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Bisphosphonate-Derivatized Liposomes to Control Drug Release from Collagen/Hydroxyapatite Scaffolds

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ABSTRACT: A drug delivery system was developed by combining composite scaffolds made up of collagen and hydroxyapatite (Col/HA) with bisphosphonate (BP)-derivatized liposomes. The Col/HA scaffold was prepared by a freezedrying method to yield a porous scaffold. The liposomes were composed of distearoylphosphocholine, cholesterol, distearoylphosphoethanolamine—poly(ethylene glycol) (DSPE-PEG), and a bone-binding bisphosphonate (BP) attached to the DSPE-PEG (DSPE-PEG-BP). By taking advantage of the specific interaction between the liposomal BP and the HA



incorporated into the scaffold, the BP-decorated liposomes (BP-liposomes) were shown to display a strong affinity to Col/HA scaffolds. Three different model drugs, carboxyfluorescein (CF), doxorubicin (DOX), and lysozyme (LYZ) were entrapped in liposomes; there were no differences in drug release from the liposomes whether the liposomes were BP decorated or not. Whereas unencapsulated drugs and drugs encapsulated in PEG-liposomes displayed rapid release from the scaffolds, the drugs entrapped in BP-liposomes showed a slower release from the Col/HA scaffolds. We conclude that the proposed system can prolong the in situ residence of model drugs and has the potential to provide a sustained drug release platform in bone regeneration and repair.

KEYWORDS: scaffold, hydroxyapatite, bisphosphonate, liposome, drug release

INTRODUCTION

Bone tissue is susceptible to a range of diseases whose treatment typically involves the stimulation of bone regeneration to replace the original tissue. Especially for critical-size bone defects where the bone tissue will not bridge the defect on its own, a bone substitute is needed to fill the defect and restore the lost tissue function. Significant concerns regarding bone grafting^{1,2} have motivated the development of tissue-engineered bone constructs and synthetic scaffolds suitable for implantation at bone sites. Bioactive molecules capable of influencing cellular events at the healing site are an integral part of this strategy,^{3,4} making it possible to accelerate or ensure healing within the defect site. However, it is difficult to deliver bioactive molecules and drugs in a controlled manner from a scaffold to facilitate bone healing and to prevent the adverse pathology associated with delayed healing.

Lipid vesicles or liposomes employed for controlled release and localization of various drugs^{5,6} could be a useful approach to control drug delivery from scaffolds. The liposomal lipid membrane formed around the drugs creates a depot of bioactive molecules, whose local release is controlled by the membrane structure. Local delivery of liposomal drug formulations could maintain appropriate drug levels at a bone site and make it possible to lower drug doses, thereby reducing adverse side-effects associated with implanting exuberant drug doses. Liposomes have been loaded into scaffolds prepared from collagen^{7–9} and gelatin,¹⁰ as well as other hydrophilic polymers.^{11–13} Cross-linking liposomes to a scaffold via functional groups can further prolong the release of the entrapped drugs.^{8,14} The reported scaffold systems, however, lacked mechanical rigidity, which limit their application in bone repair and regeneration. To obtain the desired mechanical strength, composite materials incorporating biodegradable polymers and bioceramics, including calcium phosphates, hydroxyapatite (HA), and silicate bioactive glasses, have been developed.^{1,15} Collagen/HA (Col/HA) composite scaffolds are especially promising due to the biocompatibility and biodegradability of its components, the feasibility of precisely tailoring its mechanical properties, and its compositional similarity to human bone.^{16–19} Collagen-based scaffolds have been used with human bone morphogenetic protein-2 (BMP-2) for bone regeneration in a clinical setting,^{20,21} but only a few attempts have been made to incorporate liposomes into collagen scaffolds.⁷⁻⁹ Although growth factor-containing liposomes were also reported as a means to stimulate local cellular events, 22,23 no attempts were made to incorporate these liposomes into scaffolds for bone tissue engineering.

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We²⁴ and other groups^{25,26} recently explored the utility of bisphosphonate-decorated liposomes (BP-liposomes) as drug carriers. The BP-liposomes prepared by our group was derived from a thiol-containing BP, 2-(3-mercapto-propylsulfanyl)-ethyl-1,1-bisphosphonic acid, whose synthesis and use in protein delivery was described previously.²⁷ The BP class of compounds display a strong affinity to HA due to their ability to electrostatically interact and chelate Ca²⁺ in the HA structure.²⁸ The liposomes were prepared from lipidic components that self-assembled under aqueous conditions and contained a small fraction of amphiphilic component with hydrophobic (for integration into liposomal membrane) and BP-containing hydrophilic (for display on liposomal surface) segments. The strong interactions between the HA and BP component of BP-liposomes was intended to impart a liposome affinity to bone mineral, hence serving as an osteotropic drug delivery system.²⁴⁻²⁶ However, the desired HA affinity could also make the BP-liposomes useful for incorporation into Col/ HA scaffolds to control local retention of encapsulated bioactive molecules in scaffolds. For example, it might be possible to better deliver osteogenic proteins to promote bone regeneration and anti-inflammatory drugs to suppress undesirable host reactions. Unlike the BPs used for pharmacological treatment of bone loss,²⁹ BPs present in liposomes are intended to primarily act as affinity ligands and may be pharmacologically active depending on the choice (potency) of the BP.

This study explored the feasibility of creating a liposomeincorporating scaffold for the controlled release of bioactive molecules. We hypothesized that BP-functionalized liposomes could be sequestered in mineral-containing scaffolds to better localize their drug cargo in the scaffold. A Col/HA composite scaffold was created that was subsequently loaded with BP-liposomes encapsulating different types of model drugs. The binding of the unmodified and BP-derivatized liposomes for Col/HA scaffold was characterized. The release behaviors of the liposomes from the Col/HA scaffolds, as well as the liposome-encapsulated model drugs (carboxyfluorescein, doxorubicin, and lysozyme), were investigated in detail.

MATERIALS AND METHODS

Materials. Cholesterol (CH), 5(6)-carboxyfluorescein (CF), doxorubicin hydrochloride (DOX), 1,1'-dioctadecyl-3,3,3',3'tetramethylindocarbocyanine (DiI), lysozyme (LYZ) from chicken egg white, and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). 1,2-Distearoylglycero-3-phosphatidylcholine (DSPC) and 1,2-distearoylglycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000] (DSPE-PEG) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany). The synthesis of thiolBP, (2-(3-mercapto-propylsulfanyl)-ethyl-1,1-bisphosphonic acid)²⁷ and DSPE-PEG-thiolBP were described earlier.²⁴ The preparation of synthetic HA was according to the method of Bernardi,³⁰ as previously described.³¹ Fluorescein isothiocyanate (FITC) was obtained from Pierce (Rockford, IL, USA), and it was used to label the LYZ according to previously described methodology.³² The phosphate-buffered saline (PBS, pH 7.4) was diluted from the stock of 10 \times PBS (80 g/L NaCl, 2 g/L KCl, 14.4 g/L Na₂HPO₄, and 2.4 g/L KH₂PO₄). Phosphate buffer were diluted from the stock prepared by mixing 0.5 M Na₂HPO₄ and 0.5 M NaH₂PO₄ solutions to obtain the appropriate pH. The dialysis tubing with a molecular weight cutoff (MWCO) of 12-14 kDa and 100 KDa were acquired from Spectrum Laboratories (Rancho Dominguez, CA, USA).

Distilled/deionized water (ddH_2O) used for buffer preparation and dialysis was derived from a Milli-Q purification system (Millipore, Billerica, MA).

Preparation of Col/HA Scaffold. Type-I collagen was isolated from rat tail tendons as described elsewhere³³ with minor modification. The care and use of the animals were according to institutional guidelines approved at the University of Alberta. The tendons were excised from the tails of Sprague–Dawley rats, disinfected with 70% ethanol, and stored at -20 °C. After removing other attached connective tissues, the tendons were washed with Tris-buffered saline (0.9% NaCl, 10 mM Tris) and dehydrated in serial concentrations of ethanol (50%, 75%, 95%, and 100%). The dehydrated tendons were added into precooled 0.5 M acetic acid (100 mL per 1 g wet tendon) and stirred at 4 °C for 48 h. After centrifugation at 2000 g for 1 h, the pellet was discarded, and the supernatant was precipitated with an equal volume of precooled 10% NaCl overnight at 4 °C. The collagenrich insoluble material was collected by centrifugation for 1 h at 2000 g and dissolved in 0.25 M acetic acid at 4 °C. The collagen was further purified by dialysis against 0.025 M acetic acid for 72 h (MWCO: 12-14 kDa, buffer changed 3 times a day) and ddH_2O (\times 2). The collagen was obtained by freeze-drying the dialyzed sample (48 h) and stored at 4 °C until use.

The Col/HA composite sponge was prepared by a freezedrying method as described in a previous publication.³⁴ The collagen was dissolved in 0.1 M acetic acid at 0.5 wt % by vigorous stirring at 4 °C. The blade-like HA particles $(1-10 \,\mu\text{m in size})$ were added to this solution (collagen:HA ratio of 30:70 w/w) and uniformly suspended in the collagen slurry by vigorous stirring. The mixture was degassed in a vacuum desiccator for 1 h to remove the air bubbles, transferred into a multiwell plate (BD Falcon 48-well plate) at room temperature and frozen at -20 °C overnight. Then, the samples in the multiwell mold were lyophilized by freeze-drying (-55 mmHg) for 48 h. The Col/ HA sponges were recovered from the wells by cutting them with a blade and cross-linked by dehydrothermal treatment at 120 °C under vacuum for 24 h. The sponges were then exposed to UV radiation for 8 h in a biosafety cabinet for sterilization. As a control, collagen scaffold without HA was prepared using the same method.

Characterization of Col/HA Scaffold. Scanning electron microscopy (SEM) was used to examine the microstructure of the scaffolds. Scaffold samples were cut using a sharp blade and fixed to an adhesive carbon stub. After sputter coating with gold, imaging was carried out using a Philips/FEI LaB6 SEM (FEI Company, Hillsboro, OR, USA) operated at an accelerating voltage of 20 kV. The porosity of the Col/HA scaffold was calculated using the following equation: porosity = $100 \times (1 - \rho_{\text{sample}}/\rho_{\text{material}})$, where ρ_{material} was the theoretical density of the composite material (2.12 g/cm³), and ρ_{sample} was the density of the scaffold.

Liposome Preparation. DSPC (10 μ mol), CH (5 μ mol), and 5% (molar percent of DSPC) DSPE-PEG (for PEG-liposomes) or DSPE-PEG-thiolBP (for BP-liposomes) were dissolved in chloroform and dried under reduced pressure. The resultant thin lipid film was hydrated with 2 mL of HEPES buffered saline (HBS: 10 mM HEPES, 140 mM NaCl, pH 7.4) at 60 °C, a temperature above the phase transition temperature of DSPC (55 °C), in a water bath. To encapsulate the CF, DOX, and FITC-labeled LYZ, the lipid film was hydrated with CF (0.5 mg/mL), DOX (80 μ g/mL), or FITC-labeled LYZ (1 mg/mL) solution in 2 mL of HBS buffer, respectively. The dispersions were then sonicated for 10 min in a bath-type sonicator at the same temperature as the incubation. The unencapsulated molecules were separated from the liposomal drugs by extensive dialysis against HBS (MWCO 100 kDa for FITC-labeled LYZ, 12–14 kDa for DOX) for 24 h. The encapsulation efficiency was determined by measuring the fluorescent intensity of the samples (appropriately diluted with 1% Triton X-100 in PBS) before and after the dialysis (for CF and FITC-labeled LYZ, $\lambda_{ex} = 485$ nm, $\lambda_{em} = 527$ nm; for DOX, $\lambda_{ex} = 485$ nm, $\lambda_{em} = 604$ nm), EE = 100% × [(fluorescence postdialysis)/(fluorescence predialysis)]. Initial studies demonstrated a complete removal of free drugs after dialysis in the absence of the encapsulating liposomes (not shown).

Drug Release from Liposomes. The release of CF, DOX, and FITC-labeled LYZ from the liposomes was assessed by a dialysis method.³⁵ Briefly, 0.8 mL of desired samples was added to the dialysis tubing (MWCO: 12–14 kDa for CF and DOX, 100 kDa for FITC-labeled LYZ), the tubing was immersed in a 50 mL test tube containing 40 mL of PBS and incubated on an orbital shaker (300 rpm) at 37 °C. At predetermined time points, 200 μ L of the external release medium was withdrawn and measured for fluorescence intensity. The same volume of fresh buffer was added to keep the total volume at 40 mL. The amount of released molecules was calculated based on a standard curve of free molecules in the same buffer and used to determine the cumulative release.

Binding Affinity of Liposomes to Col/HA Scaffold. To evaluate the affinity of the liposomes to scaffolds, DiI (0.2% of DSPC) was used to label the liposome membrane for quantification purposes.^{36,37} The DiI-labeled PEG-liposomes and BP-liposomes were suspended at a concentration of 0.5 μ mol/mL (equivalent phospholipid concentration) in PBS, and 1 mL of the sample was incubated with the Col/HA or collagen scaffold (cut to 9 mm in diameter and 5 mm in thickness) in a 24-well tissue culture plate for 3 h. The fluorescence intensity in the supernatant (200 μ L) was measured by a spectrofluorometer (200 μ L in 96-well black plates; $\lambda_{ex} = 536$ nm, $\lambda_{em} = 607$ nm). The percentage of liposomes bound to the scaffolds was calculated based on the fluorescence intensity of DiI in the supernatant and expressed relative to a control (i.e., equal volume of liposomal suspension incubated in the absence of a sponge): %binding = $100 \times$ (fluorescence in the control – fluorescence in the supernatant)/ fluorescence in the control.

Release of Liposomes and Encapsulated Drugs from Col/HA Scaffolds. The liposome release from the scaffolds was investigated by an immersion method. The Col/HA and collagen control scaffolds were placed in a 24-well tissue culture plate, and 100 μ L of liposome samples (PEG-liposomes or BP-liposomes) was soaked into the scaffolds for 10 min. After incubation at 37 °C in 1 mL of PBS, or phosphate buffers of different concentrations, the supernatant was removed at predetermined time points and replaced with an equal volume of fresh medium. The fluorescence intensity of DiI in the supernatant was measured by spectrofluorometer and used to calculate the cumulative release of liposome compared to the control (i.e., 100 μ L sample diluted to 1 mL with PBS but without the scaffold).

To investigate drug release, the liposomes with encapsulated CF, DOX, or FITC-labeled LYZ were incubated with the scaffolds in 24-well plates (triplicate) as described above. At indicated time points, the medium was totally removed and replaced with fresh PBS. A total of 200 μ L of the collected medium was treated with 800 μ L of 1% Triton X-100 in PBS (to disperse liposomes) and

used for fluorescence measurements. The cumulative release was calculated based on the fluorescence in the supernatant and the initial fluorescence in the control (i.e., 100 μ L sample diluted to 1 mL with PBS).

Statistical Analysis. All experimental data were collected in triplicate, and expressed as the mean \pm standard deviations (SD) of the measured parameter. Statistical analysis was performed using two-sided unpaired Student's *t*-test. Differences were considered statistically significant with a *p*-value <0.05.

RESULTS AND DISCUSSION

Col/HA Composite Scaffold. Porous Col/HA composite scaffolds were successfully prepared by a freeze-drying method. Figure 1 shows the appearance of a typical scaffold and its pore structure as observed with the SEM. The images show a connected porous structure with holes in the walls of adjoining pores and pore sizes in the range of $200-400 \ \mu$ m. HA particles were distributed (or embedded) throughout the collagen matrix, and some of the particles were clearly exposed on the surface of the pore walls. The mean porosity of the Col/HA scaffolds was calculated to be 98.3 \pm 0.3%, and the value for the collagen scaffolds was 99.2 \pm 0.2%, indicating no gross effects of HA addition on the scaffold structure. As previously described by others,^{33,34} the wetted Col/HA scaffold had extensive flexibility and elasticity upon handling and displayed a shape-recovery property after compression when inspected visually (not shown).

Porosity and pore size are important morphological properties of scaffolds intended for bone regeneration, and they can significantly influence mechanical properties of the scaffold, cell adhesion, and migration, as well as the delivery of bioactive molecules. The minimum recommended pore size for bone tissue engineering scaffolds is 100 μ m considering the size of cells that will penetrate the scaffold, but larger pores (>300 μ m) may favor direct osteogenesis by allowing vascularization and high oxygenation.³⁸ The porous Col/HA scaffolds prepared in the present study are expected to be suitable for bone regeneration, although they should be further optimized for mechanical strength, cellular adhesion, and migration by changing the collagen/HA content and parameters of the preparation process such as the freezing temperature and rate. Collagen scaffolds are usually chemically or physically cross-linked to improve their mechanical properties and to reduce the degradation rate of the scaffolds. Chemical cross-linking has the risk of unacceptable in vivo toxicity due to residual compounds left from cross-linking reactions, and therefore, physical cross-linking by thermal dehydration was utilized in this study. The mechanical properties of the scaffolds have not been quantitatively investigated in this study; however, considering that the Helistat absorbable collagen sponges (ACS) used for clinical BMP-2 implantation have minimal mechanical strength and that they perform adequately at orthopedic and maxillofacial sites,^{20,21} the mechanical properties of the designed scaffolds are not likely to be an impediment for bone tissue implantation.

Drug Encapsulation in Liposomes. To avoid self-quenching of the fluorophores at high concentrations due to intermolecular interactions, the liposome-encapsulated samples were diluted by Triton X-100 (1% in PBS) for measurement. Standard curves of fluorescence intensity vs drug concentration confirmed that the drugs freed by the Triton X-100 showed no obvious self-quenching (Figure 2). On the basis of this approach, the encapsulation efficiencies for CF, DOX, and LYZ in the PEG-liposomes were



Figure 1. Appearance of the Col/HA scaffold (a) and its pore structure observed under SEM (b and c). The SEM images demonstrate the highly porous, interconnected structure of the scaffolds (arrows in b indicate open spaces at the pore surfaces that connect the pores together) and HA particles exposed on the surface of the walls (black arrows). Scale bar: 500 μ m in b, and 50 μ m in c.

 $5.4 \pm 0.3\%$, $54.9 \pm 3.5\%$, and $10.9 \pm 0.9\%$, respectively. The values for the BP-liposomes were equivalent to the PEG-liposomes: $5.8 \pm 0.5\%$, $61.5 \pm 4.2\%$, and $12.1 \pm 2.6\%$, respectively. In a previous study,²⁴ we also investigated the encapsulation efficiency of DOX and LYZ in liposomes and the encapsulation efficiencies for DOX and LYZ reported here were consistent with our previous report. The encapsulation efficiency for CF was expectantly low since small hydrophilic CF is mainly incorporated in the aqueous phase in liposomes.³⁹ DOX and LYZ, on the other hand, are lipophilic or partially lipophilic molecules that are mainly incorporated in the liposomal bilayers.^{40,41} Encapsulation efficiency with DOX was found to range from 44% to 94% in previously reported BP-liposomes,²⁶ in line with the current



Figure 2. Standard curves for determination of CF, DOX, and LYZ concentrations. The results shown are the correlations of fluorescence intensity (\bullet : CF, \blacktriangle : DOX, and \blacksquare : FITC-labeled LYZ) vs drug concentration for the PEG-liposomes (a) and BP-liposomes (b) diluted in PBS with 1% Triton X-100.

study. It was previously found that the encapsulation efficiency of CF was proportional to the liposome diameter,³⁹ and for multilamellar vesicles and small unilamellar vesicles, the CF encapsulation efficiencies were reported to be ~27% and ~1%, respectively. Considering that the encapsulation efficiency for CF in this study was ~6%, the liposomes prepared by the lipid film hydration method were expected to be multilamellar vesicles, possibly with a fraction of small or large unilamellar vesicles. Some of the obtained encapsulation efficiencies could be low for practical purposes (e.g., ~10% for LYZ-like proteins) and might require additional optimizations for industrial applications. Process parameters, such as buffer compositions and pH, might significantly alter encapsulation efficiencies in liposomes,⁴² and this might need to be explored in the future.

Release of Model Drugs from Liposomes. Several features of liposomes, such as lipid composition, surface charge, hydrophobicity, size, and packing of lipid bilayers, are known to influence liposomal stability and release of the entrapped drugs.^{43–46} We used DSPC and CH in this study as the main component of the lipid bilayers and DSPE-PEG to stabilize the liposomes. Liposomes obtained from cholesterol and DSPC, rather than phosphotidyl-cholines with unsaturated fatty acyl chains, can minimize membrane defects, increase packing of lipid bilayers, and resist leakage in the physiological environment.^{47–49}

To evaluate the release behaviors of the liposomal drugs, three model drugs with different molecular weights and hydrophobicities were used. The fluorescent CF is the most commonly used marker to assess the rates of leakage of water-soluble molecules from liposomes. DOX is a common chemotherapeutic drug and is widely employed in release studies due to the inherent fluorescence of the DOX molecule. LYZ is a bioactive enzyme



Figure 3. Release of CF (a), DOX (b), and FITC-labeled LYZ (c) from PEG- and BP-liposomes by the dialysis method. Although the three molecules had distinctly different release rates, the BP-liposomes and PEG-liposomes displayed similar release profiles for each molecule. Values are expressed as mean \pm SD (n = 3).

with an antibacterial property, and it has similar physicochemical properties, in terms of size (\sim 14.7 kDa) and net charge (pI \sim 11.4), to some of the osteogenic proteins, such as BMP-2 (\sim 32 kDa, pI \sim 9.5). Since drug molecules can be entrapped in the aqueous core and/or adsorbed to liposomal membranes, the nature of entrapment could directly influence the release rate from the liposomes. Surface adsorbed or membrane incorporated molecules are expected to be released faster than the molecules entrapped in the core.

As shown in Figure 3, rapid CF release was seen in the first 6 h (\sim 30% released), which could be due to the release of the drug from the surface or peripheral to the surface bilayers.⁴⁶ The subsequent drug release was relatively slow in the next 48 h. The diffusion of CF from the liposomal core might be responsible for the late release profile. The DOX release from the liposomes was faster in the first 24 h (\sim 70% released). The DOX was shown to be mainly entrapped in the liposomal membrane,⁴⁰ which could account for the rapid release profile. Similar to DOX, \sim 70% of LYZ was released from the liposomes in the 48 h time period. The release of LYZ in the first 3 h was notably faster (\sim 40% released), which was probably due to the release of proteins adsorbed on liposomal membrane surfaces.⁴⁵ Interestingly, for all three encapsulants, the



Figure 4. Binding affinity of the liposomes to collagen and Col/HA scaffolds in PBS (a) and different concentrations of phosphate buffer (b). The binding affinity of PEG-liposomes to either collagen or Col/HA scaffold was very low. The BP-liposomes displayed low affinity to collagen scaffold as well, but their affinity to Col/HA scaffold was significantly higher (almost complete). The binding affinity of the BP-liposomes to Col/HA scaffold was dependent on the concentrations of phosphate buffer in the binding medium, with higher phosphate concentration leading to decreased binding (b). Values are expressed as mean \pm SD (n = 3).

release rates from the BP-liposomes were slightly faster than the PEG-liposomes, though the difference was statistically insignificant. This may be due to the thiolBP causing a minor destabilization of the lipid bilayer of BP-liposomes. In studies that employed RGD peptide⁵⁰ and Fab antibody fragments,⁵¹ it was also reported that ligand attachment to liposome surfaces resulted in faster release of the entrapped drugs.

Liposome Affinity to Col/HA Scaffolds. To obtain prolonged liposomal retention in scaffolds, liposomes have to be either chemically bound to scaffolds or modified with strong affinity to scaffold components. The latter approach was taken in this study, and the affinity of the liposomes to Col/HA scaffolds is summarized in Figure 4. Both PEG-liposomes and BP-liposomes showed weak binding to the collagen scaffolds without HA (<10%). The liposomes without thiolBP also displayed low affinity to the Col/HA scaffolds (<10%), whereas the thiolBP modification imparted significantly higher liposomal binding (>90%) to the Col/HA scaffolds, demonstrating that the thiolBP plays a critical role in the binding process. To further confirm the BP-mediated binding mechanism, the liposome binding to scaffolds was tested in buffers with different concentrations of phosphate ions (Figure 4b). The phosphate ions directly compete with BP binding to Ca²⁺ in HA.²⁷ The results indicated that the Col/HA affinity of all liposomes was dependent on the phosphate

concentrations, but the affinity of the BP-liposomes to Col/HA scaffolds showed a larger decrease when phosphate concentration was increased from 0 to 200 mM. This was likely due to the competition between phosphate ions in the buffer and BP on the surface of BP-liposomes for binding to the HA. The lower BP affinity to HA in phosphate buffers was also noted in previous studies, where an aminoBP and the thiolBP were conjugated with proteins;^{31,52} the conjugates displayed reduced HA affinity in phosphate buffer compared to that in water.

In line with our studies, other groups have also reported BPconjugated liposomes with strong HA affinity. Whereas Hangst et al. employed a cholesteryl-trisoxyethylene-bisphosphonic acid conjugate,²⁵ Anada et al. employed 4-N-(3,5-ditetradecyloxybenzoyl)-aminobutane-1-hydroxy-bisphosphonic acid to prepare the mineral-binding liposomes.²⁶ Both liposomes displayed the expected HA affinity as a function of BP substituent in the liposome preparation, suggesting that BPs could be anchored to the liposomal membranes with different moieties without loss of the desired mineral affinity. The mineral affinity of BP-liposomes in these studies was evaluated with pure HA particles, and no attempts were made to utilize them in a scaffold format. The extent of HA binding observed with these different BPliposomes was relatively similar to our results; complete binding was achieved to the Col/HA scaffold in this study, while other BP-liposomes^{25,26} demonstrated complete binding to HA under their respective experimental conditions. The BP-functionalization approach was applied to other types of particles as well, for example, nanoparticles constructed from bovine serum albumin,⁵³ gold,⁵⁴ or synthetic polymers such as poly(lactic-co-glycolic acid).⁵⁵ The improved mineral affinities by these particles were demonstrated in vitro as a result of BP functionalization, and it is likely that they could be incorporated into HA-containing scaffolds for improved local drug delivery.

Liposome Release from Col/HA Scaffold. The release of PEG-liposomes and BP-liposomes from the scaffolds is shown in Figure 5. The release of PEG-liposomes was relatively faster, regardless of the presence or absence of HA in the scaffold. The release of BP-liposomes from the Col/HA scaffolds was significantly slower than that from the collagen scaffolds (<10% until 7 days vs >90% after 24 h, respectively). Consistent with the binding results (Figure 4b), the release of liposomes from the scaffolds was enhanced with the phosphate ions, as shown in Figure 5b. At 24 h, the percentage of PEG-liposome release was 76.7% in the absence of phosphate ions, whereas 99.4% and 96.8% releases were obtained with 50 and 200 mM phosphate, respectively. For BP-liposomes, the release in 0, 50, and 200 mM phosphate buffers were 7.4%, 21.9%, and 32.2%, respectively, after the first 3 h. The release was slightly increased to 8.4%, 33.1%, and 39.8% after 7 days, respectively, indicating relatively little release in the long term.

The binding of liposomes to Col/HA scaffolds can be attributed to nonspecific as well as specific interactions.⁵¹ In the absence of buffer ions, liposome release might be dominated by diffusional release of nonbound or weakly bound liposomes, whereas both diffusion and dissociation (desorption) from the scaffolds will be important in the presence of phosphate ions. Since the size of the liposomes were much smaller than scaffold pore sizes, the PEG-liposomes, which had weak binding to both the collagen and Col/HA scaffolds, were capable of freely diffusing out of the sponges, leading to fast release immediately following exposure to the release medium. The BP-liposomes had strong affinity to Col/HA scaffolds, and even the phosphate



Figure 5. Release of liposomes from the Col/HA scaffolds in PBS (a) and the effect of phosphate concentration on the liposome release (b; for PEG-liposomes, \Box : 0 mM, \bigcirc : 50 mM, and \triangle : 200 mM; for BP-liposomes, \blacksquare : 0 mM, \odot : 50 mM, and \triangle : 200 mM). The release of liposomes was determined by measuring the fluorescence of DiI-labeled liposomes at indicated time points. Values are expressed as mean \pm SD (n = 3).

ions did not result in complete dissociation and release of BPliposomes. We noted that BP-protein conjugates displayed a more robust dissociation from the HA surfaces with phosphate ions,⁵² suggesting that other factors might contribute to liposome release, for example, factors related to liposome features (e.g., hydrophobic interactions with lipid membranes) or scaffold properties (e.g., scaffold tortuosity).^{10,11,56} Since the focus of this study was to increase liposome affinity to the HA component of the scaffold, the effects of these scaffold-related factors on the release rate were not investigated. It is not clear if the nature of the drug entrapped (or its encapsulation efficiency) in liposomes could affect the liposome release from scaffolds. As long as the drug or the drug loading level does not affect BP affinity of liposome to the HA component, liposomal release should be unaltered by the presence of the encapsulants. A factor that might influence release of BP-liposomes from Col/HA scaffolds could be the dissolution of the HA component,⁵⁶ especially under in vivo conditions, and this issue remains to be investigated in the future.

Release Behaviors of Drugs from Liposome-Loaded Scaf-folds. Although the PEG-liposomes and BP-liposomes had similar drug release rates (Figure 3), the difference in the liposomal binding to the scaffold (Figure 5) could modulate the release rate of the drugs from the scaffolds. The release profiles for CF, DOX, and LYZ from the liposome-loaded scaffolds are shown in Figure 6. For the collagen scaffolds (Figure 6a, c, and e), almost all the free drugs (>90%) were immediately released in the first 12 h period. This was indicative of a lack of affinity between the chosen model drugs and the



Figure 6. Release profiles of CF, DOX, and LYZ from the liposome-loaded collagen (a, c, and e) and Col/HA (b, d, and f) scaffolds. The liposome-loaded scaffolds were incubated in PBS at 37 °C, and the scaffolds sequestering free drugs (no liposomes) were employed as the control. The analysis for released molecules was carried out at indicated time points. Values are expressed as mean \pm SD (n = 3).

collagen scaffolds. Complete release was also observed for the drugs encapsulated in PEG-liposomes after 12 h (>90% in all cases). The delivery with the BP-liposomes led to slower release as compared to the free drugs and PEG-liposomes at all time points for CF (83.0% after 48 h period) and LYZ (81.9% after 48 h period) and for DOX (84.8% at the 12 h period).

For the Col/HA scaffolds (Figure 6b, d, and f), the free CF and PEG-liposome encapsulated CF displayed fast and complete release in 12 h (>99%), similar to the results seen with collagen scaffolds alone. The CF did not apparently display any affinity toward the HA component of the scaffold. However, the CF encapsulated in BP-liposomes was released significantly slower (e.g., 54.5% after 48 h period). A similar release profile was observed for DOX and LYZ, where the order of the release rates for the three molecules was free DOX (or LYZ) > PEGliposomes > BP-liposomes. Different from the release profiles of free CF and DOX, the free LYZ was not completely released from the Col/HA scaffolds (73.3% at 48 h), which was likely due to the inherent affinity of the protein to the HA component of the scaffolds. This observation that LYZ bound strongly to HA was noted in our previous study,⁵² due to a combined effect of both electrostatic and hydrophobic interactions.

The release profile shown in Figure 6 for each drug is a composite effect of (i) liposome affinity to scaffold (Figure 5a) and (ii) drug release from liposomes (Figure 3). Since there was no difference in drug release between the PEG-liposomes and BP-liposomes (Figure 3), the slower release for BP-liposome encapsulated drugs is believed to result from the slower release of BP-liposomes from the scaffold as a result of specific interaction between the BP on liposomes and the HA component of the scaffold.

In a related study, liposomes with collagen-binding fibronectin was prepared and used for growth hormone (GH) delivery in an animal model. Compared to liposomes without fibronectin, collagen-binding fibronectin-liposomes significantly enhanced (>30-50%) in vivo retention of GH after intramuscular injection of liposome-containing collagen gels.⁷ The gels did not contain HA and collagen was used as the binding template in this approach. Another related report investigated cross-linked gelatin gels for localized delivery of ciprofloxacin;¹⁰ the lack of liposomal affinity to gelatin matrix significantly limited the quantity of liposomes sequestered in the gel, promoting excessive liposome release. Cross-linking was needed to retain the liposomes in that study, and the results obtained in the present study were functionally comparable to that study, but with the added advantage of obviating the need for chemical cross-linking in the sustained release formulation.

Implications for Bone Tissue Engineering. The current systems employing liposome-containing collagen matrices have been mostly prepared by passively loading the liposomes during the fabrication process. The sequestered liposomes can be chemically coupled to the scaffolds; however, this technique requires both the liposomes and the scaffold to have appropriate reactive groups,^{8,14} increases the risk of inactivation of biomolecules, and may induce toxicity when utilized in vivo. The BPliposomes and HA combination described here did not involve any cross-linkers for imparting a scaffold affinity. The Col/HA scaffolds can be used as a substrate for cell attachment and proliferation¹⁸ as well as a local implant for bone repair and regeneration.^{17,19} Simply soaking the prefabricated scaffold with the liposomes for sequestering makes it convenient to entrap a variety of bioactive drugs just before therapeutic intervention. The range of drugs that can be encapsulated in the liposomes for bone diseases includes osteogenic growth factors (e.g., BMPs and TGF- β s, to accelerate extracellular matrix production and tissue integration) and anticancer, antimicrobial, anti-inflammatory, and antiresorptive agents.^{1,15,57} Although we did not assess the bioactivity of drugs entrapped in BP-liposomes, Anada et al. showed that encapsulated DOX retained its desired pharmacological activity (anticancer activity),²⁶ so that the presence of BP did not impede the desired therapeutic action. The same is expected to hold true for other drugs as well.

Current delivery strategies have been mostly focused on physical entrapment or chemical bonding of these drugs to the scaffolds. Drugs physically loaded by simply sequestering the drugs in a scaffold usually have an undesirable burst release (as seen in this study as well). Covalent conjugation of these drugs directly to a scaffold is also problematic, especially for the protein-based agents, since it might affect their bioactivity. To avoid chemical modification and overcome burst release, affinitybased drug delivery strategies utilizing interactions between the therapeutic drug and the delivery system have been recommended to control drug loading and release.⁵⁸ If these drugs can be encapsulated in liposomes and then administered to a bone site with HA-containing scaffolds, a higher drug dose could be ensured at the site while reducing nonskeletal exposure to the drugs and their undesirable side-effects. For therapies involving bone induction, the presence of BPs in scaffolds could impact subsequent tissue mineralization, since the BPs are known for modulating HA formation directly as well as impeding mineral dissolution after binding to HA crystals. The dose of BP could be minimized to reduce its effects on bone cells,²⁷ but the presence of the BPs could be also beneficial to slow down the resorption of induced mineralized tissue in the long run. This pharmacological effect of BPs in BP-liposomes will likely depend on the choice of BP; whereas the older generation BPs (with their limited pharmacological potency) could fulfill the HA-affinity requirement, the newer generation BPs (with robust pharmacological activities) could further participate in the bone turnover process in addition to fulfilling the HA-affinity requirement. The BP in the liposomal preparations will be chemically bound to other lipidic components, and it remains to be seen if the pharmacological activity of BPs will be retained in this way. Cleavable linkages might be one approach to release BP molecules from liposomes in situ. Besides the liposome-scaffold interaction, drug release rate may also depend on the structure of Col/HA

scaffolds so that scaffold optimization for composition, pore size, and degradation rate is worth investigating in the future. Although the main motivation of this study was to tailor Col/HA scaffold and BP-liposomes for bone repair, the proposed system can be also utilized in topical applications for treatment of surgical wounds and burns or for regeneration of other tissues.

CONCLUSIONS

A drug delivery system that combined a Col/HA composite scaffold and BP-modified liposomes was designed for controlled release of therapeutic agents from mineral-containing scaffolds. The Col/HA scaffold had an open and connected porous structure that is expected to be suitable for cell attachment and ingrowth at bone sites. Using several model drugs, BP-incorporating liposomes were shown to display similar drug release profiles to that of conventional liposomes without the BP moieties. The BP-liposomes loaded in the Col/HA scaffolds showed significant binding affinity to the scaffolds and prolonged the release of model drugs from the scaffolds. This was unlike the conventional liposomes that did not have a particular affinity to Col/HA implants. The reported BP-liposomes sequestered in Col/HA scaffolds are promising for application in bone tissue engineering and regenerative medicine, although detailed studies on cellular compatibility and functional tissue induction remains to be investigated with the proposed scaffolds. The versatility of the liposomes (i.e., their ability to encapsulate a variety of pharmacological agents) as well as their accepted clinical use should facilitate the application of the proposed scaffoldliposome combination in a clinical setting.

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COMMON ABBREVIATIONS

Col, collagen; HA, hydroxyapatite; LYZ, lysozyme; DOX, doxorubicin; CF, carboxyfluorescein; BP, bisphosphonate; DSPC, distearoylphosphocholine; CH, cholesterol; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetra-methylindocarbocyanine; PEG, poly(ethylene glycol); FITC, fluorescein isothiocyanate

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