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Kinetics of antibody binding to surface-immobilized antigen. Analysis of data and an empiric model

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Abstract

The kinetics of the reaction between monoclonal antibodies and surface-immobilized hapten (dinitrophenyl, DNP) was measured with in situ ellipsometry. A flow cuvette with a small volume (50 μl) and a high flow rate (3 ml/min) was used in order to avoid mass-transport limitation of the reaction. An initial linear phase was recorded by off-null ellipsometry with a time resolution of 0.1 s. The linear phase was followed by an accelerated rate of antibody binding. The time dependence of the rapid reaction exhibited an exponential form. The apparent rate of antibody binding then decreased continously with time and the surface concentration of bound antibodies was proportional to the logarithm of time. An empiric model is suggested that describes the time course and the concentration-dependence of the reaction.

Key words: Kinetics; Antibody binding; Antigen

1. Introduction

Measurements of the kinetics of antigen–antibody reactions at solid surfaces are often hampered by the effect of mass transport limitation that often restricts the reaction at plane surfaces [1]. The mass transport limitation is due to depletion of antibodies in the reaction zone close to the surface and no conclusions can be made from such measurements regarding the sticking probability of antibody molecules at the surface. This is a serious shortcoming of experimental data which hinders the development of theoretical models of antigen–antibody reactions at interfaces.

In the present study precautions were taken to avoid mass transport limitations by using a flow cuvette and fast ellipsometric detection. The kinetics of antigen–antibody reactions was measured by off-null ellipsometry in situ allowing a time resolution of 0.1 s, which makes it possible to measure the initial adsorption taking place before the diffusion layer in the solution is depleted of antibodies. The flow cuvette had a small volume (50 μl) and a high flow rate was used to give a thin unstirred layer and a continous exchange of the bulk phase to ensure constant concentration of antibodies in the solution close to the surface.

Under these boundary conditions, mass transport limitation is described by the relationship [1]:

$$dn/dt = (DC_0)/R, \quad (1)$$

where n is the mass of antibody reaching the surface, D is the diffusion constant of the antibody, C_0 is the concentration of antibodies in the

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solution, R is the thickness of the unstirred water film and t is time.

The experimental setup makes it possible to use high concentrations of antibody in the solution which further decreases the risk for depletion of antibody in the diffusion layer.

Under these experimental conditions it was indeed possible to study the whole process of antibody binding to surface-immobilized antigen.

2. Material and methods

Chemicals: Monoclonal antibodies (Mabs) directed against DNP (a generous gift from professor M. Steward, London) were dissolved in 0.05 M phosphate buffer pH 7.2 containing 0.15 M NaCl (PBS). The affinity of the Mabs for hapten in solution has been determined [2] to 4.1×10^7 M^{-1} for Mab 49; 1.6×10^7 M^{-1} for Mab 53 and 0.35×10^6 M^{-1} for Mab 57. Hexamethyldisilazane (E. Merck, Darmstadt, Germany) was used for methylization of SiO_2 surfaces (oxidized silicon wafers) as described by others [3]. Dinitrophenyl residues were coupled to serum albumin as described [4]. An epitope density of DNP_{15}-BSA was used. Surface-adsorbed BSA was used for control of unspecific andsorption of the Mab.

The antigen was immobilized at the hydrophobic methyl-silanized surfaces by adsorption from a solution of 0.1 mg/ml in PBS for 18–20 h.

Ellipsometry: A null ellipsometer (Rudolph Research model 436) was used with a He–Ne laser (632.8 nm) as the light source. The angle of incidence was 70°. A 50 μl flow cuvette (Hellma, Müllheim, Germany) was used with the back wall comprising the exchangeable silicon substrate. Buffer solution (PBS) was pumped through the cuvette at a rate of 3 ml/min. The instrument was used both as an ordinary null ellipsometer and an off-null ellipsometer. The general procedure followed was to first find the polarizer and analyzer positions resulting in a minimum of light transmission through the instrument (null ellipsometry). These settings were used as starting position for the off-null ellipsometry measurements. Any change of the thickness or refractive index of the sample surface will result in an increased light intensity, I passing through the instrument [5]. In the present study the refractive index of the film and the initial film thickness are the parameters which determine the sensitivity. The refractive index of IgG was determined to 1.6 independently by using an Abbe refractometer.

Mab-solution was then pumped through the cuvette and the change in the light intensity was recorded.

New positions of the polarizer and analyzer giving a minimum of light transmission were then sought in order to determine the change in the ellipsometric readings due to the adsorbed antibody layer. The silicon wafers used in the present study had a thin (2 nm) SiO_2 film. For such surfaces there are only small changes in the analyzer position due to antibody binding at the surface. Since the refractive index of the film is known independently we can thus use a single relation between antibody film thickness and change in polarizer position as shown in Fig. 1 in order to calibrate the light intensity versus film thickness.

Due to stray light and imperfections there is always a small residual intensity I_0 at null settings. This value is simply subtracted from the

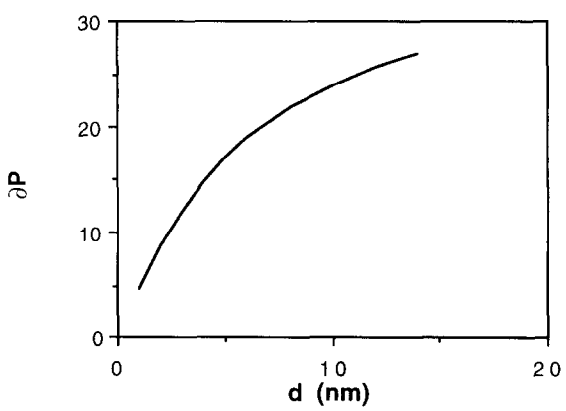

Fig. 1. Calculated change in polarizer setting ∂P from null position in null ellipsometry versus thickness of an adsorbed film with a refractive index of 1.6. The substrate is an oxidized (2 nm oxide thickness) silicon wafer with a complex refractive index of 3.858-i0.018 at the wavelength 632.8 nm. The refractive index of the oxide is 1.458 and the angle of incidence 70°.

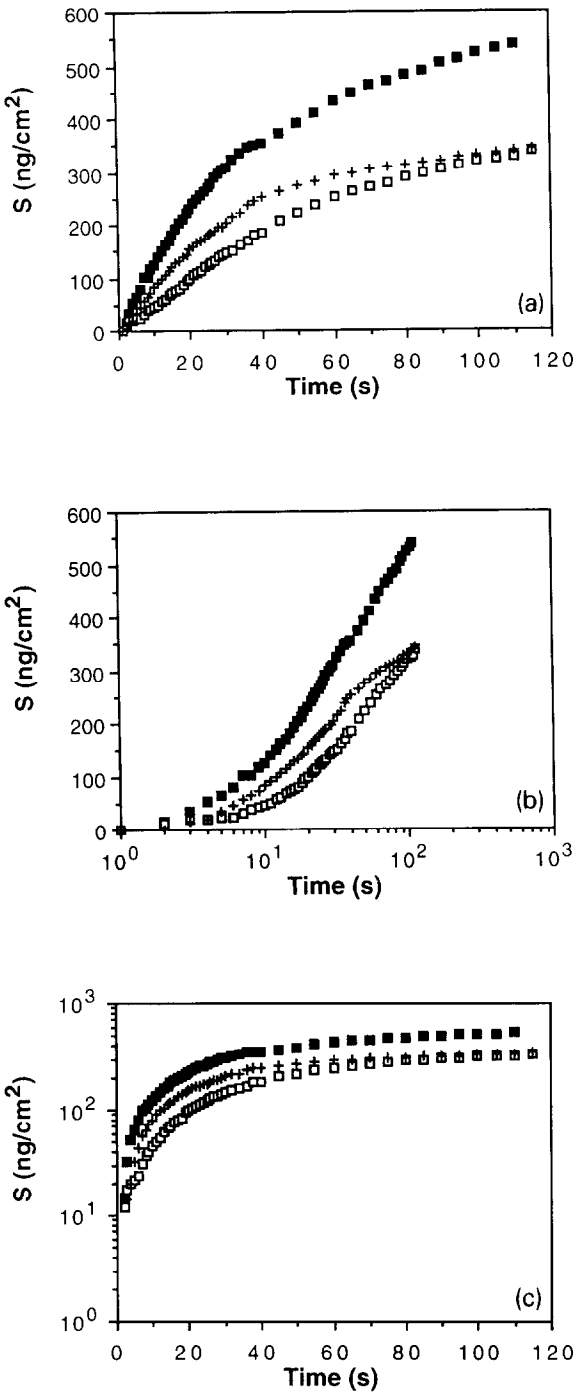

Fig. 2. Surface concentration of bound antibodies (S, ng/cm^2) versus time for three different monoclonal antibodies. (×) Mab 49; (□) Mab 53: (■) Mab 57. Concentration of antibodies in solution, $C_0 = 30$ μg/ml. (a) Linear plot. (b) Log–lin plot. (c) Lin–log plot.

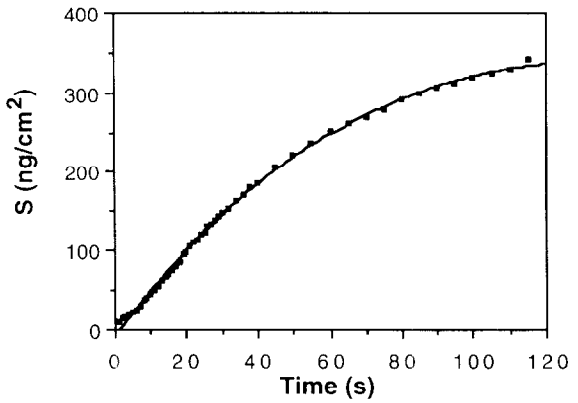

Fig. 3. Experimental curve from Fig. 2 (Mab 53) fitted to a polynoma. (■) Experimental surface concentration. (— — —) Polynoma $6t - 0.03t^2 + 0.00005t^3$ ($r = 1.00$).

intensity. The film thickness during adsorption is then given by

$$d = k\sqrt{I - I_0}\,. \tag{2}$$

The thickness d is an equivalent optical thickness. The relation between this film thickness and the amount of bound antibody can be found in different ways depending on what properties of the protein is most well characterized experimentally. With a known density of the IgG molecule, the relation

$$S = d\xi \times 100 \tag{3}$$

can be used, where S is the surface concentration (ng/cm^2) d is film thickness (nm) and ξ is the density of the IgG molecule (g/cm^3). The density of IgG used in the present study was therefore determined pycnometrically to 1.6 g/cm^3 as described [6].

3. Results

The binding of three monoclonal anti-DNP antibodies with different affinities to the DNP-hapten, measured by off-null ellipsometry, is shown in Fig. 2. A linear presentation of ellipsometry data is shown in Fig. 2a. At similar concentrations ($C_0 = 30$ μg/ml), the reaction rate of Mab 57 and 49 is higher than that of Mab 53. There is no obvious relation between antibody

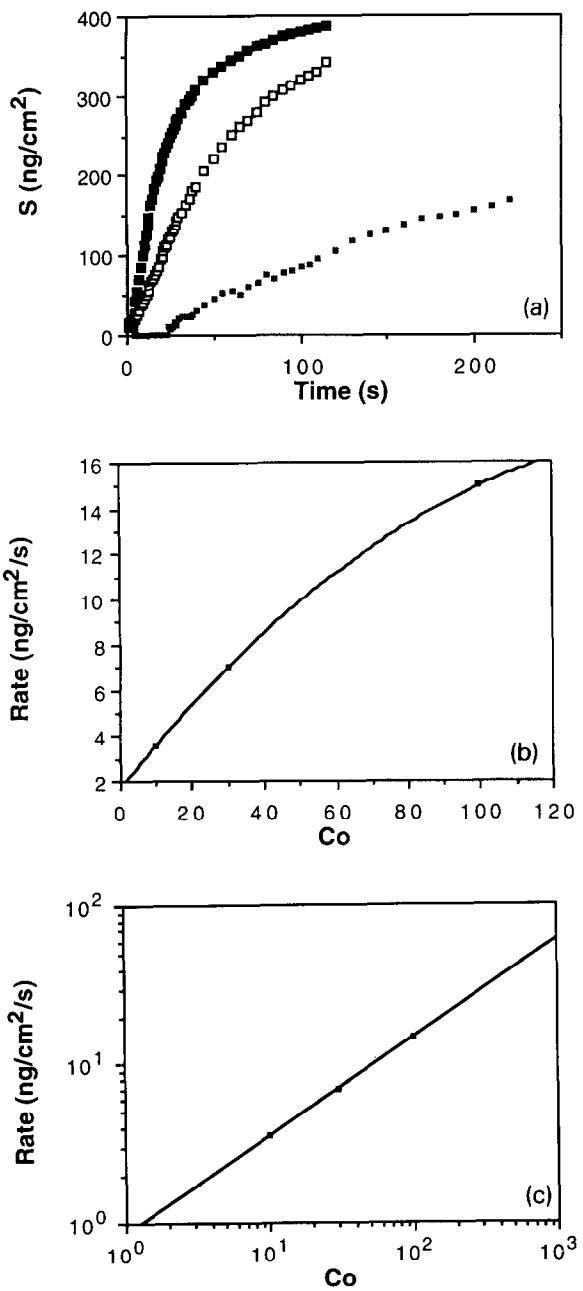

Fig. 4. The concentration dependence of binding of Mab 53 at three different concentrations. (a) Linear plot of the surface concentration (S, ng/cm2) versus time (s) for three different concentrations of antibody in solution (10 μg/ml; 30 μg/ml; 100 μg/ml). (b) The maximum rate of binding (dS/dt, ng/cm^2/s) versus concentration C_0 (μg/ml) together with curve fitting with the polynoma $0.2C - 0.0006C^2$ (———) $r = 1.00$. (c) Log–log plot of data from (b). Experimental results fitted to the function $C^{0.62}$ (———) $r = 1.00$.

affinity as measured in solution and kinetics of binding to surface-immobilized antigen. The binding rate decreases continously for all three antibodies. The low affinity antibody Mab 57 reaches the highest surface concentration within the period of measuring.

A further analysis of the reaction is made with logarithmic plots. A linear phase in the reaction is seen in the log–lin plot (Fig. 2b), followed by an exponential acceleration of binding as seen in the lin–log plot (Fig. 2c).

The continous decrease of the binding rate makes the surface concentration of bound antibodies proportional to $\log t$ over more than one decade (Fig. 2b).

Curve fitting of the experiments is shown in Fig. 3. The basic non-linearities involved in the reaction can be traced by fitting to a polynomial (Fig. 3). It can be seen that the experimental curve is well described by a polynomial containing the terms:

$$S = 6t - 0.03t^2 + 0.00005t^3 \ (r = 1.00).$$

The concentration dependence of the reaction rate is shown in Fig. 4. A linear plot of the binding of Mab 53 to surface-immobilized antigen shows that the linear phase is strongly concentration-dependent (Fig. 4a). At a concentration of 10 μg/ml the duration is 23 s. Increasing the concentration to 30 μg/ml reduces the duration of the linear phase to 2 s.

The concentration-dependence of the reaction rate is further analysed by plotting the fastest rate measured against the concentration of Mab in solution (Fig. 4b). The experimental points are well described by the polynomial $dS/dt = 0.2C_0 - 0.0006C_0^2$ ($r = 1.00$). The concentration dependence of the reaction may also be described as a fractional reaction order (Fig. 4c) of $C^{0.62}$ ($r = 1.00$).

4. Discussion

Antigen–antibody reactions at a solid surface may at a first glance appear as a trivial reaction that should be described by two reaction rate constants of opposite direction and a maximum

coverage at a monolayer. However, experimental data reveal more complexity [7,8].

In the present study two novel phenomena are presented that have to be considered in theoretical models of antigen–antibody reactions at solid–liquid interfaces – a concentration dependent linear phase followed by an accelerated binding rate. In previous studies, these phenomena have been masked by mass transport limitations of the binding rate.

Details of the kinetics of binding during the linear phase can not be obtained in the present study since it is below the detection limit of the ellipsometer (≈ 10 ng/cm^2). This means that the accelerated binding reported here starts from a surface concentration of $\approx 3.76 \times 10^9$ molecules/cm^2 or a mean distance between molecules of 160 nm.

The general appearance of the binding curve, with a rapid initial reaction followed by a slow but continous binding is in accord with early ellipsometry measurements of antigen–antibody reactions performed by Rothen [9]. The same kinetics of antibody binding has also been reported earlier using techniques with lower time resolution [4]. In earlier studies, where measurements suffered from possible mass-transport limitations, the $t^{0.5}$-like appearance of the binding kinetics has been interpreted as diffusion limitation [10]. The finding in the present study that the general appearance of the kinetics of antigen–antibody reactions are unaffected by mass-transport, calls for novel interpretations of the experimental data.

At low surface concentrations, antibodies are expected to bind independently, resulting in linear kinetics. A theoretical model of the factors determining the surface concentration of bound antibodies may thus start with the term,

$$S = k_0 C_0 t, \tag{4}$$

where S is the surface concentration of bound antibodies, k_0 is the probability of binding, C_0 is the concentration of antibodies in solution and t is time. Since we are working under boundary conditions of diffusion where the relation between antibodies reaching the surface, concentration and time is linear (Eq. (1)) C_0 will be directly proportional to C_s, which is the concentration of antibodies at the surface. Here C_0 will be used since it is the known concentration, whereas determination of C_s requires knowledge of the thickness of the unstirred layer, which is difficult to obtain.

The exponential rate of binding, due to interaction between bound and colliding antibodies can be modelled in several ways. Classically, reactions where a bound molecule creates more than one binding site for arriving molecules would be described as exponential growth [11]:

$$S = N\,\mathrm{e}^{\alpha t}, \tag{5}$$

where N is the number of nucleation sites and α is a probability of interaction between bound and arriving molecules.

Cooperative binding of hormones to receptors are often described by [12]

$$S/S_m = r_0(1 - \mathrm{e}^{-k a_0 t}), \tag{6}$$

where S/S_m is the density of occupied receptors; r_0 and a_0 are the initial concentrations of receptor and agonist and k is the association constant.

In a similar way, the cooperativity may be related to the surface concentration of bound molecules [13] by using the exponential term:

$$(1 - \mathrm{e}^{-\alpha S}). \tag{7}$$

However, as can be seen, the experimental curve is not exponential apart from an initial accelleration. The reasons for this is firstly geometrical constraints. Cooperative binding of molecules at surfaces leads to cluster formation [14] and cluster growth is limited by the cluster geometry [15]. The growth of an idealized circular 2D-cluster will be limited by t^2 [16] and the number of new sites formed will be proportional to $S^{0.5}$. Other geometries give other constraints.

Still, the binding rate decreases more than motivated by geometrical constraints.

We therefor assume that there is some dissociation during the reaction. Independent dissociation of single molecules can be described by classical first-order dissociation,

$$-k_{-0} S t, \tag{8}$$

where $-k_{-0}$ is the probability of dissociation (s^{-1}).

From literature data we know that there is no apparent dissociation of the antibodies used in the present study when rinsing with buffer, but that antibodies are exchanged with time in the presence of antibodies or antigen in solution [17]. This is partly due to rebinding of dissociated antibodies [18]. The classical dissociation term is thus only valid in the presence of colliding antibodies from the bulk,

$$-k_{-0}StC_0. \tag{9}$$

Dissociation can also be cooperative, induced by collisions with antibodies from the bulk solution [19] which can be described by

$$-k_{-1}S^n tC_0, \tag{10}$$

where n is the geometrical dimension of desorbing clusters.

The nonlinear concentration-dependence of the binding rate (Figs. 4b and 4c) is in accord with previous findings [20] and may be interpreted as a nonlinear dissociation of antibodies when the collision frequency increases. This may explain the hook-effect often seen in immunoassays.

Fractional concentration dependence of antigen–antibody reactions is nothing new. It was first reported by Biltz and Steiner in 1910 [21] and they did realize the shortcomings of describing antigen–antibody reactions according to simple equilibrium theory as suggested by Arrhenius [22].

5. Conclusion

The data presented here may serve to give a better understanding of the phenomenology of logaritmic growth and fractal kinetics. The analysis of data was designed to make further theoretical analysis possible.

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