Imparting Bone Affinity to Glycoproteins through the Conjugation of Bisphosphonates

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Purpose. To develop a novel means of conjugating bisphosphonates onto the carbohydrate moieties of glycoproteins to enhance protein affinity to bone.

Methods. 1-Amino-1,1-diphosphonate methane (aminoBP) was conjugated onto the carbohydrate moietites of oxidized fetuin by using 4-(maleimidomethyl)cyclohexane-1-carboxyl-hydrazide (MMCCH). Bone affinity of the resulting conjugates was compared to proteins obtained from another means of conjugation, whereby aminoBP was conjugated onto fetuin's lysine moieties by using succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC).

Results. The use of the MMCCH resulted in the conjugation of up to seven aminoBPs per molecule of fetuin. These conjugates gave a 2.6-, 2.0-, 30.5-, and 1.84-fold increased affinity for untreated, ashed, demineralized bone and hydroxyapatite, respectively, as compared to conjugates from the SMCC reaction. Both conjugates exhibited a pH-independent, equally slow degradation in adult bovine serum-containing media.

Conclusion. The use of the MMCCH chemistry to conjugate aminoBP onto fetuin was feasible. Furthermore, the described processes of conjugation resulted in amino-BP-dependent increase in the glycoprotein's affinity to various bone matrices in a manner that exceeds the affinity produced by the previously established method, which used SMCC.

KEY WORDS: glycoprotein; drug delivery; targeting; bone targeting; bisphosphonate.

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ABBREVIATIONS: aminoBP, 1-amino-1,1-diphosphonate methane; BP, bisphosphonate; DMF, N,N-dimethylformamide; DNP, dinitrophenylhydrazine; HA, hydroxyapatite; 2-IT, 2-iminothiolane; MMCCH, 4-(maleimidomethyl)cyclohexane-1-carboxylhydrazide; SDS, sodium dodecyl sulfate; $NaIO_4$, sodium *m*-periodate; SMCC, succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate; sulfo-SMCC, sulfo-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate; TCDG, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; TCA, trichloroacetic acid.

INTRODUCTION

Currently prescribed agents for the treatment of osteoporosis include estrogens selective estrogen receptor modulators, such as tamoxifene and raloxifene, in addition to the bisphosphonates class of molecules (1). The usefulness of these agents, however, is rather limited because they merely halt the resorption of bone and, as such, fail to reverse the progressive loss of bone mineral density associated with the disease to levels seen before its onset. One emerging prospect in the treatment of osteoporosis is the use of growth factors that are capable of stimulating deposition of new bone upon their systemic administration (reviewed in Ref. 2). Typically, these ubiquitous growth factors mediate their bone formation effects by promoting the mitogenesis and morphogenesis of cells residing at skeletal tissues. Given their pleiotropic nature, however, their exogenous administration is often associated with various unwanted side effects at extraskeletal sites (2). For example, the intravenous administration of an optimal dose of basic fibroblast growth factor (bFGF) has been shown to result in deleterious anatomic changes in both the kidney and lung (3,4). To minimize such adverse effects, which arise as a result of their nonspecific distribution throughout the body, a means of delivering these osteogenic growth factors to bone is required.

To this end, we have previously shown that direct conjugation of 1-amino-1,1-diphosphonate methane (aminoBP) onto the lysine amino acid residues of bovine serum albumin using sulfo-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) resulted in an increased affinity for various bone matrices *in vitro* (5). Subsequent studies have shown that these conjugates exhibited an enhanced retention when administered intraosseously to femurs (6) as well as an increased localization to bones (i.e., femur and tibia) when administered either intravenously or subcutaneously (7). Because the conjugates were obtained by attaching aminoBP to the protein's core, there is a possibility that this means of conjugation may inadvertently alter the protein's pharmacophore and compromise the protein's inherent bioactivity. As a result, we sought to develop an alternative means that might circumvent any difficulties that this particular means of conjugating bisphosphonate may elicit.

In delineating an alternative method, we decided to take advantage of the fact that the vast majority of mammalian proteins undergo some degree of posttranslational glycosylation (between <1% and >99% by molecular weight) (8,9). Although these carbohydrate groups mediate a variety of functions, some appear to have no biologically relevant significance (9). It was previously shown that the oxidation of carbohydrate diols yields functional aldehyde groups that can serve as the basis for subsequent chemical modifications (10). This approach has been used for antibody modifications, such as immobilization onto surfaces and radioactive or fluorescent labeling (10,11), derivatization of cell-surface glycoproteins (12), and conjugation of targeting moieties onto bioactive proteins (13,14). It was our intent to develop a chemistry that would use the oxidation of carbohydrate groups as a means through which bisphosphonates (BPs) could be conjugated onto glycoproteins. Bovine fetuin was chosen as a model glycoprotein for this study because of its relative abundance of carbohydrate groups [22% of its molecular weight

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(15)]. In addition, its 23 lysine amino acid residues also make it a suitable candidate for aminoBP conjugation via the SMCC conjugation.

Described herein is a novel method to conjugate aminoBP onto fetuin's carbohydrate moieties as a means of enhancing its bone affinity. The conjugate affinity for various bone matrices was assessed and compared to conjugates obtained from the previously established SMCC conjugation.

MATERIALS AND METHODS

Materials

4-(Maleimidomethyl)cyclohexane-1-carboxyl-hydrazide (MMCCH), succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), and sulfo-SMCC were acquired from Molecular Biosciences (Boulder, CO). Bovine fetuin (lot #59H7616), 2-iminothiolane (2-IT), bovine adult serum, sodium *m*-periodate ($NaIO_4$), trichloroacetic acid (TCA), 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (TCDG), and precast minipolyacrylamide 4–20% Tris-HCl gels were obtained from Sigma Aldrich (St. Louis, MO). $Na^{125}I$ (in 0.1 M NaOH) was obtained from Perkin Elmer (Wellesley, MA). NaCl in 0.9% solution was from Baxter Corporation (Toronto, ON). Piperazine was acquired from General Intermediates of Canada (Edmonton, AB). 2,4-Dinitrophenylhydrazine (DNP) was from Kodak (Rochester, NY). N,N-Dimethylformamide (DMF) was from Caledon Laboratories (Georgetown, ON). The Spectra/Por dialysis tubing with MW cutoff of 12,000–14,000 Da was acquired from Spectrum Laboratories (Rancho Dominguez, CA). Sodium dodecylsulfate (SDS) and Coomassie blue R-250 were acquired from Bio-Rad (Hercules, CA). Bromophenol blue was from Serva Feinbiochemica (Heidelberg, Germany). The hydroxyapatite (HA) and aminoBP were prepared as described elsewhere (5). The piperazine buffer (pH 5, 7, and 9) was prepared by mixing 0.1 M piperazine with desired amounts of 10 mM HCl. The 0.1 M phosphate (pH 7.4) and 0.1 M carbonate (pH 10) buffers were prepared as described (5), whereas the 0.1 M sodium acetate buffer was prepared as described by Chamow *et al.* (14). The SDS-glycine sample buffer for electrophoresis was prepared by adding 10% (w/v) SDS, glycerol, 0.1% (w/v) bromophenol blue, 0.5 M Tris-HCl (pH 6.8), and dH_2O in a 4:2:1:2.5:10 fashion. The SDS-PAGE running buffer was prepared by the addition of 2.9% (w/v) Trizma base, 14.4% (w/v) glycine, and 1.0% (w/v) SDS in dH_2O.

AminoBP Conjugation onto Fetuin

Fetuin–AminoBP Conjugation Using SMCC

Conjugation by SMCC was performed according to a previously published procedure (5) (Fig. 1A). Fetuin (15 mg/ml in 0.1 M phosphate buffer) was incubated for 2.5 h with 10 mM of either sulfo-SMCC or SMCC, two heterobifunctional crosslinkers with $–NH_2$ and –SH reactive groups. Separately, aminoBP was thiolated by incubating equal volumes of aminoBP (80 mM in 0.1 M phosphate buffer) with 2-IT solution (40 mM in 0.1 mM phosphate buffer) for 2.5 h. The product from this reaction was then directly added to SMCC-reacted fetuin in equal volumes for 1.5 h. In order to remove the unreacted reagents, the final conjugate was thoroughly dialyzed against 0.1 M carbonate buffer (×3) and dH_2O (×2).

Fetuin–AminoBP Conjugation Using MMCCH

The vicinal hydroxide groups within fetuin's carbohydrate moieties were selectively oxidized (14) by treating fetuin (15.5 mg/ml in 0.1 M acetate buffer) with 4 mM of $NaIO_4$ (Fig. 1B). Following a 2.5 h incubation, oxidization was halted by extensive dialysis against dH_2O and subsequently against 0.1 M acetate buffer. The oxidized fetuin was then reacted for 2.5 h with 10 mM MMCCH (stock solution dissolved as 150 mM in DMF), a heterobifunctional crosslinker whose nucleophilic hydrazine and electrophilic maleimide moieties react with aldehyde and –SH groups, respectively. Thiolated aminoBP, which had been prepared as described above, was then added to the MMCCH-reacted fetuin product at equal volumes. After 1.5 h incubation, the conjugate was dialyzed extensively against 0.1 M carbonate buffer and subsequently dH_2O to remove unreacted starting material.

Analysis of the Conjugates

Bradford Protein Assay

The protein reagent used (16) consisted of 0.01% (w/v) Coomassie blue R-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. A 50 μl sample was added to 1 ml of the protein reagent, and the absorbance was determined at 595 nm. Serially diluted bovine fetuin in dH_2O was used for the calibration curve.

DNP Assay for Aldehydes

A volume of 2.5 ml of 0.2 mM DNP (in 1 M HCl) was added to 50 μl of oxidized protein, incubated for 1 h at 37°C (17), and the absorbance of the samples was determined at 370 nm. Serially diluted formaldehyde (in dH_2O) was used for the calibration curve. Once the aldehyde and protein concentrations were quantified, the number of aldehyde groups per fetuin was calculated (mol:mol ratio).

Phosphate Assay

The phosphate assay was modified from the original procedure of Ames (18) and was previously described (5). A calibration curve based on known concentrations of aminoBP dissolved in dH_2O was used. The phosphate concentrations generated here were used in combination with the results from the protein assay to yield the number of aminoBPs conjugated onto fetuin (mol:mol ratio).

SDS-PAGE

Approximately 10 μg of protein sample was mixed with an SDS–glycine sample buffer and loaded onto a 4–20% Tris-HCl polyacrylamide gel. The samples were run at 150 V for 1.5 h in an SDS–glycine 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:acetic acid:dH_20) and scanned on a flat-bed scanner.

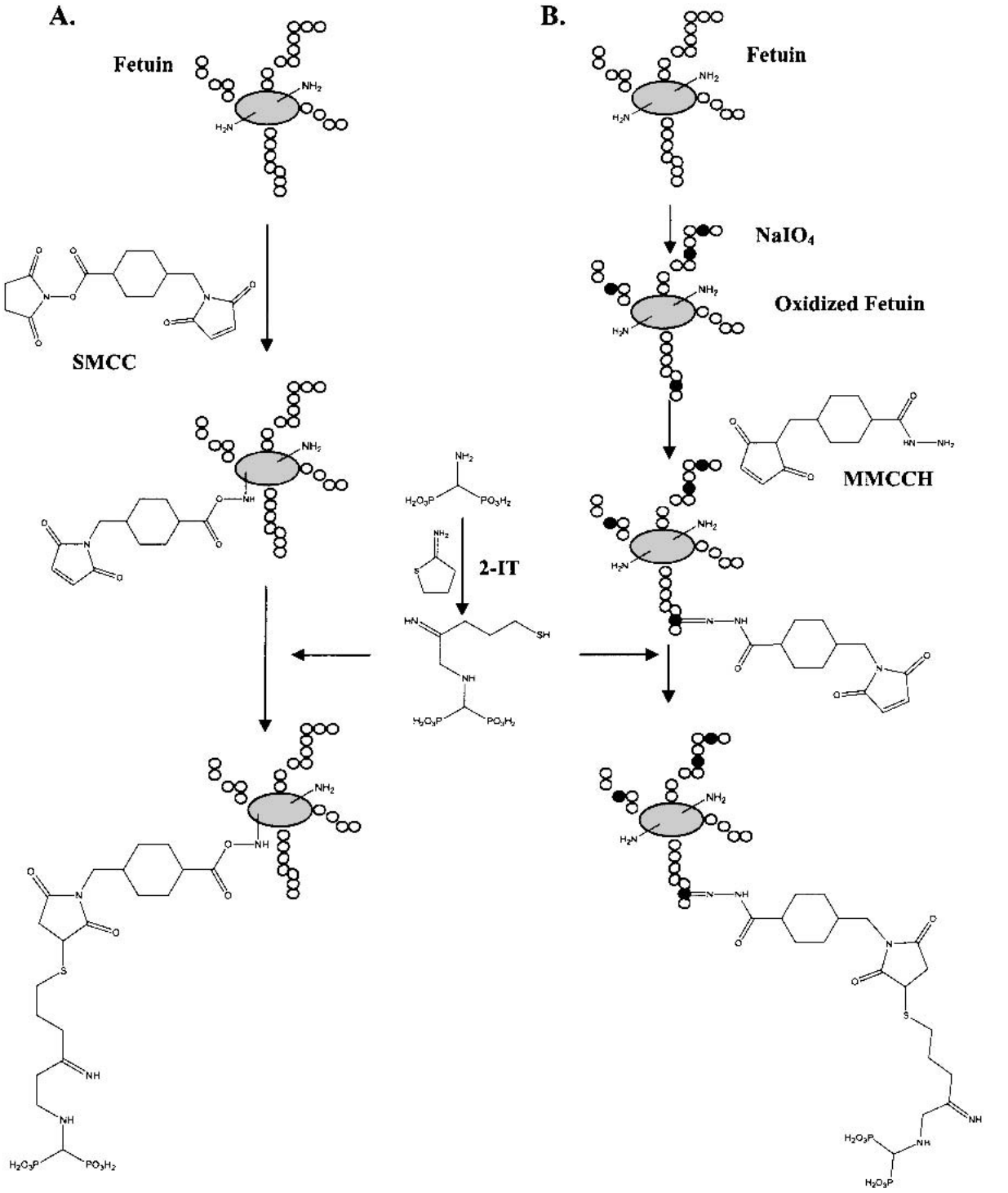

Fig. 1. A schematic representation of the (A) SMCC- and (B) MMCCH-mediated conjugation of aminoBP onto fetuin. The former crosslinker used reactive amine groups to facilitate the conjugation of aminoBP directly onto fetuin's protein backbone, whereas the latter crosslinker used fetuin's carbohydrate groups for conjugation.

Assessment of Mineral Affinity

Hydroxyapatite Binding

The details of the HA binding assay were described previously (5). Briefly, an aliquot of sample (~25 μg) was added to a microcentrifuge tube containing 10 mg of HA with 150 mM phosphate buffer (pH 7.4). This phosphate molarity had been previously determined to provide the optimal conditions for differentiating HA binding between the conjugate and unmodified glycoprotein. The samples were shaken for 2.5 h at room temperature and centrifuged, and the protein concentration in the supernatant was subsequently determined using the Bradford assay. HA affinity (expressed as percentage binding) was calculated as follows: 100% × [(protein concentration without HA) – (protein concentration with HA)] ÷

(protein concentration without HA). All binding was assessed in duplicate.

Bone Matrix Binding

The preparation of untreated, demineralized, and ashed bone was as follows. The marrow was purged from the diaphyses of 6-month-old male rat tibias and femurs, and the bones were subjected to multiple freeze–thaw cycles to ensure the nonviability of the cellular content. The samples were subsequently crushed into small fragments and thoroughly washed in dH_2O and absolute ethanol. The bones were either not processed any further (untreated bone), demineralized in 0.6 M HCl for 72 h (demineralized bone matrix), or ashed at 800°C for 48 h (ashed bone). The samples were stored at 4°C until needed.

The binding was assessed by using ^{125}I-labeled proteins. In tubes previously coated with TCDG (200 μl of 20 μg/ml TCDG in choloroform), 10 μg of protein was added to 50 μl of 0.1 M phosphate buffer (pH 7.4) and 10 μl of 0.01 mCi of $Na^{125}I$ (in 0.1 M NaOH). After reacting for 20 min, free ^{125}I was separated from the radiolabeled protein by elution on a NAP-5 column with 0.1 M phosphate buffer (pH 7.4). The use of the NAP column was not suitable for the radiolabeled-MMCCH conjugates. Consequently, these conjugates were dialyzed against 0.05 M phosphate buffer after iodination. After precipitating an aliquot of the samples with 20% TCA, we confirmed that all iodinated samples contained <5% free ^{125}I. The labeled proteins were added to cold protein to give a radioactive count of 50,000 cpm at 0.1 mg/ml protein concentration. Along with 350 μl of media (refer to figure legends for media content), the amount of binding matrix added to microcentrifuge tubes was: 50 mg of untreated bone; 50 mg of ashed bone; 10 mg of demineralized bone matrix; and 8 mg of HA. After periodic shaking over a 3-h period, the samples were centrifuged. The supernatant was collected, and the pellet, consisting of the bone matrix was washed with the binding buffer and re-centrifuged. This washing procedure was then repeated (×2), and the collected supernatant from each of these steps was subsequently measured separately. Quantification of the radioactivity was then determined using 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 was assessed in duplicate.

Conjugate Stability

To investigate the influence of pH on conjugate stability, the conjugates were dialyzed against a 0.1 M piperazine, a universal buffer, at a pH of 5, 7, or 9. At various time points over the course of 1 week, aliquots of the dialyzing samples were analyzed to assess the conjugates' affinity for HA. To determine the rate of conjugate degradation, the conjugates were prepared as described before, labeled radioactively, and subsequently dialyzed in 25% serum in saline. At various time points, aliquots were taken, and the conjugates' binding affinity for HA was determined. Binding was assessed in duplicate.

Statistical Analysis

Significant differences ($p < 0.05$) within the data were determined by student *t*-test or linear or multiple regression analysis. Statistical analysis was performed by S-PLUS Student Ed. 6.0 (Insightful Corp, Seattle, WA).

RESULTS

SMCC-Based Conjugation

Conjugation of aminoBP onto fetuin was initially attempted using sulfo-SMCC. The results had indicated that by independently augmenting the concentrations of either sulfo-SMCC (Fig. 2A) or thiolated aminoBP (Fig. 2B), the number of aminoBPs conjugated onto fetuin increased proportionally to a maximum of 17.0 aminoBPs/fetuin and 15.2 aminoBPs/fetuin when sulfo-SMCC and thiolated aminoBP concentrations were raised to 20 mM, respectively. The control samples (i.e., reactions with non-thiolated aminoBP in the case when sulfo-SMCC concentration was changed, and reaction with 0 mM sulfo-SMCC in the case when thiolated aminoBP concentration was changed), had less than 2 aminoBPs/fetuin, indicating some degree of aminoBP retention after dialysis, albeit significantly lower than the corresponding conjugates.

With the same samples, an increase in the conjugates' capacity to bind to HA was observed as the number of aminoBPs conjugated to fetuin was increased (Fig. 2C,D). All control samples, however, exhibited significantly lower binding irrespective of the concentration of thiolated aminoBP or sulfo-SMCC used in conjugations. Because the control samples in Fig. 2B, which were reacted with sulfo-SMCC, did not exhibit an HA affinity, sulfo-SMCC reaction with fetuin's amine groups did not appear to influence the HA binding of the glycoprotein. Because of cost considerations, all subsequent studies used SMCC instead of sulfo-SMCC. The final conjugation products from both crosslinkers were identical, so the substitution of sulfo-SMCC with SMCC was considered inconsequential.

MMCCH-Based Conjugation

Fetuin's intrinsic aldehyde content (i.e., 7.3 aldehydes per fetuin) was initially found to be insufficient for aminoBP conjugation. As a result, the first step in MMCCH-based conjugation was the $NaIO_4$-mediated introduction of aldehyde groups onto fetuin's carbohydrate groups. Results from this process gave a linear relationship between $NaIO_4$ concentration and the number of aldehydes introduced per fetuin (Fig. 3). Given that a concentration of 4 mM $NaIO_4$ not only yielded an adequate number of aldehyde groups (~30 aldehyde groups/molecule of fetuin) but was also considered mild enough to circumvent the complete destruction of fetuin's carbohydrate groups (19), this concentration was typically chosen for subsequent reactions. It was found that the product of this oxidation process was stable at 4°C for in excess of a month, as determined by the DNP and Bradford protein assays.

The conjugation of aminoBP to fetuin was initially attempted by reacting its $-NH_2$ directly with the aldehyde moieties of oxidized fetuin via the formation of a Schiff base. No effective conjugation was noted by this approach (data not shown). As a result, MMCCH was then used to facilitate the chemical conjugation of aminoBPs onto fetuin's aldehyde moieties. Increasing $NaIO_4$ concentrations to modulate the number of aldehydes introduced onto each molecule of fetuin

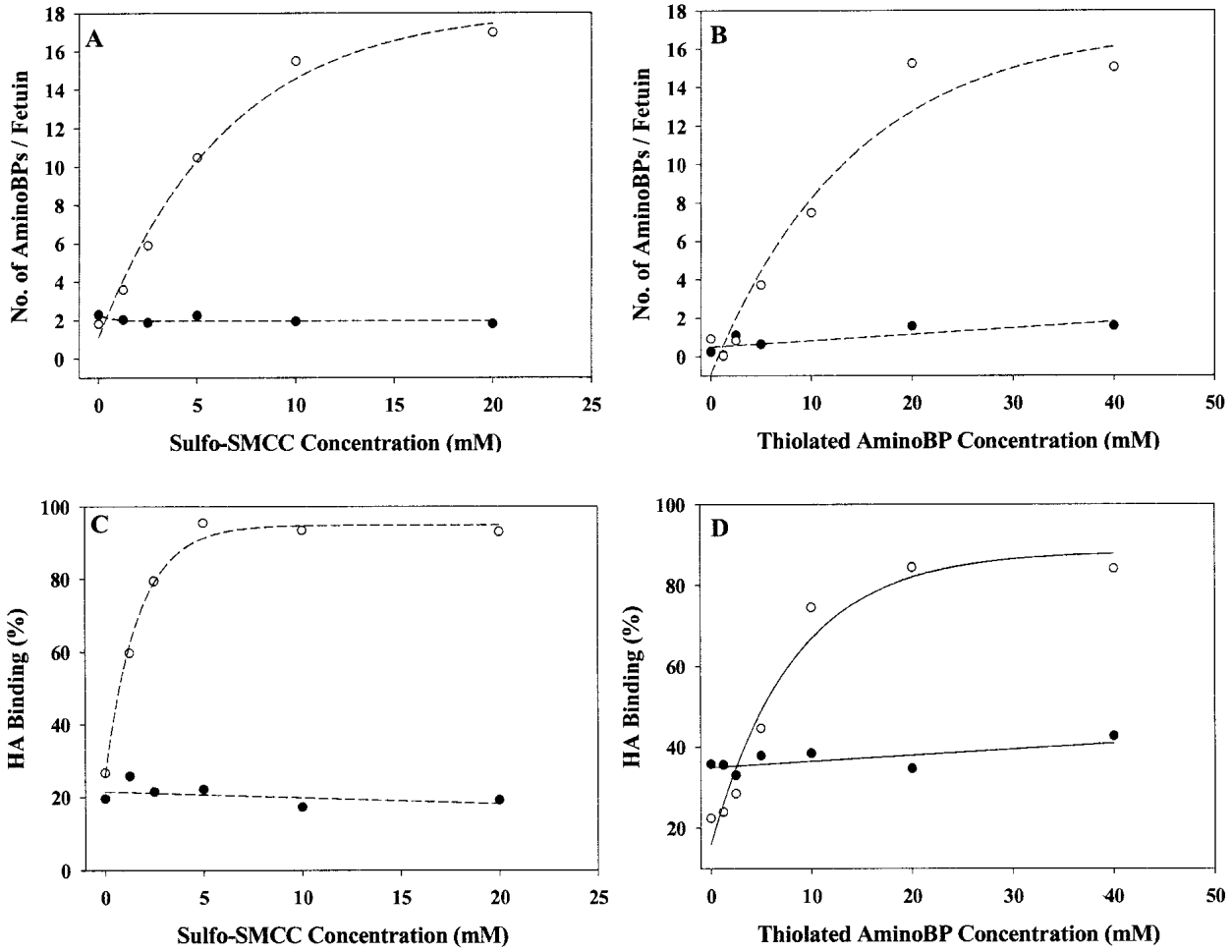

Fig. 2. The conjugation efficiency (A and B) and HA binding (C and D) for fetuin incubated with either unthiolated aminoBP (●) or thiolated aminoBP (○). The 2-IT/aminoBP concentration was maintained at 10/20 mM in A and C, whereas the sulfo-SMCC concentration was maintained at 10 mM in B and D. Increasing the sulfo-SMCC concentration increased the number of aminoBP conjugated onto fetuin (A) as well as increasing the thiolated aminoBP concentration (B). Corresponding increases in the conjugates' affinity for HA were noted for increasing sulfo-SMCC (C) and thiolated aminoBP concentrations (D).

as well as thiolated aminoBP concentrations, led to an increase in the number of aminoBPs conjugated onto fetuin (Figs. 4A and 4B, respectively). Similar to the SMCC-based chemistry, increasing MMCCH concentrations led to an increase in the number of aminoBPs conjugated onto fetuin, which corresponded to an increase in the conjugate's ability to bind to HA (data not shown). A maximum of 7.0 aminoBPs per fetuin was achieved under the experimental conditions for MMCCH conjugation. The propensity for these samples to bind to HA was then determined. As expected, the results indicated that increasing the number of conjugated aminoBPs resulted in a proportionally linear increase in the conjugates' affinity for HA (Fig. 4C,D). Typically, the number of aminoBPs conjugated per fetuin for these controls, which exhibited nominal binding to HA, was < 1. As seen in Fig. 4C, the degree of oxidation did not affect the glycoprotein's intrinsic affinity for HA. Because control samples in Fig. 4D, which were reacted with MMCHH, did not exhibit an HA affinity, MMCCH reaction with the fetuin's glycan groups did not appear to influence the HA binding of the glycoprotein.

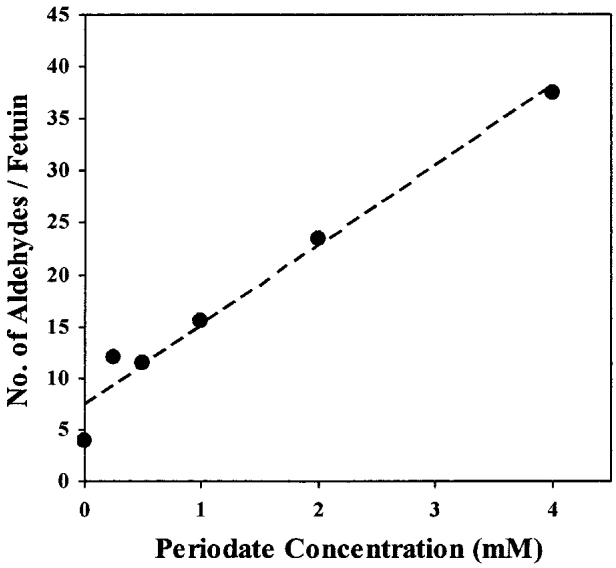

Fig. 3. Increasing the number of aldehyde groups introduced onto fetuin in a linear fashion (r^2 = 0.969) by increasing sodium periodate concentrations used for oxidation.

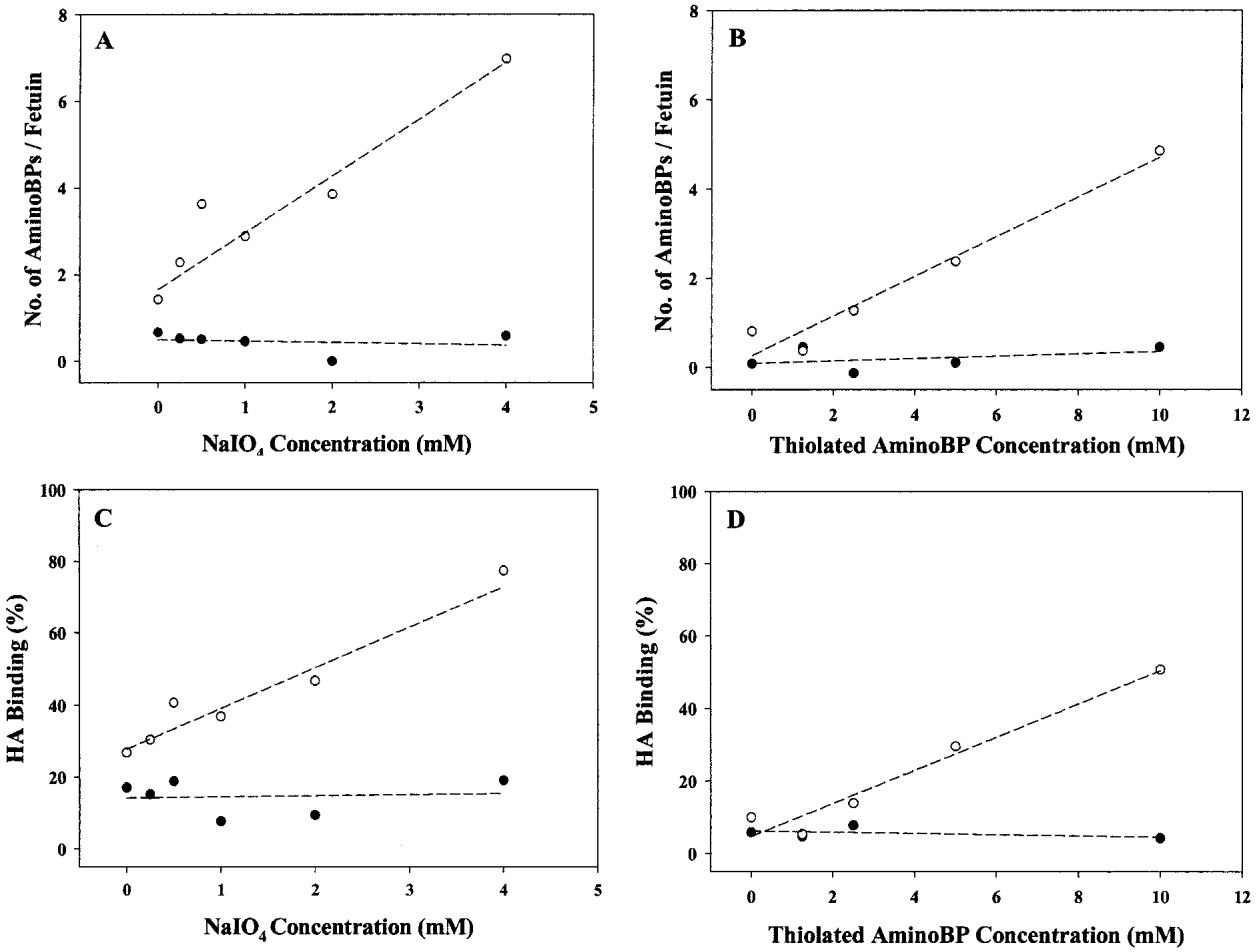

Fig. 4. The conjugation efficiency (A and B) and HA binding (C and D) for fetuin incubated with either non-thiolated aminoBP (●) or thiolated aminoBP (○). The MMCCH concentration was 10 mM for all samples. The thiolated aminoBP concentration was 10 mM for samples in A and C, whereas the samples in B and D were oxidized with 4 mM $NaIO_4$. Increasing the $NaIO_4$ concentration increased the number of aminoBP conjugated onto fetuin (A) as well as increasing the thiolated aminoBP concentration (B). Corresponding increases in the conjugates' affinity for HA were noted for increasing $NaIO_4$ (C) and thiolated aminoBP concentrations (D).

Protein Purity by SDS-PAGE

Gel electrophoresis was used to determine whether inadvertent protein–protein crosslinking was occurring as a result of using either crosslinker or through the formation of a Schiff base between fetuin $-NH_2$ and aldehyde groups (20). Because the molecular weight of fetuin is 48.4 kDa, protein–protein crosslinking would have manifested itself as ≥100 kDa protein bands on the gels. Our results indicated that there were no visible changes in the intensity of native fetuin's band of 48.4 kDa and no visible increase in the higher molecular weight bands as the concentrations of SMCC and MMCCH were increased to 10 mM and 20 mM, respectively, while thiolated aminoBP concentration used was 20 mM (data not shown). As such, any protein:protein crosslinking was deemed negligible.

Comparison of the Mineral Affinity of MMCCH and SMCC Conjugates

To compare the ability of two types of conjugates to bind to HA, each conjugate's capacity to bind to HA was plotted as a function of the number of conjugated aminoBPs (Fig. 5). The observed trend suggested that increasing the number of conjugated aminoBPs resulted in a linear increase in conjugate's affinity for HA, regardless of the crosslinker used. The MMCCH conjugates' binding to HA rose to a maximum of ~77% when a maximal number of ~7.0 aminoBPs were conjugated onto fetuin. Similarly, a binding of ~86% was achieved when ~9.0 aminoBPs were conjugated via SMCC. Increasing the number of conjugated aminoBPs past this threshold, however, did not enhance mineral affinity for the SMCC conjugates. The HA binding of the MMCCH-based conjugates could not be tested in this region because this crosslinker did not give any conjugates in this range. A comparison between the slopes in the linear regions (i.e., < 8 aminoBPs/fetuin) for each conjugate revealed no significant differences between the two conjugation approaches.

To investigate conjugate stability, the effect of pH on the conjugates' affinity to HA was determined. A comparison of the slopes (from percentage of HA binding vs. time curves) revealed no significant differences in either of the conjugates' affinity for HA when in a basic, neutral, or acidic piperazine-buffered environment (data not shown). These results suggest that the conjugates' stability, as determined by its affinity for HA, was not adversely affected by the pH of the incubating medium. To assess conjugate stability, conjugate binding to

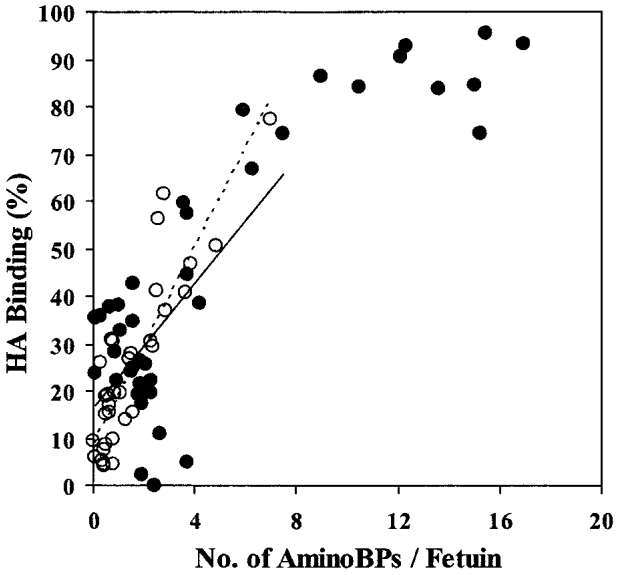

Fig. 5. Correlation between the number of aminoBPs conjugated per fetuin and the conjugate binding to HA. The MMCCH conjugates' (○) binding to HA increased in a linear fashion as the number of conjugated aminoBPs increased. Although it shared a similar linear trend, the conjugation of more than nine aminoBP/fetuin did not enhance the sulfo-SMCC conjugates' (●) capacity to bind to HA. There was no significant difference in the slopes observed in the linear region (<8 aminoBP/fetuin) for the MMCCH and the SMCC conjugates (hashed and solid trend lines, respectively).

HA in 25% adult bovine serum was determined over a 1-week time period. As shown in Fig. 6, the binding of the SMCC and MMCCH conjugates were initially ~71% and ~58%, respectively. The control samples (i.e., control for SMCC-based chemistry: fetuin incubated with thiolated aminoBP without SMCC; and for the MMCCH-based chemistry: oxidized fetuin incubated with the crosslinker without aminoBP) did not exhibit a change in HA binding over time. The conjugates' binding to HA, on the other hand, decreased gradually with time (~3% per day for both types of conjugates). Comparing the two slopes for parallelism revealed no statistically significant differences between the two conjugates' rates of degradation.

The relative stability of the conjugates in serum led us to explore their bone mineral affinity in serum, a medium that better represents *in vivo* conditions than the phosphate buffer previously used. The matrices used were untreated, ashed, and demineralized rat bone and synthetic HA in 50% bovine adult serum (Fig. 7). With the exception of demineralized bone matrix, the conjugates' binding to various bone matrices increased in response to increasing number of conjugated aminoBPs per fetuin. With demineralized bone matrix, however, MMCCH conjugates, but not SMCC conjugates, gave significant binding. For all matrices, as the number of aminoBPs/fetuin increased, it was found that the MMCCH conjugate had a statistically significantly higher affinity than the SMCC conjugate. By dividing the maximum binding capacity that the MMCCH conjugate had for a given matrix by the maximum binding capacity that the SMCC conjugate had for the same matrix, a 2.6-, 2.0-, 30.5-, and 1.84-fold difference in the conjugates' ability to bind to untreated, ashed, and demineralized bone and HA, respectively, was revealed.

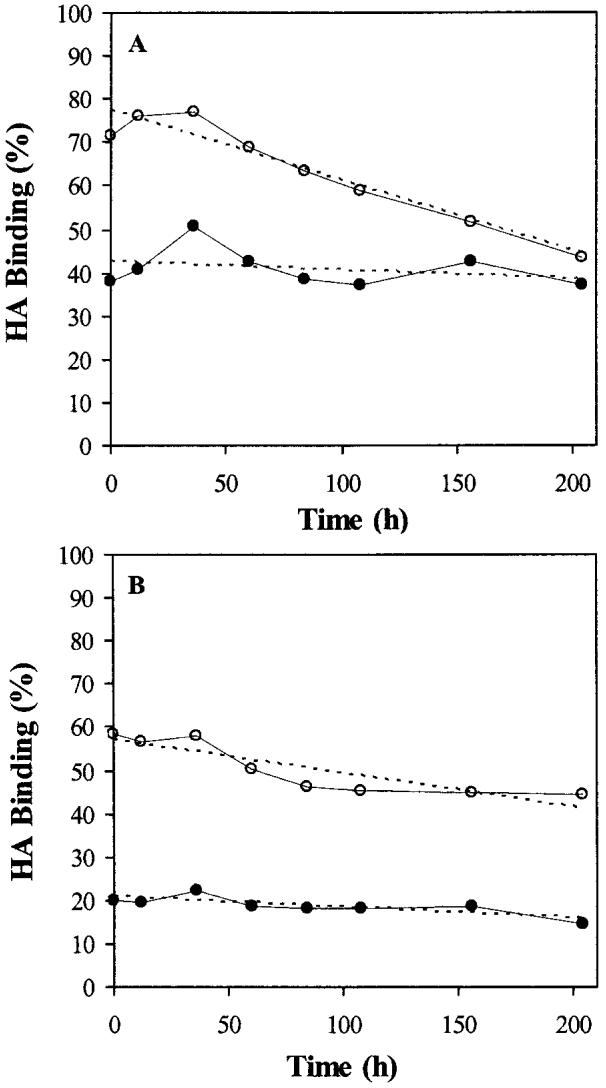

Fig. 6. The stability of the SMCC (A) and MMCCH (B) conjugates was determined as a function of their capacity to bind to HA in 25% adult bovine serum over the course of 1 week. As time progressed, the binding affinity for the SMCC and MMCCH conjugates (○) decreased in a linear fashion (r^2 = 0.95 and 0.77, respectively) while their respective controls (●) remained relatively constant. No significant difference was observed between the two conjugates' decreasing ability to bind to HA.

DISCUSSION AND CONCLUSIONS

As part of the initial phase of developing a general approach for systemic delivery of any glycosylated protein capable of eliciting a desired pharmacologic response at skeletal sites, it was the goal of this study to develop a means of conjugating bisphosphonates onto the carbohydrate moieties of a model protein, fetuin. The proposed method was designed as an alternative to the previously established SMCC-based conjugation where aminoBP was conjugated onto the protein through the direct chemical modification of the lysine amino acid residues. As a result, we were concerned that such a modification of the protein backbone may inadvertently lead to an adverse change in the protein's tertiary structure and/or interfere with its receptor binding/activation, which might culminate in the loss of the protein's desired pharma-

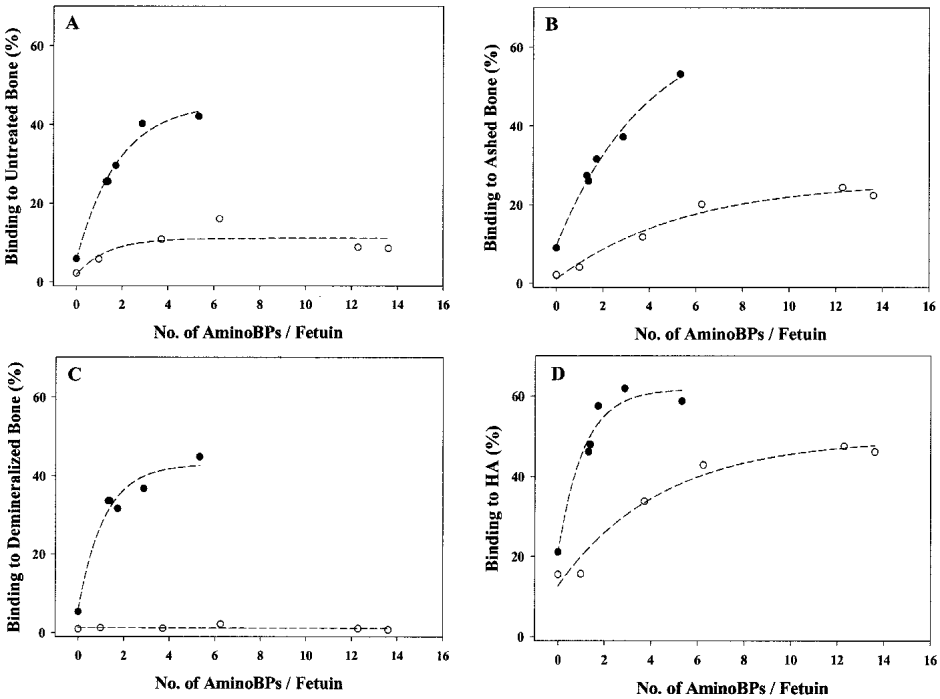

Fig. 7. Binding of SMCC (○) and MMCCH (●) conjugates to various bone matrices in 50% bovine adult serum to untreated bone (A), ashed bone (B), demineralized bone matrix (C), and HA (D). In all instances, the matrix binding of the MMCCH conjugates exceeded that of SMCC conjugates.

cologic activity on skeletal tissues. Most noncytosolic proteins undergo some degree of post-translational glycosylation endogenously. Much like the notorious heterogeneity associated with their chemical structure, the functions of these covalently attached carbohydrate groups vary widely. Some of the physicochemical and biological roles the carbohydrates mediate include protein folding, stability, and solubility; regulation of glycoprotein intracellular trafficking and localization; as well as modulation of enzyme and hormone activity (21). Based on the observed bioactivity of recombinant proteins expressed in prokaryotic systems, which are inherently incapable of performing post-translational glycosylation, it is apparent that the absence of carbohydrate residues does not have any adverse ramifications on their ability to elicit a biologic response (9). In fact, the non-glycosylated forms of numerous osteogenic proteins such as bone morphogenetic protein (BMP)-2 (22), BMP-4 (23), BMP-7 (24), BMP-14 (also known as growth and differentiation factor-5, GDF-5) (25), BMP-13 (GDF-6) (25), and BMP-12 (GDF-7) (25) have been shown to either induce effective bone formation or elicit other musculoskeletal effects typical of the protein's normal biological function. The bFGF produced in a prokaryotic system has also retained its characteristic mitogenic activity (26). Given the biologically nonessential nature of the carbohydrate moieties of these musculoskeletally active proteins, the conjugation of aminoBP onto their carbohydrate groups should not affect the proteins' inherent bioactivity.

The results described herein demonstrated that fetuin, a glycoprotein chosen for its relatively high degree of glycosylation, was an appropriate candidate to undergo SMCC-based aminoBP conjugation onto its proteinaceous backbone as well as MMCCH-based aminoBP conjugation onto its carbohydrate moieties. It was shown that the conjugation efficiency (i.e., number of conjugated aminoBPs per protein) was varied by the reagent concentrations used in either conjugation procedure. The aminoBP was used as a prototypical BP for our purposes because it is one of the simplest BPs synthesized, contains a functional $-NH_2$ group for the necessary conjugations, and acts as a model for a range of other $-NH_2$-containing BPs currently studied in the context of bone biology (e.g., pamidronate, alendronate, risedronate, and ibandronate) (5,27). Despite our desire, we were unable to use this functional group for direct conjugation with the introduced aldehyde groups on fetuin (as well as direct conjugation with succinimide ester of SMCC). Using a simple HA-binding assay that was previously shown to correlate with *in vivo* bone affinity (5,7), it was ascertained that an increase in the number of conjugated aminoBPs led to an increase in conjugate binding to HA. This was assessed under conditions (150 mM phosphate) that were not conducive for the electrostatic binding of the unmodified protein to HA. Nevertheless, a nominal degree of HA binding was noted, presumably because of protein carboxyl groups forming weak complexes with the fixed Ca^{2+} on the mineral surface (28,29). As was evident using the SMCC-mediated conjugation, an upper limit of HA binding (~95% under the experimental conditions) was reached once more than nine aminoBPs were conjugated onto fetuin. It was not possible to assess whether a similar behavior was exhibited with MMCCH-mediated conjugation because it was not experimentally possible to obtain conjugates with more than seven aminoBPs per protein. This was unexpected because an abundant (>30) number of aldehyde groups was introduced onto the glycoprotein, suggesting that MMCCH conjugation used only a small fraction of the available groups. A saturable binding kinetics was observed for both conjugates under serum conditions for HA and bone-derived matrices (40–60%

for MMCCH and 10–40% for SMCC mediated conjugations, depending on the choice of the matrix). Taken all together, these data suggest that the conjugation of aminoBPs onto fetuin was indeed responsible for imparting the observed bone mineral affinity and that modification beyond a certain threshold did not enhance the bone affinity any further.

Bone matrix binding was also dependent on the conjugation efficiency for both conjugation approaches when the binding was assessed under serum conditions. These conditions, consisting of anions, cations, and other proteins, were believed to offer a more stringent (competitive) environment for protein binding. Unlike HA binding in phosphate buffer, the MMCCH conjugate exhibited a significantly higher affinity for the bone matrices than that of the SMCC conjugate under these conditions. To explain the difference between the binding capacity of the two conjugates, it was postulated that by being conjugated onto the carbohydrate residues, the aminoBPs were further distanced from the glycoproteins' protein core where the conjugated aminoBPs would be subjected to steric interference. Furthermore, conjugation via the flexible carbohydrate moieties may afford the aminoBP molecules an increased range of motion that would enable them to access a mineral matrix more effectively. An unexpected observation was the aminoBP-dependent binding of the MMCCH conjugates to demineralized bone matrix, to which the SMCC conjugates did not bind. Although the latter observation suggested that the mineral content of the bone was minimal, the fact that the MMCCH conjugates bound to the demineralized bone in an aminoBP-dependent manner indicated that a residual bone mineral might have been left in bone matrix preparations. As with the other matrices, conjugates from the MMCCH chemistry exhibited a superior affinity for the demineralized matrix compared to the SMCC-mediated conjugation. It is also possible that the binding might be mediated by an alternative mechanism (e.g., a serum-mediated process affecting the binding of the two types of conjugates differently), which is not clear to us yet. We intend on addressing this issue in future studies by exploring the role of varying the linkage spacing between the BP and the protein molecules so as to elucidate the role of steric interference on BP-mediated protein binding to bone mineral.

Numerous studies have shown that SMCC conjugates were nondegradable in a variety of environments, including serum-containing media *in vitro* as well as in *in vivo* models (30,31). MMCCH has been previously used to conjugate STn, a mucin-associated disaccharide epitope overexpressed in human carcinomas, to keyhole limpet hemocyanin, an antineoplastic agent (32,33). Unfortunately, the stability of these conjugates was never determined (32,33). It has been shown, however, that hydrazone and thioether bonds, i.e., the linkages formed by the reactive groups of the MMCCH, were inherently stable under aqueous conditions (14). Consequently, we expected our conjugates to be stable under the experimental binding conditions, regardless of the conjugation approach. Supporting this hypothesis, the results presented herein suggest that the conjugates' mineral affinity was not influenced by the pH of the medium. Some loss of protein affinity (3% per day) was seen in 25% bovine adult serum over the course of a week, but this was similar for both proteins and was likely not to be related to the differences in the conjugation linkages. Our studies were performed for a longer duration than the previously reported studies (32,33) and may explain the discrepancy. Nevertheless, the two conjugation approaches were considered to have a similar stability when used to impart an aminoBP-mediated bone affinity.

The extent of periodate-mediated carbohydrate oxidation was dependent on the medium pH, the reaction time, the periodate concentration, and the temperature (34). As a side reaction, the process of oxidation was found to result in modification of some amino acids, such as cysteine, methionine, tryptophan, and tyrosine (10). According to a published review of the literature (10), the conditions required to attain this undesirable oxidation of amino acids (120 mM periodate for >6 h) far exceed those used in this study (<10 mM for 2 h). In fact, using periodate concentrations similar to our own, independent investigators have shown no or nominal abrogation of bioactivity for several proteins, such as horseradish peroxidase (35), gelonin (13), soluble CD4 (14), and immunoglobulin G (34). Because fetuin only served as a model glycoprotein in our studies, its bioactivity was not of interest. Studies that have examined the effects of deleting the fetuin gene in a mouse model have shown that the glycoprotein plays a role in the prevention of ectopic microcalcifications (36) and affects the process of endochondral bone formation by binding to members of TGF-β family (including BMPs) and inhibiting their bone-formation activity (37). Our long-term goal is to apply the described MMCCH approach to glycoproteins such as BMP-2, whose bone-forming activity has been unequivocally established. Although the *in vivo* targeting capability of the conjugates was not assessed in this study, it was previously shown that SMCC conjugates with increased affinity for HA also enhanced protein targeting to bone: intravenous administration of aminoBP-conjugated albumin (SMCC as the linker) led to 2.0- to 3.7-fold and 2.2- to 7.5-fold increases in bone delivery compared to unmodified albumin in normal and osteopenic rats, respectively (7). Similar results were obtained for lysozyme in the same animal models as well (7). Consequently, it is anticipated that the conjugation of aminoBP onto fetuin via the MMCCH approach will enhance the glycoprotein's *in vivo* delivery to bone in a manner similar to previous SMCC conjugates (5).

In summary, this study described the first attempt to impart a bone mineral affinity onto proteins via the modification of carbohydrate moieties. It is expected that the protein bioactivity will be better preserved by the proposed approach as compared to our previously described approach where the BPs were directed conjugated to the protein core. Based on the results presented in this study, it is also likely that aminoBP conjugation via carbohydrate moieties might exhibit a superior mineral affinity as compared to aminoBP conjugation to the protein core. Previous efforts to conjugate BPs as a way to localize molecules to bone have not been limited to proteins (38). BPs have been conjugated onto radionucleotides and bioactive drugs such as estrogen and antineoplastic and antiinflammatory agents, and the proposed approach could find applications beyond the protein class of therapeutic agents.

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