:50, and 100:0) were prepared.

Liposome preparation

Two different methods, lipid film hydration (LFH) and reverse-phase evaporation vesicle (REV) methods, were used to fabricate the liposomes. In the LFH method, DSPC (10 μmol) and CH (5 μmol) were dissolved in chloroform/methanol (85/15 v/v) and dried under reduced pressure. The resultant thin lipid film was hydrated with 2 mL of HBS buffer. For preparation of the PEG-liposomes and the BP-liposomes, 5% (molar percent of DSPC) DSPE-PEG or DSPE-PEG-thiolBP was added to the lipid solution before the formation of lipid film. To encapsulate the DOX or LYZ, the lipid film was hydrated with DOX (10 μg/mL) or LYZ (0.2 mg/mL) solution in 2 mL of HBS buffer. The preformed liposomes were extruded through a polycarbonate membrane (100 nm pore size) 11 times using a mini-extruder from Avanti Polar Lipids (Alabaster, AL, USA), and the unencapsulated DOX or LYZ was removed by dialysis as described above.

For the REV method, DSPC (10 μmol), CH (5 μmol), and 5% (percent of DSPC) DSPE-PEG (for PEG-liposomes) or DSPE-PEG-thiolBP (for BP-liposomes) were dissolved in 6 mL of diethyl ether with the help of a small amount of methanol. A total of 2 mL of DOX (10 μg/mL) or LYZ (0.2 mg/mL) in HBS (buffer only for empty liposomes) was added to the organic phase, and the two-phase system was vigorously vortexed and sonicated in a bath-type sonicator for 3–5 min until a relatively stable emulsion was formed. The organic solvent was removed by a rotary evaporator under reduced pressure, and the mixture transformed into aqueous vesicle dispersion with the entrapped drug or protein. The preformed liposomes were extruded and purified by dialysis as described above. In one set of preparations, liposomes were prepared by using different fractions of DSPE-PEG-thiolBP (0, 1, 2.5, and 5% of DSPE-PEG-thiolBP, accordingly DSPE-PEG-thiolBP/DSPE-PEG ratio, 0:100, 20:80, 50:50, and 100:0).

Characterization of micelles and liposomes

The particle sizes (or hydrodynamic diameters) and polydispersity index of the empty micelles and liposomes were measured by photon correlation spectroscopy using Zetasizer 3000HS (Malvern Instruments, Southboro, MA). The measurements were carried out at 25°C using 633 nm He-Ne laser at a scattering angle of 90°.

In order to determine the encapsulation efficiency (EE), the micellar and liposomal formulations with DOX or LYZ before and after dialysis were lysed in 1% Triton X-100. The fluorescent intensity (λ_{ex} = 485 nm and λ_{em} = 604 nm for DOX; λ_{ex} = 485 nm and λ_{em} = 527 nm for FITC-labeled LYZ) of the samples was measured by a spectrofluorometer (Thermo Labsystems, Franklin, MA) to obtain the EE: EE% = 100% × [(fluorescence postdialysis)/(fluorescence predialysis)].

***In vitro* assessment of HA binding affinity**

For the purpose of quantification, the micelles and liposomes were labeled with a lipophilic fluorescent tracer, 1,1′-dioctadecyl-3,3,3′,3′-tetra-methylindocarbocyanine (DiI, 0.2% of total phospholipid) during the preparation. An aliquot solution of micelles or liposomes were incubated with 10 mg HA suspended in 0.5 mL of 1 × PBS (total lipid concentration 0.1 μmol/mL for micelles, 0.5 μmol/mL for liposomes) in a 1.5 mL microcentrifuge tube for 3 h at room temperature. For fluorescence imaging, the HA crystals were washed three times with 1× PBS, resuspended in PBS and mounted on glass slides. The phase contrast and fluorescence images were acquired on an Olympus FSX100 epifluorescent microscope (Olympus America, Center Valley, PA). The percentage of micelles or liposomes bound to HA was quantified by a spectrofluorometer for the fluorescent intensity of DiI (λ_{ex} = 536 nm and λ_{em} = 607 nm) in the supernatant after centrifugation of the suspension at 1000 rpm for 3 min. The initial amount of micelles or liposomes was determined by samples treated in the same way but without HA (control). The percent HA binding (%) was assessed as: 100% × [(initial fluorescence – fluorescence in the supernatant)/(initial fluorescence)]. Where indicated, the binding was also determined based on the fluorescence tag of the encapsulants (i.e., autofluorescence of DOX and FITC-tag of LYZ) rather than based on DiI labeling.

***In vitro* affinity of liposomes to HA-impregnated collagen scaffold**

An HA-containing collagen scaffold composed of type-I collagen (isolated from rat tail tendons as described elsewhere[16]) and HA (Col/HA, 30/70 w/w) was prepared by freeze-drying method.[17] The DiI-labeled PEG-liposomes and BP-liposomes were suspended at a concentration of 0.5

μmol/mL (equivalent phospholipid concentration) in 1× PBS, and 1 mL of the sample was incubated with a piece of sponge (8 mm in diameter and 5 mm in thickness) in a 24-well tissue culture plate for 2 h. The percentage of bound liposomes to the scaffold was calculated by the fluorescence of DiI in the supernatant and in the control, which was equal volume of liposomal suspension without the sponge.

In vivo retention of liposomes

Six-to-eight 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 study. Although maintained in pairs in sterilized cages, rats were allowed free access to food and water 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 PEG-liposomes and BP-liposomes were prepared with DiI labeling and sterilized by filtration through a 0.45 μm Millipore filter before implantation in 18 rats in three study groups: (1) control, PEG-liposomes without DiI labeling, (2) DiI-labeled PEG-liposomes, and (3) DiI-labeled BP-liposomes. The appropriate solution for each study group was soaked into the Col/HA sponge for ~10 min (60 μL formulation per implant). Once rats were anesthetized with inhalational Metofane™ (Janssen Pharmaceuticals, Toronto, ON, Canada), two wet sponges were implanted subcutaneously into bilateral ventral pouches in each rat. At indicated time points [shown in Fig. 5(B)], two rats from each group were euthanized with CO_2, the implants were recovered, and the explanted sponges were demineralized with 1 mL of 1*M* HCl for 3 h and then the fluorescent intensity in the solution was measured. As the initial fluorescence in implant, 60 μL of solution was diluted to 1 mL by 1*M* HCl, and the fluorescence intensity in the diluted solution was determined before the implantation. The liposome retention, expressed as the remaining percentage of implanted amount, was calculated as: 100% × [(recovered fluorescence in implant)/(initial fluorescence in implant)].

Cytotoxicity

The MTT assay was performed to assess the cytotoxicity of the micelles and liposomes using human C2C12 myoblast cells. The cells were cultured as described previously.[18] After sterilization with an 0.45-μm Millipore filter, an aliquot of micellar or liposomal dispersion (50 μL) was added to the cells grown in 24-well plates (in triplicate) with 450 μL tissue culture medium (DMEM with 10% FBS, 100 μg/mL Penicillin, and 100 U/mL Streptomycin). After 48 h incubation for the micelles or 72 h for the liposomes in a humidified atmosphere with 95/5% air/CO_2 at 37°C, 0.1 mL of the MTT solution (5 mg/mL in Hanks Balanced Salt Solution, HBSS) was added to the 0.5 mL culture medium in each well. The cells were incubated for 2 h, the supernatant was removed carefully, and 0.5 mL of dimethylsulfoxide was added to the cells to dissolve the formazan crystals formed. The optical density of the solution was measured by an *EL*x800 plate reader (Bio-Tek Instruments, Winooski, VT, US) at 570 nm. Untreated cells served as reference and were taken as 100% viability.

Bioactivity assay for liposome encapsulated BMP-2

BMP-2 containing liposomes were prepared by the REV method and separated from free BMP-2 by dialysis as described above for LYZ. The formulations were sterilized with an 0.45-μm Millipore filter. Two cell lines, human C2C12 cells and rat bone marrow stromal cells (BMSCs) were incubated (in triplicate) with the liposomes at estimated concentrations of 1, 0.5, and 0.25 μg/mL BMP-2 (assuming an EE of 30% for BMP-2 similar to LYZ) in 24-well plates (0.6 mL medium/well) for 3 days, washed with HBSS solution and lysed with 0.4 mL of an ALP buffer (0.5*M* 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton-X; pH = 10.5). After 2 h, 0.2 mL of 1.0 mg/mL ALP substrate (*p*-nitrophenol phosphate) was added to 0.2 mL of cell lysate, and the ALP activity was determined by a kinetic assay on a plate reader, where the rate of change in the optical density was measured at 405 nm with 90 sec intervals for eight cycles. Untreated cells and cells treated with equivalent amount of free BMP-2 served as background and positive controls, respectively. The kinetic ALP activity was expressed as the change of optical density per unit time (mAbs/min), and the relative ALP activity was calculated by subtracting the absorbance of the untreated control from the absorbance of the samples.

Statistical analysis

All experimental data were collected in triplicate at least, and expressed as mean ± standard deviations (SD). Statistical analysis was performed using two-sided unpaired Student's *t*-test. Differences were considered statistically significant with $p < 0.05$.

RESULTS AND DISCUSSION

The DSPE-PEG-thiolBP was synthesized by conjugating thiolBP with DSPE-PEG-MAL via a reaction between the sulfydryl group of thiolBP and the maleimide group on the PEG terminal. The successful conjugation of thiolBP to DSPE-PEG-MAL was confirmed by ^{1}H NMR spectroscopy (Appendix), and the structures of DSPE-PEG-MAL and DSPE-PEG-thiolBP were shown in Figure 1. Micelles from DSPE-PEG (PEG-micelles) and DSPE-PEG-thiolBP (BP-micelles) alone or with loaded DOX or LYZ were prepared by organic solvent evaporation followed by LFH method.[19] The DSPE-PEG or DSPE-PEG-thiolBP were able to spontaneously form micelles in aqueous solution. On the other hand, stable liposomes were obtained when DSPE-PEG and DSPE-PEG-thiolBP were mixed with DSPC and CH, and the fraction of DSPE-PEG was limited to less than 10%.[20] The liposomal formulation was extruded through a 100 nm polycarbonate membrane to control the liposomal size. DSPC and CH were used to prepare the liposomes because they have good stability *in vivo*,[21] although the high gel–liquid crystalline phase transition temperature of DSPC required processing at a high temperature (>55°C).

TABLE I. Particle Size and Polydispersity Index of Micelles and Liposomes and Encapsulation Efficiency (EE%) of DOX and LYZ in Different Formulations

Formulations (methods)	Size (nm)	Polydispersity index	DOX (EE%)		LYZ (EE%)	
			LFH	REV	LFH	REV
PEG-micelle	16.1 ± 0.9	0.288 ± 0.088	45.7 ± 0.5		4.5 ± 0.2	
BP-micelle	16.9 ± 0.1	0.288 ± 0.005	38.5 ± 0.3		3.3 ± 0.1	
PEG-liposome	97.9 ± 14.5	0.146 ± 0.053	85.4 ± 3.6	32.1 ± 2.8	11.6 ± 6.1	30.8 ± 2.9
BP-liposome	104.5 ± 17.3	0.124 ± 0.066	64.9 ± 2.7	34.3 ± 4.6	13.1 ± 5.9	29.9 ± 5.4

The reported data are shown as mean ± SD derived from three independent batches.

The particle sizes and polydispersity index of the empty micelles and liposomes were summarized in Table I. The obtained sizes for the micelles (16–17 nm) and liposomes (98–105 nm) were in the expected size range, and thiolBP incorporation did not seem to affect the size of the respective carriers. The polydispersity index for all formulations was <0.3, indicating a relative homogenous distribution of the nanocarriers. We note that the thiolBP was anchored to micelles/liposomes with a DSPE-PEG of 3400 Da PEG size and the DSPE-PEG used for micelle/liposome formation contained a 2000 Da PEG; the micelles and liposomes so formed therefore had a mixture of two different PEG sizes. The importance of the PEG chain length was not explored in this study.

DOX, a clinically used anticancer drug, and LYZ (14.4 kDa, pI ~11), due to its similarity to BMP-2 (32 kDa, pI ~9) in molecular weight and net charge, were chosen as model drugs for encapsulation. DOX or FITC-labeled LYZ was entrapped in the micelles by simply adding them to the DSPE-PEG and DSPE-PEG-BP solution used for micellization, and the unencapsulated molecules were removed by dialysis. As summarized in Table 1, the micelles could encapsulate DOX with a relatively high EE (38.5 and 45.7% for PEG- and BP-micelles, respectively), whereas the EE for LYZ in micelles was quite low (<5%), indicating the unsuitability of micelles for encapsulation of LYZ. It has been reported that drugs with small molecules such as DOX and docetaxel can be easily encapsulated into the core of micelles with high EE (>80%).[19,22] The EE for DOX in this study was lower than previously reports, possibly due to the leakage of loaded DOX from the micelles during dialysis. It is difficult to entrap macromolecules such as LYZ into the micelles because of the limited space of hydrophobic core, as well as the large size and hydrophilic characteristic of the protein. The inability of the micelles to entrap the protein led us to explore liposomes for bone delivery of hydrophilic macromolecular drugs.

Two different methods were used to fabricate the liposomes. LFH method is the simplest technique to prepare liposomes and widely used to encapsulate various drugs. However, its poor capacity to entrap hydrophilic molecules limited its application in loading protein- or peptide-based bioactive therapeutics.[23] The REV method involves formation of aqueous/organic emulsions followed by solvent evaporation, and produced large trapped volumes in the liposomal aqueous core, thus leading to improved entrapment for water-soluble molecules.[24] The EEs for the two methods were summarized in Table 1; the LFH method had significantly higher EE for DOX (85.4 and 64.9% for PEG- and BP-liposomes) than the REV (32–34%) method for both PEG-liposomes and BP-liposomes, but the EEs for LYZ were reversed (30–31% for REV vs. 12–13% for LFH). The results indicated that the LFH method was advantageous for loading DOX, whereas the REV method was preferable for loading proteins.

The desired mineral affinity was imparted by incorporating the HA-binding ligand thiolBP in the nanocarriers. A longer PEG spacer was used in the DSPE-PEG-thiolBP (PEG_{3400}) conjugate than the DSPE-PEG (PEG_{2000}) to facilitate the extension of the thiolBP further from the surface.[25] The mineral affinity of the micelles and liposomes was investigated based on an *in vitro* HA binding assay. For this purpose, the micelles and liposomes were labeled with DiI during the preparation. Firstly, the HA binding of the BP-micelles and BP-liposomes was visualized by an epifluorescent microscope. As shown in Figure 2, the HA crystals treated with PEG-micelles and PEG-liposomes displayed no binding of red fluorescent dye, whereas the BP-micelles and BP-liposomes displayed strong affinity to HA, evident by the fluorescence retained on the HA crystals.

In order to identify an optimal thiolBP amount in nanoparticles, the effect of DSPE-PEG-thiolBP ratio on the HA binding of micelles and liposomes was examined. The micelles formed from the DSPE-PEG alone showed very low HA binding (<5%), whereas micelles formed with 20% of DSPE-PEG-thiolBP had increased (65%) HA binding [Fig. 3(A)]. Additional DSPE-PEG-thiolBP in the micelle formulation did not increase the HA affinity any further. The liposomes without thiolBP did not yield HA binding either (<5%), but DSPE-PEG-thiolBP incorporation (1–5%) imparted significant HA affinity to the liposomes [Fig. 3(B)]. The HA affinity of liposomes with 1–5% of DSPE-PEG-thiolBP was similar, indicating that maximum binding was reached with as little as 1% of DSPE-PEG-thiolBP in the liposomes. The HA affinity of the liposomes was dependent on the liposome concentration under the experimental conditions [Fig. 4(A)], whereas complete HA binding was seen at low liposome concentration, a lower percentage of HA binding was obtained at higher liposome concentration, possibly due to the saturation of the binding sites on HA. As the formulations were intended for *in vivo* application, the existence of plasma proteins or other serum components

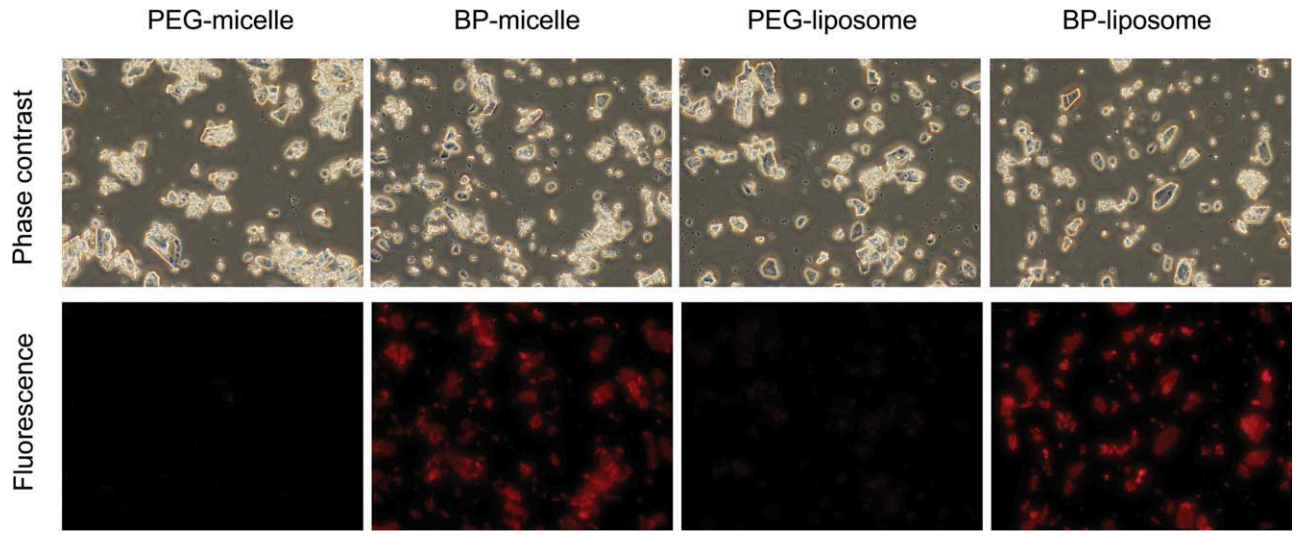

FIGURE 2. Visualization for HA binding of BP-micelles and liposomes. HA crystals were treated with micelles or liposomes (from left to right: PEG-micelle, BP-micelle, PEG-liposome and BP-liposome), and the phase contrast (top panels) and fluorescence images (bottom panels) were taken on an epifluorescence microscope. Note that HA particles visible in phase contrast (but not micelles and liposomes) was coated with the fluorescent micelles and liposomes, as seen under epifluorescence illumination. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

might affect their HA binding affinity. Therefore, the effect of serum on HA affinity was studied. As shown in Figure 4(B), the HA binding by the BP-liposomes was gradually decreased when the serum content in binding medium was increased, which was likely to reflect protein adsorption onto HA surfaces, competing with the thiolBP for surface binding. Despite of the interference effect of serum, the HA affinity of the BP-liposomes was retained significantly higher

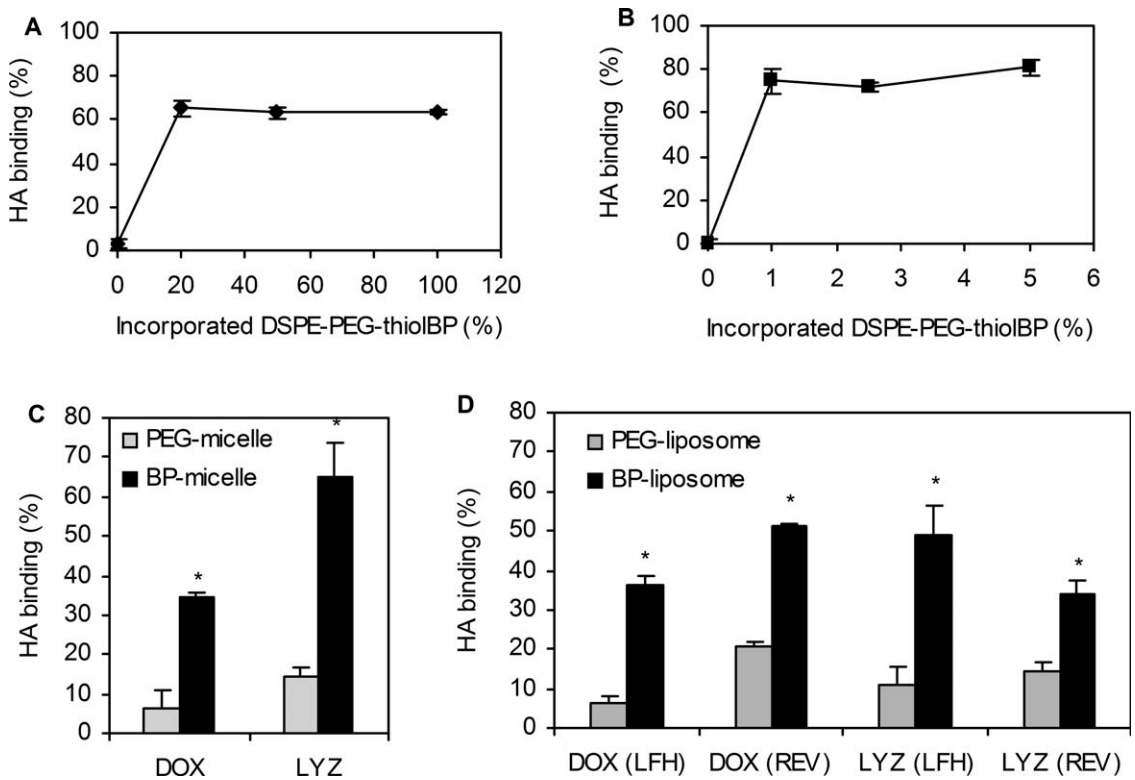

FIGURE 3. Effect of BP content on HA binding of micelles (A) and liposomes (B) and the HA affinity of encapsulated DOX and LYZ in micelles (C) and liposomes (D). The percentage of micelles or liposomes bound to HA was determined by measuring the fluorescence of DiI-labeling liposomes (A and B) or autofluorescence of DOX and FITC-tag of LYZ (C and D) in the supernatant.

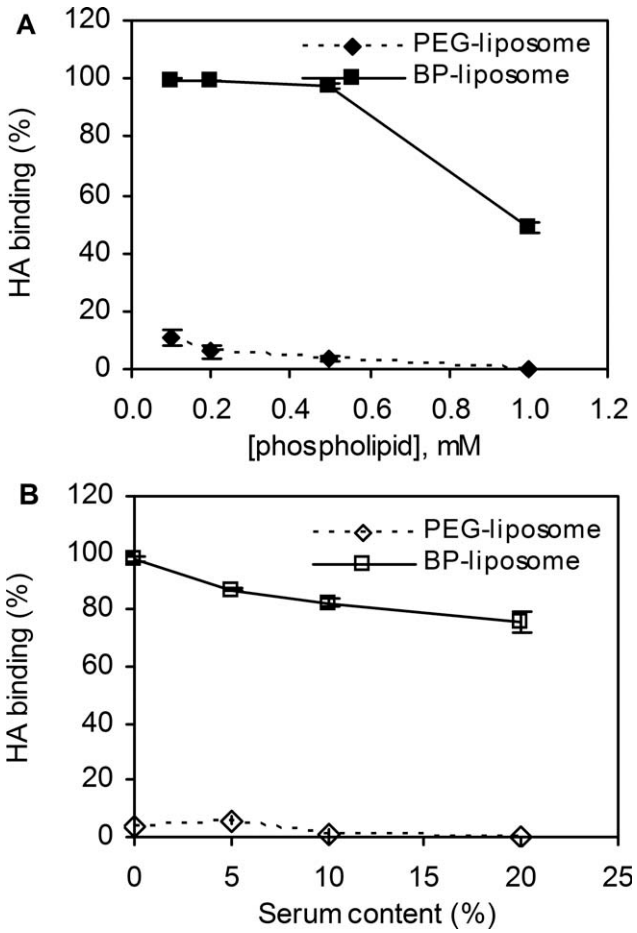

FIGURE 4. The binding of liposomes to HA in PBS (A) and serum-containing PBS (B). The HA affinity of the liposomes were investigated by changing the liposome concentration (A) or amount of rat serum (B) in the binding medium. Results are expressed as mean ± SD ($n = 3$).

than PEG-liposomes, which indicated that the serum proteins might not abolish the desired mineral affinity of the system.

The strongly HA-binding nanocarriers are expected to deposit their cargo to HA surfaces. To confirm whether the encapsulants had also displayed high mineral affinity, the HA binding was investigated based on the fluorescent encapsulants (rather than DiI labeling of the carriers). As shown in Figure 3, the results obtained by measuring the fluorescence of encapsulated DOX and LYZ also demonstrated significantly higher (2.4–5.9 fold, $p < 0.05$) HA binding ratio for the BP-micelles [Fig. 3(C)] or BP-liposomes [Fig. 3(D)], as compared to the PEG-micelles or PEG-liposomes. This was the case irrespective of the type of encapsulant (DOX or LYZ), the method of carrier fabrication (LFH vs. REV) or the extent of EE obtained. The difference between the LYZ-containing BP-liposomes and PEG-liposomes did not seem to be as significant as that for the DiI-labeled liposomes. This may be partly due to the interaction between the protein and the liposome bilayer, which may result in destabilizing a lipid membrane,[26] and/or partly due to the fractional release of the encapsulated protein during the HA binding assay, leading to nonspecific binding to HA.[8]

Particulate HA has been routinely used to investigate *in vitro* mineral affinity of a compound or nanoparticles. However, particulate HA, with a propensity to disperse from an administration site, is not suitable for *in vivo* testing. Therefore, HA-impregnated collagen sponges (Col/HA) were employed in this study in order to test the mineral affinity of the nanocarriers *in vivo*. The Col/HA scaffold displays interconnected porous structure suitable for cellular migration and ingrowth, and has been widely studied as a bone tissue engineering scaffold.[27] It was used as a substitute for bone in this study, because it was fabricated from two of the major components of bone (collagen and HA) and collagen envelopes the HA particles similar to native bone, where the endogenous HA is partly covered with extracellular matrix proteins. The affinity of the prepared liposomes to Col/HA scaffold was first evaluated by using liposomes with different DSPE-PEG-thiolBP content [Fig. 5(A)]. DiI-labeled liposomes were used for this purpose. There was a

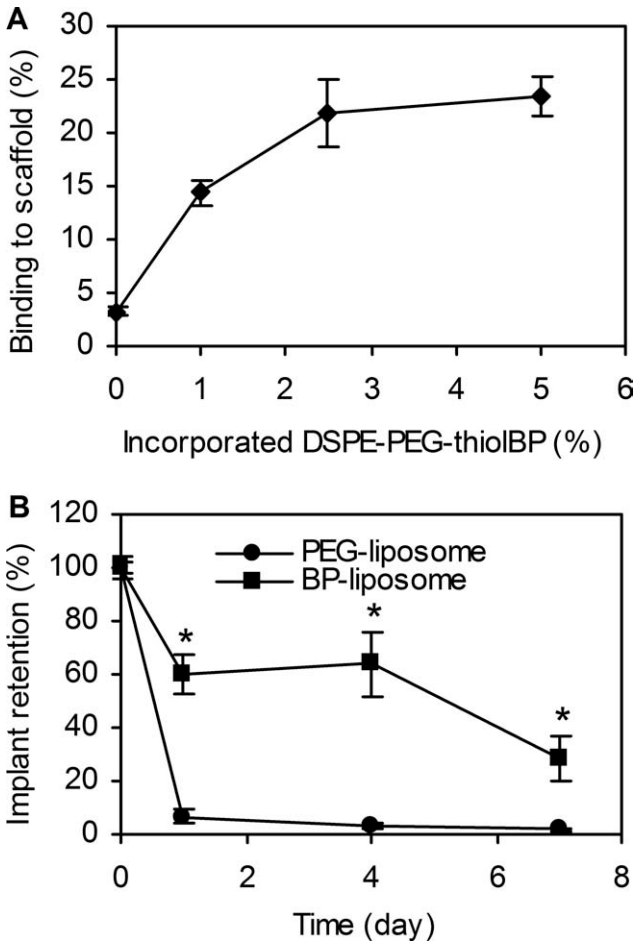

FIGURE 5. *In vitro* and *in vivo* affinity of liposomes to Col/HA scaffold. (A) *In vitro* affinity of liposomes to Col/HA scaffold, calculated based on fluorescence of DiI-labeled liposome in the supernatant after incubation with scaffold and in the control without scaffold. (B) *In vivo* implant retention of liposomes within the scaffold by subcutaneous implant model in rats. Results are expressed as mean ± SD from triplicate measurements in (A), and four implants in (B).

gradual increase in liposome binding to Col/HA scaffold when the DSPE-PEG-thiolBP content was increased from 0 to 5% in the lipid composition, which was consistent with the binding result obtained with pure HA [Fig. 3(B)]. Maximum mineral binding was obtained at 1% of DSPE-PEG-thiolBP for the HA alone, but at 5% of DSPE-PEG-thiolBP [Fig. 5(A)] for the mineralized scaffold, suggesting that the surface density of targeting ligands need to be considered for maximum affinity *in vivo*. Taken together, these results confirmed that the designed nanocarriers displayed the expected affinity to a mineral-containing scaffold, which could be in turn used for *in vivo* assessment of mineral affinity.

In vivo mineral affinity of the BP-liposomes was assessed by implanting DiI-labeled liposomes in Col/HA scaffold subcutaneously in rats. Unlabeled PEG-liposomes soaked in Col/HA was used to assess the background from the implants; the results indicated no significance fluorescence in implants for this group (not shown), indicating no interference in DiI fluorescence measurements from the endogenous components at the subcutaneous site. The retention profiles for DiI-labeled PEG- and BP-liposomes are shown in Figure 5(B). Approximately 60% of BP-liposomes was retained in the scaffolds until day 4, after which 29% retention was seen at 7 days post-implantation. The retention of PEG-liposomes was $<7\%$ at all time points evaluated. Therefore, 9–20-fold higher ($p < 0.005$) retention of liposomes was seen as a result of thiolBP incorporation into the liposomes. The *in vivo* loss of liposomes was hypothesized to be due to rapid clearance by the physiological fluid around the implant site. The PEG-liposomes were merely passively loaded into the sponges, and thus removed fast when exposed to the circulated body fluid, whereas the BP-liposomes could retain in the implantation site for a prolonged period because of the specific affinity to the HA-containing sponges. The results summarized in Figure 5 confirmed the designed BP-liposomes did exhibit the expected *in vivo* affinity to a Col/HA scaffold, and the retention of BP-liposomes at mineral-bearing sites is promising for localization and sustainable release of drugs for bone treatment. As the Col/HA scaffold has the potential for clinical application, the BP-liposomes reported in this study might be used as local delivery system for BMP-2, which can promote new bone formation in combination with the scaffold for bone repair.

Micellar or liposomal formulations are generally regarded as biocompatible systems from clinical perspective, because they are either accepted for use (liposomes[28]) or undergoing clinical trials (micelles[29]). To ensure that thiolBP addition did not alter the biocompatibility of the fabricated nanoparticles, MTT assay was used to determine the toxicity of the nanoparticles in an *in vitro* bioassay (using C2C12 cells as cell model). As expected, there was no or very low toxicity of the prepared micellar and liposomal formulations (Fig. 6). To determine the ability of the designed nanoparticles to deliver therapeutic drugs, BMP-2 was encapsulated in PEG-liposomes and BP-liposomes, and *in vitro* activities of the BMP-2 formulations were tested by alkaline phosphatase (ALP) induction assay. It was assumed that the EE for BMP-2 was 30% following the results obtained with the

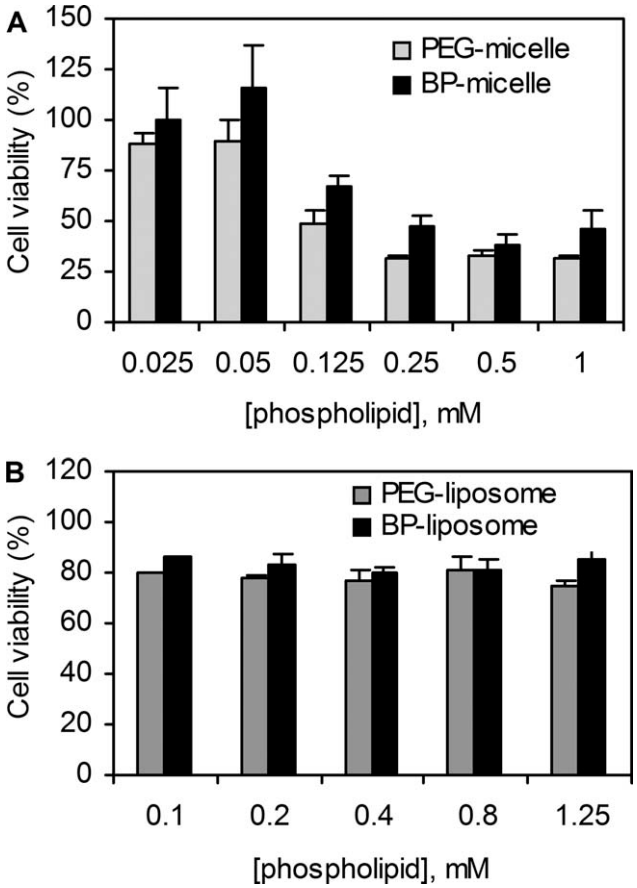

FIGURE 6. Cytotoxicity of micelles (A) and liposomes (B) on C2C12 cells. The PEG-micelles displayed no obvious toxicity at ~0.05 m*M*, but when the concentration was increased over 0.125 m*M*, the cell viability was decreased to 30–35%. The BP-micelles had a similar trend as the PEG-micelles, with no additional adverse effect of thiolBP conjugation. All cells treated with liposome suspensions displayed ~80% viability irrespective of the liposome concentration, and there was no significant difference in the viability of cells treated with PEG- and BP-liposomes. The results are shown as mean ± SD ($n = 3$).

LYZ. The BMP-2-treated cells showed increasing ALP activity as a function of BMP-2 concentration in both human C2C12 cells and rat BMSCs (Fig. 7). The BMP-2 encapsulated in PEG-liposomes and BP-liposomes displayed equivalent or higher ALP activity as compared to free BMP-2 in both C2C12 and BMSCs. The higher BMP-2 activity observed with liposomal formulations might be due to the higher EE than estimated during the fabrication or superior delivery of the protein by liposomal formulation (i.e., higher internalization[30]). The high activity of the liposome encapsulated BMP-2 indicated that the carrier fabrication procedures, including exposure to organic solvent, sonication, heating and shear force during the extrusion, did not impact the bioactivity of the BMP-2. This was in line with previous studies that showed good retention of BMP-2 activity in liposomes systems[31] and our results further showed that functionalized liposomes behaved similarly in this regard with no apparent detrimental effect due to the presence of thiolBP.

These collective results indicated the feasibility of entrapping bioactive molecules in BP-containing nanocarriers

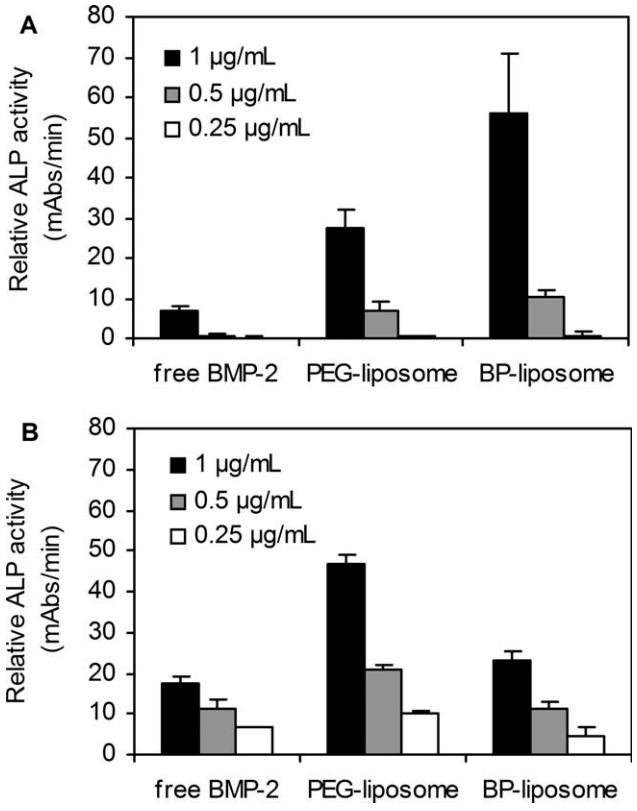

FIGURE 7. ALP activity of the BMP-2 encapsulated in the liposomes on human C2C12 cells (A) and rat BMSCs (B). The dosage shown was the concentration of BMP-2 in the wells for free BMP-2 and estimated concentration based on encapsulation efficiency of 30% for all liposomes. ALP kinetic assay was conducted after 3-days incubation of the liposomal formulations with the cells cultured in 24-well plate, and the relative ALP activity was calculated by subtracting the absorbance of the untreated control from the absorbance of the BMP-2 containing wells. Results are expressed as mean ± SD from triplicate wells.

and improving local retention of carriers in mineral-containing implants. This study paves the way for further studies to explore delivery of bioactive molecules (i.e., pharmaceutical agents such as BMP-2) using mineral-binding carriers in implanted scaffolds. Although our focus has been engineering the nanocarriers with bone mineral- binding BPs, the carriers could also be made cell specific by further modification of the liposome surface with cell-specific ligands.[32] BPs were chosen for this study due to versatility of these reagents and availability of numerous analogues with functional groups suitable for derivatizations. Other bone-seeking ligands could be also used for bone targeting, such as tetracycline used for labeling freshly deposited bone mineral,[33] and peptides such as statherin-peptides[34] and poly (aspartic acids).[35] Peptides could be particularly beneficial for conjugation to liposomes due to the convenience of creating peptide libraries for multifunctional purposes, such as cell-specific binding in addition to mineral binding. The potential of these different approaches have been explored in different labs,[3] but their relative merit in head-to-head comparisons remains to fully evaluated. Although targeting of BP-grafted nanocarriers to bone after systemic administration was not possible in our hands,[36] we showed in this study that local retention of BP-decorated nanoparticles in mineral-containing scaffolds *in vivo* (and presumably in tissues other than the subcutaneous site employed here) is feasible. This should allow better control of local drug delivery in specific diseases where local administration of mineral-binding nanaocarriers is beneficial.

CONCLUSIONS

Conjugation of the thiolBP with DSPE-PEG enabled fabrication of nanoparticulate drug delivery vehicles with significant mineral affinity. The possibility of micellar carriers capable of effectively encapsulating model drugs and displaying mineral affinity was demonstrated based on thiolBP decoration of the micelles. Taking advantage of the strong mineral affinity of BPs, the BP-liposomes had sustained release and enhanced retention in mineralized collagen scaffolds in *in vitro* studies. Furthermore, we showed for the first time that thiolBP-functionalized liposomes were retained to a higher degree in mineral-containing scaffolds in an animal implant model. With their capacity to retain the bone-inducing protein BMP-2 in a bioactive form, the prepared liposomal formulations provide potential candidates for localized or bone-targeted drug delivery system for diseases affecting skeletal tissues, especially for curing bone defects via tissue engineering approach.

The BMP-2 was kindly provided by Dr. Walter Sebald (University of Wurzburg, Germany). The authors thank Lipoid GmbH (Ludwigshafen, Germany) for providing DSPC and DSPE-PEG and Dr. Vishwa Somayaji for NMR analysis.

APPENDIX

^{1}H NMR spectrum of DSPE-PEG-thiolBP synthesized and used for the micelle and liposome formulations in this study. The original spectrum was expanded to indicate the major peaks identified in the product (shown on the schematic structure, Fig. A1).

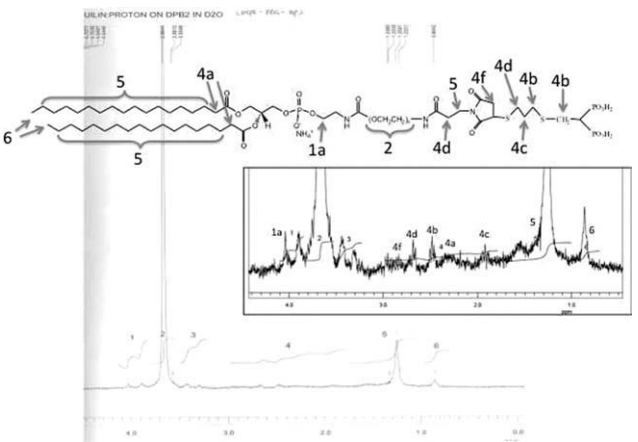

FIGURE A1.

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