UNIVERSITY OF TURKU

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University of Turku

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ABSTRACT

Bringing a new drug to the market is an expensive and long process. Typically, more than ten years and a billion dollars needs to be spent so the whole process is a big investment for a pharma company. The hit generation phase of the drug development is one of the first steps of the development and there, millions of potential molecules are screened against a known druggable target in order to generate hits, *i.e.*, molecules that have a specific activity towards the target. The hit generation phase requires high throughput screening (HTS) assays that are capable of screening millions of molecules rapidly with low expenses with platforms that are robust.

This thesis project focused on developing assays for hit generation phase for targets that catalyse protein post-translational modifications (PTMs). PTMs are regulatory mechanisms of cells that control many cellular events and are therefore involved in many disease conditions, for example, cancer. PTMs are additions or cleavages of chemical groups, typically catalysed by enzymes which activity can be affected with inhibitor or activator molecules.

Today, the techniques available for the activity monitoring of PTM-catalysing enzymes suffer from many weaknesses. Many methods, such as mass spectrometry or heterogeneous luminescence-based techniques, are not applicable for HTS, which increases the screening times of molecule libraries and therefore the cost of screening. Alternatively, some techniques are developed for one of a few different PTMs and therefore requiring a new platform for each PTM. On top of these, many methods are expensive to perform due to low sensitivity or antibodies, making the development of new assays motivating.

The work presented in this thesis expanded the Peptide-Break technology to be more well suited in PTM-detection and developed one new assay to detect cysteine-specific PTMs. The developed assays are simple homogeneous techniques applicable for HTS providing new interesting options for inhibitor screening.

KEYWORDS: PTM, HTS, drug development, TRL, TR-FRET, peptide

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TIIVISTELMÄ

Uuden lääkkeen tuominen markkinoille on kallis ja pitkä prosessi. Prosessi kestää keskimäärin yli kymmenen vuotta sekä kustantaa yli miljardi Yhdysvaltojen dollaria. Lääkekehitys alkaa lääkittävän kohteen tunnistamisella, jonka jälkeen tälle kohteelle yritetään etsiä mahdollisia lääkeainemolekyylejä miljoonien molekyylien kirjastoista. Koska seulonta on työläs työvaihe suuren lääkeainemolekyylien määrän takia, seulontamenetelmien on oltava tehokkaita, halpoja toteuttaa sekä luotettavia.

Tämä väitöskirjatyö keskittyi kehittämään peptidipohjaisia menetelmiä, jotka soveltuvat mahdollisten lääkeainemolekyylien tehoseulontaan. Tarkemmin, kohteena olivat entsyymit, jotka ovat vastuussa proteiinien jälkitranslationaalisista modifikaatioista (PTM). PTM:t ovat solunsisäisiä hallintamekanismeja, joilla solu säätelee toimintaansa. Tämän vuoksi myös erilaiset sairaudet käyttävät PTM:iä hyödykseen, kuten syövät.

Nykyään käytössä olevilla PTM:ien havaintomenetelmillä on monia heikkouksia. Monet menetelmät, kuten massaspektrometria tai heterogeeniset luminesenssipohjaiset menetelmät, eivät sovellu tehoseulontaan, jonka vuoksi suurien molekyylikirjastojen läpikäyminen niillä on kallista sekä työlästä. Tämän lisäksi joillakin käytössä olevilla menetelmillä voidaan havaita vain yhden tai muutaman PTM:n muodostuminen tai menetelmät joutuvat turvautumaan vasta-aineiden käyttöön. Mainittujen heikkouksien vuoksi uusien menetelmien kehitys on tarpeellista.

Tässä väitöskirjassa esitetyssä tutkimuksessa ”Peptide-Break” menetelmää kehitettiin monipuolisemmaksi työkaluksi PTM-detektioon sekä uusi TR-FRET-pohjainen (time-resolved Förster resonance energy transfer) menetelmä kehitettiin kysteiini-spesifisten PTM:n havainnointiin. Kehitetyt menetelmät ovat yksinkertaisia tehoseulontamenetelmiä, jotka tarjoavat mielenkiintoisia vaihtoehtoja lääkeaineseulontaan.

AVAINSANAT: peptidi, PTM, HTS, lääkekehitys, aikaerotteinen, luminesenssi, TR-FRET

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Abbreviations

ADP	adenosine diphosphate
ALPHA	amplified luminescent proximity homogeneous assay
AQUA	absolute quantification
ATP	adenosine triphosphate
CHEF	chelation-enhanced fluorescence assay
CoA	coenzyme A
CP	charge-based peptide
DELFIA	dissociation-enhanced lanthanide fluorescence immunoassay
DMF	dimethylformamide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assays
ESI	electrospray ionization
FDA	Federal Drug Administration
Fmoc	fluorenylmethyloxycarbonyl
FP	fluorescence polarization
FRET	Förster resonance energy transfer
GFP	green fluorescent protein
GTP	guanosine triphosphate
HPLC	high-performance liquid chromatography
HTRF	homogeneous time-resolved fluorescence
HTS	high throughput screening
ICAT	isotope-coded affinity tag
IMAP	immobilized metal ion affinity particle
iTRAQ	isobaric tag for relative and absolute quantification
LANCE	lanthanide chelate excitation
LMC	Lewis metal chelate
LZ	leucine zipper
MALDI	matrix-assisted laser desorption ionization
MRI	magnetic resonance imaging
MS	mass spectrometry
NAD+	nicotinamide adenine dinucleotide

PA	peptide amphiphile
PTM	post-translational modification
QRET	quenching resonance energy transfer
SA	streptavidin
SAMDI	self-assembled monolayers for matrix-assisted laser desorption ionization
SILAC	stable isotope labeling by amino acids in cell culture
SOX	sulfonamide-oxine
SPA	scintillation proximity assay
TR-FRET	time-resolved Förster resonance energy transfer
TRF	time-resolved fluorescence
TRL	time-resolved luminescence

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Kopra, K., Tong-Ochoa, N., Laine, M., Eskonen, V., Koskinen, P. J., Härmä, H. (2019). Homogeneous peptide-break assay for luminescent detection of enzymatic protein post-translational modification activity utilizing charged peptides. *Anal Chim Acta* 1055: 126–132.

II Eskonen, V., Tong-Ochoa, N., Valtonen, S., Kopra, K., Härmä, H. (2019). Thermal dissociation assay for time-resolved fluorescence detection of protein post-translational modifications. *ACS Omega* 4: 16501–16507.

III Kopra, K., Eskonen, V., Seppälä, T., Jakovleva, J., Huttunen, R., Härmä, H. (2019). Peptic fluorescent "signal-on" and "signal-off" sensors utilized for the detection protein post-translational modifications. *ACS Omega* 4: 4269–4275.

IV Eskonen, V., Tong-Ochoa, N., Mattsson, L., Miettinen, M., Lastusaari, M., Pulliainen, A., Kopra, K., Härmä, H. (2020). A single-peptide TR-FRET detection platform for cysteine-specific post-translational modifications. *Anal Chem* 92: 13202–13210

1 Introduction

In the process of drug development and screening of small drug molecules, clinical phase is the single most time consuming phase in bringing a new drug to the market, followed by the hit generation and lead discovery phase, and costs of the whole drug development process exceed hundreds of millions of dollars. Today, the estimated cost for a new drug to appear on the market is approximately 1.8 billion dollars, significantly more than, for example, 20 years ago.[1,2] The hit generation and lead discovery phase of the drug development takes place before the preclinical trials, *i.e.*, before assessing the safety of the discovered potential drug molecules, leads, and the testing the potential drug in animals and humans (Fig. 1). Hit generation follows target identification and validation, the latter two usually arising from basic research phase. The target, for example, a kinase enzyme, is first identified as a potential druggable target that is linked to a specific disease. Following identification, the validation step begins where the target is further studied. After validation of the target, hit generation phase takes place where large libraries of molecules are screened to find the drug candidates with specific activity for the target. These libraries may consist of millions of molecules but efforts have been made to reduce the sizes of compound libraries, *e.g.* target-focused compound libraries.[3] High throughput screening (HTS) assays are required to perform the screening process. Once the molecules with specific activity are found, *i.e.* hits, analogues of the molecules are further screened to discover lead molecules, which after optimization, are brought to the preclinical phase for further assessment.

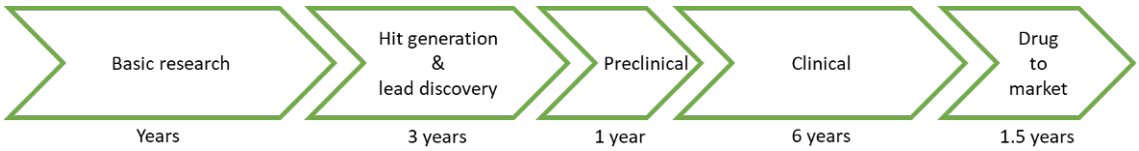

Figure 1. A simplified scheme of the drug development pipeline and the average time for drug molecule research and development phases. The development process can go back and forth phases in order to discover the potential drug candidates.

The hit generation phase is performed with primary assays in an *in vitro* HTS format. Depending on the target, *e.g.*, kinase *vs* receptor, different types of assays can be

utilized; inhibitors for receptor targets are usually screened with cell-based assay,[4] whereas with kinases, biochemical assays are typically performed.[5–7]

The requirements for the primary assays are the simple and rapid hit molecule identification, especially when the libraries are large. The assays are performed on a microtiter plate which size is typically from 96 to 1536 wells in pharmaceutical research and the screening is performed by robots capable of processing as many as 200 plates in a day.[8,9] The detection method for the assays is of high importance – the more sensitive the method, the more potential the technology is since less biomolecules are needed to perform the assays, especially if the target protein is difficult to extract. Other thing to consider is the waste produced, *e.g.*, in radiometric assays, and the overall time the assay takes to detect the binding or specific activity to the target. The techniques applied should also preferably be as universal as possible. One of the most sensitive and least expensive methods for hit molecule detection in HTS is luminescence, which is highly used in the pharma industry.

Enzymes catalyzing protein post-translational modifications (PTMs) have become one of the most important drug targets. Currently, almost 20 % of all newly approved drugs by FDA (Federal Drug Administration) per year target kinases. Kinases are enzymes that cleave a phosphate-group from adenosine triphosphate (ATP) and add it to a substrate protein, *e.g.*, as a part of signaling pathway within a cell. As the enzyme only modifies a certain amino acid in a specific amino acid sequence, this specificity can be utilized to construct simple detection platforms with peptides that include the target amino acid sequence.

Peptides have many advantages when compared to natural proteins in drug screening assays. The properties of peptides and their different structures are discussed in the next chapter but one of the most valuable advantages of peptides is that they can be synthesized with relative ease. This comes extremely useful, for example, with receptor proteins that are difficult to extract. Nevertheless, it is a delicate task since modelling the correct modification site of a protein with a short amino acid sequence may decrease the activity of the enzyme. On the other hand, the development work of a functional peptide model molecule pays off since synthesis of a peptide is considerably less expensive compared to extracting a whole protein. Peptides can also be used as an analytical tool when building new assays. An excellent example of a peptide-tool is a peptide conjugated with two terminal luminescent labels utilized in protease activity detection. Energy transfer between the two labels indicates the inhibition of the druggable target, whereas the loss of the energy transfer reveals the activity of the target. Peptides are well suited for label conjugation due to a simple conjugation and easy purification processes.

In the work presented in this thesis, peptide-based analytical assays have been developed for screening of inhibitors for PTM-catalyzing enzymes. The assays are intended for the hit generation and lead discovery phase of the drug development,

where millions of potential hit molecules are screened and HTS-compatible tools are needed, but also for the secondary screening steps that detect the potential lead molecules. In publications **I–III**, a recently published technique, Peptide-Break, is further developed and expanded to meet the needs of an end-user.[10] In the original embodiment of the Peptide-Break method, a substrate peptide, with the consensus sequence of the protein that is modified by the target enzyme, was used. In addition, the structure incorporates leucines that enable the formation of a coiled-coil leucine-zipper (LZ) structure with a detection peptide which is labeled with a Eu(III)-chelate. Upon binding of the two peptides, the time-resolved luminescence (TRL) -signal is protected from the soluble quenchers in the solution. Addition of PTMs to the substrate peptide causes the disruption of the peptide-complex and the TRL-signal is no longer protected, leading to a decreased TRL-signal. This difference in TRL-signals can be used to screen potential inhibitors for enzymes that modify the peptides. In publication **I**, the LZ-based complex formation was changed to electrostatic force-driven complex formation. The substrate peptide was designed to have an opposite charge than the detection peptide, which was kept the same as in the original Peptide-Break technology. The main goal of the publication was to alleviate problems with the substrate design that stem from the fixed leucines potentially restricting the use of natural substrates. Publication **II** tackles issues that arise from low affinity/activity enzyme targets. The low affinity or activity of a target enzyme leads to the requirement of high concentrations of reagents and therefore the price of the assays increases. In this work, we were able to overcome the potential problems with low substrate concentration in a cost-efficient way by increasing the inexpensive peptide concentration and modifying the assay setup. The developed assay was sensitive to thermal dissociation applied to the system improving the detectability of the PTMs. In publication **III**, the detection, utilizing quenching resonance energy transfer (QRET), that yields high TRL-signals when Eu-peptide is bound to the substrate peptide, was expanded to obtain a high signal readout for the unbound Eu-peptide. High signal readout from the unbound Eu-peptide is achieved by using soluble signal modulator molecules that bind only to the peptide-duplex. Therefore, the signal readout can be selected by end-user to meet the desired direction, *i.e.*, high signal at high inhibitor concentration regardless of the type of the PTM. In publication **IV**, a single-peptide time-resolved Förster resonance energy transfer (TR-FRET) assay for cysteine-specific PTMs was developed. In this application, the peptide substrate is biotinylated and therefore binds to the tetrameric streptavidin (SA) protein labeled with a Eu(III)-chelate (donor). A cysteine-specific dye (acceptor) was conjugated to the substrate forming a FRET-pair allowing the detection of the inhibited target. Enzymatically modified cysteine cannot be conjugated to the acceptor dye, leading to a low TR-FRET-signal.

2 Literature review

2.1 Peptides and their bioapplications

Peptides are molecules that are built from amino acids and they are smaller in size than proteins. Amino acids are small organic molecules with amine (-NH_2) and carboxylic acid (-COOH) groups, and a side chain, which differs from one amino acid to another. Peptides are built from amino acids when a peptide bond is formed between the carbonyl carbon and nitrogen. Traditionally, amino acid structures have been defined as peptides if the number of amino acids is between 2 and 50. Further classification can be made, so that peptides with 2 to 20 amino acids are oligopeptides, and peptides larger than 20 amino acids are called polypeptides. In this literature review, peptides larger than 50 amino acid residues are referred as polypeptides. The amino acids bound to the peptide structure are called residues and from naturally occurring amino acids 20 are encoded by eukaryotes (Fig. 2). Due to the similar nature of peptides to proteins, they display many similar properties. However, the less defined structure of peptides is the most significant difference between them. The longer the peptide chain is the more additional structure it can possess. Peptides consisting of a few amino acids do not have a secondary structure, only different conformational isomers. As the amino acid chain lengthens, different structures within the peptide become possible, such as α-helix, β-sheet, disulfide bridges between cysteine residues, and electrostatic interactions between charged amino acid residues. A few examples of different peptides and their sensor properties are presented in Table 1.

Table 1. Examples of various peptides and their utilization as sensors.

ref	peptide sequence	sensor properties
11	Ac-EACARVAib*AACEAAARQ-NH_2	light-controlled helix-stabilization
12	MRGSHHHHHHGSDDDDKWA-SGDLENEVAQLEREVRSLEDEAAELEQKVSRLKNEIEDLKAE-IGDHVAPRDTSMGGC	pH-sensitive helix structure
29	RADARADARADARADA, HEHEHKHKHEHEHKHK, VEVEVEVEVEVEVEVEVEVE	various structure formations induced by changes in ionic strenght, temperature or pH
51	hydrocarbon tail-CCCCGGGS(PO_3H_2)RGD	hydrocarbon tail conveys hydrophobicity, cysteine residues form disulfide bridges and therefore start the self-assembly, hydrophilic linker that brings flexilibility to the structure, phosphorylated serine helps direct the mineralization of hydroxyapatite, RGD is the cell adhesion ligand
59	FF	fibre-formation in aqueous solutions
62	NFGSVQ	fibre-formation in aqueous solutions
67	VPGVG	solubility in different temperatures

*Aib= α-aminoisobutyric acid

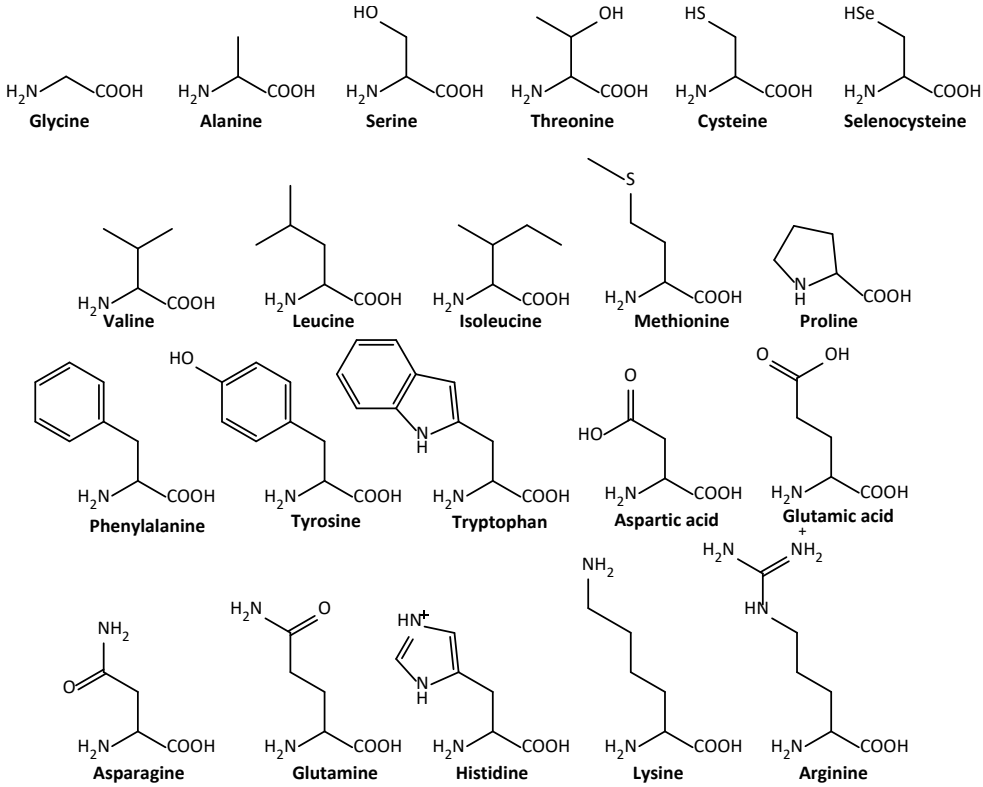

Figure 2. The structures of the 20 amino acids genetically encoded in eukaryotes.

With a defined secondary structure, peptides can be designed to respond to changes in their environments. Polypeptide structures and elastin-like sequences, can be used as analytical tools in biological systems due to their responsiveness to stimuli, *e.g.*, light, pH, ions, or temperature.[11–15] Furthermore, in some applications specific peptide solutions can act as carriers of drug molecules. After some stimulus is applied, the peptides form a hydrogel, leading to the release of the drug.[16–18] Peptides in these applications act as sensors as well as vehicles for the drug molecules. The presence of small molecules can be used to alter the secondary structure of short peptides and when using specific sequences from protein substrates, enzyme responsiveness can be achieved in the same way as the protein structure rearranges due to binding (kinases, proteases and phosphatases).[19] Secondary structures of peptides and their applications are discussed in the next chapter.

2.1.1 Peptide structures and their applications

As the number of amino acid residues increases, peptides can exhibit more defined secondary structures or form structures with other peptides, *i.e.,* tertiary structures.

This leads to peptides having many physical and chemical properties, making peptides excellent sensing tools. The 20 different amino acids found in eukaryote proteins allow a variety of different interactions between the amino acids and although these interactions are weak individually, usually multiple interactions take place simultaneously leading to stable structures. Each of these interactions require different circumstances to occur. Hydrogen bonding requires approximately 2.8 Å distance between the donor and acceptor residues and π–π-stacking requires a distance less than 3.4 Å between the aromatic moieties. The electrostatic and hydrophobic interactions are not as clearly distance dependent and vary also according to the ionic strength of the solution. In nature, the whole 3D-structure of a protein is the sum of different simultaneous interactions.

α-Helix is a secondary structure of peptides where a single peptide forms a spiral that is held together by hydrogen bonds (Fig. 3). These helices are common when a chemically similar amino acid is repeated every three or four residues in a peptide structure, corresponding to 3.6 amino acids per turn in α-helix. However, the helix-formation is dependent on the environment and the structure can be controlled. Controlled helix-formation has been accomplished with, *e.g.,* light-responsive amino acids and stabilized with a cross-linker molecule that binds between cysteine residues.[11] In this example, during the *trans* to *cis* photoisomerization of the cross-linker, the α-helical content of the peptide was observed to increase. In the described technique, the cross-linker destabilizes the helix formation in its *trans*-isomer due to the length of the cross-linker and the steric effects caused by bulky residues. The *cis*-isomer stabilizes the helix formation as the linker can bind to the cysteine residues. In this example, the linker molecule was used as a tool that stabilized the α-helix formation, the formation of which was controlled by light. Thus, the secondary structure of the peptide may alternatively be used as a tool to detect the isomerism of a small molecule.

Coiled-coil is another common helical structure found in polypeptides. In the coiled-coil structure, two or more α-helices form a superstructure where the amino acid sequence follows a seven amino acid (heptad) repeat pattern (Fig. 3). Four of these residues are responsible for keeping the superstructure together: *a*, *d*, *e*, and *g*. The residues *a* and *d* are hydrophobic amino acids in the core of the coiled-coil and stabilize the structure by Van der Waals forces. The residues *e* and *g* are either hydrophobic, hydrophilic, or charged, and contribute to the electrostatic interactions, which stabilize the structure. Switching the interacting amino acid residues to basic or acidic amino acids can alter the assembly of the coiled-coil structure, depending on the degree of protonation. Exchanging the *d* residue into leucine and the *e* and *g* residues into glutamic acid forms a homodimeric leucine zipper (LZ) coiled-coil structure. These coiled-coil structures have been attached, for example, to gold surfaces and used as pH sensors.[12,13] Similar LZ-structures are found in peptides that are able to form fibrous structures as the coiled-coils can be extended via electrostatic interactions between

peptides, so-called “sticky-ends”. The “sticky-ends” enable peptides to “stick” to each other forming the macro-scale fibers. Furthermore, these structures were responsive to different salt concentrations.[14] The LZ can also undergo a transformation into a different ordered structure in response to a specific stimulus such as temperature.[15] Other applications, such as controlling the aggregation of gold nanoparticles[20], monitoring the presence of metal ions[21–25], and Peptide-Break[10] have been reported, demonstrating the many possibilities of coiled-coil sensors.

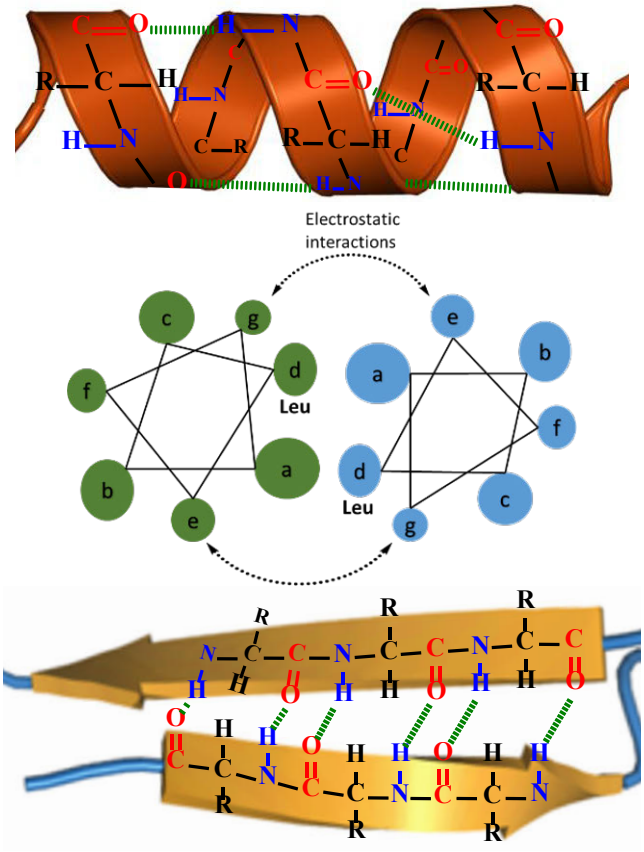

Figure 3. A schematic representation of α-helix, leucine zipper (LZ), and β-sheet structures. α-Helix (top) as well as β-sheet (bottom) structures are held together by hydrogen bonds (green) between electron-rich oxygen atoms and electron-poor hydrogen atoms bound to the electronegative nitrogen. The LZ complex structure illustrated from top (middle) has a heptad-repeat in both peptides. The leucines occupy the d-positions and the residues in a-positions are typically hydrophobic amino acids. Amino acids in e and g positions are often charged residues contributing to the stability of the structure (top and bottom figures modified from originals).[26,27]

*β-Sheet*s are formed when adjacent parallel or anti-parallel peptides are held together by hydrogen bonds or electrostatic forces (Fig. 3). The end result is a slightly curved

sheet-like structure and β-sheets are common secondary structures in natural polypeptides and proteins. One of the most interesting research area with β-sheets has been Parkinson's and Alzheimer's disease, where the amount of β-sheets in specific proteins indicate the state of neurological damage.[28] The simplest way to build a self-assembling β-sheet peptide is to have an alternating sequence of positively and negatively charged amino acid residues. A few examples of the most frequently used and simplest peptide sequences are RADA (Arg-Ala-Asp-Ala), EAKA (Glu-Ala-Lys-Ala), FEK (Phe-Glu-Lys) and KLD (Lys-Leu-Asp).[29] Depending on the amino acid residues, or branching residue strands, β-sheets with different kinds of properties can be created. Both the β-sheet and helical structures are sensitive to changes in pH or temperature, and β-sheets have been demonstrated to transform into helical structures.[30,31] In addition, β-sheets can form loose macrostructures, gels, and the formation of a gel, gelation, is often a desired result when designing tissue repairing materials. The gelation is activated by stimuli such as, temperature and light, but also with altering salt concentration and pH.[32–41] The FEK peptide, for example, can be self-assembled with Ca^{2+}-ions and increasing the temperature, or exposing the peptide solution to a specific wavelength of light.[42] β-sheet formation and the gelation can be influenced by altering the peptide sequence with different amino acid residues.[42,43] A specific structure found sometimes at the end of a β-sheet is called a β-hairpin (Fig. 4). If the peptide sequence contains amino acid residues, such as proline followed by, *e.g.*, glycine, the peptide is able to a form hairpin structure as these residues induce a turn.[44] The structure of the hairpin starts with the amino acid residue in position *i*. The residue in the $i + 1$ position, proline, initiates the curving of the peptide strand due to its rigidness. The following amino acid ($i + 2$) is conformationally flexible, such as glycine, serine or alanine[45], and the residue $i + 3$, forms a hydrogen bond with the residue *i*. Furthermore, if the following residues ($i + n$) form hydrogen bonds with residues before the hairpin, the result is a β-sheet structure.

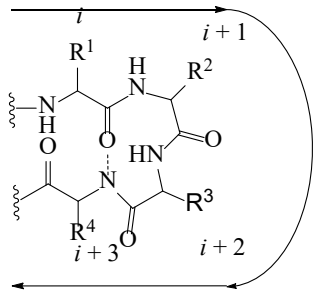

Figure 4. A schematic of a β-hairpin structure. β-hairpin can be formed when the amino acid residue in the position i + 1 is proline, which initiates the curving of the peptide backbone. The residue in position i + 2 should be a conformationally flexible amino acid, such as glycine, and the following residue in position i + 3 should be able to form a hydrogen bond with the residue in position i.

As described in the introduction, peptides have been used when designing new biomaterials, especially since peptides are able to form fibers. One such peptide is a peptide amphiphile (PA), which due to the presence of a hydrocarbon tail at the other end of the peptide has polar and apolar regions in its structure. The amino acid sequence, *i.e.*, the peptide part, is displayed on the surface of the fiber facing to the solution, while the apolar hydrocarbon chain is buried into the fiber structure.[46–48] Since the surface of these materials contains the peptide part, they make excellent responsive biomaterials. A few studies have used cysteine-rich peptides with a specific region that binds to cells.[49–52] Upon contacting cells, the peptides form a hydrogel due their ability to chelate metal ions, such as Mg^{2+} and Ca^{2+}. Moreover, pH changes can induce the self-assembly of a nanostructure. An acidic pH initiates the self-assembly process, while neutral and basic pH tend to reverse the process. After the self-assembly, other interactions between the peptides, such as disulfide bridges between cysteine residues, can help to make a more stable polymer and therefore decrease the effect of further pH changes.[49,51] Furthermore, PAs with a cell recognizing binding domain and a specific sequence that can be cleaved by enzymes, such as type IV collagenase[53], have been reported, indicating that PAs have a potential use in monitoring the enzymatic activity. Lastly, specific PAs have been applied as chelators for magnetic resonance imaging (MRI) contrast agents, using Gd^{3+}-ions.[54] Gd^{3+}-ions have the ability to increase the spin lattice relaxation rate of water protons when bound to a larger molecule, which in turn increases the contrast between tissues in imaging.

There is a huge number of different polypeptides that form large structures, such as hydrogels and fibrous structures. Short peptides with only few amino acid residues can also be used to form macrostructures, although the formation mechanism is different. Short peptides form larger ordered structures via aromatic interactions, *i.e.*, π–π-interactions between the aromatic rings of the amino acids. These π–π-interactions have previously been studied widely in organic solvent systems, *e.g.,* self-assembly of π-conjugated polymers into nanosized fibres.[55] The same π–π-interactions can be used to self-assemble peptides into different structures, since there are four natural amino acids, His, Phe, Tyr, and Trp, containing aromatic rings. In addition, π–π-interactions contribute significantly to stability of protein structures and contribute in scaffolding in DNA and RNA binding.[56,57] The length of π–π-interactions are at similar range than hydrogen bonding, most favorable distance ca. 6–7 Å, but the angle and orientation of the two interacting aromatic rings contributes to the likelihood of interaction greatly.[58] The peptides reported to form nanostructures via π–π-interactions are generally very short, a maximum of a few amino acids long, and form structures such as nanotubes[59], hollow spheres[60], and amyloid-like fibres[61,62] when diluted from fluorinated organic solvents into aqueous solutions. Self-assembling via π-stacking only can be ensured by capping the N- and

C-termini with a protecting group, *e.g.* fluorenylmethoxycarbonyl (Fmoc). Capping eliminates the electrostatic interactions between carboxylic acids and amines and therefore, only π–π-interactions contribute to the nanostructure formation.[63,64] pH sensitive systems have also been reported, where the dipeptide nanostructures can be disrupted by changing the pH.[65]

The last relevant peptide structure that is discussed in the context of this thesis is based on the natural protein elastin. Elastin is a durable biopolymer that is present in connective tissues in human body and responsible for keeping the shape of tissues after stretching or contracting.[66] Elastin is mainly built from the smallest amino acids, such as alanine, glycine, proline, and valine. The peptide monomer that forms elastin-like structures has five amino acids. The first, second, third and fifth residues are always the same, whereas the fourth residue varies. The sequence may be, for example, valine, proline, glycine and glycine, and the variable amino acid can be any amino acid, except proline. The polymers with this structure are temperature sensitive and react to temperature by aggregating or becoming less soluble. The transition temperature is dependent on the variable amino acid, the concentration of the peptide, and the polymer length.[67,68] These polymers can be employed as drug-carriers, for example, when the peptide is targeted to cancerous tissues and then mildly heated externally. In these applications, the polymer is designed to be soluble at body temperature (37 °C), but has a transition temperature at 39 °C where the aggregation begins. As the external local heating is continued to a higher temperature (~ 42 °C) in the cancer tissue, the polymer aggregates and releases the drug molecule.[16–18] This allows the exact targeting of drugs to the right tissue and only small doses of drugs can be administrated.

2.1.2 Peptide drugs and protein mimicking peptide substrates

During the hit discovery phase of drug development, as described earlier, large libraries of molecules are screened for potential new drug candidates. Peptide drugs have been developed alongside with typical small molecule drugs as well as new peptide antibiotics, which are compounds that are active against bacteria. Since the 1950's, bacterial resistance for traditional antibiotics has been reported and it has become a major concern that the selection of available antibiotics are not for long effective against bacteria. Alternatively, the rapid development of peptide antibiotics has become a much needed substitute for traditional antibiotics.[69] The high specificity and low toxicity of peptide drugs has been considered as an advantage but on the other hand, the increased proteolytic instability is a potential issue. The instability of peptide drugs may be solved by altering the peptide structure, for example, incorporating unnatural amino acids to the structure. The potential and

development of peptide drugs has been increased rapidly and peptide drugs have been performing well in clinical trials.[70] Traditionally, antibiotics refer to compounds that have originally been extracted from microbes that kill bacteria or prevent their growth. However, not all peptide drugs are antibiotics even though their structure is similar and the development of macrocyclic peptide drugs has increased during the recent years.[71] Besides antibiotics, peptides have been successfully used as substrates for target enzymes. From studying the enzyme-specific sites of substrate proteins, peptides can be synthesized to act as protein mimicking substrates and this has been used in many techniques that will be discussed in chapter 2.3.

Peptide macrocyclics have attracted a lot of interest due to their ability to target proteins surfaces that have previously been considered as undruggable. The synthesis of peptide macrocyclics is straightforward and the affinity of macrocyclic peptides to the target protein is usually higher compared to the linear peptide.[72] The peptide macrocyclic structure consists of a ring that spans over multiple amino acids and it is a common feature in peptide drugs. Fig. 5A present two peptide macrocyclics, largazole and a non-natural peptide synthesized using the macrocyclization enzyme of the cyanobactin family (PatGmac).[73,74] Constructing peptide drug libraries consisting of cyclic peptides has yielded more bioactive hits compared to linear peptides and the discovery of active compounds can be performed by *de novo* techniques using *e.g.* phase display, split-intein circular ligation of peptides and proteins (SICLOPPS) or mRNA display.[75–77] Stapling of peptides can also be used to mimic the protein regions that are involved in *e.g.* protein-protein interactions in order to create high-affinity binding peptides.[71]

Peptide antibiotics have become more common in the last 50 years with hundreds of newly found antibiotics. Much like most peptide drugs, many peptide antibiotics have cyclic regions and they contain both D- and L-amino acids, but peptide antibiotics mimicking natural antimicrobial peptides have also been developed.[78] Antimicrobial peptides have compositional differences to proteins, *e.g.*, some amino acids often found in proteins are rare in peptide antibiotics.[79] Peptide antibiotics can be roughly divided into two groups by their synthesis pathway: nonribosomally synthesized peptides, which have originated from bacteria or fungi, such as the gramicidins and the polymyxins[80,81], and secondly, natural ribosomally synthesized peptides that are produced by a variety of organisms.[82] Most of the antimicrobial peptides are cationic in nature due to the abundance of lysine and arginine residues in their backbone (Fig. 5B). Hence, they can interact with anionic surfaces of the bacteria.[83]

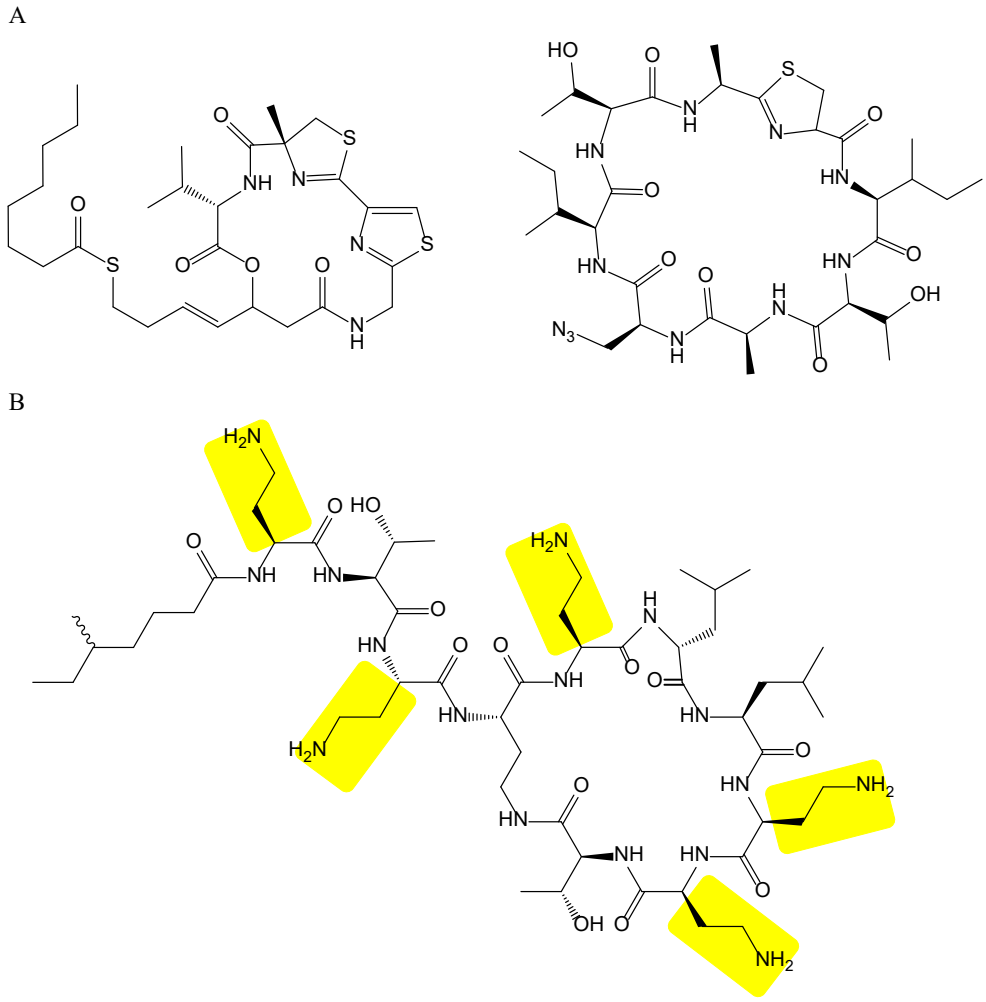

Figure 5. The structures of A) peptide macrocyclics largazole, synthesized hybrid cyclopeptide and B) antimicrobial peptide polymyxin E. The positively charged side arms of lysine residues are highlighted in yellow that can bind to the negatively charged bacterial surfaces.

Protein mimicking peptide structures are complex constructions, where a part of the protein is built from peptide chains. The target of most drugs are proteins and more specifically receptor proteins. In the case on PTM-catalyzing proteins, a major target class is kinases. Extracting pure proteins for the purposes of drug screening is often difficult and time-consuming, especially for receptor proteins with low water solubility. Furthermore, the cost of screening large libraries is expensive when whole protein substrates are used, compared to using only a fraction of the protein. As an alternative to intact proteins, peptides mimicking the proteins have been designed. Constructing a peptide structure that is a copy of the desired region of the protein

can be highly beneficial since it is possible to generate exact copies of the protein regions of interest synthetically and in high quantities. The most efficient way for designing peptides based on receptor proteins, for example, is the structure-based design that takes the 3D-structure of the binding-site into consideration.[84] Often not only the adjacent amino acid residues contribute to the binding of a drug molecule to the protein but also residues that are spatially in close proximity. The 3D-structure that mimics the protein contains unstructured and structured regions in which the amino acid sequence has α-helices or β-sheets. It is possible to create rigid peptide structures by including specific amino acids as structural tools, such as adding cysteine residues to form disulphide bridges, thus stabilizing the structure or stapling the peptide. Stapling a peptide also increases the stability of the 3D-structure by adding an additional ligand or another peptide that keeps the structure together.[85] Manufacturing these peptides is achieved by using solid-phase synthesis[86], and the different peptide chains can then be attached to a separate scaffold, such as a gold electrode.[87]

2.2 Post-translational modifications

2.2.1 Significance and function of PTMs

Protein post-translational modifications (PTMs) are molecular switches that can, *e.g.*, activate or inhibit different cellular functions. Over 200 different PTMs have been reported and the biological functions of PTMs are broad. They play key roles in, for example, gene-expression, metabolism, protein stability, signal transduction, cell division and regulation mechanisms.[88,89] In all of these cellular functions, different types of proteins are present, which are translated from 20 000–25 000 genes forming the human genome[90]. Out of these 20 000+ genes, around 100 000 proteins are translated, and one gene is capable of coding multiple proteins due to alternative splicing.[91] Furthermore, RNA splicing and polymorphism together with PTMs increase the different proteins found in different stages of a humans life cycle to millions.[92–95] There are many different types of PTMs and the PTM can be a chemical groups that is added or removed from the protein, modification of an amino acid itself (citrullination; conversion of arginine into citrulline and deamination), cyclisation of the protein structure (isoaspartate formation), disulfide bridge formation, or interchanging of functional groups.[96,97] The addition or cleavage of functional groups or molecules can vary in size from small modifications, such as methyl-groups, to large modifications, for example, ubiquitin or interferon-stimulated gene 15, ISG15, which is a small protein with a molecular weight of 17 kDa (Fig. 6).[98] The modification site is usually the reactive functional groups in the side-chain of the amino, acid such as hydroxyl groups (tyrosine and serine) amine

groups (lysine and arginine), carboxyl groups (glutamic and aspartic acid), or C- and N-terminus of the protein. As previously stated, PTMs control cellular functions, for example, controlling the protein-protein interactions that form signaling pathways. In this context, the binding of two proteins can be controlled by blocking the interaction with a PTM or alternatively, inhibiting the enzyme catalyzing the PTM. In addition, the post-translationally modified region of a protein can act as a new binding site for another protein. Unfortunately, the PTM regulated signaling pathways can also be affected by many disease conditions, and thus, PTM-enzymes have become an interest in drug screening and development.

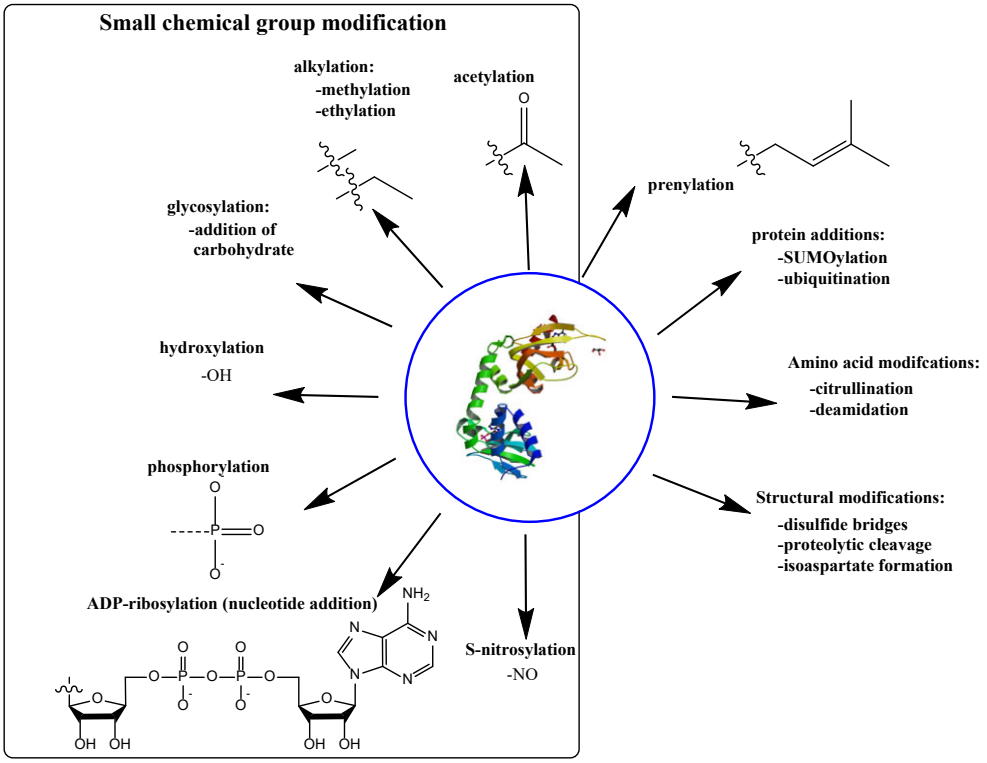

Figure 6. Examples of different protein post-translational modifications (PTMs). Most of the enzyme-catalyzed modifications are reversible, i.e., a modified protein can be unmodified by another enzyme allowing the PTMs to be used as switches that regulate cellular functions.

2.2.2 PTM enzymes as drug targets

Whilst post-translational modifications are of such a central importance in regulating multiple functions in cells, many disease conditions, such as cancer, use the PTMs of host cells to their own advantage, thus making PTM-enzyme targeted drug

development an important task.[99,100] Protein kinases are one of the most important signal transductors in eukaryotic cells and over 500 different kinase genes have been found.[101] Moreover, almost 20 % of FDA-approved drugs on year (2020) target kinases and the trend of FDA-approved kinase drugs seems to be increasing (Fig. 7). With kinase inhibitors with this large portion of the market, is estimated that kinase inhibitor market value alone will be 64.9 B$ by the year 2027.[102] Over 30 % of all human proteins can be phosphorylated by kinases.[103] Currently, kinase inhibitors are on the market for many different cancers, such as afatinib for lung cancer, axitinib for renal cell carcinoma, bosutinib for chronic myelogenous leukemia, sorafenib for kidney and liver cancer, and cobimetinib for melanoma.[104–108] In addition, inhibitors for PTM-catalyzing enzymes have been studied extensively. Histone deacetylase, DNA methyltransferase, glycosylation and ubiquitination inhibitors have been likewise developed for different cancers,[109–112] whereas ADP-ribosyltransferase inhibitors have been studied for, *e.g.,* neurological diseases and whooping cough.[113,114] The high number of enzyme-catalyzed PTMs involved in disease conditions has brought interest in developing methods to discover inhibitors for these enzymes. As the PTMs appear in specific amino acid residues, peptides have become a cost-effective substrate substitute. Despite the advancement of screening methods in the last couple of decades, more efficient and low-cost analytical tools for PTM detection are needed.

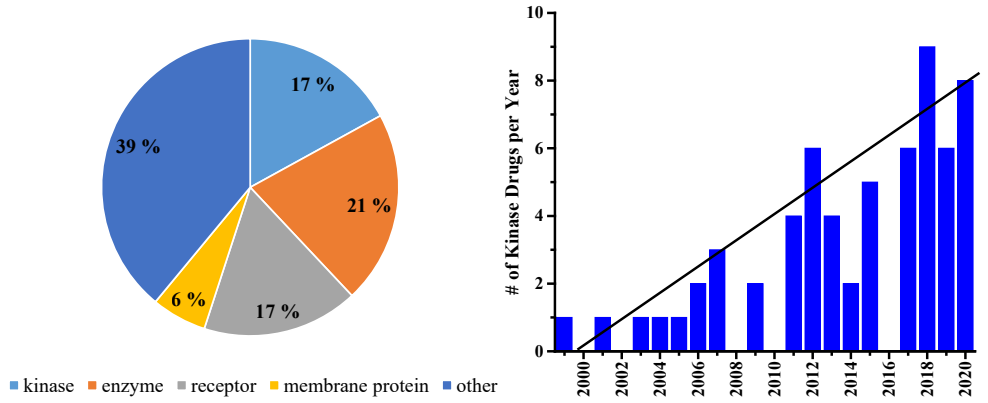

Figure 7. The targets of new FDA-approved drugs on the market (2020) and the trend of FDA-approved kinase drugs 1999–2020.

2.3 PTM detection

In the early stages of drug development and screening, it is important that the techniques for potential drug molecule detection are suitable for HTS. Such techniques has to be robust to screen millions of molecules quickly with high

efficiency and cost-efficiently. Inhibitors for PTM-catalyzing enzymes can be discovered by monitoring the activity loss of an enzyme. PTMs can be studied with many different analytical techniques and mass spectrometry and different optical detection formats, mostly luminescence, are often used. In this chapter, techniques applicable for HTS are discussed with emphasis on those, which utilize peptides as an analytical tool.

2.3.1 Mass spectrometry

Mass spectrometry (MS) is a technique where the exact mass of a molecule can be determined. Because the addition or cleavage of a PTM changes the mass of a peptide, MS is well suited for PTM-analysis. First, the sample molecules are ionized and based on the fragmentation, flight-time of the ions, and electromagnetic properties, such as oscillation frequency, the ions are separated from one another and the mass of different fragments can be calculated. MS is widely used for the analysis of proteins to determine protein masses, sequences, and modifications. Many MS instruments enable tandem mass spectrometry (MS/MS) where ions are selected from the first mass analyzer, fragmented, and further analyzed with a second MS instrument. Furthermore, the analysis of protein sequences and PTMs is regularly performed with the help of proteases, such as trypsin, that digests the protein into peptides. MS provides the *m/z*-ratio (mass-to-charge) of the studied molecules. The ions are mostly created by loss or addition of protons, thus a molecule that has a charge of ± 1 yields a peak with the exact mass of the protein ± 1. Similarly, a molecule with a charge of ± 2 has a mass to charge ratio of half of the mass of the initial molecule. PTMs can be studied when the known mass of the protein or peptide changes due to a modification. Hence, a mass change of, *e.g*. 80 Da indicates phosphorylation, whereas a mass increase of 42 Da suggests a lysine acetylation.[115] Some problems occur when determining the PTMs because not all modification are stable and degrade within the ionization process (phosphoserine and phosphothreonine). Furthermore, phosphopeptides may be difficult to identify due to the substoichiometric nature of the PTM.[116] Utilizing MS/MS for PTM detection is a powerful tool and with different scanning techniques, *e.g*., precursor ion scans and neutral loss analysis, only the selected ions can be monitored. In a situation where the mass of the modified target is known, precursor scan technique can be utilized to monitor only, for example, phosphopeptides in the sample.[117,118] The phospho-sites can also be further chemically modified to help the detection of the phosphorylation event, *e.g.*, the technique used by Steen *et al.*[119] utilizes β-elimination reaction to the phosphorylated serine and then the following Michael addition reaction adds 2-dimethylaminoethanethiol to the modified serine. Oxidation of this product with H_2O_2 creates a 2-dimethylaminoethanesulfoxide derivative,

which fragments into a sulfenic acid derivative when using low energy collision-induced dissociation and a specific signal is seen at *m/z* = 122.06. With the subsequent use of precursor ion scanning, the limit of detection can be lowered from the traditional MS-measurements. As an alternative, neutral loss analysis exploits the lability of the PTMs and yields the m/z-ratio of the modified, *e.g.*, phosphopeptide in the first MS, whereas as a result of the neutral loss of H_3PO_4, the second MS indicates the modification site as well as sequential information of the amino acid chain.[120] The quantification of the PTM substrates can be achieved by adding stable isotopes *in vivo* (AQUA)[121] or labeling the sample chemically *in vitro* at residues, such as lysine (SILAC, iTRAQ) or cysteine (ICAT).[121–124]

An essential part of the MS analysis is the ionization of the sample molecule. The ionization methods traditionally applied to protein samples are matrix-assisted laser desorption (MALDI) and electrospray ionization (ESI). MALDI is a soft ionization method that usually creates single-charged particles.[125–127] The protein or peptide is co-crystallized onto a metal surface with a compound having a high UV-absorption capability. The matrix surface is then bombarded with a UV-laser that creates the charged molecules without significant fragmentation. MALDI covers molecular weights ranging from 300 to 200 000 Daltons, which makes it suitable for protein PTM detection and furthermore, it can be applied for protein mixtures and other biomolecules. The drawback of MALDI is that it is challenging to couple directly with liquid chromatography, although LC-MALDI applications have been developed.[128] Alternatively, electron spray ionization (ESI) is preferred if the chromatographic separation, e.g. HPLC, is to be directly coupled with MS. ESI is a widely used ionization technique where a high voltage is applied to a liquid sample to create an aerosol. The ions created from the spray by the combination of desolvation and coulombic repulsion are drawn into the mass spectrometer due to difference in voltage between the capillary and the entrance to the mass spectrometer. The ion produced are often multiply charged. ESI has been widely used for peptide sequencing and for PTM-identification.

Traditional mass spectrometry has its limitations when it comes to high throughput screening of inhibitors for enzyme-catalyzed PTMs. Even though it is possible to monitor multiple different PTMs in a single analysis, and MS is the only method for PTM analysis that is able to detect all PTMs, screening of compound libraries is complicated. Moreover, the substrates, albeit modified in their natural environment, *i.e.*, cells, need to be purified with, for example, gel electrophoresis. Although MS-based screening techniques have developed during the last decade, relatively concentrated samples are usually required.[129,130] Since the MS analysis step of the assay is relatively fast and the purification of the sample is time consuming, higher throughput LC-MS analysis can be achieved with integrated systems for sample preparation and rapid chromatographic separation, such as Rapidfire. Even,

when coupled to a fast chromatography system, the throughput of MS is not as high as it is with standard luminescence-based assays. Estimates suggest that the throughput is only one-fifth.[131] Besides fast chromatography equipment, immobilization of the modified substrates onto a surface has been reported in combination with a MALDI-type of MS analysis. In this SAMDI-MS (self-assembled monolayers for matrix-assisted laser desorption/ionization MS) approach, polyhistidine-tagged protein samples self-assemble upon a surface and can be analyzed to determine whether a PTM has occurred.[130] The authors estimate that 1000 inhibitors can be screened in a day with this technique, which unfortunately, is far less than with a standard luminescence assays even though the progress with HTS-compatible MS-analysis is promising.

2.3.2 Radiometric assays

Radiometric assays for PTM-detection are performed by measuring the radiation of isotope-labelled phosphate-groups that are conjugated to peptides by enzymes. The assays rely on cofactors, *e.g.,* ATP or acetyl CoA, which are labeled with a radioisotope, usually an isotope of phosphorus. Therefore, only additions of PTMs can be monitored. Kinases are the main targets of this assay type since nucleotides, such as ATP and GTP, are easily radiolabeled with ^{32}P or ^{33}P.[132] In radiometric assays, the enzyme modifies the substrate with the radiolabeled cofactor and the, *e.g.*, phosphorylated peptide is then separated from the solution, *for example,* utilizing streptavidin-coated microtiter plates and biotinylated substrates. The excess unbound labeled cofactor is washed from the microtiter wells and the amount of radioactivity measured corresponds to the quantity of phosphorylated residues.

Scintillation proximity assay (SPA) is a wash-free radiometric technique where microbeads carrying scintillant are used.[133] The idea behind the assay is similar to the Förster resonance energy transfer (FRET) principle, where the short distance between two molecules is required to provide a readable signal. In FRET-assays, the signal is luminescence, *i.e.*, UV-VIS light, but in SPA the β-particles released by, *e.g.* ^{33}P, induce the light emission in scintillant material but in contrast to distance required in FRET (< 10 nm), distances in SPA range over one micro meter (Fig. 8). As the radiolabel-carrying substrate molecule binds onto the surface of the SPA bead, for example utilizing biotin-streptavidin-binding,[134] the β-radiation from the decay of the radioisotope stimulates the scintillants to emit detectable light. The assay does not require any wash steps, because only the beads with bound substrate emit the light. SPAs are typically measured in a microtiter plate format.

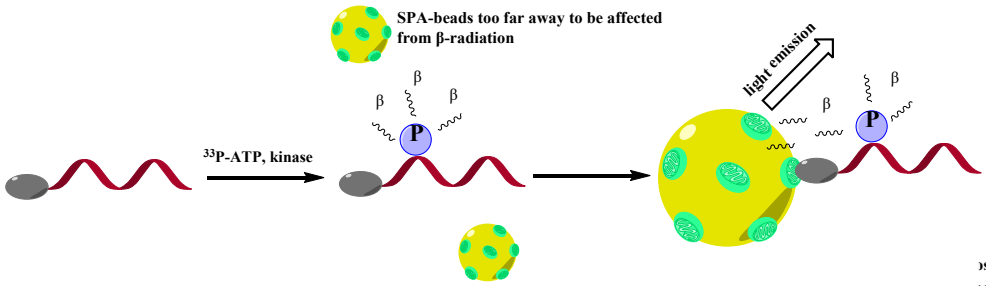

Figure 8. Principle of scintillation proximity assay (SPA). A biotinylated peptide substrate is modified with ^{33}P-labeled phosphate and binds to a streptavidin-coated microbead carrying the scintillant. The radioactive decay of ^{33}P near the scintillant induces a light emission that can be measured. The unbound ^{33}P-ATP does not induce any light emission due to the short distance travelled by β-particles.

The disadvantages of radiometric assays arise from the radioactive waste, which has to be carefully disposed, and the limitation of the modifications that can be studied. Moreover, the potential washing steps complicate the assays and the assays cannot be performed on a coated plate due to side reactions with the immobilized proteins. In addition, the shelf life of the isotopes is limited and dependent on the isotopes used. The advantage of radiometric assays is the high sensitivity. Utilizing SPA, a high sensitivity can be reached but the waste disposal remains a problem. Furthermore, only limited PTMs can be studied.

2.3.3 Luminometric assays

Most HTS-assays developed for inhibitor or activator screening of PTM-catalyzing enzymes use luminophores. Luminophores are molecules that can be excited with a specific wavelength of light and the energy transition from the excited state to the ground state releases a photon with a wavelength specific to the molecule and physiochemical conditions, such as pH.[135] Luminescence can be observed from organic molecules containing aromatic groups or conjugated C-C double bonds, or chelated lanthanide metal ions, such as Eu^{3+} or Tb^{3+}. A number of small organic molecules have been harnessed for commercial luminometric assays, such as fluorescein, Nile Red, Sypro Orange, rhodamine and cyano dyes. Large biological molecules can also be used such as, green fluorescent protein (GFP).[136] The advantage of the small organic molecules is that conjugation to large biomolecules is less likely to affect the biological functions compared to the large protein tags. On the other hand, the advantage of luminescent proteins is that they can be expressed within cells and fused with other proteins. This review of the luminometric assays employed in inhibitor screening of PTM-catalyzing enzymes focuses on *in vitro*-assays.

The most frequently used lanthanide ions in luminometric assays are Eu^{3+} and Tb^{3+}. The ions are always chelated by an organic ligand in order to collect the

excitation light more efficiently. The advantage of Ln(III) chelates are related to their long lifetime and time-resolved (TR) measurement detection mode. The millisecond-scale lifetimes of the lanthanide chelates allow delayed and gated measurements where after the initial excitation of the chelates, the luminescence signal is measured in microsecond time-scale. The TR-mode prevents the measurement of autofluorescence and scattering signals due to the long half-life (μsec-msec) of the Ln(III) chelate luminescence compared to the short nanosecond half-life of naturally occurring luminescence originating from assay compounds, used plastics, and optical components.[137] Moreover, lanthanide chelates have an apparent large Stokes shift, *i.e*. there is a minimal or no spectral overlap between excitation and emission wavelengths, which improves the reliability of the observed signal. Ln(III) chelates have a relatively low quantum yield, usually below 15 % even though over 80 % quantum efficiency has been achieved with some chelates.[138] Nonetheless, due to the time-gated measurements, high sensitivity is still achieved. Overall, the sensitivity of assays that utilize Ln(III) luminophores is higher than assays with organic luminophores. The sensitivity of the luminometric assays have brought numerous commercial assays to the market, and both heterogeneous and homogeneous methods have been developed and many of them utilizes antibodies as molecules that recognize and bind to different PTMs. Antibody-free techniques have also been developed and next, some of the most used techniques are introduced, in brief.

Heterogeneous luminescence assays are techniques where a separate surface is used to capture the analyte from the solution. Thereafter, a washing (separation) step is performed and the unbound molecules are removed. Heterogeneous luminescence assays are laborious but very sensitive due to the washing step, as the unbound molecules are removed reducing the background signal. Enzyme-linked immunosorbent assay (ELISA) and dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) are heterogeneous assays that can be utilized in PTM-detection.[139,140] The modified peptide substrate is usually biotinylated and therefore, binds to the SA-coated microtiter plate while all the unbound reagents are washed away. A labeled antibody is brought to the microtiter wells and it recognized the PTM in the bound peptide substrate while the excess antibody is again washed from the plate. The quantity of labeled antibodies correspond to bound modified substrates and the signal obtained from the well represents the substrate modification. Another option for the microtiter well coating is capture antibodies that bind a PTM-recognizing antibody. ELISA and DELFIA are similar and the main difference is the type of "sandwich" that is built on a surface. ELISA is an enzyme-tag technique that normally utilizes antibody-coated microtiter plates that recognize the PTM-carrying substrate (Fig. 9A). A detection antibody that carries an enzyme-tag, brought into the well binds to the substrate and after washing, the luminescence signal can be measured from the substance formed after the addition of the enzyme substrate.

ELISA is one of the most widely used immunoassays. DELFIA is a trademark from Perkin Elmer that utilizes a non-luminescent Ln(III)-chelate in antibody-labeling and exploits a specific luminescence enhancer solution in the detection. The enhancer solution dissociates the non-luminescent Ln(III) ion from the chelate, and after the dissociation, the ion is rechelated in the enhancer solution into a highly luminescent Ln(III)-chelate (Fig. 9B). The substrate peptide is biotinylated and the plate is coated with streptavidin, which is less expensive than antibody-coating. In addition, time-resolved fluorescence (TRF)-based ELISA has been developed by Perkin Elmer that utilize the full ELISA technique but instead of enzyme-tag, a Ln(III) chelate is conjugated to the secondary antibody.

The heterogeneous luminescence-based PTM assays are very sensitive, especially when using lanthanide labeling, since there is practically no autofluorescence. However, the disadvantage of, *e.g.*, traditional ELISA is the cost of antibodies. In addition, the multiple washing steps make the assays unsuitable for HTS.

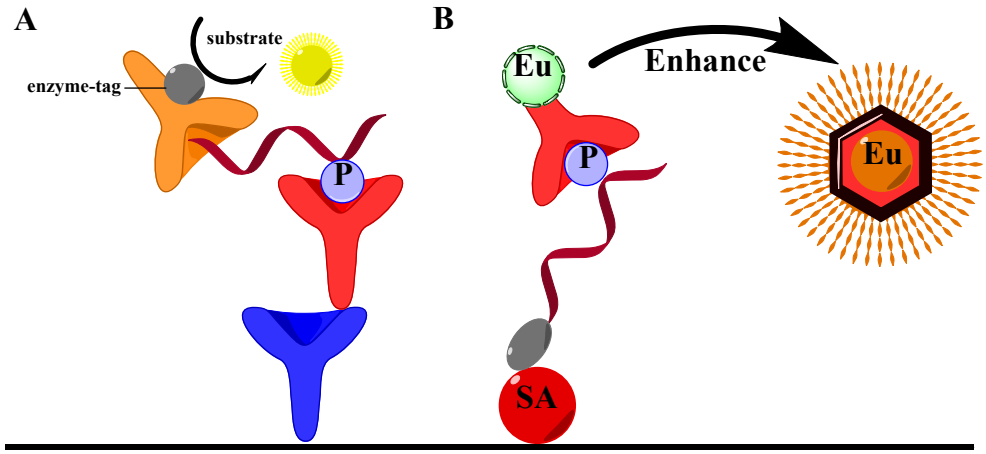

Figure 9. The principle of heterogeneous ELISA and DELFIA. A) In ELISA, the capture antibody binds a PTM-recognition antibody that recognizes the PTM. A secondary antibody carrying an enzyme-tag binds to the same PTM. After each component addition, the plate is washed to remove the excess reagents. Finally, after the enzyme-tagged antibody addition and washing step, the enzyme substrate is added and the product of the enzyme reaction is luminescent. The luminescence intensity corresponds directly to the concentration of the PTM. B) In DELFIA, the streptavidin-coated microtiter plate binds the biotinylated peptide substrate. The modified peptide allows the binding of Eu(III)-labeled antibody, and after washing steps, the bound Eu(III) is dissociated from the chelate within an enhancer solution and chelated with solution components, providing a sensitive phosphorylation detection platform with low background signal levels.

Homogeneous luminescence assays are techniques where every component of the assay is added to a single well followed by luminescence readout without any separation steps. These types of assays are further divided here into different subcategories based on the labeling of assay molecules.

Luminescent enzyme-based assays: Many nucleotide monitoring-based assays are so called luminescent enzyme-based assays where labeling of molecules is not required. Typically, luciferase enzyme is utilized to produce the light after series of reactions. There are many commercial assays available with trade names such as NAD/NADH Glo™ and PhosphoWorks.[141] The principle of these types of assays is to monitor the changing concentration of nucleotides during the enzyme-catalyzed PTM reaction and the assays are therefore suitable for, *e.g.*, kinases and ADP-ribosyltransferases. As an example, the NAD/NADH-Glo™ assay measures the changing concentration of NAD+ in the reaction solution by using a multienzyme setup (Fig. 10). Here, the Ultra-Glo recombinant luciferase enzyme emits light, the intensity of which is proportional to the concentration of NAD+ in the solution. An ADP-ribosyltransferase activity, for example, can be monitored observing the decrease of NAD+ after the enzymatic reaction.

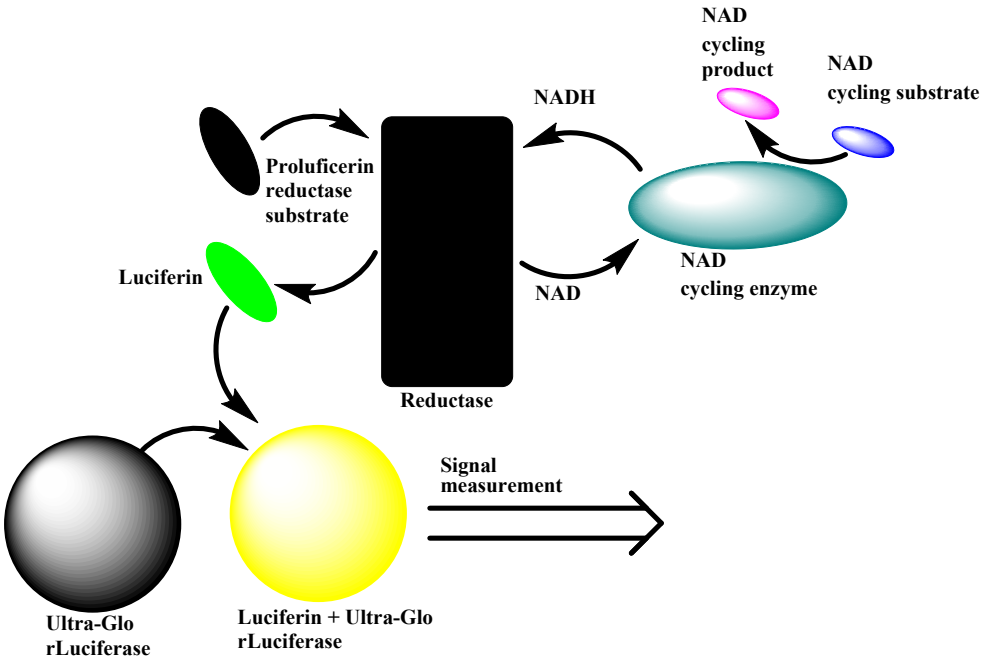

Figure 10. The principle of the NAD/NADH Glo assay. NAD cycling enzyme uses NAD to provide NADH for a reductase enzyme, which produces luciferin. Luciferin is the substrate for Ultra-Glo rLuciferase, which is highly fluorescent after binding of luciferin. The concentration of NAD is directly proportional to the amount of fluorescence monitored.

Single-label homogeneous luminescence assays: Homogeneous luminescence assays are techniques where a single assay molecule is labeled and the intensity of the luminescence is monitored. Therefore, the emission of the luminophore is typically environment sensitive and, for example, quenchers are often used. Pyrene fluorescence

has been used to study the phosphorylation and dephosphorylation of a peptide substrate.[142–144] For a tyrosine kinase PTM assay, a pyrene or tryptophan molecule was placed in the peptide sequence which fluorescence is quenched by the unmodified tyrosine residue. After the phosphorylation, an increased fluorescence intensity is detected. The mechanism of the increase in fluorescence signal was studied extensively and it was found the tyrosine quenches the pyrene/tryptophan fluorescence without other interactions, such as π–π-interaction between the aromatic ring and fluorophore.[144] The pyrene-fluorescence assay was further applied to serine kinase PKA. The serine residue has no aromatic moieties so an additional quencher molecule is therefore needed. In this Deep Quench technique, the quencher interacts with both serine and pyrene, and quenches the pyrene fluorescence effectively. Due to phosphorylation of serine and the addition of the phosphoserine binding domain 14-3-3τ, the quencher interaction is disrupted and the fluorescence intensity increases.[145]

The chelation-enhanced fluorescence assay (CHEF) utilizes a Mg^{2+}-chelating luminophore, sulfonamide-oxine (SOX), which is attached to the peptide substrate near the phosphorylation site in kinase assays.[146] Upon phosphorylation of the peptide substrate, Mg^{2+}-ion is chelated by the phosphate-group and SOX. The electronic structure of SOX is different whether an Mg^{2+} is coordinated to it. This change in electronic states will cause SOX to become highly fluorescent while coordinated to Mg^{2+} (Fig. 11). The assay is commercially available for nearly 400 wild-type kinases. Despite the well-developed SOX-assays, there are only few assays available, which use time-resolved luminescence in PTM detection. The CHEF assay has been applied successfully in a time-resolved mode, where Eu^{3+}-ions are used instead of Mg^{2+} ions, for endpoint measurements. In addition, Tb^{3+} has been used in tyrosine kinase activity monitoring.[147]

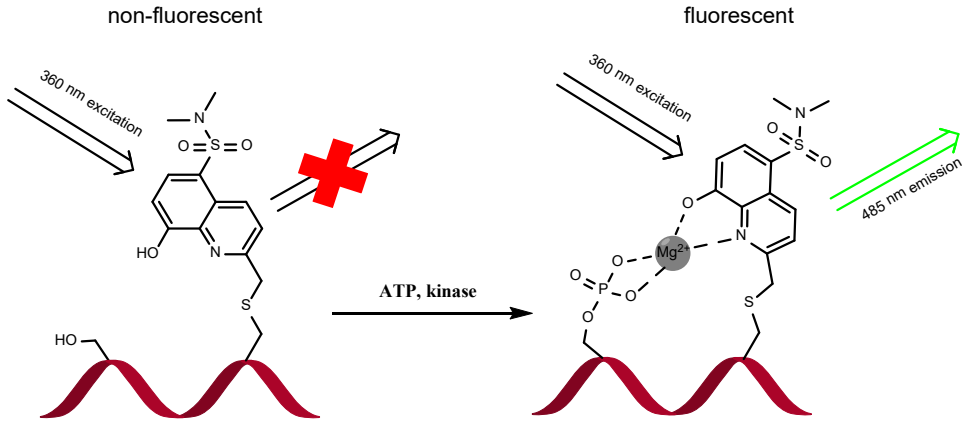

Figure 11. The principle of the CHEF assay. The peptide substrate with SOX has a low fluorescence emission, but due to phosphorylation followed by the Mg^{2+} chelation, an increase in fluorescence intensity is achieved.

Fluorescence polarization (FP) assays measure the change of polarization of the light between the excitation light and emitted light.[148] Small molecules rotate faster than large molecules. When a small molecule binds to a large molecule, the apparent rotation of the small molecule decreases. This is the principle of a FP assay when the small molecule is labeled with a luminophore. As the labeled small molecule is unbound, the plane of polarization from the excitation light to the emitted light changes rapidly due to the fast rotation. This results in a low level of polarization, *i.e.*, the emitted light is not polarized. Upon binding, the rotation of the labeled small molecule slows down and the polarization changes are small resulting in a high polarization degree, *i.e.,* the polarization of the emitted light remains similar to the excitation light (Fig. 12A). The luminophore selected for labeling of the substrate needs to be small to ensure the largest possible change is rotational velocity upon binding of the two molecules. The PolarScreen™ kinase assay, for example, utilizes a luminophore labeled phosphorylated peptide substrate as a tracer molecule in monitoring phosphorylation of added non-labeled peptide substrate.[149] Upon phosphorylation of the non-labeled substrate peptide, the competitive binding of tracer and the phosphorylated non-labeled substrate to the antibody takes place. Due to the high concentration of the non-labeled substrates compared to the tracer, the substrate binds to the antibody and low polarization is therefore observed, whereas high polarization is monitored before the phosphorylation of the substrate. In addition, in an inhibition assay of the kinase enzyme, the tracer binds to the antibody as no substrates can be phosphorylated by the kinase. Antibody-free techniques have also been developed where labeled substrates with a phosphate-group, coordinate to trivalent metal-coated particles. This immobilized metal ion affinity particle (IMAP) technology is well suitable for kinases, phosphatases, and phosphodiesterases.[150–152] Besides trivalent metal ions, cationic polyamino acids, such as polyarginine and polylysine, have also been used to bind phosphate-groups.[153,154] Furthermore, the high affinity of biotin to SA has been utilized in FP assays. The thiophosphorylated peptide substrate can be conjugated with biotin from the thiophosphate-group using iodoacetyl derivative of biotin.[155]

Quenching resonance energy transfer (QRET) is a single-label technique where, *e.g.* one peptide is labeled and the substrate peptide remains unlabeled.[10] QRET measurements monitor the luminescence intensity of the label, which is low when in contact with soluble quencher molecules in the solution. Upon binding of the labeled molecule to another molecule, the luminescent label is protected from the soluble quenchers and an increased luminescence signal is observed. QRET has been utilized for various different assays, and has also been used for PTM-detection in the work presented in this thesis which will be discussed later.[10,156–159]

Dual-label homogeneous luminescence assays: FRET is a technique where two luminophores are in close proximity with each other providing unique and specific

signaling.[160] A donor luminophore can transfer its energy to an acceptor luminophore after excitation when the molecules have overlapping emission and excitation spectra. The FRET efficiency decreases drastically when the distance between the two labels increase, as indicated in the equation 1:

$$E = \frac{1}{1+(r/R_0)^6} \quad (1)$$

where E is the efficiency of the resonance energy transfer process, r is the distance between the donor and the acceptor and R_0 is the Förster distance of the label pair where the efficiency of the energy transfer is 50 %. In practice, the distance of FRET is typically efficient up to a distance of 10 nm.[161] The principle behind FRET requires that once the donor molecule is excited, this energy is transferred to the acceptor molecule. The transferred energy causes the acceptor to become excited and the emission of the acceptor is then measured at a specific wavelength (Fig. 12A). Therefore, FRET can be used to study the binding of two label-conjugated molecules. FRET can also be used to study the interaction of two molecules with a decreasing signal output utilizing quenchers.

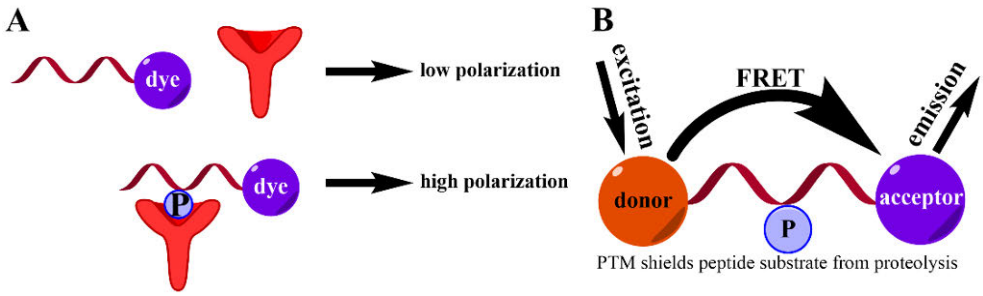

Figure 12. Principles of fluorescence polarization and Z'-LYTE kinase FRET assays. A) Fluorescence polarization assay measures the change of polarization of the luminophore emission. The small, labeled peptide substrate rotates rapidly due to its size and the polarization changes constantly. Due to the PTM, the antibody is able to bind with the peptide causing a decrease in the rotation of the peptide, thus the polarization of the luminophore does not change significantly between excitation and emission, and the PTM can be detected. B) Donor dye molecule is excited with a light pulse. The emission of the donor overlaps with the excitation of the acceptor dye molecule, thus enabling resonance energy transfer over a short distance. The emission of the acceptor dye is possible to be monitored as the phosphorylation shields the peptide substrate from proteolytic cleavage. Without phosphorylation, the peptide is cleaved and no FRET-signal is observed.

FRET is easily adapted into TR-mode by using a Ln(III)-chelate as the donor. FRET and TR-FRET are utilized in many commercial kits, *e.g.*, Z'-LYTE, LANCE (lanthanide chelate excitation), HTRF (homogeneous time-resolved fluorescence), and KinEASE-TK kinase assays.[162–165] In the Z'LYTE kinase assay, a peptide

substrate carrying terminal coumarin (donor) and fluorescein (acceptor) is phosphorylated, which prevents the digestion of the peptide substrate by a protease. Therefore, FRET-signal is observed with a phosphorylated peptide, while the non-phosphorylated peptide will be cleaved, resulting in low FRET-signal (Fig. 12B). In LANCE and HTRF kinase assays, the donor molecule is a Eu(III)-labeled antibody that recognizes the phosphorylated substrate. The peptide substrate carrying the PTM has an organic dye as an acceptor molecule and high a TR-FRET-signal is measured with the PTM-carrying peptide.

A similar technique to FRET assays is the ALPHA kinase assay (amplified luminescent proximity homogeneous assay), where instead of direct energy transfer from donor to acceptor, the excitation of the donor bead creates reactive singlet oxygen, which travels to the acceptor beads inducing chemiluminescence in molecules on the acceptor bead.[166] The chemiluminescence emission excites the molecules in the photosensitive acceptor bead and the luminescence emission is measured. Even though the technique is very similar, ALPHA assays are not FRET assays. There are currently two different ALPHA assays, ALPHAScreen and ALPHALisa, with rubrene emission and TR method with Eu(III) emission, respectively. The principle of the APLHA kinase assays is that the peptide substrate, labeled with a donor bead, is phosphorylated. A Lewis metal chelate (LMC) or antibody coated bead can then bind to the phosphorylated substrate and the excitation of the donor creates singlet state oxygen molecules that can travel up to 400 nm and upon reaching the PTM-bound bead, the reaction between the oxygen and thioxene derivative creates chemiluminescence. The chemiluminescence emission excites the luminophores in the acceptor bead and the emission can be detected at 520–620 nm with ALPHAScreen assay and at 615 nm with ALPHALisa assay (Fig. 13).

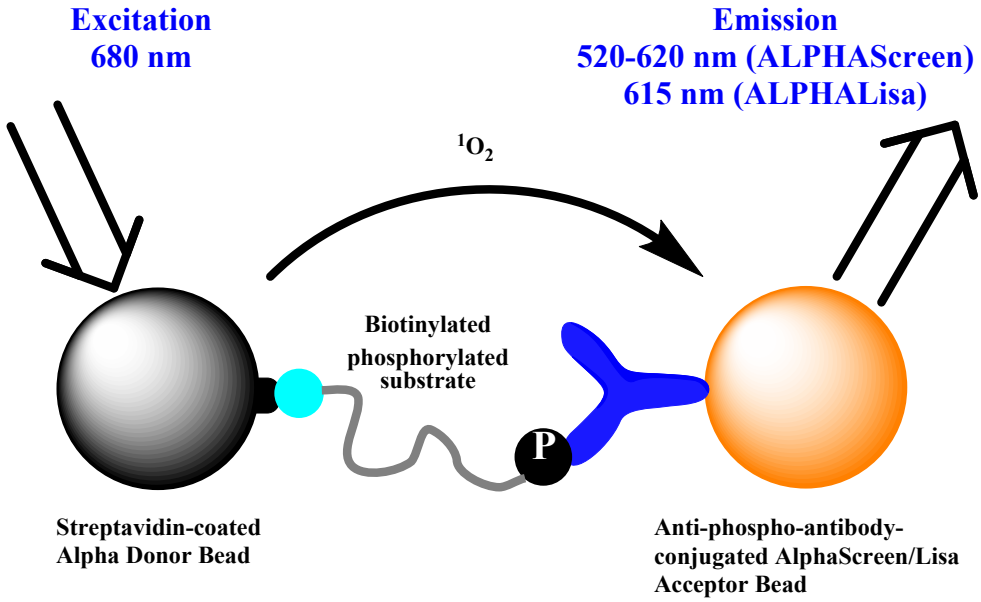

Figure 13. The principle of ALPHA kinase assays. The phosphorylation of the donor bead-labeled peptide substrate allows the binding of the acceptor bead to the peptide. Excitation of the Alpha Donor bead creates reactive oxygen species that can travel to acceptor bead and induce chemiluminescence in the thioxene derivatives. Subsequently, the chemiluminescence excites the luminophores within the Alpha acceptor bead and the luminescence is monitored.

FI assays are simple techniques that do not require washing steps. Therefore, homogeneous assays are fast and easily applicable to HTS. However, the nucleotide detection assays require expensive secondary enzymes or antibodies. Furthermore, the multienzyme methods or techniques that utilize other proteins than the PTM-catalyzing enzyme are prone to interferences. Most of the available FI-assays rely on organic luminophores and therefore, the sensitivity of the assays is poor due to the autofluorescence. The advantage of TR-mode is the increased sensitivity of the assays but there are not many such assays available. The FP assays are simple wash-free single-label assays but the sensitivity of FP is poorer than, e.g., ALPHA or TR-FRET assays. The downside of many FP assays is the requirement of antibodies. In addition, the alternative techniques that do not use antibodies, *e.g.*, IMAP, polyamino acid-based and biotin-SA-based methods, also have their weaknesses. IMAP is not compatible with substrates or other molecules that are highly negatively charged, or substrates that are prephosphorylated which limits the method functionality. Furthermore, applying polyarginine in the polyamino acid-based method suffers from decreased enzymatic activity and precipitation of biopolymer with high concentrations of negatively charged molecules, such as EDTA or ATP that are typically present in kinase assays.[154] Finally, the biotin-SA method required ATPγS

which most kinases do not recognize as ATP, therefore limiting the use of this technique.[148] Consequently, the biotin-SA method, as well as IMAP and polyamino acid-based methods, are only suitable for kinases, phosphatases, and phosphodiesterases, thus limiting the wider use of these methods.

Dual-label assays are sensitive due to low background signals. TR-mode allows even more sensitive systems and FRET and ALPHA assays are easily HTS-applicable. The dual-label system is labor-demanding due to the labeling of two molecules, or, as in the case of ALPHA assays, coating beads typically with antibodies.

2.4 Concluding remarks

Peptides have broad physical and chemical properties. This enables their use in a range of applications from protein mimicking structures to small dipeptide sensors that respond to many environmental changes. Thus, peptides are a versatile group of molecules when developing detection systems for biomedical applications.

In drug screening and development, the available methods dictate the success of the research. Large libraries of potential drug molecules require efficient techniques to reveal the potential drug candidates. The most suitable methods for screening millions of molecules are user friendly, *i.e.*, the methods are simple one-pot assays without any work-intensive steps, such as washing steps. Furthermore, the assay instrumentation must be affordable and easily accessible for researchers everywhere and this also applies to reagents required to conduct the assays. In the end, the most important aspects in the drug-screening assays are the robustness, sensitivity, and the cost of assay. Low concentration of analytes and reagents typically also provide high assay sensitivity leading to less expensive assay platforms. The scientific community has created a vast selection of different techniques from which the user can select the most suitable method. However, when considering the abundance of different enzyme-catalyzed PTMs, some techniques are much more suitable than others.

Methods, such as radiolabel or heterogeneous luminescence-based assays, are labor-intensive. The problem with these types of assays, albeit having high sensitivity, is the low throughput in screening due to washing steps. Radiometric assays also suffer from the radioactive waste produced. Other methods, *e.g.* MS, are simple to operate but even though there has been significant method development to improve the throughput, it is not yet a close competitor for luminometric techniques. In addition, the concentrations of the reagents needed for MS analysis tend to be much higher than those used in luminometric assays unless a very specialized instrumentation is at hand. Another downside many of the introduced methods is the limitations on modifications that can be studied. Radiometric assays can be utilized

only for kinase studies since the radiolabel occurs in the cofactor, such as ATP. The wash-free SPA-assay is HTS-applicable, but suffers from the same limitation.

Many different luminometric assays can be applied for drug molecule screening. With the exception of heterogeneous methods, *e.g.*, Delfia, most of the techniques are HTS-compatible. Nevertheless, the sensitivity of the heterogeneous assays makes them powerful tools. The greatest flaw that many luminometric assays have is their applicability for only a few PTMs. Assays such as pyrene fluorescence assays, CHEF, or many FP assays, can only be used to screen inhibitors for kinases, phosphatases, and phosphodiesterases. In addition, assays that rely on nucleotide-detection function only with enzymes, such as kinases, that require nucleotide cofactors, for example, ATP. Multienzyme platforms possess a high risk of interferences, *e.g.,* unspecific binding events. Majority of all the assays, *e.g.*, heterogeneous luminescence-based ALPHA, HTRF, and LANCE, utilize antibodies that recognize the modified substrate. Furthermore, several luminescence-based techniques as wells as radiolabel-assays for kinases are only functional with relatively low concentrations of ATP and tend to not work with cellular levels of ATP. This causes a high number of ATP-competitive inhibitors to be considered as hits, which do not necessarily function in physiological conditions where the concentration of ATP is 1–5 mM. Nonetheless, the potential lead molecules can still be discovered using these types of assays. Methods discussed in the literature review are compared with each other in the Table 2. Direct measurement of PTM is defined here as measuring the direct effect the PTM-formation induces.

Table 2. Comparison of different PTM-detection techniques discussed in this thesis.

Method	Multiple PTM detection	HTS-compatible	Antibody-free	Direct
MS	YES	YES*	YES	YES
Radiolabeling	NO	NO	YES	YES
SPA	NO	YES	YES	YES
ELISA	YES#	NO	NO	NO
DELFIA	YES	NO	NO	YES
Luminescent enzyme-based assays	NO	YES	YES	NO
Pyrene fluorescence	NO	YES	YES	YES
CHEF	NO	YES	YES	YES
FP	YES#	YES	YES*	YES
HTRF/LANCE/ Lantha Screen	YES#	YES	NO	YES
ALPHA	YES#	YES	NO	YES
Peptide-Break	YES	YES	YES	YES
Single-peptide TR-FRET	NO	YES	YES	YES

*only in highly specialized situations

#possible after development of specific antibodies

New enabling assays are needed for the drug discovery. The luminescence-based assays are excellent alternatives today, but the use of antibodies and limited PTMs measurable in a single assay platform increase the cost and workload of drug screening. Universal homogeneous assays with the sensitivity of heterogeneous luminescence assays are in demand as they have high sensitivity and the luminescence is possible to be read with a standard plate reader without labor-heavy steps. For the purposes of efficient, low-cost drug screening, the techniques and instrumentation used is of high importance. Currently, QRET coupled with the Peptide-Break technology, which was one of the main research topics of the work presented in this thesis, has the potential to become a universal, luminescence-based inhibitor screening platform for enzyme-catalyzed PTMs.

3 Main aims of the study

As the interest of pharmaceutical industry, as well as the academia, has increased towards post-translationally modifying enzyme-targets, the demand for new techniques to screen inhibitors against these targets has risen. There is a need for fast high throughput assays with low material-consumption. Therefore, universal methods with sensitive time-resolved luminescence detection meets these requirements.

The main aim of the work presented in this thesis was to develop a platform for enzyme-catalyzed PTM detection that utilizes peptides as a substrate for enzyme-targets. In addition, this thesis project aimed to develop methods where separation steps are not required during the PTM detection, *i.e.*, the assays developed are homogeneous. The end-result should also be applicable for different types of PTMs, with the sensitivity of the assays maximized with the incorporation of TR-mode with Ln(III)-labels. Peptide-Break technology with QRET were introduced in 2018 from the same laboratory in which the research for this PhD thesis was conducted.[10] The aim of the presented work were to further develop and improve the application of the technique, and advance the peptide-based technologies in drug screening. The original technique introduced the peptide-peptide pairing via leucine-zipper (LZ) formation. This was further investigated with an alternative pairing method allowing the use of more natural amino acid sequences. Thereafter the Peptide-Break was expanded to make screening of inhibitors against more sensitive targets possible allowing the end-user to modify Peptide-Break to meet specific needs. QRET detection coupled to the Peptide-Break technology yields high TRL-signal in the event of peptide duplex formation. Further studies aimed to utilize different signal modulator molecules, enabling the high TRL-signal monitoring from the unbound Eu-peptide. The final aim of the work in this thesis was to develop a system where only single peptide is necessary for PTM detection, allowing the use of most natural amino acid sequences compared to the LZ and CP approaches.

4 Materials and methods

A summary of used materials and methods are represented here in brief.

4.1 Labeling of biomolecules

EuLZ detection peptide used in publications **I-II**, is labeled with a nonadentate (9d) europium chelate {2,2′,2″,2‴-{[4′-(4‴-isothiocyanato-phenyl)-2,2′,6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)}tetrakis(acetate)} europium(III) from QRET Technologies and with heptadentate (7d) isothiocyanate (ITC)-TEKES-Eu(III) chelate (4-[2-(4-isothiocyanatophenyl)- ethynyl]-2,6,-bis{[N,N-bis(carboxymethyl)-amino]methyl}- pyridine) from Perkin Elmer Life Science, Wallac Oy in publication **III**. The labeling was typically performed by dissolving 1 mg of Eu(III)-chelate into 100 µl of water and mixed with 100 µl of EuLZ precursor peptide (0.5 mg) in pyridine/H2O/triethylamine solution. The conjugation reaction was incubated at room temperature (RT) for 18 h and the labeled Eu(III)-peptide was purified with HPLC. After purification, Eu(III)-peptide concentration was determined based on the Eu^{3+}-ion concentration by comparing observed luminescence signal to a commercial Eu^{3+}-standard.

Eu(III)-labeled streptavidin EuSA was used in publication **IV**. 200 µg of streptavidin was diluted in 200 µL 0.5 M carbonate buffer (Na_2CO_3/$NaHCO_3$, pH 9.8) together with 113 nmol µM heptadentate (7d) europium chelate, {2,2′,2″,2‴-{[4-[(4-isothiocyanato-phenyl)ethynyl]pyridine-2,6-diyl]-bis(methylenenitrilo)} tetrakis (acetato)}-europium(III). The reaction was incubated in dark for 1h at RT, then transferred to +4 °C overnight. The reaction was purified with Sephadex column (NAP5) with an elution buffer (1 mM Hepes, pH 8) and the right fraction was determined by UV-light.

4.2 Peptides and quenchers

A total of 16 different peptides, purchased from Pepmic and QRET technologies, were used in this study, the sequences of which are presented in Table 3. The peptides were stored in 50 % DMF in -20 °C, and used as described in the following protocols. Soluble quencher molecules were obtained from QRET Technologies.

Table 3. Peptides used in publication **1–IV**

name	sequence	application	publication
EuLZ	KDTLQAE TDQLEDE KSALQTE IANLLKE KEKLEFIL	detection	I-III
CP1	GGRRRV**S**RRVRRRV**S**RRGG	phosphorylation	I
CP2	GGRRRV**S**RRVRRRV**S**RRVRRR	phosphorylation	I
CP3	GGRRRVRRRV**S**RRVRRRVRRRVRRRV**S**RRVRRRK	phosphorylation	I
CP4	GRSG**R**GKRRSG**R**GKRRR	citrullination	I
CP5	GRSG**R***GKRRSG**R***GKRRR	citrullination	I
CP6	GRAR**K(Ac)**GARRAR**K(Ac)**GARRR	deacetylation	I,II
CP7	GRARKGARRARKGARRR	deacetylation	I,II
CP8	GRART**K**QTRRART**K**QTRRR	methylation	I,II
CP9	GRART**K(Met)**QTRRART**K(Met)**QTRRR	methylation	I,II
LZ-Y	REELRKRRAELRRR**Y**AQLRQRREQLRQR**Y**ANLRKE	phosphorylation	I,II
LZ-pY	REELRKRRAELRRR**pY**AQLRQRREQLRQR**pY**ANLRKI	phosphorylation	I-III
LZ-S	REELRKRRAELRRR**S**AQLRQRREQLRRR**S**ANLRKE	phosphorylation	III
PC10	K(Biotin)KNN LKE**C**GLY	ADP-ribosylation/S-nitrosylation	IV
PC15	K(Biotin)EEDVIIKNNLKE**C**GLY	ADP-ribosylation/S-nitrosylation	IV
RP16	K(Biotin)DGKKKKKKSKTK**C**VIM	S-nitrosylation	IV

4.3 Assay buffer selection

Buffers were selected on the basis on the requirements of the enzymes, keeping the basic buffer components the same, low concentration of HEPES and salts together with detergent Triton X-100. The buffers used in the studies are presented in Table 4.

4.4 Instrumentation and data handling

All Eu(III)-peptide purifications were carried out using reversed-phase liquid chromatography. A Dionex Ultimate 3000 LC -system from Thermo Fisher Scientific, Dionex, was used with Ascentis RP-amide C18 column from Sigma-Aldrich, Supelco Analytical. For publications **I–III**, time-resolved luminescence (TRL) emission signals were measured at 615 nm using excitation at 340 nm. For publication **III**, TR-FRET emission signals were measured at 665 nm, using excitation at 340 nm. All measurements were performed using a standard microtiter plate reader developed by Labrox Ltd or with a Victor 1420 multilabel counter. Emission and excitation spectra for EuLZ and Eu^{3+}-signal-modulating molecules (Dy1−Dy4) were recorded using Varian Cary Eclipse fluorescence spectrophotometer. TRF excitation spectra (200−500 nm) for EuLZ were recorded 615 nm emission. The TRF emission spectra (550−750 nm) for EuLZ were recorded with 340 nm excitation. Fluorescence emission spectra for Dy1−Dy4 were recorded 618 nm (Dy1), 460 nm (Dy2), 447 nm (Dy3), and 550 nm (Dy4) excitation wavelengths. Emission scans for Dy1–Dy4 were performed at 630−850, 475−850, 430−650, and 545−800 nm, respectively. Fluorescence excitation spectra for Dy1−Dy4 were recorded using 700 nm (Dy1), 740 nm (Dy2), 555 nm (Dy3), and 670 nm (Dy4) emission wavelengths, and scanning was performed at 450−680 nm (Dy1), 400−725 nm (Dy2), 400−545 nm (Dy3), and 400−650 nm (Dy4), respectively. The absorption spectra for Dy1−Dy4 were recorded using Cary 60 UV−vis spectrometer. In publication **IV**, EuSA, AlexaFluor and

Table 4. Summary of buffers used in the assays.

name	components	application	publication
Buffer 1	10 mM Hepes (pH 7.5), 0.1 mM EDTA, 0.01% Triton X-100, 5 mM NaCl, and 1 mM $MgCl_2$	kinase and phosphatase assays	I-III
Buffer 2	50 mM Hepes (pH 7.5), 25 mM NaCl, and 0.01% Triton X-100	citrullination assays	I
Buffer 3	10 mM Hepes (pH 7.5), 0.1 mM EDTA, 0.01% Triton, 25 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$	deacetylation assays	I,II
Buffer 4	20 mM Hepes (pH 7.5), 5 mM NaCl and 0.01 % Triton X-100	S-nitrosylation and ADP-ribosylation assays	IV
Buffer 5	500 mM Hepes (pH 8.5)	AlexaFluor labelling	IV
Buffer 6	50 mM Hepes (pH 7), 0.01 % Triton X-100	TR-FRET detection buffer	IV

TR-FRET spectra were recorded using Tecan Spark M20 plate reader. EuSA excitation spectrum (300–500 nm) was recorded with 615 nm emission and emission spectrum (550–800 nm) was recorded with 340 nm emission. AlexaFluor 680 excitation spectrum (550–725 nm) was recorded with 750 nm emission and emission spectrum (625–800 nm) was recorded with 600 nm excitation with 600 µs integration time without delay. TR-FRET emission spectrum (550–800 nm) was recorded with 340 nm excitation. EuSA and TR-FRET lifetimes were recorded with Varian Cary Eclipse fluorescence spectrophotometer. EuSA luminescence emission at 615 nm lifetime was recorded with 340 nm excitation. TR-FRET emission at 730 nm lifetime was recorded with 340 excitation. The luminometric measurements with the NAD/NADH Glo™ kit were performed with a Tecan Spark plate reader. TR-FRET emission signals were measured at 730 nm, using excitation at 340 nm. Delay and integration time are discussed in detail in the publications.

In all assays, the signal-to-background ratio (S/B) was calculated as μ_{max}/μ_{min} and the coefficient of variation (CV%) $(\sigma/\mu)*100$. μ is the mean value of TRL- or TR-FRET-signals, and σ is the standard deviation (SD). Limit of detection (enzyme titration in publication **IV**) was calculated as $3*\sigma_{max}$/slope. Data was analyzed using Origin 8 software and the half-maximal inhibitory concentration (IC_{50}) and the half-maximal effective concentration (EC_{50}) values were obtained using standard sigmoidal fitting functions.

4.5 Assay protocols

All reagent concentrations are reported in the final measurement volume.

4.5.1 Peptide-Break utilizing charged peptides (**I**)

All phosphorylation assays (peptides CP1–CP3 and LZ-S) were performed in Buffer 1, citrullination assays (peptides CP4 and CP5) in Buffer 2, and acetylation assays (CP6 and CP7) in Buffer 3 (Table 4).

Binding assay: 10 µL of peptide substrate (peptides CP1–CP7 and LZ-S, Table 3) (10 nM) were added to the 384 microtiter wells and mixed with 20 µL of EuLZ (10 nM) and incubated for 5 min. Next, 20 µL MT2 was added finalizing the volume to 50 µL. TRL-signals were measured at multiple time points for 60 min.

Single inhibition-point enzymatic phosphorylation assay: 4 µL of peptide substrate (CP1–CP3 or LZ-S) together with ATP (10 nM and 50 µM) were mixed with 2 µL of inhibitor H-89 (2 µM), 4 µL of protein kinase A (PKA) (0.5 nM) in the 384 microtiter wells, and incubated for 30 min. Then, 20 µL EuLZ (10 nM) and 20 µL MT2 were added, and after 5 min incubation, TRL-signals were monitored as previously.

Dose-response assay: 4 µL of peptide substrate CP1 together with ATP (10 nM and 50 µM) were mixed with 2 µL of staurosporine (0–5 µM) or H-89 (0–10 µM), 4 µL of protein kinase A (PKA) (0.5 nM) or PIM1–PIM3 (2 nM) in the 384 microtiter wells, and incubated for 30 min. Thereafter, 20 µL EuLZ (10 nM) and 20 µL MT2 were added, and after 5 min incubation, TRL-signals were monitored as previously.

4.5.2 Thermal Dissociation Peptide-Break (**II**)

Binding assay: The peptide substrate (LZ-Y, LZ-pY, and CP6–CP9) (10 µM) was added in 20 µL to the wells, followed by 30 µL of the detection solution containing EuLZ (1 nM) and quencher MT10. The plate was then heated for 3 min before TRL-measurements, which were performed at 5 °C intervals. TRL-signals were monitored until the TRL-signal reached the level of the plate background signal.

Single inhibition-point enzymatic assays: 5 µL of inhibitor (1 µM Na_3VO_4 for PTP1B and 1 µM TSA for HDAC) were added to a well and mixed with 5 µL of the corresponding enzyme (1 nM). Next, LZ-pY or CP6 (10 µM) were added and incubated for 30 min with PTP1B and 45 min with HDAC. After incubation, 20 µL of the detection solution containing 1 nM EuLZ and the soluble quencher MT10, were mixed with the reaction and incubated for 5 min. Heating and TRL-measurements were performed as described previously for the binding assay.

Dose-response assays: 5 µL of inhibitor (0–1 µM Na_3VO_4 for PTP1B and 0–1 µM TSA for HDAC) were added to a well and mixed with 5 µL of the corresponding enzyme (1 nM). After this, the assay was performed as previously described in single inhibition-point enzymatic assays.

4.5.3 Peptide-Break tunability with signal modulators (**III**)

Dose-response assays: PKA (5 nM) was incubated with LZ-S (100 nM) in Buffer 1 together with the inhibitor staurosporine or H89 (0–25 µM). The enzymatic reaction was initiated with 50 µM ATP and performed in 7.5 µL. Similarly, PTP1B (5 nM) was incubated with LZ-pY (100 nM) together with the inhibitor Na_3VO_4 (0–10 µM). 2.5 µL of the enzymatic reactions were added to the plate after 60 min incubation at RT and thereafter, EuLZ (5 nM) and soluble signal modulator/quencher molecule Dy1 (3–4 µM), Dy2 (25–100 µM), Dy3 (200–250 µM), or Dy4 (3–4 µM) was added to reach the final volume of 20 µL. After 15 min incubation at RT, Eu-signals at 615 nm and TR-FRET-signals at 665 nm were monitored.

4.5.4 A single-peptide TR-FRET detection platform (**IV**)

Assay optimization: 10–40 nM of the peptide substrate PC15 (20 µL) in Buffer 4 was mixed with 2 µL of AlexaFluor 680 (0–500 nM) in Buffer 5 and incubated for 90 min. After this, 5 µL of the reaction was mixed with 10 µL of EuSA (1–10 nM) in Buffer 6 in the microtiter wells and incubated for 5 min before measuring the TRL- and TR-FRET-signals at 615 nm and 730 nm, respectively.

S-nitrosylation: The peptide substrates (PC10, PC15, and RP16) (10 nM) were mixed with SNOG (0–3 mM) in 20 µL and incubated for 60 min in Buffer 4. Thereafter, 2 µL of AlexaFluor (100 nM) was added in Buffer 5 and the reaction was incubated for 90 min followed by the mixing of 5 µL of the reaction with 10 µL of EuSA (2.5 nM) in Buffer 6. The mixture was incubated, and the TRL- and TR-FRET-signals were measured as described previously.

Enzymatic assays: Peptide substrate PC15 (10 nM) was mixed with enzyme (wt or mutant) (0–1 µM), NAD+ (0–30 µM), and an inhibitor, nicotinamide (0–500 mM) in 20 µL and incubated for 60 min at room temperature. Next, the addition of 0.5 µL of AlexaFluor 680 dye (100 nM) was followed by 90 min incubation. Then, 10 µL of EuSA (2.5 nM) was mixed with 5 µL of the incubated enzymatic reactions media, incubated for 5 min before measuring the TRL- and TR-FRET signals at 615 nm and 730 nm, respectively.

5 Results and discussion

The background of this doctoral thesis lays in the development of Peptide-Break technology coupled with QRET detection.[10,157,167] Peptide-Break technology provides a homogeneous HTS assay platform in which a multitude of PTMs can be studied for inhibitor screening in drug discovery. As discussed in previous chapters, there is a growing need for assays to discover the novel potential drug candidates with inexpensive, sensitive, and universal methods to improve the whole drug screening process. In the work presented in this thesis, the Peptide-Break technology is improved to meet the needs of end-users by making the substrate design more user-friendly, and expanding the applicability of Peptide-Break to enzyme-targets that have low modification activity, or low affinity towards the substrate, and allowing the TRL-signal readout direction selection (publications **I–III**). Finally, a single-peptide detection technology is introduced for cysteine-specific PTMs, which further simplifies the substrate design compared to Peptide-Break technology, therefore creating an alternative sensitive platform for drug discovery (publication **IV**).

5.1 Peptide-Break technology for detection of PTMs

Peptide-Break technology provides a detection platform for screening inhibitors of PTM-catalyzing enzyme targets, such as, kinases and phosphatases. The Peptide-Break coupled with QRET-detection allows the inhibitor screening for multiple enzyme-catalyzed PTMs in a rapid, cost-effective, and homogeneous assay. The principle behind Peptide-Break is that a heptad repeat peptide substrate, which consensus sequence is derived from the natural protein substrates and embedded to the LZ-structure. The peptide substrate is manufactured with leucine residues in *d*-positions, allowing the formation of a LZ structure with a Eu(III)-labeled detection peptide (Fig. 14A). The target enzyme post-translationally modifies the substrate peptide and therefore, disrupts the LZ-formation with the Eu(III)-labeled detection peptide. The unmodified peptide substrate binds to the detection peptide and thus, the peptide-peptide structure protects the Eu(III)-label from the soluble quencher molecules. Alternatively, without the LZ-structure formation the luminescence from

the unbound Eu(III)-peptide is effectively quenched (Fig. 14B). The protected Eu(III)-label due to being shielded by the duplex structure, can be used to study the activity of the enzyme. Therefore, drug molecules affecting the activity of an enzyme can be screened. The sensitivity of Peptide-Break technology is excellent and low nanomolar target and substrate concentrations can be used in the assays. Moreover, due to the homogeneous nature of the assay format and the universal applicability to theoretically all PTMs, Peptide-Break allows the screening of inhibitors in HTS-format.[10]

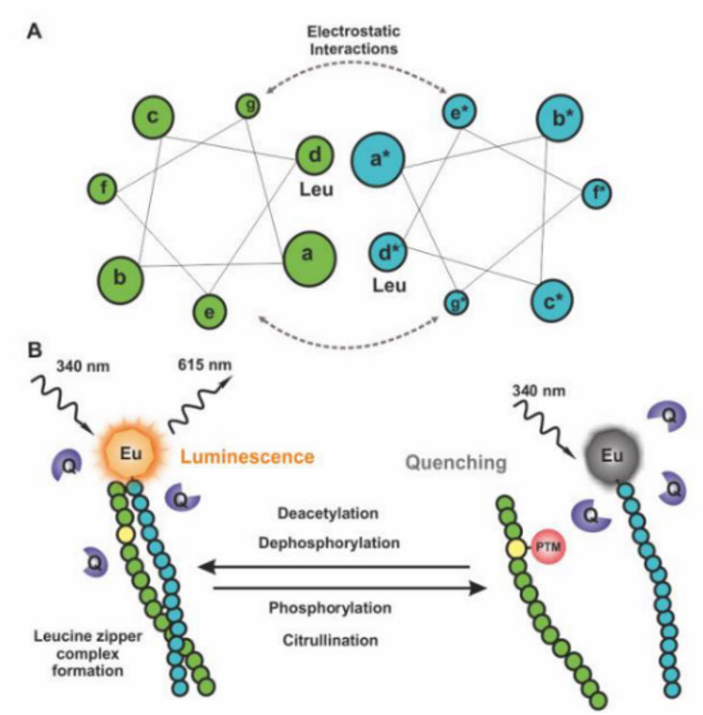

Figure 14. The principle of Peptide-Break technology.[10] A) The substrate peptide and the detection peptide are designed to form a leucine zipper (LZ) structure by placing leucine residues in fixed positions. B) The peptide substrate and the detection peptide form a strong coiled-coil structure when no PTMs cause steric effects and hence, the TRL-signal is protected from the soluble quenchers. The modification to the substrate peptide disrupts the coiled-coil structure and the luminescence signal is exposed to the quencher molecules.

Although, Peptide-Break has been demonstrated to be a powerful technique to study enzymatic activity of PTM-catalyzing targets, the design of peptide substrates following the heptad repeat can potentially be troublesome. The fixed leucine residues may not allow incorporation of the entire consensus sequence that is recognized by the enzyme and therefore, the affinity of the enzyme to the substrate

may be decreased. Moreover, some enzymes possess a naturally low affinity towards a substrate and thus, a synthetic peptide substrate may not be modified at all, thus complicating the applicability of LZ-concept. These potential issues are addressed in the following sections.

5.1.1 Peptide-Break utilizing charged peptides (**I**)

The Peptide-Break technology was introduced with a peptide design based on the use of a LZ structure formation (Fig. 14A). Thus, leucine residues have to be in fixed positions throughout the peptide sequences to enable the peptide pairing. A downside is that long consensus sequences may not be added to the substrate peptide. We tackled this potential issue by introducing charged peptides (CP) for the peptide-complex formation. The CP approach makes use of the complex formation between a positively charged peptide as the substrate and the same Eu(III)-labeled detection peptide as with the LZ-structures, which possesses a negative net charge. The principle of the CP Peptide-Break technology is presented in Fig. 15.

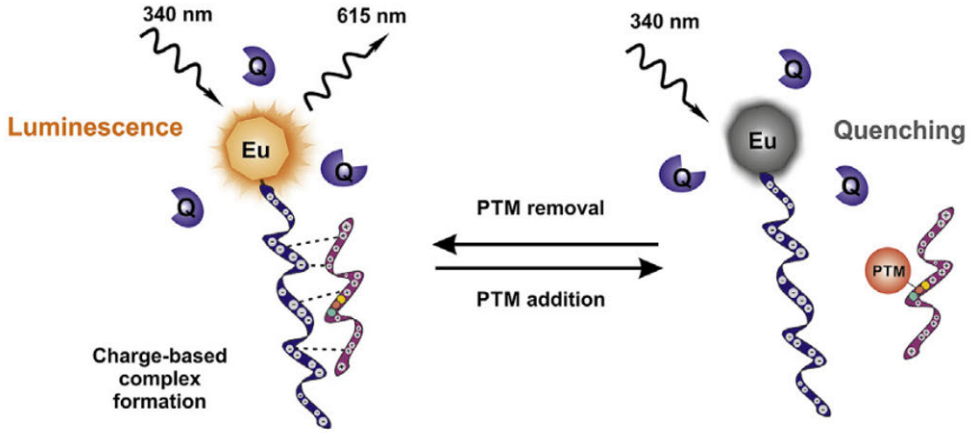

Figure 15. The Peptide-Break technology is applied to function with charged peptides.[168] The affinity of the substrate peptide towards the detection peptide can be customized by altering the overall charge of the peptides, which is a crucial part when designing the substrate. Too strong affinity will cause the peptide complex formation despite the PTM.

The binding affinity of positively charged substrates (CP1–CP3) was first limited to correspond the affinity of LZ (LZ-S). Thus, the two methods for peptide pairing were comparable having also the property to disrupt the peptide pairing via PTM-formation. The results showed that the affinity was dependent on charge, *i.e.* the higher the positive charge, the higher the affinity.

Next, a phosphorylation assay was conducted to demonstrate the applicability of CP Peptide-Break and to select a suitable peptide substrate for further assays. PKA (protein kinase A) was shown to modify the peptide substrate CP1, which was selected as the substrate for dose-response assays.

Dose-response assays were performed for PKA (0.5 nM) with two inhibitors, staurosporine and H-89 (0–5 and 0–10 µM, respectively), and for proto-oncogene serine protein kinases PIM1–PIM3 (2 nM) with the inhibitor H-89 (0–10 µM). The peptide CP1, as previously selected, was used as the substrate (10 nM), and the dose response assays with PKA inhibitors yielded sigmoidal curves with IC_{50} values of 56 ± 4 nM and 230 ± 34 nM for staurosporine and H-89, respectively (Fig. 16A). These values are in good agreement with values reported by others.[10,169] The PIM1–PIM3 kinase dose response assays gave also sigmoidal curves with IC_{50} values between 183 nM and 295 nM, which are similar to what others have reported.[170]

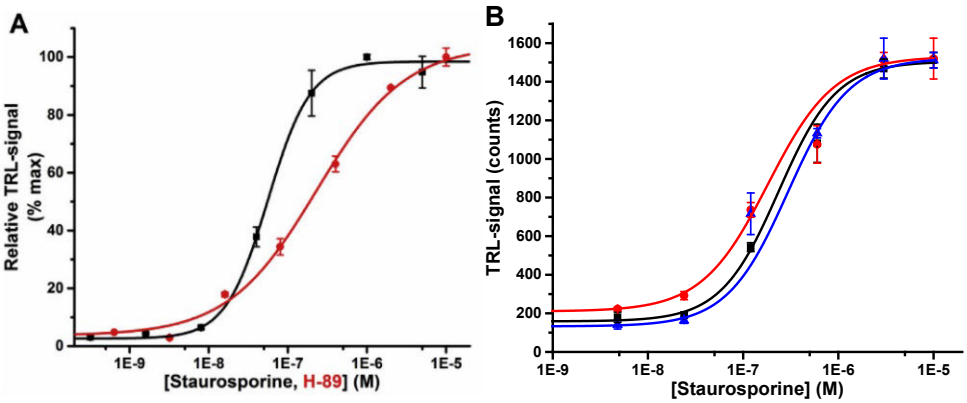

Figure 16. Dose response curves for serine kinases PKA and PIM1–PIM3.[168] Data represents mean ± SD (n = 3). A) Staurosporine and H-89 were titrated in the phosphorylation assay of 10 nM CP1 using 0.5 nM PKA. B) Staurosporine was titrated with 2 nM PIM1–PIM3 and 10 nM CP1. The obtained sigmoidal curves yield similar IC_{50} values between the three PIM-enzymes.

Following the kinase assays, deacetylation and citrullination assays were studied. First, suitable peptide substrates were assessed for the assays. The net charge of a substrate was determined earlier with kinase assays, and similar charges were thus introduced to the deacetylation peptides CP6 and CP7, and citrullination peptides CP4 and CP5. In the enzymatic assays with enzymes PAD4 (citrullination) and HDAC (deacetylation), the enzymatic activity was inhibited in the case of both enzymes with inhibitors trichostatin A (TSA) and GSK484, respectively.

Lastly, dose-response assays were performed for PAD4 and HDAC (1 and 0.5 nM, respectively) with CP-substrates (10 nM) CP6 (deacetylation) and CP4

(citrullination) side-by-side with LZ-substrates (10 nM), as control substrates. The dose-response assays yielded sigmoidal curves, as expected, with IC_{50} values of 0.35 ± 0.02 µM and 59 ± 2 nM for citrullination and deacetylation, respectively, and were in line with Arg-LZ (1.14 ± 0.16 µM) and Lys(Ac)-LZ (152 ± 5 nM) (Fig. 17).

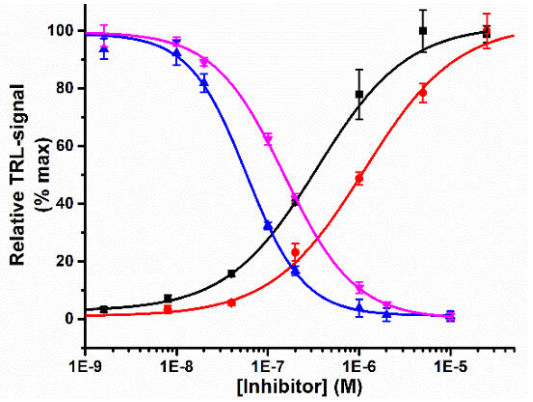

Figure 17. Citrullination and deacetylation assays with charged peptides.[168] Dose response assays were performed with deacetylase HDAC (0.5 nM) and citrullinase PAD4 (1 nM) enzymes. For citrullination, 10 nM of Arg-LZ (red) and CP4 (black) were studied side-by-side resulting in similar S/B ratios with inhibitor GSK484. For deacetylation, 10 nM of Lys(Ac)-LZ (magenta) and CP6 (blue) showed also similar S/B ratios with TSA. Data represents mean ± SD (n = 3).

In summary, CP Peptide Break technology was demonstrated to be an effective tool when monitoring the activity of PTM-catalyzing enzymes. The charged-peptide-based approach simplifies the peptide substrate design allowing the use of broader consensus sequences compared to LZ-approach. Moreover, the simple assay setup remains that was created with the LZ-based approach enabling the same functionality. The studied dose-response measurements for enzymatic targets yielded IC_{50} values that were in agreement with values reported previously and therefore demonstrating that CP approach functions as well as the original Peptide-Break with LZ approach. In addition, the affinity is now easily adaptable by increasing the number of charged residues. In contrast to the LZ-approach, increasing the affinity by can be done by inserting one or two charged amino acids into the sequence. With LZ-approach, the affinity needs to be increased by adding a whole heptad into the substrate and detection peptides.

5.1.2 Thermal Dissociation Peptide-Break (**II**)

Peptide-Break is an excellent tool for PTM detection when the enzymes have a high activity and affinity towards the peptide substrate. As shown with LZs and CPs, low

nanomolar concentrations of enzymes and substrate peptides can be applied with the system due to the active enzymes with a high affinity towards the peptides. Unfortunately, that is not always the case and therefore, monitoring the activity of low activity or affinity enzymes may become an issue. Low affinity or activity enzymes require higher concentrations of substrate or enzyme to perform well in assays. This is necessary in order to increase the probability of molecular collisions between the low affinity enzyme and substrate before the activity of enzyme decreases. Tackling this problem, we decided to increase the substrate peptide concentration, since this does not increase the assay cost significantly. However, increasing the substrate concentration introduces a new problem. The affinity between the detection peptide and the substrate peptide is typically at 10–100 nM concentration region allowing the PTM-addition to disrupt the peptide pairing. Increasing the peptide concentration to > 1 μM results in peptide complex formation regardless of the modification. We hypothesized that the problem can be solved using an elevated temperature. It is well-known fact that high temperature dissociates biomolecular interactions and thus breaks down peptide and protein structures of higher order. Increasing the temperature causes the complex with the PTM-carrying peptide substrate to break at a lower temperature and at higher rate than the peptide pair without a modification. Thus, we called this principle Thermal Dissociation Peptide-Break, as depicted in Fig. 18.

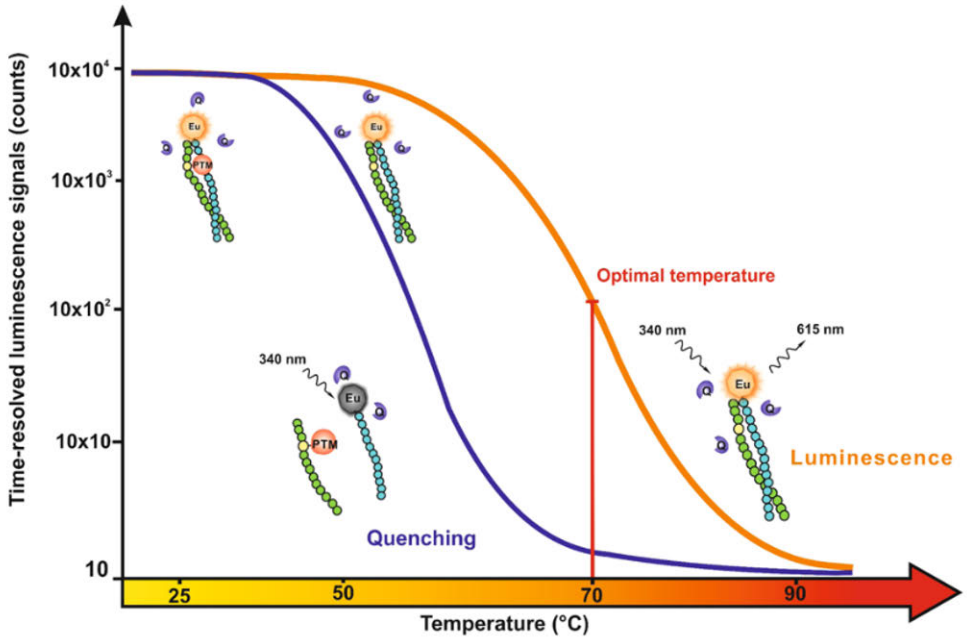

Figure 18. The Thermal Dissociation Peptide-Break technology allows the use of high substrate concentration because the higher temperature causes dissociation of the duplex.[171] The affinity difference between the unmodified and modified peptide substrates is unmeasurable at room temperature due to the high concentration, and therefore an elevated high temperature is used to disrupt the binding to achieve the separation between the modified and unmodified substrates. The modified peptide substrate dissociates from the Eu-peptide at a lower temperature and by screening a vast temperature region, an ideal temperature can be found for the assay.

The previously introduced peptides, CP6 and CP7 as well as peptide substrates LZ-Y, LZ-pY, CP8, and CP9 were studied for the method functionality (Table 3), whereas the detection peptide was the same as in the publications **I** and **II**. The assay functionality was first investigated with synthetically modified peptides and their unmodified counterparts (10 µM) determining the temperature where the maximum TRL-signal difference between the modified vs. unmodified peptide complexes takes place. The temperatures determined were 70 °C for LZ-Y/LZ-pY, and 45–50 °C for CP7/CP6 and CP9/CP8. In addition, further analysis was performed to determine the smallest population of enzymatically modified peptide that was observable from the initial peptide concentration. Approximately 1 % of synthetic dephosphorylated peptide was still measurable from the overall peptide concentration of 10 µM. Further enzymatic assays were performed with peptide pairs CP7/CP6 and LZ-Y/LZ-pY to prove the assay functionality for LZ- and CP-peptides.

The enzymatically modified peptide substrates were found to behave similarly to synthetically modified peptides regarding the dissociation curve, thus providing

the confidence to proceed to dose response assays utilizing protein tyrosine phosphatase 1B (PTP1B) and histone deacetylase (HDAC). Inhibitors Na_3VO_4 and TSA were screened for PTP1B and HDAC, respectively, yielding IC_{50} values for Na_3VO_4 and TSA of 3.8 and 1.1 nM (Fig. 19). The obtained values were keeping with our own earlier reported values, as well as others.[10,172,173]

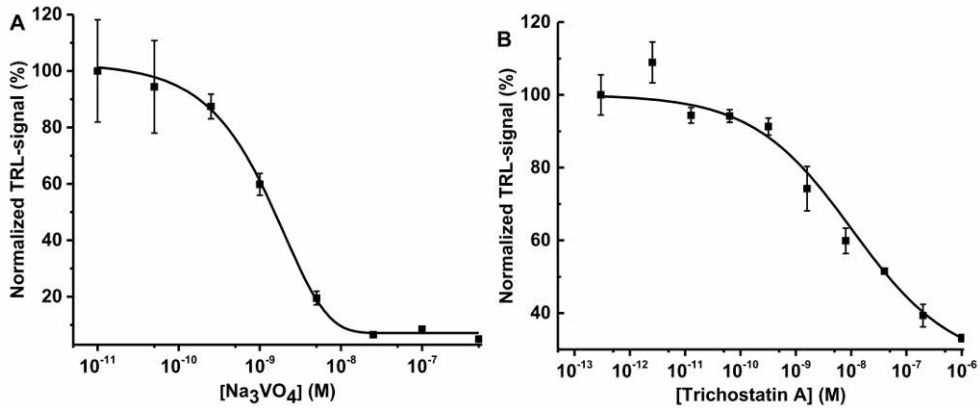

Figure 19. Dose response curves for PTP1B inhibitor Na_3VO_4 at 70 °C and HDAC inhibitor trichostatin A at 50 °C.[171] Data represent mean ± SD (n = 3). A) The enzymatic reactions with LZ-pY were heated in 5 °C intervals, and the peak maximum temperature was used to show the dose response for the used inhibitor. Based on the dose response curves, the IC_{50} value for PTP1B inhibitor Na_3VO_4 was 3.8 nM. B) From the deacetylation reaction with CP6, dose-response curve at 50 °C showed IC_{50} of 1.1 nM for HDAC3 inhibitor trichostatin A.

Finally, a single temperature flash heating protocol was developed for the assay because the heating takes a long time and we assumed the rapid heating to the temperature where the maximal separation was seen, would be sufficient. This was studied with LZ-pY and LZ-Y and the results verified that a single heating step is sufficient to create the TRL-signal difference between the peptide complexes. In addition, it was possible to measure the plate an hour afterwards when the plate had cooled, improving the user-friendliness of the assay. The heating was seen to cause a high standard deviation due to uneven cooling of the plate but this was overcome by letting the plate cool down before measuring (Fig. 20A). Finally, the Z′ factor, which depicts the robustness of an assay, was determined for both deacetylation and dephosphorylation assays. Values greater than 0.5 are generally considered as an indication of a well-performing assay and the enzymatic dephosphorylation and deacetylation assays yielded values of 0.72 and 0.62, respectively (Fig. 20B).

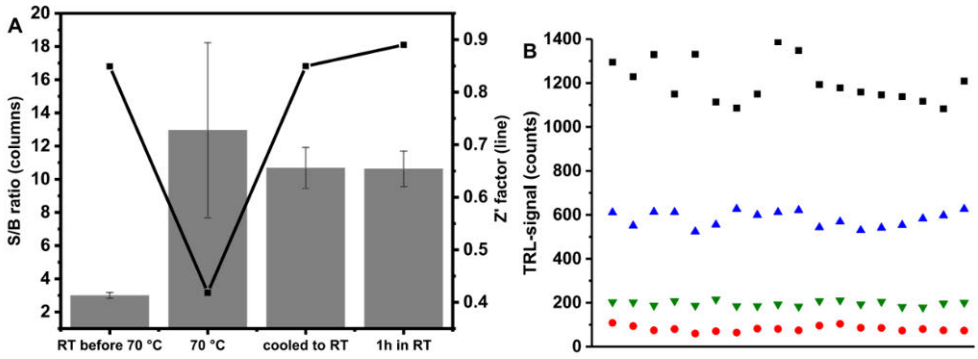

Figure 20. The results of the binding assay of peptides LZ-pY and LZ-Y and the enzymatic dephosphorylation assay for determining the Z´ factor utilizing the flash heating technique.[171] (A) Measuring the TRL-signals right after the heating step lead to low Z' factor value but provided the highest S/B ratio. Cooling the microtiter plate down to RT lowered the S/B ratio of the peptide assays approximately 20%, but due to the lower variation between replicates, the calculated Z′ factor was significantly improved. Data represent mean ± SD (n = 24). (B) Z′ factor measurements for the enzymatic dephosphorylation and deacetylation assays with 18 replicates. Dephosphorylation assay with (red) and without (black) 5 µM Na_3VO_4 and deacetylation assays with (green) and without (blue) trichostatin A gave Z′ values of 0.72 and 0.61 and S/B ratios of 14.6 and 3.0, respectively.

The Thermal Dissociation Peptide-Break assay allows the inhibitor screening for PTM-catalyzing enzymes with a fast homogeneous assay platform. Combining the benefits from the universal Peptide-Break utilizing LZ- and CP-complex formation, Thermal Dissociation Peptide-Break provides the PTM-detection even when the activity or affinity of the enzyme is low. In addition, the low enzyme concentration that has been utilized previously with the Peptide-Break can still be kept at low nanomolar level. The Z´ factors measured, demonstrated the assay robustness and the flash heating step kept the assay time short and applicable for HTS.

5.1.3 Peptide-Break tunability with signal modulators (**III**)

Thus far, the QRET detection has been based on the quenching of the unbound Eu(III)-peptide luminescence using different soluble quenchers. Therefore, the luminescence signal decreases as the substrate peptide is modified and peptide-complex formation is disrupted. In this study (publication **III**), an alternative technique was developed where the luminescence signal increases as the substrate becomes modified, *i.e.,* the Eu-peptide remains unbound. This modulated TR-FRET (mTR-FRET) method provides a high TR-FRET signal when Eu-peptide is free in solution because the modulator molecules can possibly bind only to the coiled-coil structure and therefore quench the TRL-signal. The mechanism of mTR-FRET is still unclear. The QRET method is described here as the signal-off mode as the signal

is low when the Eu-peptide is free in the solution. Conversely, mTR-FRET is described as the signal-on mode. Switching between the on and off modes is possible by changing the soluble quencher molecule into a signal modulator molecule (Fig. 21).

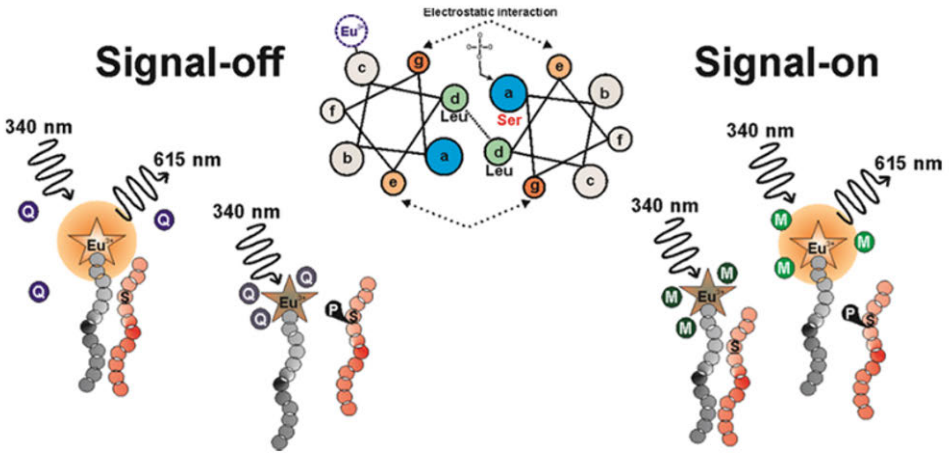

Figure 21. The principle of signal-off and signal-on Peptide-Break technology reinvented from the previous QRET detection.[159] The signal-off mode was developed earlier for the Peptide-Break detection where the peptide duplex formation shields the Eu-peptide signal preventing its quenching. In the signal-on mode, the signal readout is reversed by introducing a soluble signal modulator and high TRL signal can be obtained from the non-bound Eu-peptide.

Four different soluble molecules were selected for the quencher/modulator studies. Dy1 (1,1,3,3,3′,3′-hexamethylindodicarbocyanine iodide) and Dy2 (4-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-1-ethyl-pyridinium perchlorate) were introduced for the signal-on mode and Dy3 (6-t-7-nitrobenzofurazan-4-ylaminohexanoic acid) and Dy4 (1,4-diamine anthoaquine) for the signal-off mode. These quenchers/modulators were selected based on their spectral properties. Overlap between the EuLZ emission and D1, Dy2 and Dy4 excitation spectra was observed, and in case of Dy3, the possibility of "negative" TR-FRET was discussed. Thereafter, dose-response assays were performed for PKA and PTP1B with two PKA inhibitors, staurosporine (0–25 µM) and H-89 (0–25 µM), and one PTP1B inhibitor Na_3VO_4 (0–10 µM) (Fig. 22). LZ-peptide LZ-S (100 nM) was modified by 5 nM PKA with 50 µM ATP, and the detection was performed with 5 nM EuLZ and Dy1 (3–4 µM), Dy2 (25–100 µM), Dy3 (200–250 µM), or Dy4 (3–4 µM). Similarly, LZ-Y (100 nM) was used as a substrate for PTP1B under the same conditions.

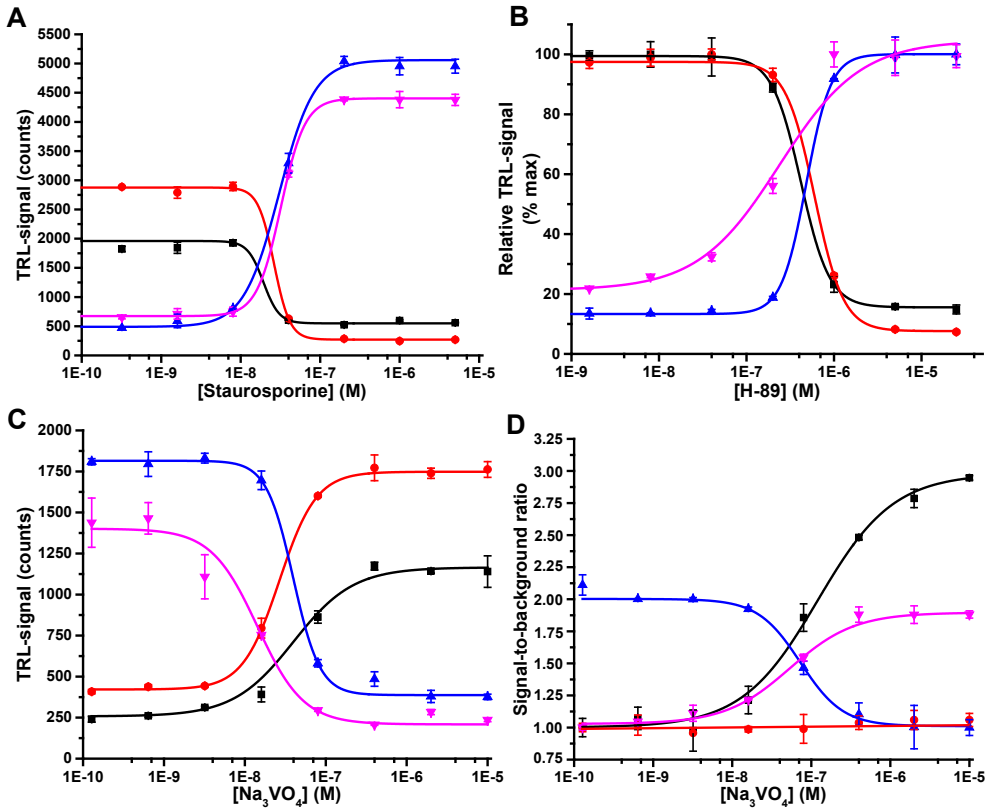

Figure 22. Dose-response assays performed for two PKA inhibitors, staurosporine and H-89, and one PTP1B inhibitor, Na_3VO_4, with quenchers Dy1 (magenta), Dy2 (blue) and modulators Dy3 (red) and Dy4 (black).[159] The PKA inhibitor titrations were performed with A) staurosporine (0–25 µM) and B) H-89 (0–25 µM). Signal quenchers Dy1 and Dy2 prevented the high TRL-signal monitored at 615 nm at low inhibitor concentrations while signal modulators Dy3 and Dy4 had the opposite effect. Dose-response assays yielded IC_{50} values between 16–32 nM and 0.24–0.60 µM, for staurosporine and H89, respectively. C) The PTP1B inhibitor titrations (0–10 µM) were performed with Na_3VO_4 under the same assay conditions but substituting LZ-S with LZ-pY. This yielded IC_{50} values between 14 and 40 nM. In this assay, the quenchers Dy1 and Dy2 prevented the high TRL-signal at high Na_3VO_4 concentrations and signal modulators Dy3 and Dy4 at low concentrations D) PTP1B inhibitor titration is also possible by TR-FRET-signal monitoring at 665 nm for Dy1, Dy2 and Dy4. No resonance energy transfer was observable with Dy3 due to lack of excitation spectrum overlapping with EuLZ emission spectrum. IC_{50} values recorded were between 53 and 120 nM. Data represents mean ± SD (n = 3).

The functionality of signal-on and signal-off method was demonstrated by screening two inhibitors against kinase PKA and one inhibitor against a phosphatase PTP1B utilizing two signal-on modulators and two signal-off modulators. The obtained IC_{50} values for PKA inhibitors staurosporine and H-89 were 16–32 nM and 0.24–0.60 µM, respectively, and for PTP1B inhibitor 14–40 nM, which are well in line reported by us and others.[10,168,169,174] The selection between signal-on and signal-off

modulators did not alter the observed IC_{50} value of the inhibitors studied for the selected targets but the S/B-ratios ranged between 3.8 to 12.1 when monitoring the Eu-emission at 615 nm. Based on the data, signal modulators Dy1 and Dy2 performed equally well with Dy3 and Dy4 but monitoring the dephosphorylation at 665 nm (TR-FRET), lower S/B ratios (1.9–3.9) were observed. In addition, Dy3 did not indicate any enzymatic activity when monitoring the reaction at 665 nm, which was expected, due to the excitation spectrum of Dy3 not overlapping with the emission spectrum of EuLZ.

The developed signal modulation technique for Peptide-Break allows the selection of the increasing signal based on the monitored reaction. Thus, the direction of signal increase in a reaction with increasing inhibitor concentration can be selected the same for PTM addition and cleaving. The change from one condition to another is done simple by changing the soluble molecule that is added alongside with the Eu-labeled detection peptide. The introduced signal-on and signal-off modulations offer the end-user an easy assay capable of switching between the increasing or decreasing luminescence signals.

5.2 Single-peptide TR-FRET PTM detection platform (**IV**)

The previous chapters described Peptide-Break and advancements that were developed to broaden its applicability and functionality. Many of the potential issues with Peptide-Break utilizing peptide complex-formation via LZ were discussed and alternative techniques were found to overcome these. However, a potential problem remains in these two-peptide systems due to the fixed-position leucines or charged amino acids required for charged-based complex-formation. Even though the charged-based complex formation is more resilient in peptide substrate design compared to LZs, the use of natural protein sequences may still be restricted. Therefore, we developed a single-peptide TR-FRET detection system for cysteine-specific PTMs that requires only a single peptide and does not require extensive consensus sequence altering.

The peptide substrate is synthesized with biotin at the N-terminus and the natural amino acid sequence containing the cysteine residue is derived from a protein. The only limitation to the peptide sequence is the length as energy transfer between the donor and acceptor has its limitations. The free cysteine residue is possible to be conjugated with a cysteine-specific dye, AlexaFluor 680 maleimide ester. Following the conjugation a TR-FRET-pair is formed as the AlexaFluor 680 acceptor is in close proximity of EuSA donor that is bound to the peptide substrate via biotin interaction. As the cysteine residue is modified, chemically or enzymatically, the AlexaFluor 680 conjugation is blocked and the TR-FRET-pair can no longer be formed. Therefore, high TR-FRET signals are monitored at 730 nm only upon the AlexaFluor

680 conjugation. The single-peptide TR-FRET detection platform was studied with enzymatic ADP-ribosylation and the peptide substrates (Table 3) were designed based on the known substrates of ADP-ribosyltransferase subunit 1 rPtxS1-wt.[175] The principle of the assay is illustrated in Fig. 23.

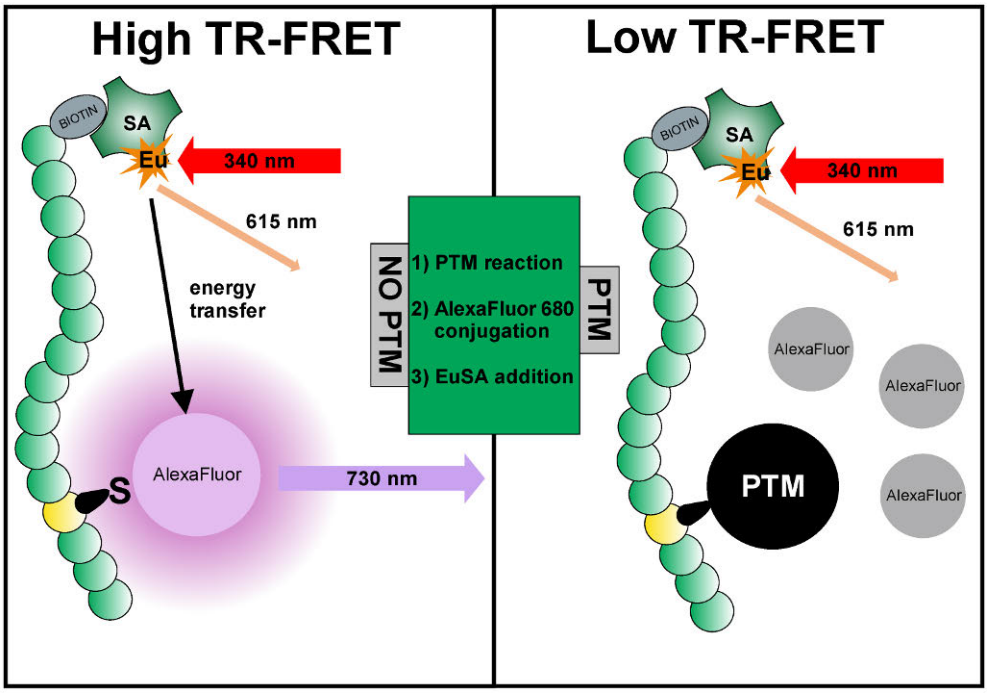

Figure 23. The single-peptide TR-FRET detection method for cysteine-specific PTMs.[176] Using a biotinylated peptide and EuSA donor, chemically or enzymatically generated cysteine modification results in a low TR-FRET-signal, since the acceptor dye (AlexaFluor) can no longer be conjugated to the occupied cysteine. Alternatively, e.g., inhibition of the cysteine-modifying enzyme leads to efficient conjugation of the thiol-reactive AlexaFluor dye, and a high TR-FRET-signal is monitored at 730 nm.

The single-peptide TR-FRET system was first optimized to reduce the background signal and to maximize the detection sensitivity. Therefore, the peptide substrate-to-EuSA ratio was screened together with the AlexaFluor 680 concentration. Peptide substrate to EuSA ratio of 4:1 was found optimal which can be anticipated as streptavidin possesses four binding sites to biotin. In addition, the AlexaFluor 680 labeling efficiency was optimal at the concentration range of 0.1–0.5 µM. These conditions were applied to further assays and next, the functionality of the method was studied with chemical S-nitrosylation where S-nitrosoglutathione (SNOG) transfers a NO group to a free cysteine residue. S-nitrosylation was studied with PC10, PC15, and RP16 substrates (Table 3) resulting in EC_{50} values of 0.56, 0.86 and 18.4, respectively.

As the detection platform proved to be functional in the chemical modification concept, enzymatic assays were developed. The ADP-ribosyltransferase S1 subunit of recombinant pertussis toxin rPtxS-wt was studied first and its ADP-ribosyltransferase activity was compared to a mutant pertussis toxin, rPtxS1-Q127D/E129D. Here it was observed that rPtxS1-Q127D/E129D does not possess any ADP-ribosyltransferase activity towards a natural protein substrate rGαi, and also that rPtxS-wt is well suited for further studies. Subsequently the best-suited peptide substrate for the TR-FRET assay was determined with a commercial NAD/NADH Glo™ assay (Fig. 10), which monitors the NAD+ concentration during a ADP-ribosyltransferase assay and out of the two possible peptide substrates PC10 and PC15, PC15 appeared to be more efficiently ADP-ribosylated by rPtx-S1-wt, as expected.[175]

Finally, PC15 was studied in the TR-FRET assays to prove the functionality of the enzyme-based method. Cofactor NAD+ was first screened (0–30 µM) with the mutant and wild-type enzymes to confirm that the enzyme does not interfere with the assay. The results revealed that the EC_{50} value for NAD+ was 3.6 µM using 333 nM rPtxS1-wt and 10 nM peptide substrate PC15 (Fig. 24A). Therefore, the concentration of NAD+ should be high (> 3.6 µM) in order to achieve effective ADP-ribosylation. No ADP-ribosylation activity was observed with the mutant enzyme, as expected. Following the NAD+ titration, a dose-response assay was performed with the inhibitor, nicotinamide (NA). NA is a precursor of NAD+ and therefore has a similar structure to NAD+. In order to better use NA as an inhibitor, NAD+ concentration was lowered to 10 µM. The ADP-ribosylation reaction was seen to be dose-dependent and yielded IC_{50} value of 82.8 mM (Fig. 24B).

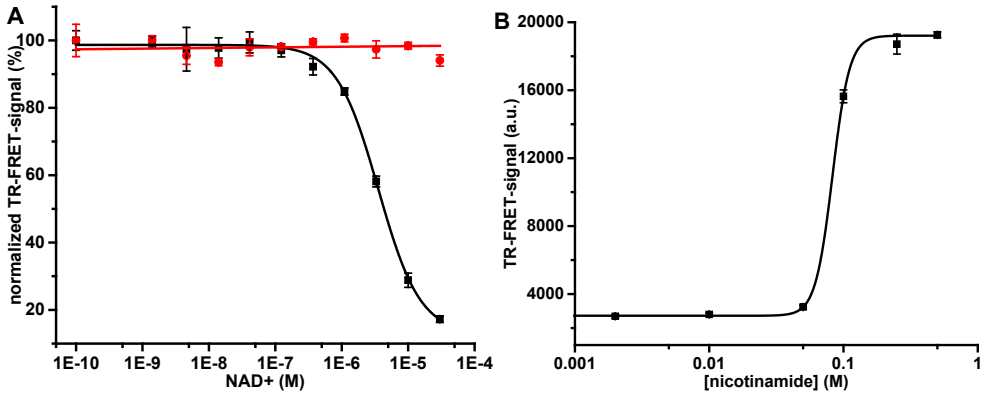

Figure 24. NAD+ titration and dose-response measurement for rPtxS1-wt inhibitor nicotinamide.[176] Data represents mean values ± SD (n=3). A) The NAD+ concentration was titrated from 0 to 30 µM, with rPtxS1-wt (black) and rPtxS1-Q127D/E129D (red) under the optimal conditions. The EC_{50} for NAD+ with rPtxS1-wt was 3.6 ± 0.2 µM. B) The inhibitor titration with nicotinamide was titrated from 0 to 0.5 mM. The IC_{50} value was determined 82.8 ± 1.4 mM.

The results of the dose-response assay prove the assay functionality in screening of inhibitors for cysteine-specific targets. Although more research needs to be conducted, chemical modification was observed with three different peptide substrates and ADP-ribosylation activity was shown with one peptide substrate. Furthermore, ADP-ribosylation activity was monitored with cofactor NAD+ screening and rPtxS1-wt inhibitor nicotinamide. This assay introduces a homogeneous universal platform that can be potentially utilized for various cysteine-modifying targets. The assay is HTS-applicable and the assay reagents, peptide substrate and EuSA, consumption is low due to low concentrations. In addition, a high NAD+ concentration that is required by the enzyme rPtxS1-wt, interferes with assays such as NAD/NADH Glo™ assay where the initial NAD+ concentration needs to be moderately low due to the fixed linear range of the assay.

The single-peptide TR-FRET detection platform can hypothetically also be applied to lysine modifications by simply changing the peptide substrate sequence from a natural target and utilizing a NHS ester dye that can be conjugated to amino groups with high specificity.

6 Conclusions

Bringing a drug to the market is a long and an expensive process. In the hope of succeeding in the discovery and development of the drug molecule, more than a billion US dollars and over ten years of intensive research has to be conducted. In order to make this process less expensive and potentially faster, the research presented in this thesis has focused on improving tools for drug discovery. New assays were developed for discovering drugs that enhance or inhibit the activity of protein PTM-catalyzing enzymes. The PTMs act as regulatory mechanisms of cells and are often targeted in disease conditions, making PTM enzymes, such as kinases, an important drug target. The developed high throughput screening assays utilize peptides and the sensitive time-resolved detection enabled by Eu(III)-chelates. The peptide consensus sequence is derived from the natural protein substrate of the target making the use of whole proteins obsolete. Moreover, the sensitive TRL-signal readout with lanthanide chelates allows to using low concentration of labeled molecules in the detection, at nanomolar scale. As the assays are performed in microtiter plates and the signals are read with a standard luminescence plate reader, these assays are well suited for HTS.

Previously, Peptide-Break technology has exploited two interacting peptides forming a leucine zipper structure (LZ) that is disrupted by a modification in the substrate peptide. The so-called detection peptide is labeled with a luminescent Eu(III)-label and is quenched by soluble quencher molecules in solution when the two-peptide structure is disrupted and the detection peptide is unbound in the solution. An intact LZ coiled-coil complex shields the Eu(III)-label from quenching and a high TRL-signal is detected. This technology was further improved by changing the peptide pairing from LZ into a charge-based approach (publication **I**). This allows improved freedom when designing the peptide substrates and the functionality of the technique was demonstrated with dose-response measurements for citrullination enzyme, deacetylation enzyme, and four target enzymes for phosphorylation. The end result is a homogeneous assay that functions at a low nanomolar scale and is applicable for HTS. The benefit of this assay when compared to the other TRL-based assays is that is provides a wash-free and antibody-free system where no additional labeling of molecules is needed because the Eu-labeled

detection peptide is applicable for all the assays. The CP-approach gives freedom to the substrate design as more natural amino acid sequences can be utilized.

As the applicability of Peptide-Break was broadened for the needs of modern drug screening, a technique was developed for PTM-catalyzing enzymes for which activity is potentially difficult to monitor. A Thermal Dissociation Peptide-Break for PTM-detection was developed where the Peptide-Break principle was used to address this problem (publication **II**). This was achieved by increasing the substrate concentration (10 µM) to make low enzyme affinity and activity measurements possible. The choice of high substrate concentration was also justified by the fact that it is the most abundant and inexpensive reagent in the assay setup. The high concentration provides the peptide-complex formation also with the modified peptide substrate and a physical force (high temperature) was therefore needed to distinguish the modified substrate from the unmodified in a homogeneous assay format. Thus, we induced the peptide-complex break with optimized high temperature. Dephosphorylation and deacetylation targets were investigated in the enzyme inhibition assays with well-known inhibitors, yielding similar IC_{50} values to those previously reported. With the developed assay, we were able to detect a small fraction of the enzyme modified peptide substrate from the large unmodified peptide pool using the same Peptide-Break technology introduced previously. The assay setup remained the same as standard Peptide-Break assays, with exception that an additional 3 min rapid heating step was introduced. This assay allows the end user to choose a suitable Peptide-Break technique depending on the enzyme target studied. The added heating step enabled the use of high substrate concentration in the assays that is obligatory for enzymes that have a low affinity or activity to the substrate.

Next, the signal readout selection was improved by allowing the high vs low signal to be chosen in the case of PTM addition or cleavage (publication **III**). Previously QRET was utilized to achieve a high TRL-signal from the bound Eu-peptide as the soluble signal modulator molecules quench the TRL-signal from unbound Eu-peptide (signal-off mode). Here, another type of soluble signal modulator molecule was introduced that enhances the signal output for the unbound Eu-peptide (signal-on mode). Two signal modulator molecules were introduced and their functionality to Peptide-Break was studied successfully. The PTM addition and cleavage was detected with all signal modulators, and the enzyme inhibition was monitored with IC_{50} values that were in agreement with values reported previously by others and us. The signal-on and signal-off modulators present the end-user a choice to select the signal readout. The increased or decreased signal can be tuned by only switching the soluble modulator molecule.

Finally, a single-peptide TR-FRET assay was developed for the detection of cysteine PTMs where the peptide sequences were directly derived from the natural

substrate protein (publication **IV**). No additional leucines or charged amino acid residues are needed and the only modification required to the sequence is the biotin-conjugation. A single peptide substrate, conjugated with biotin, binds with Eu(III)-labeled streptavidin (EuSA) providing a donor to TR-FRET. A cysteine-specific dye, an acceptor, and the PTM compete from the free cysteine residue, leading to a high TR-FRET-signal when the PTM-formation is inhibited. The functionality of the assay was shown with chemical and enzymatic modifications. As with the Peptide-Break assays, the low substrate concentrations accompanied with the homogeneous assay protocol provides a highly competitive assay when compared with the antibody-based heterogeneous methods currently used. The developed HTS-applicable assay can potentially be used in inhibitor screening for cysteine-modifying targets. Previously developed CP-approach to detect PTMs is further improved here in the case of cysteine PTMs. The peptide substrate design is made more unrestricted allowing the use of completely natural amino acid sequences as the only modification to the sequence is the biotin-conjugation.

The assays developed and presented in these studies have many qualities that top the existing assays. These qualities are compared to the techniques discussed in this thesis in Table 2. They are compatible with HTS format that is a key requirement for assays that are used to screen potential hits and eventually lead molecules. Furthermore, the assays do not require full substrate proteins but utilize peptide substrates that are designed from the protein substrate. The most suitable Peptide-Break or single-peptide method can be chosen on the basis of the PTM, the consensus sequence of the substrate, and the activity/affinity of the target enzyme in mind. Sensitive TR-detection keeps the assay costs low, and assays measured in the microtiter plate format enable a fast readout of the signal. Moreover, the assays are wash-free and homogeneous techniques were no antibodies are needed. With the current methods available for PTM-detection, as discussed in previous sections, the low-throughput or exploitation of PTM-recognition by antibodies decreases the effectiveness of the techniques.

Peptide-Break is a complete detection platform and an interesting tool for drug discovery and development. For the future, more research is needed to determine the technique robustness and other qualities for large molecule library screening. In addition, more PTMs need to be tested with Peptide-Break as for this day, only (de)phosphorylation, deacetylation, and citrullination modifications have been studied with a few targets and activators/inhibitors. Nevertheless, this amount of PTMs that can be studied in HTS-format without antibodies is exceptional. For the single-peptide TR-FRET detection platform, more cysteine modifications need to be studied with the system. As the technique relies on the modification to take place in a residue that can be specifically conjugated with a dye, lysine modifications can potentially be studied. Amine-specific NHS ester AlexaFluor dyes are commercially

available and are potentially applicable for the single-peptide TR-FRET detection platform. As the aim of this thesis was to develop HTS-applicable assays to study PTM-catalyzed enzymes and aid the hit generation phase of drug development, we hoped to peak interest of pharma to utilize these findings.

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