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Langmuir–Blodgett films of immunoglobulines IgG. Ellipsometric study of the deposition process and of immunological activity

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Abstract

The structure of the immobilized Langmuir–Blodgett films of IgG and their immunological activity were studied by means of ellipsometry. The dependence of the film thickness on the surface pressure of deposition provides evidence of the tilting of molecules with an increase in the pressure. Below pressures of 30 mN m^{-1} the thickness of the film is approximately 4 nm which coincides with the smallest dimension of the IgG molecules. At pressures between 30 and 40 mN m^{-1} the thickness increases sharply achieving a value of about 10 nm which is equal to the largest molecular dimension. A further increase of pressure does not show in terms of growth of the thickness. This means that the films are transferred from the water–air interface in the form of a 2-D ordered monomolecular layer. The dependence of the immunological activity on the pressure of deposition was shown to have a descending pattern. Different mechanisms are proposed which explain the decrease of the immunological activity of the IgG molecules in the film with an increase of the surface density such as the blocking of the active sites and the decrease of the conformation mobility of the Fab fragments.

1. Introduction

The films of antibodies are of great interest because of their possible applications as material for biosensors [1–3]. This is why attention towards the techniques of producing such films and towards the reaction ability of them is growing. Attempts were made to characterize the thermodynamics of the IgG films which yield to the proposition of optimal conditions of deposition [4]. The molecular density of the films was measured [5] and its dependence on the surface pressure was used to suggest the model of the films' molecular packing [6]. The thickness of the films was estimated by ellipsometry [4]. The antigen–antibody reaction was also studied in the aforementioned papers. It was found that the reaction ability of the films depends on the type of the immobilization of the antibodies layer on the substrate [7], on the number of monolayers in the film [5] and on the size of the antigen [6]. Since it is clear that the reaction ability of IgG molecules in the film varies with its structure and density, it is natural to suppose that these data provide evidence of the dependence of the molecular packing of the film on the conditions of the deposition and the possibility of gaining control on it.

In this paper we attempted to study two problems. What is the molecular structure of Langmuir–Blodgett (LB) films of IgG and how does it depend upon the conditions of deposition.

How does the specific reaction ability of the films depend upon these conditions and is there any possibility of improving it.

To study these problems we applied ellipsometry, a method which allows measurement of the thickness of the films with good accuracy. The thickness of the monomolecular films, consistent woth non-isometric molecules, is a structure-sensitive parameter and the measurement of it provides the possibility of considering the molecular packing of the monolayers. The measurements of the increase in the film thickness due to specific binding of antigens, allows one to evaluate the reaction ability of antibodies in LB films.

2. Experimental techniques

2.1. *Materials*

For the deposition we used rabbit anti-mouse antibodies (RAM) obtained from Gamaley Institute of Microbiology and Epidemiology (Moscow). Mouse monoclonal anti Pd-coproporphyrine antibodies were used as the antigen (Bakh Institute of Biochemistry, Moscow).

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Bovine serum albumin (BSA) was purchased from Serva. 3-glycidoxypropyltrimethoxysilane (GOPTS) was purchased from Aldrich. Undoped silicon plates were used as supports.

2.2. *Preparation of the surfaces*

Silicon plates were used as supports, having been treated with boiling chloroform, rinsed on a glass filter and dried under nitrogen. Thus, conditioned supports were silanized with 3-glycidoxypropyltrimethoxysilane under vacuum in an apparatus consisting of a silane evaporator and a 200 ml flask. Evaporated silane entered the flask volume and made contact with the heated supports. At the first stage, the silicon surface adsorbed the silane derivative. The temperature during this process was maintained at 135–140 °C. At the second stage, the evaporator was closed and the deposited silane layer was polymerized. The temperature was held at 175–180 °C for 6 h (for full details of the procedure see ref. 8).

The density of the deposited polyorganosiloxane film was controlled by the microgravimetric method [2] and its average value for GOPTS was $(1/2) \times 10^{-6}$ g cm^{-2}. Since the GOPTS polymer contain the active epoxy-groups which react with protein amino groups, no additional surface activation was required for the immobilization of the protein LB film. A schematic diagram of the surface preparation is presented in Fig. 1.

2.3. *Deposition of the films*

Antibody monolayers were formed in a Langmuir trough (MDT, Russia) with a subphase volume 0.2 l and a surface area 240 cm^2. A decimolar carbonate buffer, pH 8.6, was used as the subphase. RAM was sprayed onto the subphase in the solution of Tris-buffer (TBS) with a concentration 1.2 mg ml^{-1} and pH 7.4. The transfer of LB antibody films from the subphase surface on the reactive supports was performed by touching the support in parallel to the subphase surface (in analogy with the Langmuir–Schaefer method). After the film had been deposited the samples were dried in a nitrogen flux, incubated during 1 h under the environmental conditions, washed with deionized water and dried again in the nitrogen flux.

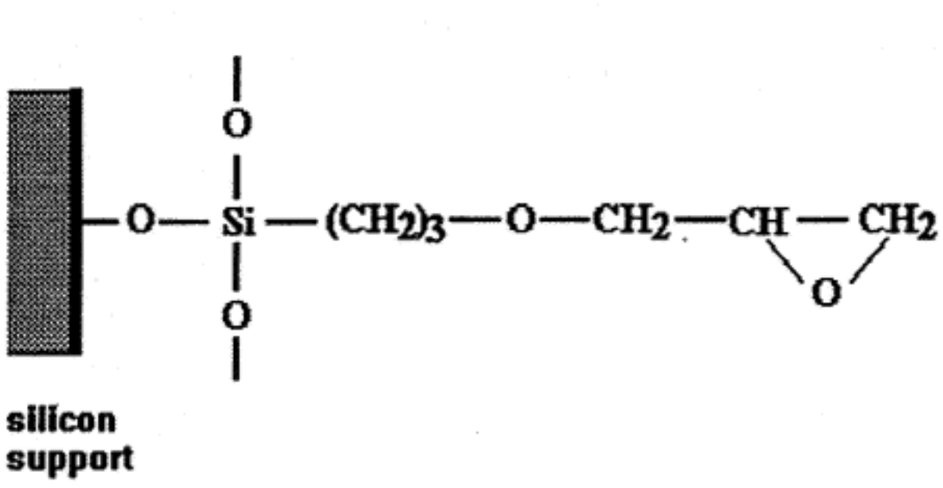

Fig. 1. Schematic diagram of the GOPTS activated surface for the immobilization of the IgG films.

2.4. *Binding procedure*

The prepared samples were incubated in the TBS (30 mM, pH 7.4) with albumin in the concentration of 0.5 mg ml^{-1} to minimize the antigen non-specific adsorption on the surface. The binding was carried out in the same buffer with the addition of the antigen in a concentration of 50 nmol. A holder with all the samples was placed in the fixed volume of antigen solution under stirring. Every 5 min the samples were withdrawn, washed with a water jet and dried with the nitrogen flux, and the thickness of the bound layer was measured. This procedure was repeated until a total exposition time of 45 min was achieved. The samples were then washed in the TBS (30 mM, pH 7.4) with 0.01% of Tween 20 for 10 min and measured again. The thickness of the bound layer of antigen had not changed after this washing, meaning that washing under the water jet, which was applied before every measurement, was strong enough to remove all the unspecific adsorbed molecules. Thus we can say that the measured thickness can be attributed only to the specific binding.

2.5. *Ellipsometry*

The measurements were carried out with a PCSA null ellipsometer using a He–Ne (633 nm) laser as a light source. The data was treated according to the two-layer model [9]. In this model the first, lower layer accounts for the imperfections which always exist on the surface of silicon substrate in the form of traces of polishing, intrusions of the polisher, a thin oxide layer, etc. In order to prove the applicability of the model multi-angle incidence measurements were carried out. The deposition-prepared substrate and one- and five-monolayer films were measured at the angle of incidence, from 50° up to 80° with steps of 5°. The measurements of the bare substrate were treated according to the one-layer model, and the resultant parameters of the surface layer were used in the treatment of the measurements of the films according to the two-layer model. To compare the applicability of the models, the data on the films were treated with the one-layer model as well. the obtained results are summarized in Table 1. The mean values of the measurements at different angles are given for the thickness and the index of refraction, while the discrepancy of the values obtained under different angles is given as an error. The errors of the one-layer treatment were too large, achieving a value of 0.5 for the refraction index. The values of the refraction index of one- and five-monolayer films differ greatly at 2.8 and 1.9 respectively. In general, the refraction index of the film can vary owing to some changes occurring

TABLE 1. Results of ellipsometric measurements of the clean silicon substrate, monolayer and five-monolayer films of IgG. Multi-angle incidence measurements, treatment according to one- and two-layer models. The discrepancies between the data obtained from different angles of incidence are indicated as the errors of determination

Substrate	One-layer model		Two-layer model	
	Thickness (nm)	Refraction index	Thickness (nm)	Refraction index
Silicon	5.3 ± 0.5	2.88 ± 0.01		
1 monolayer of IgG	10 ± 4	2.8 ± 0.2	5 ± 1	1.40 ± 0.05
5 monolayers of IgG	14 ± 1	1.9 ± 0.5	12 ± 1	1.50 ± 0.03

in the film as the number of monolayers grows, but neither the obtained value (2.8) nor the amplitude of change (0.9) can be attributed to organic matter. On the contrary, the two-layer treatment gives rather self-contained figures. The discrepancies are reasonable and the refraction index of the film remains more or less the same. The mean values almost coincided with that obtained only from the measurement at an angle of incidence of 70°, which is why this angle only was used in the main experiments. The thickness of the bound layer was determined as the difference between the total thickness and that of the RAM monolayer measured before the reaction.

3. Results and discussion

3.1. *Structure of the films*

The dependence of the thickness of the LB IgG films on the surface pressure of the deposition is shown in Fig. 2. One can easily see that this dependence has three

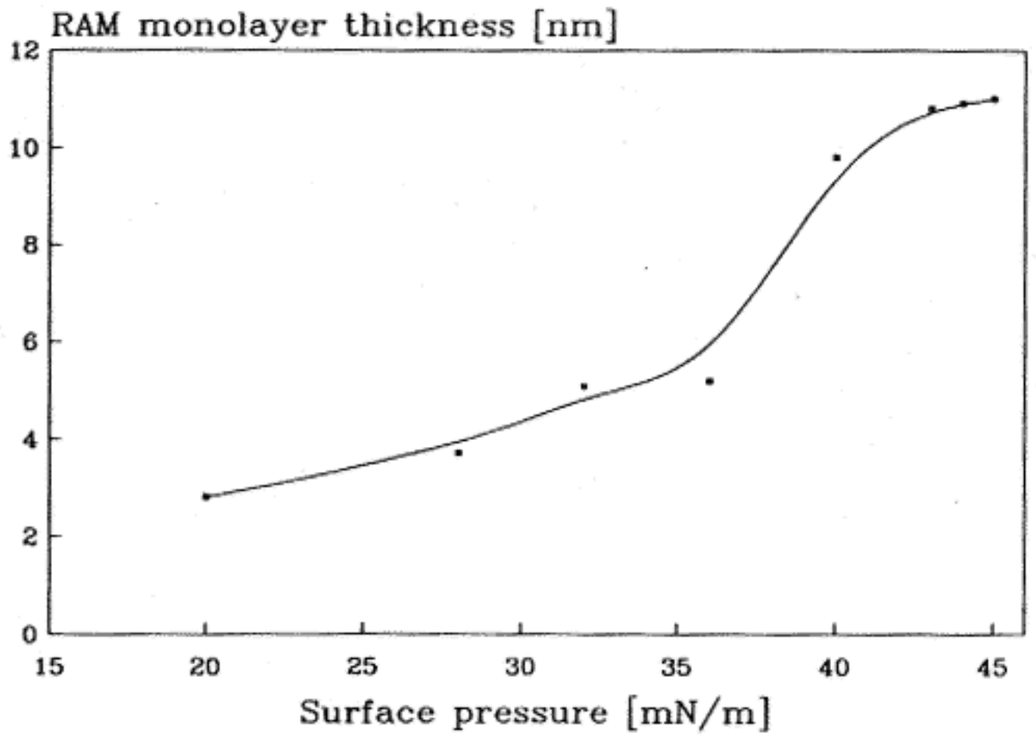

Fig. 2. Dependence of the IgG film thickness on the surface pressure of deposition ellipsometric measurements.

different ranges. The first of them corresponds to surface pressures less than 30 mN m^{-1}. The thickness in this range is about 4 nm and weakly depends on the pressure. The second range, from 30 mN m^{-1} to 40 mN m^{-1}, is one of a rather sharp increase in thickness from 4 nm up to 8 nm. In the third range (pressure greater than 40 mN m^{-1}) the thickness reaches the plateau with a thickness value of 10 nm. The deposition under pressures higher than 44–45 mN m^{-1} is almost impossible for the films of IgG even with a barrier speed greater than 1 mm s^{-1}.

The obtained results are in agreement with the model of the molecular packing of the IgG monolayer proposed on the basis of the dependence of the molecular density of the film versus surface pressure [6]. According to this model, there are three ranges of surface pressure which correspond to three different types of molecular packing of the film. The film deposited under pressures less than 20 mN m^{-1} is not dense. Molecules are "laying", which means that the $F_{ab}-F_{ab}-F_c$ plane is parallel to the surface. The increase of the pressure in this range makes the molecules come closer to each other. The pressure of 20 mN m^{-1} corresponds to the dense packing. With a further increase in pressure, the molecules begin to change their position one after the other. At a pressure of 40 mN m^{-1} the molecules achieve the vertical position. This represents the dense packing of the monolayer and a further increase of pressure does not affect the structure.

The overall pattern of the curve and the values of the thickness in the first and in the third ranges correlates with the model. In fact, when the IgG molecules are laying, the thickness of the film which they form should be about the value of the smaller dimension of the molecule, which is approximately 4 nm. The slight decrease of the thickness which occurs in the first range with the decrease of pressure may be due to an error of interpretation of the ellipsometric measurements. In fact, the value of the thickness of the monomolecular film should not be regarded literally. It is an effective parameter evaluated upon approximation of the film as a continuous homogeneous media which should be applied to the monomolecular film with definite caution. If there were no experimental errors we would obtain constant thickness and a decrease of the refractive index of the film with an increase in distance between the molecules. In the real experimental case it is very hard to resolve these two parameters of the film because the decrease of the thickness is observed with the decrease in density.

The thickness in the third range is equal to the largest dimension of the IgG molecules. This fact also agrees with the above-mentioned model. It is very important that the curve reaches the plateau of such a value in this range. If the curve does not bend or does, achieving a

value essentially higher than the largest dimension of the molecule, it would be obvious that the film is not monomolecular and all of the previous speculations about the molecular packing have no ground.

It should be noticed that monomolecular layer deposition is not the trivial one. The coefficient of the surface and volume segregation of the IgG molecules in water is rather low, especially under high pressure. The molecules may indeed form the aggregates which might cause the non-monomolecular deposition. As far as we know, he deposition of the IgG films under such pressures has not yet been studied, and therefore the plateau of either the density or the thickness has not been observed.

The thickness value of 9.3 nm reported for the film deposited under a pressure of 20 mN m^{-1} [7] does not agree with our data. It should be noted that the treatment of ellipsometric measurements in that work seems to be wrong because the authors used the approximation of a single layer. In this case, the obtained thickness of the film is increased by the thickness of the polished layer of the substrate which is always present on the surface of almost any solid material and may be comparatively large (up to 5–7 nm). The discrepancy in the thickness measured at different angles of incidence reported by the same authors [7] also indicates that the applied treatment was inadequate.

3.2. *Kinetics of the antigen–antibody reaction*

We measured the thickness of the bound layer of the antigen on the surface of the antibody film obtained under three different pressures: 20, 30 and 40 mN m^{-1}. The kinetic curves are shown in Fig. 3. The different curves correspond to the different pressures of the deposition. It is clear that both the reaction rate and the final thickness depend upon the pressure of deposition. It is very interesting that the reaction ability decreases with the increase of pressure. This is due to some

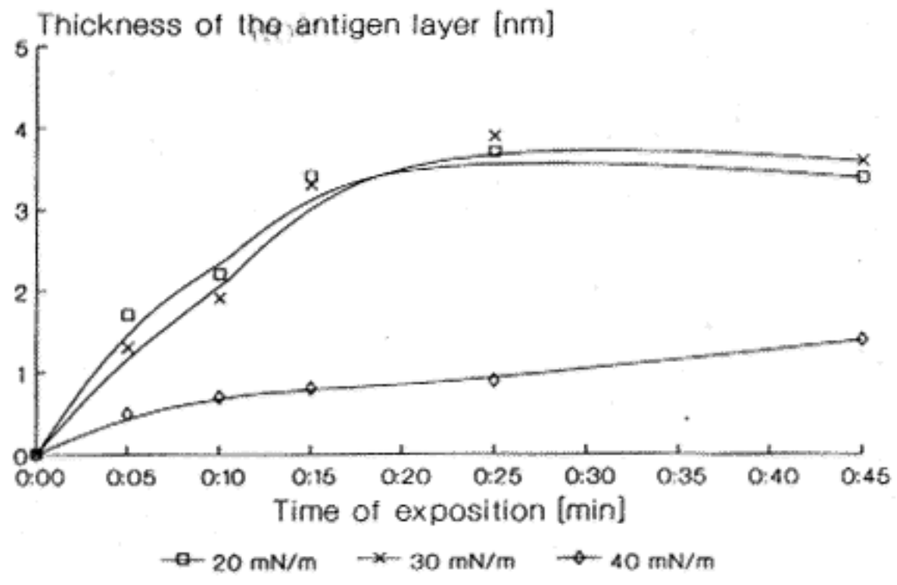

Fig. 3. Effective thickness of the bound layer of antigen at the surface of IgG monolayers deposited under different surface pressures: □, 20 mN m^{-1}; ×, 30 mN m^{-1}; ◇, 40 mN m^{-1}. Concentration of the antigen (mouse IgG) in the solution is 50 nmol.

TABLE 2. Relative molecular immunological activity of the LB film of IgG deposited under different surface pressures. The activity of the film deposited under 20 mN/m^{-1} is taken for 100%

Surface pressure mN m^{-1}	Thickness of the bound layer	Surface density of IgC monolayer[a] (pmol cm^{-2})	Immunological activity of IgG molecules in the film
20	4	0.4	100%
30	4	1.2	40%
40	2	2.1	10%

[a]Ref. 6.

structural changes in the films. The results become more striking if one bears in mind that the molecular density increases with the increase in pressure. In order to compare the molecular activities in the different films, one should divide the thickness of the bound antigen layer by the molecular density of the antibody film. The density of the films is 0.4 pmol cm^{-2}, 1.25 pmol cm^{-2} and 2.1 pmol cm^{-2} for the surface pressures 20 mN m^{-1}, 30 mN m^{-1} and 40 mN m^{-1} respectively [6], thus the final thickness of the bound antigen layer per molecule of antibody for the film obtained under 20 is 2.5 times higher than that for the film deposited under 30 mN m^{-1} and 10 times higher than in the case of 40 mN m^{-1}. In other words, if, in the case of 20 mN m^{-1} pressure, every molecule would bind one molecule of antigen, in the case of 30 mN m^{-1} there would be only 40% of the molecules that react, and only 10% would do so in the case of 40 mN m^{-1}. These results are given in Table 2.

There are several possible explanations of this phenomenon. First of all, one can imagine that in the case of a very dense packing of the binding sites, one specifically attached antigen molecule can block some binding sites of other IgG molecules. There are some observations of this phenomenon such as the influence of the size of the antigen on the binding ratio [6]. To estimate the effect of the screening we consider the form of the IgG molecule. The structure of the molecule is not rigid. The angle between F_{ab} fragments can vary over a rather large range (Fig. 4(a)). This means that the molecule can be represented as a disc, part of which is occupied by the F_{ab} fragments (binding sites shown in the figure as shaded areas) and the other part is occupied by the F_c fragment. The diameter of this disc is approximately 12 nm and the thickness 4 nm [10]. With given sizes of the molecules it is possible to imagine the situation in which one antigen molecule (mouse IgG) can block binding sites of four antibody molecules in the film (Fig. 4(b), the discs of IgG are shown from the lateral side). The other possible explanation may be that the molecules of IgG do not have any preferential orientation across the axis which is

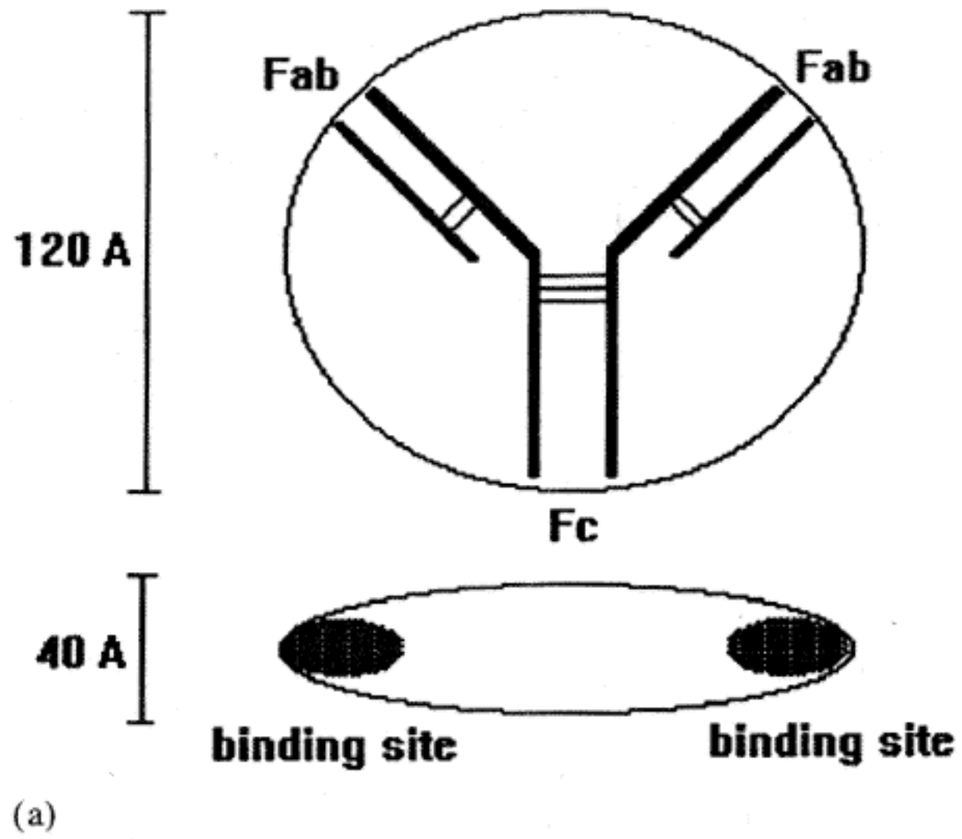

Fig. 4. Schematic diagram of the molecular packing of the IgG monolayers. (a) Characteristic dimensions of the IgG molecule. (b) and (d) Monolayer deposited under a surface pressure of $\sim 40\ mN\ m^{-1}$. (c) Monolayer deposited under a surface pressure of $\sim 20\ mN\ m^{-1}$.

perpendicular to the $F_{ab}-F_{ab}-F_c$ plane. This means that the orientation of F_{ab} fragments towards any direction has the same probability. In the case of laying molecules it does not cause any changes in the reactivity of the film because all of the F_{ab} fragments are exposed externally (Fig. 4(c)). On the contrary, in case of standing molecules a part of the F_{ab} fragments is hidden (Fig. 4(d)). Since the fragments are localized in one half of the molecule, the factor of reduction of the reactivity in this case is about two. Finally, it is possible that in the dense packing the molecule of IgG has a lower reaction ability even with F_{ab} fragments exposed. The antigen binding necessitates the molecule having some space for changing its conformation, the molecules being too dense packed locking this space. Regretfully the factor of reduction of the activity in this case is almost unpredictable.

It is likely, that the first two explanations cannot account for the observed 10 times reduction of the reactivity of the films. We have seen that they can cause only about a 3–4 times decrease. Obviously the simultaneous action of these factors does not give, as resulted reduction, the product of them, because the screening of the antibodies by the antigen is efficient only if F_{ab} fragments of all of the IgG molecules are exposed. The third explanation seems to be very reasonable. The dependence of the activity of the films on the type of immobilization of the antibody molecules at the substrate surface [7] and on the number of monolayers [5] may also serve as indirect proof of this statement

because these dependencies may be due to the variation of the mobility of the molecules. In any case, the decrease of the activity of the films, along with the increase of the pressure of deposition, is a consequence of the molecular structure of the films because all these models essentially demand molecular ordering.

4. Conclusions

1 The films of IgG, formed on the water–air interface, can be transferred onto the GOPTS activated supports in the monomolecular form with preservation of the regular structure.

2 The orientation of the molecules in the immobilized monolayers changes in dependence with the surface pressure of the deposition. Under pressures less than 25–30 mN m^{-1} the molecules are oriented parallel to the surface, while under pressures greater than 40 mN m^{-1} they are perpendicular to it.

3 Immunological activity of the monolayers of the immobilized antibodies depends essentially on the pressure of deposition. The decrease of activity with the increase in pressure is likely due to the reduction of the conformation mobility of the binding sites of the IgG.

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