

The Surface/Volume Ratio in Solid Phase Assays

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In a solid phase assay, e.g. ELISA, a larger surface to volume ratio implies a faster adsorption of molecules from the liquid phase, because the liquid is exposed to more surface. The primary aspect of a larger ratio is therefore that incubation times can be reduced.

In addition, a higher sensitivity may be obtained within a finite incubation time. However, using enough incubation time, a larger ratio will not increase the sensitivity, because there will normally be ample binding sites present on the liquid covered surface for eventual immobilization of all (scarce) analyte molecules.

In this work a quantitative relationship between the surface/volume ratio and adsorption rate is derived from simple adsorption kinetics modelling, verified by data simulation.

Adsorption kinetics model

The basis of adsorptive drainage of molecules from a liquid to a solid surface is the molecules' thermal random movements by which they will eventually come within range of the various attraction forces responsible for adsorption ¹.

In this context adsorption forces also include biospecific affinities, as those between antibody and antigen. By an adsorbing surface is meant any surface with a number of binding sites for certain target molecules, e.g. a surface coated with a specific antibody.

If we consider IgG molecules in aqueous solution and make the following assumptions:

- a molecule that hits an unoccupied surface binding site will instantaneously and irreversibly be bound and occupy that site where no other molecule can be bound,
- diffusion of molecules will keep step with adsorptive depletion so that solution homogeneity is continuously maintained (see remarks below), the rate of adsorption may be expressed by a simple combination of the kinetics for a second order chemical reaction ($A + C \rightarrow B$) and diffusion kinetics: $dB_t/dt = [(A-B_t)/A] \cdot [(C-B_t)/V] \cdot [S(kD/\pi)^{1/2} \cdot t^{1/2}]$ (1)

where:

B_t = number of bound molecules at time t

t = elapsed adsorption time = hr

A = number of molecules that can be bound

C = initial number of molecules in solution

V = liquid volume = cm^3

S = adsorbing surface area = cm^2

D = diff. constant of IgG = $1.44 \cdot 10^{-3} \text{ cm}^2 \cdot \text{hr}^{-1}$

k = dimensionless coefficient to be determined by data simulation

In equation (1) the first two factors in square brackets, derived from common second order reaction kinetics ², express that the adsorption rate at time t is proportional to, respectively, the instantaneous fraction of the surface area available for adsorption, and the instantaneous concentration of dissolved molecules. It should be noted that the first factor, in its present form, is only valid if the molecules can be adsorbed to form a monolayer.

The last factor in square brackets, derived from common diffusion kinetics ³, determines the instantaneous size of that liquid volume in which the dissolved molecules will hit the surface by diffusion during the considered time unit (1 hr).

The second assumption above is of course more controversial, since constant homogeneity may not be a valid approximation, unless the liquid is stirred; and in this case pure diffusion transport would only take place in a thin layer next to the surface. However, exact modelling of unstirred systems, taking also back-diffusion of refused molecules into account, is rather complicated and may not be more informative than simplified modelling. Indeed, failure of data simulation by a simplified model may indirectly give significant information about factors governing the particular adsorption process.

Immediately after time zero, i.e. $t =]\partial t$, eq. (1) reduces to the 'initial' adsorption rate:

$$dB_0/dt = S \cdot (C/V) \cdot (kD/\pi)^{1/2} \cdot (\partial t)^{-1/2}$$

molecules/hr (2)

from which it plausibly appears that for a given initial concentration, C/V , the initial adsorption rate may simply be proportional to the particular surface area, S .

By integration of eq. (1), one obtains a saturation function, determining the fraction, E_t , of the surface area to which adsorption has accumulated at any time.

It appears from eqs. (4) that for a given supply/capacity ratio, $C/A = F$, T_x may simply be inversely proportional to the square of the surface/volume ratio, P .

For constant P , eqs. (4a,b,c) also present T_x as a function of $0 < F < y$, which is depicted in Fig. 1 for $x = 90\%$.

$$E_t = \frac{B_t}{A} = \frac{1 - \exp\{(F-1) \cdot P \cdot 2 \cdot (kD/\pi)^{1/2} \cdot t^{1/2}\}}{1/F - \exp\{(F-1) \cdot P \cdot 2 \cdot (kD/\pi)^{1/2} \cdot t^{1/2}\}} \rightarrow \begin{cases} F & \text{for } F < 1 \\ 1 & \text{for } F \geq 1 \end{cases} \quad (3)$$

where:

$$F = C/A$$

$$P = S/V = \text{surface/volume ratio} = \text{cm}^{-1}$$

$$E_t \{F=1\} = P \cdot 2 \cdot (kD/\pi)^{1/2} \cdot t^{1/2} / [1 + P \cdot 2 \cdot (kD/\pi)^{1/2} \cdot t^{1/2}]$$

From eq. (3) one can derive the time, T_x hr, required to obtain x % of the

maximum adsorption (depending on F):

$$T_x\{F<1\} = P^{-2} \cdot (4kD/\pi)^{-1} \cdot (1-F)^{-2} \cdot [\ln\{(100-x)/F\}/(100-x)]^2 \quad (4a)$$

$$T_x\{F=1\} = P^{-2} \cdot (4kD/\pi)^{-1} \cdot [x/(100-x)]^2 \quad (4b)$$

$$T_x\{F>1\} = P^{-2} \cdot (4kD/\pi)^{-1} \cdot (1-F)^{-2} \cdot [\ln\{(100-x)/F\}/(100-x)]^2 \quad (4c)$$

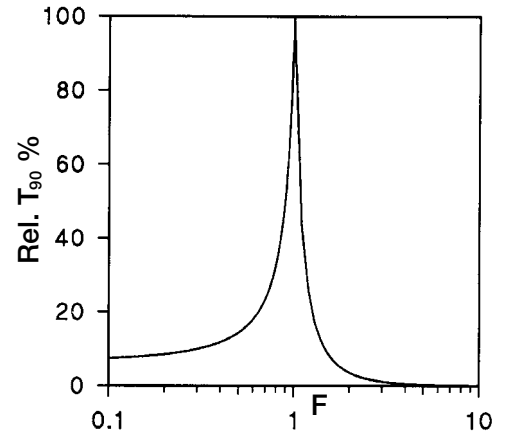


Fig. 1

The incubation time T_{90} in % of its maximum value, for obtaining 90% of maximum adsorption, as a function of the molecule supply/capacity ratio F according to eqs. (4). Note that T_{90} assumes its maximum value for $F = 1$, and approximates to a non-zero minimum value for $F \rightarrow 0$. See text for further explanation.

V cm ³	S cm ²	P cm ⁻¹	Conc. g/cm ³	F	T ₉₀ hr	T ₉₀ ·P ² hr/cm ²
0.05	0.63	12.6	0.13 · 10 ⁻⁶	0.0258	3.0	475
0.1	0.94	9.4	0.065 · 10 ⁻⁶	0.0173	5.3	470
0.2	1.54	7.7	0.0325 · 10 ⁻⁶	0.0106	7.9	466
0.2	1.54	7.7	1.11 · 10 ⁻⁶	0.36	13.1	–
0.2	1.54	7.7	3.33 · 10 ⁻⁶	1.08	59.7	–
0.2	1.54	7.7	10 · 10 ⁻⁶	3.25	1.1	–

Table 1

Parameters for the data simulations in Fig. 2 (above figures) and Fig. 3 (below figures). See text for further explanation.

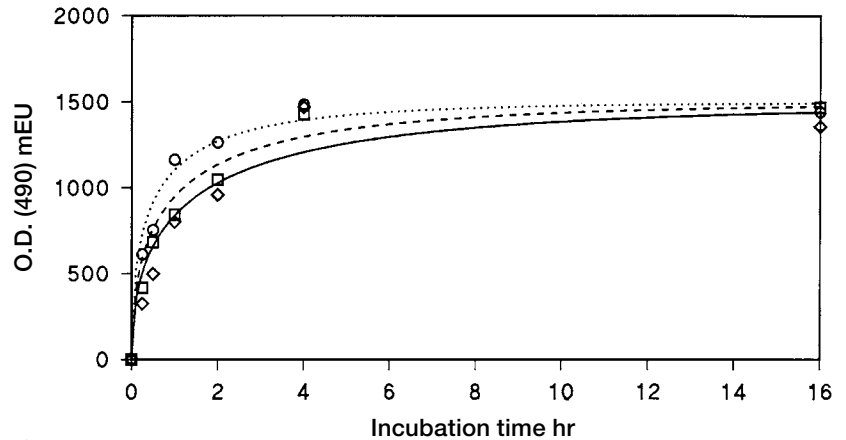


Fig. 2

Simulation curves of adsorption kinetics data from incubations of flat-bottomed MicroWells for various times with IgG-HRP conjugate, 50 µL of 0.13 µg/mL (○/···), 100 µL of 0.065 µg/mL (□/---), and 200 µL of 0.0325 µg/mL (◇/—), according to eq. (3) using the respective F and P values from Table 1. See text for further details.

This graph has a maximum at $F = 1$, indicating that adsorption completion requires the longest time, if C and A are equally large, i.e. if the molecule supply just fits the surface adsorption capacity. The steepness of the curve is striking, especially that of its right branch, indicating that the surface saturation time decreases drastically at a molecule supply just slightly above the balance concentration. The graph approximates to a lower limit value, equal to 6.5% of the maximum, for $F \rightarrow 0$. This indicates that for arbitrarily small F values adsorption will virtually be completed within a certain, relatively short time span.

The approximate constancy of T_x for small F values renders the above stated inverse proportionality between T_x and P^2 valid for a given, sufficiently small concentration (implying say $F \leq 0.1$), even though F varies with variant P for constant concentration. This is a very useful implication, concerning a given “small” concentration, meaning that if P is increased by a factor of 2, the incubation time may be reduced by a factor of 4 without reducing adsorption.

A prerequisite for the validity of these considerations is obviously that a constant value can be assigned to the coefficient k , regardless of the values of F and P .

A demand for a non-unity coefficient to the nominal D (ref. to pure water at 20°C) would not be surprising, since the operating D is depending on temperature and buffer ionic strength; but the possible influence of these parameters on k can be eliminated by just keeping them constant.

However, one could fear that k has to be changed by change of adsorption geometry, i.e. by change of P . This possibility is primarily what is to be examined by data simulation.

Data simulation and discussion

Thermo Scientific Nunc Immuno Modules F8 MaxiSorp (Cat. No. 468667) were incubated for various times with IgG-HRP conjugate (Dako P128) in three series with resp. 50 µL of 0.13 µg/mL, 100 µL of 0.065 µg/mL, and 200 µL of 0.0325 µg/mL, followed by substrate reaction with 200 µL H₂O₂/OPD solution, amply concentrated to minimize influence by substrate diffusion. These experimental series present three different adsorption systems, characterized by the parameters given in Table 1, using the common estimate of 400 ng/cm² for Nunc™ MaxiSorp™ IgG adsorption capacity.

Since the total number of molecules is the same, and F is below unity in all three systems, signals from equal incubation times should be equally large between the systems, if not for their different P values. Consequently, any observed discrepancy would reflect differing adsorption kinetics due to the diversity of P .

For optimal data simulations (Fig. 2) by equation (3) using the proper F and P values from Table 1, a common k value = 2 was estimated. Since the final signal levels should be the same, regardless of P , due to total molecule number equality between the systems, the simulations were adjusted to a common final level estimate of 1500 mEU.

The respective T_{90} values (Table 1), calculated from eq. (4a), are not exactly comparable as a function of P , since the systems' F values are also different. However, since T_{90} (for any constant P) is almost invariant within the present range of small F values (Fig. 1), the theoretical inverse proportionality between T_{90} and P^2 approximately holds from one system to the other, as demonstrated by the uniformity of the $T_{90} \cdot P^2$ figures in Table 1. The model's reasonably good fit to the data for constant k therefore demonstrates the validity of this claimed proportionality, at least for small F values.

Indeed, the model's good fit to the data in Fig. 2 might be due to the smallness of the F values, which implies that the adsorption area reduction is negligible (always less than 2.6%), whereby the systems approximate the more simple first order reaction kinetics. Therefore, experimental systems were established with F values of unity order of magnitude to test the model's simulative capability and dramatic T_{90} implications in this F regime.

Thermo Scientific Nunc MicroWell plates, as above, were incubated for various times with 200 μ L 1:100 mixtures of IgG-HRP conjugate (Dako P128) and pure IgG (Dako A008) in three series with total concentrations of respectively 10, 3.33, and 1.11 μ g/mL, followed by substrate reaction with 200 μ L H_2O_2 /OPD. These experimental series present three different systems characterized by the parameters given in Table 1.

The data were simulated (Fig. 3) by equation (3) using the proper F and P values from Table 1, together with the above estimated k value = 2π . Adjustment of the simulations to the signal units was based on a final level estimate of 400 mEU for simulation of the data having $F = 0.36$. This implies a common final level estimate of $400/0.36 \approx 1110$ mEU for the two other data series, since they both have $F > 1$, i.e. $E_t \rightarrow 1$ (cf. eq. (3)).

It is apparent that for fixed $k = 2\pi$ the model fits the data reasonably well for F up to at least $1/3$, but fails to fit the data for $F \geq 1$. Thus, for large F values there seems to be an initial excess adsorption, the more pronounced, the larger the F value, followed by some desorption during prolonged incubation. The model does not take this phenomenon into account, but it explains, by the T_{90} estimates in Table 1, the difference between the two upper data series' apparent final levels (which should be equal because $F > 1$): the difference is observed, simply because

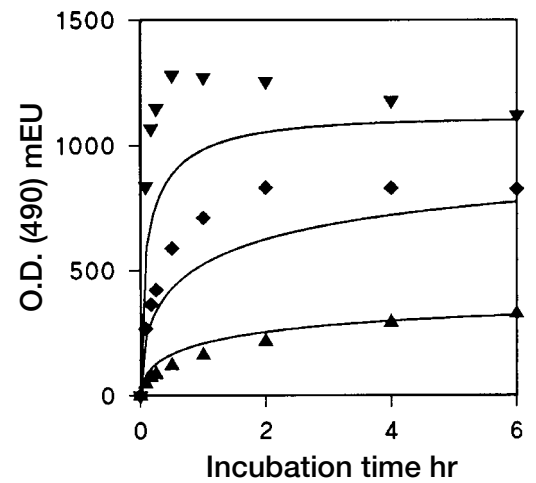


Fig. 3

Simulation curves of adsorption kinetics data from incubations of flat-bottomed MicroWells for various times with 200 μ L 1:100 mixtures of IgG-HRP conjugate and pure IgG in total concentrations of 1.11 μ g/mL (\blacktriangle), 3.33 μ g/mL (\blacklozenge), and 10 μ g/mL, (\blacktriangledown) according to eq. using the respective F and P values from Table 1. See text for further details.

complete adsorption requires an extremely long time for $F = 1.08$ compared with $F = 3.25$. Thus, the data confirm the theoretical, profound variation of T_{90} when F varies around 1, and the initial excess adsorption for the larger F values may be regarded as merely a phenomenon superimposed on basic kinetics, governed by the model. Probably the more concentrated molecules will overcrowd during their initial mass invasion of the “virgin” surface, and only after a period of molecule rearrangement and desorption of excess molecules a stable one-to-one binding between molecules and surface binding sites will be established.

Thus, it seems that for $k = 2$ the model is basically consistent with the MicroWell™ adsorption format for any P and F values of interest. However, the possibility exists that k invariance cannot be maintained for other P transitions than those established by changing the liquid volume in MicroWell plates. Therefore, the relevance of the inverse proportionality between incubation time and P^2 was tested using a different system consisting of “startubes” (⊗) vs. standard tubes (⊃).

Immuno™ StarTubes™ MaxiSorp (Cat. No. 470319) and Immuno Tubes MaxiSorp (Cat. No. 444202) were coated overnight with excess swine anti-rabbit antibody (Dako Z196), then incubated with a dilution series of rabbit antibody HRP conjugate (Dako P128), startubes for 1 hr, and standard tubes for 1, 1.5, 2.5, and 3.5 hr, followed by substrate reaction with H_2O_2/OPD solution. All liquid volumes were $350 \mu\text{L}/\text{tube}$, implying a P_{\otimes}/P_{\cup} ratio of about 1.6⁴. Consequently, by using startubes instead of standard tubes, a possible reduction of the second layer incubation time by a factor of about 2.5 without reduction of signal would be expected with the smaller concentrations.

From the data in Fig. 4 it appears that at small concentrations the 1 hr startube signals lie between the 1.5 and 2.5 hr standard tube signals. This means that the incubation time can be reduced only about twice, by using startubes instead of standard tubes, rather than 2.5 times, as expected. But from the level signals at saturating concentrations, estimated to 975 mEU for startubes, and 700 mEU for standard tubes, it appears that the working surface area ratio, S_{\otimes}/S_{\cup} , equal to the P_{\otimes}/P_{\cup} ratio (since the volumes are equal), is $975/700 = 1.4$, rather than the nominal 1.6, just implying an incubation time reduction factor of about 2 instead of 2.5.

Thus, it seems to be generally consistent for obtaining a definite adsorption of molecules from small concentrations that the adsorption time is inversely proportional to the square of the surface/volume ratio, regardless of the specific geometry. This is valid also for biospecific affinity adsorption, as demonstrated by the last experiment, provided that the adsorbing densities are the same.

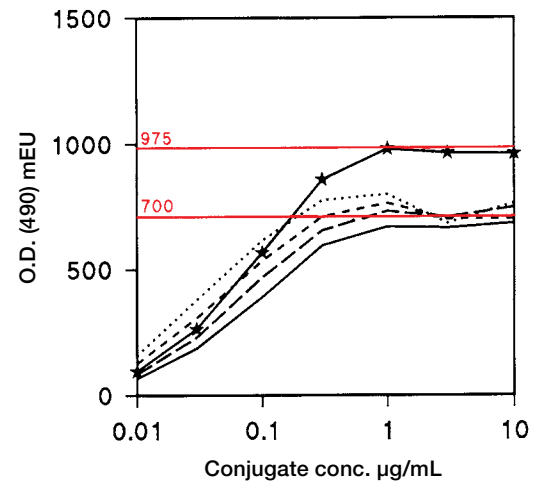


Fig. 4

Signals for immunospecific affinity adsorption obtained by incubation of startubes for 1 hr (—★—) and standard tubes for 1 hr (—●—), 1.5 hr (—◻—), 2.5 hr (—◻—), and 3.5 hr (· · · ◻ · · ·) with $350 \mu\text{L}/\text{tube}$ of a dilution series of rabbit antibody HRP conjugate after coating with swine anti-rabbit antibody. Note that at small concentrations the 1 hr startube signals lie between the 1.5 hr and 2.5 hr standard tube signals, whereas at saturating concentrations the startube signal level (= 975 mEU) exceeds the standard tube signal level (= 700 mEU) due to the larger startube surface area. See text for further explanation.

Summary

From this investigation, by means of a simple adsorption kinetics model, verified by data simulation, the following general rules related to incubation time and surface/volume ratio in solid phase assays can be derived:

1. For a given concentration of molecules to be adsorbed, the initial adsorption rate is proportional to the adsorbing surface area according to eq. (2).
2. For a given molecule concentration implying sufficiently small ratios, F , between molecule supply and surface adsorption capacity, say $F = 0.1$, the incubation time T_x required to adsorb a certain percentage x of the molecules is to a good approximation inversely proportional to the square of the surface/volume ratio, P . This means that if e.g. P is increased twice, the incubation time can be reduced four times without reducing adsorption. Analytes are often consistent with small concentrations, and the approximate time required to adsorb any particular percentage of a scarce analyte can be estimated from eq. (4a) for $F = 0$, provided that it can potentially be adsorbed to form a monolayer.
3. Surface saturation time is extremely long if the molecule supply just fits the surface adsorption capacity, i.e. if $F = 1$, but it is dramatically reduced, if F is just 1.5 times larger than 1 (Fig. 1). However, in the latter case, at least by first layer coating, a stable saturation state should be expected only after a considerable equilibration time, probably due to an initial molecule overcrowding on the surface. Therefore, the incubation time to be saved with $F > 1$ is questionable, and one might be better off with $F = 1.5$ times smaller than 1, where the adsorption time is also markedly reduced, even though the surface cannot be fully saturated. In this case, one would get a better utilisation of coating molecules within a limited incubation time.

The model's simulative capability is so far limited to conditions where molecules can be adsorbed to form a monolayer. However, this will not be the case in many situations, for instance, considering a surface coated with a specific antibody, if only part of the adsorbed antibodies are active, and/or if the target molecules are small compared with the antibodies. To adapt the model for such situations, the factor $(A-Bt)/A$ in eq. (1) has to be modified by some coefficient, implying e.g. the interesting possibility of estimating the specific activity of adsorbed molecules through estimation of the coefficient by appropriate data simulation. This will be the object of further studies of adsorption kinetics to be communicated on a later occasion.

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