Application Note

Activity of Adsorbed Antibodies

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It is well-known that bio-macromolecules may undergo conformational changes (denaturation) during passive adsorption on synthetic surfaces and thereby lose their biological activity [1].

In Thermo Scientific Nunc Bulletin No. 10 [2] it was argued that the functional activity (i.e. the active percentage) of adsorbed capture antibodies might be estimated through mathematical model fitting to appropriate data.

In the present work the activities of polyclonal capture IgG antibodies, adsorbed on the Thermo Scientific Nunc Immuno MaxiSorp and PolySorp polystyrene surfaces, were estimated by simulation of target IgG binding data using the model modified for non-monolayer binding kinetics.

Methods and Results

Nunc™ Immuno™ Plates, MaxiSorp™ F96 (Cat. No. 439454), and PolySorp™ F96 (Cat. No. 475094), were coated overnight with 200 µL/well of affinity isolated swine anti-rabbit capture IgG antibody = CAb (Dako Z 400) in PBS, pH 7.2, at 5 or 0.5 µg/mL. This established four different CAb surfaces with total antibody densities, Q' ng/cm^2, as determined by the CAb concentrations, [CAb], the surface/volume ratio (see below), and the estimates from Thermo Scientific Nunc Bulletin No. 6 [3] of 650 and 220 ng/cm^2 for the MaxiSorp and PolySorp adsorption capacities, respectively. Thus, at [CAb] = 5 µg/mL, the surfaces are saturated, i.e. Q' = 650 ng/cm^2 for MaxiSorp (MS-650), and Q' = 220 ng/cm^2 for PolySorp (PS-220), whereas at [CAb] = 0.5 µg/mL, the surfaces are not saturated, i.e. Q' = 65 ng/cm^2 for both MaxiSorp (MS-65) and PolySorp (PS-65). The coatings were followed by incubation for various times with a dilution

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$$dB_t/d_t = [(qA-B_t)/qA]\cdot[(C-B_t)/V]\cdot[S(kD/\pi)^{½}\cdot t^{-½}] \qquad \#1$$

which by integration gives:

$$E_t = \frac{B_t}{qA} = \frac{1-\exp\{(F/q-1)\cdot P\cdot 2(kD/\pi)^{½}.t^{½}\}}{q/F-\exp\{(F/q-1)\cdot P\cdot 2(kD/\pi)^{½}.t^{½}\}} \longrightarrow \begin{cases} F/q \text{ for } F < q \\ 1 \text{ for } F \geq q \end{cases} \#2$$

series of rabbit target IgG antibody = TAb (Dako A 008), then incubation for 1 hr with excess peroxidase conjungated swine anti-rabbit IgG antibody (Dako P 217), both in PBS with 0.05% Tween 20, and finally, substrate reaction with H_2O_2/OPD. Between the reaction steps, the wells were washed three times with PBS containing an extra 0.2 M NaCl and 0.05% Triton X-100. Using 200 µL/well implies a covered surface area of 1.54 cm^2, and a surface/volume ratio of 7.7 cm^{-1} . The results are shown in Fig. 1A.

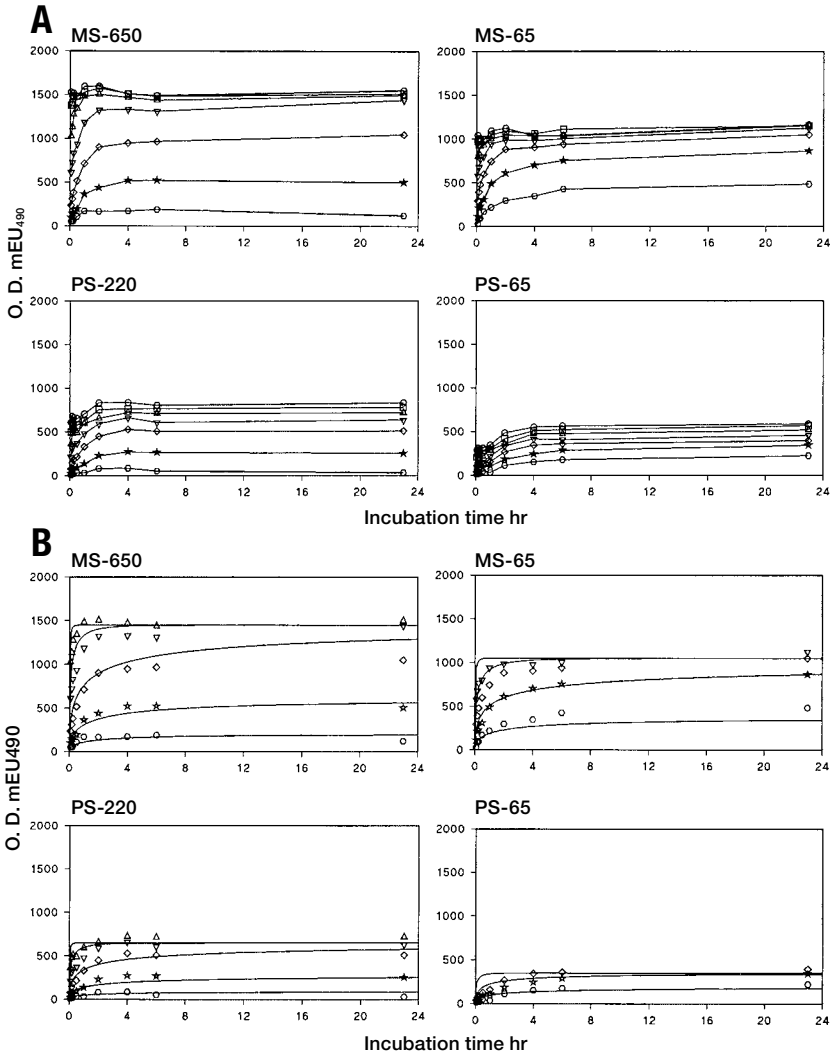

Fig. 1

A: Binding kinetics data from incubations for various times of a 1:3 target IgG dilution series, 10.0 µg/mL (○), 3.33 µg/mL (□), 1.11µg/mL (s), 0.370 µg/mL (▽), 0.123 µg/mL (◇), 0.0411 µg/mL (★), and 0.0137 µg/mL (⬭), in flat-bottomed MaxiSorp and PolySorp MicroWell plates saturated with capture IgG (resp. MS-650 and PS-220), and non-saturated (resp. MS-65 and PS-65). The bound target IgG was detected by subsequent incubation with excess anti-target peroxidase conjugate, and substrate reaction with H_2O_2/OPD. The data are the mean results from three independent experiments, each one with mutually comparable signals. All signals were subtracted by respective backgrounds from blinds without target.

B: Simulations of the data in A using eq. #2 with the parameters given in Table 1A.

See text for further explanation.

For simulation of non-monolayer TAb binding kinetics, the rate equation [2] has been modified by introduction of the parameter q into the first square bracket factor:

where:

Bt = number of bound TAb molecules at time t
t = elapsed binding time = hr
A = imaginary monolayer number of TAb molecules ~ 650 ng/cm²
q = fraction of monolayer number of molecules that can be bound
C = initial number of TAb molecules in solution
V = liquid volume = 0.2 cm^3
S = covered surface area = 1.54 cm^2
D = diffusion constant of IgG = $1.44 \cdot 10^{-3}$ $cm^2 \cdot hr^{-1}$
k = dimensionless coefficient = 2π [2]
Et = fraction of qA occupied at time t
F = C/A
P = S/V = surface/volume ratio = 7.7 cm^{-1}

The assumption is that if only a proportion, qA, of the potential monolayer CAb number is active, the TAb will accumulate to maximally the same proportion in the 2nd layer, thus reflecting the active CAb. The introduction of q into equation #1 is equivalent to assuming that a smaller monolayer TAb number than A is accumulating on a proportionally smaller surface. The interpretation of this is that on one hand the probability of an impinging TAb molecule hitting an available binding site is reduced, but on the other hand every hitting molecule will contribute proportionally more to saturation, thus the saturation rate will be proportionally increased.

Hence, the proportions of active CAb in the first layer to the total potential monolayer number were estimated by the respective q values giving the optimal TAb binding curve fittings by equation #2.

The parameters obtained by these simulations are included in Table 1A, where the F values, common to the four CAb surfaces, were determined using the previous estimate of 650 ng/cm^2 [2,3] for the densest monolayer packing of IgG. The simulations are shown in Fig. 1B.

Discussion

The estimated q values were converted into percentages of active CAb in Table 1B (ACD%) using the MaxiSorp and PolySorp adsorption capacities of 650 and 220 ng/cm^2, respectively. The active percentages are surprisingly low. For saturated MaxiSorp (MS-650) the value is only 2%, which is somewhat lower than the analogous estimate of 5-10% by others 1, whereas for saturated PolySorp (PS-220) the value is 6%. However, for non-saturated MaxiSorp (MS-65) the active percentage is increased to 8%, whereas for non-saturated PolySorp (PS-65) it remains almost unchanged (5%). This may be explained by the different IgG bedding patterns, assumed previously [3], on the two surfaces. On MS-650 the molecules are standing in tight palisades, whereas on PS-220 they have settled in a more loose pattern, implying that sterical hindrance is prevalent on the former, but eased on the latter. However, on MS-65 and PS-65 there is ample unoccupied surface, wherefore sterical hindrance is eased in both cases.

Consequently, the active percentage is much higher (four times) on MaxiSorp at the lowered CAb surface density, but almost unchanged on PolySorp. From the fractional densities, q, of active molecules, equivalent to the mass densities, Q ng/cm^2, calculated in Table 1B, it appears that MS-650 and PS-220 have the same densities of active molecules. This must, however, be seen as a coincidental peculiarity, consistent with the fact that the optimal simulations of the TAb binding kinetics on these surfaces demanded the same model parameters (except the same maximum signals, see below). Thus, the PS-220 simulation curves can be produced by multiplying the MS-650 curves by the quotient between the respective maximum signals: 650/1450. On the other hand, it appears that at the same non-saturating total densities on the two surfaces, the active density is almost twice as high on
MS-65 as on PS-65. This seems to confirm the previously proposed theory 3, that on MaxiSorp more CAb molecules will be favorably oriented than on PolySorp. The surface scenarios corresponding to these considerations are illustrated in Fig. 3 using the molecular density-distance relationship exposed in Fig. 2.

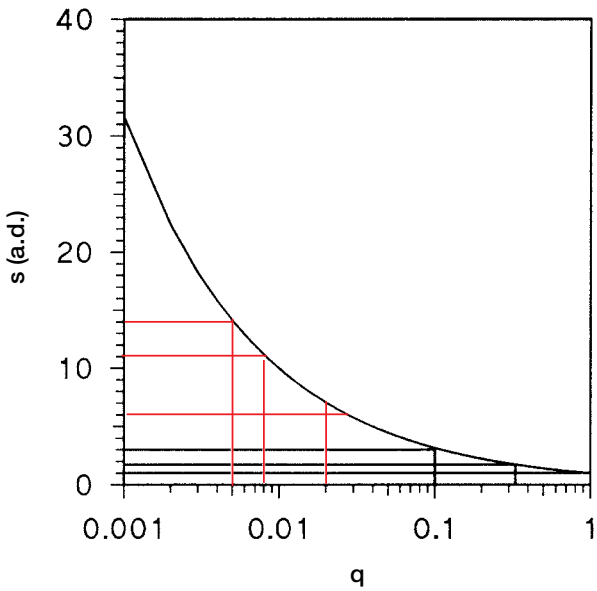

Fig. 2

Relationship between surface molecule density, q, in fraction of monolayer density, and minimum distance, s, in average IgG diameters (a.d.), based on the approximation that the molecules are globular and distributed in a hexagonal pattern, implying $s = (1/q)^{1/2}$ a.d. If the IgG Stokes radius of 5 nm [3] is used for the average IgG radius, then 1 a.d. = 10 nm. The red line interpolations give the s values corresponding to the active density q values in Table 1A; the black line interpolations give the s' values corresponding to the total density q' values of 650/650 = 1 for MS-650, 220/650 = 0.33 for PS-220, and 65/650 = 0.1 for MS-65 and PS-65, using the figure 650 ng/cm^2 for the densest monolayer packing of IgG. The interpolations have been used in Fig. 3.

More surprising and important than the observed low CAb activities are the differences in relative signal magnitudes calculated per bound TAb molecule (RTS in Table 1B). Thus, it appears that for both MaxiSorp and PolySorp the RTS is doubled by changing from the saturated to the non-saturated surface, and furthermore, the RTS is generally doubled by changing from PolySorp to MaxiSorp. Although the factor 2 seems to occur everywhere in this complex, attempts to explain this by the possibility that the binding capacity of an active CAb molecule may vary between one and two target molecules have been unsuccessful. It should be noted that this possibility has been ignored in all other considerations, assuming that active CAb molecules bind the same amount of target, regardless of the surface in question. At first, it seems most probable that these differences are due to the detection system, assuming that the conjugate access to the bound TAb molecules and/or the conjugate enzyme expression may vary from one surface to the other. The higher signals with non-saturated surfaces might be due to a better conjugate access to the TAb molecules, which are probably surrounded by more free space at sub-monolayer CAb densities, whereas the higher signals with MaxiSorp could result from a better conjugate bedding in favor of enzyme expression. Whatever the true explanations are, there seems to be a double advantage in using non-saturated instead of saturated MaxiSorp, since not only a far better utilisation of the CAb is achieved, but also a better sensitivity in the sandwich assay. Unfortunately, adsorbed molecules would generally not be stable for a prolonged time on non-saturated surfaces, especially if the surface is dried (for storage). However, in the light of the situation with PolySorp, this disadvantage can probably be remedied by co-adsorption of an appropriate amount of indifferent, supporting molecules, e.g. albumin 4.

From the empirical data in Fig. 1A it appears that with MaxiSorp all target concentrations above a certain (saturating) value yield the same maximum binding level, indicating that the amount of active CAb on this surface is independent of the amount of target (as basically anticipated). On the contrary, with PolySorp every concentration has its own level, suggesting that more and more CAb is activated with increasing amounts of target. This may be explained by the different CAb adsorption mechanisms, assumed previously [3], for the two surfaces. Thus, the MaxiSorp adsorption, including hydrogen bonds, presents a more stable orientation of the capture IgG molecules, whereas the purely hydrophobic PolySorp adsorption presents a more labile orientation, susceptible to some activating alteration by a “pull” from (abundant) target molecules. The fact that the PolySorp CAb surfaces at the higher target concentrations present binding rates with marked initial delays compared to the model kinetics (Fig. 1AB) supports this theory, since such molecular rearrangements prior to binding would probably take additional time. It also makes sense, therefore, that the activation and delays seem more pronounced with the non-saturated PolySorp, since the capture molecules would probably be more free to rearrange under these conditions.

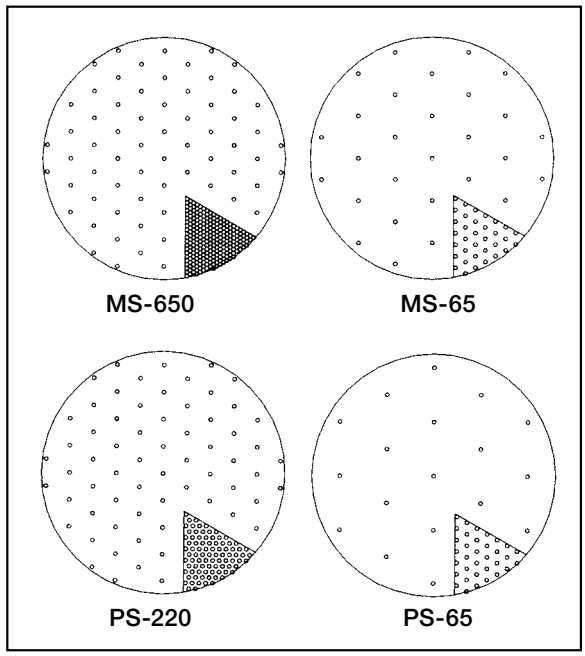

Fig. 3

Schematic illustration of the densities of active capture IgG (small circles) compared with the total densities (in sections) on approx. $25 \cdot 10^{-10}$ cm^2 (large circles) of the four capture IgG surfaces. The molecule diameters and distances are drawn to the same scale according to the interpolations in Fig. 2. Note that at the different saturating total densities, i.e. MS-650 ($s' = 1$ a.d.) vs. PS-220 ($s' = 3^{1/2}$ a.d.), the active densities are equal ($s = 7$ a.d.), whereas at the equal non-saturating total densities ($s' = 3$ a.d.) the active densities are different, i.e. MS-65 ($s = 11$ a.d.) vs. PS-65 ($s = 14$ a.d.). See Fig. 2 and text for further explanation.

This maximum level lability obviously endows the q estimations for PolySorp with considerable uncertainties implying that the derived PolySorp characteristics and their comparison with those of MaxiSorp must be taken with due reservations.

A		MS-650		MS-65		PS-220		PS-65	
MXS mEU		1450		1050		650		350	
q		0.02		0.008		0.02		0.005	
[TAb] µg/mL	F	F/q	q/F	F/q	q/F	F/q	q/F	F/q	q/F
10.0	2.00	–	–	–	–	–	–	–	–
3.33	0.666	–	–	–	–	–	–	–	–
1.11	0.222	11.1	0.0901	–	–	11.1	0.0901	–	–
0.370	0.0740	3.70	0.270	9.25	0.108	3.70	0.270	–	–
0.123	0.0247	1.23	0.810	3.09	0.324	1.23	0.810	4.94	0.202
0.0411	0.00822	0.411	2.43	1.03	0.973	0.411	2.43	1.64	0.608
0.0137	0.00274	0.137	7.30	0.343	2.92	0.137	7.30	0.548	1.82
B									
ACD	%	2		8		6		5	
	Q ng/cm²	13		5		13		3	
RTS		2		4		1		2	
s (a.d.)		7		11		7		14	

Table 1

A: Parameters estimated for the optimal simulations in Fig. 1B of the kinetics data in Fig. 1A of TAb (target IgG) binding on the four CAb (capture IgG) coated surfaces: MS-650 = saturated MaxiSorp, MS-65 = non-saturated MaxiSorp, PS-220 = saturated PolySorp, PS-65 = non-saturated PolySorp; MXS = maximum signal; q = active fraction of the densest monolayer packing (650 ng/cm²); [TAb] = TAb concentration; F = [TAb]/(0.650·P).

B: Derivatives from the data simulations in Fig. 1B; ACD% = active CAb density in % of total CAb density = active percentage = 100·q·650/(650, 220, or 65)%; Q = active CAb density in mass = q·650 ng/cm²; RTS = relative TAb signal = MXS/(Q·MXSPS-220/QPS-220); s = average minimum distance between active CAb molecules in average IgG diameters (a.d.), estimated by interpolation in Fig. 2. See text for further explanation.

Summary

Analytical simulations of the binding kinetics of rabbit target IgG (TAb) from a sandwhich assay utilizing affinity isolated anti-rabbit capture IgG (CAb) have revealed some useful indications concerning the functional activity of CAb adsorbed to the Immuno MaxiSorp or PolySorp surfaces:

1. The active CAb densities are equal on monolayer-saturated MaxiSorp and PolySorp surfaces, which translate into only 2% active CAb on MaxiSorp, but 6% on PolySorp, due to the different adsorption capacities of 650 and 220 ng/cm², estimated for MaxiSorp and PolySorp respectively [3]. This corresponds to an active CAb mass density of about 13 ng/cm² (or 0.085 pmole/cm²), meaning that with 200 µL volumes in a Thermo Scientific Nunc MicroWell, implying 1.54 cm² covered surface, the attainable maximum signal will be reached with a TAb concentration of 13 × 1.54 × 5 = 100 ng/mL (or with 0.65 pmole of an analyte) on both surfaces, assuming that only one binding site per active CAb is functional.

2. By use of non-saturated surfaces (65 ng/cm²), i.e. 0.12 × saturated MaxiSorp and 0.32 × saturated PolySorp, the active percentage is increased 4 times to 8% (corresponding to 5 ng/cm²) on MaxiSorp, but remains alomst unchanged at 5% (corresponding to 3 ng/cm²) on PolySorp. This suggests that on saturated MaxiSorp, contrary to PolySorp, there exists a heavy sterical hindrance, which is relaxed by dilution, but probably also a high stability of the tightly packed molecules. Thus, it seems that with MaxiSorp a much better utilisation of CAb can be achieved, if the surface is not saturated. However, this might create (storage) stability problems, which calls for an optimization study of the use of indifferent, stabilizing molecules (quality and quantity) for co-adsorption with the CAb.

3. The sandwich assay sensitivities are about twice as high on non-saturated as on saturated MaxiSorp or PolySorp surfaces, perhaps due to a better conjugate access to the target molecules on non-saturated surfaces. However, the phenomenon has later been approached with another explanation based on steric hindrance 5. In addition, the sensitivities are about twice as high on MaxiSorp as on PolySorp, possibly due to a better enzyme expression on MaxiSorp. Thus, compared with the other surfaces, the non-saturated MaxiSorp possesses the double advantage of a higher target detection sensitivity in addition to a better capture IgG utilization, at least for the present assay configuration. This points out the importance of including an optimization of the 1st layer coating in assay construction, and of investigating the conditions about non-saturated surfaces in general.

4. Because the active CAb percentage is higher on MaxiSorp than on PolySorp at equal levels of non-saturation, it also follows that more CAb molecules must be favourably oriented on MaxiSorp, which confirms the adsorption theory previously proposed [3]. The data also suggest that the CAb orientation is more stable on MaxiSorp, since on PolySorp it appears to be dependent on the concentration of added TAb.

In conclusion, the investigation described here indicates that a small active percentage of CAb is an inherent consequence of CAb immobilization by passive adsorption, and the percentage will be uniformly low for polystyrene surfaces of comparable quality among the various commercial manufacturers [1]. However, it should be noted that even though affinity isolated CAb has been utilized, the present active percentages hardly reflect the inactivating effect of immobilization only, since some activity has no doubt been lost during affinity purification [5]. The relevance of selecting the dedicated MaxiSorp surface for CAb immobilization has been confirmed.

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Mol. Immunol.

Application Note

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