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Imparting Mineral Affinity to Fetuin by Bisphosphonate Conjugation: A Comparison of Three Bisphosphonate Conjugation Schemes

Sébastien A. Gittens,[†,‡] Geeti Bansal,[§] Cezary Kucharski,[§] Mark Borden,[||] and Hasan Uludağ*[,†,‡,§]

Department of Chemical & Materials Engineering, Faculty of Engineering, Department of Biomedical Engineering, Faculty of Medicine & Dentistry, and Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada, and Interpore Cross International, Irvine, California

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Abstract: Protein conjugation to bisphosphonic acids (BPs), such as 1-amino-1,1-diphosphonate methane (aminoBP) and 3,5-di(ethylamino-2,2-bisphosphono)benzoic acid (diBP), was proposed as a foundation for bone-specific delivery of protein therapeutics. This study was performed to directly compare the mineral affinity of protein−BP conjugates prepared by three different approaches. Fetuin, serving as a model protein, was derivatized with BPs by the following approaches: (i) by attaching the aminoBPs onto protein lysines using succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC); (ii) by attaching the aminoBPs onto protein carbohydrates using 4-(maleimidomethyl)-cyclohexane-1-carboxyl-hydrazide (MMCCH), and (iii) by conjugating diBP to protein lysines using the carbodiimide chemistry. The results indicated that conjugation of aminoBP and diBP to fetuin by all three means unequivocally enhanced the protein's affinity for hydroxyapatite in vitro. Similarly, conjugation of aminoBP and diBP onto fetuin increased the protein's retention in a mineral-containing matrix (Pro-Osteon) when the proteins were implanted in a rat subcutaneous model. Upon parenteral administration, however, no discernible differences were found between the SMCC- or MMCCH-linked conjugates and unmodified fetuin to target to bony tissues. DiBP−fetuin conjugates, however, led to successful bone targeting after intravenous injection in rats. We conclude that all three conjugation schemes were equally effective in imparting an affinity to the proteins toward mineral-containing matrices. Bone targeting, however, was achieved only with diBP conjugation to fetuin, supportive of the superior ability of this BP with a higher density of bisphosphonic acid groups.

Keywords: Bone targeting; mineral affinity; bisphosphonate; protein conjugation; implant binding

Introduction

The need to induce the regeneration of bone, be it locally or systemically, is a challenge that physicians currently face. Numerous growth factors have been identified that can elicit de novo bone deposition and repair upon their parenteral administration.[1] Due to the detrimental effects induced by growth factors at extraskeletal sites, however, a means of targeting these proteins to bone is essential so as to circumvent these effects while concurrently taking advantage of their innate osteogenic properties. Bone targeting can be achieved if proteins exhibit a strong affinity to the bone mineral hydroxyapatite (HA). By virtue of their high affinity to HA, the conjugation of bisphosphonates (BPs) onto such growth factors was proposed as a feasible means of increasing the affinity of the proteins for bone.[2] The conjugation

* Corresponding author. Mailing address: Department of Chemical & Materials Engineering, #526 Chemical & Materials Engineering Building, University of Alberta, Edmonton, AB, Canada, T6G 2G6. Tel: (780) 492-0988. Fax: (780) 492-2881. E-mail: hasan.uludag@ualberta.ca.

† Department of Biomedical Engineering, Faculty of Medicine & Dentistry, University of Alberta.

‡ Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta.

§ Department of Chemical & Materials Engineering, Faculty of Engineering, University of Alberta.

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of 1-amino-1,1-diphosphonate methane (aminoBP) directly onto a protein's lysine residues using succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) successfully enhanced the bone mineral affinity of several model proteins in vitro, including bovine serum albumin (BSA),[3,4] lysozyme,[4] and fetuin.[5,6] Moreover, aminoBP conjugation has been shown to enhance BSA and lysozyme targeting to bone by up to ∼7-fold upon intravenous and subcutaneous administration.[4]

As a method to circumvent any potential loss of protein bioactivity elicited by the direct chemical modification of proteins' lysine amino acid residues, a second conjugation scheme was designed to conjugate aminoBPs onto protein carbohydrate moieties using 4-(maleimidomethyl)-cyclohexane-1-carboxyl-hydrazide (MMCCH).[5] This approach takes advantage of the fact that the carbohydrate moieties of several osteogenic growth factors, such as bone morphogenetic protein-2, do not play an integral role in their osteogenicity, as evidenced through the bioactivity of the prokaryotically expressed, nonglycoslyated forms of these proteins.[7] The aminoBP−fetuin conjugates prepared using the carbohydrate-attached conjugates appeared to exhibit a higher affinity in vitro than the lysine-attached conjugates.[5] To better understand the influence of aminoBP−fetuin characteristics, a subsequent study investigated the effect of tether length on conjugate bone mineral affinity. The aminoBP−fetuin conjugates prepared via shorter tethers resulted in a superior affinity for mineralized matrices in vitro as compared to conjugates prepared using longer tethers.[6] A higher BP density at the vicinity of the proteins was suggested to enhance the mineral affinity of the conjugates. To minimize tether lengths and to enhance the BP density, we recently synthesized a carboxyl-containing di(bisphosphonic acid) and conjugated it to BSA by using the carbodiimide chemistry.[8] The latter approach results in conjugation of BPs to $-NH_2$ groups of protein lysines and gives a zero tether length in final conjugates. The BSA−BP conjugates prepared with the di(bisphosphonic acid) were targeted to bone with a reduced number of conjugated BPs per protein, as compared to the conjugates prepared by aminoBP conjugation to lysines.[8]

This study was performed to directly compare the mineral affinity of protein−BP conjugates prepared by these three approaches: (i) by attaching the aminoBPs onto protein lysines using SMCC, (ii) by attaching the aminoBPs onto protein carbohydrates using MMCCH, and (iii) by conjugating di(bisphosphonic acid)s to proteins. All three approaches were employed by using fetuin as a model protein. Fetuin is an endogenous protein that participates in physiological formation of calcium/phosphate minerals. As such, it controls mineralization process at bone tissues, as well as undesired mineralization at other sites.[5,6] Since previous studies utilized different proteins with different conjugation schemes, it was not possible to directly evaluate the relative effectiveness of various strategies in imparting the desired mineral affinity. The SMCC- and MMCCH-mediated conjugations of aminoBPs to fetuin were previously described,[5,6] and we adopted the di(bisphosphonic acid) conjugation conditions developed for BSA[8] to modification of fetuin in this study. After assessment of in vitro binding to mineral-containing matrices, mineral affinity of the conjugates was assessed in vivo in a subcutaneous implantation model in rats. The proteins were also systemically administered to assess bone targeting of fetuin. Our results indicated significant differences in bone targeting ability of the conjugates depending on the scheme used to construct the BP conjugates.

Materials and Methods

Materials. Bovine fetuin (Lot No. 59H7616), 2-iminothiolane (2-IT), bovine adult serum, trichloroacetic acid (TCA), 2-(*N*-morpholino)ethanesulfonic acid (MES), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (TCDG), and 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester (Bolton−Hunter reagent) were obtained from SIGMA (St. Louis, MO). The SMCC and MMCCH were obtained from Molecular Biosciences (Boulder, CO). Precast 4−20% LongLife polyacrylamide gels were from Gradipore (Frenchs Forest, NSW, Australia). The $Na^{125}I$ (in 0.1 M NaOH) was obtained from Perkin-Elmer (Wellesley, MA). NaCl (0.9%) was from Baxter Corporation (Toronto, ON, Canada). The dimethyl sulfoxide (DMSO) and *N*,*N*-dimethylformamide (DMF) were from Caledon Laboratories (Georgetown, ON, Canada). The Spectra/Por dialysis tubing with MW cutoff of 12−14 kDa was acquired from Spectrum Laboratories (Rancho Dominguez, CA). The preparation of HA, 1-amino-1,1-diphosphonate methane, 0.1 M phosphate buffer (pH 7.4), 0.1 M carbonate buffer (pH 10), 0.1 M acetate buffer (pH 4.5), and SDS−PAGE running buffer were previously described.[3,5] The 3,5-di(ethylamino-2,2-bisphosphono)benzoic acid was synthesized as described in ref 8. Metofane (methoxyflurane), an inhalational anesthetic, was obtained from Janssen Inc. (Toronto, ON, Canada). The Pro-

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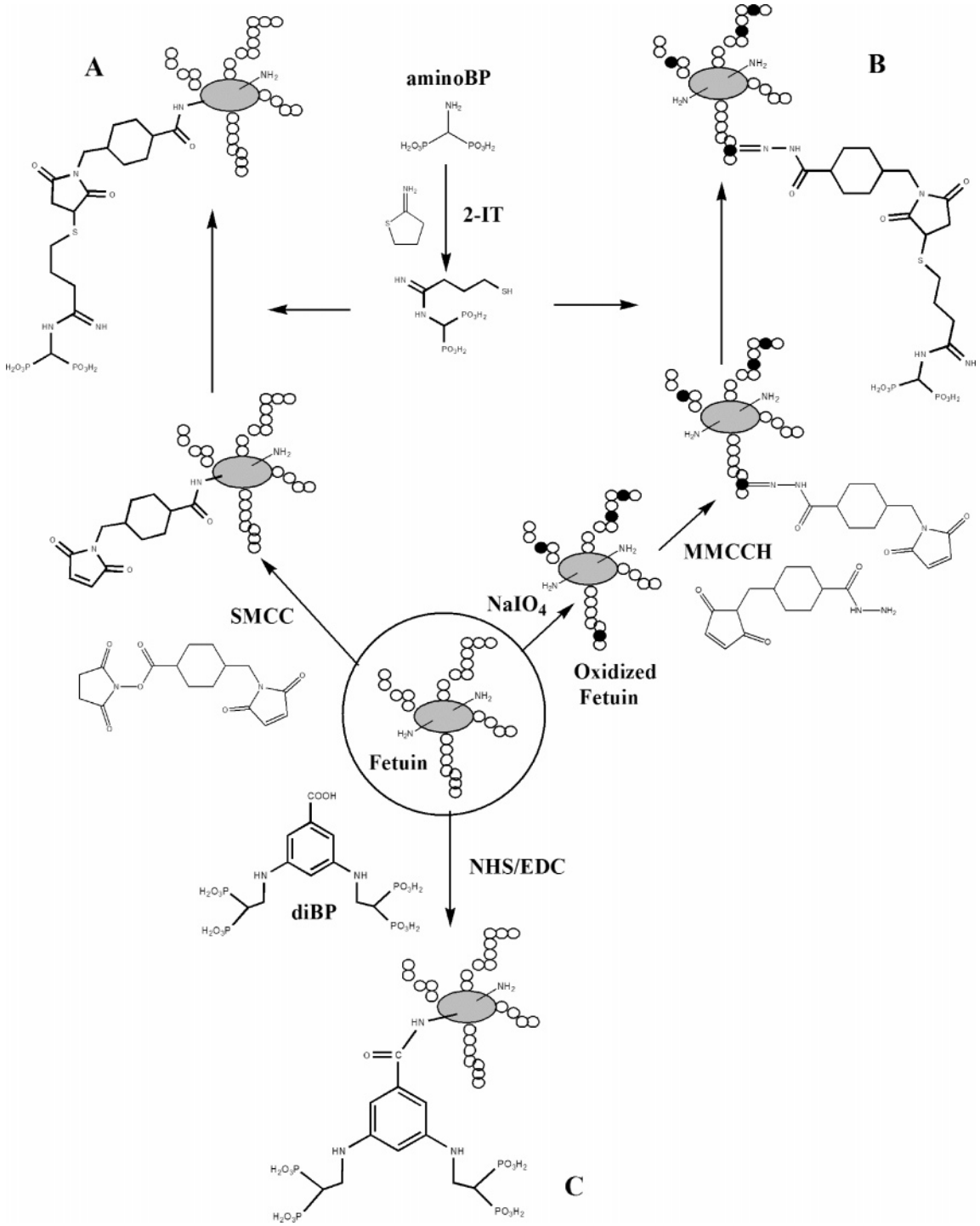

Figure 1. Conjugation schemes used in this study. (A) SMCC-mediated conjugation of aminoBP to fetuin via the protein's $-NH_2$ groups. (B) MMCCH-mediated conjugation of aminoBP to fetuin via the carbohydrate groups. (C) EDC/NHS-mediated conjugation of diBP to fetuin via the protein's $-NH_2$ groups. SMCC- and MMCCH-mediated conjugations rely on thiolation of the aminoBP with 2-IT. In scheme B, fetuin is first oxidized with $NaIO_4$ to introduce MMCCH reactive aldehyde groups onto the fetuin.

Osteon 200HA implants, which are coralline hydroxyapatite discs with three-dimensional microarchitecture similar to that of cortical bone,[9] were kindly donated by Interpore Cross International (Irvine, CA). Presterilized Helistat absorbable collagen sponges were from Integra Life Sciences (Plainsboro, NJ).

AminoBP Conjugation onto Fetuin (Figure 1). AminoBP was conjugated to fetuin through either protein lysine or carbohydrate groups at room temperature. To conjugate aminoBP to the lysine groups by using SMCC, fetuin (15 mg/mL in 0.1 M phosphate buffer) was incubated for 2.5 h with 10 mM SMCC (dissolved as 0.15 M in DMF).

Separately, aminoBP was thiolated by incubating equal volumes of aminoBP (80 mM in 0.1 M phosphate buffer) and 2-IT solutions (40 mM in 0.1 M phosphate buffer) for 2.5 h. The product from this reaction was then directly added to the SMCC-reacted fetuin in equal volumes for 1.5 h. The removal of unreacted reagents (i.e., SMCC, aminoBP, and thiolated aminoBP) was through extensive dialysis against 0.1 M carbonate buffer (×3) and dH_2O (×2). Unmodified fetuin served as a control for the SMCC conjugates.

To conjugate aminoBP to the carbohydrate groups by using MMCCH, the adjacent hydroxyl groups on carbohydrate moieties were first oxidized through the use of 4 mM $NaIO_4$ in 0.1 M acetate buffer. Following a 2.5 h incubation period, the samples were extensively dialyzed against dH_2O and 0.1 M acetate buffer. The oxidized fetuin was incubated with 10 mM MMCCH (dissolved as 0.15 M in DMF) for 2.5 h, and thiolated aminoBP, which had been prepared as described above, was added to the MMCCH-reacted fetuin at equal volumes. This reaction was allowed to proceed for 1.5 h at room temperature. The unreacted reagents, MMCCH, aminoBP, and thiolated aminoBP, were removed through extensive dialysis against 0.1 M carbonate buffer (×3) and dH_2O (×2). Oxidized fetuin was used as a control for the MMCCH conjugates.

3,5-Di(ethylamino-2,2-bisphosphono)benzoic Acid Conjugation to Fetuin (Figure 1). The 3,5-di(ethylamino-2,2-bisphosphono)benzoic acid (hereon referred to as diBP) was dissolved in 0.1 MES buffer (pH ∼4.5) and 250 μL of this 5 mM solution was mixed with 250 μL of 80 mM/80 mM EDC/NHS (1:1) solution for 45 min to activate the −COOH group of the diBP. The activated diBP was then incubated with an equal volume of fetuin solution (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) and dH_2O) (×2). Unmodified fetuin was used as a control for the diBP conjugates. In some experiments, the EDC/NHS concentrations were varied to investigate the influence of cross-linker concentration on the extent of diBP conjugation to fetuin.

Conjugation Efficiency. Protein concentrations were determined by using the Bradford Assay.[10] A 50 μL sample was added to 1 mL of protein reagent (0.01% (w/v) Coomassie Blue R-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid), and the absorbance was determined at 595 nm. A calibration curve based on the known concentrations of fetuin samples was used to obtain the protein concentration in the analyzed sample. A phosphate assay[11] was used to determine BP concentration of the samples, as described in ref 3 for aminoBP and ref 8 for diBP. A calibration curve based on the known concentrations of the respective BP samples was used to obtain BP concentrations in the analyzed samples. The BP concentration in combination with the protein concentration of a sample is used to calculate the number of BPs conjugated onto fetuin as a mole:mole ratio.

Assessment of Mineral Affinity in Vitro. The mineral affinity of the proteins was assessed in vitro by determining protein binding to synthetic HA, and to the commercially available implants, Pro-Osteon and Helistat. ^{125}I-Labeled proteins were used to quantitate the mineral binding.

(a) Preparation of Radioiodinated Proteins. The proteins were labeled by two different techniques. For TCDG-mediated labeling, which labels the tyrosine and histidine residues, microcentrifuge tubes were first coated with TCDG according to the manufacturer's suggestion, and 100 μg of protein was added to the tubes, along with 50 μL of 0.1 M phosphate buffer (pH 7.4) and 20 μL of 0.01 mCi $Na^{125}I$ (in 0.1 M NaOH). The samples were incubated for 20 min and dialyzed against 0.05 M phosphate buffer (3×). Conversely, the Bolton−Hunter technique was used to label the lysine residues. For this, microcentrifuge tubes were coated with TCDG (as above), and 50 μL of 50 mg/mL Bolton−Hunter reagent (in DMSO) was added to 50 μL of 0.1 M phosphate buffer and 20 μL of 0.01 mCi $Na^{125}I$ (in 0.1 M NaOH). After 2 min, 100 μL of DMF and 200 μL of benzene were added to the tubes to extract the labeled Bolton−Hunter reagent into an organic phase, which was isolated and dried under a stream of air. Protein solution (100 μg), diluted in ice-cold 0.1 M borate buffer (pH 8.5), was added to the tubes containing the labeled Bolton−Hunter reagent. The reaction was allowed to continue for 2 h on ice. Unreacted reagents (i.e., free ^{125}I or ^{125}I-Bolton−Hunter reagent) were separated from the radiolabeled protein via dialysis against 0.05 M phosphate buffer. Precipitating an aliquot of the samples with 20% TCA confirmed that iodinated samples contained less than 5% free ^{125}I.

To further ensure the quality of the radiolabeled proteins, 10 μg aliquots of non-radiolabeled and 10^6 cpm of radiolabeled samples were separately mixed with an SDS−glycine sample buffer, loaded onto a 4−20% Tris-HCl polyacrylamide gel and electrophoresed (150 V/1.5 h). Two halves of the gel containing the hot and the cold proteins were separated. The cold samples were stained overnight using Coomassie Blue R-250 (0.1% w/v Coomassie Blue R-250 in 10:10:80% methanol:acetic acid:dH_2O), destained with 40:10:50% methanol:acetic acid:dH_2O, and scanned on a flatbed scanner. The gel containing the hot proteins was exposed to X-ray film (XOMAT, Kodak) for 30 min, and the film was subsequently scanned on a flatbed scanner.

(b) In Vitro Binding to HA and Implants. Radiolabeled proteins were added to cold proteins in microcentrifuge tubes to give ∼10^6 cpm in 175 μL of saline and 0.1 mg/mL total

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Table 1. Summary of Bone Targeting Studies[a]

study no.	group no.	no. of rats per group	proteins injected	radiolabeling technique	sacrifice time (h)
1	1	3	fetuin	TCDG	24
	2	3	aminoBP−fetuin (SMCC)	TCDG	24
	3	3	oxidized fetuin	TCDG	24
	4	3	aminoBP−fetuin (MMCCH)	TCDG	24
2	1	6	fetuin	TCDG	6 and 48
	2	6	aminoBP−fetuin (SMCC)	TCDG	6 and 48
3	1	6	fetuin	Bolton−Hunter	72 and 144
	2	6	aminoBP−fetuin (MMCCH)	Bolton−Hunter	72 and 144
4	1	6	fetuin	TCDG	72 and 144
	2	6	diBP−fetuin	TCDG	72 and 144

[a] All injections were performed via the intravenous route (tail vein injections). Each study group corresponds to the injection of a different protein. Three rats from each study group were sacrificed at the indicated time points.

protein concentration (hot:cold ratio of ∼1:1000). Bovine adult serum (175 μL) and 5 mg of HA were then added to the microcentrifuge tubes. After shaking for 3 h, the samples were centrifuged. The supernatant was collected, and the pellet, consisting of HA, was washed with 50% bovine adult serum and recentrifuged. This washing procedure was repeated twice, and the collected supernatant from each wash was counted by a γ-counter (Wallac Wizard 1470, Turku, Finland). HA affinity, expressed as percent HA binding, was calculated as follows: 100% × {counts in HA pellet} ÷ {(counts in HA pellet) + (total counts in supernatants)}.

Protein binding to two types of implants, Pro-Osteon and Helistat, was also determined in vitro. A 50 μL aliquot of labeled protein solution, containing 2.5×10^5 cpm of proteins and 0.1 mg/mL cold protein, was applied to Pro-Osteon disks (10 mm diameter × 4 mm thickness; $n = 3$) and Helistat sponges (1 cm × 1 cm × 3 mm thickness; $n = 3$). Following a 10 min incubation, the implants were washed thoroughly (5×) with 50% bovine adult serum in saline. The counts in the supernatant of each wash, along with the matrix-bound counts, were quantified separately. The percent implant binding was calculated using the "HA affinity" formula described above.

Assessment of Mineral Affinity in Vivo. Two month old female Sprague−Dawley rats were purchased from Charles River Laboratories (Quebec City, QC, Canada). Rats were acclimated until 3−4 months of age under standard laboratory conditions (23 °C, 12 h of light/day). While maintained in pairs, rats were provided commercial chow and tap water ad libitum. All procedures involving rats were approved by the Animal Welfare Committee at the University of Alberta (Edmonton, AB, Canada).

The Pro-Osteon disks and Helistat sponges were soaked with ^{125}I-labeled proteins as described above. The initial counts in implants were determined before implantation. Once rats were anesthetized with inhalational Metofane, two implants were placed subcutaneously into bilateral ventral pouches. Each rat received the same type of implant/protein combination, and the skin incisions were closed with sterile wound clips. Each group contained either 2 rats (aminoBP conjugates) or 3 rats (diBP conjugates), giving $n = 4$ and $n = 6$ implants per group, respectively. At indicated time points, the rats were sacrificed by CO_2 asphyxiation, and the implants and thyroids were harvested from each animal. The counts associated with the thyroid and excised implants was quantified using a γ-counter. The protein retention in the implants, expressed as a percentage of the initial dose, was calculated as follows: 100% × {(final counts in implant) ÷ (initial counts in implant)}.

Assessment of Bone Targeting. Bone targeting was assessed in a series of studies outlined in Table 1. Study 1 evaluated bone targeting of aminoBP−fetuin conjugates prepared with SMCC and MMCCH linkers following 24 h postinjection. Study 2 evaluated bone targeting by SMCC-linked aminoBP−fetuin conjugates at earlier (6 h) and later (48 h) time points. Study 3 evaluated the effect of labeling technique on bone targeting of MMCCH-linked aminoBP−fetuin conjugates. Finally, study 4 evaluated bone targeting of diBP−fetuin conjugates prepared with the EDC/NHS linkers.

In all studies, 300 μL of radiolabeled protein solution (containing $\sim 10^6$ cpm of hot protein, and 0.1 mg/mL cold protein diluted in 0.9% saline) was administered intravenously by tail vein into each rat. To accurately assess the total dose administered, the counts associated with 300 μL aliquots of protein solutions were assessed in duplicate prior to injection. At indicated time points, all animals were sacrificed via CO_2 asphyxiation and a sample of blood was obtained via cardiac puncture and weighed. The bilateral femora, bilateral tibiae, bilateral kidneys, spleen, sternum, and a section of liver, which was weighed, were harvested. The counts associated with each harvested tissue were determined separately. All values were normalized with the injected dose and expressed as mean ± SD of % injected dose ($n = 3$ for spleen, sternum, and liver; and $n = 6$ for femora, tibiae, and kidneys). The liver samples were normalized by dividing the counts by the weight of excised liver. Consequently, the reported values for liver are in % injected dose/g of tissue. The volume of the blood counted was calculated by dividing the weight of the sample by the density of rat blood (1.05 g/mL[12]), and the blood counts were divided by the volume of blood obtained, to obtain % dose/mL.

To determine the TCA-precipitable counts in serum, a ~500 μL aliquot from each blood sample was centrifuged for 5 min to separate the serum from the red blood cells (RBCs). One milliliter of 20% TCA was then added to the isolated serum fractions. Once recentrifuged, the supernatant, which represented the TCA-soluble fraction, was isolated from the pellet. The radioactive counts in the supernatant and pellet, along with the RBCs, were determined separately using a γ-counter. The percentage of radioactivity in the serum was determined by using the following formula: 100% × {(counts in the supernatant)/(counts in the supernatant + counts in the pellet + counts in RBCs)}. The percentage of TCA-soluble radioactivity in the serum was determined by using the following formula: 100 × {(counts in supernatant)/(counts in the supernatant + counts in the pellet)}.

In some studies, the amount of radioactivity in urine was determined by placing the animals in metabolic cages immediately following protein administration. The urine collected from each animal at the sacrifice times was weighed, and the counts in each sample were determined. The percentage of TCA-soluble radioactivity in the urine was determined by taking a ~300 μL aliquot of urine and adding it to 200 μL of bovine adult serum (as bulking agent) and 1 mL of 20% TCA. After centrifugation, the counts within the pellet and supernatant were quantified separately and the percentage of TCA-precipitable counts was calculated as above.

Statistics. Differences in protein binding, retention, and targeting between proteins (in the in vitro and in vivo matrix studies, as well as study 1) were assessed using Tukey post hoc comparison ($p < 0.05$, S-PLUS 2000 Professional, release 2, Insightful Corp., Seattle, WA). Comparisons between fetuin and either the SMCC-linked aminoBP−fetuin conjugate group (study 2), the MMCCH-linked aminoBP−fetuin conjugate group (study 3), or the diBP−fetuin conjugate group (study 4) were determined using Student's *t* test ($p < 0.05$, two-sided).

Results

The aminoBP conjugates of fetuin were readily obtained by using SMCC and MMCCH linkers, as described in Figure 1. Previous publications from this lab reported typical conjugation efficiencies for the SMCC and MMCCH chemistries, where 1−16 aminoBPs/fetuin were obtained depending on the reagent concentrations in the reaction medium (for example, see Figures 2 and 4 in ref 4). Conjugation of diBP to fetuin was also controlled by the EDC/NHS concentration in the reaction medium, but the highest conjugation efficiency obtained was ~1.2 diBPs/fetuin (Figure 2). Unlike our expectation, using excess EDC/NHS did not yield any higher conjugation efficiency in 3 independent reactions (not shown). We, therefore, proceeded by using diBP−fetuin conjugates with only 1.2 diBPs/fetuin, since this level of conjugation was found sufficient for a significant mineral affinity (see below).

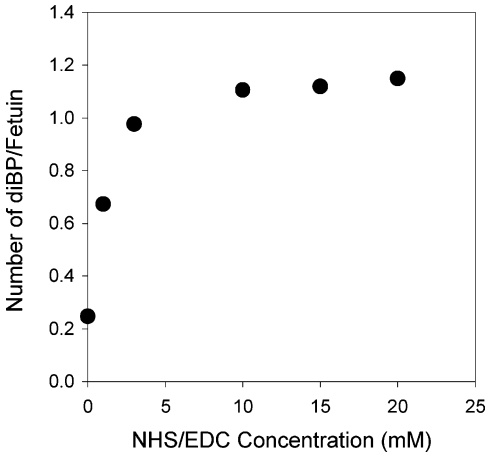

Figure 2. Conjugation efficiency (i.e., number of diBPs per fetuin) as a function of EDC/NHS concentration in the reaction medium. The protein and diBP concentrations in the reaction were maintained at 20 mg/mL and 1.25 mM, respectively. The EDC and NHS concentrations were equal. A gradual increase in conjugation efficiency was noted until ~1.2 diBPs/fetuin was obtained.

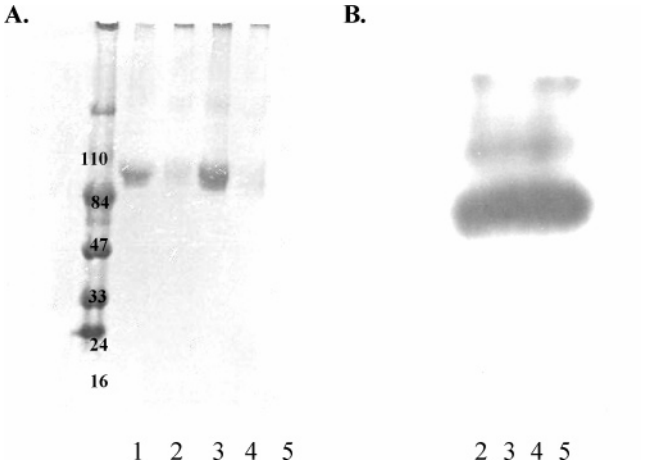

Figure 3. Gel electrophoresis of unlabeled (A) and radiolabeled proteins (B). The samples were run on 4−20% SDS−PAGE and subsequently visualized via Coomassie staining (A) or autoradiography (B), respectively. Lanes contain the following samples: 1, molecular weight standards (kDa); 2, fetuin; 3, aminoBP−SMCC−fetuin conjugate; 4, oxidized fetuin; 5, aminoBP−MMCCH−oxidized-fetuin conjugate.

Gel electrophoresis was performed to ensure that the process of iodination did not inadvertently cause protein fragmentation (Figure 3). The conjugates used in this part had 11.0 (SMCC) and 9.0 (MMCCH) aminoBPs/fetuin and were labeled with TCDG. The autoradiographic bands produced by the labeled proteins were in line with the bands visualized by the Coomassie Blue stained, unradiolabeled proteins. As no bands representing smaller molecular weight fragments were observed, radiolabeling the proteins using the TCDG method did not lead to fragmentation of fetuin, oxidized fetuin, or aminoBP−fetuin conjugates.

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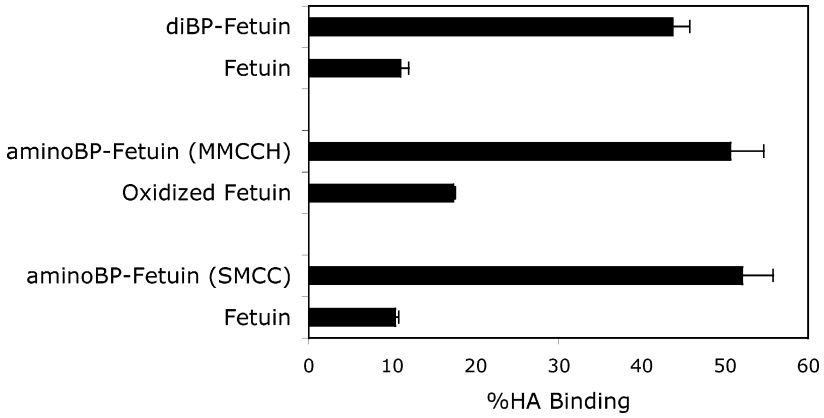


Figure 4. Binding of aminoBP and diBP conjugates of fetuin and their control proteins to HA in vitro. For aminoBP−fetuin and diBP−fetuin conjugates, unmodified fetuin served as the control protein, whereas for diBP−fetuin, oxidized fetuin served as the control protein. All proteins were labeled with ^{125}I, purified as described in Materials and Methods, and added to HA (5 mg) in 400 μL of 0.1 M phosphate buffer. After 3 h of incubation, the percentage of the proteins bound to HA was determined by quantitating the unbound protein concentration in the supernatant. The results are shown as the mean ± SD of duplicate measurements. Note that all conjugates exhibited significantly higher binding than their controls.

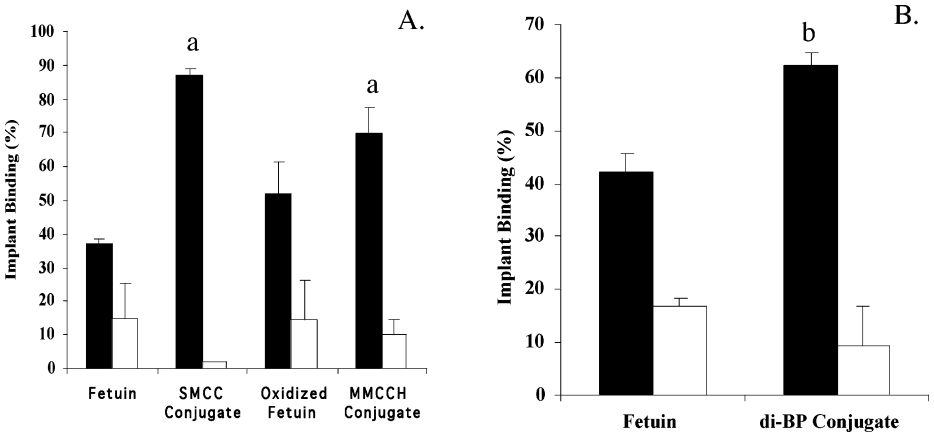


Figure 5. Binding of aminoBP and diBP conjugates of fetuin and their control proteins to Pro-Osteon (black bars) and Helistat (white bars). The binding was determined in vitro by using 50% adult bovine serum and expressed as mean ± SD of triplicate samples. (A) Binding of aminoBP−fetuin conjugates prepared with SMCC and MMCCH, and their respective controls, unmodified fetuin and oxidized fetuin. (B) Binding of diBP−fetuin conjugate and its control protein (unmodified fetuin). Note that all three conjugates exhibited significantly higher binding to the Pro-Osteon implants compared to the control proteins ((a) $p < 0.0125$; (b) $p < 0.05$). Protein binding to Helistat was lower than the binding to Pro-Osteon and similar for all proteins.

Protein Binding to HA in Vitro. The conjugates' propensity to bind to bone mineral was assessed by an HA binding assay. Using conjugates with 6.1 (SMCC) and 7.1 (MMCCH) aminoBPs/fetuin, the conjugates gave a 5.4-fold (SMCC) and 2.9-fold (MMCCH) higher HA binding as compared to the unmodified fetuin and oxidized fetuin, respectively (Figure 4). Similarly, the diBP−fetuin conjugate with 1.2 diBPs/fetuin gave a 4.0-fold increased binding to HA, as compared to the unmodified fetuin (Figure 4).

Protein Binding to Implants in Vitro. The capacity of the native fetuin and BP−fetuin conjugates to bind to mineral-based Pro-Osteon and collagen-based Helistat was subsequently assessed in vitro. The SMCC-linked aminoBP−fetuin conjugate bound 2.4-fold higher to Pro-Osteon disks than the unmodified fetuin, while the MMCCH-linked aminoBP−fetuin conjugate bound 1.3-fold higher than the oxidized fetuin (Figure 5A). The capacity of all proteins to bind to Helistat was significantly lower than their ability to bind to Pro-Osteon, and there were no significant differences among the proteins for binding to Helistat. The binding of diBP−fetuin conjugates to Pro-Osteon was also significantly higher than the binding of the unmodified fetuin (Figure 5B). Similarly to the aminoBP−fetuin conjugates, no difference was observed between the binding of unmodified fetuin and diBP−fetuin conjugate to Helistat (Figure 5B).

Protein Retention in Implants in Vivo. Conjugate retention in Pro-Osteon and Helistat implants was subsequently determined in the rat subcutaneous model. Twenty-four hours after transplantation, the retention of the SMCC- and MMCCH-linked aminoBP−fetuin conjugates in Pro-Osteon implants was 1.6-fold and 1.5-fold higher than the retention of the unmodified fetuin and oxidized fetuin,

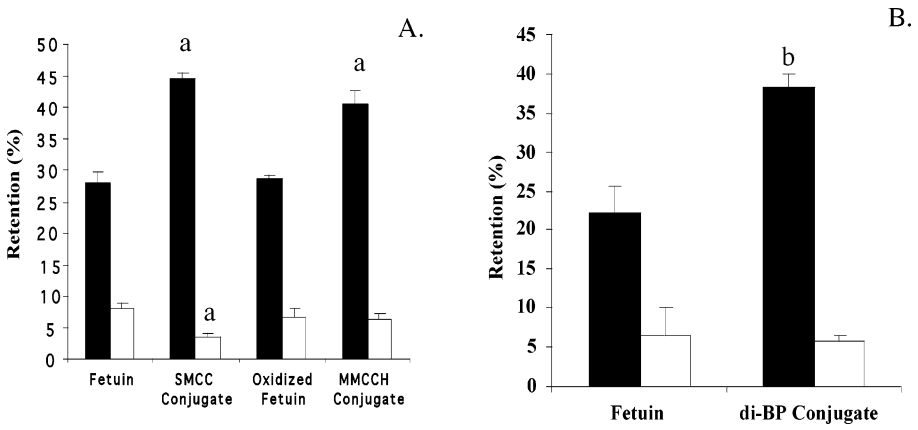


Figure 6. Implant retention of aminoBP and diBP conjugates of fetuin and their control proteins in Pro-Osteon (black bars) and Helistat (white bars). The binding was determined in vivo using the rat subcutaneous model and expressed as mean ± SD of triplicate samples. (A) Retention of aminoBP−fetuin conjugates prepared with SMCC and MMCCH, and their respective controls, unmodified fetuin and oxidized fetuin. (B) Retention of diBP−fetuin conjugate and its control protein (unmodified fetuin). Note that all three conjugates exhibited significantly higher binding to the Pro-Osteon implants compared to the control proteins ((a) $p < 0.0125$; (b) $p < 0.05$). Protein binding to Helistat was lower than the binding to Pro-Osteon and similar for all proteins, except the SMCC conjugate, which gave a significantly lower retention.

respectively (Figure 6A). The protein retention in Helistat was significantly lower compared to that in Pro-Osteon. While there were no differences among the fetuin, the oxidized fetuin, and the MMCCH-linked aminoBP−fetuin retention in Helistat, a significant decrease for the SMCC-linked conjugate retention was observed relative to unmodified fetuin. No statistically significant intergroup differences were observed in the amount of radioactivity recovered in the thyroid for either Helistat (0.17 ± 0.06% of administered dose) or Pro-Osteon (0.28 ± 0.09% of administered dose). These thyroid counts represented only a small fraction of the implanted dose.

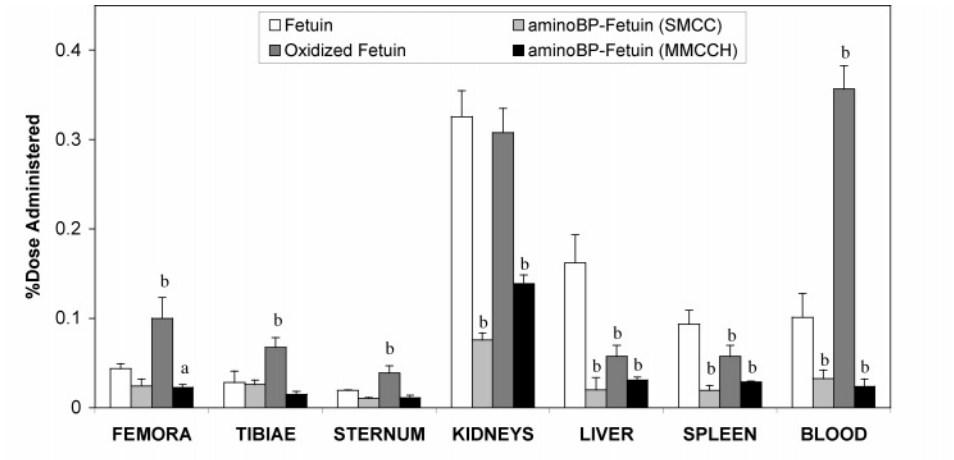
The retention of diBP−fetuin in Pro-Osteon implants was also significantly higher than the retention of unmodified fetuin (38.3 ± 3.4% vs 22.3 ± 3.4%, $p < 0.05$; Figure 6B). Protein retention in Helistat implants was significantly less (∼6%) than the retention in Pro-Osteon implants, and there was no difference between the conjugate and the unmodified protein retention.

Bone Targeting by AminoBP−Fetuin Conjugates. The SMCC-linked and MMCCH-linked aminoBP−fetuin conjugates used in the first study (study 1 in Table 1) had 6.1 and 7.1 aminoBPs/fetuin, respectively. The protein biodistribution was assessed 24 h after IV administration (Figure 7). The targeting of SMCC conjugates to femora, tibiae, or sternum was not significantly different from the unmodified fetuin. The uptake of conjugates in kidneys, liver, spleen, and blood, however, was significantly lower for the conjugate ($p < 0.0125$). With the exception of spleen, the targeting of MMCCH conjugates to all organs, including calcified tissues (i.e., femora, tibiae, and sternum), was significantly lower than that of either the unmodified fetuin or oxidized fetuin. A significant increase in the TCA-soluble counts in the serum was observed for all aminoBP−fetuin conjugates relative to their controls ($p < 0.05$; Figure 7, table).

Given the lack of bone targeting by the conjugates in study 1, we repeated study 1 by using the intraperitoneal (ip) administration route instead of the iv route (not shown in Table 1). The results, however, were similar (not shown): (1) bone deposition of aminoBP conjugates was significantly less than that of the unmodified fetuin and oxidized fetuin, and (2) TCA-soluble counts in serum were higher for conjugates (29.3% and 30.6% for SMCC and MMCCH conjugates, respectively) than their respective controls (5.3% and 4.5% for fetuin and oxidized fetuin, respectively).

Next, bone targeting was assessed at shorter (6 h) and longer (48 h) time points following iv administration (study 2 in Table 1). Only the SMCC-linked aminoBP−fetuin conjugate (6.1 aminoBPs/fetuin) was used in this study. Although fetuin localization was significantly higher ($p < 0.0125$) in the kidneys after 6 h, differences in the biodistribution between fetuin and SMCC conjugates were not significant in any other organs (Figure 8). In addition, no significant differences were observed in the thyroid uptake between the fetuin (8.1 ± 1.1 % dose) and the SMCC conjugate (9.4 ± 2.6 % dose) groups. At 48 h postadministration, the concentration of fetuin was significantly higher in femora, tibiae, kidneys, and blood than that of the SMCC conjugate. The spleen had a higher amount of SMCC conjugate than fetuin (Figure 8). No differences were found in the thyroid counts between the fetuin (8.3 ± 1.3 % dose) and the conjugate group (8.8 ± 3.2 % dose). Likewise, no differences were observed in urinary parameters (i.e., % dose recovered and TCA-soluble counts) between the two groups (Figure 8, table). Although there was no difference in TCA-soluble counts in blood at 48 h, the SMCC-conjugate group had higher level of TCA-soluble counts in blood at the 6 h time point.

The next study (study 3 in Table 1) assessed the biodistribution of fetuin and the MMCCH-linked aminoBP−fetuin



Protein	%TCA Soluble Counts in Serum	Protein	%TCA Soluble Counts in Serum
Fetuin	9.3 ± 3.6	Oxidized Fetuin	3.5 ± 0.9
aminoBP-Fetuin (SMCC)	23.4 ± 1.9	aminoBP-Fetuin (MMCCH)	23.6 ± 1.9

Figure 7. Organ distribution of aminoBP−fetuin conjugates (prepared with SMCC and MMCCH) and the control proteins. The radioactive count at each site was determined 24 h after iv administration and was normalized by the average dose administered per animal. The counts at femur, tibia, and kidney were determined for both organs. The % administered dose to liver and blood was further normalized by the weight of the tissue and by the volume obtained, respectively. Means ± SD of 3 animals are shown. Listed in the table is a summary of the TCA-soluble fraction in the blood. Note the higher localization of oxidized fetuin to bones and the higher TCA-soluble counts in the blood for the conjugates. (a) $p < 0.05$, (b) $p < 0.025$ vs fetuin.

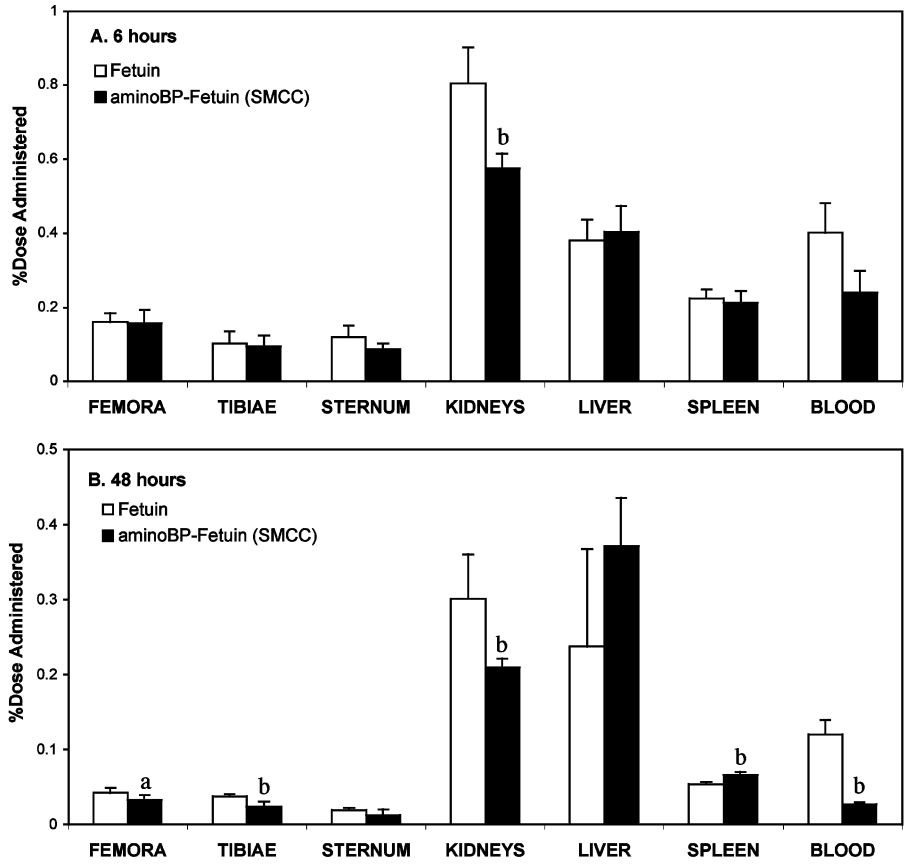
conjugates (4.1 aminoBP/fetuin) radiolabeled with the Bolton−Hunter reagent. Longer time points (72 and 144 h) were used for assessment since our previous studies indicated that the difference between unmodified proteins and BP conjugates was better revealed at longer times.[4] At 72 h, there were no differences between fetuin and conjugate localization to the femora, tibiae, sternum, spleen, and blood (Figure 9A). Increased conjugate localization was observed in the liver, while increased fetuin uptake was observed in the kidneys. The thyroid counts were significantly ($p < 0.05$) higher for the fetuin (2.8 ± 0.8 % dose) than the aminoBP−fetuin (1.0 ± 0.4 % dose) group. Similarly, no statistically significant differences were observed in protein localization to the femora, tibiae, sternum, or blood after 144 h (Figure 9B). A significant difference in protein uptake by the spleen, kidney, and liver was observed at the 144 h time point. However, no differences were found in the thyroid counts between the fetuin and aminoBP−fetuin groups (1.0 ± 0.1 vs 1.2 ± 0.2 % dose, respectively), % dose recovered in the urine, % counts in serum, and % TCA-soluble counts in serum (Figure 9, table).

Bone Targeting by DiBP−Fetuin Conjugates. The protein biodistribution of diBP−fetuin and its control protein (unmodified fetuin) was assessed 72 and 144 h following iv administration. The targeting of diBP−fetuin conjugates to the mineralized tissues, femora, tibiae, and sternum, as well as to the other clearance organs (kidneys, liver, and spleen), was significantly higher than the targeting of unmodified fetuin at both time points (Figure 10A,B). The conjugate level in blood was significantly reduced at each time point compared to fetuin ($p < 0.025$ and $p < 0.05$ at 72 and 144 h, respectively). Although the % TCA-soluble counts in serum were higher for the diBP−fetuin than for the unmodified fetuin, the differences did not reach significant levels at both 72 h (15.6 ± 6.4 % vs 6.2 ± 2.4 %; $p > 0.11$) and 144 h (14.0 ± 8.0 % vs 6.7 ± 5.8 %; $p > 0.25$). Similarly, there were no differences between the thyroid counts at the 72 h (6.1 ± 0.2 vs 6.3 ± 1.3 % dose for fetuin and diBP−fetuin, respectively) and the 144 h time points (3.8 ± 0.8 vs 3.5 ± 1.4 % dose for fetuin and diBP−fetuin, respectively).

Discussion

Conjugation of BPs onto proteins was designed to enhance the affinity of proteins to calcium/phosphate-based minerals. Detailed characterization of the conjugates performed in previous studies suggested that their affinity for hydroxyapatite-based matrices was increased proportionally to the extent of aminoBP and diBP conjugation.[5,8] This study further evaluated the in vivo mineral affinity of BP−fetuin conjugates prepared using both aminoBP (with SMCC- and MMCCH-based reactions) and diBP. The conjugates' binding and retention to Pro-Osteon was determined prior to assessment of their capacity to target to bone upon parenteral administration. Proteins with high affinity for mineralized matrices are expected to be clinically beneficial in two aspects: (i) improved protein retention to mineralized implants; and (ii) enhanced targeting of proteins to bone upon parenteral administration.

Implant Retention by BP Conjugates of Fetuin. Due to the limitations of biologically derived grafts in repair of large osseous defects, the utilization of synthetic mineralized matrices is becoming increasingly more prevalent. To improve the osseointegration of HA implants, numerous studies have examined the effects of implant treatment with



Group	0-6 hour			6-24 hour		24-48 hour		
	Dose in Urine (%)	TCA-Soluble Counts in Urine (%)	TCA-Soluble Counts in Blood (%)	Dose in Urine (%)	TCA-Soluble Counts in Urine (%)	Dose in Urine (%)	TCA-Soluble Counts in Urine (%)	TCA-Soluble Counts in Blood (%)
Fetuin	33.5 ± 1.0	90.4 ± 1.1	11.7 ± 1.7	14.8 ± 7.0	89.0 ± 0.8	3.8 ± 1.1	90.0 ± 2.1	2.69 ± 3.3
AminoBP-Fetuin	17.2 ± 18.8	89.7 ± 1.3	57.2 ± 6.6 [a]	19.0 ± 2.6	89.5 ± 0.8	2.0 ± 0.8	88.3 ± 2.2	8.7 ± 12.7

Figure 8. Organ distribution of fetuin and aminoBP−fetuin conjugates (prepared with SMCC) 6 h (A) and 48 h (B) after injection. The radioactive count at each site was normalized by the average dose administered per animal. The counts at femur, tibia, and kidney were determined for both organs. The % administered dose to liver and blood was further normalized by the weight of the tissue and by the volume obtained, respectively. Means ± SD of 3 animals are shown. Listed in the table is a summary of the counts recovered in the urine at 6 h (i.e., between 0 and 6 h), 24 h (i.e., between 6 and 24 h), and 48 h (i.e., between 24 and 48 h), as well as the TCA-soluble fractions in the blood and urine. Note the lack of bone targeting by the aminoBP−fetuin conjugate and the higher TCA-soluble counts in the blood for the conjugates. (a) $p < 0.05$, (b) $p < 0.025$ vs fetuin.

an osteogenic growth factor. For example, the treatment of a porous HA with recombinant human bone morphogenetic protein-2 (rhBMP-2) significantly improved bone regeneration in a calvaria of rabbit.[13] Similarly, the treatment of (i) biphasic HA/tricalcium phosphate implants with rhBMP-2 in a spinal fusion[14] and (ii) HA-coated titanium rods with recombinant human transforming growth factor-β_2[15] enhanced radiological and histological indices of osseointegration over control groups. Recently, a correlation was demonstrated between a protein's osteoinductivity and its retention in various matrices in vivo.[16] In light of these observations, it was postulated that BP conjugation might enhance protein retention in mineral-containing matrices,

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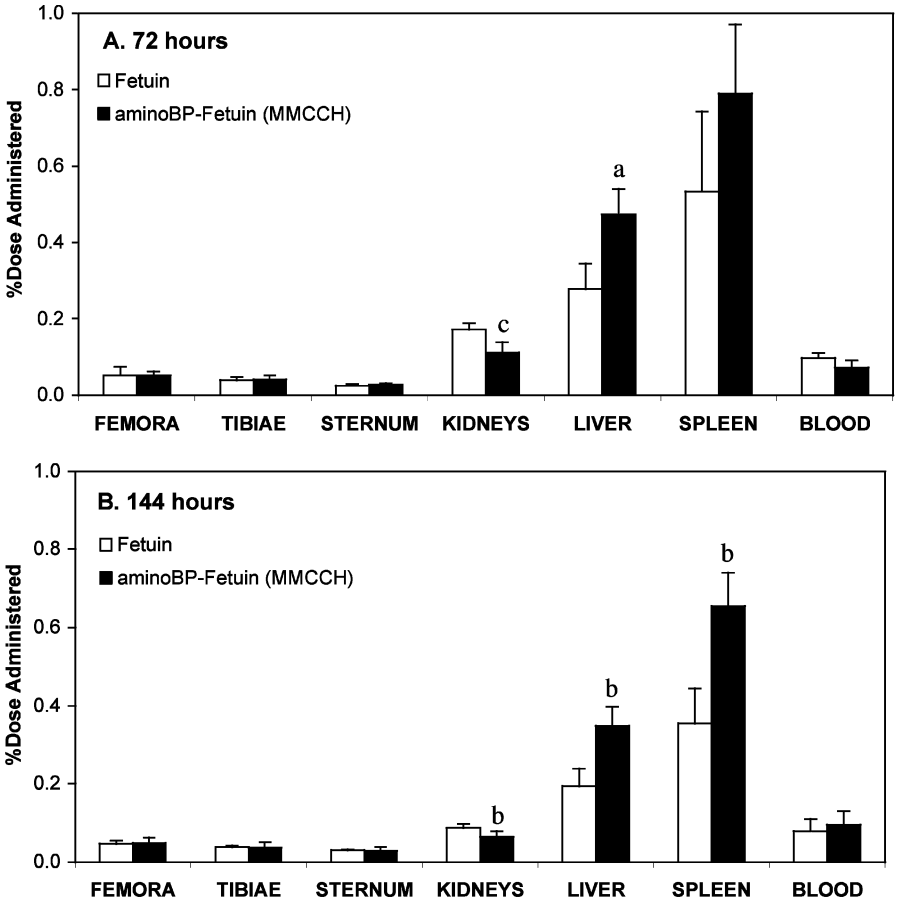

Group	0-72 hour			72-144 hour		
	Dose in Urine (%)	^{125}I in Serum (%)	TCA-Soluble Counts in Serum (%)	Dose in Urine (%)	^{125}I in Serum (%)	TCA-Soluble Counts in Serum (%)
Unmodified Fetuin	28.9 ± 4.6	71.5 ± 4.8	5.5 ± 3.5	3.2 ± 1.4	62.0 ± 2.7	13.2 ± 4.4
AminoBP-Fetuin (MMCCH)	25.1 ± 5.2	82.0 ± 5.7	15.8 ± 10.4	2.4 ± 0.5	64.2 ± 3.0	10.4 ± 3.9

Figure 9. Organ distribution of fetuin and aminoBP−fetuin conjugates (prepared with MMCCH) 72 h (A) and 144 h (B) after injection. The proteins were labeled with ^{125}I using Bolton−Hunter reagent. The radioactive count at each site was normalized by the average dose administered per animal. The counts at femur, tibia, and kidney were determined for both organs. The % administered dose to liver and blood was further normalized by the weight of the tissue and by the volume obtained, respectively. Means ± SD of 3 animals are shown. Listed in the table is a summary of the counts recovered in the urine at 72 h (i.e., between 0 and 72 h) and 144 h (i.e., between 72 and 144 h), as well as the TCA-soluble fractions in the serum and urine. Note the lack of bone targeting by the aminoBP−fetuin conjugate and the higher TCA-soluble counts in the blood for the conjugates. (a) $p < 0.05$, (b) $p < 0.025$, (c) $p < 0.0125$ vs fetuin.

potentially improving the performance of growth factor treated implants.

To this end, the capacity of MMCCH- and SMCC-linked aminoBP−fetuin conjugates, as well as diBP−fetuin conjugates, to bind to Pro-Osteon was assessed in vitro as well as in vivo. Pro-Osteon is utilized in clinics to support bone fusion.[9] As expected, BP conjugation to fetuin by all three approaches resulted in a significant increase in fetuin's capacity to bind and be retained in Pro-Osteon implants. An initial concern in these studies was the impact of BP conjugation on protein solubility under physiological conditions. Through the use of BMP-2, chemical modification of

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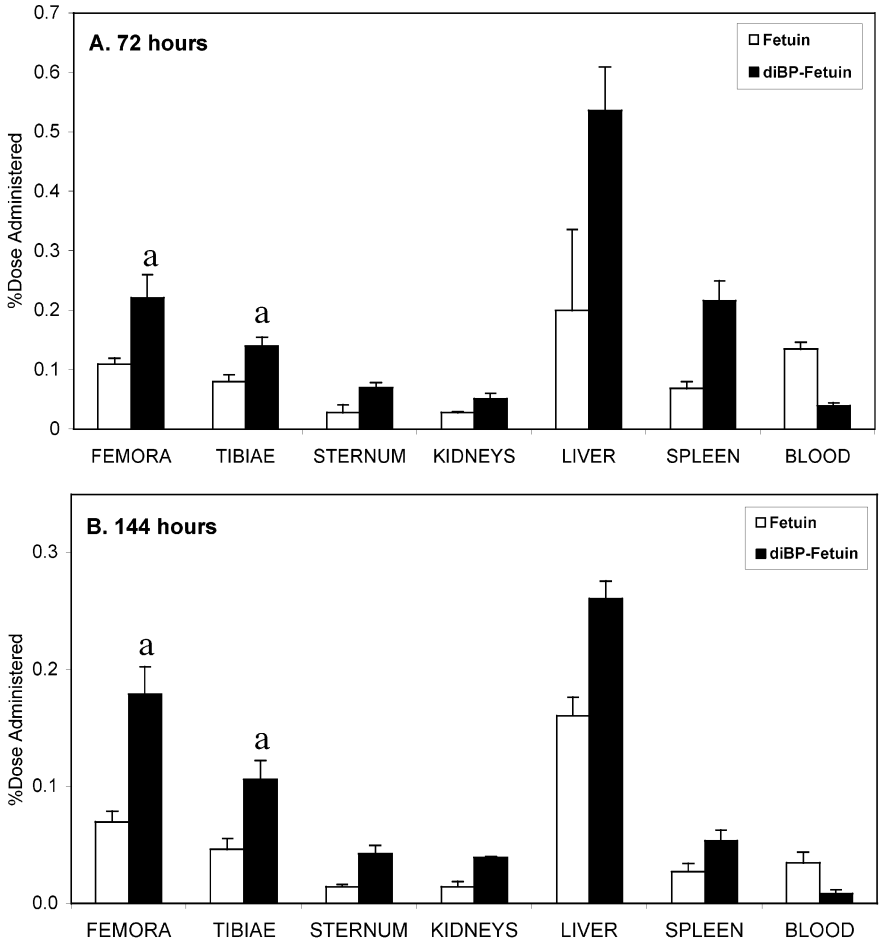

Group	0-72 hour			72-144 hour		
	Dose in Urine (%)	^{125}I in Serum (%)	TCA-Soluble Counts in Serum (%)	Dose in Urine (%)	^{125}I in Serum (%)	TCA-Soluble Counts in Serum (%)
Unmodified Fetuin	26.9 ± 1.6	90.9 ± 2.6	6.2 ± 2.4	2.9 ± 0.3	76.2 ± 19.4	6.7 ± 5.8
diBP-Fetuin	28.2 ± 2.0	83.4 ± 1.1[c]	15.6 ± 6.4	2.2 ± 1.1	74.6 ± 17.5	14.0 ± 8.0

Figure 10. Organ distribution of fetuin and diBP−fetuin conjugates 72 h (A) and 144 h (B) after injection. The radioactive count at each site was normalized by the average dose administered per animal. The counts at femur, tibia, and kidney were determined for both organs. The % administered dose to liver and blood was further normalized by the weight of the tissue and by the volume obtained, respectively. Means ± SD of 3 animals are shown. Note the higher targeting of diBP to femur and tibia compared to fetuin at both time points. (a) $p < 0.0125$, (c) $p < 0.025$ vs fetuin.

a protein was noted to alter its physiological solubility.[16] This is significant as an increase in BMP-2's inherent solubility, as a result of a modification, resulted in rapid protein loss from various implants, including collagenous and mineral implants. On the basis of these results, a causal relationship between a protein's solubility and its rate of release from implants was proposed.[16] A reduction in protein solubility as a result of BP conjugation might have been erroneously perceived as an increased mineral affinity, due to lower solubility and, consequently, higher retention in implants. Both the in vitro binding results and in vivo protein retention results with Helistat suggested that neither the process of oxidation (with MMCCH chemistry) nor the BP attachment resulted in any differences in fetuin solubility under physiological conditions. As such, changes in solubility were considered negligible in influencing conjugate retention in Pro-Osteon; thus the increase in conjugate retention to matrices was due to BP conjugation to fetuin. Only a single diBP conjugation was sufficient to yield a significant mineral affinity, compared to conjugation of >6 aminoBPs (both in vitro and in vivo). These results confirm the expected benefit of using diBP for imparting mineral affinity, as was the case with two other proteins, albumin and IgG, in our previous study.[8] These results also indicate that the in vitro experimental setup described in this study may be used to accurately predict the retention of proteins in Helistat and Pro-Osteon in vivo. In fact, further analysis of the binding data revealed a significant linear correlation between in vitro

binding and in vivo retention in Helistat ($r^2 = 0.8956$, $p < 0.05$) and Pro-Osteon ($r^2 = 0.9214$, $p < 0.025$).

It was interesting to note that BP conjugates exhibited higher binding capacity in vitro to particulate HA than to Pro-Osteon; i.e., the differences in binding between the BP conjugates and the control proteins were more pronounced with HA as the binding matrix. The HA and Pro-Osteon are expected to differ in several aspects, including (i) chemical nature; (ii) geometric features (particulate vs single piece), especially tortuosity; (iii) surface area (being particulate, HA is likely to exhibit a higher surface area/volume than the Pro-Osteon); and (iv) extent of hydration (higher for HA on the basis of our qualitative visual observations). One or more of these factors have ultimately resulted in lower nonspecific (i.e., unmodified protein) binding in combination with stronger binding of BP conjugates to HA. Whether such a difference between the HA and Pro-Osteon also existed in vivo was not explored, since implanting particulate HA was expected to cause difficulties in implant recovery. Nevertheless, the physicochemical properties of mineral matrices seem to be important for binding to BP-modified proteins, and identifying critical features will be important in the design of mineral-containing matrices for better retention proteins.

Lack of Bone Targeting by AminoBP−Fetuin Conjugates. Among the three conjugation schemes, the delivery of proteins to bone was achieved with only diBP-based conjugates. Conjugation of aminoBP to fetuin using either MMCCH or SMCC chemistry did not result in bone targeting. This observation was unlike our previous studies on bone targeting of BSA and lysozyme, where significant bone targeting was readily demonstrated with the aminoBP conjugation approach.[4] Because the BSA and lysozyme conjugates contained 11.0 and 3.9 aminoBPs/protein, respectively, the extent of aminoBP conjugation onto fetuin (i.e., between 4 and 7 aminoBPs/fetuin) was not the likely reason for lack of bone targeting. As unmodified fetuin is ~48 kDa, while albumin and lysozyme are ~66 kDa and ~14 kDa, respectively, the glycoprotein's size was also not a likely reason. The possibility of a significant barrier to leave the vascular system for the intravenously injected fetuin and modified fetuins was considered. The ip administration of the proteins, however, essentially led to the same result as the iv administration, indicating that lymphatic uptake from the peritoneum[17] was not a superior route for bone targeting. The initial results suggested that the oxidation of fetuin enhanced its affinity for osseous tissues (as levels of radioactivity in the femora, tibiae, and sternum were significantly greater for oxidized fetuin; Figure 7). Because levels of oxidized fetuin were significantly higher in the blood, these results should be interpreted with caution, as this phenomenon may simply be due to an increase in the oxidized protein's systemic bioavailability. More importantly, the uptake of the proteins in all tissues assessed was significantly less in the aminoBP−fetuin groups (both SMCC and MMCCH conjugates) than that of the control fetuins. Increased levels of TCA-soluble counts in the blood of the aminoBP−fetuin injected rats suggested that either (i) increased disassociation of the radiolabel from the conjugates or (ii) enhanced degradation of conjugates into low molecular weight species[18] may be responsible for low levels of organ uptake. To differentiate between these two possibilites, urine was collected in subsequent experiments. No significant differences between fetuin and the conjugate groups were found in any of the urinary parameters assessed (i.e., % dose, and TCA-soluble counts in urine). Because the TCA-soluble counts in serum were significantly higher in the conjugate-treated groups, while both the urinary parameters and the amount of thyroid counts were the same among fetuin and conjugate groups, the radioactivity quantified in the serum was likely from the products of protein metabolism and not dissociated ^{125}I.

Radiolabeling is the most convenient method of assessing protein pharmacokinetics. As oxidative radiolabeling with the TCDG method has been shown to alter the biochemical properties of several proteins,[19−23] it was thought that iodination using this technique may be a reason for negating the aminoBP−fetuin's inherent affinity to bone once administered parenterally. The Bolton−Hunter technique was chosen as an alternate means of radiolabeling as this method had not adversely altered the biochemical properties of IgM[20], IgG1,[21] and placental insulin receptors,[22] while the TCDG method had. As above, no differences between the Bolton−Hunter-radiolabeled fetuin and MMCCH-linked aminoBP−fetuin conjugate were found in their capacity to target to bone following iv administration. Consequently, the radiolabeling techniques employed in this study did not seem to affect the conjugates' incapacity to target to bone.

Exploring fetuin's innate properties may offer some insight into explaining the inability of aminoBP conjugation to target fetuin to bone. The negative charges of fetuin's β-sheet in

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its cystatin-like domain D1 imparts an ability to bind to basic calcium phosphate (BCP).[24] Through its ability to bind to BCP, it is thought that the formation of 30−150 nm colloidal "calciprotein particles" consisting of up to 100 molecules of fetuin bound to apatitic microcrystals causes a delay in growth of calcium microcrystals and facilitates their mobilization and subsequent removal from circulation via the hepatic and splenic phagocytes in the reticuloendothelial system.[24] Corroborating its ability to prevent precipitation of calcium and phosphate in vitro,[24−26] fetuin has been shown to be an integral component in mitigating aberrant mineralization in vivo.[27−30] As the aminoBP−fetuin conjugates had a higher capacity to bind to HA than unmodified fetuin, it was likely that the fetuin conjugates may be more efficient in forming "calciprotein particles" in vivo than either fetuin or oxidized fetuin. This may serve to explain the conjugates' increased susceptibity to the liver and spleen and the significant decrease in the blood levels of the aminoBP−fetuin conjugate groups relative to fetuin at shorter time points (i.e., <48 h) following administration (Figures 7). Studies by Hirabayashi et al. also reported faster accumulation of a diclofenac−BP conjugate in the liver and spleen as a result of faster infusion[31] and higher administration doses.[32] Formation of calcium complexes and subsequent precipitation of such complexes was postulated as the underlying basis for the higher spleen and liver uptake. The proteins (i.e., lysozyme and albumin) previously targeted to bone in our studies had no such physiological functionality.

Successful Bone Targeting by DiBP−Fetuin Conjugates. Unlike the aminoBP−fetuin conjugates, bone targeting of diBP−fetuin conjugates was significantly higher than that of the unmodified fetuin. The degree of modification for these conjugates was relatively low (∼1.2 diBPs/fetuin), but significant bone targeting was achieved despite the low conjugation efficiency. Although fetuin conjugates with higher degrees of substitution were desired, this was not possible under the experimental conditions in this study. This was a unique observation particular to fetuin, since both BSA and IgG were derivatized with diBP up to ∼7 diBPs/protein in our previous studies.[8] Our studies with BSA were also supportive of the notion that a relatively lower degree of substitution was needed (e.g., ∼2.7 diBPs/BSA) to achieve successful bone targeting in rats.[8] The advantage of diBP was likely the higher ligand density on fetuin, since our previous studies showed that higher ligand densities resulted in superior mineral affinities and implant retention in vivo.[6] However, three sets of studies, namely, HA binding in vitro, implant binding in vitro, and implant retention in vivo, did not provide any indication about the reasons for the success of bone targeting by diBP conjugates; aminoBP−fetuin and diBP−fetuin conjugates behaved the same way in all of these binding and retention studies. It appears that these experimental setups can successfully predict whether the modified fetuin exhibits a superior mineral affinity compared to the native protein, but cannot predict the relative efficiency of the fetuin−BP conjugates to seek bone after systemic administration. It remains to be seen whether this observation is applicable solely to fetuin, or whether the same holds true for other proteins as well. Experimental data on other proteins is not extensive enough at this stage to shed light on this issue.

A likely explanation of the ability of diBP−fetuin to target bone was the relatively lower catabolism of conjugates in the systemic circulation. As with aminoBP conjugates, liver and spleen uptake of diBP−fetuin conjugates was relatively higher than that of the unmodified fetuin, suggesting that the diBP−fetuin conjugates still had a propensity to be preferentially removed by the major clearance organs. The systemic availability was also lower for the diBP−fetuin conjugates, as was the case for aminoBP−fetuin conjugates. However, unlike aminoBP conjugates, where the blood analysis indicated significant catabolism, the degree of conjugate breakdown was less for the diBP−fetuin conjugates, and was not significantly higher than for control fetuin. The relatively lower extent of BP substitution (1.2 diBPs vs 4−7 aminoBPs/fetuin) was likely the underlying basis of the

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lower breakdown of diBP conjugates, which enabled the systemic diBP−fetuin conjugates to bind bone. On the basis of the limited data set, the rate of protein loss (i.e., over a 72 h period between the 72 and 144 h sampling points) for the diBP conjugates was lower than the rate of fetuin loss: 19.0% vs 36.2% in femora, and 23.8% vs 42.2% in tibiae. This was according to previous observations on BP conjugates, where the BP−protein conjugates yielded not only an initially higher extent of bone localization, but also subsequently a lower rate of protein loss at the mineralized tissues.

More preclinical studies will be needed on the pharmacological properties of the newly synthesized diBP. Although conventional bisphosphonates, such as etidronate, pamidronate, and alendronate, are known to be relatively safe, the full pharmacological profile of diBP remains to be determined. Organ specific toxicities of diBP on protein conjugates are currently unknown. In addition, reason(s) for increased deposition of diBP conjugates to other soft tissues need to be better understood to minimize nonspecific deposition while increasing specific bone trageting of dibP conjugates. Finally, it will be important to explore the stability of the conjugates in vivo with respect to the cleavage of conjugate tethers. The amide- and thioether-based tethers in the current conjugates are generally regarded as stable linkages, unlike ester or disulfide-based linkages used in cleavable conjugates.[33] The aminoBP and diBP conjugates of fetuin, therefore, should not differ in this respect. Nevertheless, it is feasible that differences in the stability of conjugate linkages might contribute to differences in in situ residence time when the proteins are implanted in mineral matrices or reach the bone tissue. The data generated in this study did not allow us to explore this issue directly, and such studies are currently under exploration (see Reference 33).

Conclusions

The results of this study indicated that conjugation of aminoBP and diBP to fetuin unequivocally enhanced the protein's affinity for HA in vitro. Similarly, conjugation of aminoBP and diBP to fetuin increased the protein's retention in a mineral-containing matrix (Pro-Osteon) when the proteins were implanted in a subcutaneous model. These results confirmed that the BP conjugation was a feasible means to enhance the retention, and possibly the biological effect, of osteogenic proteins when implanted with a mineral-based matrix. Upon parenteral administration, however, no discernible differences were found between the SMCC- or MMCCH-linked conjugates and unmodified fetuin to target to bony tissues. This was unlike other proteins previously targeted to bone (BSA and lysozyme), suggesting that the capacity of aminoBP conjugation to enhance protein targeting to bone might be protein-specific. A higher level of degradation was seen as a result of aminoBP conjugation to fetuin, which was considered the likely reason for the lack of bone targeting of fetuin conjugates. Conjugating a diBP with a higher density of bisphosphonic groups, however, led to successful targeting of fetuin to bone. These results were supportive of the superior ability of the diBP to target proteins to bones, previously observed with the BSA and IgG conjugates of diBP. Our future studies will focus on elucidating the range of proteins appropriate for bisphosphonate-based bone targeting.

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