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FROM MOLECULAR BLOCKS TO BIOANALYTICAL ASSAYS:

Combining Lanthanide Luminescence and Bioaffinity Binders for Protein Detection

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LIST OF PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals (I-V). The publication I is a review and publications II-V are original publications.

I Henna Päkkilä and Tero Soukka (2011). Simple and inexpensive immunoassay-based diagnostic tests. *Bioanal Rev* **3**:27-40.

II Henna Päkkilä, Minna Ylihärsilä, Satu Lahtinen, Liisa Hattara, Niina Salminen, Riikka Arppe, Mika Lastusaari, Petri Saviranta, and Tero Soukka (2012). Quantitative multianalyte microarray immunoassay utilizing upconverting phosphor technology. *Anal Chem* **84**:8628-8634.

III Henna Päkkilä, Sami Blom, Kari Kopra, and Tero Soukka (2013). Aptamer-directed lanthanide chelate self-assembly for rapid thrombin detection. *Analyst* **138**:5107-5112.

IV Henna Päkkilä, Eeva Malmi, Satu Lahtinen, and Tero Soukka (2014). Rapid homogeneous immunoassay for cardiac troponin I using switchable lanthanide luminescence. *Biosens Bioelectron* **62**:201-207.

V Henna Päkkilä, Riikka Peltomaa, Urpo Lamminmäki, and Tero Soukka. Precise construction of oligonucleotide-Fab-fragment conjugate for homogeneous immunoassay using HaloTag technology. Submitted manuscript.

In addition, unpublished data are included.

ABBREVIATIONS

Bio	Biotin
BSA	Bovine serum albumin
DARPin	Designed ankyrin repeat protein
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPL	Expressed protein ligation
Fab	Antigen-binding fragment of antibody
HER2	human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
NHS	N-hydroxy succinimide
UCNP	Upconverting nanoparticle
Mab	Monoclonal antibody
MIP	Molecularly imprinted polymer
nt	Nucleotide
FRET	Förster resonance energy transfer
LH	Luteinizing hormone
LFIA	Lateral flow immunochromatographic assay
LED	Light emitting diode
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PSA	Prostate specific antigen
SA	Streptavidin
SH3	SRC homology 3 domain
scFv	Single-chain variable fragment
SELEX	Systematic evolution of ligands by exponential enrichment
TSH	Thyroid stimulating hormone
UV	Ultraviolet

ABSTRACT

Measuring protein biomarkers from sample matrix, such as plasma, is one of the basic tasks in clinical diagnostics. Bioanalytical assays used for the measuring should be able to measure proteins with high sensitivity and specificity. Furthermore, multiplexing capability would also be advantageous. To ensure the utility of the diagnostic test in point-of-care setting, additional requirements such as short turn-around times, ease-of-use and low costs need to be met. On the other hand, enhancement of assay sensitivity could enable exploiting novel biomarkers, which are present in very low concentrations and which the current immunoassays are unable to measure. Furthermore, highly sensitive assays could enable the use of minimally invasive sampling. In the development of high-sensitivity assays the label technology and affinity binders are in pivotal role. Additionally, innovative assay designs contribute to the obtained sensitivity and other characteristics of the assay as well as its applicability.

The aim of this thesis was to study the impact of assay components on the performance of both homogeneous and heterogeneous assays. Applicability of two different lanthanide-based label technologies, upconverting nanoparticles and switchable lanthanide luminescence, to protein detection was explored. Moreover, the potential of recombinant antibodies and aptamers as alternative affinity binders were evaluated. Additionally, alternative conjugation chemistries for production of the labeled binders were studied. Different assay concepts were also evaluated with respect to their applicability to point-of-care testing, which requires simple yet sensitive methods.

The applicability of upconverting nanoparticles to the simultaneous quantitative measurement of multiple analytes using imaging-based detection was demonstrated. Additionally, the required instrumentation was relatively simple and inexpensive compared to other luminescent lanthanide-based labels requiring time-resolved measurement. The developed homogeneous assays exploiting switchable lanthanide luminescence were rapid and simple to perform and thus applicable even to point-of-care testing. The sensitivities of the homogeneous assays were in the picomolar range, which are still inadequate for some analytes, such as cardiac troponins, requiring ultralow limits of detection. For most analytes, however, the obtained limits of detection were sufficient. The use of recombinant antibody fragments and aptamers as binders allowed site-specific and controlled covalent conjugation to construct labeled binders reproducibly either by using chemical modification or recombinant technology. Luminescent lanthanide labels were shown to be widely applicable for protein detection in various assay setups and to contribute assay sensitivity.

TIIVISTELMÄ

Proteiinien mittaaminen biologisesta näytteestä, kuten plasmasta, on kliinisen diagnostiikan perustehtäviä. Kliinisessä diagnostiikassa käytettävien bioanalyyttisten määritysten tulisi olla herkkiä ja tarkkoja, jotta ne voivat mitata proteiineja näytteestä luotettavasti. Lisäksi olisi hyödyllistä pystyä mittaamaan samasta näytteestä monta analyyttiä yhtäaikaisesti. Vieritestaukseen tarkoitettujen testien tulisi edellä mainittujen ominaisuuksien ohella olla nopeita, helppokäyttöisiä ja edullisia. Toisaalta nykyistä merkittävästi herkempien määritysten kehittäminen mahdollistaisi uusien, hyvin pieninä pitoisuuksina esiintyvien biomerkkiaineiden käytön diagnostiikassa. Erittäin herkät määritykset voisivat myös mahdollistaa helposti saatavilla olevien näytemateriaalien käytön. Määrityksen herkkyyteen ja muihin ominaisuuksiin vaikuttavia tekijöitä ovat käytetty leimateknologia sekä sitojamolekyylit. Lisäksi määrityskonseptin valinnalla voidaan vaikuttaa testin suorituskykyyn ja sen soveltuvuuteen esimerkiksi vieritestausolosuhteisiin.

Tässä väitöskirjatyössä tutkittiin kahden luminoivan lantanidileimateknologian soveltuvuutta proteiinien mittaamiseen. Yhdessä osatyössä tutkittiin upkonvertoivien eli käänteisviritteisten luminoivien nanopartikkelien käyttökelpoisuutta monianalyyttimäärityksiin ja kolmessa osatyössä kehitettiin nopeita erotusvapaita määrityksiä mallianalyyteille hyödyntäen kytkeytyvää lantanidiluminesenssia. Osatöissä selvitettiin myös rekombinanttisten vasta-ainefragmenttien ja aptameerien soveltuvuutta proteiinipitoisuuksien mittaamiseen. tutkittiin erilaisia konjugointitapoja leiman ja sitojamolekyylin yhdistämiseksi. Lisäksi arvioitiin eri määritystapojen soveltuvuutta vieritestaussovelluksiin.

Väitöskirjatyössä osoitettiin, että käänteisviritteisiä nanopartikkeleita voidaan hyödyntää usean analyytin yhtäaikaiseen mittaamiseen käyttämällä kuvantavaa mittaustapaa. Tällöin voidaan käyttää yksinkertaisempaa laitteistoa, sillä toisin kuin muut luminoivat lantanidileimat, käänteisviritteiset nanopartikkelit eivät edellytä aikaerotteista mittaustapaa. Kehitetyt erotusvapaat kytkeytyvää lantanidiluminesenssia hyödyntävät määritykset olivat nopeita ja yksinkertaisia suorittaa, ja siten soveltuvia jopa vieritesteihin. Erotusvapaiden määritysten havaintorajat olivat pikomolaarisella alueella, mikä ei ole riittävän herkkä kaikille analyyteille kuten esimerkiksi sydänspesifisille troponiineille, joiden mittaamiseen vaaditaan erittäin herkkiä testejä. Vasta-ainefragmenttien ja oligonukleotidipohjaisten aptameerien käyttö mahdollisti paikkaspesifisen ja kontrolloidun leima-sitojamolekyyli-konjugaatin muodostamisen joko kemiallisesti tai rekombinanttiteknologiaa käyttäen. Käänteisviritteisten luminoivien nanopartikkelien ja kytkeytyvien lantanidileimojen osoitettiin soveltuvan proteiinien detektioon monenlaisissa määrityskonsepteissa.

1 INTRODUCTION

The complexity of blood is exceptional and any changes in a person's health are reflected in blood. This allows the health monitoring simply by measuring occurrence of or changes in the concentration of biomarkers in the blood. Together with small molecules and nucleic acids, proteins are central biomarkers. The variety in protein concentrations in blood is enormous ranging from femtomolar to millimolar range, which sets high demands for assay technologies (Zichi *et al.*, 2008). Despite the advances in recent decades, more sensitive assays are still needed to enable the reliable measurement of low-abundance analytes. Highly sensitive assays could also pave the way for the use of novel biomarkers. Additionally, highly sensitive assays could allow the use of minimally invasive sample matrices that are more distant from the tissue of biomarker origin and thus contain lower concentrations of the biomarker in question (Landegren *et al.*, 2012; Giljohann and Mirkin, 2009).

Ideal diagnostic tests are sensitive and selective *i.e.* they are able to specifically find the correct biomarker among the huge variety of molecules present in the sample. Furthermore, diagnostic tests should be cost-effective and have potential for automatization and even multiplexing. In addition to clinical laboratories, diagnostic tests are also needed in point-of-care testing, the significance of which is expected to increase. Point-of-care testing enables rapid diagnosis and effective treatment at doctor's office and even in resource-poor settings. Furthermore, point-of-care tests allow self-testing at home for the monitoring of both the disease and the effectiveness of treatment (Giljohann and Mirkin, 2009). However, point-of-care tests should possess additional features compared to tests intended for central laboratory-based testing. They should be user-friendly, inexpensive and robust and require minimal instrumentation (Peeling *et al.*, 2006). Immunoassays are traditionally used for measuring low abundance biomarkers, and they are still invaluable to for diagnostics despite the evolvement of other methods, such as biomedical imaging or tandem mass spectrometry (Landegren *et al.*, 2012). Immunoassays are based on the ability of antibodies to bind their target molecules with high specificity and affinity. Other specific interactions between biomolecules can also be utilized to develop bioaffinity assays.

Label molecules producing *e.g.* luminescent or electrical signal are commonly attached to antibodies in order to obtain measurable signal as a response to the binding event. Label-free detection methods, such as surface plasmon resonance, also exist (Homola, 2008). In label-free methods, the assay components are simpler to produce but label-free detection requires expensive and delicate instrumentation, which limits the robustness of the technology. Furthermore, the non-specific binding of matrix components significantly compromises the analytical sensitivity. Some labels, such as colloidal gold, produce a signal that is detectable by naked eye. This is very convenient for especially point-of-care applications but the sensitivity of detection is compromised and no quantitative results are obtained. For the development of highly sensitive assys, a measurement device is often necessary. (Faulstich *et al.*, 2009.)

The choices of a label and a binder as well as the overall assay design are the key factors in the development of immunoassays and other bioaffinity assays (Kricka *et al.*, 1994). Assay sensitivity can be improved by using novel label technologies with high specific signal or by minimizing the background signal originating from the non-specific binding of the labeled assay component. Autofluorescence originating from the sample matrix and the reaction vessel is an additional source of background signal in fluorescence-based detection (Tegler *et al.*, 2011; Kricka, 1994). Moreover, development of small and low-cost instrumentation enhances the applicability of novel tests to point-of-care applications. Furthermore, high binder affinity and selectivity are essential for the development of sensitive assays. Optimal binders are able to recognize the low-abundance targets in the sample despite the high abundance of other, possibly cross-reacting biomolecules with similar epitopes (Landegren *et al.*, 2012). Development of specific and well-controlled conjugation methods enables correct orientation of molecules in the assay and does not interfere with the activity of the label nor the binder. It enhances also uniform and reproducible quality of the assay components. (Gold *et al.*, 2012.)

2 REVIEW OF THE LITERATURE

Development of diagnostic assays requires combination of knowledge related to various fields of life sciences. This literature review presents different assay concepts utilized in the bioaffinity assays. Particular emphasis is in affinity binders for proteins, luminescent lanthanide labels and conjugation methods that can be used for coupling binders and labels or immobilizing binders. Label-free detection methods and affinity-binders for nucleic acids, small molecules and carbohydrates are out of the scope of this thesis.

2.1 Bioaffinity assay concepts

Various assay concepts have been developed for efficient analyte concentration measurements. In competitive reagent-limited assays, analyte molecules in the sample compete with the labeled analyte analog from limited amount of binding sites whereas non-competitive assays are based on using an excess of reagents in the assay (Davies, 2013; Ekins and Chu, 1991). Bioaffinity assays can also be classified as heterogeneous or homogeneous assays. Heterogeneous assays contain many steps and include washing steps whereas homogeneous assays are separation-free and are thus significantly simpler to perform (Davies, 2013) (Fig. 1). Heterogeneous assays employ binders immobilized in solid phase, which is commonly a microtiter well or a glass slide. This allows using washing steps to get rid of the non-bound reagents in order to achieve high assay performance. The simplest assays involve only the analyte and the binder, but if the analyte is large enough, two antibodies are typically used in the assay. In these two-site immunometric assays or sandwich assays, one antibody is immobilized in the solid phase to capture the analyte whereas the other antibody is labeled and acts as a tracer. Thus two separate recognitions of the analyte are required to obtain signal, which improves the selectivity of recognition and minimizes the background signal. (Davies, 2013; Ekins and Chu, 1991.) In heterogeneous assays, the high binding capacity of the solid phase together with the properties of the labeled binder is particularly important for assay sensitivity (Wild and Kusnezow, 2013; Ylikotila *et al*., 2006). Heterogeneous assays can be multiplexed by printing the arrays on solid phase (Fig. 1b). This type of array contains separate spots for each analyte and the analytes are recognized by their site in the array. Thus single label is enough for multiplexed detection, which simplifies also the required detection instrumentation. (Ellington *et al.*, 2010.)

Homogeneous assays are simple mix-and-measure type of separation-free assays performed in liquid phase. They allow rapid detection due to the fast kinetics and the avoidance of several time-consuming incubation and washing steps. Homogeneous assays are simple to perform making them well applicable to point-of-care testing. Additionally, they are cost-effective because the required instrumentation is less complicated than that used for the heterogeneous assays. The disadvantages are compromised sensitivity and limited linear range (Ekins and Chu, 1991). In homogeneous assays, the analyte detection is based on signal modification occurring due to analyte recognition. Förster resonance energy transfer (FRET) is often applied to the luminescence-based detection of homogeneous assays (Fig. 1c). In FRET, two fluorophores are employed, one acting as a donor and the other one as an acceptor. A

spectral overlap of the excitation spectrum of the acceptor with the emission spectrum of the donor is required for FRET to occur. Furthermore, FRET is distance-dependent requiring close proximity of the donor and the acceptor to allow non-radiative energy transfer (Geissler *et al.*, 2014). For an effective FRET-based homogeneous assay, small-sized binders and controlled binder labeling are particularly important. Also, homogeneous bead-based suspension multianalyte assays, such as the Luminex xMAP technology, exist (Fig. 1d) (Ellington *et al.*, 2010). These assays require different label for each analyte, but their reproducibility is better than that of heterogeneous multianalyte arrays because labeled binders can be prepared in large batches.

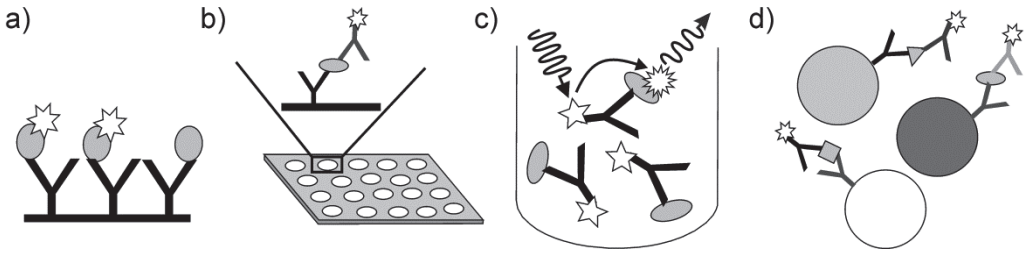


Figure 1. Various bioaffinity assay concepts. a) Heterogeneous competitive assay b) Heterogeneous two-site immunometric assay in a multianalyte array format. c) Homogeneous competitive FRET-based assay d) Bead-based suspension multianalyte assay based on two-site immunometric assay principles.

2.2 Bioaffinity binders for protein detection

High-affinity binders are necessary for the development of sensitive assays. The robustness of binders is important in order to obtain reliable and reproducible results. This is particularly important in the case point-of-care tests, which need to tolerate varying conditions, especially in low-resource settings. In this section, binders used for detection of protein analytes are covered. Antibodies and their use in combination with DNA oligonucleotides are presented. Furthermore, nucleic acid-based aptamers and novel alternative protein binders are described. The emphasis of this section is on the applications developed for protein detection.

2.2.1 Antibodies

Antibodies can be considered the foundation of the modern biotechnology. They are well-established and employed virtually all branches of biotechnology and biomedicine. They are used as basic research tools to study biochemical processes and interactions as well as in applications including clinical diagnostics and bioimaging. Furthermore they are used as therapeutic agents mainly in various types of cancers but some are also targeted for the treatment of allergy, transplant rejection, or cardiovascular diseases. (Weiner *et al.*, 2009; Revets *et al.*, 2005.)

There are five antibody classes out of which IgG antibodies are the most common. They consist of two identical heavy and two identical light chains forming the characteristic Y-shape structure (Fig. 2). Altogether six hypervariable loops, three from VH and three from VL, are responsible for the configuration of the binding site in the

antibody forming a flexible continuous interaction surface with the antigen (Liddell, 2013; Skerra, 2000). The affinities of antibodies are commonly in the nano- and picomolar range and the antibody molecular weight is approximately 150 kDa (Holliger and Hudson, 2005). For biotechnology applications, antibodies are commonly produced in hybridoma cells, which are formed by hybridization of tumor cells with spleen cells of Balb/c mice immunized with the target antigen (*e.g.* Lilja *et al.*, 1991). The hybridoma technology allows monoclonal antibodies to be produced in large quantities. Therefore monoclonal antibodies are preferred in applications over polyclonal antibodies, which are mixture of antibodies recognizing different analyte epitopes and thus suffer from poor reproducibility (Skerra, 2000).

Antibodies have several limitations despite their vast use in biotechnology and medical applications. One of the main drawbacks of antibodies is their large size, which causes steric hindrances in diagnostic assays and results in limited tissue penetration thus compromising their applicability to imaging purposes (Ylikotila *et al.*, 2006; Skerra, 2000). Furthermore, the Fc-region promotes the non-specific binding of the antibodies in immunoassays because components of the biological sample matrix, such as rheumatoid factors or human anti-animal antibodies, bind to it. This may cause interferences in immunoassays leading to either false negative or false positive results (Selby, 1999; Kricka, 1999). However, antibodies of non-human origin can be humanized to avoid these interferences in immunoassays and especially in therapeutic applications (Liddell, 2013). The antibody structure composed of four polypeptide chains involving disulfide bonds and heavy chain glycosylations is complex, site-specific modifications are difficult to include in the structure and antibody production in transfected mammalian cell lines is expensive and labor-intensive. Moreover, antibodies are not robust and antibody-based multifunctional fusion proteins are difficult to produce. Additionally, intellectual property issues impede their further development (Moore *et al.*, 2012; Gebauer and Skerra, 2009; Holliger and Hudson, 2005). On the other hand, the benefits of antibodies over other binders include bivalency and a long serum-half-life beneficial especially in therapeutic applications. (Binz *et al.*, 2005.)

DNA-antibody conjugates

Recently, DNA-oligonucleotides conjugated with antibodies have enabled novel assay configurations with excellent limits of detection. Oligonucleotide-antibody conjugates have been used to ensure correct orientation in surface-bound immunoassay formats (Washburn *et al.*, 2011) and to allow the self-assembly of multianalyte biosensors (Boozer *et al.*, 2006; 2004) and protein microarrays (Ng *et al.*, 2007). Furthermore, immuno-PCR (McDermed *et al.*, 2012; Sano *et al.*, 1992) as well as assays utilizing proximity ligation (Fredriksson *et al.*, 2002) or proximity extension (Lundberg *et al.*, 2011) for detection are all based on the DNA-antibody conjugates as binders.

In proximity ligation, the antibodies bind to the analyte thus bringing the two probe oligonucleotides into proximity. This allows the hybridization of the connector oligonucleotide to the probe pair to occur. The formed DNA strand is ligated and the ligation product replicated by nucleic acid amplification after which the amplification product is detected. In proximity extension assays, the ligation step is omitted and DNA

polymerase first extends the hybridized probe pair, after which the formed template is amplified. Proximity ligation- and proximity extension-based assays have reached ultra-high sensitivities in the femtomolar range and possess high multiplexing capacity (Assarsson *et al.*, 2014; Hammond *et al.*, 2012; Darmanis *et al.*, 2010). DNA-antibody conjugates have also been used to enable effective signal generation in homogeneous sandwich-type assays based on FRET (Heyduk *et al*., 2008). The distance-dependency of FRET usually impedes the use of sandwich-type assays because the donor and the acceptor are not brought close enough to each other for efficient energy transfer to occur. By using complementary oligonucleotides conjugated to antibodies, sandwich-type FRET-based assays with sub-nanomolar sensitivities can be created. In these assays, the analyte-induced hybridization brings the labels into close proximity and facilitates efficient energy transfer. Assays for insulin and cardiac troponin I have been demonstrated (Heyduk *et al.*, 2010; 2008). DNA-antibody conjugates have also been utilized in a heterogeneous assay for prostate specific antigen (PSA), where the DNA acted as a bio-barcode and was amplified by polymerase chain reaction (PCR) to enable the sensitive detection of PSA. The assay was approximately 300 times more sensitive compared to the commercial assays, enabling PSA measurement in attomolar concentrations (Thaxton *et al.*, 2009; Nam *et al.*, 2003).

Antibody fragments

To overcome the drawbacks of monoclonal antibodies, different antibody fragments containing the antigen-binding portion have been developed and employed as binders (Fig. 2). $F(ab)_2$-fragments can be obtained from monoclonal antibodies by enzymatic digestion in the hinge region using pepsin or bromelain (Bjerner *et al.*, 2002; Rousseaux *et al.*, 1983) while Fab-fragment is produced, *e.g*,. by using papain (Coulter and Harris, 1983). The main functional difference between $F(ab)_2$ and Fab is that the bivalency of the monoclonal antibody is maintained in $F(ab)_2$ while Fab is monovalent. The ability of Mabs and $F(ab)_2$s to bind two antigens simultaneously increases their functional affinity *i.e.* avidity. Single chain variable fragment (scFv) contains only the VH and VL domains connected by a flexible linker to prevent dissocation (Holliger and Hudson, 2005).

In the production of large amounts of antibody fragments, however, digestion from the monoclonal antibodies is not convenient or cost-effective. Display technologies enable a relatively simple method for the screening and production of recombinant antibody binders from modern affinity-binder protein libraries. Selecting new binders is also relatively fast because no immunization is needed. Furthermore, binders for cavity epitopes can be generated by using recombinant technologies. Phage display is the most widely used display technology, but yeast display and ribosome display are also well established. The display technologies are based on linking the phenotype and genotype which enables straightforward production once a binder with the desired characteristics has been selected. Binder libraries may contain up to 10^7-10^{13} variants, and display technologies allow the screening of novel binders with affinities typically in the sub-nanomolar range. The affinity of antibody variants can be increased by mutagenesis and affinity maturation, and affinities even in femtomolar range have been reported. To reduce the interference caused by heterophilic antibodies, the humanization of the antibody fragments is also possible (Hoogenboom, 2005; Boder *et al.*, 2000).

In diagnostic assays, the use of Fabs instead of monoclonal antibodies (Mab) has allowed more sensitive assays due to the elimination of the Fc-portion related interferences (Hyytiä *et al.*, 2013). Also, more dense binding surfaces can be obtained due to the smaller size of the binder, which contributes to the binding capacity of the solid phase and subsequently to the obtained signal levels (Ylikotila *et al.*, 2006). Modifications are introduced more easily and with higher control allowing the production of , *e.g.*, optimally oriented binder surface as a result of site-specific conjugation. Moreover, fusion proteins containing both the binder and the label, such as scFv fusion with green fluorescent protein, can be produced with recombinant technology thus avoiding a separate time-consuming labeling step (Hink *et al.*, 2000).

In addition to Fabs and scFvs, engineered antibody variants have been developed. Bi- and trivalent Fabs have been generated by using chemical crosslinking, and multimeric biabodies, triabodies and tetrabodies have been generated via self-assembly from scFvs by controlling the linker length. These variants are employed mostly in therapeutic and imaging applications (Holliger and Hudson, 2005).

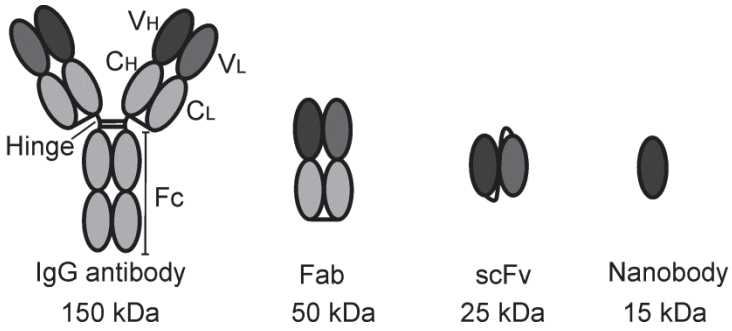


Figure 2. Different antibody formats. (Modified from Holliger and Hudson 2005.)

Nanobodies or VHH domains are single domain binders that originate from camelid antibodies consisting of only two heavy chains. The advantages of nanobodies include easy and low-cost manufacturing, high affinity and selectivity, lack of aggregation tendency, low immunogenicity, high stability, and high tissue penetration. The current applications of nanobodies are mostly intended for therapeutics but nanobodies have also been used in imaging studies where a genetic fusion molecule composed of a nanobody and green fluorescent protein has been shown to cross the blood-brain-barrier enabling brain imaging (Li *et al.*, 2012). Nanobodies have also been employed as binders in microarrays for detection of DNA-protein interactions (Nguyen-Duc *et al.*, 2013) and immunoassays for fibrinogen and prostate specific antigen demonstrating analytical sensitivities even in the picomolar range (Campuzano *et al.*, 2014; Saerens *et al.*, 2005).

2.2.2 Aptamers

Aptamers are relatively short (15-50 nt) RNA or single-stranded DNA sequences that are synthetically produced and have the capability to bind their targets with high sensitivity and specificity (Radom *et al.*, 2013). The three-dimesional structure of aptamers is responsible for the binding capacity and aptamers also recognize three-

dimensional shapes instead of specific epitopes contrary to antibodies (Famulok and Mayer, 2011). The secondary structure of aptamers is easily predicted from the nuleic acid sequence based on the complementary base pairing and structures such as hairpin, G-quadruplex and pseudoknot are common. However, modeling of the three-dimensional structure of aptamers has proved difficult but it can be defined experimentally using crystallography or nucleic magnetic resonance spectroscopy. (Radom *et al.*, 2013.) Additionally, aptamers can be modified to e.g. enhance their resistance to nucleases by derivatization of the 2'-ribose. The introduction of phosphorothioate to the DNA backbone has also been shown to increase the stability of aptamers and enhance their cellular availability (Mayer, 2009).

Nanomolar affinities are commonly obtained in the aptamer selection process (Famulok and Mayer, 2011) but even picomolar affinities have been described (Mosing *et al.*, 2005; Ruckman *et al.*, 1998). Aptamers are also very selective as they are able to distinguish molecules even on the basis of differences in their functional groups (de los Santos-Alvarez *et al.*, 2007). The benefits of aptamers over antibodies include their small size. Aptamers can also be selected *in vitro* in a relatively fast process and they do not trigger immune response *in vivo*. In addition, aptamers can be selected towards haptens and toxins, and there is no batch-to-batch variation in their activity. Their properties can be easily tuned by introducing modifications to the sequence. Additionally, aptamers are more stable than antibodies, and their tertiary structure can be completely renatured after denaturation (Radom *et al.*, 2013).

Aptamers are easily synthesized both in laboratory and commercial scale using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method. Different variants of SELEX have emerged to better fulfill various needs but the main principle is the same in all of them. Aptamers are selected for their affinities for target molecule from oligonucleotide libraries containing optimally 10^{13}-10^{16} different oligonucleotide sequences (Mayer, 2009; Tuerk and Gold, 1990). First, the library is subjected to immobilized target molecules. After incubation, all non-bound sequences are washed off and the bound sequences are eluted and amplified by PCR. Finally, the DNA strands corresponding to the original library are separated from the double-stranded amplification products and exposed to the next round of selection. Typically, 10-15 selection rounds are needed to obtain high quality binders, and as the selection proceeds the selection conditions become more stringent (Radom *et al.*, 2013). The selection conditions can also be tuned to guarantee the proper performance of the aptamers in desired conditions. Furthermore, the diversity of potential aptamers can be expanded by using chemically modified nucleotides (Gold *et al.*, 2012).

Aptamers have been utilized in a broad range of applications. They can be utilized as stationary phase in the affinity chromatography purification of proteins and small molecules (Kokpinar *et al.*, 2011; Deng *et al.*, 2003), and they also enable target-specific chiral separation (Michaud *et al.*, 2004). The therapeutic applications of aptamers are widely studied but so far only one aptamer, Pagaptanib, has been approved by FDA for vascular ocular disease treatment (Ng *et al.*, 2006). In addition, aptamers are potential drug delivery agents and they can be used in bioimaging applications (Wu *et al.*, 2013; Ai *et al.*, 2012).

In bioanalytical assays, the unique features of aptamers have enabled the development of innovative assay concepts. The conformational change occurring upon analyte binding allows signal modification, thus enabling also homogeneous assays (de-los-Santos-Alvarez *et al.*, 2007). The ability for conformational change has been used in numerous FRET-based assays (Chi *et al.*, 2011; Heyduk and Heyduk, 2005; Hamaguchi *et al.*, 2001), as well as in colorimetric (Zhu *et al.*, 2010) and electrochemical assays (Xiao *et al.*, 2005). Depending on the assay design, the binding of the analyte can cause either a signal increase or decrease. However, not all aptamers undergo a conformational change upon analyte binding but they retain the tertiary structure instead. Therefore, strand displacement assays involving competitor oligonucleotides have been developed (Nutiu and Li, 2003; Xiao *et al*., 2005). In strand-displacement assays, the competitor oligonucleotide dehybridizes from the aptamer in the presence of an analyte creating a measureable change in the signal. The use of competitor oligonucleotides decreases also the assay background (Tang *et al*., 2008). Some aptamers can be split into two fragments, which can be utilized in development of specific assays for small molecules. It demonstrates also the high verstility of aptamers. The binding of the analyte shifts the equilibrium from separate aptamer fragments toward complete folded aptamer complex resulting in a change in the signal (Fig. 3). (Zuo *et al*., 2009) Furthermore, the assay can be based on the use of intercalating dyes to avoid the need for labeling of the aptamers prior to the assay (He *et al*., 2010). However, these methods often suffer from high background and they lack the multiplexing capability.

The analytical sensitivity of aptamer-based assays is often in the nanomolar range (Babu *et al*., 2013; Shimada *et al*., 2012) but also picomolar and even attomolar analytical sensitivities have been obtained (Müller *et al*., 2011; Wang J. *et al*., 2011). However, the most sensitive assays are often heterogeneous assays involving washing steps. Aptamers have also been used as binders in multiplexed assays (Wu *et al*., 2014).

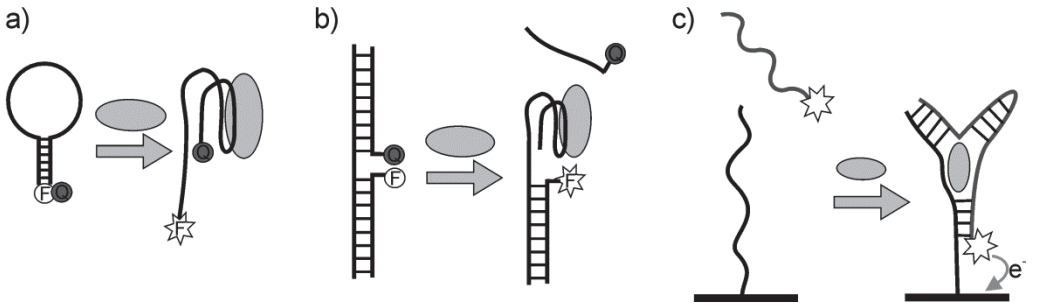


Figure 3. Assay principles utilizing aptamers as binders. a) FRET-based assay. b) Strand-displacement assay. c) Electrochemical split aptamer assay. (Modified from Hamaguchi *et al., 2001,* Xiao *et al.,* 2005, Zuo *et al.,* 2009).

The main limitation of aptamer technology is the lack of aptamers for clinically relevant analytes. This explains the numerous applications existing for the detection of thrombin, platelet-derived growth factor, or cocaine, for which there are well-characterized aptamers available. Moreover, two separate aptamers for different recognition sites of a single analyte are particularly rare. However, the lack of aptamers

for various analytes allows an easy comparison of different assay architectures and detection technologies (Table 2). Further disadvantage of aptamers is that the SELEX process is not standardized and is often conducted in non-physiological conditions. Therefore the performance of aptamers may be compromised in the assay buffer and in biological samples (Cho *et al*., 2006). Thus it is essential that the intended use and sample matrix should be taken into account when selecting new aptamers.

Table 1. Characteristics of aptamer-based thrombin assays.

Assay type	Label	Analytical sensitivity	Reference
Quenching-type homogeneous FRET-based assay	$[Ru(dpsphen)_3]^{4-}$ doped silica nanoparticles and Dabcyl	4 nM	Babu *et al*., 2013
Homogeneous assay based on isothermal cascade signal amplification	Fluorescein and Dabcyl	0.17 nM	Ma *et al.,* 2012
Homogeneous FRET-based assay	Quantum dots and DNA intercalating dye BOBO-3	1 nM	Chi *et al*., 2011
Homogeneous FRET-based assay	Upconverting nanoparticles and carbon nanoparticles	0.18 nM	Wang Y *et al*; 2011
Homogeneous FRET-based assay	Eu^{3+} chelate and Cy5	50 pM	Heyduk *et al*., 2005
Homogeneous electronic detection	Methylene blue	6.4 nM	Xiao *et al*., 2005
Quenching-type homogeneous FRET-based assay using aptamer beacon	Fluorescein and Dabcyl	0.11 nM	Li *et al*., 2002
Heterogeneous fluorescence-based assay	Alkaline phosphatase	11 nM	Shimada *et al*., 2012
Heterogeneous fluorescence-based assay	7-amino-4-methylcoumarin	0.47 pM	Müller *et al*., 2011
Heterogeneous electrochemiluminescence-based assay	semiconductor nanocrystals and gold nanoparticles and CDS thin film	26 aM	Wang J *et. al*., 2011
Heterogeneous electrochemiluminescence-based assay	$Ru(bpy)_3^{2-}$-gold nanoparticles and ferrocene	8.0 fM	Zhang *et al.,* 2011

2.2.3 Alternative protein binders

Antibodies are not the only class of affinity binders as also alternative protein scaffolds such as designed ankyrin proteins (DARPins), affibodies, knottins, and Src homology 3 domains (SH3) (Table 1) exhibit selective binding. Protein scaffolds are polypeptide frameworks that can be engineered by adding mutations or insertions without reducing the stability or affecting the structure (Skerra, 2000). Although originating from various sources, these binders share many common features. They are thermostable

and small. Moreover, most of them do not contain disulfide bonds to ensure correct folding as in the case of antibodies. Thus they can be expressed to cytosol bacterial expression hosts enabling high yields (Banta *et al.*, 2013). Because of their small size, binders with novel target specificities can be easily selected from recombinant protein libraries using display techniques, and the binding properties can be engineered by using error-prone PCR or random peptide insertion, or by rational design (Binz *et al.*, 2005). Furthermore, they do not aggregate, which is advantageous in *in vitro* applications enabling an efficient selection process. Additionally, multivalent binders can be created by connecting the binders at genetic level via a linker (Theurillat *et al.*, 2010). The advantage in bioanalytical assays is that their site-specific coupling to label molecules or solid surfaces is possible by simply adding, e.g., cysteine residue to the protein (Boersma and Pluckthun, 2011). Long shelf-life and low production costs due to high expression levels are also important aspects when considering diagnostic applications.

Table 2. Properties of alternative protein binders.

Alternative protein binder	Size (amino acids)	Structure	Affinity	Reference
Affibody	58	Three α-helices	Picomolar	Banta *et al.*, 2013; Orlova *et al.*, 2006
DARPin	10-50/ repeat	Monomeric binder comprised of commonly 4-6 repeats of β-sheet and two α-helices	Picomolar	Schilling *et al.*, 2014; Theurillat *et al.*, 2010
Knottin	30-50	Resembles a knot and is formed when a disulfide bond threads through the peptide backbone circle	Sub-nanomolar	Moore *et al.*, 2012
SH3 domain	60	Two perpendicular β-sheets and a groove formed by two surface loops	Sub-picomolar	Lehto *et al.*, 1988, Järviluoma *et al.*, 2012

By using non-immunoglobulin scaffolds as binders, immunoassay interferences caused by, for instance, heterophilic antibodies and human anti-animal antibodies can be avoided (Binz *et al.*, 2005). Alternative binders are not affected by immobilization to solid phase. Furthermore, the dynamic range of the biosensor can be tuned by choosing binders with different affinities for the same molecule. This is valuable when measuring analytes with broad concentration range. Because of the high thermal stability of alternative binders, the binding surfaces in assays can also be regenerated (Albrecht *et al.*, 2010). Additionally, modifications can be added to alternative binders to allow self-assembly of monolayers enabling the simple, yet controlled coating of solid phases and particulate labels. For example, metal-affinity driven self-assembly has been used for coating of quantum dots with hexahistidine-tagged peptides to create an assay for measuring enzyme activity (Ghadiali *et al.*, 2010).

Several examples of assays based on the use of alternative binders exist. The applicability of affibodies has been demonstrated for interferon γ using immuno-rolling circle amplification (Akter *et al.*, 2012). Affibody-β-galactosidase fusion protein has been employed in enzyme-linked immunosorbent assay (ELISA) for human IgA as a model analyte (Ronnmark *et al.*, 2003). Additionally fusion protein composed of an affibody and green fluorescent protein has been used in an assay measuring antibodies for *Mycoplasma pneumouniae* (Yang *et al.*, 2012). Affibodies are also applicable as binders in microarrays. In an array for six different protein analytes, multimeric affibodies were immobilized to carboxymethyl dextran slide from their C-terminal cysteine residue. Six proteins labeled with Cy3 were detected resulting in analytical sensitivities between 70 fM and 60 pM depending on the analyte. The capability of affibody microarrays to recognize analytes from complex samples was demonstrated with TNF-α and IgA (Renberg *et al.*, 2007).

DARPins have been exploited in the development of homogeneous biosensor: solvatochromic dye was site-specifically conjugated to DARPins to allow a change in fluorescent signal upon analyte recognition. MalE protein from E. coli was used as a model analyte and the analytical sensitivity was in the low nanomolar range (Miranda *et al.*, 2011). In another application, DARPins were used as binders in proximity ligation-based assay for the detection of human epidermal growth factor receptor 2 (HER2), which is a tumor antigen (Gu *et al.*, 2013). In a histochemistry microarray, DARPins have demonstrated higher specificity than antibodies indicating their potential for future applications (Theurillat *et al.*, 2010).

Because of their remarkably small size, knottins can be produced even by chemical synthesis. However, disulfide bonds may result in aggregation of knottin multimerization and also hamper the production in bacterial expression systems. The resistance to proteolytic degradation could enable oral administration instead of intravenous administration making knottins attractive especially for therapeutic applications. In addition to single domain protein binders, other proteins have also been studied as alternative binders. Variants of TEM-1 β-lactamase with target specificities for streptavidin and ferritin have been reported and their applicability to ELISA assays has been demonstrated (Legendre *et al.*, 2002). Also HEAT and ARM repeat proteins have been proposed as binders (Boersma and Pluckthun, 2011). β-roll motifs are another type of scaffolds that could be used to create a switchable binder. β-roll motifs consist of tandem repeats of nine-amino-acid-long repeating sequences. The β-roll motif remains unstructured in the absence of calcium but calcium addition results in the folding into a β-helix structure composed of two parallel β-sheet faces. This dependence on calcium could be used to create a conformational change in an active binder (Banta *et al.* 2013; Blenner *et al.*, 2010).

2.2.4 Molecularly imprinted polymers

Molecularly imprinted polymers (MIPs), sometimes called plastic antibodies, are synthetic recognition elements that are co-polymerized from interacting monomers using the analyte molecule as a template. When the template is removed a pocket fully

matching its size, shape and interactions is formed on the polymer and can be employed as a specific binder. The main benefits of MIPs are their robustness, tolerance toward different temperatures and pH values, and a long shelf-life. These features make them a potential alternative for antibodies especially in diagnostic tests intended for resource-poor settings, where extreme conditions are encountered. Moreover, MIPs can be generated for virtually any molecule. (Kryscio and Peppas, 2012) The MIP dissociation constants are often in the micromolar range, but values as low as $2.4x10^{-12}$M have been reported (Karimian *et al*., 2013).

There are numerous different MIP synthesis methods, which can be roughly classified into covalent and non-covalent methods depending on how the target molecule is immobilized in the polymer (Haupt 2010). Also the physical form of MIPs can vary from monoliths (Matsui *et al*., 1998) to particles (Cakir *et al*., 2013; Yoshimatsu *et al*., 2007) or thin surfaces (Barrios *et al*., 2012) depending on the intended application. Various synthesis methods have been extensively reviewed elsewhere (Biffis *et al*., 2012).

MIPs have been employed mostly in bioanalytics and especially as solid phase in purification applications, such as solid phase extraction and high-performance liquid chromatography (HPLC) (Alexander *et al*., 2006). The first immuno-like assay with MIPs was developed for theophylline and diazepam using radiolabels (Vlatakis *et al*., 1993) while later applications have exploited MIPs in combination with detection methods such as surface plasmon resonance (Kugimiya and Takeuchi, 2001), quartz crystal microbalance (Reimhult *et al*., 2008), electrochemical (Huang *et al*., 2007), and fluorescent detection (Zdunek *et al*., 2012). Both homogeneous and heterogeneous assays have been developed. Sandwich-type assays are difficult to implement with MIPs due to steric hindrances and, therefore, competitive assays using labeled analyte analogs are common. Alternative approach is to embed label molecules into the MIPs. In this case, the binding of an analyte modifies the signal thus enabling analyte detection. The drawback of MIPs is the slow diffusion of proteins into the MIP cavities. In addition, the non-specific binding is relatively high. Furthermore, many of the MIP-based sensors or assays rely on the use of organic solvents, and performance in aqueous solution or real clinical samples have not been demonstrated. (Moreno-Bondi *et al*., 2012)

Despite a number of immuno-like assays published, only a few assays exist for protein analytes. The large size, complexity, and conformational flexibility, as well as the requirement of aqueous solution set additional challenges for creating MIPs for proteins (Haupt *et al*., 2012). However, MIP-based assays for cardiac troponin T using electrochemical detection have been published, and a analytical sensitivity of 9 ng/L has been obtained by using cyclic voltammetry and an MIP-modified gold electrode. This result is comparable to the existing high-sensitivity immunoassays for cardiac troponin T (Karimian *et al*., 2014; Apple *et al*., 2012; Moreira *et al*., 2011). Assays for bovine serum albumin (BSA) (Kryscio and Peppas, 2012) and insulin have also been reported (Schirhagl *et al*., 2012). The analytical sensitivity for insulin using label-free quartz crystal microbalance was 1 µg/mL and the linear range was 2 orders of

magnitude. However, the assay sensitivity was enhanced by using double imprinted antibody replicae, which are created by first imprinting template nanoparticles using antibodies as models. Then the antibody replicae are imprinted by using the template nanoparticles as models. The antibody replicae recognize the same epitopes of the target as the original antibodies but are more robust.

2.3 Bioconjugation techniques

The high-quality binders and labels alone are not sufficient to guarantee reliable high-sensitivity assays, but the methods used for labeling and immobilizing the biomolecules have a considerable impact on the final assay performance. Predictability and reproducibility of the conjugation process, rapid reaction rate as well as biomolecule orientation and activity are key features in achieving high quality bioconjugates (Algar *et al*., 2011). Low control over the conjugation reaction compromises the activity of the biomolecules and labels resulting in heterogenic products and reduced assay performance. Furthermore, conjugation methods affect the reproducibility of the assay because of the potential batch-to-batch variation (Kalia and Raines, 2010; Kricka, 1994). The significance of assay component quality has been widely realized in recent years, and an increasing number of papers concerning site-specific conjugation and solid-phase immobilization have been published (Warden-Rothman *et* al., 2013; Bundy and Swartz, 2010; Niemeyer, 2010). In addition, interest in bio-orthogonal chemistry has increased (Sletten and Bertozzi, 2009).

Most of the criteria for successful conjugation apply both to immobilizing biomolecules on solid phase and conjugation of biomolecules to other biomolecules or labels. Still, some differences exist. In labeling of biomolecules, the label is commonly used in excess while in immobilization, the amount of biomolecule depends on the assay type while. Immobilization may cause steric hindrances and changes in conformation, which impede the proper function of the biomolecule and prevents antibodies from recognizing their antigens. In labeling reactions, steric hindrances are caused by excessive labeling or labeling of the analyte-binding site of the binder molecule. In immobilizations, steric hindrances are caused by the attachment of biomolecules to solid phase so that the active site becomes unavailable. Furthermore, the removal of unattached biomolecules is easy in immobilizations because the biomolecules can be simply washed off from the solid phase and the remaining surface blocked in order to prevent non-specific binding. (Wild and Kusnezow, 2013.)

2.3.1 Non-covalent conjugation methods

Non-covalent conjugation techniques have been commonly used for producing the assay components and they are particularly prevalent in immobilizing binders on solid-phases. To generate solid phases for immunoassays, the binders have commonly been immobilized simply by adsorption to solid phases, such as polystyrene microtiter wells and glass slides. Binder immobilization by passive

adsorption is easy and relatively rapid, but the activity of the binders is often decreased due to adsorption (Wild & Kusnezow, 2013; Butler *et al*., 1992). Affinity-based non-covalent conjugation techniques include, for example, HisTag, leucine zippers, and proteins such as FLAG. However, the non-covalent interactions are cleavable and highly dependent on assay conditions, which affect the stability and reproducibility of the conjugates. Additionally, the interaction may be inefficient if the concentration of biomolecules is relatively low. Thus affinity tags are mostly used for protein purification applications (Zhao *et al*., 2013; Shimada *et al*., 2012; Schweller *et al*., 2008; Ohiro *et al*., 2007).

Interaction between avidins and biotin (bio) is a prominent and widely used non-covalent conjugation technique due to the extremely high affinity of avidins to biotin (dissociation constant 10^{-15} M). Streptavidin (SA) is the most commonly used avidin because its nonspecific binding is lower compared to that of avidin. (Hermanson, 2013a; Schetters, 1999.) The use of SA-bio interaction in binder immobilization on solid phase allows more optimal binder orientation and maintains the activity of the binders thus enhancing the binding capacity of solid phases (Saerens *et al*., 2005; Collet *et al*., 2005; Välimaa *et al*., 2003). The interaction enables also creating a universal binding phase, which can be easily customized to different needs by adding various biotinylated molecules (Välimaa *et al*., 2003). For creating the universal binding surface, SA is immobilized to solid phase and the binder molecule is biotinylated using the conventional covalent conjugation methods that are used for labeling of the protein binders. In addition to using SA-bio interaction for immobilization of biomolecules, it has been utilized in producing labeled binders and in coating of particulate labels with binders. However, the use of SA-bio interaction is not optimal for homogeneous FRET-based systems, where the signal is distance-dependent. Also, the tetrameric nature of streptavidin compromises the controllability and reproducibility of the reaction (Sano *et al*., 1996).

Applications using DNA-directed immobilization have increased in recent years because utilization of the specific base pairing of DNA enables well-controlled conjugation and even self-assembly of array surfaces (Pippig *et al*., 2014; Niemeyer, 2010). Furthermore, reversible sensor surfaces can be easily created by utilizing DNA hybridization (Niemeyer *et al*., 1999). However, additional conjugation methods are required to attach the DNA strands to solid surface and to proteins.

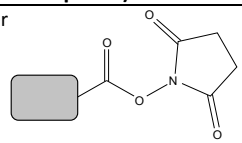
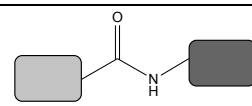
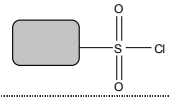
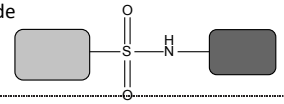
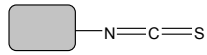
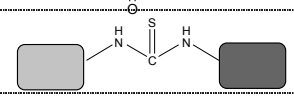
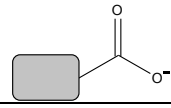
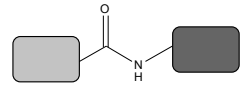
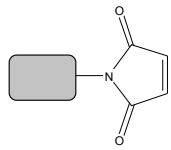
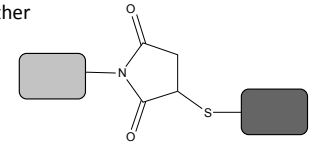
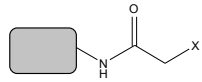
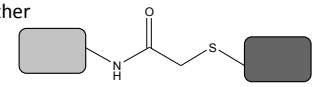
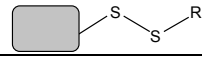
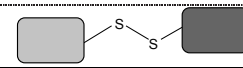
2.3.2 Conventional covalent conjugation

Covalent approaches to conjugation are preferred because they provide a more stable conjugation resulting in more robust assays and improved assay performance (Saerens *et al*., 2005). Therefore, they are reviewed here in more detail than non-covalent methods. Traditional methods for protein conjugation rely on using the primary amines in lysine residues or thiol groups in cysteine residues as targets (Table 3). Conjugation to cysteines provides good site-specificity because cysteines are the second least common amino acids in proteins. Functional groups that react with thiol include haloacetamides, maleimides, and disulfides, although the applicability of disulfides is

limited to *in vitro* applications due to biological reducing agents such as glutathione (Kalia and Raines, 2010). The advantage of a reaction between maleimide and thiol is its selectivity at near-neutral pH enhancing its applicability to biomolecule conjugations (Algar *et al*., 2011). However, maleimides hydrolyze spontaneously, and the hydrolysis competes with the Michael addition to thiol resulting in the heterogeneity of the product. At high concentrations, the selectivity of maleimides and iodoacetamides to thiols is decreased and they can also react with lysine and histidine products (Kalia and Raines, 2010).

Although lysine is much more common in proteins than cysteine, the advantage of using lysine residues is the wide variety of methods available for primary amines. Amino group reacts with N-hydroxysuccinimide activated esters, sulfonyl chlorides, isocyanates, or isothiocyanates producing amides, sulfonamides, ureas, or thioureas, respectively (Sletten and Bertozzi, 2009). Isothiocyanates are widely used for protein labeling, *e.g.*, in labeling lanthanide chelates to antibodies (*e.g.*, von Lode *et al*., 2003). Additionally, an amino group is often used to couple proteins to moieties containing carboxyl groups by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS). This method has been widely applied to biomolecule conjugation to nano- or microparticles (Wang *et al*., 2014; Algar *et al*., 2011; Härmä *et al*., 2001). The drawback of these conventional labeling methods is that a high excess of label is required for obtaining proper yields. Additionally, the reactions are not site-specific resulting in variance in conjugation degree in the product and between batches. Excess labeling can also interfere with the analyte detection of antibodies.

Table 3. Conventional conjugation chemistries. (Modified from Sletten and Bertozzi, 2009.)

Reactive group in biomolecule	Reagent (e.g. another biomolecule, label moiety or solid phase)	Product
H_2N—	NHS ester 	Amide 
	Sulfonyl chloride 	Sulfonamide 
	Isothiocyanate 	Thiourea 
	Carboxyl group with EDC (and (Sulfo-)NHS) in reaction 	Amide 
HS—	Maleimide 	Thioether 
	Haloacetamide X= I, Br or Cl 	Thioether 
	Disulfide 	Disulfide 

Bifunctional linkers allow the conjugation of biomolecules between amine-amine, amine-thiol, or thiol-thiol groups. In homobifunctional linkers, the two reactive groups are identical whereas heterobifunctional linkers contain two different reactive groups. The drawback of homobifunctional linkers is that the resulting product is a mixture where conjugates between two different molecules form only a part of the product and conjugates between two similar molecules form unwanted side products. The yield of the optimal product is particularly low if the conjugation is performed in one-step reaction, where all the reagents are added at once. Heterobifunctional crosslinkers allow better control because of the different specificities of the two reactive groups. However, to achieve the best possible result, the conjugation should be performed in two separate steps including a purification step between them, making the method complicated and time-consuming. (Hermanson, 2013b.) Unnatural amino acids containing, *e.g.*, alkyne or azide functionality can also be introduced to recombinant proteins to insert alternative reactive groups, thus avoiding the use of bifunctional crosslinkers (Bundy and Swartz, 2010). Furthermore, novel methods have been developed for conjugation to lysine and cysteine. Also tyrosine, tryptophan, and the N-terminus of proteins have emerged as possible sites for conjugation. (Sletten *et al*. 2009, Cserep, 2013)

2.3.3 Novel bioorthogonal and chemoselective conjugation methods

Chemoselective and bioorthogonal conjugation chemistries have emerged in recent years. These methods provide efficient product formation with high selectivity and control over conjugation. Moreover, the conjugations can be performed at physiological conditions in complex matrices, such as serum (Best, 2009). In chemoselective reactions, no protective groups are needed for the covalent labeling of proteins or other biomolecules (Agten *et al*., 2013). Several chemoselective reactions and a few bioorthogonal reactions exist and the most prominent of these in bioanalytical applications are discussed below (Table 4).

In cycloaddition reactions, two unsaturated molecules yield a cyclic addition product. Huisgen 1,3-dipolar cycloaddition is rapid and highly regioselective in the presence of Cu(I) salt, thus it is also called copper-catalyzed azide-alkyne cycloaddition. (Algar *et al*., 2011; Best 2009.) It is the most prominent example of the so-called click-chemistry, which strives for the generation of building blocks for easy covalent conjugation. It defines also the optimal characteristics for reactions resulting in C-X-C heteroatom links (Kolb *et al*., 2001). However, the cytotoxicity of Cu(I) limits the applicability of Huisgen cycloaddition in biological systems. Therefore, a copper-free alternative, in which the reactive moiety is a strained alkyne, such as cyclooctyne, has been developed. Its applicability to the effective labeling of cells *in vivo* has been demonstrated. (Agard *et al*., 2004.) However, compared with the Cu(I)-catalyzed reaction, the specificity of copper-free cycloaddition may be compromised because the strained alkyne is more reactive also to other nucleophiles (Algar *et al*., 2011). Furthermore, the synthesis of reagents for copper-free cycloaddition is complex and many derivatives suffer from poor water solubility (Devaraj *et al*., 2008). Heterobifunctional linkers or unnatural amino acids are needed to introduce the desired functional group to the biomolecule because azides and alkynes do not exist in living organisms. This also provides a possibility for selective labeling. For oligonucleotides and peptides, the reactive group can be included during the synthesis. Recombinant technology and unnatural amino acids can be used to introduce the desired reactive groups to proteins to enable cycloaddition (Bundy and Swartz, 2010). Despite the challenges, cycloaddition reactions have been successfully utilized for the biofunctionalization of silica nanoparticles and other silica-coated surfaces, as alkyne or azide groups can be easily added to the surface via modified silanes (Mader *et al*., 2010; Achatz *et al*., 2009) Additionally, cycloadditions have been utilized in the production of microarrays (Uszczynska, 2012), and protein and oligonucleotide labeling (Cserep *et al*., 2013; Hong *et al*., 2009), as well as in drug discovery (Kolb and Sharpless *et al*., 2003).

Tetrazine ligation is another biorthogonal cycloaddition reaction and it occurs between tetrazine and cyclooctene derivatives. No catalyst is required and the reaction rate is fast when micromolar reactants are used. In these conditions, the reaction efficiency is close to 100%. (Blackman *et al*., 2008.) Tetrazine ligation has been employed in, *e.g.*, antibody labeling (Devaraj *et al*., 2008), oligonucleotide ligation (Seckute *et al*., 2013), and preparation of small molecule microarrays (Zhang *et al*., 2013).

In Staudinger ligation, azide reacts with a modified phosphine in mild conditions to produce an aza-ylide intermediate, which further hydrolyzes to produce an amide bond under aqueous conditions. The reaction is rapid and it can be performed at room temperature resulting in high yields of the product. Furthermore, bioconjugation causes only minimal interference to the biomolecule due to the small size of azides. (Saxon and Bertozzi, 2000). The main limitation of Staudinger ligation is its relatively slow kinetics, causing the need of high concentrations of triarylphosphine (Sletten and Bertozzi, 2009). In addition, phosphines can be oxidized under ambient conditions or by metabolic enzymes, but this can be effectively avoided by using an excess of reagents (Algar *et al*., 2011). Moreover, in alkaline conditions, azides can react with thiols and disulfides often present in biomolecules. In physiological pH, however, the competitive reaction is very slow affecting the Staudinger ligation only slightly. Therefore, Staudinger ligation can be considered bioorthogonal and it has been utilized in cell cultures as well as *in vivo* environments (Sletten and Bertozzi, 2009). Staudinger ligation has been exploited in multiple applications ranging from protein synthesis to preparation of glycomicroarrays (Ma *et al*., 2012; Nilsson *et al.*, 2003).

Carbonyl condensations with hydrazide and oxyamine compounds produce hydrazones and oximes, respectively. Both ligation reactions are chemoselective and can be performed in mild conditions (Agten *et al*., 2013). The drawback of the methods is that the reactions are reversible. Nevertheless, bioconjugations are commonly performed in aqueous solutions where the reaction products hydrazine and oxime are favored. Still, the hydrolysis can affect the shelf life of the conjugates making this reaction less than optimal for bioconjugates intended for diagnostics. The reaction rates of hydrazine and oxime ligations are also relatively slow at neutral pH and for efficient ligation aniline is needed as catalyst (Algar *et al*., 2011). However, the reversibility of the reaction can be exploited in drug delivering systems, where the slightly acidic pH of the endosomes increases the hydrolysis rate upon cellular uptake resulting in drug release (Aryal *et al*., 2010). Hydrazone and oxime ligations have been used, *e.g.*, in the coating of polymers with proteins (Grotzky *et al*., 2012). Recently, hydrazone ligation has been further developed to allow faster reaction rates at neutral pH and to enhance product stability (Agarwal *et al*., 2013), and a more efficient catalyst, m-phenylenediamine, has been shown to enhance oxime ligation 15-fold more efficiently than aniline (Rashidian *et al*., 2013). These improvements enhance the applicability of hydrazine and oxime ligations.

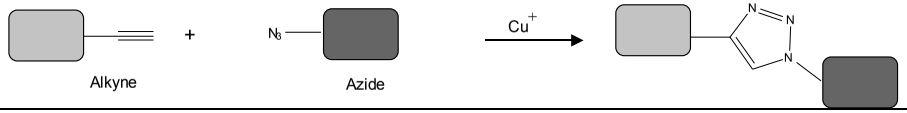
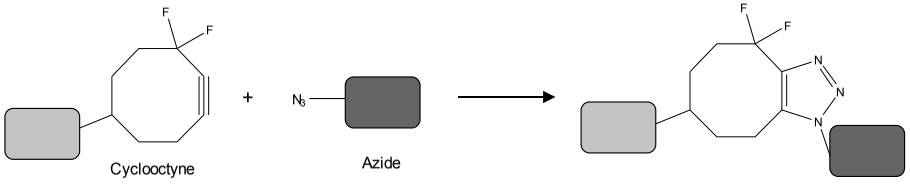
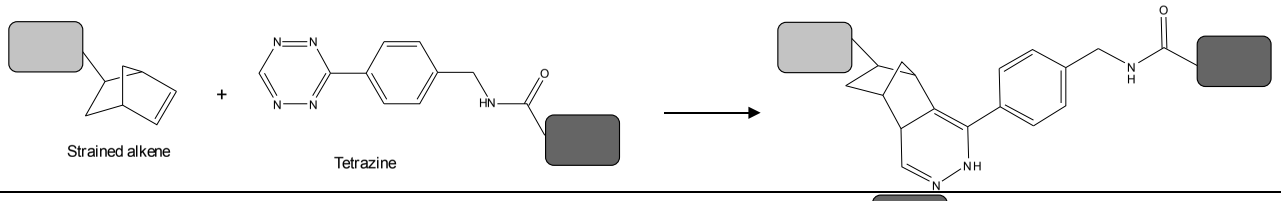
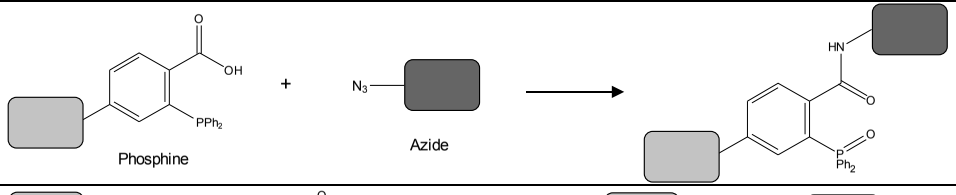
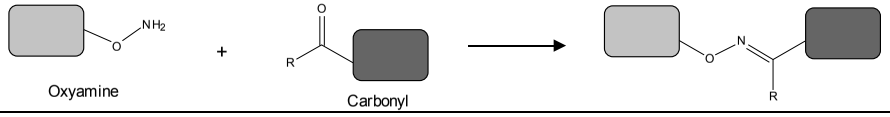
Protein tags such as SNAP-tag, CLIP-tag, and HaloTag enable covalent conjugation to proteins by exploiting their modified enzymatic activity and synthetic ligands. In these methods, the modified enzyme and the target protein are produced as a recombinant fusion protein. The synthetic ligand is coupled to, *e.g.*, a label and a covalent bond is formed due to selective enzymatic activity. SNAP-tag is derived from human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT). The primary substrate of AGT is O^6-alkylguanine DNA, but AGT reacts also with O6-benzylguanine, the derivatives of which are exploited in SNAP-tag technology (Keppler *et al*., 2003). CLIP-tag is a mutant of AGT and it reacts with O^2-benzylcytosine. Despite their common origin, they do not cross-react significantly and thus they can be used for

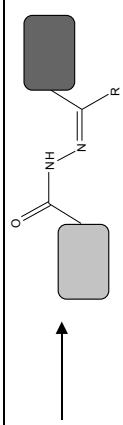
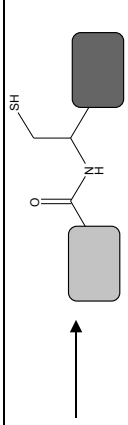
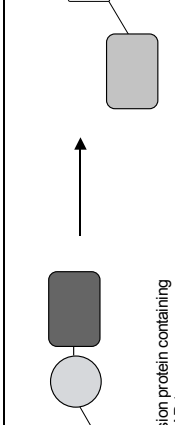
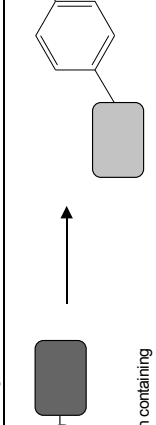
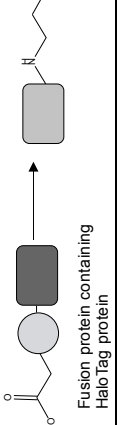
labeling different proteins in the same application. The conjugation reaction of protein tags is fast due to its enzymatic nature, and rate constants of 2.8×10^4 $M^{-1}s^{-1}$ and 1.1×10^3 $M^{-1}s^{-1}$ have been reported for SNAP-tag and CLIP-tag, respectively. (Gautier *et al*., 2008.) HaloTag has been modified from bacterial haloalkane dehalogenase and it uses chloroalkane derivatives as substrates. Cross-reactions in *in vivo* experiments may be lower with HaloTag compared to other tags because its enzymatic reaction is foreign to mammalians whereas SNAP-tag and CLIP-tag are of human origin (Los *et al*., 2008).

The relatively large size of the protein tags may cause steric hindrances in some applications. Furthermore, the recombinant fusion needs to be generated separately for every protein. Once a gene construct has been generated, however, the production of the recombinant protein is relatively simple and reproducible. More importantly, the labeling using protein tags is stoichiometric (Keppler *et al*., 2003). Protein tags were initially developed to enable covalent self-labeling of proteins *in vivo*, which was necessary for protein localization and interaction studies. To date, they are well-established in these applications (Gu *et al*., 2013; Gautier *et al*., 2009; Hartzell *et al*., 2009; Hurst *et al*., 2009). Additionally, protein tags have been exploited in applications aimed at tumor detection and therapeutic applications (Hussain *et al*., 2011; Kosaka *et al*., 2009). They have also been employed in biochemical affinity purification (Hata *et al*., 2007) as well as in the production of microarrays (Noblin *et al*., 2012; Nath *et al*., 2008). Still, only a few examples exist of using fusion proteins consisting of protein tags and antibody fragments, or alternative affinity binders, such as DARPins (Gu *et al*., 2012; Hussain *et al*., 2011; Kampmaier *et al*., 2009).

Native chemical ligation is a chemoselective reaction between thioesters and N-terminal Cys peptides forming an amide bond. Native chemical ligation is widely used for introducing tags, labels, or other macromolecules, such as DNA and proteins. It is particularly useful for conjugating peptides to other peptides or proteins to allow protein semi-synthesis. However, converting recombinant proteins into thioesters is a demanding task and limits the applicability of native chemical ligation. Expressed protein ligation (EPL) resolves this problem by using self-splicing enzymes called inteins, which are fused to the protein of interest. When the intein is spliced from the fusion protein in the presence of sodium 2-mercaptoethane sulfonate, the formed thioester is ligated with a peptide containing C-terminal cysteine (Hackenberger and Schwarzer, 2008). However, the requirement for reducing conditions may limit the applicability of the method. EPL can also be expensive because a large excess of cysteine-containing reagent is needed. EPL has been used for the conjugation of recombinant proteins to proteins, imaging agents, and nanoparticles (Warden-Rothman *et al*., 2013).

Table 4. Chemoselective reactions for protein modification. (Modified from Sletten and Bertozzi, 2009, Algar *et al*., 2011, Keppler *et al*., 2003, Los *et al*., 2008.)

Conjugation method	Reaction
Copper-catalyzed azide-alkyne cycloaddition	
Copper-free azide-alkyne cycloaddition	
Tetrazine ligation	
Staudinger ligation	
Oxime ligation	

Conjugation method	Reaction
Hydrazone ligation	
Native chemical ligation	
SNAP-tag	
CLIP-tag	
HaloTag	

2.4 Luminescent lanthanide labels

Luminescence is the emission of photons *i.e.* light that arises from an electronically excited state of a substance. On the basis of the excited states, luminescence can be classified as fluorescence and phosphorescence (Lakowicz, 2006). Photoluminescent labels are widely used in diagnostic tests and bioimaging. Upon excitation, typically in the ultraviolet (UV) range, they emit light at a wavelength characteristic to each label. Photoluminescent labels can be either organic molecules or proteins. The advantage of luminescent labels over other label technologies is that the signal can be measured outside the reaction vessel thus avoiding the contamination of the measurement system. Although the photoluminescent signal is commonly in the visible range of the spectrum, a measurement device is required to obtain sensitive detection and quantitative results. Multianalyte assays can be developed using different labels for each analyte, but high degree multiplexing is difficult to obtain due to partly overlapping emission peaks (Geissler *et al*., 2013). Moreover, autofluorescence and scattering interference limit the assay sensitivity when conventional fluorophores are utilized. Autofluorescence background signal originates from the fluorescent components in the sample matrix and reaction vessels that are excited simultaneously with the luminescent label (Diamandis and Christopoulos, 1990).

Luminescent lanthanide labels possess exceptional features that make them effective for diagnostic assays. The main advantage of luminescent lanthanide labels over conventional fluorescent labels is their long luminescence lifetime, which enables elimination of the autofluorescence background signal and thus facilitates the development of high-sensitivity assays. By measuring the signal of lanthanide labels using a delay after the excitation pulse, the short-lifetime autofluorescence has already decayed and does not interfere with the measurement. The problems related to high background signal can also be overcome by using two-photon excitation. However, this method is expensive because simultaneous absorption of two photons is required and it can be achieved only with high-power pulsed lasers (Gorris and Wolfbeis, 2013; Hänninen *et al*., 2000). Advantages of luminescent lanthanide labels include also narrow emission peaks and large spectral differences between excitation and emission peaks. Therefore efficient emission measurement without interference from the excitation radiation is possible (Soini and Lövgren, 1987). This section covers various luminescent lanthanide labels and their applications.

2.4.1 Lanthanide chelates

As such, lanthanide ions are weakly luminescent because of their low absorption of excitation irradiation. Furthermore, their luminescence is quenched by water molecule coordination to lanthanide ions in aqueous solutions. (Hemmilä, 1985.) Organic ligand structure, however, can significantly enhance the fluorescent properties of lanthanides. The ligand contains a chromophore that is excited in the UV region and can further excite the lanthanide ion via internal energy transfer (Fig. 4). The 4f resonance levels of Sm^{3+}, Eu^{3+}, Tb^{3+}, or Dy^{3+} correspond to the triplet state of the ligand thus allowing effective ion fluorescence (Soini and Lövgren, 1987). Additionally, the ligand

coordinates to the lanthanide ion shielding the ion from quenching effects. Chelate derivatization with reactive groups, such as isothiocyanate, is also necessary for the coupling of the chelate to biomolecules. However, ligands that can both efficiently excite the lanthanide ion and shield it from the quenching effects of water are challenging to create. Dissociation-enhanced luminescence overcomes this problem by using two ligands. First, a non-fluorescent chelate conjugated to the biomolecule is used in the assay. After the formation of the immune complex, the lanthanide ion is dissociated from the chelate using low pH. It is successively chelated by a second ligand, which enables high intensity lanthanide luminescence in hydrophobic micellar solution. The specific signal of the label is high, and dissociation-enhanced europium fluorescence can be quantified down to $5x10^{-14}$ M (Hemmilä *et al*., 1984). Furthermore, the linear range of the label extends to 10^{-7} M concentrations and it has been exploited in numerous immunoassays (Pettersson *et al*., 2000; Qin *et al*., 1997; Hemmilä *et al*., 1988; Hemmilä *et al*., 1987).

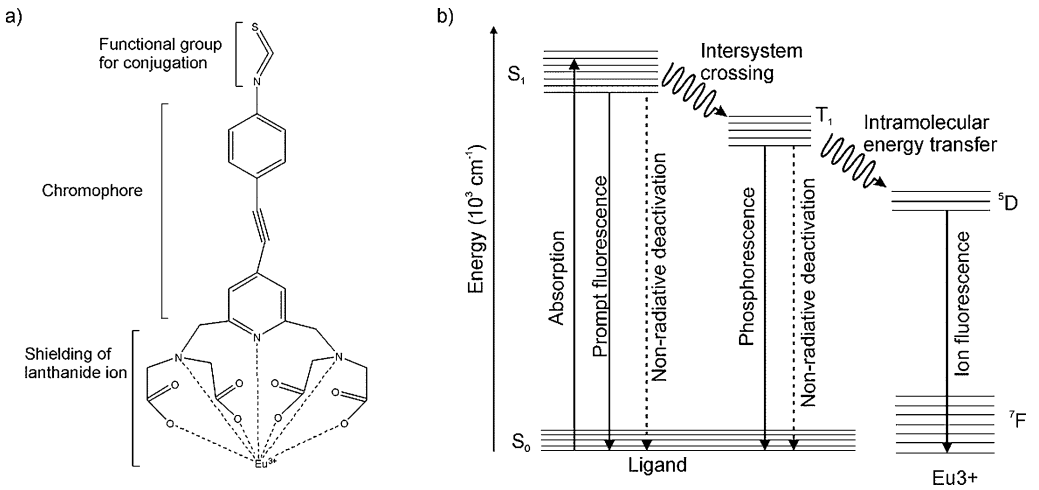


Figure 4. a) Schematic figure of a lanthanide chelate showing the different functionalites of the ligand. (Modified from Takalo *et al.* 1994.) b) Simplified energy level diagram demonstrating the excitation and emission process of europium chelate. (Modified from Bünzli and Piguet, 2005; Soini and Lövgren, 1987.)

Despite the challenges, directly luminescent chelates and cryptates have also been developed and utilized in bioanalytical assays and in cellular imaging applications (Gahlaut *et al*., 2010; Hanaoka *et al*., 2007; von Lode *et al*., 2003; Yuan *et al*., 1998; Takalo *et al*., 1994; Prat *et al*., 1991). The use of directly luminescent chelates simplifies the assay protocol because additional enhancement step is avoided. Moreover, directly luminescent chelates are essential for homogeneous FRET-based assays, where separate enhancement step is unfeasible. However, the signal intensities are higher with dissociation-enhanced lanthanide luminescence.

Several homogeneous FRET-based assays using lanthanide chelates as donors have been demonstrated. The technology has also been commercialized and is intended especially for high-throughput screening. (Härmä *et al*., 2009; Appelblom *et al*., 2007;

Karvinen *et al*., 2002; Heyduk *et al*., 2008; Blomberg *et al*., 1999.) Homogeneous assays relying on conventional fluorophores for detection have often suffered from insufficient signal modulation, which has limited the assay performance. They are also prone to background interference and scattering. (Hemmilä 1985.) In homogeneous FRET-based assays, the use of lanthanide labels as donors affects also the lifetime of the acceptor. The intensity of FRET-sensitized acceptor signal is increased because of the long decay time of the luminescent lanthanide labelsh. This allows multiple excitations of a single short-lifetime acceptor and results in brighter acceptor emission and potentially more sensitive assays. (Geissler *et al*., 2014.) Additionally, donor emission crosstalk and the direct excitation of the acceptor are avoided, and time-resolved measurement excludes autofluorescence enabling sensitive assays (Hildebrandt and Geissler, 2012).

Lanthanide chelates are potential labels also for multianalyte assays. Several dual-label assays utilizing two different lanthanide ions and dissociation-enhanced luminescence have been published and the analytical sensitivities have been in the picomolar range (Zhu *et al*., 2003; Eriksson *et al*., 2000; Hemmilä *et al*., 1987). Also a quadruplex assay using different lanthanide ions and dissociation-enhanced lanthanide luminescence for the simultaneous detection of thyroid stimulating hormone, 17 α-hydroxyprogesterone, immunoreactive trypsin, and creatine kinase MM has been demonstrated with nanomolar limits. The spectral crosstalk from other lanthanides to the measurement window increases the background signal and compromises the obtained limits of detection in multilabel applications. Furthermore, other lanthanides are not as brightly luminescent as Eu^{3+}, which affects the analytical sensitivity (Xu *et al*., 1992). Homogeneous FRET-based multianalyte assays resulting in picomolar sensitivities have also been developed for analytes such as tumor markers by using lanthanide chelates as donors and conventional organic fluorophores or quantum dots as acceptors (Wegner *et al*., 2013; Geissler *et al*., 2013; Kokko *et al*., 2008). Ratiometric measurement can be used to eliminate interferences caused by the sample medium (Wegner *et al*., 2013), and crosstalk correction algorithm can be used to avoid spectral crosstalk in assays with higher multiplexing degree (Geissler *et al*., 2013).

Recently, novel lanthanide chelates that can be excited using lower energy have been developed. Higher excitation wavelengths enable the use of inexpensive filters and possibly even light emitting diode (LED) excitation thus decreasing the cost of the instrumentation required for the measurement of lanthanide luminescence. The use of a novel ligand in dissociation-enhanced lanthanide luminescence facilitated the efficient excitation of Eu^{3+} in micellar solution using wavelengths over 365 nm (Valta *et al*., 2012.) A directly fluorescent Eu^{3+} chelate has also been developed and it has been utilized in the detection of proteins in Dot Blot and SDS-PAGE with low femtomole sensitivities using high-power UV-LED excitation (Zuchner *et al*., 2009).

A relatively novel modification of lanthanide chelates is the switchable lanthanide luminescence reporter technology, which enables sensitive homogeneous detection with a wide dynamic range. In switchable lanthanide luminescence, the luminescent chelate is divided into two non-luminescent label moieties, which are coupled to two

separate binders. When the binders recognize their target, the label moieties are brought into close proximity and a luminescent mixed chelate complex is formed (Fig. 5). By dividing the luminescent chelate into light-harvesting antenna ligand and ion carrier chelate, issues causing background signal in FRET-based assays, such as donor crosstalk, radiative energy transfer, and direct acceptor excitation can be avoided. Additionally, time-resolved luminescence measurement avoids autofluorescence. (Karhunen *et al.*, 2010.) The detection is specific and the assay background is low because of the two separate recognition events required for obtaining the signal. The principle of dividing the chelate into two parts was first published in 1990, but the signal modulation in the original nucleic acid hybridization assay was very moderate. (Oser and Valet, 1990.) The signal modulation in the following hybridization assays was not significantly improved although different components for sensitized lanthanide luminescence were employed (Wang *et al*., 2001). The applicability of template-directed lanthanide chelate formation to colorimetric qualitative assay for single nucleotide polymorphism was demonstrated by using ethylenediaminetetraacetic acid (EDTA) and phen(1,10-phenanthroline) conjugates (Kitamura *et al*., 2008). Sensitized lanthanide luminescence has also been achieved by utilizing tryptophan residue in a protein. It enabled the intermolecular sensitization of Tb^{3+} , which was brought into proximity with the protein by using a protein-binding peptide (Pazos *et al*., 2008). Further, surface-assisted sensitized lanthanide luminescence has been demonstrated on self-assembled surfaces using EDTA-based ligands and a naphthalene-based antenna (Hsu *et al*., 2009). The high potential of switchable lanthanide luminescence has been demonstrated in nucleic acid hybridization assays, where the reporter technology has shown negligible background and exceptionally high signal modulation, wide dynamic range, and sensitive detection (Karhunen *et al*., 2013, 2011, 2010). Moreover, switchable lanthanide luminescence has been applied to homogeneous nucleic acid amplification, and also the possibility of duplex PCR has been demonstrated (Lehmusvuori *et al*., 2013, 2012).

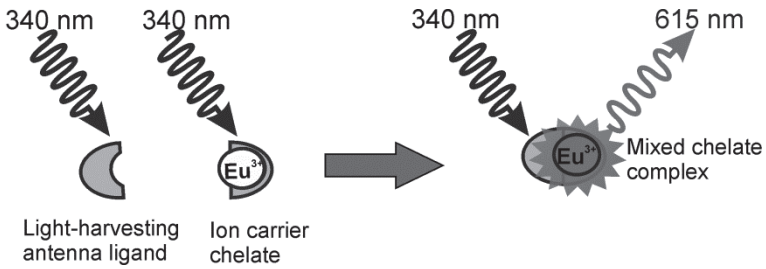


Figure 5. Principle of switchable lanthanide luminescence. Lanthanide ion carrier chelate and light-harvesting antenna ligand are intrinsically non-luminescent but they self-assemble into a luminescent mixed chelate complex when brought into close proximity.

Today, lanthanide chelates are well-established, and mature label technology with numerous applications also commercially available. Vast majority of the lanthanide chelate -based assays for protein or small molecule detection employ antibodies or Fab fragments as binders. In addition to immunoassays, lanthanide chelates have been employed in a variety of applications, such as in nucleic acid hybridization assays,

homogeneous nucleic acid amplification, and sequencing (Ollikka *et al*., 2008; Ylikoski *et al*., 2004; Nurmi *et al*., 2002; Samiotaki *et al*., 1997).

2.4.2 Lanthanide chelate-dyed nanoparticles

The luminescence intensity of lanthanide chelates is drastically lower compared to conventional organic fluorophores. However, this can be compensated by embedding numerous lanthanide chelates inside a polystyrene sphere. This is possible because self-quenching of lanthanide chelate luminescence is not observed even in millimolar concentrations (Härmä *et al*., 2000, 2001). Another advantage is that within the nanoparticles the lanthanide chelates are in a hydrophobic environment and, therefore, shielded from interference by water, metal ions, or pH variations, which are known to affect lanthanide chelate luminescence (Kokko *et al*. 2007). Thus the nanoparticulate labels enable even more sensitive assays than dissociation-enhanced luminescence (Härmä *et al*., 2000). Furthermore, when the nanoparticles are coated with biomolecular binders, the avidity of the binders is increased and the assay performance enhanced (Soukka *et al*., 2001). Eu^{3+} nanoparticles are commercially available and contain carboxyl groups for the covalent conjugation of biomolecules. EDC and sulfo-NHS chemistry is commonly used for coupling the biomolecules via their amine group onto the nanoparticle (Kokko *et al*., 2004; Soukka *et al*., 2001) The main challenge of nanoparticulate lanthanide labels is their non-specific binding, which is difficult to eliminate and increases the background signal limiting the potential sensitivity of the assay (Näreoja *et al*., 2014).

Most of the bioanalytical applications are based on the use of Eu^{3+} nanoparticles although applications using nanoparticles doped with other lanthanides also exist (Davydov *et al*., 2012; Huhtinen *et al*., 2005; Ye *et al*., 2004). Heterogeneous assays using antibody-coated lanthanide chelate-doped nanoparticles have enabled the development of rapid and ultrasensitive assays. Analytical sensitivity in the femtomolar range corresponding to the zeptomole amount of the analyte has been demonstrated for PSA (Härmä *et al*., 2000). By using Eu^{3+} nanoparticles coated with chimeric Fab-fragments, a rapid one-step heterogeneous sandwich-type assay for cardiac troponin with a analytical sensitivity of 0.41 ng/L (17 fM) and a dynamic range of over four orders of magnitude has been developed (Hyytiä *et al*., 2013). The applicability of Eu^{3+} nanoparticles to pathogen and toxin detection as well as for diagnosing infectious diseases has been extensively studied (Talha *et al*., 2013; Myyryläinen *et al*., 2010; Tang *et al*., 2009; Jaakohuhta *et al*., 2007; Valanne *et al*., 2005a). In addition to polystyrene-based particles, silica nanoparticles and self-assembled protein nanoparticles have also been used as hosts to lanthanide chelates. Utilizing protein nanoparticles composed of genetic fusions of human ferritin and scFv makes it possible to avoid a separate conjugation step of adding binders to nanoparticles (Davydov *et al*., 2012; Jääskeläinen *et al*., 2008; Xu and Li., 2007; Ye *et al*., 2004).

Eu^{3+} nanoparticles have also been utilized as donors in homogeneous FRET-based assays with small organic fluorophores as acceptors. A competitive assay for estradiol with a analytical sensitivity in the picomolar range has been published and this assay is

20 times more sensitive compared to an assay utilizing water-soluble chelates (Kokko *et al*., 2008, 2004). Also larger fluorescent microparticles can be employed as acceptors, *e.g.*, in non-competitive sandwich-type assays for PSAwith the detection limit in the low picomolar range. In the assay, however, FRET was not the only mechanism to excite the acceptor but proximity-based reabsorptive energy transfer significantly contributed to the overall signal. Moreover, an assay based on double FRET has been developed for the measurement of enzymatic activity. Peptide substrates containing an acceptor and quencher were attached to the surface of the nanoparticles. When the enzyme activity was inhibited, no acceptor signal was detected because the energy from the europium excitation was further transferred from the acceptor to the quencher molecule. When the enzyme was active, it cleaved the peptide substrate and the moiety containing the quencher was released enabling fluorescence of the acceptor (Valanne *et al*., 2008).

Eu^{3+} nanoparticles have also been utilized in lateral flow immunochromatographic assays (LFIA) together with a measurement device, and they have provided a analytical sensitivity that is 7-300 times improved compared to colloidal gold depending on the assay and binders used (Juntunen *et al*., 2012; Xia *et al*., 2009). Because of the large Stokes shift inherent to lanthanides, the quantification of the results can be performed with a simple instrumentation employing only an excitation source, such as LED, and a camera or photodiode for collecting the emission. Time-resolved measurement is impractical and unnecessary because the non-specific binding of the label is the most significant source of the background signal (Juntunen *et al*., 2012). A quantitative LFIA assay for leucocyte counts based on the measurement of their surface proteins has been reported. In the study, a small-sized reader was used for the quantification of the signal intensities. The obtained sensitivities were in the low nanomolar range, and the linear range was over two orders of magnitude (Rundström *et al*., 2007). However, the LFIAs for hepatitis B surface antigen and PSA were significantly more sensitive demonstrating picomolar sensitivities (Juntunen *et al*., 2012; Xia *et al*., 2009). Microchips and lab-on-a-chip applications are more sophisticated and complex alternatives for point-of-care diagnostics compared to simple lateral flow assays. Recently, a microchip platform for HIV diagnostics was demonstrated using HIV-1 p24 antigen as an analyte and Eu^{3+} nanoparticles as labels. A sub-picomolar sensitivity was obtained with this platform **(**Liu *et al*., 2014). However, a lot of manual work was required to perform the assay, making it inconvenient and prone for errors. In order to be applicable to point-of-care diagnostics in resource-limited settings, the tests need to be very simple and inexpensive so that even a non-technician is able to perform them. Although the use of a reader for measurement of luminescence of lanthanide labels increases the costs of an assay, it also enables quantitative and sensitive detection. Examples of simple photoluminescence readers, which can be incorporated even to smart phones, already exist indicating the applicability of lanthanide-based nanoparticles also for point-of-care diagnostics (Faulstich *et al*., 2009, Wei *et al*., 2013).

2.4.3 Inorganic lanthanide-doped nanoparticles

Inorganic lanthanide-doped nanoparticles are crystals composed of inorganic host lattice embedded with lanthanide ions (Soukka *et al*., 2005). Materials such as Y_2O_2S (Beverloo *et al.,* 1990), $CePO_4$ (Kömpe *et al.*, 2003) and $NaGdF_4$ (Chen *et al.*, 2012) have been utilized as host lattice. The photostability of inorganic lanthanide-doped nanoparticles is significantly higher than that of organic fluorophores and lanthanide chelates. Compared to lanthanide chelates and cryptates, the lanthanide ions are also more effectively shielded from the quenching effects of water and thus their quantum yield is higher. Due to their high photostability, inorganic lanthanide-doped nanoparticles have been utilized especially in various bioimaging applications both *in vitro* and *in vivo*. Applicability of these particles has been demonstrated for immunocytochemistry and microscopy. In the measurements, time-resolved measurement can be utilized for elimination of autofluorescence background. (Beverloo *et al.*, 1990; Chen *et al*., 2012). Furthermore, photoluminescence employing near-infrared wavelengths both for excitation and emission has enabled imaging in a window where biological substances are optically transparent (Chen *et al.*, 2012). Applicability for bioaffinity assays has also been demonstrated with proteins, nucleic acids and small molecules and analytical sensitivity of 0.5 ng/mL was obtained in competitive immunoassay for atrazine (Feng *et al*., 2003; Beverloo 1992).

Upconverting nanoparticles (UCNPs) are inorganic lanthanide-doped nanoparticles that are capable of converting infrared radiation to emission at visible wavelengths due to sequential absorption of two or more photons (Fig 6a). They have raised wide interest as attractive labels in bioanalytical assays and bioimaging because autofluorescence and scattered excitation radiation have minimal effect on upconversion luminescence. Autofluorescence occurs at higher wavelengths than the excitation. Thus, it commonly overlaps with the emission wavelengths of luminescent labels and interferes with the measurement. In the case of upconversion process however, the emission is observed in the visible range of the spectrum, which is at lower wavelengths compared to the excitation. Thus autofluorescence is inherently avoided and time-resolved is not required as opposed to lanthanide chelates, cryptates and other inorganic lanthanide-doped nanoparticles. (Anderson *et al*., 2014; Soukka *et al*., 2005; Auzel, 2004.) The excellent photostability allows continuous excitation and additionally, inexpensive laser diodes and LEDs are efficient enough to excite UCNPs. Due to these features the required instrumentation is relatively simple and inexpensive. Furthermore, UCNPs are non-toxic and they do not blink contrary to quantum dots. (Haase and Schäfer 2011.)

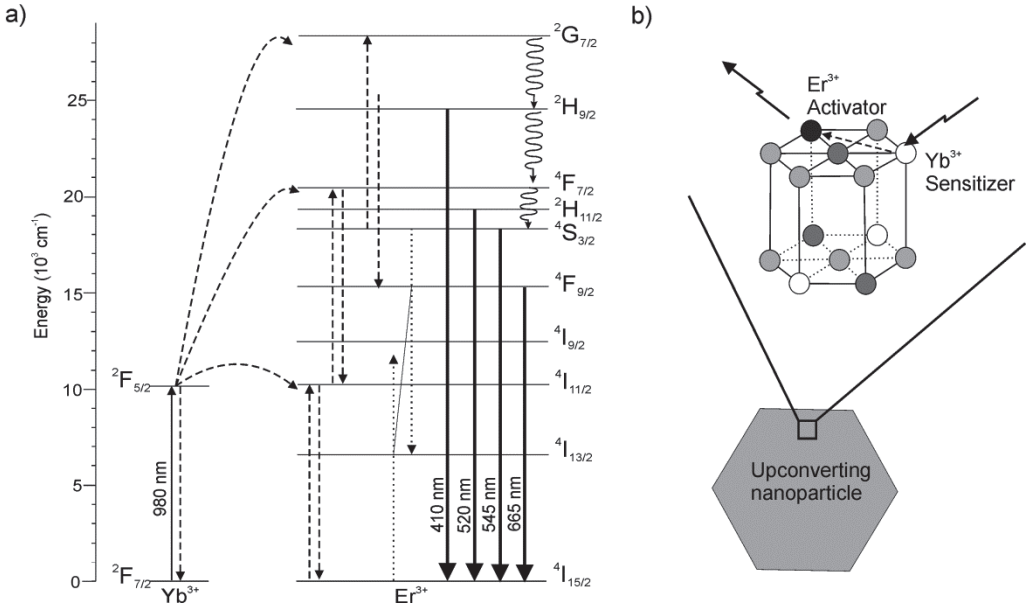


Figure 6. a) Simplified energy-level diagram of energy transfer upconversion processes. Only emissions in the visible range of the spectrum are presented. Full, dashed, dotted and curly arrows represent radiative energy transfer, non-radiative energy transfer, cross-relaxation, and multiphonon relaxation, respectively. (Modified from Anderson *et al.*, 2014; Gorris and Wolfbeis, 2013.) b) Schematic figure of Yb^{3+},Er^{3+}-doped upconverting nanoparticle. Yb^{3+} acts as a sensitizer that absorbs IR radiation and excites Er^{3+} acting as an activator and emitting at visible and near-infrared light.

The currently preferred synthesis method for UCNPs is solvothermal synthesis in the presence of oleic acid and octadecene due to the high yields and the simplicity of the method, (Wang et al., 2010). Hexagonal $NaYF_4$ has been the most commonly used host material as it produces the highest upconversion signals and is 10 times brighter than cubic phase (Krämer *et al.*, 2004). Yb^{3+} is most often used as a sensitizer ion transferring its energy to activator ion thus exciting it. The activator ion emits a photon when returning to the ground state and the commonly used activator ions are Er^{3+}, $Tm^{3+,}$ and Ho^{3+} because of their compatible energy levels (Fig. 6) (Gorris and Wolfbeis, 2013).

After the synthesis, further surface modifications are required to make the particles dispersible in aqueous solution and utilizable in bioanalytical applications. Also functional groups, typically NH_2 or COOH, need to be introduced to the particle surface to allow the covalent conjugation of biomolecules. The surface modifications can be done via ligand exchange when the UCNPs are synthesized in the presence of oleic acid (Doughan *et al.*, 2014; Chen *et al.*, 2013; Liu *et al.*, 2013; Boyer *et al.*, 2010; Chen *et al.*, 2008). However, the stability of the ligand exchange may be compromised as the oleic acid is only adsorbed to the particle surface. Polyethyleneimine can also be used for surface modifications but oleic acid seems to prevent the quenching of the luminescence more efficiently (Liu *et al.*, 2013). Silica-coating is a common method to generate water-dispersible UCNPs and it is often obtained using either Stöber method (Stöber, 1968), or

microemulsion, which allows the formation of a thinner silica layer (Wilhelm *et al*., 2013; Abdul Jalil *et al*., 2008). Functional groups are introduced by using a small portion of amino- or carboxysilanes in the reaction (Fig 6a). A silica-coating is stable and inert. Additionally, it shields the UCNPs against the possible environmental quenching effects and thus retains their luminescent properties (Bogdan *et al*., 2011; Saleh *et al*., 2011; Achatz *et al*., 2011). Another alternative for surface modifications is absorbing polyacrylic acids on the particle surface (Wang Y *et al*., 2011; Kuningas *et al*., 2006). However, the coating is susceptible to detaching making the UCNPs prone to compromised binding capacity and stability. This may affect the reproducibility of the assays. Biomolecules are commonly covalently attached to the modified UCNP surface via EDC and sulfo-NHS chemistry (Liu Y *et al*. 2013; Zhang *et al*., 2011; Chen Z *et al*., 2008; Kuningas *et al*., 2006) or by click-chemistry (Mader at al., 2010).

The inherent drawbacks of UCNPs are the non-linear dependency on excitation power, low quantum yield (0.005-3 %), and possible quenching effects by water molecules. Quenching is even more prominent with small particles due to increased surface area (Boyer and van Veggel, 2010). However, numerous studies have been published in the field of tuning the luminescence properties. Core-shell structure, doping of cations, such as K^+, Mn^+, and Gd^{3+}, Nd^{3+}, to the host material or production of hybrid nanomaterials with gold have been reported to increase the luminescence or modify the excitation wavelengths and the red-to-green ratio of UCNP emission (Wang and Liu, 2014; Wen *et al*., 2013; Xie *et al*., 2013; Li *et al*. 2011; Paudel *et al*. 2011;Wang *et al*. 2011; Boyer and van Veggel 2010). Also new host materials for enhanced quantum yield and photon upconversion luminescence have been proposed (Huang *et al*., 2014). The potential of photon upconverting particles in heterogeneous bioanalytical assays has been widely demonstrated with different analytes ranging from small molecules and proteins to bacteria and nucleic acids (Zhou *et al*., 2014; Ukonaho *et al*., 2007; Niedbala 2001). The applicability of photon upconverting particles to FRET-based homogeneous assays has also been established (Wang *et al*., 2005, Kuningas *et al*., 2006): in these assays, larger submicrometer-sized particles were used. Upconverting particles have also been shown to enable assay sensitivities comparable to those achieved with Eu^{3+} nanoparticles. However, the labels used in the study by Ukonaho *et. al.* (2007) were irregular in shape and had a wide size-distribution in the sub-micron range. These features caused significant non-specific binding of the label molecules to the assay well and resulted in compromised sensitivity. Moreover, upconversion luminescence has been demonstrated to enable homogeneous assays using whole blood as sample matrix. This can be achieved because blood has minimal absorption in the wavelengths exploited in upconversion luminescence technology (Kuningas *et al*., 2007). The unique ability to measure analytes from optically challenging samples makes UCNPs effective for point-of-care testing as it enables simple assays and instrumentation. Furthermore, the applicability of upconversion particles to LFIAs and paper-based assays has been widely demonstrated with different analytes (Zhou *et al*., 2014; Corstjens *et al*., 2011, 2008, 2007; Hampl *et al*., 2001), and the inexpensive read-out systems have enabled sensitive and quantitative pathogen detection (Chen *et al*., 2013; Corstjens *et al*., 2013; van Dam *et al*.2013; Liu *et al*. 2009).

In the early applications, submicron-sized upconverting particles were used because the controlled synthesis of nanosized particles was not well established. Today, monodispersed, uniform nanoparticles with narrow size distribution are routinely synthesized and used in applications. The recent applications have focused more on imaging, whereas bioanalytical applications for protein detection are relatively rare. The obtained analytical sensitivities are commonly in the nanomolar range and ultra-low sensitivies have not been achieved despite the great advantages of the label technology. This highlights the importance of assay design, surface chemistry of the UCNPs, and the impact of instrumentation in enabling sensitive assays.

In addition to immunoassays and imaging applications, multiplexing applications based on planar array-type applications have been published for DNA hybridization assays (Ylihärsilä *et al*., 2013, 2011; van de Rijke *et al.,* 2001). The potential of UCNPs for multiplex assays relying on spectral differentiation and barcoding has also been demonstrated (Zhang *et al*., 2104; Gorris *et al*., 2011; Zhang *et al*., 2011) but their applications in bioanalytical assays are still scarce. The unique properties of UCNPs offer entirely new applications and enable effective point-of-care testing. For example, UCNPs embedded in a poly(acrylamide) film were used as nanolamps to excite fluorescein-labeled glucose oxidase, the fluorescence properties of which change due to an enzymatic reaction with glucose. This could eventually lead to the development of a sensor for the continuous monitoring of glucose and other biomarkers by using an implanted sensor. (del Barrio *et al*., 2014.)

2.4.4 Future trends

Lanthanide-binding peptides are a potential alternative to lanthanide chelates and complexes. The peptides are small in size, composed of fewer than twenty amino acids, and they have been developed from calcium-binding proteins (MacManus, 1990). By using recombinant fusion proteins containing lanthanide-binding peptide, it is possible to avoid the chelate synthesis and bioconjugation steps. The applicability of lanthanide-binding peptide to FRET-assays has been demonstrated and, on the basis of that, a protease assay has been developed (Vuojola *et al*., 2013; Arslanbaeva *et al*., 2010).

Molecular upconversion provides an option for UCNPs. The possibility of molecular upconversion has already been demonstrated, although the measurements were performed in an anhydrous solvent and the phenomenon has not yet been applied to analyte detection. The structure enabling molecular upconversion resembled typical lanthanide chelates in a sense that it consisted of an Er^{3+} chelate and a light-harvesting antenna capable of efficient excitation at 808 nm. Molecular upconversion is highly promising as it avoids the limitations of particulate labels, such as problems with reproducible synthesis, colloidal stability, high non-specific binding of the label, and steric hindrances. Furthermore, the conjugation of the molecular label to the affinity binder is easier. Most importantly, the molar absorptivity of molecular dye-sensitized upconversion is significantly higher compared to that of the UCNPs (Hyppänen *et al*., 2014).

3 AIMS OF THE STUDY

The overall objective of the study was to assess the impact of assay components on the performance of affinity-based protein detection assays. The effect of reporter technology, affinity binders, and their conjugation chemistry were evaluated in both homogeneous and heterogeneous assays. The characteristics of the developed assays were analyzed with the objective of meeting the demands for simple and rapid yet sensitive point-of-care testing. More specifically, the aims were:

1. To demonstrate the multiplexing capability of UCNPs for quantitative protein detection using imaging approach. For this purpose, a heterogeneous two-site immunometric assay for three model analytes, i.e. thyroid stimulating hormone, prostate specific antigen and luteinizing hormone, was developed.

2. To study the applicability of switchable lanthanide luminescence to protein detection. Three homogeneous assays using thrombin, cardiac troponin I, and prostate specific antigen as model analytes were developed to demonstrate the wide applicability of the reporter technology.

3. To study alternative binders and bioconjugation methods of affinity-binders and labels especially in homogeneous assays. Antibodies, antibody fragments and aptamers were used as binders. Furthermore, the binders were conjugated to oligonucleotides or label moieties using either chemical methods or HaloTag technology. The used conjugation chemistrues included the conjugation of amino groups to isothiocyanates, thiols to maleimides and amino to carboxyl groups via EDC and sulfo-NHS. In addition, streptavidin-biotin interaction was used for immobilization of binders to solid phase.

4. To assess the applicability of different assay concepts to point-of-care testing. In publication I, existing technologies were evaluated in respect to the ASSURED criteria developed by World Health Organization. In original publications, the the main objective was to develop assays that would contribute in resolving the challenges related to point-of-care tests, particularly those concerning sensitive detection, ease-of-use and affordable detection instrumentation.

4 SUMMARY OF MATERIALS AND METHODS

4.1 Assay designs

4.1.1 Quantitative multianalyte microarray immunoassay

In order to study the applicability of UCNPs for quantitative multianalyte protein detection, a heterogeneous multianalyte assay was developed. The assay consisted of four two-site immunometric assays that were carried out simultaneously in a microtiter well. A single label was required for the detection because the analytes were identified by their location in the array. However, separate coating batches of the UCNP were required in order to allow specific recognition of each analyte. Luteinizing hormone (LH), thyroid stimulating hormone (TSH), and prostate specific antigen (PSA) were used as model analytes while the fourth spot in the array containing polyclonal rabbit anti-mouse antibody acted as a positive control (Fig. 7). The assay results were measured using image-based detection.

For the assay, biotinylated capture antibodies for each analyte were printed in a four-spot array in white streptavidin-coated 96-well microtiter wells that were contact printed at the VTT Medical Biotechnology Centre using their proprietary technology. White wells were used to enhance the obtained UCNP signal intensities (Kuningas *et al*., 2005). The standard dilutions (0-200 mU/L, 0-60 ng/mL and 0-60 U/L for TSH, PSA and LH, respectively) were pipetted into wells in 10 µL together with 30 µL buffer containing antibody-coated UCNPs that were able to recognize either TSH or PSA. The anti-LH antibody-coated UCNPs (10 µL) were pipetted after a 10-min incubation because the tracer antibody for LH recognizes the alpha chain of the LH molecule and an identical alpha chain is also present in TSH. Sequential addition was necessary to avoid the binding of anti-LH-coated UCNPs also to the TSH spots. The concentration of each set of UCNPs was 20 µg/mL in reaction. Total reaction volume was 50 µL. After 30-min incubation, the wells were washed to remove the unbound reporters, and the arrays were imaged for analyte quantification. The performance of the multianalyte assay was compared to both single analyte assays performed in the array-wells and single analyte assays performed in regular microtiter wells. The multianalyte assay and the reference assays performed in regular microtiter wells were measured both using the Anti-Stokes photoluminescence imager and the plate reader while the single analyte assays performed in the array-well were measured only using the imager.

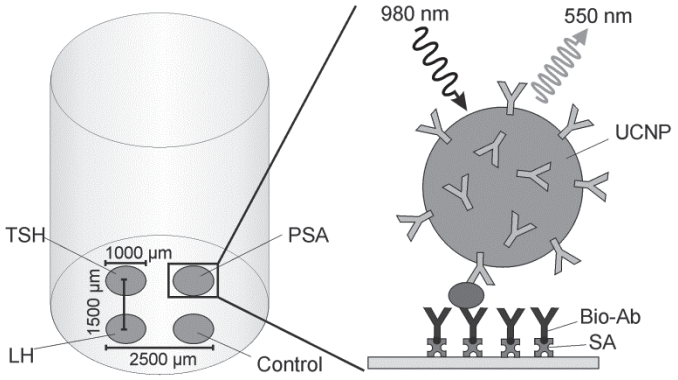


Figure 7. Principle of the quantitative multianalyte protein assay.

4.1.2 Homogeneous protein detection assays

Switchable lanthanide luminescence was utilized for protein detection in homogeneous assays in publications III-V. Bearing in mind the demands for point-of-care tests, the assays were designed to be simple to perform. Thrombin, PSA and cardiac troponin I (cTnI), were utilized as model analytes to demonstrate the wide applicability of the reporter technology. In every assay, two separate binders were employed, and the formation of mixed luminescent lanthanide chelate was assisted by reporter oligonucleotides. They were attached to the binders and had partly complementary sequences with each other. The recognition of an analyte by the binders brought the reporter oligonucleotides in close proximity enabling the hybridization of the complementary reporter stem, which subsequently triggered the complementation of the label moieties into a mixed luminescent chelate complex (Fig 8).

The length of the complementary region forming the reporter stem was optimized in publications III and IV. In publication III, aptamers with oligonucleotide extensions were used, whereas Fabs and a second pair of assisting linker oligonucleotides were exploited in publications IV and V. These linker oligonucleotides connected the binders to the reporter oligonucleotides containing the label moieties. In publication IV, the Fabs were conjugated site-specifically to the linker oligonucleotides, which further hybridized to the reporter oligonucleotides. In publication V, the Fabs were coupled to linker oligonucleotides using HaloTag technology enabling controlled and stoichiometric conjugation. The assays were straightforward mix-and-measure assays, where the reactants were pipetted into low fluorescence background microtiter wells and incubated for a few minutes before measurement. The concentrations of labeled binders were optimized for each assay. Furthermore, the kinetics of the assays was measured to define the optimal incubation times. The reaction volume in the assays in publications III-V was 60 µL. Other assay details are presented in Table 5.

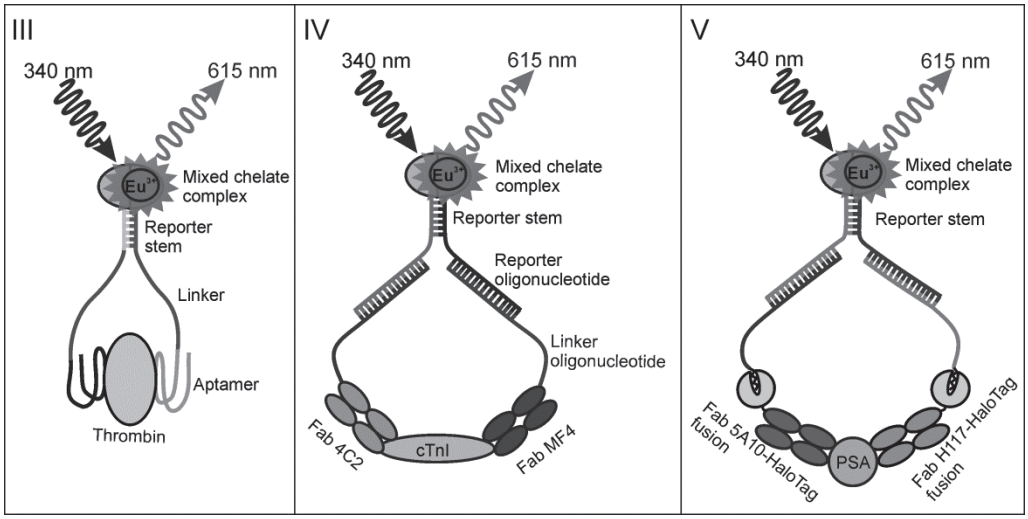


Figure 8. Principles of homogeneous assays. In all the assays (III-V), biomolecular recognition of an analyte results in the formation of the reporter stem, which brings the label moieties into close proximity thus assisting in the self-assembly of the mixed chelate complex.

Table 5. Assay characteristics in publications III-V.

Publication	III	IV	V
Analyte	Thrombin	cTnI	PSA
Binders	Aptamers	Fabs	Fabs
Concentration of labeled binder (nM)	5 nM	2.5 nM	2.5 nM
Incubation time (min)	5	6	30
Incubation and measurement temperature (°C)	RT	36	36

Furthermore, a heterogeneous assay for PSA was demonstrated using the labeled binders developed for publication V (unpublished data). In the assay, SA-coated wells were pre-washed and 150 ng of biotinylated Mab H50 (60 μL) (Piironen *et al*., 1998, Qin *et al*., 1997) was pipetted into the microtiter wells. The wells were incubated for 1 h at room temperature in slow shaking after which they were washed twice. PSA-standards (10 concentrations in the range of 0-100 ng/mL) were pipetted into the wells in three replicates except six replicates for the blank sample. The wells were incubated for 45 min at 36 °C in 900 rpm shaking and then washed twice. The linker oligonucleotide-Fab conjugates and reporter oligonucleotides forming the labeled binders were all pipetted into the wells in a total volume of 60 μL. The concentration of the components was 10 nM each. The wells were incubated for 1 h at 36 °C in 900 rpm shaking and then washed twice before the measurement of time-resolved luminescence.

Possible interference of sample components to the formation of the mixed chelate complex was studied in a hybridization test using complementary 20-nt long oligonucleotides. One oligonucleotide was labeled with ion carrier chelate and the other one with light-harvesting antenna ligand. The oligonucleotides were mixed to

TSA buffer, pH 7.75, containing 30 μM diethylene triamine pentaacetic acid to final concentration of 10 nM each. After a 5-min incubation at room temperature, the time-resolved luminescence from the formed mixed chelate complex was measured. Furthermore, the effects of BSA and yeast extract on switchable lanthanide luminescence were studied with the thrombin assay. BSA or yeast extract were added to the reaction to a final concentration of 0.5 mg/mL, which corresponds the protein concentration in 1/100-diluted plasma. Otherwise, the test was carried out similarly as described above.

4.2 Binders

A 15-mer thrombin binding aptamer 1 (TBA1) recognizing the fibrinogen binding exosite and a 29-mer TBA2 recognizing heparin-binding exosite were used as binders in publication III. The dissociation constants for TBA1 and TBA2 were 30 nM and 0.5 nM, respectively. (Tasset *et al*., 1997; Bock *et al*., 1992.) Additional 3-8 nucleotides and a terminal amino modification were inserted to the 3' end of TBA1 and 5' end of TBA2 to enable hybridization with the complementary sequence in the other aptamer and assist in signal generation. Additionally, linkers consisting of five hexaethylene glygol units or 18 nt long poly-dT-sequence were evaluated as linkers connecting the aptamers and the complementary sequences (Table 6). The oligonucleotides were ordered from Biomers.net (Germany).

Table 6. Sequences of the thrombin binding aptamers including complementary sequences of different lengths.

TBA1		
Aptamer sequence	**Linker**	**Complementary sequence**
5'-GGT TGG TGT GGT TGG	TTT TTT TTT TTT TTT TTT	CGC T*-3'
5'-GGT TGG TGT GGT TGG	TTT TTT TTT TTT TTT TTT	CGC A T*-3'
5'-GGT TGG TGT GGT TGG	TTT TTT TTT TTT TTT TTT	CGC AT T*-3'
5'-GGT TGG TGT GGT TGG	TTT TTT TTT TTT TTT TTT	CGC ATC T*-3'
5'-GGT TGG TGT GGT TGG	TTT TTT TTT TTT TTT TTT	CGC ATC GT*-3'
5'-GGT TGG TGT GGT TGG	TTT TTT TTT TTT TTT TTT	CGC ATC GGT*-3'
5'-GGT TGG TGT GGT TGG	-(H(OCH$_2$CH$_2$)$_6$OH)$_5$-	CGC ATC T*-3'
TBA2		
Complementary sequence	**Linker**	**Aptamer sequence**
5'-*GCG	TTT TTT TTT TTT TTT TTT	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'
5'-*T GCG	TTT TTT TTT TTT TTT TTT	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'
5'-*AT GCG	TTT TTT TTT TTT TTT TTT	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'
5'-*GAT GCG	TTT TTT TTT TTT TTT TTT	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'
5'-*C GAT GCG	TTT TTT TTT TTT TTT TTT	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'
5'-*CC GAT GCG	TTT TTT TTT TTT TTT TTT	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'
5'-*GAT GCG	-(H(OCH$_2$CH$_2$)$_6$OH)$_5$-	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'

* - 5'/3' –terminal aminomodification with six carbon aliphatic spacer.

In publications II-V, antibodies and antibody fragments were used as binders. These are presented in Table 7. Fab fragment HaloTag fusion proteins were produced for the publication V. Antibody binders used in publications II-IV were obtained ready for labeling and thus their production is not included in this thesis.

Table 7. Antibodies used in the study.

Antibody	Specificity	Publication	Source	Reference*
5404 Fab	TSH	II	University of Turku	Ylikotila *et al.*, 2005
5409 Mab	TSH	II	Medix Biochemica, Finland	
8D10 Mab	LH	II	Wallac/PerkinElmer, USA	
H117 Mab	PSA	II	Abbott, USA	Eerola *et al.*, 1997; Piironen *et al*. 1998
H50 Mab	PSA	II, V	Abbott, USA	Eerola *et al.*, 1997; Piironen *et al*. 1998
M21241Mab	LH	II	Fitzgerald Industries International, USA	
RaM, polyclonal	Molecules of mouse origin	II	DakoCytomation, Denmark	
4C2 Fab	cTnI	IV	University of Turku	Ylikotila *et al.*, 2006
MF4 Fab	cTnI	IV	University of Turku	Ylikotila *et al.*, 2006
5A10 Fab	PSA	V	University of Turku	Eriksson *et al.*, 2000
H117 Fab	PSA	V	University of Turku	Eriksson *et al.*, 2000

*References describing the details of the antibody production or characterization in the University of Turku.

Fusion proteins containing the HaloTag protein and either Fab5A10 or FabH117 were produced for publication V. Standard molecular biology techniques were used for preparing the constructs for overexpressing the fusion proteins in *E. coli*. The genes encoding the Fabs were cloned to separate vectors of pAK600-series, and HaloTag protein gene and the hexahistidine tag were cloned from the commercial His_6HaloTag T7 vector and inserted downstream from the Fab gene so that the HaloTag protein was translated together with the heavy chain of the Fab while the light chain was translated separately. The complete fusion protein was folded from the polypeptides after they were transported to periplasmic space.

Plasmid expression vectors were transformed into *E. coli* BL21 cells by electroporation. The *E. coli* B21 strains containing the plasmid were inoculated to 25 mL pre-cultures. LB medium containing 0.2% glucose and 25 µg/mL chloramphenicol was used for the pre-cultivation of the *E. coli* cells, and the growth was monitored spectrophotometrically. The LB medium used for the main cultures contained 0.05% glucose and 25 µg/mL chloramphenicol. Isopropyl β-D-1-thiogalactopyranoside (100 µM) was used for the induction of fusion protein overexpression, and then the cultures were transferred to 26 °C for 15 h. To stop the cell growth, the cultures were transferred on ice for 10 min, after which the cells were collected by centrifugation at 7000 g for 15 min at 4 °C. In order to release the periplasmic fusion protein, the cell pellet was suspended to 100 mL lysis buffer containing 0.2 mg/mL lysozyme, 1:1000 diluted EDTA-free protease inhibitor cocktail set III (Merck), 750 units bentsonase, and 1 mM $MgCl_2$. The cells were sonicated on ice for 3 min and then centrifuged. Finally the supernatant was filtered through 0.45 µm filter membrane and purified using Ni-NTA matrix (Korpimäki *et al*., 2004).

4.3 Preparation of label conjugates

4.3.1 Antibody-coated upconverting nanoparticles

Single-crystal $NaYF_4$:Yb^{3+}, Er^{3+} upconverting nanoparticles with crystallite size 25-28 nm were used as binders in publication II. The synthesis was based on the method using oleic acid (Wang *et al*., 2010). The UCNPs were prepared by adding methanol solutions of rare earth metal chlorides to a flask containing 9 mL oleic acid and 21 mL octadecene. The total concentration of rare earth metals was 0.2 M and the ratios of Yb^{3+} and Er^{3+} were 17 mol% and 3 mol%, respectively. The solution was stirred and heated to 160°C. After a 30-min heating, the solution was let cool down to room temperature before the addition of 14 mL methanol solution containing 0.18 g NH_4F and 0.12 g NaOH. The solution was stirred for 30 min at room temperature, then heated to 300°C and maintained there for 3 hours before cooling down to room temperature. The formed particles were collected from the solution by the addition of ethanol and subsequent centrifugation.

After the synthesis, UCNPs were silanized in toluene to provide functional groups for the covalent conjugation of biomolecules and to protect the UCNPs from environmental effects (Achatz *et al*., 2011; Bogdan *et al*., 2011). Briefly, 16 mg of UCNPs in cyclohexane were suspended in 250 µL of toluene, and cyclohexane was evaporated from the suspension by heating at 80 ˚C. Next, 10 mL of toluene, 25 µL tetra-methyl-orthosilicate, and 50 µL of (N-(3-trimethoxysilyl)propyl)ethylene diamine were added to the suspension, and the reactants were mixed by bath sonication and vortexing and incubated at 70 ˚C for 40 min. The silanized particles were collected by centrifuging after which they were washed once both with toluene and ethanol.

UCNPs were collected from the aqueous solution by centrifuging (17000 g, 30 min) and suspended in 400 µL of pyridine. Glutaric anhydride corresponding to a ten-fold excess compared to the mass of UCNPs was dissolved in 320 µL of pyridine and immediately added to the UCNP suspension, which was then bath-sonicated for 5 min. Incubation was continued for 5 h at 50°C and another batch of glutaric anhydride in pyridine was added in the halfway of the incubation. Ethanol (500 µL) was added to reaction to enable proper pellet formation during centrifugation (10000 g, 7 min). The UCNPs were washed twice with ethanol and deionized water to remove pyridine and excess glutaric acid. Finally, the carboxylated UCNPs were suspended in deionized water.

For each analyte, separate UCNP batches were conjugated with corresponding antibodies. The carboxyl groups on the surfaces of the UCNPs (2 mg for each conjugation) were activated using 20 mM EDC and 30 mM sulfo-NHS in a total reaction volume of 260 µL. The reactants were incubated for 45 min at room temperature, after which the particles were washed once and suspended in a 20 mM 4-morpholineethanesulfonic acid buffer, pH 6.1. Mabs (1mg/mL) were added to the activated UCNP suspension resulting in a total reaction volume of 250 µL. The reactants were incubated for 2.5 h in slow rotation, after which the reaction was

stopped by adding glycine (pH 11) to a final concentration of 50 mM. The reaction tube was incubated for 30 min at room temperature in slow rotation. The Mab-coated UCNPs (Fig. 9) were washed once and finally suspended in a storage buffer containing 5 mM borate, pH 8.5, 0.05% Tween 20, 0.5% BSA, and 0.05% sodium azide.

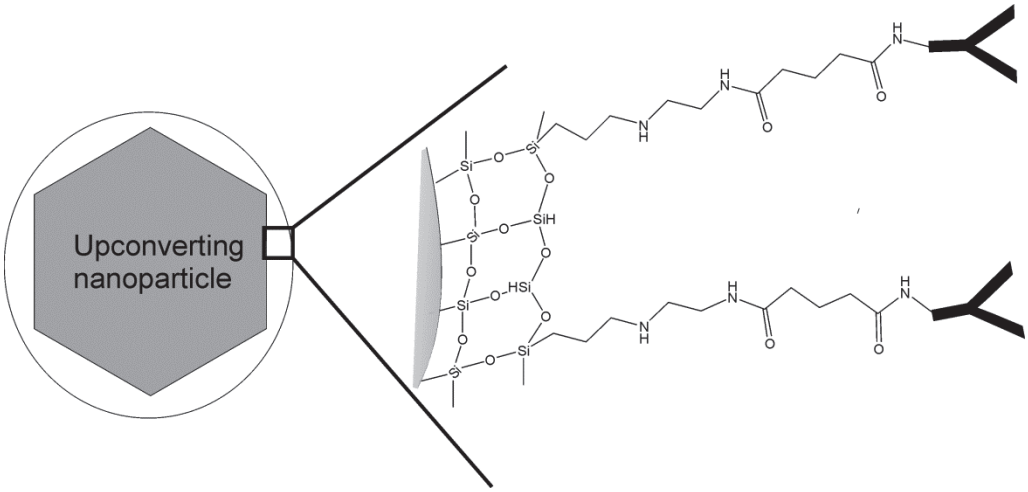


Figure 9. Mab-coated UCNP. UCNP is coated with a thin silica layer, and the monoclonal antibodies are covalently attached to the silica layer.

4.3.2 Switchable lanthanide luminescence bioconjugates

Isothiocyanate-activated lanthanide ion carrier chelate and light-harvesting antenna ligands (Fig. 10) were covalently coupled to the terminal amino modifications of aptamer oligonucleotides in publication III and reporter oligonucleotides in publications IV and V. In brief, the procedure was as follows: The oligonucleotides were dissolved in 50 mM carbonate buffer, pH 9.8, and a label moiety was added to the reaction. The concentration of oligonucleotides was 200 µM in reaction and for europium(III) carrier chelates, a 20-fold molar excess was used, whereas for antenna labelings, a 100-fold molar excess of label moiety and 63 mM carbonate buffer, pH 9.8, were employed. The reactants were incubated overnight at 37 ºC with slow shaking. The crude excess of the unconjugated label moieties was removed in a NAP 5 column before the labeled conjugates were purified using reverse phase HPLC.

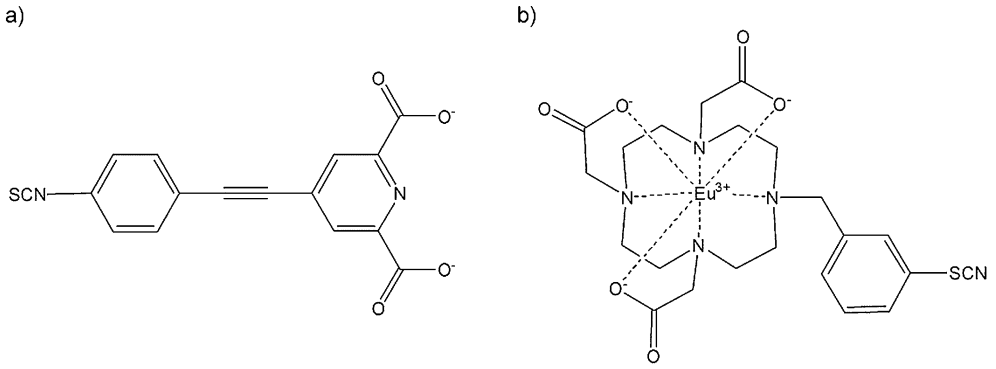


Figure 10. a) Light-harvesting antenna ligand. b) Europium ion carrier chelate.

For publications IV and V, linker oligonucleotides were used to connect the reporter oligonucleotides to the Fabs. In publication IV, the linker oligonucleotides were site-specifically coupled via the terminal maleimide modification to Fabs, which were genetically engineered to contain a cysteine residue at the carboxyterminal end of the constant region. In the conjugation reaction, 50 µM linker oligonucleotide and Fab were conjugated in 20 mM phosphate buffer 20 mM, pH 7.4, at room temperature for 1.5 h and then transferred to 4 °C for incubation overnight. The Fab-linker oligonucleotide conjugates were purified using gel filtration and 20 mM Tris buffer, pH 7.5, 300 mM NaCl, for both equilibration and elution. For publication V, the linker oligonucleotide was coupled to Fab using HaloTag technology. The HaloTag succinimidyl ester ligand was conjugated to the terminal amino modifications of the two linker oligonucleotides. The concentration of linker oligonucleotides was 400 µM, and a 20-fold molar excess of HaloTag ligand was used in the conjugation reaction performed overnight in 100 mM sodium tetraborate, pH 8.5, at room temperature. The reaction was stopped by adding 500 mM Tris-HCl, pH 7.2, to a final concentration of 100 mM in reaction. The excess of HaloTag ligand was removed from the solution using a NAP 5 column after which the conjugate was purified using reverse phase HPLC and linear gradient. The oligonucleotide concentrations of the fractions were determined by measuring the absorbance at 260 nm and calculating the concentration based on the assumption that the optical density at wavelength 260 nm equals one for a 33 µg/mL solution of single-stranded DNA.

A linker oligonucleotide containing a terminal HaloTag ligand was conjugated to a Fab-HaloTag fusion protein while the fusion protein was captured in protein G matrix. The fusion protein was loaded to a HiTrap protein G HP column. The column was washed for 10 min to get rid of all misfolded fusion protein and free HaloTag protein. Then, 30 nmol (20-fold excess) of the linker oligonucleotide featuring HaloTag ligand was loaded to the column using a syringe. The column was incubated for 2 h at room temperature to allow the oligonucleotide conjugate to Fab via HaloTag technology. Thereafter, the column was washed before the oligonucleotide-protein conjugate was eluted with 0.1 M glycine-HCl, pH 2.8. From the elution, 1.5 mL fractions were collected to tubes containing 90 µL 1 M Tris-buffer, pH 9.0, to neutralize the elution buffer.

4.4 Instrumentation

In publication II, an in-house developed desktop prototype of a charge-coupled device (CCD) sensor-based imager was used for measuring the anti-Stokes photoluminescence signals of the protein microarrays. The instrument was built on a Plate Chameleon microplate reader (Hidex, Turku, Finland), and a fiber-coupled 976 nm laser diode was used for excitation. The laser beam was collimated using a lens and then directed through a 850 nm longpass filter to a hot mirror, which reflected the excitation beam to the microtiter well. The green emission light of UCNPs was collected using a 650 nm shortpass filter and two 50 mm lenses, and the image was captured on a CCD-camera intended for low light imaging. The access of scattered excitation irradiation to CCD-sensor was blocked by using two shortpass filters. A laser power of 7W, an exposure time of 1 s, and 2x binning were used for the measurements. ImageJ software version 1.43n was employed for analyzing the images (http://rsbweb.nih.gov/ij/index.html) (Ylihärsilä 2011).

The arrays were also measured with a modified Plate Chameleon microplate reader, which was used as a reference. The plate reader was equipped with 980 nm laser excitation and 535 nm emission bandpass filter (Soukka *et al*., 2005). The arrays were scanned using a 7 x 7 raster, a 0.8 mm distance between the measurement points, and 2 s reading time. In the raster, four measurement points were selected to represent each analyte spot. The single analyte reference assays were measured with the modified Plate Chameleon plate reader in addition to the measurement with the imager. In order to get a representative signal from the entire well bottoms, a 3 x 3 raster with 1 mm spacing between the measurement points was used and the average from the nine measurement points was calculated. Furthermore, the single analyte whole well reference assays were also imaged with the microwell imager using the same specifications as for the measurement of arrays.

For publication III, a Victor 1420 Multilabel Counter was used for the measurement of time-resolved luminescence signal. The measurements were performed at room temperature using a 400 µs delay time and 400 µs window time. For publications IV and V, and for the heterogeneous PSA assay, a Victor X4 Multilabel Plate Reader was utilized, and the measurements were done at 36 °C with a delay time of 250 µs and a window time of 750 µs. Both Victor multilabel readers were from Wallac/Perkin-Elmer Life and Analytical Sciences (Waltham, USA). For all measurements, factory-provided filters for 340 nm excitation and 615 nm emission were utilized.

5 SUMMARY OF RESULTS AND DISCUSSION

The ASSURED criteria developed by World Health Organization describe the desired characteristics of diagnostic tests intendeded for use in resource-poor settings. The acronym ASSURED stands for affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable (Peeling *et al*., 2006). Moreover, multiplexing capacity would be advantageous. In publication I, these requirements were compared to the characteristics of the current rapid immunoassay-based tests. The main limitation of the existing diagnostic tests aimed at point-of-care testing especially in low-resource settings was the lack of sensitivity and quantitative results or the high cost of the test due to the instrumentation required. Furthermore, achieving high sensitivity and low cost in a single test was proved to be extremely challenging. Aspects contributing to the development of affordable yet sensitive bioaffinity-based diagnostic tests were studied in the assays developed in this thesis. In particular, the possibility for quantitative multiplexed protein detection using affordable image-based detection was examined. In addition, the option of using homogeneous assay principle was studied. The compromised sensitivity of homogeneous assays compared to heterogeneous assays has impeded their use in point-of-care tests although the assays performance themselves are simple to perform and thus applicable for many point-of-care applications.

5.1 Bioconjugates

In publication II, the antibodies were contact printed in microtiter wells to form an array. SA-bio interaction was used in printing of the array in order to enhance the stability of immobilized antibodies (Välimaa *et al*., 2003). Additional advantage of using SA-bio interaction is the universal nature of the produced solid phase, which allows the easy modification of the array as other antibodies can easily be introduced in the array (Välimaa et al., 2003). UCNPs were used as labels in publication II. According to transmission electron microscopy, the crystals were nanosized (25-28 nm) and monodispersed before the silica-coating and the conjugation of biomolecules. Although UCNPs were feasible for bioaffinity assays, their drawback was the extensive surface modifications required. The silica-coating, carboxylation, and bioconjugation are time-consuming processes, which means that the particles are expensive and thus currently not compatible with point-of-care applications. A separate carboxylation step could be avoided if carboxy-silanes were used instead of aminosilanes. Also, the centrifugation is not a particularly efficient method for separating the coated UCNPs. The use of magnetic field could be an alternative method for collecting the particles in the various steps of producing biocompatible UCNPs (Arppe *et al*., 2013).

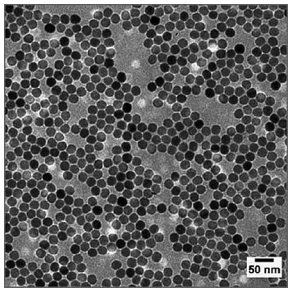


Figure 11. Transmission electron microscopy image of monodispersed UCNPs.

In publications III-V, the labeled binders were produced in a controlled manner to avoid any loss of activity of the binder and to ensure high reproducibility of the conjugates. The label moieties were conjugated to oligonucleotides using conventional conjugation reaction between amino and isothiocyanate groups. Reaction between maleimide and thiol groups was also employed in publication IV for conjugation of linker oligonucleotides to Fabs. These methods are well-established but required high excess of label moiety in the reaction.

In publications IV-V, additional linker oligonucleotides were used to connect the reporter oligonucleotides to an antibody fragments. Although this assay concept was effective, it could be further simplified by omitting the linker oligonucleotide with double-labeling of a single oligonucleotide. This would probably also facilitate the optimization of the assay because conditions for several oligonucleotide hybridizations would not have to be considered. However, double-labeling would require a careful optimization of the labeling protocol and possibly two purifications, which would decrease the product yield. Also, the selection of different functional groups commercially available in oligonucleotide synthesis is still relatively limited and, therefore, specific labeling is a demanding task. The use of aptamers in publication III simplified the production of the labeled binder because separate conjugation of a binder molecule to a linker oligonucleotide was not needed. Furthermore, the specific labeling of aptamers was easy as reactive groups could be added during the synthesis with moderate cost. The drawback of aptamers was that the number of available aptamer sequences is very limited. For switchable lanthanide chelate labeling, building blocks that could be added to the oligonucleotide during the chemical synthesis could enable the use of single oligonucleotides (Jaakkola *et al*., 2006). Furthermore, the label could be added site-specifically to the oligonucleotide during the synthesis. This would further simplify the production protocol of the labeled binder.

The HaloTag technology exploited in publication V required a separate process for each protein of interest to HaloTag protein, which was relatively laborious. However, the production of the recombinant proteins is rather simple once the clones are ready. The yield of correctly folded fusion protein from E. coli BL21 cultures was very low, being only in microgram levels. Still, the dual purification with Ni-NTA and protein G was effective, because the final product was highly active. Moreover, the protein G column provided a convenient method for conjugating HaloTag-ligand to HaloTag

protein while the fusion protein was immobilized to protein G matrix. After conjugation, the non-reacted ligand was simply washed off prior to elution, and additional purification steps were avoided. The most significant advantage of HaloTag technology was that it enabled a controlled and stoichiometric conjugation. However, the relatively large size of the HaloTag protein compared to linkers and functional groups used in conventional conjugation chemistries may cause steric hindrances in some applications.

5.2 Quantitative multiplexed immunoassay using UCNPs

The applicability of UCNPs to multianalyte protein detection was demonstrated. Quantitative response was obtained from all analytes in the multianalyte assay. The analytical sensitivities, calculated as 3 x standard deviation of the blank, were 0.64 mU/L, 0.45 U/L, and 0.17 ng/ml (5.0 pM) for TSH, LH, and PSA, respectively (Fig. 12). The obtained sensitivities were comparable to single analyte assays performed in array wells and measured using the imager. The performance of the reference assays carried out in a regular microtiter wells and measured with the plate reader were also in the same range. These results indicated high applicability of UCNPs for array-based multianalyte protein detection.

The dynamic range of the multianalyte assay was approximately two orders of magnitude and the analytical sensitivities were in clinically relevant range. However, TSH assays with ultrahigh sensitivities are needed for the measurement of hyperthyroidism and, for that purpose, the analytical sensitivity of the current assay was approximately 10 times worse than what is required. The assay performance in the multianalyte assay was not affected by the presence of the reporters for other analytes because the analytical sensitivities of the multianalyte assay were comparable to those of the reference assays. However, the assay sensitivities were limited by the non-specific binding of the label, which was significant particularly in the case of TSH assay. The employed antibodies had high affinity and, earlier, they have enabled the development of a highly sensitive TSH assay with a analytical sensitivity of 0.003 mU/L (Ylikotila *et al*., 2005). However, the production of the solid phase was different in publication II compared to the earlier study and this may explain the compromised sensitivity. Additionally, the particulate nature of the reporter most likely affected the extent of the non-specific binding. Although the non-specific binding is an inherent feature of antibodies and cannot be completely eliminated, it can be minimized by a careful optimization of antibody combinations and assay conditions (Ellington *et al*., 2010). Furthermore, the surface chemistry used for the coating of the UCNPs and the assay conditions may affect the colloidal stability of UCNPs during the assay, which increases the non-specific background signal (Soukka *et al*., 2008).

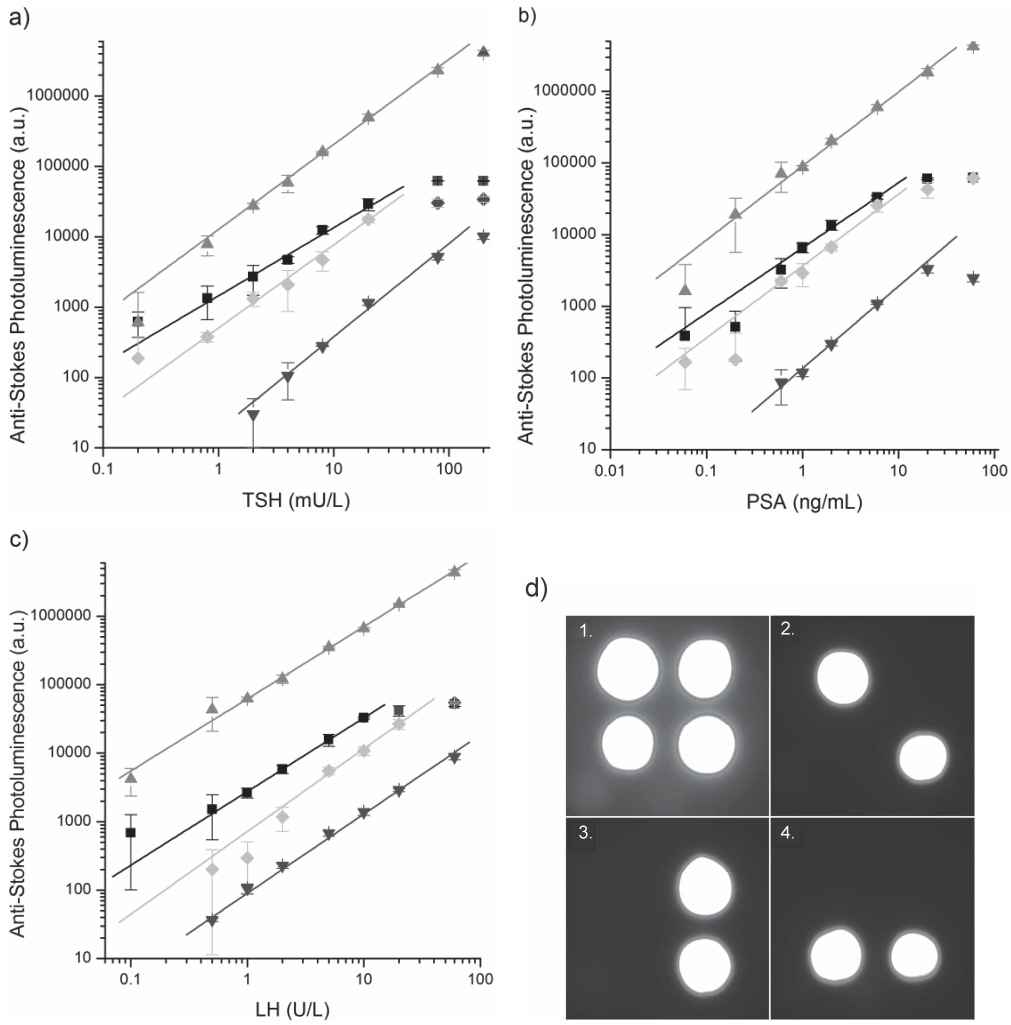


Figure 12. a-c) Standard curves for TSH (a), PSA (b) and LH (c) in different assay formats. Upward triangles, squares, diamonds and downward triangles represent reference assay measured with plate reader, multianalyte assay, single analyte assay in array well and reference assay measured with imager, respectively. The equations of the fittings of the multianalyte assay were y=0.909x+3.16 (R^2=0.936), y=0.911x+3.82 (R^2=0.985) and y=1.08x+3.44 (R^2=0.998) for TSH, LH and PSA, respectively. d) Fluorescence image of the array containing 1) all analytes 2) TSH 3) PSA and 4) LH. The analyte concentrations in the images were 200 mU/L, 60 ng/mL and 60 U/L for TSH, PSA and LH, respectively.

The advantage of using imaging for detection was that the measurement was significantly faster compared to scanning and any inhomogeneities were easily discovered. The drawback of imaging-based detection was that the dynamic range was limited because of the maximum value of approximately 65000 for each pixel in the CCD-chip. However, the exceptional photostability of the UCNPs was exploited to increase the dynamic range by employing various exposure times (Fig. 13). This feature further increases the applicability of UCNPs to imaging-based detection. With other label technologies, the increase of background has impeded the use of longer

exposure times in measuring immunoassays (Ahmad *et* al., 2011; Momeni *et al*., 1999). Earlier, the dynamic range has been increased, *e.g.*, by using solid phase with high binding capacity, by desensitizing the assay with added detection antibody, or by using a combination of high- and low-affinity antibodies (Hyytiä *et al*., 2013; Ylikotila *et al*., 2005; Ohmura *et al*., 2003). Utilization of different exposure times was convenient because the sensitivity was not compromised and no modifications to the assay itself were required. Additionally, the same array well can be imaged several times using different exposure times.

Another thing affecting the dynamic range of the array-based assays was the spots themselves, because their binding capacity was limited compared to the whole wells. This was demonstrated by scanning the multi-analyte array wells with a plate reader. No increase in the dynamic range was observed contrary to the whole well assays. This indicates that the reason for the signal saturation was the spots and not the CCD-chip of the imager. Additional drawback of imaging-based detection is that the sensitivity of CCD-chip is generally not as good as with the photomultiplier tube. This was evident in the case of reference assays, which analytical sensitivity were significantly worse when measured with the imager. This could be partly explained by the fact that UCNP concentration of the reference assays was not optimized for imaging-based detection.

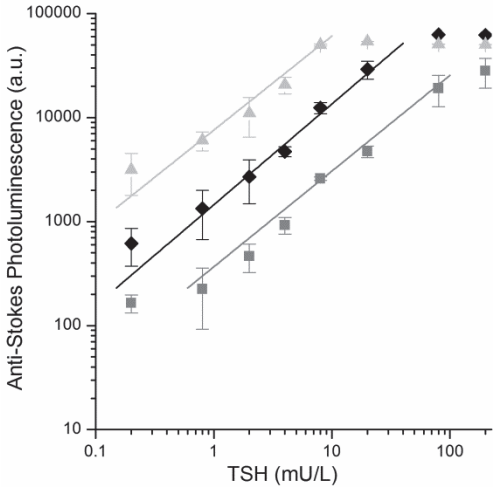


Figure 13. Effect of exposure time on dynamic range. Exposure times of 0.2 s (grey squares), 1 s (black diamonds) and 5 s (light grey triangles) were compared.

The combination of analytes in the array was not clinically relevant. By choosing different antibodies, the array could enable effective diagnosis of, for instance, cardio-vascular diseases. Moreover, multiplexing capacity is not limited to three analytes but could be increased if highly specific binders were found. As an example of higher multiplexing capacity, an oligonucleotide array for adenovirus typing of seventeen different genotypes has been demonstrated using the same technology (Ylihärsilä *et al*., 2013). The antibody-coated binders were added to the array in two steps due to the tracer antibody of LH, which recognized the alpha chain present in both TSH and LH. The two-step addition of

the label is not feasible in point-of-care tests, but this could be avoided by using a different antibody for detecting LH. Many challenges still remain. The assay included sequential addition of reagents and separate washing steps in between. However, multiple steps increase the complexity of the assay, require more dedicated instrumentation, and prolong the assay times. Additionally, the incubation time of the multianalyte protein array was 40 min, which is not adequate compared to the demands of point-of-care tests (von Lode, 2005). The possibility of testing multiple analytes simultaneously alleviates this deficiency and may ultimately result in faster diagnosis compared to performing several tests separately when searching for the reason to patient's symptoms.

In addition to enabling accurate and timely diagnosis, multianalyte assays are also cost-effective because of the reduced hands-on time. Furthermore, the effective use of the sample is patient-friendly, which is important especially with small children. The developed multianalyte assay was a planar array, which identifies the analyte on the basis of its position in the array surface. The drawback of planar arrays compared to bead-based suspension arrays, such as Luminex's xMAP technology, is the printing of the arrays. It is time-consuming, requires careful optimization, and is susceptible to variations between batches thus compromising the reproducibility (Ellington *et al*., 2010; Vignali 2000). On the other hand, only single label is required in planar arrays. This enables relatively simple instrumentation, because only a single excitation source is required and different emission wavelengths do not have to be separated. This is essential in point-of-care applications. The multiplexing capacity is also significantly higher compared to homogeneous FRET-based assays with luminescent lanthanide labels (Xu *et al*., 1992; Kokko *et al*., 2008).

5.3 Protein detection using switchable lanthanide luminescence

In publications III-V, homogeneous assays for three different model analytes were demonstrated. The assays were straightforward mix-and-measure type assays, which were very simple to perform. In addition, the potential of switchable lanthanide luminescence for heterogeneous protein detection was demonstrated (unpublished data). The developed assays fulfilled many requirements of the ASSURED criteria because the assays were sensitive, rapid, and simple to perform and, therefore, applicable to point-of-care testing. The use of aptamers or alternative protein binders could solve the stability issues associated with immunoassays making the tests more robust, but currently high-affinity aptamers or alternative protein binders are available for only a few analytes. Furthermore, the instrumentation required for the measurement of switchable lanthanide luminescence is complex and too expensive for resource-poor settings. In an optimal situation, no instrumentation is required for detection but, unfortunately, that commonly leads to compromised assay performance (Faulstich *et al*., 2009; Weigl *et al*., 2009).

5.3.1 Aptamer-based thrombin assay

Short complementary oligonucleotide sequences were added to the thrombin binding aptamers in order to assist in formation of the mixed chelate complex in the presence of

the analyte. Poly-dT-linkers and polyethylene glycol (PEG) linkers were used to connect the reporter sequences to the actual aptamer sequences. Additionally, the linkers ensured that the hybridization of the reporter stem and the formation of the mixed chelate complex did not interfere with the analyte-binding activity of the aptamer.

The optimal length of the reporter stem was 6 nucleotides (nt), because it resulted in highest signal-to-background ratios. With a shorter reporter stem, the specific signal in the presence of thrombin was low, whereas with a longer reporter stem sequence the hybridization occurred even without the presence of the analyte. The optimal labeled binder concentration was 5 nM, which resulted in the highest signal-to-background ratios. With higher labeled binder concentrations, the signal levels increased, but the background signal was elevated even more thus affecting the analytical sensitivity.

The kinetics of the assay was studied with TBAs having 4, 6, or 8 nt-long complementary sequences (Fig. 14). The maximum signals were obtained already after 2, 3, and 9 minutes, respectively. With the 4 nt complementary sequence, the signal level remained low, whereas with the 8 nt complementary sequence, the background signal was elevated compromising the signal-to-background ratios. The highest signal-to-background ratios were obtained with the oligonucleotides containing 6 nt-long complementary sequence after 5 minutes of incubation.

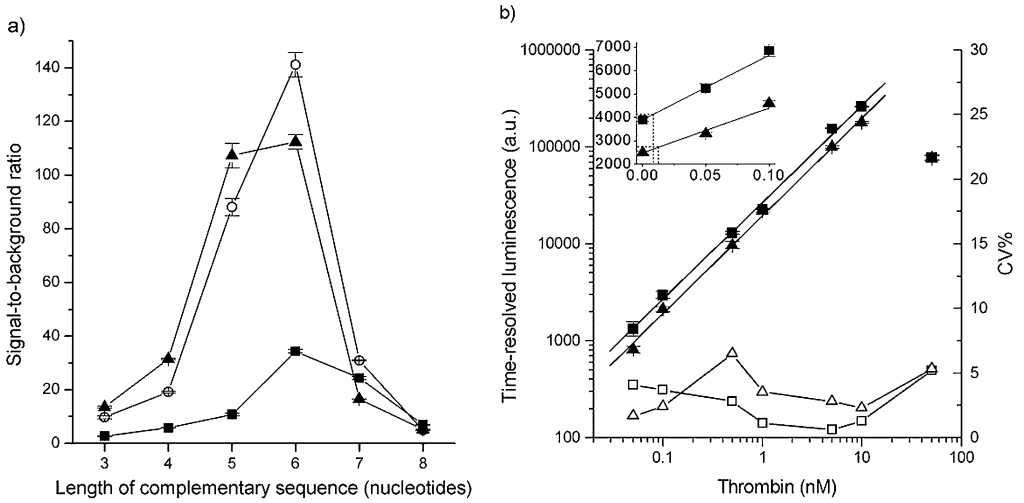


Figure 14. a) Optimization of the length of the complementary sequences using 0 mM (black squares), 50 mM (open circles) or 150 mM (black triangles) NaCl in buffer. b) Standard curves with poly-d(T)-linker (black squares) and PEG-linker (black triangles). The equations for fitted standard curves using poly-d(T)-linker and PEG-linker were y=4.44+0.999x (R=0.999) and y=4.29+1.01x (R=0.999), respectively. Open symbols represent coefficients of variation. In the inset, the signals of the blank and the two smallest calibrators are shown in linear scale. The limits of detection are presented in dashed lines.

The analytical sensitivity was defined as 3 x standard deviation of the blank in all assays. In publication III, two alternative linkers were compared: the poly-dT linker contained 18 nucleotides and the PEG-linker consisted of five hexaethylene glycol units. The analytical sensitivities using thrombin-binding aptamers with PEG-linkers and poly-dT linkers were 0.44 ng/mL (12.0 pM or 0.72 fmol) and 0.29 ng/mL (7.8 pM or 0.48 fmol), respectively, and the maximum signal-to-background ratios were approximately 70, which were excellent for a homogeneous assay. Thus the linker did not significantly affect assay performance. The sensitivities were among the best obtained for homogeneous thrombin assays even though multiple aptamer-based thrombin assays have been developed. In these applications the analytical sensitivities have generally been in the nanomolar range (Doughan *et al*., 2014; Ma *et al*., 2012; Chi *et al*., 2011; Wang Y. *et al*., 2011; Heyduk *et al*., 2005). Furthermore, the dynamic range of the developed assay was almost three orders of magnitude, which is good for a homogeneous assay. This indicates the potential of switchable lanthanide luminescence for sensitive homogeneous protein detection. However, the obtained analytical sensitivity was still several orders of magnitude higher than the best heterogeneous thrombin assays with sensitivities in the picomolar or even attomolar range (Wang J. *et al.*, 2011; Zhang *et al*., 2011). Thus the requirement for equal sensitivity for rapid tests compared to more complex tests was not yet met. Nevertheless, the assay was exceptionally simple and rapid to perform because the assay components were incubated for only 5 min before measurement and, therefore, the proposed assay concept has potential for point-of-care applications.

5.3.2 cTnI assay using site-specifically conjugated Fabs

The reporter stem length was also optimized for cTnI assay in publication IV. Similarly to publication III, the reporter oligonucleotide containing the ion carrier chelate had one nucleotide overhang because this had improved the signal-to-background ratios in publication III. Using the same reporter oligonucleotides employed in publication IV, the highest obtained signal-to-background ratio was 25 at cTnI concentration of 50 ng/mL. It was achieved using a 6 nt-long reporter stem. The use of longer reporter stem region increased the background signal significantly compromising the signal-to-background ratios and the analytical sensitivity. This suggested that the complementation of mixed lanthanide chelate occurred to some extent even without the presence of an analyte. Despite the use of DINAmelt Web Server oligonucleotide analysis software (Markham and Zuker, 2005) for designing the oligonucleotides, the prediction of melting temperature Tm turned out to be challenging. This was because of light-harvesting antenna ligands and ion carrier chelates, which have some affinity towards each other contributing to the reporter stem hybridization. This may partly explain why the background signal increased with longer reporter stems even though their Tm was significantly lower than the used assay temperature. The assay was performed and measured at 36 °C and the calculated Tm of the 6 nt reporter stem was 11 °C according to DINAmelt Web Server. In theory, the assay could be performed at lower temperatures, such as room temperature, by shortening the reporter oligonucleotides. This would minimize non-specific signal. However, if the reporter

sequences are too short, they are not able to effectively bring the label moieties together thus impairing the assay performance.

In publication IV, a labeled binder concentration of 2.5 nM was chosen for the assay, although the signal-to-background ratios were higher with 1.3 nM binders. With 2.5 nM, however, the signal intensities were higher. This allowed reliable measurement at the low end of the calibration curve and thus the achieved analytical sensitivity was also better. Higher concentrations of the labeled binders were not evaluated in this assay, because in publication III they had already been discovered to have even lower signal-to-background ratios due to the elevated background. A further point of interest was whether the use of an excess amount of the labeled binder containing the antenna ligand would enhance the signal levels without increasing the background. A twofold excess (5 nM) increased the assay levels at low cTnI concentrations and, therefore, it was chosen for the final assay.

In publication IV, the assay kinetics was also very rapid and maximum signal-to-background ratios were obtained already after 6 min (Fig. 15). In fact, the maximum signal levels were reached even faster, already after 3 min of incubation, but the background signal was high at the beginning of the assay compromising the initial signal-to-background ratios. The increased background of the assay arouse from the non-analyte-dependent hybridization of the reporter stem, which occurred because the pre-heated reagents had time to cool down during the pipetting. The signal-to-background levels seemed to decrease slowly after a 15-min incubation. This could result from the degradation of the cTnI and subsequent decomposition of the luminescent mixed chelate complex (Katrukha *et al*., 1998).

The analytical sensitivities of the homogeneous assay and heterogeneous reference assay were in the same range, 380 ng/L (16 pM) and 260 ng/L (11 pM), respectively, indicating the high potential of switchable lanthanide luminescence for homogeneous detection. The same Fabs were used in both assays ensuring comparable results. In publication IV, the maximum signal-to-background ratio of the homogeneous assay was 50. In the heterogeneous reference assay, the obtained signal levels were still higher, and the maximum signal-to-background ratio was as high as 250. The difference between the signal levels in the homogeneous assay and heterogeneous reference assay can be partly explained by the structure Eu^{3+} chelate employed in the heterogeneous assay (von Lode *et al*., 2003). The 9-dentate Eu^{3+} chelate contained two chromophores allowing a more efficient excitation of the Eu^{3+} ion compared to a single chromophore in the mixed chelate complex formed in switchable lanthanide luminescence. The dynamic range of the homogeneous assay was over 2 orders of magnitude and it was limited by the hook effect occurring at high cTnI concentrations. The heterogeneous assays do not suffer from hook effect, and the dynamic range of the reference assay was over three orders of magnitude.

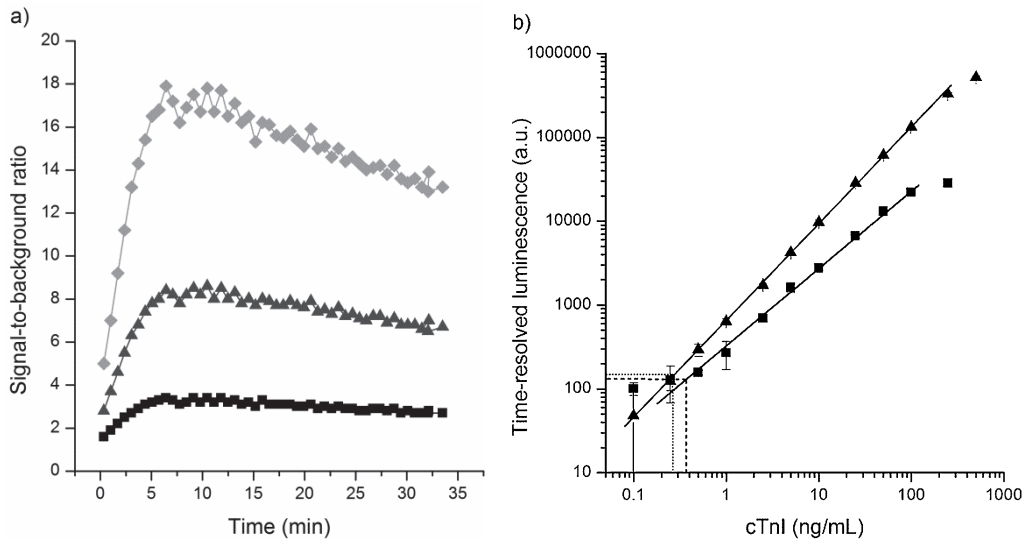


Figure 15. a) Kinetics of the homogeneous measured with cTnI concentrations of 7.5 ng/mL (squares), 20 ng/mL (triangles) and 50 ng/mL (diamonds). The concentrations of labeled binders containing antenna ligand and Eu^{3+} carrier chelate were 5 nM and 2.5 nM, respectively. b) Standard curves of homogeneous assay (squares) and heterogeneous reference assay (triangles). Equations of the fitted standard curves for homogeneous and heterogeneous assays were $y=326x^{0.921}$ ($R^2=0.998$) and $y=660x^{1.15}$ ($R^2=0.999$), respectively. Analytical sensitivities are marked as dashed lines.

Even though the picomolar analytical sensitivity was reasonably low and adequate for measuring many analytes in clinically relevant range, the sensitivity was not adequate for all analytes, such as cTnI requiring ultra-low sensitivities. Currently the limits of detection of the high-sensitivity troponin assays are in the low ng/L range (Hyytiä *et al*., 2013; Apple *et al*., 2012; Järvenpää *et al*., 2012), indicating that they are approximately three orders of magnitude more sensitive than that of the developed homogeneous assays utilizing switchable lanthanide luminescence. Other attempts to develop a simple and rapid troponin test have also been made, but also these assays have managed to achieve only picomolar sensitivities despite inclusion of washing steps and used longer incubation times (Song *et al*., 2011). This indicates the serious challenge of developing rapid assays with superior performance. The sensitivity of the cTnI assay developed in publication IV could probably be improved by using more optimal binders. The Fabs MF4 and 4C2 employed in publication IV bind to the N- and C-terminal regions of the cTnI molecule, which are known to be prone to proteolysis (Katrukha *et* al., 1998). Usually, binders recognizing the mid-fragment of the cTnI are used to overcome this problem (Apple *et al*., 2012; Panteghini, 2006). In publication IV, however, Fabs recognizing the terminal ends of the cTnI molecule were used due to limited avalailability of Fabs.

5.3.3 PSA assays using Fab-HaloTag fusion proteins as binders

In publication V, a PSA assay was demonstrated with the labeled binders created using HaloTag technology and conjugation during the immobilization of binders in the

column. A labeled binder concentration of 2.5 nM was used in the assay as it resulted in the best analytical sensitivity. However, the kinetics was faster with higher concentrations of labeled binders. With the optimal labeled binder concentration, analytical sensitivity of 0.27 ng/mL (8.2 pM) was obtained using 30-min incubation time. The obtained results were comparable to homogeneous FRET-based PSA assays utilizing lanthanide chelates or nanoparticles as donors (Kokko *et al*., 2008; Valanne *et al*., 2009).

With Fab-HT protein fusions, it was also demonstrated that lanthanide chelate complementation can be used in heterogeneous assays (unpublished data). In theory, the switchable lanthanide luminescence should enable very sensitive heterogeneous assays because of the minimal background signal due to the fact that three separate binding events are needed to signal generation. In the heterogeneous assay, 10 nM binders were used, because it was hypothesized that the washing steps would allow the use of a larger labeled binder concentration without increasing the background. Any chelate complexes formed non-specifically without binding to the PSA would be washed off. The washings, indeed, reduced the assay background and variation between replicates, but the signal intensities at low PSA concentrations also decreased. The analytical sensitivity was 1.1 ng/mL, which is comparable to the analytical sensitivity of the homogeneous assay carried out using 10 nM binders (Fig. 16). However, the slope of the calibration curve was steeper in the heterogeneous assay indicating that the PSA concentrations differed better from each other.

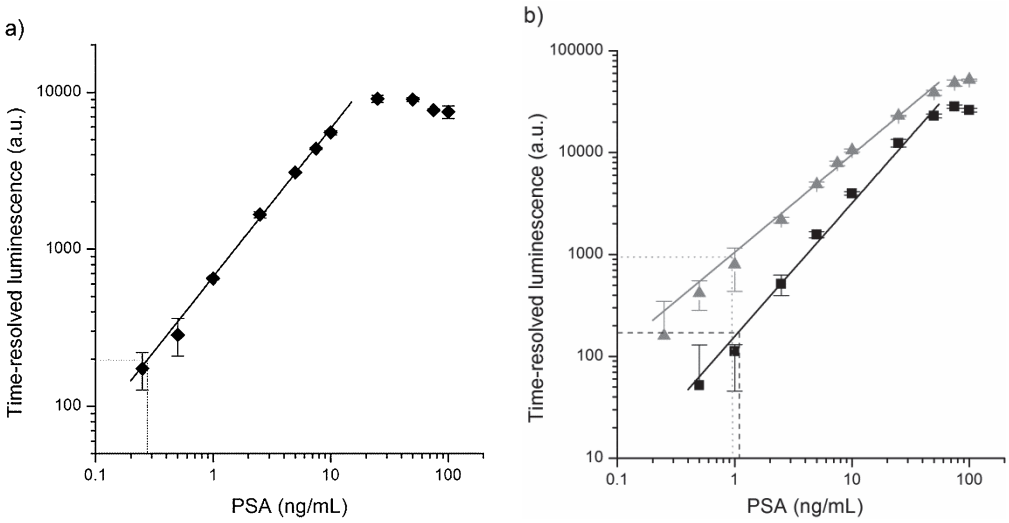


Figure 16. Standard curves of PSA assays utilizing switchable lanthanide luminescence. a) Homogeneous PSA assay with 2.5 nM labeled binders. The equation of the fitting was $y=667x^{0.945}$ (R^2=0.999). b) Homogeneous (grey triangles) and heterogeneous assays (black squares) with 10 nM labeled binders. The equations of the fittings of homogeneous and heterogeneous assays were $y=1050x^{0.957}$ (R^2=0.994) and $y=157x^{1.31}$ (R^2=0.929), respectively. Analytical sensitivities are marked as dotted and dashed lines, respectively.

Switchable lanthanide luminescence could perhaps be improved by developing novel label moiety structures. A light-harvesting antenna ligand with lower affinity to ion carrier chelate would be beneficial in further diminishing the background signal. That would increase the specificity of the signal, which would then be generated only when the analyte is present in the sample, and any nonspecific formation of a chelate complex could be avoided. That would also allow the use of higher concentrations of label moieties resulting in higher signal levels without an increase in non-specific background signal. In developing novel label moieties, a higher absorptivity of the antenna ligand would be beneficial. The main challenge in finding applications for switchable lanthanide luminescence is that it requires exact label positioning. This may limit its applicability or at least careful selection of bindes is required.

5.3.4 Assay interference

So far, the applicability of switchable lanthanide luminescence has not been demonstrated in real samples. However, multivalent metal ions were shown to impair the switchable lanthanide luminescence probably by coordinating to the antenna ligand and preventing the formation of a mixed chelate complex (Fig. 17) (unpublished data). This interference can be evaded by using chelators, *e.g.*, EDTA, in the assay buffer. Quenching of luminescence was not related specifically to switchable lanthanide luminescence, because regular chelates are also known to be affected by the bivalent metal ions (Kokko *et al*., 2007). To enhance the applicability of switchable lanthanide luminescence to diagnostic tests, the development of ion-selective ligands would better allow the use of clinical sample matrices. Then the cations in the biological samples (Mg^{2+}, Zn^{2+}, Ca^{2+}) would not interfere the formation of the mixed chelate complex (Qin *et al*., 2014; Carolan *et al*., 2013).

As an indication to the applicability of switchable lanthanide luminescence to clinical samples, it was observed that a high abundance of non-specific proteins did not interfere with the signal generation of switchable lanthanide luminescence (IV, V, unpublished data). The PSA and cTnI assays were performed in a buffer containing a high excess of BSA and bovine γ-globulin compared to the analyte. The selectivity of the thrombin assay was studied by adding BSA and yeast extract to the assay. Although yeast extract is not comparable to biological sample matrices, its complexity allows the preliminary screening of several possible interfering substances simultaneously. The BSA and yeast extract altered the obtained signal levels. However, the signal-to-background ratios remained the same indicating that the analytical sensitivity of the assay as well as the capability to distinguish different concentrations were not affected.

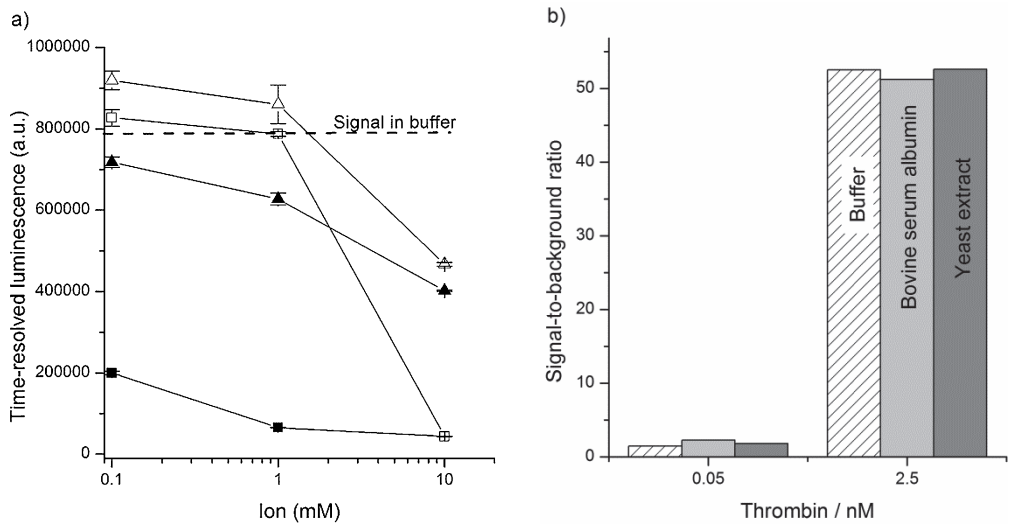


Figure 17. a) Effect of Ca^{2+} (squares) and Mg^{2+} (triangles) ions on switchable lanthanide luminescence. The cation concentration was 1 mM. Open symbols represent signals when 1 mM EDTA was added in solution. b) Effect of BSA and yeast extract on thrombin assay.

The reason for high background in assays can partly be due to reporter oligonucleotides and the formed reporter stems. The sequences were designed by using a DINAMelt Server that calculates parameters for hybridized oligonucleotides. In our applications (III-V), however, the oligonucleotides were not free in solution after the analyte recognition by the binders but in proximity to each other. Thus the oligonucleotides distantly resemble intramolecular hairpin structures, which have higher melting temperatures compared to the hybridization of two separate strands.

6 CONCLUSIONS

Luminescent lanthanide labels are already well-established in diagnostic assays and their main advantage over other labels is the possibility of the elimination of autofluorescence. Novel applications of lanthanide luminescence strive to resolve the remaining limitations, such as expensive instrumentation and non-specific binding of the labels, and to enable effective but affordable diagnostics.

In this thesis, the applicability of two alternative lanthanide label technologies, upconverting nanoparticles and switchable lanthanide luminescence, was studied. Upconverting nanoparticles were utilized in a heterogeneous multianalyte assay while switchable lanthanide luminescence was exploited in homogeneous assays. Different bioconjugation strategies and alternative binders were also studied.

The conclusions based on the publications are

1 Upconverting nanoparticles were effective labels in quantitative imaging-based multiplex protein array detection. The advantage of upconverting nanoparticles over other luminescent lanthanide labels was that no time-resolved measurement was required and therefore the detection instrumentation was relatively affordable. Imaging-based detection was also faster and simpler compared to scanning, which is commonly used for measuring arrays. An additional benefit of upconverting nanoparticles was that they did not photobleach, which enabled long exposure times. Furthermore, the possibility to increase the dynamic range of the assay by using several exposure times was demonstrated.

2 The use of switchable lanthanide luminescence enabled rapid homogeneous protein detection with analytical sensitivities in the picomolar range. This was demonstrated with three different model analytes in publications III-V. In publications III and IV, the assay was very rapid demonstrating a potential for point-of-care applications. An excess of other proteins in the buffer did not interfere with the analyte recognition indicating the selectivity of the assay.

3 Site-specific and stoichiometric conjugation of the label molecules to the binders enabled highly controlled production of the assay components in publications III-V. Thus it can be expected to increase the reproducibility of the assays. High-quality assay components are also essential to robust assays applicable to point-of-care testing.

4 Aptamers were studied as alternative binders in publication III. Because of their oligonucleotide nature, the modification of the binder was straightforward. Site-specific and stoichiometric labeling was easily obtained with aptamers using well-established chemical labeling methods. The analytical sensitivity obtained with aptamers in publication III was in the same range compared with the other assays (IV-V), which were based on antibody binders. This demonstrated that aptamers are highly potential alternatives for antibodies.

5 This study explored aspects related to assay development with the emphasis on point-of-care testing. Fulfilling every requirement presented in the ASSURED criteria is extremely challenging and, in practice, compromises between the criteria are currently unavoidable. Therefore the criteria need to be prioritized for each application while bearing in mind the intended use and the end-user. However, the results of this thesis indicate that there are several possibilities to enhance the performance of the rapid diagnostic tests.

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