The Development of Receptors and Chemosensors for the Recognition of Proximally Phosphorylated Motifs

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Abstract

Protein phosphorylation is an important post-translational modification central to controlling protein activity and function. While single residue phosphorylation is common, di- or poly-phosphorylation on proximal residues has emerged as a functionally significant motif often implicated in certain diseased states. Despite the relevance of these motifs, there has been limited effort towards the development of tools to target this type of phosphosite. To advance the understanding of this field, a metal-based triethylbenzene receptor was developed with the goal of selectively targeting proximally phosphorylated motifs over mono-phosphorylated motifs on peptides. While this receptor scaffold was not sufficiently suitable for this aim, it was successfully applied towards the recognition of small molecule phosphoanions ATP and PP$_i$ and was used for their selective sequestration in a phosphopeptide detection assay. The conclusion of these findings prompted a change in the initial receptor-based recognition strategy of di-phosphorylated motifs, which resulted in the development of a lanthanide-based chemosensing approach. Tb$^{3+}$ luminescence was used for the selective detection of a specific subset of proximally phosphorylated peptide motifs over mono-phosphorylated peptides and off-target phosphoanions. This chemosensing strategy has the potential to be applied as a high-throughput screening tool for different subsets of the phosphoproteome and could be of significant value in elucidating diseased states.
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Chapter 1

1 Proximally phosphorylated motifs

1.1 Phosphorylation

Phosphate-containing molecules are vital components of all living cells. Phosphate esters are found in multiple biological constituents including lipids, nucleic acids, nucleotides, nucleosides, and sugars and in small molecules such as pyrophosphate and on the surface of proteins. The advantageous chemical properties of phosphate justify its ubiquitous presence in several cellular components. Phosphate is highly abundant and highly water soluble with a large hydration shell.\footnote{1} It has three pKa values (2.2, 7.2 and 12.4) and can form mono, di, and tri esters and anhydrides.\footnote{1} Phosphate esters are stable in water at physiological pH 7 with a half life of $10^{12}$ years at 25 °C.\footnote{1} While the phosphate ester linkage is the hallmark of polymeric nucleic acids responsible for the storage of genetic information (ribonucleic acid, RNA and deoxyribonucleic acid, DNA), phosphate esters as a versatile post-translational modification on proteins is particularly significant.

1.1.1 Protein phosphorylation

Protein phosphorylation holds a central role in the regulation of life and has evolutionarily emerged as one of the most prominent types of post-translational modification (Figure 1.1).\footnote{2,3} The process of phosphorylation commonly involves the reversible phosphate ester modification of the three hydroxyl amino acids, serine (pS), threonine (pT) and tyrosine (pY) residues found on the surface of proteins.\footnote{2} It is estimated that 30% of all proteins in a cell are phosphorylated at any given time.\footnote{4} The diversification of protein surfaces generated by newly formed phosphorylated amino acids creates new entities that are distinct from the negatively charged amino acids, aspartic acid (Asp) and glutamic acid (Glu), which both have one negative charge and smaller hydration shells.\footnote{5} The rate and level of phosphorylation is tightly regulated in cellular systems, with the transfer of a phosphate to protein surface sites largely accomplished by kinase enzymes.
The functional significance of protein phosphorylation lies in its numerous roles in controlling a proteins’ activity status and function.\textsuperscript{2,5} Phosphorylation can affect a protein’s structural properties\textsuperscript{6} by inducing conformational changes\textsuperscript{7} and can further regulate protein-protein interactions (PPIs)\textsuperscript{8} as well as cellular localization.\textsuperscript{9} It can also play a direct role in the prevention of protein degradation\textsuperscript{10,11} and can effect its destination for degradation\textsuperscript{12}. Of critical importance, protein phosphorylation can affect the activity of a protein by regulating transcription factors and modulating protein translocation.\textsuperscript{9,13} A common PPI of interest is the dimerization of signal transducer and activator of transcription proteins (STATs). A specific example of this regulation is the phosphorylation of tyrosine 705 on the STAT3 protein which is recognized by the Src Homology 2 (SH2) domain of another STAT3, inducing the formation of a reciprocal dimer complex.\textsuperscript{14} STAT3 dimerization induces its translocation to the nucleus and can in turn promote the transcription of target genes.\textsuperscript{14} The SH2 domain is an important phosphorylation recognition module found in over 110 human proteins.\textsuperscript{15} As a recognition pocket, the SH2 domain binds to the pY705 of STAT3 and interacts simultaneously with polar residues within the pocket via hydrogen bonding and electrostatic interactions as well as additional interactions with flanking residues.\textsuperscript{16} STAT3 is a known oncogenic transcription
factor, which is found to be hyperactivated in numerous human cancers including breast, prostate, ovarian, leukemia and lymphoma.\textsuperscript{17}

The intra- and intermolecular effects of a single phosphate modification are important, especially in the context of disease processes. Given the significance of protein phosphorylation, there is a great need for the development of versatile methods and molecular probes capable of the selective recognition of phosphorylation sites over other anionic species or sites.

1.1.2 Proximal phosphorylation on proteins

While the activation of some proteins is mediated by single residue phosphorylation, others can become activated by di- or poly-phosphorylation on proximal sites. For example, in the Janus kinase 2 (JAK2) protein, proximal di-phosphorylation can occur on the tyrosine 1007 and tyrosine 1008 residues within the activation loop of the kinase domain (Figure 1.2A).\textsuperscript{18} The trans- or auto-phosphorylation of both tyrosine residues is critical to its kinase activity.\textsuperscript{18,19} The JAK2 protein is implicated in the JAK/STAT signalling pathway as the kinase responsible for STAT phosphorylation.\textsuperscript{20} Thus, the constitutive activation of JAK2 can result in the hyperactivation of numerous STAT proteins, which dimerize, translocate to the nucleus and activate the transcription of target genes. The over-activation of protein activity within this pathway is associated with many types of cancers such as breast, prostate, head, neck and several lymphomas.\textsuperscript{20} Similar to the JAK2 protein, proximal di-phosphorylation of tyrosine 525 and tyrosine 526 on the spleen tyrosine kinase (Syk) occurs within the activation loop of the kinase domain (Figure 1.2B).\textsuperscript{21,22} Syk holds key roles within immune signalling cells (e.g. B lymphocytes, mast cells, and macrophages) and its deregulated activity is implicated in various types of autoimmune disorders such as rheumatoid arthritis and allergic rhinitis.\textsuperscript{22} Syk has also been linked to the development and maintenance of hematological malignancies.\textsuperscript{21} Another important family of protein kinases, mitogen activated protein kinases (MAPKs), mediate and control a wide range of fundamental processes such as cell growth, proliferation, differentiation, death, migration and invasion.\textsuperscript{23,24} Like JAK2 and Syk, MAPKs are activated by proximal di-phosphorylation, specifically on threonine and tyrosine residues in a conserved pT-X-pY motif (X = any amino acid) within the activation loop of the kinase domain.\textsuperscript{25,26} As with JAK2 and Syk, the dual phosphorylation of these residues is critical for activity.\textsuperscript{25} This is shown with the MAPK isoforms of extracellular signal related protein kinase (ERK), ERK1 and ERK2, where
di-phosphorylation of threonine183 and tyrosine185 residues (Figure 1.2C) leads to over 1000-fold activation. Abnormalities in MAPK signalling pathways are highly implicated in human cancer progression and development. For example, deregulation of activity within the p38 MAPK family contributes to the progression of prostate, breast, bladder, liver and lung cancers as well as leukemia and transformed follicular lymphoma. The deregulation of ERK2 and p38MAPK protein family members is also linked to the hyperphosphorylation of the Tau protein. Tau is a microtubule-associated protein that contains numerous phosphosites (~79), many of which are proximal to one another (Figure 1.2D). It is highly enriched in the neurons (mostly neuronal axons) of the central nervous system (CNS). Under normal conditions, there is a partial phosphorylation of the Tau protein in the human adult brain, which leads to the stabilization of microtubules. However, aberrant hyperphosphorylation of the Tau protein results in its misfolding and accumulation within neurons termed as ‘fibrils’ of the tau protein. This leads to the formation of insoluble neurofibrillary tangles (NFTs), which can lead to synaptic and neuronal deficits that are characterized in numerous tauopathies such as Alzheimer’s disease (AD), Pick’s disease, progressive supranuclearpalsy (PSP), Lytico-Bodig disease and corticobasal degeneration (CBD). Based on these examples, it is clear that proximally phosphorylated motifs have broad impact on intracellular signalling in both normal and diseased states.
Figure 1.2 Proximally phosphorylated motifs on A) Jak2$^{18}$, B) SYK$^{21}$, C) ERK$^{27}$, and D) Tau$^3$ proteins. Figures adapted from cited references.

The importance of proximally phosphorylated motifs in these proteins and their link to disease onset and progression prompted further investigation on the prevalence of these motifs in the human phosphoproteome. We conducted an analysis of the distribution of proximally phosphorylated motifs using the post-translational modifications database PhosphoSitePlus® and filtered for human phosphosites. To start, we searched pS(X)$_n$ pS and pY(X)$_n$ pY motifs where X = any amino acid and n = the number of residues in between phosphosites. The results in Figure 1.3 indicate that for both pS(X)$_n$ pS and pY(X)$_n$ pY di-phosphorylated motifs, *proximal* phosphorylation is most common with approximately 35,000 sites for pS(X)$_n$ pS and 3200 for pY(X)$_n$ pY where X = 0-4 residues. As the space between residues increases (X = 10-160 residues) the number of sites decreases with ~17,000 for pS(X)$_n$ pS and 1700 for pY(X)$_n$ pY. While it can be seen that the occurrence of pY(X)$_n$ pY (Figure 1.3B) is significantly less than that of pS(X)$_n$ pS (Figure 1.3A), this is to be expected as tyrosine phosphorylation is less common as compared to threonine and serine protein phosphorylation.$^{31}$ Although phosphotyrosine is
relatively in low abundance, its central role in critical signalling pathways that are implicated in cancer highlights its importance.\textsuperscript{6,32}

![Bar chart A]

**Figure 1.3** The distribution of A) $pS(X)_n pS$ and B) $pY(X)_n pY$ motifs (human phosphoproteome) taken from PhosphoSitePlus\textsuperscript{®}.

We next performed a search of $pX(X)_n pX$ motifs where $pX = $ either $pT$, $pY$ or $pS$, $X = $ any amino acid and $n = 0, 1, $ or $2$. Figure 1.4 shows that motifs containing the $pS(X)_n pT$ or $pT(X)_n pS$ phosphosites were the most abundant. Similar to the findings in Figure 1.3, the results in Figure 1.4 indicate that in most cases, $pY$ containing motifs were the lowest in abundance. Overall, $pX(X)_n pX$ motifs were highly abundant (~34536 sites in total).
Figure 1.4 The distribution of pX(X)_n pX motifs (human phosphoproteome) taken from PhosphoSitePlus\textsuperscript{3}, with A) n = 0, B) n = 1, and C) n = 2.

In addition to our findings, a study conducted in 2010 based on the statistical assessment of phosphosite distribution within the eukaryotic phosphoproteome found that proximal phosphorylation or phosphosite clustering is common and occurs primarily on S and T residues (Figure 1.5A).\textsuperscript{33} In contrast to our findings, the study reported that phosphosite clustering was
proportionally less common for Y residues compared to S and T residues (Figure 1.5B).\textsuperscript{33} We assume that this discrepancy could arise from the author’s analysis of the eukaryotic phosphoproteome as opposed to our subset analysis of the human phosphoproteome. Nonetheless, taken together, these results support the notion that proximal phosphosites are common. While not much is known about the functional relevance of these motifs, their implication in diseases suggests that their detection could be of importance.

![Figure 1.5](image)

**Figure 1.5** The distribution of distances between A) phosphorylated serine and threonine sites and B) phosphorylated tyrosine sites (eukaryotic phosphoproteome). Figure adapted from \textsuperscript{33}.

### 1.2 Targeting phosphorylation sites

Given the enormous importance of protein phosphorylation and its fundamental role in biological systems, a great amount of work has been done in developing methods for the selective detection
of this modification. For example, antibodies have been used to specifically recognize the phosphorylated forms of proteins.\textsuperscript{34} Chemical derivitization techniques have also been useful for investigating protein phosphorylation and the functional role of specific phosphoproteins.\textsuperscript{35,36} Additionally, most analytical phosphoproteomic studies are commonly employed via phosphoprotein enrichment methods in combination with mass spectrometry.\textsuperscript{37-39} Aside from these common methods, much work as been done around developing more high-throughput and inexpensive phosphorylation detection techniques based on receptor and chemosensor design strategies. Along with single residue phosphorylation, an emerging aim is to develop recognition techniques specific for proximally phosphorylated motifs in order to elucidate their functional relevance and their implication in disease.

1.2.1 Metal-based receptors and chemosensors for protein and peptide phosphorylation

The achievement of efficient molecular complementarity between the host (receptor/chemosensor) and guest (phosphorylated motif) is central to receptor and chemosensor design approaches that target protein phosphorylation. Phosphorylated protein recognition in nature involves forming multiple hydrogen bonds and electrostatic interactions between the anionic phosphate and amino acid residues. These interactions are possible with proteins because the anion binding pockets are characterized by multiple interactions that are shielded from the aqueous environment, changing the local dielectric constant of the binding interface. In addition, proteins can adjust their conformation upon binding to avoid entropic penalties, which would favour interaction and recognition of phosphosites.\textsuperscript{40} It would be tempting to design large, sophisticated molecules to mimic these recognition pockets, however this can be synthetically laborious and achieving adequate complementarity using this strategy would be difficult. While a number of anionic receptors that utilize hydrogen bonding and electrostatic interactions have been reported,\textsuperscript{41,42} very few are able to form stable complexes in aqueous solution. This is due to the fact that the phosphate anion is strongly hydrated, thus any complexation event would involve desolvation of both the phosphate group and anionic receptor with a concomitant large energetic cost. Since water competes for hydrogen bonding sites, hydrogen-bonding interactions within synthetic receptors are greatly attenuated. In addition, electrostatic interactions are weakened due to the dielectric constant of water (~80), which serves to shield charges. Phosphate recognition in nature has evolved to mitigate these challenges, and the aim in
designing synthetic receptors and chemosensors to target phosphorylation involves competing with natural receptors in maintaining similar interactions in aqueous solutions.

One emergent strategy in anion recognition is the use of vacant metal coordination sites to mediate reversible bond interactions with high affinity.\(^{43}\) This metal-centred recognition is also seen in metalloenzymes such as alkaline phosphatase which posses two zinc ions in its active site to bind phosphate anions.\(^{44}\) The direct coordination of a cationic metal centre to an anion has a high enthalpy.\(^{43}\) Since water is a poor Lewis base, it interferes less with metal coordination-based anion recognition systems than with hydrogen bond based scaffolds and opens up the opportunity to construct useful metal-based receptors.\(^{43,45}\)

The groups of Hamachi, Koike and Konig have pioneered advances in the application of metal coordination based receptor and chemosensor complexes in phosphoprotein/peptide recognition. Of several divalent metals investigated, zinc (II) and manganese (II) transition metal ions were most commonly employed with dipicolylamine (DPA) and 1,4,7, 10 tetracyclododecne (cyclen) chelate derivatives for the recognition of phosphate modifications.

In 2002, the Hamachi group employed the use of anthracene-based bis- DPA chemosensors for the detection of mono-phosphorylated peptides, which displayed an increase in fluorescence intensity upon binding (Figure 1.6).\(^{46}\) The chemosensors contained two zinc (II) coordinated groups and showed a higher apparent affinity for pY peptide (\(K_a = 10^7 \text{ M}^{-1}\)) than pS peptide (\(K_a = 10^4 \text{ M}^{-1}\)). The receptors were tested against a set of different mono-phosphorylated peptides and showed an affinity in correlation with the overall negative charge of the peptide. The additional negative charge on peptides arose from glutamic acid and aspartic acid residues on the peptide and the most negatively charged peptide displayed the highest affinity.\(^{47}\) The detection of phosphoproteins in aqueous solution and on polyacrylamide (PA) gels has also been reported for these compounds.\(^{48}\)
Our group, Gunning et al., sought to derive binding selectivity amongst mono-phosphorylated peptides using metal-DPA chelate complexes \(^{49-52}\). The work was focused on designing receptors that would achieve binding selectivity via the recognition of pX and additional flanking amino acid residues, pX + X. Di-topic substituted bi-phenyl (Figure 1.7A) \(^{50,51}\) and benzothiazole scaffold derivatives (Figure 1.7B) \(^{52}\) were synthesized and tested in hopes of deriving binding affinity via the simultaneous interaction of Zn\(^{2+}\)-DPA units with the pX residue and the appended R group with variable proximal amino acid residue, pX + X in order to enhance selectivity amongst peptides. The results from the studies indicated that there was an overall preference of receptors towards negatively charged peptide sequences regardless of the nature of the R group.

**Figure 1.6** Mono-phosphorylated peptide detection. A) Proposed mode of action of Hamachi chemosensors. B) Chemical structure of Hamachi chemosensors.

**Figure 1.7** Chemical structures of receptors synthesized by the Gunning group containing A) substituted biphenyl or B) benzothiazole scaffolds.
The Konig group also targeted mono-phosphorylated peptide motifs using di-topic Zn\textsuperscript{2+}-cyclen receptors. Receptors were designed to interact with phosphorylated peptides varying in i + 3 amino acid residues. Within the library of compounds synthesized, receptors containing bis-Zn\textsuperscript{2+}-cyclen triazine phosphate binding units tethered to guanidinium (Figure 1.8A) or Zn\textsuperscript{2+}-nitrilotriacetic acid (NTA) (Figure 1.8B) groups displayed nanomolar affinities to select peptides.\textsuperscript{53} A follow-up study focused on the synthesis and testing of a larger library of Zn\textsuperscript{2+}-cyclen receptor complexes and the most potent receptors were used to inhibit mono-phosphorylated peptide binding to STAT1 and Chk2 proteins.\textsuperscript{54}

**Figure 1.8** Chemical structures of receptors designed by the Konig group.

The Koike group also explored the use of Zn\textsuperscript{2+}-DPA complexes starting in 2004 with the synthesis of a bridged alkoxide binuclear Zn\textsuperscript{2+}-DPA complex termed PhosTag (Figure 1.9).\textsuperscript{55} This complex displayed a high affinity (K\textsubscript{d} = 25 nM) to p-nitrophenyl phosphate in aqueous solution with the structure confirmed by X-ray crystal analysis to be a phosphate bound by two bridged Zn\textsuperscript{2+} ions. This molecule was also shown to be selective for the phosphate monoester over sulfate, acetate, chloride and bis (phenyl)phosphate.\textsuperscript{55} From this preliminary work, the PhosTag molecule was used in many phosphopeptide and phosphoprotein applications such as immobilized metal ion affinity chromatography (IMAC),\textsuperscript{56} mass spectrometry,\textsuperscript{57} and SPR.\textsuperscript{58} It was taken further in applications with the Zn\textsuperscript{2+} complex functionalized with biotin for the detection of phosphoproteins on polyvinylidene fluoride (PVDF) membranes via interaction with streptavidin-labelled horse radish peroxidase (HRP).\textsuperscript{59} The Mn\textsuperscript{2+} derivative was used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) phosphoprotein shift assays.\textsuperscript{59} The SDS shift assay with the Mn\textsuperscript{2+} complex was further applied within *in vitro* kinase profiling, in cell kinase profiling and within *in vitro* kinase drug screens.\textsuperscript{60} Previous work has also used
phosphate affinity SDS-PAGE bound with PhosTag to separate different phosphoprotein isotypes and reported that the same number of phosphorylated sites can have distinct migration patterns.\textsuperscript{61} The same method was successfully applied to a 2D gel separation of phosphoproteins.\textsuperscript{62} Through changing the percentage of polyacrylamide and agarose this technique was adapted specifically for the separation and analysis of large phosphoproteins of molecular masses greater than 200 kDa.\textsuperscript{63}

\begin{center}
\textbf{Figure 1.9} Chemical structure of PhosTag.
\end{center}

With the aim of more advanced applications in phosphopeptide and phosphoprotein detection, the Koike group further synthesized mono-, bis-, and tetrakis-biotinylated derivatives of the original PhosTag. (Figure 1.10) The dodeca(ethylene glycol) spacer derivative (BTL-111) displayed favourable results in Western blot analysis, in quartz-crystal microbalance analysis and in peptide microarray techniques.\textsuperscript{64} The development and application of PhosTag is a prime example of the potential use of metal-based receptors in phosphoprotein detection.
Figure 1.10 Chemical structures of PhosTag derivatives.

The commercialization and application of numerous molecular tools of this type (Figure 1.11) further underscores the significant potential of these metal-based receptors in phosphopeptide and phosphoprotein recognition and detection.
1.2.2 Metal-based receptors and chemosensors for proximally phosphorylated motifs

The few studies focused on the emergent aim of the recognition of proximally phosphorylated motifs also employ a metal-based approach.\textsuperscript{65-68} A common challenge with this aim is the selective recognition of proximally phosphorylated motifs over mono-phosphorylated motifs. To this end, the Hamachi group employed a cross-linked, two-point interaction approach with the synthesis of rigid bis-\(\text{Zn}^{2+}\)-DPA complexes (Figure 1.12).\textsuperscript{65} They proposed that the rigid structure of the receptor would display preference to proximally di-phosphorylated motifs based on the restrictive spacing of the \(\text{Zn}^{2+}\) binding units (Figure 1.12). The first examples of this approach were the use of small libraries of synthesized receptors that were tested for binding affinity against mono- and di-phosphorylated peptides.\textsuperscript{65,66} The results demonstrate that select receptors have 10- 20-fold selectivity in binding affinity for di-phosphorylated peptides over their mono phosphorylated counterparts (Figure 1.13). Overall, the results indicate that receptors containing two-\(\text{Zn}^{2+}\)-DPA sites can target di-phosphorylated motifs if the distance between the two \(\text{Zn}^{2+}\) centres is sufficient to match the two phosphates within the di-phosphorylated peptide.
Figure 1.12 Cross-linking, two-point interaction approach proposed by Hamachi group.

Figure 1.13 Chemical structures of cross-linking receptor by Hamachi group.

This strategy was taken further by the Hamachi group with the synthesis of a turn-on fluorescent chemosensors selective for proximally di-phosphorylated motifs, where an increase in the fluorescence intensity of the reporter group would preferentially arise in the presence of di-phosphorylated over mono-phosphorylated motifs (Figure 1.14). A rigid bis- Zn$^{2+}$-DPA scaffold linked to a BODIPY fluorescent reporter was synthesized (Figure 1.15A) and used to target proximally phosphorylated motifs of the tau protein. This turn-on fluorescent chemosensor demonstrated selectivity for proximally phosphorylated tau peptide motifs in an $i$, $i+4$ position. On the other hand, mono-phosphorylated motifs and other di-phosphorylated motifs at $i$, $i+2$ and $i$, $i+6$ positions could not induce an increase in fluorescence intensity. This highlights the distance-based discrimination of the chemosensor, as it is able to show preferential signal amongst proximally phosphorylated motifs. This chemosensor was also applied to detect
hyperphosphorylated tau proteins within *in vitro* assays and in the staining of fixed brain slices of Alzheimer’s patients. The group also designed a different turn-on chemosensor for the same aim using a rigid diazastillbene reporter group (Figure 1.15B). When applied to proximally phosphorylated peptides, the authors cite that the chemosensor was selective for the pTpS (*i, i*+1) di-phosphorylated motif over mono-phosphorylated motifs and other di-phosphorylated motifs with positions ranging *i, i*+2 to *i, i*+6. However, it must be noted that the *i, i*+1 peptide sequence used was different from the other peptides tested, as it possessed the most overall negative charge.

**Figure 1.14** Mechanism of turn-on response from a chemosensor as proposed by the Hamachi group. Due to the rigid scaffold between zinc (II) centres, this chemosensor shows low affinity and signal when bound to A) a mono-phosphorylated peptide, compared to enhanced binding affinity and signal when bound to B) a proximally phosphorylated peptide.
As of 2012, to the best of our knowledge, these were the only published studies focused on targeting proximally phosphorylated motifs using a metal-based recognition approach. As a follow up to these studies, we sought to build upon the cross-linking strategy with the design of different metal-based receptors to target proximally phosphorylated motifs.

### 1.3 Central aims of research

The phosphorylation of protein surfaces is an important process in the maintenance of life. Its role in several cellular processes is central to controlling the activity status and function of proteins. Different levels of aberrant functioning of this process are implicated in the onset and progression of disease states. Thus, the development of methods to target protein phosphorylation is important area of interest. To this end, metal-based receptors have been successfully employed and have been shown to be versatile in their application. It has also become increasingly apparent that proximally phosphorylated motifs are functionally relevant to diseased states. However, despite the need to further understand proximally phosphorylated motifs, there are few methods to target these specific phosphosites. Building upon the works of the pioneers in metal-based receptor development, we sought to develop metal-based methods for the selective targeting of proximally phosphorylated over mono-phosphorylated motifs on proteins and peptides under aqueous conditions. We initially rationalized that the design of alternative receptors based on the cross-linking, two-point interaction strategy could selectively target proximally di-phosphorylated motifs with high affinity.
1.4 Contributions

The PhosphoSitePlus® searches, filtering and analysis was performed by Eugenia Duodu and Dziyana Kraskouskaya.
Chapter 2

2 The development of a tripodal receptor scaffold for the recognition of proximally phosphorylated motifs

2.1 The use of triethylbenzene receptors for the recognition of proximally di-phosphorylated motifs

As described in Chapter 1, the development of metal-based methods to target proximally phosphorylated motifs is an emerging area of research. The principal objective of this work was to design and synthesize a receptor capable of preferential binding to proximally phosphorylated motifs over their mono-phosphorylated counterparts. We sought to employ a cross-linking, two-point interaction strategy similar to that demonstrated by the Hamachi group. In this approach, the use of a rigid receptor molecule positions the Zn$^{2+}$-binding units at a defined distance from one another. This restrictive spacing yields preference for variably spaced proximally phosphorylated motifs over mono-phosphorylated motifs (Figure 2.1).

Figure 2.1 Cross-linking, two-point interaction approach proposed by the Hamachi group.

While the Hamachi group used rigid bi-phenyl, BODIPY and diazastillbene bis Zn$^{2+}$- DPA scaffolds in their receptor design, we sought to use an alternative scaffold to achieve the selective
interaction with proximally phosphorylated motifs. We decided to use the well-characterized triethylbenzene scaffold (Figure 2.2). Since its first discovery,69 extensive work has been done to synthetically optimize this receptor scaffold for binding various target analytes with high affinity and selectivity.70-72 The key feature of this scaffold and one reason for its widespread use, is the arrangement of the substituents on the hexasubstituted benzene ring, which favours an alternating “three-up, three down” conformation by ~ 4 kcal mol\(^{-1}\), compared to the next stable conformation.73,74 This conformation is favoured via the minimization of unfavourable electrostatic and steric interactions between substituents. We reasoned that this feature could be exploited in the selective recognition of proximally phosphorylated motifs by positioning phosphate-binding units in a manner to match the projection of phosphorylated residues on the peptide. We opted to investigate if this approach could result in binding discrimination amongst phosphopeptides based on the number of and the relative distance between phosphorylated sites \((i, i + X, \text{where } X= \text{the number of amino acid residues})\). We postulated that the designed receptor would bind select proximally phosphorylated peptides with a higher affinity than mono-phosphorylated peptides based on a two-point interaction.

![Figure 2.2](image)

**Figure 2.2** Chemical structure of proposed tripodal receptor scaffold, shown in the more favoured “three-up, three-down” conformation.

### 2.2 The design and synthesis of a tripodal receptor to target di-phosphorylated peptides

#### 2.2.1 Tripodal receptor design considerations

Given the success of metal-based receptors in protein phosphorylation recognition, we opted to use two Zn\(^{2+}\) phosphate recognition units in our design to bind proximal phosphate anions on peptides. The third “R” group of the scaffold was designed for the possible incorporation of a reporter group or functionality to modify binding affinity amongst phosphorylated motifs. We
proposed that binding to specific proximally phosphorylated motifs would be favoured over their mono-phosphorylated counterparts based on increased affinity with a two-point interaction as observed in previous research (Figure 2.3A and Figure 2.3C). In addition, the distance between recognition units could favour binding to certain proximally phosphorylated motifs over others depending on phosphorylated residue spacing (Figure 2.3B and Figure 2.3C).

Figure 2.3 Proposed mode of selectivity with the tripodal receptor scaffold using a cross-linking, two-point interaction approach. A) A weak affinity interaction with mono-phosphorylated peptide, B) a weak affinity interaction with a di-phosphorylated peptide with phosphorylated sites too far apart for a two-point interaction with receptor recognition units and C) a high affinity interaction with di-phosphorylated peptide with phosphorylated sites at a distance that matches a two-point interaction with receptor recognition units.
2.2.2 Tripodal receptor scaffold design and synthesis

A tripodal receptor was designed incorporating two Zn\(^{2+}\)-cyclen binding units and a methyl group tethered by indole groups (Figure 2.2). As demonstrated in the receptors designed by the König group,\(^{53,54}\) we opted to use Zn\(^{2+}\)-cyclen groups as phosphate binders in our design for a less hydrophobic receptor than that with DPA chelate groups. Indole groups were used to tether the recognitions units to the scaffold to provide a more rigid linker system. We hypothesized that the tripodal Zn\(^{2+}\)-cyclen indole receptor (Figure 2.2) would bind to select di-phosphorylated motifs with an increased affinity compared to mono-phosphorylated motifs. The tripodal receptor was synthesized using a developed synthetic procedure (Scheme 2.1). Briefly, the bromomethylation of 1,3,5-triethylbenzene with ZnBr and paraformaldehyde in HBr/AcOH resulted in 1,3,5 tris(bromomethyl)2,4,6-triethylbenzene 1. This product was then alkylated using three equivalents of methyl indole 4-carboxylate and NaH in THF to afford a tri-indole substituted compound 2 in high yield. Methyl ester hydrolysis of 2 using NaOH in 3:1:1 (THF:MeOH:H\(_2\)O) furnished the tri-acid 4. The selective benzyl protection of a single indole carboxylic acid was achieved using benzyl bromide and KOTbu in DMF yielding 5. Two equivalents of tri-Boc protected cyclen were coupled to 5 using TBTU and DIPEA in DMF to yield 6. To obtain a site for functionalization, the benzyl-protecting group was then removed via Pd/C catalysed hydrogenolysis and the resulting free acid was coupled to methylamine hydrochloride to yield 7. Global deprotection using 1:1 (TFA, DCM) resulted in the final ligand 8 which was metallated using Zn(OTf)\(_2\) in MeOH to afford the final compound 9.

Figure 2.4 Triethylbenzene scaffold receptor 9.
Scheme 2.1: a) (CH$_2$O)$_n$, ZnBr$_2$, HBr in AcOH, 90 °C, 10h, 95%; b) methyl indole-4-carboxylate, NaH, THF, 0 °C to RT, overnight, 80%; c) NaOH, 3:1:1 (THF, MeOH, H$_2$O), 50 °C, 1 h, 90%; d) KOTBu, BnBr, DMF, RT, 6 h, 17%; e) Boc$_3$cyclen, TBTU, DIPEA, DMF, RT, overnight, 67%; f) H$_2$, Pd/C, MeOH, RT, overnight, 55%; g) CH$_3$NH$_2$, TBTU, DIPEA, DMF, RT, 16 h, 56%; h) 1:1 (TFA, DCM), 0.5 h, 62%; i) Zn(OTf)$_2$, MeOH, RT, overnight.
2.3 Evaluation of tripodal receptor binding affinity to di-phosphorylated peptides

In order to test our hypothesis, we evaluated the binding affinity of receptor 9 to target proximally phosphorylated peptides and off-target mono-phosphorylated peptides. We used fluorescence intensity (FI) and isothermal titration calorimetry (ITC) as biophysical techniques to measure binding affinity.

2.3.1 Fluorescence intensity assay: Results and discussion

We opted to use a previously developed fluorescence intensity assay\textsuperscript{50,51} as a preliminary approach to assess the receptor to peptide interaction. This assay was shown to be a fast technique to measure receptor to peptide binding affinity, results from which correlated well with those obtained by other biophysical techniques.\textsuperscript{50,51} The proposed mode of action of this assay to the testing of our receptor scaffold and peptide motifs is demonstrated in Figure 2.5. A constant concentration of fluorescently labelled (TAMRA fluorophore) phosphorylated peptide is titrated with a varying concentration of the receptor (Figure 2.5). Upon complexation of receptor with a peptide, there is a concentration-dependant decrease in the fluorescence intensity of the labelled peptide. The binding affinity of the receptor to the peptide can then be derived from the resulting titration curve.
**Figure 2.5** Representative curve indicating receptor:peptide complexation using a fluorescence intensity assay that reports the quenching of TAMRA-labelled peptide signal.

Using this approach, we ran a preliminary screen of receptor 9 against TAMRA-labelled peptides (full sequences in Section 6.1) that varied in the number of phosphorylated residues, the nature of the phosphorylated residue (pS or pY) and the distance between phosphorylated residues. For a controlled comparison of proximal phosphorylation alanine-based peptides were used which only differed in phosphorylated residue. Receptor 9 (24 nM - 50 µM) was titrated into a solution of labelled peptide (20 nM) in 50 mM HEPES buffer at pH 7.5 containing 5% DMSO. The fluorescence emission of the TAMRA peptide was measured at 580 nm upon excitation at 540 nm using a TECAN Infinite M1000. The affinity of each peptide to the receptor was calculated using a dose-response fitting model in ORIGIN and is presented as $K_a$ in Figure 2.6.
Figure 2.6 Binding affinities ($K_a$) of receptor 9 to phosphopeptide as measured by the fluorescence intensity assay.

We hypothesized that receptor 9 would display preferential binding affinity to select proximally phosphorylated peptide motifs over mono-phosphorylated motifs, according to the proposed binding modes described in Figure 2.3. Inconsistent with our hypothesis, among pY-containing peptides, receptor 9 displayed no significant selectivity between mono- and di-phosphorylated peptide sequences, however slight increases in affinity were observed with increasing spacing between pY sites (e.g. pYpY to pYAApY). In contrast to the results with pY containing peptides and consistent with our hypothesis, the receptor displayed a lower affinity to the mono- pS peptide ($10^5$ M$^{-1}$) than to the di-phosphorylated pS peptides ($10^6$ M$^{-1}$). A higher affinity for mono-pY over mono-pS peptides was also observed. We presumed that this could be a result of the increased distance of the anionic phosphate from the peptide backbone in pY as compared to pS making the phosphate more accessible for interaction.

Overall, the majority of the binding affinity values of the receptor to phosphopeptides obtained from the fluorescence intensity assay were on the same order of magnitude. The results indicate that there is little preference for proximally phosphorylated motifs over mono-phosphorylated counterparts in the case of pY-containing peptides. Based on the previous literature reports of 10-20-fold selectivity of receptors to di-phosphorylated over mono-phosphorylated peptides$^{65,66}$ more significant differences were expected. In order to further investigate the results of the
fluorescence intensity assay, we opted to use isothermal titration calorimetry (ITC) as additional method to evaluate the binding affinity of the receptor to peptides of interest.

2.3.2 Isothermal Titration Calorimetry (ITC)

We chose to use isothermal titration calorimetry (ITC) as a next step in elucidating the binding affinity of the receptor scaffold to phosphorylated peptides. ITC is a powerful universal detection technique, in which the thermodynamic parameters of molecular interactions are determined by measuring the heat evolved (exothermic) or absorbed (endothermic) during a binding event. An ITC experiment can simultaneously quantify the binding stoichiometry (n), binding constant (K), binding enthalpy ($\Delta H^\circ$) and the binding entropy ($\Delta S^\circ$) associated with a binding event.

2.3.2.1 Overview of the technique

The heat evolution or heat consumption as a result of a molecular binding interaction is measured in a micro-calorimeter. A standard isothermal micro-calorimeter is composed of two coin shaped cells that are permanently seated in an insulated adiabatic jacket typically set to 5-10°C above the experimental temperature (Figure 2.7A). The reference cell is filled with the solvent (i.e. water or buffer) and the sample cell is filled with one partner in the binding reaction to be studied. The other binding partner, usually 10-20 fold higher in concentration, is titrated in aliquots from a syringe inserted into of the sample cell. The power supplied to heat the reference cell is kept constant to set a temperature difference of about 0.01 °C over that of the insulating jacket. A similar power supply is connected to the sample cell and is automatically regulated by a feedback mechanism to minimize the temperature difference ($\Delta T$) between the two cells in order to keep $\Delta T = 0$. As the injection syringe titrates aliquots of one binding partner into the sample cell, the association of binding partners results in heat release or absorption that in turn either raises or lowers the temperature of the sample cell. This change in temperature triggers the feedback regulator associated with the sample cell to adjust the power supply in order to maintain identical differences in temperature between both cells (power compensation technique). The change in the feedback current is the signal recorded by the instrument.

In an exothermic binding event, less heat is needed to keep the temperature between cells constant resulting in a drop in power supplied to the sample cell in order to keep $\Delta T = 0$. The drop in the power supplied is characterized by a downward pulse in signal and as the
temperatures of the two cells re-equilibrate, the signal returns to its original position (Figure 2.7B). The opposite trend is true for endothermic binding events, where an upward peak reflects an increase in power to the sample cell in order to maintain $\Delta T = 0$ in the system. The microcalorimeter measures this power compensation to the sample cell and plots the changes in power ($\mu$cal s$^{-1}$) vs. time (min) (Figure 2.7B). As ligand is titrated with each injection, the molar ratio between ligand and receptor increases and the amount of receptor available to bind ligand decreases, causing smaller changes in heat. Once the receptor becomes saturated with ligand, minimal heat changes upon injection occur, corresponding to heats of dilution. The area of each peak is integrated and plotted vs. molar ratio of ligand to receptor and the resultant isotherm can be fit to a binding model that can solve for $K_a$ (Figure 2.7C). From the isotherm, $\Delta H^0$ is equal to the height of the sigmoid, $K_a$ is proportional to the slope of the sigmoid and the stoichiometry ($N$) is the vertical extrapolation at the inflection point of the sigmoid.

**Figure 2.7** Isothermal Titration Calorimetry. A) Schematic of the components of a microcalorimeter. B) Raw data and C) Labelled binding titration curve resulting from a typical exothermic binding event, as recorded using a micro-calorimeter.
2.3.3 Isothermal titration calorimetry: Results and discussion

As described in Section 2.3.2, ITC can provide direct quantitative information about the thermodynamic parameters that characterize a receptor-ligand binding interaction. Therefore we conducted ITC experiments to determine the binding affinity of receptor 9 to alanine- based unlabelled pYpY (Ac-ApYpYAA-NH₂) and pY (Ac-ApYpYAA-NH₂) peptides in 50 mM HEPES, pH 7.5 buffer solution (dilution of pYpY and pY peptide into 50 mM HEPES, pH 7.5 buffer, Figure 6.1). Initial experiments involved the titration of the pYpY peptide from the injection syringe into the sample cell containing receptor 9 with all resultant titration curves fit to a “one-site” binding model in ORIGIN software. The first ITC experiment was performed using initial concentration of 0.375 M of pYpY peptide (in syringe) and 0.025 M of receptor 9 (in sample cell). This generated the titration curve shown in Figure 2.8A and the corresponding thermodynamic data is summarized in Table 2.1. The binding affinity of the receptor-peptide interaction was calculated to be $7.77 \times 10^4$ M$^{-1}$ with $N = 0.2$. However, the titration curve did not reach saturation. This may have occurred because the concentrations of the receptor and peptide were too low. Thus, in order to reach saturation and more accurately calculate the binding affinity, the concentration of the receptor and peptide were increased in subsequent experiments (Figure 2.8 B, C, and D). Each of the resultant curves was endothermic and the binding affinities at different concentrations did not change considerably ($K_a = 5.05 \text{ - } 7.83 \times 10^4$ M$^{-1}$). The corresponding N values increased from 0.2 to 0.4 at 0.1 M of receptor 9 and 1.5 M of pYpY peptide (Table 2.1). These N values indicate that the stoichiometry of the interaction is less than 1:1. Overall, increasing the concentration did not result in reaching the signal saturation. While these results supported that the affinity value obtained in the first ITC experiment was reproducible at different conditions, we sought to investigate the reason for the inability to achieve saturation.
Figure 2.8 ITC traces of receptor 9 with pYpY peptide at varied concentrations of both receptor and peptide.
Table 2.1 ITC thermodynamic binding data for receptor 9 complexation with pYpY peptide in 50 mM HEPES buffer, pH 7.5, 298.15 K. $\Delta G^\circ$, $\Delta H^\circ$ and $T\Delta S^\circ$ are in units kcal mol$^{-1}$. ITC trace labels correspond to Figure 2.8.

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<th>$\Delta G^\circ$</th>
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</tbody>
</table>

As saturation could not be reached by increasing the concentration of receptor and peptide it was assumed that the binding interaction had a low affinity. In addition to this, since the corresponding N values indicated a stoichiometry less than 1:1, it was hypothesized that factors outside of the direct binding partners could affect the binding interactions. Thus, we decided to study the system in more detail. Based on the chemical structure of the receptor, we reasoned that non-specific hydrophobic interactions could occur amongst receptor molecules as well as between the receptor and target peptide. Such “higher-order” complexes have been previously reported for receptors of this type.$^{75}$ As the heat change measurements in ITC represent a global response of many interactions occurring simultaneously, these additional interactions could complicate the receptor to peptide interaction. Therefore, as a way to probe non-specific hydrophobic interactions, 10% methanol was added to the original buffer (50 mM HEPES, pH 7.5), and 0.1 M receptor 9 was titrated with 1.5 M pYpY peptide. The resultant titration curve (Figure 2.9) shows that saturation was again not achieved. The receptor-peptide binding affinity in methanol-containing buffer decreased slightly in comparison to the analogous experiment without methanol ($K_a = 2.37 \times 10^4$ M$^{-1}$ and $K_a = 5.05 \times 10^4$ M, respectively). However, the N value increased from 0.4 (without 10% methanol) to 0.7 (with 10% methanol) indicative of a stoichiometry closer to 1:1 (Table 2.2).
Figure 2.9 ITC trace of receptor 9 with pYpY peptide in 50 mM HEPES, pH 7.5, 10% methanol.

Table 2.2 ITC thermodynamic binding data for receptor 9 complexation with pYpY peptide in 50 mM HEPES, pH 7.5, 10% methanol. ΔG°, ΔH° and TΔS° are in units kcal mol⁻¹. ITC trace labels correspond to Figure 2.9.

<table>
<thead>
<tr>
<th>ITC Trace Label</th>
<th>[receptor] (M)</th>
<th>[pYpY peptide] (M)</th>
<th>N</th>
<th>K_a (x 10⁴ M⁻¹)</th>
<th>ΔG°</th>
<th>ΔH°</th>
<th>TΔS°</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.1</td>
<td>1.5</td>
<td>0.7</td>
<td>2.37±0.16</td>
<td>-5.94</td>
<td>5.52±0.25</td>
<td>11.47</td>
</tr>
</tbody>
</table>

A graphical representation of the summarized thermodynamic data in Table 2.1 provides additional information on receptor 9 and pYpY peptide complexation (Figure 2.10). It can be seen that upon increasing the receptor and peptide experimental concentrations, there is a decrease in the values of the entropic term (less positive) with a decrease in the value of the enthalpic term (more negative) (Figure 2.10). This suggests that upon increasing receptor and peptide concentrations, complexation becomes less entropically driven and less enthalpically...
unfavourable. The results of experiments conducted in 10% methanol buffer are the least entropically driven and most enthalpically favourable as compared to results obtained in non-methanol containing buffer (Figure 2.10).

![ITC Experiment](image)

**Figure 2.10** A graphical representation of the ITC thermodynamic data presented in Table 2.1 and Table 2.2.

We hypothesized that the reason for an entropy-driven complexation between the receptor and peptide might arise from desolvation upon interaction. In solution, water molecules can form ordered ‘solvation spheres’ around the receptor and peptide (Figure 2.11). A binding interaction between the receptor and peptide could result in the displacement of ordered water molecules into the bulk solution (Figure 2.11). Thus, the process of desolvation would result in releasing restricted water molecules, which in turn results in an overall increase in entropy of the system. While it is presumed that the interaction between the receptor Zn$^{2+}$ binding units and the anionic phosphate of the peptide upon binding would be associated with a favourable enthalpic term, other studies have shown that this metal to anion interaction displays entropically driven interactions. The above results also indicate that in the presence of 10% methanol the interaction is less entropically driven than without methanol. One of several explanations for this
effect could be the decreased solvating ability of water molecules. Thus, with less ordered water molecules, desolvation becomes less favourable and the interaction is less entropically favoured.

**Figure 2.11** The process of desolvation in the formation of a receptor to ligand complex

According to our proposed binding mode, we hypothesized that the affinity of receptor 9 to pY peptide would be weaker than with the pYPY peptide. However, according to the FI experiments, the affinity of receptor 9 to both pY and pYPY peptides was on the same order of magnitude. The results of the ITC experiments of receptor 9 to pYPY peptide indicate an estimated affinity of $K_a = 10^4$ M$^{-1}$. Thus, we next sought to use ITC to determine the affinity of receptor 9 to mono-phosphorylated peptide. To test this, we ran ITC experiments with receptor 9 and pY in 50 mM HEPES, pH 7.5 and all resultant titration curves were fit to a “one-site” binding model in ORIGIN software. In order to achieve saturation, the first experiment was run at higher concentrations than cited in initial experiments for pYPY peptide with a receptor concentration of 0.1 M and pY peptide concentration of 1.5 M (Figure 2.12A and Table 2.3). The results indicated receptor to pY peptide affinity of $1.63 \times 10^4$ M$^{-1}$ with $N = 0.8$, indicative of a 1:1 stoichiometry, however complete saturation was again not achieved (Figure 2.12A and Table 2.3). In order to reach saturation the receptor concentration was increased to 0.2 M and the pY peptide concentration was increased to 3.0 M (Figure 2.12B). The titration curve generated did not reach complete saturation ($K_a = 0.85 \times 10^4$ M$^{-1}$, $N = 0.7$ Table 2.3). Repeating the experiment
conditions in Figure 2.12A (receptor 9 = 0.1 M, pY peptide = 3.0 M) in 10% methanol in 50 mM HEPES, pH 7.5 solution yielded the titration curve in Figure 2.12C. Once again, complete saturation was not achieved in 10 % methanol buffer and there was negligible difference in binding affinity compared with the experiment without methanol (K_a = 1.80 x 10^4 M^{-1} and K_a = 1.63 x 10^4 M^{-1}, respectively) along with a slight increase in the N value (0.8 and 1.1, respectively). The thermodynamic parameters listed in Table 2.3 indicate very little difference in entropic and enthalpic terms between experimental conditions. However, as with the ITC experiments with the pYpY peptide, the titration curves of receptor 9 and pY peptide appear to be endothermic and indicative of an entropy-driven process.
**Figure 2.12** ITC traces of A) receptor 9 with pY peptide, B) at varied concentrations of peptide and receptor, and C) with 10% methanol added to the buffer solution.
Table 2.3 ITC thermodynamic binding data for receptor 9 complexation with pY peptide. $\Delta G^o$, $\Delta H^o$ and $T\Delta S^o$ are in units kcal mol$^{-1}$. ITC trace labels correspond to Figure 2.12.

<table>
<thead>
<tr>
<th>ITC Trace Label</th>
<th>[receptor] (M)</th>
<th>[pY peptide] (M)</th>
<th>N</th>
<th>$K_a$ ($x 10^4$ M$^{-1}$)</th>
<th>$\Delta G^o$</th>
<th>$\Delta H^o$</th>
<th>$T\Delta S^o$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.1</td>
<td>1.5</td>
<td>0.8</td>
<td>1.63±0.31</td>
<td>-5.72</td>
<td>0.77±0.11</td>
<td>6.50</td>
</tr>
<tr>
<td>B</td>
<td>0.2</td>
<td>3.0</td>
<td>0.7</td>
<td>0.85±0.09</td>
<td>-5.33</td>
<td>1.23±0.12</td>
<td>6.61</td>
</tr>
<tr>
<td>C</td>
<td>0.1</td>
<td>1.5</td>
<td>1.1</td>
<td>1.80±0.37</td>
<td>-5.78</td>
<td>0.57±0.06</td>
<td>6.38</td>
</tr>
</tbody>
</table>

Overall, the results from the ITC experiments indicate that receptor 9 has a binding affinity to both pY and pYpY peptides on the same order of magnitude ($K_a = 10^4$ M$^{-1}$). The ITC titration curves were endothermic and indicated an entropy-driven process. These results are in agreement with other reported examples of entropy-driven endothermic titration curves from ITC experiments with metal-based receptors and phosphopeptides.$^{47,66}$ Importantly, the calculated binding affinity of receptor 9 to the target proximally phosphorylated pYpY peptide is notably weaker as compared to previously designed receptors aimed at targeting proximal di-phosphorylated motifs.$^{65,66,68}$ The result from both the FI and ITC experiments reasonably suggest that the receptor 9 does not display preferential binding to pYpY peptide over mono-phosphorylated pY peptide. This indicated that the design of this receptor might not be suitable for the targeting of proximally di-phosphorylated motifs in contrast to what was originally hypothesized.

2.4 The implications of the triethylbenzene scaffold design in the recognition of di-phosphorylated peptide motifs

The results of this study provided insight into the prerequisites for achieving the original aim of selectively targeting proximally phosphorylated motifs.

The FI results displayed marginal differences between the binding affinities of receptor 9 to proximally phosphorylated peptides. Although the binding constants calculated from ITC were lower than those generated from the FI experiments, the trend was consistent, both indicating minimal difference between the binding affinity of receptor 9 to pY and pYpY peptides. While
the ITC results provided valuable information about the binding interaction, the propensity of receptor 9 to aggregate in solution at concentrations necessary for ITC measurements in 50 mM HEPES pH 7.2 buffer hindered further uses of this technique.

Based on the preliminary results, we reconsidered the ability of this receptor scaffold to preferentially bind proximally phosphorylated motifs via the two-point interaction approach. In receptor design, the triethylbenzene scaffold demonstrates a balance between preorganization and flexibility generating more induced-fit binding interactions with guest analytes. In many cases, this additional flexibility could result in decreased binding selectivity (as measured by the magnitude of the binding constant) of the receptor to analytes with a lower binding affinity as compared to more preorganized receptor systems. This property is advantageous in the development of pattern-based recognition sensor array systems. However, since our aim was to design a receptor capable of discriminating between phosphorylated peptides based on number of, and distance between, phosphate residues, the use of the triethylbenzene scaffold may not be suitable. We reasoned that these differences between peptides would be too subtle to achieve measurable distinctions in binding affinity and in turn adequate selectivity, which might have been the case with our observed results. Thus, as previously in Section 1.2.2, in order to attain selectivity for proximally phosphorylated over mono-phosphorylated motifs based on the cross-linking, two-point interaction strategy, designed receptors should be fairly rigid and highly preorganized. The challenge in this approach remains achieving notable selectivity between proximally phosphorylated residues that differ based on residue spacing and the nature of residue phosphorylated (pS, pT or pY). An additional requirement in receptor design also previously discussed is the incorporation of sensing/reporter unit that can detect measurable differences in phosphopeptides. Rigid chemosensors capable of discrimination between proximally phosphorylated motifs and monophosphorylated motifs have been described. However these chemosensors are also limited in the degree of discrimination based on the peptides tested, highlighting a need for the continued development of more robust sensing techniques. In retrospect, all of the information garnered with the synthesis and testing of the triethylbenzene receptor scaffold encouraged a shift in future approaches to achieving our aim in detecting proximally phosphorylated motifs.
2.5 Conclusions and future directions

In this study, we presented the synthesis and testing of a triethylbenzene receptor scaffold that was designed to preferentially target select proximally phosphorylated peptide motifs over mono-phosphorylated ones based on a two-point interaction approach. The preliminary results of the biophysical assays used indicate that this particular receptor scaffold might not be effective in selectively targeting proximally phosphorylated peptide motifs. While the results presented here did not lead to the desired outcome, they were used as a vantage point to reconsider the approach in achieving our aim. We reasoned that for future work, receptor designs should include a reporter unit or “sensing” unit capable of producing a selective turn-on response in the presence of target proximally phosphorylated motifs. Along with this, proposed receptors designs should maintain high-affinity interactions with the phosphorylated target and be amenable to testing via high-throughput techniques.

2.6 Contributions

Joel Drewry designed the triethylbenzene scaffold and formulated the idea to use this approach. Joel Drewry and Eugenia Duodu optimized the synthesis of the triethylbenzene receptor. Eugenia Duodu performed the FI and ITC experiments with assistance from Dziyana Kraskouskaya.
Chapter 3

3 The testing and application of a triethylbenzene receptor in the recognition of small molecule phosphoanions

In Chapter 2, a triethylbenzene receptor scaffold was synthesized for the purpose to selectively bind to proximally phosphorylated over mono-phosphorylated motifs on small peptides. However, the results indicated that the receptor scaffold was not suitable for achieving this aim. Therefore, the work presented in this Chapter describes the repurposing of the triethylbenzene scaffold to target small molecule phosphoanions using an indicator displacement assay and its application.

3.1 The recognition of biologically relevant anions

The selective recognition of biologically relevant anions is established area of study. Various anions such as adenosine triphosphate (ATP), pyrophosphate (PP$_i$), inositol triphosphate (IP$_3$) and citrate (Figure 3.1) hold fundamental roles in cell signalling, bioenergetic and metabolic processes. An extensive amount of work has been done in the development of receptors that selectively recognize small molecule phosphoanions. However, there are numerous challenges that need to be considered when selectively targeting anions in aqueous solution such as varying size of anions, differing geometries, pH sensitivity and solvation. With the specific aim of targeting anions in aqueous solution, receptor scaffolds have evolved to mediate anion recognition through electrostatic interactions, hydrogen bonds or metal coordination. Receptors that include metal-cation centres are often applied to anion recognition in aqueous solution. As previously stated (Chapter 1), anion to metal-receptor association in aqueous solution has a higher affinity as compared to hydrogen bond receptor systems due to the poor Lewis basicity of water, thus it interferes less with the anion to metal-based receptor association than in anion-hydrogen bond receptor association. Therefore, the identification of a receptor scaffold capable of forming pre-organized, high affinity contacts with target anions mediated by metal-based recognition in aqueous solution is a growing area of interest.
In Chapter 2 we described the synthesis of a Zn\(^{2+}\)-based triethylbenzene receptor. We postulated that the synthesized scaffold, based on the assumption that it formed a binding ‘cavity’, could potentially bind to different biologically relevant phosphate-containing anions that are smaller in size yet possess a high negative charge to facilitate interaction with the metal centres. Considering the numerous studies which use triethylbenzene receptors scaffolds for this aim,\(^70\)-\(^72\),\(^79\) we decided to test the efficacy of receptor 9 (Figure 3.2) (Chapter 2) for the recognition of small molecule phosphoanions. We hypothesized that select phosphoanions could form favourable interactions with the receptor.

Figure 3.1 Various biologically relevant anions.

Figure 3.2 Chemical structure of receptor 9.
3.2 The use of an indicator displacement assay to test the binding of phosphoanions to triethylbenzene receptor

Anslyn and co-workers pioneered advances in monitoring anion binding with the triethylbenzene scaffold using indicator displacement assays (IDA). In an IDA, the indicator dye reversibly binds to the receptor and subsequently the competitive analyte in question is added. If the analyte has sufficient affinity to the receptor, it displaces the bound indicator into bulk solution. This displacement modulates a change in optical signal based on changes in the photophysical properties of the indicator dye (Figure 3.3). By monitoring the absorbance change of the chromophore, target analytes that interact with the receptor can be identified. The main advantages of using a colorimetric IDA are: it does not require the incorporation of signalling functionality (i.e. fluorophore) into the receptor; it is cost effective; and it is colorimetric, where most changes can be first assessed with the naked eye. Thus, a colorimetric indicator displacement (IDA) assay was chosen to evaluate the binding of receptor 9 to a range of analytes.

Figure 3.3 Basic schematic of an indicator displacement assay.

3.2.1 Indicator displacement assay using pyrocatechol violet

One way that a change in the photophysical properties of an indicator dye can occur is through pH changes in the microenvironment of the chromophore. In this case, the change is mediated by the interaction of the dye with the receptor, causing a shift in the absorption of the dye and resulting change in its colour depending on whether or not it is bound by the receptor. In order to apply the IDA to test the efficacy of phosphoanion binding to receptor 9, the indicator dye, pyrocatechol violet (PV), was chosen based on its widespread use in IDA applications (Figure 3.4).
Figure 3.4 Structure of indicator dye, pyrocatechol violet (PV)

The mechanism of anion-specific signal modulation with respect to PV can first be explained by considering its colour changes associated with pH. PV can undergo various colour changes based on differing pH conditions (Figure 3.5). Increases in pH cause changes in the structure of PV via the sequential deprotonation of its acidic functionalities, and can result in shifts in the absorbance ($\lambda_{\text{MAX}}$) of PV, which results in changes in its visible colour.

Figure 3.5 Visible colour changes of PV with associated changes in pH, adapted from 85.
In the same way, the binding of metals to PV induces changes in colour similar to what is seen from neutral to alkaline pH values (Figure 3.6). Metal binding through chelation causes changes in the microenvironment of the bound catechol group resulting in spectral shifts in absorbance to a visible blue to blue-violet colour.\textsuperscript{85}

\textbf{Figure 3.6} Visible colour changes of PV with metal binding. Adapted from\textsuperscript{85}.

Since receptor 9 contains Zn\textsuperscript{2+} binding sites, which are assumed to mediate binding to anionic targets, we reasoned that the colour changes associated with PV to metal binding could be applied to our IDA. Briefly, PV in the unbound state would cause the solution to appear yellow to the naked eye with an absorbance peak at 450 nm (Figure 3.7). Upon the association of PV with the Zn\textsuperscript{2+}-cyclen of receptor 9, the solution would appear blue to the naked eye with a bathochromic shift in absorbance to 650 nm (Figure 3.7). We proposed that in the scheme of our PV-IDA the bound PV could be displaced from receptor 9 in the presence of an analyte that competes for binding to the receptor (Figure 3.8). This would cause a visible colour change in solution from blue to yellow and a hypsochromic shift in absorbance from 650 nm to 450 nm.
**Figure 3.7** Colour changes in PV induced by proposed interaction with receptor 9.

**Figure 3.8** Schematic of an IDA using PV, tripodal receptor and competitive anions, and the associated hypsochromic shift that should be observed in the absorbance of PV in the bound to unbound state upon displacement.
3.3 Indicator displacement assay: Results and discussion

From our IDA we first determined the affinity of PV to receptor 9. A constant concentration of PV (20 µM) was titrated with a varying amount of receptor 9 (0-200 µM) in 50 mM HEPES buffer, 5% DMSO pH 7.2 and an absorbance spectral scan (300-800 nm) of each concentration was taken (Figure 3.9) using a BioTek Cytation 3. The resultant data shows a bathochromic shift with an increase in absorbance at 650 nm and a concomitant decrease at 450 nm upon increasing receptor concentration. There was also a gradual visible colour change from yellow to blue. Both results are indicative of PV bound to receptor 9. The apparent dissociation constant ($K_d$) of PV to the tripodal receptor was determined using the Hill equation at 650 nm (Figure 6.2) and calculated to be 16.5 µM. From the fit of the curve and the Hill coefficient of 3.8 (Figure 3.9 Inset) it was apparent that the stoichiometry of the binding of the receptor to PV was not a not a simple 1:1 interaction. Thus, the results of these IDA studies were treated exclusively as a qualitative analysis.

![UV-Vis spectra](image)

**Figure 3.9** UV-Vis spectra of 20 µM PV titrated with receptor 9 (0-200 µM) in 50 mM HEPES buffer, 5% DMSO pH 7.2.

In order to identify the preferential analyte targets of receptor 9, the relative binding potencies of receptor 9 to various anions (sodium salts of adenosine triphosphate (ATP), adenosine monophosphate (AMP), pyrophosphate (PP$_i$), sulphate (SO$_4^{2-}$), phenyl phosphate (PhoP)) and phosphopeptides (Ac-ApYpYAA-NH$_2$ and Ac-ApYpYAA-NH$_2$) were determined using the PV-
IDA. 20 µM of PV and 20 µM of receptor were pre-incubated in 50 mM HEPES buffer, 5% DMSO pH 7.2. A five-fold excess (100 µM) of each anion or peptide were added to the mixture and compared to a standard blank containing only PV and receptor 9 for visible changes in colour (Figure 3.10).

![Image of the PV:receptor complex](image)

**Figure 3.10** Image of the PV:receptor 9 complex (20 µM each) after the addition of 100 µM of analyte representative of the displacement of PV from receptor 9 in the presence of various analytes in excess.

As can be seen from Figure 3.10, only ATP and PP

\[ PP_i \]

were able to induce a visible colour change from blue to yellow indicative of preferential binding of the receptor compared to other anions. All other analytes including the phosphorylated peptides (pYpY and pY) were incapable of displacing the PV indicator at 5-fold excess. We reasoned that preference for ATP and PP

\[ PP_i \]

over all other analytes was based on favourable interactions between these anions and receptor 9. The high negative on ATP from the triphosphate group may increase its affinity to the Zn\(^{2+}\) cyclen binding groups within receptor 9. The adenine ring and ribose sugar can also presumably make additional favourable interactions with the receptor scaffold,\(^{86}\) and perhaps increase the affinity of the anion. PP

\[ PP_i \]

also has a high negative charge; therefore the favourable interaction between the di-phosphate of PP

\[ PP_i \]

and the Zn\(^{2+}\) cyclen anchor groups on receptor 9 might explain its affinity and PV displacement. These observations were consistent with our hypothesis that receptor 9 could mediate preferential contacts with certain small molecule phosphoanions.

In order to determine the possible applications for receptor 9, we used the PV-IDA to probe the limit of detection for ATP and PP

\[ PP_i \]

compared to the other analytes tested in the first screen. We performed a screen of 20 µM of PV incubated with 20 µM receptor 9 against various concentrations of anions (100 to 400 µM) and the relative absorbance of the mixtures were determined. An absorbance max at 650 nm is representative of the PV: receptor 9 complex. Thus, the maximum intensity of the PV: receptor 9 divided by the observed absorbance resulting
from the addition of the anion was determined at 650 nm ($I_{\text{max}}/I_{\text{obs}}$) and depicted in Figure 3.11. It can be seen from Figure 3.11, that only ATP and $\text{PP}_i$ anions induce significant changes in $I_{\text{max}}/I_{\text{obs}}$ at excess analyte concentrations. Notably, no binding of receptor 9 to $\text{pY}$ and $\text{pYpY}$ was observed at concentrations up to 400 µM. Adenosine diphosphate (ADP) was also tested in this screen. From the results, ADP displayed slight disruption of the PV:receptor complex as compared to the other analytes but to a less extent than ATP and $\text{PP}_i$. Overall, this result further demonstrated that receptor 9 displays a clear preferential affinity for ATP and $\text{PP}_i$ and that there is potential its for future applications.

![Figure 3.11 Titration of PV:receptor 9 complex (20 µM each) with various concentrations of analytes. Absorbance was measured at 650 nm using the BioTek Cytation 3. $I_{\text{max}}$ is the maximum absorbance of the PV:receptor 9 complex in the absence of analyte; $I$ is the observed absorbance of the PV:receptor complex in the presence of analyte.](image)

We were encouraged by these results for a number of reasons. ATP and $\text{PP}_i$ are often off-target contaminants in receptor-based assays aimed at detecting protein phosphorylation. Since many receptor designs are primarily based on affinity to anionic phosphate, the presence of phosphate groups on ATP and $\text{PP}_i$ could compete for binding to the receptor and/or induce an off-target signal from a chemosensor. Thus, the ability of receptor 9 to preferentially bind ATP and $\text{PP}_i$ and
over pY or pYpY peptides could be of use in phosphorylation detection assays in order to preclude off-target detection.

3.3.1 Indicator displacement assay: Conclusions

The PV-IDA employed in this study provided a facile qualitative analysis of the efficacy of receptor 9 against biologically relevant anions. The results of the IDA experiments show that receptor 9 displays preferential binding to ATP and PPi anions from 100-400 µM. From the PV-IDA, none of the other anions and peptides tested were able to displace PV from receptor 9 at excess concentrations (100-400 µM). Thus, we identified ATP and PPi as the target phosphoanions to bind to receptor 9 and postulated that this preference was based on favourable interactions between the receptor and anion. Based on these results, we sought to test whether receptor 9 could selectivity bind to these phosphoanions over phosphopeptides in an established phosphopeptide detection assay.

3.4 Application of tripodal receptor in a ProxyPhos sensing assay

Since the PV-IDA assay indicated that receptor 9 displayed preferential binding to ATP and PPi anions over pY and pYpY peptides, we opted to test its ability to sequester off-target anions in a phosphopeptide detection assay. We hypothesized that in a mixed anion/phosphopeptide environment, receptor 9 could bind to ATP or PPi in preference to phosphopeptides. In the case of a chemosensor, we presumed that the presence of receptor 9 could minimize the off-target signal from the ATP or PPi anions without interfering with phosphopeptide detection. We decided to test this using the established in-solution ProxyPhos assay.

3.4.1 ProxyPhos sensing assay

The ProxyPhos sensing assay was developed by our group for the selective detection of proximally di- or poly-phosphorylated motifs on peptides and proteins. A turn-on fluorescence-based chemosensor was designed based on pyrene-mediated excimer emission. While this chemosensor has been taken forward in numerous applications (data not published), one issue with this excimer-based sensing approach has been the occurrence of an off-target signal from PPi (Figure 3.12). This could pose as a problem in some applications of ProxyPhos where contaminant concentrations of PPi could interfere with the signal for the intended proximally di-
phosphorylated motif target. Considering the preferential binding of receptor 9 to PP$_i$ over pYpY peptides we sought to test its efficacy with decreasing off-target detection within the ProxyPhos assay. We proposed that the addition of receptor 9 to the ProxyPhos assay could selectively sequester off-target PP$_i$ without interfering with the ProxyPhos signal for pYpY (Figure 3.12).

**Figure 3.12** Scheme of the selective sequestration of PP$_i$ by receptor 9 in a ProxyPhos assay.

### 3.4.2 Receptor 9 in a ProxyPhos Assay: Results and discussion

In order to probe our hypothesis, we first determined the relative signal of ProxyPhos in the presence of pYpY and/or PP$_i$ in the absence of receptor 9. A control experiment was conducted where 40 µM of the ProxyPhos chemosensor was incubated with either target pYpY peptide (10 µM), PP$_i$ (10 µM) or a mixture of pYpY and PP$_i$ (both at 10 µM) in 50 mM HEPES buffer, 5% DMSO, pH 7.5. The fluorescence emission of ProxyPhos was measured using TECAN Infinite M1000 using 350 nm excitation (5nm bandwidth) and 476 nm emission (20 nm bandwidth) and the results are presented in Figure 3.13. By analysing the signal from ProxyPhos without receptor 9 in Figure 3.13A (blue bars), it can be seen that PP$_i$ induces a greater ProxyPhos signal enhancement than the target pYpY peptide. The influence of PP$_i$ on ProxyPhos emission is further seen with the PP$_i$ and pYpY mixture where the signal from pYpY is over-estimated in the presence of PP$_i$. Therefore, PP$_i$ can induce a large off-target signal in the ProxyPhos assay. We next sought to measure the signal of ProxyPhos in response to analytes in the presence of receptor 9. 40 µM of the ProxyPhos chemosensor along with 40 µM receptor 9 was incubated either with the target pYpY peptide (10 µM), PP$_i$ (10 µM) or a mixture of pYpY and PP$_i$ (both at 10 µM) in 50 mM HEPES buffer, 5% DMSO, pH 7.5. The ProxyPhos emission was measured and presented in Figure 3.13 (red bars). The results of the signal from ProxyPhos in the presence of receptor 9 indicate that PP$_i$-induced signal enhancement is significantly decreased as
compared to the target pYpY peptide, which only decreases minimally (Figure 3.13A, red bars). The ProxyPhos signal is also decreased in the PP$_i$ and pYpY mixture and is not greatly affected by the presence of receptor 9 alone, without any analytes. These results confirmed that receptor 9 is capable of the selective sequestering of PP$_i$ in a ProxyPhos sensing assay with minimal interference to the ProxyPhos phosphopeptide detection signal.

Figure 3.13 The effect of receptor 9 on ProxyPhos excimer emission in the presence/absence of analytes. ProxyPhos (40 µM) signal in the presence of pYpY (10 µM), PP$_i$ (10 µM) and pYpY+PP$_i$ (10 µM each) without (blue bars) and with (red bars) 40 µM receptor 9.

3.5 Conclusions

We successfully identified ATP and PP$_i$ as the target phosphoanions for receptor 9 using a PV-based IDA. We took the results of this qualitative assay to test the application of receptor 9 to a ProxyPhos assay where PP$_i$ is a contaminant off-target analyte that is detected by ProxyPhos. Receptor 9 was able to sequester PP$_i$ and decrease ProxyPhos off-target signal without markedly affecting the signal for the target pYpY peptide. Overall, the work presented in this Chapter represents the successful repurposing of receptor 9 to target phosphoanions and its application in a ProxyPhos assay. We presume that future applications of this receptor scaffold could involve its use to sequester ATP or PP$_i$ within the in-solution ProxyPhos assay with protein samples.
3.6 Contributions

Eugenia Duodu designed and performed the experiments of the PV-IDA with receptor 9. The ProxyPhos experiment design and implementation were carried out by Eugenia Duodu, Dziyana Kraskouskaya and Mohammed Ali.

The author would like to acknowledge Dr. Drew Woolley for his assistance in optimizing and understanding the IDA.
Chapter 4

The majority of the work presented in this Chapter was published in the article listed below. Unpublished data is also presented.


4 Selective detection of tyrosine-containing proximally phosphorylated motifs using an antenna-free Tb$^{3+}$ luminescent sensor

The work presented in this Chapter exemplifies a pivot in our approach to the original aim of developing a method to selectively target proximally phosphorylated motifs over mono-phosphorylated motifs. As stated previously (Chapter 1 and 2), since the high affinity of chemical probes to proximally phosphorylated targets is often mediated by metal-based anionic phosphate interactions, there is minimal discrimination in the presence of certain phosphoanions. These off-target species can also presumably form high affinity interactions and decrease the effective concentration of the receptor or induce an off-target signal in chemosensors. Thus, the development of methods that can minimize these off-target signals is an attractive approach in the chemosensor field. To this end, we sought to develop a technique that incorporated a sensing unit to monitor the presence of the target phosphosite with minimal off-target signal from mono-phosphorylated motifs and other phosphoanions. In addition, the proposed chemosensor included a binding unit capable of forming favourable interactions with target proximally phosphorylated motifs in an aqueous environment. This Chapter presents the use of lanthanide luminescence to target a subset of proximally phosphorylated motifs.

4.1 Targeting proximally di-phosphorylated pY-containing peptide motifs

Protein phosphorylation on tyrosine residues is not a commonly occurring post-translational modification as compared to on serine and threonine residues. Thus, in the area of proximal phosphorylation, tyrosine-containing motifs are least common. However a variety of
perturbations associated with phosphotyrosine sites are commonly implicated in the onset and progression of many diseases. Proximally di-phosphorylated pY-containing motifs can be found in the activation loops of JAK2 (pYPY) and MAPK protein family members (pT-X-pY, T = threonine, X = any amino acid), whose aberrant activities are known contributors to cancer progression. Similar to JAK2, Syk kinase, whose deregulated activity is implicated in autoimmune diseases, requires phosphorylation on two neighbouring tyrosine residues for its activation. An in-depth analysis of a post-translational modifications database (PhosphoSitePlus®) presented in Chapter 1, (Figure 1.3 and Figure 1.4), highlights the large number of di-phosphorylated motifs present in the human phosphoproteome. A reanalysis of this subset of motifs suggests that over a 13,000 proximally di-phosphorylated pY-containing motifs are present in the human phosphoproteome (Table 4.1). Thus, it appears that these motifs have broad impact in intra-cellular signalling, and their detection could be of importance.
Table 4.1 The number of sites with proximally di-phosphorylated pY-containing motifs within the human phosphoproteome as filtered using the online database, PhosphoSitePlus®.

<table>
<thead>
<tr>
<th>Specific motif</th>
<th>Number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYpY</td>
<td>965</td>
</tr>
<tr>
<td>pTpY</td>
<td>725</td>
</tr>
<tr>
<td>pYpT</td>
<td>753</td>
</tr>
<tr>
<td>pSpY</td>
<td>1236</td>
</tr>
<tr>
<td>pYpS</td>
<td>1558</td>
</tr>
<tr>
<td>pYXpY</td>
<td>755</td>
</tr>
<tr>
<td>pTXpY</td>
<td>576</td>
</tr>
<tr>
<td>pYXpT</td>
<td>687</td>
</tr>
<tr>
<td>pSXpY</td>
<td>1285</td>
</tr>
<tr>
<td>pYXpS</td>
<td>1323</td>
</tr>
<tr>
<td>pYXXpY</td>
<td>789</td>
</tr>
<tr>
<td>pTXXpY</td>
<td>545</td>
</tr>
<tr>
<td>pYXXpT</td>
<td>503</td>
</tr>
<tr>
<td>pSXXpY</td>
<td>1144</td>
</tr>
<tr>
<td>pYXXpS</td>
<td>1061</td>
</tr>
</tbody>
</table>

4.2 Lanthanide based luminescent chemo-sensors

With the focus on targeting this specific subset of the phosphoproteome, we sought to employ a technique for the selective recognition of pY-containing proximally phosphorylated motifs over mono-phosphorylated motifs. We opted to use a metal centre as the key phosphate-binding site
based on the reasons outlined in Chapter 1. As stated, several luminescent chemosensors incorporating transition metals with vacant coordination sites have been employed in the detection of mono-phosphorylated peptide and protein sequences \(^{46,47,92,93}\) as well as the selective detection of proximally di-phosphorylated peptide and protein motifs over mono phosphorylated motifs.\(^{65,67,68,87}\) Although transition metal-based luminescent phosphoprotein sensors have demonstrated to be effective and versatile in their applications, they cannot be readily optimized to recognize among the specific residues that are phosphorylated, i.e. pY versus pS or pT. On the other hand, while the use of lanthanide-based sensors for the detection of phosphopeptides is less explored, several examples have shown that terbium (Tb\(^{3+}\))-based sensors are capable of the selective detection of pY-containing mono-phosphorylated peptides.\(^{94-100}\) This is due to the unique photophysical properties of lanthanides that give rise to their luminescence also making them excellent candidates for the development of selective chemosensors.

### 4.2.1 Lanthanide luminescence

Lanthanide luminescence holds key advantages over organic luminescent counterparts. In contrast to fluorescence, lanthanide luminescence has narrow emission bands (<10 nm at half max.), long luminescent lifetimes (\(\mu s\) to ms), large Stokes shifts (>150 nm \(c.f.\) fluorescein \(~25\) nm and rhodamine \(~20\) nm), and a relative insensitivity to photobleaching.\(^{101-104}\) The unique characteristics associated with lanthanide luminescence are attributed to their photophysical properties.

The luminescence properties of trivalent lanthanide ions, Ln\(^{3+}\), primarily arise from the f-f transitions in the 4f\(^n\) subshell of the [Xe]4f\(^n\) (\(n = 0 - 14\)) electronic configurations.\(^{101}\) First, these configurations give rise to well-defined energy levels generated from the shielding of the 4f orbitals by the filled 5s\(^2\)5p\(^6\) subshells. This shielding causes the 4f orbitals to have little participation in chemical bonding (5-7% covalency), resulting in sharp, narrow f-f emission bands from the minimal perturbation of the surrounding ligand.\(^{101}\) This narrow-band emission is advantageous as it allows for efficient spectral separation.\(^{101}\) Secondly, since the f-f transitions are parity ‘forbidden’ by the spin and Laporte rule, emission lifetimes are long in the \(\mu s\) to ms range in contrast to the ns average lifetimes of organic fluorophores.\(^{101}\) This allows for the use of time-gated or time resolved detection techniques that hold advantages in enhancing the signal-to-noise ratio by eliminating short-lived auto- and background-fluorescence interferences.\(^{105}\)
the emission lifetimes of lanthanide ions are long, the intrinsic luminescence is weak due to their low molar absorptivity ($\varepsilon = 0.1 - 1 \text{ M}^{-1} \text{ cm}^{-1}$), a consequence of the orbitally ‘forbidden’ intra-configurational f-f transitions.\textsuperscript{101,106,107} Thus, intense light sources, such as lasers, are required for significant luminescence enhancement, making the direct excitation of lanthanide ions impractical for numerous applications.\textsuperscript{101,106,108} Indirect sensitization of lanthanide centres via the interaction with an organic chromophore, often referred to as the \textit{antenna effect}, has emerged as a phenomenon to circumvent this limitation.\textsuperscript{101,105,108-110}

4.2.1.1 Antenna sensitization

A simplified scheme in Figure 4.1 describes the process of antenna-based luminescence sensitization of lanthanide ions. First, light is absorbed by the chromophore ‘antenna’ to its short-lived singlet excited state ($S_0 \rightarrow S_1$), which can then undergo intersystem crossing (ISC) to the longer-lived triplet state ($S_1 \rightarrow T_1$) (Figure 4.1). This ISC energy transfer process is induced by the lanthanide ion, which acts as an acceptor and increases spin-orbit coupling as a result of the heavy atom effect.\textsuperscript{111} The transfer of energy from the $T_1$ state of the antenna populates the lowest $^5D_J$ excited state of the lanthanide resulting in its sensitization (Figure 4.1). Radiative transitions from the $^5D_J$ excited state to the $^7D_J$ ground state of the lanthanide ion produce luminescent emission illustrated by a series of narrow bands spanning visible and near-IR wavelengths.\textsuperscript{108} More specifically, the Tb$^{3+}$ ion emits green light emission wavelengths.\textsuperscript{112}
Figure 4.1 A simplified scheme showing the sensitization of lanthanide luminescence via an antenna chromophore.

In the scope of the work presented here, two main mechanisms for the transfer of energy from the T₁ state of the antenna to the Ln³⁺ are described: the Dexter/exchange (Figure 4.2) and the Förster (Figure 4.3) energy transfer mechanisms. The Dexter/exchange mechanism is a double-electron transfer that occurs through orbital overlap with energy transfer efficiency proportional to 1/eʳ, where r is the distance between the Ln³⁺ and the antenna. The Förster or dipole-dipole mechanism involves the coupling of the dipole moments of the T₁ state of the antenna and the 4f orbitals of the Ln³⁺ with the efficiency of transfer being 1/r⁶.
The additional advantages of lanthanide luminescence arise from this described sensitization process via the antenna effect. Although lanthanides display negligible Stokes shifts, antenna-based sensitization produces pseudo-Stoke’s shifts that are larger than organic fluorophores. These pseudo-Stoke’s shifts allow for the ease of spectral discrimination, as the absorption
spectrum is that of the antenna rather than the lanthanide itself and the excitation spectrum occurs at wavelengths greater than 500 nm. Additionally, since the lanthanide ion acts as a good quencher of T\textsubscript{1} states of the antenna chromophore, Ln\textsuperscript{3+} complexes are relatively insensitive to photobleaching allowing for prolonged detection.\textsuperscript{108}

The simplified model for sensitization generates the following expressions to describe the total quantum yield, $\Phi_{Tot}$ and brightness, $B$ for the lanthanide complex\textsuperscript{105}:

$$\Phi_{Tot} = \Phi_{Ln}\eta_{ISC}\eta_{ET} \quad \text{Equation 4.1}$$

$$B = \varepsilon_{Ant}\Phi_{Tot} \quad \text{Equation 4.2}$$

In $\Phi_{Tot} = \Phi_{Ln}\eta_{ISC}\eta_{ET}$ Equation 4.1, the total quantum yield is expressed as the intrinsic quantum yield of the lanthanide ion ($\Phi_{Ln}$) and the overall sensitization efficiency which is defined as the product of the efficiency of ISC between the antenna excited states ($\eta_{ISC}$) and the T\textsubscript{1} $\rightarrow$ $5\text{D}_J$ energy transfer ($\eta_{ET}$). Thus, the emission intensity or brightness in $B = \varepsilon_{Ant}\Phi_{Tot}$ Equation 4.2 is the product of the molar extinction coefficient of the antenna ($\varepsilon_{Ant}$) and the total quantum yield. The intricate process of sensitization lends to the meticulous development of lanthanide chemosensors that maximize these terms by the choosing the appropriate antennas. Firstly there must be a small S\textsubscript{1}$\rightarrow$T\textsubscript{1} energy gap for efficient ISC with ISC being favoured over the competing radiative (antenna fluorescence) and non-radiative relaxation back to the ground state, S\textsubscript{0} (Figure 4.1). This contributes to an increased efficiency of ISC, $\eta_{ISC}$. Secondly, the energy gap of the T\textsubscript{1} state for the antenna must be slightly higher than the $5\text{D}_J$ state of the lanthanide for efficiency of energy transfer, $\eta_{ET}$. This energy transfer must be favoured over T\textsubscript{1}$\rightarrow$S\textsubscript{0} phosphorescence and non-radiative dissipation. The $\eta_{ET}$ can be further increased with an appropriate antenna to lanthanide distance for energy transfer as per the requirements of the Dexter and Förster energy transfer mechanisms (Figure 4.2 and Figure 4.3).\textsuperscript{105}

### 4.2.2 Terbium luminescence sensitization using phosphotyrosine

As previously mentioned, several studies have been reported that demonstrate the use of Tb\textsuperscript{3+-} based sensors for the selective detection of pY-containing mono-phosphorylated peptides.\textsuperscript{94,100,113} For example, Sames et al. demonstrated the use of neighbouring group-assisted Tb\textsuperscript{3+} binding with an iminodiacetate group in a synthetic mono-phosphorylated peptide-sensor (Figure
Terbium and europium analogues of this sensor were applied to monitor the activity of enzymatic phosphorylation/dephosphorylation. In another study, a Tb\(^{3+}\)-DOTA-M(2,2',2''- (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayltetraacetamide) complex was used as a turn-on luminescence sensor to detect the highly negatively charged phosphorylated mono-phosphorylated peptide, Ac-EEIpYEEFD-NH\(_2\) over its non-phosphorylated counterpart (Figure 4.4B). The same group followed up their initial findings with the use of a di-Tb\(^{3+}\)-DOTA-M complex to target to the same mono-phosphorylated nonapeptide and applied the sensor to monitor the phosphorylation of the peptide in an enzymatic assay (Figure 4.4C). A different study reported the use of unchelated Tb\(^{3+}\) in detecting the negatively charged mono-phosphorylated peptide, Ac-GGDEEdYEEPDEPGK\(_6\)GG-NH\(_2\) over its mono-phosphorylated counterpart, which was also applied in monitoring enzymatic activity (Figure 4.4D).
Figure 4.4 Summary of studies using turn-on Tb$^{3+}$-based sensors for the detection of pY-containing mono-phosphorylated peptides.

The signal selectivity of Tb$^{3+}$ for phosphotyrosine, pY, arises from the luminescence sensitization of the Tb$^{3+}$ ion by the tyrosine antenna. As a Lewis acid, Tb$^{3+}$ can bind to negatively charged phosphate groups found on pY, pS and pT residues of phosphopeptides. Upon binding to pS- and pT-containing peptides, no significant luminescence enhancement
occurs. On the other hand, the phenyl ring of the bound pY can act as an antenna and efficiently transfer its excitation energy to the Tb$^{3+}$ centre inducing “luminescence sensitization” of Tb$^{3+}$ (Figure 4.5). This leads to an enhanced, long-lived lanthanide luminescence at well-defined wavelengths.

**Figure 4.5** A schematic representation of a Tb$^{3+}$ sensor proposed mode of action with di-phosphorylated peptide motifs via pY induced antenna sensitization.

Based on previous studies, we sought to employ Tb$^{3+}$ luminescence sensitization via phosphotyrosine. Considering the importance of pY signalling pathways described in Chapter 1, we sought to determine if Tb$^{3+}$ luminescence could be applied for the detection of proximally di-phosphorylated peptides containing at least one pY residue. We hypothesized that this could be achieved through the simultaneous association of a single Tb$^{3+}$ ion with more than one phosphate group found on neighbouring residues, which we rationalized could lead to a more stable complex as compared to that of a mono-phosphorylated site (Figure 4.5) in similar manner to the neighbouring group-assisted Tb$^{3+}$ binding effect reported by Sames et al. (Figure 4.4D).
4.3 Tb$^{3+}$ luminescence for the detection of proximally di-phosphorylated pY-containing peptide motifs: Results and discussion

4.3.1 Proof of concept

In order to test our hypothesis, we monitored Tb$^{3+}$ luminescence in the presence of pY-containing proximally di-phosphorylated peptides. Thus, we measured the change in Tb$^{3+}$ luminescence in response to target alanine-based peptides containing pYpY or pTApY (full sequences in Section 6.3) on a TECAN Infinite M1000 using time-resolved detection settings (60 $\mu$s delay time and 1.5 ms integration time). Varied concentrations of peptides (0.244 - 500 $\mu$M) were titrated into 50 $\mu$M of TbCl$_3$ in an aqueous buffer (50 mM HEPES, 50 mM NaCl, pH 7.5) and following incubation the luminescence intensity (450-650 nm, 2 nm steps) was measured upon irradiation at 263 nm. As expected, luminescence enhancement at Tb$^{3+}$-specific wavelengths was directly correlated with the concentration of target peptides in solution (Figure 4.6 A and B). The apparent dissociation constants of the peptides to Tb$^{3+}$ were found to be approximately 20 $\mu$M (Figure 4.6C and D) with a 4 $\mu$M detection limit (Figure 4.7). It was surprising that pTApY peptide exhibited greater luminescence enhancement than pYpY peptide considering that the pYpY peptide possesses a second antenna group. The significant signal enhancement in response to pTApY suggested that of the two neighbouring phosphorylated residues, only one is required to bear an antenna. Additionally, since the relative spacing of phosphorylated residues is different between the two peptides, it could be suggested that the specific orientation of phosphates in relation to one another contributes to the luminescence enhancement of Tb$^{3+}$. 
Figure 4.6 Luminescence intensity of Tb$^{3+}$ (50 µM) in the presence of A) pTApY peptide and B) pYpY peptides (500-0.244 µM). The titration data for the luminescence enhancement of Tb$^{3+}$ (50 µM) for C) pTApY and D) pYpY peptides (500-0.244 µM) at 544 nm (20 nm bandwidth) fit to the Hill equation using Origin Software for to derive apparent dissociation constants (displayed at the bottom of each graph in µM). Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.
Figure 4.7 Detection limit of Tb$^{3+}$ with pY, pYPY and pTApY peptides. Luminescence intensity spectra of 50 µM of TbCl$_3$ titrated with 0.244 – 500 µM peptides at 544 nm (20 nm bandwidth, 60 µs delay time, 1.5 ms integration time). Dashed lines indicate the Tb$^{3+}$ ion limit of detection for target peptides, pTApY and pYPY as compared to the off-target mono-phosphorylated pY. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

We hypothesized that the mode of binding of the Tb$^{3+}$ and the di-phosphorylated peptide would be a neighbouring group assisted interaction (Figure 4.5). In order to investigate the binding interaction of the peptide target to Tb$^{3+}$ a Job’s Plot analysis was performed by measuring the luminescence enhancement of 100 µM of TbCl$_3$ in the presence of 100 µM of pTApY peptide and the luminescence intensity at 544 nm was measured upon irradiation at 263 nm. The binding of Tb$^{3+}$ to pTApY peptide was shown to be 1:1 (Figure 4.8A), suggesting that the observed signal enhancement might require association of Tb$^{3+}$ with both phosphate groups simultaneously as previously proposed. A noted advantage of lanthanide luminescence-based sensors has been stability to photobleaching. In order to test if this sensing system could sustain prolonged detection, an experiment was performed where Tb$^{3+}$ (50 µM) was incubated with target peptides (50 µM) and the luminescence intensity at 544 nm was measured continuously upon irradiation at 263 nm in 5-minute intervals over 90 minutes. The results show the sensing system to be stable to photobleaching and amenable to prolonged detection techniques (Figure
4.8B). Encouraged by these results, we proceeded with more peptide experiments to test the efficacy of the proposed sensing system.

![Figure 4.8](image)

**Figure 4.8** A) Job plot of Tb$^{3+}$ (100 µM) with pTApY peptide (100 µM), luminescence intensity at 544 nm (20 nm bandwidth) upon irradiation at 263 nm B) Luminescence intensity of Tb$^{3+}$ (50 µM) in the presence of target peptides (50 µM) at 544 nm (20 nm bandwidth) measured continuously upon irradiation at 263 nm in 5 minute intervals over 90 minutes. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

We next sought to test our hypothesis that di-phosphorylated motifs would be preferentially targeted over mono-phosphorylated ones based on our proposed mode of action. To explore the observed Tb$^{3+}$ signal selectivity for the target pY-containing proximally di-phosphorylated motifs, a screen against variably phosphorylated short peptides was performed under time resolved settings (Figure 4.9). As hypothesized, Tb$^{3+}$ displayed significant signal selectivity for a peptide proximally di-phosphorylated on tyrosine residues over its mono-phosphorylated counterpart of identical primary amino acid sequence (Ac-AYYAA-NH$_2$). This supported the notion that *di*-phosphorylation of peptide motifs was necessary for significant luminescence enhancement and that the dual interaction was necessary for signal selectivity. However, as the pTAY peptide did not induce significant signal enhancement, the results indicated that direct phosphorylation on the tyrosine residue antenna may be required for luminescence sensitization. The increased luminescence sensitization presumably as a result of the interaction of the
phosphate group bearing the phenyl ring antenna with the Tb$^{3+}$ is in agreement with previous works highlighting that sensitization efficiency ($\eta_{ET}$) can be improved by using an antenna that can directly coordinate to the metal centre.\textsuperscript{116} Such coordinating antennae can undergo both Förster and Dexter energy transfer mechanisms via a decreased $r$ term and increased orbital overlap.\textsuperscript{110} Additionally, a peptide proximally di-phosphorylated on serine residues (pSpS; lacking an antenna) did not induce significant Tb$^{3+}$ luminescence enhancement. This result further confirmed that sensitized luminescence arose from the coordinating phenyl ring of the phosphotyrosine residue. Consistent with previous studies,\textsuperscript{94,95,98} we observed no Tb$^{3+}$ luminescence enhancement in response to some biologically relevant phosphoanions including ATP, AMP, and PP$_I$ (Figure 4.9). This sensing selectivity likely arose from the inability of adenine base, present in ATP and AMP, to sensitize Tb$^{3+}$ and the absence of a sensitizing antenna in PP$_I$. We were encouraged by this result as both ATP and PP$_I$ pose as a major interfering off-targets for other sensing techniques as they are highly abundant in biological systems\textsuperscript{1} and possess a similar anionic phosphate charge density as di-phosphorylated targets. This result highlights another major advantage of lanthanide luminescence in the selective sensing of our di-phosphorylated targets.

\textbf{Figure 4.9} Change in luminescence intensity of 100 µM of Tb$^{3+}$ in the presence of various analytes (100 µM) at 544 nm (20 nm bandwidth) upon irradiation at 263 nm. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired
using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

This preliminary screen further supported the proposed mode of interaction and source of signal selectivity. We next sought to probe the signal selectivity amongst differently spaced proximally di-phosphorylated motifs separated by a number of amino acids residues. We also wanted to probe the spatial requirements of the proximal phosphorylated groups within target peptide motifs that could result in enhanced luminescence intensity. We chose to further probe the signal selectivity of Tb$^{3+}$ with di-phosphorylated peptides variably spaced with alanine residues by monitoring the change in luminescence intensity of 100 µM of TbCl$_3$ in the presence of variably spaced peptides (100 µM) (Figure 4.10). While the precise secondary structure of these peptides in solution was not predicted, there were significant differences in luminescence enhancement in relation to the spacing of the pY residues. From the screen it seemed as though the most significant luminescence enhancement arose from the pTApY peptide. As the number of alanine residues in between the pY residues increased, the luminescence intensity signal decreased. We rationalized that the orientation of the two flanking phosphate residues could contribute to the luminescence sensitization of the Tb$^{3+}$. Additionally, the pYpY peptide generated the least luminescence enhancement as compared to the other peptides, which further indicated from this study that the most optimal distance between phosphate residues is one amino acid. This result further confirmed our proposed mode of binding of Tb$^{3+}$ and outlined that there are specific spatial requirements between neighbouring phosphorylated residues in order to achieve maximum luminescence enhancement.
Figure 4.10 The effect of variably spaced di-phosphorylated peptide motifs on Tb$^{3+}$ luminescence enhancement. Change in luminescence intensity of 100 µM of Tb$^{3+}$ in the presence of variably spaced peptides (100 µM) at 544 nm (20 nm bandwidth) upon irradiation at 263 nm. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

From the screen in Figure 4.9, Tb$^{3+}$ in the presence of pSpS peptide does not produce a signal. Although luminescence enhancement was not observed, Tb$^{3+}$ was expected to bind the pSpS phosphorylated site, which is a highly abundant di-phosphorylated motif within cells. Thus, one would expect that the signal from the target peptide, pYpY/pTApY, would be reduced in the presence of pSpS due to the decrease in the amount of Tb$^{3+}$ available for binding the target. Thus, a competition experiment was performed in order to further probe the signal selectivity of Tb$^{3+}$ for target pY- containing di-phosphorylated peptides. Variable concentrations of TbCl$_3$ were added to peptide solutions (100 µM of pTApY or pYpY in the presence of 200 µM of pSpS). In order to quantify the effect of competing off-target species on the Tb$^{3+}$ luminescence signal, we calculated the ratios of Δ luminescence intensity for pTApY or pYpY alone and in the presence of the pSpS competing peptide (Figure 4.11; ratios are expressed as percentages on the graph). As expected, the signal enhancement for both target peptides (pYpY and pTApY) was greatly decreased in the presence of pSpS peptide. However, the signal for the target peptides
was readily recovered by the addition of excess Tb$^{3+}$ (Figure 4.11). Successful detection of the target motif in the presence of an off-target species competing for Tb$^{3+}$ binding was encouraging, given the higher relative abundance of phosphorylation on S and T residues within a cellular environment.³

**Figure 4.11** Change in luminescence intensity of varying concentrations of Tb$^{3+}$ with 100 µM pTApY or pYpY target peptides in the absence of pSpS peptide (red bars) and the presence of pSpS peptide (200 µM, blue bars) at 544 nm (20 nm bandwidth) upon irradiation at 263 nm. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

To assess if other detection techniques were compatible with this sensory approach, we repeated the screens described in (Figure 4.6 and Figure 4.9) and generated images using a short-wave UV-lamp (Figure 4.12). As expected, the selectivity trend among the analytes tested was comparable for both detection techniques as only the target peptides were able to induce a signal (Figure 4.12A). The visible detection limit for both peptides obtained using a UV-lamp was 30 µM (Figure 4.12B). Although this was inferior to that obtained on the microplate reader (4 µM, Figure 4.7), the compatibility of this system with a conventional UV-lamp extends the application of this approach beyond microplate analysis.
4.3.2 Tb\(^{3+}\) luminescence signal selectivity: Protein studies

While the results with short-peptides were very encouraging, as a next step we wanted to assess the efficacy of Tb\(^{3+}\) in sensing target pY-containing di-phosphorylated motifs on full size proteins. However, in the search for the appropriate control proteins, we found that the majority of commercially available pY-containing di-phosphorylated motif proteins were very expensive and could not be used in these studies. Additionally, the degree of site-specific phosphorylation is not provided by the commercial sources supplying these proteins. Thus, we chose to take a different approach to testing with full-size proteins. Since the results of the peptide-based competition experiment previously described demonstrated that sensing of the target motifs could be retained in the presence of off-target peptides which compete for binding to Tb\(^{3+}\) (Figure 4.11), we opted to test if the sensing of target motifs could be retained in the presence of full size unphosphorylated or variably phosphorylated proteins. Tb\(^{3+}\) (50 µM) was added to a mixture of 40 µM of target peptides (pTApY and pYPY) incubated with or without 40 µM of a protein (BSA, lysozyme, ovalbumin, D-α-casein, or β-casein). We expected that Tb\(^{3+}\) would display a decreased luminescence enhancement to the target peptide in the presence of competing proteins. Analogous to the peptide competition experiment, the detection signal from Tb\(^{3+}\) luminescence decreased in the presence of a competing protein (Figure 4.13A). This was
expected as Tb\(^{3+}\) likely interacts with the proteins’ surface, particularly with acidic residues. In most cases, increasing the concentration of Tb\(^{3+}\) from 50 to 200 µM resulted in a significant recovery in the detection signal (Figure 4.13B). Interestingly, D-\(\alpha\)-casein induced significant Tb\(^{3+}\) luminescence in the absence of target peptides. This was unexpected as D- \(\alpha\)-casein is partially dephosphorylated and contains two pS residues.\(^9\) Thus the observation could be a result of Tb\(^{3+}\) binding to a site on the protein proximal to an appropriate amino acid antenna (tyrosine or tryptophan), inducing its luminescence sensitization.\(^1\)\(^1\) The observed off-target signal is not likely a result of Tb\(^{3+}\) binding to either of the two phosphorylated serine residues on D-\(\alpha\)-casein,\(^9\) since the signal induced by incubation with the ovalbumin protein, which also bears two phosphorylated serine residues,\(^3\) was minimal. In addition, the recovery of signal towards target peptide was not as efficient in the presence of \(\beta\)-casein and BSA as compared to other proteins tested (Figure 4.13B). This is likely due to the presence of five phosphorylated serine residues on \(\beta\)-casein\(^9\) and a metal binding site potentially present on BSA.\(^1\)\(^1\) On the other hand, incubating Tb\(^{3+}\) with the highly positively charged non-phosphorylated protein lysozyme alone, which was not expected to significantly interact with Tb\(^{3+}\), did not induce significant luminescence enhancement. Among the proteins tested, lysozyme interfered the least with the Tb\(^{3+}\) luminescence originating from the interaction with the target peptides (Figure 4.13 A and B). Therefore we demonstrated that at appropriate concentrations of Tb\(^{3+}\), target di-phosphorylated pY-containing peptides could be detected in the presence of proteins.
**Figure 4.13** A) Change in luminescence intensity of TbCl$_3$ (50 µM) in the presence and absence of different analyte mixtures. B) Change in luminescence intensity of TbCl$_3$ (200 µM) in the presence and absence of different analyte mixtures at 544 nm (20 nm bandwidth) upon irradiation at 263 nm. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

A noted advantage of Tb$^{3+}$ luminescence sensing in the detection of pY-containing di-phosphorylated motifs is the minimization of signal from major off-target anions, such as ATP and PP$_i$, that plague many methods using chemosensors by interfering with the detection of the analyte of interest. However, another potential off-target anionic species could be highly negatively charged mono-phosphorylated pY peptide motifs that contain acidic aspartic acid (D) and glutamic acid (E) residues. While phosphate has a larger charge density than E or D residues, two or more of these amino acids proximal to one another can mimic a phosphorylated residue and can therefore interfere with the detection of target phosphosites. To monitor this effect, we tested Tb$^{3+}$ (50 µM) against increasing concentrations of negatively charged mono-phosphorylated pY peptides along with positive (pTApY) and negative (pY) control peptides (0.244-500 µM) in 50 mM HEPES buffer, 50 mM NaCl, pH 7.5. The results in Figure 4.14 show that the variably charged mono-phosphorylated peptides induce significant luminescence...
enhancement of Tb\(^{3+}\). In line with our predications, the most negatively charged peptide, EEDQDpYD, induced the highest signal enhancement among all peptides tested. The positive control pTApY peptide had a lower signal than the DADpYDLS peptide, but a greater signal than the QDpYDSL peptide. These results were consistent with previous reports the use of Tb\(^{3+}\) in detecting highly negatively charged mono-phosphorylated peptide motifs in monitoring kinase activity.\(^{99,100}\)

**Figure 4.14** The effect of negatively charged peptides on Tb\(^{3+}\) luminescence. Luminescence intensity of 50 µM of TbCl\(_3\) titrated with 0.244 - 500 µM peptides at 544 nm (20 nm bandwidth) upon irradiation at 263 nm. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

We hypothesized that varying the pH and ionic strength of the buffer might decrease the off-target peptide signal while retaining signal for the target peptide motif. To test this, the pH was varied in 50 mM HEPES (between pH 6.5 and 7.5) and in 50 mM NaOAc (between pH 5.5 and 4.5) buffers. The peptides indicated above (125 µM) were then incubated with Tb\(^{3+}\) (50 µM) and the resultant luminescence intensity at 544 nm was recorded (Figure 4.15). As the pH decreased there was an overall decrease in luminescence intensity from the DADpYDLS and EEDQDpYD peptides. With the first and second ionization of the phosphate monoester being 2.2 and 7.2, at pH 6.5 a lower fraction of double negatively charged phosphates would be present. Thus, this
trend was indicative of a weaker Tb$^{3+}$ to phosphate interaction as a result of the overall lower fraction of negatively charged residues being present at lower pH. For pTApY and QDpYDLS peptides the resultant luminescence intensity did not decrease significantly going from pH 7.5 to 6.5 but decreased intensity was observed at the lower pH values 4.5 and 5.5 (Figure 4.15). From this, we reasoned that pH 6.5 was best to minimize the off-target signal while maintaining the signal for the target pTApY peptide.

**Figure 4.15** The effect of pH in the Tb$^{3+}$ sensing of negatively charged peptides. Luminescence intensity of 50 µM of TbCl$_3$ with 125 µM peptides at different pH values was measured at 544 nm (20 nm bandwidth) upon irradiation at 263 nm. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

We next sought to test the variation of ionic strength on minimizing off-target detection. The same peptides (125 µM) were incubated with Tb$^{3+}$ (50 µM) in 50 mM HEPES buffer at pH 7.5, NaCl (0-1000 mM). The resultant luminescence intensity at 544 nm was recorded (Figure 4.16). The overall trend observed with increasing salt concentration was a decrease in luminescence enhancement from the peptides (Figure 4.16). This was expected as increased salt concentrations increase the polarity of the solvent and can attenuate electrostatic interactions, thus weakening Tb$^{3+}$ to phosphate interactions. However, the salt concentration screen at pH 6.5 affected
peptides to a lesser extent than at pH 7.5 (Figure 4.17). In both cases, the minimization of off-target signals did not result in a greater selectivity for the target pTApY peptide. While our preliminary optimization studies show some promise, it is clear that off-target signals from negatively charged mono-phosphorylated pY peptide motifs pose as a challenge to be addressed in future work.

**Figure 4.16** The effect of ionic strength in the Tb$^{3+}$ sensing of negatively charged peptides at pH 7.5. Luminescence intensity of 50 µM of TbCl$_3$ with 125 µM peptides at increasing NaCl concentrations (0-1000 mM) was detected at 544 nm (20 nm bandwidth) upon irradiation at 263 nm. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.
Figure 4.17 The effect of ionic strength in the Tb\(^{3+}\) sensing of negatively charged peptides at pH 6.5. Luminescence intensity of 50 µM of TbCl\(_3\) with 125 µM peptides at increasing NaCl concentrations (0-1000 mM) was detected at 544 nm (20 nm bandwidth) upon irradiation at 263 nm. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

4.4 Tb\(^{3+}\) chelate experiments

While the use of “free” Tb\(^{3+}\) for the detection of proximally di-phosphorylated peptide motifs is a simple and straightforward approach, the use of the un-chelated lanthanide ion has its weaknesses. For any future application to biological systems, lanthanides are typically chelated with multi-dentate ligands to diminish the toxicity of free lanthanide ions.\(^{118,119}\) For luminescent applications chelation can also reduce sensitivity to competing off-target anions and can protect the metal from solvent coordination-induced quenching.\(^{120}\) While Tb\(^{3+}\) and Eu\(^{3+}\) are known to be less sensitive to signal quenching by solvent,\(^{118}\) the coordination of water molecules to Ln\(^{3+}\) ions can shorten the Ln\(^{3+}\) ion excited state lifetime through non-radiative vibrational energy transfer to the high-frequency O-H oscillator (Figure 4.18).\(^{101,107}\) Chelates can minimize the number of water molecules coordinated to Ln\(^{3+}\) centre through increased steric bulk and coordinative saturation which in turn would attenuate solvent quenching and maximize the \(\Phi_{\text{Ln}}\) term, increasing luminescence emission.\(^{117}\) Given the entropy-driven complexation of Ln\(^{3+}\) in water,
the large hydration enthalpy of Ln\(^{3+}\) and their hard acid nature, multi-dentate ligands that incorporate hard bases such as amides, carboxylic acids and pyridines are commonly employed.\(^{105,106}\) Macrocycles such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and its derivatives as well as open diethylene triamine pentaacetic acid (DTPA) derivatives are commonly used as lanthanide chelates.\(^{105}\)

**Figure 4.18** General scheme for luminescence quenching by coordinated water molecule through vibrational energy transfer to the O-H oscillator.

We chose to test the efficacy of Tb\(^{3+}\) sensing against target peptides within chelates complexes based on the advantages of using chelated Tb\(^{3+}\) compared with unchelated ions. As a first step we used DOTA and DTPA chelates (each 50 µM) and incubated them with increasing concentrations of pTApY and pY peptides (0.244-500 µM) with the resultant luminescence intensity recorded at 544 nm (Figure 4.19). We hypothesized that the luminescence intensity of the chelated complex would be significantly lower than that of the free Tb\(^{3+}\) as the chosen
chelates decrease available sites for phosphate binding. Consistent with our expectations, the DOTA and DTPA chelates abolished target pTApY peptide luminescence enhancement compared to the free Tb$^{3+}$ (Figure 4.19). This result indicated that the appropriate ligand must be chosen with a fine balance between binding the lanthanide with high affinity to reduce solvent quenching but also leaving an available site for binding to the target peptide motifs. Future work is focused on identifying and testing Tb$^{3+}$ chelates more appropriate for pTApY sensing based on these findings.

**Figure 4.19** Luminescence intensity of 50 µM of free TbCl$_3$ and chelated complexes Tb(DOTA) and Tb(DTPA) titrated with 0.244-500 µM peptides was recorded at 544 nm (20 nm bandwidth) upon irradiation at 263 nm. Experiments were performed in 50 mM HEPES, 50 mM NaCl, pH 7.5 and luminescence spectra were acquired using time resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN Infinite M1000.

### 4.5 Conclusions

We have demonstrated that Tb$^{3+}$ luminescence can successfully be employed in the sensing of pY-containing proximally di-phosphorylated peptide motifs with phosphotyrosine as a sensitizing antenna. Unlike many other sensors designed for di-phosphorylated motifs, Tb$^{3+}$ luminescence holds advantages in minimal signal interference from major off-targets such as ATP and PP$_1$. We have also demonstrated that at the appropriate concentrations, Tb$^{3+}$ displays
selective luminescence enhancement for target peptides in the presence of pSpS competing peptide and protein off-targets. This facile approach towards the detection of an important subset of phosphorylated motifs warrants further exploration and could be of potential value to the phosphopeptide/phosphoprotein detection field. This work represents the successful development of a technique that incorporated a sensing unit to monitor the presence of a target proximally phosphorylated motif with minimal off-target signal from mono-phosphorylated motifs and other phosphoanions.

4.6 Contributions

All of the work that was presented in this chapter was done by the contributing authors on the publication: Duodu, E., Kraskouskaya, D., Campbell, J., Graca-Lima, G., & Gunning, P. T. (2015). Selective detection of tyrosine-containing proximally phosphorylated motifs using an antenna-free Tb$^{3+}$ luminescent sensor. *Chemical Communications, 51*(30), 6675–6677.

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5 Conclusions and future directions

Protein phosphorylation is a fundamentally important post-translational modification in several cellular processes central to controlling a protein's activity status and function. The abnormal functioning of this process is a key hallmark in disease onset and progression. Therefore, a significant amount of work has been focused in the area of targeting protein phosphorylation. Similarly, the phosphorylation of proximal residues has emerged as a functionally relevant motif that has been implicated in certain disease states. Despite this, not much work has been done in developing methods to target these specific phosphorylated motifs. Thus, the aim of this thesis has been to develop methods for the selective recognition of proximally phosphorylated motifs on proteins and peptides.

Chapter 2 described the application of a two-point interaction approach to target proximally phosphorylated motifs on peptides. A metal-based triethylbenzene scaffold receptor was proposed to preferentially target select proximally phosphorylated motifs over mono-phosphorylated motifs on peptides based on a favourable two-point interaction between Zn$^{2+}$ cyclen binding units and proximal phosphate groups. The synthesis and testing of the receptor outlined the efficacy of the approach. Unfortunately, the FI results indicated that the receptor scaffold did not show preferential selectivity in affinity amongst the phosphorylated peptides tested. Furthermore, the results from the ITC experiments illustrated the lack of selectivity in affinity between the pY and pYpY peptides and indicated that the receptor to peptide interaction was relatively weak. Overall, the results of the biophysical assays used, concluded that the metal-based triethylbenzene receptor scaffold was likely not suitable for selectively targeting proximally phosphorylated peptide motifs over mono-phosphorylated ones.

Chapter 3 sought to utilize the synthesized receptor scaffold described in Chapter 2 in the application of targeting phosphate-containing small molecule anions. A PV-IDA was used to determine the preferential phosphoanions that bound a putative “anionic cavity” in this receptor, in which ATP and PP$_i$ were identified as targets. Interestingly enough, the proximally
phosphorylated pYPY peptide did not demonstrate binding to our receptor, which reaffirmed the results obtained in Chapter 2. The results of the PV-IDA led to the application of this receptor scaffold to a ProxyPhos assay that was originally designed to detect proximally phosphorylated motifs but suffered from off-target signal activity arising from contaminant PP$_i$. The metal-based triethylbenzene receptor from Chapter 2 was able to selectively target and potentially sequester PP$_i$ in solution leading to a decrease in the off-target signal shown by ProxyPhos without significantly affecting its signal for the target pYPY peptide. The major significance of this work was the repurposing of this receptor scaffold in the recognition of a different target (PP$_i$) and its subsequent application in a mixed anion assay. We presume that future applications of this receptor scaffold could be in the binding of contaminant phosphoanions in conjunction with receptor-based assays targeting protein phosphorylation in order to minimize off-target signals that could confound the data obtained from these sensing assays.

Chapter 4 represents a return to our original aim, the selective recognition of proximally phosphorylated motifs. The findings of Chapter 2 initiated a deviation from our initial approach. We sought to develop a facile sensor-based technique to target proximally phosphorylated motifs in an aqueous environment. In Chapter 4, we demonstrated the successful use of Tb$^{3+}$ luminescence in the sensing of pY-containing proximally di-phosphorylated peptide motifs with phosphotyrosine serving as a sensitizing antenna. This approach demonstrated signal selectivity for di-phosphorylated motifs over mono-phosphorylated counterparts, as well as displayed minimal signal interference from major off-target phosphoanionic species such as ATP and PP$_i$. Furthermore, at the appropriate concentrations, Tb$^{3+}$ displayed selective luminescence enhancement for target peptides in the presence of competing pSpS peptide and competing off-target proteins. The results arising from Tb$^{3+}$ luminescence experiments with highly negatively charged peptides and Tb$^{3+}$ chelate complexes provided a focus for future assay and chelate development. Overall, the shift from the two-point interaction strategy presented in Chapter 2 to the use of Tb$^{3+}$ luminescence represents a change in the detecting strategy employed by our group without changing our ultimate goal. We presume that the work presented in this Chapter will provide a foundation for future work in understanding different subsets of the phosphoproteome and could be of potential value to the phosphopeptide/phosphoprotein detection field.
In particular, recent work published by Wang and co-workers reaffirms our technique in using Tb$^{3+}$ luminescence for the detection of proximally phosphorylated motifs.\textsuperscript{121} Specifically, a Tb$^{3+}$-Zn$^{2+}$ complex was used as a chemosensor for proximally phosphorylated pS peptides using time resolved settings. Two 8-aminoquinoline groups were used as sensitizing antennas and the mechanism of turn-on luminescence was proposed by the authors to occur through a photo-induced electron transfer (PET) quenching effect (Figure 5.1). Binding to proximally phosphorylated motifs was achieved through Zn$^{2+}$ sites and this modulated a luminescence signal enhancement from Tb$^{3+}$. The chemosensor displayed a detection limit of 62 nM and selectivity for the pSpS peptide sequence of a tau protein (TPKSPpSpS) over more proximally spaced sequences (TPPKpSpSS and TPPKpSPSpS) and a mono phosphorylated sequence (TPPKpSPSS). The target peptide was also detected in the presence of protein extractions from brain homogenates of mice in tris-buffer. Along with these results, the authors cited a significant signal enhancement of the chemosensor in the presence of PP$_1$, which can be an off-target issue in some assays. Overall, the work presented by Wang and co-workers further supports the notion that proximally phosphorylated site selective recognition is important and that Tb$^{3+}$ luminescence is a viable approach that can be used in its targeting.

\textbf{Figure 5.1} Chemosensor for proximally phosphorylated peptides by Wang et al. Figure from\textsuperscript{121}.

One drawback of our Tb$^{3+}$-based method is the use of high excitation wavelengths that limit its use to only a few techniques beyond micro-plate time resolved fluorescence experiments. However, the future applicability of the method shows promise. A review published in 2013
summarizes the advances made with lanthanide (terbium and europium) luminescent peptide probes in monitoring the activity of kinases and phosphatases.\textsuperscript{100} Numerous examples of such probes show promise in the development of high-throughput kinase assays, which serve as an important tool for therapeutic drug development by monitoring the effectiveness of kinase inhibitors. More recent reports depict conjugation free and click-conjugation Tb$^{3+}$-based detection approaches for the real-time monitoring of tyrosine kinase activity using monophosphorylated substrate peptides using chemosensor scaffolds described in Section 4.2.2 (Figure 5.2).\textsuperscript{96,122}

![Conjugation-free Click-Conjugation](image)

**Figure 5.2** Conjugation-free and click-conjugation Tb$^{3+}$ chemosensors for analysis of kinase activity.

Based on these recent findings, we that propose Tb$^{3+}$ luminescence detection of proximally phosphorylated pY-containing motifs have the potential application in the development of peptide-based probes for kinase activity. The use of proximally phosphorylated motif peptides as substrates allows the Tb$^{3+}$ luminescent signal enhancement to detect kinase activity as it relates to proximal phosphorylation (Figure 5.3). As previously discussed, this technique could provide a means of understanding proximally phosphorylated motifs especially pY-containing subsets, which are of high importance in disease progression. As the development of detection methods to selectively study proximal protein phosphorylation have thus far been limited, the presented findings of this thesis has hopefully contributed to the further development of this field.
Figure 5.3 Proposed real-time detection of tyrosine kinase activity with proximally phosphorylated peptide substrates.
Chapter 6

6 Materials, methods and supplementary information

6.1 Chapter 2

General Methods: All reagents and solvents were purchased from Sigma–Aldrich. Silica gel chromatography was performed with Silica Gel 60 (particle size 40–63 µm) obtained from EMD. Thin layer chromatography (TLC) plates were obtained from EMD.

General: Synthesis and Characterization: NMR spectra were recorded on a Bruker Advance III spectrometer at 23 °C, operating at 400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR spectroscopy in either CDCl$_3$ or CD$_3$OD. Chemical shifts (δ) are reported in parts per million (ppm) referenced to residual isotopic solvent. Coupling constants (J) are reported in Hertz (Hz). Low Resolution Mass Spectrometry (LRMS) was performed on a Waters Micromass ZQ model MM1. Purifications by prep-HPLC were performed using Atlantis Prep T3 10 µm C18 (2) 250 x 19 mm column run at 20 mL/min (preparative) using gradient mixtures of water with 0.1% TFA and 10:1 acetonitrile/water with 0.1% TFA. The crude mixture was injected as a solution 4:1 0.1% TFA in water / acetonitrile.

From Scheme 2.1:

1,3,5-Tris(bromomethyl)-2,4,6-triethylbenzene (I): Triethylbenzene (8.01 g, 9.68 mL, 49.3 mmol), paraformaldehyde (15.5 g, 517.8 mmol) were dissolved in hydrobromic acid solution 33 wt. % in acetic acid (98 mL). Zinc bromide (17.7 g, 78.9 mmol) was added to the solution slowly at room temperature. The solution was heated to 90 °C for 10 hours. The reaction was then cooled to room temperature and the reaction mixture was filtered using vacuum filtration. The resulting white precipitate was washed several times with water and left to dry on the vacuum for 10 hours (20.79 g, 95%). δ$_H$ (400 MHz, CDCl$_3$) 1.34 (t, $J = 8.3$ Hz, 9H), 2.94 (q, $J = 7.6$ Hz, 15.2 Hz, 6H), 4.58 (s, 6H); δ$_C$ (100 MHz, CDCl$_3$) 15.5, 22.6, 28.4, 132.5, 144.9; LRMS (ESI+) m/z calc’d for C$_{15}$H$_{21}$Br$_3$[M + H]$^+$ 438.92, found 438.87
Trimethyl,1,1′,1′′-((2,4,6-triethylbenzene-1,3,5-triyl)tris(methylene))tris(1H-indole-4-carboxylate) (2): 1,3,5-tris(bromomethyl)-2,4,6-triethylbenzene (1) (10.0 g, 22.6 mmol) and methyl indole-4-carboxylate (12.5 g, 68.0 mmol) were dissolved in anhydrous THF (226 mL). The reaction was cooled to 0 °C and sodium hydride 60% w/w (4.5 g, 113.0 mmol) was slowly added. The solution was heated to room temperature for 48 hours, at which TLC confirmed that the reaction had gone to completion. The solution was quenched with methanol and concentrated in vacuo. The residue was extracted several times into EtOAc from neutral water after which the organic layers were combined and washed once with water. Following drying and concentration in vacuo, the product was purified by flash chromatography (2:1 hexanes:EtOAc) to afford (2) (13.1 g, 80%): δ_H (400 MHz, CDCl_3) 0.964 (t, J = 7.52 Hz, 9H), 2.61 (q, J = 7.4 Hz, 6H), 3.98 (s, 9H), 5.38 (s, 6H), 6.74 (d, J = 3.8 Hz, 3H), 7.09 (d, J = 4.0 Hz, 3H), 7.32 (t, J = 7.7 Hz, 3H), 7.70 (d, J = 8.3 Hz, 3H), 7.96 (d, J = 7.6 Hz, 3H); δ_C (100 MHz, CDCl_3) 15.3, 23.3, 43.5, 51.7, 102.8, 113.6, 121.0, 121.9, 123.5, 127.4, 128.4, 130.7, 137.1, 146.5, 167.8; LRMS (ESI+) m/z calc’d for C_{45}H_{45}N_3O_6 [M + Na]^+ 746.33, found 746.56

1,1′-((2,4,6-Triethylbenzene-1,3,5-triyl)tris(methylene))tris(1H-indole-4-carboxylic acid) (3): 2 (6.4 g, 8.85 mmol) was dissolved in THF: MeOH: H_2O (3:1:1 88 mL) and sodium hydroxide was added (1.4 g, 35.5 mmol). The solution was then heated to 65 °C for 5 hours at which TLC confirmed that the reaction had gone to completion. The solution was then extracted several times into EtOAc from 0.1 M HCl solution after which the organic layers were combined and washed once with water. Following drying and concentration in vacuo, the product was collected as a white solid (3) (5.6 g, 90%). δ_H (400 MHz, CD_3SO) 0.76 (t, J = 6.8 Hz, 9H), 2.58 (q, J = 6.8 Hz, 14.6 Hz, 6H), 5.45 (s, 6H), 6.87 (d, J = 3.2 Hz, 3H), 6.95 (d, J = 3.2 Hz, 3H), 7.27 (t, J = 7.9 Hz, 15.4 Hz, 3H), 7.76 (d, J = 7.5 Hz, 3H), 7.94 (d, J = 8.3 Hz, 3H), 12.62 (s, 3H); δ_C (100 MHz, CD_3SO) 15.2, 23.3, 43.7, 102.6, 114.9, 120.9, 122.3, 123.2, 128.1, 128.3, 131.1, 137.2, 145.8, 168.6; LRMS (ESI-) m/z calc’d for C_{42}H_{39}N_3O_6 [M + Na]^− 704.28, found 704.27

1,1′-((5-((4-((Benzyloxy)carbonyl)-1H-indol-1-yl)methyl)-2,4,6-triethyl-1,3-phenylene)bis(methylene))bis(1H-indole-4-carboxylic acid) (4): 3 (5.6 g, 8.05 mmol), potassium tert-butoxide (0.99 g, 8.86 mmol) and benzyl bromide (1.05 mL, 8.86 mmol), were dissolved in DMF (161 mL) at room temperature. The reaction was left at room temperature for 10 hours. The solution was then extracted several times into EtOAc from 0.1 M HCl solution after which the organic layers were combined and washed once with water. Following drying and
concentration in vacuo, the product was purified by flash chromatography (50% dichloromethane, 50% (1% acetic acid, 7% methanol, 92% dichloromethane)) and collected as a dark orange oil. (1.10 g, 17%) δ_H (400 MHz, CDCl_3) 1.01 (t, J = 7.5 Hz, 6H), 1.15 (t, J = 7.0 Hz, 3H), 2.45 (q, J = 7.6 Hz, 16.0 Hz, 2H), 2.67 (q, J = 7.6 Hz, 15.3 Hz, 4H), 5.40 (s, 4H), 5.41 (s, 2H), 5.44 (s, 2H), 6.67 (d, J = 3.4 Hz, 2H), 6.77 (d, J = 3.6 Hz, 1H), 7.10 (d, J = 3.2 Hz, 1H), 7.24 (d, J = 33.2 Hz, 2H), 7.31-7.42 (m, 6H), 7.49 (d, J = 7.2 Hz, 2H), 7.74 (d, J = 8.1 Hz, 2H), 7.79 (d, J = 8.13 Hz, 2H), 8.00 (s, 1H), 8.06 (d, J = 7.68 Hz, 2H); δ_C (100 MHz, CDCl_3) 15.2, 15.4, 23.3, 43.4, 50.4, 66.3, 103.1, 113.7, 120.9, 121.7, 122.7, 123.6, 123.8, 127.2, 127.6, 128.1, 128.4, 128.7, 130.5, 136.2, 137.0, 145.9, 146.2, 147.0, 172.8 LRMS (ESI-) m/z calc’d for C_{49}H_{45}N_{3}O_{6} [M + H] 794.33, found 794.35

Hexa-tert-butyl 10,10’-(1,1’-((5-((4-(benzyloxy)carbonyl)-1H-indol-1-yl)methyl)-2,4,6-triethyl-1,3-phenylene)bis(methylene))bis(1H-indole-4,4’-carbonyl))bis(1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate) (5): 4 (1.03 g, 1.33 mmol) and TBTU (1.4 g, 4.25 mmol) were dissolved in DMF (13.3 mL) at room temperature and left for 10 minutes. Boc_3Cyclen (2.0 g, 4.25 mmol) and DIPEA (1.15 mL, 6.65 mmol) were added to the reaction mixture and the reaction was left at room temperature for 48 hours. The solution was then extracted several times into EtOAc from a saturated sodium bicarbonate solution after which the organic layers were combined and washed once with water. Following drying and concentration in vacuo, the product was purified by flash chromatography (4:1, EtOAc: hexanes) and collected as a yellow solid. (1.51 g, 67%) δ_H (400 MHz, CDCl_3) 0.94 (t, J = 3.0 Hz, 9H), 1.43 (s, 5H), 1.45 (s, 31H), 1.46 (s, 18H), 2.59 (q, J = 8.5 Hz, 7.9 Hz, 6H), 3.32 – 3.50 (m, 32H), 5.30 (s, 4H), 5.36 (s, 2H), 5.41 (s, 2H), 6.39 (d, J = 3.2 Hz, 2H), 6.63 (d, J = 3.3 Hz, 2H), 6.70 (d, J = 3.7 Hz, 1H), 7.10 (d, J = 7.5 Hz, 3H), 7.22 – 7.40 (m, 6H), 7.47 (d, J = 8.4 Hz, 4H), 7.69 (d, J = 8.4 Hz, 1H), 7.99 (d, J = 8.4 Hz, 1H); δ_C (100 MHz, CDCl_3) 13.7, 15.1, 15.2, 19.1, 23.2, 28.0, 28.3, 28.4, 31.5, 43.5, 53.3, 61.7, 71.0, 71.6, 79.6, 80.2, 100.4, 109.9, 117.9, 121.5, 125.7, 126.1, 127.1, 127.6, 128.7, 128.9, 130.5, 136.4, 137.0, 146.0, 156.7

1-(2,4,6-Triethyl-3,5-bis((4-(4,7,10-tris(tert-butoxycarbonyl)-1,4,7,10-tetraazacyclododecane-1-carbonyl)-1H-indol-1-yl)methyl)benzyl)-1H-indole-4-carboxylic acid (6): 5 (1.51 g, 0.90 mmol) was dissolved in methanol (7.9 mL) and Pd/C (0.15 g, 10% wt.) dissolved in 1 mL methanol was
added slowly to the solution. The solution was purged several times with H₂ and reacted at room temperature under H₂ for 16 hr. The resulting solution was filtered through celite and the product was dried and concentrated in vacuo. The product was purified by column chromatography (70% dichloromethane. 30% (1% H₂O, 12% methanol, 70% dichloromethane)) and collected as an off-white solid. (0.789 g, 55%) δ_H (400 MHz, CDCl₃) 0.96 (t, J = 6.9 Hz, 9H), 1.16 (s, 17H), 1.46 (s, 20H), 1.51 (s, 17H), 2.62 (q, J = 6.9 Hz, 7.5 Hz, 6H), 3.20 – 3.75 (m, 32H), 5.33 (s, 4H), 5.38 (s, 2H), 6.42 (d, J = 2.6 Hz, 2H), 6.65 (d, J = 3.5 Hz, 2H), 6.73 (d, J = 3.1 Hz, 1H), 7.13 (s, 2H), 7.24 – 7.36 (m, 4H), 7.49 (d, J = 8.3 Hz, 2H), 7.73 (d, J = 8.3 Hz, 1H), 8.02 (d, J = 7.9 Hz, 1H); δ_C (100 MHz, CDCl₃) 13.8, 15.1, 15.3, 19.1, 23.2, 28.1, 28.3, 28.4, 31.5, 43.4, 53.3, 61.7, 65.7, 71.0, 71.6, 79.6, 80.2, 100.4, 109.9, 117.9, 121.5, 125.7, 126.1, 128.7, 128.9, 130.5, 136.4, 137.0, 146.0, 156.7; LRMS (ESI+) m/z calc’d for C₈₈H₁₂₃N₁₁O₁₀⁺ [M+Na]⁺ 1613.91, found 1613.16.

Hexa-tert-butyl 10,10'-(1,1'-(2,4,6-triethyl-5-((4-(methylcarbamoyl)-1H-indol-1-yl)methyl)-1,3-phenylene)bis(methylene))bis(1H-indole-1,4-diyl-4-carbonyl))bis(1,4,7,10-tetraazacyclododecane-1,4,7-triacrylate) (7): 6 (80.0 mg, 0.05 mmol) and TBTU (32 mg, 0.1 mmol) were dissolved in DMF (0.5 mL) at room temperature and left for 10 minutes. Methylamine hydrochloride (3.09, 0.1 mmol) and DIPEA (43.3 µL, 0.25 mmol) were added to the reaction mixture and the reaction was left to react at room temperature for 16 h. The product was then extracted several times into EtOAc from a saturated sodium bicarbonate solution after which the organic layers were combined and washed once with water. Following drying and concentration in vacuo, the product was purified by flash chromatography (10% MeOH in DCM) and collected as a yellow oil 7 (72.6 mg, 56%) δ_H (400 MHz, CDCl₃) 0.96 (t, J = 7.6 Hz, 9H), 1.15 (s, 17H), 1.45 (s, 20H), 1.49 (s, 17H), 2.62 (q, J = 6.9 Hz, 7.5 Hz, 6H), 3.04 (s, 3H), 3.15-3.83 (m, 32 H), 5.31 (s, 4H), 5.34 (s, 2H), 6.40 (d, J = 2.6 Hz, 2H), 6.65 (d, J = 3.5 Hz, 2H), 6.75 (d, J = 3.1 Hz, 1H), 7.13 (s, 2H), 7.24 – 7.36 (m, 4H), 7.49 (d, J = 8.3 Hz, 2H), 7.73 (d, J = 8.3 Hz, 1H), 8.02 (d, J = 7.9 Hz, 1H); δ_C (100 MHz, CDCl₃) 13.7, 15.1, 15.2, 19.1, 23.2, 28.0, 28.3, 28.4, 31.5, 43.5, 53.3, 61.7, 71.0, 71.6, 79.6, 80.2, 100.4, 109.9, 117.9, 121.5, 125.7, 126.1, 128.7, 128.9, 130.5, 136.4, 137.0, 146.0, 156.7; LRMS (ESI+) m/z calc’d for C₈₉H₁₂₆N₁₂O₁₆ [M+Na]⁺ 1625.90, found 1625.95

1-(3,5-Bis((4-(1,4,7,10-tetraazacyclododecane-1-carbonyl)-1H-indol-1-yl)methyl)-2,4,6-triethylbenzyl)-N-methyl-1H-indole-4-carboxamide (8): 7 (72.6 mg, 0.05 mmol) was dissolved
in 50% (v/v) TFA/DCM and allowed to react for 0.5 h. The solvents were then removed *in vacuo* and the crude product purified by rp –HPLC to produce the product 8 as a clear oil (62.7 mg, 62%) δH (400 MHz, CD3OD) 0.93 (t, J = 7.9 Hz, 6H), 0.93 (t, J = 7.9 Hz, 6H), 2.33-2.85 (bm, 24H), 2.91-3.42 (bm, 12H), 3.46-3.98 (bm, 5H), 5.47 (s, 4H), 5.51(s, 2H), 6.66-6.86 (m, 2H), 6.38-6.45 (m, 3H), 7.06-7.81 (m 9H), 7.97 (d, J = 7.9 Hz, 1H) δC (100 MHz, CD3OD) 14.0, 14.1, 22.7, 25.2, 43.3, 44.6, 46.8, 99.6, 110.2, 111.6, 111.6, 117.5, 120.7, 121.0, 121.2, 125.5, 126.5, 126.6, 128.4, 130.7, 130.8, 131.5, 136.5, 137.0, 145.8, 145.9, 174.0; LRMS (ESI+) m/z calc’d for C59H78N12O3 [M+Na]+ 1025.63, found 1025.67

**Metallated receptor 9: 8** (28.9 mg, 0.03 mmol) was dissolved in anhydrous methanol (0.3 mL) and zinc(II) trifluoromethanesulfonate (20.9 mg, 0.06 mmol) was added and allowed to stir for 0.5 h at ambient temperature. The reaction was filtered and the acetonitrile was then removed *in vacuo* to yield the final product as an off-white solid (35.9 mg)

**Fluorescence Intensity Assay:** A TECAN Infinite M1000 microplate reader was used for all solution fluorescence intensity measurements at 400 Hz in black 384 well, flat bottom plates. For 384 well plates a total sample volume of 60 µL was used throughout. Samples were excited at 540 nm and luminescence intensity was recorded an emission wavelength of 580 nm with the bandwidth of 5 nm. All experiments were performed in triplicate with data averaged to ensure accurate Kₐ values. Results were presented at Kₐ values. Binding constants were obtained using dose response fitting in Origin 8.

All peptides were purchased from CanPeptide at 95% purity as lyophilized powders.

TAMRA peptides full sequences:

5- TAMRA- A pY pYA-NH₂
5- TAMRRA- A pYApY-NH₂
5- TAMRA- A ApSA-NH₂
5- TAMRA- A pS pYA-NH₂
5- TAMRA- A pSApYA-NH₂
5- TAMRA- A pYApYA-NH₂

5- TAMRA- A pSApSA-NH₂

5- TAMRA- A pSpSA-NH₂

5- TAMRA- A ApYA-NH₂

**ITC Experiments**

Isothermal titration calorimetry (ITC) experiments were used to measure the binding of our metal complexes to various substrates, and were performed at 25 °C (298 K) using a Microcal VP-ITC titration microcalorimeter. All solutions prior to experiments were degassed before being added to the calorimeter cell. The peptides were injected in 10 µL increments into the reaction cell (cell volume 1.49 mL) containing receptor. A 250 µL injection syringe with 310–400 rpm stirring was used to give a series of 10 µL injections at 3.5-minute intervals. Control experiments for heats of mixing and dilution were performed under identical conditions and used for data correction in subsequent analysis. Data acquisition and subsequent non-linear regression analysis were done in terms of a one-site binding model using the Microcal ORIGIN software package.

All peptides were purchased from CanPeptide at 95% purity as lyophilized powders.

Full-length sequences for purchased peptides:

\[ pY = \text{Ac-AYpYAA-NH₂} \]

\[ pYpY = \text{Ac-ApYpYAA-NH₂} \]
Figure 6.1 ITC traces for A. pYPY peptide and B. pY peptide into 50 mM HEPES, pH 7.5 buffer

6.2 Chapter 3

Indicator Displacement Assay: HEPES free acid was purchased from BioShop Canada (cat. HEP005). ATP, ADP, AMP, NaSO₄ and PPᵢ were purchased from Sigma Aldrich. Pyrocatechol violet was purchased from Sigma Aldrich. All peptides were purchased from CanPeptide at 95% purity.

Full-length sequences for purchased peptides:

pY = Ac-AYpYAA-NH₂

pYPY = Ac-ApYpYAA-NH₂

All absorbance measurements were taken on a BioTek Cytation 3 instrument.
Figure 6.2  Absorbance max of PV (20 µM) and receptor 9 (0-200 µM) at 650 nm.

ProxyPhos Assay:

A TECAN Infinite M1000 microplate reader was used for all solution fluorescence intensity measurements at 400 Hz in black 384 well, flat bottom plates. For 384 well plates a total sample volume of 60 µL was used throughout. Samples were excited at 350 nm and luminescence intensity was recorded an emission wavelength of 476 nm with the bandwidth of 5 nm. All experiments were performed in triplicate.

6.3 Chapter 4

**General:** Bovine serum albumin (BSA), lysozyme, ovalbumin, dephosphorylated α-casein and β-casein proteins were purchased as lyophilized powders from Sigma Aldrich. TbCl₃ (99.0 % purity) was purchased from Sigma Aldrich. HEPES free acid was purchased from BioShop Canada (cat. HEP005). ATP, AMP and PPi were purchased from Sigma Aldrich.

All peptides were purchased from CanPeptide at 95% purity.

Full-length sequences for purchased peptides:

\[ pY = \text{Ac-AYpYAA-NH}_2 \]
pYpY = Ac-ApYpYAA-NH₂

pSpS = Ac-ApSpSAA-NH₂

pTAY = Ac-ApTAYA-NH₂

pTApY = Ac-ApTApYA-NH₂

pYAApY = Ac-ApYAApYA-NH₂,

pYAAApY = Ac-pYAAApY-NH₂

pYAAAApY = Ac-pYAAAApY-NH₂

pYAAAAApY = Ac-pYAAAAApY-NH₂.

**Luminescence experiments:** A TECAN Infinite M1000 microplate reader was used for all solution fluorescence intensity measurements at 100 Hz in black 384 well, flat bottom plates, unless otherwise indicated. For 384 well plates a total sample volume of 60 µL was used throughout. All time resolved luminescence measurements were taken at a delay time of 60 µs and an integration time of 1.5 ms, unless otherwise indicated. All experiments were performed in 50 mM HEPES, 50 mM NaCl buffer, pH 7.5 in triplicate. Tb³⁺ and analytes were incubated for 30 min and following this, samples were excited at 263 nm and luminescence intensity was recorded from 450 to 650 nm or at the analytical emission wavelength of 544 nm with the bandwidth of 20 nm.

Δ luminescence intensity represents the ratio of Tb³⁺ luminescence intensity in the presence of analytes and in the absence of lanthanides.

All images were acquired upon excitation with a short-wave UV lamp and captured with a digital camera. Images were taken on black 96 well plates with a total sample volume of 300 µL.

The following Hill equation was used for fitting titration data:

\[ Y = \frac{V_{\text{max}} \times X^n}{(k^n + X^n)} \]
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