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Assessment of affinity constants by rapid solid phase detection of equilibrium binding in a flow system

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Abstract

We present a method for the determination of affinity constants based on equilibrium binding between an analyte and an antibody in liquid phase by a heterogeneous phase detection scheme. Equilibrium concentration of free antibody binding sites was probed kinetically by direct optical detection of specific binding to an immobilised analyte derivative. The additional binding signal due to dissociation of the analyte-antibody complex during detection was minimised by the use of fast flow-through conditions. The concentration of free antibody binding sites was titrated by adding increasing analyte concentrations. The affinity constant was derived from the titration curve by a non-linear least square fit of a model function. The affinity of monoclonal triazine antibodies to several *s*-triazine pesticides and a relevant metabolite was investigated. Kinetic determination of equilibrium concentration of free binding sites was carried out by reflectometric interference spectroscopy (RIfS) using flow injection analysis. The capabilities of the model were investigated using different analyte-antibody pairs and various antibody concentrations. Both bivalent IgG and monovalent Fab fragments were used to compare different binding models. The applied model corresponds well to the titration curves for affinity constants of 10^7 M^{-1} and higher. For lower affinity constants significant deviations due to dissociation of the analyte-antibody complex during detection were observed.

Keywords: Antibody affinity; Affinity constant; Label-free affinity probe; Monoclonal anti-*s*-triazine antibody

1. Introduction

The strong and specific binding of target molecules to the binding sites of an antibody plays an important role in several fields of analytical chemistry. The interaction between these target molecules (termed ‘analytes’

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in the following) with the antibody is characterised by its affinity constant K defined by the equilibrium concentrations of analyte-antibody complex c_{anab}, free analyte c_{an} and free antibody binding sites c_{ab}:

$$K = \frac{c_{anab}}{c_{an} \cdot c_{ab}} \quad (1)$$

The assessment of affinity constants is of fundamental importance, in particular for the characterisation of monoclonal antibodies with respect to their analytical performance and cross reactivity (Steward and Lew, 1985). Methods for this task are usually based on the determination of the equilibrium concentrations of the reactants. Equilibrium concentrations are classically determined by dialysis, by quenching or transfer of fluorescence, by immunoprecipitation of radio-labelled analytes or by centrifugation and filtration techniques (Steward and Steengaard, 1983). All these methods require specific properties of the analytes (certain molecular size, fluorophors, radio-label) and are not practical when fast and efficient screening is demanded.

During the last 5 years a new concept for characterising affinity interactions by real-time monitoring of the binding event at a transducer surface has become popular. The association and dissociation rate constants of the interaction are derived from the binding curves. The affinity constant is determined from the ratio of the rate constants or the equilibrium coverage (Karlsson et al., 1991; O'Shannessy et al., 1993; and others). Numerous applications have been reported since a commercial system for this technique was introduced by Pharmacia in 1991 (e.g. Jönsson et al., 1991; Fägerstam et al., 1992; Fisher and Fivash, 1994). A basic requirement of this method is the immobilisation of one of the interacting compounds. This is a critical factor, in particular when low molecular compounds are involved. For a direct investigation of the interaction either a derivative of the low molecular compound has to be immobilised (which can strongly influence the interaction properties) or indirect assay formats are required that have to be optimised thoroughly and lack the main advantages of this technique (Karlsson, 1994). Furthermore, various systematic deviations and limitations of this technique have recently been reported (O'Shannessy, 1994; Nieba et al., 1996; O'Shannessy and Winzor, 1996).

Another prominent and more universally applicable approach to the assessment of affinity constants is the determination of free antibody concentration in equilibrium with the analyte by detecting antibody binding to an immobilised analyte. Immobilisation of the interacting compounds is not required and therefore the true homogeneous phase affinity constant is obtained. The basic problem of this approach is the fact that for free antibody is removed from the solution leading to a perturbation of the equilibrium, dissociation of the analyte-antibody complex takes place and additional free antibody is generated. The apparent free antibody concentration determined in such assays is therefore higher than the true equilibrium concentration. Hence, solid phase detection schemes are only appropriate for the detection of equilibrium concentrations of free antibody if either

1. a very small part of the free antibody is bound at the surface and the perturbation of the equilibrium is negligible or
2. the signal due to the equilibrium free antibody significantly exceeds the signal due to free antibody additionally generated by dissociation.

Several ELISA-based test formats are described that use the first of these two principles for the determination of free antibody concentrations. The most simple methods directly estimate relative affinity constants from the test midpoints of inhibition assays (Nieto et al., 1984; Rath et al., 1988). A decade ago a more systematic approach to the assessment of antibody affinity by a heterogeneous test format was reported (Friguet et al., 1985). In this procedure free antibody concentration at equilibrium were determined by the detection of specific antibody binding to an immobilised analyte. Excess concentrations of free analyte were used to minimise the perturbation by the immobilised analyte. This method was applied frequently to characterise monoclonal antibodies (e.g. Portnoi et al., 1986; Ternynck and Avrameas, 1986) and was further optimised (Stevens, 1987; Schots et al., 1988) by applying more complex mathematical models initially developed by Feldman (1972). Approaches using microspot immobilisation for reducing the amount of immobilised analyte have been discussed by Ekins (1994) but, recent investigations (Hetherington, 1990; Seligman, 1994) have demonstrated

that the results of the ELISA-based method are significantly affected by both the concentration and species of the analyte at the surface.

Here we present a method for heterogeneous phase detection of equilibrium antibody concentrations using the second principle mentioned above. The additional signal due to dissociation of the analyte-antibody complex is minimised by kinetic detection in a flow-through system. Continuous replacement of the interacting volume in the flow cell allows a maximum binding rate of the free antibody and restricts perturbation of the equilibrium to a diffusion layer at the surface. Thereby the ratio of 'true' signal to perturbation signal is maximised.

Kinetic detection of antibody binding at a solid phase requires monitoring of the binding event. This is achieved by using a transducer for direct (i.e. label-free) detection of affinity interactions. Various methods for direct affinity interaction analysis at interfaces have been introduced in recent years. Many of them are based on optical detection, e.g. surface plasmon resonance (SPR) (Liedberg et al., 1983), grating coupler (Lukosz, 1991), frustrated total reflection (Cush et al., 1993) and reflectometric interference spectroscopy (RIfS) (Gauglitz et al., 1993). Since these methods do not require labelling of the antibody, simple test formats for screening applications are feasible.

In this paper we describe the application of such detection technology for probing equilibrium free antibody concentrations in a flow through system. Reflectometric interference spectroscopy (RIfS) is used for direct kinetic detection of antibody binding to an immobilised analyte. Affinity characterisation of monoclonal antibodies for the immunoassay of *s*-triazine analytes (Piehler et al., 1995) was investigated in detail. The concentration of free antibody binding sites was titrated using various analyte concentrations over a range of 2–3 orders of magnitude. Affinity constants were determined by fitting a model to the titration curves taking the valence of the antibody into account.

2. Theoretical considerations

2.1. Titration models

Although the terms 'analyte' and 'antibody' are used throughout these considerations the models described are valid for any ligand-binder system.

The following notation is used for the discussion of the titration model:

$c_{0,an}$: initial analyte concentration
c_{an}: concentration of free analyte
$c_{0,ab}$: initial antibody concentration
c_{ab}: concentration of free antibody binding sites
$c_{ab,full}$: concentration of fully occupied IgG molecules
$c_{ab,bind}$: concentration of antibodies with binding sites
c_{anab}: concentration of analyte-antibody complex
K: affinity constant

The equilibrium concentrations of analyte c_{an}, free antibody binding sites c_{ab} and the analyte-antibody complex c_{anab} are determined by the affinity constant K in the law of mass action (Eq. (1)). The equilibrium concentration of the analyte-antibody complex c_{anab} is calculated from the initial analyte concentration $c_{0,an}$, the total binding site concentrations $c_{0,ab}$, and the affinity constant K by

$$c_{anab} = \frac{c_{0,ab} + c_{0,an} + \frac{1}{K}}{2} - \sqrt{\frac{\left(c_{0,ab} + c_{0,an} + \frac{1}{K}\right)^2}{4} - c_{0,ab} \cdot c_{0,an}} \quad (2)$$

The detection method is based on the detection of antibody binding to an analyte attached to the surface. This permits the determination of the concentration of antibodies with free binding sites $c_{ab,bind}$. For monovalent antibodies such as Fab fragments this concentration is directly correlated to the concentration of occupied antibody binding sites:

$$c_{ab,bind} = c_{0,ab} - c_{anab}$$

respectively

$$c_{ab,bind} = \frac{c_{0,ab} - c_{0,an} - \frac{1}{K}}{2} + \sqrt{\frac{\left(c_{0,ab} + c_{0,an} + \frac{1}{K}\right)^2}{4} - c_{0,ab} \cdot c_{0,an}} \quad (3)$$

For IgG molecules the distribution of the analyte to the two binding sites has to be taken into account. The probabilities of occupying two, one or none of the IgG binding sites is given by a standard binomial distribution if no interaction between the binding sites is assumed (Stevens, 1987). The concentration of doubly occupied antibodies $c_{ab,2}$ depends on the total concentration of the analyte-antibody complex c_{anab} and the total concentration of antibody binding sites $c_{0,ab}$ (which is twice the concentration of antibody molecules)

$$c_{ab,full} = \frac{c_{anab}^2}{2 \cdot c_{0,ab}}$$

Only fully occupied IgG molecules are effectively inhibited and are not able to bind to the surface attached analyte. The concentration of antibody with free binding sites $c_{ab,bind}$ is equivalent to the remaining antibody

$$c_{ab,bind} = \frac{c_{0,ab}}{2} - \frac{c_{anab}^2}{2 c_{0,ab}}$$

or

$$c_{ab,bind} = \frac{c_{0,ab}}{2} - \frac{\left[\frac{c_{0,ab} + c_{0,an} + \frac{1}{K}}{2} - \sqrt{\frac{\left(c_{0,ab} + c_{0,an} + \frac{1}{K}\right)^2}{4} - c_{0,ab} \cdot c_{0,an}}\right]^2}{2 \cdot c_{0,ab}} \quad (4)$$

using Eq. (1).

The change in concentration of antibodies with free binding sites with increasing analyte concentrations are compared for monovalent and bivalent antibodies in Fig. 1. An affinity constant of 1×10^9 M^{-1} and an initial concentration of 10 nM antibody binding sites is assumed. At the midpoint of the titration curve (50% inhibition of the antibody binding sites) a maximum deviation of the curves of 25% corresponding to the maximum signal is reached. Because the method described here uses the full range of the titration curve for the determination of the affinity constant, it is important to take the valence of the antibody into account.

To obtain a maximum of information from the titration curves, antibody concentrations in the range of the reciprocal affinity constants should be used. The effect of using higher antibody concentrations is demonstrated in Fig. 2. Calculated titration curves for several antibody concentrations for an affinity constant of 1×10^9 M^{-1} are shown in comparison to titration curves corresponding to an infinite antibody affinity where all analyte in solution is bound by the antibody. These infinite affinity titration curves do not contain information about the affinity constant since the law of mass action is not valid. At antibody concentrations exceeding the reciprocal affinity constant, the titration curves for finite affinity constants become similar to the corresponding titration

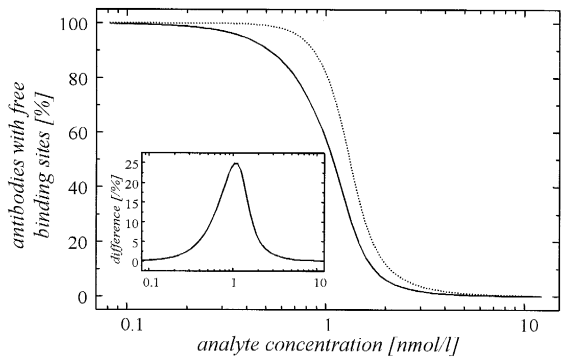

Fig. 1. Calculated concentration of antibodies with free binding sites as a function of the analyte concentration for IgG (· · ·) and Fab fragments (—). The inset shows the difference between the two curves.

curve for an infinite affinity constant. The higher the difference between these two curves the higher the amount of information available from the titration curve. A rapid decrease in the maximum of the difference curve with the antibody concentration above the reciprocal affinity constant and a strong shift to higher analyte concentrations can be observed.

2.2. Kinetic aspects

The determination of free antibody binding site concentrations is carried out by monitoring the kinetics of antibody binding to a surface in a flow-through system. Hence, the concentration of free antibody binding sites in a diffusion layer adjacent to the surface is decreased leading to a perturbation of the equilibrium in this region. To define the potential of the method this perturbation has to be analysed taking kinetic considerations into account.

The kinetics of formation and the decomposition of the analyte-antibody complex is determined by the

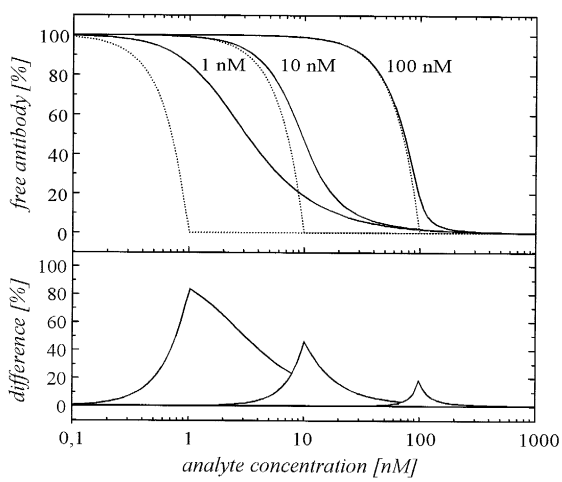

Fig. 2. Calculated change of effective IgG concentration as a function of analyte concentration for an affinity constant of 1×10^{9} $M^{-}1$ (—) compared to an infinite affinity constant (· · ·). The difference of the curves is decreasing rapidly with antibody concentrations above the reciprocal affinity constant.

association rate constant k_{ass}, the dissociation rate constant k_{diss} and the actual concentrations of free analyte c_{an}, free antibody c_{ab} and the analyte-antibody complex c_{anab}:

$$\frac{dc_{anab}}{dt} = k_{ass} c_{an} c_{ab} - k_{diss} c_{anab} \quad (5)$$

Equilibrium is reached when the dissociation rate equals the association rate:

$$k_{ass} c_{an} c_{ab} = k_{diss} c_{anab}$$

Association rate constants for binding of low molecular weight analytes to antibodies lie in the range of 5×10^6–5×10^8 M^{-1} s^{-1} for many analyte-antibody systems which is near to the diffusion limited rate (Tijssen, 1985). Therefore the difference in affinity is mainly ascribed to different dissociation rate constants. A typical association rate constant of 2×10^7 M^{-1} s^{-1} is assumed for the following considerations. The dissociation rate constant is then for a given affinity constant K

$$k_{diss} = \frac{k_{ass}}{K}$$

The formation of free antibody by dissociation of the analyte-antibody complex during exposure to the sensor leads to additional antibody binding to the surface. Therefore a higher signal than expected is detected for the equilibrium free antibody. For the application of the titration model described above this effect should be negligible compared to the original response. In the following we estimate this effect for the worst case.

To achieve maximum binding rates an analyte derivate with high affinity to the antibody and with a high association rate constant is immobilised at the sensor surface at a high concentration. For low antibody concentrations and low surface loadings ($< 20\%$ of the saturation), the binding of the antibodies at the surface is much faster than the mass transport by diffusion to the surface (Eddowes, 1987; Glaser, 1993). All antibodies reaching the surface by diffusion are immediately bound by the excess of immobilised analyte derivate, leading to a maximum concentration gradient. The binding rate is then limited by the maximum diffusion rate to the surface. Assuming a linear decrease of the concentration c_{ab} in a diffusion layer of constant thickness δ this maximum binding rate is given by

$$\left(\frac{d\Gamma}{dt}\right)_{max} = D \cdot \frac{c_{ab}}{\delta}$$

We observe in our flow system with a cavity depth of 50 μm and laminar flow rates of approximately 5 mm/s diffusion limited binding rates of approximately 1–2 pg/(mm^2 · s) for antibody concentrations of 1 μg/ml. Assuming a typical diffusion coefficient of 6×10^{-11} m^2/s for IgG this binding rate corresponds to an average diffusion layer thickness of a few 10 μm.

Due to the gradient of free antibody concentration in the diffusion layer the equilibrium is perturbed in this region. The dissociation rate exceeds the association rate (Eq. (5)) and additional free antibody will be generated. This perturbation of equilibrium extends only over the diffusion layer as equilibrium concentrations are maintained by the flow system.

In the following example we assume that only a small part of the analyte-antibody complex dissociates during detection which is a prerequisite to the detection of any inhibition effect. This allows us to assume

1. a constant concentration of analyte and analyte-antibody complex in the diffusion layer and
2. a linear decrease of the free antibody concentration down to zero at the surface in the diffusion layer

Then the effective dissociation rate dc_{ab}/dt within the diffusion layer at a distance r from the surface is given by

$$\frac{dc_{ab}}{dt}(r) = k_{diss} \cdot c_{anab} - k_{ass} \cdot c_{an} \cdot c_{ab}(r) = k_{diss} \cdot c_{anab} \cdot \left(1 - \frac{r}{\delta}\right)$$

Table 1
Binding rates caused by additionally generated free antibody following dissociation of the analyte-antibody complex. An initial antibody concentration of 1 μg/ml is assumed

k_{diss} (s)	$(\mathrm{d}\frac{\Gamma}{\mathrm{d}t})_{diss}$ (pg/mm^2/s)
0.01	0.025
0.1	0.25
0.2	0.5

If the worst case is assumed that all additionally generated antibody reaches the surface a binding rate of

$$\left(\frac{\mathrm{d}\Gamma}{\mathrm{d}t}\right)_{diss} = \frac{k_{diss} \cdot c_{anab} \cdot \delta}{2}$$

will be detected. This additional binding rate is given in Table 1 for various dissociation constants assuming 50% inhibition $c_{anab} = 0.5 \cdot c_{0,ab}$ and the same antibody concentration $c_{0,ab}$ as above (1 μg/ml).

If the original maximum binding rate of 1–2 pg $\cdot$ mm^{-2} s^{-1} for this antibody concentration is taken into account dissociation constants below 0.02 s^{-1} are required to reduce the additional signal to less than 10% of the maximum non-perturbed signal. This is valid for the worst case assumption that all additionally generated antibodies are detected. In reality only a minor part of the additionally generated free antibodies will reach the surface due to mass transport limitations and a significantly lower perturbation effect is then detected.

These considerations show that the critical parameter for this detection method of equilibrium is the thickness of the diffusion layer δ. On the one hand, the maximum binding rate is limited by this parameter whilst on the other hand perturbation of the equilibrium only occurs within the diffusion layer and can be reduced by a decreased thickness of this layer. Diffusion layer thickness can be reduced by increased flow rates and optimised flow regimes.

3. Materials and methods

3.1. Materials

Common chemicals and biochemicals were purchased from Sigma, Deisenhofen/Germany and Fluka, Neu-Ulm/Germany. *s*-Triazine standard solutions were purchased from Riedel de Haën, Seelze/Germany. The triazine derivative 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6′-amino)caproic acid (atrazine caproic acid, ACA) was a gift from Ram Abuknesha, GEC London. Interference transducers (10 nm Ta_2O_5 and 500 nm SiO_2 on float glass) were produced by Schott, Mainz/Germany in a plasma impulse CVD process.

3.2. Antibody preparation

Monoclonal triazine antibodies were prepared as described in detail by Giersch and Hock (1990), Giersch (1993) and Kramer et al. (1994). BALB/c mice were immunised by analyte-BSA and -KLH conjugates and fused with myeloma cells PAI-B_3AgI8. The hybridoma supernatant was assayed for anti-triazine antibodies and the cultures which proved positive were cloned by single cells.

Antibodies were produced in serum free cell culture and purified by affinity chromatography on a protein A column. The concentration of purified antibodies was determined from the absorbance at 280 nm.

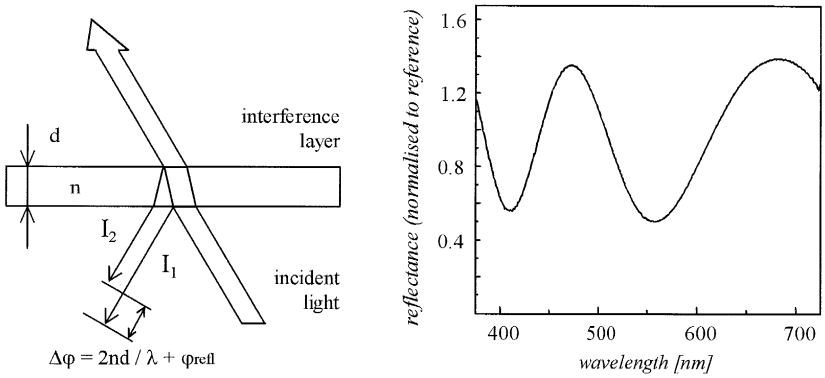

Fig. 3. Detection principle of RIfS: The increase in thickness of a thin layer is detected as a change in the pattern of the interference spectrum.

Fab fragments from mab K4E7 were prepared by papain cleavage according to Zierz (1990). The optimum digestion time was 3 h at 37°C. The purification of Fab fragments was carried out by ion exchange chromatography (DEAE Sephacel from Pharmacia) to remove residual IgG and Fc fragments. Purity was checked by SDS-PAGE.

The affinities of the antibodies to various *s*-triazines were estimated from test midpoints of standard direct competitive ELISA. The antibodies from the culture medium were immobilised on microtiter plates pre-coated with goat anti-mouse IgG. The sample and an enzyme tracer (*s*-triazines labelled with peroxidase or alkaline phosphatase) were added sequentially to compete for the antibody binding sites. After adding the enzyme substrate absorbance values were determined using a microtiter plate reader (Giersch and Hock, 1990).

3.3. Antibody detection

The equilibrium concentration of antibodies with free binding sites was determined from binding to a solid phase attached *s*-triazine derivative in a flow-through system. Reflectometric interference spectroscopy (RIfS) allows label-free monitoring of binding events at surfaces (Gauglitz et al., 1993). The detection principle is demonstrated in Fig. 3.

RIfS is based on white light interference at a weakly reflecting Fabry-Perot cavity. Molecules binding at the surface of the cavity increase the optical thickness of the cavity which is detected as a shift of the reflectance spectrum. The application of this method for immunosensing in a flow system has been described in detail by Brecht et al. (1993).

The experimental set-up for monitoring affinity interactions is shown in Fig. 4. Light from a tungsten light source (20 W) was guided to the transducer using bifurcated fibre optics (PMMA, 1 mm diameter from Microparts, Dortmund/Germany). The reflected light was collected in the same fibre and detected in a diode array spectrometer (MCS 210 and 410 series, 512 diodes, 350–780 nm, and 16 bit nominal resolution, Carl Zeiss, Jena/Germany). Samples were introduced by flow injection (ASIA, Ismatec, Wertheim-Mondfeld/Germany) using a sample loop of 500 μl. A flow cell of approximately 200 nl volume, 50 μm depth and 2 mm width was used with a standard flow rate of approximately 30 μl/min. This corresponds to a velocity of approximately 5 mm/s in the cavity.

Interference spectra were evaluated on-line by a parabolic fit to an interference minimum at about 550 nm. Data assessment, controlling the FIA and evaluation was achieved using a personal computer with self written software.

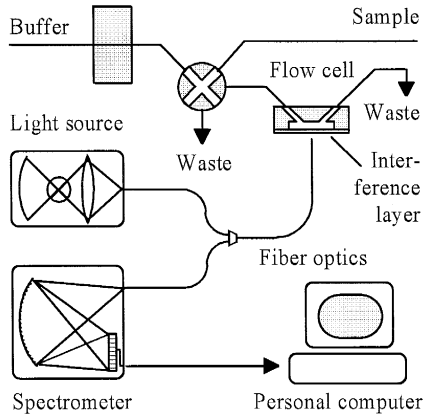

Fig. 4. Schematic set-up for monitoring of affinity interactions by RIfS.

3.4. Sensor preparation

Efficient detection of antibody binding requires appropriate modification of the sensor surface. High concentrations and accessibility of the attached analyte was achieved by covalent coupling of an analyte derivative to an amino-dextran modified surface as recently described (Piehler et al., 1996). For the detection of *s*-triazine antibodies 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6′-amino)caproic acid (atrazine caproic acid, ACA) was used as a derivative for coupling chemistry. Negligible non-specific protein adsorption was observed at such modified surfaces even at high protein concentrations (Piehler et al., 1996). A high surface concentration of the immobilised analyte derivative and a high binding capacity due to the flexible dextran matrix permitted high maximum loading by antibody (more than 10 ng/mm^2, data not shown). Under these conditions mass transport limited binding was observed for surface loadings up to 30% of the maximum loading. As described above, this behaviour guaranteed maximum binding rates that did not depend on the affinity of the antibody for the immobilised derivative. Constant binding rates were obtained permitting simple evaluation of the linear binding curves by linear regression. In such plots the slope directly corresponds to the concentration of free antibody. This also provides a simple method for the assessment of antibody concentrations in complex matrices (e.g. culture supernatants) by calibration with antibody solutions of defined concentration. This determination of the antibody concentrations is important for the further evaluation of the titration curves.

3.5. Test format

For the assessment of titration curves the equilibrium concentration of free antibody binding sites has to be determined for a constant antibody concentration and various analyte concentrations. An interaction period of 900 s for the pre-incubation of the antibody with the analyte was found to be sufficient to reach equilibrium binding for all analyte antibody pairs investigated. After longer incubation times no significant difference in assay response was detected. The pre-incubated solution was exposed to the sensor surface to monitor antibody binding by RIfS. After the binding event the sensor was regenerated by injecting pepsin (2 mg/ml, pH 2) to prepare it for the next cycle. This enzymatic digestion was found to be the fastest and mildest method to remove antibodies from the surface (Piehler et al., 1996). The time protocol for the whole test scheme is given in Table 2.

Table 2
Time protocol for a test cycle of binding site determination

Step	Time (s)	Action	Monitoring
1	Pre-run	Pre-incubation	–
2	0–120	Loading of the sample loop	Baseline
3	120–520	Injection of sample	Binding curve
4	520–600	Rinsing	Baseline
5	600–780	Injection of pepsin	Regeneration
6	780–900	Rinsing	Baseline

3.6. Model fitting

The model function describing the concentration of antibodies with free binding sites was fitted to the titration curve by a Marquart-Levenberg non-linear least squares algorithm (software ORIGIN from Microcal, Northampton/USA). The stability of the fitting procedure was investigated using simulated data sets modified by random noise of different scales. The model was fitted to these modified data sets and the parameters were compared to the original values. An antibody concentration of the reciprocal affinity constant was assumed. The data sets were calculated for 11 different logarithmically equidistant analyte concentrations starting from one tenth of the antibody concentration. A blank value was also determined. Noise of 5, 10 and 20% of the blank value (maximum signal) was generated randomly for the calculated values (see Fig. 5 for an example).

The deviations of the calculated affinity constant K from the original value for different fitting procedures are shown in Table 3. If only the affinity constant is fitted as a free parameter, deviations of less than 5, 10 and 20% from the theoretical value were obtained for the different noise levels. The same fitting results were obtained using different start parameters for the affinity constant.

If both the initial antibody concentration $c_{0,ab}$ and the affinity constant K were fitted higher deviations of about 10, 15 and 25% were observed for the affinity constant. The coefficient of correlation between the affinity constant and the initial antibody concentration was typically less than 0.5. Nevertheless, different fitting results were obtained when the start parameters were changed (deviations of about 5–10%). Therefore it is preferable that only the affinity constant is fitted to obtain more reliable results. For this purpose the initial antibody concentration has to be determined in a prior step. This can be carried out conveniently by calibration of the

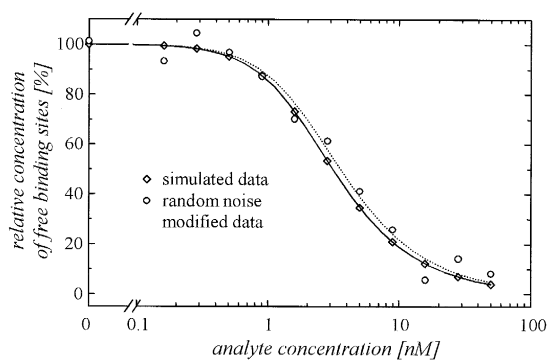

Fig. 5. Comparison of the fitting results for the original simulated data set (—) and data set randomly modified with 20% noise (· · ·). An affinity constant of 1×10^{9} l/mol was assumed. By fitting the model to the modified data set an affinity constant of $(8.5\pm0.8)\times10^{8}$ l/mol was obtained. The error is calculated from the mean square error of the fitted curve from the data points.

Table 3
Deviations of the fitting result observed for different noise in the data points of the titration curve

Noise of data points (titration curve)	Deviation of the calculated K from the original value	
	Variation of K	Variation of K and $c_{0,ab}$
5%	5%	10%
10%	10%	15%
20%	20%	25%

slope of the binding signal obtained for the blank sample with known antibody concentrations as described above.

4. Results and discussion

4.1. Antibody detection

The concentration of free antibody was determined by flow-through detection of antibody binding to an immobilised analyte derivative. A typical binding curve for an antibody at the modified transducer surface and the regeneration by pepsin monitored by RIfS is shown in Fig. 6. A linear increase of the signal during incubation of the antibody was observed indicating mass transport limited binding. The concentration of free antibody was derived from the slope of the curve. The residuals of the linear regression of the binding curve demonstrate that only statistical deviations from linearity were observed.

Non-specific binding of proteins in the sample matrix at the transducer surface strongly interferes with direct detection methods as it cannot be distinguished from binding caused by specific antibody. For a reasonable detection of the inhibition of free antibody binding sites non-specific adsorption has to be suppressed efficiently. This was achieved successfully by shielding the surface by covalent attachment of a dextran matrix. The absence of significant non-specific binding even during incubation of hybridoma cell culture supernatants containing serum is demonstrated in Fig. 7. For the diluted supernatant (1/10 in PBS) a typical linear increase due to mass transport limited binding of the antibody was observed. The slope of the binding curve corresponds to a concentration of 0.7 μg/ml active antibody. After suppressing specific binding by adding 3 μM atrazine caproic acid to the same supernatant no residual binding was observed. This shows that even with such complex sample matrices there was no significant non-specific binding.

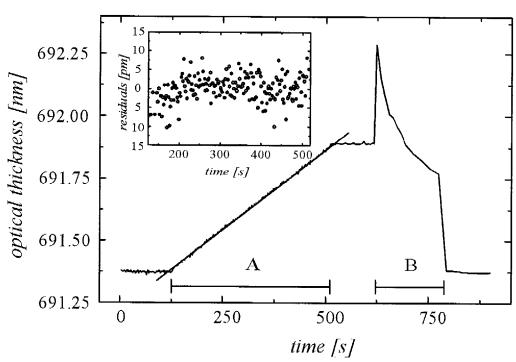

Fig. 6. Typical test cycle for the determination of free antibodies binding sites. Injection of 0.75 μg/ml mab K4G2 without any analyte (section A) and regeneration by incubation of 2 mg/ml pepsin pH 2 (section B). The inset shows the residuals of a linear regression of the binding curve.

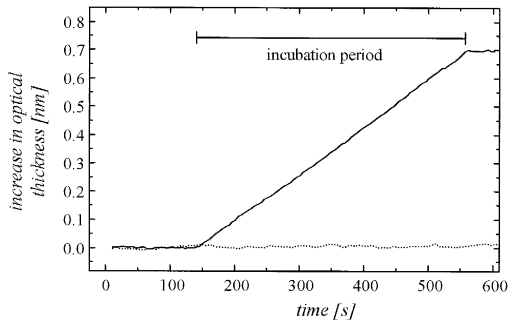

Fig. 7. Investigation of non-specific binding effects: Incubation of hybridoma culture (clone K4G2) supernatant diluted 1/10 in PBS (—) and the same supernatant with excess of a high affinity analyte (1 μg/ml ACA) to inhibit specific binding ($\cdots$).

4.2. Affinity titration

The change of sensor response due to titration of the free binding antibody sites by several concentrations of the analyte is demonstrated in Fig. 8 for the antibody K1F4 (IgG) and the analyte terbuthylazine. The slope of the binding curves decreases with increasing concentration of the analyte and is reduced to zero for high analyte concentrations.

The titration curve obtained from the slope of these binding curves normalised to the blank value and the fit of the IgG model for IgG binding are shown in Fig. 9.

At high analyte concentrations the signal in this analyte-antibody system is reduced to less than 5% of the blank value suggesting that dissociation during detection does not play a significant role. For this reason this titration is appropriate for the determination of the total concentration of available antibody binding sites. First the model was fitted to the data by estimating a binding site concentration of 6 nM and only changing the affinity constant. This gave an affinity constant of 9.0×10^8 M^{-1}. In a further step both the affinity constant and the concentration of antibody binding sites were fitted using the results of the first fit as start parameters. While the affinity constant did not change significantly a refined binding site concentration of 5 nM was obtained. Very good correspondence of fit and data was observed. Coefficients of variation of less than 10% were determined for the affinity constant and the antibody concentration from the mean square errors of the fitting curve.

A titration curve for simazine using the same antibody clone and antibody concentration, and the corresponding fit of the model is shown in Fig. 10. An affinity constant of 3.8×10^7 M^{-1} was determined by fitting only

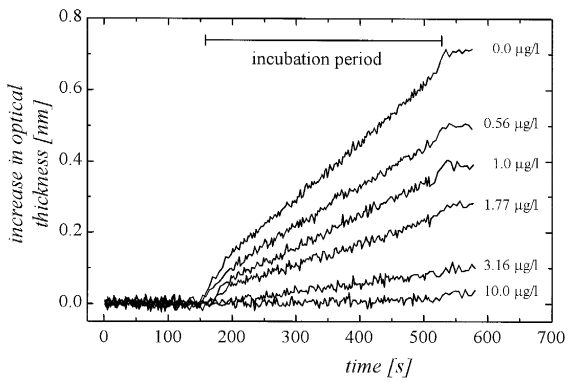

Fig. 8. Binding curves for 0.5 μg/ml K1F4 at various concentrations of terbuthylazine.

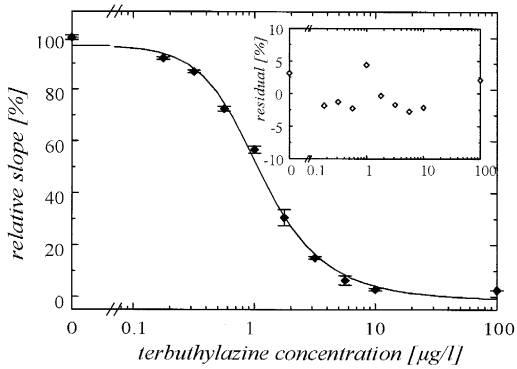

Fig. 9. Titration curve of terbuthylazine with antibody K1F4, 1 µg/ml, and fitting by the model described above for IgG (line). The residuals of the fit are shown in the inset.

the affinity constant and using the concentration of antibody binding sites determined above. The correlation of data and model was still reasonable and no significant systematic deviation was observed. Even at high simazine concentrations (100 µg/l corresponds to a 100-fold molar excess) antibody binding was detected.

For lower affinity constants significant deviation of the fit curve from the experimental values is observed. An example is the titration of K4G2 by simazine shown in Fig. 11.

Even at very high analyte concentrations antibody binding of approximately 20% of the blank value was detected suggesting a perturbation signal due to a high dissociation rate. An apparent affinity constant of 5.5×10^6 M^{-1} was determined by a fit of the model described above. The residuals of the fit are shown in the bottom. The true affinity constant of the antibody was higher since the model does not take dissociation into account. Furthermore a higher concentration of the free binding sites was detected for high concentrations of the analyte-antibody complex than are predicted by the model.

For the above reason two modified fitting models were applied to this titration curve. First, a constant residual binding term was introduced which simulates a constant binding rate due to dissociation. This curve fits much better to the data than the simple model. A higher affinity constant of 1.3×10^7 M^{-1} was obtained. Second, only the first part of the titration curve (0–10 µg/l) was evaluated because the concentration of the analyte-antibody complex is low in this part and dissociation does not play an important role. The problem of this method is that only a few points are used for the fit which affects the accuracy of the results. The residuals in the evaluated part of the titration curves are low. This curve corresponds to the ideal case when no

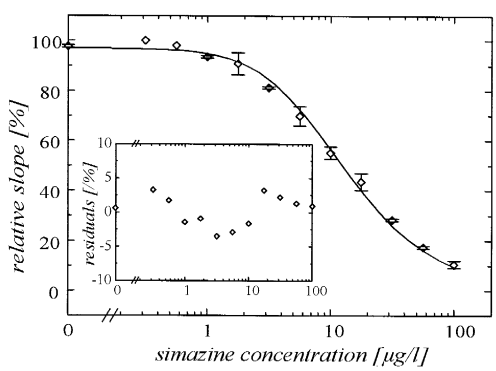

Fig. 10. Titration curve of antibody K1F4 by simazine and fit of the free binding site model. The residuals of the fit are shown in the inset.

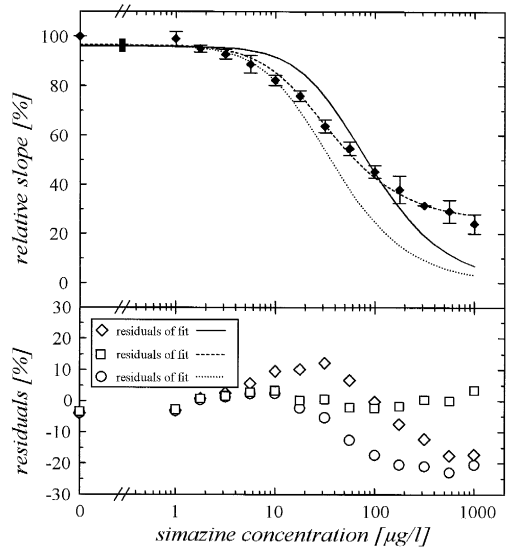

Fig. 11. Titration of antibody K4G2 by simazine and fitting of simple model (—), a model taking an offset into account (---) and a fit of the beginning (0–10 μg/l) of the titration curve ($\cdots$). The residuals of all three fits are shown in the bottom diagram.

dissociation occurs during detection. Using this method an affinity constant of 1.1×10^7 M^{-1} was obtained. These results are more adequate and correspond well to each other.

These results demonstrate that refinement of the model is necessary for low affinity systems taking into consideration the dissociation during detection. The determination of affinity constants below 10^6 M^{-1} will still be critical because then most of the signal will be due to perturbation by dissociation.

In Fig. 12 the titration of antibody clone K4E7 by the analyte atrazine at two different antibody concentrations are compared. Since this is a high affinity system the concentration of antibody binding sites and the affinity constant were determined by a fit of the model as described for the system K1F4/terbuthylazine. Similar affinity constants of 1.3×10^9 M^{-1} and 1.2×10^9 M^{-1} respectively were obtained with corresponding antibody concentrations of 16 nM and 3 nM. This result compares well to the slopes of the binding curves of the blank values for the different antibody concentrations.

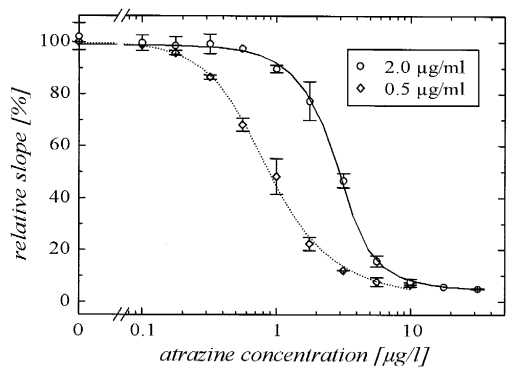

Fig. 12. Titration of free binding site concentration for two different antibody concentrations (K4E7): approx. 0.5 μg/ml (◇) and 2 μg/ml (○) (protein concentration determined photometrically).

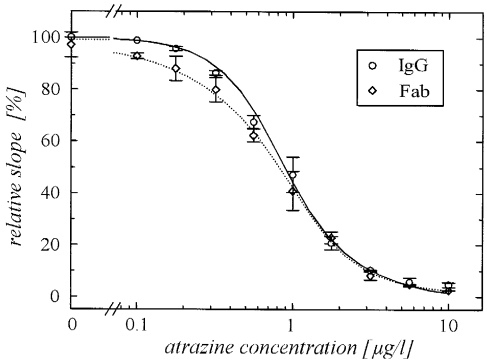

Fig. 13. Comparison of titration curves for IgG and Fab of the same monoclonal antibody (K4E7). The binding site concentration is about 5 nM for both systems.

The different shapes of the two titration curves illustrate how antibody concentration influences the assessment of affinity information from the curve. At the higher antibody concentration the decrease of antibody binding from maximum to minimum occurs over a very small concentration range (less than one order of magnitude). At the lower antibody concentration this range is significantly broader (more than one order of magnitude). This generates a problem for two reasons:

1. the titration curve contains less information about the affinity of the antibody as demonstrated above;
2. the increased slope of the titration curves leads to a higher statistical error in the data points.

For these reasons the best results for affinity titrations are achieved using antibody concentrations in the range of the reciprocal affinity constant.

This consideration permits an estimation of the upper limit of the range of affinity constants that are accessible by this detection method. It is given by the detection limit of antibody concentrations by the RIfS transducer. The current set-up allows the detection of about 30 ng/ml (20 pmol/l) antibody. A tenfold concentration of the detection limit corresponding to an antibody concentration of 0.2 nM is required to obtain a titration curve. This concentration permits a reasonable titration of affinities up to 10^{10}–10^{11} M^{-1}.

In Fig. 13 the titration curves for IgG and Fab of the monoclonal antibody clone K4E7 for the analyte atrazine are compared. The initial binding site concentration for both species was in the range of 5 nM. By fitting the corresponding models to the data, affinity constants of 1.2×10^9 M^{-1} for the IgG and 1.0×10^9 M^{-1} for the Fab fragment were obtained. This difference is not significant taking a typical error of about 10% into account. The difference in the shape of the titration curves for IgG and Fab is due to the number of binding

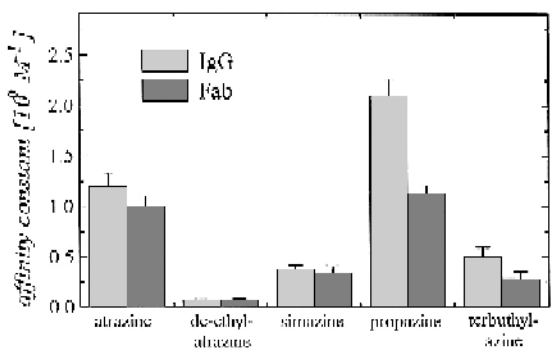

Fig. 14. Affinity constants of several analytes for antibody K4E7. Results for IgG and Fab fragments are compared.

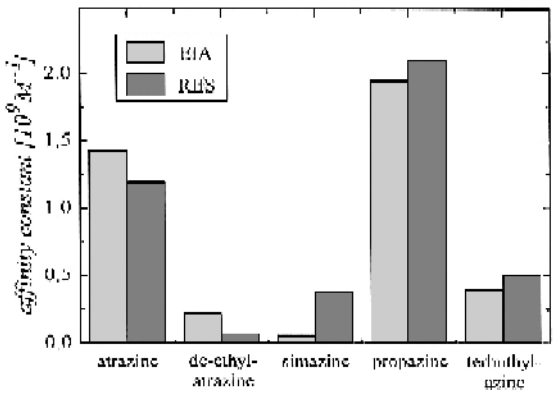

Fig. 15. Comparison of the affinity parameters obtained by the different test schemes used for ELISA and RIfS detection for the antibody K4E7: reciprocal test midpoints for the competitive immunoassay and affinity constants for the kinetic detection of equilibrium binding.

sites per molecule. This effect predicted by the binding model for low analyte concentration is clearly seen in this comparison.

A comparison of the affinity constants obtained for various analytes is shown in Fig. 14. A slightly decreased affinity constant for the Fab fragments is observed for most of the analytes investigated but is not significant if all sources of error are taken into account. The good correspondence of the results for different antibody species and models demonstrates the potential of this method.

4.3. Comparison to ELISA

Convenient access to affinity information from heterogeneous competitive immunoassays gives the test midpoint (50% inhibition of the antibody) of an optimised test scheme (Nieto et al., 1984, Rath et al., 1988). The reciprocal test midpoints obtained for a set of five different analytes by a direct ELISA test format were compared to the affinity constants calculated from a fit to the titration curves shown in Fig. 15. There was a general correspondence of the results obtained by these different methods and test formats. A notable exception was the analyte simazine which strongly differed in this respect. The significantly lower value calculated from the ELISA test midpoint was probably due to the fact that the test parameters were not fully optimised. Herein lies one major advantage of our method since it allows investigation of different analyte-antibody pairs using the same test parameters.

Similar results were obtained for four other monoclonal *s*-triazine antibodies (data not shown).

5. Summary and conclusions

We have introduced a new method for the determination of affinity constants in homogeneous phase using a direct affinity probe for the rapid detection of equilibrium antibody concentrations in a flow through system. This detection scheme is superior to conventional ELISA because equilibrium distribution of the antibody to free and surface attached analyte does not occur. Influences by the affinity and the surface concentration of the immobilised ligand are therefore avoided in this method. In principle this method can be applied with any other flow-through detection system but label-free detection systems allow very simple and flexible procedures. The method has distinct advantages which are superior to real-time kinetic analyses if high affinity systems with small ligands are investigated. No modification of the ligand for immobilisation is required which can alter the affinity. Furthermore, the interaction takes place under homogeneous phase conditions permitting high associa-

tion rates which cannot, for fundamental reasons, be observed during heterogeneous phase interactions (diffusion controlled interactions).

Good correspondence of the titration curves and the fitted model are observed for analyte-antibody systems with interaction constants between 4×10^7 M^{-1} and 2×10^9 M^{-1}. Analyte-antibody systems with lower affinities show strong systematic deviations of the experimental titration curve from the model. This effect is well explained by the generation of additional free antibodies by dissociation of the analyte-antibody complex in the diffusion layer during the detection event. More reliable results are obtained by refinement of the fitting model.

The model was confirmed using antibodies at different concentrations, from different cell lines and with different valencies. No optimisation of the test parameters was required to obtain reliable results. General correspondence between information on affinities based on optimised ELISA test midpoints and the affinity constants determined by our method were found for several antibody clones.

An estimation of the range of affinity constants that can be determined by this method has been made possible by these results. For low affinity constants the applicability of this methods is limited by the dissociation of the analyte-antibody complex. Although this is a kinetic parameter that depends on the system and not directly on the affinity constant, a lower limit of about 10^6 M^{-1} may be estimated. For high affinity systems the minimum antibody concentration which can be detected limits the range to affinity constants of about 10^{10}–10^{11} M^{-1}. Extension of these limits is possible by improving the flow system since mass transport is the critical factor both for the detection of low antibody concentrations and perturbation at low affinity constants. As additional free antibody is generated by dissociation only in the diffusion layer, a decrease of the contribution by dissociation is possible by reducing the thickness of this layer. At the same time mass transport will be increased, thereby allowing the detection of lower antibody concentrations. Improvement of the mass transport to the surface is feasible by optimised flow conditions in the flow cell (e.g. by using a wall-jet design).

Modification of the binding model is necessary to improve the applicability of the method to systems with lower affinity constants (i.e. higher dissociation rate constants). The dependence of the dissociation kinetics on the concentration of the analyte-antibody complex has to be taken into account by an additional term instead of an (arbitrary) constant binding offset term.

Acknowledgements

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References

Brecht, A., Gauglitz, G. and Polster, J. (1993) Interferometric immunoassay in a FIA-system: a sensitive and rapid approach in label-free immunosensing. Biosens. Bioelectron. 8, 387–392.

Cush, R., Cronin, J.M., Stewart, W.J., Maule, C.H., Molloy, J. and Goddard, N.J. (1993) The resonant mirror: a novel optical biosensor for direct sensing of biomolecular interactions. Part I: principle of operation and associated instrumentation. Biosens. Bioelectron. 8, 347–353.

Eddowes, M.J. (1987) Direct immunochemical sensing: Basic chemical principles and fundamental limitations. Biosensors 3, 1–15.

Ekins, R. (1994) Immunoassay: recent developments and future directions. Nucl. Med. Biol. 21, 495–521.

Fägerstam, L.G., Frostell-Karlsson, A., Karlsson, R., Persson, B. and Rönnberg, I. (1992) Biospecific interaction analysis using surface plasmon resonance detection applied to kinetic, binding site and concentration analysis. J. Chromatogr. 597, 397–410.

Feldman, H.A. (1972) Mathematical theory of complex ligand binding systems at equilibrium. Some methods for parameter fitting. Anal. Biochem. 48, 317.

Fisher, R.J. and Fivash, M. (1994) Surface plasmon resonance based methods for measuring the kinetics and binding affinities of biomolecular interactions. Curr. Opin. Biotechnol. 5, 389–395.

Friguet, B., Chaffotte, A.F., Djavadi-Ohaniance, L. and Goldberg, M.E. (1985) Measurements of the true affinity constant in solution of analyte-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods 77, 305–319.

Gauglitz, G., Brecht, A., Kraus, G. and Nahm, W. (1993) Chemical and biochemical sensors based on interferometry at thin (multi-)layers. Sens. Act. B 11, 21–27.

Giersch, T. (1993) A new monoclonal antibody for sensitive detection of atrazine with immunoassay in microtiter plate and dipstick format. J. Agric. Food Chem. 41, 1006–1011.

Giersch, T. and Hock, B. (1990) Production of monoclonal antibodies for the determination of *s*-triazines with enzym immunoassay. Food Agric. Immunol. 2, 85–97.

Glaser, R.W. (1993) Antigen-antibody binding and mass transport by convection and diffusion to a surface: A two-dimensional computer model of binding and dissociation kinetics. Analyt. Biochem. 213, 153–161.

Hetherington, S. (1990) Solid phase disruption of fluid phase equilibrium in affinity assays with ELISA. J. Immunol. Methods 138, 195–202.

Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfas, S., Persson, B., Roos, H., Rönnberg, I., Sjölander, S. Stenberg, E., Stahlberg, R., Urbaniczky, C., Östlin, H. and Malmquist, M. (1991) Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. Biotechniques 11, 620–627.

Karlsson, R. (1994) Real-time competitive kinetic analysis of interactions between low molecular weight ligands in solution and surface-immobilized receptors. Anal. Biochem. 221, 142–151.

Karlsson, R., Michaelsson, A. and Mattson, L. (1991) Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. J. Immunol. Methods 145, 229–240.

Kramer, K., Giersch, T. and Hock, B. (1994) Magnetic bead selection of hybridomas producing pesticide antibodies. Food Agric. Immunol. 6, 5–16.

Liedberg, B., Nylander, C. and Lundström, I. (1983) Surface plasmon resonance for gas detection and biosensing. Sens. Act. 4, 299–304.

Lukosz, W. (1991) Principles and sensitivities of integrated optical and surface plasmon sensors for direct affinity sensing and immunosensing. Biosens. Bioelectron. 6, 215–225.

Nieba, L., Krebber, A. and Plückthun, A. (1996) Competition BIAcore for measuring true affinities: Large differences from values determined from binding kinetics. Analyt. Biochem. 234, 155–165.

Nieto, A., Gaya, A., Jansa, M., Moreno, C. and Vives, J. (1984) Direct measurement of antibody affinity by hapten inhibition enzyme immunoassay. Mol. Immunol. 21, 537–543.

O'Shannessy, D.J. (1994) Determination of kinetic rate and equilibrium binding constants for macromolecular interactions: A critique of the surface plasmon resonance literature. Curr. Opin. Biotechnol. 5, 65–71.

O'Shannessy, D.J. and Winzor, D.J. (1996) Interpretation of deviation from pseudo-first-order kinetic behaviour in the characterisation of ligand binding by biosensor technology. Analyt. Biochem. 236, 275–283.

O'Shannessy, D.J., Brigham-Burke, M., Soneson, K.K., Hensley, P. and Brooks, I. (1993) Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: Use of non-linear least squares analysis methods. Analyt. Biochem. 212, 457–468.

Piehler, J., Brecht, A., Kramer, K., Hock, B. and Gauglitz, G. (1995) Multi-analyte determination with an optical multi-antibody detection system. In: T. Vo-Dinh and R. Nießner (Eds.), Environmental Monitoring and Hazardous Waste Site Remediation. Proc. SPIE 2504, 185–193.

Piehler, J., Brecht, A. and Gauglitz, G. (1996) Surface modification for direct immunoprobes. Biosens. Bioelectron. 11, 579–590.

Portnoi, D., Freitas, A., Holmberg, D., Bandeira, A. and Coutinho, A. (1986) Immunocompetent autoreactive B lymphocytes are activated cycling cells in normal mice. J. Exp. Med. 164, 25–35.

Rath, S., Stanley, C.M. and Steward, M.W. (1988) An inhibition enzyme immunoassay for estimating relative antibody affinity and affinity heterogeneity. J. Immunol. Methods 106, 245–249.

Schots, A., Van der Leede, B.J., De Jongh, E. and Egberts, E. (1988) A method for the determination of antibody afffinity using a direct ELISA. J. Immunol. Methods 109, 225–233.

Seligman, S.J. (1994) Influence of solid phase antigen in competition enzyme-linked immunosorbent assays (ELISAs) on calculated antigen-antibody dissociation constants. J. Immunol. Methods 168, 101–110.

Stevens, F.J. (1987) Modification of an ELISA-based procedure for affinity determination: corrections necessary for use with bivalent antibody. Mol. Immunol. 24, 1055–1060.

Steward, M.W. and Lew, A.M. (1985) The importance of antibody affinity in the performance of immunoassays of antibody. J. Immunol. Methods 78, 173–190.

Steward, M.W. and Steengaard, J. (1983) Antibody Affinity: Thermodynamic Aspects and Biological Significance. CRC Press, Boca Raton, FL.

Ternynck, T. and Avrameas, S. (1986) Murine natural monoclonal antibodies – a study of their polyspecifities and their affinities. Immun. Rev. 94, 99–112.

Tijssen, P. (1985) Practice and Theory of Enzyme Immunoassay. Elsevier Science Publisher, Amsterdam.

Zierz, R. (1990) Herstellung immunreaktiver Fragmente aus monoklonalen Mausantikörpern. in: J.H. Peters and H. Baumgartner (Eds.), Monoklonale Antikörper, Herstellung und Charakterisierung. Springer Verlag, Heidelberg.