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Bisubstrate luminescent probes, optical sensors and affinity adsorbents for measurement of active protein kinases in biological samples

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Author's contribution

Paper I: The author participated in the planning of the experiments, designed and performed the synthesis of affinity support and was responsible for the writing of the respective part of the manuscript.

Paper II: The author participated in the planning of the experiments, performed SPR and TR-FRET experiments and was responsible for the writing of the respective part of the manuscript.

Paper III: The author participated in the planning of the experiments, in the characterization of the luminescent probes and in writing the manuscript.

Paper IV: The author planned and performed most of the experiments and was responsible for writing the manuscript.

ABBREVIATIONS

2DE	two-dimensional gel-electrophoresis
2-D DIGE	2-D fluorescence difference gel electrophoresis
6His-tag	hexahistidine tag
Adc	adenosine 4′-dehydroxymethyl-4’-carboxylic acid moiety
ADP	adenosine 5′-diphosphate
Ahx	6-aminohexanoic acid moiety
AKAP	A-kinase anchoring protein
akt/PKB	protein kinase B
ALK	anaplastic lymphoma receptor tyrosine kinase
AMTH	5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid moiety
AMSE	5-(2-aminopyrimidin-4-yl)selenophene-2 -carboxylic acid moiety
ARC	adenosine analogue-oligoarginine conjugate
ARC-341	Adc-Ahx-$(LArg)_6$-NH_2
ARC-342	Adc-Ahx-$(LArg)_6$-LLys-NH_2
ARC-347	Adc-Ahx-$(LArg)_6$-LLys(biotin)-NH_2
ARC-583	Adc-Ahx-$(DArg)_6$-DLys(TAMRA)-NH_2
ARC-668	AMTH-Ahx-(DArg)-Ahx-$(DArg)_6$-DLys-NH_2
ARC-704	Adc-Ahx-$(DArg)_6$-DLys(PEO-biotin)-NH_2
ARC-904	Adc-Ahx-$(DArg)_6$-DLys-NH_2
ARC-1023	Adc-Ahx-$(DArg)_6$-DLys(Ahx_2)-NH_2
ARC-1024	Adc-Ahx-$(DArg)_6$-DLys(Ahx_2-biotin)-NH_2
ARC-1063	AMTH- Ahx-(DArg)-Ahx-$(DArg)_6$-DLys(Alexa Fluor 647)-NH_2
ARC-1138	AMTH- Ahx-(DArg)-Ahx-$(DArg)_6$-DLys-NH_2
ARC-1139	AMTH- Ahx-(DArg)-Ahx-$(DArg)_6$-DLys(PromoFluor-647)-NH_2
ARC-Fluo	ARC labelled with a fluorescent dye
ARC-Lum	luminescent ARC-based probe
ATP	adenosine 5′-triphosphate
BCR-ABL	fusion protein consisting of the BCR (breakpoint cluster region) protein and the Abl (Abelson) tyrosine kinase
CA125	cancer antigen 125
CA19-9	carbohydrate antigen 19-9
CaMK IV	calcium/calmodulin-dependent protein kinase type IV
cAMP	cyclic adenosine 3′,5′-monophosphate
CEA	carcinoembryonic antigen
Cy3	indocarbocyanine
Cy5	indodicarbocyanine
DDR1	discoidin receptor tyrosine kinase
ELISA	enzyme-linked immunosorbent assay
FA	fluorescence anisotropy
FDA	Food and Drug Administration of the United States of America
FITC	fluorescein isothiocyanate
FRET	Förster resonance energy transfer
GPCR	G protein-coupled receptor
GST	glutathione-S-transferase

H89	N-{2-[(p-bromocinnamyl)amino]ethyl}-5-isoquinolinesulfonamide
HA-1077	1-(5-isoquinolinesulfonyl)-homopiperazine
Her2/neu	human epidermal growth factor receptor 2
HTS	high-throughput screening
IC_{50}	half-maximal inhibitory concentration
k_{+1}	association rate constant for the formation of enzyme:substrate complex
k_{-1}	dissociation rate constant for dissociation of enzyme:substrate complex
k_{cat}	the rate constant of product formation
K_d	equilibrium dissociation constant determined from a displacement assay
K_D	equilibrium dissociation constant determined from a direct binding assay
K_i	equilibrium dissociation constant determined from an inhibition assay
KIT	tyrosine-protein kinase Kit
K_M	Michaelis constant
k_{obs}	observed association rate constant
k_{on}	association rate constant of complex formation
k_{off}	dissociation rate constant of complex dissociation
MALDI-TOF	matrix-assisted laser desorption/ionisation time-of-flight
MS	mass spectrometry
MSK1	nuclear mitogen- and stress-activated protein kinase 1
PEO	polyethylene oxide moiety
PF647	PromoFluor-647
PKI	heat stable inhibitor of PKA
PKA	cAMP-dependent protein kinase
PKAc	catalytic subunit of cAMP-dependent protein kinase
PKAr	regulatory subunit of cAMP-dependent protein kinase
PKC	protein kinase C
PRKX	the human X chromosome-encoded protein kinase X
PSA	prostate-specific antigen
ROCK II	Rho-associated protein kinase 2
RU	response unit
S6K1	ribosomal protein S6 kinase I
SDS	sodium-laurylsulphate
SPR	surface plasmon resonance
TAMRA	carboxytetramethylrhodamine
TGL	time-gated luminescence
TR-FRET	time-resolved Förster resonance energy transfer
V_{max}	maximum velocity of the enzyme catalysed reaction

INTRODUCTION

A human organism is an extremely complicated system consisting of innumerable different compounds ranging from ones as small and simple as water to those being as complicated multi-subunit structures like proteins with molecular weights over a hundred kilodaltons. These molecules make up extremely complicated intra- and intercellular signalling pathways and the disharmony of functioning of the molecules at any stage of these pathways may result in the progression of a disease. In past decades much effort has been put into understanding the functioning of normal signalling pathways and the origins of different diseases. This has created the possibility for development of artificial regulators of components of signalling cascades that will support the diseased organism to manage with normal functioning. The development of new and better drugs depends highly on the finding out and understanding the role of disease-related proteins. Thus new and better methods for monitoring of cellular functioning of proteins as well as methods for screening of new regulators of these proteins are of great importance. Moreover, methods enabling the division of patients into groups based on genetic and proteomic information related to the special person support the understanding of the exact mechanism of the disease and enable the personalized tailoring of the therapy according to this information leading to better outcome of the treatment.

The present thesis describes the development of bioanalytical methods for monitoring the activity of protein kinases in biological samples. High-affinity adsorbents were worked out and used for the enrichment of protein kinases. Novel non-metal long lifetime luminescence probes were discovered that were used for full thermodynamic and kinetic characterization of un-labelled inhibitors of protein kinases in biochemical assays and for monitoring kinase activity in live cells. A novel homogeneous assay based on the measurement of time-gated luminescence was used for the quantification of a putative cancer biomarker ECPKA in crude plasma samples.

REVIEW OF THE LITERATURE

Proteins as biomarkers

In normally functioning tissues the cellular signalling pathways are firmly controlled by external messages. In contrast, pathogenic tissues generate false, mimicked proliferation and angiogenesis signals for themselves. Probably the most explored group of diseases caused by flaws in signalling pathways is cancer [Hanahan and Weinberg 2011]. Hence, the present thesis is focused on cancer related protein biomarkers. However, the described methods generally can be used for the detection and analysis of protein biomarkers related to other diseases as well.

The beginning of the Human Genome Project in 1990 and the completion of sequencing of the genome in 2003 opened a new era in the identification and analysis of disease-related proteins. The knowledge of the amino acid sequence of all encoded proteins provides means for the identification of proteins based on the masses and amino acid sequences of the peptides derived from those proteins. This has triggered a massive proteomics research to discover the disease-related alterations in the protein expression and activation patterns by comparing samples from healthy and diseased tissues [Boja and Rodriguez 2011]. Moreover, the genomic changes and alterations in the protein expression patterns can be not only causes but also consequences of failures in signalling pathways. On one hand the alternatively expressed proteins are the major targets for the development of new drugs. On the other hand the abundances of disease-related proteins can be used for the development of screening programs for early detection of diseases. Identifying markers that allow subtyping the patients with similar clinical phenotype based on different mechanisms responsible for causing the disease will also significantly improve the outcome of new targeted (personalized) therapies.

Great efforts have been channelled into discovery of substances designated as biomarkers that could signal about emerging illnesses in early stages. Generally, a biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [Atkinson *et al.* 2001]. Cancer biomarkers can be analysed from biopsy samples but also from samples of blood or other body fluids [Hanash *et al.* 2008; Huijbers *et al.* 2010; Wu *et al.* 2010; Yamada *et al.* 2008].

Analysis of blood serum or plasma samples well suits for clinical diagnostics due to minimal harm caused to the patients, stability of the concentration levels of markers and cost-effectiveness. Cancer researchers continue to work on improving the sensitivity and specificity of methods for determination of serum-based markers, with the goal of achieving the highest level of diagnostic accuracy possible for the detection of cancer at an early stage when successful curative interventions are still possible [Hanash *et al.* 2011]. The tests target circulating tumour cells, proteins, RNA and DNA in plasma, and autoanti-

bodies. Recent studies suggest that combining several selected tumour markers, possibly in a multiplexed analytical mode, could lead to more specific assays for the early detection of cancer [Hanash *et al.* 2011]. Approximately 200 protein analytes, equivalent to 1% of the human proteome, are currently measured in plasma or serum for clinical purposes [Anderson 2010].

Based on the information they provide biomarkers are divided into three main categories:

- Diagnostic biomarkers are used as tools for making the diagnosis, *e.g.,* elevated blood glucose levels signal about emerging diabetes. Diagnostic biomarkers can be rather general simply indicating the presence of a disease or specific determining the exact cause or mechanism of the disease.
- Prognostic markers give the estimation of the outcome of therapy or average life expectancy, *e.g.*, the cancer's likelihood to give metastases.
- Predictive biomarkers indicate the patient's likelihood to benefit from treatment with a specific drug or set of drugs. One of the most successful predictive biomarkers that set the grounds for personalized medicine was the Her2/neu receptor status [Carney *et al.* 2007].

One of the most widely used circulating protein biomarker measured in patient's blood samples for cancer screening purposes is the prostate-specific antigen (PSA) for the diagnosis of prostate cancer [Hoffman 2011]. However, there are major concerns arising as the PSA tests in general have limited ability to discriminate between malignant cancers and other benign prostate conditions and therefore possess rather low sensitivity and specificity. Moreover, a substantial proportion of PSA-detected cancers are considered over-diagnosed because they would not cause clinical problems during a man's lifetime.

Serum- or plasma-based cancer-related protein biomarkers currently in clinical use include CA125 (cancer antigen 125) for ovarian cancer, CA19-9 (carbohydrate antigen 19-9) for pancreatic cancer and CEA (carcinoembryonic antigen) for colon cancer [Huijbers *et al.* 2010]. However, similarly to PSA all these biomarkers have limited value with respect to their use for screening owing to low sensitivity and specificity as they are present at increased levels in benign disease as well [Polanski and Anderson 2007]. Still, these markers are valuable for monitoring cancer progression and response to therapy. Hence, it is proposed that only the application of multiple biomarker panels (either proteomic, genetic, or both) has the potential to be developed into clinically useful diagnostic methods [Malinowski 2007], meaning that for a definitive diagnosis, the concentration of several cancer-related markers has to be established.

Since the majority of pathological states result from intra- or intercellular communication disorders and dysregulation of cellular signalling cascades, most of the recently identified disease related proteins are transduction related proteins, including protein kinases [Bianco *et al.* 2006]. Therefore the development of assays for protein kinase research and disease diagnostics is of great importance.

Protein Kinases

Protein kinases form a family of proteins that play a key role in signal transduction pathways inside the cells by catalysing transfer of the γ-phosphate group from ATP to the hydroxyl group of a serine, threonine, or tyrosine residue of a substrate protein. The addition of negative charges to the substrate protein changes its conformation [Steichen *et al.* 2010], activity and localization and therefore affects the processes that are downstream in the signalling cascade. About one third of all human proteins (including protein kinases) contain residues that can be phosphorylated [Cohen 1999]. Phosphorylated proteins in turn are substrates for phosphatases that catalyse the removal of phosphate group from the protein.

From the human genome sequence 518 protein kinase-coding genes were identified which constitutes about 2% of genes [Manning *et al.* 2002]. The structure, activation and inhibition mechanisms of many kinases have been extensively studied. The function of others still remains to be discovered as the expression of some proposed kinases on protein level has never been proved. In addition to those 518 proposed protein kinase structures, for several protein kinases various splice variants have been identified diversifying the protein kinase family even further.

Both the size and structure of protein kinases vary widely [Hanks and Hunter 1995] as do the ligands that activate the kinases. Protein kinases consist of catalytic and regulatory moieties which are typically either a part of a continuous polypeptide chain or a part of a stable multi-subunit structure. Similarities in sequence define the conserved catalytic domain which consists of 250–300 amino acid residues and catalyses the phosphorylation event [Hanks *et al.* 1988]. Other domains in protein kinases vary widely and are important for functions such as regulation of kinase activity, linking to other signalling modules and subcellular localization [Manning *et al.* 2002; Taylor *et al.* 1990].

Nearly all aspects of cell life are controlled by reversible phosphorylation of proteins: cellular signalling cascades rely on the phosphorylation status of proteins in their pathways [Manning *et al.* 2002] playing critical role in many regulatory mechanisms such as signal transduction, metabolism, transcription, cell cycle progression, cytoskeletal rearrangement, cell differentiation, movement, growth and apoptosis [Shabb 2001]. Therefore protein phosphorylation is a key regulatory mechanism of functioning of eukaryotic cells [Cohen 2000].

It has been shown that malfunctions such as kinase over-activation *via* mutation or overexpression in signalling pathways can have pathological consequences such as cancer, diabetes, rheumatoid arthritis, hypertension and inflammation [Cell Signaling Technology 2012; Cohen 1999; Tsatsanis *et al.* Jul 2007]. This has made protein kinases one of the most important protein targets for drug discovery of the past decade. Therefore, the need for methods that would facilitate the study of the role of kinases in a wide range of biological processes arises.

PKA – cAMP-dependent protein kinase

The cAMP-dependent protein kinase (PKA) was one of the first protein kinases discovered [Cohen 2002a; Walsh et al. 1968] and since then it has been under thorough investigation. In the absence of cAMP, PKA is an enzymatically inactive tetrameric holoenzyme consisting of two regulatory (PKAr) and two catalytic (PKAc) subunits. Resent data point to substantially higher expression levels of PKAr isoforms compared to these of the catalytic subunit PKAc isoforms in normal (heart) tissue that is required for tight regulation of PKA activity [Aye et al. 2010]. PKAc is released from PKAr and becomes active only as the result of a regulated rise of cAMP concentration. Binding of two cAMP molecules to each regulatory subunit alters their affinity for the catalytic subunits and promotes dissociation of the holoenzyme into a dimer of regulatory subunits and two active monomeric catalytic subunits [Skalhegg and Tasken 2000]. The regulatory subunits have been classified into two types, PKArI and PKArII that are expressed as a variety of isoforms (PKArIα, PKArIβ; PKArIIα, PKArIIβ) that differ in the level of expression, subcellular localisation and tissue distribution. The regulatory subunits are anchored to the cellular compartments by A-kinase anchoring proteins (AKAPs) [Carnegie *et al.* 2009; Huang *et al.* 1997]. Four forms of the catalytic subunit have been identified in mammalian tissues with different amino acid sequences, PKAcα, PKAcβ, PKAcγ and PRKX [Beebe *et al.* 1990; Zimmermann *et al.* 1999; Uhler *et al.* 1986a; Uhler *et al.* 1986b]. Cα appears to be expressed in most cells, Cβ is mainly expressed in brain [Uhler *et al.* 1986b], Cγ is expressed in human testis [Beebe *et al.* 1990]. Cα and Cβ possess 91 % identity in amino acid sequence; Cγ and PRKX are less identical with Cα and Cβ [Gamm *et al.* 1996]. Both the amino acid sequence and the size of the catalytic subunit are highly conserved between species.

PKAc is a relatively small (40 kDa) nearly globular protein that contains in addition to the catalytic core that is conserved throughout the family of protein kinases only short flanking regions at the N- and C-termini. The ATP-binding site is located deep inside the catalytic core between the small amino-terminal and large carboxy-terminal lobes. The large lobe is additionally responsible for substrate protein binding [Taylor *et al.* 1990; Taylor *et al.* 1993]. Crystal structures have elucidated how the catalytic cleft opens and closes to grasp the adenine moiety at the base of the active site cleft and how the glycine-rich loop and the catalytic loop position so that both MgATP and the substrate protein come into contact for catalysis in the cleft between the two lobes [Taylor *et al.* 2005; Taylor *et al.* 2004].

The catalytic mechanism appears to be rather similar for all of protein kinases. Therefore PKAc serves as a prototype for a large and highly diverse family of protein kinases. The simplicity of PKAc makes it an ideal template for modelling of more complex protein kinases.

PKAc as a cancer biomarker

In 2000 it was established that cancer cells originating from various tumour tissues excrete active PKAc, designated as extra-cellular PKA (ECPKA) into the conditioned medium [Cho *et al.* 2000b]. Furthermore, it was confirmed that ECPKA was present in the bloodstream of patients ill with different types of cancer (colon, breast, *etc*.) [Cho *et al.* 2000b; Cvijic *et al.* 2000; Wang *et al.* 2007]. It was also shown that ECPKA existed as an active catalytic subunit (PKAc) in patient's blood serum, its catalytic activity was not further increased by addition of cAMP and its activity could be inhibited by the PKA-specific inhibitor PKI [Cho *et al.* 2000a]. Later the increase of concentration of ECPKA autoantibodies was detected in blood serum of cancer patients [Nesterova *et al.* 2006a; Nesterova *et al.* 2006b]. Although the activity level of ECPKA in the blood serum of cancer patients was increased by 5-fold compared to healthy people [Cho *et al.* 2000a], the concentration of PKA in serum samples was still very low and thus not detectable with routine liquid chromatography/tandem mass spectrometry analysis [Berna and Ackermann 2009].

Later studies have confirmed that ECPKA may function as a cancer marker for various types of human cancer and thus can be used for cancer detection and for monitoring response to therapy together with other screening or diagnostic techniques [Cho *et al.* 2000b; Cvijic *et al.* 2000; Kita *et al.* 2004; Wang *et al.* 2007]. If reliable, ECPKA as a generic cancer marker could be of great value for wider screening of population for diagnosis of cancer in early stage.

The studies related to the analysis of ECPKA in blood samples have used kinetic radio-assays based on the quantification of radioactive product formed in the course of transfer of radioactive phosphoryl group from labelled ATP to the substrate peptide Kemptide [Cho *et al.* 2000a; Cho *et al.* 2000b; Cvijic *et al.* 2000; Kita *et al.* 2004; Wang *et al.* 2007]. The quantification of the phosphor-peptide product was performed with a liquid scintillation spectrometer or a phosphor imager. Although sufficiently sensitive, these methods are not well suitable for use in a clinic or an analytical laboratory by virtue of personal and environmental hazards and bad suitability for automation.

Inhibitors of protein kinases

Diverse biochemical functions and generally good druggability [Hopkins and Groom 2002] have made protein kinases an important class of drug targets and objects of intensive biochemical research. Small-molecule inhibitors of protein kinases have been considered as possible drugs for many kinase-related diseases as they directly decrease the activity of the over-activated or over-expressed kinases. However, only a few small cell-permeable molecules have been developed that inhibit one kinase specifically without giving rise to unwanted side effects [Cohen 2002b]. Highly selective inhibitory potency has been assigned to clinically applied protein kinase inhibitor Imantinib (Gleevec) binding to the ATP binding pocket of inactive BCR-ABL tyrosine kinase [Druker *et al.* 1996; Schindler *et al.* 2000]. The drug is indicated for the treatment of chronic myeloid leukaemia [Deininger and Druker 2003]. Still, recent data reveal comparable or even better affinity of Imatinib towards some other protein kinases, *e.g.*, DDR1 and KIT [Davis *et al.* 2011]. By 2011 10 small-molecule kinase-inhibiting compounds had been approved by the Food and Drug Administration of the United States of America (FDA) as cancer drugs, and about 150 compounds had been in clinical testing as drug candidates against different diseases [Fedorov *et al.* 2010]. In 2011 two new protein kinase inhibitor-based drugs received approval from FDA: Vemurafenib (Plexxikon/ Roche's Zelboraf; PLX4032) for advanced melanoma and Crizotinib (Pfizer's Xalkori), a multi-targeted kinase inhibitor for treatment of ALK-driven lung cancer.

Inhibitors of protein kinases are widely used in signal transduction research. Correctness of interpretation of the results obtained with these reagents critically depends on their selectivity. Therefore it is extremely important to determine selectivity of the inhibitors for adequate interpretation of the obtained data. The selectivity of an inhibitor is usually assessed in parallel enzymatic assays with a set of recombinant protein kinases [Manning *et al.* 2002]. Commercial testing panels incorporate increasing number of kinases, the widest panel for kinase inhibitor profiling and screening is KINOMEscan™ kinase assay from DiscoveRx company that currently includes assays for 451 kinases [KinomeScan], a substantial portion of all 518 human protein kinases.

Still a significant demand for cost-effective and amenable to automation high-throughput methods for analysis of protein kinases and their inhibitors exists. Tools for intracellular tracking of kinase functioning are especially valuable [Allen *et al.* 2006].

Although allosteric inhibitors have gained wider interest in recent years [Dar and Shokat 2011], most inhibitors bind to the active site of protein kinases.

ATP-competitive inhibitors

The majority of characterized protein kinase inhibitors are ATP-competitive. A potent inhibitor of protein kinases Staurosporine (AM-2282), a naturally occurring alkaloid, was isolated from the bacteria *Streptomyces staurosporeus* more than 30 years ago [Omura *et al.* 1977; Tamaoki *et al.* 1986]. The analysis of the crystal structure of Staurosporine in complex with the catalytic subunit of PKA confirmed that the high binding affinity towards protein kinases is realized *via* interactions with the ATP binding pocket [Prade *et al.* 1997]. A recent study demonstrated that Staurosporine inhibited the majority of the tested 442 protein kinases with K_d values in the sub-nanomolar and nanomolar range [Davis *et al.* 2011].

Derivatives of isoquinolinesulfonamides, commonly known as Hidaka-series compounds, form another class of protein kinase inhibitors [Hidaka *et al.* 1984]. Although the first representatives of these compounds possessed micromoloar activity towards several protein kinases of the AGC group, the further structural modifications resulted in significant improvements in affinity and selectivity [Ono-Saito *et al.* 1999]. The most thoroughly investigated representative of Hidaka's inhibitors H89 was for some time considered to be a PKAc-selective inhibitor with a K_i value of 50 nM. However, recent reports have shown that H89 inhibits also other kinases of the AGC group (S6K1, ROCK II and MSK1) with nanomolar potency [Lochner and Moolman 2006; Murray 2008]. Furthermore, the first clinically applied protein kinase inhibitor Fasudil (HA-1077) is rather selective towards ROCK II and is used in Japan for the treatment of cerebral vasospasm after subarachnoid haemorrhage [Olson 2008].

Despite the conserved ATP binding sites of protein kinases, numerous rather selective ATP-competitive inhibitors have been developed [Davis *et al.* 2011] that are under clinical trials for signal transduction therapies [Kéri *et al.* 2011; Kéri *et al.* 2006].

A serious disadvantage of designing ATP-competitive inhibitors as drugs is the fact that ATP-competitive inhibitors need to compete with high intracellular concentration of ATP that has been estimated to be in the range of 1 to 5 mM [Gribble *et al.* 2000; Traut 1994].

Substrate protein-competitive inhibitors

Inhibitors competing with the peptide or protein substrate of the kinase are also in wider use [Bogoyevitch *et al.* 2005; Kemp and Pearson 1991; Kemp *et al.* 1991; Lawrence and Niu 1998]. Inhibitors of this class have mostly been comprised of peptides containing the substrate consensus motif that makes it possible to generate greater specificity. Unfortunately, substrate peptide-based inhibitors have usually relatively weak inhibitory potency (K_i in the micromolar range). However, these compounds may express positive cooperativity with ligands binding to the ATP site and hence possess enhanced affinity in the

presence of ATP [Masterson *et al.* 2008]. Usually, longer peptidic structures are needed for achieving better inhibitory potency, which leads to problems with cellular transport and stability of the compounds [Gumireddy *et al.* 2005]. Protein kinase inhibitor protein (PKI) is an endogenous thermo-stable peptide that inhibits PKAc with sub-nanomolar K_i in the presence of ATP [Dalton and Dewey 2006]. The shorter versions of PKI have been synthesized; however shorter peptides show somewhat lower affinity towards PKAc and lack specificity [Glass *et al.* 1986; Pearce *et al.* 2010; Wen and Taylor 1994].

Bisubstrate inhibitors

The understanding that both ATP and the substrate protein are simultaneously present in the active site of a kinase and the transfer of the phosphoryl group takes place directly between these two substrates without the involvement of a covalent intermediate with the enzyme, has led to the construction of bisubstrate inhibitors (also known as substrate analogue inhibitors) [Parang and Cole 2002]. Bisubstrate inhibitors can give a greater number of interactions with the target protein that may result in higher affinity and selectivity of the compounds [Lavogina *et al.* 2010a].

The first bisubstrate inhibitors contained both substrates: a nucleotide or a nucleoside mimic and a substrate peptide connected *via* a linker chain. However, ATP-competitive inhibitors can be used as a nucleotide binding pocket targeted part of the bisubstrate inhibitor and inhibitory peptide fragments can be applied instead of substrate peptides [Ricouart *et al.* 1991; Schneider *et al.* 2005].

The bisubstrate character of inhibitors is generally confirmed by three methods [Lavogina *et al.* 2010a]:

- X-ray analysis of the structures of the protein kinase: inhibitor co-crystals
- kinetic analysis of the competitiveness of the inhibitor versus both substrates
- displacement of the bisubstrate probe from its complex with protein kinase by either substrate

Bisubstrate inhibitors have been used as biochemical and structural probes of mechanism and such an approach has led to the development of compounds with powerful therapeutic properties [Yu *et al.* 2006].

ARC-type bisubstrate inhibitors

Adenosine-oligoarginine conjugates (ARCs) are a good example of a successful realization of bisubstrate inhibitor approach for basophilic kinases (protein kinases catalysing phosphorylation of proteins at Ser/Thr residues which close proximity is rich in basic amino acid residues, arginine and lysine [Pinna and Ruzzene 1996]). More than 50 protein kinases of the AGC group, many members of the CaMK group and several other kinases fall into this category, including PKAc and kinases of therapeutic interest Akt/PKB and ROCK II.

ARC-type inhibitors consist of a nucleoside-resembling part (an adenosine mimic or a fragment of small-molecular-weight inhibitor binding to the ATP-site of the kinase) coupled to a peptidic moiety (an arginine-rich peptide) *via* a flexible linker chain [Enkvist *et al.* 2006].

The sub-micromolar affinity of the first generation ARC-type inhibitors towards basophilic protein kinases PKAc and PKC [Loog *et al.* 1999] was significantly improved by replacing the L-amino acid residues by D-amino acids in the linker and peptidic moiety [Enkvist *et al.* 2006]. In addition to improving the binding affinity the application of D-amino acids in the linker and in the peptidic moiety of ARCs increases their stability towards proteolysis. The affinity and selectivity of ARCs towards basophilic protein kinases can be tuned by application of different nucleosidic fragments, linkers, and peptide moieties [Enkvist *et al.* 2009; Enkvist *et al.* 2007]. Very high affinity of ARCs (K_D-values reaching picomolar range) towards basophilic kinases was achieved based on extensive structure-activity studies and X-ray analysis of crystals of complexes of inhibitors with PKAc [Lavogina *et al.* 2009; Lavogina *et al.* 2010b; Pflug *et al.* 2010]. These studies enabled the optimization of the structure of the linker fragment that now plays a critical role in realization of the affinity of these compounds by providing crucial interactions with the target kinase and directing the peptidic chain into the right orientation for important interactions with the kinase.

The high binding affinity is preserved after labelling of ARCs with PEG [Viht *et al.* 2003] or fluorescent dyes [Vaasa *et al.* 2009], or immobilization of ARCs onto solid surfaces [Viht *et al.* 2007]. Furthermore, the arginine-rich peptide moiety makes the conjugates plasma membrane-permeable [Uri *et al.* 2002; Viht *et al.* 2003] enabling the use of these probes for cellular experiments [Vaasa *et al.* 2010]. All these remarkable properties enable the application of ARCs as bioprobes and -sensors in a large variety of assays.

Protein analysis methods

Methods for the identification of potential protein biomarkers

Great effort has been put into the development of methods that enable comparative and quantitative analysis of benign and malignant tissue samples for the identification of altered levels of proteins and their post-translational modifications related to the progression of cancer. On one hand the detected disease-related proteins are major drug targets for the targeted therapies. On the other hand these proteins could serve as biomarkers for disease diagnosis, prognosis and prediction of the therapeutic outcome on an individualized basis. However, tissue samples are obtained from the biopsy samples when the first symptoms of cancer have emerged. Therefore blood based biomarkers are considered more valuable as the collection of the sample is easier and less invasive. Furthermore, blood based cancer biomarkers can be used for cancer screening before the first

symptoms appear and for the monitoring of the disease recurrence before the tumour can be visualized with other methods.

The numerous molecular mass-based methods enabling the identification of proteins and their posttranslational modifications related to their amino acid sequence have guided the discovery of new potential biomarkers in the recent years. Generally the biomarkers are discovered by comparing the relative abundances of proteins in biological samples like biopsy samples or body fluids. The first method used for differential protein expression profiling was two-dimensional gel-electrophoresis (2DE) that enabled the separation of proteins by their isoelectric point in one dimension and molecular weight in the second dimension [Gorg *et al.* 2004]. The differential staining profiles in healthy and diseased tissue were compared and the differentially expressed proteins were identified by mass spectrometry. However, the need for manual aligning of the protein spots on two gels has restricted the usability of 2DE for biomarker research. Therefore methods for covalent labelling of proteins from healthy and diseased tissue samples prior to 2DE with two labels with similar chemical and physical properties like radio-isotopes with different gamma-ray emission energies (*e.g.,* ^{125}I and ^{131}I in ProteoTope technology [Cahill *et al.* 2003; Poznanovic *et al.* 2005; Wozny *et al.* 2007]) or spectrally resolvable fluorescent dyes with similar mass and charge (*e.g.,* Cy3 and Cy5 in 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) [Kondo *et al.* 2003]) have been developed for pairwise comparison of two samples on a single gel. Another major disadvantage of 2DE based methods is the wide dynamic range of protein concentrations in biofluids. Hence, the spots of low abundance proteins (possible biomarkers) are often masked by high abundance proteins. Therefore for the detection of blood based biomarkers methods for the depletion of high abundance proteins like albumin are applied [Bjorhall *et al.* 2005; Echan *et al.* 2005]. However, the depletion procedure may deplete some of the potential biomarkers as well [Granger *et al.* 2005]. Therefore the development of methods for affinity enrichment of target class of proteins is of great importance.

Although, the combination of 2DE and mass spectrometry is a sensitive and well suitable method for identification of new potential biomarkers such methodology is expensive and time consuming and hence, not well suitable for clinical screening purposes. Therefore affinity based methods for the screening purposes are developed.

Bioaffinity assays for the measurement of protein biomarkers

The most common assays used in clinical laboratories for the detection of proteins are immunoassays. The principle of an immunoassay is based on the antibody-antigen complex formation providing assays with required high specificity and sensitivity. Furthermore, immunoassays are easy to perform which has contributed to their success and widespread use.

In the so-called sandwich-ELISA (Enzyme-Linked ImmunoSorbent Assay, Figure 1A) [Crowther 2001], the target protein is captured from the sample to the surface *via* the formation of the complex with a surface-bound antibody (capture antibody) or another molecule with high affinity towards the target protein. The solution of another target protein-specific antibody (primary detection antibody) is added after removal of unbound material, and the amount of the detection antibody bound to the target protein on the surface is quantified. For the quantification purposes the detection antibodies or secondary detection antibodies (recognizing the primary detection antibody) are labelled with an enzyme (*e.g.,* horse-radish peroxidase or alkaline phosphatase) which catalyses fluorogenic or luminogenic reactions leading to the signal enhancement. As the amount of each biomarker has to be determined in a separate assay the number of biomarkers determined from one sample is rather limited.

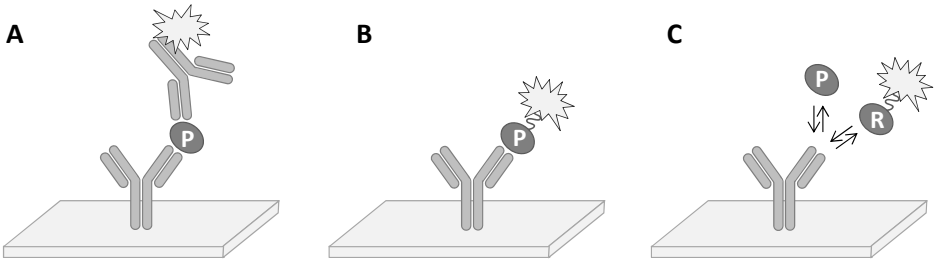


Figure 1. Schematic representation of heterogeneous immunoassays for the measurement of concentration of proteins. **(A)** Sandwich immunoassay. Capture antibodies are immobilized on the surface and incubated with test sample containing the target protein *P*; detection is carried out by means of a labelled secondary antibody. **(B)** Antibody microarray. After labelling of all proteins of the sample with a fluorescent dye the test sample is incubated on the surface. The amount of target protein interacting with capture antibody (after unbound material has been removed by washing) is proportional to fluorescence intensity. **(C)** Reporter displacement assay. After the sample has been incubated on the surface the detection reagent *R* (containing the fluorescently labelled epitope of the target protein) is added. The amount of target protein can be quantified from the reduction of fluorescence intensity.

Antibody-microarrays enabling simultaneous (multiplexed) detection of several biomarkers in a single assay have been developed. Basically, antibodies binding different target proteins are spotted on the bottom of an assay plate or colour coded beads and the amount of target proteins can be determined by numerous label-free and label-based detection systems [Chandra *et al.* 2011; Glokler and Angenendt 2003]. The simplest antibody-microarray as depicted on Figure 1B requires the development of a single monoclonal antibody against each target protein and allows analysing more samples in parallel. The proteins in the samples are covalently labelled with fluorescent dyes or radio-isotopes. Specific antibodies spotted to the glass or filter surfaces bind their target proteins *via*

specific interactions and the unbound material can be removed by washing. Although allowing the pairwise analysis of two samples (if two samples are treated with different fluorescent dyes or radio-isotopes) the labelling of all proteins in the sample is rather tedious and reporter displacement formats of antibody-microarrays (Figure 1C) for diagnostic purposes have been introduced. For increased selectivity the antibody-microarray can be performed in sandwich format familiar from the sandwich-ELISA using detection antibodies labelled with either radioisotopes, fluorescent or luminescent dyes, *etc*. for the quantification.

Despite the numerous possibilities in different antibody-microarray setups, the number of different biomarkers determined in a single antibody-microarray is still limited by the inherent cross-reactivity of antibodies [Michaud *et al.* 2003]. Additionally, the application of sandwich immunoassays is retarded by the need for the development of two orthogonal monoclonal antibodies binding their target protein with high affinity. Hence, alternative capture agents like peptoids (oligo-N-substituted glycines) [Reddy and Kodadek 2005], aptamers (short short single-stranded DNA or RNA oligonucleotides that fold into a well-defined three-dimensional structure can bind to a wide range of target molecules with high affinity and specificity [Gold *et al.* 2010; Walter *et al.* 2008]), affibody molecules (small protein bundles generated by combinatorial protein engineering with specificity for a wide range of targets [Renberg *et al.* 2007]), *etc.* replacing the capture antibodies in sandwich array formats have been introduced.

The greatest advantage of heterogeneous immunoassays is the possibility to remove the non-target proteins by washing. However these procedures add several steps to the analysis procedure and therefore homogeneous methods have been introduced. Homogeneous immunoassays, similarly to heterogeneous assays, take advantage of antibodies as bioaffinity reagents. Although simultaneous analysis of multiple biomarkers is generally not possible the homogeneous format has several advantages over the heterogeneous formats as well, like homogeneity, usability in single-step format, quickness, and amenability to automation.

The setup of the homogeneous immunoassays requires the detection procedure to be performed directly in the sample which on one hand ensures that the target protein is not removed by washing procedures. On the other hand, the excess of the detection reagent cannot be removed by washing and hence simple radio-isotope- or fluorescence dye-based detection methods widely used in heterogeneous assays are not applicable. Therefore Förster-type resonance energy transfer- (FRET)-based (discussed in the next section) sandwich- or reporter displacement-type assays have been developed (Figure 2). Furthermore, as the measurements in blood liquids plasma and serum are especially demanding because of strong fluorescence background of the samples [Morgner *et al.* 2011], probes with longer luminescence lifetimes like luminescent lanthanide complexes enabling the short-lived background interferences to be

removed *via* time-gated (time-resolved) acquisition have been developed [Hagan and Zuchner 2011]. The homogeneous assays based on the measurement of long lifetime luminescence of plasma and serum samples are now available from biotech companies (*e.g.*, Cisbio, B·R·A·H·M·S GmbH) for several protein-type biomarkers. These assays use two orthogonal antibodies, one labelled with a luminescent lanthanide chelate (luminescence donor) and the other with a fluorescent dye (acceptor) [Hagan and Zuchner 2011]. Simultaneous association of both antibodies with the antigen induces FRET from the donor to the acceptor that enables the quantification of the antigen in homogeneous solution without need for separation and washing steps.

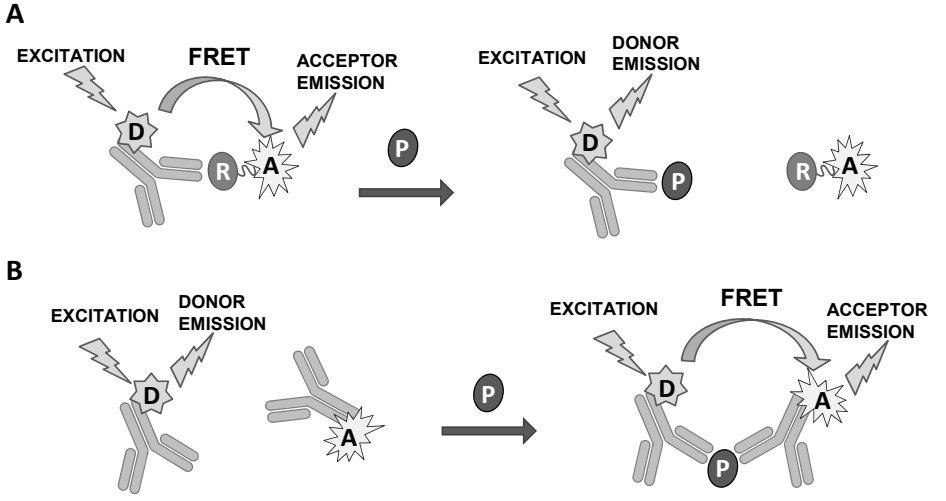


Figure 2. The schematic representation of homogeneous immunoassays. **(A)** Reporter displacement assay. In the absence of the analyte an antibody labelled with RET donor *D* forms a complex with the reporter protein *R* labelled with an acceptor dye *A*. Upon excitation of the donor the energy is transferred to the acceptor *via* FRET and only the emission of the donor is observed. Upon adding a sample containing the target protein *P* the reporter is displaced from its complex with antibody and the FRET does not occur, thus only emission at the donor is observed. **(B)** Homogeneous sandwich immunoassay. Two orthogonal antibodies are labelled with either a donor or an acceptor dye. The FRET occurs only if both of the antibodies are brought to close proximity by interacting with the target protein.

Photoluminescence

In the recent years photoluminescence has become a major methodology used in biochemical assays. Photoluminescence is an optical emission of photons from molecules that have been excited to higher energy levels by absorption of light. As a photoluminescent molecule (luminophore) absorbs a photon, its outer electrons are raised from the ground state (S_0) to some of its higher energy levels (S_1 or S_2). After spending some time in the electronically excited state the luminophore returns to the ground state either by emitting a photon (fluorescence) or by any of the following non-radiative mechanisms: non-radiative dissipation of energy (as heat), intersystem crossing (ISC) to triplet state, resonance energy transfer to another molecule (quenching), photobleaching (light-induced destruction of a dye while in the excited state), *etc.* (Figure 3). The intersystem crossing from S_1 to T_1 occurs *via* spin conversion resulting in paired spins of the electrons at the excited and ground states and hence, the energy relaxation from T_1 to S_0 is a forbidden process.

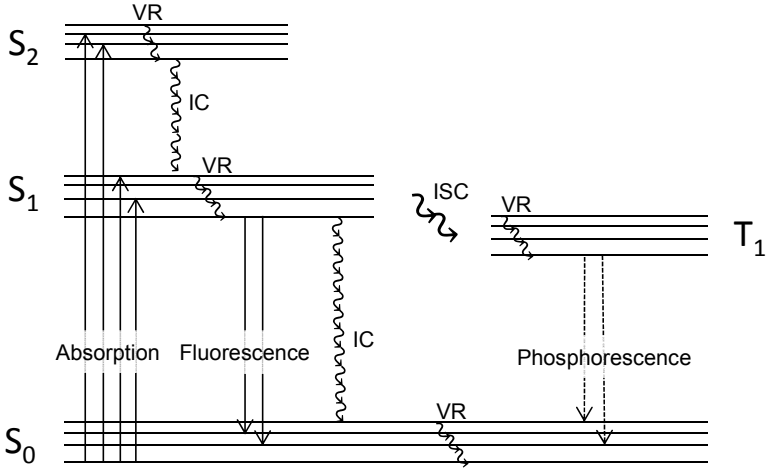


Figure 3. The Jablonski diagram for photoluminescence (adopted from [Hemmilä 1991]). A luminophore absorbing a photon is excited from its ground state (S_0) to any of the vibrational levels of the singlet exited states (S_1 and S_2). The molecule rapidly relaxes to the lowest vibrational level of the lowest excited state by vibrational relaxation (VR) or by internal conversion (IC) from S_2 to S_1 if higher energy level was obtained upon excitation. The molecule returns to its ground state either non-radiatively by IC or by emitting a photon (fluorescence). Additionally molecules in the S1 state can undergo a spin conversion to the first triplet state (T_1) *via* mechanism termed as intersystem crossing (ISC) which is followed by the radiative dacay from the triplet excited state to the singlet ground state (phosphorescence).

The average time the luminophore spends in the exited state is described by luminescence lifetime (τ) that is related to the rate constants of the radiative (k_f) and the sum of non-radiative (k_{nr}) processes:

$$\tau = \frac{1}{k_f + \sum k_{nr}} \quad (1)$$

The fluorescence lifetimes of most organic fluorescent molecules are in the range of 1 – 10 nanoseconds [Lakowicz 2006]. Transition from T_1 to S_0 is forbidden and as a result the rate constants for triplet emission (phosphorescence) are significantly smaller than those for the singlet emission (fluorescence) and hence the luminescence lifetime is in microsecond and millisecond range.

The luminescence intensity (*I*) follows a single exponential decay model if only florescence is observed (equation 2) where I_0 is the intensity at $t = 0$:

$$I(t) = I_0 e^{-t/\tau} \quad (2)$$

As during the excitation and emission processes some of the energy is always lost as a result of vibrational relaxations the energy of the photons required for the excitation is greater than that of the emitted photons. Hence, the excitation of the luminophore occurs at lower wavelengths than the emission is observed. The difference between the maximums of the excitation and emission spectra is termed as the Stokes shift.

Homogeneous bioaffinity assay formats do not allow the separation of the unbound and complexed with the biological target detection reagents. Thus optical methods that afford differentiating of signals from unbound and bound interacting partners are required. One of the most popular methods for the detection of molecules in close proximity is based on the measurement of intensity of Förster resonance energy transfer (FRET). FRET is a distance-dependent non-radiative energy transfer from the exited state $S(D)_1$ of FRET donor molecule to the exited state $S(A)_1$ of an acceptor molecule [Lakowicz 2006]. As a result of the energy transfer both the fluorescence lifetime and the intensity of the emitted fluorescence of the donor are decreased and the increase in the emission intensity of the acceptor (sensitized emission) is observed. In addition to distance between the donor and acceptor FRET efficiency (*E*) is related to several other factors including the overlap of the donor emission and acceptor excitation spectra and the relative orientation of the dyes. However, as some of the parameters affecting *E* are difficult to determine FRET efficiency is often calculated based on the change of the luminescence lifetimes (equation 3) or intensities (equation 4) of the donor in the absence and presence of the acceptor:

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (3),$$

where τ_A and τ_{DA} are the luminescence lifetimes of the free acceptor and the acceptor in complex with the donor, respectively, or:

$$E = 1 - \frac{FI_{DA}}{FI_D} \quad (4),$$

where FI_A and FI_{DA} are the luminescence intensities of the free acceptor and the acceptor in complex with the donor, respectively. The occurrence of FRET and hence the interaction between the proteins labelled with either donor or acceptor can be determined either by measuring the change in the fluorescence intensities at the donor and acceptor wavelengths or by determining the change in the luminescence lifetime of donor emission.

Although measurements of FRET intensity between organic dyes, fluorescent proteins or their combinations are widely used both in biochemical and cellular measurements for the analysis of ligand-protein and protein-protein interactions, these measurements are often disturbed by the background autofluorescence of biological samples. The autofluorescence usually has decays on the nanosecond timescale as do most of organic fluorophores that reduces the sensitivity of detection. To overcome this shortcoming probes with longer luminescence lifetime have been described [Soini and Hemmilä 1979].

The lanthanides are uniquely luminescent metals that display emission in aqueous solutions and decay times in the range of 0.5 –3 ms. The emission results from transitions between *d* and *f* orbitals, which are formally forbidden transitions [Hemmilä and Laitala 2005; Werts 2005]. Hence the emissive rates are slow resulting in long lifetimes. Lanthanides display line spectra from individual atoms [Moore *et al.* 2009]. As the extinction coefficients of lanthanides are extremely low, less than 10 $M^{-1}cm^{-1}$, for practical measurements in bioassays lanthanides are not excited directly but rather through chelated organic ligands. It is proposed that the organic acceptor is first excited to S_1, followed by its transition to T_1 state and thereafter the energy transfer to the lanthanide ion occurs [Soini and Hemmilä 1979]. As a result of the cascade of transfers the absorption spectrum resembles that of the chelating ligand and not of the lanthanide ion itself. Besides increasing the excitation efficiency of lanthanides the chelators displace the water molecules coordinating the lanthanide ion in aqueous solutions where water molecules would act as quenchers.

Lanthanide-ligand complexes have found widespread use as luminophores in highly sensitive analytical methods, particularly for homogeneous immunoassays [Hagan and Zuchner 2011]. Luminescent lanthanide complexes have exceedingly long-lived luminescence in comparison with conventional organic

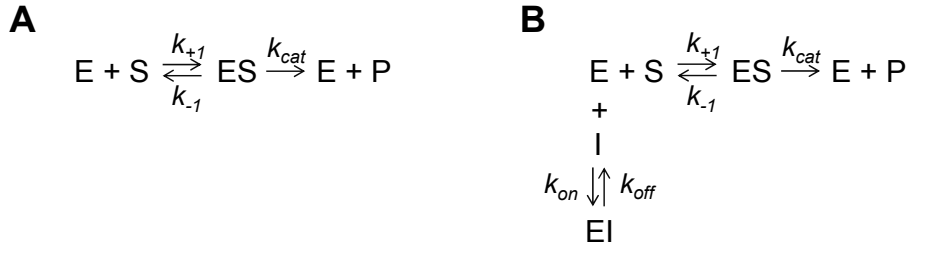
fluorophores, enabling the short-lived background interferences to be removed *via* time-gated (time-resolved) acquisition, thus delivering greater assay sensitivity and a broader dynamic range [Hagan and Zuchner 2011]. Moreover, as lanthanides possess several narrow emission bands over the wavelength range of several hundred nanometers numerous different organic dyes and fluorescent proteins with different excitation spectra can be used as FRET acceptors.

Another advantage of the usage of lanthanide chelates as FRET donors is the possibility of using double wavelength detection for the analysis of unprocessed complex samples (like plasma or serum) containing numerous proteins and small molecules absorbing a significant proportion of the exciting photons [Morgner *et al.* 2011]. The emissions of both the lanthanide donor luminophore and the acceptor fluorophore are assumed to be affected to the same degree [Bazin *et al.* 2001; Degorce *et al.* 2009] by optical properties of the sample. Hence the luminescence intensity at the donor lanthanide chelate emission peak is used as the internal reference while the emission from acceptor fluorescent dye is used as the indicator of the amount of the molecular complex formed that brings the luminescence donor and acceptor to close proximity [Mathis 1993]. Moreover, the multiple emission wavelengths of the lanthanide chelates can be used for simultaneous excitation of multiple acceptors with different excitation spectra and hence multiple analytes can be measured simultaneously in a single step format using either organic dyes or quantum dots as acceptors [Geissler *et al.* 2010; Kokko *et al.* 2009; Kokko *et al.* 2008; Myyrylainen *et al.* 2010].

Although numerous different optical setups and detection principles have been successfully used in immunoassays, there are still biological requirements for further improvements of these assays. A disadvantage of the immunoassays is the high cost of development and production of monoclonal antibodies with reasonable affinity towards target proteins. Another major disadvantage is the fact that antibodies can be used for the determination of the total (molar) amount of the target protein and they are usually not suitable for the quantification of the activity level of target enzymes (*e.g.,* protein kinases) which knowledge may be crucial for the assessment of the status of cellular signalling pathways under investigation.

Methods for monitoring of protein kinase activity

Protein kinase activity can be determined by monitoring the formation of the product formed in course of enzymatic reaction. As protein kinases are multi-substrate enzymes the catalysis mechanism is rather complicated and therefore the enzymatic activity assays are commonly performed under the conditions where one substrate is taken in excess and the formalism of a monosubstrate enzymatic reaction can be adapted for the description of the process (Scheme 1A).



Sheme 1. (A) The mechanism of a monosubstrate enzymatic reaction, where E, S and P denote the enzyme, substrate and product, respectively; the active complex is denoted as ES; k_{+1} and k_{-1} are the association and dissociation rate constants, respectively, and k_{cat} is the rate constant of product formation. **(B)** The mechanism of a monosubstrate reaction in the presence of a competitive inhibitor, where I denotes a competitive inhibitor, and k_{on} and k_{off} are the association and dissociation rate constants of EI complex formation and dissociation, respectively.

The velocity of a monosubstrate enzymatic reaction is determined by values of the rate constants [Segel 1993]:

$$v = \frac{k_{cat}[\mathrm{E_t}][\mathrm{S}]}{\frac{k_{-1}}{k_{+1}}+[\mathrm{S}]} \quad (5),$$

where $[\mathrm{E_t}]$ represents the total concentration of the enzyme. If $k_{cat}[\mathrm{E_t}]$ is substituted by V_{max}, the limiting maximal velocity that would be observed when all the enzyme is present as ES and if k_{-1}/k_{+1} is substituted by K_M, the Michaelis-Menten constant[1] of the substrate, the classical Michaelis-Menten equation (6) is obtained [Segel 1993]. It should be noted that V_{max} and K_M are characteristic to a specific substrate [Price and Stevens 1999].

$$v = \frac{V_{max}[\mathrm{S}]}{K_M+[\mathrm{S}]} \quad (6)$$

If the monosubstrate enzymatic reaction is performed under the conditions where the concentration of the substrate significantly exceeds that of the Michaelis-Menten constant ($[S] \gg K_M$), the reaction velocity is only dependent on the concentration of the enzyme:

[1] In biochemistry all the coefficients are by convention referred to as constants (*e.g.*, dissociation constant) although, the values are not determined under standard conditions but in the relevant conditions to biochemistry (30 °C or 37 °C, pH = 7) and the values are dependent on the temperature, pH, ionic strength *etc*. Moreover the values are not dimensionless (*e.g.*, the dissociation constant possesses a dimension of [mol/L]).

$$v \cong k_{cat}[\mathrm{E_t}] \quad (7)$$

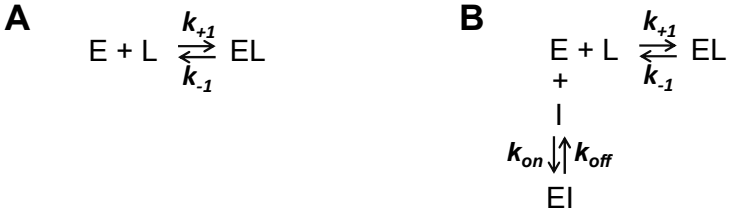
These conditions have been named as stoichiometric binding conditions, as no molecule of enzyme is present as a free enzyme. The assessment of inhibitory potencies of competitive inhibitors (Scheme 1B) is generally performed under these conditions.

The most commonly used method for the assessment of protein kinase activity has been the phospho-cellulose paper method in which the incorporation of radioactive phosphate from [^{32}P]ATP into the substrate peptide or protein is determined [Glass *et al.* 1978; Witt and Roskoski 1975]. As radiometric methods have several drawbacks (personal risks, environmental pollution and short half-life of ^{32}P) several non-radiometric alternatives have been developed and the radioactive assay is applied only if no better assay is available [Zaman *et al.* 2003]. The alternative detection techniques are often based on the conjugation of the substrate peptide or protein to a fluorescent dye and the chromatographic [Viht *et al.* 2005; Wu *et al.* 2006] or electrophoretic separation of the fluorescently labelled product from the non-phosphorylated substrate. Additionally, immunoassays (*e.g.,* ELISA) based on monoclonal antibodies recognizing the phosphorylated amino acid are available [Alberta and Stiles 1997]. Several versions of kinetic assays based on the measurement of the decrease of the concentration of ATP or the formation of ADP are also in active use now [Fan and Wood 2007; Huss *et al.* 2007; Kunzelmann and Webb 2010; Srinivasan *et al.* 2004].

The activity of protein kinases (like other enzymes) is usually expressed in the "enzyme unit" scale (U), where 1 U of enzyme activity corresponds to the amount of protein kinase that catalyses the phosphorylation of 1 nanomole of the defined substrate in one minute. Specific activity is defined as *U of activity per milligram protein* [Segel 1993]. However, even the patches of purified recombinant proteins always contain a mixture of a fully active and inactive (*e.g.,* lacking necessary posttranslational modifications or degraded during sample preparation procedures) forms of the kinase. The situation is even more complicated when biological samples containing proteases, phosphatases, *etc.* are to be analysed. Hence, the specific activity depends on the origin of the sample and sample preparation procedures, and for comparison of samples the originally defined substrate has to be used for monitoring the phosphorylation reaction to avoid recalculations [Price and Stevens 1999].

Equilibrium binding assays

The concentration of the active enzyme can be determined by titration of the enzyme with a compound binding to the active form of the enzyme (Scheme 2A). The concentration of the active form of an enzyme does not depend on the affinity of the ligand used for the titration procedure and hence, is a more basic characteristic of an enzyme than its specific activity.



Scheme 2. (A) The mechanism of equilibrium binding, where E and L denote the enzyme and ligand, respectively; and the formed complex is denoted as EL; k_{+1} and k_{-1} are the association and dissociation rate constants, respectively. **(B)** The mechanism of a ligand displacement assay in the presence of a competitive inhibitor, where I denotes a competitive inhibitor, and k_{on} and k_{off} are the association and dissociation rate constants of EI complex formation and dissociation, respectively.

The equilibrium dissociation constant K_D of the enzyme:ligand complex can be determined as follows:

$$K_D = \frac{k_{-1}}{k_1} = \frac{[E] \times [L]}{[EL]} = \frac{([E_t] - [EL]) \times ([L_t] - [EL])}{[EL]} \quad (8),$$

where $[E_t]$ and $[L_t]$ represent the total concentration of the enzyme and ligand, respectively. However, in the ligand binding assay usually the amount of [EL] is quantified and hence equation 8 can be rearranged to obtain equation 9 for fitting the equilibrium binding data.

$$[EL] = \frac{[E_t] + [L_t] + K_D - \sqrt{([E_t] + [L_t] + K_D)^2 - 4[E_t][L_t]}}{2} \quad (9)$$

If the titration of the protein kinase active site is performed under the conditions $[L] >> K_D$, the initial part of the curve ([E] < [L]) follows linear dependency and the curve is tilted at the point where the concentration of active sites equals to the concentration of the ligand [Tetin and Hazlett 2000]. When concentration series of an enzyme is titrated with two different concentrations of the ligand ($[L] >> K_D$ and $[L] \leq K_D$) both the K_D and the fraction of the active protein kinase can be determined [Vaasa *et al.* 2009].

The ability of a compound to bind to its target kinase provides important information of the interaction but often the compounds most interesting from the pharmacological point of view are not labelled with tags that would allow the determination of the affinity of the compound from a direct binding assay. Therefore sensors or probes that allow the screening of libraries of low molecular compounds in displacement assay in HTS format are developed (Scheme 2B).

Surface plasmon resonance

Surface plasmon resonance (SPR) spectrometry is an optical technique which measures refractive index change at the sensor–fluid interface layer [Zimmermann *et al.* 2002]. The ligand (or the protein) is covalently coupled to the carboxymetylated dextran matrix on a gold-coated glass chip and the binding of the analyte to the sensor surface results in an increase in refractive index which is detected by a shift in the resonance angle (Figure 4). The signal generated in arbitrary response units (RU) is proportional to a change in mass: *e.g.,* signal difference equal to 1 RU is obtained if 1 pg of a protein is adsorbed to 1 mm^2 area of the sensor surface [Stenberg *et al.* 1991].

This versatile technology enables the measurement of both the equilibrium dissociation constant K_D and kinetic parameters, the binding on- and off-rates, in real time without the need of labelling the analyte. A wide range of affinities can be characterized with high reproducibility, high sensitivity and low sample consumption. Simultaneous monitoring of different surfaces makes it possible to subtract the signal resulting from non-specific binding and to account for refractive index changes as well as matrix effects.

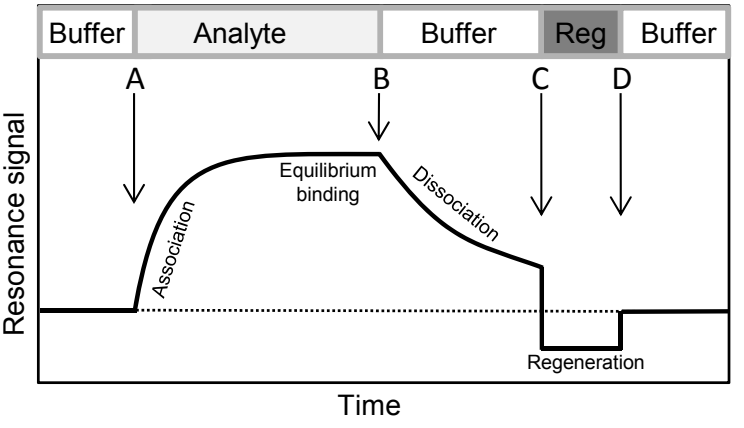


Figure 4. A typical binding cycle observed with an optical biosensor. At time point A a solution of the analyte is passed over the sensor surface: as the analyte binds to the immobilized ligand, the refractive index at the sensor surface increases, causing an increase in the resonance signal. At time point B the analyte solution is replaced by a running buffer and the ligand–analyte complex starts to dissociate and the resonance signal begins to decrease. In between time points C and D a regeneration solution is used to disrupt the binding on the surface and regenerate the free ligand on the sensor surface.

Analysis of the binding in the association phase gives the observed association rate (k_{obs}). If the concentration of the analyte is known, the association rate constant of the interaction (k_{on}) can be determined. Analysis of the dissociation

phase gives the dissociation rate constant (k_{off}) for the interaction. The affinity of the interaction can be calculated from the ratio of the rate constants ($K_D = k_{off}/k_{on}$) or by fitting of the response at equilibrium binding against varying concentrations of the analyte [Moll *et al.* 2006].

If a small inhibitor is coupled to the surface its selectivity towards different biomolecules can be measured (Figure 5A). As an advantage of this approach, large biomolecules give measurable responses even at their very low concentrations and low surface densities. In most cases the resulting surface with immobilized small organic molecules is very stable and quite harsh regeneration solutions can be used. Unfortunately, pure proteins are needed to conduct such experiments correctly.

If large biomolecules are immobilized on the sensor surface the binding of different inhibitors can be measured (Figure 5B). Since the SPR signal depends on the mass of the material bound to the sensor surface, small analytes (Mr <1000) give very small responses. Although instruments have been developed (Biacore 3000 and S51, both from GE Healthcare) that are capable of directly detecting substances possessing low molecular weights, high surface concentration of the active immobilized ligand is needed. Furthermore, at such high ligand densities accurate kinetic analysis is not possible due to mass-transport limitations and re-binding of the analyte. Another downside of this approach is the fact that the biomolecules tend to lose their activity during immobilization procedure and measurements.

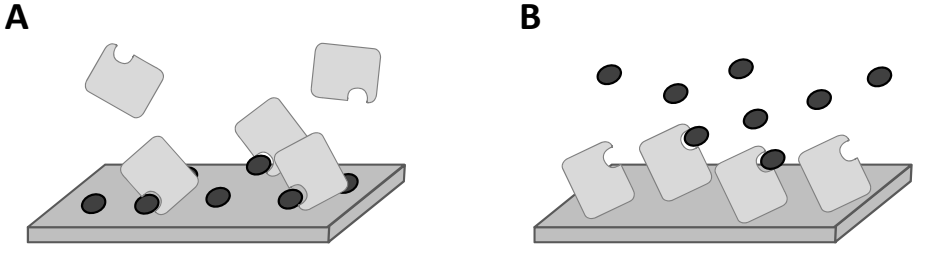


Figure 5. Alternative schemes of SPR measurements. **(A)** A small inhibitor (dark grey) is immobilized on the surface and the binding of a biomolecule (light grey) is monitored. **(B)** A biomolecule is immobilized on the surface and the binding of an inhibitor is monitored [Zimmermann *et al.* 2002].

Competition assays [Karlsson 2004] are useful alternatives to the aforementioned approaches. Two assay formats, solution competition (Figure 6A) and surface competition (Figure 6B) are possible. Competition assays can easily be automated and are especially useful for the analysis of inhibitory potency and selectivity of low molecular weight substances; however, rate constants cannot be determined from the surface competition assays.

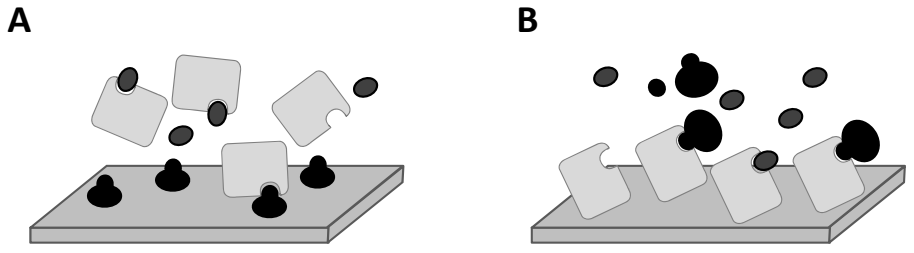


Figure 6. Experimental setup of the competition experiments. The apparent IC_{50}-values can be measured by varying the concentration of the competitor. Solution competition (A): competitor molecules bind to analytes obstructing the binding of the immobilized ligand. Surface competition (B): competitor molecules compete with analytes for the same binding site of an immobilized ligand. This assay setup requires the occurrence of significant mass differences between the analyte and the competitor [Zimmermann *et al.* 2002].

Another advantage of SPR measurements is the ability to couple this method with mass spectrometry. The sensor chip is used to capture the analyte, which may exist as a minor species in a milieu of cellular proteins. The matrix is added directly to the sensor surface and the sensor chip is mounted into a MALDI-TOF mass spectrometer. Mass spectroscopy is then used to analyze the molecular weight of the captured molecule directly on the chip. The advantage of this type of analysis is that there is the opportunity to examine the material actually bound to the surface of the sensor chip without any further manipulation of the sample [Krone *et al.* 1997; Nelson *et al.* 2000].

Methods for analyzing protein kinase activity in live cells

The easiest way to monitor protein kinase activity in live cells is monitoring the incorporation of [^{32}P] into kinase substrate proteins in the presence of [^{32}P]ATP. After incubating the cells in the presence of protein kinase activators or inhibitors the phosphorylated proteins are separated by SDS gel electrophoresis. Although the assay can be applied for the investigation of a broad range of kinases and protein kinase activators and inhibitors, the assay is often avoided in the research institutions as the methodology involves many environmental and health hazards.

Another opportunity is the identification of phosphoproteins by Western plot analysis with phospho-specific antibodies [Chen *et al.* 2005]. Whereas the antibodies against phosophotyrosine are readily available the analysis of both phosphoserine- and phosphotreonine-containing proteins needs the raising of specific antibodies against specific phosphopeptide sequences.

Both of the described assays are end point assays and hence do not allow the constant monitoring of kinase activity in living cells. Most of the assays

designed for the monitoring of protein kinase activity in real time are based on genetically encoded biosensors that require the over-expression of the target protein as a fusion with a pair of fluorescent proteins (e.g., variants of GFP) [Zhang *et al.* 2001; Yeh *et al.* 2002]. These sensors often take advantage of the FRET phenomenon.

AIMS OF THE STUDY

The aim of the current study was the development and assessment of ARC-type inhibitor-based high throughput methods for the determination of concentration of active protein kinases and evaluation of inhibitors of protein kinases.

More specifically the objectives of the studies that are summarized in the present thesis were the following:

- The development of ARC-based affinity supports for the selective enrichment of active protein kinases from crude biological samples.
- The characterization of immobilized ARCs in surface plasmon resonance measurements and the application of the developed sensors for analysis of activity of protein kinases and affinity of their inhibitors.
- The elaboration of a homogeneous high throughput photoluminescence method for measurement of protein kinases in biological samples.
- The application of the developed photoluminescence assay for the analysis of a putative cancer biomarker ECPKA in the patient's blood samples.

SUMMARY OF RESULTS AND DISCUSSIONS

Development of affinity supports for enrichment of protein kinases and characterization of protein kinase inhibitors (Papers I, II and unpublished data)

Tissue samples contain an enormous number of different proteins whose concentration differs by more than six orders of magnitude. Therefore a severe need exists for the development of methods capable of selective enrichment of low abundance proteins like protein kinases from crude tissue samples.

To start the development of enrichment procedures and assay methods the possibility of using ARC-type inhibitors as baits for the enrichment of protein kinases from crude samples was investigated. An ARC-type inhibitor of the first generation [Lavogina *et al.* 2010a] ARC-342 was covalently immobilized on agarose beads. The obtained affinity adsorbent was successfully applied for the pull-down of protein kinases ROCK II and PKAc from cell lysates (Paper I, Figure 4C). Although ARC-341 (the precursor of ARC-342) possesses similar affinity (K_D values in the submicromolar region) towards both tested protein kinases (Paper I Table 1; Table 2 in [Vaasa *et al.* 2009]), the differences in the binding and elution patterns from the affinity resin Affi-Gel-ARC-342 indicate that the immobilization of the inhibitor affects the binding of these protein kinases differently.

In order to investigate the binding and dissociation kinetics of protein kinases to immobilized ARC-type inhibitors ARCs were immobilized on surfaces of biosensor chips. Two strategies were used for the surface preparation. Firstly, ARC-type inhibitors of the second generation (ARC-904 or ARC-1023) were covalently bound to the biosensor surface *via* the free amino group on the side chain of lysine residue. The second approach required the covalent coupling of streptavidin to the biosensor surface and successive binding of a biotinylated ARC (ARC-704 or ARC-1024) to streptavidin.

Both, the covalent immobilization of ARC-904 and the attachment of biotin-containing ARC-704 on the streptavidin-conjugated surface, resulted in sensor surfaces with similar affinity towards PKAc. The addition of 2 extra 6-amino-hexanoic acid residues into the immobilization linker arm of ARC-1024 and ARC-1023 only slightly improved the affinity of ARC-surfaces towards PKAc. The association phase of the binding curves measured on the covalently bound ARC-904 and ARC-1023 surfaces reached a stable value at equilibrium binding and fitted better to the 1:1 Langmuir interaction model as compared with the biotinylated ARC surfaces (Figures 7A, B, C). The titration of the latter surfaces with PKAc did not lead up to a stable plateau signal, whilst the dissociation rate constants for the kinases on covalently immobilized ARC-904 and ARC-1023 surfaces were somewhat greater (Figures 7B, C).

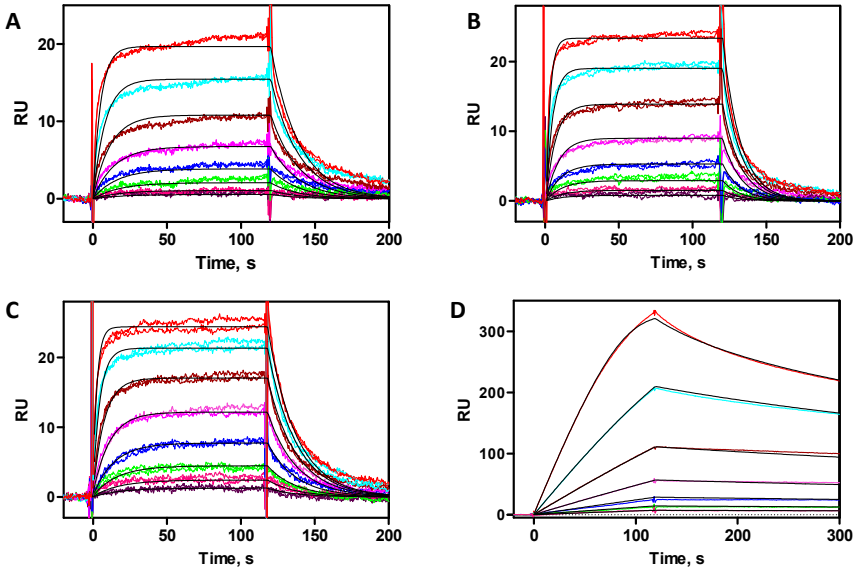


Figure 7. The binding of protein kinases to the ARC-conjugated SPR sensor surfaces: PKAc (from top to bottom two-fold dilutions starting from 80 nM) binding to **(A)** ARC-704 bound to streptavidin conjugated CM4 sensor chip **(B)** ARC-904, and **(C)** ARC-1023 bound covalently to CM4 sensor surface; **(D)** ROCK II (from top to bottom two-fold dilutions starting from 7.5 nM) binding to high density ARC-1023 surface.

High binding affinity of the catalytic domains of both CaMK IV and ROCK II expressed as fusions with a GST-tag to ARC-1023 surface could be detected ($K_{D\ (\mathrm{ROCII\text{-}GST})}$ = 0.4 nM and $K_{D\ (\mathrm{CamK\ IV})}$ = 5 nM) as well as much slower dissociation kinetics as compared to that of PKAc (Figure 7D). In order to prove that the slow dissociation results from the binding of catalytic domain of the kinase and not from binding of the purification tag, the experiments were performed with the catalytic domain of ROCK II possessing a 6His tag and similar results were obtained ($K_{D\ \mathrm{ROCII\text{-}6His}}$ = 0.6 nM). The affinity towards the surface was so high that the surface regeneration conditions used for PKAc measurements (regeneration with 1 M amintoethanol) did not dissociate ROCK II from the surface and additional regeneration steps were used [regeneration with successive injections of glycine (pH 1.5), 1 M NaCl in borate (pH 8.5) and glycine (pH 2.5)]. The affinity towards ARC-704 and ARC-1024 was not determined as the regeneration of the surfaces (complete dissociation of the kinase from the surface) was not achieved without destroying the sensor surface.

These results confirmed the previous finding that coupling of an ARC-type inhibitor to the surface reduces its affinity towards PKAc, but the binding affinity towards ROCK II is not significantly affected by the immobilization of the inhibitor.

The finding that several kinases bind to ARC-surfaces indicates that ARC-based affinity supports can be used for the selective pull-down of protein kinases from crude biological samples for further analysis. Thereafter post-translational modifications and expression patterns of surface-bound proteins can be established with MS analysis. Moreover, as ARC-type inhibitors bind to the active centre of protein kinases and do not recognize inactive kinases, ARC-based affinity supports could be used for separating the active form of a kinase from its inactive state counterpart. Therefore the method provides an excellent complement to methods measuring the total amount of protein kinases (*e.g.*, most antibody-based assays).

In addition to the possibility of selective enrichment of protein kinases ARC-based biosensor was used for the characterization of binding affinities of protein kinase inhibitors in competitive assay formats by monitoring the efficiency of an inhibitor to reduce the binding of PKAc to the ARC-sensor chip (Paper II, Figure 2). The values of binding constants determined for the tested compounds covered a wide range (K_d values from 500 pM to 1.0 mM), and were in good agreement with the values that had been obtained with other assay technologies. The main drawbacks of the ARC-based SPR assay for inhibitor testing are the risk of nonspecific binding of test compounds to the chip surface and the low throughput potential. However, the heterogeneity of the assay offers some specific advantages; for instance, the biosensor with immobilized ARC can be used in many experiments without the consumption of the probe and several kinases can be tested on one chip in selectivity studies.

Development and application of homogeneous photoluminescence-based assays for the determination of concentration of protein kinases (Papers II and III)

ARC-type inhibitors labelled with fluorescent dyes (ARC-Fluo probes) have been successfully applied for the development of a fluorescence anisotropy-(FA)-based assay that enables the determination of the concentration of active protein kinase [Vaasa *et al.* 2009] by performing the active site titration with high concentration of the ARC-Fluo probe ($[L_t] >> K_D$). However, if the K_D value of the probe:protein kinase complex is not in the subnanomolar range, high concentrations of the probe are required, which in turn leads to the increased amount of the protein kinase needed for the titration procedure. Moreover, the FA assays cannot be performed in crude samples as the highly abundant proteins non-specifically interacting with the fluorescence dye will contribute to the anisotropy signal.

Therefore it was decided to adopt an assay with TR-FRET readout (Figure 8A). The lysine side chain of ARC-904 was labelled with Lumi4 Terbium chelate (Lumi4Tb) [Moore *et al.* 2009] and PKAc was covalently labelled with

an organic dye FITC. The efficiency of FRET between PKAc-FITC and ARC-583 (ARC-904 labelled with organic dye TAMRA) was used as a comparison. The assay window (difference between the signals obtained in the absence and presence of the complex of FRET pair) obtained with TR-FRET pair ARC-Lumi4Tb:PKAc-FITC was significantly greater as compared with the assay window obtained with PKAc-FITC and ARC-583 FRET pair (Paper II, Figure 5). The result indicates that ARC-Lumi4Tb and PKAc-FITC pair-based assay could be used in a reporter displacement assay format for the quantification of protein kinases.

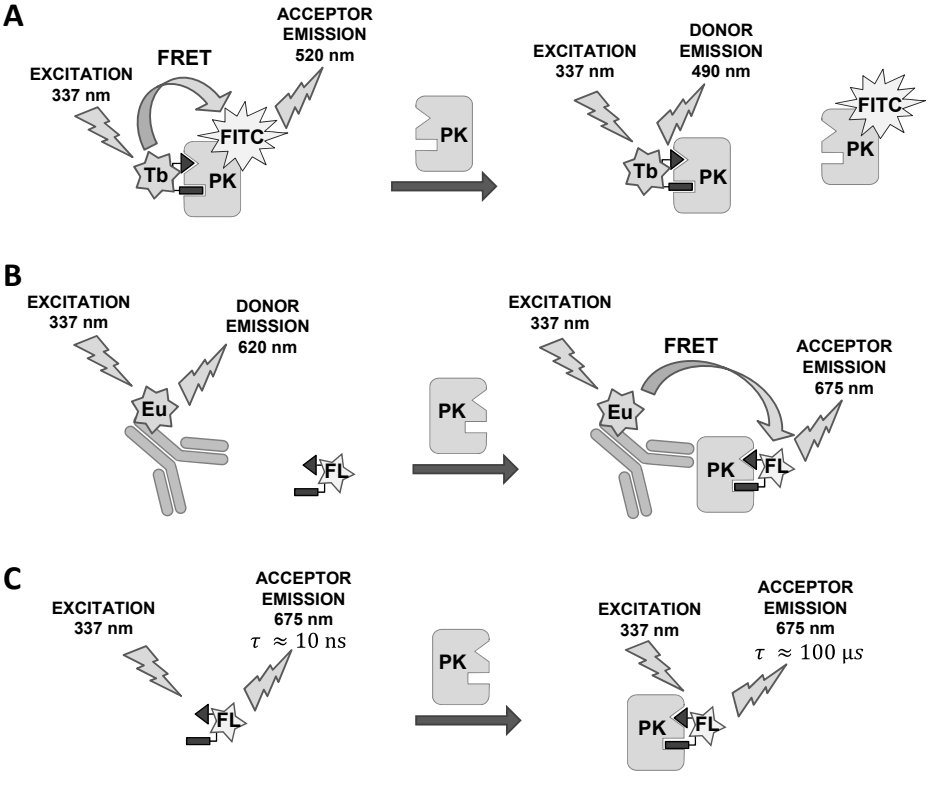


Figure 8. The schematic representation of homogeneous ARC-based methods for detection of protein kinases. **(A)** FRET between ARC-Lumi4Tb and PKAc-FITC is disrupted when PKAc-FITC is displaced from its complex by unlabelled PKAc, leading to increase in donor (Lumi4Tb) emission (490 nm) and reduction in the acceptor (FITC) emission (520 nm). **(B)** FRET between Eu-Ab and ARC-Fluo probe (FL) occurs when the donor (Eu) and acceptor (Alexa Fluor 647) are brought in close proximity by forming a triple complex with a PK, leading to the reduction in the donor's emission intensity (620 nm) and increase in the acceptor's emission intensity (675 nm). **(C)** Upon excitation at 337 nm luminescence with microsecond range lifetime is emitted only if ARC-Lum probe is bound to a PK.

Next it was tried to establish a three component assay for the analysis of protein kinases in biological samples (Figure 8B). An antibody binding to 6His-tag of the target protein was labelled with an Europium cryptate (Eu-Ab) as the TR-FRET donor and ARC-Fluo probe was labelled with a luminescence acceptor fluorophore Alexa Fluor 647 (ARC-1063). Surprisingly, in the course of these studies a long lifetime luminescence signal was detected as the assay was performed with the protein kinase not possessing a 6His tag. Furthermore, a luminescence signal was detected even if ARC-1063 was titrated with a protein kinase in the absence of the FRET donor Eu-Ab (Figure 8C). The luminescence intensity of ARC-1063 probe in the absence of a protein kinase was negligible and the measured luminescence intensity was proportional to the amount of the complex ARC-1063:protein kinase. It was later established that the phenomenon was distinctive only for probes containing a thiophene or a selenophene moiety in the ATP-competitive fragment of the inhibitor whereat no luminescence signal was detected in the case of ARC-based probes containing an adenosine moiety. Hereafter, ARC-probes with protein induced long lifetime luminescence property were designated as ARC-Lum probes.

Characterization of optical properties of ARC-Lum probes (Papers III and IV)

The following mechanism of protein induced luminescence of ARC-Lum probes was proposed. The thiophene-fragment-containing ARCs absorb light in the region of the TRF excitation filter (337 nm) with an absorption maximum at 340 nm and hence, upon flash-excitation the thiophene fragment is excited to singlet exited state $S(D)_1$ followed by ISC to the triplet excited state of the fragment $T(D)_1$ when the probe is bound to the active site of a protein kinase. The interactions with the ATP-binding pocket of the kinase probably restrict the molecular movements of the thiophene fragment and hence, the triplet excitation state is not annihilated by molecular motions. Thereafter the energy is passed to the singlet excited state of the conjugated acceptor fluorescent dye $S(A)_1$, followed by radiation at the emission wavelength of the dye (Paper III Figure 1D).

For the confirmation of the hypothesis the thiophene moiety in ARC-Lum probes was replaced by a selenophene moiety that contained a heavier selenium atom instead of sulphur. Heavier atoms have been shown to favour the triplet excitation state formation [Kuijt 2003]. Indeed, while titrated with protein kinases the probes containing a selenium atom gave greater long lifetime luminescence intensities as compared with their sulphur-containing counterparts. The long lifetime luminescence signal (excitation in the range of 300–350 nm, emission at 650–700 nm) was induced by binding of all tested protein kinases of the AGC group (Paper III, Table 1, Figures 2A, B) to either the thiophene-containing probe ARC-1063 or its selenophene-containing counterpart ARC-1139 (Paper III, Figure 1A). Interestingly, both the intensity and lifetime of

emitted light of complexes of different protein kinases with ARC-Lum probes were dependent on the origin of the probe and the binding kinase (Paper III, Figures 2A, B, Table 1).

From the absorption spectra of organic fluorescent dyes it is evident that most of them can be directly excited in the wavelength range of the TRF excitation filter (300–350 nm). It was observed that excitation of ARC-Lum and ARC-Photo probes with TRF modules in steady state fluorescence intensity mode resulted in the direct excitation of the fluorescent dye. However, only in case of ARC-Lum probes in complex with a protein kinase the luminescence signal was detected in the case of pulse excitation and time-gated (more than 50 microseconds) detection of luminescence intensity, indicating that the signal with long luminescence lifetime does not result from the direct excitation of the organic dye.

All tested fluorescent dyes possessing strong absorption in the wavelength range of 500 - 650 nm could be used as effective sensitizing acceptors (Paper III, Supplementary Figures 3, 4A, and 5A). Moreover, the light emitted at 630(40) nm by a selenopehene-containing compound ARC-1138 (not labelled with a fluorescent dye) in complex with PKAc was strong enough to be used for measurement of the concentration of the kinase bound to the probe (Paper III, Supplementary Figure 6), although the compound did not contain a sensitizing fluorescent luminescence acceptor. Still, the intensity of the emitted light was 20- to 100-fold weaker than that of ARC-Lum probes labelled with sensitizing dyes pointing to considerable sensitizing effect of the acceptor dye.

It was confirmed that energy transfer between the donor and acceptor luminophores was also possible intermolecularly as the transfer occurred if PKAc chemically labelled with PromoFluor-647 (PKAc-PF647) was titrated with thiophene- or selenophene-containing conjugates ARC-668 and ARC-1138, respectively (Paper III Figure 2D). Titration of PKAc-PF647 with 5-(2-aminopyrimidin-4-yl)selenophene-2-carboxylic acid (the small-molecule precursor of ARC-1138) resulted in concentration dependent long lifetime luminescence signal (Paper III Supplementary Figure 7), indicating that the phenomenon of protein-induced luminescence is characteristic not only of bisubstrate inhibitors but occurs also in the case of small ATP-competitive compounds containing a selenophene or a thiophene fragment.

Application of the ARC-Lum probes for determination of concentration of active kinases (Paper III and unpublished data)

The usability of ARC-Photo probes for the assessment of the fraction of catalytically active form of the protein kinase in the sample was previously described for the assay with FA readout (Paper II Figure 1, [Vaasa *et al.* 2009]). ARC-Lum probes can be used in a similar assay using either FA- or TGL-based detection (Figure 9). Comparison of the two detection methods reveals relatively smaller standard deviations of replicate samples of the TGL-based

detection at the lower concentrations of the probe as compared to the FA-based readout if the measurements are performed in the same samples (Figure 9).

The concentration of the catalytically active form of the protein kinase and K_D values can be obtained from TGL measurements if the data are fitted to equation 10, where B is the background signal and M is the luminescence intensity of the Kinase:ARC-Lum compex at 1 nM concentration and [EL] is defined by equation 9.

$$TGL = B + M \times [EL] \tag{10}$$

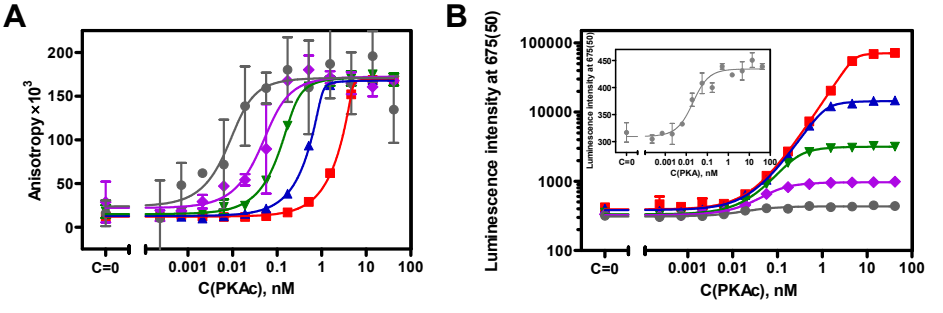


Figure 9. The titration of ARC-1063 [5 nM (■), 1 nM (▲), 0.2 nM (▼), 0.05 nM (♦) and 0.01 nM (●)] with PKAc using **(A)** FA-based or **(B)** TGL-based detection. Inset: the magnification of the 0.01 nM ARC-1063 titration curve. All samples were measured on a PHERAstar plate reader (BMG) using either FA module (excitation at 590(50) nm, emission at 675(50) nm) or TRF module [TRF excitation filter (BMG 337), emission at 675(50) nm (C), excitation with 200 flashes, delay 80 microseconds and gate 400 microseconds]. The mean and standard deviation of two replicate samples is plotted in the graphs.

Additionally the affinity of the ARC-Lum probe and the fraction of catalytically active form of protein kinase could be determined by performing the titration of the protein at fixed concentration with varying concentration of the probe (Paper III Supplementary Figure 5A) which was possible because of a negligible signal of the unbound probe. Both titration variants resulted in coincident values of the dissociation constants. The heights of plateaus of the binding curves in the case of the titration with kinases were in linear correlation with the concentration of ARC-1063 in the solution (Paper III Supplementary Figure 5B).

Development of an assay for the determination of the concentration of a putative cancer marker ECPKA (Paper IV)

The unique properties of ARC-Lum probes encouraged us to the development of a homogeneous assay for the determination of the concentration of a putative biomarker PKAc from the patient's blood samples. First, it was established that the probe ARC-1063 bound to PKAc with picomolar affinity (K_D = 10 pM) exceeding the affinity of most antibodies widely used in immunological methods by two orders of magnitude allowing quantitative binding to picomolar concentrations of PKAc without the need of using the probe at high concentrations. It was confirmed that the intensity of protein-induced luminescence determined at different concentrations of PKAc well correlated with the enzyme activity determined with the PKAc-catalysed peptide phosphorylation assay (Paper IV Figure 1). The limit of detection and the limit of quantification obtained for the assay were 4 pM and 11 pM, respectively, and the relative standard deviation of the assay did not exceed 15%. The upper limit of the quantification of the assay depends on the concentration of the ARC-Lum probe used in the assay.

Although the luminescence lifetimes of ARC-Lum:PKAc complexes were significantly shorter than that of an Eu-cryptate the protein-induced luminescence intensity of ARC-Lum:PKAc complexes under the assay conditions was comparable to that of Eu-cryptate widely used as a donor in TR-FRET based assays (Paper IV, Figure 4) supporting the hypothesis that ARC-Lum-based homogeneous assay could be performed with a wide range of instrumental setups developed for lanthanide-based assays.

The ARC-Lum-based binding assay revealed a good linearity between the luminescence intensity and concentration of recombinant PKAc added to the plasma sample regardless of the presence of large amounts of non-target proteins (*e.g.,* albumin) and fluorescent blood components (Paper IV Figure 5). However, the slope of luminescence intensity concentration dependency and the maximum value of the luminescence intensity determined at high concentrations of recombinant PKAc were highly dependent on the shape of the absorption spectrum of the specific blood sample (Paper IV Figure 6). Therefore two-point normalization was applied for the analysis of the concentration of ECPKA that took into account the background and the maximal luminescence intensity of each individual plasma sample (Paper IV, Scheme 2, and Figure 8).

The success of developed assay is based on high luminescence intensity and long luminescence lifetime of the ARC-Lum:PKAc complex leading to the lack of background noise from the short lifetime autofluorescence of plasma samples. This enables the use of the assay for analysis of crude biological samples without the need of depletion of highly abundant proteins. The assay can be performed with instruments that are equipped for time-gated measure-

ments and are suitable for example for lanthanide-based immunoassays. Moreover, the homogeneity, usability in single-step format, quickness, lack of need for special substrates or capricious antibodies make the assay amenable for automation and support the use of the assay for high throughput protein kinase analysis in clinics, small analysis laboratories and HTS departments of big pharmaceutical companies.

Additional applications of ARC-Lum probes (papers II and III)

Application of ARC-Lum probes for the characterization of protein kinase inhibitors (Paper III)

Although high-affinity ARC-Fluo probes have been previously successfully used for the characterization of inhibitors of PKAc in the displacement assay with a FA-based readout [Vaasa *et al.* 2009], the range of resolvable binding affinities in this assay format is limited by the affinity of the fluorescent probe [Huang 2003]. If the assay is performed with protein kinases possessing lower affinity (K_D > 5 nM) towards the ARC-Fluo probe, higher concentration of the protein kinase is used to ensure that a significant portion of the probe is in complex with the protein kinase as the portion of the bound probe defines the span of the assay window. However, the assay does not produce reliable inhibitory affinities if it is performed under the tight-binding conditions of the inhibitors (concentration of the enzyme close or higher than the binding constant of the inhibitor [Copeland 2005]). Another disadvantage of FA displacement assay is the necessity to know the exact concentration of the protein kinase used in the assay in order to convert the obtained IC_{50}-values into K_d values [Cer *et al.* 2009; Nikolovska-Coleska *et al.* 2004].

Based on the results of experimental testing it was confirmed that induced by binding of protein kinases the long lifetime luminescence signal of ARC-Lum probes was abolished by displacement of the probe by various ATP-competitive, protein substrate-competitive, or bisubstrate inhibitors. The obtained displacement IC_{50}-values were in a very good correlation with affinities and inhibitory potencies of the competing compounds as determined in the binding/displacement assay with fluorescence anisotropy readout [Vaasa *et al.* 2009] and/or enzyme inhibition experiments [Viht *et al.* 2005], respectively (Paper III Figure 3a). The time-delayed format of the assay enabled the determination of affinities of strongly fluorescent compounds without the interference from the increasing concentration of fluorescent dye conjugated to the tested inhibitor (Paper III Supplementary Figure 9).

Lack of a signal from the unbound probe allowed the usage of ARC-1063 at relatively high concentration in the displacement assay that on one hand enables the determination of affinities for highly potent compounds by shifting their displacement curves away from the tight-binding region. On the other hand,

using high concentration of the reporter probe supports use of the Cheng-Prusoff equation [Cheng and Prusoff 1973] for simplified calculation of K_d values from displacement curves (Paper III Figure 3B) and makes the results of measurements almost independent of the concentration of active protein present. This is a clear advantage of ARC-Lum-based luminescence intensity assays compared to fluorescence anisotropy-based measurements where the knowledge of protein concentration is required [Huang 2003]. The possibility of application of the reporter ligand at higher concentrations widens the list of kinases that can be analysed with a single probe as at higher concentration of the probe protein kinases possessing lower affinity still have an adequate fraction of the kinase bound to the probe.

A simple assay for the determination of dissociation rate constants for protein kinase inhibitors based on the application ARC-Lum probes was developed (Paper III Figure 4A). The inhibitor under examination was mixed with the protein kinase at a concentration that resulted in binding of at least 90% of the kinase. The ARC-Lum probe was added to the solution in great excess (final concentration 100–300 nM), and the increase of long lifetime luminescence signal was monitored. In these conditions the formation of ARC-Lum:protein kinase complex is determined by the rate of release of the protein kinase from the complex with the inhibitor.

Altogether, with the help of an ARC-Lum probe a full and reliable kinetic/thermodynamic characterization of the inhibitor:kinase complex can be obtained. Differently from widely used SPR method ARC-Lum-based assays are homogeneous, amenable to automation and can be used for high throughput analysis of drug candidates with the aim of finding inhibitors with suitable residence times. Taking into account the unique bisubstrate character of ARC-Lum probes, in addition to characterization of ATP-competitive inhibitors the probes are also applicable for characterization of compounds binding to the protein substrate binding site of the kinase.

The scope of the assays can be extended to other kinases within the selectivity profile of new ARC-based probes [Enkvist *et al.* 2009].

Application of ARC-Lum probes for the characterization of protein kinase activity in live cells (Papers II and III)

It had been previously shown that ARC-Fluo probes could cross the plasma membrane and therefore they were applicable in a FRET assay for the monitoring of cAMP accumulation (Paper II, Figures 5, 6; [Vaasa *et al.* 2010]). However, genetic modification of cells leading to over-expression of the PKAc-YFP fusion protein is required for the FRET assay.

In the present study it was confirmed that ARC-Lum probes can be used for monitoring the activity of protein kinases in native live cells. The increase in luminescence signal in ARC-Lum treated cells was detectable if the accumulation of cAMP and successive activation of PKA was induced by activating

adenylate cyclases (ACs) with Forskolin or by treating the cells expressing β-adrenergic receptors (GPCRs that activate the G_s-protein inducing AC activation) with Isoproterenol (Paper III Figure 5). The luminescence intensity was decreased after a cell permeable PKAc inhibitor H89 was added to the cells.

The main advantage of the application of ARC-Lum probes is the possibility of real-time monitoring of activity of protein kinases in cells possessing native expression level of protein kinases with no need for recombinant overexpression of a fusion of the protein kinase with a fluorescent protein that is required for other cellular cAMP concentration and protein kinase activity sensors [Allen *et al.* 2006; Nikolaev *et al.* 2004; Zaccolo *et al.* 2000].

CONCLUSIONS

This thesis describes the application of ARC-type bisubstrate inhibitors for the analysis of protein kinases and the characterization of protein kinase inhibitors.

The main results of the present research can be summarized as follows:

- Novel ARC-type inhibitor based affinity supports were prepared and used for the pull-down of protein kinases PKAc and ROCK II. The results point to the applicability of such affinity surfaces for the selective enrichment of active protein kinases from crude samples.
- ARC-type bisubstrate inhibitors immobilized on chips of surface plasmon resonance-based biosensor were successfully used for both characterization of affinity surfaces and determination of affinity of competing inhibitors of protein kinases PKAc and ROCK II. The binding and dissociation rate constants for binding of the protein kinases to ARC surfaces were determined.
- Non-metal small-molecule ARC-Lum probes which binding to protein kinases induces long lifetime photoluminescence signal were discovered. The probes were applied for the determination of affinities of non-labelled inhibitors and rate constants of their dissociation from complexes with protein kinases.
- The applicability of ARC-Lum probes for the real-time monitoring of protein kinase activity in living cells was demonstrated.
- An ARC-Lum probe was used in a homogeneous high throughput binding assay with time-gated luminescence intensity readout for the measurement of concentration of putative generic cancer biomarker ECPKA at essential picomolar concentrations in samples of blood plasma.

SUMMARY IN ESTONIAN

Bisubstraatsed luminestsentssondid, optilised sensorid ja afiinsusadsorbendid aktiivsete kinaaside määramiseks bioloogilistes proovides

Proteiinkinaasid on looduses laialdaselt levinud ensüümid, mis osalevad paljudes rakusisestes signaaliülekanderadades. Vead signaaliradades viivad mitmete raskete haiguste tekkeni. Tõsiasi, et enamiku protiinkinaaside aktiivsust on võimalik reguleerida madalmolekulaarsete ühendite abil, on muutnud proteiinkinaasid üheks põhiliseks ravimiarenduse sihtmärkidest. See omakorda on tinginud vajaduse arendada biokeemilisi meetodeid, mis võimaldaksid uurida proteiinkiniaaside aktiivsuse muutust patsientide koeproovides leidmaks haigusi põhjustavaid proteiinkinaase. Teisalt on oluline arendada kliinilises praktikas kasutatavaid meetodeid, mis võimaldaksid kiiresti ning odavalt haigusi diagnoosida nende varajases staadiumis ning oleksid eelduseks hinnangu andmisel, kas ja millisele kinaasi aktiivsust reguleerivale ravimile antud haigus kõige paremini allub.

Käesoleva töö põhieesmärgiks oli töötada välja metoodika, mis võimaldaks määrata aktiivsete proteiinkinaaside hulka koeproovides. Metoodika arendamisel lähtuti ARC-tüüpi bisubstraatsetest inhibiitoritest, mis seonduvad proteiinkinaaside aktiivtsentrisse kõrge afiinsusega. Inaktiivse kinaasiga ARC-tüüpi inhibiitorid ei seondu ning seetõttu võimaldavad ARC-tüüpi inhibiitoritel põhinevad meetodid määrata erinevalt immunokeemilistest sidumismeetoditest ka muutusi kinaaside aktiivsuses.

Töö esimeses etapis töötati välja metoodika ARC-tüüpi inhibiitoritel põhinevate afiinsusadsorbentide valmistamiseks. Afiinsuskandjat rakendati edukalt Rho-sõltuva proteiinkinaasi (ROCK II) ja proteiinkinaas A katalüütilise alaühiku (PKAc) väljapüüdmiseks rakulüsaadist. Immobiliseeritavaid ARC-tüüpi inhibiitoreid kasutati pinna plasmonresonantsspektroskoopia (SPRS) sensorpindade valmistamiseks. Näidati, et nii ROCK II kui ka PKAc seonduvad sensorpinnaga suure afiinsusega (dissotsiatsioonikonstandid vastavalt 0,5 nM ja 12 nM). Pinnale sidumine ei muutnud oluliselt inhibiitori afiinsust ROCK II suhtes, ent kahandas inhibiitori afiinsust PKAc suhtes ühe suurusjärgu võrra, võrreldes vaba inhibiitoriga lahusefaasis. ARC-põhine SPRS on hea meetod aktiivsete proteiinkinaaside selektiivseks sidumiseks ja kontsentreerimiseks bioloogilistest proovidest. Lisaks saab ARC-tüüpi inhibiitoriga kaetud pinda kasutada ravimikandidaatide afiinsuse ja selektiivsuse uurimiseks.

Sensormeetodid omavad küll suurt tundlikkust, kuid nende kasutamine kiiranalüüsimeetodites on seotud mitmete probleemidega. Seetõttu arendati välja üheetapiline Försteri resonantsenergia ülekandel (FRET) põhinev meetod proteiinkinaaside määramiseks otse koeproovides. FRETi doonorina kasutati pika luminestsentsi elueaga lantaniidi kelaadiga märgistatud antikeha ning FRETi aktseptorina lühiksese elueaga orgaanilise fluorestsentsvärviga märgistatud ARC-tüüpi inhibiitorit. Töö käigus avastati, et mõnede ARC-tüüpi ühendite

(ARC-Lum) kompleksid proteiinkinaasidega omavad varemkirjeldamata optilisi omadusi. Nende komplekside ergastamisel kiirgusega lähis-UV alas emiteerivad nad punases spektriosas valgust, mille sumbumise poolestusaeg on vahemikus 14–170 mikrosekundit – 10000 korda pikem kui tavalistel fluorestsentsvärvidel.

Töötati välja ülitundlik ARC-Lum-sondide kasutamisel põhinev meetod vähi biomarkeri, rakuvälise PKAc (ECPKA), määramiseks inimese vereplasmas. Uus meetod on üheetapiline, automatiseeritav, odav, teostatav levinud aparatuuri kasutades ning võimaldab mõõta pikomolaarseid ECPKA kontsentratsioone eeltöötlemata plasmaproovides.

ARC-Lum sonde kasutati ka rakusiseselt proteiinkinaaside aktivaatorite ja inhibiitorite uurimiseks reaalajas. Näidati, et ARC-Lum sondid sisenevad imetajarakkudesse ning pika elueaga luminestsentssignaal sõltub kinaaside aktiivsusest. ARC-Lum sondid võimaldasid uurida madalmolekulaarsete inhibiitorite rakku sisenemist ja nende võimekust rakusiseseks proteiinkinaaside inhibeerimiseks. Samuti näidati, et ARC-Lum sonde on võimalik rakendada proteiinkinaase aktiveerivate G-valk-seotud retseptorite agonistide toime uurimiseks. Seejuures võimaldab kirjeldatud metoodika katsed läbi viia geneetiliselt muundamata rakkudes, mida teised reaalajas kinaaside aktiivsuse uurimiseks rakendatud meetodid ei võimalda.

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Teaduspublikatsioonid

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