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Conjugation of a dipicolyl chelate to polypeptide conjugates increases binding affinities for human serum albumin and survival times in human serum

Aleksandra Balliu [b] and Lars Baltzer [a]

Abstract: The affinity for human serum albumin (HSA) of a series of 2-5 kDa peptides covalently linked to 3,5-bis[[bis(2-pyridylmethyl)amino]methyl]benzoic acid, a dipicolyl chelator with μM affinity for Zn^{2+}, was found by surface plasmon resonance to increase in the presence of 1μM $ZnCl_2$ at physiological pH. The dependence on polypeptide hydrophobicity was found to be minor, suggesting that the conjugates bound to the metal binding site and not to the fatty acid binding site. The affinity of the conjugates increased strongly with the positive charge of the polypeptides suggesting proximity to the negatively charged protein surface surrounding the metal binding site. The survival times of the peptides in human serum were extended as a consequence of stronger binding to HSA suggesting that Zn^{2+} ion chelating agents may provide a general route to increased survival times of peptides in serum in therapeutic and diagnostic applications without significantly increasing their molecular weights.

Introduction

Recombinant proteins and peptides are promising pharmaceutical and diagnostic agents although they suffer from poor survival times in blood due to fast renal clearance and in the case of peptides also from rapid proteolytic degradation [1]. The molecular weight cut-off for renal clearance is believed to be 30-50 kD and small organic molecule drugs would be excreted rapidly unless they bound tightly to circulating plasma proteins [2] Efforts to reduce the rate of clearance of peptides and recombinant proteins are based either on increasing the molecular mass, i.e through protein fusion, glycosylation or the addition of polyethylene glycol polymers [3] or on the development of approaches that aim to improve the half-life during in vivo drug exposure[4]. Noncovalent association with human serum albumin (HSA) provides long circulation times in the blood and protection from immune cells, thus improving the efficacy and pharmacokinetic behavior. There is a fine balance between affinity and dissociation rate. Strong binding and a slow off-rate from HSA may impair rather than improve binding whereas too fast an off-rate will not prevent rapid renal clearance. Efforts to take advantage of and understand the structure and function of HSA–drug complexes are therefore of considerable interest [2, 5]

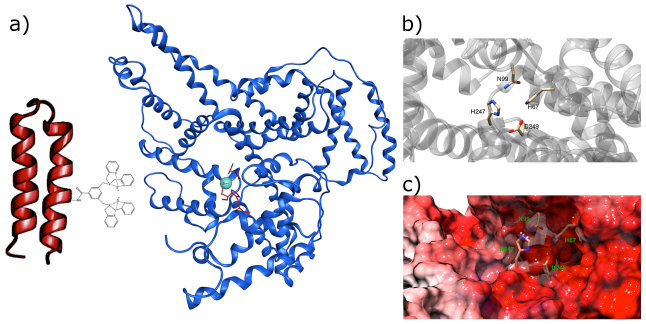
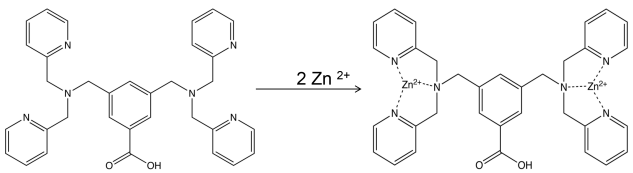


Figure 1. Illustration of the concept. a) Crystal structure of human serum albumin (HSA) (PDB ID code 4K2C) and cartoon of polypeptide conjugate formed from a 42-mer polypeptide and 3,5-bis[[bis(2-pyridylmethyl) amino] methyl]benzoic acid (PP1). The PP1 group, covalently linked to the side chain of Lys-8 of the polypeptide, is known to form a chelate with Zn^{2+} ions. The chelate hypothetically coordinates to the Zn^{2+} ion of the Zn-binding site of HSA and the polypeptide provides further interactions with the protein. b) Detail from crystal structure showing key Zn^{2+} binding residues in Zn^{2+} binding site (His-67, Asn-99, His-247, Asp-249) located between domain I and II of HSA. c) Molecular surface representation with charge distribution of negatively charged HSA Zn2+ binding site. Deep red colour indicates charge of -10. Polypeptide conjugate not drawn to scale.



Scheme 1. Structure of 3,5-bis[[bis(2-pyridylmethyl) amino] methyl]benzoic acid (PP1) and its complex with Zn^{2+} ions.

HSA is a single–chain plasma protein with a number of physiological functions. The high plasma concentration of 0.6 mM of HSA ensures high ligand binding capacity and HSA transports a vast number of endogenous biomolecules including fatty acids (FA), amino acids, hormones, metals and toxic metabolites in the blood stream [5b]. It is synthesized and secreted from liver tissue as a non-glycosylated single polypeptide chain with 585 amino acids and a molecular mass of

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66.5 kDa. Structurally, HSA is mainly composed of α-helices with nine extended loops in three homologous domains, domain I (residues 1–195), domain II (residues 196–383) and domain III (residues 384–585), Figure 1. Each domain possesses two long loops and one shorter loop and is further divided into subdomains A and B. HSA contains 35 cysteine residues, and all of them except Cys-34 are involved in disulfide bond formation stabilizing the tertiary structure. Subdomains with separate helical structures bind various endogenous and exogenous ligands in high affinity binding sites. Sites I and II are the two most important binding sites located in subdomains IIA and IIIA, respectively. Crystallographic studies have shown that the IIIA subdomain is a potential binding site for fatty acids, whereas drug binding to HSA usually occurs in the IIA subdomain. Site I in subdomain IIA is a large hydrophobic cavity that facilitates the simultaneous binding of several drug molecules [5a, 6]. The majority of drugs that interact with HSA are anionic or neutral. Nonetheless, recent studies have shown that a number of cationic antimicrobial peptides as well as few cationic drugs bind HSA with significant affinity [7].

In addition to the high binding capacity of HSA for exogenous drugs, its affinity for a variety of essential and toxic metal ions have been well characterized. HSA possesses a distinct binding site for Zn^{2+} and assists in the delivery of this essential element to cells and tissues[8]. The total concentration of Zn^{2+} in plasma is approximately 19 μM and close to 80% of Zn^{2+} exists in the form of a complex with albumin with an apparent dissociation constant of 30 nM [8-9]. X-ray crystallographic and NMR spectroscopic studies revealed a five-coordinate Zn^{2+} binding site at the interface between domains I and II containing the highly conserved amino acids His-67, His-247, Asn-99, and Asp-249, Figure 1[8-10]. It is interesting to note that the surface area close to the Zn^{2+} binding site is highly negatively charged.

While it may appear tempting to modify bioactive peptides to increase the level of positive charge or the hydrophobicity as a strategy to increase the binding affinity for HSA, the risk is considerable that affinities will be decreased due to impaired interactions with the target protein. In contrast, the alternative strategy to increase the affinity for HSA by conjugating small organic molecules that target specific HSA binding sites does not entail modifications to the peptide sequence. A small organic molecule conjugated to a peptide via a linker can be far enough removed from the binding interface not to interfere with peptide-protein interactions. While one should not underestimate the complexity of targeting proteins, and thus that even a linker may interfere with binding interactions, the present approach allows for very moderate disturbance as well as tunability of those interactions. Binding sites of particular interest are those that sequester metal ions, as small organic metal chelating agents are readily incorporated into peptides without severely increasing the molecular weight and because these sites have not been addressed previously for HSA binding. Addressing the Zn^{2+} binding site would therefore increase the available toolbox for complexation of HSA in bioanalytical and biomedical applications. In addition, it leaves other sites free for the simultaneous binding of ligands with other molecular properties.

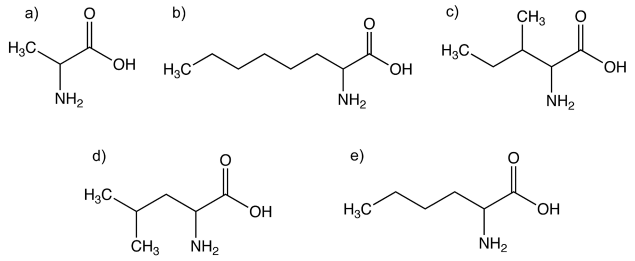
Here we report on a metal-binding site targeting strategy based on the covalent incorporation of a dipicolyl Zn^{2+} chelating agent, 3,5-bis[[bis(2-pyridylmethyl)amino] methyl]benzoic acid (PP1), Scheme 1,[11] into a set of polypeptides of varying charge, size and hydrophobicity. We report on the effect of chelator incorporation on HSA affinity as well as on the effect on the survival times in human serum.

Results and Discussion

In order to investigate by surface plasmon resonance (SPR) analysis the effect on HSA affinity of incorporating a small organic molecule Zn^{2+} ion chelating agent into peptide sequences, a number of 42-residue polypeptides were selected from a previously reported set [12], Figure 2. The sequences were chosen to allow an evaluation of HSA binding as a function of charge as well as of hydrophobicity. Each of the polypeptides were covalently linked at the side chain of Lys-8 to PP1, a ligand known to form a chelate with Zn^{2+} ions with dissociation constants in the μM range, Scheme 1. As a control, one of the polypeptides, 4-C15L8, was prepared without the PP1 ligand, and the side chain of Lys-8 was instead acetylated to form 4-C15L8-Ac. Also, the polypeptide corresponding to 4-C15L8-PP1 was synthesized with norleucine, isoleucine and leucine residues replaced by L-2-aminooctanoic acid, Scheme 2.

1-C15L8
Ac-NEADL EA**K**IR HLAE*K* LEARG PEDCE QLAEQ LARAF EAFAR KG-OH
2-C15L8
Ac-NEADL EA**K**IR HLAE*K* LAARG PVDCA QLAEQ LARAF EAFAR KG-OH
3-C15L8
Ac-NAADJ EA**K**IR HLAE*K* JAARG PVDCA QJAEQ LARRF EAFAR KG-NH_2
4-C15L8
Ac-NAADJ EA**K**IR HLRE*K* JAARG PRDCA QJAEQ LARRF ERFAR KG-NH_2
5-C15L8
Ac-NAARL EA**K**IR RLRE*K* LAARG PRDCA QLREQ LARRF ERFAR KG-NH_2
VI
Ac-NAADAoc EA**K**AocR HAocRE*K* JAARG PRDCA QJAEQ LARRF ERFAR KG-NH_2
VII Ac-DAocEA**K** AocRHAocR G-NH_2

Figure 2. The amino acid sequences in the one letter code of polypeptides selected for conjugation to PP1 in order to evaluate binding to human serum albumin. Lysine residues where PP1 was incorporated are shown in boldface, lysine residues where coumarin was introduced are shown in italics and L-2-aminooctanoic acid (Aoc) is colour coded red for clarity. No coumarin was incorporated in **VII.** Orthogonal protection of the Cys residue in position 24 and of the Lys residue in position 41 was obtained using Acm and Tfa groups, respectively.



Scheme 2. *Chemical structures of a) alanine, b) L-α-aminooctanoic acid, c) isoleucine, d) leucine and e) norleucine.*

All peptides were synthesized on the solid phase using standard Fmoc synthesis protocols, purified by reversed phase HPLC and identified by MALDI-TOF mass spectrometry. They were conjugated to PP1 in solution after cleavage from the resin and purification by HPLC.

The design of the 42-mer polypeptides has been described in detail, previously [12]. In short they were designed to form two amphiphilic helices connected by a short loop. A total of eight hydrophobic residues were introduced and conserved in all sequences, whereas the distribution of charged residues as well as the site of incorporation of the small molecule ligand was varied, each parameter in four steps, giving a total of sixteen sequences. Polypeptide total charges were varied from -7 to +2 in steps of 3 and the ligand was introduced at the side chain of Lys-8, Lys-16, Lys-22 or Lys-34. The polypeptides selected in the present investigation from the original set of sixteen were all conjugated to PP1 at the side chain of Lys-8, and the total peptide charges varied from -7 to +2. In addition, as the role of the charge turned out to be quite significant, a polypeptide with the total charge of +5, the sequence 5-C15L8, was also included. In aqueous solution the polypeptides dimerize to form four-helix bundle proteins at high μM concentrations but form unordered monomeric structures at nM concentrations [12]. Conjugated to small organic molecules known to bind proteins they form binder molecules with dramatically increased affinities as well as increased selectivities compared to those of the small molecules [13]. We hypothesize that their sequences can adopt conformations that are complementary to many protein structures and therefore provide enhanced affinities for a large number of different proteins. Their capacity for protein recognition and thus their potential for biomedical applications suggested that these polypeptides would be suitable candidates for an evaluation of molecular properties that govern HSA binding. The incorporation of PP1 into the 42-mer polypeptide 4-C15L8 to form 4-C15L8-PP1 was previously shown to give rise to a polypeptide conjugate capable of binding glycogen phosphorylase a with nM affinity [17]. The bidentate Zn^{2+} chelate was shown to bind the side chain of the phosphorylated Ser-15 residue while the polypeptide interacted with the protein surface providing additional binding energy. PP1 binds Zn^{2+} ions with dissociation constant in the μM range and we hypothesized that the chelator would coordinate also to the Zn^{2+} ion in the Zn^{2+} binding site of HSA and the polypeptides interact with the protein to a larger or lesser extent depending on charge and hydrophobicity. The concentration of Zn^{2+} ions found in human serum [9] is above the level required for chelate formation and the chelate would therefore be expected to form also in vivo.

The observation that cationic peptides bind tightly to HSA has been reported previously [7b, 7c]. In order to compare the relative influence of charge versus metal ion chelation the 42-mer polypeptide conjugates 1-C15L8-PP1, 2-C15L8-PP1, 3-C15L8-PP1, 4-C15L8-PP1 and 5-C15L8-PP1 as well as the polypeptide, 4-C15L8-Ac where the side chain of Lys-8 was acetylated and did not carry the PP1 ligand, were examined by SPR with regards to HSA binding, Figure 3, Figure 4. The charge of the selected polypeptide conjugates varied from -7 to +5 in steps of 3. A Biacore 2000 instrument was used to record the sensorgrams. HSA was immobilized on the chip and the polypeptide conjugates were included in the running buffer. Precautions were taken not to overload the chip and immobilization of HSA was limited to an upper level of 7000 RUs. The relative amounts of binding were measured at 4, 20, 100 and 500 nM concentrations of polypeptides and polypeptide conjugates in the presence or absence of 1 μM $ZnCl_2$ in 10mM HEPES buffer at pH 7.4. The amount of binding was considered a reliable qualitative measure of relative affinities when large differences were observed. An equation describing a 1:1 model was fitted to the experimental results, SI, Table S2. The dissociation rate constants that do not depend on concentration were used in combination with the amount bound to rank the polypeptide conjugates with regards to affinity. For the association rate constants the fitting gave rise to large errors, most likely because the state of aggregation in solution varied with polypeptide as well as with concentration. We do not emphasize the measured dissociation constants for that reason.

Judging from the amount of bound polypeptide conjugate the more positively charged the polypeptide, the stronger the binding to HSA, in agreement with previous reports on cationic peptide-protein interactions. While quantitative data would be preferred, the weak binding of 1-C15-L8-PP1, 2-C15L8-PP1 and 3-C15L8-PP1 prevents the fitting of an equation to the experimental data. However, the very large differences between the amount bound provide strong evidence for the ranking of the polypeptide conjugates. The sensorgrams of the tightest binders, 4-C15L8-PP1 and 5-C15L8-PP1 were recorded also in the absence of $ZnCl_2$, Figure 5. The reduced amount of bound peptide observed in the absence of Zn^{2+} ions demonstrated that PP1 conjugation increased the affinity for HSA of the cationic polypeptides. In a control experiment, the affinity for HSA of 4-C15L8-Ac in the presence and absence of Zn^{2+} ions was evaluated by SPR, Figure S2. No binding of HSA was detected in either case and the increase in affinity for HSA of the polypeptide conjugates could therefore not be due to the presence of Zn^{2+} ions alone. The combined observations that polypeptides conjugated to PP1 bind more strongly in the presence than in the absence of $ZnCl_2$ and that a polypeptide conjugated to the PP1 group binds more strongly than a polypeptide conjugated to an acetyl group in the presence of $ZnCl_2$ further suggests that the PP1 in fact facilitates binding to the Zn^{2+} binding site of HSA. The effect of PP1 conjugation on the negatively charged polypeptides could not be measured as no binding was observed for the most negatively charged polypeptide conjugates, 1-C15L8-PP1 and 2-C15L8-PP1 even at 500 nM concentration. The highly negatively charged surface in close proximity to the Zn^{2+} binding site is likely to account for the observed strong interactions with cationic polypeptides as well as the poor interactions with negatively charged ones, Figure 1. The results suggest that weakly cationic peptides may benefit the most from PP1 conjugation. It has been reported previously that there is a cooperative effect of metal ions on the binding of hydrophobic compounds to HSA, and the structural influence of Zn^{2+} ions could in principle also play a role in the binding of hydrophobic peptides [14]. This effect is unlikely to play a significant role in the present case since the acetylated polypeptide 4-C15L8-Ac was not found to bind HSA in the presence of $ZnCl_2$, Figure S2. The observation is compatible

with a model where the PP1 conjugates do not bind to the hydrophobic binding site.

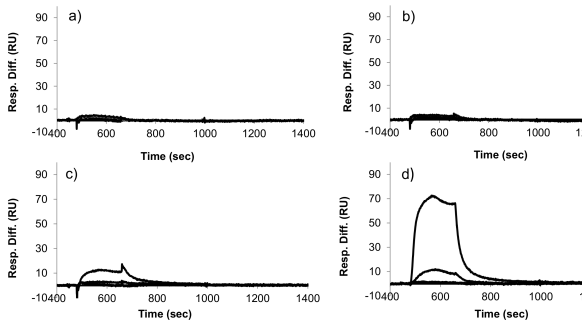


Figure 3. SPR sensorgrams showing interactions between immobilized HSA and a) 1-C15L8-PP1, b) 2-C15L8-PP1, c) 3-C15L8-PP1 and d) 4-C15L8-PP1 in 10mM HEPES buffer at pH 7.4 The concentrations of polypeptide conjugates were 4nM, 20 nM, 100 nM and 500 nM. Sensorgrams recorded of 1μM $ZnCl_2$ solution in buffer were subtracted from those of polypeptides in 1 μM $ZnCl_2$ solution.

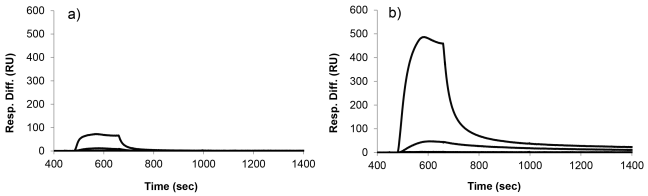


Figure 4. SPR sensorgrams showing interactions between immobilized HSA and a) 4-C15L8-PP1 and b) 5-C15L8-PP1 in 10 mM HEPES buffer at pH 7.4. The concentrations of polypeptide conjugates were 4nM, 20 nM, 100 nM and 500 nM. All measurements were carried out in the presence of 1 μM $ZnCl_2$. Sensorgrams recorded of 1μM $ZnCl_2$ solution in buffer were subtracted from those of polypeptides in 1 μM $ZnCl_2$ solution. Note. Due to the strong binding of 5-C15L8-PP1 the scale is different from that of Figure 3.

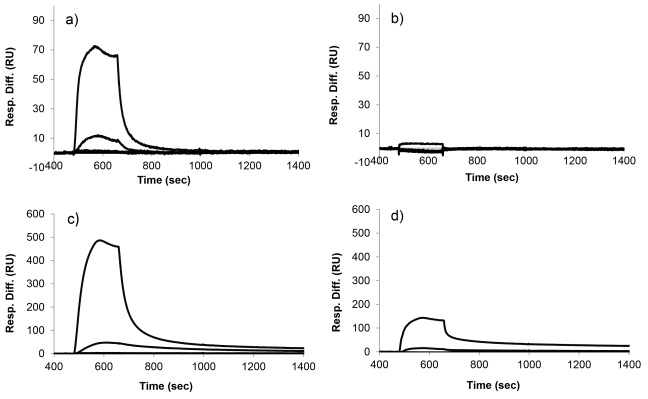


Figure 5. SPR sensorgrams showing the interactions between immobilized HSA and a) 4-C15L8-PP1 in the presence of 1μM $ZnCl_2$ b) 4-C15L8-PP1 without $ZnCl_2$, c) 5-C15L8-PP1 in the presence of 1μM $ZnCl_2$ and d) 5-C15L8-PP1 without $ZnCl_2$. The concentrations of polypeptide conjugates were 4nM, 20nM, 100nM and 500 nM in 10mM HEPES buffer at pH 7.4. Sensorgrams recorded of 1μM $ZnCl_2$ solution in buffer were subtracted from those of polypeptides in 1 μM $ZnCl_2$ solution. Note! Different scale between top and bottom panels.

In a control experiment, a conjugate with higher affinity for Zn^{2+} than PP1 was incorporated into 4-C15L8 and the interactions of the conjugate with HSA evaluated by SPR, SI, Figures S4 and S5. No difference between the two conjugates in binding to HSA was observed and the affinity for Zn^{2+} of the conjugates of PP1 is not a limiting factor.

Having established the significance of the Zn^{2+} chelate of the polypeptide conjugates, the role of hydrophobic residues in the polypeptide structure was evaluated. It was shown recently that the replacement of the commonly occurring hydrophobic amino acid residues Ile, Leu, Nle and Phe by an amino acid with a larger aliphatic side chain, L-2-aminooctanoic acid (Aoc), increased the affinity for glycogen phosphorylase a dramatically [17]. This strategy was used in an evaluation of the role of hydrophobicity in combination with the targeting of the metal binding site of HSA.

The polypeptide 4-C15L8-PP1 was modified to form **VI**-PP1 as Nle-5, Ile-9 and Leu-12 were replaced by Aoc residues. The interactions of **VI**-PP1 with HSA were evaluated by SPR analysis. No significant effect on the amount of bound polypeptide conjugate nor on the dissociation rate constants were observed, Figure 6, SI, Table S2. It appears that hydrophobicity is not an important parameter in the interaction with HSA, when a metal binding ligand is incorporated into the polypeptide. The results suggest that the use of the PP1 ligand has a larger and potentially more useful effect on binding than hydrophobic groups in peptides with close to neutral total charge

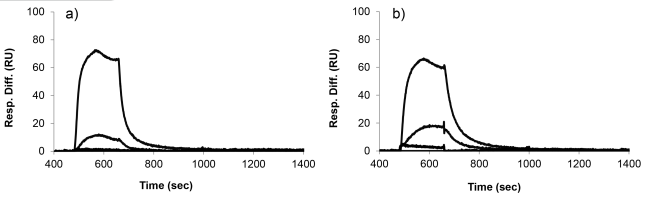


Figure 6. SPR sensorgrams showing the interactions between immobilized HSA and a) 4-C15L8-PP1 and b) **VI**-PP1. The concentrations of polypeptide conjugates were 4nM, 20nM, 100 nM and 500 nM in 10mM HEPES buffer at pH 7.4. Measurements were carried out in the presence of 1 μM $ZnCl_2$. Sensorgrams recorded of 1μM $ZnCl_2$ solution in buffer were subtracted from those of polypeptides in 1 μM $ZnCl_2$ solution.

The importance of molecular weight is particularly relevant for peptides in therapeutic applications, due to the increased risk of immunogenicity at higher molecular weights[15]. Based on the sequence of **VI** an 11-mer sequence, **VII**, was prepared with a molecular weight of 1960D in comparison to that of **VI**, which was 5860D. The interactions with immobilized HSA were evaluated by SPR, SI, Figure S3, Table S2. Due to the lower molecular weight of the 11-mer compared to that of the 42-mer, corresponding to approximately 25% of that of the parent peptide, the SPR response was correspondingly smaller. The dissociation rate constant was, however, comparable to that of 4-C15L8-PP1 as well as to that of **VI**-PP1, suggesting that the affinity of the 11-mer peptide was comparable to that of the 42-mer. Although the estimate is clearly a qualitative one the introduction of the chelator improves binding also for the 11-mer peptide conjugate.

The conjugation of polypeptides to the Zn^{2+} chelator PP1 increased their affinity for HSA at concentrations of $ZnCl_2$ lower

than the concentrations of Zn^{2+} ions found in human serum. The effect of PP1 conjugation on HSA binding was therefore evaluated under conditions relevant to those of peptide drugs in vivo. HSA complexation serves to prevent renal clearance of small organic molecule drugs, and the kinetics of complexation are highly important. Assuming that on-rates are diffusion controlled, which would be likely for any small molecule, nM dissociation constants correspond to off-rates that approach the minute time scale. Dissociation constants in the pM range become even more problematic. However, dissociation constants in the medium nM to low μM range are associated with dissociation rate constants that allow drugs to circulate in the blood stream rather than being excreted while being readily accessible for protein targets. The affinity enhancements provided by the introduction of a Zn^{2+} ion chelator as demonstrated here do not give rise to prohibitively slow off-rates.

Binding in human serum is difficult to measure but the expected outcome of HSA binding would be longer survival times that are readily measured by HPLC since tight binding to HSA protects from proteolytic degradation. In addition, although this was not measured, binding to HSA would be expected to protect peptides from immune cells and thus improve the performance of peptide drugs by reducing the risk of eliciting an immune response. The polypeptide conjugates 4-C15L8-PP1 and 5-C15L8-PP1 as well as the corresponding polypeptides 4-C15L8-Ac and 5-C15L8-Ac where the side chain of Lys-8 did not carry a PP1 group, were incubated in 25% human serum at 310K and samples analyzed by analytical reverse phase HPLC at regular intervals after the initiation of incubation. Identities of the fractions were secured by MALDI-TOF mass spectrometric analysis. In addition, 11-mer peptides with and without PP1 were analyzed in the same manner, Figure 7. In all cases did PP1 conjugation increase the survival times in serum as evidenced by the appearance of the peaks in the HPLC chromatograms. Typical estimated half-lives of unconjugated polypeptides were on the order of 200-250 min, whereas those of the PP1 conjugates were 2-10 times longer. Most dramatically, the half-life of 4-C15L8-PP1 was estimated to be extended by approximately an order of magnitude, quite a significant improvement for a peptide in human serum.

Conclusion

The conjugation of the Zn^{2+} chelating agent PP1 to a set of polypeptides increased their binding affinities for HSA as evidenced by increased amounts of bound polypeptide conjugates to immobilized HSA as well as by reduced dissociation rate constants. The significance of these findings is that a small organic molecule with a molecular weight of 544D is capable of increasing the affinity of a peptide for HSA that circulates in blood at high concentration, and thus to provide a vehicle for protecting peptide drugs from proteolytic degradation and renal clearance. While other circulating as well as extracellular membrane bound metal binding proteins can compete for the modified peptide, thus making it non-specific, the high concentration of HSA ensures that a large fraction of the PP1 conjugate would bind the carrier protein. The exact location of the binding site of the polypeptide in the absence of structural evidence has not been identified but the cooperativity between positive charge and metal ion chelation strongly suggests that the polypeptide conjugates bind in close proximity to, and probably to, the Zn^{2+} binding site of HSA. The increases in survival times are within an order of magnitude and are likely to be of use in bioanalytical as well as in biomedical applications.

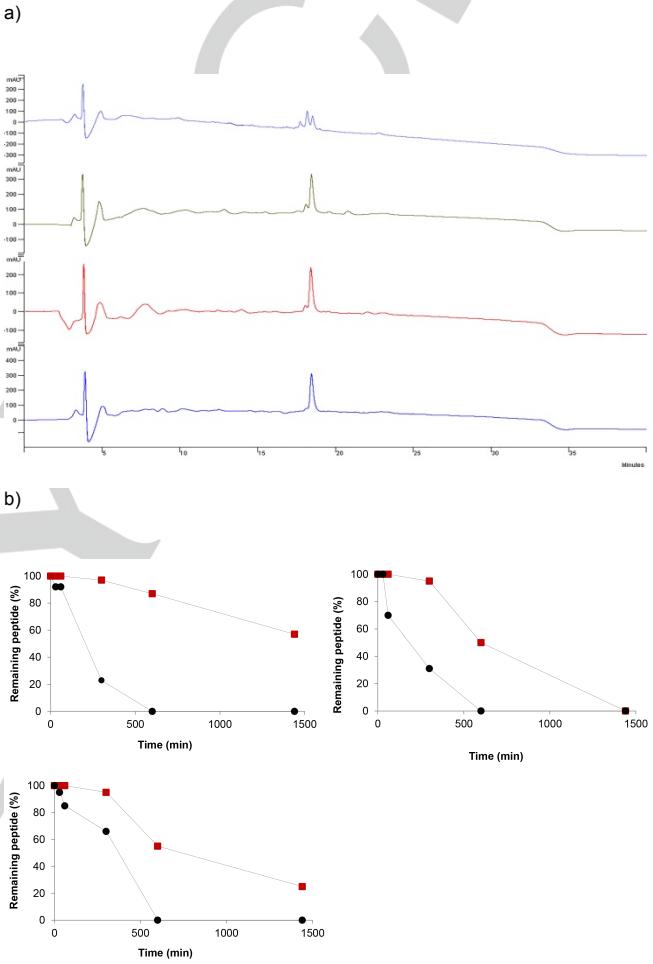


Figure 7. *HPLC analysis of polypeptide and polypeptide conjugate stability in human serum. a) Panel of chromatograms recorded after 0, 30, 60, and 300 min incubation of polypeptide 5-C15L8-Ac in 25% human serum albumin at 37 °C. b) Time dependence of degradation of 5-C15L8 (upper left), 4-C15L8 (upper right) and **VI** (bottom panel). Red symbols indicate PP1 conjugates, blue symbols indicate acetylated polypeptides.*

Experimental Section

Synthesis of ligand: The synthesis, identification and purity analysis of 3,5-Bis[[(bis(2-pyridylmethyl) amino) methyl)]]benzoic acid (PP1) has been described previously[19]. For ^{1}H NMR spectrum and mass spectrometric analysis, see SI, Figure S1.

Synthesis of peptides and conjugations: All reagents and solvents were purchased from commercial sources and used without further purification. All peptides were synthesized on an automated peptide synthesizer using standard solid-phase Fmoc protocols, with O-(6-chlorobenzotriazol-1-yl)-N,N,N,N-tetramethyluronium hexafluorophosphate (HCTU) diisopropylethylamine (DIPEA) as activeting agents. Peptides were synthesized on a 0.1mmol scale using a H_2N-RinkAmide-ChemMatrix resin (Iris Biotech GmbH) and a Fmoc-Gly-PEG-PS (Iris Biotech GmbH) resin for amidated and unmodified C-terminal peptides, respectively. Amino acids were added in 5-fold excess. The Fmoc protecting groups were simultaneously removed by 20% piperidine in dimethyl formamide (DMF). The side chains of each residue were protected with base-stable groups including Pfb (2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl) for Arginine, tert-butyl ester for Aspartic acid and Glutamic acid, and trityl for Histidine, Asparagine, and Glutamine. To enable further modifications of the peptides, the residues Lysine 8, Lysine 15 and Cystein 24 were orthogonally protected with tert-butyloxycarbonyl (Boc), 4-methoxytrityl (Mmt), and acetmidomethyl (Acm) respectively.

Deprotection of the Mmt group was performed by washing of the peptide resin with a mixture of DCM/TFA/TIS (triisopropylsilane) (95:1:1 (v/v) until no yellow colour of the washing solution was observed. The resin was further washed sequentially with DMF and DCM and dried under vacuum.

Coupling of 7-Methoxycoumarin-3-carboxylic acid to the amino group of the lysine at position 15 was performed by in N-Methyl-2-pyrrolidone (NMP) for 2h at room temperature, with 4-fold excess of the acid being activated by a mixture containing HCTU and DIPEA in the ratio 4:8. After the coumarin incorporation the resin was washed with DMF and DCM and desiccated.

For the peptide cleavage and simultaneous removal of sidechain protecting groups, the peptide resin was treated with trifluoroacetic acid (TFA) in presence of triisopropylsilane (TIS) and water in a ratio of TFA/TIS/H2O (95/2,5/2,5% v/v). After filtration and concentration, the peptide was precipitated by the addition of cold diethyl ether, centrifuged, redissolved in water, and lyophilized. Purification of the crude peptides was performed by reversed phase HPLC on a preparative hypersil C-18 column using a gradient from 20-80% of 0.1% aqueous TFA (solvent A) to 0.1% TFA in 95% aqueous acetonitrile (solvent B). All the peptides were identified with the aid of MALDI-TOF mass spectrometry (Applied Biosystems, Voyager-DE PRO) using a-cyano-4-hydroxy-cinnamic acid as matrix, see SI.

Conjugation of PP1: The purified peptide (3mg) possessing a free lysine amine group was dissolved in anhydrous DMSO (200µl) and a mixture of 3,5-bis[(bis(2-pyridylmethyl)amino)methyl)]benzoic acid (PP1, 4 equivalents), HCTU (5 equivalents) and DIPEA (20 equivalents) in DMSO (100µl) was added. The reaction progress was followed by analytical HPLC using a gradient from 20%-80% of 0.1% aqueous TFA to 0.1% TFA in 95% acetonitrile for 40 min. After completion of the reaction cold diethyl ether was added and the lower layer containing the peptide was separated and dissolved in a small amount of TFA (0.1 ml) after which cold diethyl ether was again added. The mixture was centrifuged, and the pellet was redissolved in water and purified by preparative HPLC using a GraceVydac, MS, gradients and solvents as described above.

Surface Plasmon Resonance analysis: Analysis of peptide-HSA interactions were performed using a Biacore 2000 instrument (GE Healthcare, Uppsala, Sweden) at 298K. The reagents, running buffers as well as the CM5 were supplied by GE Healthcare Bio-Science, Uppsala, Sweden. HSA was dissolved to a concentration of 0.1 mg/ml in sodium acetate coupling buffer at pH 5.0 and covalently immobilized on a CM5 sensor chip, using standard amine coupling with running buffer HBS-EP (10mM, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% P_2O). The carboxylated surface was activated for 7 min by injection of EDC/NHS (1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide/N-hydroxysuccinimide). After activation, the HSA solution was allowed to react for 7min with the activated surface, followed by 7 min reaction of ethanolamine hyrochloride to block the remaining active groups. The final HSA immobilization level corresponded to 7000 RU.

For interaction experiments, peptides were diluted in HBS-P (10mM, pH 7.4, 150 mM NaCl, and 0.005% P_2O) running buffer, in the presence of 1µM $ZnCl_2$, in 5-fold serial dilution, ranging from 4 to 500nM. Each peptide solution was injected over the immobilized surface for 3min, at a flow rate of 50µl/min. All concentrations of stock peptide solutions were estimated by subtracting 30% of the determined weight of the peptide due to the presence of water, as determined by quantitative amino acid analysis. After 15 min of dissociation the surface was regenerated by allowing the HBS-P running buffer to flow over the surface at a flow rate of 50ul/min for 30min. Blank injections containing 1µM $ZnCl_2$ in HBS-P buffer were included for each measurement series and the sensorgrams subtracted from the sensorgrams recorded in the presence of $ZnCl_2$. For each peptide, SPR analyses were performed several times and their interactions with HSA evaluated according to observed uptakes as well as off-rates. When not in use the sensor chips with the immobilized protein were stored at 277K for a maximum of three weeks.

In vitro peptide stability assay: Measurements of peptide stability in human serum has been described in detail previously [16]. Stock solutions containing peptides with and without incorporated PP1 ligand were freshly prepared in a HEPES buffer (10mM HEPES, 150mM NaCl pH 7.4). Buffer solutions containing 25% human serum (Male AB, Sigma-Aldrich) and 1µM $ZnCl_2$ were centrifuged at 14.000rpm for 1min in order to remove lipids and the supernatants were collected and incubated at 37°C for 10min. Peptides were added to the serum solution to final peptide concentrations of 450 µM. Aliquots of 20 µL were removed at time intervals of 0, 30, 60, 300, 600 and 1440min and mixed with 40 µL of 95% ethanol (EtOH) to precipitate serum proteins. Following centrifugation at 14,000 rpm for 10 min, the supernatants were collected and stored at −20°C. The assay for each peptide was performed twice. The reaction supernatants were analysed utilizing preparative RP-HPLC, using a gradient from 0-90% of 0.1% aqueous TFA (solvent A) and 0.1% TFA in 95% aqueous acetonitrile (solvent B) for 40 min. The injection volume of all the peptide conjugate samples was 20ul. The relative percentages of the remaining stable peptides were analysed by measuring the absorbance maxima of the peptides at 210 nm. For time course evaluations, see SI, Figures S6-S10.

Keywords: Peptides • Zinc Binding Site • Human Serum Albumin • Affinity • Biosensor

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