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A Fokker–Planck description of multivalent interactions

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Abstract

A dynamic model is presented which uses a mean first passage time to characterize multivalent binding as the continuum limit of a one-step stochastic process. The use of a stochastic model instead of a model based on rate equations permits consideration of fluctuations in the number of bonds formed. The importance of fluctuations to the problem of multivalent interactions, especially low affinity interactions, is discussed. Assumptions for the theory are based on data in which liposomes were used as a model system for multivalency. The data suggest a linear sequential process of bond rupture, and thus a high degree of cooperativity in the microscopic events associated with multivalent liposomal binding.

Keywords: Multivalency; Fokker–Planck equation; Liposomes; Mean first passage time

1. Introduction

The occurrence of multiple simultaneous interactions between biomolecules at surfaces is important in many diverse areas of study, from cell biology to biosensors. Stimulation of immune cells, serum complement fixation, cell–cell interaction, protein adsorption, cell adherence and motility, drug targeting, and immunoassays are all influenced by the occurrence of multiple site interactions. Multivalent cross-linking has been shown to enhance T-cell proliferation [1], and to stimulate B-cell activation [2,3]. Hapten density has been shown to effect immunogenicity of liposomes [4], and complement lysis of red blood cells [5]. The effect of multivalency on immune response has stimulated interest in synthetic multivalent antigens for vaccines [6]. Other recent studies have included discussions of the effect of epitope density on antibody affinity and immunoassay sensitivity [7], on the interaction between ligates and solid phase ligands [8], and on the effect of receptor density on the adhesion of cells to surfaces [9].

Theoretical approaches to the formation of multivalent interactions frequently involve assigning rate equations for individual bond-making and bond-breaking events [10,11]. For multivalent interactions, there are by definition many individual rate equations which would have to be assigned. In the theory presented here we treat the process of multivalent interaction as a continuum of discrete binding and rupture events. The result is a stochastic model which describes multivalent interactions as a diffusion process with drift in

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bond space. Compared to the use of kinetic equations, this is an economical approach which combines all unknown kinetic constants into two probabilities, the probability of making a bond and the probability of breaking a bond. This approach produces a generalized form for dealing with different kinetic influences, such as cooperativity, without requiring detailed reworking of a large matrix of individual rate equations or the solution of a complex master equation. This approach has the additional advantage over the use of rate equations by taking into account the contribution from fluctuations in the number of bonds formed with the surface. We show how fluctuations in bond space may play a significant role in surface retention of multivalent species.

To provide a framework for this theoretical discussion, we present here an initial systematic study of an experimental model system for multivalency: the binding of multivalent liposomes to particle-immobilized antibodies. The data which will be shown are of too low a resolution to provide direct evidence for fluctuations in bond space; however, they provide a basis for the theoretical assumptions. Liposomes containing phospholipid derivatized with the small hapten, theophylline, were allowed to react with anti-theophylline antibodies which were covalently immobilized on solid glass spheres. Liposomes are an excellent model system for this purpose, since they can be prepared with known and variable amounts of antigen in their bilayer. In addition, the valency of liposomes is itself of great interest particularly in their application as drug delivery agents [12,13], immunization agents [14,15], and as immunoassay reagents, as previously demonstrated in work from this laboratory [16–18].

2. Experimental observations and theoretical assumptions

2.1. Preparation and characterization of antigenic liposomes and immobilized antibody

Liposomes were prepared, by the methods previously described [17], with dimyristoyl phosphatidylcholine, cholesterol, dicetyl phosphate and variable amounts of the antigen, a theophylline–phospholipid conjugate (theophylline–DPPE), in their membranes; and with 0.1 *M* 5-(and 6-) carboxyfluorescein (CF) encapsulated in their aqueous interior. Theophylline–DPPE comprised from 0.0008 to 3.1 mole% of the total liposomal lipid. Since theophylline–DPPE was dispersed as a solution in the bulk lipid solution prior to evaporation of solvent and preparation of liposomes, we assume that it was homogeneously distributed in the liposome population. Quasi-elastic light scattering indicated an average diameter of liposomes containing theophylline–DPPE of 135 ± 65 nm. The lipid concentration of liposome preparations was determined by phosphorous analysis according to the method of Bartlett [19]. Using these measured values and assuming a surface area per lipid molecule in the liposomes of 50 Å^2 [20], we calculated that the stock preparation contained approximately 4×10^{12} liposomes mL^{-1}. The number of theophylline–DPPE molecules present on the outer surface of liposomes was estimated by assuming that liposomes were composed of single bilayer membranes, and that there was a homogeneous distribution of theophylline–DPPE in the liposome population, and between the inner and outer leaflets of the membrane.

Protein A-purified monoclonal anti-theophylline antibody was immobilized onto 210–250 μm diameter solid glass spheres as previously described [21]. The density of active antibody sites, as determined by ^{3}H-theophylline binding, was approximately 4×10^{-15} mol/mm^2 of glass bead. Using a rough estimate of 15 nm^2 to accommodate an antibody molecule, we calculate that about 30% of the total surface area was covered by active antibody protein.

2.2. Association of multivalent liposomes with immobilized antibody

Two types of liposome binding measurements were performed: equilibrium binding, and dissociation kinetics. A schematic of the experimental system is shown in Fig. 1. The complete data set is shown in Table 1, where the functional affinity constants and dissociation rate constants of lipo-

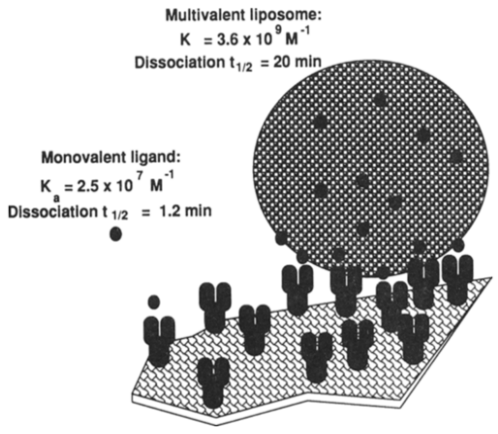

Fig. 1. Schematic of a multivalent liposome binding to surface-immobilized antibodies. The data suggest that a liposome can bind to the surface by forming a number of bonds simultaneously with surface receptors. Monovalent ligand, shown on the left, interacts with only one receptor at a time, and as a result, with a smaller free energy.

somes containing varying concentrations of theophylline–DPPE are compared to that for univalent theophylline.

2.2.1. Equilibrium binding measurements

To measure the equilibrium binding constant between theophylline–DPPE liposomes and surface anti-theophylline, different concentrations of liposomes in 0.15 mL volumes were incubated with 0.1 mL of beads (containing $\approx 5 \times 10^{-12}$ moles of anti-theophylline binding sites) in a test tube on a rocker for 1 h at room temperature. Following several washing steps, the liposomes that were bound to the beads were disrupted by the addition of 1.5 mL of 6.25 mg/mL 1-*O*-octyl-β-D-glucopyranoside, and the associated CF fluorescence was quantified by fluorometry. As a control for nonspecific adsorption or incomplete washing, the fluorescence from liposomes which remained associated with beads derivatized with bovine γ-globulin instead of anti-theophylline was determined. This was calculated as a percentage of the total fluorescence added (less than 5%), and the data for immunospecific binding were adjusted accordingly. The fluorescence intensity was related to the concentration of liposomes. The effect of multivalency was determined by repeating this experiment with liposomes prepared with varying concentrations of theophylline–DPPE in their membranes. A maximum of $\approx 1 \times 10^{-14}$ moles of liposomes bound to the beads. The area of the beads which is covered by this many liposomes with a diameter of 135 nm is 108 mm^2, or about 6% of the total surface area of the beads. This is in contrast to the estimate of 30% of the surface area being covered by active protein. It cannot be assumed that antibody molecules are homogeneously distributed over the surface, and some binding sites may be available to the small theophylline molecule but not to the liposome bound theophylline–DPPE due to steric considerations. This percentage of surface area coverage suggests that steric limitations to liposome interactions with the surface are probably negligible. By these estimates, an average of ≈ 500 antibody binding sites is available per liposome.

The data were analyzed by nonlinear fitting of the concentration of bound liposomes as a func-

Table 1

Liposome interaction with immobilized antibodies. Effect of membrane antigen density on binding and kinetic constants

Theophylline–DPPE (mol%)	No. of Theophylline–DPPE/outer leaflet	K_a (M^{-1})	k_- (min^{-1})	$t_{1/2}$ (min)	$\Delta G°$ (kcal/mol)
0.0008	1	6.8×10^7	0.19	3.65	−10.5
0.008	9	6.9×10^8	0.12	5.77	−11.9
0.08	90	2.2×10^9	0.21	2.66	−12.6
0.8	900	1.8×10^9	0.048	14.1	−12.5
3.1	3500	3.6×10^9	0.034	20.4	−12.9
Theophylline		2.5×10^7	0.553	1.25	−9.9

tion of total liposome concentration according to the mass action law:

$$K_a = \frac{[AbAg]}{[Ab] \times [Ag]}$$

where, in the case of a monovalent antigen, K_a is the intrinsic affinity constant as defined by Hornick and Karush [22], [Ab] is the concentration of unbound antibody binding sites, [Ag] is the concentration of unbound antigen, and [AbAg] is the concentration of antigen complexed to antibody. Total antibody concentration, $[Ab]_{total}$, is the sum of [Ab] and [AbAg], and $[Ag]_{total} = [AbAg] + [Ag]$.

In the case of binding of multivalent liposomes to immobilized antibody, K_a is an apparent, or functional affinity constant [22], [Ag] is the concentration of unbound liposomes, and [Ab] is the concentration of unbound potential liposome binding sites. [AbAg] is the concentration of liposomes bound to immobilized antibodies. The mass action equation was rearranged to the following quadratic equation:

$$0 = [AbAg]^2 - [AbAg] \times \left([Ab]_{total} + [Ag]_{total} + \frac{1}{K_a} \right) + [Ab]_{total} \times [Ag]_{total}$$

and was solved by nonlinear least squares regression analysis for K_a and $[Ab]_{total}$.

Since the mass action equation appeared to adequately describe the binding data regardless of the valency of the liposome population, it was used to determine equilibrium binding constants (K_a). Figure 2 shows the results of a representative experiment in which immobilized anti-theophylline was incubated with increasing concentrations of multivalent theophylline–DPPE liposomes. The concentration of bound liposomes as a function of total liposomes added is plotted along with the best fit to the mass action equation by nonlinear regression analysis. The effect of

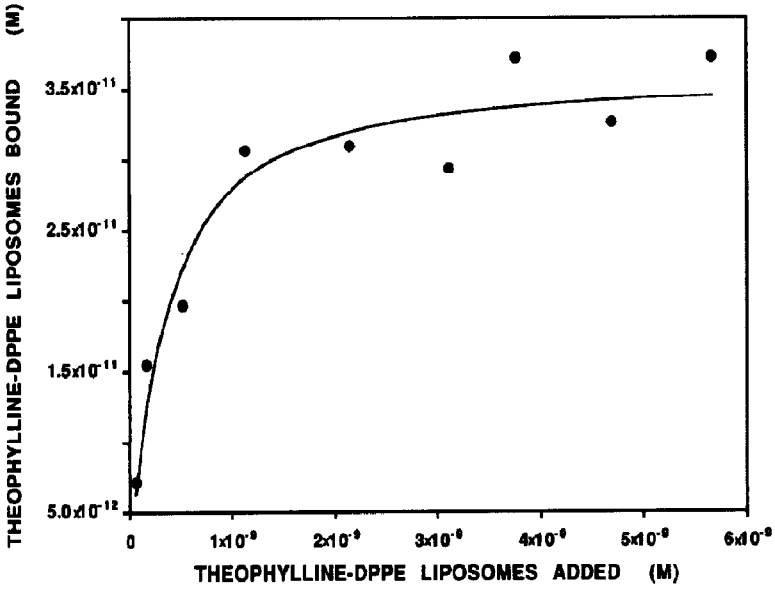

Fig. 2. Different concentrations of liposomes containing 3 mol% theophylline–DPPE in their membranes were incubated with glass beads to which anti-theophylline antibody was covalently coupled. The fluorescence associated with bound liposomes was quantified and related to liposome concentration in the reaction volume. The solid line is the fit by nonlinear regression to a quadratic form of the mass action equation, solving for $[AB]_{total}$ and K_a.

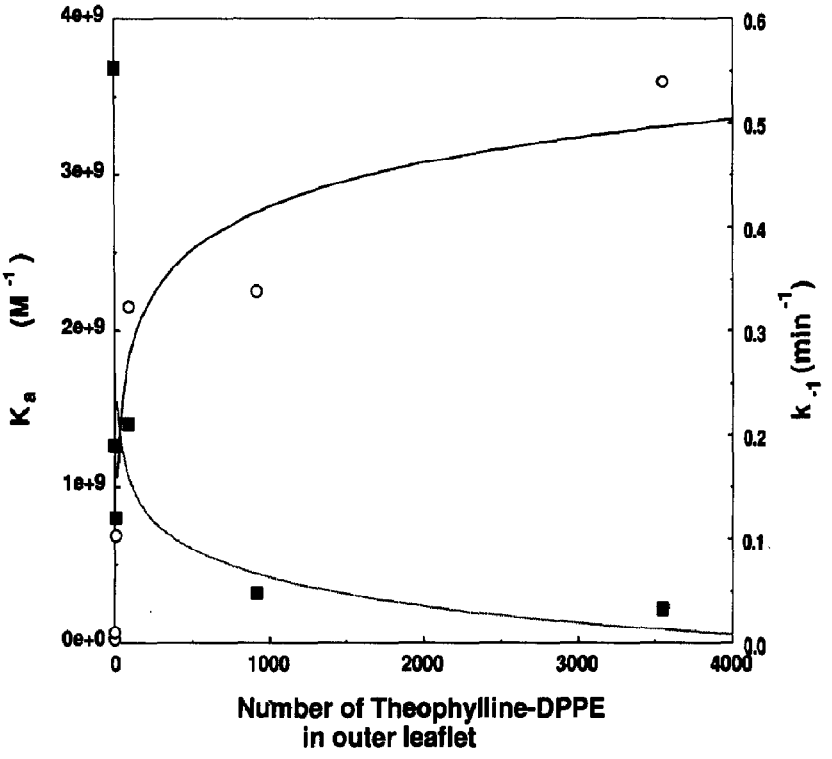

Fig. 3. Apparent binding constants (○) and dissociation rate constants (■) are plotted as a function of the number of theophylline–DPPE molecules present in the outer leaflet of liposome membranes. The lines are drawn by eye. The general trend of the data is discussed in Section 2.

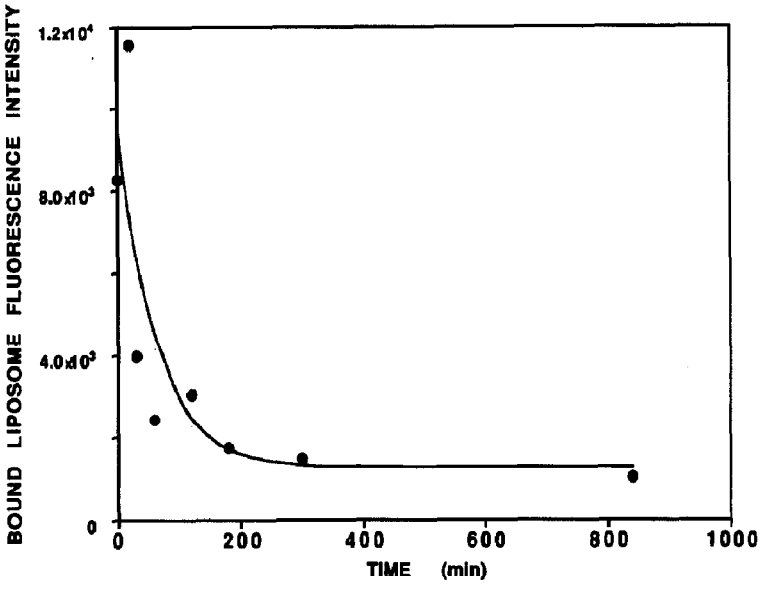

Fig. 4. Liposomes containing 3 mol% theophylline–DPPE were allowed to bind to samples of anti-theophylline derivatized glass beads. To initiate dissociation, unbound liposomes were removed and a 1000-fold molar excess of theophylline was added. At specified times, incubation was terminated, and the fluorescence associated with liposomes remaining bound to beads was quantitated. The solid line is the nonlinear least squares regression fit to a single exponential decay.

valency is shown in Table 1 and Fig. 3 where K_a is plotted as a function of the number of theophylline–DPPE per liposome. The data show that the affinity constants determined for multivalent liposomes are significantly greater than the constant measured for the monovalent antigen in similar experiments using ^{3}H-theophylline. In addition, the effect of multivalency on affinity is greatest at lower hapten densities and appears to plateau at higher densities. It is reasonable to conclude that the increase in free energy of the interaction is the result of multiple bonds being formed simultaneously between liposomes and the surface.

2.2.2. Rate constants for dissociation of liposomes from the solid phase

The rate constants for dissociation of theophylline–DPPE liposomes from immobilized anti-theophylline were determined using the following experimental protocol: theophylline–DPPE liposomes were incubated with anti-theophylline beads for sufficient time to approach equilibrium, unbound liposomes were removed, and then the kinetic experiment was initiated by addition of a 1000-fold molar excess of free theophylline to the beads for varying times. The presence of excess free theophylline was intended to produce conditions such that when a bond between a liposome and the surface ruptured, reformation of that bond or formation of a new bond was unlikely. In addition, when a liposome detached from the surface, it was highly unlikely to reattach. However, even this large excess of free theophylline does not insure that bond formation cannot occur. In the case of liposomes with a large valency, there may be bonds with the surface which are sterically shielded from solution and not exposed to excess free theophylline. (In fact, there may be bonds for which rupture can only be preceded by rupture of neighboring bonds.) Thus in our model we do not assume that even under the experimental conditions described here that the probability of bond formation is zero; however, it is probably very small. Figure 4 shows a representative dissociation experiment for theophylline–DPPE liposomes. For all experiments, first order dissociation rate constants, k_-, were determined by nonlinear least squares regression analysis of the fluorescence associated with the beads as a function of time. The effect of valency on retention mirrors the effect on the binding constants, as is shown in Fig. 3 and in Table 1. Dissociation rate constants decrease with increasing valency, and range from 0.5 min^{-1} for theophylline ($t_{1/2}$ = 1.2 min), to 0.034 min^{-1} ($t_{1/2}$ = 20 min) for liposomes containing 3% theophylline–DPPE.

2.3. Stochastic kinetic model

We have used liposomes as a model system for multivalent binding because of the ease of preparing populations with different valencies but otherwise identical characteristics. In addition, because lipids diffuse rapidly in membranes, lipid molecules which can bind with the surface will migrate in the plane of the bilayer to maximize the number of bonds with the surface [23].

We have observed that the interaction of multivalent liposomes with surface-immobilized antibodies has several predominant features. Regardless of the valency, the steady state interaction between liposomes and the surface can be described by the mass action law. The interaction appears to be uncomplicated by cooperativity between liposomes, and looks like a simple bimolecular event. Furthermore, regardless of valency, dissociation of liposomes from the surface occurs with apparent first order kinetics, as though the breaking of a single bond is the rate determining step in dissociation. However, other aspects of the data clearly indicate the presence of multiple bonds. We observe that as the valency increases, there is an increase in the equilibrium free energy of binding, and a decrease in dissociation rate constants. This is consistent with the simultaneous occurrence of multiple bonds with the surface, where the number of bonds formed increases as the valency increases; this is made possible by the diffusion of theophylline–DPPE in the membrane. Finally, we observe that the response to increasing valency is larger for smaller hapten densities and appears to saturate at higher hapten densities. We assume that there is a limit due to steric considerations that determines the

maximum number of bonds which can be formed between a liposome and a surface.

From this starting point, we have developed a stochastic model for the dissociation of a multivalent species from a surface. In this report we consider only the kinetic response of multivalency; the equilibrium response will be considered in a future publication. Our kinetic experimental protocol involved initiating dissociation by addition of a large concentration of competing hapten. This addition altered the transition probabilities for bond breaking and bond formation that existed during the equilibrium period. However, the theory which we present is not restricted to the specific experimental conditions we employed. This model allows us to explore in depth some general microscopic aspects of multivalent binding, such as the microscopic probabilities of bond-making and bond-breaking, and the relative importance of the first vs. the subsequent bonds formed during a multivalent interaction.

For all binding species studied, including the monovalent antigen and the liposomes of different valency, the same functions for the kinetics and the equilibrium binding constants appear to be valid, suggesting that the determinant steps in the multivalent binding process involve the making and breaking of single bonds. We have chosen a stochastic model since, in the most general case, if a liposome remains attached to the surface and is able to break and make new bonds until the last bond is broken, statistical fluctuations in the number of surface bonds cannot be neglected; i.e., the number of bonds formed between a liposome and the surface can be small, and so diffusion in bond space is always important. Classical kinetic models generally ignore these fluctuations. Fluctuations are allowed in the stochastic model because we deal with transition probabilities as opposed to rate constants. This approach is similar to the one taken by Szabo et al. [24], who described association reactions as a first passage time (in real space) based on the Smoluchowski equation. Our approach involves a mean first passage time in bond space.

We describe the dissociation of multivalent liposomes from surface-immobilized antibody as the continuum limit of the master equation for one-step stochastic processes (see Appendix A). The result is a Fokker–Planck-like equation (the nomenclature used by van Kampen [25] is preserved):

$$\frac{\partial}{\partial t}P(n,t) = \frac{\partial}{\partial n}[g(n) - r(n)]P(n,t) + \frac{1}{2}\frac{\partial^2}{\partial n^2}[g(n) + r(n)]P(n,t) \tag{1}$$

In this approach, we treat discrete events (i.e., individual bond making and bond breaking events) as a continuum of events involved in the multivalent process. We define n as the number of bonds formed between a liposome and the surface, $P(n, t)$ as the probability of realizing n bonds at time t for a single liposome, and $g(n)$ and $r(n)$ as the bond breaking and bond formation probabilities per unit time, respectively. By rescaling time, g and r can be interpreted as microscopic transition rates which are assumed to be positive, smooth functions of n. We can define D as a kind of diffusion coefficient in bond space, which characterizes the random fluctuations in the number of bonds,

$$D(y) = \frac{g(y) + r(y)}{2} \tag{2}$$

Thus, D mimics the effects of random collisions in a condensed phase. Cooperative (and anti-cooperative) effects between individual bond formation and bond breaking events can be incorporated by assuming various forms for $g(n)$ and $r(n)$. In a model without cooperativity, g and r would be linear functions of n; i.e., the rate of bond breaking would be proportional to the number of bonds, and the rate of bond making would be proportional to the number of available binding sites. However, we will consider in our model the case where g and r are constants. This is a highly cooperative model which corresponds to a linear sequential process of bond rupture and bond making, and is a model which adequately describes the data. For an example of a noncooperative discrete stochastic model for protein folding, see Zwanzig et al. [26]; the predictions from

their model are significantly different from what we will present here.

We define $Q(n, t)$ as the probability that a liposome having n surface bonds at $t = 0$ has not detached from the surface after time t has passed. The probability distribution of first passage times (see Appendix B; also [27]) is then given by

$$\mathrm{T}(n, t) = -\frac{\partial}{\partial t}Q(n, t) \tag{3}$$

where we have assumed irreversible detachment from the surface following rupture of the last bond. (This condition was experimentally achieved in the present case by the presence of a large molar excess of free ligand; however, the same condition occurs under continuous flow.) If L is the number of liposomes initially bound to the surface, then the exact kinetic equation for liposomal detachment is

$$L\frac{\partial Q(n, t)}{\partial t} = -L\mathrm{T}(n, t) \tag{4}$$

where $Q(n, t)$ satisfies the adjoint (see Appendix B and Riskin [27]) of eq. (1):

$$\frac{\partial Q(n, t)}{\partial t} = -[g(n) - r(n)]\frac{\partial}{\partial n}Q(n, t) + \left[\frac{g(n) + r(n)}{2}\right]\frac{\partial^2}{\partial n^2}Q(n, t) \tag{5}$$

Although eq. (4) is exact within the confines of a continuum, small fluctuation approximation, its solutions are in general quite complex (i.e., Q decays in time as an infinite sum of exponentials). A much simpler and useful "low resolution" kinetic description, based on an average retention time, can be obtained from the approximation

$$\mathrm{T}(n, t) = \frac{1}{T(n)}Q(n, t) \tag{6}$$

where $T(n)$ is the mean first passage time, i.e., the average time required for a liposome to reach $n = 0$ bonds from n bonds for the first time. (This passage time is the inverse of our experimentally determined k_{-1}, or $t_{1/2}$.) Since $T(n)$ is defined by

$$T(n) = \int_0^\infty \mathrm{d}t\, t\mathrm{T}(n, t) = \int_0^\infty \mathrm{d}t\, Q(n, t) \tag{7}$$

$T(n)$ can be expressed *explicitly* as a function of $g(n)$ and $r(n)$.

By substituting eq. (3) into eq. (6), the kinetic equation can be further simplified to

$$\frac{\mathrm{d}Q(n, t)}{\mathrm{d}t} = -\frac{1}{T(n)}Q(n, t) \tag{8}$$

Thus, although an exact general solution predicts that the actual kinetic behavior is a sum of many single exponentials, "low resolution" kinetic data will appear to be a single exponential decay. This is what we observed experimentally. For situations in which uncertainties in the data are rather large, which is the case in our experiments, fitting data to a single exponential may be preferable to a direct numerical integration of the raw kinetics data as in eq. (7). In fact, the single exponential approximation is expected to become exact for stochastic processes having a single rate-limiting step such as a passage over a sharp, high potential barrier. Furthermore, this approximation is exact in the coarse-grained sense of time averaging; i.e., one obtains an identity if eq. (8) is substituted into eq. (5) and the result is integrated over time. This implies that an optimized fit of detachment kinetics data to a single exponential can be used to determine the mean first passage time for the process.

We now introduce N as the valency and therefore the maximum number of bonds which can exist between a liposome and the surface, and we assume a reflecting boundary at $n = N$. Thus, liposomes can bind to the surface by n bonds, where n is between 1 and N. For our kinetic experiment protocol, liposomes are first allowed to bind to the surface and establish n bonds. For convenience, we will call this the equilibration step, although technically, equilibrium binding is not required. At time $t = 0$ unbound liposomes

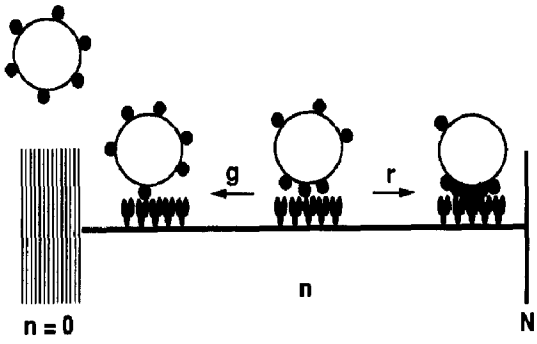

Fig. 5. Schematic of boundary conditions for a multivalent liposome binding to surface-immobilized antibodies. The data suggest that a liposome can bind to the surface by forming a number of bonds simultaneously with surface receptors. n = number of bonds between a liposome and the surface, $n = 0$ marks the absorbing boundary, N = liposome valency and marks the reflecting boundary, g = probability per unit time of bond breaking, r = probability per unit time of bond formation.

are removed and competing hapten is added. Thus, at $t = 0$ we have changed the relative magnitudes of the transition probabilities compared to the equilibrium step: because of the addition of excess free hapten, the relative magnitude of the probability of bond formation, r, is decreased, and the probability of bond breaking, g, is increased. Under these conditions, we have an absorbing boundary at $n = 0$, assuming irreversible detachment from the surface when all bonds are broken due to the presence of a large molar excess of free hapten. Figure 5 depicts these reflecting and absorbing boundaries, and the relationship between g and r.

It is appropriate here to comment on the possibility of alternative experimental protocols. Unlike the above conditions, one might design an experiment in which the transition probabilities g and r are the same during the equilibration step as the kinetic step. This could be achieved simply by replacing a reflecting boundary at $n = 0$ with an absorbing boundary at $t = 0$, such as would occur by equilibrating in a nonflowing condition, and then introducing a flow stream. Other alternatives might involve transition probabilities which differ from the g and r which appear in our equations. In addition, the state variable, n, instead of corresponding to an average over an equilibrium distribution, may be a non-equilibrium steady state average, or even an average over some fraction of the initial distribution.

Standard manipulations [28] on eq. (1) or eq. (5) yield an expression for the mean first passage time from n bonds to zero bonds:

$$T(n, N) = \int_0^n \mathrm{d}y \exp[U(y)] \int_y^N \mathrm{d}z \frac{\exp[-U(z)]}{D(z)}$$

$$0 \leqslant n \leqslant N \tag{9}$$

where $D(n)$ is as defined in eq. (2), and $U(n)$ is a potential energy surface in bond space:

$$U(y) = 2\int_0^y \mathrm{d}x \frac{[g(x) - r(x)]}{[g(x) + r(x)]} \tag{10}$$

If g and r are constants, depending on whether $g > r$ or $g < r$, U scales as y or $-y$, i.e., a ramp potential. If we assume cooperativity in the form of a linear sequential process so that both g and r are taken as constants, this immediately leads to

$$T(n, N) = \frac{n}{(g-r)} - \frac{(g+r)}{2(g-r)^2} \exp\left[-2\left(\frac{g-r}{g+r}\right)N\right] \times \left\{\exp\left[+2\left(\frac{g-r}{g+r}\right)n\right] - 1\right\} \tag{11}$$

Experimental conditions make it unlikely that there is much difference between n and N. For liposomes in solution, theophylline–DPPE is probably homogeneously dispersed within the liposome membrane, but because lipids diffuse rapidly in the plane of the bilayer, when exposed to a surface with which they bind, the distribution is expected to change in order to maximize the number of bonds with the surface. Thus the number of bonds formed, n, could be equal to the liposome valency, N. At some point, however, N can be larger than the maximum number of bonds that can be formed between a liposome and the surface, given steric constraints. In the case of liposomes, these constraints involve membrane packing density, liposome distortion, surface antibody density, etc. We define N_{sat} as the maxi-

mum number of bonds which can be formed within these constraints. Therefore, we focus on $T(N, N)$ for $N < N_{sat}$, and $n \approx N$,

$$T(N, N) = \frac{N}{(g-r)} - \frac{(g+r)}{2(g-r)^2} \times \left\{1 - \exp\left[-2\left(\frac{g-r}{g+r}\right)N\right]\right\} \quad (12)$$

We anticipate that different experimental conditions will effect the relative magnitude of g, the bond breaking probability, and r, the bond formation probability. Depending on the relationship between g and r, the model predicts very different responses in $T(N)$ as a function of N. There are three conditions to consider $g \gg r$, $g \ll r$, and $g \approx r$. The experimental conditions under which we collected the data shown in Figs. 3 and 4 involved the addition of excess competing ligand to liposomes bound to the solid phase. Competing ligand is expected to bind to free sites on the solid phase. Under these conditions, the probability of liposomes forming new bonds with the surface is practically zero, while the probability of liposomes breaking bonds with the surface is larger than zero; thus $g \gg r$ under these conditions. Nevertheless, it is interesting to examine some alternative conditions. From eq. (12) we can see that when $g \ll r$ (bond formation is favored), and N is large, both $T(N)$ and $\partial T/\partial N$ diverge as $\exp(N)$ as N approaches infinity. This is the relationship which we would expect to see when the association rate is larger than the dissociation rate. For the condition where $g \approx r$, such as in cases where association and dissociation rates are not very different, diffusion, i.e., fluctuations, in bond space dominates the kinetics, and the mean retention time is expected to be a quadratic function of N:

$$T(N, N) = \frac{N^2}{(g+r)} \quad (13)$$

Thus, for low affinity interactions, multivalency affects retention times primarily as a result of fluctuations in bond space.

Finally, for the condition of $g \gg r$ where N is large, this expression yields the following drift-dominated regime:

$$T(N, N) = \frac{N}{(g-r)} \quad (14)$$

This last expression predicts that under conditions where g and r are constant, and $g \gg r$ such as in the presence of a large molar excess of free hapten, $T(N)$ will increase linearly with N for $N < N_{sat}$, and the change in the mean retention time with N will be approximately equal to the inverse of the microscopic dissociation rate, i.e., $1/g$. Since we have created here experimental conditions where the kinetics are dominated by drift and fluctuations are suppressed, this is also the result that one expects from a classical kinetics description which neglects fluctuations in the number of bonds. The preliminary data shown here for liposomes suggest an approximately linear relationship between retention and valency (certainly not an exponential or quadratic function), before the effect of valency begins to saturate (Fig. 3). This observation is not consistent with what would be predicted using the noncooperative model of Zwanzig et al. [26]. The noncooperative or mean field model predicts an exponential increase in T with N (for large N).

3. Discussion

Increasing the number of theophylline–DPPE molecules per liposome resulted in an increase in the binding constant of liposomes for immobilized antibodies, and a corresponding decrease in dissociation rate constants. For a prediction of the continuum effect of increasing the valency, N, (for $N \leqslant N_{sat}$), we can take the partial differential of eq. (9) with respect to N while keeping the initial number of bonds, n, fixed:

$$\left(\frac{\partial T(n, N)}{\partial N}\right)_n = \frac{\exp[-U(N)]}{D(N)} \int_0^n dy \exp[U(y)] \geqslant 0 \quad (15)$$

This treatment shows that if N is allowed to vary under conditions where n is fixed, $T(n, N)$ remains sensitive to the form of U even as $n \to N$. This implies that increasing the valency significantly increases the retention time of the multivalent species, even if the number of bonds formed with the surface is not increased. As N is increased, a liposome can now explore a larger number of possible conformations, i.e., there are a larger number of ways to achieve n bonds. This may be visualized as the ability of a multivalent species to break a bond and make a new bond with a previously unbound ligand, an example of which is the movement of cells on surfaces. Association with the surface is prolonged, even though the number of bonds remains the same.

The effect of multivalency of liposomes appears to saturate at larger N. In our experiments, the effect on binding is greatest up to approximately 100–200 haptens per liposome, (a concentration of theophylline–DPPE of less than 0.2 mol% of total lipid), above which the increase in free energy of binding continues at a slower rate. This could be due to the steric limitations on the maximum number of bonds which liposomes can form simultaneously with the surface. The combined area taken up by 100 to 200 densely packed theophylline–DPPE molecules (assuming approximately 70 Å^2 each) is between 70 and 140 nm^2. A circle formed from this many theophylline–DPPE would have a diameter that is between 7 and 10% of the diameter of the liposome. It is reasonable that steric constraints on the packing density of reactive lipid and on the extent of deformation of the liposome must limit the maximum area of interaction, thereby defining N_{sat}.

We have assumed that steric considerations are responsible for the leveling off of the effect of increasing valency. We have thus far accomplished this by imposing an artificial reflecting boundary that limits n, the number of bonds per liposome which initially form with the surface during the equilibration period. We can investigate the influence of this boundary on the mean retention time after initiation of dissociation by the following treatment: if we differentiate eq. (9) with respect to n, while keeping the functional forms of U and D for the kinetic process fixed, we obtain

$$\left(\frac{\partial T(n, N)}{\partial n}\right)_N = \exp[U(n)] \int_n^N \mathrm{d}z \frac{\exp[-U(z)]}{D(z)} \tag{16}$$

This treatment allows us to consider cases of different initial distributions in n. In other words, we perturb n without altering U. We see that for $0 \leqslant n \leqslant N$, $\partial T/\partial n \geqslant 0$. However, if we take the limit of this expression for $n \approx N$, and define $\Delta = N - n$, then

$$\left(\frac{\partial T(n, N)}{\partial n}\right)_{n \to N} = \frac{1}{D(N)} \Delta \tag{17}$$

When $n = N$, $\Delta = 0$ and no more bonds will form. Likewise, if $N > N_{sat}$, no more then N_{sat} bonds will form, and $\partial T/\partial n$ will go to zero as n goes to N_{sat}. The effect of increasing the number of bonds on the mean retention time is thus greatest when the number of bonds formed between a liposome and the surface is much smaller then N, and will naturally go to zero as $n \to N$ or N_{sat}. Thus the effect of bond formation on retention is not equivalent for all bonds formed, even when their microscopic rates do not change.

Also of interest is that with increasing number of bonds formed, i.e., as $n \to N$, the terms containing the potential cancel, and we are left with only the diffusion term, D (see eq. 2). The retention time thus becomes entirely independent of the relative magnitude of the microscopic rates, g and r, and is determined instead by their sum, the unbiased rate of fluctuations in the number of bonds. For a multivalent interaction where fluctuations are smaller, as the number of bonds formed approaches the maximum, each additional bond formed has a larger effect on retention than would occur under conditions of larger fluctuations. If we compare two hypothetical multivalent systems where the bond formation probability is the same, for the case where the rate of bond breaking is smaller, we see from eq. (17) that increasing the number of bonds will have a greater effect on retention. The converse is also true: for two otherwise identical multivalent systems, the case with the smaller bond formation

probability will show a larger effect of increasing number of bonds on retention.

It is of interest to note that from eq. (17), we predict that the effect of increasing bond formation on retention decreases as $n \to N$. However, we also see from eq. (17) that the dependence of D on N could cause the sensitivity of retention time with respect to n to be influenced by the valency. Thus the contribution to the multivalent interaction afforded by each additional bond need not be the same for every multivalent species, due to changes in microscopic rate constants with changes in the valency of the species. Thus, empirically it is impossible to determine the mechanism of saturation in the effect of multivalency on retention without measuring microscopic rate constants. However, if one examined only the microscopic constants, the measurement of fluctuations (the diffusion coefficient, $D(N)$) would dominate, and nothing would be learned about retention.

Another possible cause of saturation in $T(N)$ at large N could, of course, be anti-cooperativity. Consider eq. (16) for the case where the maximum number of bonds formed between a multivalent structure and a surface is infinitely large $(N \to \infty)$,

$$\lim_{N\to\infty}\left(\frac{\partial T(n,N)}{\partial n}\right)_N = \exp[U(n)]\int_n^\infty \mathrm{d}z\,\frac{\exp[-U(z)]}{D(z)} \qquad (18)$$

We can see that, in contrast to eq. (17), $\partial T/\partial n$ can now be quite sensitive to the form of the potential. For instance, consider the case where $D(z)$ takes the constant value D_0 and $U(z)$ assumes the form

$$U(z) = +z^p \quad (p = 2, 3, 4, \ldots)$$

where p can be regarded as the order of anti-cooperativity. The integral in eq. (18) now has the form of a generalized complementary error function [29] and the decay of $\partial T/\partial n$ for large n describes a natural saturation effect due to anti-cooperative binding.

4. Conclusion

We observe that the dissociation rate constants for desorption of multivalent liposomes from an antibody-coated surface vary approximately linearly with valency, N. We have presented a model which indicates that first order rate constants for this process are good estimates, and may be exact solutions, for mean first passage times. The linear relationship between T and N indicates the presence of a ramp potential in the dissociation process. Thus, dissociation of multivalent liposomes from a surface appears to be a highly cooperative sequential process.

The Fokker–Planck continuum description allows us to make mathematically concise predictions concerning how retention is effected by valency, the number of bonds formed, and the relative magnitudes of the microscopic rates. Given the limits of a reflecting and an absorbing boundary, we have shown how retention can change with n, the number of bonds initially formed. Space constraints prohibit exhaustive consideration of all parameters which could effect multivalent interactions. For example, we have treated n as if it were the average over some very sharply peaked initial distribution, but inhomogeneity in liposome size or in the distribution of reactive lipid within the liposome population, or inhomogeneity in the distribution of surface binding sites will effect the width of the distribution of n. These spurious inhomogeneities introduce additional uncertainties into the interpretation of kinetics data, and additional efforts should be made to minimize them in future experiments.

We discussed the effect of the relative magnitudes of the microscopic rates, and we showed how the relationship between T and N is a valuable diagnostic tool for determining the relative magnitudes of g and r, and can also provide a criterion for the importance of statistical fluctuations in the number of bonds. These relationships will provide useful guidelines for future experiments involving liposomes and other multivalent systems where statistical fluctuations in bond space should be considered.

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Appendix A

Let us introduce the one-step raising (E) and lowering (E^{-1}) operators by

$$Ef(n) = f(n+1)$$
$$E^{-1}f(n) = f(n-1) \qquad \text{(A.1)}$$

The master equation for one-step stochastic processes may then be written as

$$\frac{\partial P(n,t)}{\partial t} = (E-1)g(n)P(n,t) + (E^{-1}-1)r(n)P(n,t) \qquad \text{(A.2)}$$

where $P(n, t)$ is the probability of the system being in state n at time t and where g and r are bond rupture and bond formation transition probabilities which are considered to be functions of the discrete state variable n. The first term on the right hand side of eq. (A.2) is the probability flux into state n from $(n+1)$ while the second term is the flux into n from $(n-1)$. Suppose the state space is bounded such that $0 \leqslant n \leqslant N$, where N is the system size, and we introduce a system size scaling by $N^* = \alpha N$ and $n^* = \alpha n$, where α is a large dimensionless number. Further suppose that g, r, and P are scale-invariant or intensive quantitites; i.e.,

$$g(n^*) = g(n),\ r(n^*) = r(n)$$
$$P(n^*, t) = P(n, t),\ (N \to N^*) \qquad \text{(A.3)}$$

Now consider the exponential representations of E and E^{-1}:

$$E = \exp\left(\frac{\partial}{\partial n}\right),\ E^{-1} = \exp\left(\frac{-\partial}{\partial n}\right) \qquad \text{(A.4)}$$

along with the corresponding scaled operators

$$E_* = \exp\left(\frac{\partial}{\partial n^*}\right) = \exp\left(\frac{1}{\alpha}\frac{\partial}{\partial n}\right)$$
$$E_*^{-1} = \exp\left(\frac{-\partial}{\partial n^*}\right) = \exp\left(\frac{-1}{\alpha}\frac{\partial}{\partial n}\right) \qquad \text{(A.5)}$$

We can perform a Taylor expansion of the exponentials in eq. (A.5) to achieve the Fokker–Planck system size expansion of the master equation for one-step processes. Making use of eq. (A.3), in the following equation we consider the two lowest order terms in $(1/\alpha)$ in the expansion of the scaled master equation eq. (A.2):

$$\frac{\partial P(n,t)}{\partial t} = \frac{1}{\alpha}\frac{\partial}{\partial n}[g(n)-r(n)]P(n,t) + \frac{1}{2\alpha^2}\frac{\partial^2}{\partial n^2}[g(n)+r(n)]P(n,t) + O\left(\frac{1}{\alpha^3}\right) \text{(for large } \alpha) \qquad \text{(A.6)}$$

A similar scaling argument and Fokker–Planck equation can be constructed with the bivariate quantities

$$g(n^*, N^*) = g(n, N),$$
$$\text{(similarly for } r, P), (N \to N^*) \qquad \text{(A.7)}$$

which are no longer intensive system properties insofar as the transition probabilities now depend on the system size, but which obey scale invariance.

It should be noted that size scale invariance may not hold for systems with very long range correlations. For such cases it becomes necessary to revert back to the master equation, even if the system size becomes infinite. Our assumption of scale invariance rests on the intuitive notion that cooperative bond formation and rupture events involve short or finite range correlations in bond space.

As a final point, it should be noted that for any discrete state Markov process the Fokker–Planck approximation to the master equation which describes the evolution of $P(n, t)$ is expected to break down at very short times or for small n. Noise in our data precludes our accurate resolu-

tion of short-time behavior; but in addition, the inclusion of an absorbing boundary condition requires the Fokker–Planck approximation to be identical to the exact master equation at $n = 0$. Thus, as n approaches 0, $P(n, t)$ goes to 0 in both cases.

Appendix B

Let $p(n, t \mid n^*, 0)$ be the probability density for a liposome starting with n^* surface bonds at $t = 0$ to reach n bonds after time t has elapsed. The conditional probability p, as well as $P(n, t)$, satisfies the Fokker–Planck equation [27]

$$\frac{\partial p}{\partial t} = \frac{\partial}{\partial n}[g(n) - r(n)]\, p + \frac{1}{2}\frac{\partial^2}{\partial n^2}[g(n) + r(n)]\, p,$$

$$p(n, 0 \mid n^*, 0) = \delta(n - n^*), \tag{B.1}$$

and

$$p(n, t \mid n^*, 0) = 0 \text{ for } n^* = 0 \tag{B.2}$$

where the absorbing boundary condition at $n^* = 0$ assumes irreversible detachment following rupture of the last surface bond. The probability of realizations $Q(n^*, t)$ beginning at n^* which have *not* reached 0 after time t has elapsed is then

$$Q(n^*, t) = 1 - p(0, t \mid n^*, 0) = \int_0^N p(n, t \mid n^*, 0)\,dn \tag{B.3}$$

where the integral accounts for all realizations with $n \neq 0$ and where we assume the existence of a reflecting upper boundary at $n = N$. It follows that the fraction $-dQ$ of those kinetic trajectories that *do* reach $n = 0$ in the time interval $(t, t + dt)$ is

$$-dQ(n^*, t) = -[Q(n^*, t + dt) - Q(n^*, t)] = -\left[\int_0^N \frac{\partial p}{\partial t}\,dn\right]dt \tag{B.4}$$

The distribution function $T(n^*, t)$ of first passage times for liposomal detachment kinetics is therefore given by

$$T(n^*, t) = -\frac{\partial Q(n^*, t)}{\partial t} \tag{B.5}$$

where $T(n^*, t)$ is the probability that the time interval t has elapsed for the irreversible detachment (at precisely time t) of a liposome initially having n^* surface bonds. Furthermore, it can be shown that Q satisfies the adjoint Fokker–Planck equation

$$\frac{\partial Q(n^*, t)}{\partial t} = -[g(n^*) - r(n^*)]\frac{\partial}{\partial n^*}Q(n^*, t) + \left[\frac{g(n^*) + r(n^*)}{2}\right]\frac{\partial^2}{\partial n^{*2}}Q(n^*, t),$$

$$Q(0, t) = 0 \tag{B.6}$$

This follows from the time translation invariance of the conditional probability p; i.e.,

$$p(n, t \mid n^*, 0) = p(n, 0 \mid n^*, -t) \tag{B.7}$$

As a consequence of this symmetry it can be shown that p is a solution of *both* the Fokker–Planck (FP) equation and its adjoint ($\dagger$) [27]:

$$\frac{\partial p}{\partial t} = L_{FP}(n)\, p, \quad p = 0 \text{ for } n = 0 \tag{B.8a}$$

$$\frac{\partial p}{\partial t} = L_{FP}^{\dagger}(n^*)\, p, \quad p = 0 \text{ for } n^* = 0 \tag{B.8b}$$

where L_{FP} is the ordinary FP operator defined by eq. (1) and $L_{FP}^{\dagger}$ is defined by eq. (B.6). It follows from eq. (B.3) that $Q(n^*, t)$ also satisfies eq. (B.8b). Eq. (B.6) is particularly convenient for calculating recursion relations between the first and higher order moments of the first passage time distribution $T(n, t)$. In this paper, however, we are concerned only with the first moment of T; i.e., the mean first passage time for liposomal detachment.

References

1 R.B. Pepinsky, L.L. Chen, W. Meier and B.P. Wallner, The increased potency of cross-linked lymphocyte function-associated antigen-3 (LFA-3) multimers is a direct consequence of changes in valency, J. Biol. Chem. 266 (1991) 18244–18249.

2 K.C. Allison, W. Strober and G.R. Harriman, Induction of IL-5 receptors on normal B cells by cross-linking surface Ig with anti-Ig-dextran, J. Immunol. 146 (1991) 4197–4203.

3 R.Z. Dintzis, M. Okajima, M.H. Middleton, G. Greene and H.M. Dintzis, The immunogenicity of soluble haptenated polymers is determined by molecular mass and hapten valence, J. Immunol. 143 (1989) 1239.

4 T. Tadakuma, T. Yasuda, S.C. Kinsky and C.W. Pierce, The effect of epitope density on the in vitro immunogenicity of hapten-sensitized liposomal model membranes, J. Immunol. 124 (1980) 2175–2179.

5 T. Borsos, R.M. Chapuis and J.J. Langone, Distinction between fixation of C1 and the activation of complement by natural IgM anti-hapten antibody: effect of cell surface hapten density, Mol. Immunol. 18 (1981) 863–868.

6 T. Toyokuni, B. Dean and S. Hakomori, Synthetic vaccines: I. Synthesis of multivalent Tn antigen cluster-lysyl-lysine conjugates, Tetrahedron Lett. 31 (1990) 2673–2676.

8 L. Goldstein, A. Niv and S.A. Yankofsky, The biotinylcellulose–avidin system: A model for interaction between solid-phase ligands and multivalent high molecular weight ligates, Ann. N.Y. Acad. Sci. 613 (1990) 54–67.

9 C. Cozens-Roberts, J.A. Quinn and D.A. Lauffenburger, Receptor-mediated adhesion phenomena. Model studies with the Radial-Flow Detachment Assay, Biophys. J. 58 (1990) 107–125.

10 B. Goldstein, R. Posner, D.C. Torney, J. Erickson, D. Holowka and B. Baird, Competition between solution and cell surface receptors for ligand, Biophys. J. 56 (1989) 955–966.

11 C. DeLisi and H.W. Hethcote, Chromatographic theory and application to quantitative affinity chromatography, in: Analytical Affinity Chromatography, ed. I.M. Chaiken, (CRC Press, Boca Raton, FL, 1987) pp. 1–64.

12 R.A. Schwendener, T. Trüb, H. Schott, H. Langhals, R.F. Barth, P. Groscurth and H. Hengartner, Comparative studies of the preparation of immunoliposomes with the use of two bifunctional coupling agents and investigation of in vitro immunoliposome-target cell binding by cytofluorometry and electron microscopy. Biochim. Biophys. Acta Bio-Membr. 1026 (1990) 69–79.

13 T.D. Heath, R.T. Fraley, J. Bentz, E.W. Voss Jr., J.N. Herron and D. Papahadjopoulos, Antibody-directed liposomes. Determination of affinity constants for soluble and liposome-bound antifluorescein, Biochim. Biophys. Acta 770 (1984) 148–158.

14 B. Frisch, S. Muller, J.P. Briand, M.H.V. Van Regenmortel and F. Schuber, Eur. J. Immunol. 21 (1991) 185–193.

15 H.M. Therien, E. Shahum and A. Fortin, Cell. Immunol. 136 (1991) 402–413.

16 A.L. Plant, M. Gray, L. Locascio-Brown and W.T. Yap, Hydrodynamics of liposomes and their multivalent interactions with surface receptors, in: Liposome technology, ed. G. Gregoriadis, (CRC Press, Boca Raton, FL, 1992) chap. 25.

17 L. Locascio-Brown, A.L. Plant, V. Horvath and R.A. Durst, Liposome flow injection immunoassay: Implications for sensitivity, dynamic range, and antibody regeneration, Anal. Chem. 62 (1990) 2587–2593.

18 W.T. Yap, L. Locascio-Brown, A.L. Plant, S.J. Choquette, V. Horvath and R.A. Durst, Liposome flow injection immunoassay: Theoretical analyses of competitive immunoreactions involving univalent and multivalent ligands, Anal. Chem. 63 (1991) 2007–2011.

19 G.R. Bartlett, Human red cell glycolytic intermediates, J. Biol. Chem. 234 (1959) 449.

20 J.N. Israelachvili, D.J. Mitchell and B.W. Ninham, Theory of self-assembly of lipid bilayers and vesicles, Biochim. Biophys. Acta 470 (1977) 185–201.

21 A.L. Plant, L. Locascio-Brown, W. Haller and R.A. Durst, Immobilization of binding proteins on nonporous supports: Comparison of protein loading, activity, and stability, Appl. Biochem. Biotech. 30 (1991) 83–98.

22 C.L. Hornick and F. Karush, Antibody affinity—III. The role of multivalence, Biochemistry 9 (1972) 325–340.

23 R.J.H. Ho, B.T. Rouse and L. Huang, Destabilization of target-sensitive immunoliposomes by antigen binding—a rapid assay for virus, Biochem. Biophys. Res. Commun. 138 (1986) 931–937.

24 A. Szabo, K. Schulten and Z. Schulten, First passage time approach to diffusion controlled reactions, J. Chem. Phys. 72 (1980) 4350–4357.

25 N.G. van Kampen, Stochastic processes in physics and chemistry (North-Holland, Amsterdam, 1981) chap. VIII.

26 R. Zwanzig, A. Szabo and B. Bagchi, Levinthal's paradox, Proc. Natl. Acad. Sci. 89 (1992) 20–22.

27 H. Riskin, The Fokker–Planck equation (Springer Verlag, New York, 1989) chap. 4.

28 G.H. Weiss, Adv. Chem. Phys. 13 (1967) 1.

29 M.A. Abramowitz and I.A. Stegun, Handbook of mathematical functions, National Bureau of Standards Applied Math Series Vol. 55, (U.S. Govt. Printing Office, Washington, DC, 1964) pp. 297.