

Stability of Disulfide-Linked Fetuin-Bisphosphonate Conjugates for Increased Mineral Affinity

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Introduction: An effective therapeutic agent intended for the treatment of bone diseases is expected to exhibit a high affinity to bone. Hydroxyapatite (HA) is the major component, and an essential ingredient of normal bone. It can be utilized to act as an “affinity template” for proteins desired for bone targeting [1]. To develop bone-specific protein delivery systems, chemical derivation of proteins with bisphosphonates (BPs) was designed to impart mineral affinity to proteins and improve their therapeutic potential. BPs linked to proteins with cleavable linkages were considered desirable over the conjugates linked with stable linkages, since proteins could be released in free form *in situ* to facilitate the interactions between proteins and their intended biological targets on cellular surfaces. The main objective of this study was to investigate the conjugation of 2-(3-mercaptopropylsulfonyl)-ethyl-1,1-bisphosphonic acid (thiolBP) to fetuin, an important circulating inhibitor of calcification *in vivo* [2], by disulfide linkage. This linkage was readily cleaved in the presence of three physiological thiols, *L*-cysteine, *DL*-homocysteine, and *L*-glutathione (reduced), which are most abundant in serum. These results are preparatory to further studies designed to evaluate the potential application of the fetuin conjugates *in vivo*.

Methods: The thiolBP was synthesized from tetraethyl methylenebisphosphonate (Fig.1). The structure was confirmed by ¹H-NMR [3]. The fetuin-thiolBP conjugates were prepared by using the disulfide-containing crosslinker N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Fig.1). The extent of BPs substitution per fetuin (no. of thiolBP/fetuin) was determined by a phosphate assay [4] and the Bradford protein assay [5]. The mineral affinity of the samples was determined using a HA binding assay [6]. *L*-Cysteine, *DL*-homocysteine and *L*-glutathione (reduced) were studied for the cleavage of the disulfide linkage of the conjugates (Fig.2). For conjugate cleavage in solution, the protein were incubated with various concentrations of chosen thiols for 24 hours, and then dialyzed against 50 mM Tris buffer (pH=7.0) to remove the thiols and the cleaved thiolBP. In addition, the conjugate stability after binding the conjugates to HA was determined by initially binding the conjugates to HA for 3 hours and then

incubating the bound proteins with various concentrations of thiols. The samples were centrifuged and aliquots of the supernatant were taken at various time points to determine the protein concentrations.

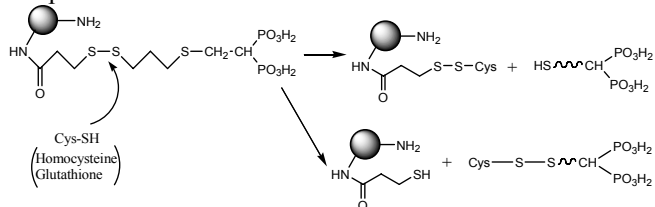


Fig. 2 Cleavage of the conjugates with three physiological thiols

Results / Discussion: The extent of thiolBP substitution on fetuin can be controlled by varying the concentration of crosslinker SPDP. The HA binding was also proportional to the extent of BP substitution. All three thiols chosen for this study cleaved the conjugates (Fig.3). *DL*-homocysteine was found to be most reactive towards the disulfide cleavage, possibly due to less steric hindrance of the thiolate anion for the disulfide attack. The imparted bone affinity as a result of thiolBP conjugation, as assessed by HA binding *in vitro*, was eliminated upon the cleavage of the disulfide linkage (Fig.3). Similar to cleavage in solution, fetuin-thiolBP conjugates adsorbed to HA were also cleaved when the three thiols were present in the binding medium, *DL*-homocysteine being the most reactive again. Comparing the outcome of cleavage in solution and on HA surface, there was no significant difference between these two ways of cleavage ([thiol]<1mM), suggesting that adsorbed fetuin-thiolBP conjugates are freely accessible to the thiols.

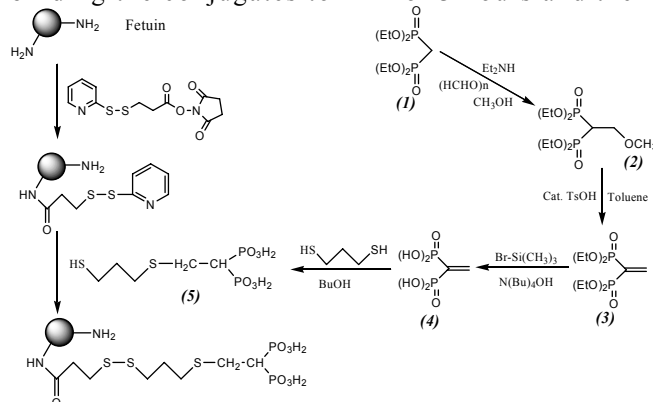


Fig. 1 Synthesis of thiolBP and fetuin-thiolBP conjugate

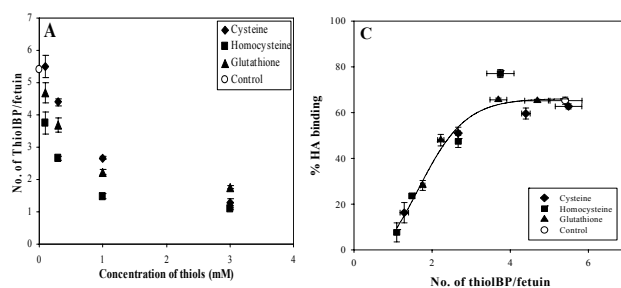


Fig.3 Cleavage of fetuin-thiolBP conjugates (left) in solution after incubating with thiols for 24 hours. The correlation between %HA binding and extent of BPs substitution for conjugates treated with the thiols (right).

Conclusions: In conclusion, it was possible to impart mineral affinity to a model protein (fetuin) by using BPs via a disulfide linkage. Three thiols most abundant in serum were all effective in cleaving the disulfide linkage to release the protein. Future studies will focus on *in vivo* delivery of protein-BPs conjugates in order to assess cleavage kinetics of the disulfide-linkage under physiological conditions.

References: [1] Uludag et al., J Biomed Mater Res 2000; 50:227-238. [2] Price et al. (2003) J. Biol. Chem. 24: 22144-22152. [3] Bansal et al. JBMR: Part A, in press. [4] Ames BN. Meth. Enzymol. (1966) 8, Academic Press, NY, pp.115-117. [5] Bradford MM. (1976) Anal. Biochem. 72: 248-254. [6] Uludag et al., Biotech Prog 2000; 16:258-267.