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Exploring molecular interactions between polypeptide conjugates and protein targets

Manipulating affinity by chemical modifications

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Abstract

Balliu, A. 2017. Exploring molecular interactions between polypeptide conjugates and protein targets. Manipulating affinity by chemical modifications. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1534. 69 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0016-0.

In this thesis molecular interactions between polypeptide conjugates and protein targets were investigated. Polypeptides were derivatized with small organic molecules, peptides and oligonucleotides. New strategies were developed with the aim to increase affinities for proteins of biological interest.

A 42-residue polypeptide (4-C15L8) conjugated to a small organic molecule 3,5-bis[[bis(2-pyridylmethyl)amino]methyl]benzoic acid (PP1), was shown to bind glycogen phosphorylase a (GPa) in the presence of zinc ions. Under the assumption that hydrophobic interactions dominated the binding energy, the hydrophobic residues of 4-C15L8-PP1 were systematically replaced in order to study their contribution to the affinity enhancement. The replacement of the Nle, Ile and Leu residues by Ala amino acids reduced affinities. The introduction of non-natural *L*-2-aminooctanoic acid (Aoc) residues into the peptide sequence enhanced the binding affinity for GPa. A decreased K_D of 27nM was obtained when Nle5, Ile9 and Leu12 were replaced by Aoc residues, in comparison to the K_D value of 280nM obtained for the unmodified 4-C15L8-PP1. It is evident that there are non-obvious hydrophobic binding sites on the surfaces of proteins that could be identified by introducing the more hydrophobic and conformationally flexible Aoc residues. The downsizing of the 42-mer peptide to an 11-mer and the incorporation of three Aoc residues gave rise to a K_D of 550 nM, comparable to that of 4-C15L8-PP1 suggesting that bioactive peptides can be downsized by the introduction of Aoc.

Aiming to improve in vivo stability, the affinity for human serum albumin (HSA) of hydrophobic, positively and negatively charged polypeptide-PP1 conjugates was evaluated. Increased hydrophobicity due to the introduction of Aoc residues did not significantly increase the affinity for HSA. No binding was observed in the case of the most negatively charged polypeptides whereas the slightly negatively and positively charged polypeptides conjugated to PP1 bound HSA with affinities that increased with the positive charge. It was found that polypeptide-PP1 conjugates target the zinc binding site of the HSA. Affinity enhancement was obtained due to the incorporation of PP1 and increased by charge to charge interactions between the positively charged amino acids of the polypeptide and the negatively charged residues of HSA, in close proximity to the HSA zinc binding site. The survival times of the peptide-PP1 conjugates in human serum were extended as a result of binding to HSA. Zn^{2+} ion chelating agents can be incorporated in potential peptide therapeutics with a short plasma half-life, without increasing their molecular weights.

Keywords: polypeptide conjugates, protein targets, molecular recognition, affinity enhancement, conjugation

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Dedicated to my family

As you set out for Ithaka
hope your journey is a long one
full of adventure, full of discovery

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Balliu, A. and L. Baltzer, Exploring Non-obvious Hydrophobic Binding Pockets on Protein Surfaces: Increasing Affinities in Peptide–Protein Interactions. *ChemBioChem,* **2017**. 18(14): p. 1396-1407.

II Balliu, A. and L. Baltzer, Conjugation of a Dipicolyl Chelate to Polypeptide Conjugates Increases Binding Affinities for Human Serum Albumin and Survival Times in Human Serum. *ChemBioChem*, **2017**. 18(14): p. 1408-1414.

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Publication not included in this thesis

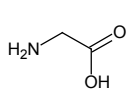
III Sakka, M., A. Balliou, A. Marianou, D. Krikorian, M. Tsirogianni, A. Tsiligianni, K. Vartzeli, A.-I. Koukkou, M. Sakarellos-Daitsiotis and E. Panou-Pomonis. "Synthesis and Antimicrobial Evaluation of Amphipathic Peptide Models." *Letters in Drug Design & Discovery*, **2016**. 13(7): 715-724.

Contents

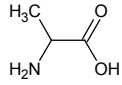
Abbreviations

Acm	Acetamidomethyl
ACN	Acetonitrile
ADP	Adenosine diphosphate
AKT1	RAC-alpha serine/threonine-protein kinase
Alloc	Allyloxycarbonyl
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Boc	*tert*-Butyloxycarbonyl
CD	Circular dichroism
DCM	Dichloromethane
DIC	Diisopropylcarbodiimide
DIPEA	*N,N*-Diisopropylethylamine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
Fmoc	9-Flourenylmethoxycarbonyl
GPa	Glycogen phosphorylase a
HCA	Human carbonic anhydrase
HCA IX	Human carbonic anhydrase isoform IX
HPLC	High performance liquid chromatography
MALDI-TOF	Matrix-assisted laser desorption ionization time of flight
NMP	*N*-Methyl-2-pyrrolidone
Mmt	4-Methoxytriphenylmethyl
MS	Mass spectrometry
NHS	*N*-Hydroxysuccinimide
OtBu	*tert*-Butoxy
OVA	Ovalbumin
PEG	Polyethylene glycol
PLA	Proximity ligation assay
RU	Response units

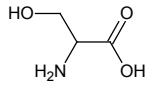
SPPS	Solid phase peptide synthesis
SPR	Surface plasmon resonance
TCEP	tris(2-carboxyethyl)phosphine
TFA	Trifluroacetic acid
Trt	Trityl
TIS	Triisopropylsilane
UV-Vis	Ultraviolet-visible



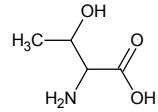
Glycine (Gly, G)



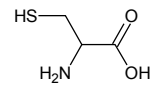
Alanine (Ala, A)



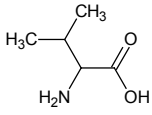
Serine (Ser, S)



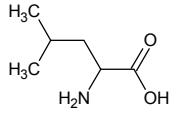
Threonine (Thr, T)



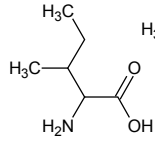
Cysteine (Cys, C)



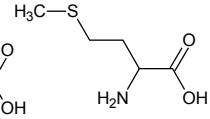
Valine (Val, V)



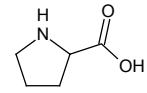
Leucine (Leu, L)



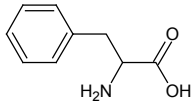
Isoleucine (Ile, I)



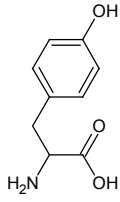
Methionine (Met, M)



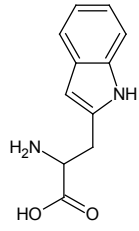
Proline (Pro, P)



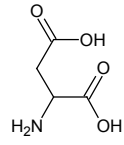
Phenylalanie (Phe, F)



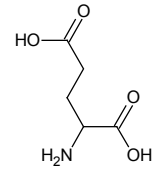
Tyrosine (Tyr, Y)



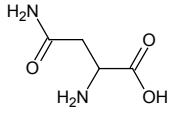
Tryptophan (Trp, W)



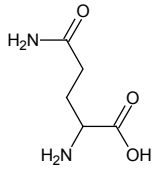
Aspartic acid (Asp, D)



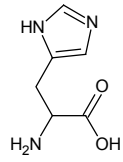
Glutamic acid (Glu, E)



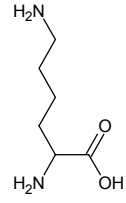
Asparagine (Asn, N)



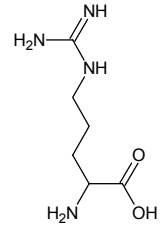
Glutamine (Gln, Q)



Histidine (His, H)

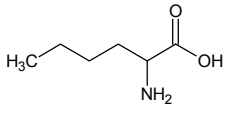


Lysine (Lys, K)

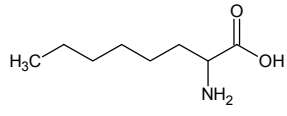


Arginine (Arg, R)

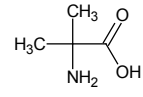
Non-natural *L*-amino acids relevant to the present thesis



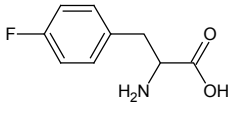
Norleucine (Nle, J)



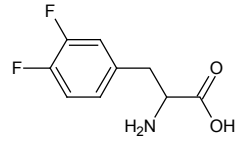
2-aminooctanoic acid (Aoc)



Aminoisoburyric acid (Aib)



4-fluorophenylalanine (4-F_1Phe)



3,4-difluorophenylalanine (3,4-F_2Phe)

Introduction

For over half a century there has been significant development in researching biological macromolecules, including their physical and chemical properties as well as the molecular mechanisms and metabolic processes that they are involved in. Even though information of all cell processes is stored within DNA and RNA, proteins remain the agents that access and execute nearly all genetic programs. Originated from the Greek word "πρώτειος" (proteios) meaning "primary", proteins accelerate a number of molecular processes, such as protein folding, chemical transformations, intracellular communication, etc. Owing to their importance as drug targets, proteins have been the subject of study aided by the conceptual and technological advances in molecular cell biology, biochemistry and biophysics.

For the reasons stated above, the identification and the quantification of the expression levels of proteins are essential in diagnostic and bioanalytical applications. Comprehensive strategies for high-affinity binding of protein targets remain an attractive research area.

Currently the most used protein binders can be classified into two categories: synthetic small organic molecules with molecular weights of <500Da and large biologicals (antibodies, recombinant proteins and nucleic acids). Due to their small size organic molecule drug candidates often suffer from reduced target selectivity, whereas biological therapeutics tend to be very specific to their targets. However, low bioavailability, metabolic instability, high cost and time-consuming development remain major drawbacks with the latter.

Peptide-based binders have become popular in many diagnostic and therapeutic applications offering several advantages over antibodies. Peptides are associated with lower production complexity and cost in comparison to antibodies. Recent advances in peptide chemistry and in conjugation techniques have made possible the design of conformationally controlled peptides or peptide templates with additional suitable functions.

In this thesis, a peptide conjugation strategy was explored and developed to improve the recognition of protein targets by small organic molecules and peptides. Molecular interactions between chemically synthesized polypeptides and phosphorylated proteins were elucidated in Paper I.

In paper II, human serum albumin (HSA) was used to improve the life time of peptides in the blood stream. Polypeptides conjugated to a zinc chelating ligand were shown to bind to the HSA zinc binding site.

In addition, a variety of polypeptide conjugates were prepared for applications in biotechnology.

1. Peptide-protein interactions

During the last few decades there has been significant development in the understanding of the fundamental role of peptides in the biological processes of life[1]. Advances in peptide research and synthesis marked the beginning of a new era where peptide-based therapeutics appeared and tools were developed for use in bioanalytical and diagnostic applications[1b, 2].

Within drug development, a vast number of peptide therapeutics have been approved (Zoladex, Sandostatin, Lupron etc.) and 400-600 peptides are in preclinical studies[1b, 2c]. Synthetic peptides have systematically been used in immunological studies[3], as antimicrobial peptides[4], as substrates[5] and inhibitors to investigate and influence enzymatic reactions and to modulate protein-protein interactions[6].

Structural determinations applied in combination with biochemical and biophysical methods generate important information about ligand–receptor complexes and guide the identification of key residues involved in molecular recognition[7]. The rational design of peptides with high affinity for a given receptor based on such information can provide peptide therapeutics with improved efficacy and stability[2b, 8].

Among non-rational design methods, phage-display is the most popular technique and used for the screening of random peptide libraries[9]. Through affinity selection (biopanning) of phage-displayed peptides almost 10^{11} peptide leads have been identified and used in target validation and additional applications[10].

Rational peptide design is based on studies of structure-activity relationships and identification of essential residues. Chemical modifications have enabled the development of multifunctional peptide conjugates with improved physicochemical properties and in vivo stability[1b, 2b].

The molecular interactions between a peptide and a protein are non-covalent and the affinities can be evaluated to determine the thermodynamic parameters including the kinetic rate constants[11]. The main types of non-covalent interactions that provide binding energy are charge-charge interactions, hydrogen bonding and hydrophobic effects (van der Waals interactions and the release of water molecules)[11-12].

The strength of charge-charge interactions depends on the distance between the two charged groups, and on the nature of the environment. The binding energies of hydrogen bonds are typically between 12 kJ/mol to 38

kJ/mol, and the strength is dependent on the distance between the hydrogen donor and the acceptor. In aqueous solution charge-charge interactions and hydrogen bonding are weaker because of the high dielectric constant and hydrogen bonding capacity of water. If water is excluded they become stronger. However, the contribution of charge-charge and hydrogen bonding to binding selectivity is important[11].

Hydrophobic interactions are entropically favoured due to the displacement of ordered water molecules layer from both peptide and protein hydrophobic surfaces. It is often the strongest force between molecules in aqueous solution[11, 13]. The hydrophobic effect dominates in protein folding[11]. The active sites of proteins and the large interfaces of protein-protein interactions are frequently hydrophobic in nature[14].

Both the entropic and the enthalpic component of the binding free energy need to be known to fully understand the binding interactions between the peptide and the target protein. Accurate evaluation methods are essential to determine the binding contributions[15].

Techniques for the study of peptide-protein interactions include sequence modifications, NMR spectroscopy and X-ray crystallography as well as computational predictions and measurements of binding kinetics with the aid of SPR (surface plasmon resonance)[7].

For sequence modifications experiments, the experiments need to be sufficiently refined to make sure that the observed effects are only those caused by the modification of the desired residue, and are not caused by secondary changes to the overall protein structure or stability[16].

In the case of NMR experiments, the large number of atoms in a protein creates difficulties in differentiating the different peaks in the spectra, making full assignment of signals possible only for small proteins and ligands[17].

Crystallography remains the dominant structural method for proteins although it is not always easy to crystallize a protein-ligand complex[18].

The many degrees of freedom of a peptide as well as of a protein create challenges in modelling the interactions[19]. In addition, non-specific binding and identification of the binding site are major issues during experiments[7].

Measurements of binding kinetics are of considerable interest to obtain a complete understanding of the interactions, these are based largely on the power of surface plasmon resonance (SPR)[20].

2. The polypeptide conjugate concept

Aiming to investigate new binder candidates with molecular weight in between small drug-like molecules and proteins, and combining the advantages of small organic molecules with those of peptides, a new binder concept has been developed based on synthetic, designed polypeptides that are conjugated to small organic molecules or peptides[21]. The resulting conjugates have higher affinity and specificity in comparison to those of the small molecules alone. The small molecule binds to the protein and the conjugated polypeptide interacts with the protein to provide additional binding energy[21a, 22]. The designed polypeptides contain 42 amino acid residues and are selected from a set of only 16 sequences, which are neither pre-organized to recognize proteins nor based on sequences that occur in nature. The organic molecule drives the recognition, whereas the polypeptide scaffold improves target affinity and selectivity. The polypeptide has a variety of hydrophobic and charge residues that offer many possible binding modes for a large number of proteins[21a, 23].

2.1 The polypeptide scaffold

The design of a 16-membered library with 42 residue polypeptides was based on the development of polypeptide sequences with a propensity to form helix-loop-helix motifs. Each helix has the propensity to adopt an amphiphilic conformation with a hydrophobic and a hydrophilic side in the folded state[21a, 24]. In terms of the heptad repeat pattern $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$[25], hydrophobic residues are introduced in the a and d positions to form the hydrophobic core. Charged residues are incorporated at the dimer interface i.e. the b and e positions, and in the solvent exposed, c, f, and g positions, Figure 1. Throughout the sixteen sequences there are eight conserved hydrophobic residues that are believed to provide the bulk of the binding energy. Charged residues are placed close to the hydrophobic residues for selectivity enhancement by charge-charge interactions and H-bonding[21a], Table 1.

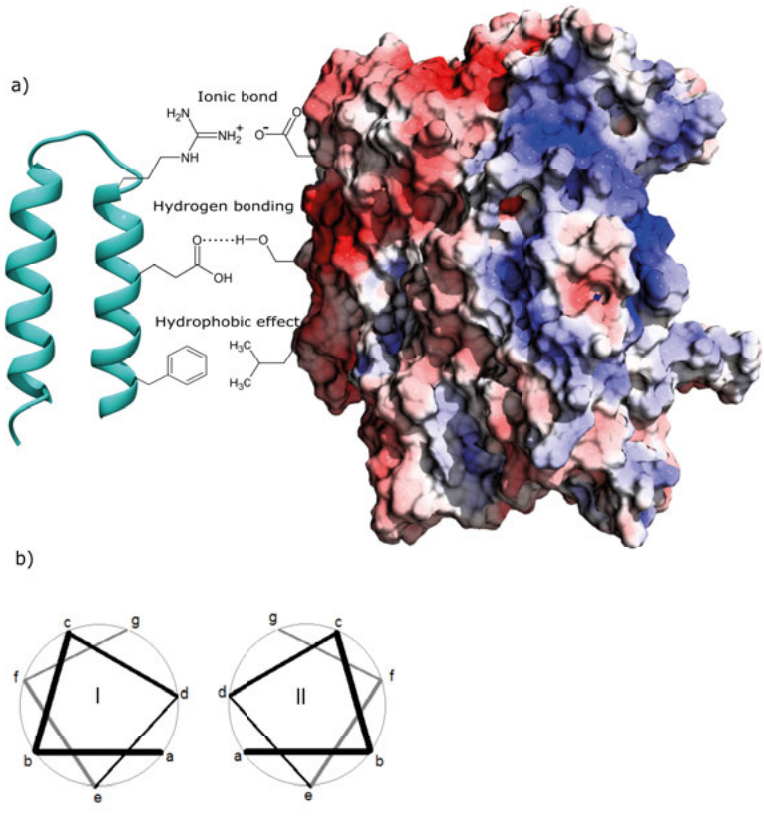


Figure 1 Schematic representation of peptide-protein interactions. a) Demonstration of hydrophobic, charge-charge interactions and hydrogen bonding between the amino acids of a peptide with a helix-loop-helix conformation and the residues of a protein target (PDB code: 1A8I). b) The heptad repeat pattern and the orientation of the amino acids in a helix-loop-helix conformation.

Structural data obtained by CD and ^{1}H NMR spectroscopy verify the propensity of the peptides to form helix-loop-helix dimers at high concentrations in aqueous solution, whereas at lower concentrations the amount of unordered monomers dominates. On the basis of NMR spectra, melting temperatures and concentration dependent CD spectra it was concluded that the polypeptides exist in rapid exchange between a large number of similar conformations, and are usually referred to as molten globules[24].

The 42-residue peptides have four different total charges of -7, -4, -1 and +2, and four different positions where a small molecule ligand can be incorporated[21a]. In this thesis, for purpose of simplicity, the polypeptides with total charges of -7, are referred to as the 1-series, the polypeptides with total charges of -4 are referred to as the 2-series etc. The four peptides of the 1-series with a total charge of -7 form the most negatively charged sequences of the set with the ligand incorporated at positions 8, 17, 22 and 34, Table 1.

1-C15L8	Ac–NEADLEAKIRHLAE*K*LEARGPEDCEQLAEQLARAFEAFARAG-COOH
1-C10L17	Ac–NAADLEAAI*K*HLAEALKERGPEDCEQLAEQLARAFEAFARAG-COOH
1-C25L22	Ac–NEADLEAAIRHLAEALEARGPKDC*K*QLAEQLARAFEAFERAG-COOH
1-C37L34	Ac–NEADLEAAIRHLAERLEARGPADCAQLAEQLAAKFE*K*FARAG-COOH
2-C15L8	Ac–NEADLEAKIRHLAE*K*LAARGPVDCAQLAEQLARAFEAFARAG-COOH
2-C10L17	Ac–NAADLEAAI*K*HLAEALKARGPVDCAQLAEQLARAFEAFARAG-COOH
2-C25L22	Ac–NEADLEAAIRHLAEALAARGPKDC*K*QLAEQLARAFEAFARAG-COOH
2-C37L34	Ac–NAADLEAAIRHLAERLAARGPVDCAQLAEQLAAKFE*K*FARAG-COOH
3-C15L8	Ac–NAADJEAKIRHLAE*K*JAARGPVDCAQJAEQLARRFEAFARAG-NH_2
3-C10L17	Ac–NAADJEARI*K*HLAERJKARGPVDCAQJAEQLARAFEAFARAG-NH_2
3-C25L22	Ac–NAADJEAAIRHLAERJAARGPKDC*K*QJAEQLARAFEAFARAG-NH_2
3-C37L34	Ac–NAADJEAAIRHLAERJAARGPVDCAQJAEQLARKFE*K*FARAG-NH_2
4-C15L8	Ac–NAADJEAKIRHLRE*K*JAARGPRDCAQJAEQLARRFERFARAG-NH_2
4-C10L17	Ac–NAADJEARI*K*HLRERJKARGPRDCAQJAEQLARAFERFARAG-NH_2
4-C25L22	Ac–NAADJEARIRHLRERJAARGPKDC*K*QJAEQLARAFERFARAG-NH_2
4-C37L34	Ac–NAADJEARIRHLRERJAARGPRDCAQJAEQLARKFE*K*FARAG-NH_2

Table 1 The 16 membered set of 42-residue sequences in the one letter code and the variation in terms of charge and the site of the conjugated ligand. Negatively charged residues, Asp and Glu are coloured green and positively charged Arg residues are red. The 1-series of polypeptides, top, contain the most negatively charged polypeptides with a total net charge of -7, the 2-series has a total charge of -4 and the 3-series a total charge of -1. The 4-series contains the most positively charged groups, with a total charge of +2. Lys residues where the small molecule ligand is incorporated are coloured blue and Lys residues carrying a fluorophore (for binding measurement purpose) are shown in italic. The Cys residues in position 24 are orthogonally protected with Acm groups.

The unfavourable entropy of binding of the ligand is not experienced also by the polypeptide as the latter is covalently linked to the small molecule ligand[21a]. Consequently, even weak interactions between protein and polypeptide can provide considerable affinity enhancements. A few hydrophobic contacts between the polypeptide and the protein can, for example, contribute around 20 kJ/mol of binding energy and raise the affinity of the polypeptide conjugate ten thousandfold over that of the small molecule ligand[11]. The peptide possessing 42 residues can adapt to a large number of conformations and a large number of possible interactions with a large number of proteins[23].

With regards to the utilization of this small polypeptide library for the recognition and binding of protein targets, further modifications including the incorporation of non-natural building blocks and conjugation of ligands with distinct functionalities remain to be explored.

3. Polypeptide conjugation

Site-selective chemistry performed on peptides and proteins such as the introduction of pharmacophores and other groups has the potential to improve the pharmacokinetic and pharmacodynamic properties of peptide therapeutics as well as the properties of molecular diagnostic tools[1b, 26].

Conjugates of polyethylene glycol (PEG) are well-known and used in the clinic; PEGylation increases in vivo stability and reduces immunogenicity[27]. Fluorescent probes, biotin and reporter enzymes such as alkaline phosphatase and horseradish peroxidase are conjugated to peptides and used in titrations, as imaging tools and for detection in binding assays[28]. The molecular structures that can be conceived represent a high level of complexity. Members of the polypeptide library were covalently linked to small molecule ligands and peptides to obtain increased affinity and selectivity. They were linked to enzymes and a variety of labels such as fluorophores and radionuclide chelators for the identification of proteins, for measurements of affinity and for imaging applications.

Synthetic engineering of peptide sequences may confer beneficial properties such as improved binding affinity and selectivity for bio-molecular targets, protease resistance, and enhanced bioavailability[26a, 29]. One approach for improving efficacy, chemical and enzymatic stability of peptides is the introduction of non-natural amino acids. While they do not count as conjugates per se, they can be used to introduce functional groups not available in the common residues and alter the biophysical and chemical properties of the peptide[29-30]. For example, fluoroalkyl amino acid substitution has been proven to substantially increase peptide activity and stability and fluorine substitution is a well-known strategy in medicinal chemistry[31]. Substitution of key natural residues by non-natural amino acids e.g. 2-naphthylalanine, ornithine, 2,3-diaminopropionic acid etc. led to enhanced enzymatic peptide stability[32]. The stapling of a tumor-suppressing peptide in combination with the introduction of unnatural amino acids, led to a more stabilized helix and an increased affinity for the MDM2 and MDMX proteins[33].

Systematic studies of how such non-natural amino acids can interact with natural amino acids of native protein targets may yield important information that can facilitate the prediction of the properties of novel non-natural peptide therapeutics or biomaterials.

Peptides can be immobilized onto solid supports by chemoselective reactions for affinity purification of proteins or for bioanalytical and diagnostic

applications. Ample examples of such supports are nitrocellulose, polystyrene plates or particles; beaded agarose/polyacrylamide resins the supports being activated for direct coupling to a ligand[34].

3.1 Peptide conjugation strategies

Peptides are usually synthesized in organic solvents which allow great versatility in the selection of reagents for modification[1a].

Amino, carboxyl, sulfhydryl or carbonyl groups can be reacted with a large number of reagents. Site-specific conjugation is achieved most often using cystein or lysine residues[35]. However there are also recently developed methods that are based on the non-natural olefin functional group and the metathesis reaction[36] or on the `click´ reaction that utilizes an azide and an alkyne group[37].

Amide bonds are thermodynamically very stable and amide-type bond formation is often preferred in conjugation reactions. There are a number of chemical reagents available for conjugation reactions at lysine side chains. These include fluorophenyl and NHS esters, isocyanates, isothiocyanates, and acyl azides[38], Figure 2. Additionally, the formation of amide bonds can be achieved by the usual methods of peptide synthesis, such as with carbodimides in the presence of additive reagents or phosphonium/aminium/uronium salts etc.[1a].

The derivatization of peptides utilizing the side chain of cystein residues is usually achieved by maleimide-, iodoacetamide- or disulphide-containing compounds[38], Figure 2. In the reaction with maleimides, thiolates undergo a Michael addition reaction to form succinimidyl thioethers[39]. The reactions with iodoacetamide and maleimide electrophiles are carried out under carefully controlled reaction conditions of pH and reagent concentrations due to the risk of unwanted covalent modifications with primary amines[38].

Homo- and heterobifunctional crosslinkers with a variety of spacer sizes and types are available for most of the peptide functional groups. Most often, however, amine-to-carboxylic acid or sulfhydryl-to-amine strategies are employed[40], Figure 3.

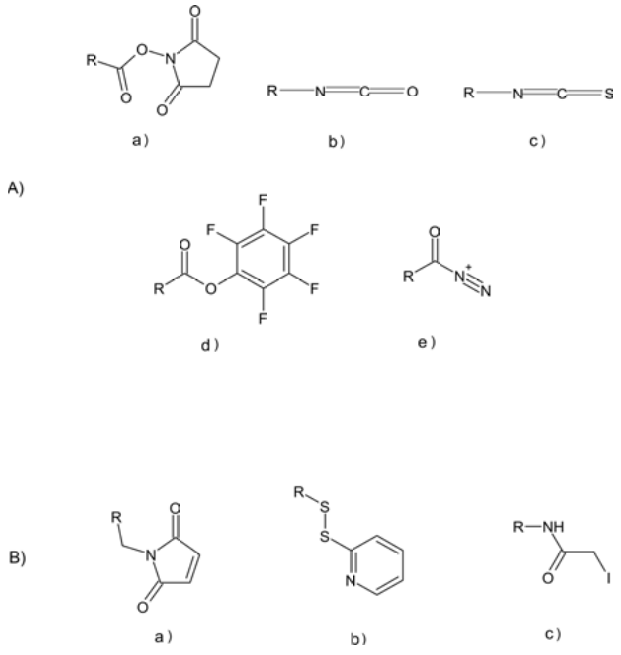


Figure 2 Chemical structures of amine- and sulfhydryl- reactive reagents. A) Amine reactive reagents a) NHS esters, b) isocyanates, c) isothiocyanates, d) fluorphenyl esters, e) acyl azides. B) Thiol reactive reagents a) maleimides b) pyridyl disuphides c) iodoacetamides

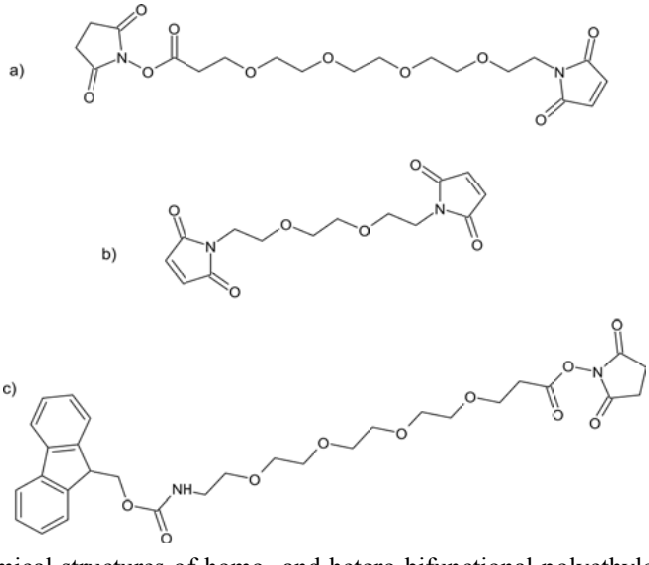


Figure 3 Chemical structures of homo- and hetero-bifunctional polyethylene glycol (PEG) crosslinkers a) NHS-PEG_4-Maleiminde, b) bismaleimide-PEG_2, c) Fmoc-NH-PEG_4-NHS

3.2 The synthesis of polypeptide conjugates

Multiple site-specific derivatizations of the 42-residue polypeptide sequences using orthogonal protection strategies were carried out to provide functionalized recognition elements for a number of applications including target identification, biochemical assays and in vivo imaging, Figure 4.

An automated peptide synthesizer was used for the solid phase peptide synthesis (SPPS) of 42-residue sequences using Fmoc methodology. To enable site-selective incorporation of ligands, orthogonally protected Lys and Cys residues were incorporated. Fluorescein, small organic molecules, oligonucleotides and proteins were conjugated to polypeptides on the solid phase as well as in solution after cleavage/purification of the peptide. The progress of the conjugation reactions was monitored by analytical RP-HPLC and MALDI-TOF mass spectrometry.

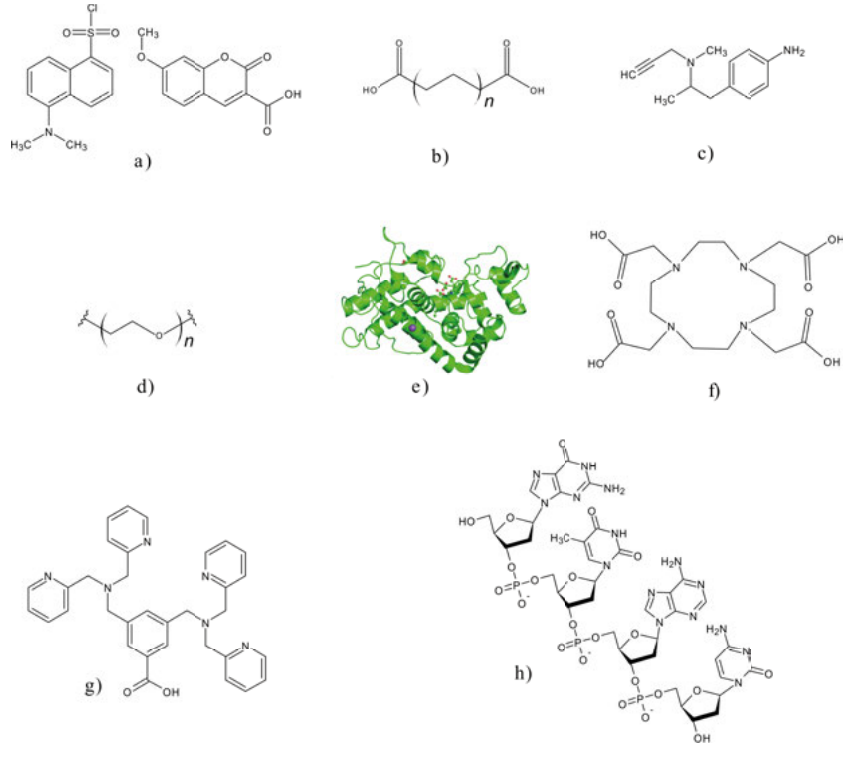


Figure 4 The reagents conjugated to the polypeptide sequences. a) The fluorescent probes 5-dimethyl-aminonaphthalene-1-sulfonyl chloride (Dansyl) (left) and 7-methoxycoumarin-3-carboxylic acid (Coumarin) (right) b) aliphatic dicarboxylic acids spacer, c) (R/S)-2-(N-propargyl-N-methyl)amino-1-phenylpropane (d-deprenyl), d) polyethylene glycol $(PEG)_n$ crosslinker, e) horseradish peroxidase enzyme (HRP) (PDB, code 1W4W), f) 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), g) 3,5-bis[[bis(2-pyridylmethyl)amine]methyl]benzoic acid (PP1), h) deoxyribonucleic acid (DNA).

3.2.1 Polypeptide-deprenyl conjugation

It was discovered previously that a phenylpropylamine (d-deprenyl) derivative accumulates in rheumatoid arthritis knees[41]. The target/receptor is unknown, but is assumed to be a protein and its identification would be of considerable interest as a target for drug development. We hypothesized that a polypeptide-d-deprenyl conjugate could be used to detect the protein in a non-denaturing western blot assay.

Four 42-residue sequences were selected for evaluation (Table 2) and covalently conjugated to a d-deprenyl active ester derivative, designed and synthesized in-house (Prof. T. Norberg). Two peptide modifications were introduced through the reactions of active esters with orthogonally deprotected amino groups of lysine residue side chains. First, the d-deprenyl derivative was reacted with a heterobifunctional linker, *o*-flurophenyl-pentafluorophenyl adipate[42], to give a reactive acylating agent, Figure 5.
The resulting p-aminodeprenyl *o*-fluorophenyl ester was then reacted with each of the four peptides in the presence of 2% *N,N*-diisopropylethylamine (DIPEA) and 10% pyridine, in DMSO solution, Figure 6. The addition of the stronger base (DIPEA) was needed to drive the reaction of the less reactive *o*-fluorophenyl ester[42]. After two days reaction, the desired deprenyl-polypeptide conjugate was obtained in 85% yield.

4-Nal15L8	Ac–NAADJEA**K**IRHLRENalJAARGPRD<u>K</u>AQJAEQLARRFERFARAG-NH_2
4-Nal10L17	Ac–NAADJEARINalHLRERJ**K**ARGPRD<u>K</u>AQJAEQLARAFERFARAG-NH_2
4-Nal25L22	Ac–NAADJEARIRHLRERJAARGP**K**D<u>K</u>NalQJAEQLARAFERFARAG-NH_2
4-Nal37L34	Ac–NAADJEARIRHLRERJAARGPRD<u>K</u>AQJAEQLAR**K**FENalFARAG-NH_2

Table 2 Four 42-mer polypeptide sequences conjugated to the d-deprenyl derivative for protein detection in a western blot assay. The Lys residues where the ligand was incorporated are shown in bold and the Lys residues where a PEG crosslinker was introduced are underlined. The unnatural naphthylalanine (Nal) residue was introduced in the peptides instead of a coumarine or dansyl probe.

The base labile trifluoroacetyl (Tfa) protecting group at the side chain of Lys24 was then removed by treating the peptide with 20% piperidine in aqueous solution. A heterobifunctional PEG derivative with two ethylene glycol units, equipped with a pentafluorophenyl ester and a Fmoc protected amino group, was reacted with the deprotected Lys side chain. The Fmoc group was removed in the presence of 15% trimethylamine (TEA) in DMF and a pre-activated form of horseradish peroxidase (NHS-HRP enzyme) was reacted with the polypeptide (J. Rydberg), Figure 7.

The resulting enzyme-peptide conjugate was used for the development of a Western blot assay (K. Fromell). Protein samples were selected from synovial fluids from patients with rheumatoid arthritis but no biomacromolecule

could be detected using the polypeptide conjugate for detection. The receptor protein remains unidentified.

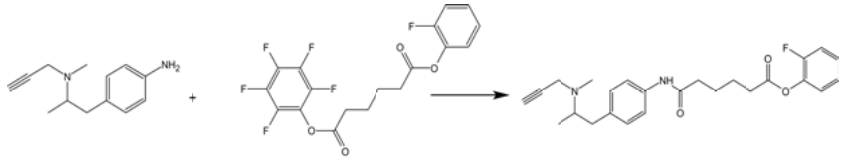


Figure 5 Synthesis of p-aminodeprenyl *o*-flurophenyl ester. Conditions: 10% pyridine, dry DMF, (r.t.).

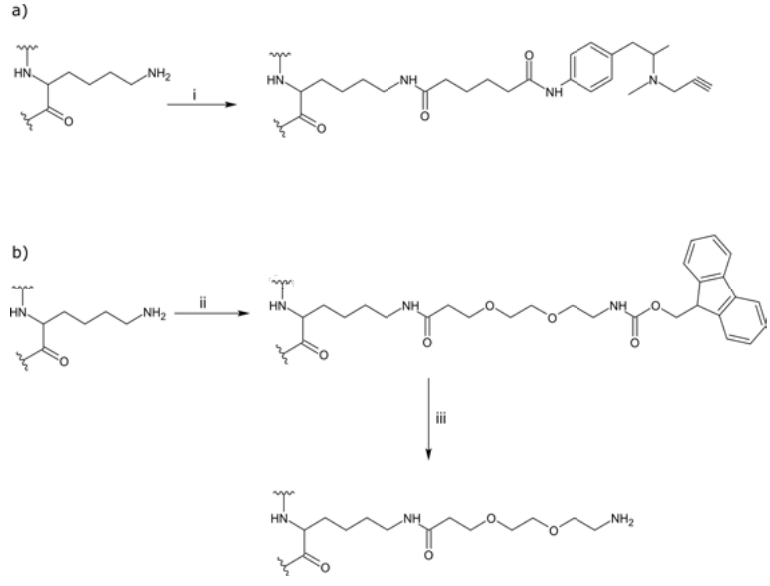


Figure 6 Synthesis of peptide-deprenyl conjugates equipped with a bifunctional PEG crosslinker. Each peptide carried two orthogonally protected Lys residues. Reagents and conditions: (i) p-aminodeprenyl *o*-flurophenyl ester (6 equiv.), 2% DIPEA, 10% pyridine, dry DMSO, (r.t.), (ii) Fmoc-NH-PEG_2-NHS ester (4 equiv.), DIPEA (8 equiv.), DMSO, (r.t.), (iii) 15% TEA, dry DMF, (r.t.).

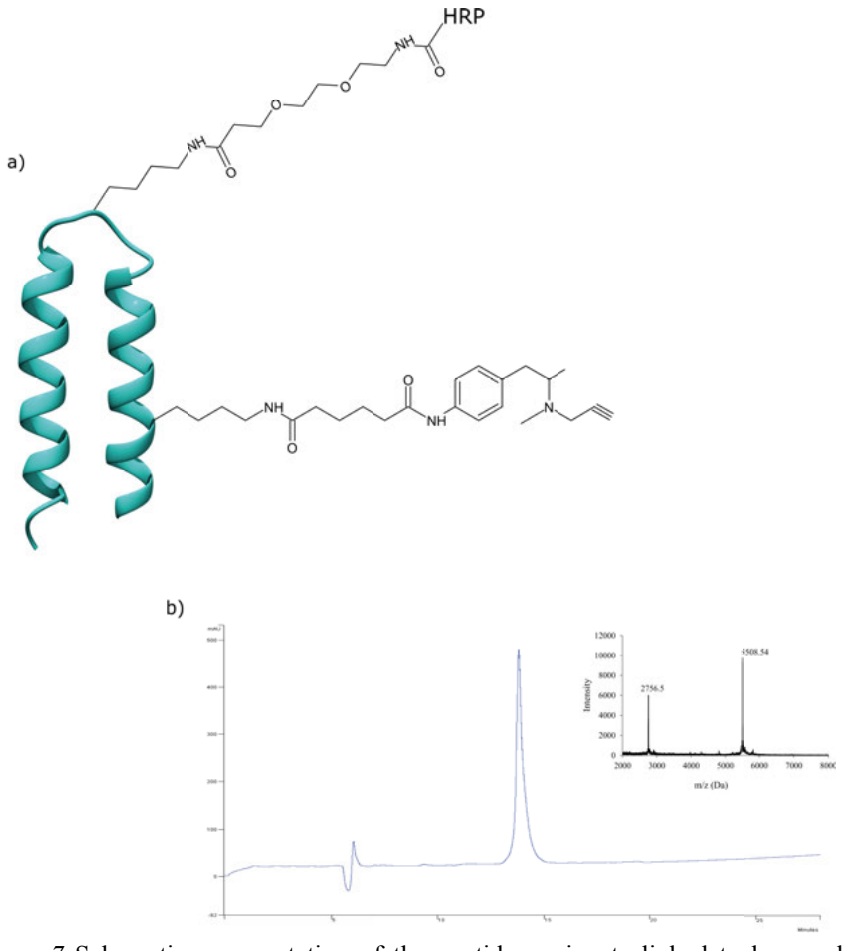


Figure 7 Schematic representation of the peptide conjugate linked to horseradish peroxidase (HRP). Analytical HPLC chromatogram and MALDI-TOF mass spectrum of the polypeptide conjugated to d-deprenyl and the Fmoc deprotected PEG_2 crosslinker show pure reaction product prior to HRP conjugation.

3.2.2 Polypeptide-DOTA conjugation

Carbonic anhydrases (CAs) are metalloenzymes that catalyze the reversible hydration of carbon dioxide to form carbonic acid, in the form of bicarbonate ions and protons at physiological pH. First identified in 1933, carbonic anhydrases are frequently found in plants and animals and are classified in six categories: α-, β-, γ-, δ-, ζ-and η-CAs[43]. Mammal CAs belongs to the alpha family and exists in nearly fifteen forms, differing as to their subcellular location and tissue-specific distribution. Structural studies have shown that the active site of the enzymes is located in a deep cavity, where a zinc ion is

tetrahedrally coordinated by three residues and one water molecule [43-44]. Each enzyme has a number of cellular functions and their absence or abnormal function can be a factor in disease. The overexpression in man of the membrane-bound isoenzyme human Carbonic Anhydrase IX (HCA IX) is correlated with tumor progression[45].

Acetazolamide (AZA) is a small molecule drug used to treat glaucoma, epilepsy, periodic paralysis, and heart disease. It is a CA inhibitor which binds to the active site of the enzyme[46].

An acetazolamide-polypeptide conjugate (4-C10L17-AZA) was previously shown to bind HCA IX with a dissociation constant of 90pM (Yang et al, submitted for publication), thus being a potential conjugate for imaging and therapeutic applications.

4-C10L17-AZA equipped with a DOTA chelating agent was synthesized for its evaluation as a possible in vivo imaging agent targeting overexpressed HCA IX in tumors. Such a construct could be radiolabelled with several nuclides such as ^{111}In[47].

Due to the increased complexity of the polypeptide conjugate, in comparison to that of 4-C10L17-AZA, a synthesis protocol was developed that deviated from the previous one. A 19-mer peptide corresponding to residues Lys17-Gly42 of the 42-mer polypeptide was synthesized on resin with Lys17 orthogonally protected by the 4-methoxytriphenylmethyl (Mmt) group and the *N*-terminal amino group Fmoc protected.

The acetazolamide pentafluorophenyl ester derivative was obtained after acetazolamide hydrolysis, followed by the reaction with an aliphatic dicarboxylic acid spacer, and subsequent esterification (J. Yang).

After selective removal of the Mmt group under mild acidic conditions, the AZA derivative was reacted with the side chain of Lys17 in the presence of 8equiv. DIPEA in DMF. The reaction progress was monitored by cleavage of a small amount of resin with a mixture of TFA/TIS/H_2O (95:2.5:2.5 v/v) and the developing conjugates identified via MALDI-TOF mass spectrometry. A peptide-acetazolamide conjugate was obtained after 1h. The Fmoc group of the *N*-terminal amino group was then removed and the peptide elongated to a 42-mer. Lys10 was deprotected and reacted with dansyl chloride. A number of byproducts were unfortunately obtained at the end of the synthesis and the desired peptide conjugate was only produced in low yield.

The protocol was modified (Figure 8) and the synthesis of the 42-mer repeated with Lys10, Lys17 and Lys41 orthogonally protected by Mmt, *tert*-butyloxycarbonyl (Boc) and Tfa groups respectively. The conjugation of the dansyl probe to the peptide was carried out on solid phase after the deprotection of Lys10 in presence of 6equiv. DIPEA, in DMF. The reaction was complete after 2h and the dansylated peptide was obtained in a good yield (95%). After cleavage of the peptide from the resin and simultaneous deprotection of all side chain protecting groups except Tfa, in TFA/TIS/H_2O

(95:2,5:2,5 v/v) solution, the peptide-dansyl conjugate was purified by RP-HPLC. The AZA pentafluorophenyl active ester was reacted with the amino group of the Lys17 side chain in the presence of DIPEA, in DMSO. The active site of HCA IX is located at the bottom of a cavity and a decanedioic acid spacer was introduced to allow simultaneous binding of the polypeptide and the AZA group.

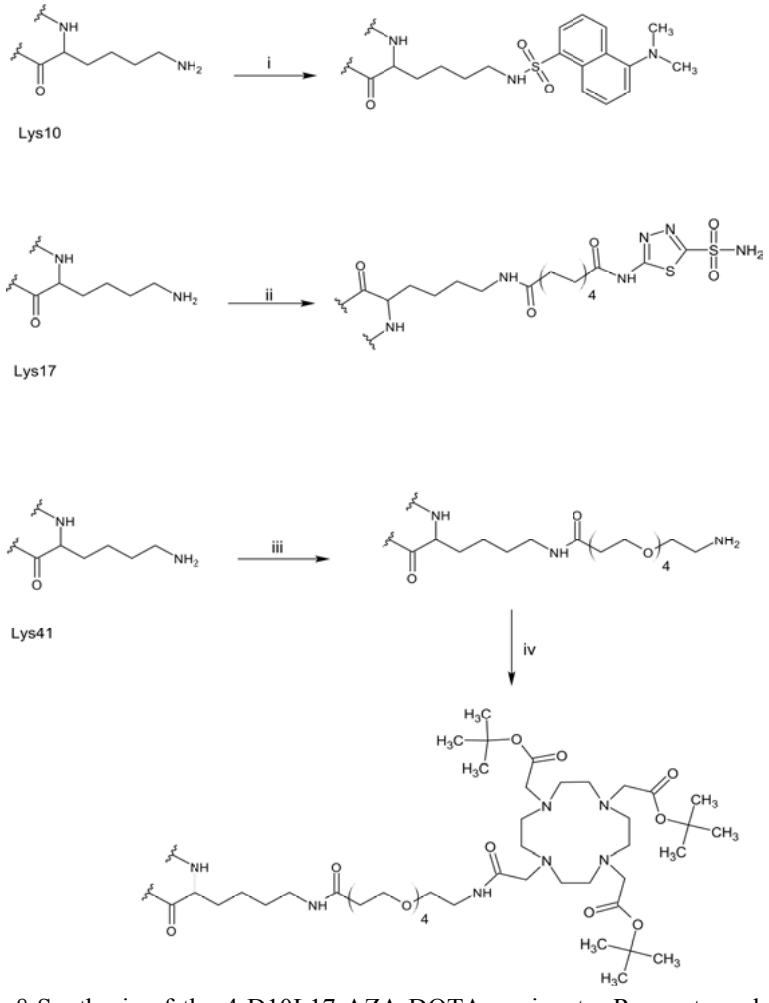


Figure 8 Synthesis of the 4-D10L17-AZA-DOTA conjugate. Reagents and conditions: (i) dansyl chloride, 6equiv. DIPEA, DMF, (r.t), on solid phase. (ii) AZA equipped with spacer and pentafluorophenyl ester end group (4 equiv.), DIPEA (8equiv.) dry DMSO, (r.t.), in solution phase, (iii) Fmoc-NH-PEG_4-NHS ester (4 equiv.), DIPEA (8 equiv.), dry DMSO, (r.t.), (iv) DOTA-tris(*t*Bu) NHS ester (4equiv.), DIPEA (6equiv), dry DMSO, (r.t).

After removal of the Lys41 Tfa protecting group under basic conditions a heterobifunctional PEG_4 NHS ester with an Fmoc protected amino group

was reacted with the deprotected Lys side chain, Figure 8. The Fmoc group was then removed (15% TEA in DMF) and the tris-*tert*-butyl protected and NHS-activated DOTA was reacted with the deprotected Lys residue of the peptide. Finally hydrolysis of the DOTA *t*-butyl esters (TFA/TIS/H_2O) gave the desired dansyl-acetazolamide-DOTA-peptide conjugate, Figure 9. For all the conjugation reactions, the yields were estimated to be in the range of 80-95%, according to the relative intensities at 210 nm of the chromatography peaks of the modified polypeptides and the peaks of the starting materials. Each polypeptide conjugate (intermediate) was purified by preparative RP-HPLC, before further polypeptide modifications.

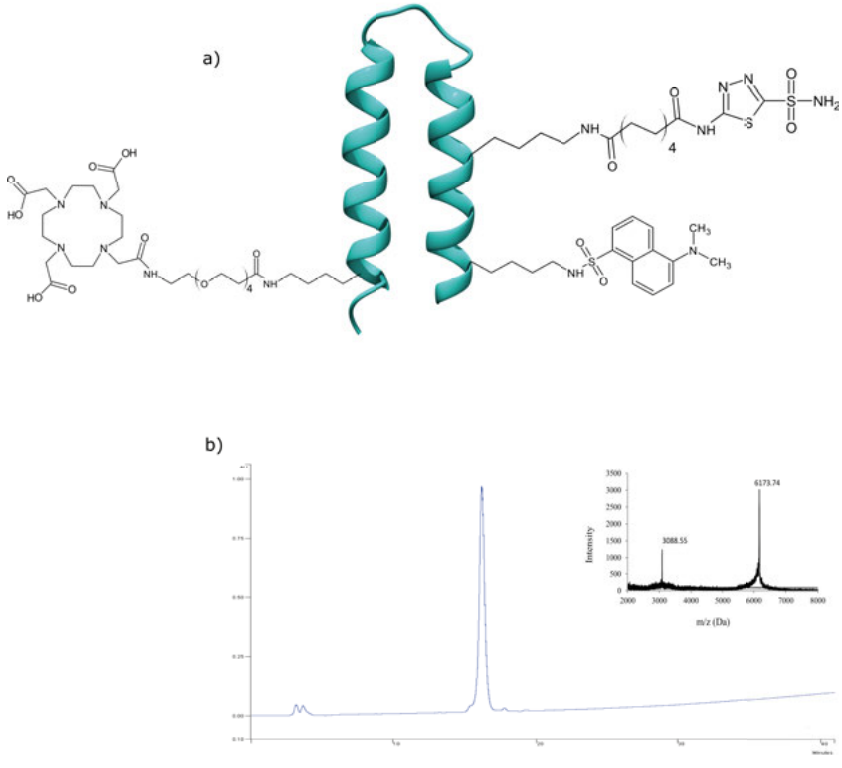


Figure 9 Schematic representation of the AZA-dansyl-DOTA-polypeptide conjugate. Analytical HPLC chromatogram and MALDI-TOF mass spectrum of the polypeptide conjugate show pure product.

3.2.3 Polypeptide-DNA conjugation

Proximity ligation assay (PLA) is a technique used for protein recognition and analysis. The method is based on the conjugation of DNA strands to reagents e.g. antibodies that can detect and bind a target protein[48]. When two or more such DNA-reagent conjugates are in close proximity to one another on a protein surface, the oligonucleotide tails ligate together, providing an amplicon that can be detected by real-time PCR or other amplification methods. PLA was developed using antibody-DNA conjugates that either bind to discrete subunits of a protein dimer or a monomer[49].

In order to evaluate whether polypeptide conjugates were suitable recognition elements in PLA analyses peptide-based affinity molecules for the recognition of the phosphorylated proteins Smad3 and glycogen phosphorylase a (GPa) were conjugated to oligonucleotide tags, Table3. A 42-mer polypeptide (4-C15L8) conjugated to a phosphate-specific Zn chelator (3,5-bis[[bis(2-pyridylmethyl)amine]methyl]benzoic acid, (PP1)) was designed to bind a variety of phosphorylated proteins with high affinity, in the presence of zinc ions[23c]. The incorporation of PP1 in the peptide was carried out in solution in the presence of 3equiv. HCTU and 6 equiv. DIPEA. The peptide-PP1 conjugate was obtained in 95% yield after 30min reaction. The two 14-mer oligonucleotides derivatized with aldehyde functional groups were conjugated to the polypeptide through hydrazone bond formation at the side chain of Cys24, Table3, Figure 10.

Oligonucleotide 1	4FB-C_6-AGACCAGCGAACAC-OH
Oligonucleotide 2	ACAACACGTCAGAG-C_6- 4FB

Table3 The 14-mer oligonucleotides used for the formation of polypeptide-oligonucleotide conjugates. Both sequences were modified with 4-formylbenzamide (4-FB) groups to enable the reaction with the hydrazine functionalized 42 residue polypeptide

The acetamidomethyl (Acm) protecting group was removed from the Cys side chain by silver trifluoromethanesulfonate (AgOTf) in acidic aqueous solution. To obtain the functionalized peptide 5-maleimido-2-hydraziniumpyridine hydrochloride (MHPH) was reacted with the deprotected sulfhydryl group to form a thioether linkage. The conjugation reaction was monitored by analytical HPLC and MALDI-TOF mass spectrometry.

Oligonucleotides were obtained from a commercial supplier (Solulink) premodified with a benzaldehyde moiety (4FB-C_6-) and reacted with the peptide conjugate in DMSO, Figure 10.

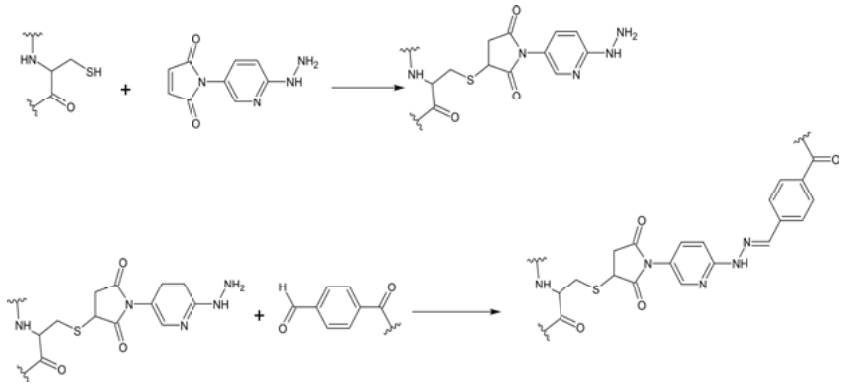


Figure 10 Reaction of the hydrazine functionalized peptide conjugate with the aldehyde functionalized oligonucleotide to form the hydrazone bound. Reagents and conditions: (i) MHPH (6equiv.), dry DMSO, (r.t). (ii) 4-FB-C_6-oligonucleotide, dry DMSO, (r.t).

The identification of the DNA-peptide conjugate by MALDI-TOF mass spectrometry is difficult due to the complexity of the product. Following the reaction progress by analytical HPLC, a peak observed at a different retention time than the peaks corresponding to the starting materials was assumed to be due to the DNA-peptide conjugate. Both peptide and DNA absorbances at 210nm and 260nm, respectively, were used to monitor the relative percentage of the reacting compounds. The oligonucleotides of the two resulting probes were further elongated by DNA ligase to 74 bases and the elongated polypeptide-DNA conjugates were analyzed by nucleic acid gel electrophoresis (M. Hammond). The presence of bands with molecular weights larger than those of the polypeptide-14-mer oligonucleotide conjugates and larger than that of the 60-mer oligonucleotide suggested that the product was in fact a polypeptide conjugated to a DNA single strand that had been enzymatically elongated, Figure 11.

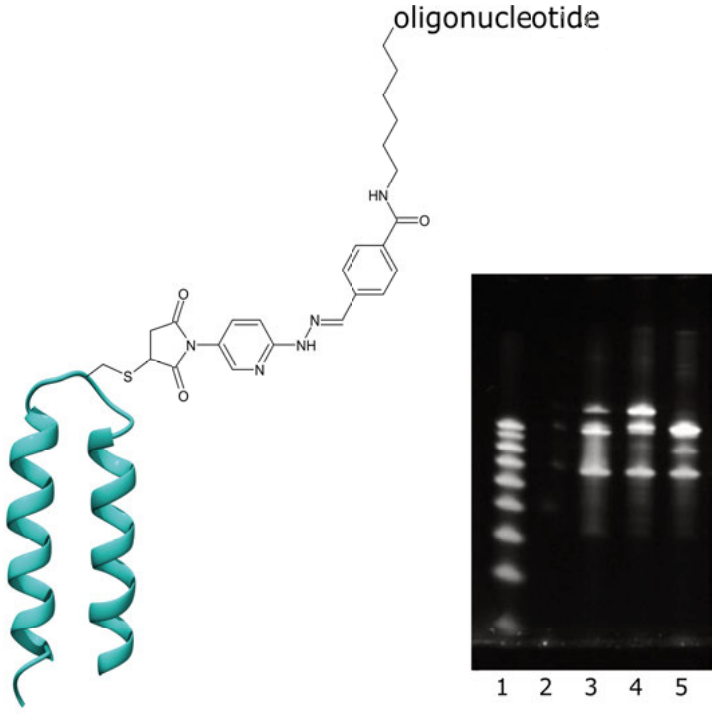


Figure 11 Schematic representation of the DNA-polypeptide conjugate (left) and 10% TBE-urea gel of single-stranded DNA and DNA-peptide conjugate (right). Lane 1. Oligonucleotide size marker (20-100 bases). Lane 2. Polypeptide conjugated to 14-mer oligonucleotide. Lane 3. Polypeptide conjugated to 14-mer oligonucleotide and enzymatically ligated with a 60-mer oligonucleotide, at a ratio of 1:1. Lane 4. Polypeptide conjugated to 14-mer oligonucleotide and enzymatically ligated with a 60-mer oligonucleotide, at a ratio of 1:5 (excess polypeptide-oligonucleotide conjugate). Lane 5. negative control (60-mer oligonucleotide without the polypeptide-oligonucleotide conjugate).

Unfortunately, preliminary in situ PLA experiments were not successful maybe because methodology developed for antibody conjugates need further adaption to become compatible with the chemical and physical properties of polypeptide-DNA conjugates.

Real-time PCR experiments were carried out to measure the affinity of the peptide-DNA conjugate for GPa in comparison to an anti-GPa antibody. The data suggested that the affinity of the peptide conjugate for GPa was lower than that of the antibody.

4. Recognition of phosphorylated proteins

4.1 Protein phosphorylation

Protein phosphorylation refers to a fundamental class of post-translational modifications of proteins that regulate many aspects of cell life, including cell cycle, differentiation, metabolism and neuronal communication[50]. Reversible phosphorylation is largely achieved by protein kinases (PKs) that catalyse the transfer of a phosphoryl group either to serine, threonine or tyrosine residues within eukaryotic organisms. A large number of PKs are encoded in the human genome and nearly 30% of all proteins are phosphorylated. Phosphorylation of serine residues dominates in comparison to threonine and tyrosine phosphorylation[50c].

The mutation of protein kinases and phosphatases, as well as that of naturally occurring toxins and pathogens, may lead to abnormal phosphorylation and result in many human ailments, ranging from diabetes, rheumatoid arthritis, cardiovascular and neurodegenerative disorders, to inflammatory diseases[51]. Recent research has shown that abnormal activity of kinases in signal transduction pathways is a factor in many types of cancer[51b].

Phosphoproteins and the kinases that induce their phosphorylation are attractive drug targets. They have been intensely investigated to understand what networks they form. The challenge, however, remains in the clarification of individual phosphorylation events and in the development of more efficient methods that generate proteomic information[52].

A number of experimental approaches have been applied to the detection and characterization of phosphorylated proteins, including western blot analysis, Enzyme-Linked Immunosorbent Assay (ELISA) and mass spectrometric analysis[53]. Mass spectrometry has been applied to the identification of phosphorylated sites in a variety of tissues. However, a number of crucial proteins are not detectable by this technique because of their low abundance, particularly since phosphorylation is a transient modification[53-54].

Enrichment technologies including immunoprecipitation, immobilized metal affinity chromatography (IMAC) and Phos-Tag based extraction have been applied for more efficient phospho-protein analysis by MS[55].

Immunoprecipitation is based on the employment of phosphate-specific monoclonal antibodies raised against phosphorylated proteins and peptides. However, while commercially available antibodies used during immunoprecipitation have advanced the identification of tyrosine-phosphorylated amino

acids they do not bind with the same efficiency to phosphoserines and phosphothreonines[55b, 56].

IMAC chromatography is a well-known technique and much data for protocol improvement is available, despite its partly low specificity[55-56].

Another method for the identification and quantification of both phosphoproteins and phosphopeptides in bioanalytical applications comprises the design of a small organic molecule named Phos-Tag, developed by Kinoshita et al. The Phos-Tag, 1,3-bis[bis(pyridin-2-ylmethyl)-amino]propan-2-olate dizinc (II) complex interacts with the oxygen atoms of the phosphate groups through charge-charge interactions. The Phos-Tag binds phosphorylated proteins with dissociation constants in the μM range[57].

4.2 Phosphorylated model proteins

AKT1 or protein kinase B is a serine/threonine kinase involved in the PI3K-AKT signal transduction pathway existing in all eukaryotic cells. There are three highly homologous isozymes named AKT 1, 2 and 3 each of them with a different cellular function. Structurally, AKT kinases consist of three conserved domains named *N*-terminal PH domain, kinase catalytic CAT domain and *C*-terminal hydrophobic motif (HM). AKT1 is activated by the phosphorylation of Thr308 and Ser473 that mediates the subsequent phosphorylation of a range of intracellular proteins and regulation of down-stream responses including cell growth, survival, migration and proliferation. The aberrant function of AKT1 through hyper-activation is implicated in the pathogenesis of several human cancers, rendering it an attractive target for the design of potential inhibitors in drug discovery[58].

Glycogen phosphorylase (GP) is an allosteric enzyme responsible for regulating the degradation rate of glycogen and retention of blood glycose levels. It catalyses the phosphorolytic cleavage of 1,4 glycosidic bonds of glycogen and the production of glucose-1-phosphate monomers; a process referred to as glycogenolysis. Glycogen phosphorylase exists in two interconvertible forms termed GPa (R-state) and GPb (T-state) associated with the high and low level of the glycogenolytic activity, respectively. In the presence of AMP and IMP effectors (which bind at the allosteric nucleotide binding site) and through phosphorylation of Ser15, the dimer phosphorylase b undergoes conversion to the tetramer phosphorylase a.

Human GP is identified in three isoforms located in metabolically active tissues, i.e. brain, liver and skeletal muscle. Abnormal activity of GPa induces a number of ailments including type 2 diabetes associated with hyperglycemia. From a drug discovery perspective, a large diversity of synthetic molecules is being investigated as GP potential inhibitors[59].

Ovalbumin (OVA) the major protein in chicken egg-whites (70%), is a monomeric phosphorylated glycoprotein of 385 residues with a molecular weight of 45kDa. It is a protein rich in glutamic and aspartic amino acids, and contains six cysteines, but only a single disulfide bond between Cys74 and Cys121, and an acetylated amino terminus. OVA undergoes several posttranslational modifications including *N*-terminal acetylation, serine phosphorylation, and glycosylation. Due to its availability, ovalbumin was used as a model antigen in immunological studies. Characterization of antibodies binding to OVA helped in mapping the immunogenic epitopes and in characterizing structure–function relationship of ovalbumin antigen[60].

4.3 The polypeptide-bis(di-(2-picolyl)amine) conjugate

A phosphate-specific organic molecule was designed for the recognition of phosphorylated proteins based on the propensity for Zn^{2+} ions of polyamines and on the affinity of Zn^{2+} ions for phosphate ions. Two di-(2-picolyl)amino groups 2,2'-dipicolylamino were linked to 3,5 dimethyl benzoic acid leading to the formation of a 3,5-bis[[bis(2-pyridylmethyl)amino]methyl]-benzoic acid (PP1)[23c, 61]. In the presence of Zn^{2+} PP1 binds inorganic phosphate with an affinity in the μM range mainly by charge-charge interactions between the zinc ions of the chelate and the negatively charged oxygen atoms of the phosphate, Figure 12.

The zinc-chelating molecule was conjugated by amide formation to the side chain of lysine residues in eight of the 42-mer polypeptides with total charges of -1 and +2. The conjugate found to have the highest affinity for α-casein, had a dissociation constant of 17 nM. The phosphate-specific peptide conjugate was observed to bind a number of phosphorylated proteins including ERK1 and β-casein but weak or no binding was observed for the mono- and di-phosphorylated OVA and AKT1 proteins[21a, 23c].

Further investigations were undertaken to understand the scope and limitations of phosphoprotein recognition and binding by polypeptide-PP1 conjugates.

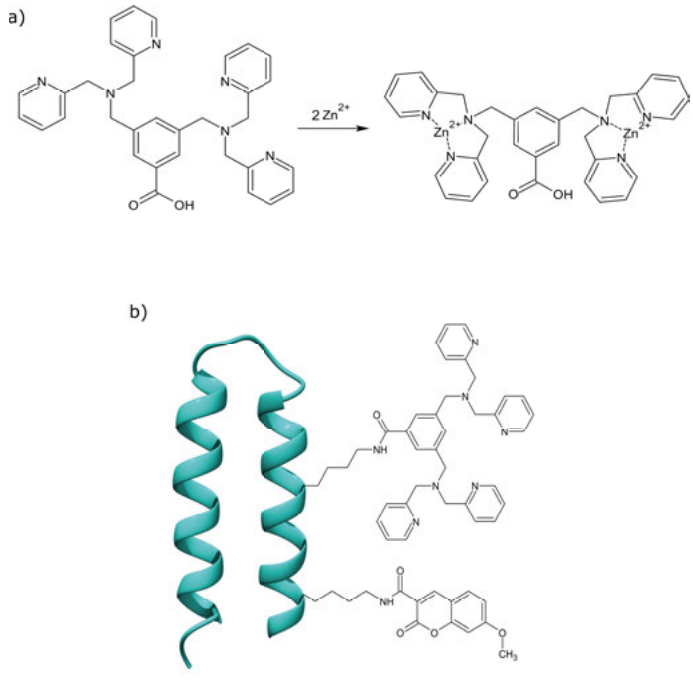


Figure 12 Schematic representation of the polypeptide-PP1 conjugate for the recognition of phosphorylated proteins (b). PP1 and a coumarin probe were incorporated at side chains of lysine residues in the peptide through amide bond formation. The ligand forms a chelate with Zn^{2+} ions (a).

4.3.1 The recognition of AKT1

The phosphate-specific PP1 ligand was conjugated to all 42-mer sequences of the 16-membered polypeptide set. The coumarin probe was attached to all of the polypeptides for preliminary affinity screening by fluorescence.
The binding of AKT1 was evaluated via sandwich competitive ELISA assay. A more detailed description of the assay is included in chapter 8.

In brief, ELISA is a test for the quantification of proteins usually based on antibodies. The sandwich ELISA is a method where a capture antibody is immobilized on a surface through non-specific absorption, the sample is applied and the protein captured. Next, a detecting antibody is added and a secondary antibody (antibody-enzyme conjugate) that recognizes the detecting antibody binds and provides a signal that is proportional to the amount of captured protein. Several washing steps are included to ensure that the signal arises only due detection of the intended protein.

In a competitive ELISA, the detecting antibody is pre-incubated with the molecule that is to be measured. When the sample is applied the detecting antibodies are displaced by the molecules in the sample, reducing the signal

with increased molecules concentration[62]. The following experiment was set up to compare the affinities of the polypeptide conjugates for AKT1 to that of a commercially available anti-phosphoserine antibody.

A sandwich ELISA was carried out to validate the experimental setup and to determine the concentration of the AKT1 protein. Stock solutions of polypeptide conjugates dissolved in HEPES buffer containing 4μM $ZnCl_2$ were diluted in steps of 2 to give final concentrations in the range 100-5000nM. AKT1 was incubated for 2h at room temperature in commercially available ELISA well plates (Sigma-Aldrich) coated with the capture antipan-AKT1 antibody and blocked with blocking buffer. Excess protein was discarded and the wells washed four times with washing buffer. In the competition experiment, solutions containing the detecting antibody (anti-phospho-AKT1, Ser73) at a fixed concentration and the polypeptide conjugates over a range of concentrations were added to the well plates and incubated for 1h at room temperature. Well plates containing the AKT1 protein and the anti-phospho-AKT1 antibody and wells without any protein were used as positive control and blank respectively. The wells were washed and solutions of horseradish peroxidase (HRP)-conjugated to an anti-rabbit IgG antibody added. After incubation for 1h and washing as above, the reaction of HRP was stopped by the addition of a H_2SO_4 solution. The bound HRP-antibody conjugate was quantified by the addition of tetramethyl benzidine (TMB) and the measurement of the absorbance at 450 nm using an ELISA plate reader. The assay was performed in triplicates for each polypeptide conjugate. The absorbance is proportional to the relative affinities for AKT1 of the polypeptide conjugates and the antibody, Figure 13.

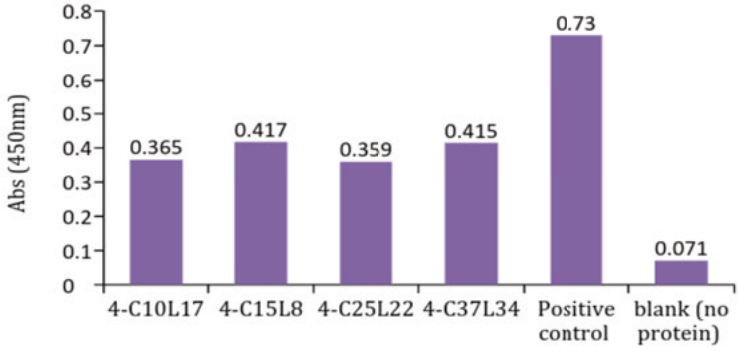


Figure 13 The absorbance at 450nm measured after incubating the anti-phos (Ser73) AKT1 antibody in the presence of 5 μM polypeptide conjugates in a competitive sandwich ELISA format. In the, positive control there is no competing polypeptide conjugate, and in the blank there is no AKT1 protein.

The affinities of the polypeptides for AKT1 were low in comparison to the anti-phospho-AKT1antibody. The polypeptides could compete with the antibodies, reducing the absorbance to approximately 50% only at a 5 μM concentration. In the case of the AKT1 protein chemical modifications of the polypeptide was clearly necessary to enhance the affinity, Figure 13.

4.3.2 Polypeptide dimerization

Hydrophobic interactions provide more binding free energy in aqueous solution than charge-charge interactions and hydrogen bonds, and dominate the interactions between the polypeptide conjugates and proteins. A single Leu amino acid can provide as much as 20 kJ/mol of free binding energy at room temperature, but only if it is perfectly embedded in a hydrophobic cavity[11]. To improve the binding performance of the 42-residue polypeptides the number of hydrophobic amino acids was increased by peptide dimerization.
A bismaleimide diethylene glycol homobifunctional crosslinker ($BM(PEG)_2$) was used for the peptide dimerization by reacting it with the sulfhydryl groups of Cys residues of the polypeptides, Figure 14. Prior to the conjugation reaction, disulphide bonds between Cys residues were reduced using DTT reducing reagent. The reaction was conducted in DMSO solvent, introducing the $BM(PEG)_2$ crosslinker at half the concentration of the polypeptide in order to prevent the formation of by-products.

The coumarine fluorophore was attached to the polypeptide conjugates to allow the evaluation of affinity by fluorescence titration. For accurate results, concentrations of stock solutions of the polypeptide conjugates were determined by amino acid analysis. Fluorescence titration experiments were performed using a microtiter plate reader and 96 well microtiter plates.

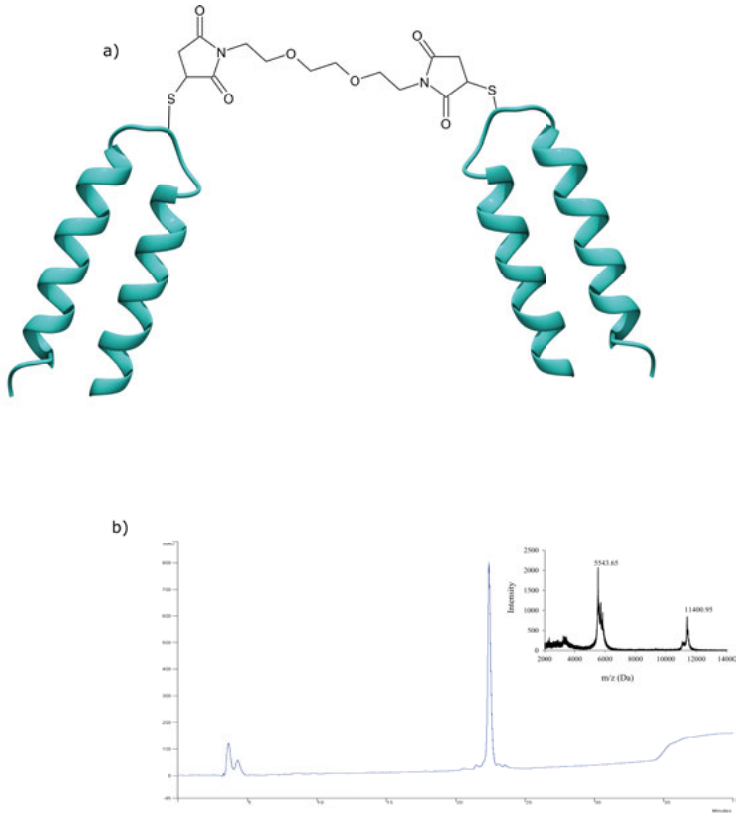


Figure 14 Schematic representation of the polypeptide dimer. The dimerization of the peptide was attained by the reaction of free sulfhydryl groups of the cystein residues in the polypeptide sequences with the maleimide groups of the $BM(PEG)_2$ crosslinker. Analytical HPLC chromatogram and MALDI-TOF mass spectrum of the dimer conjugate shows pure product.

The affinity of OVA and GPa proteins was evaluated. Each peptide dissolved in HEPES buffer at pH 7.4 in the presence of 4μM $ZnCl_2$ was added to the wells of the microtiter plate to give a final concentration of 500 nM and the protein was added to give final concentrations of 0nM, 500nM, 1000nM and 1500nM. The change of fluorescence intensity upon addition of the protein in equimolar amounts with no further change in intensity upon further addition of protein was taken as evidence of "strong" binding. A gradual change of fluorescence intensity as a function of protein concentration was taken as evidence of "weak" binding. The experiment was performed three times for each peptide conjugate. In addition, fluorescence intensity measurements of the protein in buffer solution were used as a negative control.

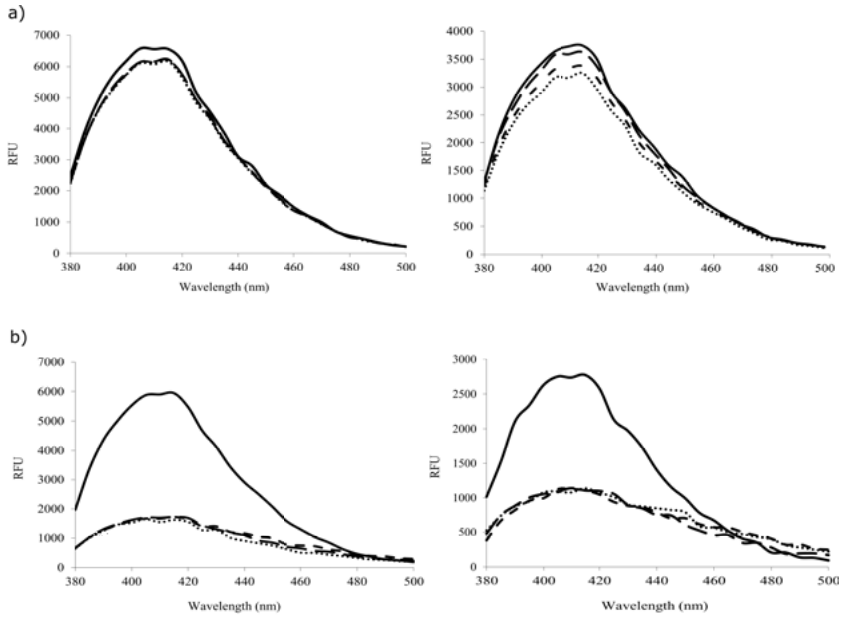


Figure 15 Fluorescence emission titrations of binding to a) ovalbumin (OVA) and b) glycogen phosphorylase a (GPa) by the 4-C15L8-PP1 dimer (left panels) and the 4-C15L8-PP1 monomer (right panels). The concentration of the polypeptide conjugates was 500 nM and the concentrations of GPa and OVA were 500 nM, 1000 nM and 1500 nM respectively. The dimer binds OVA better than the monomer. The monomer binds GPa strongly and at the concentrations used improved binding by the dimer is not visible.

As a result of dimerization, all the four polypeptide conjugates exhibited enhanced affinity for GPa and OVA in comparison to those of the monomers, Figure 15. In comparison to the other three monomers, 4-C15L8-PP1 was also identified as the polypeptide conjugate with the highest affinity for GPa. The difference between 4-C15L8-PP1 and the monomers 4-C10L17-PP1, 4-C25L22-PP1 and 4-C37L34-PP1 illustrated the significance of which position was used for ligand incorporation**.**

5. Polypeptide conjugates as facilitators of increased affinity for protein targets

5.1 Scouting protein surfaces for non-obvious hydrophobic binding pockets. (Paper I)

Interfaces between phosphorylated proteins in protein-protein interactions are frequently hydrophobic with large non-polar flat surface areas[14]. Hence, hydrophobicity is the main driving force of such interactions. Phosphorylation occurring outside of an interface may cause long-range conformational changes through allosteric mechanisms and directly influence the binding of the partner; a classic example is glycogen phosphorylase [63]. It has also been shown that flexible regions as well as intrinsically disordered proteins have a tendency to be phosphorylated, which might induce both disorder-to-order and order-to disorder protein transitions [64]. Methods for the identification of hydrophobic patches close to the binding site of small organic molecules of proteins in general could therefore be developed based on the phosphorylation site of GPa.

The polypeptide conjugate 4-C15L8-PP1 which was previously found by fluorescent titration experiments to bind GPa was selected as the analytical tool in the search for non-obvious hydrophobic binding pockets in proximity to the phosphorylated Ser15 residues of the protein. A number of modifications to the 4-C15L8-PP1 conjugate were introduced including the systematic reduction of peptide hydrophobicity by replacing hydrophobic residues Nle, Ile and Leu with Ala. Peptide hydrophobicity was also increased by the introduction of *L*-2-aminooctanoic acid (Aoc) and mono- and di-fluorinated phenylalanine residues, Table 4. In all designed peptides, the Zn^{2+} chelating agent PP1 was covalently attached to the side chain of a Lys residue.

4-C15L8	
	Ac-NAADJEA**K**IRHLRE*K*JAARGPRDCAQJAEQLARRFERFARKG-NH_2
I	Ac-NAADAEA**K**ARHARE*K*JAARGPRDCAQJAEQLARRFERFARKG-NH_2
II	Ac-NAADJEA**K**IRHLRE*K*AAARGPRDCAQAAEQAARRFERFARKG-NH_2
III	Ac-NAADAEA**K**IRHLRE*K*JAARGPRDCAQJAEQLARRF ERFARKG-NH_2
IV	Ac-NAADJEA**K**ARHLRE*K*JAARGPRDCAQJAEQLARRFERFARKG-NH_2
V	Ac-NAADJEA**K**IRHARE*K*JAARGPRDCAQJAEQLARRFERFARKG-NH_2
VI	Ac-NAADJEA**K**IRHLRE*K*AAARGPRDCAQJAEQLARRFERFARKG-NH_2
VII	Ac-NAADJEA**K**IRHLRE*K*JAARGPRDCAQAAEQAARRFERFARKG-NH_2
VIII	Ac-NAADJEA**K**IRHLRE*K*JAARGPRDCAQJAEQLARRF_4ERF_{34}ARKG-NH_2
IX	Ac-NAADJEA**K**IRHLRE*K*JAARGPRDCAQJAEQLARRAERAARKG-NH_2
X	Ac-NAADAocEA**K**AocRHAocRE*K*JAARGPRDCAQJAEQLARRFERFARKG-NH_2
XI	Ac-NAADJEA**K**IRHLRE*K*AocAARGPRDCAQAocAEQAocARRFERFARKG-NH_2
XII	Ac-NAADJEA**K**IRHLRE*K*JAARGPRDCAQJAEQLARRAocERAocARKG-NH_2
XIII	Ac-DJEA**K**IRHLRG-NH_2
XIV	Ac-DJEAib**K**IRAibLRG-NH_2
XV	Ac-DAocEA**K** AocRHAocRG-NH_2
XVI	Ac-AA_{C5}AAocEA_{C5}**K**AocRGAocRG-NH_2
XVII	Ac-A**K**AocRHAocRE*K*AocG-NH_2

Table 4 Amino acid sequences of polypeptide conjugates designed for GPa binding. PP1 was incorporated at the side chain of Lys residues shown in boldface, coumarin was conjugated at Lys residues shown in italic and the Ala residues introduced to reduce hydrophobicity are shown in red. The non-naturally occurring residues Aoc, F_4 and $F_{3,4}$ are green, Aib and AC_5 are blue. Cys24 and Lys41 were orthogonally protected with Acm and Tfa groups, respectively.

The evaluation of binding interactions was carried out by Surface Plasmon Resonance (SPR) interaction analysis[65] using a Biacore 2000 instrument (Chapter 8). The model protein GPa from rabbit muscle, was immobilized on a sensor chip and the peptide conjugates were included in the running buffer in the concentration range from 1nM to 125 nM. The HEPES buffer at pH 7.4 contained 4μM $ZnCl_2$.

Accurate measurements of dissociation constants were obtained by conjugating fluorescein to the side chain of Cys24 to enable the titration of polypeptide conjugate-GPa complex formation. The polypeptide conjugate equipped with the fluorophore was kept at constant concentration whereas that of GPa was titrated in the range from 1 nM to 20 μM.

For the identification of hydrophobic residues that interact with GPa, the hydrophobic amino acids Nle, Leu, Ile, and Phe were replaced by Ala residues. As a result, the affinity for GPa decreased, showing that peptide hydrophobicity contributed to the affinity enhancement of the polypeptide conjugate-protein interactions, Figure 16. In contrast, the replacement of Phe35 and Phe38 with Ala residues did not have an observable effect on affinity.

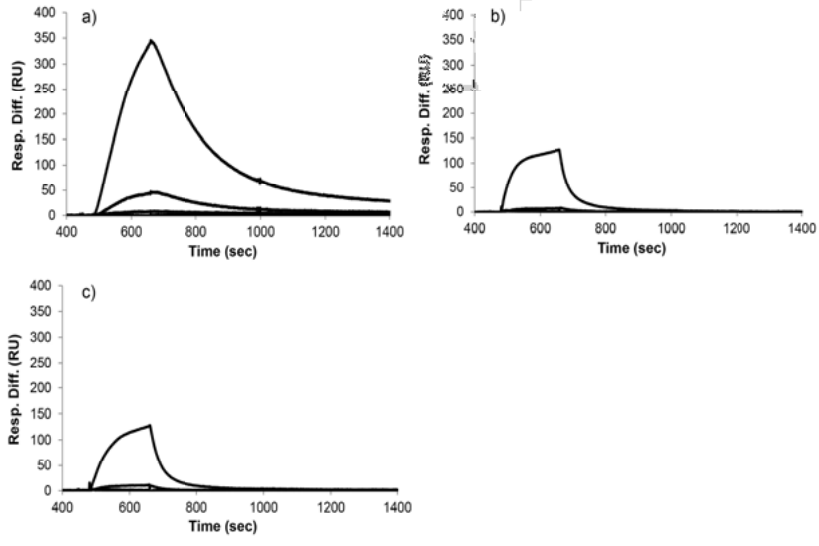


Figure 16 SPR sensorgrams showing the interactions between immobilized GPa and a) 4-C15L8-PP1, b) I-PP1, and c) II-PP1. Polypeptide conjugates were injected at 1, 5, 25 and 125 nM concentration in HEPES buffer in the presence of 4μM $ZnCl_2$, at 25°C. The sensorgram obtained by a separate injection of 4μM $ZnCl_2$ solution was subtracted from each of the ones obtained from the injection of polypeptide conjugates.

The identification of the hydrophobic residues of 4-C15L8-PP1 that were involved in binding interactions, suggested that affinity could be enhanced by the introduction of residues with increased hydrophobicity. The strength of hydrophobic interactions depends on the number of carbon atoms and the shape of the hydrophobic group. In comparison to branched residues which can cause steric hindrance, linear side chains of amino acids can adopt a variety of conformations and interact in a more flexible way[11]. Fluorine is more hydrophobic than carbon[66].

Nle, Ile and Leu residues were replaced by *L*-2-aminooctanoic acid (Aoc), whereas the Phe residues were replaced by the fluorinated derivatives 3,4-difluoro-*L*-phenylalanine and 4-fluoro-*L*-phenylalanine, Table 4.

The introduction of three Aoc residues increased the binding affinity, as demonstrated by a dramatically reduced off-rate and by binding observed at lower concentrations than those of other conjugates, Figure 17. The introduction of Aoc5, Aoc9 and Aoc12 gave rise to a dissociation constant of 27 nM as compared to that of 4-C15L8-PP1 of 280 nM, an order of magnitude enhancement, Table 5.

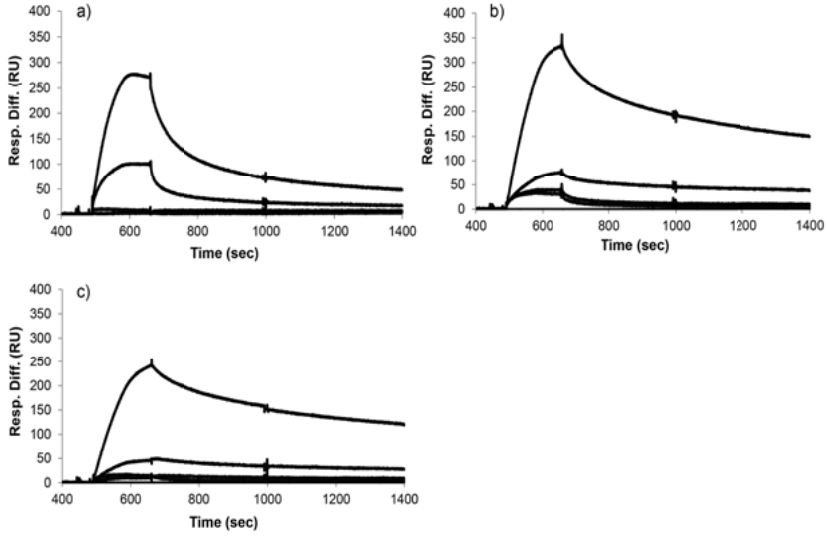


Figure 17 SPR sensorgrams showing the interactions between immobilized GPa and a) 4-C15L8-PP1, b) X-PP1, and c) XI-PP1. Polypeptide conjugates were injected at concentrations of 1, 5, 25 and 125 nM in HEPES buffer in the presence of 4μM $ZnCl_2$, at 25°C. The sensorgram obtained by a separate injection of 4μM $ZnCl_2$ solution was subtracted from those obtained at injections of polypeptide conjugates.

The affinity reduction observed for the fluorophenylalanine modified polypeptide conjugates was not found for the corresponding Aoc derivatives where instead the dissociation rate was remarkably reduced. The reason might be that fluorines induce higher propensity for peptide-peptide interactions and favour homodimerization over protein interactions. On the other hand, Aoc residues due to their size are able to identify binding pockets further removed from Ser15, alternatively bind better due to their conformational flexibility.

The observation that the interactions in close proximity to PP1 were the most important for affinity, and that affinities increased with the introduction of Aoc residues suggested that polypeptides could be miniaturized. The size of the polypeptide was therefore reduced from 42 residues to 11 with the sequences based on residues Asp4-Arg13 of 4-C15L8, Table 4. As expected, the affinity of the 11-mer sequence with the original residues was reduced in comparison to the 42-mer, the dissociation constants were 2 μM and 280 nM, respectively, Table 5.

The introduction of three Aoc residues increased the affinity of the 11-mer to within a factor of 2 of the 42-mer, suggesting a convenient approach to downsizing, Table 5.

It has been shown that helical folded peptides with controlled conformations bind well to protein surfaces. Aib residues were introduced and a stapling protocol employed to control the conformation of the 11-mer pep-

tide and induce helix formation[67]. However, CD spectroscopic measurements did not show helix formation for any of the 11-mers and there was no observed affinity increase.

In contrast, the replacement of residues Nle2, Ile6 and Leu9 with the more flexible Aoc derivative led to the formation of a peptide conjugate with a K_D of 550 nM, Table 5. Affinity was shown to be more efficiently enhanced by flexible hydrophobic amino acid side chains than by control of conformations.

Peptide	K_D (nM)
4-C15L8-PP1	280 ± 60
I-PP1	590 ± 190
X-PP1	27 ± 5
XIII-PP1	2040 ± 200
XV-PP1	550 ± 100

Table 5 Dissociation constants of the polypeptide conjugate-GPa complexes measured by fluorescence spectroscopy. Titrations with GPa were carried out at constant concentrations of polypeptide conjugate in HEPES buffer at pH 7.4, in the presence of 4μM $ZnCl_2$. The conjugates were equipped with the strongly emitting fluorophore fluorescein-5-maleimide (5-MF).

6. Conjugation of a dipicolyl chelate to polypeptides increases binding affinity for human serum albumin and survival times in human serum (Paper II)

6.1 Human serum albumin (HSA)

Most peptides have a short half-life in vivo as a result of rapid renal clearance and proteolytic degradation. Efforts to increase survival times include peptide modifications, glycosylation and conjugation to PEG polymers [26a]. The pharmacokinetic properties in vivo of small molecule drugs are linked to their association with plasma proteins. HSA, one of the most abundant plasma proteins, has been shown to bind a number of drugs and the properties of drug-HSA complexes are therefore of great interest[68].

HSA is a non-glycosylated 66.5 kDa monomer, composed of 585 amino acids, which is synthesized and secreted from the liver. It possesses three homologous highly α-helical domains, domain I, II and III. Each domain is divided into two subdomains A and B that mediate binding with a large number of ligands. There are two major binding sites in HSA, site I and II, which are located in the subdomain IIA and subdomain IIIA respectively. Site I has a high binding affinity for heterocyclic compounds and is referred to as a warfarin site. Site II interacts through electrostatic and hydrophobic interactions with small aromatic or acidic ligands. In addition, there are important metal ion binding sites in all domains[69].

Previous studies have shown that the affinity of various molecules for HSA is increased in the presence of metal ions[70]. This observation suggests that new HSA binding sites in proximity to the metal binding site can be identified and that strategies can be developed to improve the binding capacity of weak binders for HSA, e.g. peptides. Structural studies have revealed a zinc binding site at the interface between domains I and II. It is a four coordinate site that comprises the conserved residues His67/Asn99 and His247/Asp249 from domain I and II respectively[71].

The conjugation of peptides to a zinc chelator was therefore investigated as a potential strategy for improved binding of peptides to HSA.

6.2 Effect on HSA affinity by conjugation of a zinc chelator to peptides

A series of previously reported peptide-PP1 conjugates were selected based on their charge and hydrophobicity and ranked for their affinity to HSA by SPR analysis, Table 6.

1-C15L8	Ac-NEADLEA**K**IRHLAE*K*LEARGPEDCEQLAEQLARAFEAFARKG-COOH
2-C15L8	Ac-NEADLEA**K**IRHLAE*K*LAARGPVDCAQLAEQLARAFEAFARKG-COOH
3-C15L8	Ac-NAADJEA**K**IRHLAE*K*JAARGPVDCAQJAEQLARRFEAFARKG-NH_2
4-C15L8	Ac-NAADJEA**K**IRHLRE*K*JAARGPRDCAQJAEQLARRFERFARKG-NH_2
5-C15L8	Ac-NAARLEA**K**IRRLRE*K*LAARGPRDCAQLREQLARRFERFARKG-NH_2
VI	Ac-NAADAocEA**K**AocRHAocRE*K*JAARGPRDCAQJAEQLARRFERFARKG-NH_2
VII	Ac-DAocEA**K**AocRHAocRG-NH_2

Table 6 Amino acid sequences of polypeptides conjugated to PP1 and ranked for HSA binding presented in the one-letter code. For clarity the Aoc residues are coloured blue, the Lys residue to which PP1 was conjugated is shown in boldface and the Lys residue where coumarin was incorporated is shown in italic. The Cys residue and Lys41 were orthogonally protected with Acm and Tfa groups respectively.

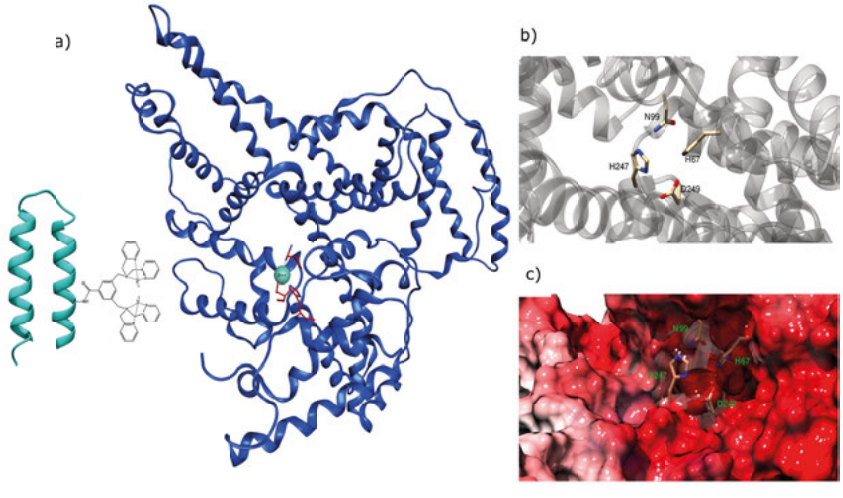


Figure 18 Illustration of the interaction between the polypeptide conjugate and HSA. a) Schematic structure of the 42-mer polypeptide conjugated to PP1 and the crystal structure of HSA (PDB ID code 4K2C). The PP1 ligand incorporated at the side chain of a Lys residue interacts with the Zn^{2+} ion in the zinc-binding site of HSA and the peptide contributes further binding energy by interactions with the residues of the protein surface in close proximity to the zinc binding site. b) Detail of crystal structure of the zinc binding site of HSA. Zinc ions are tetrahedrally coordinated with the conserved residues (His-67, Asn-99, His-247, Asp-249) located between domains I and II. c) Molecular surface representation with charge distributions of HSA Zn^{2+} binding site. Deep red colour indicates charge of -10.

The protein was covalently immobilized on a sensor chip and the peptide conjugates were injected in the concentration range from 4nM to 500nM in HEPES buffer at pH 7.4 in the presence of 1μM $ZnCl_2$.

Control measurements of peptides without the PP1 ligand or in the presence of the Zn^{2+}chelating agent EDTA provided evidence that the polypeptide conjugates targeted the zinc binding site of the HSA.

Polypeptide-PP1 conjugates with charges ranging from -7 to +5 were evaluated with regards to affinity for HSA and shown to bind stronger the more positive the charge, Figure 19, Figure 20. The strong dependence on charge suggests interactions between the positively charged polypeptide and the negatively charged surface area next to the zinc binding site of HSA. It appears that the PP1 ligand and the positively charged peptide bind cooperatively to HSA, enhancing the overall affinity over that of the peptide alone, Figure 18.

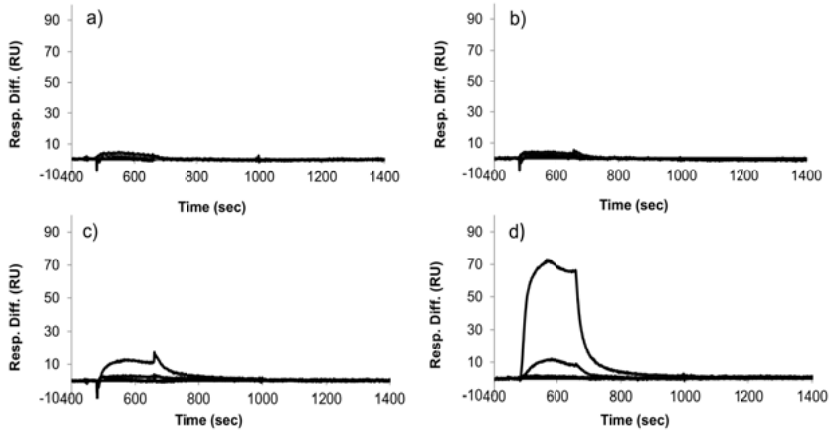


Figure 19 SPR sensorgrams showing the interactions between immobilized HSA and a) 1-C15L8-PP1, b) 2-C15L8-PP1, c) 3-C15L8-PP1 and d) 4-C15L8-PP1 polypeptide conjugates. Polypeptide conjugates were injected at 4, 20, 100 and 500 nM concentration in HEPES buffer in the presence of 1μM $ZnCl_2$, at 25°C. A sensorgram obtained by a separate injection of 1μM $ZnCl_2$ solution was subtracted from those of polypeptide conjugates.

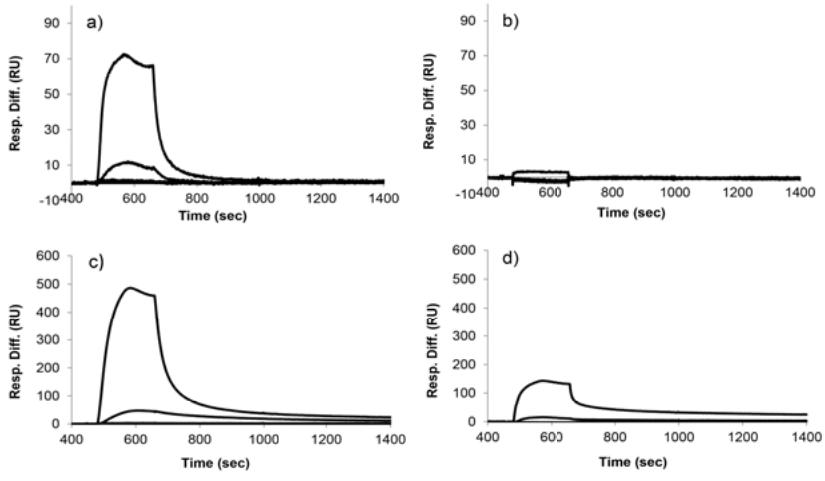


Figure 20 SPR sensorgrams showing the interactions between immobilized HSA and a) 4-C15L8-PP1 in the presence of 1μM $ZnCl_2$ b) 4-C15L8-PP1 without $ZnCl_2$, c) 5-C15L8-PP1 in the presence of 1μM $ZnCl_2$ and d) 5-C15L8-PP1 without $ZnCl_2$. Polypeptide conjugates were injected at 4, 20, 100 and 500nM concentration in HEPES buffer in the presence of 1μM $ZnCl_2$, at 25°C. A sensorgram obtained by a separate injection of 1μM $ZnCl_2$ solution was subtracted from those of polypeptide conjugates.

It is known from previous investigations that synthetic antimicrobial peptides interact with HSA through hydrophobic interactions[72]. The introduction of the hydrophobic Aoc residues into the peptide scaffold did, however, not give rise to increased affinity for HSA in comparison to the unmodified peptide. Most likely different binding sites are involved.

Protein and peptide therapeutics can stimulate immune responses and modifications of the peptide as well as size reductions have been the main approaches to reduce the risk of immunogenicity[73]. The molecular weight of the modified sequence VII was 1960Da and although the affinity was somewhat reduced the 11-mer bound HSA even at 100nM concentration probably due to the effect of the zinc chelator. The conjugation of a Zn chelator may thus be beneficial for peptides in vivo applications.

6.3 In vitro serum stability

Synthetic peptides often lack the in vivo stability required for a successful drug. A number of ways to prevent renal clearance and proteolytic degradation have been developed[26a, 74]. The peptide conjugates 4-C15L8-PP1, 5-C15L8-PP1 and the VII-PP1 which bound to HSA were incubated in human serum and samples analyzed by analytical RP-HPLC with regards to degradation as a function of time, Figure 21.

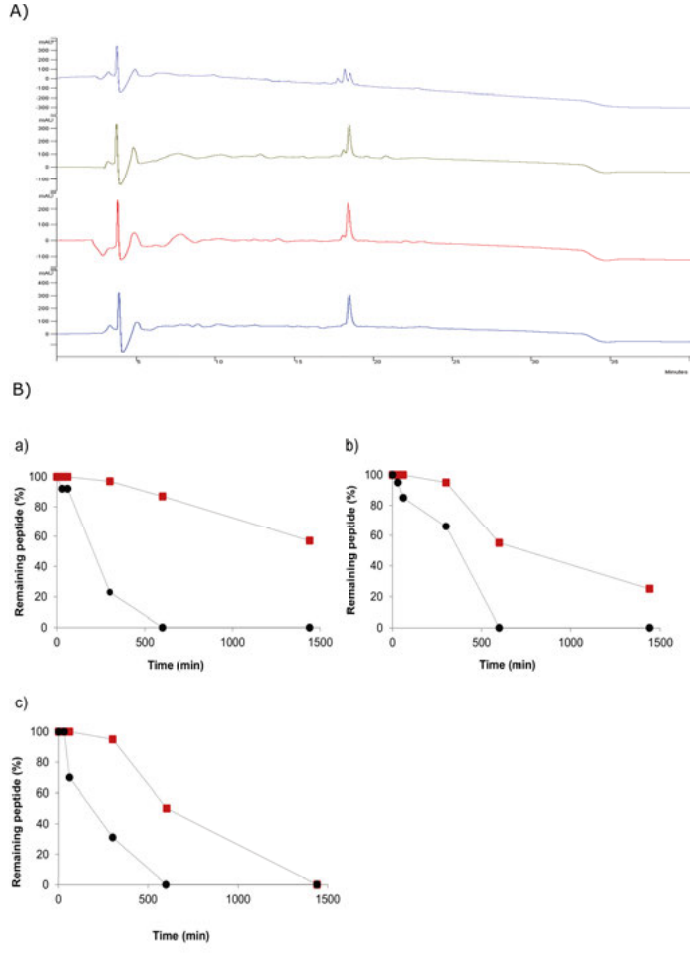


Figure 21 Analytical HPLC analysis of polypeptides conjugated to PP1 and polypeptides without PP1, incubated in 25% human serum, 75% HEPES buffer at pH 7.4 as a function of time at 37°C. (A) Chromatograms recorded 0, 30, 60, and 300 min after incubation of 5-C15L8-Ac. (B) Time dependence of degradation of a) 5-C15L8, b) 4-C15L8 and c) VII. Red symbols indicate PP1 conjugates, black symbols indicate acetylated polypeptides.

The peptide conjugates were incubated in human serum at 37°C and samples collected at time intervals for analysis. The reaction was stopped by the addition of 95% ethanol, precipitating larger serum proteins while leaving peptide conjugates soluble for HPLC analysis.

The PP1 conjugates that were shown by SPR to bind to HSA showed an increased survival time in serum in comparison with the corresponding peptides that did not bind HSA. i.e. that did not carry the PP1 ligand, Figure 21. In addition, the 5-C15L8 sequence showed a longer survival time in comparison to the other peptide conjugates in agreement with previous reports that HSA binds cationic peptides. The use of PP1 would thus be the most useful for peptides with close to neutral charge.

Conclusion and Outlook

The overall aim of this thesis was to provide new approaches to enhance affinities for proteins based on polypeptide conjugation to small organic molecules. Design strategies to identify non-obvious hydrophobic protein binding pockets and modulate binding affinities have been developed. Uncommon amino acids, such as Aoc, were shown to enhance peptide-protein binding affinities, to a level where a peptide downsized from 42 residues to 11 exhibited similar affinity for GPa with values of dissociation constants within a factor of 2. The discovery provides a route to the downsizing of peptides for biomedical applications, and to improve affinities of weakly interacting peptides in general.

Among drug classes, peptides are of considerable current interest in clinical research. Since the efficacy and the bioavailability of the peptides depend on their noncovalent association with serum proteins, the identification of further HSA binding sites can expand the toolbox available to drug developers. The conjugation of the Zn^{2+} chelator PP1 to positively charged 42-mer polypeptides improved the binding capacity for HSA, indicating that new binder molecules can be designed targeting the albumin zinc binding site. Charge-charge interactions between the peptide and the protein, in proximity to the zinc binding site, were additional driving forces for affinity enhancement.

The peptide conjugates discussed could be used to guide the design of bioanalytical tools or the rational design of peptide therapeutics with improved in vivo stability.

7. Peptide synthesis

First introduced by Merrifield in 1963[75], solid phase peptide synthesis (SPPS) remains the major automated synthesis method used for the production of synthetic peptides. Successful SPPS depends on the selection of the solid support and the linker (covalently attached between the solid support and the peptide under synthesis). Additionally, the selection of suitable protecting groups for the amino acids, the coupling method applied as well as the conditions used for the cleavage of the peptide from the resin and detachment of side chain protecting groups is important[1a].

Currently, there is an extensive number of commercially available solid supports for SPPS, composed of polystyrene (PS), polyethylene glycol polystyrene (PEG-PS) or hydrophilic PEG-based resins. Peptide-resin synthesis is performed using active bifunctional molecules (linkers) which form covalent bonds with the peptides. The design and application of each linker is based on the peptide synthesis conditions and on the basis of the desired C-terminus functionality, e.g. free-carboxyl-terminus peptide, amide, alcohol, ester, etc. Two resins were utilized for SPPS in this thesis; a H_2N-Rink-Amide resin and a Fmoc-Gly-PEG-PS resin for C-terminal amidated and unmodified peptides respectively. For both solid supports the final peptide-resin cleavage was performed in TFA solution[1].

During the repetitive synthetic cycle (N^{α}-terminal deprotection and amino acid coupling) either the acid-labile *tert*-butyloxycarbonyl (Boc) or the base-labile fluorenyl-9-methoxycarbonyl (Fmoc) groups are used as temporary N^{α}-amine protecting groups.

For Boc chemistry the use of liquid hydrogen fluoride (HF) for the final side chain deprotection, remains one of the main drawbacks when applied to large scale peptide synthesis[76].

In Fmoc SPPS the peptide-resin cleavage and the final deprotection of the amino acid side chains occur simultaneously in TFA solution. Additionally, in Fmoc chemistry during the deprotection of the N^{α}-amino group, the fluorene released has strong UV absorption properties facilitating the monitoring of peptide synthesis[77].

The selection of the persistent protecting groups of the amino acid side chains depends on the nature of the N^{α}-protecting group (Boc or Fmoc group) and the sequence of the peptide to be synthesized. For additional orthogonality, a number of different protecting groups may be applied to Lys and Cys residues, to access further peptide modifications.

All peptide sequences presented in this thesis were synthesized using standard solid-phase Fmoc protocols[1]. The amino acid side chain protecting groups used are shown in Table 7.

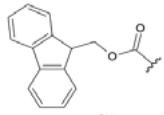
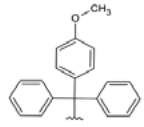
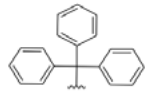
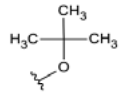
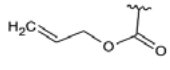
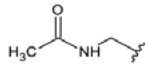
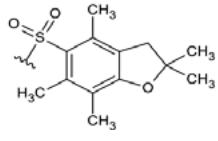
Name	Structure	Protecting group for
Fmoc 9-Fluorenylmethyloxycarbonyl		amine N-terminus
Mmt Monomethoxytrityl		amine
Trt Triphenylmethyl		thiol, amine
Tfa Trifluoroacetyl		amine
tBuO t-Butoxy		carboxylic acid
Alloc Allyloxycarbonyl		amine
Acm S-Acetamidomethyl		Thiol
Pbf 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl		amine

Table 7 Protecting groups used for the polypeptide synthesis

Coupling occurs upon activation of the α-carboxyl group of the amino acid to be reacted with the free *N*-terminal amino group of the peptide on the resin. A number of activation reagents are used, including carbodiimides and phosphonium/aminium/uronium salts.

The two most widely used carbodiimides are *N,N*-dicyclohexyl-carbodiimide (DCC) and diisopropylcarbodiimide (DIC) (Rich and Singh,

1979). A number of additive reagents such as 1-hydroxy-7-azabenzo-triazole (HOAt) or Oxyma Pure (Sigma Aldrich) are employed in order to prevent racemization and to accelerate the coupling reaction.

Ample examples of phosphonium and aminium/uronium based coupling reagents are the (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and the O-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HCTU). Since the above derivatives react only with carboxylate anions a base (*N,N*-diisopropylethyl-amine (DIPEA)) is added to the reaction mixture in order to drive the reaction.

For solid phase Fmoc chemistry the final peptide-resin cleavage and the deprotection of side chain protecting groups is performed in TFA/TIS/$H_2$0 (95/2,5/2,5 v/v) solution. In order to prevent undesired reactions of sensitive amino acids with carbocations (generated during the cleavage process) scavengers such as triisopropylsilane (TIS) and H_2O are added.

7.1 Purification and characterization of peptides

The diversity of the peptides in terms of shape/conformation, charge and size, in addition to the presence of byproducts produced during peptide assembly can create challenges in peptide purification. The most commonly used methods for the separation and purification of peptides involve gel-filtration chromatography, high performance liquid chromatography (HPLC) and ion exchange chromatography[1a].

All the peptides in this thesis were purified and analyzed by preparative and analytical reverse phase HPLC. According to the mechanism, the peptides are continuously partitioned between the hydrophobic stationary phase and the mobile phase. A C_{18} or C_8 carbon-bonded silica gel is the most popular type of reversed-phase HPLC column packing material. By gradually increasing the concentration of an organic solvent in the mobile phase the peptide-hydrophobic surface interactions are desorbed and the peptide is eluted from the column. The more hydrophobic the peptide, the more strongly it will bind to the stationary phase, and the higher the required concentration of the organic solvent. The hydrophilic compounds will pass through the column and are eluted first[78]. As mobile phase water and acetonitrile was used in the presence of an ionic regulator such as TFA (0.1%). The separation of each compound was monitored by a UV/VIS spectroscopy detection system.

The characterization of each peptide fragment can be obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The MALDI samples are co-crystallized with an organic matrix compound on a metallic plate. The matrix absorbs ultraviolet light (337 nm wavelength) and ionizes the analyte. The ionized molecules will be accelerated with the aid of a short voltage gradient and ions with lower m/z

ratio will move faster through space to reach the detector, whereas ions with larger ratio will travel more slowly. Consequently, the time for each ion flight is dependent on the mass-to-charge ratio (m/z). This mass spectrometry phenomenon is called Time of Flight Mass Spectrometry (TOF MS) [79].

8. Methods

8.1 Surface Plasmon Resonance (SPR) spectroscopy

Surface plasmon resonance (SPR) analysis is a powerful technique for the detection of biomolecular interactions in real-time, in a label-free environment. Through SPR efficient characterization of the kinetics and the mechanisms of biomolecular interaction can be obtained[20, 80].

Typical SPR sensor chips consist of a golden film coated on a glass plate. The metal surface is derivatized with a carboxymethyl-dextran (CM-dextran) hydrogel to facilitate the immobilization of biomolecules[81].

The SPR phenomenon occurs when a beam of light, strikes the surface of the sensor chip at a specific angle through a prism. Photon plasmon surface electromagnetic waves are created at the metal dielectric interface. The light is reflected at a reduced intensity and can be detected by an optical detection system. The angle θ between the incident and the reflected light is dependent on the refractive index of the medium or on the mass changes occurring at the sensor surface e.g. on complex formation of the analyte and the immobilized receptor biomolecules, Figure 22. The angle changes are reported as resonance units (RU)[80, 82].

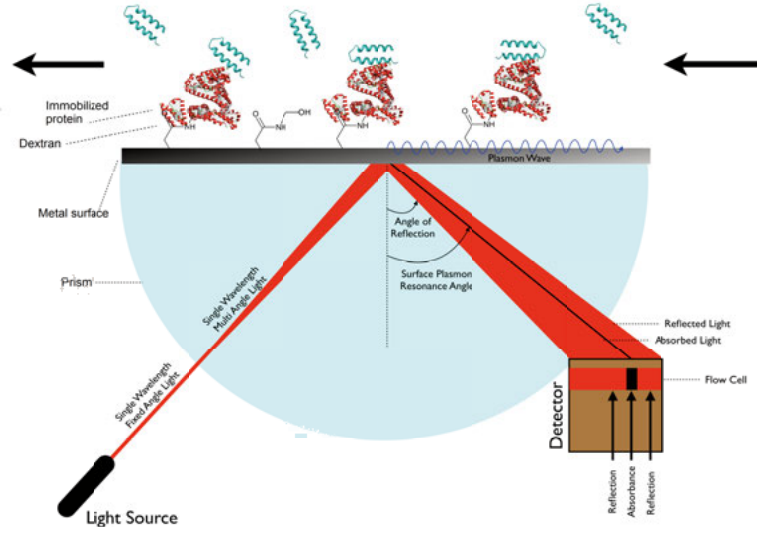


Figure 22 Schematic structure of surface plasmon resonance (SPR) biosensor[83]

The real-time responses of an SPR experiment are presented in the form of a sensorgram. According to the sensorgram, there are four phases within each experiment. During the association phase, the injection of the analyte into the flow cell and the interaction with the immobilized receptor causes an increase in the signal intensity (change in the refractive index). When the interaction between the analyte and the receptor reaches equilibrium (steady state) the RU values correspond to the concentration of the ligand-receptor complex. During continued buffer flow the analyte starts to dissociate and the binding sites of the receptor become unoccupied (dissociation phase). During the regeneration phase all the analyte is removed and a new experiment can be performed[84] .

The sensorgrams obtained from a set of different concentrations of the injected analyte can be simultaneously fitted to procure accurate association rate constants (k_{on}) and dissociation rate constants (k_{off}). The equilibrium dissociation constant can be calculated from the ratios of rate constants or estimated by fitting the steady-state responses (RU) as a function of the different concentrations of the analyte[85].

In this work SPR was used to determine the binding affinities of the peptide conjugates. Due to the fact that the peptide-protein binding did not reach saturation the K_D values could not be evaluated according to the 1:1 predefined binding model.

8.2 Circular dichroism (CD) spectroscopy

Circular dichroism is a technique used to obtain information about the secondary structure of peptides and proteins. The method is based on the propensity of a chiral medium to differently absorb the left and the right circular polarized light[86]. The difference in absorption can be calculated according to the Lambert-Beer's law (equation 1).

$$\Delta A = (\varepsilon_{left} - \varepsilon_{right}) * l * C = (\Delta\varepsilon) * l * C \qquad (1)$$

Where: ΔA (delta absorbance) is the absorbance difference of the left-handed circular polarized light (LCPL) and the right-handed circular polarized light (LCPL), ε_{left} and ε_{right} are the molar extinction coefficients for LCPL and RCPL, C is the molar concentration of the medium and l is the path length in centimeters (cm).

The difference in extinction coefficients ($\Delta\varepsilon$) is proportional to the circular dichroism of the chiral medium. The most commonly used CD unit is the so called molar ellipticity [θ] which is proportional to $\Delta\varepsilon$[87].

A CD spectrum represents the molar ellipticity (θ) values obtained as a function of the wavelength. The shape of the curves, called either plane

curves or curves possessing positive and negative cotton effects, are correlated to the structure of the medium. CD spectra recorded between 260 to 180 nm wavelength can be used to detect secondary structural conformations of peptides and proteins; α-helices, β-sheets and random coils. A α-helical component is characterized by two negative bands observed at 222nm and 108nm and a positive band observed at 192 nm. The most commonly used units to evaluate the helical content of a peptide is the mean residue ellipticity $[\Theta]_{222nm}$ (deg cm^2 $dmol^{-1}$) measured at 222nm wavelength[88].

Secondary structures of the proteins are sensitive to the environment, pH and temperature. CD spectroscopy was used to estimate the secondary structures of the peptide conjugates.

8.3 Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme Linked Immunosorbent Assay (ELISA) is a biochemical, microtiter plate-based assay technique used for the detection and quantification of peptides, proteins and antibodies. The ELISA experiments are typically performed in polystyrene microtiter plates, which can passively bind antibodies and proteins. Enzymes conjugated to specific detecting antibodies produce readout signals by reactions with chromogenic substrates.

The general procedure of an ELISA experiment involves the immobilization of a capture antibody in the wells of the microtiter plate, the blocking of unbound surface area to prevent false positive results and the capture of the protein by the immobilized antibody, followed by washing steps. A detecting antibody conjugated to an enzyme is added and the reaction of the enzyme with its substrate will create a detectable and quantifiable signal. There are four types of ELISA; direct, indirect, sandwich and competitive ELISA[62].

In the sandwich competitive ELISA, a capture antibody is immobilized on a solid support and the protein of interest incubated with the capture antibody. A solution containing the enzyme-labelled detecting antibody and the unlabelled (competing) ligand is added. The competition for the protein is evaluated by measuring the amount of bound antibody (labelled), as a function of the concentration of competing ligand[62].

In order to evaluate the binding capacity of the polypeptide conjugates for phosphorylated proteins, a sandwich competitive ELISA assay was performed.

8.3 Fluorescence spectroscopy

Fluorescence spectroscopy is considered to be a primary research tool in the biochemical and biological sciences as it is highly sensitive, efficient and safe. It is based on the fluorescence of a large variety of mostly aromatic molecules (fluorophores)[89].

When light of a specific wavelength has been absorbed by the fluorophore, the electrons are excited to energetically higher states. Vibrational and rotational relaxation results in the transition of the electrons to lower energetic levels and loss of energy. Fluorescence emission at a longer wavelength compared to that of the absorbed light is a phenomenon known as Stokes shift. The dipole moments of solvent molecules surrounding the excited fluorophore can cause changes in the energy levels and in the Stokes shift. This is the solvent relaxation process and is dependent on solvent polarity[90].

The fluorescence emission spectrum is a plot of the fluorescence intensity versus the wavelength of the emitted light. The intensity of the fluorescence emission in dependent on the chemical structure of the fluorophore and the solvent in which it is dissolved. Interactions between the fluorophore and the solutes such as proteins create changes in the fluorescence emission and a shift in the fluorescence intensity[89-90].

In order to evaluate the K_D values of the polypeptide conjugates a strongly emitting fluorophore (fluorescein) was incorporated in the peptide sequence. The fluorescence intensities were monitored at 520nm and plotted against the total protein concentrations. The K_D for each peptide was determined by numerical fitting of the experimental data.

Summary in Swedish-Sammanfattning på svenska

Molekylär igenkänning av proteiner utgör ett attraktivt forskningsområde med tillämpningar inom läkemedelsforskning, diagnostik och bioanalytisk metodutveckling. En bindarteknologi baserad på konjugering av små molekyler eller korta peptider till en uppsättning polypeptider med 42 aminosyror har utvecklats för igenkänning av proteiner. Den lilla molekylen förankrar konjugatet genom interaktion med ett specifikt bindningssäte medan polypeptiden förbättrar affinitet och selektivitet genom elektrostatisk och hydrofob växelverkan med proteinets yta i närheten av den lilla molekylens bindningssäte.

Forskningen i denna avhandling är fokuserad på utveckling av peptidkonjugat för igenkänning av proteiner av biologiskt intresse såväl som på bioanalytiska tillämpningar. Nya tillvägagångssätt med syfte att förbättra peptidkonjugeringens affinitetshöjande effekt för proteiner har utarbetats och utvärderats.

För igenkänning av fosforylerade proteiner inkorporerades den zinkbindande molekylen (3,5-bis[[bis(2-pyridylmetyl) amino] metyl] bensoesyra, PP1, i fyra polypeptidsekvenser med total laddning av +2. PP1 har tidigare visat sig binda fosforylerade proteiner med mikromolar affinitet i närvaro av zinkjoner. 4-C15L8-PPl visades binda glykogenfosforylas a (GPa) med en dissociationskonstant K_D av 280 nM. För att undersöka bidragen från de hydrofoba aminosyrorna till den totala bindningsenergin gjordes ett antal peptidmodifieringar genom alaninsubstitution och genom introduktion av icke naturligt förekommande aminosyror. Utbyte av de naturligt förekommande hydrofoba aminosyrorna isoleucin, leucin och norleucin mot alanin reducerade affiniteten för GPa. Introduktion av den icke naturligt förekommande och hydrofoba aminosyran *L*-2-amino-oktansyra ökade peptidkonjugatets affinitet till en K_D av 27nM. Således finns det icke uppenbara hydrofoba bindningssäten på ytan av GPa i närheten av den fosforylerade Ser15. Introduktion av tre Aoc rester i en peptid med 11 aminosyror, i huvudsak motsvarande aminosyrorna 4-14 hos 4-C15L8, gav ett peptidkonjugat med en jämförbar affinitet till den hos 4-C15L8-PP1. Resultatet antyder att bioaktiva peptider kan reduceras i storlek genom introduktion av icke naturliga aminosyror vilket reducerar risken för immunogenicitet och förenklar synteserna.

In vivo stabiliteten hos peptiderga läkemedelskandidater förbättras genom association med plasmaproteiner och en strategi för att öka peptiders affinitet för humant serumalbumin (HSA) utvecklades därför. HSA är ett bärarprotein som binder till en rad molekyler med olika fysikaliska egenskaper. HSA binder också zinkjoner. Polypeptid-PPl-konjugatet som binder Zn^{2+} joner befanns binda till HSAs zinkbindningssäte. På grund av den negativt laddade ytan nära HSAs zinkbindningssäte visade de positivt laddade polypeptiderna en högre affinitet för HSA än de som var negativt laddade. Den kombinerade effekten av förmågan hos konjugaten att binda zink och att interagera väl med den negativt laddade ytan gav hög affinitet för HSA. Överlevnadstiden i humant serum förlängdes, sannolikt genom deras starka bindning till HSA.

Peptidkonjugaten som beskrivits i denna avhandling kan användas i bioanalytiska applikationer såsom klinisk avbildning eller som verktyg för utveckling av potentiella peptidterapeutika med förbättrad biologisk aktivitet.

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Happiness is only real when shared

There is no greater gift in life than a true friendship and I have been lucky enough to have received it from amazing people that I have met:

Esli, Klev, Kosta, Emily, Alba, Monali, Petro, Mariatzela and Aggela thank you for giving me a heart full of joy! Looking back in time I can see that some of the best memories are all the simple moments I have spent with all of you. Thank you for always being by my side! I wish you all very good luck in career and life!

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Αγαπημένες μου αδελφές, *Αναστασία* και *Λάουρα* η αγάπη μου για εσάς δεν περιγράφεται με λόγια. Ως μεγαλύτερη αδελφή, μου δόθηκε η ευκαιρία να σας βλέπω να μεγαλώνετε και να ομορφαίνετε κάθε οικογενειακή στιγμή με την προσωπικότητα σας! Ευελπιστώ ότι αυτό το βιβλίο θα φτάσει καποια στιγμή στα χέρια της *Μαρκέλλας* και της *Ίριδας*! Η φαντασία και η αγάπη σας με βοήθησε να καταλάβω ότι ο κόσμος είναι πολύ πιο λιτός αρκεί να τον δείς μέσα απο τα μάτια ενός μικρού παιδιού.

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