# Synthesis and in Vitro Hydroxyapatite Binding of Peptides Conjugated to Calcium-Binding Moieties

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To confer bone-binding properties to proteins and other biological agents that lack specific targeting capacity, model peptide-based molecules were synthesized containing poly(aspartic acid), poly(glutamic acid), or a bisphosphonate (pamidronate). These motifs have well-documented affinities to hydroxyapatite, a property desirable for the targeting of molecules to bone for drug delivery and tissue engineering applications. Model peptides of increasing molecular mass (5−33 amino acids) were directly conjugated to eight aspartic acids ($Asp_8$), eight glutamic acids ($Glu_8$), or pamidronate, purified by high-performance liquid chromatography, and characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy. The modified peptides were incubated with hydroxyapatite in phosphate-buffered saline at physiological conditions over 24 h. This study revealed a significant amount (>90%) of conjugated peptides adsorbed to the hydroxyapatite as compared to unmodified peptides (<5%). It was found that while there were significant differences between the different hydroxyapatite-binding and control groups for all time points, the size of the peptide had no statistical effect on peptide−hydroxyapatite binding. These results demonstrate that bisphosphonate and oligopeptide conjugates hold great promise for the development of new bioactive molecules for bone-specific applications.

## Introduction

With an ever-increasing need for organ and tissue replacements, bone loss due to disease or trauma remains a particularly serious health concern. In the field of orthopedic tissue engineering, the central paradigm consists of combining cells, osteogenic growth factors, and a biocompatible scaffold to direct the repair and replacement of tissue.[1] While choosing the appropriate growth factor(s) is of paramount importance, the delivery mechanism and rate also play a critical role in the recruitment and differentiation of cells and the development of new tissue. Most present tissue engineering strategies rely on diffusion of growth factors from a degrading polymeric system, which can lead to the spread of molecules systemically as well as a loss of function during prolonged delivery.[2] The systemic or extraskeletal delivery of many drugs is undesirable due to possible adverse effects at unintended reception sites as well as the obvious inefficiency in drug delivery. Specifically targeting drugs to bone is difficult because of its high mineral content and lack of natural biological target molecules.

Extensive research has been conducted on various bisphosphonates, especially with regard to their affinity for and effects on bone. Bisphosphonates and their drug conjugates are hydrophilic and resistant to chemical and enzymatic degradation.[3] Bisphosphonates present a popular therapy for bone pathologies including osteoporosis and osteogenesis imperfecta.[4] It is believed that internalized bisphosphonates disrupt the mevalonate pathway in the cell metabolism, leading to apoptosis.[5] These effects were seen in higher rates for osteoclasts (bone-resorbing cells) and metastatic tumor cells than osteoblasts (bone-forming cells). Bisphosphonates also reduce the resorption of mineralized bone during the revascularization of a bone defect.[6] In addition to an uncanny attraction to bone tissue, nitrogen-containing bisphosphonates have shown pro-osteoblastic potential in vitro.[7−9] Because osteoblasts are responsible for incorporating the mineral component into mature bone tissue, differentiation of migrating mesenchymal stem cells from the bone marrow down the osteoblast lineage should lead to increased bone formation. This is a favorable property given the ultimate goal of this research: bone regeneration.

Bisphosphonates have demonstrated a remarkable attraction to bone. This affinity arises from the attraction of the diphosphonate moiety to calcium ions present in HA crystal, the mineral component of bone.[10] Specifically for amino-bisphosphonates like pamidronate and alendronate at physiological pH, the bisphosphonate is a zwitterion, possessing negative charges on each phosphonate group while the terminal amine is protonated.[11] The protonated form of pamidronate is shown in Figure 1. Plasma concentrations drop 95% over the 6 h following injection, indicating rapid excretion or bone-binding. Also of great importance, alendronate (like most bisphosphonates) has no natural metabolism and therefore will remain active for its duration of time in the body.[12] At pH 7, the affinity of alendronate for crushed bone particles is described by an apparent dissociation constant of 1 mmol/L and a binding constant of 100 nmol/mg.[13] These high bone-binding, pro-osteoblastic characteristics make nitrogen-containing bisphosphonates, such as pamidronate, a promising candidate for use in bone-targeting applications. Nevertheless, there are some concerns reported with the heavy systemic use of bisphosphonates that must be addressed before the development of any bisphosphonate-based treatment.[14]

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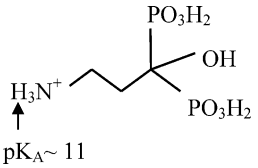


**Figure 1.** Pamidronate fully protonated under acidic conditions.

Poly(aspartic acid) ($Asp_8$) and poly(glutamic acid) ($Glu_8$) oligopeptides (eight consecutive residues of aspartic acid and glutamic acid, respectively) have also exhibited a remarkable affinity to hydroxyapatite (HA) mineral. Not only do they show preferential attraction to bone but also they are enzymatically degraded, do not form colloids with metallic ions, and have no long-term health effects.[3] It has been shown that, with nonspecific conjugation to 6−8 consecutive negatively charged amino acid residues, molecules readily adsorb to certain calcium-containing surfaces.[3,15] Molecules modified with $Asp_6$ exhibited high accumulation in bone in vivo after systemic delivery, while others conjugated to $Asp_8$ showed greater than 90% binding to HA in vitro.[16−17] An inherent advantage to using these oligopeptides is that no unnatural, synthetic chemicals are necessary to achieve the desired calcium affinity for the proposed drug conjugates. The conjugation chemistry of these molecules to peptides is also established and relatively straightforward.

Previous work has been conducted linking molecules to bisphosphonates and acidic oligopeptides in order to confer their bone-binding properties to the conjugate. Uludag and coworkers[10,18] have utilized numerous methods to link bisphosphonic acid moieties to proteins, including the synthesis of small bisphosphonate functional groups and tethering of such groups to larger molecules using thiol and *N*-hydroxysuccinimide (NHS) chemistry. However, that work required bisphosphonates that are not registered as pharmaceutically actively agents (such as alendronate and pamidronate), as well as use of biologically unnatural chemical linkers. The only work to use an FDA-approved, medically prescribed bisphosphonate (alendronate) required 11.2−18.4 mol of nitrogen-containing bisphosphonate/mol of conjugate product.[17] The present work made use of a recognized pharmaceutically active bisphosphonate (pamidronate) and more efficient conjugation chemistry for the development of bone-specific bioactive molecules.

The primary objective of this study was to assess the effects of peptide size on the HA-binding capacity of $Asp_8$, $Glu_8$, and pamidronate bound to peptides. It is hypothesized that these groups, attached at the N-terminus of all peptides, will provide modified drugs with their previously observed high affinity for mineralized surfaces. Another goal of this work was to determine variation in HA binding between the different moieties ($Asp_8$, $Glu_8$, and pamidronate) and control groups. A depiction of the chemical structures of these peptide-bound agents is provided in Figure 2.

## Materials and Methods

**Materials.** Amino acids (L isomers only, when applicable), water-soluble carbodiimide (WSC), *N*-hydroxysuccinimide (NHS), *o*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBT), Wang resins, and other peptide synthesis supplies were purchased from NovaBiochem (San Diego, CA). Anisole, calcium hydroxide, dichloromethane (DCM), dicyclohexylcarbodiimide (DCC), diisopropylethylamine (DiEA), dimethylformamide (DMF), phosphoric acid, tetrafluorophenol (TFP), triethylamine (TEA), trifluoroacetic acid (TFA), and triisopropylsilane (TIS) were purchased from Sigma−Aldrich (St. Louis, MO) and used as received. HA granular microparticles were prepared from calcium hydroxide and phosphoric acid in a pH-controlled reaction.[19,20] Pamidronate disodium salt was synthesized and graciously provided by the University Hospital, University of Regensburg, Germany.

**Peptide Synthesis.** Peptides were produced by solid-state, fluorenylmethoxycarbonyl- (Fmoc-) based peptide chemistry on an Advanced Chemtech Apex 396 peptide synthesizer (Louisville, KY). Acylation was obtained by reacting the N-termini of the peptides with 2 equiv of HOBT, 2 equiv of HBTU, and 4 equiv of DiEA in DMF at room temperature. For peptides conjugated to pamidronate, Fmoc-Glu(O-2-PhiPr)-OH was added as the final amino acid. Its side-chain protecting group was removed with 1% TFA in DCM with mild shaking for 1 h.

**Bisphosphonate−Peptide Conjugation.** To provide a carboxylic acid for reaction with pamidronate, the peptide (still bound to its synthesis resin) was extended by addition of a special glutamic acid [Fmoc-Glu(O-2-PhiPr)-OH]. This glutamic acid (abbreviated E′) possessed a very weak protecting group on its carboxylic acid side chain (R-group) that can be selectively removed with a 1% TFA solution while the Fmoc remains to protect the terminal amine. It is also important to note that the protecting groups remain intact for all other amino acids of the peptide to prevent side reactions and the formation of undesired products.

Resin-bound peptides with deprotected Glu residues (as described above) were dissolved in 5 mL of a DMF/chloroform solution, to which 2 molar equiv of TFP was added. One molar equivalent of DCC was dissolved in 1 mL of the DMF/chloroform solution and added dropwise to the reaction mixture. The solution was shaken at room temperature for 2 h, and then the liquid phase was removed from the resin-bound peptide by vacuum filtration. Resin-bound peptides with a TFP-activated carboxylic acid (TFP-peptides) were dried by vacuum filtration and stored at 0 °C until further reaction.

In a separate vessel, 2 molar equiv of pamidronate disodium were dissolved in 4 mL of Millipore water and 0.5 mL of TEA and sonicated for 10 min. The amount of TEA was based on bisphosphonate concentration such that the final solution pH was greater than 11. The amounts of each reactant used in the conjugation trials can be seen in Table 1. After the bisphosphonate was totally dissolved, the solution was added to TFP-peptides, and the mixture was vortexed for 2 min and shaken at room temperature. To maximize the yield, 2 molar equiv of WSC and NHS were added after 2 h of shaking to reactivate any remaining hydrolyzed or unconjugated carboxylic acids. Exposure of successfully conjugated peptides to WSC does not reverse the reaction, as the amide bond between pamidronate and the peptide was very stable. After a total of 24 h, the liquid was removed by vacuum filtration and the peptide was washed twice with 10 mL of DCM. The resin-bound conjugates were again stored at 0 °C until the time of cleavage. This method was performed twice for each model peptide group to verify reproducibility.

**$Asp_8$– and $Glu_8$−Peptide Conjugation.** Unlike bisphosphonate conjugation, the addition of $Asp_8$ or $Glu_8$ can be achieved in organic solutions. For resin-bound peptides, the addition of $Asp_8$ or $Glu_8$ was achieved by extending the peptide with eight additional aspartic acid or glutamic acid molecules, respectively, by use of standard solid-state peptide synthesis techniques. Like bisphosphonate conjugates, $Asp_8$- and $Glu_8$-conjugated peptides were washed, dried, and stored at 0 °C until cleavage from the resin.

**Product Cleavage, Purification, and Characterization.** Peptide products were cleaved from the resin by exposure to a TFA−TIS−anisole−water (91:3:3:3 by volume) solution for 1 h. The peptide-containing solution was collected via vacuum filtration and dried in a rotory evaporator (37 °C, <25 mmHg). The product was precipitated by addition of cold diethyl ether, centrifuged at 2500 rpm for 5 min, filtered, dried under vacuum, and stored at 0 °C. The crude product was dissolved in DI water to a concentration of 0.5 wt % and purified by high-performance liquid chromatography (HPLC) with a C18 column and using a linear gradient of acetonitrile and DI water containing 0.05% TFA. Peptides and conjugates were analyzed by matrix-assisted laser

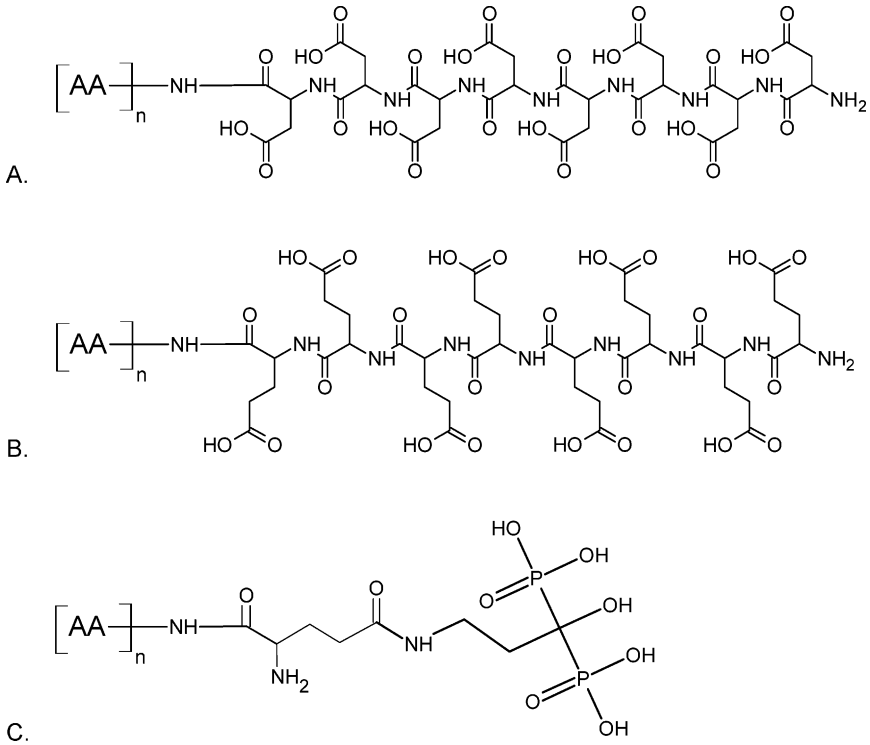


**Figure 2.** Structures of peptides (*n* amino acids in length) modified with (A) $Asp_8$, (B) $Glu_8$, or (C) Glu-pamidronate. Each modification was performed on the terminal amine group of the resin-bound peptide.

**Table 1.** Peptide−Pamidronate Conjugation Reactants

| reactant | amount (mmol) | mass (mg) |
|---|---|---|
| peptide | 0.15 | *a* |
| TFP | 0.3 | 50 |
| DCC | 0.176 | 36 |
| pamidronate | 0.32 | 100 |
| WSC | 0.15 | 40 |
| NHS | 0.15 | 20 |

[a] Varies with sequence.

desorption ionization time-of-flight (MALDI-TOF) mass spectrometry to determine molecular mass. MALDI-TOF was also utilized to verify the addition of pamidronate, $Asp_8$, and $Glu_8$ to the peptide conjugation products.

**In Vitro Binding of Peptides and Peptide Conjugates to HA.** Peptides were synthesized with an aromatic residue as a signaling agent (UV absorbance calibrated to determine peptide concentration) and a semirandom repeat pattern. Four different peptide sizes were tested, from 5 to 33 amino acids in length. The sequences were Y*GNAE, Y*GNAEGNAR, Y*$[GNAEGNAR]_2$, and Y*$[GNAEGNAR]_4$ (Y* represents tyrosine for peptides in the control, $Asp_8$, and $Glu_8$ groups, and tryptophan for the pamidronate group), yielding 16 total groups. Hydrophobic/hydrophilic interactions were balanced by use of glycine and alanine (hydrophobic residues) with asparagine, glutamic acid, and arginine (hydrophilic residues). To balance the net charge within the model peptide sequence, glutamic acid (negative at physiological pH) and arginine (positive) were alternated. All tests were performed in triplicate ($n = 3$).

Peptides were dissolved (0.25 mM) in a phosphate-buffered saline solution (PBS, pH 7.4) at 37 °C. In Eppendorf centrifuge tubes, 10 mg of HA (100−300 $\mu$m microparticles) and 200 $\mu$L of peptide solution were mixed via vortex for 1 min and placed in a shaker bath at 37 °C. Each formulation was cultured for 0.5, 1, 2, 4, 8, and 24 h. After the prescribed time, tubes were removed from the water bath and centrifuged for 2 min at 2500 rpm. Aliquots (100 $\mu$L) of the supernatant solution were removed and placed in 96-well flat-bottom plates. Standard peptide solutions were also prepared from 0.01 to 1 mM to generate a concentration calibration curve. Concentrations for each solution were determined by UV absorbance on a Tecan GENios plate reader (Durham, NC) at 280 nm.

**Statistics.** Data were interpreted by two-way analysis of variance (ANOVA) for the effects of each binding group (control, $Asp_8$, $Glu_8$, and pamidronate) and peptide length on HA binding at all time points. Tukey's HSD test was performed for factors determined to have a significant effect on HA binding. ANOVA and Tukey's tests were conducted with JMP IN version 5.1 statistical discovery software.

## Results

**Bisphosphonate−Peptide Conjugation.** Pamidronate was covalently linked to resin-bound peptides via amide bond between the primary amine of the bisphosphonate and the deprotected carboxylic acid on the peptide's final glutamic acid residue. Peptides before and after bisphosphonate conjugation were analyzed via MALDI-TOF to determine their molecular mass. MALDI-TOF spectra for the WGNAEE−pamidronate and W$[GNAEGNAR]_4$E−pamidronate conjugated products are shown in Figure 3. The shift in primary peaks was roughly 216 Da (corresponding to the molecular mass addition of the bisphosphonate and loss of one water molecule) for each peptide, with a secondary peak shifted approximately 22 Da more due to sodium ionization. The lack of peak at the molecular mass of the respective unmodified peptide should also be noted.

**Effects of Peptide Size on HA Binding in Vitro.** Modified peptides exhibited immediate binding to HA, with over 90% of initial peptides bound after only 2−4 h (binding data for the smallest and largest peptide sequences are shown in Figures 4 and 5). The concentration of unmodified and conjugated peptides (5−33 amino acids in length) was measured over 24 h in culture with HA. It was observed that, for each HA-seeking domain, molecular mass of the peptide had no significant effect on the conjugate's binding rate ($p > 0.05$). The only contributor to significant differences in binding was the HA binding group.

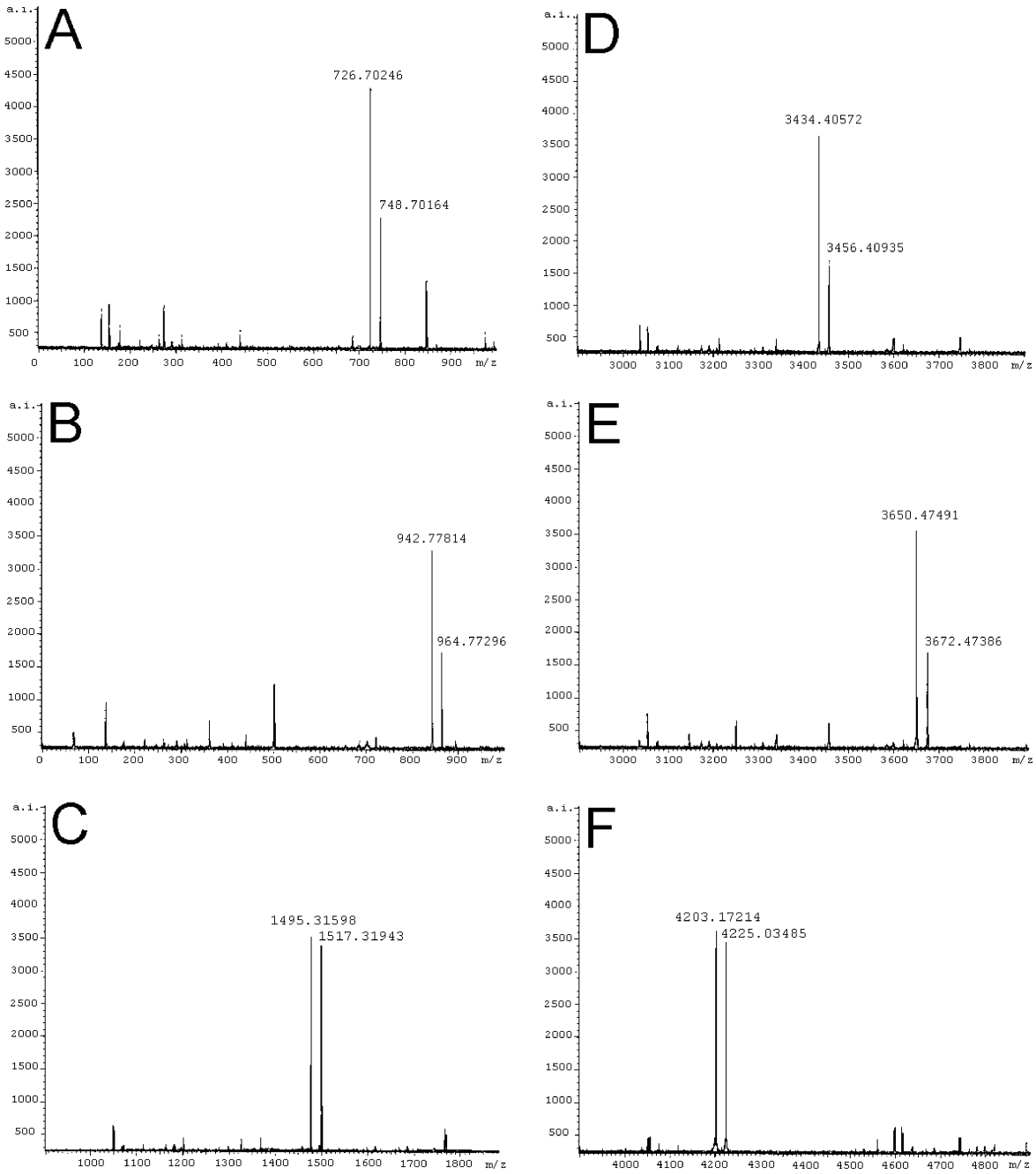


**Figure 3.** MALDI-TOF mass spectrographs indicating molecular masses of the smallest and largest peptide groups, as well as the corresponding peptide−bisphosphonate and peptide−$Asp_8$ conjugates examined: (A) WGNAE−E, (B) WGNAE−E-pamidronate, (C) YGNAE−$D_8$, (D) W[GNAEGNAR]$_4$−E, (E) W[GNAEGNAR]$_4$−E-pamidronate, and (F) Y[GNAEGNAR]$_4$−$D_8$.

**Comparing the HA Affinities of $Asp_8$-, $Glu_8$-, and Pamidronate-Conjugated Peptides.** The choice of HA binding group had a significant effect on the percentage of bound peptides ($p < 0.01$). For each peptide length, the bisphosphonate conjugate initially exhibits less HA binding than the oligopeptide analogues (the difference between pamidronate and $Asp_8$/$Glu_8$ conjugates was statistically significant at 0.5 and 1 h time points). From 2 to 24 h, all conjugates for each model sequence (all lengths) are not statistically different from one another. All modified peptides reached equilibrium between 90% and 95% bound by 24 h. Unmodified control peptides showed no greater than 4% mean binding. The binding of various-sized peptides for each targeting moiety at 0.5 and 24 h is shown in Figures 6 and 7.

## Discussion

The objective of this research was to produce model peptides, chemically linked to calcium-binding moieties, that demonstrate high attraction to bone. While bones contain few biologically active targets, the compounds examined in this study can utilize this perceived targeting disadvantage in order to adhere (and anchor any conjugated agents) to bone's mineral surface. Early studies employing similar strategies with proteinlike molecules for systemic skeletal delivery have shown great promise in bone binding in vivo.[21]

The exact mechanisms for the preferential binding bisphosphonates and poly(aspartic acid) to bone have never been completely explained in the literature. It is hypothesized that the structures provide favorable ionic interactions between the

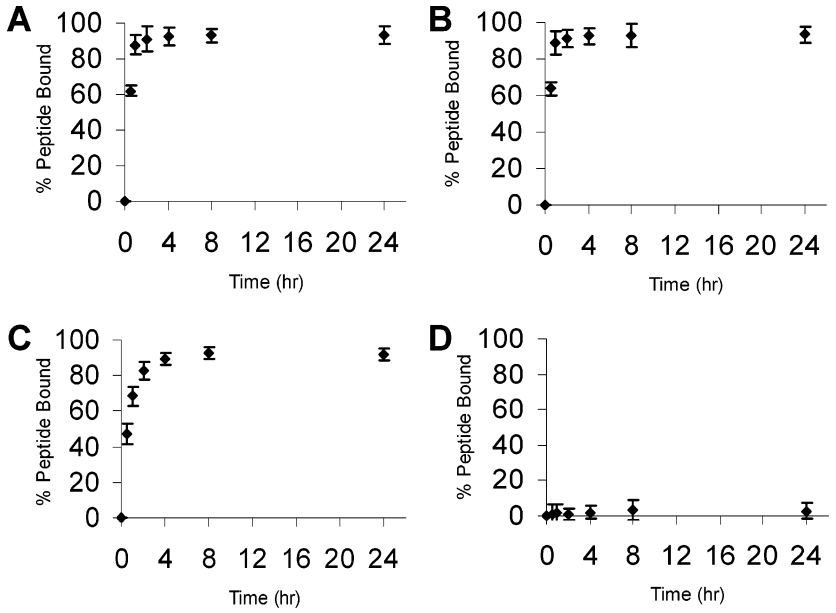


**Figure 4.** Change of percent peptide bound to hydroxyapatite for short (5 amino acids, Y*GNAE) peptides conjugated to (A) $Asp_8$, (B) $Glu_8$, and (C) pamidronate and (D) unmodified peptides. Error bars designate standard deviation for $n = 3$.

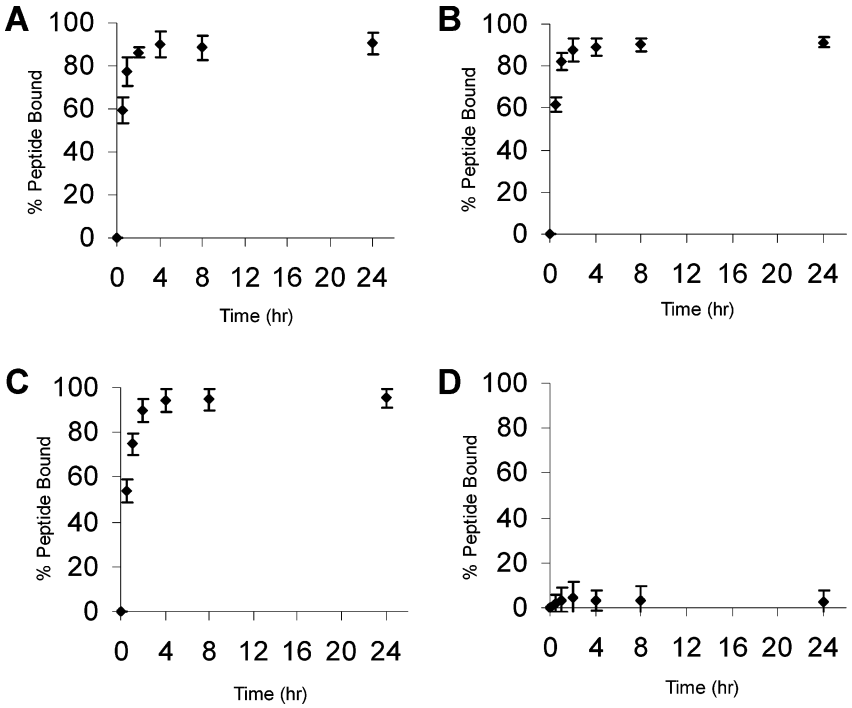


**Figure 5.** Change of percent peptide bound to hydroxyapatite for long (33 amino acids, Y*$[GNAEGNAR]_4$) peptides conjugated to (A) $Asp_8$, (B) $Glu_8$, and (C) pamidronate and (D) unmodified peptides. Error bars designate standard deviation for $n = 3$.

negative charges of these groups ($Asp_8$, $Glu_8$, and pamidronate) and calcium ions within the mineral component of bone (hydroxyapatite) at physiological pH.[22] This study was not intended to discover the mechanisms of this attraction but rather to impart the known bone-binding properties of these chemicals to model peptides.

Pamidronate was successfully coupled directly to peptides via an amide bond between the primary amine of the bisphosphonate and a carboxylic acid on the terminal amino acid of the resin-bound peptides. The method described here used a 2:1 bisphosphonate to peptide ratio with only an amide bond between the molecules, which is significantly more efficient than previously reported methods.[17] Significant obstacles presented themselves in conjugating pamidronate to a resin-bound peptide. First, this design strategy calls for linkage of the primary amine of pamidronate to a terminal amino acid of the model peptide via amide bond (highly stable under physiological conditions). Unfortunately, solid-state peptide synthesis is based on building peptides from the carboxyl to the amine terminus, meaning the amine is the exposed functionality at the end of the model peptide. Amine−amine conjugation is generally nonspecific, meaning some peptides would conjugate to each other, as would bisphosphonate molecules.

A more selective method explored in this work transformed the terminal functional group of the peptide to a carboxylic acid to form a stable amide bond with the amine of the bisphosphonate. An available carboxylic acid on the terminal amino acid was activated with an electronegative leaving group, making

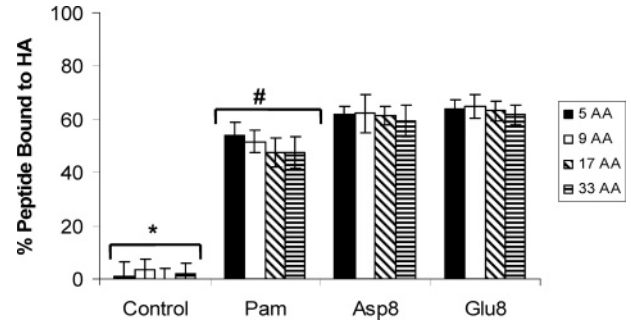


**Figure 6.** Percentage of peptides bound to hydroxyapatite at 0.5 h for control, $Asp_8$-, $Glu_8$-, and pamidronate-modified groups (all peptide sizes). Error bars indicate standard deviation for $n = 3$. *Controls were significantly different than all binding groups for each peptide size; #pamidronate conjugates were significantly different than $Asp_8$ and $Glu_8$ groups for each peptide size.

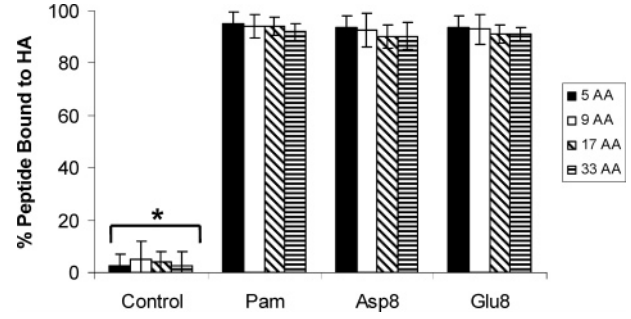


**Figure 7.** Percentage of peptides bound to hydroxyapatite at 24 h for control, $Asp_8$-, $Glu_8$-, and pamidronate-modified groups (all peptide sizes). Error bars indicate standard deviation for $n = 3$. *Controls were significantly different than all binding groups for each peptide size.

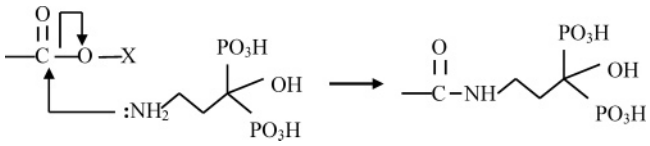


**Figure 8.** Conjugation reaction of pamidronate to the carboxylic acid of glutamic acid (X = leaving group).

the carbon atom susceptible to nucleophilic attack by the pamidronate amine's electron pair. A depiction of this scheme is shown in Figure 8. To achieve this, Fmoc-Glu(O-2-PhiPr)-OH was added to the N-terminal end of resin-bound peptides as described in the Materials and Methods section.

An alternative method for generation of a new carboxylic acid group was conversion of the terminal amine of the peptide. The reaction of succinic anhydride with primary amines is an established method to achieve this conversion. Model peptides were reacted in this manner to form corollary conjugates (data not shown), but this approach was deemed less desirable due to the use of biologically unnatural materials (succinic anhydride) and the requisite extra mass added to the final product.

Another challenge of this chemistry was the lack of swelling of the polystyrene peptide resins in water. Bisphosphonate salts were insoluble in mixtures of >95% organic solvents, rendering traditional peptide chemistry techniques ineffective. When the bisphosphonate conjugation was performed in an aqueous solution, the polystyrene resin can fold on itself to minimize thermodynamically unfavorable contact with water. This compacted resin left many or most of the peptide chains unexposed to the reaction solution. To overcome this dilemma, the standard Wang resin was replaced with a NovaSyn TGA resin (NovaBiochem, San Diego, CA). The NovaSyn resin contained a poly(ethylene glycol) (PEG) spacer between the polystyrene and the cleavage site prior to the first amino acid. This PEG spacer was soluble in both aqueous and organic solutions, ideal for peptide synthesis and conjugation in dual phases.

The most critical impediment to this conjugation chemistry is the highly acidic nature of pamidronate (and alendronate) sodium salts. There are five ionizable protons on these bisphosphonates (two on each phosphonate group and one on the primary amine).[23] While the primary amine is protonated, it will not act as a nucleophile to attack the activated carboxylic acid and form a peptide bond. The $pK_a$ of the amine's proton is above 11, meaning an equal or higher reaction pH is necessary for the conjugation. Typical amino acid coupling by amide bond formation is conducted by reaction of the carboxyl group with an activating molecule such as DCC or WSC. The activation agent is electron-withdrawing, making the carboxyl vulnerable to nucleophilic attack by the amine's electron pair under mildly basic conditions. The primary amine of pamidronate is protonated (as $-NH_3^+$) below pH 11 and unable to attack the carboxyl without its free electron pair. However, at such a high pH, standard carboxyl activating agents are hydrolyzed and rendered useless. To overcome this dilemma, the deprotected carboxylic acid of E′ is activated to a significantly more stable intermediate with TFP by a modified version of a previously described reaction.[24] The TFP intermediates were considered very stable and could be preserved for substantial amounts of time and in different solvents. Although the $Asp_8$ and $Glu_8$ peptide conjugates synthesized in this study employed previously established peptide chemistry methods (addition during synthesis), use of the TFP-activated intermediate technique can be applied to the conjugation of poly(Asp) and poly(Glu) oligopeptides to primary amines without the requirement of elevated pH or an aqueous reaction solution used in the reaction with pamidronate.

Peptides of four different lengths/molecular masses were conjugated to $Asp_8$, $Glu_8$, or pamidronate as described previously. Each group was incubated with HA in PBS over 24 h to determine the effects of (a) the different calcium-binding moieties and (b) peptide molecular mass on the conjugate's HA affinity. These studies were conducted at physiological conditions (pH 7.4, 37 °C) to draw conclusions about the likely binding properties of modified peptides in vivo. All modified peptides demonstrated rapid adsorption during the first 2 h. Unmodified peptides exhibited practically no affinity for HA, especially when compared to the conjugated versions. The molecular mass of the peptides had no statistically significant effect on the molecule's attraction to HA. $Asp_8$- and $Glu_8$-conjugated peptides appeared to show greater or more rapid binding than bisphosphonate-incorporated molecules for all peptide masses, with differences between pamidronate and $Asp_8$/$Glu_8$ conjugates statistically significant at 0.5 and 1 h time points. By 8 h, all modified groups exhibited greater than 90% binding and approached their respective binding plateaus. The added HA affinity of peptides modified with $Asp_8$, $Glu_8$, or pamidronate, compared to unmodified counterparts, was significant at all time points and for all peptide lengths and is promising for the modification of other biologically active molecules.

The precise mechanisms for the early binding advantage of the $Asp_8$ and $Glu_8$ oligopeptides over pamidronate are not yet fully understood, but it could be a result of the larger region of negative charges to associate with calcium in the mineral crystal. The greater total binding at later time points for bisphosphonate conjugates could be a result of more specific binding to HA than for the $Asp_8$- or $Glu_8$-conjugated molecules. Regardless of the mechanism, the combining of all these molecules to

larger-sized peptides resulted in the conference of HA attraction to the product.

The addition of $Asp_8$, $Glu_8$, and bisphosphonates such as pamidronate to larger molecules to confer HA affinity is promising for the future of bone drug delivery, both systemically and locally. Some drugs influence cells via interactions with receptors on the cell surface and need not be released from the chemical anchoring them to bone. For drugs that must be internalized by the cell or require conformational flexibility, it is envisioned that hydrolytically or enzymatically degradable linkers will be used to connect the drug with the bone-binding moiety.

## Conclusion

In order to more efficiently deliver molecules to bone tissue, moieties with high calcium affinity were covalently linked to model peptides. The ability of $Asp_8$, $Glu_8$, and pamidronate to confer their attraction to HA in vitro to larger conjugated molecules was observed for peptides ranging from 5 to 33 amino acids in length. $Asp_8$ and $Glu_8$ conjugates exhibited a faster initial binding than those of pamidronate, but after 2 h the bisphosphonate-linked peptides achieved equivalent binding to their counterparts (>90% bound) from hours 4 through 24. The differences between the bisphosphonate and oligopeptide conjugates were significant only at the 0.5 and 1 h time points. The molecular mass of the peptide was found to have no statistically significant effect on HA binding. It can be reasoned that such HA affinity can be provided to other molecules of similar (and potentially larger) size and molecular mass of these model peptides. The findings of this work are critical in the development of future drugs with the capacity to localize in bone tissue as well as improved bone tissue engineering scaffolding materials.

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