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Use of phage display methods to identify heptapeptide sequences for use as affinity purification 'tags' with novel chelating ligands in immobilized metal ion affinity chromatography

Jane T. Mooney [1], Dale Fredericks, Milton T.W. Hearn *

ARC Special Research Centre for Green Chemistry, Building75, Monash University, Clayton, Victoria 3800, Australia

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ABSTRACT

This study describes the screening of a peptide phage display library for amino acid sequences that bind with different affinities to a novel class of chelating ligands complexed with Ni^{2+} ions. These chelating ligands are based on the 1,4,7-triazacyclononane (TACN) structure and have been chosen to allow enhanced efficiency in protein capture and decreased propensity for metal ion leakage in the immobilized metal ion affinity chromatographic (IMAC) purification of recombinant proteins. Utilising high stringency screening conditions, various peptide sequences containing multiple histidine, tryptophan, and/or tyrosine residues were identified amongst the different phage peptide sequences isolated. The structures, and particularly the conserved locations of these key amino acid residues within the selected heptapeptides, form a basis to design specific peptide tags for use with these novel TACN ligands as a new mode of IMAC purification of recombinant proteins.

1. Introduction

Random peptide phage display libraries offer a highly efficient means to evaluate the binding behaviour and affinity of large numbers (typically 10^7–10^9) of peptides (ranging in size from 4- to 40-mers) with 'receptor-like' molecules. The sequences of these peptides can be readily determined from the encoding DNA that is inserted into the genome of a filamentous bacteriophage [1]. To select a subpopulation of such peptide sequences, the library is subjected to a series of 'biopanning' screening steps. This technique involves several rounds of incubation of the phage library with the target molecule(s) followed by washes of varying stringency to remove unbound or weakly bound phage. Amplification of the eluted bound phage via infection of *E. coli* host cells then serves as the input material for the next round of selection. Such procedures have found wide application in medicinal chemistry and molecular immunology.

When utilising such screening methods several experimental considerations need to be addressed, namely the importance of maintaining a balance between the stringency of the selection pressure used in the 'biopanning' steps, and the yields required to isolate the most likely binding candidates. Further, the manner in which the target 'receptor' molecules are presented is important, with a high density of immobilized targets enabling multivalent binding, favouring the isolation of peptides of lower binding affinity but possibly higher avidity [2]. Conversely, high levels of stringency can be used with monovalent phage peptide displays and a low density of target 'receptors', favouring binders with higher affinity [1,3]. By placing the random peptide sequence within the context of a conformational constraint, the isolation of peptide sequences with enhanced binding can similarly be achieved [4], but at the price of increased limitations in diversity of the peptide sequences so identified [5].

Phage displayed random peptide libraries have been widely used as screening tools for the discovery of mimotopes or linear epitopes recognised by monoclonal antibodies [1,6–9]. Other studies have involved investigations into the substrate specificity of various enzymes [4,10–12], applications in drug discovery [13], gene delivery agents [14], and detection of putative DNA binding peptides [15]. In contrast, characterisation of phage library-derived peptide sequences and their use as affinity tags for protein purification by immobilized metal ion affinity chromatography (IMAC) has attracted only very limited attention. Patwardhan and colleagues [16,17] have described the use of a phage display peptide library for the selection of several peptides that interact with Cu^{2+} ions chelated to immobilized iminodiacetic acid (IDA), whilst Glokler has reported [18] a similar approach with immobilized IDA complexed with Fe^{3+}, Co^{2+}, Ni^{2+} and Zn^{2+} ions.

* Corresponding author. Tel.: +61 3 9905 4547; fax: +61 3 9905 8501.
E-mail address: milton.hearn@sci.monash.edu.au (M.T.W. Hearn).
[1] Current address: Sensologix, La Trobe University, 15/2 Park Drive, Bundoora, Victoria 3083, Australia.

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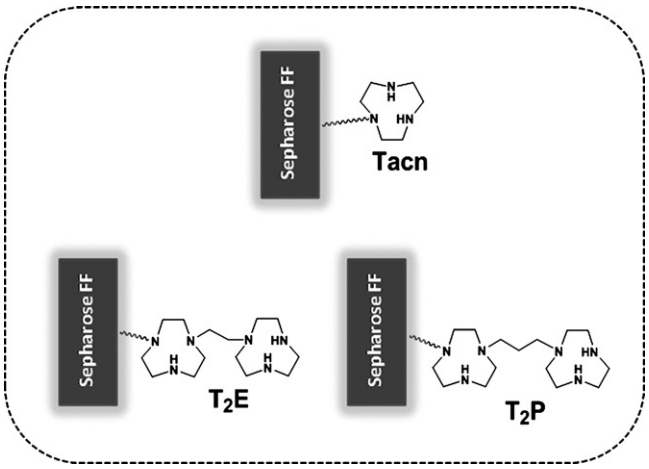

Fig. 1. Schematic representation of the immobilized TACN, *bis*-(TACN)-ethane (DTNE), T_2E, and *bis*-(TACN)-propane (DTNP), T_2P.

The ability of metal ions to co-ordinatively bind to electron donating atoms of amino acids accessible at the surface of proteins forms the basis of IMAC [19–21]. In IMAC, the so-called 'borderline' metal ions, e.g. Cu^{2+} or Ni^{2+}, are usually complexed with chelating ligands such as iminodiacetic acid (IDA), nitrilotriacetic acid (NTA) or tris-(carboxymethyl)-ethylenediamine (TED) [21–24]. Borderline metal ions exhibit a preference for coordination with nitrogen atoms, but will also bind to oxygen and sulphur [20,21,25]. This property has been exploited for many years by taking advantage of the affinity of histidine residues with immobilized 'borderline' metal ions [21,26], including the well-known N- or C-terminal histidine tags ((His)*n* where $n = 2–6$), which serve as affinity handles for the purification of recombinant proteins [27–30]. However, inefficiencies at the capture stage, limitations in the purity of the product under scale up conditions, metal ion leakage with non-constrained chelating ligands or ligand toxicity have limited the industrial and pharmaceutical application uses of IMAC [28]. With histidine-containing proteins the characteristics of surrounding amino acid residues are known to influence the binding ability of the histidine residue to IMAC resins, via a combination of steric and electronic effects leading to a change in the pK_a of the histidyl side chain. These structural effects have yet to be fully incorporated into the design of peptide tags for recombinant protein purification [28,31,32].

In this study, we have investigated the ability of random heptamer sequences to bind to Ni^{2+} ions complexed to a new class of immobilized chelating ligand, 1,4,7-triazacyclononane (TACN) and two analogues. TACN is a tridentate, macrocyclic ligand that forms complexes with metal ions with very high stability constants, β, e.g. with Cu^{2+} and Ni^{2+} ions β-values are $>10^5$ times larger compared to IDA or NTA [32,33]. Furthermore, two TACN can be covalently linked via a suitable spacer group to form bis(macrocycles) in which each TACN moiety can independently co-ordinate a metal ion. For example, *bis*-(TACN)-ethane (DTNE) has TACN macrocycles bridged by a linking ethyl group whilst *bis*-(TACN)-propane (DTNP) contains a propyl bridge (Fig. 1). Unlike other chelating ligands used in IMAC, TACN does not contain any carboxylate or phosphate pendant groups, and when bound with divalent metal ions, cannot be protonated. As a result, non-specific interactions between TACN complexed with M^{2n+} and proteins due to ionic effects are virtually eliminated over a large pH range (i.e. pH 4.0–9.5) [31,33]. The aim of this study was thus to identify unique peptide sequences that recognise these TACN-related moieties and which can be used to develop high affinity tags for the efficient IMAC purification of recombinant proteins with Ni^{2+}- (or other borderline M^{2+}-) chelating ligand systems, thus circumventing some of the earlier constraints.

2. Experimental

2.1. Phage displayed random peptide library

A Ph.D.-7 phage displayed random peptide library was used (New England Biolabs, Ipswich, MA). The library contains 2×10^9 electroporated sequences which correspond to 1.28×10^9 (i.e. 20^7) possible peptide sequences, each of 7 amino residues in size and displayed in a linear (unconstrained) fashion. These sequences were amplified once to produce approximately 100 copies of each sequence per 10 μl of library. The mature peptides are expressed, as fusion proteins, at the N-terminus of pIII, the minor coat protein of M13 bacteriophage containing a short spacer sequence (Gly-Gly-Gly-Ser) used to link the peptide and wild-type pIII sequence.

2.2. Metal chelate preparation

The TACN or the bis(TACN) ligands, ethylbis(TACN) (DTNE) and propylbis(TACN) (DTNP), were prepared and immobilized onto Sepharose CL-6B by procedures described previously [31,32]. The naked chelating gels were then incubated with 50 mM $Ni(NO_3)_2{\cdot}6H_2O$ (9 times volume) for 90 min at room temperature. The Ni^{2+}-loaded resins were filtered and washed with 3 volumes of 50 mM acetic acid, pH 4 (to remove any non-specifically bound Ni^{2+} ions), followed by washing with 3 volumes of Milli-Q water. The gels were stored in 20% ethanol at 4 °C until used.

2.3. Serial biopanning of the phage displayed random peptide library

In order to identify suitable heptapeptide targets, two biopanning strategies were employed. In the first, the three different IMAC resins (the immobilized Ni^{2+}–TACN, Ni^{2+}–DTNE or Ni^{2+}–DTNP resins) were each biopanned against the random peptide phage display library using 50 mM imidazole for the elution of the bound phage. In the second strategy, the Ni^{2+}–TACN resin, was panned against the library using four elution conditions, namely with 50 mM, 150 mM and 250 mM imidazole and also 200 mM malonic acid. Four rounds of serial biopanning were conducted, with the same elution buffer used in each round, with the exception of the 250 mM imidazole experiment, which was carried out as a step wise elution in the first round of positive selection, whereby the more weakly bound phage were first eluted with 150 mM imidazole followed by elution of the more strongly bound phage with the 250 mM imidazole. Each round of serial biopanning consisted of a positive selection step for phage bound to the Ni^{2+}–TACN (or the Ni^{2+}–DTNE and Ni^{2+}–DTNP) resin, followed by a negative selection step which removed phage that had bound only to the Sepharose CL-6B support material, followed by amplification of these selected target phage.

For the positive selection steps of the biopanning the following protocol was used; the Ni^{2+}–TACN, Ni^{2+}–DTNE or Ni^{2+}–DTNP resin (50 mg) was suspended in 500 μL of the washing buffer (10 mM Na_2HPO_4, 150 mM NaCl, 0.1% Tween 20 [rounds 1–3] or 10 mM Na_2HPO_4, 150 mM NaCl, 0.5% Tween 20 [round4]). The suspended gel was then centrifuged for 3 min at 5000 rpm and the resultant supernatant discarded. In the first round, the Ph.D.-7 library (2 μL) was added to the gel and the volume made up to 500 μL with the wash buffer. In subsequent rounds, the amplified phage (100 μL) from the previous round of biopanning were added to a fresh sample of the washed Ni^{2+}–TACN, Ni^{2+}–DTNE or Ni^{2+}–DTNP resin. The gels were then incubated overnight at 4 °C with continuous gentle

mixing. The solutions were then centrifuged at 5000 rpm for 3 min and the supernatant discarded. The gels were then washed 10 times with 500 μL of wash buffer to remove the unbound phage. Bound phage were eluted from the gels in 200 μL of the chosen elution buffer (50 mM imidazole, 150 mM imidazole, 250 mM imidazole or 200 mM malonic acid). After incubation at room temperature for 5 min, the gels were centrifuged at 5000 rpm for 3 min and the supernatent was (subsequently referred to as the positive selection eluate) stored at 4 °C.

The negative selection process consisted of the following steps: 50 mg of dried Sepharose CL-6B was washed 3 times in 500 μL of the washing buffer and then 130 μL of the eluate from the positive selection steps added to the gel and the volume made up to 500 μL with incubation buffer (10 mM Na_2HPO_4, 150 mM NaCl, 0.1% Tween 20 [rounds 1–3] or 10 mM Na_2HPO_4, 150 mM NaCl, 0.5% Tween 20 [round4]). The resins/phage eluates were then incubated with gentle mixing overnight at 4 °C. The samples were then centrifuged at 5000 rpm for 3 min and the supernatant was removed and stored at 4 °C. This supernatant is subsequently referred to as the negative selection eluate.

To amplify the selected phage after each round of biopanning, the eluate from the negative selection was passaged through *E. coli* strain ER 2537. The phage particles were then either purified by PEG precipitation and subjected to another round of positive and negative selection, or their DNA isolated for sequencing following transduction and culturing of single phage plaques.

2.4. DNA sequencing analysis

DNA sequencing reactions were conducted using the Sequenase Version 2.0 T7 DNA Polymerase sequencing kit (Amersham, Life Science, Cleveland, OH, USA) with 0.5 pmol of the −28 gIII primer (5′GTATGGGATTTTGCTAAACAAC3′). The sequencing reactions were run on 4.5% polyacrylamide sequencing gels and the DNA sequences were determined following exposure of X-ray films to the dried gels. Approximately 20 clones for each selection condition were sequenced. To negate issues relating to any potential biases in the observed frequency of individual amino acids within the isolated sequences that may arise from biological pressures such as codon usage and/or technical issues relating to poor amplification of some peptide sequences, the relative frequency (RF) of amino acids from selected peptides was determined. The results were then expressed as the ratio (Obs. f aa/Exp. f $aa_{[naïve]}$) of the observed frequency of amino acids within the isolated peptides (Obs. f aa) to their expected frequency (Exp. f $aa_{[naïve]}$) as determined by sequencing 24 randomly selected peptides from a 'naïve' phage libary i.e. one that had not been exposed to the target Ni(II)-chelated TACN-related ligands. The choice of the 24 randomly selected phage peptides from a naïve phage library was made according to the criteria discussed by Szardenings [33], and also to allow optimal use of the equipment available to rapidly carry out the DNA sequencing reactions and the polyacrylamide sequencing experiments.

2.5. BIAcore analysis

The BIAcore 3000 (Pharmacia Biosensors Uppsala, Sweden) with a sensor chip containing immobilized Ni^{2+}–TACN was used to examine the molecular interactions between various samples and the Ni^{2+}–TACN. The TACN was amine linked to the dextran polymer of the sensor chip in flow channel 1 (FC_1) employing EDC/NHS chemistry, which resulted in an increased baseline signal of 300 RU. The surface was then deactivated with ethanolamine–HCl and Ni^{2+} bound to the TACN. The flow channel 2 (FC_2) was similarly treated, with the exception that the TACN immobilisation step was not carried out, and served as a reference channel. The reactivity of various samples of phage displayed peptides selected after biopanning with Ni^{2+}–TACN was normalised using the filamentous phage, f1, to 5×10^{13} pfu/mL. Various dilutions (between 1/100 and 1/1000) of the phage preparations [corresponding to 5×10^{11} and 5×10^{10} pfu/mL respectively] were prepared in 1× HBS buffer (10 mM N-[2-hydroxyethyl]piperaz-ine-N-[2-ethanesulfonic acid] {Hepes}, 300 mM NaCl, 0.005% P20 detergent (Amersham, Life Science, Cleveland, OH, USA), pH 4). Alternatively, the phage samples were diluted 1 in 2 before the samples were PEG precipitated with 0.25 volume of 20% PEG 8000/2.5 M NaCl. The resulting pellets were re-suspended in 150 μL HBS buffer containing 005% P20. Samples (100 nM) of N-terminal histidine tagged glutathione S-transferase, 0.2 mg/mL) were diluted in HSB buffer or TE buffer (10 mM Tris, 1 mM EDTA, pH 8). All buffers were filter sterilised through 0.26 mm filters and de-gassed prior to use.

Before the samples were injected, the baseline for the biosensor detection was established using the running buffer (1× HBS buffer). Samples (30 μL) were then injected at a flow rate of 1 μL/min, based on an established protocol [34]. Following passage of the sample pulse, the flow of running buffer was resumed at a rate of 1 μL/min for 1800 s. The injected analyte was then eluted from the sensor chip with the addition of 30 μL of malonic acid buffer (1× HBS buffer containg 0.2 M malonic acid, 0.005% P20, pH 4.0). The running buffer was then passed over the biosensor until the pre-sample baseline returned. If this proved inadequate to re-establish the baseline, 0.5% SDS (20 μL sample at a flow rate of 20 μL/min) was injected to remove any remaining bound sample. The interactions were monitored in real time with sensorgrams generated for FC_1, FC_2 and FC_{1-2}. RU measurements were taken over an interval of 900 s after the beginning of the dissociation phase, and overlay plots generated.

2.6. Cloning, expression, purification and SDS-PAGE analysis of the recombinant tagged green fluorescent protein

The gene corresponding to the tagged green fluorescent protein, containing the N-terminal peptide sequence HHHNSWDHDANR was subcloned into the plasmid pTRC99a [35] using established procedures for all recombinant DNA manipulations [36] and expressed in *E. coli* strain BL-21. Expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and the bacterial cells cultured at 37 °C for 5 h, based on methods described previously [36]. Bacterial cells were harvested by centrifugation at 4000 × *g* using a Sorvall Evolution centrifuge (Thermo Scientific, Waltham, MA, USA). Cell pellets were washed with sterile water to remove residual culture medium and re-suspended in 10 mM imidazole, 10 mM NaH_2PO_4, 500 mM NaCl, pH 7.6 (20 mL). The suspension was lysed using a Misonix Sonicator 3000 (Farmingdale, NY, USA), the lysate centrifuged at 16,060 × *g* for 20 min and the supernatant filtered through a 0.2 μm syringe filter. An aliquot of the filtrate (10 mL) was loaded onto a column (1 mL), packed with the Cu^{2+}–TACN Sepharose Fast Flow equilibrated with 50 mM Tris, 500 mM NaCl, pH 7.5. Elution was achieved using a linear gradient from 0 mM imidazole to 300 mM imidazole, 50 mM Tris, 500 mM NaCl, pH 7.6, carried out using an Äkta XPress system (GE Biosciences, Sydney, Australia) with dual wavelength detection set at 280 nm and 488 nm.

SDS-PAGE analysis of the crude lysate and recovered fractions following the chromatographic purification of the tagged green fluorescent protein was carried out based on methods described previously for tagged recombinant proteins [37,38] with the molecular weight standards obtained as a Benchmark™ 10747-012 kit from Invitrogen (Australia) Pty. Ltd. Protein bands were detected using an established silver stain method [37,38].

3. Results and discussion

3.1. General considerations

In this study, a phage displayed random linear, heptapeptide library was used to select by serial biopanning methods [39] a set of peptide sequences that have the propensity to bind to novel metal ion TACN-related chelates immobilized onto a chromatographic support material. These peptide sequences were then analysed to establish key structural characteristics useful for the design of universal peptide tag sequences to be employed in downstream IMAC applications with tagged recombinant proteins.

In the first aspect of this work, biopanning was carried out with the phage heptapeptide library against Ni^{2+} ions complexed to immobilized TACN or the TACN derivatives DTNE and DTNP, using the same incubation, wash and elution protocol, namely with 50 mM imidazole employed at the elution stage to recover the bound phage. In the second approach, Ni^{2+}–TACN was chosen to examine how different elution conditions affected the selective recovery of the phage displayed heptapeptides, i.e. whether peptides of different amino acid sequences were enriched in the various desorbed fractions. In this latter case, four separate biopanning cycles were conducted in parallel using four different elution conditions, namely four rounds of positive and negative selection with the stringency of the selection process increased after the third round of biopanning. The inclusion of the negative selection steps was important to allow identification of phage peptides that bound preferentially to the support gel rather than to the target immobilized metal ion chelate, Ni^{2+}–TACN; a process that is vital if specificity is to be achieved for peptide fusion tags with recombinant proteins in downstream IMAC applications.

Analysis of the biopanning selected peptides to determine the frequency of individual amino acids within these sequences concentrated on factors known to be important in influencing protein binding to immobilized borderline metal ions, namely, the number and position of histidine residues [40] and the nature of the flanking amino acids [20]. Other factors taken into consideration included the fact that the frequency of amino acids incorporated into phage displayed peptides is a function of the library construction and differs from that theoretically expected, i.e. the third base of each codon is restricted to a choice of two bases (reviewed in Ref. [41]), and that biological pressures are known to result in some peptide sequences being selected against [41]. As a result all amino acid residues are not equally represented in the resultant library, consequently 24 wild-type clones were sequenced from a naïve library (i.e. one that has not seen any ligand) to ascertain the actual expected frequency of each residue. In order to establish the significance of the selected sequences from the biopanning of the phage library used in this study, the observed frequencies of each amino acid was compared to the aforementioned expected frequencies. These results can be expressed as a relative frequency (RF), and evaluated from the ratio Obs. f aa/Exp. f $aa_{(naïve)}$. Accordingly, a RF value of 10 for the amino acid tyrosine meant that this residue was enriched 10-fold in the selected peptide sequences obtained from the biopanning process compared to the expected value derived for 24 randomly selected naïve sequences.

3.2. Comparison of sequences derived from Ni^{2+}-immobilized to TACN, DTNE and DNTP

The relative frequencies of the amino acids present in the phage peptides selected by Ni^{2+}–TACN and the TACN derivatives with the same incubation, wash and 50 mM imidazole elution protocol are shown in Fig. 2. The percentage and relative frequency of histidine and other amino acids associated with these peptide–ligand interactions are given in Table 1. The following observations relate to the

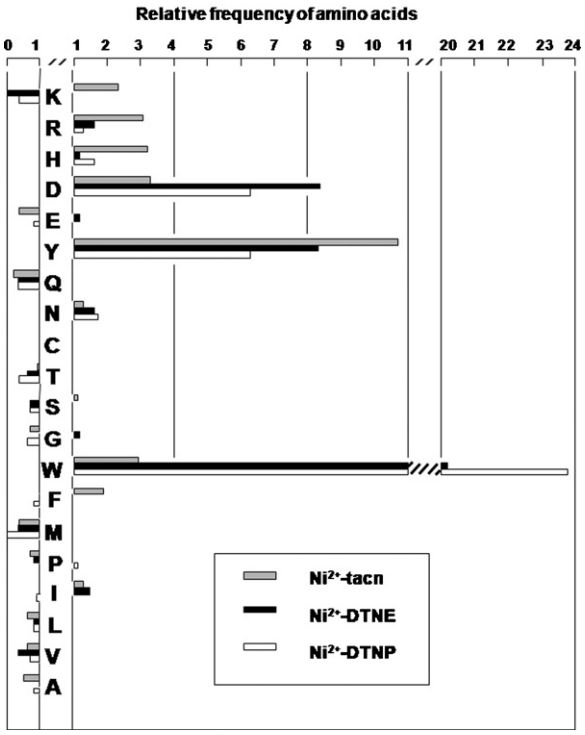

Fig. 2. Relative frequency of amino acids in the peptides isolated following panning of the random phage display library against Ni^{2+}–TACN, Ni^{2+}–DTNE and Ni^{2+}–DTNP using 50 mM imidazole elution buffer. Amino acids are represented as their single letter code.

Ni^{2+}–TACN data. Overall comparisons of the relative frequencies of the amino acids (Fig. 2) revealed increased amounts of histidine, arginine, aspartic acid and tryptophan within the selected peptides, with all of these observed enhancements approximately 3 times greater than levels present in the naïve library. There was also a large increase (10.6 times) in the representation of tyrosine residues in the sequences. Interestingly, tyrosine, occurred in peptide sequences containing 1 or no histidine residues with 60% of the sequences containing no histidine residues containing tyrosine, whilst tryptophan and aspartic acid each occurred in 20% of these peptide sequences.

For the sequences that contained histidine, 44% contained a single histidine, which occurred directly at the amino terminus in 75% of the cases. Similarly, 17% of the sequences contained 2 histidine residues some of which were N-terminal and these sequences also contained tryptophan and aspartic acid. One phage peptide sequence that bound to the immobilized Ni^{2+}–TACN had three his-

Table 1
Comparison of the types of peptide sequences eluted with 50 mM imidazole following panning against the three immobilized Ni^{2+} metal chelate complexes. Relative frequency (RF) of each amino acid residue from the selected peptide sequences equals their observed frequency (Obs. aa f) divided by their expected frequency in a naïve library as assessed by sequencing analysis (Exp. aa $f_{naïve}$).

	Ni^{2+}–metal chelate complex		
% Sequences containing	TACN	DTNE	DTNP
Histidine (H) residues	67	30	37
N-terminal histidine (H) residues	33	5.0	5.3
RF (Obs. aa f/Exp. aa $f_{naïve}$)			
Tyr (Y)	10.6	8.3	6.3
Trp (W)	2.7	20.2	23.8
Asp (D)	3.3	8.4	6.3
His (H)	3.2	1.2	1.6
Arg (R)	3.1	1.6	1.3

tidines, one of which was again N-terminal but did not contain tyrosine, tryptophan or aspartic acid.

The types of peptide sequences identified from the phage heptapeptide library to bind to the immobilized Ni^{2+}–DTNE and Ni^{2+}–DTNP were markedly different from those isolated following panning against Ni^{2+}–TACN. Firstly, these sequences had an extremely high relative frequency of tryptophan (Fig. 2) followed by very high levels of aspartic acid and tyrosine. There also appears to be conservation in relation to the position of the tryptophan in a large number of examples with this amino acid residue occurring as the third amino acid in the linear heptapeptide sequences. Secondly, the number of histidine residues in these sequences was low, having a relative frequency similar to the expected frequency of this residue within the naïve library sequences (Table 1). Interestingly, similarly to the Ni–TACN observations, the low histidine representation correlated with higher relative frequencies of tyrosine as well as tryptophan and aspartic acid. Of the sequences that did contain histidine, only one of the phage binding to Ni^{2+}–DTNE and two binding to Ni^{2+}–DTNP contained more than one histidine.

One explanation for the low frequency of histidine seen in the sequences of some of the phage peptides that interacted with the immobilized Ni^{2+}–bisTACN macrocycles is that the elution condition trialed (50 mM imidazole) was not sufficiently strong to successfully compete with these other histidine containing peptides for binding to the metal ion coordinated to the immobilized chelate. Moreover, it can be noted that some bisTACN macrocycles, e.g. ortho-xylylene bridged bisTACN macrocycles, can form both open structures, involving two coordinated metal ions, as well as sandwich structures, involving only a single coordinated metal ion. When the former situation prevails, multi-point binding will be favoured for peptide binding, whilst when the latter case prevails, due to the sequestering of additional coordination sites, the peptide could interact through single point binding or not even bind, depending on the coordination state of the metal ion. Metal ion analysis of samples of the immobilized Ni^{2+}–DTNE and Ni^{2+}–DTNP Sepharose CL-6B indicate that these complexes do not form sandwich structures involving only a single Ni^{2+}-ion under the buffer conditions employed in this study. Moreover, multipoint binding of synthetic peptides containing several histidine residues to binucleated Ni^{2+}–bisTACN macrocycles is known [32,41] to result in increased binding strength due to the presence of the two nickel ion co-ordination sites with these immobilized macrocycles. Such multipoint binding is dependent on the orientation, proximity and accessibility of the amino acid residues to the ligand [42,43], and as such with these new IMAC systems the location of the histidine residues within linear peptides may be critical in the design of affinity tags based on such sequences.

Moreover, as noted above, after four rounds of serial biopanning of the phage displayed random peptide libraries using 50 mM imidazole to elute the bound phage from Ni^{2+}-immobilized to TACN, DTNE and DNTP at each round of positive selection, 18–20 clones were recurrent for each ligand system, with the occurrence of tyrosine more frequent than aspartic acid or histidine for the immobilized Ni^{2+}–TACN, whilst tryptophan was more frequent followed by aspartic acid and tyrosine with the immobilized Ni^{2+}–DTNE or Ni^{2+}–DNTP systems. Of the various peptides expressed on the phage that contained at least two histidine residues, the following seven heptapeptide sequences were identified, namely (i) HHHNSWD; (ii) HTNIHQD, (iii) LDRAHDH; (iv) SLHEHH; (v) THYNAVH; (vi) DIHHWTD and (vii) HYSHTAH to satisfy the requirements of relatively high binding affinity 'peptide tags' for use with borderline metal ions, such as Cu^{2+} or Ni^{2+}, with these new macrocyclic chelating ligand IMAC systems for the purification of recombinant fusion proteins (cf Section 3.5).

Table 2
Comparison of the types of peptide sequences eluted with either 50, 150 or 250 mM imidazole following panning against the immobilized Ni^{2+}–TACN chelate complex. Relative frequency (RF) of each amino acid residue from the selected peptide sequences equals their observed frequency (Obs. aa *f*) divided by their expected frequency in a naïve library as assessed by sequencing analysis (Exp. aa $f_{naïve}$.).

% Sequences containing	Imidazole concentration		
	50 mM	150 mM	250 mM
Histidine (H) residues	67	74	95
N-terminal histidine (H) residues	33	39	84
N-terminal HW motif	0	4	26
One histidine residue	44	61	42
Two histidine residues	17	13	37
Three histidine residues	6	0	16
RF (Obs. aa *f*/Exp. aa $f_{naïve}$)			
Tyr (Y)	10.6	7.3	20.1
Trp (W)	2.7	8.3	17.6
Asp (D)	3.3	4.7	1.9
His (H)	3.2	3.0	5.6

3.3. Comparison of sequences derived from Ni^{2+}-immobilized to TACN using various elution conditions

In order to examine the possible composition of peptides with higher affinity for Ni^{2+}–TACN, further biopanning was performed against this ligand using step changes in imidazole concentration up to 250 mM and 200 mM malonic acid at the elution/desorption stage. As expected, as the strength of the elution buffer increased the percentage of peptide sequences containing histidine increased up to 95% (Table 2). This trend reflected both an increase in the overall number of histidines present in each sequence, as well as the high number of phage peptides containing a N-terminal histidine residue or a N-terminal histidine–tryptophan dipeptide (Table 2). When the 200 mM malonic acid buffer was used for the elution steps, the number of sequences containing histidine increased to 85%, with the majority containing a N-terminal histidine residue. Collectively, these results are consistent with the conclusion that both the number and position of the histidine residues influence the binding of the phage peptide to these immobilized Ni^{2+}–TACN and–bisTACN complexes. The increased presence of the N-terminal His-Trp dipeptide motif within these selected phage heptapeptides is consistent with the previously reported enhanced binding to other types of IMAC adsorbents by other peptides containing this motif [27,44]. It is also of interest to note from the above findings that peptides with only two His residues were found to be superior in their binding than hexa-histidine sequences to Cu^{2+}IDA resin [16,17].

None of the peptide sequences identified contained more than three histidine residues. Given that polyhistidine, e.g. His_6, tags bind to IMAC adsorbents, such as Ni^{2+}-NTA [reviewed in Ref. [20]], it is possible that some phage peptides, containing more than three histidine residues, are still bound to the metal chelated TACN-related gels, even under the more stringent elution conditions examined. Alternatively, it is feasible that sequences containing higher numbers of histidine were selected against due to issues relating to biological selection pressures associated with the use of the phage library. This conclusion is supported by the observation that desorption conditions with more forceful eluents (malonic acid) failed to recover any other phage peptides containing more than three histidine residues. Concordant with earlier conclusions [39,43,45], these results indicate that it is the position and surface accessibility of the histidine residues within the peptide and not the absolute number that are the most influential factors determining the strength of the binding.

The relative frequency values for the selected peptides isolated with stronger elution conditions also showed very large levels of tyrosine, tryptophan and to a lesser extent aspartic acid (Fig. 3 and

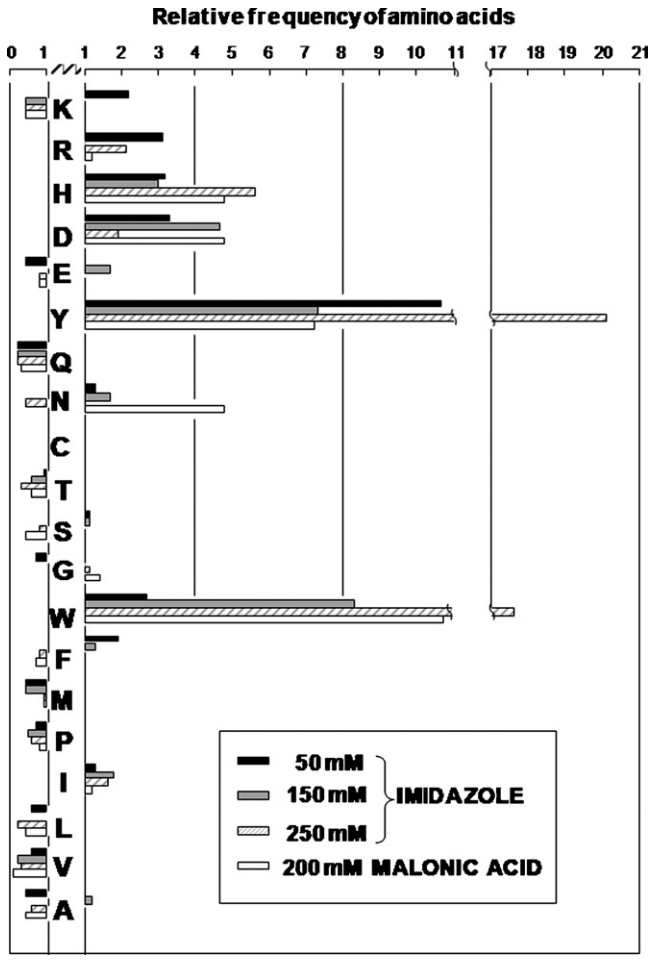

Fig. 3. Relative frequency of amino acids in the peptides isolated following panning of the random phage display library against Ni^{2+}–TACN, using 50 mM, 150 mM, 250 mM imidazole or 200 mM malonic acid elution buffer. Amino acids are represented as their single letter code.

Table 2). Tyrosine was present with high frequency for each elution condition and increased dramatically in the sequences eluted at the highest concentration of 250 mM imidazole, followed in rank order by tryptophan and then aspartic acid (Fig. 3 and Table 2). With the highest imidazole elution condition (250 mM), a number of peptides also contained a His-Tyr motif. The binding interaction of these motifs may be favoured by the high electron density of the tyrosine residues, which result in increased affinity of the N-electron donor imidazolyl group of the histidine. The high relative frequency of tyrosine and to some extent tryptophan residues present in phage eluted by the stronger elution conditions suggests that these amino acids are significant contributors to the interaction.

Previously, it has been proposed that the effect of tryptophan residues on the affinity of peptides for IMAC resins [27] may be due to hydrophobic interactions with the support material. However, in the case of TACN-macrocycles negligible interactions of proteins or tryptophan-containing peptides are known to occur in the absence of the chelated metal ion [31]. This would argue against hydrophobic interactions per se being a dominant cause behind the selection of peptides containing tryptophan with this phage library used. Nevertheless, the high incidence of tryptophan in many of these biopan-selected peptides is a novel finding, and may indicate that dipole–dipole effects could play a more significant role in these coordination interactions that hitherto appreciated. Clearly, this observation warrants further investigation if a rigorous scientific explanation is to be determined.

Table 3
Quantification of the change in RU following BIAcore analysis for the positive GST control, peptide display phage and wild-type phage samples.

Sample	Change in baseline RU @ 900 s
GST (His tag)	562.1
Phage displaying peptide (HAIYPRH)	61.6
Wild-type phage	−8.2

One important observation was the occurrence of identical heptapeptides isolated when the 250 mM imidazole elution conditions were used. In particular, four phage clones had the peptide sequence HWGMWSY and four other phage clones bore the sequence HAIYPRH. These sequences were also represented amongst the peptides selected when using 200 mM malonic acid as the elution buffer. These sequences exhibited higher binding constants than the other sequences, a finding that reflects the relative position of the histidine and other important residues within these sequences. These peptides may therefore represent sequences with particularly desirable IMAC properties as potential candidates upon which to base the amino acid sequences of IMAC affinity tags.

3.4. Independent verification of peptide binding to the IMAC adsorbents

When either serial or parallel biopanning of a phage-displayed random peptide library is carried out, the possibility for non-specific binders to interact with the support material must be excluded from the resultant selected population of phage peptides. The negative selection step incorporated into the biopanning protocol used in this study was therefore included to diminish the occurrence of non-specific binders being isolated. In addition, the specificity of one of the selected phage sequences was independently verified by automated biosensor-based methods to measure in real time the molecular interactions of the phage peptide with the immobilized Ni^{2+}–TACN and –bisTACN complexes by exploiting the surface plasmon resonance (SPR) phenomenon [46,47]. The selected phage clone bearing the sequence . . .HAIYPRH. . ., which was isolated using the high strength elution buffers containing 250 mM imidazole or 200 mM malonic acid was applied to the BIAcore sensorchips containing the immobilized Ni^{2+}–TACN complex using protocols adapted from previously employed methods for the direct application of phage displayed peptide immunogens to BIAcore sensorchips bearing immobilized antibodies [33,46,47]. As a positive control for binding, a sample of GST with a hexahistidine tag was also investigated (Table 3). The increase in the RU values 900 s after passage of the test samples relative to the pre-sample baseline was used to give a measure of the extent of binding. As can

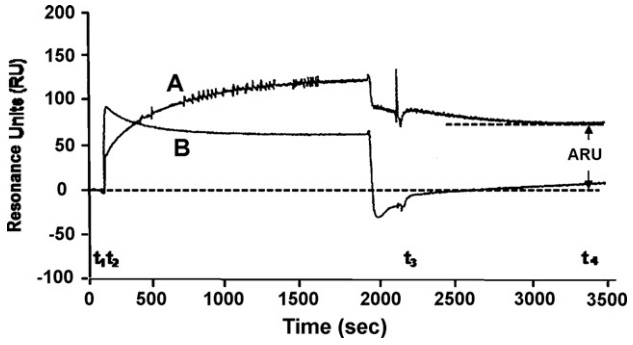

Fig. 4. Overlay plots of sensograms obtained from passing samples of either the phage clone displaying the sequence HAITPRH (A) or the wild type phage (B). Baseline measuremant occurred at t1. Sample injection point (t2), Cessation of sample pulse and running buffer was resumed (t3). Baseline levels 900 s post t3 (t4). The bound sample was observed as an increase in RU at t4. The positive control, namely His_6-glutathione transferase gave a change of 562.1 RU units.

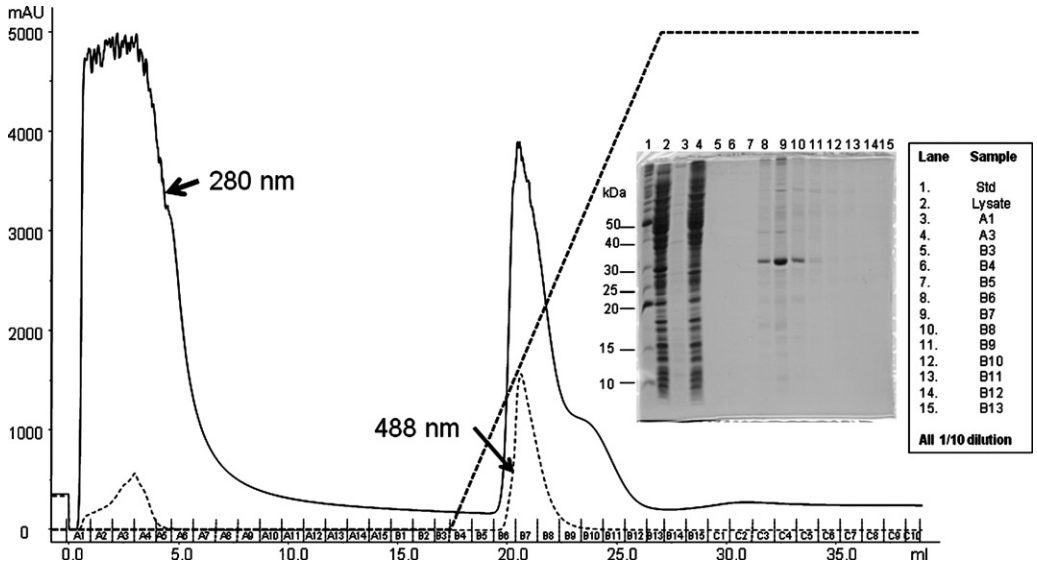

Fig. 5. Chromatographic profile and corresponding SDS-PAGE results for the purification of the peptide-tagged green fluorescent protein, using the Cu^{2+}–TACN Sepharose Fast Flow adsorbent (column size 1 mL), equilibrated with 50 mM tris, 500 mM NaCl, pH 7.5 and eluted with a linear gradient from 0 mM imidazole to 300 mM imidazole, 50 mM tris, 500 mM NaCl, pH 7.5, at a flow rate of 1 mL/min. The solid profile line corresponds to detection at 280 nm and the dashed line to detection at 488 nm. Other details are given in Section 3.5.

be seen in Fig. 4, binding was observed for the selected phage clone whilst no binding resulted with the sample of wild-type phage (Fig. 4 and Table 3). Importantly, this binding study demonstrates specific binding of the selected peptides to the immobilized metal chelate thus validating the approach taken in this study. These results also exclude the possibility that the binding is due to interaction between the phage coat proteins and the metal immobilized metal-chelating ligand complex, with the binding confined to the selected phage displayed peptide(s). These results parallel other experiences whereby the affinity of phage displayed peptide antigens can be ranked by comparison of their dissociation curves [33].

3.5. Application of peptide tags in the purification of recombinant proteins

Fig. 5 demonstrates the potential of the selected heptamer peptides as affinity tags for the purification of recombinant proteins, In this case, a peptide tag containing the amino acid sequence HHHNSWD. . . was incorporated as a peptide fusion construct via its corresponding oligonucleotide sequence, which was ligated to the 5′-end of the gene for green fluorescent protein (GFP) using established cloning methods with the *E. coli* strain DH5α serving as the plasmid host. The hybrid gene was sub-cloned into the plasmid pTRC99a and then expressed in *E. coli* strain BL-21. Following induction of expression with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), the bacterial cells were cultured at 37 °C for 5 h, harvested by centrifugation, lysed by sonication and the N-terminally tagged GFP present in the crude lysate purified using a Cu^{2+}–TACN adsorbent. The elution profile and corresponding SDS-PAGE of the recovered fractions (Fig. 5) clearly demonstrate a very efficient purification for this tagged protein by this novel IMAC procedure.

4. Conclusions

In this study, a phage displayed random heptapeptide library has been used to identify peptides that selectively bind to immobilized Ni^{2+} metal ions complexed to TACN or bis(TACN) macrocycles. Analysis of the identified peptide sequences indicated that the immobilized Ni^{2+}–TACN behaved differently to the immobilized Ni^{2+}–DTNE or Ni^{2+}–DTNP complexes under low stringency conditions as the majority of peptide sequences identified as binders with the immobilized Ni^{2+}–TACN contained histidine residues, whilst this was not the case with the immobilized Ni^{2+}–DTNE or Ni^{2+}–DTNP complexes. In addition, the relative frequency of histidine and the other amino acids for the phage peptide binders for the immobilized Ni^{2+}–DTNE or Ni^{2+}–DTNP complexes were very similar, but quite different to the immobilized Ni^{2+}–TACN system. These results may indicate the occurrence of multipoint binding with the immobilized Ni^{2+}–DTNE or Ni^{2+}–DTNP complexes, a process known to occur with these macrocyclic complexes [20].

The library was also serial biopanned against immobilized Ni^{2+}–TACN under several different elution conditions. The sequence analysis of the selected phage peptides revealed that as the strength of the eluent was increased the number of peptide sequences containing histidine, the number of peptide sequences having a N-terminal histidine residue, and the number of peptide sequences having a N-terminal His-Trp motif, all increased. The very high relative frequencies also observed for the occurrence of tryptophan and tyrosine residues suggest that these residues play a more significant role in peptide adsorption to IMAC adsorbents than the literature would suggest [20,43]. The higher levels of tyrosine observed, particularly in sequences deficient in histidine, may reflect a mixed mode binding with the Ni^{2+}–TACN based ligands. Furthermore, the sequence patterns identified for the selected phage peptides support the conclusion that the adsorption of these peptides to the immobilized Ni^{2+}–macrocycle complexes is greatly influenced by their structural arrangement, a finding that has precedent in other studies on the binding of synthetic peptides to IMAC adsorbents [30]. In addition, it is apparent from the investigation described above with the tagged green fluorescent protein as well as from other studies [37,38,48–50], these phage-derived peptides when coupled with the use of TACN-derived IMAC adsorbents represent a useful and alternative approach for the purification of tagged recombinant proteins.

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