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PAPER

The molecular recognition of phosphorylated proteins by designed polypeptides conjugated to a small molecule that binds phosphate†

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The conjugation of polypeptides from a designed set to the small molecule ligand 3,5-bis[[bis(2-pyridylmethyl)amino]methyl]benzoic acid, which in the presence of Zn^{2+} ions binds inorganic phosphate, has been shown to provide a polypeptide conjugate that binds α-casein, a multiply phosphorylated protein, with a dissociation constant K_D of 17 nM. The measured affinity is more than three orders of magnitude higher than that of the small molecule ligand for phosphate and the binding of 500 nM of α-casein was not inhibited by 10 mM phosphate buffer, providing a 2000-fold excess of phosphate ion over protein. The selectivity for phosphoproteins was demonstrated by extraction of α-casein from solutions of various complexity, including milk and human serum spiked with α-casein. In addition to α-casein, β-casein was also recognized but not ovoalbumin. Conjugation of a polypeptide to the zinc chelating ligand was therefore shown to give rise to dramatically increased affinity and also increased selectivity. A set of polypeptide conjugates is expected to be able to capture a large number of phosphorylated proteins, perhaps all, and in combination with electrophoresis or mass spectrometry become a powerful tool for the monitoring of phosphorylation levels. The presented binder can easily be attached to various types of surfaces; here demonstrated for the case of polystyrene particles. The example of phosphoproteins was selected since posttranslational phosphorylation is of fundamental importance in cell biology due to its role in signaling and therefore of great interest in drug development. The reported concept for binder development is, however, quite general and high-affinity binders can conveniently be developed for a variety of proteins including those with posttranslational modifications for which small molecule recognition elements are available.

Introduction

Small organic molecules that recognize and bind proteins are attractive goals in pharmaceutical and bioanalytical chemistry, yet notoriously difficult to develop to the highest levels of performance. New molecular strategies that enable us, with less effort, to increase affinities and selectivity for protein targets are therefore of considerable interest. Recently we reported a novel concept for protein recognition and binding, based on the conjugation of small organic molecules to polypeptides where the resulting conjugates were shown to have affinities several orders of magnitude higher than those of the small molecules and to have improved selectivity.[1–3] Binder molecules based on this concept are readily modified by conjugation techniques and readily immobilized on solid support for chip-based and continuous flow applications.

The concept provides an opportunity to form specific, high-affinity binders for proteins taking advantage of compounds that would otherwise be considered poor or failed, or at an early stage of development. In particular, conjugation to a polypeptide has been shown to induce a considerable difference in affinity for proteins to which the small molecule binds with equal strength, suggesting that specificity can be substantially improved.[2]

While high-affinity binders for specific proteins are extremely important, binder molecules for specific functional groups that arise, for example, as a result of post-translational modifications are also of considerable interest. The concept behind polypeptide conjugate binder molecules is that the small molecule warhead dominates the interactions between binder and protein, and that the attached polypeptide enhances affinity as well as selectivity.[1] Under the condition that the small molecule warhead is selective for a specific functional group rather than a specific protein surface epitope or active site, the resulting binder molecules are expected to be capable of recognizing large fractions of the proteins carrying such functional groups. A panel of binders could be developed that bind all proteins modified to carry that particular functional group, in a biological sample. This application is especially important in proteomics in the search for proteins that are not known previously to undergo posttranslational modification, where antibody based detection cannot be used. In this context, the extractions of groups of proteins, followed by

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mass spectrometric analysis or pull-down experiments followed by Western blots are attractive goals. In a first application of this strategy we have addressed the recognition of proteins modified by phosphorylation.

Phosphorylation is a reversible covalent modification controlled by kinases and phosphatases and acts as a "molecular trigger" for signal transduction cascades, apoptotic progression, metabolic changes and gene expression.[4] Whenever abnormal phosphorylation occurs, mutagenic, neuropathogenic or cancerogenic activities are initiated and kinases are therefore important drug targets. The interest in phosphotyrosines from a pharmaceutical perspective stems largely from their involvement in cell signaling and therefore in cancer.[5] Phosphoserines and phosphothreonines are approximately 2000-fold more abundant and their phosphorylation occurs downstream from that of tyrosines.[6] The relatively poor performance of anti-phosphoserine and anti-phosphothreonine antibodies suggest that there is a need for high-affinity, selective, robust and efficient binders for proteins phosphorylated at the side chains of serines and threonines.

Phospho-specific antibodies are routinely used to recognize phosphorylated epitopes in immunoprecipitation[7–9] and although antiphosphotyrosine antibodies show satisfactory efficiency[10,11] antiphosphoserine and antiphosphothreonine antibodies are as a rule only moderately specific.[12] Non-specific capturing of phosphate groups is used in combination with mass spectrometry, using immobilized metal ion chromatography (IMAC) or metal oxides such as TiO_2.[12–14] Upon modification or elimination of phosphate groups in proteins, chemical tags, such as biotin, can be introduced for extraction and identification of phopshorylated proteins.[15] The latter methods typically require that the proteins are digested by trypsin before capture and mass spectrometric analysis.

Here, we have addressed the problem of recognition and binding of phosphorylated proteins, and in a proof-of-principle demonstration applied the binder concept to the capturing of the model proteins α-casein, β-casein and ovalbumin.[16,17] Both caseins carry multiple phosphate groups that may enhance the probability for binding and thus their avidities. The binder molecules have been evaluated with regards to affinity, binding mechanism and selectivity in media of variable complexity. The results suggest that these binder molecules are strong candidates for diagnostic applications in the cases where phosphorylation events play vital roles.

Experimental

A detailed description of the organic synthesis, the solid phase peptide synthesis, the deprotection and purification of polypeptides as well as the conjugation of the small organic molecule and fluorophor to the polypeptides is provided in the supporting information.

Fluorescence screening

Fluorescence measurements were carried using standard 96 well plates, coated to prevent non-specific binding as described in the supporting information, and a microtitre plate reader. Three solutions were prepared: the first one contained 500 nM of 4C15*L8-PP1 (coumarin attached to the lysine at position 15) in HEPES buffer (pH = 7.2, 10 mM HEPES, 150 mM NaCl), the second one contained 500 nM of 4C15*L8-PP1 and 500 nM α-casein in HEPES buffer (pH = 7.2, 10 mM HEPES, 150 mM NaCl), the third one contained 500 nM of 4C15*L8-PP1 and 1000 nM α-casein in HEPES buffer (pH = 7.2, 10 mM HEPES, 150 mM NaCl). Each solution was prepared immediately prior to measurement. Each series of the three solutions was measured in triplicate and the mean value of the three measurements was reported. The fluorescence emission of the solution containing only protein dissolved in the buffer was registered but the response was only at the noise level (not reported).

Affinity determination by fluorescence spectroscopy

A series of solutions were prepared, where each one contained the binder 4C15L8-PP1* (fluorescein attached to the cysteine at position 24) at a concentration of 100 nM and the concentration of α-casein was varied from 1nM to 10 μM. All solutions were prepared in HEPES buffer (pH = 7.2, 10 mM HEPES, 150 mM NaCl). The fluorescence at 525 nm was determined as a function of total protein concentration and the dissociation constant K_D was determined by fitting eqn (1) to the experimental results.

$$F_{obs} = \frac{F_{bound}[\alpha C] + F_{free} K_D}{[\alpha C] + K_D} \quad (1)$$

In eqn (1), F_{obs} is the observed fluorescence intensity, F_{bound} is the fluorescence of the peptide-binder bound to the α-casein, F_{free} is the fluorescence of the free peptide and [αC] is the concentration of the free α-casein. [αC] can be derived from eqn (2), where $[P]_{tot}$ is the total concentration of peptide and $[\alpha C]_{tot}$ is the total concentration of the α-casein.

$$[\alpha C] = -\frac{[P]_{tot} + K_D - [\alpha C]_{tot}}{2} + \sqrt{\left(\frac{[P]_{tot} + K_D - [\alpha C]_{tot}}{2}\right)^2 + K_D[\alpha C]_{tot}} \quad (2)$$

Numerical fitting was done with IGOR Pro 4.03 (WaveMetrics Inc.).

Pull-down experiments

Polystyrene beads (0.5 mg–1 mg) coated and functionalized with polypeptide conjugate binder molecules as described in the supporting information were incubated with buffer, milk or serum (0.5 mL–1 mL). The suspensions were shaken end-over-end for 90 min and centrifuged (14 000 rpm for 7 min). The resulting bead plug was washed at least 4 times by means of sequential resuspension in 1 mL of HEPES buffer (pH = 7.2, 10 mM HEPES, 150 mM NaCl), followed by centrifugation (14,000 rpm for 7 min). In between the washing procedure an occasional sonication was conducted in order to diminish unspecific binding to the surface. After the washing procedure, the beads were centrifuged and the plug was resuspended in the DTT solution (1 mM) in HEPES buffer (pH = 7.2, 10 mM HEPES, 150 mM NaCl) (20 μL) and incubated for 1 h with occasional sonication. Thereafter, the suspension was centrifuged and the supernatant was analysed by SDS-PAGE and stained using Silver Staining. Samples for gel electrophoresis were prepared according to the manufacturer's instructions.

"Catch and release" column experiment

The polystyrene beads were coated and functionalized with binder molecules as described above. Functionalized beads (3 mg) were applied on the Biosphere Filter Tips as a suspension in HEPES buffer (pH = 7.2, 10 mM HEPES, 150 mM NaCl). The beads were washed with HEPES buffer containing 150 mM NaCl at pH 7.2 and 300 µL of the analyte was applied to the Filter Tips. The beads were washed with 50 µL of the HEPES buffer four times and the eluate from the fourth washing was collected for further analysis. In order to release the bound proteins the beads were washed once with 20 µL of 0.5% TFA and the eluate was collected. Both the fourth washing and the eluate were analysed by SDS-PAGE.

Results and discussion

Binder design and synthesis

In general, the binder concept is based on a set of 16 designed polypeptide sequences[1–3] and for each target protein a selective small molecule warhead is selected and covalently linked to each member of the set, Fig. 1. Depending on the target a spacer may be incorporated to link the small molecule to the polypeptides.

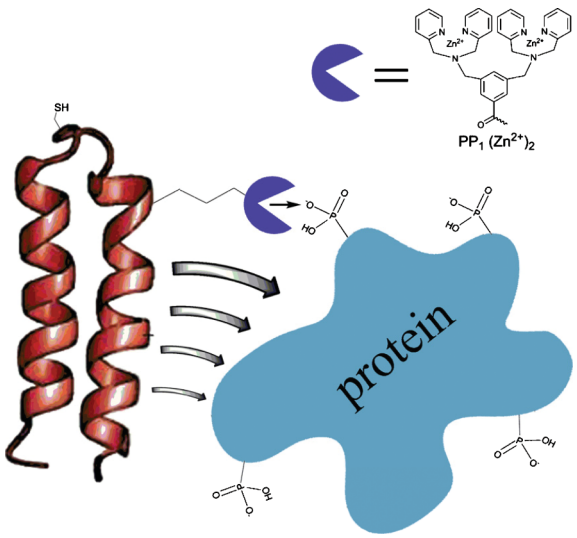


Fig. 1 Illustration of the binder concept and the interactions between the polypeptide conjugate binder and a schematic phosphorylated protein in the presence of Zn^{2+}. No crystal structure of α-casein exists and the protein is therefore presented in a schematic way. Hydrophobic and charge–charge interactions between polypeptide and the phosphorylated protein give rise to affinity and selectivity that is considerably increased in comparison to that between the small molecule and the protein. The Cys residue in the loop was used for immobilization on beads.

The polypeptides were designed to have some propensity for the formation of amphiphilic helices connected by a short loop.[1,3] They dimerize to form molten globule like four-helix bundles at µM concentrations but dissociate to unordered monomers at nM concentrations.[18] The polypeptides were not designed to form preorganized structures but to be able adapt to the protein surface interactions between the polypeptide and the target protein in addition to those between the small molecule and the protein.[1,2,19,20] The major part of the binding energy between protein and peptide is believed to arise from hydrophobic interactions between the protein and Leu, Ile or Phe residues on the hydrophobic face of the amphiphilic helices.[3] The enhanced selectivity arises as a consequence of electrostatic interactions between amino acid residues in the polypeptide and those exposed on the protein surface. The total charge of the peptides varies between −7 and +2 and the majority of the charged residues were introduced close to the hydrophobic face of each helix to bring them in close contact with residues on the protein surface. In the present investigation eight sequences were singled out for binder development, Fig. 2, whereas in other cases the full set of 16 sequences was screened to identify the best binders with the highest affinity and selectivity.[1,2] Here the polypeptides with total charges of −1, the so called 3-series, and +2, the so called 4-series, were used.

3-C15L8 Ac-NAADJEA**K**IRHLAE*K*JAARGPVDCAQJAEQLARRFEAFARAG-NH2
4-C15L8 Ac-NAADJEA**K**IRHLRE*K*JAARGPRDCAQJAEQLARRFERFARAG-NH2
3-C10L17 Ac-NAADJEARI*K*HLAERJ**K**ARGPVDCAQJAEQLARAFEAFARAG-NH2
4-C10L17 Ac-NAADJEARI*K*HLRERJ**K**ARGPRDCAQJAEQLARAFERFARAG-NH2
3-C25L22 Ac-NAADJEAAIRHLAERJAARGP**K**DC*K*QJAEQLARAFEAFARAG-NH2
4-C25L22 Ac-NAADJEARIRHLRERJAARGP**K**DC*K*QJAEQLARAFERFARAG-NH2
3-C37L34 Ac-NAADJEAAIRHLAERJAARGPVDCAQJAEQLAR**K**FE*K*FARAG-NH2
4-C37L34 Ac-NAADJEARIRHLRERJAARGPRDCAQJAEQLAR**K**FE*K*FARAG-NH2

Fig. 2 Amino acid sequences in one-letter code of eight polypeptides used for binder development. Lysine residues used for conjugation to PP1 are shown in bold and lysine residues used for conjugation of coumarin are shown in italics.

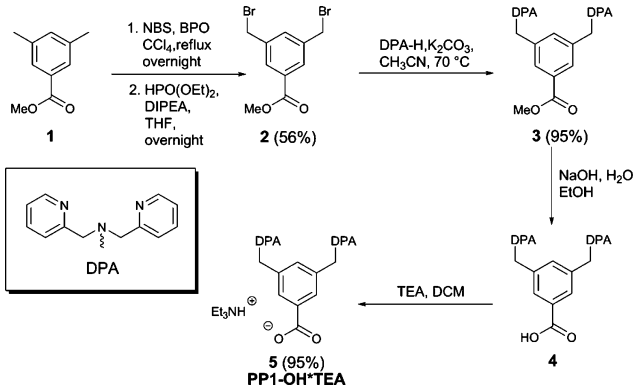
By design each peptide was equipped with three orthogonal anchoring positions that could be addressed independently, two lysine residues and one cysteine, Fig. 2. Selective modification of the lysines was achieved using orthogonal protection groups. The cysteine is located in the loop region and is conveniently used for immobilization on beads or attachment of further functional groups. Modifications can be performed both on the solid phase during peptide synthesis and in solution after cleavage and purification. An illustration of the folded structure and its interactions with a protein is shown in Fig. 1.

Among many available molecules that bind the phosphate group[21] a Zn(II)–2,2′-dipicolylamine (DPA) complex, reported previously by Hamachi *et al.*, to bind the phosphate anion was used.[22,23] We selected a molecule composed of two DPA groups linked by dimethylbenzoic acid for binder development, Fig. 1. It has been used previously in the construction of phosphate receptors and binds two Zn^{2+} ions that coordinate to the phosphate oxygens. The complex has been reported to bind phosphopeptides with dissociation constants in the µM range.[24] The 3,5-bis[[bis(2-pyridylmethyl)amino]methyl]benzoyl group, PP1, was attached to the polypeptide by amide formation to the side chain of a lysine residue.

The synthesis of 3,5-bis[[bis(2-pyridylmethyl)amino]methyl]-benzoic acid is outlined in Scheme 1.[25] The synthesis route was optimized, see supporting information, and the target compound produced in very good yield compared to that reported in the literature.[17]

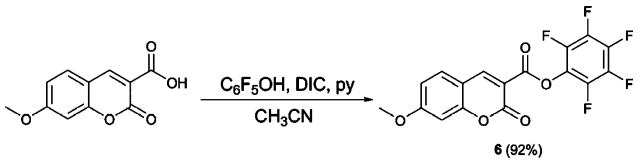
The binder molecules were formed in a reaction between **5** and the lysine side chain of the peptide attached to the solid support using PyBOP (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate) to ensure a high yield. The binder was cleaved from the resin and purified by HPLC.

For affinity determination, the binder molecules were equipped with fluorophores. For the initial studies, 7-methoxycoumarin-3-



Scheme 1 Outline of the synthesis of 3,5-bis[[bis(2-pyridylmethyl)amino]-methyl]benzoic acid triethylammonium salt.

carboxylic acid was conjugated to the lysine side chain through the reaction of 7-methoxycoumarin-3-carboxylic acid pentafluorophenyl ester (**6**) in DMSO (dimethyl sulfoxide)/pyridine (py) solution. The synthesis of **6** is outlined in Scheme 2.



Scheme 2 Outline of synthesis of 7-methoxycoumarin-3-carboxylic acid pentafluorophenyl ester.

For accurate affinity determination using a binder concentration of 100 nM, fluorescein-5-pyrrolidine-2,5-dione was conjugated to the deprotected thiol side chain of the cysteine residue through the reaction with the commercially available fluorescein-5-maleimide.

Phosphorylation takes place on the surface of folded proteins and therefore no spacer was included, PP1 was conjugated to the side chain of a lysine residue.

Structural characterization

The secondary structure content of the set of sixteen unmodified polypeptides has been reported previously.[1,2] In order to determine whether the introduction of PP1 and Zn^{2+} ions affected the solution conformation of the polypeptides the binder molecule 4C15L8-PP1 at various stages of assembly was investigated by circular dichroism spectroscopy at 1, 10 and 50 μM concentration in 10 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer at pH 7.2 (Supporting Information: SM Table 1†). All peptides showed helical signatures with minima at 209 and 222 nm suggesting that they are at least partly dimerised to form four-helix bundles at these concentrations.[18] The parent peptide 4-C15L8 and the fully decorated 4-C15L8 carrying the Zn^{2+} complex of PP1 showed a modest concentration dependence indicating a monomer–dimer equilibrium whereas 4-C15L8 functionalized with PP1 did not show concentration dependence in the absence of Zn^{2+}. We conclude that regardless of modification the polypeptides fold into helix–loop–helix motifs that dimerize to four helix bundles at concentrations in the μM range, and that their solution structures are not strongly affected by the introduction of the functional groups.

Binder affinity

In a proof-of-concept demonstration we studied the binding of the model proteins α-casein, β-casein and ovalbumin, where α-casein is probably the most widely investigated and well characterized phosphoprotein. It has between 8 and 13 phosphate groups whereas β-casein has 5 and ovalbumin one. In these proteins phosphorylation occurs at the side chains of serine residues. They are readily available from commercial sources and therefore suitable for systematic investigations. Both caseins and ovalbumin have isoelectric points of around 5, with an IEP of 4.6 for α-casein, 5.1 for β-casein, and 4.6 for ovalbumin.

For the determination of affinity, fluorescent probes were employed that were expected to respond with altered quantum yields to changes in the environmental hydrophobicity resulting from binding. In a preliminary and simple assay executable in microtitre plate format, each of the eight binder candidates were titrated with α-casein in three steps (Supporting Information SM Fig. 1 and SM Fig. 2†). To peptide stock solutions of approximately 5 μM concentration as determined from the absorbance of the coumarin probe at 345 nm, was added $Zn(NO_3)_2$ to a concentration of 12 μM. Aliquots from stock solutions were transferred to the wells of microtitre plates and the final concentrations of binders were 500 nM in 10 mM HEPES buffer containing 150 mM NaCl at pH 7.2. Each of the binder molecules were equipped with the fluorophor 7-methoxycoumarin-3-carboxylic acid, Fig. 2, and the fluorescence emission spectra were recorded in the absence of protein, as well as in the presence of 500 nM, 1000 nM and 1500 nM protein, Fig. 3.

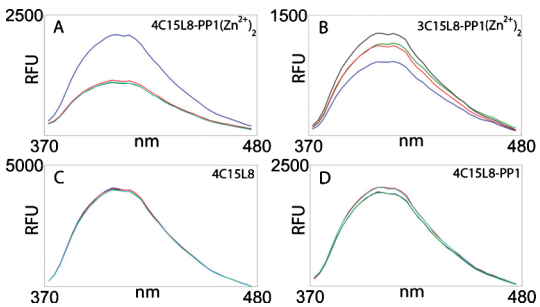


Fig. 3 Titration of 500 nM solutions of binder molecules with α-casein in 10 mM HEPES buffer containing 150 mM NaCl at pH 7.2. A. The fluorescence emission of the tight binder 4C15L8PP1$(Zn^{2+})_2$ decreases upon addition of one equivalent of α-casein and is unaffected by further additions. B. The emission increase of the weak binder 3C15L8PP1$(Zn^{2+})_2$ does not reach saturation upon addition of one equivalent of α-casein but increases with further additions. C. The fluorescence emission of the polypeptide 4C15L8 is not affected by the presence of protein and does not bind α-casein (lysine side chain acetylated). D. The fluorescence emission of 4C15L8-PP1 in the absence of Zn^{2+} does not change showing that Zn^{2+} is required for binding.

The 4-series of binder molecules showed nearly identical fluorescence emission spectra regardless of the concentration of α-casein suggesting that binding is saturated at 500 nM concentration each of binder and protein, Fig. 3. From the fluorescence titrations, assuming that the experimental error is not higher than 10%, the 4-series of binder candidates were all estimated to have dissociation constants of around 5 nM or lower. The

3-series of binders with the possible exception of 3C10L17-PP1, (Supporting Information, SM Fig. 2†) did not reach saturation at the protein concentrations used here. We conclude that the 3-series binders have dissociation constants in the high nM to the μM range. The nearly identical affinities within the 3-series and within the 4-series are probably fortuitous. As negative controls the unmodified polypeptide 4C10L17 and 4C10L17-PP1 in the absence of Zn^{2+} were titrated with α-casein. No effects on the fluorescence emission spectra were observed as a function of protein concentration.

The affinity of free polypeptide for α-casein is clearly low and from a comparison between the affinity of the polypeptide conjugate and the published dissociation constant of PP1 one can conclude that conjugation of the polypeptide to PP1 gives rise to a binder molecule that binds on the order of three orders of magnitude stronger than PP1 and consequently, that the polypeptide contributes significantly to binding when covalently linked to PP1. For a more accurate determination of the dissociation constant of 4C15L8-PP1 in the presence of Zn^{2+}, titration with α-casein in 10 mM HEPES buffer and 150 mM NaCl at pH 7.2 was carried out, Fig. 4. At a binder concentration of 100 nM a more strongly emitting probe was required and fluorescein was attached to the side chain of Cys-24 by reacting the maleimide group of fluorescein-5-maleimide with the free thiol of the polypeptide after removal of the acetamidomethyl (Acm) protection group. The binder concentration was kept constant at 100 nM and a series of samples were prepared where the concentration of α-casein was varied. The best fit of an equation describing the dissociation of a 1 : 1 complex to the experimental results gave an apparent dissociation constant of 17 nM, although the description of the complex dissociation behaviour of α-casein with 8–13 phosphate groups and one or more copies of 4C15L8-PP1 in terms of a single dissociation constant is obviously an oversimplification. It is not known, for example, how many binder molecules bind to the phosphate groups of α-casein, and it is not known if they are associated with different affinities, although most likely they are since the electrostatic profile of the protein surface is expected to vary between phosphorylated sites.

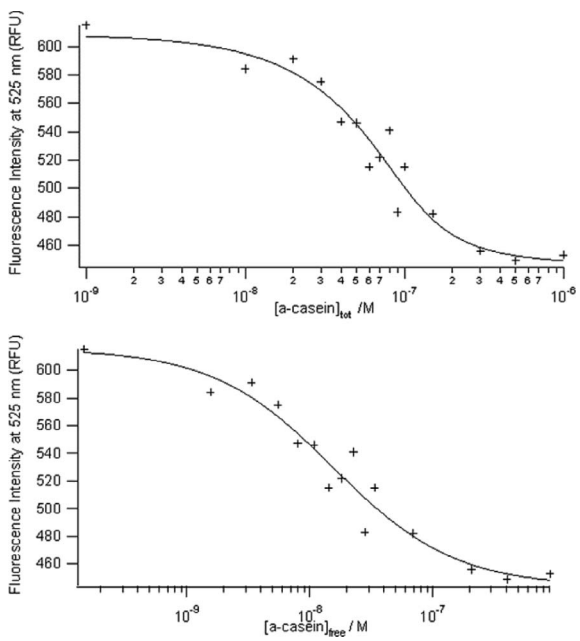


Fig. 4 Fluorescence intensity *versus* total concentration of α-casein (upper panel) and free concentration of α-casein (lower panel). A dissociation constant of 17 nM was obtained from the best fit to the experimental results of an equation describing the dissociation of a 1 : 1 complex.

The measured value is probably an underestimate of the true K_D. In order to obtain the most reliable value of a dissociation constant, the measurements need to be carried out at a concentration that is similar to the value of the dissociation constant. In this case it was not feasible since the changes in fluorescence emission were not large enough to permit measurements at or below 17 nM concentration with the required accuracy. Also the concentration of α-casein is lower than the concentration of 4C15L8-PP1 at around the inflexion point and below, and the change in fluorescence emission therefore underestimates binding strength. Unfortunately due to practical limitations linked to fluorophore emission, we were unable to establish more accurate values of K_D.

In order to rank the affinities of the binder molecules pull-down experiments with α-casein were instead carried out using the binders with the highest affinities. Polystyrene latex nanoparticles were functionalized with[26] all the members of the 4-series of binders by reacting deprotected Cys side chains in the loop regions of the polypeptides with activated coated particles. Free thiols react readily with pyridylsulfide (PDS) groups to form disulfide bridges. Particles coated with Pluronic F-107 carrying PDS groups were incubated with 10 nM and 100 nM solutions of α-casein, washed three times with buffer and separated from the supernatant after each wash by centrifugation. After three washes the particles were treated with DL-dithiothreitol (DTT) to release the binders and captured proteins from the beads and the resulting supernatants were analyzed by SDS-PAGE (Supporting Information: SM Fig 3†). All 4-series peptide conjugates successfully extracted α-casein at a concentration of 100 nM whereas only the binders 4C10L17-PP1 and 4C15L8-PP1 extracted α-casein from solutions with a protein concentration of 10 nM. These two binders were the most potent, clearly due to the best shape and charge complementarity to α-casein.

The interactions between the binder 4C15L8-PP1 and α-casein in the presence of Zn^{2+} were further probed by competition experiments and analysed by pull-down followed by SDS-PAGE, Fig. 5. Although PP1 chelated with Zn^{2+} was designed to bind phosphorylated amino acid side chains through coordination to the phosphate group, 10 mM PBS buffer corresponding to an excess of phosphate anions over phosphoserines by three orders of magnitude, did not inhibit the capture of α-casein at a protein concentration of 500 nM. This opens up a wide range of diagnostic applications in media with high concentrations of phosphate anions.[27–29] The capture of α-casein from a 500 nM solution by 4-C15L8-PP1 was not inhibited by a synthetic peptide with one phosphotyrosine side chain, PhosPep, even at a PhosPep concentration of 400 μM. Only when the concentration of α-casein was decreased to 100 nM and the concentration of PhosPep was 80 μM did binding appear to be suppressed. Commercially available dephosphorylated α-casein with a phosphorylation level of 20% was also readily extracted by the binder, see supporting information.

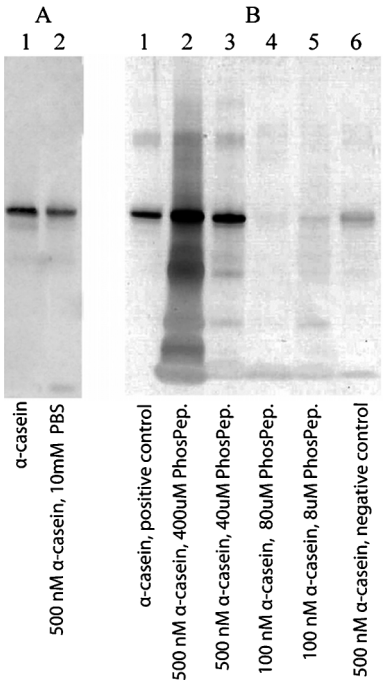


Fig. 5 SDS-PAGE analysis of α-casein extraction by 4C15L8-PP1in competition with phosphate anion in PBS (A) and in competition with the peptide PhosPep where one phosphotyrosine residue has been incorporated in the sequence, in 10 mM HEPES, 150 mM NaCl at pH 7.2. (B). Panel A. Lane 1. α-casein, positive control. Lane 2. Extract from 500 nM α-casein in 10 mM PBS. Panel B. Lane 1. α-casein, positive control. Lane 2. Extract from 500 nM α-casein and 400 μM PhosPep. Lane 3. Extract from 500 nM α-casein and 40 μM PhosPep. Lane 4. Extract from 100 nM α-casein and 80 μM PhosPep1. Lane 5. Extract from 100 nM α-casein and 8 μM PhosPep. Lane 6. Extract from 500 nM α-casein using beads coated with Pluronics without binder molecules, negative control.

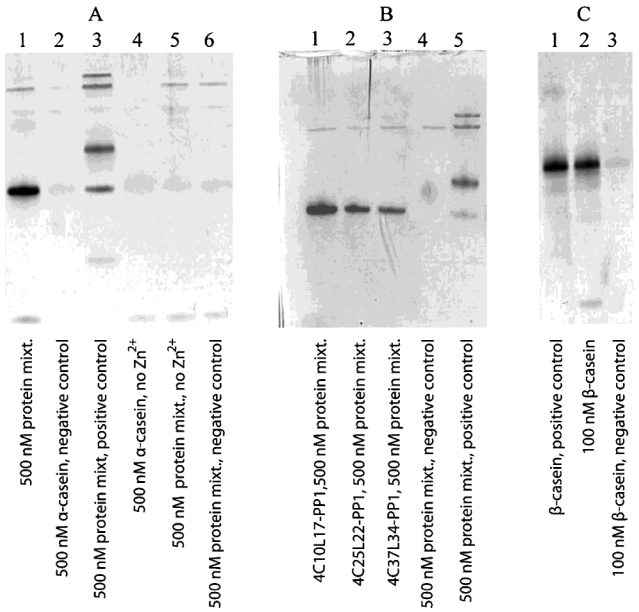


Fig. 6 SDS-PAGE analysis of proteins extracted by binder molecules immobilized on beads coated with Pluronics from protein mixture containing 500 nM lysozyme (15 kDa), 500 nM phosphorylase B (97 kDa), 500 nM β-galactosidase (116 kDa), 500 nM α-casein (23.6 kDa) and 500 nM ovalbumin (45 kDa). Panel A) Extract by 4C15L8-PP1 Lane 1. Extract from 500 nM protein mixture. Lane 2. Extract from 500 nM α-casein by Pluronic coated beads, negative control. Lane 3. 500 nM protein mixture, positive control. Lane 4: Extract from 500 nM α-casein by 4C15L8-PP1 in the absence of Zn^{2+}, negative control. Lane 5. Extract from 500 nM protein mixture by 4C15L8-PP1 in the absence of Zn^{2+}, negative control. Lane 6. Extract from protein mixture by 4C15L8 acetylated at Lys8, negative control. Weak bands at the bottom of gel is binder molecule, MW 5 kD. Panel B) Extracts from 500 nM protein mixt. by binder molecules. Lane 1. 4C10L17-PP1 Lane 2. 4C25L22-PP1 Lane 3. 4C37L34-PP1. Lane 4. Extract from protein mixture by beads coated with Pluronic, negative control. Lane 5. Protein mixture (Lysozyme not visible), positive control. Panel C) Extract from 100 nM solution of β-casein in 10 mM HEPES buffer, 150 mM NaCl at pH 7.2 Lane 1. β-casein, positive control. Lane 2. Extract by 4C15L8-PP1. Lane 3. Extract by beads coated with Pluronics, negative control.

Binder selectivity

The analysis of extracts from protein mixtures containing 500 nM each of α-casein, ovalbumin and the non-phosphorylated proteins lysozyme, phosphorylase B and β-galactosidase was undertaken to investigate the level of selectivity that could be obtained, Fig. 6. Three negative controls were used for detection of unspecific binding, beads coated with Pluronic, beads coated with Pluronic conjugated to 4C15L8 where the lysine at position 8 was acetylated and beads coated with Pluronic conjugated to 4C15L8-PP1 and used in the absence of Zn^{2+} ions. To make sure that Zn^{2+} ions were removed from the complex the extraction was carried out in the presence of 1 mM EDTA.

All 4-series binders were capable of extracting α-casein selectively from the mixture, with insignificant non-specific uptake. A band corresponding to the non-phosphorylated protein phosphorylase B was observed but a control experiment showed that phosphorylase B binds non-specifically to the Pluronic coated beads. It was also confirmed that Zn^{2+} is essential for α-casein binding since no α-casein was extracted in the presence of 1 mM EDTA. The binders 4C10L17-PP1 and 4C15L8-PP1 provided the highest intensity α-casein bands in comparison with those of the other binder molecules.

To evaluate binding also of phosphoproteins with a lower level of phosphorylation than α-casein the extraction of β-casein from a 100 nM solution was demonstrated, Fig. 6. This protein has approximately half the number of phosphate groups of α-casein. The mono-phosphorylated protein ovoalbumine at 100 nM concentration could not be extracted at a detectable level under the experimental conditions. The difference in extraction performance may be due to differences in affinity, since the avidity for poly-phosphorylated proteins is expected to be higher than for a mono-phosphorylated one. It may also be due to less optimal interactions between polypeptide and protein, and could possibly be improved by selecting a different polypeptide.

The selectivity of 4C15L8-PP1 in more complex media was evaluated by pull-down experiments in bovine milk and human serum, Fig. 7. Bovine milk contains, in addition to many other proteins and lipids, a number of casein isoforms where α-casein is the most abundant.[30] Human serum contains thousands of different proteins and extraction from human serum provides a critical evaluation of binder selectivity of relevance to the measurement of phosphorylated proteins in biomedical applications. Human serum was selected as the medium although it does not contain

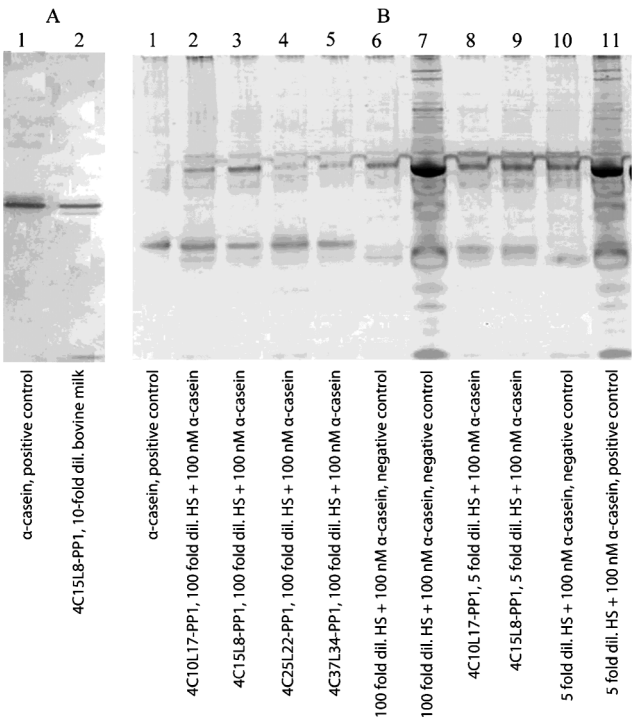


Fig. 7 Pull-down of α-casein from milk (A) and human serum (B). Panel A). Extract from 10-fold diluted bovine milk. Lane 1. Control, α-casein. Lane 2. Extract by 4-C15L8-PP1. Panel B. Extracts from 100-fold diluted human serum spiked with 100 nM α-casein (lanes 2–5) and 5-fold diluted human serum spiked with 100 nM α-casein (lanes 8–9). Lane 1. Positive control α-casein, Lane 2. Extract by 4C10L17-PP1. Lane 3. Extract by 4C15L8-PP1. Lane 4. Extract by, 4C25L22-PP1. Lane 5. Extract by4C37L34-PP1. Lane 6. Extract by beads coated with Pluronic, negative control. Lane 7. 100 nM α-casein in 100-fold diluted human serum, positive control. Lane 8. Extract by 4C10L17-PP1. Lane 9. Extract by 4C15L8-PP1, Lane 10. Extract by beads coated with Pluronic, negative control. Lane 11. Five-fold diluted human serum spiked with 100 nM, positive control.

α-casein, which was therefore added to a concentration of 100 nM. Pull-down of α-casein from bovine milk was clearly demonstrated and only a minor band corresponding to β-casein accompanied the main α-casein band. Pull-down of α-casein by all four binders of the 4-series from a 100 nM spiked solution in 100-fold diluted human serum was successfully demonstrated and pull-down from spiked neat human serum was successful although several bands appeared on the gel. It is not possible to avoid non-specific uptake by the beads in neat human serum and it is thus not possible to clearly define the level of selectivity of 4-C15L8-PP1 in neat serum. However, it was demonstrated that α-casein was extracted in competition with thousands of proteins by the synthetic binders of the 4-series in 5-fold diluted serum spiked with 100 nM α-casein. Some non-specific uptake is observed but otherwise a remarkable selectivity by 4C15L8-PP1 whereas the other two binders give rise to somewhat lower intensity. This result is in good agreement with observations reported above.

In order to evaluate the possibility to utilize this technology in continuous flow systems we prepared a set of columns. These columns were packed with the beads functionalized with our binders in the analogous way to the ones that were used in pull-down experiments. For technical reasons, we were forced to use beads with a higher mean diameter in comparison with the beads used in pull-down experiments. The binder molecules 4C15L8-PP1 and 4C10L17-PP1 were selected as they were shown to be the most efficient in capturing α-casein in pull-down experiments. A mixture containing dilute human serum enriched with α-casein was passed through the columns followed by washing. The release of the bound proteins was achieved by 0.5% aqueous TFA and aliquots were collected and analysed by SDS-PAGE, Fig. 8. Extraction of α-casein from human serum was demonstrated at very low concentration. Although unspecific binding to the beads that was observed in pull-down experiments was also observed in this experiment, a clear and dominating α-casein band was found on the gel. To demonstrate that α-casein was extracted specifically by the binder molecule, the final washing solution was analysed by SDS-PAGE, Fig. 8. No α-casein band was observed, proving that the captured protein was released only by the change of pH and that it was extracted exclusively by specific interactions with the binder molecule. The experimental results open a wide

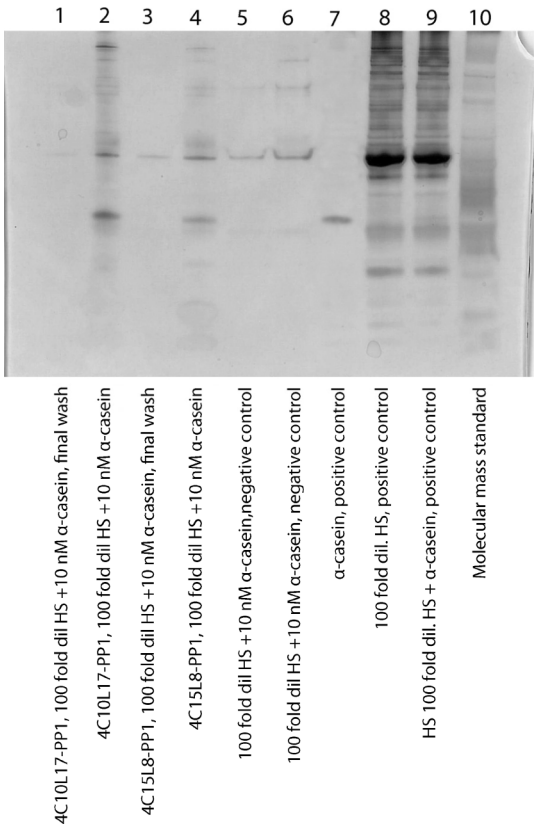


Fig. 8 SDS-PAGE analysis of fractions collected from columns packed with polystyrene beads coated with Pluronic and binder molecules 4C15L8-PP1 and 4C10L17-PP1. Analysed solution contained 100 fold diluted human serum (HS) spiked with 10 nM α-casein, Lane 1. Final wash from 4C10L17-PP1 column. Lane 2. Eluate released with 0.5% aqueous TFA from 4C10L17-PP1 column. Lane 3. Final wash from 4C15L8-PP1 column. Lane 4. Eluate released with 0.5% aqueous TFA from 4C15L8-PP1 column. Lane 5. Extract by beads coated with Pluronic, negative control. Lane 6. Extract by beads coated with Pluronic, negative control. Lane 7. α-casein, positive control Lane 8. Human serum, positive control Lane 9. Human serum spiked with 10 nM α-casein, positive control. Lane 10. Molecular mass standard.

range of new applications, especially in combination with mass spectrometric detection.

Conclusion

The binders reported here show high affinity and selectivity for the model phosphoproteins α-casein and β-casein in buffer as well as in milk and serum and provide strong support for the hypothesis that the set of polypeptide sequences used to develop the binders for phosphoproteins and previously for the C-reactive protein and human Carbonic Anhydrase II is of general applicability in developing binders for proteins. The concept is different from that of biological binders such as antibodies and aptamers where highly complex and preorganized structures with high molecular weights are required and offer an alternative for capturing and detecting proteins in solution as well as on solid support. This type of binder is highly suitable for a wide range of applications in biotechnology and biomedicine where robust capturing technologies can extend shelf-lives and areas of application quite significantly. The binders can be stored without special precautions at room temperature and are readily derivatized using well established chemical methods and reagents. The possibility of conjugating fluorescent probes and radionuclide sites specifically makes the technology highly attractive in bioanalytical applications. We believe that the binder will be capable of acting as an imaging agent, *in vitro* and *in vivo*, due to the flexibility with which organic molecules can be covalently attached without perturbing binding performance. The binder molecules reported here are developed especially for the purpose of monitoring phosphorylation events involving serine and threonine. Enrichment of the biological samples prior to mass spectrometric analysis is an attractive application for binders that capture phosphorylated proteins bearing in mind that phosphorylated proteins are less ionizable in comparison to the corresponding non-phosphorylated ones. The binder also performs very well in "pull-down" experiments and is expected to have a large number of applications in monitoring cellular phosphorylation events as a result of exposure to pharmaceuticals. The fact that the casein proteins are both multiply phosphorylated may have contributed to the efficient extraction achieved by immobilized binder molecules, if the loading is high. However, in solution only minor avidity effects are expected as the polypeptides do not dimerize at the low concentrations used for titrations.

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