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Reaction kinetics of a two-photon excitation microparticle based immunoassay—from modelling to practice

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Abstract

Real time observation of reaction kinetics is one of the key features of the newly developed microparticle based two-photon excitation fluorescence immunoassay system (TPX). By observing binding reactions at the surface of individual microparticles during the incubation of an assay, the binding constants of an assay become apparent. This paper describes the use of the new system in quantifying the reaction parameters of human thyroid stimulating hormone (hTSH) assay. A mechanistic reaction model for the assay is presented. The reaction model is further shown to precisely predict the behaviour of the assay kinetics over a wide range of analyte concentrations.

Keywords: Assay kinetics; Immunoassay; Two-photon excitation; Multiphoton; Microparticle; Fluorescence

1. Introduction

Characterization of the reaction components of an immunoassay is a key issue in developing an assay kit [1]. *Affinity*–the 'strength' of binding of an antibody–antigen reaction can be described as the ratio of association and dissociation constants. The association constant is proportional to the average probability of the reaction to take place once the two free molecules collide whereas dissociation constant is proportional to the average lifetime of the complex. Although affinity is usually given by the reagent manufacturer, the kinetic constants remain unknown. Moreover, the affinity can be expected to alter if reagent molecules are linked with label molecules or a surface.

Several attempts have been made to quantify reaction constants of antigen–antibody pairs. Exact evaluation under true assay conditions is possible only in a single-step immunoassay that enables monitoring of reaction products during the assay incubation [2,3]. By combination of these results with theoretical assessment of the assay, the associated reaction constants can be revealed.

In the following, a simple mechanistic model for a sandwich type immunoassay is presented. The precision of the model is then verified by using a newly developed two-photon excitation microparticle assay system (TPX) [4–6] that enables *on-line* monitoring of the binding reactions.

The TPX system utilizes two-photon excitation of fluorescence and observation of individual

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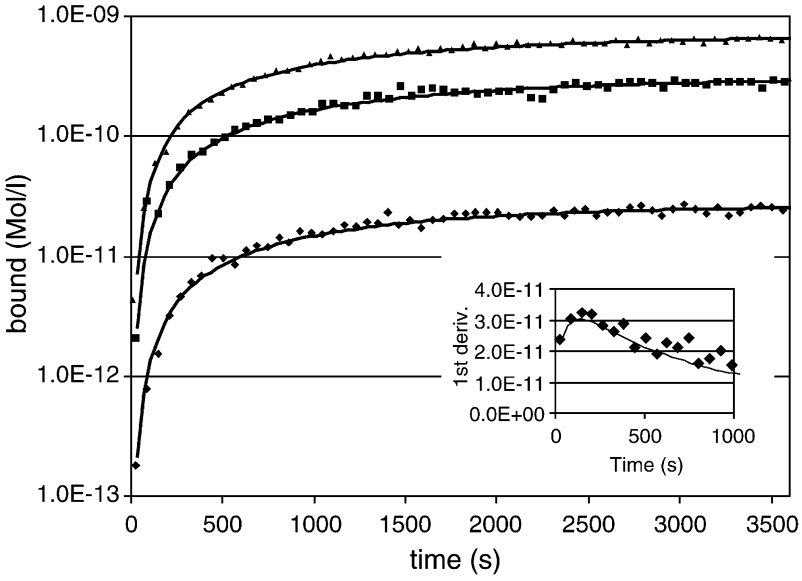

Fig. 1. Solid phase bound complexes as a function of time for incubation concentrations of 4 mIU (◇), 50 mIU (▭) and 150 mIU (△) of hTSH standard and fitted to the theoretical curves (solid). The insert shows the first derivatives of 150 mIU measured and modelled data.

microparticles from reaction suspension—in a way the system may be viewed as an automatic two-photon excitation microscope that searches for microparticles from the reaction suspension and once found, measures their fluorescence individually. In a sandwich type immunoassay, the primary binding reagent (Ab) has been attached to microparticle surface. When target molecules (Ag) and a secondary, fluorescently labeled reagent (Ab′) specifically bind to the microparticle surface, the amount of fluorescence signal from individual microparticles becomes representative of the target antigen concentration. Since these, now brightly fluorescent microparticles are observed individually, the background fluorescence from unbound secondary antibody (Ab′) is suppressed and there is no need for separation steps between bound and unbound fractions.

2. Mechanistic assay model

A typical immunodiagnostic assay, targeted for determination of analyte molecule concentration uses two analyte specific antibodies that have specificity against different epitopes of the analyte molecule. The reaction scheme for this type of a sandwich immunoassay may be written into form:

$$Ag + Ab \underset{k2}{\overset{k1}{\rightleftarrows}} AgAb$$

$$Ag + Ab' \underset{k4}{\overset{k3}{\rightleftarrows}} AgAb'$$

$$Ab + AgAb' \underset{k6}{\overset{k5}{\rightleftarrows}} AbAgAb'$$

$$Ab' + AgAb \underset{k8}{\overset{k7}{\rightleftarrows}} AbAgAb'$$

Ab denoting the primary binding antibody, Ag the sample antigen and Ab′ the secondary antibody against another epitope of the antigen. It is possible now directly from the equations to create a mathematical model for the assay. For simplicity we have made the following assumptions:

– there are no other reactions such as cross-reactivity with another antigen or non-specific

binding taking place (i.e. equations are the full representation of all the reactions);
- possible steric effects are ignored;
- individual reagent and analyte molecules remain evenly distributed throughout the reaction volume; and
- the physical limits of the reaction volume do not alter the results

In a mechanistic model of the reactions, a set of differential equations as a function of time for each component in the reaction mixture may be written:

$$\mathrm{d}\frac{[Ag]}{\mathrm{d}t} = k2\cdot[\mathrm{AgAb}] - k1\cdot[\mathrm{Ag}][\mathrm{Ab}] + k4\cdot[\mathrm{AgAb'}] - k3\cdot[\mathrm{Ag}][\mathrm{Ab}]$$

$$\mathrm{d}\frac{[\mathrm{Ab}]}{\mathrm{d}t} = k2\cdot[\mathrm{AgAb}] - k1\cdot[\mathrm{Ag}][\mathrm{Ab}] + k6\cdot[\mathrm{AbAgAb'}] - k5\cdot[\mathrm{Ab}][\mathrm{AgAb'}]$$

$$\mathrm{d}\frac{[\mathrm{Ab'}]}{\mathrm{d}t} = k4\cdot[\mathrm{AgAb'}] - k3\cdot[\mathrm{Ag}][\mathrm{Ab'}] + k8\cdot[\mathrm{AbAgAb'}] - k7\cdot[\mathrm{Ab'}][\mathrm{AgAb}]$$

$$\mathrm{d}\frac{[\mathrm{AgAb}]}{\mathrm{d}t} = k1\cdot[\mathrm{Ag}][\mathrm{Ab}] - k2\cdot[\mathrm{AgAb}] + k8\cdot[\mathrm{AbAgAb'}] - k7\cdot[\mathrm{Ab'}][\mathrm{AgAb}]$$

$$\mathrm{d}\frac{[\mathrm{AgAb'}]}{\mathrm{d}t} = k3\cdot[\mathrm{Ag}][\mathrm{Ab'}] - k4\cdot[\mathrm{AgAb'}] + k6\cdot[\mathrm{AbAgAb'}] - k5\cdot[\mathrm{AgAb'}][\mathrm{Ab}]$$

$$\mathrm{d}\frac{[\mathrm{AbAgAb'}]}{\mathrm{d}t} = k7\cdot[\mathrm{Ab'}][\mathrm{AgAb}] - k8\cdot[\mathrm{AbAgAb'}] + k5\cdot[\mathrm{AgAb'}][\mathrm{Ab}] - k6\cdot[\mathrm{AbAgAb'}]$$

where the respective association constants are denoted as k1,3,5,7 and the dissociation constants as k2,4,6,8. [Ag], [Ab], [Ab′]… are the respective concentrations of the reaction components. Looking at the first equation the concentration of the unbound antigen [Ag] is reduced by binding with the antibodies Ab, Ab′ and increased by dissociation of the complexes AgAb and AgAb′. The rate of increase and decrease is determined by the products of respective reaction constants and concentrations. Intuitively the set of equations may be viewed as equations defining the changes in concentrations of reaction components within an infinitesimally short time-interval—in fact this is how the classical *Euler's* method is used computationally in solving sets of differential equations: the new concentrations are repeatedly calculated in small time steps until the desired time point has been reached.

In their work Zuber et al. [2] used this type of mechanistic model in evaluation of a liquid phase homogeneous immunoassay, Cryptor™. They suggested that the mechanistic model could also be used in studying macromolecular interactions. It has been commonly assumed that the inherent complexity of solid-phase kinetics prevents one from obtaining reliable kinetic information about the reactions by similar models to those of liquid phase immunoassays [7]. We argue, however, that the presented equations may be safely used to model a solid phase immunoassay with the restriction that acquired constants k_n will only hold for a given reaction environment, but still regardless of the analyte concentration. To verify this we have measured the kinetic behaviour of a model assay in a newly developed single step TPX-system [5]. Due to its' single-step format, the TPX-system enables binding reactions and their kinetics to be followed on-line during the incubation similarly to the Cryptor™ system.

3. Measurement of kinetic parameters

3.1. The measurement set-up

The TPX instrument has earlier been described in its single channel [4] and multichannel [5] form. As an addition for the instrument we have included a piezoelectric mixer consisting of an actuator (PA50/12NV, Jena Piezotechnic, Jena, Germany) and a stainless steel head that inserted directly to the liquid. The mixing was performed to ensure homogeneous microparticle suspension throughout the measuring of kinetic reactions.

3.2. Assay set-up

To assess the kinetic parameters of an immunoassay we chose one of the most common immuno-

diagnostic targets: the human thyroid stimulating hormone (hTSH) as the model assay.

The microparticles (3.2 μm carboxy modified, Bangs Laboratories, Fishers, IN) were coated by first passively coating the particles with the antibody (Medix Biochemica, Espoo, Finland, clone 5404, $K_a = 2 \times 10^{10}$ M^{-1}) and then generating the covalent bonds by EDC (*N*-(3-dimethyl-aminopropyl)-*N*′-ethylcarbodiimide, Pierce, Rockford, IL) reaction [6]. The particles were then checked for the active surface density by titration of the particles by hTSH standard (Scripps Laboratories, San Diego, CA, catalogue No: T0133, 2.4 IU/mg) and checking the unbound fraction of the hTSH standard. From the derived Scatchard plot we determined each microparticle to carry 580 000 ± 10% active binding sites and the affinity of the solid phase binding to be the same as given by the manufacturer (2×10^{10} M^{-1}). For each assay, 25 000 microparticles in total assay volume of 20 μl were used yielding a solid phase concentration of 1.2 nM. The number of particles/volume unit was checked by counting the particles under a microscope in a Burker chamber. As a tracer we used succidinimidyl ester of the orange fluorescent dye BF 560.8 ($\lambda_{ex} = 560$ nm, $\lambda_{em} = 580$ nm) (Arctic Diagnostics Oy, Turku, Finland) linked to an antibody against another epitope of the hTSH molecule (Medix Biochemica, Espoo, Finland, clone 5409, $K_a = 1 \times 10^{10}$ M^{-1}). The tracer concentration was 1.2 nM in the assay.

The reactions were started by mixing the microparticle-tracer suspension with the standard. The exact time point of mixing was recorded and a measuring cycle of 30 s measurements and 30 s pauses in between measurements was started. The reactions were followed for 2 h. The used final concentrations of hTSH standard were 4, 50, 150 and 1200 mIU.

4. Results

For the theoretical results the set of differential equations was solved numerically by using the second order Runge–Kutta method [8]—a computer program was written and integrated into a LabVIEW™ (National Instruments Inc.) interface to evaluate the accuracy of the model. Modelling results were compared by fitting with the actual data. The fitting process was performed by the usual mean-square-error (MSE) method in OpenOffice 1.0, but to all acquired kinetic curves at the same time adjusting the forward kinetic constants and the affinity of the labelled tracer in the model to achieve the best possible fit. The solid phase binding affinity was calculated from the previously described Scatchard plot measurement. At the final point small manual adjustments of the model input values were made for the amount of tracer and surface to improve the fitting results: The tracer concentration was reduced to 1 nM and both subunits of the IgG molecule were assumed to be active reflecting as a double concentration of the label for the modelling calculations (2 nM). The amount of surface was reduced from 1.2 nM to 0.9 nM. These small changes can be accounted for experimental errors and reflect well the precision of the model: simultaneous fitting to several experimental curves produce not only the kinetic parameters but reveal possible errors in the input parameters. The parameters that the fitting procedure yielded were the following:

$$k_1 = k_5 = k_7 = 9 \times 10^5 \frac{\text{liter}}{\text{mole} \times \text{s}},$$
$$k_2 = k_6 = k_8 = 4.5 \times 10^{-5} \text{s}^{-1}, \quad (K_a = 2 \times 10^{10}\ \text{M}^{-1})$$
$$k_3 = 8 \times 10^6 \frac{\text{liter}}{\text{mole} \times \text{s}},$$
$$k_4 = 1.33 \times 10^{-3} \text{s}^{-1}, \quad (K_a = 6 \times 10^9\ \text{M}^{-1})$$

The forward kinetic constants in the liquid phase ($k_{1,5,7}$) were approximately nine-fold over the solid phase constants reflecting, as expected, the reduced motility of microparticle bound antibodies. The affinity of the labelled antibody appears to have somewhat reduced by the labelling procedure: the value derived from the fitting procedure is 6×10^9 M^{-1} whereas the respective manufacturer's value for this clone (5409) is 1×10^{10} M^{-1}.

The plot of Fig. 1 shows the concentrated calibrated TPX-instrument signals from three different hTSH concentrations as a function of time starting from the mixing of all reaction components. The respective concentrations were 4, 50 and 150 mIU the highest concentration giving the

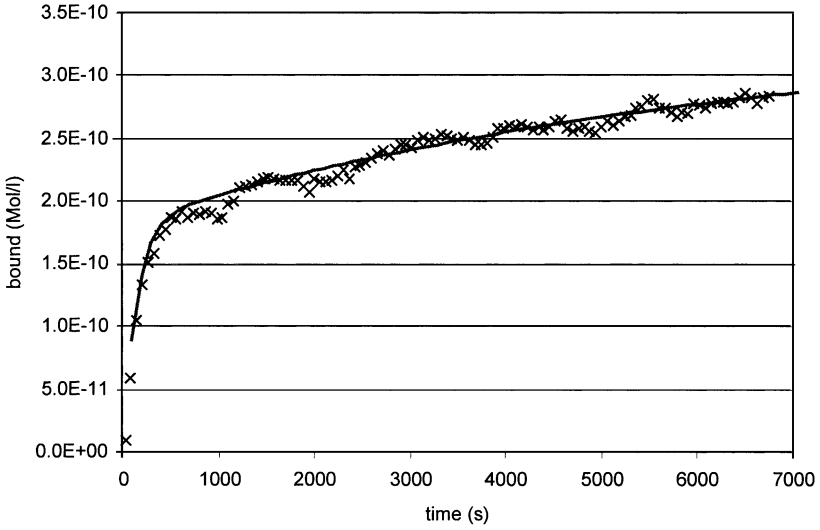

Fig. 2. Measured (+) and modelled (solid) data for 1200 mIU final hTSH concentration.

highest signal. The solid lines represent the respective results obtained from modelling. The insert of Fig. 1 shows the first derivatives of the theoretical and measured curves at 150 mIU representing the typical inclination point [2] at the early stage of incubation.

Fig. 2 shows an interesting phenomena from a measurement at 1200 mIU concentration of the hTSH standard until 2 h of incubation time. The analyte concentration (approx. 8.6 nM) was well beyond the capacity of assaying reagents. The two-fold kinetic behaviour of the curve can only be explained by the differences in kinetics of liquid and solid phase—regardless of the performed mixing. This is also reflected in the modelled curve (solid). The noisy appearance of the kinetic curve is in our belief largely due to complex biochemical phenomena that repeatedly became pronounced at excess-dose conditions.

When these results are compared with our earlier publication data of a C-reactive protein (hCRP) immunoassay [6], the measured hTSH kinetic constants appear slightly slower on the solid phase as compared with that of the hCRP with the liquid phase kinetics being approximately the same. This kind of differences can in our belief be largely accounted for differences in analyte molecular structure: the hCRP molecule is pentameric in structure with five identical binding sites—this increases the likelihood of binding when two molecules collide thus increasing the apparent association constant. Our studies with the CRP kinetics, however, are not as complete as described here for the thyroid stimulating hormone and are a matter of future work. Also, the model presented here is not fully valid for analyte molecules with several equal binding sites—an equivalently larger set of differential equations would need to be written to account for all possible reactions.

5. Discussion

From the presented theoretical data and practical measurements it is clear that the simple mechanistic model predicts well the kinetic behaviour of a microparticle based immunoassay system. Although the acquired kinetic parameters do not represent the actual but the apparent values for a single binding site, we argue that these values may nevertheless be used in predicting the behaviour of the assay under varying conditions saving time and money in the development phase of an assay.

An important key point of the results is that the TPX-system enables evaluation of kinetic para-

meters under true assay conditions, as they would appear in the final immunodiagnostic assessment of patient samples.

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