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SIMULATION METHODS IN THE MODELLING OF BIOAFFINITY ASSAYS

by

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To my family

ABSTRACT

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Simulation methods in the modelling of bioaffinity assays
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Computational model-based simulation methods were developed for the modelling of bioaffinity assays. Bioaffinity-based methods are widely used to quantify a biological substance in biological research, development and in routine clinical *in vitro* diagnostics. Bioaffinity assays are based on the high affinity and structural specificity between the binding biomolecules. The simulation methods developed are based on the mechanistic assay model, which relies on the chemical reaction kinetics and describes the forming of a bound component as a function of time from the initial binding interaction. The simulation methods were focused on studying the behaviour and the reliability of bioaffinity assay and the possibilities the modelling methods of binding reaction kinetics provide, such as predicting assay results even before the binding reaction has reached equilibrium. For example, a rapid quantitative result from a clinical bioaffinity assay sample can be very significant, e.g. even the smallest elevation of a heart muscle marker reveals a cardiac injury.

The simulation methods were used to identify critical error factors in rapid bioaffinity assays. A new kinetic calibration method was developed to calibrate a measurement system by kinetic measurement data utilizing only one standard concentration. A node-based method was developed to model multi-component binding reactions, which have been a challenge to traditional numerical methods. The node-method was also used to model protein adsorption as an example of nonspecific binding of biomolecules. These methods have been compared with the experimental data from practice and can be utilized in *in vitro* diagnostics, drug discovery and in medical imaging.

Keywords: bioaffinity assay, binding reaction, reaction modelling, kinetics

TIIVISTELMÄ

Pilvi J. Ylander
Simulaatiomenetelmiä bioaffiniteettimääritysten mallintamiseen
Biofysiikan laboratorio, Biolääketieteen laitos, Turun yliopisto
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Väitöskirjatyössä on kehitetty tietokoneavusteisia matemaattisia menetelmiä bioaffiniteettimäärityksiin, jotka perustuvat biomolekyylien spesifiseen sitoutumiseen, rakenteeseen ja korkeaan affiniteettiin. Nämä määritysmenetelmät ovat laajasti käytössä biologisten näytteiden kvantitatiivisissa mittauksissa, tutkimuksessa sekä *in vitro* –diagnostiikassa. Esimerkkinä näistä on vasta-aineen ja antigeenin sitoutumisreaktio. Työssä kehitetyt simulaatiomenetelmät perustuvat mekanistiseen malliin, joka ottaa huomioon biomolekyylien kemiallisen sitoutumisreaktiokinetiikan ja jonka parametrit ovat helposti ymmärrettävissä, kuten aika, pitoisuus ja sitoutumis- ja erkaantumisvakiot. Simulaatiomallin avulla pystytään kuvaamaan minkä tahansa sitoutumisreaktioon osallistuvan komponentin konsentraatio ajan funktiona.

Työssä keskityttiin reaktiomallinnuksen tarjoamiin mahdollisuuksiin, kuten tulosten ennustamiseen ja virhelähteiden arvioimiseen kineettisissä menetelmissä. Esimerkiksi hyvin pieni kohoama sydänlihasmerkkiainearvoissa on osoitus tulevasta sepelvaltimotautikohtauksesta ja siksi havaitseminen on erityisen tärkeää. Työssä kehitettiin kinetiikkaan perustuva kalibraatiomenetelmä, jonka avulla voidaan suorittaa kalibraatiomääritys vain yhtä standardikonsentraatiota ja tämän kineettistä vastetta apuna käyttäen. Hyvin monimutkaiset sitoutumisreaktiot ovat haastavia perinteisille ratkaisumenetelmille. Tämän vuoksi on kehitetty solmupohjainen simulaatiomenetelmä, jossa jokainen solmu edustaa erikseen yhtä sitoutumisreaktiota. Näistä solmuista rakennetaan verkko, joka kuvaa kaikkia sitoutumisreaktioita tietyllä ajan hetkellä. Solmumenetelmää on sovellettu proteiiniadsorption mallinnuksessa, joka perustuu epäspesifiseen sitoutumiseen. Nämä matemaattiset menetelmät on todennettu ja vastaavat laboratoriossa mitattuja tuloksia. Tutkimuksen tarkoitus on osoittaa kineettisten mittausten, mallinnuksen sekä simulaatioiden tarjoamia mahdollisuuksia tulevaisuudessa. Tutkimuksessa syntyneitä matemaattisia menetelmiä voidaan soveltaa yleisesti *in vitro* diagnostiikan mittauksiin, lääketutkimukseen sekä biolääketieteen kuvantamiseen.

Avainsanat: bioaffiniteettimääritys, sitoutumisreaktio, reaktiomallinnus, kinetiikka

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ABBREVIATIONS

Ab	antibody
Ab'	labelled antibody
Ag	antigen
BLI	biolayer interferometry
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
CME	chemical master equation
CRP	C-reactive protein
cTnI	cardiac troponin I
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EU	European Union
Fab	antibody binding fragment
Fc	**(**antibody) crystallizable region fragment
FCS	fluorescence correlation spectroscopy
FDA	U.S. Food and Drug Administration
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer förster resonance energy transfer
Fv	(antibody) variable fragment
GNLS	generalized nonlinear least square
GPL	general public licence
HTS	high throughput screening
hTSH	human thyroid stimulating hormone
IgG	immunoglobulin G (also A, D, E, M)
in vitro	Latin for *'in glass'*, in a test tube, outside the living body or in artificial environment

in vivo	Latin for *'within the living'*
IRMA	immunoradiometric assay
KRYPTOR™	time-resolved energy transfer assay concept
LabVIEW™	graphical programming environment (by National Instruments NI)
MM	Michaelis-Menten
MSE	mean square of error
NSB	nonspecific binding
ODE	ordinary differential equation
ODESOLVE	ordinary differential solver library package in R
PCFIA	particle concentration fluorescence immunoassay
PDE	partial differential equation
PDF	probability density function
POC	point of care
R	R-programming language and software environment, GPL-software
RIA	radioimmunoassay
RNA	ribonucleic acid
SPA	scintillation proximity assay
SPR	surface plasmon resonance
SSA	stochastic simulation algorithm
SSR	sum square of residuals
TPE	two-photon excitation
TPX	technology that utilises two photon excitation of fluorescence
TRACE	time-resolved amplified cryptate emission
TST	transition state theory
QCM(-D)	quartz crystal microbalance (with dissipation)
4PL	four-parameter logistic

LIST OF ORIGINAL PUBLICATIONS

I Pilvi J. Ylander, Zoltán Bicskei, Pekka Hänninen and Juhani T. Soini, Theoretical assessment of errors in rapid immunoassay – how critical is the exact timing and reagent concentrations. *Biophys. Chem.* 123 (2006) 141-145

II Zoltán Bicskei, Pilvi Ylander and Pekka Hänninen, Calibration of bioaffinity assays using kinetic data. *J. Biochem. Biophys. Methods* 67 (2006) 75-85

III Pilvi J. Ylander and Pekka Hänninen, Modelling of multi-component immunoassay kinetics – a new node-based method for simulation of complex assays. *Biophys. Chem.* 151 (2010) 105-110

IV Pilvi J. Ylander, Pekka Hänninen and Sari Pihlasalo, An experimental and a theoretical study of the complex kinetic behaviour of particle adsorption - a multi-component model based on the NODE-method. (Manuscript submitted to *Biophys. Chem.)*

1 INTRODUCTION

Bioaffinity assays are widely used in biology, biomedicine and in related research fields to recognize qualitatively or to quantify the presence of a biological substance in a sample volume. A bioaffinity assay is based on the binding interaction between biomolecules, which have a high affinity and structural complementarity e.g. antibody and antigen or ligand and receptor. The behaviour of the binding reaction can be studied by binding reaction kinetics. During recent decades, the development of sensitive separation free bioaffinity assays and analytical detection techniques together with computerization have improved system robustness, sensitivity and stability. Several technological advantages e.g. rapid assays, simplified protocols providing high throughput screening (HTS) and real-time measurement systems have been established to provide quantitative biological measurement data with quality and speed.

Computer-assisted mathematical methods have been used to model the behaviour of biological systems in many related fields in biology. The benefits the modelling methods provide are clear: it is possible to simulate what-if scenarios, to predict results without performing the actual measurements and to estimate the parameter dependency by sensitivity analysis. Thus, simulation methods save time, costs and labour. Molecular binding reactions have been widely studied theoretically on the single molecule level, but there still remains the high potential of modelling and simulation methods that could be utilized in experimental measurements and data analysis of bioaffinity assays by data processing.

In *in vitro* diagnostics the bioaffinity assays are used to quantify protein concentrations such as C-reactive protein (CRP), thyroid stimulating hormone (TSH) and cardiac troponin (cTnI) protein specific to heart muscle in serological samples. Reaching for a quantitative result from clinical tests may take time, though the benefits of rapid assay response are obvious, e.g. the elevations in specific cardiac muscle proteins can detect minor heart muscle injury and predict a major heart attack. Thus, research on the binding reaction kinetics of biomolecules creates the background for inventions concerning accurate and rapid diagnosis in clinical health care and also in drug discovery by speeding up the research of targeted binders and their binding kinetics through modelling methods.

This thesis highlights the possibilities that the modelling methods and simulation tools provide for the research and evaluation of binding reaction kinetic data. The kinetic binding reaction models presented are based on the mechanistic assay model, which obeys the mass action law and provides understandable parameters for simulations, such as time, concentrations and kinetic rates. Simulation methods are used to estimate the errors in rapid response immunoassays due to inaccurate timing and antibody concentrations. A calibration method, which requires only one measurement point in order to calibrate a bioaffinity assay measurement system, is presented. A new method was developed to model complex multi-component binding reactions by decomposition

of binding reactions in nodes. The node-method was used to model the complex binding behaviour of a multi-component assay based on adsorption as an example of the nonspecific binding of biomolecules. This work concentrates mainly on immunoassays as an example of bioaffinity assays. Experimental data utilizing label-based two-photon excitation fluorescence detection technology (TPX-technology) from practice was used to confirm our models.

2 REVIEW OF LITERATURE

2.1 Mathematical modelling in biomedicine and biology

A model is an imitation of reality and a mathematical model is a particular form of representation (Hangos and Cameron, 2001). Mathematical modelling is used to gain a deeper understanding of the system's behaviour and help in decision-making, optimization and predicting. Simulations based on validated models point out how sensitive the dynamics of the system can be to changes in parameters, which is often difficult and usually important in sensitivity analysis (Murray, 2002). Mathematical modelling has been on the rise and enhanced by computerization during recent decades. Model-based computational methods are important in various ways as they provide powerful computing tools that contribute to all fields of science. Simulations can be easily carried out instead of many time-consuming expensive experiments, including facility, material and labour costs. They also provide the possibility of studying cases which may be very difficult, dangerous or even impossible to carry out by experimental methods. Thus, the benefits of mathematical modelling are clear and undisputed.

Mathematical modelling is widely utilized in the biology and biomedicine research fields in order to describe the structure and the function of a biological system. Biological systems are often more difficult to model in comparison to systems in mechanical or electronic engineering. This is due to the complexity of the biological mechanisms on several scales, sensitivity to time-variant environmental conditions (e.g. light, humidity and pH) and unknown factors causing errors, greater in magnitude than instrumental errors (Zheng and Sriram, 2010). Though the problems in physics, chemistry and engineering sciences sound very different, the algorithms and procedures are suitable for solving the problems in biomedicine and biology.

Mathematical modelling in medicine is applied to various scales of size and time from the nanometre to the metre scale and from the nanosecond to the month and year scale, depending upon the system being modelled, see Figure 1 (Sloot and Hoekstra, 2009; Southern *et al.*, 2008). On the large scale the modelling methods in medicine comprise population models (e.g. epidemiology models for infectious disease in a population (Anderson and May, 1979; Garnett, 2002)), models for a function of a single organ such as heart and brain (Šterk and Trobek, 2005; Markham, 2006) and for a complex system such respiratory and circulatory systems (Aittokallio *et al.*, 2006; Conlon *et al.*, 2006). On a small scale, the modelling methods are used to describe the behavior of single cells, proteins and viruses in inter- and intracellular systems in *in vivo* and tissue samples *in vitro* e.g. in diagnosing and observing the progression of a disease (Louzoun, 2007; Cohn and Mata, 2007; Siepmann and Siepmann, 2008). In the modelling of gene expression, the research on a genetic disease can be narrowed down to a certain gene (Peltonen *et al*.,

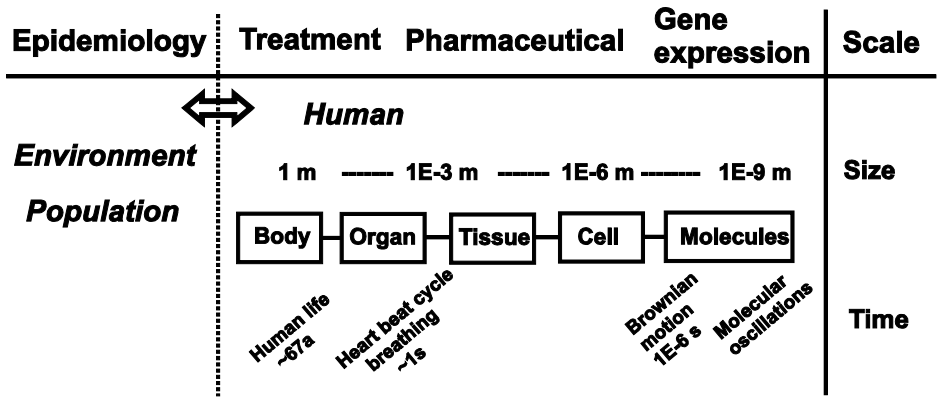

Figure 1. Mathematical modelling in medicine (Modified after: Sloot and Hoekstra, 2009; Southern J *et al.*, 2008).

2006; Rios *et al*., 2010). In drug discovery, the modelling of structural aspects together with interaction behaviour could shorten the preclinical phase of research by providing a reliable tool to make predictions about the effects of drugs (Bersani *et al.*, 2008). Thus, mathematical methods and simulations provide advantageous tools in biomedicine and biology research. However, a mathematical model must be constructed carefully in creating a theoretical basis that meshes with possible data from experiments, hand in hand, in order to produce reliable predictions.

2.1.1 Modelling and simulation processes

It is important that the model is correctly posed and represented properly in order to operate as a reliable simulation tool. A simplified modelling process is illustrated in Figure 2. It includes four main steps: the formulation of the problem in a mathematical manner (construction of a model), the solution created by the model, its interpretation and its validation (Hangos and Cameron, 2001). The key question in formulating the problem is achieving an adequate level of detail, the recognition of entities, parameters, mechanisms and theories behind the problem and boundary conditions (Crampin *et al.*, 2004). The solution created by the model is interpreted in a non-mathematical manner for comparison with the data from the real world. The modelling process can be considered finished in the validation process, when the criteria for the model are fulfilled. Thus, a validated theoretical model with prior knowledge of the system's behaviour from experimental data provides a tool for simulations. The reliability of a simulation prediction depends on the accuracy of the model, precise quantitative details of boundary conditions and on the observed range of experimental data for comparison. Modelling processes in biomedicine and biology may also require a mandatory quality assurance and software validation step in order to meet the EU- and FDA- regulations and other standards prior to the implementation in practice e.g. in medical care.

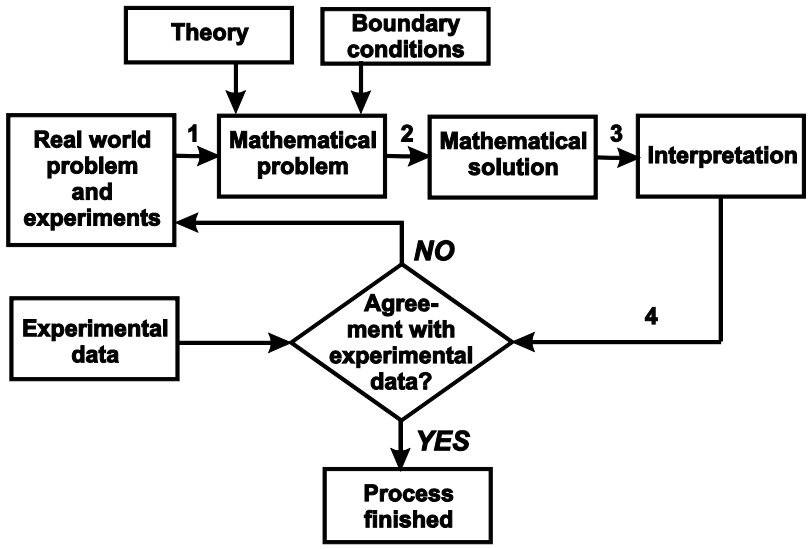

Figure 2. A simplified modelling process with comparison to experimental data (Modified after: Hangos and Cameron, 2001).

2.1.2 Modelling approaches in mathematical modelling

There are different modelling approaches available. The characteristics of, first the descriptive and mechanistic and then, second, the deterministic and stochastic approaches are introduced and compared. It is important to choose the appropriate approach for building the model concerning the purpose.

Descriptive and mechanistic models

Models can be divided into descriptive and mechanistic based on their level of understanding of the system mechanisms. The descriptive model depicts the system studied, but does not explain the system's behaviour. It represents the results of an experiment or an observation and characterise what the system does, but is a hypothesis of the system. A descriptive model can be considered to be a 'black box' model, in which the variables and their relations have little or no physical meaning at all. Thus, this model may be unsuitable for extrapolating and predicting results outside the validated observed range. However, descriptive models are widely used when the mechanism underlying the system is poorly understood.

A mechanistic model is constructed explicitly by considering the first principles of the system and the identifiable variables (e.g. time, mass and volume). Therefore, a deep insight into the fundamental behaviour of the system is required. The mechanistic model has a clear cause-effect relationship, and a pure mechanistic model can be considered as a 'white box' model. It is dynamic and describes how the system behaves in different cases and it can be used to predict results from experiments. Thus, mechanistic models are used to increase understanding of the system's behaviour, extrapolating and studying

cases, which are hard to perform in real life. In most cases, a purely descriptive or a purely mechanical model does not exist, but a combination of these two, hence a 'grey box' model can be found (Tan and Li, 2002).

Deterministic and stochastic (probabilistic) models

Simulation methods can be divided into the categories deterministic and stochastic. Deterministic models are constructed and solved by analytical and numerical methods based on classical mathematical analysis. Analytical methods provide solutions for closed-form problems described by analytical equations without any degree of uncertainty. However, when the complexity of the mathematical problem increases, the numerical methods have to be developed to overcome technical problems, e.g. stiffness in solving ordinary differential equations (ODEs) (Érdi and Tóth, 1989). In deterministic models, the state of the calculation process is determined by the former state of the process, and the response is always the same for an identical set of initial parameters and conditions. Thus, deterministic models can predict, and they are reproducible.

In stochastic modelling approach, the process is ruled by probabilistic equations based on fluctuations, noise or random event factors, which are stochastic by nature. Stochastic approaches are used in everyday life, e.g. in weather forecasting, insurance mathematics and risk management, and in theoretical studies of discrete random event processes, e.g. collisions between single molecules and particle events in detectors at the molecular level. Arguments for stochastic models for chemical reactions involve the consideration of the discrete or random character of the parameter, the large number of coupled degrees of freedom and appropriateness for describing small systems and instability (Turner *et al.*, 2004; Raj and van Oudenaarden, 2008). Stochastic methods are widely used in sensitivity analysis and to analyse methods and models (Thakur *et al.*, 1980; Gottschalk and Dunn, 2005).

In stochastic modelling, single simulations for an identical set of initial conditions differentiate from each other. This is why stochastic models are not used to yield a unique quantity but they are used instead to produce a collection of responses representing a probability function. A great number of simulations, hundreds or even thousands, are required in order to yield a reliable probability distribution of events in the model space. The most famous stochastic algorithm developed is the Monte Carlo method, which produces a collection of simulations to mimic the output range of experiments by using randomizing in sampling and then providing a probabilistic distribution (Metropolis and Ulam, 1949; Reiter, 2008). Therefore, stochastic modelling requires a reliable random number generator and enough processing time. The stochastic process which does not concern the previous state or how it has proceeded to the present state is defined as a Markov process (e.g. Brownian motion).

Notably, in many cases it is possible to describe the same system by both deterministic and stochastic models; a historical example of this is the predator-prey population

model (Abundo, 1991). Sometimes a hybrid model can be constructed by combining deterministic and stochastic parts. A hybrid model for Brownian dynamics has a deterministic part for modelling the motion equations and stochastic part for the random process of particle collisions.

2.2 Bioaffinity assays

Bioaffinity assays are based on the binding reactions between binding partners, which have propensity to bind specifically with complementary structures in favourable conditions. The strength of the binding is defined by the affinity. Bioaffinity assay technologies are widely used in studies of biological phenomenon in environmental and food chemistry, in health science and medical care (Price and Newman, 1991) Their methodology is to provide a tool to detect the presence (qualitative analysis) or the quantity of exact amount of specific biochemical substance in a test sample (quantitative analysis). Therefore bioaffinity assays play an important role in science. The vast majority of bioaffinity assays in biomedicine are immunoassays, cell-based assays or DNA-probe assays. The last two are introduced only briefly here and immunoassays are introduced in depth as an example of a bioaffinity assay.

The cell-based assays play a vital role in the pharmaceutical industry by providing information about the functional impacts and activity of a compound in cellular environment e.g. in drug screening. Cell-based bioaffinity assays can be used to screen and study e.g. the cell surface, to recognize proteins and viruses and to probe molecular interactions on cell surfaces, cell staining and to detect cellular responses due to inter- /intracellular activities. (Day and Schaufele, 2005; Gschwind *et al.*, 2004; Ariel Michelman-Ribeiro *et al.*, 2009) Other than for bioaffinity cell assays are used to study of cytotoxicity (how toxic a chemical is to cells), viability (to determine the ratio of dead or living cells based on the total amount of cells or the growth of cells) and apoptosis (programmed cell death).

The DNA-probe assay is based on identifying a predetermined complementary sequence of nucleic acids by a labelled DNA-probe. The benefit of the DNA-probe assay is that it can identify genetic disorders, oncogenes, microbes and viruses. The DNA-probe assays can be used when conventional microbiological methods are not appropriate.

2.2.1 Binding theory of bioaffinity assays

Binding reactions in bioaffinity assays are equilibrium processes and are governed by the binding strength. A bioaffinity assay constitutes of the binding partners, the binder (e.g. an antibody) and the ligand molecule (e.g. an antigen or an analyte). The binding partners taking part in any binding reaction in assay are referred to later as binding components. The binding components are introduced from the immunoassay binding reaction point of view, and a short overview of binding theory is given in this chapter.

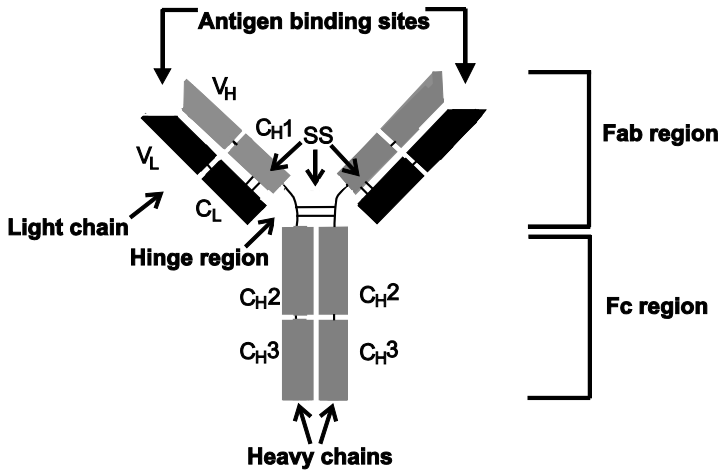

Figure 3. The structure of an antibody. (Modified after Wild, 1994).

The principles of binding reactions are explained further in, for instance, Lehninger Principles of Biochemistry (Nelson DL and Cox MM, 2004).

Antibodies

Antibodies (immunoglobulins, Ig) are proteins produced by plasma cells (B-lymphocytes) in response to foreign targets (e.g. molecules, viruses and bacteria) in bodily fluids. They constitute the natural defence system in a human body, the immune system. There are five (5) types (classes) of immunoglobulins for mammalians: IgA, IgD, IgG, IgE and IgM. Immunoglobulin G (IgG) accounts for 70-75% of the Ig pool. The classes of immunoglobulins have different biological properties, functional locations and ability to deal with various antigens. The immunoglobulin classes are divided by the heavy chain type present and the number of Y-shaped Ig-units, which are composed of four polypeptide chains: two large ones, called heavy chains, and two light chains, linked by noncovalent and disulfide bonds. The complex structure of monomer antibody (e.g. IgG) is divided into two regions: antigen binding fragments Fab(s) and crystallizable region fragment Fc; see Figure 3. The antigen binding fragments are identically composed of variable (Fv) and constant domains of heavy and light chains. The antigen binding site has a complementary structure to the antigenic determinant; hence, it can recognize the antigen. These recognition parts of the antibody (i.e. paratopes) locate at the end of the Fab-arms. The Fv region is hypervariable, which provides a huge diversity of antibodies based on recombination. The immune system can therefore recognize a variety of antigens. The binding may also require conformational changes in the binding site for optimal induced fit. Thus, the interaction between the antibody and antigen is highly specific.

The functional purpose of the Fc region is to ensure an appropriate immune response for the specific antigen (Heymann, 1996), and the Fc region also binds to immune cell Fc

receptors *in vivo* and *in vitro*. In *in vitro* diagnostics, the Fc-region is used to anchor the antibody to a carrier, in other words a plate or polystyrene microparticle in immunoassay processes and direct conjugated antibodies can be labelled in the Fc region.

Physicochemical interactions between antigen and antibody

Like all biological interactions, antigen and antibody interactions are stereochemical. They require complementary structures in order to recognize, match and interact. In the binding reaction, the active site structures of the binding components may also undergo conformational changes and result in an induced fit. The binding specificity of an antibody is determined by the amino acid residues in the variable domains of its heavy and light chains. The chemical complementarity between the antigen and its specific binding site is defined in terms of the shape and the location of the charged, nonpolar, and hydrogen-bonding groups involved in binding interaction (Nelson and Cox, 2004). The major primary interaction is ionic bonding between the oppositely-charged antibody binding site and the antigen (Price and Newman, 1991). The secondary interaction by hydrogen bonds may be formed between hydrophilic groups, though in aqueous media the water competes with hydrogen bonding. Long-range van der Waals interactions, which are due to dipole polarization, may play a significant role in drawing the binding partners closer in primary and secondary bonding. Hydrophobic interactions are caused by the effects of hydrogen bonding and van der Waals (Price and Newman, 1991). Hydrophobic interactions occur when apolar or partly apolar molecules are surrounded by water and the water molecules reorganize. This is a result of the free energy of cohesion of water molecules when the expulsion of interstitial water forces the binding molecules closer creating a stronger bond caused by van der Waals forces (van Oss, 1995). Thus, the binding interaction is composed of a multiple combination of weak interactions, which are collectively strong; see Table 1 for effective distances of interactions. On a microscopic, i.e. molecular level, the binding interaction is proposed to rely on molecular reaction dynamics, which considers the discrete state space of energy levels and the probability distribution that the particle is present at a specific location at a specific time. The reaction rate constants (introduced later) for single molecules have been commonly studied by the time- and temperature-dependent Fokker-Planck equation approach or by the temperature independent approach by Kramers (Levine, 2005; Pollak and Talkner, 2005). According to these approaches, the reactant particle is considered to be a damped oscillator driven by a random force and assumes that the reaction occurs at the time the peak energy between initial reactants and product states is reached (Pollak and Talkner, 2005). However, the statistical behaviour of a large number of single molecules deduces the behaviour at the macroscopic level.

The energetics of the binding interaction at the macroscopic level is characterized by reaction thermodynamics. The binding interactions, like all reactions, obeys the law of Gibbs free energy change ΔG (1). The standard free energy change ΔG^{o} (2), characteristic to each reaction, defines the thermodynamic equilibrium constant, which shows the tendency of the reaction as the ratio of concentrations of products and initial

Table 1. The effective distances of different bonding interactions (Price and Newman, 1991).

Bonding/ Interaction	Effective distance up to / Å
Ionic	100
Van der Waals	100 - 1000
Hydrogen	1.5 - 5
Hydrophobic	short

reagents. The transition state (from reagents to products or vice versa) determines the amount of activation energy (ΔG^{+}) required to overcome the potential barrier in order to react, bind or break, respectively. The chemical reaction kinetics of binding interaction is defined by this activation energy according to law of Arrhenius, which represents the rate of the reaction k (3).

$$\Delta G = \Delta H - T\Delta S \tag{1}$$

$$\Delta G^{o} = -RT \ln K_a \tag{2}$$

$$k = \mathrm{A}\exp(E_a / RT) \tag{3}$$

In equations (1), (2) and (3), the symbols are used as followed: enthalpy H, temperature T entropy S, molar gas constant R , equilibrium association constant K_a, reaction rate k, activation energy E_a and A for steric factor of the reaction.

The binding reaction is temperature dependent. The temperature dependence of bimolecular binding interactions for equilibrium and transition state thermodynamics were studied and reviewed by Weiland and Molinoff (Weiland and Molinoff, 1981).

The binding reaction between an antibody (Ab) and an antigen (Ag) is described by the association rate constant k_{on}, which defines the reaction velocity for this second-order association reaction (k_{on} [Ab][Ag]) and the dissociation rate constant k_{off} for the reverse reaction (4):

$$Ag + Ab \underset{koff}{\overset{kon}{\rightleftharpoons}} AgAb \tag{4}$$

The measure of binding strength in a single bond between the antibody binding site and the antigen determinant is called affinity: the more complementary interactions the stronger affinity. Thermodynamically, the strength of the binding interaction between the antibody (Ab) and the antigen (Ag) can be determined by the binding affinity association constant K_a or by the dissociation constant K_d, the reciprocal of K_a. The binding strength according to the association constant is defined by the ratio of concentrations of the binding components Ab and Ag and the bound complex AbAg at the equilibrium, see (5):

$$K_a = \frac{[AbAg]}{[Ab][Ag]} = \frac{1}{K_d} = \frac{k_{on}}{k_{off}} \tag{5}$$

In bioaffinity assays, the affinity of the antibody for its antigen is the most important parameter determining the assay performance for monovalent binding interactions. However, when a component with a bivalent binding site binds to a component with a multivalent structure, the 'true affinity' cannot be characterized in such a simple way. A more informative measure for binding is avidity, which is also known as 'functional affinity' or 'apparent affinity' and is a measure of antibody-antigen binding strength, considering the affinity and the number of antigen binding sites (Hemmilä, 1991; Ong and Mattes, 1993). These terms were introduced to describe more complex interactions, to approximate the true situation and to provide a basis to compare different antibodies, e.g. monoclonal antibodies, or to improve the performance of traditional assays (Xie *et al.*, 2005). However, the concept of functional affinity has been criticized and discussed owing to validity reasons by Ong and Mattes (Ong and Mattes, 1993; Mattes, 1997; Mattes, 2005). They point out that the binding interactions cannot be described in terms of functional affinity, serving only to obscure the true interactions and that enhanced model alternative will be useful in the further development of improved monoclonal antibodies.

Structural modelling

The function of a binding component depends on its three-dimensional structure and conformation. Knowledge of molecular structures at high resolution is important. The structural information can be used first identifying epitopes and paratopes and then in modifying the antigen and antibody binding affinity. It is possible to detect very small differences in binding kinetics due to changes in remote residues from the paratope in the structure using high precision bioaffinity measurements (Van Regenmortel *et al.*, 1998).

The structural aspects can be determined experimentally by X-ray crystallography and NMR- methods (Nuclear magnetic resonance). X-ray crystallography is the most important technique for studying protein structures reaching high resolutions (1-2 Å). X-ray crystallography requires samples in solid phase crystal -form with a rigid, precisely ordered and repeating structure. However, X-ray powder diffraction, which does not require a solid crystal, has also been used to solve protein structures (van der Kamp *et al.*, 2008). The multidimensional NMR- method is suitable for smaller proteins (50 – 100) kDa. It provides the possibility of determining the structure of proteins, localizing the binding site and studying molecular recognition, low affinity interactions and activity even in liquid phase. The NMR- method builds up a list of distance constraints, by which the three-dimensional structure can be determined. Today, additional structural studies (protein structure prediction, protein docking) can be performed to some extent by computational biological approaches e.g. molecular mechanics (MM), molecular dynamics (MD) and software packages for molecular modelling (e.g. Rosetta), together with databank libraries, e.g. Protein Data Bank (PDB) (Tramontano, 2006; Kaufmall *et al.*, 2010).

The inexpensive production of recombinant antibodies in large quantities is a major advantage for the drug discovery research on recombinant antibodies. The identification of efficacious antibody drugs against viruses has benefitted from the development of libraries for synthetic recombinant antibodies. As a result, there has been more rapid manufacturing of new therapeutic antibodies and designing of drugs (Holt *et al.*, 2000; Schofield *et al.*, 2007; Bregenholt *et al.*, 2006).

2.2.2 Bioaffinity assay formats

Bioaffinity assays can be divided into qualitative (recognition) and quantitative competitive and non-competitive assays. The latter is also known as a sandwich assay or immunometric assay, in the case of non-competitive immunoassay. The first sensitive immunoassay detection technique, radioimmunoassay (RIA) was introduced to measure the concentration of antigens in 1960 by Yalow and Berson, and by Ekins, independently (Yalow and Berson, 1960; Ekins, 1960). Yalow received the Nobel Prize in Physiology and Medicine for the development of radioimmunoassay (competitive) for peptide hormones, in 1977. The first immunometric assay using immunoradiometric assay (IRMA) technology for detecting allergen antibodies was developed by Wide et al. (Wide *et al.*, 1967). Radioactive label techniques have been partially replaced by non-isotopic techniques, such as enzyme and molecular labelling (Jolley *et al.*, 1984). Enzyme immunoassay (EIA) and Enzyme-linked immunosorbent assay (ELISA) were developed independently by two research groups (Schuurs and van Weemen, 1971; Perlmann and Engvall, 1972; van Weemen, 2005). In EIA/ELISA, the enzyme works as a reporter label, converting a substrate into a detectable signal, e.g. a colour change. Luminescence-based labelling and different sensor techniques were developed as non-isotopic alternatives to radioimmunoassay (RIAs), though in early years their sensitivity and cost were often comparable (van Emon J, 2007).

Competitive (reagent limited) assay

In a competitive binding assay the presence or the concentration of a substance is measured by competitive binding of the free and the labelled molecule to the target immobilized molecule. The competitive assay format is presented in Figure 4, where antibodies are

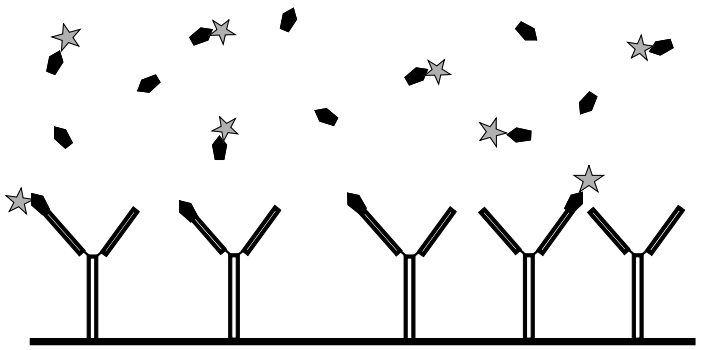

Figure 4. Competitive assay on a solid phase surface.

immobilized on the surface of a solid support (e.g. tube, bottom of a microtiter well or microparticle), depending on the measurement technique. In competitive assays, the number of binding sites is limited in order to provide the conditions for competition. In a low sample concentration, the signal induced is high, since the labelled molecules bind to the immobilized binding pair. In a high concentration, the sample molecules block the binding sites on solid surface and the signal will be low. Competitive assays are used to measure small molecules (< 10 kDa). A dose-response curve for a competitive assay is presented in Figure 6A.

Non-competitive (reagent excess) assay

In a non-competitive assay, a sample molecule must have (at least) two epitopes, one for binding to the immobilized molecule on the solid surface and another for binding to the free labelled molecule. Therefore, a non-competitive assay is suitable for large sample molecules. In Figure 5, the free analyte binds specifically to the immobilized antibody and to the labelled antibody with the other epitope, and a three-component complex is formed. The measurement signal obtained from a non-competitive assay is proportional to the sample concentration. An excess of reagents are used in non-competitive assays in order to capture all the analytes in the sample. Non-competitive assays are considered inherently more sensitive than competitive assays, because they label the bound target (Ekins and Chu, 1991). Therefore, the immunometric assay method is preferred whenever possible.

In the two-site immunometric single step assays, a relevant working range is important due to the consequence of excess sample molecule concentration. When the sample molecule concentration exceeds the antibody concentration, the assay has passed over the dose-response saturation point, and the concentration of the three-component complex will begin to decrease due to the excess analytes binding to both antibodies, thus preventing the signal -producing three-component complex from forming. This

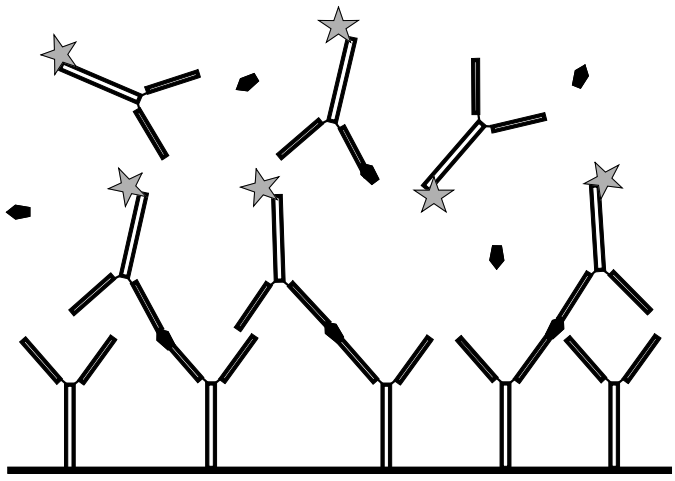

Figure 5. Immunometric assay on a solid surface.

phenomenon is called the 'hook effect' (Rodbard *et al.*, 1978). A dose-response curve for a sandwich assay is presented in Figure 6B.

2.2.3 Separation and single-step bioaffinity assays

The first bioaffinity assays were separation (heterogeneous) assays, which required several incubation and washing steps to enhance the assay performance: specificity, sensitivity and signal-to-noise ratio. An assay procedure for a conventional separation immunometric assay is described in Table 2. The drawbacks of separation assays are the time consuming labwork required due to the pipetting and washing steps, chemical waste and restrictions on volume minimization, due to the washing steps. Single-step (homogeneous) assays are established to provide simplified mix-and-measure assay protocols. an example of such a single-step assay protocol for immunometric assay is described in Table 2. These single-step assays require a sensitive detection technique in order to detect the signal in the presence of other binding components. They also can be performed in smaller (microliter) volumes and they are more applicable to automated high throughput screening (HTS) than separation assays. Single-step bioaffinity assays can be performed in both a heterogeneous environment, with a solid surface phase for immobilizing a binding component, and in a homogeneous environment, in which the reactions and detection are performed in liquid phase. Homogeneous environments are provided by the use of small particles as solid support carriers on nano or micro scale. However, single-step assays require a measurement instrumentation that is able to detect the signal from bound in the presence of other binding components without a separation step.

The binding interactions in a three-component non-competitive assay between an immobilized antibody (Ab), a labelled antibody (Ab') and the sample antigen (Ag) in a single-step assay format can be described by the law of mass conservation. The reactions can be described by a set of equations (6); where binding reactions are taking place in solid phase (independent of the heterogeneous and homogeneous environment), this is marked with (s) and reactions present in liquid phase with (l):

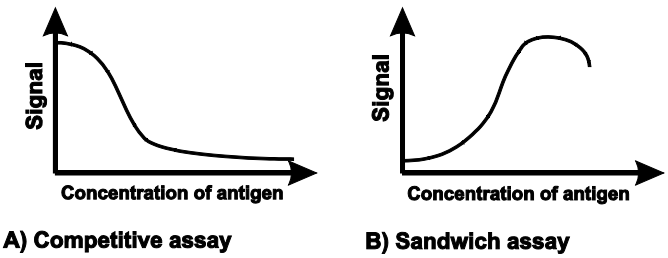

Figure 6. Dose-response curves for A) a competitive assay and B) a sandwich assay, representing also the 'hook effect', the bend in the curve being due to the concentration of antigen exceeding the antibody concentrations.

Table 2. Procedures for separation and single step non-competitive (immunometric) assays

Separation (heterogeneous) assay	Single-step (homogeneous) assay
Incubation of immobilized antibody and the sample antigen	Incubation of all reagents: immobilized antibody, sample antigen and labelled antibody
Washing (removal of unbound), several times	
Incubation of immobilized bound complex and labelled antibody	
Washing (removal of unbound), several times	
MEASUREMENT	

$$
\begin{aligned}
&Ag + Ab \underset{k2}{\overset{k1}{\rightleftarrows}} AgAb && \text{(s)}\\
&Ag + Ab' \underset{k4}{\overset{k3}{\rightleftarrows}} AgAb' && \text{(l)}\\
&Ab + AgAb' \underset{k6}{\overset{k5}{\rightleftarrows}} AbAgAb' && \text{(s)}\\
&Ab' + AgAb \underset{k8}{\overset{k7}{\rightleftarrows}} AbAgAb' && \text{(s)}
\end{aligned}
\qquad (6)
$$

In the set of equations (6), k_{2n-1} stands for the association and k_{2n} for the dissociation rate constants, respectively. It is notable that the first reaction in (6) would represent the first incubation step and that the last reaction would represent the second incubation in a separation assay. An assay scheme for a three-component binding assay can be seen in Figure 7.

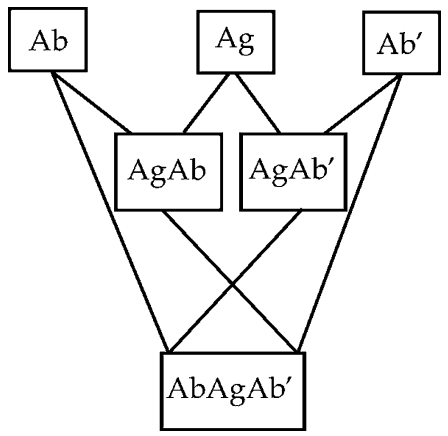

Figure 7. Assay scheme for a three-component binding assay between an immobilized antibody (Ab), a labelled antibody (Ab') and the sample antigen (Ag) in a single-step assay format.

2.2.4 Kinetic behaviour of bioaffinity assays

The binding interaction of binding components forming bound complexes seeks an equilibrium. Each binding reaction can be described by a kinetic curve, which illustrates the rate of the reaction and the amount of bound complex forming as a function of time, see Figure 8. In the beginning, the initial binding components are added to the assay volume. The components, which enters the binding reaction, will be mixed last in the assay at time $t = 0$. The bound complex is formed according to the association rate and dissolved by the dissociation rate of the binding reaction. Eventually the reaction will reach its equilibrium. At equilibrium, the association and dissociation rates are equal. However, there may be binding reactions with fast and slow reaction rates present, since the reaction rates are considered faster in liquid phase compared with the reaction rates in solid phase (Stenberg and Nygren, 1988; Soini, 2002b). A rapid real-time evaluation of binding interaction is possible only with a single-step bioaffinity assay with a measurement system, which enables online-monitoring of a signal from a bound complex. Before the single-step assays, the kinetics was studied by several separate assays by interrupting the incubation after different durations in order to cover the incubation time range until the reaction reached its equilibrium.

Experimental binding reaction behaviour of bioaffinity assay kinetic data obtained by an on-line measurement system provides several advantages and possibilities over a conventional equilibrium endpoint assay. By observing the binding reactions during the incubation time, the kinetic association and dissociation rates become apparent. However, the kinetic rates resulting from different assay formats are not straightforwardly comparable to each other since the assay system with immobilized binding particles may suffer from geometry dependent diffusion limitations (Stenberg *et al.*, 1988).

2.2.5 The dose-response of a bioaffinity assay

A dose-response curve is also known as the calibration curve or standard curve, see Figure 7. The dose-response curve illustrates the responses of equilibrium assays as a function of studied analyte concentration. A dose-response curve is used to back-calculate the concentrations of samples (unknown concentrations). The standard

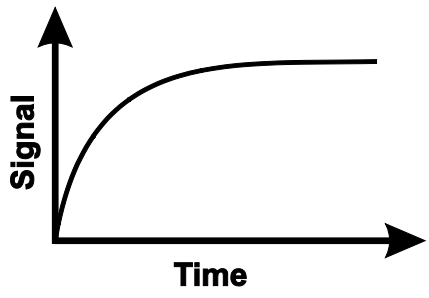

Figure 8. Reaction kinetic curve as a signal induced by the bound complex as a function of time.

analyte concentrations are chosen in order to cover the working range evenly, from the preliminary estimates of the lower limit and upper limit of quantitation. The entire assay performance, the clinical relevance of the dynamic range (working range), the minimum (detection limit) and maximum levels of reliable assay measurement and the assay sensitivity need to be considered carefully.

Dose-response curve estimation methods

Today, the dose-response curves are often constructed in laboratory by a commercial instrument that has all the functionality "built-in" for assay calibration, which includes software for curve fitting. Earlier, this fitting was made by hand, but the choices of curve-fitting method, weighting of data, acceptance criteria and outlier rejections are a part of the automated bioaffinity assay calibration systems today (Sadler, 2008). However, still little interaction by the user is required regarding curve fitting and acceptance. Hence, the technical experience of the user is needed for quality control (Daniels, 1994). The fitting methods used are based on interpolation, e.g. linear and curvilinear interpolation, and spline or descriptive empirical graphical methods, e.g. hyberbolic and polynomial functions and four-parameter logistic (4PL) and five-parametric logistic (5PL) models (Dudley *et al.*, 1985; Anthony and Cox, 1989; Ragatt, 1991; Gottschalk and Dunn, 2005). The problem in using a program, which automatically fits data to thousands of equations and then presents the best fit, is that there is no connection to the scientific context of the experiment and to the underlying mechanisms of the reaction in a quantitative way (Ragatt, 1991, Motulsky and Christopoulos, 2003).

2.2.6 Error and sensitivity limiting factors in bioaffinity assays

A bioaffinity assay, like any experimental process, has several error- and sensitivity-limiting factors, from the production of reagents through the measurement system to the interpretation of data. An increase of assay sensitivity and reliability is an asset for providing quantitative results with quality. The most critical point in the assay process should be found in order to minimize the variation, increase the reliability and attain system robustness. Errors can be classified into three kinds: systematic, statistical and catastrophic errors (Ragatt, 1991) and should be recognized during the assay validation procedure. The reaction kinetics in immunometric assays (Ekins, 1991; Rodbard, and Feldman, 1978; Rodbard *et al.*, 1978) are affected by many different factors, some of which are more easily controlled than the others. The variations within initial materials, assay preparation and incubation timing as well as nonspecific binding, diffusion (mass transport), and matrix effects of the sample must be considered as a probable cause of error in the immunoassay response. There are three major reasons for designing new immunoassay strategies: (1) to improve assay sensitivity, (2) to improve assay reliability by devising more reproducible sources of key reagents, and (3) to simplify the assay in order to achieve a direct readout of the result (Kricka, 1994).

Temperature variations

Binding reactions are dependent on thermodynamics (Weiland and Molinoff, 1981). Temperature variations in incubation affect the binding kinetics and contribute to assay drift, because the reaction rate approximately doubles for each 10 °C rise in temperature (Wild, 1994; Van Regenmortel, 1998). In laboratory work, it should be noted that assay performance may be affected if the reagents are not stored and handled at the proper temperature: e.g., pipetted volumes may not be equal if the reagents are at different temperature. However, error factors due to temperature variations are easily avoided by careful and stable laboratory conditions, practice and proper storage temperature. Although these requirements are standard, one needs to be aware of situations arising, for instance, in mobile laboratories at remote locations where all requirements may not be met.

Nonspecific binding (NSB)

Bioaffinity assays are based on specificity between the binding species. However, there may exist substances with physicochemical similarities to the binding components in the assay. These substances interfere with specific binding by adsorption on the binding surface causing nonspecific binding (NSB). Protein adsorption on solid surfaces can be due to two major classes: 1) solute conditions such as temperature, pH and viscosity and 2) protein properties such as charge, size and hydrophobicity (van Emon J, 2007; Kubiak-Ossowska and Mulheran, 2010). The NSB may play a major role in assays in which the structural issues of components, such as artefacts in drug discovery and serological assays, are unknown (Furusawa *et al.*, 2009). The optimal conditions for maximal bioaffinity assay sensitivity can be found by minimizing the nonspecific binding. In a competitive assay, this is carried out by a measuring assay without the antiserum and in a non-competitive assay by using a zero-concentration sample as an analyte (Wild D, 1994). However, there may be a dependence between specific and nonspecific binding since the nonspecific binding is often proportional to specific binding and increases in relation to the specific binding of the binding component concentrations (Soukka *et al.*, 2001).

Timing errors in kinetic measurements

The incubation of a conventional bioaffinity assay may take hours until the binding reaction reaches the equilibrium phase and a reliable response can be read. Rapid quantitative bioaffinity assays rely on interrupted incubation and the assay response will be read from the non-equilibrium phase of the binding reaction. From the kinetic curve (Figure 8), it can be seen that the greater change of response in the non-equilibrium phase results in greater error due to error in timing. The measurement timing should start exactly when the binding reaction starts. Incubation timing is therefore critical in rapid bioaffinity assays. Reading the result from non-equilibrium phase is also proposed to be more susceptible to variation in incubation time and assay conditions resulting in error in assay results, especially in heterogeneous format (Soukka, 2003).

Instrumentation and measurement technique related errors

Errors due to instrumentation, such as laboratory equipment, are inevitable. In laboratory work, pipetting error is the major contributor to the overall measurement error in an immunoassay (van Emon, 2007). The errors in pipetting result mainly from inadequate precision in pipette calibration and/or human errors e.g., lack of competence. Bioaffinity assays are often carried out by using a plate reader. The plate variations and the well position on the plate should be checked by carrying out intra and inter assays. The errors caused by the detection system are dependent upon the measurement technique and should be studied individually with consideration of the possible pitfalls of each technique. Common error sources and limiting factors are inadequate calibration and instrumental noise.

Miscellaneous error and sensitivity limiting factors

The characteristics of binding components (e.g. stability) affect the assay performance. The binding component (e.g. a protein) may be denatured and undergo conformational changes upon adsorption at solid-liquid interfaces, which may affect the irreversibility and change protein activity and function (Nakahishi *et al.*, 2001). The shelf-life expectations for assay products when stored at optimal recommended conditions are often underestimated to guarantee assay performance. However, degradation or loss in affinity of binding components during storage can be caused by denaturation or degradation. Thus degradation of assay products leads to poorer assay performance over time and this fact must be taken into account when results are compared.

2.3 Enzyme kinetics

In clinical chemistry, the interest is not always in the concentration of a substance in a sample. In many cases, the interest is focused on the biological activity which has clinical significance. The activity of a reaction can be catalyzed by enzymes, which are highly specific to the reaction they catalyse due to complementary structure, charge and hydrophilic/hydrophobic properties. Enzymes are proteins or RNA molecules which may require a functional group, such as amino acid residues with a substituent group (coenzymes) and/or metal-ions (cofactors) for their catalysis (Nelson and Cox, 2004). The purpose of enzymes is to increase the reaction rate; they do not to affect the equilibrium of the reaction and are not consumed in the reaction. The enzymes can accelerate the reactions from five (5) to seventeen (17) orders of magnitude (Nelson and Cox, 2004). The measurement of enzyme activity in a sample is important in the diagnosis of certain illnesses and many biological effects of drugs result from processing by enzymes. Further, enzyme kinetics and kinetic parameters are used to study the role of individual amino acids in enzyme structure and the reaction mechanism and also to compare the activities of different enzymes. The regulation of enzyme activity is determined by the amount of the product needed. The enzyme activity can be lowered by inhibitors in order to maintain the balance of chemical reactions. Enzyme reactions

often form a preferable reaction pathway, in which the regulation of enzyme activity is controlled by feedback inhibition and/or allosteric regulation (Pardee and Reddy, 2003).

2.3.1 Enzyme-catalysed reactions

According to generalized transition state theory, a reaction requires a fixed amount of activation energy in order to reach the transition state at which proceeding to a substrate or a product is equally probable. The enzymes lower this activation energy of the reaction, which they catalyse. The enzyme-catalysed reactions can be divided into two steps, binding and catalysing. In a simplified enzyme-catalyzed reaction, the reaction progresses from substrate S and enzyme E through intermediates of ES (enzyme-substrate) and EP (enzyme-product) before conversion into product P. The binding interaction of a substrate and an enzyme is due to weak interactions and structural complementarity in the transition state. The formation of weak interactions in intermediates is accompanied by a release of a small amount of free energy, and the activation energy is lowered, thus providing a lower energy pathway (Copeland, 2000). Therefore, the energy for enzymatic rate enhancements is mainly contributed by the formation of weak interactions between the substrate and enzyme in the transition state. The kinetics of enzyme-catalysed reactions, which will be introduced next, have been widely researched, but the protein dynamics and a detailed understanding of the entire trajectory of the enzymatic catalysis are still a challenge (Schramm, 2005; Eisenmesser *et al.*, 2005).

The substrate concentration determines the velocity of the enzyme reaction. At low concentrations of substrate, the initial rate of reaction increases linearly until the inflection point and saturates to its maximum. At higher substrate concentrations, the changes in substrate concentrations do not affect the rate due to the lack of free enzymes, see Figure 9. The velocity of the catalytic reaction in enzymatic reaction is characterized by the steady state approach. The steady state describes the time period during which the rate of formation of intermediate ES equals its decay rate to free enzyme and substrate (E + S), and the catalytic rate of the reaction can be derived (Copeland, 2000). This dependence can be expressed by the Michaelis-Menten kinetic model, see equation (7), where K_m stands for the Michaelis constant representing the ratio between the kinetic rates of reverse (dissociation and catalytic rate) and forward (association rate) reactions considering the intermediate (ES):

$$V_0 = \frac{V_{\max}[S]}{K_m + [S]} \tag{7}$$

The model applies for single-substrate kinetics and the kinetic constants in enzyme-catalyzed reactions K_m and V_{max} are not appropriate parameters to compare different enzyme-catalyzed reactions since the values vary from enzyme to another. The maximum velocity at which the reaction can proceed is defined by the limiting rate of the reaction. In enzyme kinetics the overall rate of catalysis for collective chemical steps is the turnover

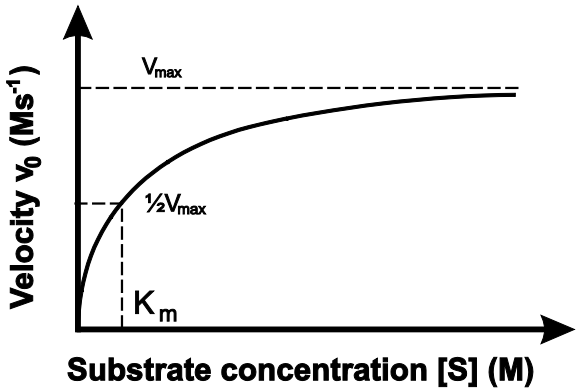

Figure 9. Michaelis-Menten (MM) curve. The MM curve illustrates the dependence of substrate concentration on the reaction rate in an enzyme-catalyzed reaction. The curve converges to the maximum velocity, V_{max} and the substrate concentration at half the maximum velocity ($½V_{max}$) represents the Michalis-Menten constant K_m.

number k_{cat}, which is the maximum number of substrate molecules that an enzyme can convert to product per catalytic site per unit of time (Copeland, 2000). The catalytic efficiency is considered the best measure of substrate specificity and is described by the ratio of kinetic constant K_m and the turnover rate k_{cat} (8).

$$\frac{k_{cat}}{K_m} \tag{8}$$

Both enzymatic and receptor binding are characterized by hyperbola saturation curves. However, they may require a different interpretation and a direct translation of receptor equilibrium by using the knowledge from enzyme kinetics leads to flawed analysis (Krohn and Link, 2003).

2.3.2 Enzymatic reaction pathways and biological networks

Enzymatic processes may require a large collection of intermediates and activated phases until the substance is transformed into the final product. These enzymatic reaction pathways, as well as metabolic (in cell) and signalling pathways, are widely studied to gather all information to illustrate and summarize all the potential reaction pathways to the final product (Sinanoğlu, 1975; Lanzeni *et al.*, 2008; Conradi *et al.*, 2005). Interest has lain mainly in the activation mechanisms and qualitative properties of the enzymatic reaction system. The pathways are usually large and complex processes including feedback control in the regulation system and are described as reaction networks. The computational modelling of reaction pathways has been widely carried out by differential equation methods (Orton *et al.*, 2005). However, switch-like behaviour bistability and multistationarity issues, due to the complex reaction network, are the key concerns in the modelling of enzyme reaction systems (Crampin *et al.*, 2004; Craciun *et al.*, 2006).

During recent decades the stochastic simulation approaches have been gaining more ground on modelling biological reaction networks (Qian and Bishop, 2010).

Various researches have considered the reaction network theories promising and beneficial for systems biology. The network theories are also used to construct models which describe the biological system by means of a network structure and not by individual parameters. This kind of approach is considered beneficial since sparse time course data from experiments and lack of general standards for measurements provide qualitative rather than quantitative data for direct modelling (Orton *et al.*, 2005). The complexity of the biological model is thereby reduced, but the results are then based on the network structure itself. There are network theories developed for systems biology, such as the chemical reaction network theory (CRNT) and artificial neural network (ANN), (Feinberg, 1979; Feinberg and Horn, 1974; Baş *et al.*, 2006a; Baş *et al.*, 2006b). The CRNT was developed in the 70s to study the reaction mechanisms considering the dynamics of the chemical system and the complex network structure (Feinberg, 1979; Feinberg and Horn, 1974). The CRNT has been applied to the study of simple cases of enzyme activation mechanisms and it provides an identification and an exclusion of multistationary states in the pathway system, though limited to tens of complexes involved (Conradi *et al.*, 2005; Conradi *et al.*, 2007). ANN is composed of inter-connected adaptive processing elements (artificial neurons), which allow high parallel computing for data processing. The other benefits of ANNs in biological network modelling, according to Baş *et al*., are nonlinearity, robustness, learning, the ability to handle imprecise information and the capability to generalize. The ANN method has been applied to find a proper kinetic model and the kinetic constants of enzymatic reactions by training the network using biological experimental data until the minimum level of variation is reached (Baş *et al.*, 2006a; Baş *et al.*, 2006b).

In immunology, the Jerne interaction networks were used in the development of mathematical immunology and led to the proposing of the existence of regulatory immune networks (Jerne, 1972). However, the interest in Jerne networks faded over time due to the lack of a good correlation between the theoretical models and proper experimental validation (Louzoun, 2007). To my knowledge and according literature studied, network theories have not been widely applied to bioaffinity assays in practice.

2.4 Reaction modelling of bioaffinity assays

In bioaffinity assays, the modelling is focused on the behaviour of binding partner populations, not on the binding behaviour of a single binding pair. The main challenge in constructing a reliable reaction model is to combine theoretical concepts of binding interactions and experimental measurement data, as stated earlier. This highlights the importance of qualified quantitative biological measurement data, correct assumptions and boundary conditions. In the reaction modelling of bioaffinity assays, the appropriate

approach depends on the boundary conditions, limitations and the requirements for the model or simulation tool: e.g. if delivering a quantitative result rapidly is an asset for a simulation tool, then the time consumed in computing must be considered.

The binding reaction kinetics of biomolecules can be studied according to transient state kinetics and the law of mass action. According to transition state theory, the kinetic rates are dependent on the activation energy. Theoretical studies on binding interaction predictions based on binding and conformational energetics can be carried out by computational methods, since structural models are used to study the specificity of a binding pair (Morozov *et al.*, 2005). According to the law of mass action, the reaction kinetic analysis is restricted and valid for a closed system where there is no exchange of energy or mass between the system and the environment. In this thesis, the bioaffinity assays are performed under isothermal conditions; therefore, temperature variations are not considered. However, the temperature dependence of binding interaction parameters can be determined by thermodynamic analysis for simple binding reactions (van Regenmortel *et al.*, 1998; Tamil Selvi *et al.*, 2002).

This chapter summarizes the reaction modelling approaches and their usability in the modelling of bioaffinity assays by examining the pros and cons. Bioaffinity assays are performed in different environments (e.g. homogeneous media, solid phase platforms and microfluidic tubes) which affect the binding interactions. Therefore, rate limiting factors are considered in homogeneous and heterogeneous reactions in bioaffinity assays. A short review of the history of mathematical modelling concerning bioaffinity assays is presented in the light of immunoassays: from separation assays to real time binding reaction kinetics.

2.4.1 Homogeneous and heterogeneous reactions

The reaction modelling of bioaffinity assay has its intrinsic basis in the binding behaviour of the binding partners. However, the bioaffinity assay reflects the binding behaviour of a large number of binding partners and components in the total assay volume environment. A proper model for an assay also considers error and sensitivity limiting factors, presented in Section 2.2.6, and assay platform issues concerning heterogeneous reactions at solid-liquid interfaces and homogeneous reactions in liquid phase. The rate at which the end product (measurable complex) appears is determined by the slowest reaction - limited either by the diffusion or kinetic (forward) reaction rate. The effect of diffusion in heterogeneous and homogeneous reactions has been studied carefully by Stenberg *et al*. and Nygren *et al.* (Stenberg *et al.*, 1986; Nygren and Stenberg, 1989). Further, the effect of the assay platform has been studied theoretically and it has been concluded that the reactions occurring on small spheres such as cell surfaces (micrometer scale) in well-mixed conditions do not easily become diffusion-rate limited. These homogeneous reactions can be reaction rate limited, while reactions at solid surfaces are diffusion limited for the same initial conditions (Stenberg *et al.*, 1986). Thus, the binding reactions in molecular and particle based assays can be considered

kinetic reaction rate limited, whereas at the solid-liquid boundary the very first binding reactions are diffusion rate limited, though the intrinsic reaction is limited by the kinetic reaction rate. The kinetic reaction rates can be estimated when the reaction is limited by reaction kinetics (Nygren and Stenberg, 1989). The geometry of the incubation chamber may have a crucial effect on the reaction mechanism in heterogeneous reactions at solid-liquid interfaces (Kusnezow *et al.*, 2006a). For instance, microspot arrays which would allow small assay volumes with single-step protocol and multi-analyte assay have been reported to have a strong dependence on the mass transport (Klenin *et al.*, 2005; Kusnezow *et al.*, 2006a; Kusnezow *et al.*, 2006b). The mass transport and diffusion rate limited binding are modelled by reaction diffusion theory, which considers separately the binding reaction and the transport of reactant in the liquid phase to the surface (Nygren and Stenberg, 1989; Kusnezow *et al.*, 2006b). More comprehensive theoretical studies on the diffusion-controlled irreversible and reversible binding in microspot assay are provided by Klenin *et al.* (Klenin *et al.*, 2005).

It is widely assumed that the surface of the solid-phase is homogeneous and bound antibodies are distributed evenly and randomly across the surface. However, there is some evidence that antibodies may form self-similar fractal clusters, high densities of highly organized antibodies in the solid phase (Wild, 1994). In the surface, the diffusion-limited reactions on these clusters are expected to express fractal-like kinetics (Kopelmann, 1988). Fractal analysis of diffusion rate limited binding kinetics is more often utilized in *in vivo* conditions (Schnell and Turner, 2004) and rarely in *in vitro*. However, fractal analysis has been carried out in studying binding in the liquid-solid boundary of biosensor surface (Butala *et al.*, 2003).

2.4.2 Approaches in binding reaction modelling

There are both descriptive and mechanistic model approaches used in modelling bioaffinity assays. Descriptive models have been used to model both dose-response curves and binding reaction kinetic curves (Zuber *et al.*, 1997a). In the reaction modelling of bioaffinity assays the interest is in the response due to changes of parameter or initial value. Therefore experimentally accessible parameters (i.e. concentration, time and kinetic association and dissociation rate constants) and their dependence on the reaction mechanism are required for a precise model. Such mechanistic modelling approaches have been used to study the kinetic behaviour of the assay system (Rodbard and Feldman, 1978; Rodbard *et al.*, 1978; Zuber *et al.*, 1997b; Hänninen *et al.*, 2003)

The deterministic mechanistic approach in the modelling of binding reactions

The classical, deterministic mechanistic approach to modelling association and dissociation reactions in bioaffinity assays has at its core the law of mass action, which was proposed by Arrhenius in 1907 (Arrhenius, 1907), and has proven to be extremely successful in the *in vitro* environment (Turner *et al.*, 2004). The model considers the errors of the initial components to be Gaussian-distributed; therefore, the parameters are

considered to be average values of the population. The models are based on constructing a set of ordinary differential equations (ODEs) for each binding component as a function of time. The mechanistic modelling approach is presented in more detail later in this chapter (2.4).

The advantages of the deterministic mechanistic approach:
(1) Identifiable parameters
(2) Continuous, wholly predictable process

The limitations and requirements for deterministic approaches:
(1) Restricted to well stirred, homogeneous environments, populations large enough to result in an average behaviour and without being disturbed by single fluctuations
(2) Do not result in a low level of details (in comparison to thestochastic approach, presented later)
(3) Parameter dependence must be identified in the reaction mechanism

Stochastic approaches in the modelling of binding reactions

The arguments for stochastic models for chemical reactions involve the consideration of the discrete or random character of the component or the phenomenon, in accordance with the theories of thermodynamics and stochastic processes, and their appropriateness for describing small systems and instability (Turner *et al.*, 2004). Stochastic approaches are inherently suited to *in vivo* conditions, such as in cellular systems (Gillespie, 2007; Nicolau and Burrage, 2008).

Efforts and developments to mathematically accommodate the stochastic nature of well-stirred chemically reacting systems and molecular dynamics were made by McQuarrie and later by Gillespie (McQuarrie, 1967; Gillespie, 1976; Gillespie, 1977). The stochastic approach to chemical reaction kinetics can be described by the probability density function (PDF), the time evolution of chemical reactions by differential-difference equation, the chemical master equation (CME) or stochastic simulation algorithm (SSA). The PDF describes the entire expected distribution which a continuous random variable has in a given interval. It is also used to describe the relative likelihood for a random variable at a certain point in a distribution curve. The CME determines the equation of probabilities and summarizes all the fluctuations of the system in the time evolution (Gillespie, 1976; Érdi and Tóth, 1989). Thus, the CME describes the molecular system by determining all the possible behaviour cases of each molecular species. Therefore, it is usually difficult to solve the CME analytically (Gillespie, 2007). The SSA was introduced by Gillespie in 1976 to overcome the problems that resulted from the CME. The SSA is a Monte Carlo procedure for numerically generating time trajectories of the molecular species and can be implemented when the CME cannot be solved. The SSA results are in exact accordance with the CME (Gillespie, 1977; Gillespie, 1992). However, the SSA approach requires massive computing and is therefore time consuming. A review of these stochastic approaches has been collected by Gillespie (Gillespie, 2007).

The advantages of the stochastic approach are listed as follows: (Gillespie, 1977; Nicolau and Burrage, 2008; Louzoun, 2007; Turner *et al.*, 2004):

(1) possible to take full account of the fluctuations and low level details (such as random behaviour of single molecules)
(2) never approximates the infinitesimal time increments but by finite time steps,
(3) easy to code for any specified set of chemical reactions

The limitations of and requirements for the stochastic approach:

(1) Requires several iterations and therefore excessive computer time and costs,
(2) Detailed information in the simulation result may be lost in the noise
(3) Requires a random number generator

Thus, the general advantage of stochastic approach is the possibility of including a great number of details; however, the drawback is the difficulty in understanding the basic mechanisms affecting the dynamics (Louzoun, 2007; Turner *et al.*, 2004).

The deterministic-stochastic hybrid approach

The appropriate approach, stochastic or deterministic, has been argued, though it would have been more fruitful to focus on the complementary aspects of the deterministic and stochastic computational methods instead of the competitive aspects (Gillespie, 1977). Since there are inevitable benefits to both approaches, hybrid models have been introduced for solving chemical systems (Hellander and Lötstedt, 2007). A combination of both approaches can be applied by separating the components into subsets: those which can be treated as normally distributed, and those which require the stochastic treatment. This separation could also be carried out by using biological insight. This is proposed in order to either introduce stochasticity in some components of the reaction rate equation or to improve the efficiency of stochastic algorithm by reducing the number of species in the system vector (Hellander and Lötstedt, 2007).

2.4.3 Modelling of binding reaction kinetics – from equilibrium assays to non-equilibrium assays

The development of the mathematical modelling of bioaffinity assays is presented here from the immunoassay point of view. Theoretical models developed for radioimmunoassays (RIAs) and immunoradiometric assays (IRMAs) were first constructed to optimize the assay conditions and estimate the assay performance, such as the minimal detectable dose, co-operativity and sensitivity (Rodbard and Feldman, 1978; Rodbard *et al.*, 1978). Graphical methods were first utilized in studying the binding parameters based on experimental data and knowledge from the ratio of bound and free ligands in assay under equilibrium conditions (Yalow and Berson, 1960). In immunoassays, the binding site occupancy was studied by constructing a Scatchard plot (Pennock, 1973; Walker, 1977). In the Scatchard plot, the ratio of bound and free ligands was plotted against the

bound complex and the dissociation constant was estimated from the plot, see equation (9) and Figure 10.

$$B = \frac{nL}{K_d + L} \tag{9}$$

In equation (9), B represents the bound concentration, n the total concentration of binding sites, L the free component (ligand) concentration and K_d the dissocation constant.

However, the use of Scatchard plot was criticized due to fitting data in straight line by linear regression, though the shape of the plot was often more complicated; e.g., ligand heterogeneity increases the degree of curvilinearity and results in a hyperbolic curve (Pennock, 1973; Thakur *et al.*, 1980; Mendel *et al.*, 1985). The effects of variations in assay parameters and conditions (affinity constant (*K*), specific activity (radionuclides), concentrations and duration of the reaction) were studied by simulation methods (Rodbard and Weiss, 1973). Notably, at that time there were only equilibrium separation assays available and effort was therefore put into the equilibrium binding studies and separation steps. The separation step, the separation of free components from bound components, was a concern because the secondary reaction could perturb the primary reaction (Rodbard and Catt, 1972).

Theoretical time course analysis of binding reactions in immunoassays began around 1970s (Rodbard and Weiss, 1973; Vassent, 1974; McPherson and Zettner, 1975). The study of binding interactions was based on a binding pair, and the reaction schemes were simple due to separation steps. The following preliminary theoretical limitations and assumptions were considered in a model in order to obey the law of mass action (Price and Newman, 1991):

(1) Both antigen and antibody have to be present in homogeneous form, consisting of only one chemical species each
(2) Both antigen and antibody must be univalent
(3) There should be no effects to modify the reactivity of the binding partners

The solution for the binding reaction was carried out by constructing time derivative of the bound component, e.g. for competitive binding of single ligand species (P) and specific binder molecule (Q) in equations (10-12). This approach was used to graph the

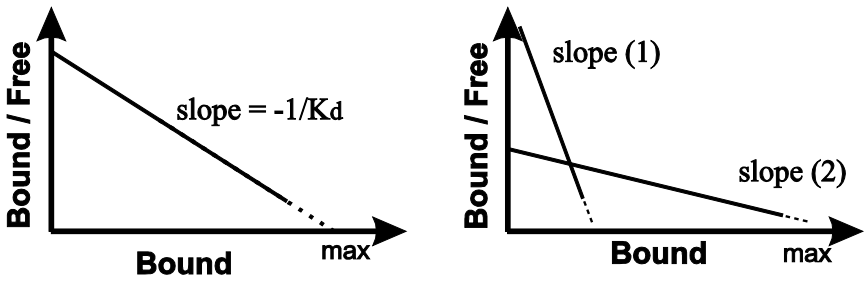

Figure 10. Scatchard plot for one binding specie (on left) and for two binding species (on right).

simple kinetic behaviour of binding and to estimate the incubation time for equilibrium (McPherson and Zettner, 1975):

$$\frac{d[PQ]}{dt} = k_{on}[P][Q] - k_{off}[PQ] \tag{10}$$

If initial concentrations of P and Q are set to be p and q, then at any time:

$$p = [P] + [PQ] \text{ and } q = [Q] + [PQ] \tag{11}$$

Substituting (11) into (10):

$$\frac{d[PQ]}{dt} = k_{on}[PQ]^2 - \left(k_{on}p + k_{on}q + k_{off}\right)[PQ] + k_{on}pq \tag{12}$$

The modelling of the radioimmunometric (sandwich) assay was considered more difficult due to the higher number of parameters and experimental variables involved (Rodbard and Weiss, 1973). Rodbard and Weiss (Rodbard and Weiss, 1973) wrote the reaction scheme for an immunometric assay and solved it by numerical methods. These deterministic mechanistic assay models are the basis for modelling bioaffinity assays today.

Since the 1970s, computer simulations have been used in studying the equilibrium behaviour of binding complexes in simple cases, e.g. divalency. Most of the models in those days were 'deterministic' rather than stochastic in approach, dealing with systematic rather than random errors (Rodbard and Catt, 1972). In the 1980's, the computer-based approaches were employed for the characterization of equilibrium assay systems for dose-response data and calibrating the assay curve using LIGAND software (Munson and Rodbard, 1980).

2.4.4 Modelling of binding reaction kinetics – single-step bioaffinity assays

The single-step (homogeneous) assay method and the developments in detection technologies (both label-based and biosensor-based) together with real-time monitoring techniques have promoted the bioaffinity assay technologies. They provide the ability to monitor binding reaction kinetics in a nonequilibrium state without interrupting the reaction. This provides a more rapid way of obtaining measurement data (results) for the comparison of binding affinities and kinetics resulting from different binding conditions.

In single-step assays, the number of component species (initial components, intermediates and (final) bound products) present in the assay reaction increases the complexity of the binding interaction. From a modelling point of view, the larger number of parameters requires more computational capacity and advanced numerical methods. The mechanistic assay model based on the law of mass action for three component immunometric assay reaction scheme (equation 6) is constructed by writing a set of differential equations, in which the time derivative, the rate of change, is determined for each concentration of binding component present, see equation 13. In equation 13, the kinetic rate constants for

association reactions are associated with odd numbers (k1, k3, k5…) and for dissociation associated with even numbers (k2, k4, k6…).

By constructing a Jacobian matrix, which is a multivariable matrix consisting of all the partial derivatives of the vector in the system, the behaviour of the system can be simulated. This requires the initial concentrations, kinetic parameters and the duration of the reaction, i.e. the end time point. The calculation is performed by an ordinary differential equation solver (odesolver), which one can program oneself or use a ready-made solver provided by several program libraries, such as in R (R Development Core Team, 2005; Setzer, 2004). The odesolvers use numerical methods such as Runge-Kutta, which is an iterative method for approximating a solution.

The mechanistic assay reaction model has been shown to predict the behaviour of the assay kinetics over a wide range of analyte concentrations (Hänninen *et al.*, 2003; Zuber *et al.*, 1997b). Zuber *et al.* also studied the kinetic characteristics, such as the inflexion point and the kinetics steepest slope. They suggested that the inflexion point could be used as an indicator for samples suffering from analyte excess, the 'hook effect' (presented in Figure 6B). The time of occurrence was seen constant as long as the analyte concentration did not exceed the smallest antibody concentration. The steepest slope of the kinetic curve was correlating with the analyte concentration, which indicates the possibility of assaying higher analyte concentrations than can be done by classical end-point assay methods (Zuber *et al.*, 1997b).

As can be seen, mathematical modelling of reaction kinetics becomes more complicated when the number of binding complexes increases. Therefore, modifying the model by rewriting the equations in a large matrix becomes exceedingly cumbersome and error-prone. The mechanistic assay model has been criticized for its dependence on kinetic parameters, which have to be well-defined. However, in practice, by using a well-standardized assay system and a validated reaction model, early access to the results (signal level at equilibrium) is possible. This can be carried out by following on-line measurement and predicting the equilibrium signal level from non-equilibrium

$$
\begin{aligned}
\frac{d[Ag]}{dt} &= k2\cdot[AgAb]-k1\cdot[Ag][Ab]+k4\cdot[AgAb']-k3\cdot[Ag][Ab'] \\
\frac{d[Ab]}{dt} &= k2\cdot[AgAb]-k1\cdot[Ag][Ab]+k6\cdot[AbAgAb']-k5\cdot[Ab][AgAb'] \\
\frac{d[Ab']}{dt} &= k4\cdot[AgAb']-k3\cdot[Ag][Ab']+k8\cdot[AbAgAb']-k7\cdot[Ab'][AgAb] \\
\frac{d[AgAb]}{dt} &= k1\cdot[Ag][Ab]-k2\cdot[AgAb]+k8\cdot[AbAgAb']-k7\cdot[Ab'][AgAb] \\
\frac{d[AgAb']}{dt} &= k3\cdot[Ag][Ab]-k4\cdot[AgAb']+k6\cdot[AbAgAb']-k5\cdot[AgAb'][Ab] \\
\frac{d[AbAgAb']}{dt} &= k7\cdot[Ab'][AgAb]-k8\cdot[AbAgAb']+k5\cdot[AgAb'][Ab]-k6\cdot[AbAgAb']
\end{aligned}
\tag{13}
$$

reaction kinetics with prior knowledge of the reaction rates. This can all be done without interrupting the measurements as the binding reaction proceeds and the accuracy of the assay result increases upon longer incubation. Thus, by using model-based methods and a validated assay system, it is also possible to already predict the sample concentration from the non-equilibrium phase.

2.5 Detection technologies for kinetic measurement of bioaffinity assays

There exist several technologies which can be utilized to detect real-time bioaffinity interactions and measure kinetic data. The general prerequisites for these technologies are single-step protocols, sensitivity and a real-time monitoring system which enables prompt observation of the signal from the bound component. The technologies introduced here are divided into biosensor-based and label-based techniques.

2.5.1 Biosensor-based methods in bioaffinity assay techniques

There are several biosensor-based technologies utilized in measuring binding reaction kinetics and determining the kinetic rate constants for interactions (Rich and Myszka, 2007; Rich *et al.*, 2009). Here, the following technologies are presented: surface plasmon resonance (SPR), biolayer interferometry (BLI) and quartz crystal microbalance (QCM). The basic idea in biosensor-based methods is the sensor surface, which is coated by immobilized binding molecules. Dependent on the detection system the sample is brought into contact with the sensor and the binding of the sample molecules and the immobilized target molecules on surface is detected. These detection methods are label- and radiation free.

The most critical points in biosensor techniques are related to the biosensor surface area: immobilization of molecules on the surface and nonspecific binding (Chen and Sadana, 1996; Deng *et al.*, 2006). Another criticism is that the surface-based sensors suffer from mass transport, which alters the binding constants (Fong *et al.*, 2002). The biosensor -based methods require larger reagent volumes due to microfluidistics compared to small volume fluorescence based techniques. This causes costs and waste.

Surface plasmon resonance (SPR)

The surface plasmon resonance (SPR) based biosensor technique was introduced in the early 1990s (Patrick Englebienne *et al.*, 2003). The surface plasmon resonance biosensor involves a detection surface immobilized with a binding component (the ligand). The immobilization is carried out by either a direct ligand immobilization with covalent attachment or by a ligand -capturing method. In ligand -capturing method, a specific molecule is immobilized on the sensor surface to capture the ligand. The biosensor is located inside a microfluidic channel through which the analyte (the sample) flows and binds to the ligand. The biosensor is attached to an optical set-up, see Figure 11.

The principle of the SPR method is to evoke the resonance-phenomenon of the surface plasmons, excitations in the metal-dielectric interface caused by the incoming beam of light with specific intensity, wavelength and angle of incidence. The photons of p-polarized light interact with the free electrons of the metal layer. The excited surface plasmons induce surface plasmon resonance and the electromagnetic waves propagate parallel to the dielectric-metal surface. Due to the loss of energy, the intensity of the reflected light is suppressed at a specific wavelength angle, and the change in refractive index due to binding causes a shift in the resonance angle. The refractive index changes at the sensor surface are detected as an SPR signal. The use of a functionalized metal surface, the high energy concentration in the near field of surface plasmons and high sensitivity makes it possible to detect adsorption of molecular monolayers on the surface (*Maier, 2007; Homola, 2006*). The SPR system consists of either an evaporated metal layer on dielectric media, e.g. glass (Kretsch mann configuration), or a metal layer isolated by air (Otto configuration) (*Homola, 2006*). The former set-up is generally used due to advantages in the freedom of design of the liquid handling system and is introduced in Figure 11.

SPR biosensors have been used to carry out equilibrium analysis, determine the binding constants and thermodynamic properties. The association rate can be determined from the SPR signal, when the analyte binds to the immobilized ligand and reaches its equilibrium. After this a solution without analyte (buffer) is injected in the flow cell and the dissociation rate can be determined from the decreasing SPR sig-nal. The effect of nonspecific binding in SPR biosensor is estimated by measuring a reference cell which does not include the sample analyte. Higher throughput screening has been increased by increasing the number of flow cells and by using a microspot array plate as a sensor surface (Rich and Myszka, 2007). However, combining flow cell microfluidics with the array plate system causes transport rate deviations between the spot positions on the array plate (Rich *et al.*, 2008).

There are several manufacturers of commercial SPR biosensors e.g. BiaCore AB (part of GE Healthcare), which manufactured the first commercial SPR biosensor launched.

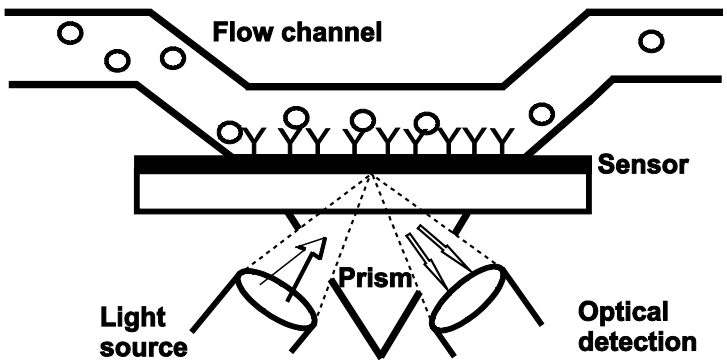

Figure 11. A schematic figure of an optical biosensor using the surface plasmon resonance technique.

There are also Bio-Rad Laboratories' ProteOn, Reichert's SR7000 DC and BioNavis' SPR Navi™ (Rich *et al.,* 2008; www.bionavis.com).

Bio-Layer Interferometry (BLI) Technology

Bio-Layer Interferometry (BLI) is a label-free technology based on an optical fibre biosensor technique for detecting and quantifying molecular interactions in real time (Abdiche *et al.*, 2008). BLI analyses the changes in refractive index using the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor, and an internal reference layer. Any change in the number of molecules bound to the biosensor causes a shift in the interference pattern that can be measured in real-time using optical interferometry. Therefore, binding between a ligand immobilized on the biosensor surface and an analyte in the solution produces an increase in the optical thickness of the biosensor, which in turn results in a shift of the interferogram and is a direct measure of the change in thickness of the biological layer (www.fortebio.com).

A commercialized BLI based biosensor called Octet is manufactured by ForteBio (Menlo Park, CA, USA), shown in Figure 12. The Octet biosensor is a dip-and-read assay with a single-use sensor tip customized for the binding assay. The Octet platform also offers a parallel set of assay measurements when sensor tips are delivered to the sample wells and no extra microfluidics is required (Abdiche *et al.*, 2008).

Quartz crystal microbalance (QCM)

Quartz crystal microbalance (QCM) was developed to analyse mass and viscoelastic changes on rigid surfaces 50 years ago, first in gas and vacuum, and later in liquid media (King, 1964). The quartz crystal microbalance-based sensor technique utilises an oscillating piezoelectric quartz crystal, the resonance frequency change of which is proportional to the mass change of deposited material on the quartz's surface (Schaible *et al.*, 2004). The detection device is connected to an oscillator with a real-time measurement system. A schematic of aQCM-based biosensor can be seen in Figure 13. This method

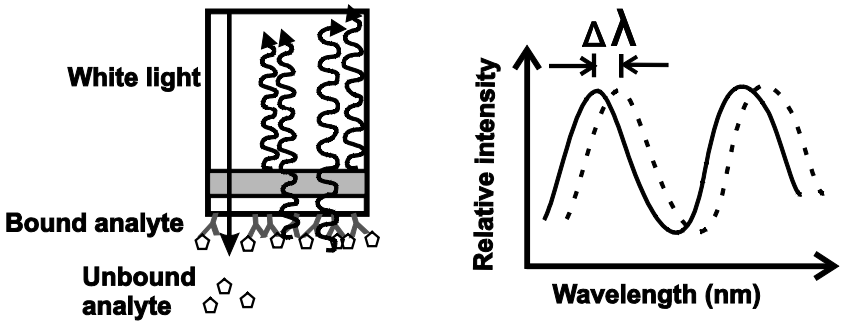

Figure 12. Application of the Bio-Layer Interferometry based biosensor, the Octet. The biosensor tip is coated with a biocompatible matrix and customized for binding assays. The changes in the sensor surface thickness are detected by a shift of the wavelength in the interference pattern due to binding interaction (www.fortebio.com).

provides a nanogram-sensitive measurement technique for mass and viscoelastic changes and can therefore be utilised as a biosensor for measuring the binding of biomolecules on-line. In the biosensor method, the biological molecules bind to the immobilized target molecules on the surface of quartz crystal and the changes of frequency are monitored during the binding process. The quartz crystal microbalance method has been developed for screening purposes and for evaluating the binding kinetic and equilibrium constants of binding reactions (Schaible *et al.*, 2004; Deng *et al.*, 2006; Másson *et al.*, 1995). A commercial QCM-based biosensors is available the Q-Sense QCM-D system by BiolinScientific AB.

2.5.2 Label-based methods in bioaffinity assay techniques

Label-based methods are widely used among bioaffinity assays detecting various physical phenomena **(**Hemmilä, 1991; Wilumsen *et al.*, 1997). The principle of label-based techniques is to attach a detectable (tracer) label to one of the binding components of interest, observe the detectable signal during the binding reaction and determine the concentration of the sample. In the 1950s, the radioisotopic labelling technique provided higher sensitivity and shorter assay times compared to the other detection methods in diagnostics in those days. Alternative nonisotopic labels were also developed, such as enzyme labels, based on enzymatic reactions (change of colour) and luminescent labels, based on the energy source used for excitation: fluorescence, electroluminescence and chemiluminescence (Hemmilä, 1991). The reasons for this were the radioactive waste problems, slow performance times unless high doses of label were used, health risks and the public attitude against radioactive isotopes. However, the development of radioisotopic labelling based techniques resulted in e.g. scintillation proximity assay (SPA) using beta-radiation from low energy isotopes which provides high throughput, low sample volume and single-step assays (Hart and Greenwald, 1979; Cook, 1996).

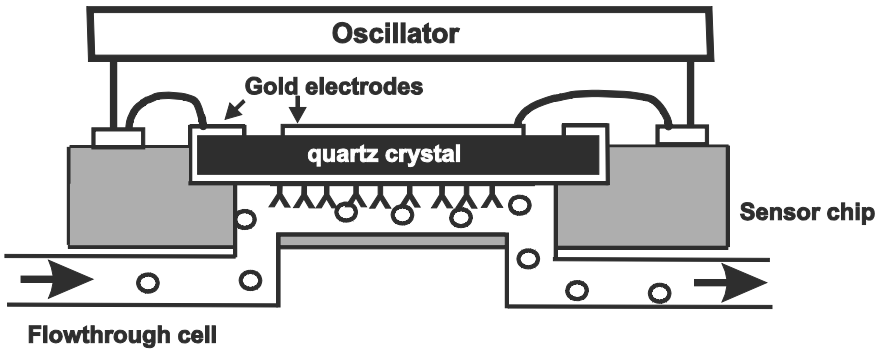

Figure 13. A schematic of a quartz crystal microbalance (QCM)-based biosensor. Gold plating provides the electrical contact to the oscillator and support for the biomolecule immobilization. It contains a quartz crystal with immobilized ligand and free analyte in the flowthrough cell (Schaible *et al.*, 2004).

Fluorescence is theoretically the most sensitive analytical technique (Bazin *et al.*, 2002). The first fluorescent-based marker application, the immunofluorescent staining technique, was developed in 1941 (Coons *et al.*, 1941). The developments and number of applications based on fluorescence techniques have significantly increased during the recent decades. The development of efficient high peak power lasers has been a prerequisite for several sensitive fluorescence techniques today. Applying confocal microscopy, the measurement volume can be reduced to a femtoliter and the sensitivity reaches down to the level of single molecule detection (Jäger *et al.*, 2003).

Fluorescence simultaneously yields several parameters of interest, e.g. intensity, lifetime, anisotropy and spectral characteristics, which will respond to environmental changes. Fluorescent labels, labels with delayed fluorescence and lanthanide chelates provide long-lifetime fluorescent labelling for single-step bioaffinity assays. However, there are several requirements for the label. The label should be optimal, contain a reactive group for covalent attachment to molecule, be biocompatible (pH, solubility, hydrophilicity), have appropriate physical properties in the assay (introduce minimum perturbation to the host molecule) and have appropriate photo-, electro- and physico-chemical properties for the excitation system (Meltola *et al.*, 2005).

The advantages of the homogeneous fluorescence-based bioaffinity assays are cost reductions due to microvolume samples, simplified assay protocols and the possibility offer multiplexing measurements for high-throughput screening (HTS). The usual disadvantages and sensitivity limiting factors among fluorescence techniques are autofluorescence, quenching and label-related issues, e.g. photobleaching. The signal-to-noise ratio and the background signal are also considered critical factors. However, background signal discrimination can be improved by instrumentation and optimal labelling chemistry.

The following fluorescence-based methods are introduced: Fluorescence correlation spectroscopy (FCS), Fluorescence polarization (immunoassay) (FPIA), time-resolved Förster resonance energy transfer (TR-FRET) and two-photon fluorescence excitation (TPX).

Fluorescence Correlation Spectroscopy (FCS)

The fluorescence correlation spectroscopy (FCS) technique has been developed for measuring molecular dynamics and concentrations at the single molecule level (Qian and Elson, 1991; Sterrer S and Henco K, 1997). FCS embedded within a confocal microscopy system is a sensitive technique to measure fluorescence intensity and fluctuations in microscopic detection volumes. The principle of FCS is to illuminate a focal volume and to excite fluorescent molecules, which will emit bursts of fluorescence photons. The FCS system is capable of discriminating between small and large molecules and the interaction between small fluorescent ligand and a large macromolecule can be determined and quantified from retarded diffusion. For example,

the molecules in solution diffuse through the focal volume by Brownian motion, and the time that it takes to diffuse is proportional to physical size. The rapid and sensitive photon detection system detects the fluorescence intensity fluctuations and the FCS software calculates the autocorrelation function, which decays according to the intensity fluctuation of diffusion time. This autocorrelation function is characteristic of molecular processes causing fluorescence changes, e.g. diffusion, reaction kinetics and photophysical triplet state (Visser and Hink, 1999; Michelman-Ribeiro *et al.*, 2009). The sensitivity limiting factors in FCS are the mass dependence (in order to discriminate between the labelled molecules), autofluorescence and fluorescence quenching.

Fluorescence polarization (FP)

Fluorescence polarization (FP) primarily provides information on the mobility and the orientation of the substance in the sample. In the fluorescence polarization assay method, the sample is illuminated by polarized light. The molecules that have their absorption dipoles (absorption transition moments) aligned with electric vector of polarized light are effectively excited. The electric field oscillates along a predetermined direction, and any change in the orientation of the transition moment during the excited state lifetime of the molecule will cause a decrease and depolarization of fluorescence (Lakowicz, 1983). Thus, the fluorescent-labelled analyte is bound to an antibody, the sample is excited by polarized light, the free and bound components have different rotational rates, and the emission of polarized light is detected from the bound labelled component. The competitive assay format is used in most cases of fluorescence polarization immunoassay (FPIA) in order to measure the increase in fluorescence polarization of fluorescent labelled antigens when bound by a specific antibody and decreasing when competiting with the sample antigens. FPIA provides a simple and single-step detection method which is suited for high-throughput screening (Smith and Eremin, 2008). A fully automated instrument and kits are commercially provided by, for instance, Abbott IMx™ (Shipchandler and Moore, 1995).

Time-resolved fluorescence resonance energy transfer (TR-FRET)

Fluorescence (or Förster) resonance energy transfer (FRET) is a distance-dependent interaction between two chromophores, an acceptor-donor pair with overlapping emission and absorption bands. The principle of FRET is to excite the donor, which will transfer the excitation energy from its excited state to the acceptor. This energy transfer occurs through dipole-dipole coupling within less than 100 Å distance, and the spectroscopic technique detects the decrease in the fluorescence intensity of the donor and the concomitant increase in the fluorescence intensity of the acceptor. The phenomenon of FRET indicates that the acceptor-donor pair is in proximity within a complex. A characteristic value for each FRET pair is the Förster distance at which the energy transfer efficiency is 50 % (Selvin, 2000). The FRET technique can be utilized in detecting changes in molecular proximity (DNA rulers), conformational changes of

single biological molecules and studying interactions between biomolecules (proteins, motifs of DNA and RNA) including real-time kinetics (Ha, 2001; Singh *et al.*, 1999: Mank *et al.*, 2006).

The time-resolved measurement system with fluorescence resonance energy transfer (TR-FRET) and homogenous fluoroassay technique, is applicable for measuring reaction kinetics (Bazin *et al.*, 2002). The commercially available high throughput, homogeneous, separation-free FRET-based systems, TRACE and HTRF technologies utilises the cryptate-based assay technique and LANCE technology utilises lanthanide-chelates (Zuber *et al.*, 1997b; Hemmilä, 1999). The FRET technique may also be applicable to different labels, such as bioluminescence resonance energy transfer (BRET), which is an variation of FRET, but with a bioluminescent label, i.e. luciferase as donor (Xu *et al.*, 2003).

TPX-technology

The TPX-technology is based on the two-photon excitation (TPE) microscopy technique with a pulsed near infra-red laser (e.g. 1064 nm). The two-photon excitation constitutes of simultaneous absorbance of two photons by a fluorescent label molecule, which corresponds to double the energy of a single photon. The advantages of this technology are the restricted small focal volume of about 1 fl (corresponding to the size of a microsphere), the optical pressure and trapping by Gaussian distributed and high average power of laser beam, and the background signal suppression. The optical pressure guides the microparticle through the centre of the laser beam focus, which is monitored by a microfluorometer. The fluorescence signal from out of the focus is suppressed during detection due to the properties of two-photon excitation (Hänninen *et al.*, 2000; Soini *et al.*, 2000a). The TPX technology utilizes biochemically-activated polystyrene microparticles as solid phase carriers, which correspond to the size of the focal volume, see Figure 14. Microparticles are continuously monitored and tracked three dimensionally by an optical scanner which stops when the backscattering amplitude rises above a pre-set threshold level, indicating the presence of a microparticle in the close vicinity of the focal volume. The fluorescence signal is detected and measured from the surface of microparticle. The fluorescence emitted at visible wavelengths from the labelled binding complexes on the surface of a single microsphere is related to the concentration of the analyte present. The optical set-up discriminates between the fluorescence signals of the unbound tracer molecules. This is achieved by the small focal volume and activating the measurement while a single microparticle appears at the focal point (Soini, 2002a; Hänninen *et al.*, 2000; Soini *et al.*, 2000a; Soini, 2002b).

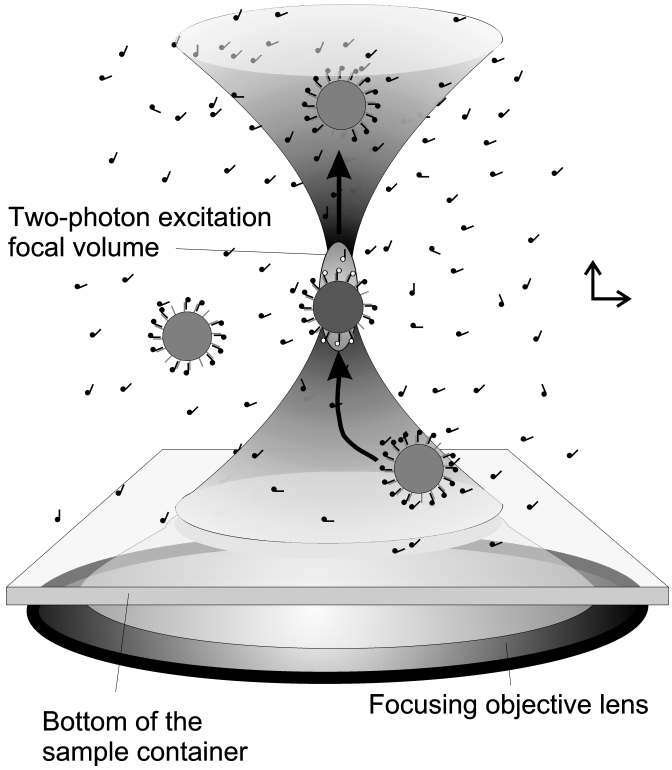

Figure 14. Fluorescence excitation of a single particle in TPX system. The signal is measured from the focal volume and is suppressed from outside the focus. The arrows indicate the optical pressure guiding the microparticles through the focal volume. (Figure courtesy of Juhani Soini and Marko Mäenpää.)

3 AIMS OF THE WORK

The aim of this work has been to develop computational model-based methods and simulation tools for bioaffinity assays, based on chemical reaction kinetics and the law of mass action. This research explains the possibilities that the modelling of binding reaction kinetics together experimental data provide. The methods developed in this thesis can be used to research and predict the behaviour of binding interactions (I-IV) and estimate the reliability of the bioaffinity assay results (I). The methods were confirmed by experimental kinetic data and are widely applicable also to other bioaffinity assays.

In brief, the objectives of the work were (articles indicated by Roman numerals according to which article the subject relates):

1) To estimate the reliability of rapid immunoassay concerning initial concentrations and incubation timing by mechanistic modelling and simulation methods (I)

2) To develop a new calibration method from assays that use kinetic data (II)

3) To develop a new node-based method and simulation tool for the modelling of complex multi-component assays, which can be applied to study specific binding in bioaffinity assays (III) and protein adsorption as a case study of modelling nonspecific binding (IV)

4 MATERIALS AND METHODS

Materials and methods used in the studies are described here briefly. Details of the studies are presented in original publications (I-IV).

4.1 Materials and kinetic measurement data

The binding reaction kinetic data measurements had been carried out earlier in our laboratory and published, for example, the kinetics of human thyroid stimulating hormone, hTSH (Hänninen *et al.*, 2003) (I, II, III) and the kinetics of human C-reactive protein, hCRP (Waris *et al.*, 2002)(II). The data were used for confirmation and comparison purposes in articles as indicated.

In the research on nonspecific binding, in this case protein adsorption (IV), several protein candidates for adsorption on polystyrene microparticle, were studied. In the end, the hen egg white lysozyme was selected due to several favourable properties: it is stable, globular (slightly ellipsoidal) in shape, small in size and hard protein, which does not undergo significant conformational change upon adsorption (Kubiak-Ossowska and Mulheran, 2010; Ravichandran, 2001; Wahlgren *et al.*, 1995, Lundin *et al.*, 2010). The reagents used in this study (lysozyme from hen egg white, polyethylene glycol *p*-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100) and hydrochloric acid) were purchased from Sigma-Aldrich (St. Louis, MO). Carboxylate modified polystyrene particles 3.098 µm in diameter were purchased from Seradyn, Inc. (Indianapolis, IN). Glycin was sourced from Merck KGaA (Darmstadt, Germany). High purity MilliQ water was used to prepare all aqueous solutions. Lysozyme was conjugated with dipyrrylmethene-BF_2 530, succinimidyl ester (BF530) (Arctic Diagnostics Oy, Turku, Finland).

4.2 Measurement methods for real-time kinetic data

All real-time binding reaction kinetic data was measured by TPX technology, presented as a detection technology in Section 2.5.2 ‘Label-based techniques’.

Reliable kinetic data requires careful incubation timing. The delay in assay measurement is period between adding the last binding component, which starts the incubation and binding process (t=0), and starting the TPX-measurement (t>0). This delay, duration of incubation, was timed separately in this study and considered by adding the exact delay time in the TPX measurements in the kinetic data analysis.

4.3 Mathematical modelling methods

The modelling and simulations of the binding reactions have been written using open source GPL-software R for constructing mathematical models based on differential equations (I, II, III) and the LabVIEW™-program for constructing mathematical models using the newly developed node-method (III, IV).

The R provides a statistical software and user interface with library R packages (R Development Core Team, (2005); Woodrow Setzer, 2004), for instance, random number generator and solvers for differential equations. The following R packages were utilized:

- ODESOLVE was utilized to solve ordinary differential equations with the function *lsoda*.

- NLME was utilized in weighted fitting with the function *gnls*.

The National Instruments' LabVIEW™ software was adopted for constructing the NODE-method to solve multicomponent binding reaction kinetics. LabVIEW™ provides a graphical programming environment with a user interface which is easily constructed and customizable by modifying built-in blocks. Thus, a network of functions was implemented rapidly with LabVIEW™.

5 RESULTS

The main results of this work consist of the following four original publications referred to by Roman numerals (I-IV).

The modelling approach in this work is mechanistic. The fluctuations in initial parameters and assay measurements are considered Gaussian-distributed due to the high number of occurrences. The temperature during assay performances was constant.

5.1 Theoretical assessment of errors in rapid immunoassays – how critical is the exact timing and reagent concentrations?

In *in vitro* diagnostics, obtaining rapid and reliable quantitative assay results is a great asset in patient care, contributing to correct treatment. The reliability of rapid immunoassay is a concern due to incomplete incubation to a non-equilibrium state. In the first article (I) the effect of error and variation of initial parameters in rapid immunoassays was studied in order to provide theoretical information about how the magnitude of error affects the response. Special attention was paid to timing and initial concentrations of antibodies, focusing on the early phase of incubation.

The behaviour of rapid assays was predicted by simulations using a carefully-constructed mechanistic assay model, based on antibody-analyte binding reaction kinetics and the law of mass action. This antibody-analyte binding reaction model was constructed for a three-component immunometric assay, and the kinetic rate constants were chosen to be those determined experimentally for an hTSH immunometric assay (Hänninen *et al.*, 2003). The range of the analyte concentration covered the dynamic range, while one concentration was selected outside these limits to represent a sample with excess analyte concentration and therefore suffering from the 'hook effect'. The magnitudes of errors in the input parameters were estimated using knowledge from practical immunoassays.

The following simulations were carried out and compared with an ideal binding reaction kinetic curve, which describes the assay reaction without errors. The deviations of these modelled kinetic curves were given in percentages of relative error.

1. The effect of error induced by variation of initial concentrations: antibody concentration on microparticle solid phase surface [Ab] and labelled antibody concentration [Ab']. The kinetic curves are modelled for reduced (-10 %) and extra (+10 %) antibody concentrations.

2. The effect of exact timing of the incubation adding an absolute error of 6 seconds.

3. The effect of Gaussian-distributed variation in incubation timing (standard deviations of 3s, 6s and 12 s) for different interrupted incubation times (60s, 120s,

300s, 600s). A collection of 100 randomized simulation responses represents 100 assay measurement replicates.

According to the simulations, the inaccuracy in the solid phase antibody concentration resulted in a higher error response than that due to the inaccuracy in the labelled antibody concentration (Figure 15). Reduced concentrations resulted in a higher error in assay response than increase of both antibody concentrations. A local minimum can be seen for a concentration of 150 mIU/l in the percentage of relative error at 1000 s of incubation. According to the incubation timing simulations, the absolute error of 6 s in incubation timing resulted in errors during the early phase of the incubation while error decreased after the first hundred seconds (Figure 16). The effect of Gaussian-distributed variation in incubation timing on reading the result from the non-equilibrium state (respective to interrupted incubation) was studied by predicting a collection of one hundred randomised assay responses for each interrupted incubation time point. In the simulations, the standard deviations of randomized variance in timing were 3 s, 6 s and 12 s. The interrupted incubation time points selected were 60 s, 120 s, 300 s and 600 s. The results of these simulations are presented as percentage of the coefficient of variation (% CV), which is widely used to describe the inter-assay performance of replicate measurements (Figure 17). According to the simulations, the inaccurate incubation timing adds error to the results at very short incubation times, especially at low analyte concentration level, but the error diminishes significantly after the first hundred seconds.

The first derivative curves of the modelled kinetic assay curves were calculated and the inflexion points were studied. This highlighted early detection of the out-of working range concentration suffering from the 'hook effect'. The inflexion point was reached after 70 s by out-of-range concentration, and after approximately 200 s by in-range concentrations.

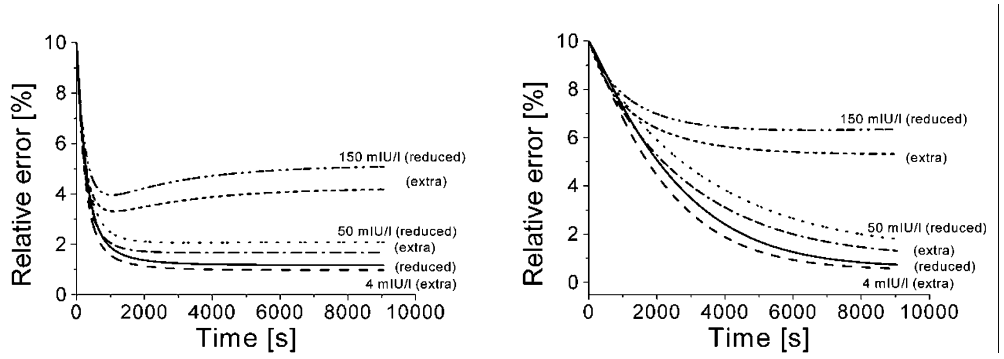

Figure 15. Relative error in percentage caused by inaccuracy in A) labelled antibody concentrations [Ab'], and B) solid phase antibody concentrations [Ab]. The error induced was -10% for reduced and +10% for elevated initial concentrations, respectively. (Modified with permission after: Pilvi J. Ylander, Zoltán Bicskei, Pekka Hänninen and Juhani T. Soini (2006) Theoretical assessment of errors in rapid immunoassays – how critical is the exact timing and reagent concentrations? *Biophys. Chem.* 123: 141-145).

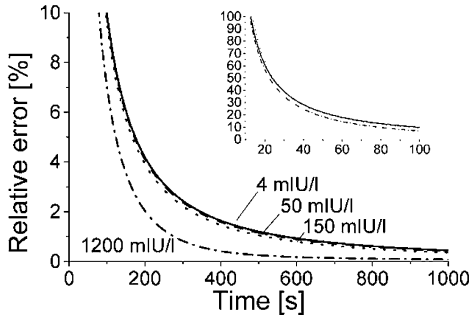

Figure 16. Relative error in assay response, when there is an absolute error of 6 seconds in timing. (Modified with permission after Pilvi J. Ylander, Zoltán Bicskei, Pekka Hänninen and Juhani T. Soini (2006) Theoretical assessment of errors in rapid immunoassays – how critical is the timing and reagent concentrations? *Biophys. Chem.* **123:** 141-145).

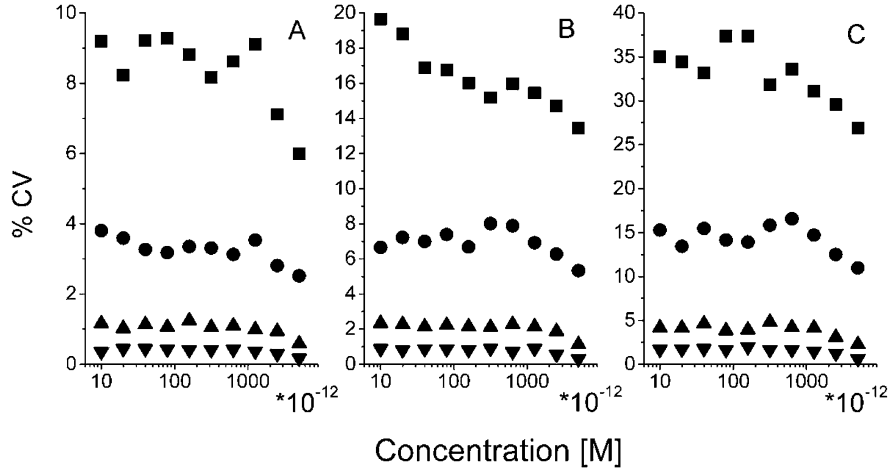

Figure 17. Percentage of the coefficient of variation (% CV) of assay response as a function of analyte concentration range due to randomised Gaussian-distributed variation in incubation timing for the following standard deviations A) 3s, B) 6s, C) 12s. The results are read after the following incubation time points: 60 s (square), 120 s (circle), 300 s (triangle) and 600 s (upturned triangle). (Modified with permission after Pilvi J. Ylander, Zoltán Bicskei, Pekka Hänninen and Juhani T. Soini (2006) Theoretical assessment of errors in rapid immunoassays – how critical is the exact timing and reagent concentrations? *Biophys. Chem.* **123:** 141-145).

5.2 Calibration method for bioaffinity assays using kinetic data

Bioaffinity assays are usually calibrated by conventional methods, constructing a dose-response (standard) curve using replicates of standard concentration measurements. The curve is fitted to a descriptive model of a certain shape; however, these descriptive empirical models do not take into account the underlying binding reaction mechanism. In this article (II), a new calibration approach based on the mechanistic assay model is

presented. This approach considers the reaction kinetics and requires only one standard concentration and one non-equilibrium kinetic data point in order to calibrate the assay system. This new calibration method is quick to perform, whereas the conventional methods would require six standard concentrations over the dynamic range measured to the equilibrium.

This calibration method was constructed by two iterative processes, one for the actual calibration process and another one for calculating the concentrations of unknown samples, see Figure 18. The first process calculates the reaction response using the required constants: initial concentrations for standard measurements [Ab_0], [Ag_0] and [Ab'_0] and estimates for kinetic reaction rates k_i. The reaction modelling is carried out by a mechanistic assay model. The measured signal is scaled by an instrumentation scaling factor converting the signal counts to concentration of bound component (AbAgAb'). The kinetic reaction rate constants are refined by weighted minimization of the difference between the scaled measurement signal and the modelling response concentration in the time domain. The minimization is carried out by weighted Generalized Nonlinear Least Squares (GNLS) fitting. Special attention has been paid to time points in early phase binding and near the equilibrium using larger weights in order to fix the start and equilibrium plateau. The refined rate constants k_i are used in numerically solving the ordinary differential equations of the reaction model for the minimization procedure until the stopping criteria are reached: high number of iterations and a limit for error margin**.**

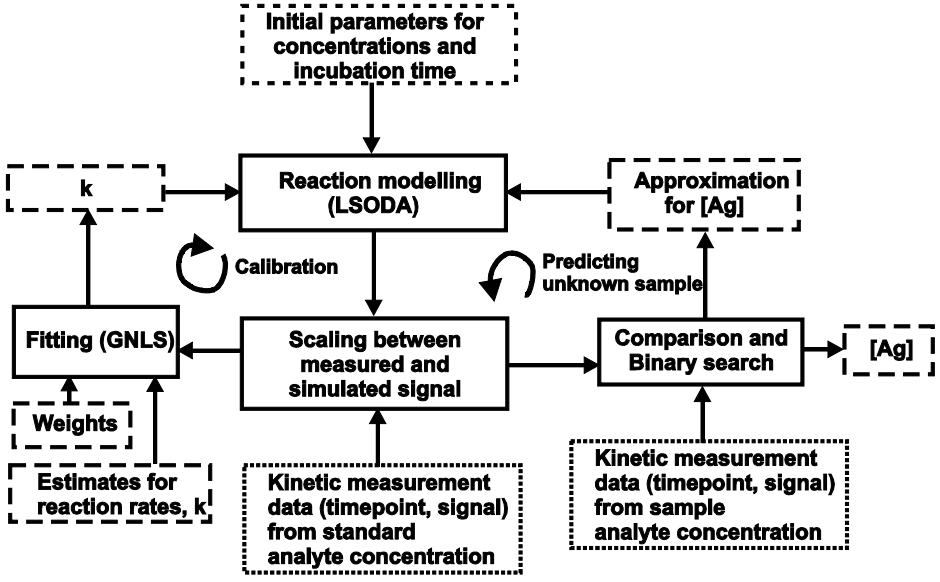

Figure 18. Procedures for calibration (left) and predicting a sample concentration (right). (Redrawn and modified with permission after: Zoltán Bicskei, Pilvi Ylander and Pekka Hänninen, Calibration of bioaffinity assays using kinetic data. *J. Biochem. Biophys. Methods* 67 (2006) 75-85).

The calculation of concentration for analyte samples is carried out by reading the kinetic data (timepoint and signal) at any time during incubation. A binary search algorithm is used to approximate the analyte concentration by comparing the measured signal with the reaction model response for the approximate analyte concentration. The method was evaluated by experimental data from hTSH and C-reactive protein (CRP) assays, see Figure 19 for calibration of an hTSH assay. The calibrated standard curves were constructed after three different incubation times: 1000 s, 2000 s and 4000 s. The simulated dose-response curves were seen to fit well with the measured signals. The tolerance of the method was tested by reducing the data set from 100 measurement points to 30 points, but keeping the incubation time constant. The reduction of data points did not affect the quality of the calibration curve.

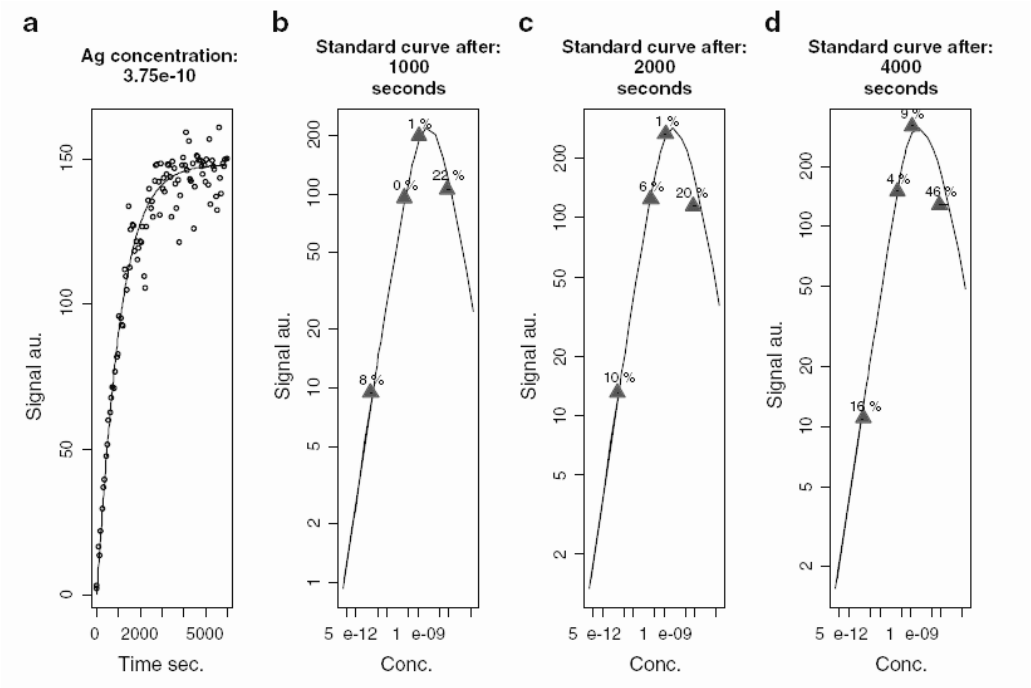

Figure 19. Calibration performed by a) hTSH assay kinetic data using analyte concentration of 50 mIU/l. Calibration curves after b) 1000 s c) 2000 and d) 4000 are presented. (Modified with permission after: Zoltán Bicskei, Pilvi Ylander and Pekka Hänninen, Calibration of bioaffinity assays using kinetic data. *J. Biochem. Biophys. Methods* 67 (2006) 75-85).

5.3 A node-based method for simulation of multi-component binding assays

The modelling of binding reactions may become too complicated for traditional numerical methods, if multivalent components are present in the binding reaction. This leads to binding complexes, which are not considered in the basic three-component assay model,

and as a result this model does not meet the complexity of experimental data. A new easy-to-use method was constructed for modelling complex multi-component bioaffinity assays (III), avoiding the large matrix when modelled by the mechanistic assay model. This method is a variant of the mechanistic assay model; it obeys the laws of mass action and utilizes the same initial parameters: initial concentrations, incubation time and kinetic reaction rate constants. The NODE-method will calculate a binding reaction network (of any shape) by decomposing the reaction network into separate binding reactions and small time steps according to Euler's approach using predetermined fixed time steps. Each binding reaction is represented by a node with two associating components and one dissociating complex, see Figure 20 for an example of a node and a three-component assay model scheme presented by the NODE-method. The network of nodes is calculated in a loop representing an indefinite small incubation time step.

This method was confirmed and compared with a conventional mechanistic assay model and an hTSH experimental binding data. The kinetic rate constants were chosen to be those determined for hTSH in immunometric assay using experimental kinetic data (Hänninen *et al.*, 2003). Simulations carried out by these two methods resulted in consistent binding curves. The effect of the time-step size used in the NODE-method was studied by comparing the kinetic curves delivered by the NODE-method, using different step sizes (1 s, 0.1 s and 0.01 s) with kinetic curves from the mechanistic assay model. The relative error % curves were calculated as a function of incubation time, see Figure 21. The reduction of step size from 0.1 s to 0.01 s minimized the relative error in results only by 1 % and was considered insignificant in comparison to the typical experimental errors.

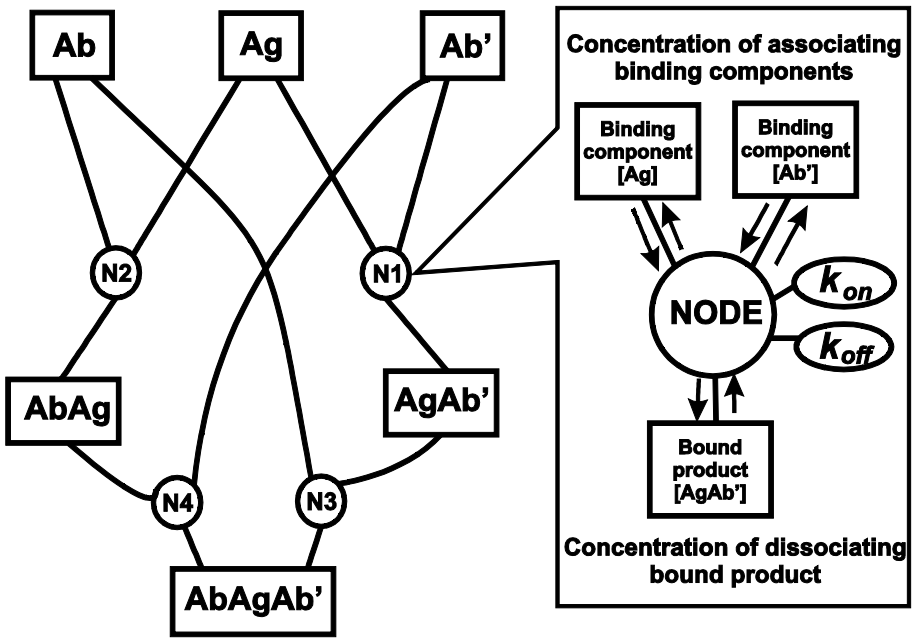

Figure 20. The reaction scheme of a three-component binding reaction based on the NODE-method. An example of a node is shown on right.

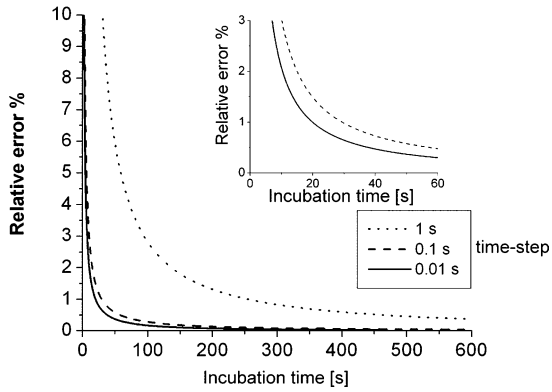

Figure 21. The effect of the time-step (0.01s, 0.1s and 1s) in the NODE-method compared to the mechanistic assay model as a function of incubation time. The insert allows comparison of the deviation between the two models during the first minute of incubation. (Reproduced with permission after: Ylander PJ and Hänninen P, Modelling of multi-component immunoassay kinetics – a new node-based method for simulation of complex assays. *Biophys. Chem.* 151 (2010) 105-110).

5.4 A study of the kinetic behaviour of protein adsorption based on the NODE-method

Nonspecific binding (NSB) is one of the sensitivity limiting factors in bioaffinity assays. In this article (IV), the protein adsorption of lysozyme onto the surface of polystyrene microparticles was studied as a case of nonspecific binding. The measurements were carried out by competitive assay protocol using labelled and unlabelled small globular (slightly ellipsoidal) lysozyme and single-step microparticle-based TPX technology. The kinetic behaviour was modelled by constructing a theoretical node net model utilizing our previously presented NODE-method and comparing the model with the experimental data. The number of microparticles present in the assay volume was converted mathematically to a concentration value, based on the free surface space on a microparticle for lysozymes to attach to. The lysozyme was considered to be globular and end-on or side-on combinations were not therefore considered separately.

The reversibility/irreversibility of lysozyme adsorption was monitored by incubating labelled lysozyme with microparticles until equilibrium was reached and adding 1000-fold unlabelled lysozyme to the assay volume. The signal dropped to 1/3rd of the maximum reached at equilibrium. This indicated that the adsorption is partly irreversible. The model was first constructed for single layer binding and compared with an experimental data set comprising various lysozyme concentration measurements, see Figure 23 for data plots. The model that resulted was too simple and did not meet the complexity of the adsorption reaction. The model was reconstructed by considering the multilayer structure of lysozyme proposed by Lundin *et al*. (Lundin *et al*., 2010), which led us to

study the possibility of the second layer structure and the dimerization of lysozymes, which can represent one of the reaction paths to the second layer structure. Thus, the node net was constructed to study the following adsorption processes: lysozyme adsorption onto the microparticle surface, lysozyme adsorption onto lysozyme already adsorbed onto microparticle (second layer structure) and lysozyme dimerization, see Figure 22 for node network. The purpose of this study was not to find exact kinetic rate constants but to show the possibilities of the NODE-method. All the measurements indicated rapid kinetics as seen in the experimental kinetic data plot in Figure 23. The primary reaction of lysozyme adsorption onto the surface of the microparticle had a major contribution to the shape of the reaction curve. The dimerization of lysozyme was insignificant in the adsorption process since a 1000-fold increase in the kinetic rate parameters of dimerization resulted in a less than 1 % deviation in the response signal for the studied range of lysozyme concentrations. This was also stated in the literature according to Wilson *et al.* and Carlsson *et al.* [Wilson *et al.*, 1996; Carlsson *et al.*, 2001]. The adsorption of lysozyme onto lysozyme, which is already bound to the microparticle surface, can be due to favourable conditions resulting from changes that lysozyme undergoes upon adsorption onto the microparticle in the first place. According to the results the proportion of the signal induced from the second layer components increased as a function of lysozyme and resulted in a 25 % proportion of the signal in the measurement of the lowest lysozyme concentration (603 nM). See Figure 23 for the experimental and modelled adsorption kinetic curves. An error analysis was carried out by statistical methods for estimating the best fit in terms of overall fit (sum of square

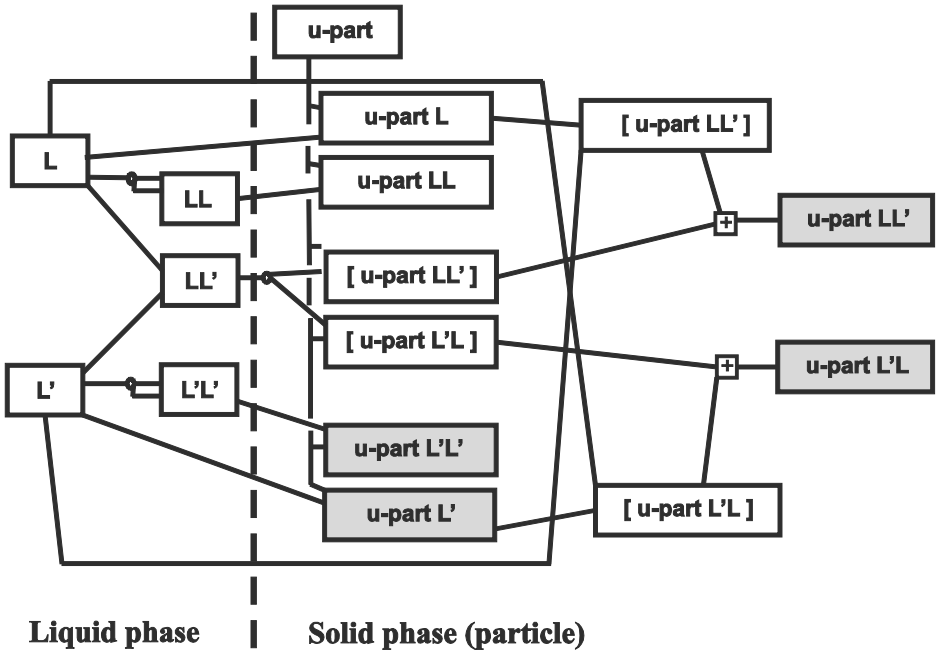

Figure 22. Network for modelling competitive adsorption of lysozyme (L) and labelled lysozyme (L') on polystyrene microparticles (here u-part). Complexes with shaded background represent the signal inducing components.

residuals, SSR), best unbiased estimation of error (mean square of error, MSE) and the goodness of fit in the non-equilibrium state (the coefficient of determination,). A precondition for the best fitted group was also to have a conveniently low error for the upper and lower concentration ranges. The simulated best-fitted group of model curves for various concentrations of unlabelled lysozyme agree with the experimental data to a reasonable extent. The purpose of this study was not to find exact kinetic rate constants but to combine modelled and experimental data and to show the possibilities of the NODE-method.

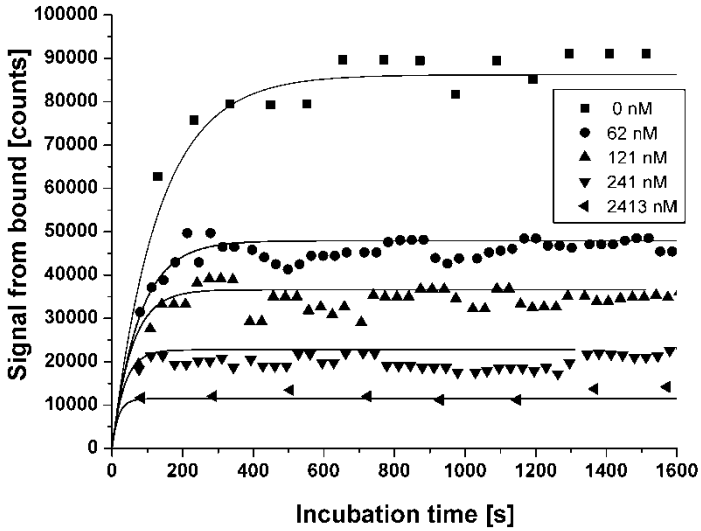

Figure 23. Experimental data (data plot) and modelled kinetic curves (solid lines) for a competitive lysozyme adsorption assay. The concentration of labelled lysozyme was constant in well (62 nM) and assay responses for various unlabelled lysozyme concentrations are presented as kinetic curves.

6 DISCUSSION

Computational model-based methods and simulation tools were developed for bioaffinity assays. These methods and tools were compared and verified with experimental bioaffinity assay data. The benefits and possibilities of the simulation methods in bioaffinity assays are demonstrated in assay reliability studies (I), calibration (II) and modelling of complex multi-component reaction kinetics (III, IV). This thesis emphasizes the importance of providing rapid and quantitative results concerning all the binding components present in an assay environment over the incubation time. The modelling approach was deterministic-mechanistic and the error factors and fluctuations in parameters are considered to be Gaussian-distributed. The kinetic measurement data obtained by sensitive TPX technology were an asset in developing the simulation methods in compliance with experimental data. This work can be considered as a continuation research of modelling reaction kinetics done by Rodbard, a pioneer who used the conventional separation assay data in the 1970s (Rodbard and Catt, 1972; Rodbard and Weiss, 1973; Rodbard and Feldman, 1978; Rodbard *et al.*, 1978), and also by Zuber, who used real-time, time-resolved fluorescence-detection-based TRACE ^{R}-technology in the 1990s (Zuber *et al.*, 1997b) and by Hänninen in the 2000s, who carried out the first modelling studies using data from real-time two-photon excitation fluorometry (Hänninen *et al.*, 2003). In the future, applications considering patient care and on-line monitoring systems can benefit from the further developments and results of mathematical biology and modelling methods.

6.1 Theoretical assessment of errors in rapid immunoassays – how critical is the exact timing and reagent concentrations?

Simulation methods based on a mechanistic assay model were used as a theoretical tool to study the effects of inaccuracies in incubation timing and errors in initial concentrations in rapid immunoassays (I). In rapid immunoassays, predicting the assay response result from the non-equilibrium state is proposed to be susceptible to error factors causing variance (Soukka, 2003).

For theoretical assessment an error of 10 % was introduced into initial antibody concentrations. The relative error is more critical in the early phase of the incubation in both antibody concentrations: the solid phase antibody [Ab] (antibody immobilized in microparticle) and the labelled antibody [Ab']. The solid phase antibody concentration Ab was seen to be more detrimental to the assay response during the whole incubation time. However, this result shows that using an excess concentration of labelled antibody in sandwich assays does not add a significant error in assay response. An excess of labelled antibody is sometimes used in order to speed up the binding reaction. One element is that the use of this excess amount of labelled antibody should be considered,

since it may reduce the signal-to-noise ratio, if the background signal is not suppressed in detection.

Theoretical assessments of errors in timing were studied by introducing an absolute error of six seconds (6 s) and randomized variation. The result revealed that for the early phase of the incubation timing is the most critical issue, and even more critical for low reagent concentrations. In the most critical case (incubation time 60 s and variance 6 s), the assay response approaches the limit of functional sensitivity (20 % CV). However, the absolute error and variance in timing decreased significantly during the first hundred seconds of incubation. In practice, the timing errors in a laboratory environment are usually caused by the users. With careful work, this error can be estimated to be 1 second. Considering this, the error caused by timing is small compared to the other error factors in assay measurement.

6.2 A calibration method for bioaffinity assay using kinetic data

Bioaffinity assays are usually calibrated in conventionally; fitting a set of standard measurement results to an empirical dose-response curve without concerning the reaction mechanisms. This newly invented calibration method carries out the calibration of an assay system using binding reaction kinetics and only one standard concentration measurement in a non-equilibrium phase. Thus, the method provides a fast calibration approach.

The reaction scheme in the calibration model presented a three-component binding reaction, which corresponds to the binding scheme of an hTSH sandwich assay The calibration method developed was confirmed with experimental data from practice, and simulated dose-response curves compared well with the signals measured from standards. The model also seemed to predict pentameric protein binding CRP well, though the complexity of the model does not correctly represent the actual reaction scheme of a sandwich assay of the pentameric protein binding

This calibration methodology is applicable for assay systems, for which the reaction mechanism, rate constants and instrument scaling are known, and for technologies which provide a real-time monitoring system. In the calibration process, the critical factor for refining the kinetic reaction rate constants was timing. In order to yield adequate rate constants, the kinetic measurement must be accurately timed. The benefits of this calibration method are rapid response time, low cost materials and decreased labour intensity, which are also key issues in POC diagnostics. Thus, the calibration method enables rapid diagnostics when accompanied by automated bioaffinity analyzer.

6.3 A node-based method for modelling multi-component binding reactions and the kinetic behaviour of protein adsorption

A new node-based method was developed to model multi-component binding reaction kinetics in bioaffinity assays. This method was compared with the mechanistic assay

model using the same initial parameters (III). The method was later applied to modelling nonspecific binding kinetics due to protein adsorption and compared with experimental data (IV). This method provides an easy and a quick way to study complex binding reactions utilising a simulation network structure which is simple to construct and modify by extension or shrinkage of the network.

The NODE-method developed (III) resulted in congruent kinetic curves when compared with the mechanistic assay model. The difference between these two methods is in the time-step defined in the calculation: dynamic (used by the solver for ordinary differential equations in a mechanistic assay model) or fixed (NODE-method). The comparison of the different time-step size in the results of the NODE-method (1s, 0.1s, 0.01s) revealed that the deviation between the results from the two methods (the conventional mechanistic assay model and the NODE-method) decreased as the incubation proceeded and using a shorter time-step resulted in more rapid convergence. The relative error between these two methods decreased to 1 % in 30 seconds with a step size of 0.1 second and in 20 seconds with a step size of 0.01 seconds. This error in results is insignificant compared to other errors in bioaffinity assay measurements. The error in the early phase of incubation was studied carefully given the results from article (I), which stated that the early state of incubation is sensitive to error factors.

The modelling of protein adsorption behaviour was studied as a case of nonspecific binding. The NODE-method presented above was utilized in modelling a competitive assay of labelled and unlabelled lysozyme adsorption onto the surface of microparticles. A simple single-layer model did not meet the complexity of the protein adsorption when compared with experimental data, and the model was reconstructed by adding the second layer structure, considering also the reaction pathways in the model. The purpose of this study was not to find the exact kinetic parameters for the model, but to identify the possibilities of the NODE-method compared to the conventional numerical methods. The NODE-method was seen as a valuable tool in studying the behaviour of bioaffinity assays and reaction mechanisms. The complexity of the reaction was easy to enlarge with the model scheme. The number of parameters was increased in this study with several intermediate components and the most effective way to benefit from the NODE-method is when some of the kinetic parameters are already known and only a few variables are studied.

7 SUMMARY

Mathematical modelling and simulations were shown to be valuable tools in studying the behaviour of bioaffinity assays. Bioaffinity-based methods are widely used to quantify a biological substance in biological research, development and in routine clinical *in vitro* diagnostics. The development of single step bioaffinity assays and real-time monitoring detection technologies provide bioaffinity-based binding kinetic data. Experimental methods together with the valuable tools of computational model-based methods can be developed for studying the binding behaviour of bioaffinity assays and predicting quantitative assay results. This thesis demonstrates the possibilities and benefits of mathematical models and simulation methods in the field of bioaffinity assay and reaction kinetics. The research studies combine the theoretical modelling approaches and measurement data practices performed. This emphasizes the value of the results.

The mathematical models and simulation methods developed were based on mechanistic models, which rely on binding reaction kinetics and the law of mass action. Two different simulation methods were developed and confirmed by experimental data: the calibration method and node-based modelling method. The calibration method was developed to calibrate a bioaffinity assay system by utilizing non-equilibrium kinetic measurement data from only one standard concentration. This method can also be used to predict an unknown sample concentration from the non-equilibrium kinetic phase. The NODE method was developed to model complex multi-component binding reactions, which have been a challenge for traditional numerical methods. The NODE method provides an easy-to-use, network-based modelling approach, which decomposes the binding reactions present in multi-component assays into smaller partial problems. This method has been compared with the conventional mechanistic model and proved to result in a congruent assay response. These methods were shown to be applicable to bioaffinity assays in, for example, point-of-care and drug discovery in order to hasten the processes. Separate simulation studies were carried out in order to discover the most critical error factor in rapid immunoassays, concentrating on the incubation timing and initial reagent concentrations. These simulations revealed that the early phase of the assay is critical. However, they also showed that the assay recovers from the initial errors after a few hundredths of seconds of incubation. The NODE-method was applied to modelling the behaviour of protein adsorption, proving useful for complex reactions that require several intermediates and end-products be considered.

The simulations and simulation methods in this thesis demonstrate the excellence of single-step technologies and the possibilities they are able to provide. In this study, the kinetic binding reaction data, obtained by sensitive, single-step two-photon excitation fluorescence based technology, played an important role. Thus, mathematical models and simulations provide important information on the binding reaction kinetics in bioaffinity assays, but together with new inventions in high-sensitive detection methods and assays, novel developments can be achieved in practice.

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9 REFERENCES

Abdiche Y, Malashock D, Pinkerton A and Pons J (2008) Determining kinetics and affinities of protein interactions using a parallel real-time label-free biosensor, the Octet. *Anal. Biochem.* **377:** 209-217.

Abundo M (1991) A stochastic model for predator-prey systems: basic properties, stability and computer simulation. *J. Math. Biol.* **29:** 495-511.

Aittokallio T, Gyllenberg M, Polo O and Virkki A (2006) Parameter estimation of a respiratory control model for noninvasive carbon dioxide measurements during sleep. *Math. Med. Biol.* **24:** 225-249.

Anderson RM and May RM (1979) Population biology of infectious diseases: Part I. *Nature* **280:** 361-367.

Anthony HM and Cox MG (1989) An automatic algorithm for immunoassay curve calibration using controlled quadratic spline. *Math. Med. Biol.* **6:** 91-110.

Arrhenius S (1907) Immunochemistry, MacMillan New York.

Baş D, Dudak FC and Boyacı İH (2006a) Modeling and optimization III: Reaction rate estimation using artificial neural network (ANN) without a kinetic model. *J. Food Eng.* **79:** 622-628.

Baş D, Dudak FC and Boyacı İH (2006b) Modeling and optimization IV: Investigation of reaction kinetics and kinetic constants using a program in which artificial neural network (ANN) was integrated. *J. Food Eng.* **79:** 1152-1158.

Bazin H, Trinquet E and Mathis G (2002) Time resolved amplification of cryptate emission: a versatile technology to trace biomolecular interactions. *Rev. Mol. Biotech.* **82:** 233-250.

Bersani AM, Bersani E and Mastroeni L (2008) Deterministic and stochastic models enzymatic networks – application to pharmaceutical research. *Comput. Math. Appl.* **55:** 879-888.

Bostrom J, Yu S-F, Kan D, Appleton BA, Lee CV, Billeci K, Man W, Peale F, Ross S, Wiesmann C, Fuh G (2009) Variants of the antibody herceptin that interact with HER2 and VEGF at the antigen binding site. *Science* **323***:* 1610-1614.

Bregenholt S, Jensen A, Lantto J, Hyldig S and Haurum JS (2006) Recombinant Human Polyclonal Antibodies: A New Class of Therapeutic Antibodies Against Viral Infections. *Curr. Pharm. Des.* **12:** 2007-2015.

Butala HD, Ramakrishnan A and Sadana A (2003) A mathematical analysis using fractals for binding interactions of estrogen receptors to different ligands on biosensor surfaces. *Sens. Actuators* **B 88:** 266-280.

Carlsson F, Malmstein M and Linse P (2001) Monte Carlo simulations of lysozyme self-association in aqeous solution. *J Phys. Chem.* **B 105:** 12189-12195.

Chen Z and Sadana A (1996) An analysis of antigen-antibody binding kinetics for biosensor applications utilized as a model system: influence of non-specific binding. *Biophys. Chem.* **57:** 177-187.

Cohn M and Mata J (2007) Quantitative modeling of immune responses. *Immunolog. Rev.* **216:** 5-8.

Conlon MJ, Russell DL and Mussivand (2006) Development of a mathematical model of the human circulatory system. *Ann. Biomed. Eng.* **34:** 1400-1413.

Conradi C, Saez-Rodriguez, Gilles E-D and Raisch J (2005) Using chemical reaction network theory to discard a kinetic mechanism hypothesis. *IEE Proc.-Syst. Biol.* **152:** 243-248.

Conradi C, Flockerzi D, Raisch J and Stelling J (2007) Subnetwork analysis reveals dynamic features of complex (bio)chemical networks. *Proc. Natl. Acad. Sci. USA* **104:** 19175-19180.

Cook ND (1996) Scintillation proximity assay: a versatile high-throughput screening technology. *Drug Discovery Today* **1:** 287-294.

Coons AB, Creech HJ and Jones, RN (1941) Immunological properties of an antibody containing a fluorescent group. *Proc. Soc. Expt. Biol. Med.* **47:** 200-202.

Copeland RA (2000) Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, 2nd Edition, Wiley-VCH, Inc, New York.

Cracuin G, Tang Y and Feinberg M (2006) Understanding bistability in complex enzyme-driven reaction networks. *Proc. Natl. Acad. Sci. USA* **103:** 8697-8702.

Crampin EJ, Schnell S and McSharry PE (2004) Mathematical and computational techniques to deduce complex biochemical reaction mechanisms. *Prog. Biophys. Mol. Biol.* **86:** 77-112.

Daniels PB (1994) The fitting, acceptance, and processing standard curve data in automated immunoassay systems, as exemplified by Serono SR1 analyzer. *Clin. Chem* **40:** 513-517.

Day RN and Schaufele F (2005) Imaging molecular interactions in living cells. *Mol. Endocrinol.* **19:** 1675-1686.

Deng T, Li J-S, Huan S-Y, Yang H-F, Wang H, Shen G-L and Yu R-Q (2006) Quartz crystal microbalance bioaffinity sensor for biotin based on mixed self-assembled monolayers and metastable molecular

complex receptor. *Biosens. Bioelectron.* **21:** 1545-1552.

Dudley RA, Edwards P, Ekins RP, Finney DJ, McKenzie IG, Raab GM, Rodbard and Rodgers RP (1985) Guidelines for Immunoassay Data Processing. *Clin. Chem.* **31:** 1264-1271.

Eisenmesser EZ, Millet O, Labeikovsky W, Korzhnev DM, Wolf-Waltz M, Bosco DA, Skalicky JJ, Kay LE and Kern D (2005) Intrinsic dynamics of an enzyme underlies catalysis. *Nature* **438:** 117-121.

Ekins RP (1960) The estimation of thyroxine in human plasma by electrophoretic technique. *Clinica Chemica Acta* **5:** 453-459.

Ekins RP and Chu FW (1991) Multianalyte microspot immunoassay – microanalytical "compact disk" of the future. *Clin. Chem.* **37:** 1955–1967.

Ekins R (1991) Immunoassay Design and Optimisation, in Principles and Practice of Immunoassay. Stockton Press, New York.

van Emon J (2007) Immunoassay and other bioanalytical techniques, CRC Press.

Englebienne P, van Hoonacker A and Verhas M (2003) Surface plasmon resonance: principles, methods and applications in biomedical sciences, *Spectroscopy* **17:** 255-27.

Érdi P and Tóth J (1989) Mathematical models of chemical reactions. Princeton University Press, Princeton NJ.

Feinberg M (1979) Lectures on chemical reaction networks, Notes of lectures given at the Mathematics Research Center of the University of Wisconsin, 1979. Available from: http://www.che.eng.ohio-state.edu/~FEINBERG/LecturesOnReactionNetworks.

Feinberg M, Horn FJM (1974) Dynamics of open chemical systems and the algebraic structure of the underlying reaction network. *Chem. Eng. Sci.* **29:**775-787.

Fong C-C, Wong M-S, Fong W-F, and Yang M (2002) Effect of hydrogel matrix on binding kinetics of protein-protein interactions on sensor surface. *Anal. Chim. Acta* **456:** 201-208.

Furusawa C, Ono N, Suzuki S, Agata T, Shimizu H and Yomo T (2009) Model-based analysis of non-specific binding for background correction of high-density oligonucleotide microarrays. *Bioinformatics* **25:** 36–41.

Garnett GP (2002) An introduction to mathematical models in sexually transmitted disease epidemiology. *Sex. Transm. Inf.* **78:** 7-12.

Gillespie DT (1976) A general method for numerically simulating the stochastic time evolution of coupled chemical reactions. *J. Comput. Phys.* **22:** 403-434.

Gillespie DT (1977) Exact stochastic simulation of coupled chemical reactions. *J. Phys. Chem* **81:** 2340-2361.

Gillespie DT (1992) Rigorous derivation of the chemical master equation. *Physica A* **188:** 404-425.

Gillespie DT (2007) Stochastic simulation of chemical kinetics. *Annu. Rev. Phys. Chem.* **58:** 35-55.

Gottschalk PG and Dunn JR (2005) Determining the error of dose estimates and minimum and maximum acceptable concentrations from assays with nonlinear dose-response curves. *Comput. Meth. Prog. Bio.* **80:** 204-215.

Gschwind A, Fischer OM and Axel Ullrich A (2004) The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat. Rev. Cancer* **4:** 361-370.

Ha T (2001) Single-Molecule Fluorescence Resonance Energy Transfer. *Methods* **25:** 78-86.

Hangos K and Cameron I (2001) Process modelling and model analysis. Academic Press London.

Hart HE and Greenwald EB (1979) Scintillation-proximity assay of antigen-antibody binding kinetics: concise communication. *J. Nucl. Med.* **20:**1062-1065.

Hänninen P, Soini A, Meltola N, Soini J, Soukka J and Soini E (2000) A new microvolume technique for bioaffinity assays using two-photon excitation. *Nat. Biotechnol.* **18:** 548-550.

Hänninen P, Waris M, Kettunen M and Soini E (2003) Reaction kinetics of a two-photon excitation microparticle based immunoassay—from modelling to practice. *Biophys. Chem.* **105:** 23–28.

Hellander A and Lötstedt P (2007) Hybrid method for chemical master equation. *J. Comput. Phys.* **227:** 100-122.

Hemmilä I (1991) Applications of Fluorescence in Immunoassays, Wiley & Sons New York.

Hemmilä I (1999) LANCE™: Homogeneous assay platform for HTS. *J. Biomol. Screen.* **4:** 303-308.

Heymann B (1996) Complementary and Fc-receptors in regulation of the antibody response. *Immunol, Lett.* **54:** 195-199.

Holt LJ, Enever C, de Wildt RMT and Tomlinson IM (2000) The use of recombinant antibodies in proteomics. *Curr. Opin. Biotechnol.* **11:** 445-449.

Homola J (2006) Surface Plasmon Resonance Based Sensors, Springer Series on Chemical Sensors and Biosensors 4, Springer-Verlag Berlin Heidelberg.

Jerne NK (1972) Towards a network theory of the immune system. *Ann. Immunol. Inst. Pasteur* **125C:** 373-385.

Jolley ME, Chao-Huei J. Wang C-HJ, Ekenberg SJ, Zuelke MS and Kelso DM (1984) Particle Concentration Fluorescence immunoassay

(PCFIA): a new, rapid immunoassay technique with high sensitivity. *J. Immunol. Methods* **67:** 21-35.

Jäger S, Brand L and Eggeling C (2003) New fluorescence technique for high-throughput drug discovery. *Curr. Pharm. Biotech.* **4:** 463-476.

Kaufmann KW, Lemmon GH, Deluca SL, Sheehan JH and Meiler J (2010) Practically useful: what the Rosetta protein modeling suite can do for you. *Biochemistry,* **49:** 2987-2998.

King WH (1964) Piezoelectric Sorption Detector. *Anal. Chem.* **36:** 1735-1739.

Klenin KV, Kusnezow W, Langowski J (2005) Kinetics of protein binding in solid-phase immunoassays: Theory. *J. Chem. Phys.* **122:** 214715.

Kopelmann R (1988) Fractal reaction kinetics. *Science* **241:** 1620-1626.

Kricka LJ (1994) Selected Strategies for improving sensitivity and reliability of immunoassays. *Clin. Chem.* **40:** 347-357.

Krohn KA and Link JM (2003) Interpreting enzyme and receptor kinetics: keeping it simple, but not too simple. *Nucl. Med. and Biol.* **30:** 819-826.

Kubiak-Ossowska K and Mulheran PA (2010) Mechanism of Hen egg white lysozyme adsorption on a charged solid surface. *Langmuir* **26:** 15954-15965.

Kusnezow W, Syagailo YV, Rüffer S, Baudenstiel N, Gauer C, , Hoheisel JD, Wild D and Goychuk I (2006a) Optimal design of microassay immunoassays to compensate for kinetic limitations. *Molecular & Cellular Proteomics* **5:** 1681-1696.

Kusnezow W, Syagailo YV, Rüffer S, Klenin K, Sebald W, Hoheisel JD, Gauer C, Goychuk I (2006b) Kinetics of antigen binding to antibody microspots: Strong limitation by mass transportation to the surface. *Proteomics* **6:** 794-803.

Lakowicz JR (1983) Principles of fluorescence spectroscopy. Plenum, New York.

Lanzeni S, Messina Enza and Archetti F (2008) Graph models and mathematical programming in biochemical network analysis and metabolic engineering design. *Comput. Math. Appl.* **55:** 970-983.

Levine RD (2005) Molecular reaction dynamics. University Press, Cambridge, UK.

Louzoun Y (2007) The evolution of mathematical immunology. *Immunolog. Rev.* **216:** 9-20.

Lundin M, Elofsson UM, Blomberg E, Rutland MW (2010) Adsorption of lysozyme, β -casein and their layer-by-layer formation on hydrophilic surfaces: Effect of ionic strength. *Coll. Surf. B* **77:** 1-11.

Maier SA (2007) Plasmonics, Fundamentals and Applications, Springer 2007.

Mank M, Reiff DF, Heim N, Friedrich M, Borst A and Griesbeck O (2006) A FRET-Based Calcium Biosensor with Fast Signal Kinetics and High Fluorescence Change. *Biophys. J.* **90:** 1790-1796.

Markham H (2006) The blue brain project. *Nature Rev. Neurosci.* **7:** 153-160.

Másson M, Yun K, Haruyama T, Kobatake E, and Aizawa M (1995) Quartz Crystal Microbalance Bioaffinity Sensor for Biotin. *Anal. Chem.* **67:** 2212-2215.

Mattes MJ (1997) Binding parameters of antibodies reacting with multivalent antigens: functional affinity or pseudo-affinity. *J. Immunol. Methods* **202:** 97-101.

Mattes MJ (2005) Binding parameters of antibodies: pseudo-affinity and other misconceptions. *Cancer Immunol. Immun.* **54:** 513-516.

McPherson RA and Zettner A (1975) A Mathematical analysis of incubation time in competitive binding systems. *Anal. Biochem.* **64:** 501-508.

McQuarrie DA (1967) Stochastic approach to chemical kinetics. *J. Appl. Prob.* **4:** 413-478.

Meltola NJ, Kettunen MJ and Soini AE (2005) Dipyrrylmetheneboron Difluorides as Labels in Two-Photon Excited Fluorometry. Part I - Immunometric Assays. *J. Fluorescence* **15:** 221-232.

Mendel CMM, Licko V and Kane JP (1985) The Effect of Ligand Heterogeneity on the Scatchard Plot. *J. Biol. Chem.* **250:** 3451-3455.

Metropolis N and Ulam S (1949) The Monte Carlo Method. *J.Am. Stat. Assoc.* **44:** 335-341.

Michelman-Ribeiro A, Mazza D, Rosales T, Stasevich TJ, Boukari H, Rishi V, Vinson C, Knutson JR and James McNally J (2009) Direct Measurement of Association and Dissociation Rates of DNA Binding in Live Cells by Fluorescence Correlation Spectroscopy. *Biophys. J.* **19:** 337-346.

Morozov AV, Havranek JJ, Baker D and Siggia ED (2005) Protein-DNA binding specificity predictions with structural models. *Nucleic Acids Research* **33:**5781-5798.

Motulsky H and Arthur Christopoulos A (2003) Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting. Graph Pad software

Munson PJ and Rodbard D (1980) Ligand; a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107:** 220-239.

Murray JD (2002) Mathematical biology: I. An Introduction. 3ed. Springer.

Nakahishi K, Sakiyama T and Imamura K (2001) On the Adsorption of Proteins on Solid Surfaces, a Common but Very Complicated Phenomenon. *J. Biosci. Bioeng.* **91:** 233-244.

Nelson DL and Cox MM (2004) Lehninger Principles of Biochemistry 4th edition, W. H. Freeman.

Nicolau DV Jr. and Burrage K (2008) Stochastic simulation of chemical reactions in spatially complex media. *Computers and Mathematics with Applications* **55:** 1007-1018.

Noble M, Sinha Y, Kolupaev A, Demin O, Earnshaw D, Tobin F, West J, Martin JD, Qiu C, Liu W, DeWolf WE Jr., Tew D and Goryanin II (2006) The kinetic model of the shikimate pathway as a tool to optimize enzyme assays for high-throughput screening. *Biotech. Bioeng.* **95:** 560-571.

Nygren H, Czerkinsky C and Stenberg M (1985) Dissociation of antibodies bound to surface-immobilized antigen. *J. Immunol. Methods* **85:** 87-95.

Nygren H and Stenberg M (1989) Immunochemistry at interfaces. *Immunology* **66:** 321-327.

Ohmura N, Tsukidate Y, Shinozaki H, Lackie SJ and Saiki H (2003) Combinatorial Use of Antibody Affinities in an Immunoassay for Extension of Dynamic Range and Detection of Multiple Analytes. *Anal. Chem.* **75: 104-110**.

Ong GL and Mattes MJ (1993) Re-evaluation of the concept of functional affinity as applied to bivalent antibody binding to cell surface antigens. *Mol. Immunol.* **30:** 1455-1462.

Orton RJ, Sturm OE, Vyshermirsky V, Calder M, Gilbert DR and Kolch W (2005) Computational modelling of the receptor-tyrosine-kinase-activates MAPK pathway. *Biochem. J.* **392:**249-261.

O'Shannessy DJ (1994) Determination of kinetic rate and equilibrium binding constants for macromolecular interactions: a critique of the surface plasmon resonance literature. *Curr. Opinion in Biotech.* **5:** 65-71.

van Oss CJ (1995) Hydrophobic, hydrophilic and other interactions in epitope-paratope binding. *Mol. Immunol.* **32:** 199-211.

Pardee AB and Reddy GPV (2003) Beginnings of feedback inhibition, allostery, and multi-protein complexes. *Gene* **321:** 17-23.

Peltonen L, Perola M, Naukkarinen J, Palotie A (2006) Lessons from studying monogenic disease for common disease. *Hum. Mol. Genet.* **15:** R67-R74.

Pennock BE (1973) A Calculator for Finding Binding Parameters from A Scatchard Plot. *Anal. Biochem.* **56:** 306-309.

Perlmann P and Engvall E (1972) Enzyme-linked immunosorbent assay, Elisa III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J Immunol.* **109:** 129-135.

Pollak E and Talkner P (2005) Reaction rate theory: What it was, where is it today, and where is it going? *Chaos* **15:** 026116.

Price CP and Newman DJ (1991) Principles and practice of Immunoassay. Stockton Press, New York, U.S.A.

Qian H and Bishop LM (2010) The chemical master equation approach to nonequilibrium steady-state of open biochemical systems: Linear single-molecule enzyme kinetics and nonlinear biochemical reaction networks. *Int. J. Mol. Sci.* **11:** 3472-3500.

Qian H and Elson EL (1991) An analysis of confocal laser-microscope optics for 3-D fluorescence correlation spectroscopy. *Applied Optics* **30:** 1185-1195.

R Development Core Team (2005). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.r-project.org.

Ragatt P (1991) Data processing, principles and practice of immunoassay. Stockton Press, NY

Raj A and van Oudenaarden A (2008) Nature, Nurture, or Chance: Stochastic Gene Expression and Its Consequences. *Cell* **135:** 216-226.

Reiter D (2008) The Monte Carlo method, an introduction. In: Fehske H, Schneider R and Weiße A (Eds), *Computational Many-Particle Physics* Lect. Notes Phys. **739:** 63-78, Springer, Berlin Heidelberg.

Rich RL and Myszka DG (2007) Higher-througput, label-free, real-time molecular interaction analysis. *Anal. Biochem.* **361:** 1-6.

Rich RL, Cannon MJ, Jenkins J, Pandian P, Sundaram S, Magyar R, Brockman J, Lambert J and Myszka DG (2008) Extracting kinetic rate constants from surface plasmon resonance array systems. *Anal. Biochem.* **373:** 112-120.

Rich RL, Papalia GA, Flynn PJ, Furneisen J, Quinn J, Klein JS, Katsamba PS, Waddell MB, Scott M, Thompson J, Berlier J, Corry S, Baltzinger M, Zeder-Lutz G, Schoenemann A, Clabbers A, Wieckowski S, Murphy MM, Page P, Ryan TE, Duffner J, Ganguly T, Corbin J, Gautam S, Anderluh G, Bavdek A, Reichmann D, Yadav SP, Hommema E, Pol E, Drake A, Klakamp S, Chapman T, Kernaghan D, Miller K, Schuman J, Lindquist K, Herlihy K, Murphy MB, Bohnsack R, Andrien B, Brandani P, Terwey D, Millican R, Darling RJ, Wang L, Carter Q, Dotzlaf J, Lopez-Sagaseta J, Campbell I, Torreri P, Hoos S, England P, Liu Y, Abdiche Y, Malashock D, Pinkerton A, Wong M, Lafer E, Hinck C, Thompson K, Di Primo C, Joyce A, Brooks J, Torta F, Hagel ABB, Krarup J, Pass J, Ferreira M, Shikov S, Mikolajczyk M, Abe Y, Barbato G, Giannetti AM, Krishnamoorthy G, Beusink B, Satpaev D, Tsang T, Eric Fang E, Partridge J, Brohawn S, Horn J, Pritsch O, Obal G, Nilapwar S, Busby B, Gutierrez-Sanchez G, Gupta RD, Canepa S, Witte K, Nikolovska-Coleska Z, Cho YH, D'Agata R, Schlick K, Calvert R, Munoz

EM, Hernaiz MJ, Bravman T, Dines M, Yang M-H, Puskas A, Boni E, Li J, Wear M, Grinberg A, Baardsnes J, Dolezal O, Gainey M, Anderson H, Peng J, Lewis M, Spies P, Trinh Q, Bibikov S, Raymond J, Yousef M, Chandrasekaran V, Feng Y,Emerick A, Mundodo S, Guimaraes R, McGirr K, Li Y-J, Hughes H, Mantz H, Skrabana R, Witmer M, Ballard J, Martin L, Skladal P, Korza G, Laird-Offringa I, Lee CS, Khadir A, Podlaski F, Neuner P, Rothacker J, Rafique A, Dankbar N, Kainz P, Gedig E, Vuyisich M, Boozer C, Ly N, Toews M, Uren A, Kalyuzhniy O, Lewis K, Eugene Chomey E, Pak BJ and Myszka DG (2009) A global benchmark study using affinity-based biosensors. *Anal. Biochem.* **386:** 194-216.

Rios J, Stein E, Shendure J, Hobbs HH and Cohen JC (2010) Identification by whole-genome resequencing of gene defect responsible for severe hypercholesterolemia. *Hum. Mol. Genet.* **19:** 4313-4318.

Rodbard D and Catt KJ (1972) Mathematical theory of radioligand assays: the kinetics of separation of bound from free. *J. Steroid Biochem.* **3:** 225-273.

Rodbard D and Weiss GH (1973) Mathematical Theory of Immunoradiometric (Labelled Antibody) Assays. *Anal. Biochem.* **52:** 10-44.

Rodbard D and Feldman Y (1978) Kinetics of two-site immunoradiometric ('sandwich') assays—I. Mathematical models for simulation optimization, and curve fitting. *Immunochemistry* **15:** 71–76.

Rodbard D, Feldman Y, Jaffe ML, Miles LEM (1978) Kinetics of two-site immunoradiometric ('sandwich') assays—II. Studies on the nature of the 'high-dose hook effect'. *Immunochemistry* **15:** 77–82.

Sadler WA (2008) Error models for immunoassays. *Ann Clin Biochem* **45:** 481-485.

Schaible U, Liss M, Prohaska E, Decker J, Stadherr K, and Wolf H (2004) Affinity Measurements of Biological Molecules by a Quartz Crystal Microbalance (QCM) Biosensor. In: J. Decker and U. Reischl editors, *Methods in Molecular Medicine, Molecular Diagnosis of Infectious Diseases* **94:** 321-330.

Schnell S and Turner TE (2004) Reaction kinetics in intracellular environments with macromolecular crowding: simulations and rate laws. *Prog. Biophys. Mol. Biol.* **85:** 235-260.

Schofield DJ, Pope AR, Clementel V and Buckell J (2007) Application of phage display to high throughput antibody generation and characterization. *Genome Biology* **8:** Article R254:1-18.

Schramm VL (2005) Enzymatic transition states and transition state analogues. *Curr. Opin. Struct. Biol.* **15:** 604-613.

Schuurs A and van Weemen B (1971) Immunoassay using antigen-enzyme conjugates. *FEBS Lett.* **15:** 232-236.

Selvin PR (2000) The renaissance of fluorescence resonance energy transfer. *Nature Struct. Biol.* **7:** 730-734.

Setzer W (2004) Odesolve: Solvers for ordinary differential equations. R package version 0.5-12.

Shipchandler MT and Moore EG (1995) Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott Imx analyzer. *Clin. Chem.* **41:** 991-994.

Siepmann J and Siepmann F (2008) Mathematical modeling of drug delivery. *Int. J. Pharm.* **364:** 328-343.

Sinanoğlu Oktay (1975) Theory of chemical reaction networks. All possible mechanism or synthetic pathways with given number of reaction steps or species. *J Amer Chem Soc* **97:** 2309-2320.

Singh KK Parwaresch R and Krupp G (1999) Rapid kinetic characterization of hammerhead ribozymes by real-time monitoring of fluorescence resonance energy transfer (FRET). *RNA* **5:** 1348-1356.

Sloot PMA and Hoekstra AG (2009) Multi-scale modelling in computational biomedicine. *Brief. Bioinform.* **2:** 142-152.

Smith DS and Eremin SA (2008) Fluorescence polarization immunoassays and related methods for high-throughput screening of small molecules. *Anal. Bioanal. Chem.* **391:** 1499-1507.

Soini JT, Soukka JM, Meltola NJ, Soini AE, Soini E, and Hänninen PE (2000a) Ultra sensitive bioaffinity assay for micro volumes. *Single Molecules* **1:** 203-206.

Soukka T, Härmä H, Paukkunen J and Lövgren T (2001) Utilization of kinetically enhanced monovalent binding affinity by immunoassays based on multivalent nanoparticle-antibody bioconjugates. *Anal.Chem.* **73:** 2254-2260.

Soukka T (2003) Immunoassay Designs and Potential of Particulate Photoluminescent

Lanthanide Reporters, Annales Universitatis Turkuensis AI 304, Doctorate thesis, University of Turku, Painosalama Oy.

Soini JT (2002b) Development of instrumentation for single-step, multiplexed, microvolume bioaffinity assays. Doctorate thesis, University of Turku. Annales Universitatis Turkuensis AI 281, Painosalama Oy.

Southern J, Pitt-Francis J, Whiteley J, Stokeley D, Kobashi H, Nobes R, Kadooka Y and Gavaghan D (2008) Multi-scale computational modelling in biology and physiology. *Prog. Biophys. Mol. Biol.* **96:** 60-89.

Stenberg M, Stiblert L and Nygren H (1986) External diffusion in solid-phase immunoassays. *J. Theor. Biol.* **120:** 129-140.

Stenberg M and Nygren H (1988) Kinetics of antigen-antibody reactions at solid-liquid interfaces. *J. Immunol. Methods* **113:** 3-15.

Stenberg M, Werthén M, Theander S and Nygren H (1988) A diffusion limited reaction theory for a microtiter plate assay. *J Immunol. Methods* **112:** 23-29.

Šterk M and Trobek R (2005) Biomedical simulation of heat transfer in a human heart. *J. Chem. Inf. Model.* **45:** 1558-1563.

Sterrer S and Henco K (1997) Fluorescence correlation spectroscopy (FCS) – A highly sensitive method to analyze drug/target interactions. *J. Recept. Signal Tr. R* **17:** 511-520.

Tamil Selvi P, Ashish B and Murthy GS (2002) Determination of thermodynamic parameters of antigen-antibody interaction from real-time kinetic studies. *Curr. Science* **82:** 1442-1448.

Tan KC and Li Y (2002) Grey-box model identification via evolutionary computing. *Control Engineering Practice* **10:** 673-684.

Thakur AK, Jaffe ML and Rodbard D (1980) Graphical analysis of ligand binding systems: evaluation by Monte Carlo Studies. *Anal. Biochem.* **107:** 279-295.

Tirronen E and Salmi T (2003) Process development in fine chemistry. *J. Chem Eng.* **91:** 103-104.

Tortajada-Genaro LA and Romero M (2008) Immunoradiometric determination of thyroglobulin in serum samples by time calibration transfer. *Clin Chem Lab Med* **46:** 1416-1422.

Tramontano A (2006) The role of molecular modelling in biomedical research. *FEBS Lett.* **580:** 2928-2934.

Turner TE, Schnell S and Burrage K (2004) Stochastic approaches for modelling in vivo reactions. *Comput. Biol. Chem.* **28:** 165-178.

van der Kamp MW, Shaw KE, Woods CJ and Mulholland AJ (2008) Biomolecular simulation and modelling: status, progress and prospects. *J. R. Soc. Interface* **5:** 173-190.

Van Regenmortel M (1998) Thermodynamic parameters in immunoassay. *Clin. Chem. Lab. Med.* **36:** 353–354.

Van Regenmortel MHV, Altschuh D, Chattelier J, Christensen L, Rauffer-Bruyère, Richalet-Secordel P, Witz J and Zeder-Lutz G (1998) Measurement of antigen-antibody interactions with biosensors. *J. Mol. Recognit.* **11:** 163-167.

Visser AJWG and Hink MA (1999) New Perspectives of Fluorescence Correlation Spectroscopy. *J. Fluorescence* **9:** 81-87.

Wahlgren M, Arnebrandt T and Lundström I (1995) The adsorption of lysozyme to hydrophilic silicon oxide surfaces: comparison between experimental data and models for adsorption kinetics. *J. Colloid Interf. Sci* **175:** 506-514.

Walker WHC (1977) The Scatchard plot in immunometric assay. *Clin. Chem.* **23:** 588-590.

Waris ME, Meltola NJ, Soini JT, Soini E, Peltola OJ and Hänninen PE (2002) Two-photon excitation fluorometric measurement of homogeneous microparticle immuno assay for C-reactive protein. *Anal. Biochem.* **309:** 62-74.

van Weemen BK (2005) The rise of EIA/ELISA. *Clin. Chem***. 51:** 2226.

Weiland GA and Molinoff PB (1981) Quantitative analysis of drug-receptor interactions: I. Determination of kinetic and equilibrium properties. Life Sciences **29:** 313-330.

Wide L, Bennich H and Johansson SG (1967) Diagnosis of allergy by an in-vitro test for allergen antibodies. *Lancet* **2:**1105-1107.

Wild D (1994) The Immunoassay Handbook. Wild D, editor. MacMillan Press Ltd., London UK.

Wilson LJ, Adcock-Downey L and Pusey ML (1996) Monomer concentrations and dimerization constants in crystallizing lysozyme solutions by dialysis kinetics. *Biophys J.* **71:**2123-2129

Wilumsen B, Christian GD and Ruzicka J (1997) Flow injection renewable surface immunoassay for real time monitoring of biospecific interaction. *Anal. Chem.* **69:** 3482-3489.

Woodrow Setzer R (2004) Odesolve: Solvers for Ordinary Differential Equations. R package version 0.5-12.

Xie L, Jones RM, Glass TR, Navoa R, Wang Y and Grace MJ (2005) Measurement of the functional affinity constant of a monoclonal antibody for cell surface receptors using kinetic exclusion fluorescence immunoassay. *J Immunol. Methods* **304:** 1-14.

Xu Y, Kanauchi A, von Arnim AG, Piston DW and Johnson CH (2003) Bioluminescence resonance energy transfer: Monitoring protein-protein interactions in living cells. *Meth. Enzymol.* **360:** 289-301.

Yalow RS and Berson SA (1960) Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest* **39:** 1157-1175.

Zheng Y and Sriram G (2010) Mathematical Modelling: Bridging the Gap between Concept and Realization in Synthetic Biology. Hindawi Publishing Corporation. *J. Biomed. Biotechnol. 2010:* Article ID 541609.

Zuber E, Rosso L, Darbouret B, Socquet F, Mathis G and Flandrois JP (1997a) A Descriptive model for the kinetics of a homogeneous fluorometric immunoassay. *Journal of Immunoassay* **18:** 21-47.

Zuber E, Mathis G and Flandrois J-P (1997b) Homogeneous two-site immunometric assay kinetics as a theoretical tool for data analysis. *Anal. Biochem.* **251:** 79-88.