No. 6

BULLETIN

Principles in Adsorption to Polystyrene

When considering the binding capacity of adsorbant plastic surfaces for biomacro-molecules, one must distinguish between the total amount of molecules that can be bound to the surface and the amount that can be bound and still remain biologically active. Both quantities are very much dependent on the nature of the molecules and the character of the surface.

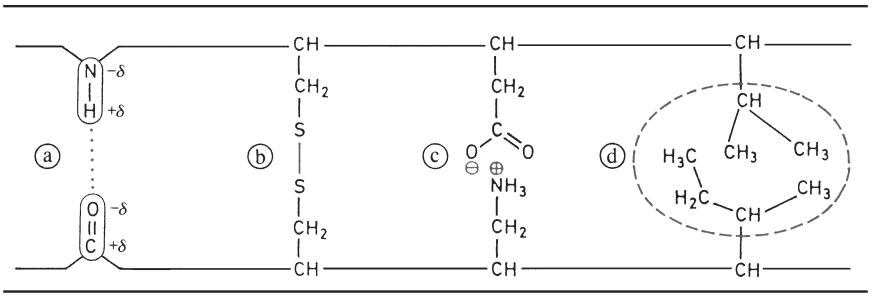

Fig. 1. The four main types of possible bonds between macromolecules. »True« chemical bonds are represented by a covalent disulphide bond (b) and an ionic bond between a carboxyl ion and an amino ion (c). Van der Waals mediated bonds are represented by a hydrogen bond between two dipoles (a) and an alternating polarity bond between hydrocarbon residues protruding from the macromolecules' backbones (d), where the encircled area indicates a water-deprived zone. See text for further explanation.

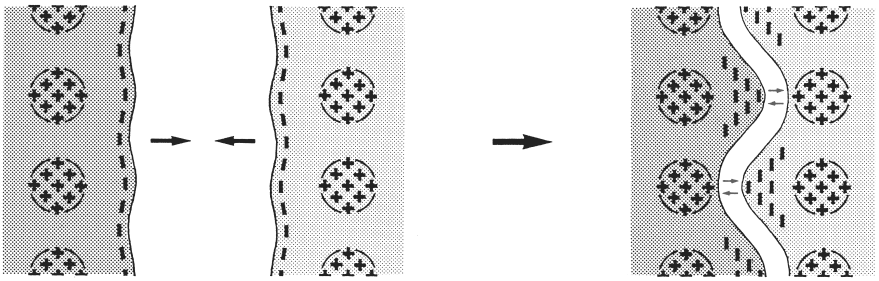

Fig. 2. Schematic illustration of how synchronously alternating polarities (AP), created by reciprocal electron cloud disturbances in approaching molecules, can establish a bond between the molecules. Transient, minus-charged electron cloud condensations in one molecule will attract reciprocally exposed, plus charged nuclear regions in the other molecule.

Adsorption Forces

The adsorption of molecules to a polystyrene surface is due to inter-molecular attraction forces (van der Waals forces), to be distinguished from »true« chemical bonds, i.e. covalent bonds (through electron share) and ionic bonds (through stoichiometric charges of opposite signs), see Fig. 1. *Inter*molecular attraction forces are based on *intra*molecular electric polarities of which two types can be distinguished: alternating polarities (AP) and stationary polarities (SP), i.e. dipoles. AP arises when molecules approach each other, thereby creating disturbances in each other's electron clouds. This causes synchronously alternating polarities in the molecules, which may establish a bond between them, as illustrated in Fig. 2.

AP mediated binding is a common substance property, which is obviously the stronger, the larger the molecules implied. This is demonstrated by the fact that melting and boiling points increase with number of carbon atoms in the non-polar hydrocarbon series. Indeed, it is due to this force that non-polar molecules at all aggregate into liquids and solids.

In addition to the AP attraction forces, molecules may possess SP (stationary polarity) through which they can bind to each other simply by bedding dipole against dipole, as illustrated in Fig. 1a.

Compared with SP, AP attraction decreases drastically with increasing distance between the molecules. Thus, AP attraction is inversely proportional to the seventh power of the distance, whereas SP attraction is inversely proportional to only the second power of the distance. Hence, the former has a much shorter range than the latter.
In general, van der Waals mediated bonds are about 100 times weaker than ionic and covalent bonds. However, among SP mediated bonds the *hydrogen bond* takes up an exceptional position because it is up to 10 times stronger than the others and because of its crucial importance for the properties of water and for the specific behaviours of bio-molecules.
Chemical groups, which can take part in hydrogen bonding, in particular –OH, =O, $-NH_2$, =NH, ≡N, are called *hydrophilic,* as opposed to hydrophobic groups lacking this ability. Accordingly, hydrogen bonds may be called hydrophilic bonds, as opposed to AP mediated bonds, which are called hydrophobic bonds. The AP mediated attraction is also called *hydrophobic interaction.*

Adsorbing Surfaces

The Nunc-Immuno™ programme includes different types of adsorbant polystyrene surfaces, including the PolySorp™ and the MaxiSorp™ surface. While PolySorp predominantly presents hydrophobic groups, MaxiSorp has in addition many hydrodrophilic groups, which results in a fine patchwork of hydrophobic and *hydrophilic* binding sites. In aqueous medium, a repelling effect exists between the PolySorp surface and hydrophilic macromolecules (i.e. rich in hydrophilic groups), because these molecules will rather tend to intermingle with the water molecules (i.e. be dissolved) by the strong hydrogen bonds than bind to the surface by the weak hydrophobic bonds.
On a MaxiSorp surface, however, adsorption of hydrophilic macromolecules will be greatly facilitated, because not only can this surface compete with the water molecules for binding the macromolecules by hydrogen bonds, but the molecules can also be captured from a much longer distance by the long-range hydrogen bond forces for establishment of both hydrogen bonds and eventually hydrophobic bonds (see Fig. 3).
On the other hand, hydrophobic macromolecules (i.e. deficient in or lacking hydrophilic groups) can only be loosely adsorbed to MaxiSorp, because this surface tends to bind water molecules by hydrogen bonds, against which the macromolecules cannot compete and therefore exhibit poor ability for displacing water molecules and hydrophobic adsorption free from water pocket interruptions.
On a PolySorp surface, however, no hindrance exists for stable hydrophobic adsorption of hydrophobic molecules, except that they may not be applicable in purely aqueous medium, wherefore addition of or substitution with detergent or organic solvents (e.g. ethanol or hexane) may be needed.
In summary, when no attention is paid to maintenance of specific activities, hydrophobic compounds bind preferably to PolySorp, and hydrophilic compounds preferably to MaxiSorp (see Table 1). However, with MaxiSorp binding events are more likely to occur, which means that adequate incubation conditions are more easy to establish – a fact that may extend the MaxiSorp application range well into the theoretical PolySorp domain. Nevertheless, considering the maintenance of the specific activities of the molecules (e.g. enzymatic, immunologic), which of course is crucial, the specific sites may well be obscured, impaired or destroyed through the binding to the surface. Therefore, when constructing a solid phase assay, it is generally recommended to try adsorption to MaxiSorp first. If this does not work satisfactorily, it may be due to molecular malfunction, and one should then try PolySorp whereby specific activities may be maintained because of the different binding mechanism to this surface. However with PolySorp, where molecules must come very close to the surface to establish hydrophobic bonds, one must anticipate more demanding

Fig. 3

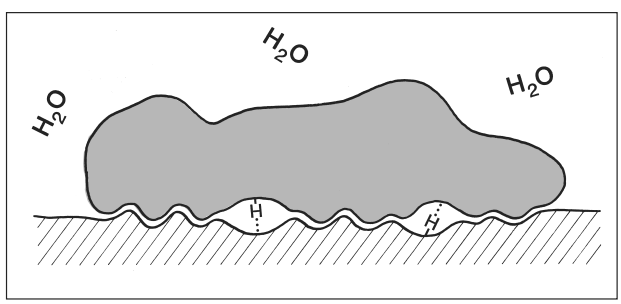

Fig. 3
Schematic illustration of how a hydrophilic macromolecule can be firmly adsorbed to MaxiSorp by »squeezing« out the water between the molecule and the surface through the combined action of hydrogen bond and AP bound forces. See text for further explanation.

Table 1
Theoretical PolySorp and MaxiSorp preferences for adsorption of various bio-macromolecules.

PolySorp	MaxiSorp
←	→
	Proteins & Peptides*
Lipoproteins	Glycoproteins
Compound lipids	Compound polyglycans
Lipids	Polyglycans

** Surface preference is dependent on predominance of hydrophobic or hydrophilic amino acid residues in the molecules.*

Fig. 4

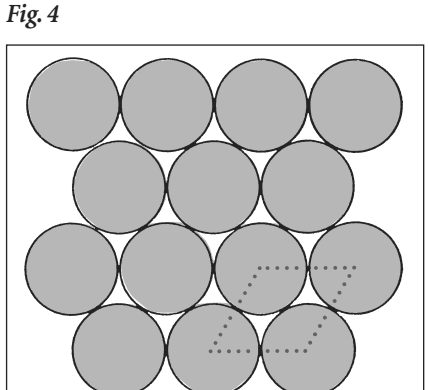

Fig. 4
The densest monolayer packing of globular molecules seen from above. The factor $2/\sqrt{3}$ in the text formulas for surface binding capacities originates in this non-quadratic pattern.

incubation conditions, such as higher reactant concentration, longer duration, higher temperature, (more) agitation, to obtain adsorption efficiency comparable with MaxiSorp.
As mentioned above, van der Waals mediated bonds are relatively weak, wherefore they may be insufficient for stable binding when they are few in number, i.e. when the molecules are small. For binding of small molecules strong chemical bonds are needed. Ionic bonds would not do, because they normally dissociate in aqueous solution, leaving covalent bonds as the only possibility for direct, stable binding of small molecules. However, this difficulty may be overcome by using small molecules linked to (indifferent) carrier macromolecules. Small molecules would in this context be e.g. peptides of less than 10 amino acids (corresponding to about 1500 dalton).

Geometric Estimation

Before making any experimental estimates of binding capacities on solid phase surfaces, it is worth making an estimate from geometric considerations of how many molecules can maximally be packed in one layer on a surface. Taking immunoglobulin G antibody (IgG) as an example, and assuming that it is globular and packed in the densest monolayer packing (Fig. 4), the amount Q_{GLOBE} per cm² will be:

$$Q_{GLOBE} = \frac{2}{\sqrt{3}} \cdot \frac{MW}{N} \cdot \frac{1}{(2r)^2} \cdot 10^9 \text{ ng/cm}^2 = 300 \text{ ng/cm}^2$$

where:

MW = molecular weight of IgG = 153,000 g v mole^{-1}

N = Avogadro's number = $6 \cdot 10^{23}$ mole^{-1}

r = Stokes radius of IgG = $\dfrac{R \cdot T_{20}}{6 \cdot \pi \cdot \eta_{20} \cdot D_{20} \cdot N}$ cm

R = gas constant = $8.3 \cdot 10^7$ g · cm² · sec^{-2} · °K^{-1} · mole^{-1}

T_{20} = room temperature (20°C) = 293°K

η_{20} = viscosity of water at 20°C = $1 \cdot 10^{-2}$ g · cm^{-1} · sec^{-1}

D_{20} = diff. coeff. of IgG ref. to water at 20°C = $4 \cdot 10^{-7}$ cm² · sec^{-1}

However, according to various sources, the IgG molecule is rather a lense-shaped spheroid with a diameter, d, of about 15 nm and a thickness, t, of about 3 nm as illustrated in Fig. 5. Assuming the densest packing of these spheroids in »upright« or »lying« position **(Fig. 6),** the respective Q_{LENSE} values will accordingly be:

$$Q_{LENSE} = \frac{2}{\sqrt{3}} \cdot \frac{MW}{N} \cdot 10^9 \cdot \begin{cases} 1/td = 650 \text{ ng/cm}^2 \text{ at upright position} \\ 1/d^2 = 130 \text{ ng/cm}^2 \text{ at lying position} \end{cases}$$

Fig. 5

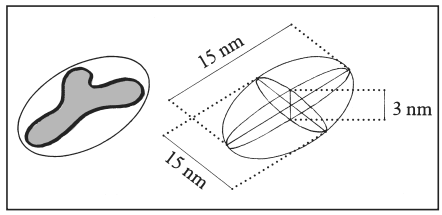

Fig. 5
The Y-shaped IgG antibody will approximately take up the volume of a lenseshaped spheroid with a diameter of 15 nm and a thickness of 3 nm.

Fig. 7

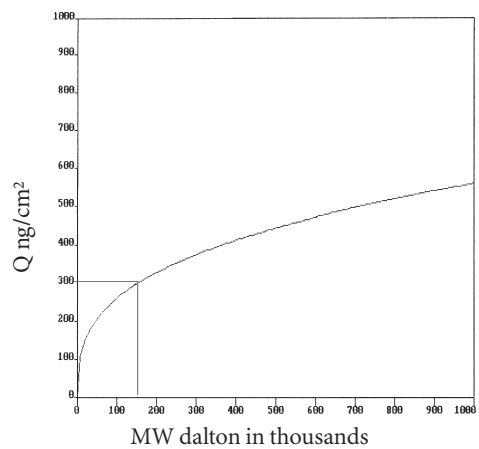

Fig. 7
Relationship between monolayer weight density (Q) and molecular weight (MW) of globular molecules illustrating that within a 10-factor MW range Q will roughly vary within only a 2-factor range. The curve is extrapolated on the basis of an idealized IgG molecule with an MW of 153,000 (red lines). See text for further explanation.

Fig. 6

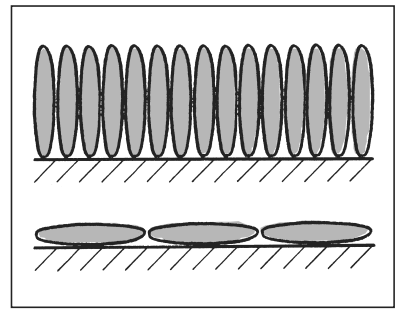

Fig. 6
Profiles of the densest IgG packings on a surface illustrating the density ratio of 5 to 1 between molecules packed in upright position (above) and in lying position (below).

Fig. 8

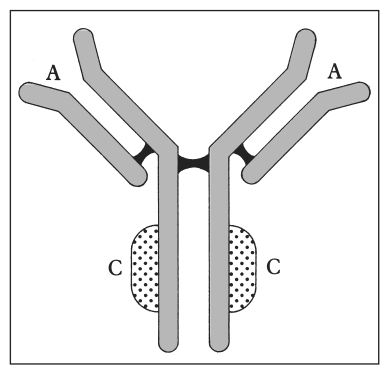

Fig. 8
Schematic illustration of the IgG antibody structure. Note the carbohydrate moiety (at C) associated with the leg opposite the antigen binding sites (at A) of the molecule.

So, for geometrical reasons alone, the maximum amount of monolayer IgG that can be bound on a surface is 650 ng/cm². If an average is taken between the two Q_{LENSE} figures, the final estimate would be 400 ng/cm².
Assuming that molecular weight is proportional with volume, Q will not change considerably within wide molecular weight limits, other things being equal, because of the low power relationship between volume and profile area of a body. **Fig. 7** illustrates the relationship between Q and molecular weight for globular molecules.

Experimental Estimation

Let us, as an example, stay with IgG, a glycoprotein with a structure shown schematically in Fig. 8.
On a MaxiSorp surface, one would expect an orientated adsorption in favour of exposing the antigen recognizing

Fig. 9

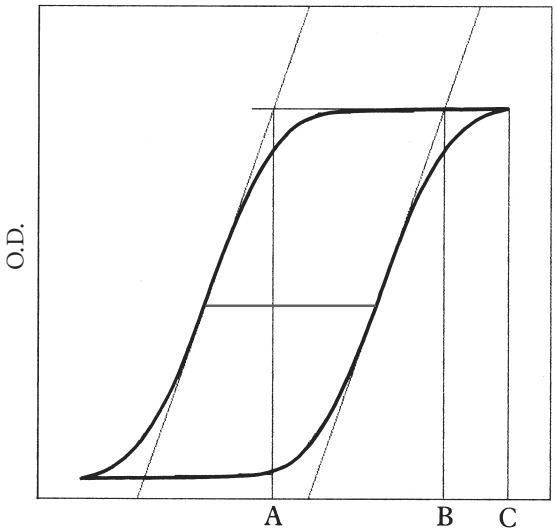

Fig. 9
Expected results from ELISA experiments with a dilution series of first layer specific IgG-antibodies, starting with a concentration C well above surface saturation concentration S (left sigmoid curve), or with a corresponding dilution series mixed with unspecific IgG to a constant total of C (right sigmoid curve). The ratio between S and C is A/B, which is represented by the distance between the two curves (red line). See text for further explanation.

sites, because this surface would favour a binding through the hydrophilic carbohydrate moiety associated with the non-recognizing leg of the molecule.
On a PolySorp surface, on the other hand, one would expect an adsorption in favour of obscuring the antigen-recognizing sites, because of the repelling effect between this surface and the carbohydrate moiety.
To investigate the actual adsorption conditions, the following experiment was designed (see Fig. 9).
PolySorp and MaxiSorp MicroWell™ surfaces were coated with a dilution series of specific antibodies, starting with a concentration C well above saturation concentration, or with a corresponding dilution series mixed with unspecific antibodies to a constant total of C. The relative amount of specific antibody adsorbed in each case was determined by a sandwich ELISA for the antigen in question using excess antigen and excess HRP-conjugated specific antibodies.

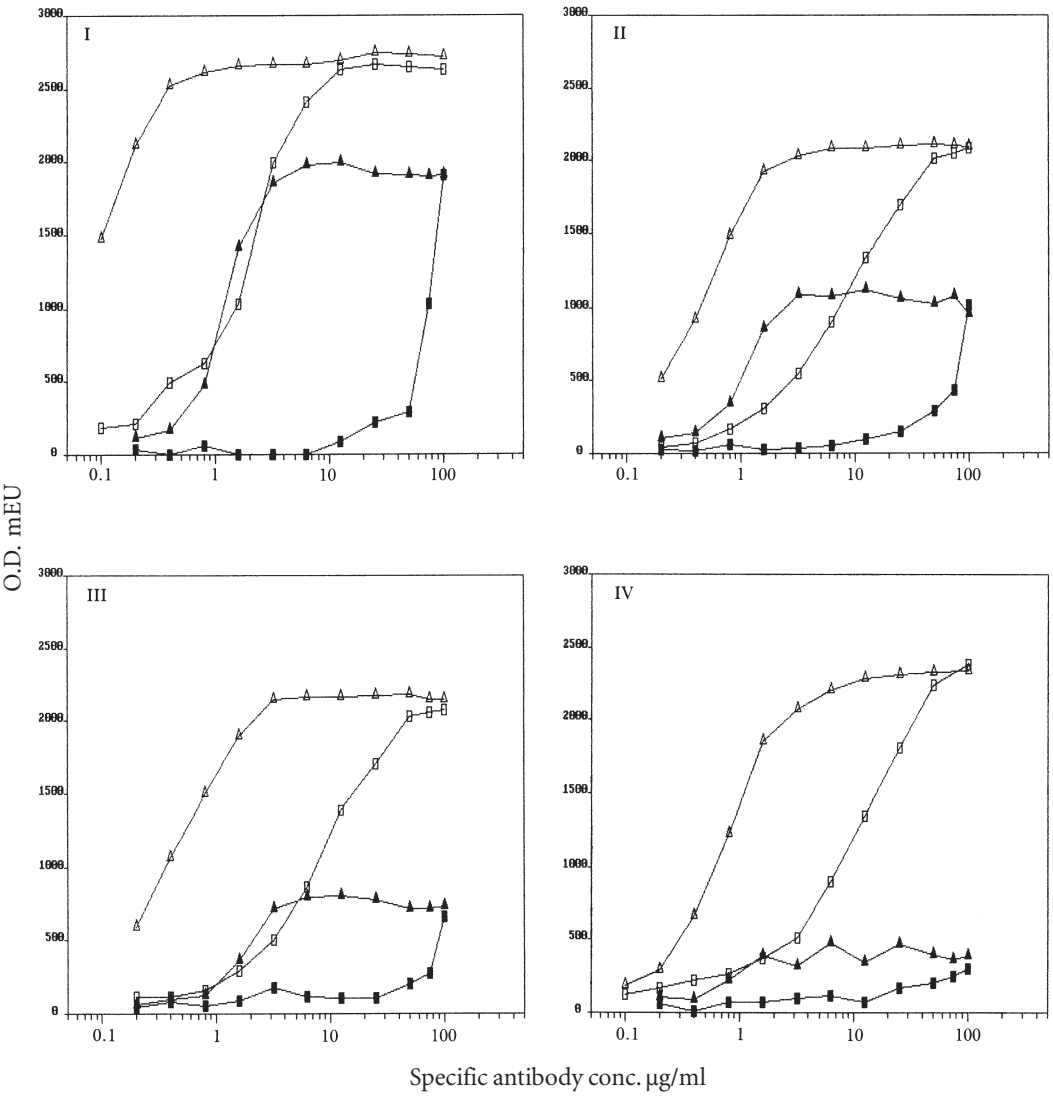

Fig. 10
Results from experiments prospected in Fig. 9 with four different antibody/antigen systems on MaxiSorp (open symbols) and on PolySorp (filled symbols).
I: ferritin antigen (MW 440,000);
II: fibronectin antigen (MW 450,000);
III: thyroglobulin antigen (MW 670,000);
IV: AFP (a – foetoprotein) antigen.
(MW 70,000). Note the seemingly constant curve distances for MaxiSorp and PolySorp respectively, regardless of the system in question.
See text and Fig. 9 for further explanation.

When it is assumed that equal signals mean equal amounts of specific antibody adsorbed in both dilution series, the quantity B/C is the fraction of the saturation concentration S formed by the maximum signal concentration A, i.e. $A = S \cdot B/C$, or:

$$S = \frac{A}{B} \cdot C$$

In **Fig. 10** are shown the results from experiments, designed as above, with four antibodies of different specificities. From the seemingly constant curve distances for MaxiSorp (MS) and PolySorp (PS) respectively, it is concluded that the adsorptions are independent of antibody specificity, and that they amount to the following quantities:

$$Q_{MS} = \frac{A_{MS}}{B_{MS}} \cdot C \cdot \frac{V}{F} \cdot 10^3 = 650 \text{ ng/cm}^2$$

$$Q_{PS} = \frac{A_{PS}}{B_{PS}} \cdot C \cdot \frac{V}{F} \cdot 10^3 = 220 \text{ ng/cm}^2$$

where:

$A_{MS}/B_{MS} = 1/20$

$A_{PS}/B_{PS} = 1/60$

C = max. IgG conc. = 100 µg/ml

V = reactant volume = 0.2 ml

F = surface area = 1.54 cm²

Fig. 11

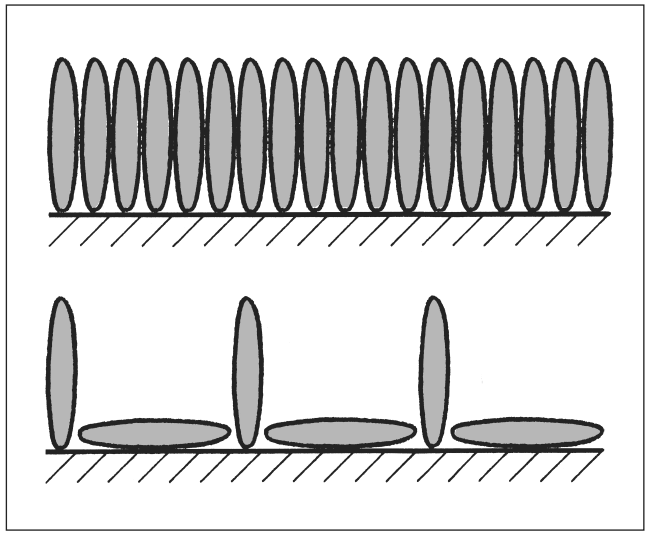

Fig. 11
Profiles of IgG adsorption patterns on MaxiSorp (above) and PolySorp (below) which can explain the experimentally found ratio of 3 to 1 between the densities on the respective surfaces.

Fig. 12

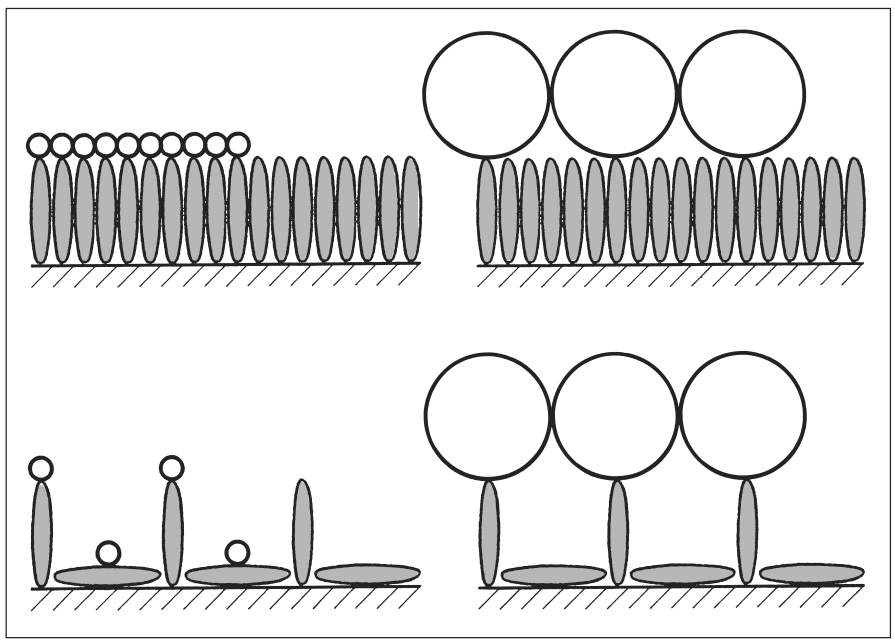

Fig. 12
Profiles of second layer (antigen) binding to antibody-coated MaxiSorp (above) and PolySorp (below) surfaces illustrating how the presumptive difference between the IgG adsorption patterns may imply a PolySorp decrease in bound amounts of small antigen molecules (left), but not of large antigen molecules (right). It should be noted that the third layer consisting of HRP-conjugated antibodies would hardly influence the detection of this phenomenon, as HRP is a relatively small molecule (MW 40,000). See text for further explanation.

Discussion

Whereas Q_{MS} is identical with the geometric maximum estimate for upright molecules, Q_{PS} is only one third hereof, which can be explained by assuming that on PolySorp upright and lying molecules are present in equal numbers, as illustrated in Fig. 11. Because each IgG antibody can maximally bind two antigen molecules, this PolySorp decrease in number of adsorbed antibodies would have the greater effect, the smaller the antigen molecules are compared with the antibodies, as illustrated in **Fig. 12.**
This could, partly at least, explain the very low PolySorp signals for AFP, which has a molecular weight of less than half the weight of IgG, whereas the other antigens have 3-5 times the weight of IgG.
In addition, the variation of PolySorp-to-MaxiSorp maximum signal ratios may be due to differently obstructed affinities through the antibody adsorption to PolySorp, and/or due to different antibody-antigen affinities from one system to the other. In the ferritin system, the affinity seems relatively high since there is a long delay before maximum signal decline on MaxiSorp, which may be consistent with the high PolySorp maximum signal, i.e. the higher the affinity, the less it is obstructed by antibody adsorption to PolySorp.

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Stability of Nunc-Immuno MaxiSorp Surfaces

Nunc-Immuno™ MaxiSorp™ products are the result of extensive research in order to produce surfaces with a high and uniform immunoglobulin binding capacity.
However, the elaborate adsorption qualities of MaxiSorp surfaces may change if the product is handled incorrectly, e.g. by exposure to UV light or high temperatures. This report describes the temperature influence on Nunc-Immuno MaxiSorp products stored for various periods of time.

Materials and Methods

Nunc-Immuno Plates MaxiSorp F96 from one batch were used in all experiments, except in the batch to batch variation analysis. After exposing the plates to different temperatures for various periods of time, the surface stability was tested using Nunc's binding uniformity test (described in Nunc Bulletin No. 4). In short, a mixture of enzyme-conjugated and unconjugated antibodies in carbonate buffer, pH 9.6, was added to each well (200 µl/well). After incubation overnight, each well was washed 3 times with PBS containing 0.05% Triton X-100.
Colour development by substrate reaction was read on an Immuno Reader NJ 2000. The mean, standard deviation, and CV were calculated using in-house software on an IBM-AT computer.
Storage at the following temperatures was used to simulate various storage/transport conditions: +60°C, +37°C, +4°C, room temperature (+20°C), –70°C, and outdoor temperature variations.

Results

Plates produced in a five year period were compared in order to make sure that storage at room temperature did not change the binding properties of the MaxiSorp surfaces. The results from this batch to batch comparison are shown in Figure 1. Seven different batches were tested, and only a slight variation in the binding capacity was observed.
This means that under Nunc's standard storage conditions at room temperature, MaxiSorp products can be kept in stock for at least 4 years without affecting the surface binding capacity.
All other results are expressed as a percentage of values obtained simultaneously from plates kept at room temperature.
Transport during winter in cold climates may lead to exposure to extremely low temperatures for short periods of time, e.g. during air transport. In Figure 2 are shown the results of plates stored at –70°C. Plates were stored for up to one month, and as indicated in the figure, no significant change occurred when compared with plates stored at room temperature.
In hot climates the products may be exposed to temperatures well above +40°C for short or long time periods. The effect of exposure to high temperatures was demonstrated by storing plates at +60°C (results are shown in Figure 2). The signal level decreased by 30% after three days of storage but remained thereafter constant for the rest of the observation period (42 days).
Plates stored at room temperature, +4°C, +37°C, and plates stored outdoor (packed in a plastic bag) were followed for more than one year. The results are shown in Figure 3.
There was no significant difference between the results obtained for plates stored at +4°C, at room temperature, or outdoor. However, after being stored at +37°C for 150 days, the plates showed a 10% decrease in signal as compared to those stored at room temperature. But after this period no further decrease could be observed for up to one year of storage.

Conclusion

From these experiments it can be concluded that Nunc-Immuno MaxiSorp surfaces can be stored for at least one year at temperatures below +37°C without changing their high adsorption properties. Storage at +37°C will reduce the signal level by 10% during the first 150 days, but after that no further decrease can be observed.
Storage at high temperatures, even for short periods, should be avoided as the signal level decreases rapidly at +60°C. However, like plates stored at +37°C, the decrease in signal level will not continue, but will stop at a temperature specific level. For plates stored at +60°C, the signal level decreases to 70% of the value for plates stored at room temperature.
It should be noted that these studies were performed exclusively with IgG. The adsorption of other molecules might respond differently to storage temperature.

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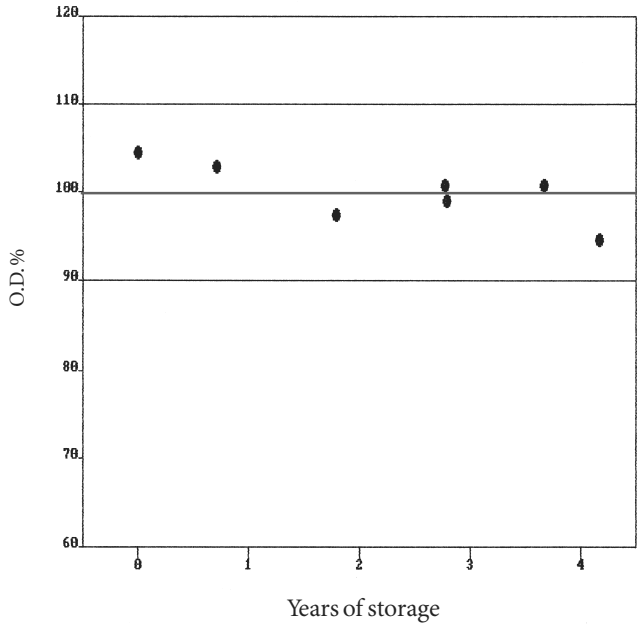


Fig. 1
Simultaneous adsorption performances of individual plate batches (●) produced fin a 5 year periode and stored under Nunc standard conditions at room temperature. The values are given in percent of their common mean (red line). Note that the values stay between +/–5% from the mean during storage for more than 4 years.

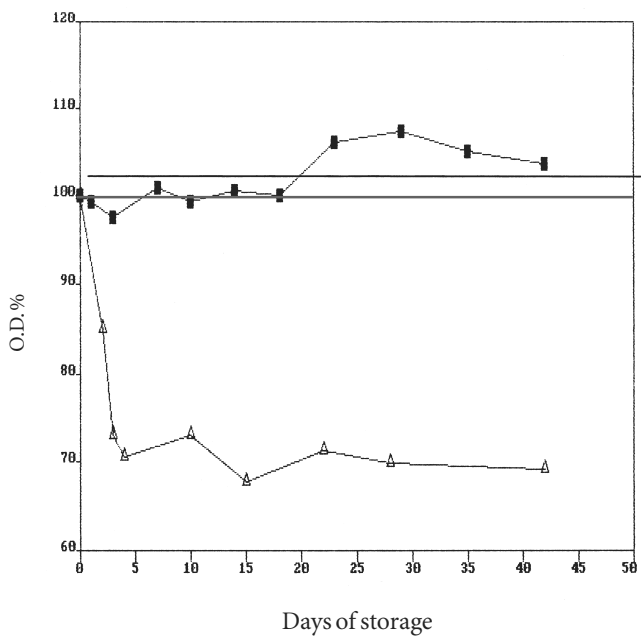


Fig. 2
Adsorption by plates stored at –70°C (m) and at +60°C (■) in percent of adsorption by plates kept at room temperature (red line). Whereas cold storage implies no change, storage at +60°C quickly reduces adsorption to a constant level at about 70% of the initial value.

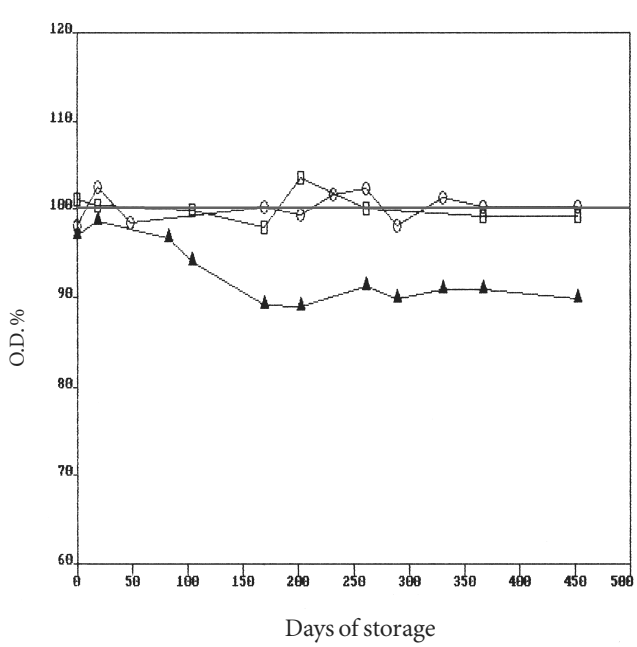
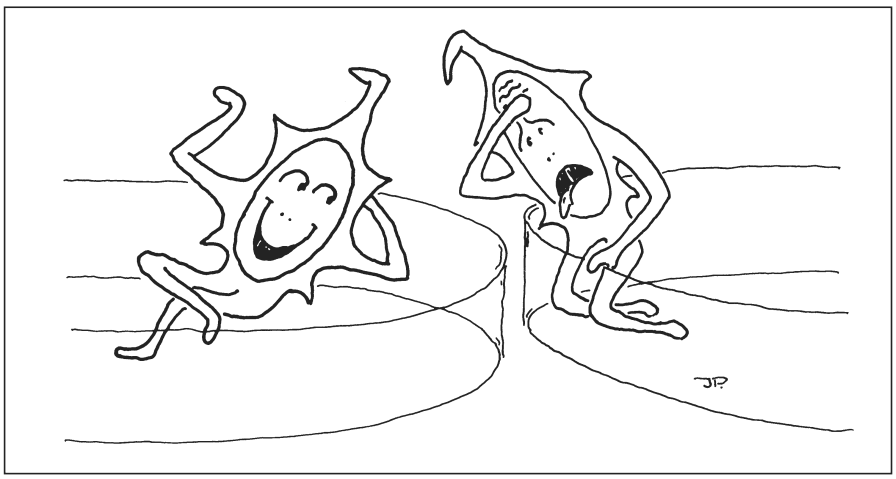


Fig. 3
Adsorption by plates stored at +4°C (□), outdoor (V), and at +37°C (▲) in percent of adsorption by plates kept at room temperature (red line). Whereas storage at +4°C or outdoors implies no change, storage at +37°C slowly reduces adsorption to a constant level at about 90% of the initial value.



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