Expert Opinion on Therapeutic Patents

Review

Biologicals & Immunologicals

Kinetics of biomolecular interactions

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Recent advances in instrumentation, in particular the development of biosensors, have greatly simplified the measurement of the kinetics of molecular interactions. Previously, while it was realised that the kinetics of biomolecular interactions were important factors in the successful design of molecules for clinical, industrial or scientific purposes, the difficulties in measuring the kinetics of these interactions meant that, in most cases, they were ignored. This review will briefly explain the principles behind kinetic interactions (more fully reviewed in [1-5]), then summarise the nature of optical biosensors used to measure kinetic interactions and, finally, give some selected and limited case studies to illustrate why measuring these interactions can be important in drug development.

Keywords: *affinity, biosensor, bispecific antibodies, cell signalling, kinetics, serum antibodies*

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1. Kinetics of molecular interactions

1.1 Equilibrium reactions

The interaction of molecules lies at the heart of biology: simply considering proteins, they interact with other proteins, with peptides, lipids, carbohydrates, nucleic acids as well as with non-organic molecules. Much of drug design is aimed either at creating novel interactions or at disrupting unwanted interactions, thus influencing the behaviour of the system. In addition, biomolecular interactions are vital in industrial applications where manufacture and purification of biomolecules is performed. One fundamental description of these interactions relies on describing the kinetics or the affinity of the interactions. Any non-covalent interaction between two molecules (A and B) can be described as an equilibrium reaction:

$$A + B \rightleftharpoons AB \quad (1)$$

where AB is the product of the interaction. (In this review we will assume, unless otherwise stated, that A and B are homogeneous species which interact with each other in a monovalent manner. More complex kinetic analyses are required to describe more complex interactions.)

If one mixes A and B then, initially, only the forward reaction occurs (from left to right). As this occurs, AB is formed, and the concentrations of A and B decrease (**Figure 1**). However, as time proceeds, and the concentration of AB increases, then the reverse (right to left) reaction becomes significant. At some time (theoretically infinite, though in most biological systems this practically can be considered to take as little as a few seconds or as long as overnight, depending on the kinetics and system under study) the two reactions (forward and reverse) proceed at the same rate. This is termed the equilibrium, as, although AB continues to be formed from A and B, and AB continues to dissociate into A + B, the concentrations of A, B and AB are constant.

At equilibrium the concentrations of A, B and AB ([A], [B] and [AB]) are related by the Law of Mass Action:

$$[AB] = K_a[A][B] \quad (2)$$

Figure 1 illustrates a typical reaction between two molecules, A and B, that associate in a monovalent manner to form the complex AB (as in equation 1). At time t = 0 equal amounts of A and B are mixed. The concentration of A, B and AB as a function of time are shown in **Figure 1a** (the concentration is given as a percentage of the initial concentration of A, the time is indicated in arbitrary units). As can be seen [A] and [B] decrease with time, while [AB] increases. After some time the reaction reaches equilibrium, and [A], [B] and [AB] remain constant. The rates of the reaction are shown in **Figure 1b**; initially the rate of the forward reaction (A+B→AB) is high, as [A] and [B] decrease, and [AB] increases, this rate falls, and the rate of the reverse reaction (AB→A+B) increases until equilibrium is reached and the forward and reverse reactions proceed at the same speed. The inset shows the same graph, but using a log scale. As can be seen at equilibrium, while the concentration of the reactants remains constant, the association and dissociation reactions continue.

Figure 1a: Equilibrium reactions: concentrations of A, B and AB as a function of time.

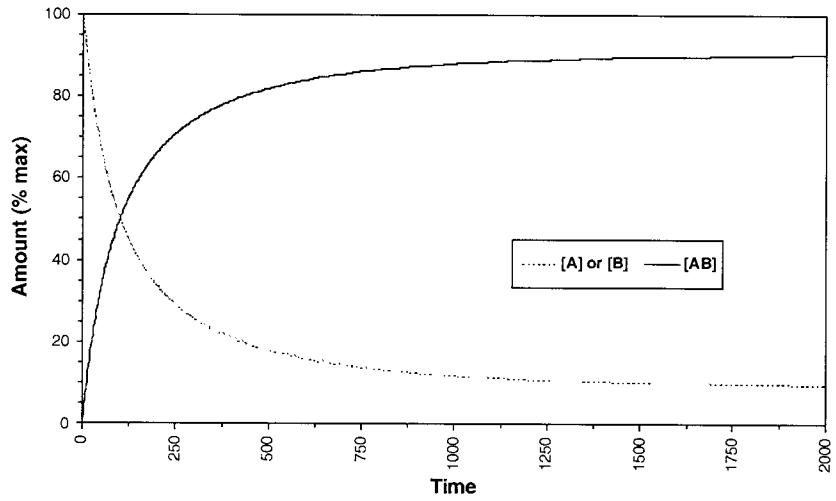
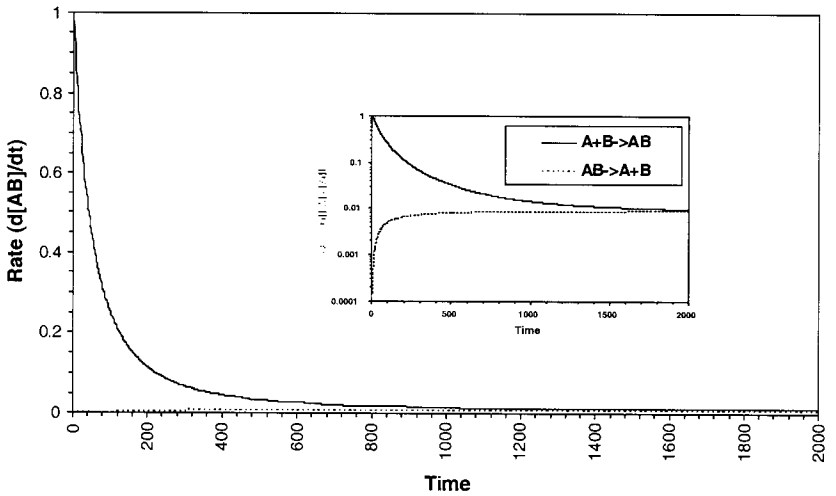


Figure 1b: Equilibrium reactions: rates of the reaction.



This equation simply states that as you increase the concentration of A or B you increase the concentration of AB. If you decrease [A] or [B] then you decrease [AB]. The constant in the reaction, K_a, is termed the association equilibrium constant. It has units of M^{-1} and is frequently given other abbreviations, including K, K_{+1}, K_1, K_0. The K_a is a description of the affinity of the interaction between the two molecules; if K_a is high then the affinity of the interaction is high. Thus, for given concentrations of A and B, the concentration of AB will be higher for an interaction with a high K_a than one with a low K_a. It should be noted that the value of K_a is dependent on other factors, such as the temperature, pH and the ionic strength of the buffer.

Equation (2) can be rearranged to give:

$$K_a = \frac{[AB]}{[A][B]} \qquad (3)$$

This allows the affinity of an antibody to be determined by measuring the concentration of AB, A and B at equilibrium. Note that [A] and [B] (the concentrations of unbound, free A and B) do not equate to the concentrations of A and B added to the system ($[A]_{total}$ and $[B]_{total}$), as some of A and B will be contained in the complex, AB.

In addition to K_a, the dissociation equilibrium constant (K_d, K_D, K_{-1} etc.) is frequently used to describe the affinity of the interaction (especially for antibody-antigen interactions).

$$K_d = \frac{1}{K_a} = \frac{[A][B]}{[AB]} \quad (4)$$

The units of K_d are M, and the lower the K_d the higher the affinity. The popularity of the K_d is that it gives a term that is readily understood. If one considers a reaction in which, at equilibrium, half of B is found in the complex then, by definition;

$$[B] = [AB] \quad (5)$$

$$\therefore K_d = [A] \quad (6)$$

Thus the K_d is the concentration of A at which half of B is complexed in AB. Thus, if we consider an antibody-antigen interaction; if one was to titrate the amount of antibody (A) to get half maximal reactivity in the assay system (e.g. in an ELISA or cell staining), then the K_d is given by the [A] (remember that [A] is the concentration of free A, not $[A]_{total}$. However, in many experimental situations, $[A] \approx [A]_{total}$. For example, if one uses a relatively large volume of antibody solution to stain a tissue section then only a negligible proportion of the antibody will be contained in the antibody-antigen complex).

1.2 Kinetics

The affinity of protein-protein interactions gives information about what the situation is at equilibrium, i.e., what the relative concentrations of the three components (A, B and AB) are at that time. This is clearly very important information as, given knowledge of the concentrations of A and B in a particular situation, this indicates how much of the complex, AB, will be formed. Thus the affinity will allow one to determine the extent to which molecules will interact in any particular system.

However, it gives no information about the rate or kinetics of the reaction. This is provided by the rate constants of the forward and reverse reactions; k_{ass} (association rate constant, variously known as k_a, k_{+1}, k_{on}; units $M^{-1}sec^{-1}$) and k_{diss} (dissociation rate constant, or k_d, k_{-1}, k_{off}; units sec^{-1}). The rate of the forward reaction is simply given by the product of k_{ass}, and the concentrations of the reactants, A and B.

$$\text{forward reaction} = k_{ass}[A][B] \quad (7)$$

The reverse reaction is dependent on k_{diss} and the concentration of AB.

$$\text{reverse reaction} = k_{diss}[AB] \quad (8)$$

$$\therefore \frac{d[AB]}{dt} = k_{ass}[A][B] - k_{diss}[AB] \quad (9)$$

At equilibrium the forward and reverse reactions occur, by definition, at the same rate, so

$$k_{ass}[A][B] = k_{diss}[AB] \quad (10)$$

This can be rearranged to give:

$$\frac{k_{ass}}{k_{diss}} = \frac{[AB]}{[A][B]} = Ka \quad (11)$$

It is thus clear that two interactions could have the same affinity (K_a) but different rate constants (k_{ass} and k_{diss}). Such interactions could be characterised as 'fast on-fast off' and 'slow on-slow off'. As shown in **Figure 2**, interactions that have the same affinity, but different kinetics, reach the same equilibrium point, however 'fast on-fast off' reactions reach equilibrium sooner than the 'slow on-slow off' interactions. In addition, at equilibrium, the rates of the forward and reverse reaction are much higher for the 'fast on-fast off' reaction (**Figure 2b**).

As shown in **Figure 2** the rate constants give information about how fast equilibrium is reached. They also inform one about the stability of the complex, AB. Thus, if one of the reactants is removed, the complex AB will dissociate at a rate determined by k_{diss}. This equates to the dissociation of an antibody-antigen complex on a tissue section or ELISA plate after washing out unbound antibody. The dissociation is illustrated in **Figure 3** for interactions exhibiting the 'fast' and 'intermediate' kinetics considered shown in **Figure 2**. The half life of the AB complexes ($t_{1/2}$) can be determined from the k_{diss} as

$$t_{1/2} = \frac{\ln 2}{k_{diss}} \quad (12)$$

The importance of kinetics in a range of settings will be discussed in more detail below, and is well reviewed in [4]. For the present, we will consider the simple case of using an antibody to bind to an antigen

Figure 2: The effect of kinetics on the formation of a complex between two molecules interacting as described in equation (1). **Figure 2a** illustrates the formation of the complex is shown for three reactions, each of which has the same equilibrium constants (K_a/K_d), but vary in their rate constants. As in **Figure 1**, time is given in arbitrary units and the concentration of AB is given as a percentage of the starting concentration of A. The slow and fast reactions have kinetics that are 10 times slower or faster than those of the intermediate reaction. As can be seen all reactions reach the same equilibrium point (though in the case of the slow reaction that is not reached in the time indicated on the graph). However, the time it takes to reach equilibrium is dependant on the rate constants of the reaction. **Figure 2b** shows the reaction rates for the intermediate and fast kinetics (shown using a log scale). At equilibrium the reaction rates are faster for the fast kinetics, indicating that there is greater turnover of A, B and AB than is seen in reactions with slower kinetics.

Figure 2a: Effect of kinetics on equilibrium reactions.

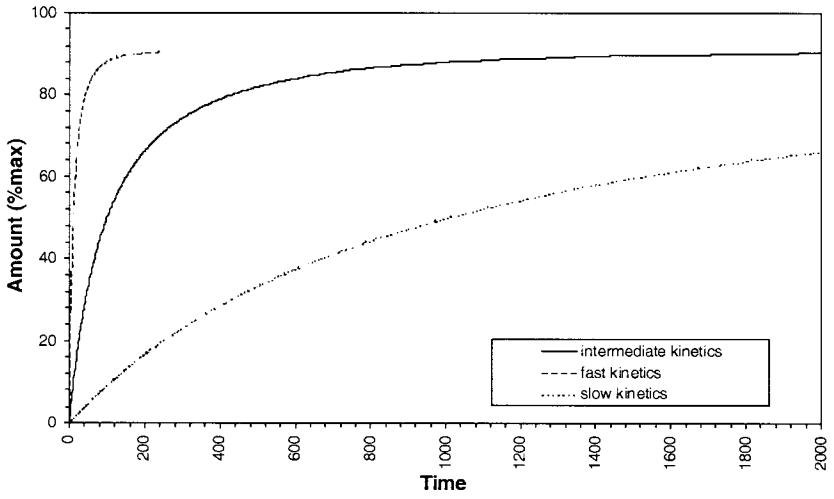


Figure 2b: Effect of kinetics on equilibrium reactions.

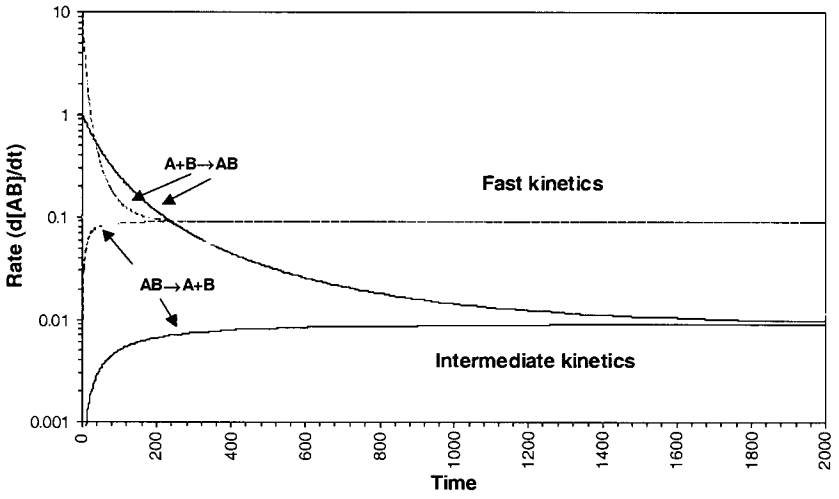


Figure 3: Effect of kinetics on dissociation of complexes. This figure illustrates the dissociation of the complex AB following removal of free A and free B for interactions with fast, intermediate and slow kinetics (varying by a factor of 10 as in **Figure 2**). The [AB] is expressed as a percentage of the starting concentration. As can be seen the complex is stable for reactions with slow kinetics, but rapidly dissociates in the case of the fast kinetics.

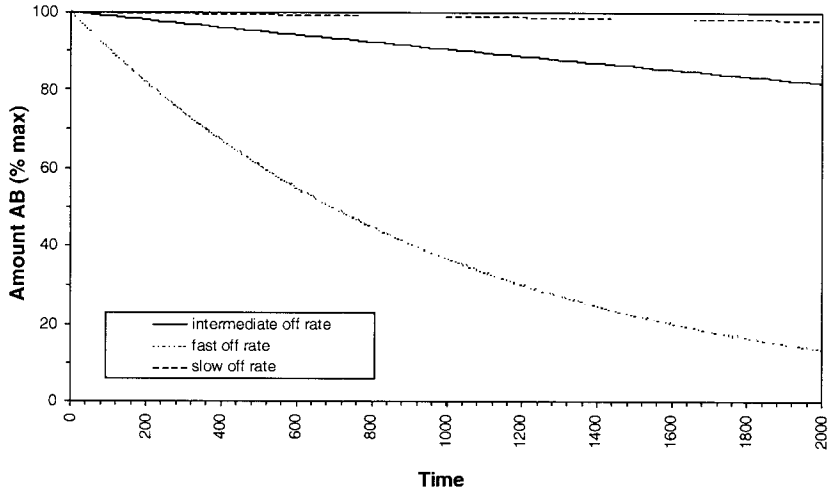
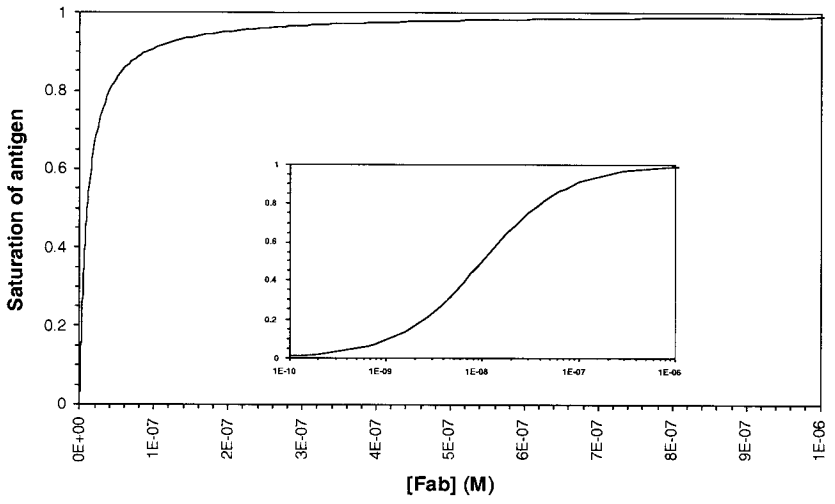


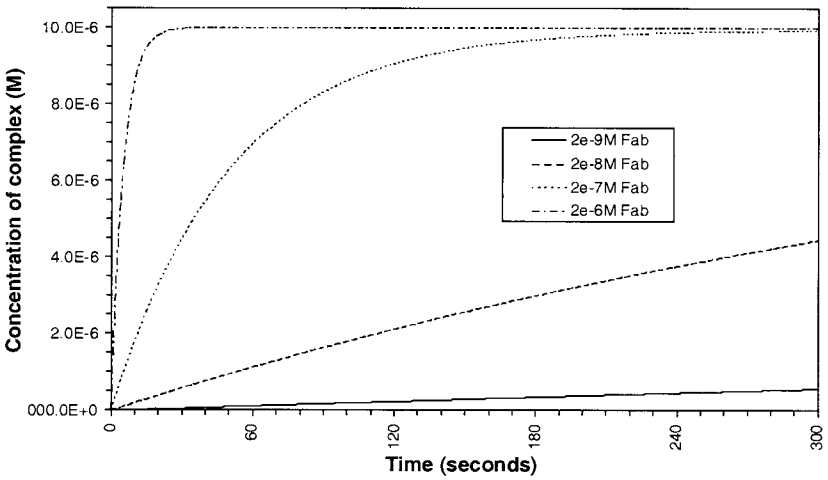
Figure 4: Saturation of antigen by Fab fragments: effect of concentration. This graph illustrates the proportion of the available antigenic epitopes bound by a Fab antibody at different concentrations of the Fab. The affinity (K_d) of the interaction is taken to be 10^{-8} M, and it is assumed that the antibody is in considerable excess. Half the antigenic sites are bound when the [Fab] = K_d. The same graph is shown in the inset, but using a log scale for the [Fab].



on a cell surface, with a view to analysing the expression of that antigen. In a typical experiment, the antibody will be mixed with the cell and incubated for a period of time. The cells will then be washed, removing free, unbound, antibody. The preparation will then be subjected to further manipulations (for example, addition of a secondary antibody, placing in a fluorescence cell analyser) before the levels of antibody binding to the surface can be determined.

The concentration and affinity of the antibody is important in determining the proportion of the antigenic sites on the cell that are bound by the antibody (we will assume, for simplicity, that we are using Fab fragments throughout - thus preserving monovalent

Figure 5: Association as a function of concentration. This figure models the formation of a complex between an Fab antibody fragment and its antigen. The two molecules interact with k_{ass} 10^5 $M^{-1}s^{-1}$, k_{diss} $10^{-4}s^{-1}$ (resulting in a K_a of 10^9 M^{-1}). The antigen is present at 10^{-5} M, while four different concentrations of Fab are modelled (ranging from 2 x 10^{-9} M to 2 x 10^{-6} M - or ~0.1 - 100 μg/ml). As can be seen the higher concentrations of Fab not only reach a higher level of equilibrium binding, but approach equlibrium faster than lower concentrations. It is instructive to note that the concentration of complex at equilibrium for the lowest concentration of Fab would be 6.67 x 10^{-6} M; however, inspection of the graph reveals that it would take a considerable time to reach this.



binding). If we assume an affinity (K_d) of 10^{-8} M (or a K_a of 10^8 M^{-1}), which is reasonable for a Fab-antigen interaction, then if the antibody were used at 10^{-8} M, half the antigenic sites on the cell surface would be bound by the antibody (**Figure 4**). This concentration of antibody is equivalent to ~0.5 μg/ml. As one increases the concentration the proportion of B bound also increases, with the binding tending towards saturation at high concentrations of Fab. Thus, at a concentration 10 times the K_d, (~5 μg/ml) ~91% of the antigenic sites will be bound. If one assumes the amount of the antibody is much greater than the number of antigenic sites, so that $[Fab] \approx [Fab]_{total}$, the degree of saturation of the antigen will be given by

$$P_{sat} = \frac{[Fab]}{([Fab] + K_d)} \qquad (13)$$

where [Fab] is the concentration of Fab and P_{sat} the proportion of antigenic epitopes bound to the Fab. Therefore, if you use two antibodies with different K_d values (e.g. 10^{-6} M and 10^{-9} M) you will need different concentrations of these antibodies to reach 90% saturation (around 10^{-5} M and 10^{-8} M respectively).

The k_{ass} and k_{diss} determine the rate of the complex formation, together with the concentration of the antibody, according to equation (9). The total amount of complex formed after time t is given by:

$$[FabAg]_t = [FabAg]_\infty (1 - e^{-(k_{ass}[Fab] - k_{diss})t}) \qquad (14)$$

(assuming that the Fab is in considerable excess to the Ag) $[FabAg]_t$ is the concentration of the immune complex at time t, $[FabAg]_\infty$ is the concentration at equilibrium, which is given by:

$$[FabAg]_\infty = \frac{[Fab][Ag]_0}{[Fab] + Kd} = \frac{k_{ass}[Fab][Ag]_0}{k_{ass}[Fab] + k_{diss}} \qquad (15)$$

(See reference [6] for derivation of these equations).

The typical k_{ass} for an antibody-antigen interaction will be in the range 10^5-10^6 $M^{-1}s^{-1}$ [5].

Figure 5 illustrates the formation of an antibody-antigen complex assuming that the k_{ass} and k_{diss} of the Fab fragment for its antigen are 10^5 $M^{-1}s^{-1}$ and $10^{-4}s^{-1}$, respectively. The concentration of the Fab is varied from between 2 x 10^{-9} M and 2 x 10^{-6} M (0.1 - 100 μg/ml, typical hybridoma culture supernatant will contain IgG antibody at 5 - 10 μg/ml). The amount of antigen in the system is assumed to be constant (10^{-5} M). As can be seen, at the higher two concentrations, the reaction is relatively fast, with the reaction approaching equilibrium well within the typical times used for staining reactions (less than 5 min). However, at lower concentrations not only is the equilibrium level of binding lower, but the time taken to approach equilibrium is longer. If the interaction has slower kinetics then, in order to obtain a high degree of antibody binding, it is necessary to either increase the incubation period, or to increase the concentration of the antibody, thus driving the forward reaction faster.

Figure 6: Dissociation as a function of k_{diss}. The graph models the dissociation of a complex formed between an antigen and four Fab fragments with k_{diss} ranging between 1 x $10^{-2}s^{-1}$ and 1 x $10^{-5}s^{-1}$. The $t_{1/2}$ of the complexes (given by equation 12) are shown in **Table 1**.

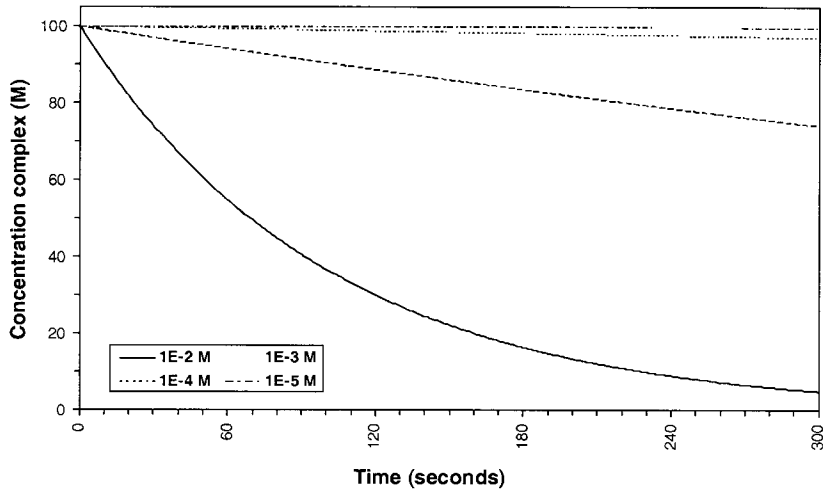


Table 1: Half life of complexes formed by monovalent interactions with different k_{diss}.

k_{diss} of complex (s^{-1})	$t_{1/2}$ of immune complex
1 x 10^{-2}	69.3 s
1 x 10^{-3}	11.6 min
1 x 10^{-4}	1.9 h
1 x 10^{-5}	19.3 h

Once the cells are washed, and free, unbound Fab fragments are removed, then the equilibrium of the reaction is driven in the reverse direction, and only dissociation of the antibody-antigen complex occurs (as there is no free antibody present to drive the forward reaction - clearly rebinding of dissociated antibodies can occur, but in many experimental situations this is negligible). The dissociation of the antibody-antigen complex is given by equation 8. In the case of Fab fragments of antibodies, the typical k_{diss} range from 10^{-2} - $10^{-5}s^{-1}$ [5]. Most of the differences in affinity for different antibodies is as a result of differences in the k_{diss}, and affinity maturation of an antibody response is normally associated with a reduction in k_{diss}. The amount of complex remaining at different times after the removal of the free antibody is shown in **Figure 6**, with the $t_{1/2}$ shown in **Table 1**, and given by the following equation:

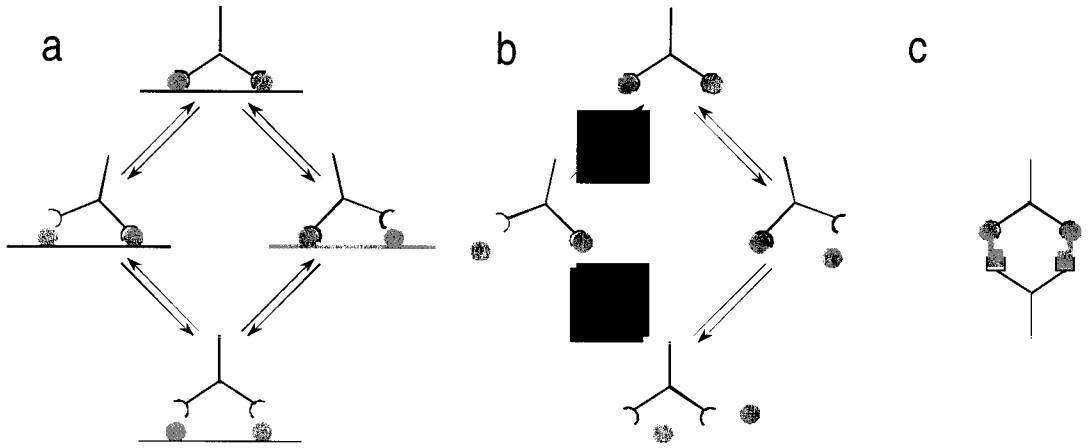
$$[FabAg]_t = [FabAg]_0.e^{-k_{diss}t} \quad (16)$$

Clearly, if one has an antibody-antigen with high k_{diss}, then the complex is unstable. In such a case it is important to reduce the period of time that the molecules are allowed to dissociate (for example during the washing of the cells). In one extreme example that we have studied, the k_{diss} of a Fab' antibody-antigen complex was 7.7 x $10^{-3}s^{-1}$ [6]. This gives a $t_{1/2}$ of 90 s. Thus, if one took 15 min to wash and analyse the cells, < 0.1% of the antibody-antigen complex would remain. In most cases the k_{diss} of the molecules are not as high as this, and the typical 15 - 30 min washing period is satisfactory. But one should be aware that this can be a problem. In particular it has been noted that antibodies isolated from phage display libraries can have high k_{diss}, [7,8], necessitating short washes when using these as immunohistochemical reagents.

1.3 Avidity

No discussion of the kinetics of molecular interactions would be complete without mention of avidity. Up to this time, we have considered monovalent interactions between A and B. However, if the interaction between these molecules is multivalent then there is a considerable rise in the functional affinity of the interaction, which is termed the avidity. This is largely as a result of a decrease in the k_{diss} of the interaction, consequent on the reduced ability of the molecules to dissociate. Thus, in the case of an IgG antibody molecule binding to a cell surface antigen using both antigen binding sites, for the molecule to dissociate from the cell surface both of the sites must dissociate; dissociation of just one site is not enough (**Figure 7a**). In the case of other molecules, such as IgM with 10 potential

Figure 7: Avidity - consequence of multivalent binding. When an interaction occurs between an IgG antibody and immobilised antigen (for example on cell surface or immobilised to an ELISA plate), both antigen-binding sites can bind to antigen (**Figure 7a**). Once such a multivalent interaction has been formed, dissociation of the antibody from the antigen requires first dissociation of one of the antigen binding sites from the antigen, and then subsequent dissociation of the second site before rebinding of the first site occurs. This is a relatively rare event, so the dissociation rate of the IgG antibody occurs more slowly than dissociation of the univalent Fab fragment. This avidity advantage is not seen when the antigen is monovalent and in solution (**Figure 7b**), as the two antigen binding sites act independently, there is no advantage to be obtained by the bivalent nature of the IgG molecule. However, if polyclonal antibodies are used, which can recognise different epitopes on the antigen, then co-operative binding events are possible (**Figure 7c**).



antigen binding sites, the potential avidity gain is immense. Note that this avidity only occurs if both molecular species are multivalent, if a monoclonal antibody binds to a monovalent antigen in solution then there is no avidity advantage as the antigen is free to dissociate (**Figure 7b**). This is the explanation why many IgM antibodies work for staining tissue sections (where the antigen is multivalent and there is an avidity advantage) but cannot immunoprecipitate the antigen from solution (where the antigen is monovalent, and the off rate of the reaction such that the complex dissociates too rapidly to allow isolation of the antigen). Polyclonal antibodies, which recognise multiple epitopes on the same antigen, can cross link the antigenic molecules in solution, so providing for an avidity advantage (**Figure 7c**). This is one of the explanations as to why polyclonal antibodies function well in immunoprecipitation assays (the others include the ability to form a lattice of precipitation).

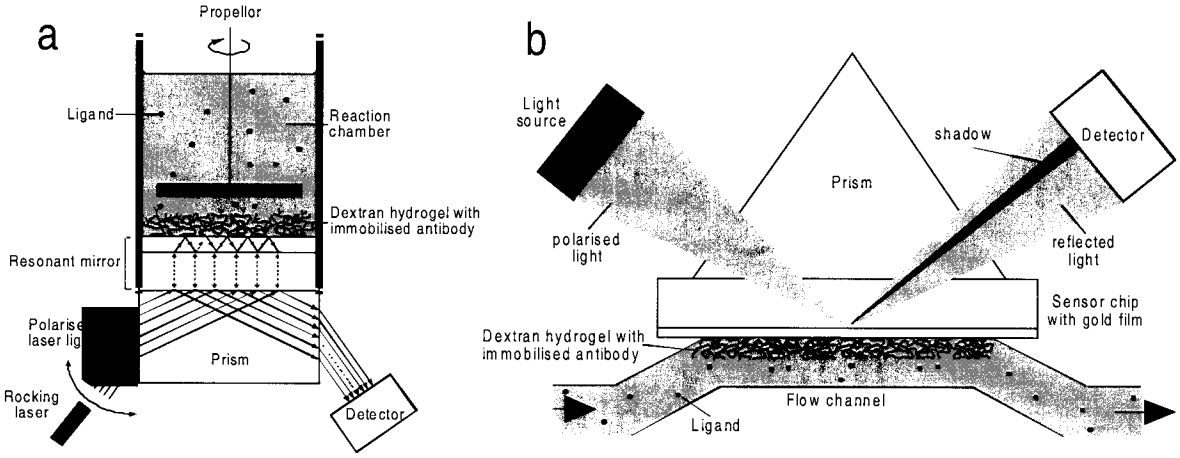
The difference between affinity and avidity is frequently exploited in physiological systems. For example, many of the receptors for the Fc region of IgG are of relatively low affinity, such that they are poor at binding circulating IgG found in the serum. However, if the Fc regions of the IgG are aggregated, for example as a result of the antibodies binding a cell surface antigen, then the Fc receptors (which are themselves multimeric as a result of being on a cell surface) will have an avidity advantage and so be able to efficiently bind the IgG molecules. This way the action of the Fc receptors is targeted to where it is useful, without being 'distracted' by circulating antibodies.

2. Biosensor technology

The concept that the kinetics of biomolecular interactions might be important will seem commonplace to any enzymologist, who in characterising any enzyme will seek to determine its kinetics. However, in many other fields the ability to determine kinetics of molecule interactions has been limited by the technology. The equilibrium constants K_a and K_d can, in most cases, be readily determined but, until recently, most methods for analysing k_{ass} and k_{diss} have been either too laborious, too specialised or too inaccurate for common use. This review will not discuss methods of determining K_a and K_d from equilibrium studies, they are well reviewed elsewhere [1-3].

This situation has changed thanks to the advent of new techniques that allow rapid and simple determination of the rate constants [9-11,5,12]. In this review, we will concentrate on optical biosensors, as they are most widely used. In particular the BIAcore family [13,14] (marketed by BIAcore, formerly Pharmacia Biosensor) and the IAsys instruments [15-17] (Affinity Sensors, formerly FAST). However, there are other types of biosensor becoming available, as well as other technologies such as fluorescence correlation spectroscopy [18] which may become more commonly used over the next few years.

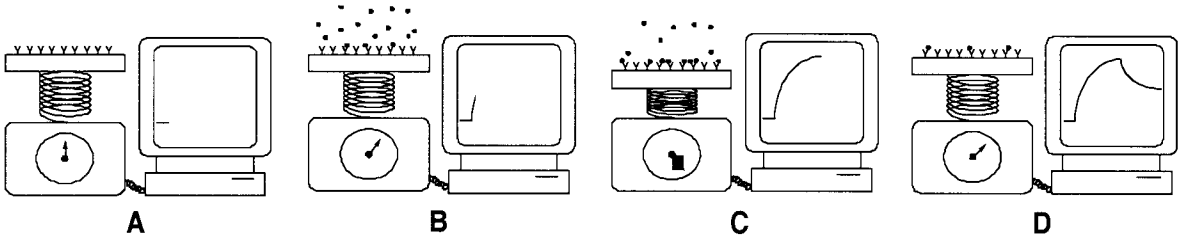
Figure 8: How biosensors work.
Figure 8a gives a schematic representation of the IAsys resonant mirror biosensor. The sample cuvette contains a reaction chamber, into which the sample is added, a dextran hydrogel surface to which the ligate is immobilised (represented in this case by Y shaped antibodies), the resonant mirror, which is made of up to ~100 nm of high refractive index Si_3N_4 on top of 500 nm of SiO_2, and a prism. Other surfaces can be used as alternatives to the dextran hydrogel. The cuvette is placed within the IAsys instrument, and the contents of the reaction chamber are efficiently stirred with a propeller. Laser light is directed at the prism at a range of angles. At one angle (the resonant angle) the light tunnels through the SiO_2 and propagates along the Si_3N_4 layer before tunnelling out of the mirror. This resonant angle can be readily detected owing to a shift in the phase of the resonant light. Alterations in the amount of material bound to the dextran affect the position of the resonant angle, and so the binding of the antibody to the antigen can be followed by determining the resonant angle. Figure adapted from reference [33].
Figure 8b shows the flow cell and optical system of the BIA series of instruments. These rely on the surface plasmon resonance effect to detect binding of ligand to ligate. In a similar manner to the IAsys the ligate is immobilised onto the sensing surface (in most cases a carboxymethylated dextran hydrogel). The ligand or test solution is flowed past the sensing surface (controlled by a microfluidic system). A wedge-shaped beam of polarised light is focused onto the gold film that forms part of the sensing surface, and is reflected off this surface. At one angle the intensity of the reflected light is reduced, producing a shadow. This reduction in intensity is caused by surface plasmon resonance, or the interaction of the evanescent wave with delocalised surface electrons in the metal film at the interface with the fluid phase. The angle at which surface plasmon resonance occurs is dependent on the refractive index of the fluid layer immediately adjacent to the gold surface, and so is affected by the binding or dissociation of the ligand from the immobilised ligate. Measurement of the angle at which the light intensity drops can therefore be used to determine the amount of material bound to the surface. An alternative strategy, exploited by the BIACORE probe, is to measure the wavelength, rather than angle, at which surface plasmon resonance occurs.



All biosensors consist of a biological detector coupled to a signal transducer that provides a signal when the biological surface interacts with an appropriate molecule [11,12]. For kinetic analysis, one of the molecules involved in the interaction is immobilised onto the sensing surface, and the second molecule added in solution (we will term the immobilised molecule the ligate and the molecule in solution the ligand, though different groups use different nomenclature. General usage [including that of Affinity Sensors and BIAcore] is not consistent and care should be taken in reading the relevant publications). The interaction of the ligand and ligate is then monitored using the signal transducer. There are two types of signal transducer in widespread commercial application, those that rely on surface plasmon resonance (used in the BIAcore series of instruments) and waveguide devices (such as the resonant mirror used in the IAsys machines) (**Figure 8**). In this review we will not cover the principles behind these technologies, they are extensively reviewed elsewhere [11,12]. For the purposes of understanding the data produced using biosensors (or indeed for operating the machines!) the salient feature is that they use the signal transducer to detect the interaction between the ligand and ligate. In many ways, the machinery could be considered to be an affinity column mounted on a very sensitive balance, when a molecule binds to the affinity column the weight of the column increases, and when the molecule dissociates the weight decreases (**Figure 9**).

There are differences in the configuration of the different machines. They can be manual or automatic (with regards to handling the reagents). In the IAsys machines the reactions take place in a rapidly stirred microcuvette, while in the BIAcore machines the sample flows over the sensing surface. The sensing surface can be different, most commonly consisting of a carboxymethylated dextran hydrogel that allows immobilisation of the ligate using standard chemistries,

Figure 9: Biosensors for biologists. This cartoon illustrates the principle behind both the plasmon resonance and resonant mirror biosensors. In all cases one of the molecules (the ligate, in this case shown as Y shaped antibody molecules) is immobilised on a sensing surface. The weight of the surface is measured using a balance. This is the function of the plasmon resonance or resonant mirror, they serve, as a first approximation, to measure the weight of material bound to the surface. The balance is connected to a computer that collects data. This is shown on the screen as a graph, with time on the x axis and the weight of the surface on the y axis. Part **A** shows the set up before the experiment begins, the situation is in equilibrium and the computer indicates that there has been no change in weight. Part **B** is drawn just after some antigen (black circles) has been added. Some of the antigen molecules have started to bind to the surface, and the computer has registered an increase in weight. A while later (Part **C**) the reaction has nearly reached equilibrium, there is some free antigen still present, but the rate of dissociation of the antibody/antigen complexes is nearly the same as the rate of association. At this stage all free antigen is removed from the system, and so dissociation of the complex occurs, which is detected (Part **D**) as a decrease in the weight of the surface.



such as linkage through ε amino groups of lysine [19,15]. Other surfaces employ biotin or streptavidin for immobilisation, and 'flat' surfaces that lack the hydrogel may have important applications. In addition, alternative chemistries may have advantages in certain circumstances, for example introduction of a thiol group into the carbohydrate region of the Fc portion of an antibody molecule allows immobilisation of the antibodies in the 'correct configuration', with the antigen binding sites presented away from the surface [101].

Each potential user must come to a judgement as to which type of machine is most appropriate for their applications. The data shown in this paper was generated using both the manual IAsys and its automated counterpart (IAsys Plus) as they are in use in the authors' laboratory, however, similar results would have been obtained with BIAcore machines.

The data obtained from a typical experiment is shown in **Figure 10**. In this experiment, saporin (a plant derived toxin) was immobilised onto the sensing surface of the machine. An antibody to the saporin was then added (point A). The binding of the antibody to the saporin was then followed for a period of time (the association phase of the reaction). At point B the surface was washed, removing any unbound antibody. As a result no association can occur, and the equilibrium of the reaction is changed and so just the dissociation of the antibody-antigen complex is seen. Typically, the experiment is performed at different concentrations of ligand. The values for k_{ass} and k_{diss} are then obtained by curve fitting the curves obtained for the association and dissociation to appropriate equations that describe the reactions (**Figure 11**).

In the example we have given above the interaction of an antigen and antibody are described. However, other ligand-ligate interactions can be measured using biosensors, not only those between different proteins, but also those involving nucleic acids, carbohydrates and peptides [20].

3. Kinetic case studies

In order to illustrate why the measurement of kinetics can be important, a number of case studies will be given in which such measurements either have been or may be useful. These are examples primarily taken from work carried out in our laboratory, and so are biased towards immunological problems. However, they should illustrate some of the important points in this work.

3.1 Interaction of antibodies with saporin

Saporin is a ribosome inactivating protein that is isolated from the seeds of *Saponaria officinalis* [21]. It has been developed as a component in both immunotoxins and other forms of targeted therapy [22]. Of interest to this study is the use of bispecific antibodies to target the toxin to neoplastic cells [23-25]. Such antibodies have a dual specificity for the toxin and for a tumour cell, so directing the toxin to the cell surface (**Figure 12**) [26]. This approach has the theoretical advantage of not requiring chemical conjugation of the toxin to the targeting molecule, thereby reducing damage to the molecules, as well as allowing indirect targeting strategies.

Figure 10: Binding of anti-saporin antibody to immobilised saporin. This figure illustrates part of a typical experiment to measure the kinetics of monoclonal antibodies. Saporin (the antigen or ligate in this example) has been immobilised on the surface of the dextran hydrogel of an IAsys cuvette. At point (a) an Fab′ fragment of an anti-saporin antibody (MPD1-14) at 2 x 10^{-6} M was added. The molecules were allowed to associate until point (b), where the cuvette was washed with buffer (PBS containing 0.05 % Tween detergent). The dissociation was then followed. At the end of the experiment the cuvette was washed with acidified glycine (pH 2) (c) to regenerate the surface. After washing with PBS-Tween (d) the response returns to the baseline, ready for the next cycle of analysis. The time of the experiment is shown on the x-axis, and the relative response on the y-axis. The units are arc seconds, a measure of the resonant angle. Changes in the resonant angle are produced either by binding or dissociation of molecules from the sensor surface, or by changes in the buffer as a result of differing refractive indices of the solutions. These produce 'bulk shift' responses, which are near instantaneous changes in the resonant angle, at some of the washing stages.

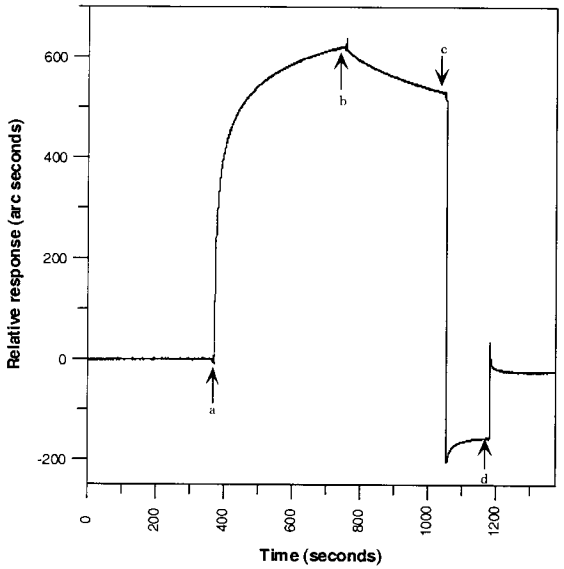


Figure 11: Analysis of biosensor data. These figures illustrate how, using the FASTfit software provided with the IAsys resonant mirror biosensor, analysis of the data can be used to determine the k_{ass}. Similar non-linear curve fitting procedures are used by the BIA series of instruments. The experimental set-up is as in **Figure 10** (but using DB7-18 Fab′). **Figure 11a** illustrates the association portion of the data for different concentrations of anti-saporin Fab′ (ranging in doubling dilutions from 2 x 10^{-7} - 7.8 x 10^{-10} M). Each of these curves is then fitted to the appropriate equation using an iterative curve fitting procedure (illustrated for one of these responses in **Figure 11b**). In this case the curve is fitted to the equation (though other equations can be used):

$$R_t = R_0 + E(1 - e^{-k_{obs}t}) \qquad (17)$$

where R_t is the response at time t, E is the extent of the reaction (equivalent to R), and k_{obs}, the rate constant for the reaction. The small circles in the figure show the primary data, the solid line (largely obscured by the data points) shows the fitted curve.

$$k_{obs} = k_{ass}[\text{Fab}] - k_{diss} \qquad (18)$$

And so a plot of k_{obs} against the concentration of Fab will produce a straight line, whose slope is k_{ass}. The k_{diss} can be obtained directly by curve fitting the dissociation phase of the data. For fuller explanation of data analysis used in the IAsys series of instruments see [6].

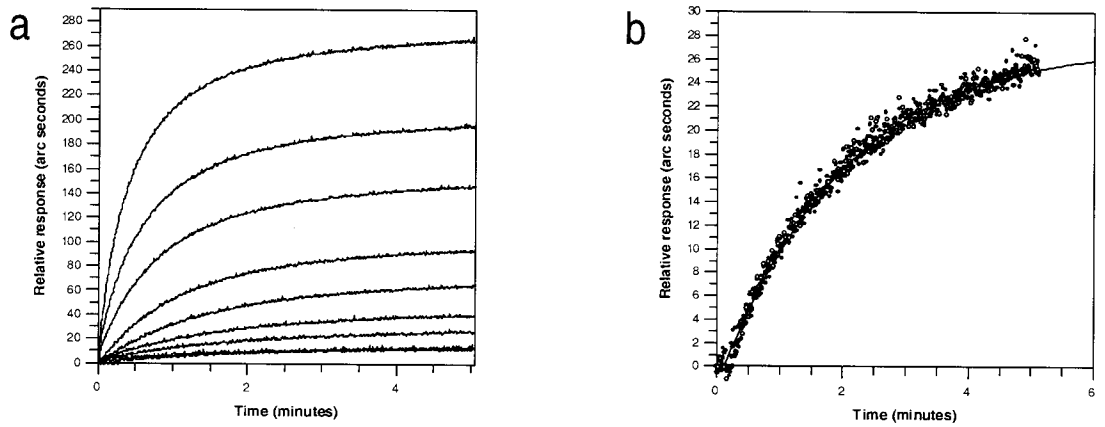
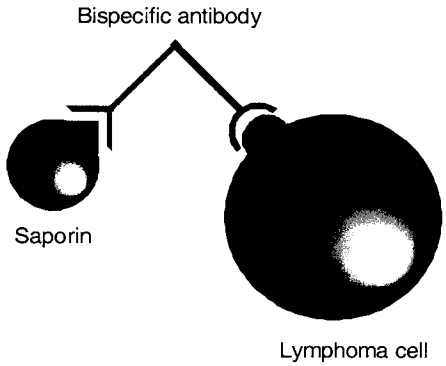


Figure 12: Bispecific antibodies to target saporin. Bispecific antibodies with a dual specificity for saporin, a plant derived toxin, and lymphoma cells can be used to target the molecule to the surface of lymphoma cells, where the molecule can be internalised and kill the tumour cell.

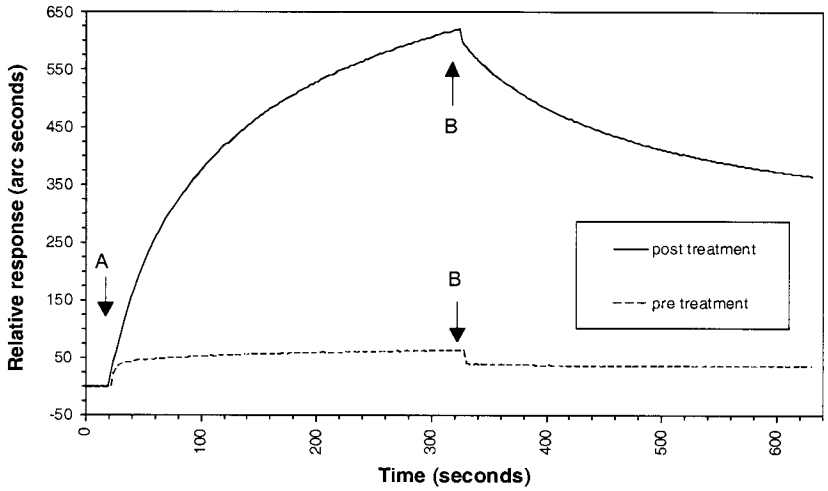


In initial studies to make bispecific antibodies, monoclonal antibodies were raised against saporin. These were then used to construct $F(ab')_2$ bispecific antibodies, using a chemical approach, with dual specificity for lymphoma cells and saporin [23-25]. The affinity of the anti-saporin antibodies were determined using conventional equilibrium techniques, and their ability to target cytotoxicity determined *in vitro* [25]. As might be expected the anti-saporin antibodies with the highest affinity showed the greatest ability to target cytotoxicity *in vitro*, when incorporated into bispecific antibodies, and were taken on for further development, leading eventually to their use in preliminary clinical trials [27].

However, somewhat surprising data was obtained in animal studies, which showed that the best bispecific antibodies *in vivo* were not necessarily those with the highest affinity, or those that performed best *in vitro* [25]. The reason for this became clear only when the kinetics of binding of the antibodies were measured using the IAsys resonant mirror biosensor [6]. This demonstrated that Fab' fragments from all the anti-saporin antibodies had a relatively high k_{diss} for saporin ($1.37 \times 10^{-3}s^{-1}$ - $7.69 \times 10^{-3}s^{-1}$). The half life of the Fab'-saporin complexes was therefore very low (90 - 500 s). The molecules that performed best *in vivo* had the lowest k_{diss} (so formed the most stable complexes). This discrepancy between the *in vivo* and *in vitro* data is best explained when the nature of the two experiments is considered. The *in vitro* assessment of toxicity was done using fixed concentrations of all the reagents in the assay. Under these conditions, equilibrium was rapidly established, and the amount of saporin targeted to the cell surface by the antibody was constant, determined by the equilibrium constants (K_a/K_d) of the interaction. However, when the bispecific toxin conjugates are administered to an animal (or indeed a patient) they are rapidly diluted in a large volume (the blood pool), and so the equilibrium is shifted from right to left, and the relative concentration of the complexes is reduced. As a consequence the major factor determining the efficacy of the bispecific antibodies is the stability of the complexes *in vivo*, which is determined by the k_{diss}. When cocktails of bispecific antibodies are used, recognising different epitopes on the toxin, the therapeutic benefit increases due to an avidity advantage on the surface of the tumour cells [25].

It is therefore clear that the ability to determine the kinetics of the interaction can be vital in the selection and development of antibody molecules for therapeutic or diagnostic purposes. We went on to use this technology to identify antibodies against gelonin that might be suitable for therapy [28]. In addition, the biosensor can be used to rapidly epitope map the antibodies against the same antigen, again facilitating the rapid selection of suitable molecules for further development [28]. The kinetics of interaction of antibodies, recombinant antibody fragments and other targeting molecules are all of great importance in determining their efficacy, and biosensor technology is going to be of increasing importance in this field. In addition we have found that the measurement of the binding kinetics of recombinant antibodies is an important tool in assessing the behaviour of these molecules following modification (for example by fusion to other molecules) [29-31]. In the patent literature it is increasingly common to find reports in which the binding kinetics of novel recombinant antibodies [e.g. 102] or modified natural antibodies [e.g. 103] have been determined using biosensor technology. In the cases given above the new molecules were shown to have an increased affinity (and therefore, probably, an increased clinical utility) by virtue either of genetic mutations [102] or by chemical crosslinking of monomeric antibodies [103]. Even when monoclonal antibodies are generated using conventional hybridoma technology it is common, as part of their characterisation, to detect their binding using a biosensor [104].

Figure 13: Detection of human anti-mouse antibody. In the course of clinical trials of immunotherapy, patients with ovarian carcinoma were treated with injections of the murine HMFG1 monoclonal antibody. As a result of this they produced antibodies to the murine antibody (human anti-mouse antibody, HAMA). This graph shows the trace obtained with the serum from one patient (JS) before and after treatment with the antibody. The HMFG1 antibody was immobilised onto the sensor surface, and a diluted serum sample added (A). The association of the antibody was then followed until the surface was washed with buffer, removing any unbound serum (B) and the dissociation followed. As can be seen the sample taken before treatment shows only low levels of background binding to the immobilised HMFG1, while after treatment high levels of binding were seen, indicating that this patient had developed a HAMA response.



3.2 Detection and measurement of serum antibodies

In addition to measuring the binding of artificial antibodies, biosensors can be used to detect the interaction of serum antibodies. These include autoantibodies, such as anti-glomerular basement membrane antibodies that are found in Goodpasture's disease [32] and also the human anti-mouse antibodies (HAMA) that develop in patients treated with monoclonal antibodies [33]. An example is shown in **Figure 13**, which illustrates the binding of HAMA present in the serum of a patient with ovarian carcinoma, which has been treated with an antibody (HMFG1) against polymorphic epithelial mucin. A serum sample taken before treatment shows only modest binding to the HMFG1 antibody that has been immobilised on the sensor surface. A similar sample, but taken after treatment, shows considerably increased binding to the therapeutic antibody, indicating that this patient has developed HAMA to the murine antibody.

This approach to detecting serum antibodies has a number of potential uses. First, it may allow a more rapid diagnosis of the presence of antibodies or other serum molecules than is currently possible. It is likely, therefore, that biosensor technology may replace the conventional immunoassay in clinical laboratories. Thus, biosensors have been used to measure metalloproteases and heparan sulphate endoglycosidase, in both cases as a marker of cancer metastasis and malignancy [105-106], and it is likely that similar assays will be rapidly developed for other serum markers.

Second, useful information may be obtained from kinetic analysis of the antibody binding. This analysis is complicated by the polyclonal nature of the antibodies in the serum, but we are developing methods to estimate the average affinity and kinetics of these molecules (unpublished observations). It is likely that such information would be of considerable clinical benefit. It has been shown that the pathogenicity of autoantibodies in diseases such as systemic lupus erythematosus and some vasculitides correlates with their avidity [34-36]. Similarly the affinity of anti-viral antibodies has been used as an indicator of whether they were formed by a primary- or a secondary-memory immune response [37-40]. This is important in determining in pregnant women whether the antibodies are an indication of a concurrent rubella infection, or whether they represent a previous infection. Similar data can be useful in the management of cytomegalovirus and herpesvirus infection in transplant recipients. Currently, all the routine methods used to measure the affinity of the antibodies are very crude, and difficult to reproduce. The development of more accurate and simple methods to measure the kinetics will allow them to be applied more generally. It is probable that the affinity and/or kinetics of other antibodies for their antigens will prove to be of clinical importance.

In addition the biosensor may be useful in measuring an immunological response to an administered drug or other therapeutic molecule. Such a situation is seen with the HAMA detected in patients following treatment with monoclonal antibody [33]. We have also used the biosensor to epitope map the autoantibody repertoire in patients with autoimmune disease, thus highlighting the parts of the molecule involved in the disease [32]. This can provide useful information for the development of therapeutic strategies.

4. Unravelling signalling pathways

Biosensors have been widely used to study the interaction of molecules involved in transmembrane signalling (for example [41-47]). This allows the kinetics of interaction of the molecules to be determined. It might appear that such information is of academic interest only, however, increasingly kinetic models of signalling are being developed in which the outcome of the signalling event is seen as being critically dependent on the kinetics of the pathways that mediate the signalling across the membrane and into the cell [48-50].

One example is to be found in the signalling of the T cell receptor following ligation with its ligand, a complex formed of the major histocompatibility antigen and antigenic peptide (MHC-peptide). The recognition of antigen by the T cell receptor is central to immunology, and manipulation of the signalling event would be desirable in a range of conditions including transplantation, autoimmune disease, vaccination and antitumour immune responses. However, the signalling event is complex. Interaction of the receptor with the MHC-peptide complex can have a number of outcomes depending on the exact nature of the ligand [51]. If the MHC-peptide forms an agonist, the T cell receives a positive signal and goes on to be activated. If the complex forms a null ligand, no response is seen. However, if the MHC-peptide forms an 'altered peptide ligand', then the signal received by the T cell is a partial signal that will either cause only partial activation or may even antagonise a negative signal [52,53,51].

Recently, we have proposed a model, termed the kinetic differentiation model, to explain these different signalling events [50]. This model assumes that the kinetics of interaction (in particular the k_{diss}) of the T cell receptor with the ligand determine the outcome of the signalling event. There is strong data to support this [54]. We propose that there are multiple signalling pathways across the membrane, each of which have different kinetics. These pathways are only capable of signalling while the receptor engages its ligand. If we consider a system with just two pathways, one with fast kinetics producing a product F and the other slower pathway that yields a product S, then the cell will be able to determine whether it has bound a ligand with a high k_{diss} or a low k_{diss} by virtue of the relative proportions of S and F. If the k_{diss} is very high, then the receptor ligand complex will dissociate before any signalling event occurs. This will produce a null signal. If the k_{diss} is lower, then F will be formed, but there will not be enough time for S to have formed. This will therefore lead to a partial (and possibly antagonistic) signal. Finally, if the k_{diss} is sufficiently low, both S and F will have time to form, and a fully activating positive signal will be delivered. We have gone on to mathematically simulate this model and show that it closely approximates the situation seen *in vivo* [50].

Such a model is speculative at present. However, it is likely that as this and other models are developed which stress the importance of the kinetics of both the ligand-receptor interaction and also the kinetics of the signalling pathways [48-50], increasing emphasis will have to be placed on measuring the kinetics of interaction of the various components of the system. Such an approach is vital for drug discovery programmes. Thus, if the kinetics of the ligand-receptor interaction are important, they will direct the design of soluble receptor antagonists. Similarly, in the case of the T cell, such studies will indicate which steps are critical in the signalling process, again guiding drug development. For example if the model that we propose turns out to be correct, it would suggest that strategies to block or inhibit the production of S might be especially useful in the development of immunosuppressive agents. It might even suggest that promoting the formation of F might lead to a negative signal in some circumstances. In addition, once the critical molecular interaction has been identified, biosensors can be used as a research tool in their own right, for example to aid in the discovery of small molecules that interfere with (or, possibly, enhance) the interaction. Similar work has already been performed in the search for IgE antagonists, with a biosensor being used to help identify peptides that block the interaction of IgE with its receptor [107].

5. Expert opinion

The measurement of biomolecular interactions is becoming increasingly accessible with the advent of biosensors. As a result there is an increasing realisation of the importance of kinetics in a full understanding of biomolecular interactions. These include not only protein-protein interactions, but also those between proteins, nucleic acids, carbohydrates and synthetic molecules - all of which have been investigated using biosensor technology. Interactions on the biosensor

have also been used to probe structural alterations in proteins following chemical manipulation [55]. It is likely that the development of novel molecules for therapeutic or diagnostic purposes will increasingly include an assessment of the kinetics of their interaction at an early stage. In addition, a fuller appreciation of the importance of the speed and stability of molecular interactions will prove important in the identification of suitable targets for intervention.

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- •• of considerable interest

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