A Di(Bisphosphonic acid) for Protein Coupling and Targeting to Bone

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ABSTRACT: Proteins intended for treatment of bone diseases should ideally exhibit a high bone affinity, so that they are preferentially deposited to bones after systemic administration. This can be achieved by combining molecules having a high affinity to bone with the proteins. Bisphosphonates (BPs) are chemical analogs of pyrophosphate that possess exceptional bone mineral affinity. To this end, we synthesized a novel BP, 3,5-di(ethylamino-2,2-bisphosphono)benzoic acid (**6**), which contains two BP moieties on a single molecule, unlike conventional BPs that contain one BP moiety per molecule. **6** was then conjugated to two model proteins, bovine serum albumin and nonspecific bovine immunoglobulin G by the carbodiimide chemistry. By varying the reagent concentrations, the conjugation efficiency (i.e., number of **6** per protein) was readily controlled under the experimental conditions. The protein-**6** conjugates exhibited an *in vitro* mineral affinity that was proportional to the number of conjugated **6**. The **6**-conjugates of both bovine serum albumin and immunoglobulin G were found to be bone seeking in rats, based on the increased concentration of **6**-conjugated proteins in bone tissue after intravenous administration. We conclude that the novel BP synthesized (**6**) can serve as a carrier for bone delivery while reducing the extent of protein modification necessary for bone targeting. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 93:2788–2799, 2004

Keywords: conjugation; drug delivery; protein

INTRODUCTION

Protein-based therapeutic agents are being actively pursued for treatment of skeletal diseases.[1] Being an endogenous regulator of cellular activity, the proteins have the potential to modulate cellular activity at skeletal sites in order to regulate endogenous tissue responses. For example, proteins capable of stimulating new bone formation or inhibiting cell activity responsible for accelerated bone loss will be beneficial in treatment of osteoporosis and arthritis. Despite the demonstrated efficacy of several proteins in preclinical models of these skeletal diseases,[2] the clinical entry of most proteins has been hampered, because of two fundamental barriers. First, the recombinant proteins have a short half-life in circulation because of rapid uptake by the liver, kidneys, and spleen. Basic fibroblast growth factor, for example, is a potential therapeutic agent for systemic stimulation of bone formation

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but it has a systemic half-life of 1–5 min.[3,4] Second, the high doses that need to be administered, due to rapid clearance from the circulation, result in significant deposition of the proteins at extraskeletal sites. Because the proteins are ubiquitous modulators of cellular activity, pharmacological levels of bioactive proteins at extraskeletal sites result in undesired side-effects at these sites. Systemic lowering of blood pressure[5] and functional kidney failure[6] are such effects seen when proteins are attempted for augmentation of bone mass. If proteins could be engineered to exhibit a strong bone affinity, then systemic administration of the proteins would deliver a significant protein dose to skeletal sites with reduced distribution to extraskeletal sites. This would reduce the efficacious dose that needs to be administered, as well as the undesired side effects at other anatomical sites.

To achieve bone-specific delivery of proteins, proteins can be chemically modified with bisphosphonic acids (BPs), a class of compounds with exceptionally high affinity to the bone mineral hydroxyapatite (HA). BPs are chemical analogs of endogenous pyrophosphate where the central, hydrolytically labile P–O–P linkage is replaced by the hydrolysis-resistant P–C–P bond. Intravenous administration of BPs results in significant deposition (20–50% of injected dose) of the molecules to bone.[7,8] The exceptional mineral affinity of BPs has been utilized to deliver radiopharmaceutical and imaging agents to skeletal tissue.[9,10] The concept of chemically modifying proteins with BPs was initially disclosed in a patent application, where protein conjugation to BPs was proposed by using hydrophilic polyethylene glycol linkers.[11] No data were provided whether the conjugation chemistry was successful or whether the conjugates exhibited a high affinity to bone. To realize this vision, we devised a conjugation scheme in which 1-amino-1,1-diphosphonate methane (aminoBP) was thiolated with 2-iminothiolane. The thiolated aminoBP was subsequently incubated with a heterofunctional crosslinker to link the thiol group on the BP to an amine group on proteins.[12–16] *In vitro* HA affinity of proteins was shown to be proportional to the extent of aminoBP conjugation, when aminoBP was conjugated onto the protein's $-NH_2$[12] and carbohydrate groups.[15] Intraosseous injection of the aminoBP conjugates confirmed the high bone affinity of the conjugates *in vivo*,[13] as well as their capability to "seek" bone after systemic injection in rats.[14] A critical observation in our studies was the importance of tether length on the HA affinity of conjugates; the shorter tether lengths were found to give conjugates with higher HA affinity compared with conjugates formed with longer tether lengths.[16] A higher ligand density at the vicinity of proteins, and/or feasibility of secondary interactions between protein side-chains and HA were postulated for beneficial effect(s) of shorter tethers. Irrespective of the specific mechanism(s), these results pointed out the need to minimize the tether length between a protein and a BP.

This study was conducted to determine whether a bone affinity could be imparted to proteins by using a conjugation approach that provides a minimal tether length (Fig. 1). A novel BP was synthesized for this purpose that is composed of two BP moieties linked via a benzene ring and contains a $-COOH$ moiety (**6**, Scheme 1). This compound provides a higher BP density on proteins compared with previous conjugates, because we can attach two BPs per protein site. Moreover, the presence of the $-COOH$ group on the BP allows one to utilize the aqueous carbodiimide chemistry for BP coupling to proteins. Unlike the commercially available linkers, such as the one used in previous studies, this chemistry provides the desired zero tether length between the protein and BP. Herein, we demonstrate feasibility of

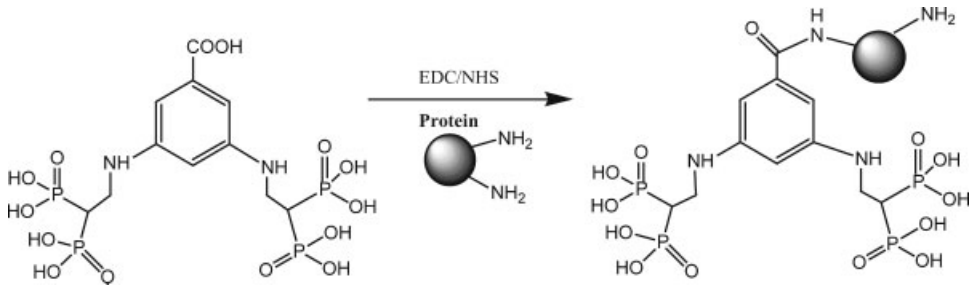
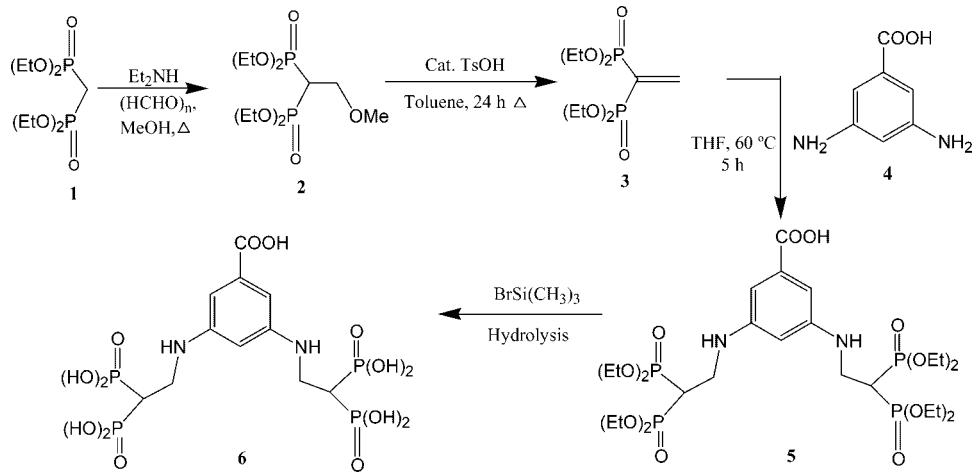


Figure 1. Protein conjugation approach which provides a minimal tether length.



Scheme 1.

designing proteins with high bone affinity by carbodiimide-mediated coupling of a –COOH-containing BP to proteins.

MATERIALS AND METHODS

Materials

Tetraethyl methylenebisphosphonate, diethylamine, paraformaldehyde, calcium hydride (CaH_2), *p*-toluenesulfonic acid monohydrate, and *N*-hydroxysuccinimide (NHS) were acquired from Aldrich (Milwaukee, WI). Bromotrimethylsilane [$BrSi(CH_3)_3$] was obtained from Acros Organics (Fairlawn, NJ) whereas CH_3OH, CH_2Cl_2, and $CHCl_3$ were from Fisher (Fair Lawn, NJ). Tetrahydrofuran and hexane from EM Science (Gibbstown, NJ) and toluene from BDH (Toronto, ON) were used. 1-Ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC), bovine serum albumin (BSA; lot 118H0563), bovine immunoglobulin G (IgG; lot 042K9023), bovine adult serum, trichloroacetic acid (TCA), 2-(*N*-morpholino)ethanesulphonic acid (MES), and 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (TCDG) were obtained from Sigma (St. Louis, MO). Precast 4–20% Long Life polyacrylamide gels were from Gradipore (Frenchs Forest, Australia), whereas $Na^{125}I$ (in 0.1 M NaOH) was obtained from PerkinElmer (Wellesley, MA). NaCl 0.9% was from Baxter Corporation (Toronto, ON). The Spectra/Por dialysis tubing with molecular weight (MW) cutoff of 12–14 kDa was acquired from Spectrum Laboratories (Rancho Dominguez, CA). Sodium dodecylsulfate (SDS) and Coomassie Blue R-250 were obtained from Bio-Rad (Hercules, CA). The HA used for protein binding studies was prepared as described.[12] The phosphate (pH 7.4) and carbonate (pH 10) buffers were prepared as described.[13] The SDS-glycine sample buffer for electrophoresis was prepared as previously described.[16] The SDS-polyacrylamide gel electrophoresis (PAGE) running buffer was prepared by the addition of 12.1% (w/v) Trizma Base, 23.8% (w/v) HEPES, and 1.0% (w/v) SDS in deionized water.

Synthesis of 3,5-Di(ethylamino-2,2-bisphosphono)benzoic Acid (6) (Scheme 1)

Diethylamine (0.29 g, 4 mmol) and paraformaldehyde (0.606 g, 20 mmol) were mixed in 12 mL of CH_3OH and the mixture was warmed until clear. Tetraethyl methylenebisphosphonate (**1**) (1.163 g, 4 mmol) was added after removing the heat. The mixture was refluxed for 24 h, and methanol was removed *in vacuo*. Toluene was added and the solution was again concentrated to remove residual methanol. The intermediate, tetraethyl 2-methoxyethylene-bisphosphonate (**2**), was diluted with toluene and catalytic amount of *p*-toluenesulfonic acid monohydrate (0.003 mg) was added.[17] The mixture was refluxed using a Soxhlet apparatus containing CaH_2 and concentrated after 14 h. The product was diluted with chloroform (25 mL) and washed with water (3×15 mL). The organic layer was dried over $MgSO_4$ and concentrated to give oily residue. The crude product was purified by silica gel chromatography (60–270 mesh; 4:1 $CHCl_3$/hexane as eluent) to give 0.79 g (71%) of tetraethyl ethenylidenebis(phosphonate) (**3**). **3** (0.48 g, 1.6 mmol)

and 3,5-diaminobenzoic acid (**4**, 0.12 g, 0.8 mmol) in 15 mL of THF were heated at 60°C for 5 h to give 3,5-di(tetraethyl ethylamino-2,2-bisphosphonate)benzoic acid (**5**). THF was removed under vacuum. **5** (0.45 g, 0.59 mmol) was dissolved in CH_2Cl_2 (5 mL) and $BrSi(CH_3)_3$ (2.01 g, 13.1 mmol) was added dropwise using a syringe at 0°C. The mixture was then stirred at room temperature for 48 h. CH_2Cl_2 was evaporated and 3,5-di(ethylamino-2,2-bisphosphono)benzoic acid (**6**) was precipitated using acetonitrile and acetone. The precipitate was washed with acetone and dried under vacuum to give 0.1704 g (54%) of off-white-colored hygroscopic solid. The indicated intermediates and the final product were characterized by ^{1}H, ^{13}C, ^{31}P NMR and mass spectra. Elemental analysis was not conclusive, because of the hygroscopic nature of the final product, as was the case for other BPs reported in the literature.[17]

Conjugation of 6 onto BSA and IgG

In a typical reaction, **6** was dissolved in 0.1 MES buffer (pH 4.5) and 500 μL of this solution was combined with 250 μL solutions of NHS and 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (both in MES buffer) for 45 min to activate the −COOH of **6**. The activated **6** was then incubated with equal volumes of the protein solutions (BSA and IgG; 20 mg/mL in MES buffer) for 3 h, after which the unreacted components were removed by dialysis against 0.2 M carbonate buffer (×4, pH 10) and dH_2O (×2). Table 1 summarizes different concentrations of **6** and EDC/NHS combination used for this study. The BSA and IgG mixed with **6**, but without the addition of EDC/NHS, were used as the controls.

Analysis of Conjugates

Bradford Protein Assay[18]

To obtain protein concentrations, 50-μL of sample was added to 1 mL of the protein reagent (in duplicate), which consisted of 0.01% (w/v) Coomassie Blue R-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. The sample's absorbance was subsequently determined at 595 nm. Serially diluted BSA and IgG in dH_2O were used for the calibration curve for the respective protein conjugates. The protein concentrations were expressed in millimolar by assuming a BSA MW of 66 kDa and IgG MW of 150 kDa.

Phosphate Assay

Phosphate concentration in samples was assessed by the method of Ames.[19] Thirty microliters of sample and 18 μL of 10% $Mg(NO_3)_2$ in 95% alcohol were added to a glass test tube (in duplicate) and ashed over a flame. Three hundred microliters of 0.5 N HCl was then added to the tubes, and the tubes were boiled for 15 min. Ten percent w/v ascorbic acid (in dH_2O) and 0.42% w/v $(NH_4)_6Mo_7O_{24} \cdot H_2O$ (in 1 N H_2SO_4) were mixed in a ratio of 1:6, and 700 μL of this mixture was added to the hydrolyzed samples. Absorbance was read at 820 nm after 1.5 h of incubation at 37°C. As a calibration curve, unconjugated **6** was dissolved in dH_2O, serially diluted, and analyzed as above. The phosphate concentrations of the samples were used in combination with the results from the protein assay (phosphate concentration in millimolar divided by protein concentration in millimolar) to yield the average number of **6** conjugated onto BSA and IgG (mol/mol ratio).

SDS-PAGE

Approximately 10 μg of protein sample was mixed with an SDS-glycine sample buffer and loaded onto 4–20% Tris-HEPES gels. The samples were run at 150 V for 45 min in an SDS-HEPES running buffer. The gels were then stained overnight using a Coomassie Blue R-250 (0.1% w/v Coomassie Blue R-250 in 10:10:80 methanol/

Table 1. Summary of **6** and EDC/NHS Concentrations Used in Protein Conjugation Experiments

Experiment no.	Protein	**6** Concentration(s)	EDC/NHS concentration(s)
1	BSA	0.25, 0.62, 1.25 mM	0, 1.25 mM
2	BSA	1.25 mM	0, 1.25, 3.0, and 10 mM
3	IgG	0.25, 0.62, 1.25 mM	0, 1.25 mM
4	IgG	1.25 mM	0, 1.25, 3.0, and 10 mM

The concentrations indicated are the final concentration after all reagents were combined. The proteins incubated with 0 mM EDC/NHS served as controls, where no **6** conjugations were expected.

acetic acid/deionized water), destained, and scanned on a flat-bed scanner.

Assessment of HA Binding

The HA binding of proteins[20] was assessed by incubating the proteins with 5 mg of HA in a microcentrifuge tube (in duplicate) containing 400 μL of buffer (protein concentration: 0.5 mg/mL). The binding buffer used was 0, 50, 100, and 200 mM phosphate buffer.[12] After 2.5 h, the tubes were centrifuged and the protein concentration in the supernatant was determined. As reference, each protein was incubated in microcentrifuge tubes without the HA. The percent HA binding was calculated as: [(protein concentration without HA/MW of protein) − (protein concentration with HA/MW of protein)]/[(protein concentration without HA/MW of protein)] × 100%. All binding experiments were performed in duplicate and the binding results were summarized as mean ± SD.

Assessment of Bone Targeting

Bone targeting was determined by using ^{125}I-labeled proteins, injected intravenously in rats.[12–14] To obtain radiolabeled proteins, 10 μg of protein was added to microcentrifuge tubes previously coated with TCDG (200 μL of 20 μg/mL TCDG in chloroform), along with 50 μL of 0.1 M phosphate buffer (pH 7.4). Ten microliters of 0.01 mCi of $Na^{125}I$ (in 0.1 M NaOH) was then added to the tubes and labeling was allowed to proceed for 20 min. Free ^{125}I was separated from the radiolabeled protein via dialysis against 0.05 M phosphate buffer. After precipitating an aliquot of the samples with 20% TCA, it was confirmed that the purified proteins had >95% TCA-precipitable counts, indicating the radiolabel was protein-bound. The labeled protein was used for (i) *in vitro* HA binding and, (ii) intravenous injection in rats.

In Vitro HA Binding

The radiolabeled proteins were added to unlabeled (cold) proteins to give a radioactive count of $\sim 10^6$ cpm at a 0.1 mg/mL protein concentration (hot/cold ratio 1:100). Along with 175 μL of 50% bovine adult serum (diluted with 0.9% saline), 5 mg of HA was added to microcentrifuge tubes. This serum containing binding medium was used because it better represented physiological conditions than the phosphate buffers, where excess concentrations of protein are expected to compete for the HA binding. After periodical shaking during a 3-h period, the samples were centrifuged (3 min at 50*g*). The supernatant was collected, and the pellet, consisting of the mineral matrix, was washed with the binding buffer used, then recentrifuged. This washing procedure was then repeated twice more, and the collected supernatant from each of these steps was subsequently counted separately by a γ-counter (Wallac Wizard 1470; Turku, Finland). Matrix affinity, expressed as percentage matrix binding, was calculated as follows: 100% × (counts in matrix pellet)/[(counts in matrix pellet) + (counts in supernatants)]. All binding results were assessed in duplicate and presented as mean ± SD.

Intravenous Injection in Rats

Preapproval for all animal studies was obtained from the Animal Welfare Committee of the University of Alberta. The housing and care of animals were according to the guidelines of the Canadian Council on Animal Use. The animals were allowed free access to food and water at all times. Female Sprague-Dawley rats 6–8 weeks old (200–225 g; Biosciences, Edmonton, AB) were used to investigate the capability of **6**-conjugated proteins to be targeted to bone. All rats were injected with ^{125}I-labeled proteins while restrained in plexiglass holders. The proteins for injection were dissolved in saline at 0.1 mg/mL (hot/cold ratio of 1:100), and 300 μL of the protein solution was via tail vein. The biodistribution of each protein was evaluated in three rats. After 48 h, rats were sacrificed by CO_2 asphyxiation and their femora and tibiae on both sides, along with both kidneys, a portion of liver (2–3 g), sternum, and spleen were harvested. Blood samples were obtained from heart by using an 18-gauge needle and weighed. The harvested organs along with weighed blood sample were directly counted with a γ-counter.

The radioactive counts of harvested organs were divided by the originally injected counts to calculate the percentage of injected dose found in each organ. In the case of liver and blood, the measured counts were normalized with the weight (g) of the liver tissue excised or the weight of the blood sample collected. All results were summarized as mean ± SD of either $n = 3$ organs (for liver, spleen, and sternum) or $n = 6$ organs (for tibia, femur, and kidney). Significant differences ($p < 0.05$) among protein delivery to the organs were determined by analysis of variance (Tukey

post hoc comparison) using S-PLUS 6.0 (Insightful Corp., Seattle, WA).

RESULTS AND DISCUSSION

Synthesis of 3,5-Di(ethylamino-2,2-bisphosphono)benzoic Acid (Scheme 1)

Compound **6** was synthesized in a four-step synthesis starting from **1** which underwent methoxymethylation in the presence of diethylamine and paraformaldehyde to give an intermediate **2** (Step 1). Refluxing of intermediate **2** with a catalytic amount of *p*-toluenesulfonic acid (Step 2) led to elimination of methanol to give **3**. ^{1}H NMR spectra for **2**, δ_H ($CDCl_3$): 4.19 (m, 8H), 3.88 (td, 2H, $J = 5.4$ and 16.2), 3.36 (s, 3H), 2.68 (tt, 1H, $J = 10.8$ and 24), and 1.31 ppm (t, 12H, $J = 9.3$; the H on P–C_A–P carbon resonates as a triplet of triplets with coupling to 2 P and to 2 H on the adjacent C_B atom whereas the 2 H on the C_B couples with 2 P nuclei to give a triplet that splits into doublet by the H on C_A). The small peak at 6.95 ppm (dd) is due to the formation of **3**. The characteristic peaks in **3**, δ_H ($CDCl_3$): 6.98 (dd, 2H, $J = 34.2$ and 33.9), 4.15 (m, 8H), and 1.32 ppm (t, 12H, $J = 6.9$); δ_C ($CDCl_3$): 149.01, 132.03, 63.12, and 16.2 ppm. These chemical shifts were consistent with the reported values in the literature.[17] The desired compound was obtained by anti-Markovnikov addition of the amino groups of **4** to intermediate **3** (Step 3) to give the coupled product **5**. The ^{1}H NMR of **5**, δ_H ($CDCl_3$): 6.92 (s, 2H), 6.15 (s, 1H), 4.17 (m, 16H), 3.75 (m, 4H), 2.75 (tt, 2H, $J = 17.7$ and 23.4), 1.36 ppm (t, 24H, $J = 11.1$), showed the addition of two tetraethyl ethenylidenebis(phosphonate) groups to two $-NH_2$ groups of 3,5-diaminobenzoic acid. The phosphonate esters of **5** were hydrolyzed by dealkylating agent, bromotrimethyl silane (Step 4) to give free acid **6**. ^{1}H NMR for **6** (Fig. 2), δ_H (D_2O): 6.75 (s, 2H), 6.45 (s, 1H), 3.98 (m, 1H), 3.76 (m, 4H), 2.56 (tt, 2H, $J = 13.35$ and 21.9), and 1.21 ppm (t, 1.5H, $J = 14.4$); ^{13}C NMR: δ_C (D_2O) 166.95, 148.69, 132.66, 108.27, 61.22, 35.66, 30.67, and 16.32 ppm; ^{31}P NMR: δ_P 17.16 ppm; MS *m/z* 263 (M^{2-}), 175 (M^{3-}), 277 $(M + Et)^{2-}$ peak due to unhydrolyzed ethyl ester. ^{1}H NMR typically showed a lack of phosphonate esters, but occasionally a minor peak in ^{1}H and ^{31}P NMR showed the presence of a small amount of unhydrolyzed ethyl ester in **6** (δ_P 17.9 ppm; ~5% in one preparation).

We are aware that several groups have reported the synthesis of tetraphosphonates,[21–23] but only two studies reported synthesis of a di-bisphosphonate compound.[24,25] In one study,[24] the di-bisphosphonate contained a functional $-NO_2$ group, but the compound was obtained as a phosphonate ester, not as a di(bisphosphonic acid) that is necessary for HA affinity. In the other study,[25] two BPs were linked with 4–10 methylene groups, but without any other functional groups suitable for subsequent conjugations. Unlike previous di(phosphonates), the di(phosphonic acid) synthesized in this study provides a $-COOH$ group for convenient coupling to proteins and is present in acid form necessary for the display of mineral affinity.

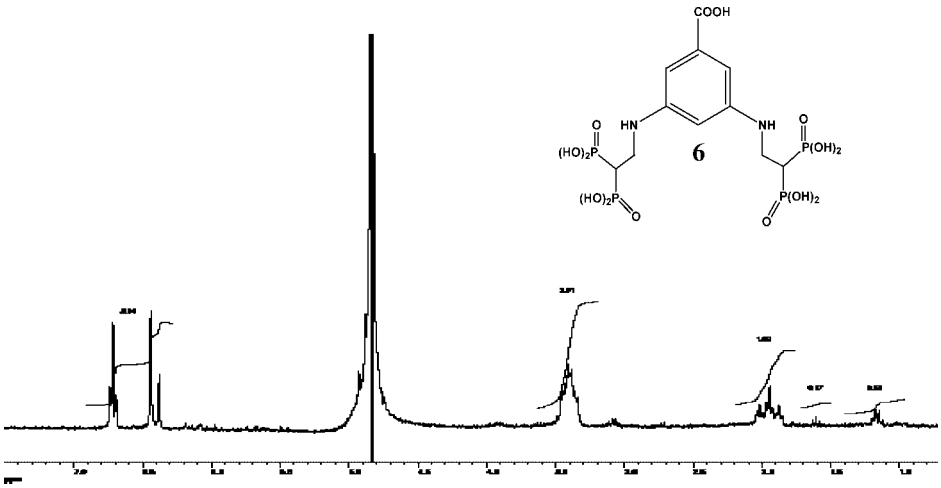


Figure 2. ^{1}H NMR spectrum of **6**.

Conjugation of 6 to Proteins and Resultant HA Affinity

Conjugation of **6** onto BSA and IgG was performed by using aqueous carbodiimide chemistry. In one set of studies, EDC/NHS concentration was kept constant at 1.25 mM, whereas **6** concentrations were varied between 0.25 to 1.25 mM, and in another set of studies, **6** concentration was kept constant at 1.25 mM, whereas EDC/NHS concentrations were varied between 1.25 to 10 mM. The results indicated that the conjugation efficiency was increased linearly for both BSA (Fig. 3A, ●) and IgG (Fig. 3A, ○) as the concentration of **6** was increased. The conjugation efficiency for both BSA (Fig. 3B, ■) and IgG (Fig. 3B, □) was also increased as the concentration of EDC/NHS was increased. The increase in conjugation efficiency was not linear between 3 and 10 mM EDC/NHS, possibly approaching maximum conjugation efficiency under these conditions. Further increase in EDC/NHS concentration was not attempted because of the possibility of obtaining protein–protein crosslinking (see SDS-PAGE results below). Maximal conjugation efficiency accomplished was 2.7 and 6.3 **6** per protein in case of BSA and IgG, respectively, at the highest EDC/NHS concentration. The control samples had no detectable **6** (<0.1/protein), consistent with a lack of association between **6** and the proteins in the absence of EDC/NHS linkers. As expected, the larger protein IgG, where more abundant $-NH_2$ groups (90 lysines/protein) were present, gave correspondingly higher conjugation efficiency compared with BSA (59 lysines/protein),[26] in both cases (Fig. 3A and B).

Gel electrophoresis was used to determine whether inadvertent protein–protein crosslinking occurred during the process of conjugation. Because the MW of BSA is 66 kDa, BSA–BSA crosslinking would have manifested itself as >66 kDa protein bands on the gels. For the ~150 kDa IgG, protein bands >150 kDa would have been indicative of IgG–IgG crosslinking. In both cases, a corresponding decrease in the intensity of the native protein band was expected to result when the proteins were loaded at equal amounts onto the gels. The results indicated that the control BSA and **6**-BSA conjugates migrated as a single band at the expected MW region (Fig. 4A). No other protein bands in the gel or at the loading level were seen, and the protein band at the 66 kDa level was equivalent in all samples, all indicative of lack of protein–protein crosslinking. A similar result was seen with IgG (Fig. 4B), except the **6**-IgG conjugate prepared at the highest EDC/NHS concentration (10 mM) had ~10% of the protein retained at the loading level. Because of the presence of this band, no higher concentrations of EDC/NHS were explored in this study to avoid any crosslinked proteins in the samples.

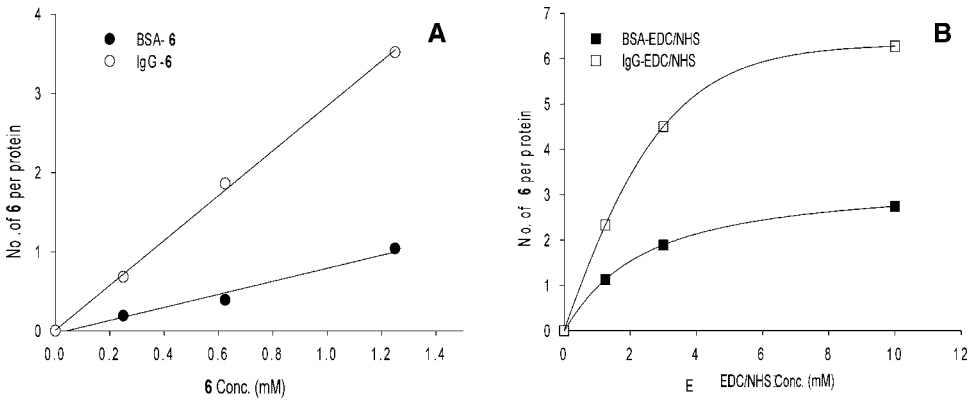


Figure 3. The dependence of conjugation efficiency on the reagent concentrations. (A) The EDC/NHS concentration was maintained at 1.25 mM and **6** concentration was varied between 1.25 and 0.25 mM. There was a linear relationship between the conjugation efficiency and the concentration of **6** for both BSA (●) and IgG (○). (B) The EDC/NHS concentration was increased from 1.25 to 10 mM whereas the **6** concentration was kept constant at 1.25 mM. An increase in conjugation efficiency was evident with increasing EDC/NHS concentration for both BSA (■) and IgG (□). The increase in conjugation efficiency, however, was not linear especially after 3 mM EDC/NHS, which might be indicative of reaching a plateau for the conjugation efficiency.

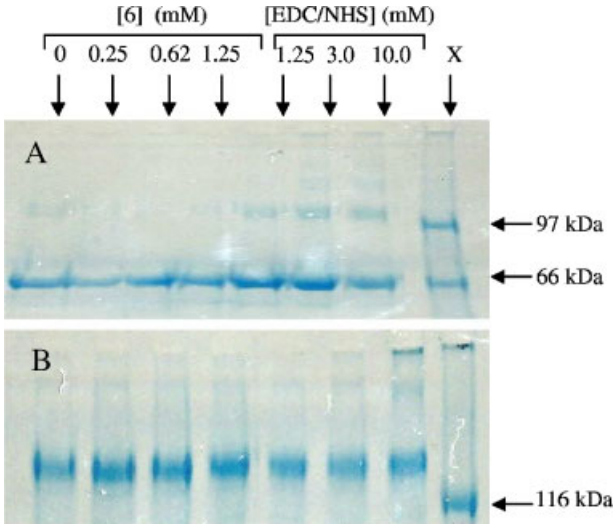


Figure 4. SDS-PAGE analysis of protein-**6** conjugates. For both BSA (A) and IgG (B), the resultant proteins migrated at the expected MW region as that of native (unmodified) proteins. Lane X indicates the lane in which known molecular weight markers were loaded. Only in the IgG sample with the highest concentration of EDC/NHS (B), a small fraction of protein did not enter the gel, indicating some level of protein–protein cross-linking. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

The control of conjugation efficiency obtained in this study was analogous to the control of conjugation efficiency from our previous approach, in which the conjugation efficiency was modulated depending on the BP and the SMCC linker concentrations in reaction medium.[12,15] The similarity between the two approaches was expected because the previous approach and the present carbodiimide chemistry both utilized the $-NH_2$ groups on the proteins for BP attachment. An advantage of the previous approach, however, was the fact that the NHS-activated reagents were commercially available, which eliminated the use of carbodiimides in the presence of proteins, and, hence, eliminated the possibility of obtaining protein–protein linking. It is possible to preactivate **6** with EDC/NHS and then to obtain the activated **6** ester suitable for protein coupling. Although this was attempted in our laboratory, we were not able to purify the NHS-ester of **6** because of the small amount of **6** available to us at this time (i.e., the sample was lost during the work-up). With larger quantities of **6** synthesized in the future, the activated NHS-ester of **6** is expected to be the appropriate reagent of choice for BP conjugation to proteins.

HA Affinity of 6-Protein Conjugates *In Vitro*

The HA binding of **6**-protein conjugates was studied at increasing phosphate buffer concentrations. This binding buffer was previously optimized to establish conditions in which the strength of protein binding is reproducibly manipulated.[12] All proteins, including the controls and conjugates, strongly bound to HA in 0 mM phosphate (Fig. 5). For proteins, in which the EDC/NHS concentration was maintained constant and **6** concentration was varied (Fig. 5A,B), the protein binding was gradually reduced as the concentration of phosphate in binding medium was increased. At the highest (100 mM) phosphate concentration, both BSA (A) and IgG (B) gave an increasing percent HA binding with the increasing **6** in the reaction medium. A similar binding profile was seen for samples in which the EDC/NHS concentration was varied, with conjugates at the highest EDC/NHS concentration giving the most robust binding at the highest (200 mM) phosphate concentration used in binding studies (Fig. 5C,D). The control proteins exhibited insignificant binding (<8%) at the highest phosphate concentrations.

To probe a correlation between conjugation efficiency and HA affinity, the conjugates' capacity to bind with HA in 100 mM was plotted as a function of conjugation efficiency (number of **6** per protein). The observed trend (Fig. 6) suggested that an increase in the number of conjugated **6** resulted in a linear increase in conjugates' binding capacity for HA. Both BSA and IgG gave a similar binding affinity per **6** conjugated, indicating that the size/nature of the protein was not a significant factor in imparting the HA affinity *in vitro*.

This excellent correlation between the conjugation efficiency and HA affinity was also noted in our previous conjugates obtain with SMCC-based conjugation,[12] as well as for the conjugates in which the BPs were attached to the carbohydrate moieties.[15] The correlation is directly indicative of the BPs being responsible for the imparted mineral affinity.

Bone Targeting

Bone targeting was assessed by using ^{125}I-radiolabeled proteins. Four proteins were selected for this study: (i) control BSA, (ii) BSA-**6** conjugate

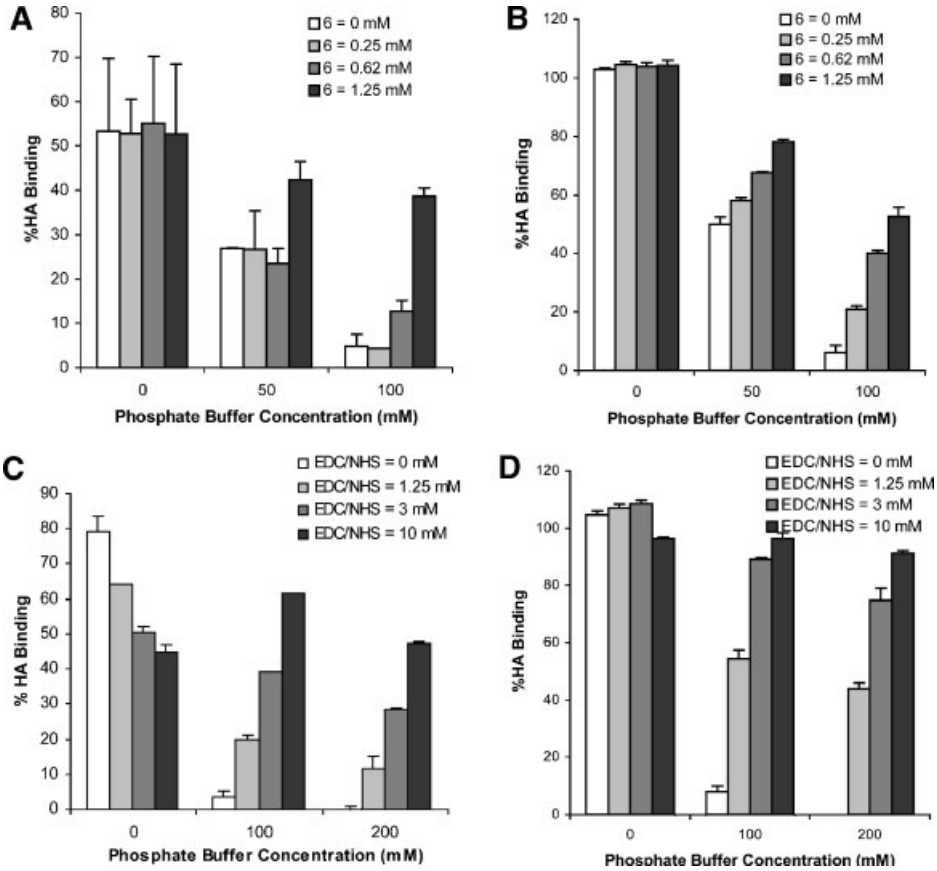


Figure 5. Binding of protein-**6** conjugates to HA in 0, 50, 100, and 200 mM phosphate buffer. The results are expressed as mean ± SD of duplicate measurements. (A) (BSA) and (B) (IgG) summarize the results in which the concentration of **6** was varied, whereas (C) (BSA) and (D) (IgG) summarize the results in which the concentration of EDC/NHS was varied. Note that all proteins bound strongly to HA in 0 mM phosphate buffer and percent HA binding generally decreased with the increasing phosphate buffer concentration in the medium. The HA affinity of protein-**6** conjugates increased with the increasing crosslinker (EDC/NHS) and **6** concentration as the buffer concentration was increased in the binding media.

with 2.7 **6** per BSA, (iii) control IgG, and (iv) IgG-**6** conjugate with 6.3 **6** per IgG. The labeled proteins were first tested for *in vitro* HA binding, to ensure that labeling procedure did not adversely affect the HA affinity of the conjugates. Binding was assessed in the presence of 50% serum to mimic the protein background found *in vivo*, which is expected to compete with the binding of conjugates to bone mineral. The ^{125}I-labeled protein-**6** conjugates exhibited strong affinity to HA (≈64% binding for both BSA and IgG, Fig. 7A) compared with control BSA (≈11%) and control IgG (≈2%). The labeling procedure clearly did not alter the ability of the conjugates to strongly bind to HA. The extent of protein binding for controls and **6**-conjugates observed in the presence of serum was comparable to the level of binding in the absence of serum (i.e., in phosphate buffer). These results also indicated the ability of **6**-conjugates to exhibit mineral affinity in the presence of a high concentration of proteins competing for mineral binding.

Bone targeting was assessed after intravenous injection of the ^{125}I-labeled proteins in normal rats (Fig. 7B). The organ distribution of the administered proteins was assessed after 2 days, an optimal timeframe based on previous studies in the authors' laboratories.[10] At the femurs, both BSA-**6** and IgG-**6** had significantly higher levels of accumulation of radioactivity compared with the control proteins (2.9- and 1.5-fold, respectively; $p < 0.001$ and $p < 0.01$, respectively; analysis of

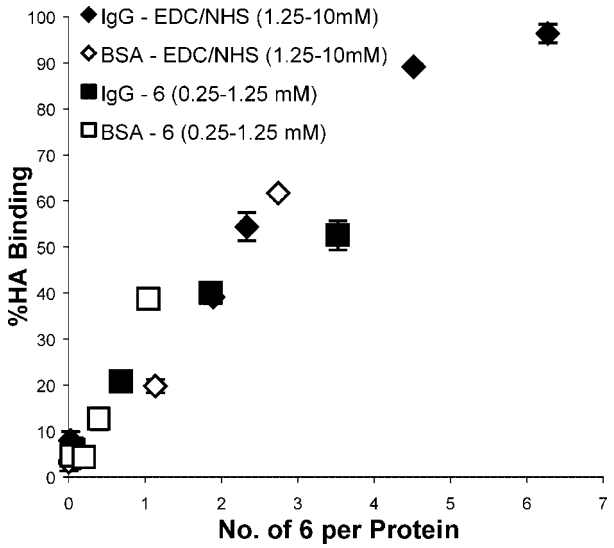


Figure 6. Correlation between the conjugation efficiency and percent HA binding (mean ± SD of duplicate measurements) in 100 mM phosphate buffer for the protein samples in Figure 3. Note the linear relationship between the number of **6** conjugated per protein and percent HA binding, especially for proteins with lower conjugation efficiencies.

variance with Tukey *post hoc* analysis). A similar result was obtained at the tibiae (3.7- and 2.6-fold for BSA-**6** and IgG-**6** conjugates, respectively; $p < 0.001$ for both proteins). The protein levels at nonskeletal tissues (kidneys, spleen, and liver) were significantly lower for the conjugates. Blood in particular had 8.6- to 10.1-fold decreased levels of the conjugates ($p < 0.01$ for IgG-**6** versus IgG, but $p > 0.05$ for BSA-**6** vs. BSA), and NH_4SO_4 precipitation of proteins in blood indicated relatively small amount of free label ($0.5 \pm 0.2\%$ vs. $6.7 \pm 0.9\%$ for IgG vs. IgG-**6**, respectively; $15.7 \pm 9.2\%$ vs. $18.9 \pm 9.1\%$ for BSA vs. BSA-**6**, respectively). Reduced distribution to extraskeletal sites was consistent with the increased delivery of protein-**6** conjugates to bone tissues.

Taken together, these results demonstrated that the **6**-conjugated proteins were delivered to bone tissues at higher levels than the unmodified proteins. It is noteworthy that this is the first study that a protein as large as IgG (≈150 kDa) was specifically targeted to bone. Our previous studies utilized smaller proteins (66.5 kDa BSA and 14.4 kDa lysozyme) and this study extends the size range of proteins that could be targeted to bone. The fact that an antibody was capable of being targeted to bone also points to the feasibility of using antibodies specific for pathological conditions (such as anti-RANK antibodies for the reduction of osteoclastic activity or anti-ILs for the reduction of inflammation) for therapeutic use in skeletal diseases. In addition, our previous studies utilized BSA with 11.0 aminoBP per protein for bone targeting,[14] unlike this study which utilized proteins with lower conjugation

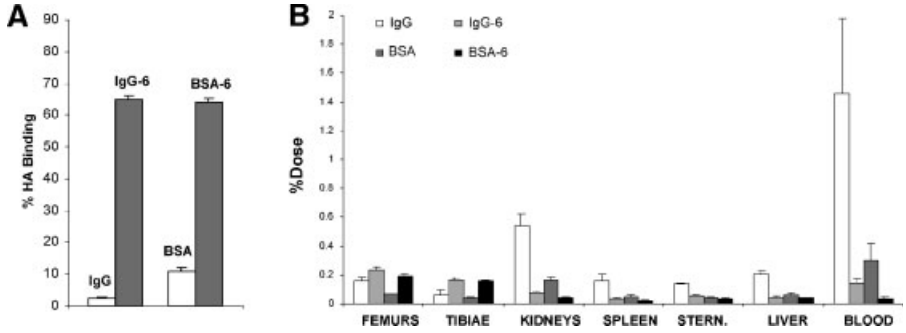


Figure 7. (A) percent HA binding (mean ± SD of duplicate measurements) of control and **6**-conjugated proteins *in vitro* after ^{125}I-labeling. Both IgG and BSA conjugates bound strongly to HA compared with the control samples. (B) Biodistribution of control (unmodified) and **6**-conjugated proteins in rats 2 days postinjection. At this time point, the rats were sacrificed and the indicated organs were excised. The radioactivity (counts per minute, cpm) associated in each organ was determined by a γ-counter and the results are expressed as %Dose in each organ (i.e., cpm in each organ divided by the total cpm injected). In the case of femurs, tibiae, and kidneys, both bilateral organs were excised and the results were averaged per organ. In the case of liver and blood, the measured cpm was normalized with the weight (g) of the liver tissue excised or the weight of the blood sample collected. Note the increase in %Dose in femurs and tibiae for both BSA-**6** and IgG-**6** compared with unmodified BSA and IgG (see text for statistical analysis). Nonskeletal organs gave reduced conjugate levels compared with control proteins.

efficiency (2.7 per protein). Our unpublished results indicated that BSA with ≈ 6 aminoBPs per protein was not targeted to bone [BSA delivery to tibia and femur: $0.10 \pm 0.02\%$ and $0.15 \pm 0.06\%$ ($n = 3$), respectively; BSA-BP delivery to tibia and femur: $0.02 \pm 0.01\%$ and $0.02 \pm 0.01\%$ ($n = 3$), respectively]. In this regard, the newly synthesized di(bisphosphonic acid) seems beneficial over the previously used conjugation approaches by enabling successful protein targeting to bone with less protein modification.

We are currently pursuing more in-depth studies probing further correlation(s) between conjugate properties (protein size, overall charge, and the conjugation efficiency) and bone targeting. Based on our previous structure-property studies on BP-protein conjugates,[12–16] the BP prepared for this study (**6**) is expected to be beneficial in two aspects: (i) because of its ability to load a higher number of BPs per protein attachment site (2 vs. 1), and (ii) because of the introduction of a minimal tether length to link the BPs to the protein. It will be important to probe both of these aspects of the new BP so as to elucidate the relative contributions of each aspect. We also noted that the *in vitro* binding for the conjugates, especially in high phosphate and serum-containing media, was exceedingly high compared with the unmodified proteins (>10-fold difference at times). In rats, however, the difference in bone targeting was not as much: 1.5- to 3.7-fold differences at the 2-day assessment point. Our previous experience[14] indicated that the differences between the unmodified proteins and the BP-conjugates usually became more significant at later time points (>4 days), because of faster elimination of unmodified proteins from the bone sites. It will be important to assess bone targeting at longer postinjection times to determine whether the targeting efficiency is increased for the **6**-conjugates.

CONCLUSIONS

In conclusion, a COOH-containing di(bisphosphonic acid) (**6**) was synthesized and conjugated to model proteins. The conjugation efficiency was controlled by the reagent concentrations in the reaction medium. The conjugates exhibited an *in vitro* HA affinity that was proportional to the extent of **6** conjugated on a protein. The conjugates were bone-seeking, based on the increased concentration of **6**-conjugated proteins in bone tissue after systemic administration in rats. This is the first report on the feasibility of targeting proteins as large as antibodies (≈ 150 kDa), which is expected to facilitate the evaluation of antibody-based therapeutic agents for bone diseases. The synthesized di(bisphosphonic acid) allows attachment of multiple copies of BPs per protein site, in addition to giving a minimal distance between the protein attachment site and the BP moieties. Further studies are underway to prepare dendritic BPs in which a controlled number of BPs can be conjugated onto a single protein site.

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REFERENCES

1. Puleo D. 2003. Biotherapeutics in orthopaedic medicine: Accelerating the healing process? BioDrugs 17:301–314.
2. Gittens SA, Uludag H. 2001. Growth factor delivery for bone tissue engineering. J Drug Target 6:407–429.
3. Whalen GF, Shing T, Folkman J. 1989. The fate of intravenously administered bFGF and the effect of heparin. Growth Factors 1:157–164.
4. Cueves P, Fernandez-Ayerdi A, Carceller F, Colins S, Mascarelli F, Munoz-Willery I, Gimenez-Gallego G. 1996. CNS distribution of fibroblast growth factor injected into the blood stream. Neurol Res 18:267–272.
5. Cueves P, Carceller F, Ortega S, Zaro M, Nioto I, Gimenez-Gallego G. 1991. Hypotensive activity of fibroblast growth factor. Science 254:1208–1210.
6. Mazue G, Newman AJ, Scampini G, Della Torre P, Hard GC, Iatropoulos MJ, Williams GM, Bagnasco SM. 1993. Histopathology of kidney changes in rats and monkeys following intravenous injection of massive doses of FCE26184, human basic fibroblast growth factor. Toxicol Pathol 21:490–501.
7. Porras AG, Holland SD, Gertz BJ. 1999. Pharmacokinetics of alendronate. Clin Pharmacokinet 36: 315–328.
8. Blake GM, Park-Holohan SJ, Cook GJ, Fogelman I. 2001. Quantitative studies of bone with the use

of 18F-fluoride and 99m Tc-methylene diphosphonate. Semin Nucl Med 31:28–49.
9. De Ligny CL, Gelsema WJ, Tji TG, Huigen YM, Vink HA. 1990. Bone seeking radiopharmaceuticals. Nucl Med Biol 17:161–179.
10. Uludag H. 2002. Bisphosphonates as a foundation of drug delivery to bone. Curr Pharm Des 8:1929–1944.
11. Bentz H, Rosen D. 1992. Targeted delivery of bone growth factors. EP 0512 844A1.
12. Uludag H, Kousinioris N, Gao T, Kantoci D. 2000. Bisphosphonate conjugation to proteins as a means to impart bone affinity. Biotechnol Prog 16:258–267.
13. Uludag H, Gao T, Wohl GR, Kantoci D, Zernicke RF. 2000. Bone affinity of a bisphosphonate-conjugated protein *in vivo*. Biotechnol Prog 16:1115–1118.
14. Uludag H, Yang J. 2002. Targeting systemically administered proteins to bone by bisphosphonate conjugation. Biotechnol Prog 18:604–611.
15. Gittens SA, Matyas JR, Zernicke RF, Uludag H. 2003. Imparting bone affinity to glycoproteins through the conjugation of bisphosphonates. Pharm Res 20:978–987.
16. Gittens SA, Kitov PI, Matyas JR, Löbenberg R, Uludag H. 2004. Impact of tether length on bone mineral affinity of protein-bisphosphonate conjugates. Pharm Res 21:608–616.
17. Degenhardt CR, Burdsall DC. 1986. Synthesis of ethenylidenebis(phosphonic acid) and its tertaalkyl esters. J Org Chem 51:3488–3490.
18. Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.
19. Ames BN. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8: 115–117.
20. Bernardi G. 1971. Chromatography of nucleic acids on hydroxyapatite columns. Methods Enzymol 31: 96–97.
21. Rajendran JG, Eary JF, Bensinger W, Durack LD, Vernon C, Fritzberg A. 2002. High-dose 166Ho-DOTMP in myeloablative treatment of multiple myeloma: Pharmacokinetics, biodistribution, and absorbed dose estimation. J Nucl Med 43:1383–1390.
22. Zeevaart JR, Jarvis NV, Louw WKA, Jackson GE. 2001. Metal-ion speciation in blood plasma incorporating the tetraphosphonate, *N*,*N*-dimethylenephosphonate-1-hydroxy-4-aminopropily-denediphosphonate (APDDMP), in therapeutic radiopharmaceuticals. J Inorg Biochem 83:57–65.
23. Banerjee S, Samuel G, Kothari K, Unni PR, Sarma HD, Pillai MR. 2001. Tc-99m and Re-186 complexes of tetraphosphonate ligands and their distribution pattern in animal models. Nucl Med Biol 28:205–213.
24. Winckler W, Pieper T, Keppler BK. 1996. Preparation of octaethyl-3-amino-pentane-1,1,5,5-tetrakisphosphonate by catalytic hydrogenation of the corresponding 3-nitro-compound. Phosphorus Sulfur Silicon Relat Elem 112:137–141.
25. Van Gelder JM, Breuer E, Schlossman A, Ornoy A, Monkkonen J, Simila J, Klenner T, Stadler H, Krempien B, Patlas N, Golomb G. 1997. *In vitro* and *in vivo* effects of tetrakisphosphonates on bone resorption, tumor osteolysis, ectopic calcification, and macrophages. J Pharm Sci 86:283–289.
26. Chen JP, Yang HJ, Hoffman AS. 1990. Polymer-protein conjugates. I. Effect of protein conjugation on the cloud point of poly(*N*-isopropylacrylamide). Biomaterials 11:625–630.