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Immunological activity of IgG Langmuir films oriented by protein A sublayer

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Abstract

The immunological reaction ability of IgG Langmuir–Blodgett monolayers organized by means of a protein A sublayer has been studied by the gravimetric technique. In order to discriminate the effects of molecular orientation and to preserve the native structure of IgG molecules using the protein sublayer, the kinetics of specific and non-specific binding at an immunoglobulin monolayer deposited onto different substrates, such as a silanized aluminium surface and a surface covered by ovalbumin and protein A sublayers, are compared. It is shown that the prevailing effect is molecular orientation. A sublayer of protein A appeared not only to increase the specific sensitivity of the IgG monolayer but to decrease the non-specific binding as well. For this structure the sensitivity of a monolayer of rabbit anti-mouse IgG towards mouse IgG is 10 pM. The sensitivities in the case of a bare metal surface and ovalbumin sublayer are 10 and 100 times less, respectively. Protein A and ovalbumin sublayers are obtained by the Langmuir–Schaeffer technique. The formation of monolayers on the water–air interface and the deposition onto the solid substrate are studied by means of compression isotherms, gravimetric and ellipsometric techniques. It is shown that the solubility of protein A depends on the surface pressure increasing sharply after a certain value of the pressure. It is also shown that protein A can be deposited onto the substrate in the form of dense two-dimensional monolayers.

Keywords: Immunoglobulin; Langmuir–Blodgett films; Protein A

1. Introduction

Biosensors — a combination of specifically active biological molecular assemblies with a certain physical transducer — have been gaining increasing interest during the last two decades. Molecules of immunoglobulins are often used in such devices as sensing elements. One of the most important points in the design of an immunosensor is the proper choice of the immobilization method that will retain the activity of an antibody molecule. The utilization of a flow-injection scheme in an immunoassay procedure requires the use of antibody monolayers covalently bound to the surface of the sensor chip. In order to enhance the sensitivity of the sensor, the distance between the transducer surface and the immobilized film of antibodies should be minimized and the monolayer on the surface should possess a two-dimensional order. In the bidimensionally ordered film the binding irregularities of antigen to antibody are negligible and cannot give rise to a non-uniform distribution of bound antigen molecules in the layer. The most promising technique for producing dense monolayers of immunoglobulins, IgG in particular, is the Langmuir–Blodgett procedure [1]. A number of papers describe the ways of producing such films and their biological activity (see, for example, Refs. [2,3]). In Ref. [4] the possibility of controlling the orientation of the IgG molecules in Langmuir monolayers has been demonstrated by changing the surface pressure of the deposition process of a thin film. Subsequently, we tried to exploit this opportunity to obtain the molecular packing of a Langmuir film of IgG with molecules oriented in the direction normal to the surface to which they were attached by their Fc fragments in order to achieve the highest sensitivity [5]. However, such packing can be obtained only under very high surface pressure and the density of the film thus produced is also very high. It was shown that an increase in density above a certain limit decreases the reactivity of the film, since the molecules lack the conformation mobility that is necessary for antigen binding. Thus, the problem of

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obtaining an orientation of the IgG molecules with their Fab fragments exposed is still very important.

One of the well-known ways to orient an IgG molecule on a solid substrate is adsorption on a sublayer of protein A, the protein that binds the Fc fragment of IgG relatively specifically [6,7]. There is one more advantage in IgG deposition onto a protein sublayer if compared to a direct deposition onto a solid substrate: the sublayer serves as a protector for the IgG molecules from the effects of the substrate that might denature the deposited protein molecules. This has been described elsewhere [8,9]. A promising technique seems to be the combination of this approach with Langmuir–Blodgett (LB) deposition, since this combination might provide enhanced properties of the film, such as: a high degree of molecular orientation (due to the specific binding to the protein A sublayer); sufficient molecular density, since with the LB technique even the most dense packing of IgG molecules can be obtained; preserved nativity of IgG molecules (due to the protective effect of the protein A sublayer).

In the present paper we attempt a comparative study of the immunological activity of the IgG films obtained by different techniques. The schemes of structures produced and studied are shown in Fig. 1. Structure (a) consists of an LB film of IgG immobilized on a silanized solid substrate. Structure (b) consists of the same IgG film deposited onto the sublayer of ovalbumin treated with glutaraldehyde, which is immobilized on the substrate, and the last structure (c) has a protein A sublayer under an IgG film. In the last case the IgG films were produced both by means of LB deposition and by adsorption from the solution. Confronting the activity of these structures, the effects mentioned above were estimated: the orientation capability of protein A, the screening of the solid substrate by the protein sublayer and the increase in density of an IgG monolayer. The specific and non-specific immunoreactions of the structures were studied in order to evaluate their real sensitivity and applicability in biosensor devices. Part of the paper is devoted to problems of producing Langmuir films of protein A and ovalbumin.

A microgravimetric technique was used for measuring the mass of the deposited compounds, be it protein layers or specifically or non-specifically bound molecules. This technique is promising not only for the investigation of immunoassays but for the manufacturing of biosensors as well [10]. Ellipsometry was used for studying the LB deposition of protein A layers by measuring the thickness of the resulting films.

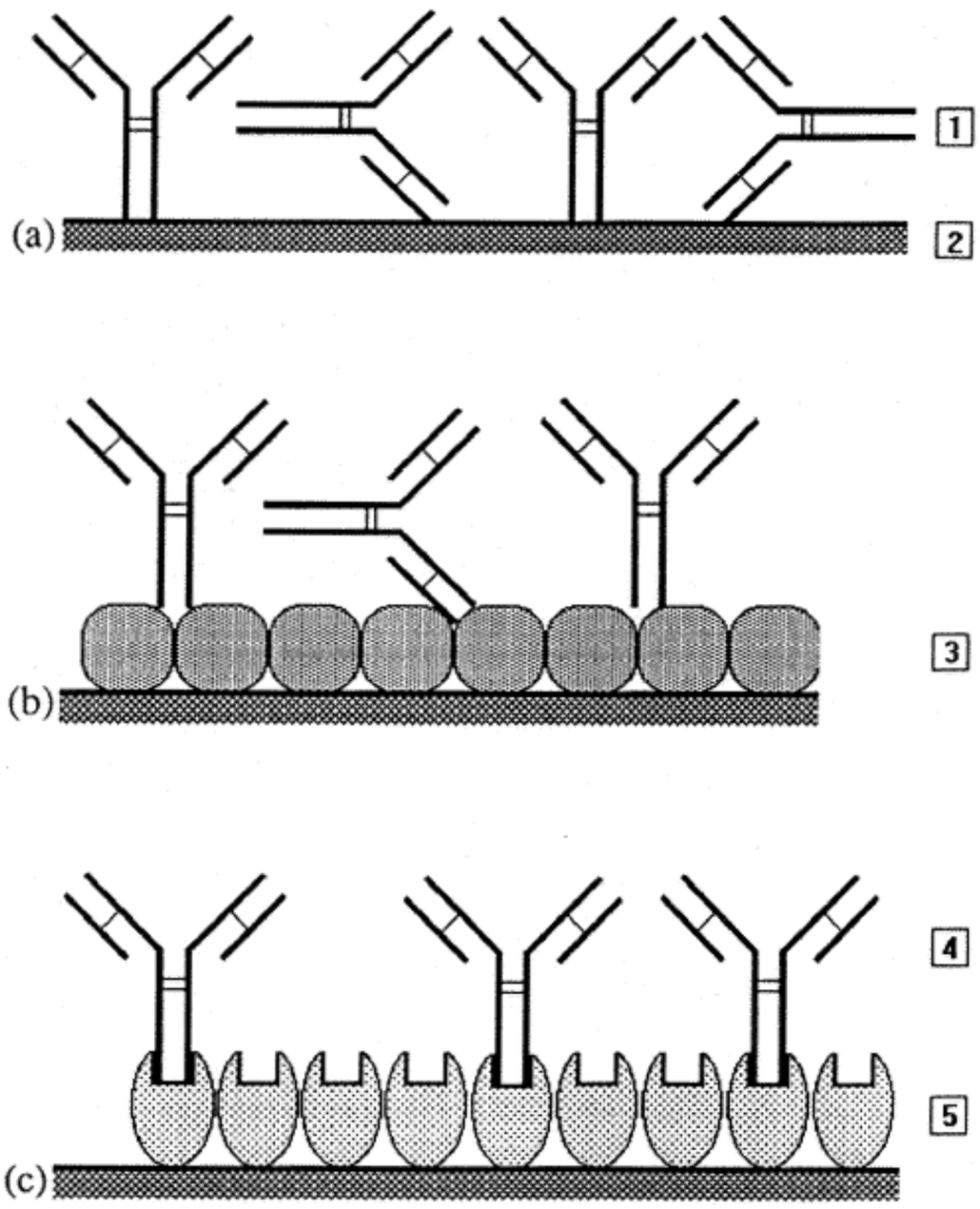

Fig. 1. Scheme of the prepared structures: (a) IgG monolayer deposited onto the silanized aluminium surface; (b) IgG monolayer deposited onto ovalbumin sublayer; (c) IgG monolayer oriented by protein A sublayer. 1, RAM antibody monolayer; 2, GOPTS- activated surface; 3, ovalbumin sublayer; 4, IgG molecules oriented by protein A; 5, protein A sublayer.

2. Experimental

2.1. Materials

Rabbit anti-mouse (RAM) antibodies were used for deposition, obtained from the Institute of Microbiology and Epidemiology (Moscow). Mouse monoclonal anti-Pd-coproporphyrin antibodies were used as antigen. These antibodies were a kind gift from the Molecular Immunology Laboratory of the Bakh Institute of Biochemistry (Moscow). Hen egg ovalbumin (mol. wt. 67 000), blocker BSA and affinity-purified protein A from *Staphylococcus aureus* (mol.wt. 42 000) were purchased from Pierce. 3-glycidoxypropyltrimethoxysilane (GOPTS) was purchased from Aldrich.

2.2. Modification of the crystal surface

Standard commercially available quartz resonators (2B,SGS, Italy) with a resonance frequency of 10 MHz and aluminium-plated polished electrodes were used for microgravimetric measurements.

The resonators have been treated with boiling chloroform, rinsed on a glass filter and dried under nitrogen. The crystals conditioned in this way have been silanized with GOPTS under vacuum in an apparatus described elsewhere [11].

The surface density of the deposited polyorganosiloxane film was controlled gravimetrically and its average

value for GOPTS was found to be 1.1 μg cm^{-2}. Since the GOPTS polymer contains the active epoxy groups that react with protein amino groups, no additional surface activation for the immobilization of protein LB films was required.

2.3. Microgravimetric measurements

The most important theoretical considerations dealing with sensitivity were published by Sauerbrey [12] and Kanazawa and Gordon [13]. Sauerbrey derived the following equation describing the frequency-to-mass relationship:

$$\Delta F = -2.3\times10^{-6}F^2M/A$$

where ΔF is the change in fundamental frequency of the coated crystal, F is the resonance frequency of the crystal, A is the area coated and M is the mass deposited.

For the calibration of resonators, the frequency shift induced by the deposition of cadmium arachidate bilayers was measured (Fig. 2). The bilayers were deposited by the Langmuir–Blodgett technique at a deposition pressure of 25 mN m^{-1}. Using the data on the molecular weight of the $(CH_3(CH_2)_{18}COO^-)_2\cdot Cd^{2+}$ complex and on the area per molecule at the bilayer (20.5 Å^2, [14]), it is possible to calibrate the weight gauge. A sensitivity of 2.95 ng Hz^{-1} cm^{-2} was found. This method was described in Ref. [15].

2.4. Ellipsometry

Ellipsometric measurements were performed using a PCSA null ellipsometer LEPh-2 (Special Design and Production Bureau for Scientific Devices of the Siberian branch of the Russian Academy of Sciences, Novosibirsk) at a wavelength of 633 nm. The accuracy of the device is 0.02° in ψ and δ. The angle of incidence was 70°. The data were processed according to the two-layer model, in which the lower layer accounts for the imperfections of the silicon substrate, and the upper one represents the deposited film. The applicability of such a model for the interpretation of the measurements of thin layers deposited onto the silicon substrate has already been shown [5]. The optical parameters of the lower layer were determined before deposition according to the one-layer model.

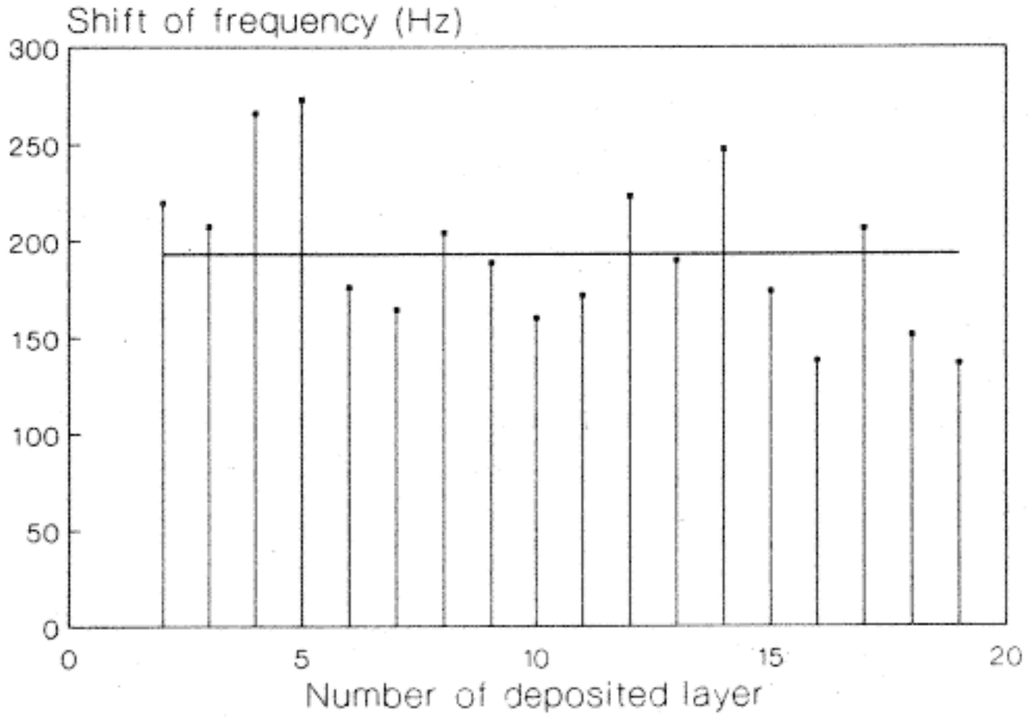

Fig. 2. Frequency shift of the resonator after deposition of 19 bilayers of cadmium arachidate. The straight line is the average value of the frequency shift after one bilayer deposition (193.2 Hz).

2.5. Langmuir–Blodgett films

Monolayers of RAM antibodies were formed in a Langmuir Teflon trough (MM-MDT Corp., Russia, subphase volume 40 ml and surface area 45 cm^2) by spreading 200 μl of Tris-buffer (100 mM, pH 7.3) solution with a concentration of 1 mg ml^{-1}. 100 mM $NaHCO_3$ buffer pH 8.6 was used as the subphase. Under these conditions, the protein primary amino groups suitable for covalent binding with the support are more deprotonized. The subphase temperature was 22 °C. Protein A and ovalbumin films were formed on the surface of the subphase containing homogeneously distributed proteins with a concentration of 50 nM. After a 40 min incubation, the formed film was compressed with a barrier speed of about 0.1 mm s^{-1}.

The transfer of protein monolayers from the subphase surface onto the reactive supports was performed by touching the support in parallel to the subphase surface according to the Langmuir–Schaefer method [16]. After film deposition, the resonators were dried in a nitrogen flux, incubated for 4 h at +4 °C, rinsed thoroughly with deionized water and dried again in the nitrogen flux. In the case of ovalbumin deposition, after drying the resonators were treated with glutaraldehyde vapour under vacuum.

Binding of RAM IgG to protein A sublayer was performed in Tris-buffer solution (100 mM, 250 mM NaCl, pH 8.3) containing 0.5% BSA (bovine serum albumin) and 2 μM IgG for 1 h with gentle stirring at a temperature of 22°C. After their immobilization IgG layers were exposed to the blocker buffer (30 mM Tris–HCl, 100 mM NaCl, 1% BSA 0.02% NaN_3, pH 7.4) for 15 min with gentle stirring, rinsed thoroughly with water and dried in nitrogen flux.

3. Results and discussion

3.1. Deposition of Langmuir–Blodgett films

Protein A

The deposition of a protein A monolayer directly onto the solid substrate surface prior to antibody binding provides the positioning of the IgG with the antigen binding sites directed outwards, and thus capable of binding antigen molecules present in the mobile phase.

The compression isotherms of protein A monolayers were measured at different temperatures. The curves are shown in Fig. 3. Similar isotherms were obtained by Turko et al. [17]. As is evident from Fig. 3, the surface pressure versus area isotherm is rather unusual in its shape. The pressure reaches a certain value and does not increase with a further decrease in the area. This means that after a definite pressure value the protein starts to leave the surface, i.e., its solubility grows. However, protein A molecules are known to have pronounced hydrophobic regions [18]. The process of self-formation of the film results in a marked increase in surface pressure (up to 8 mN m^{-1}), which proved that molecules are partially hydrophobic and prefer the location at the water/air interface. Thus it is obvious that the solubility of the molecules undergoes drastic changes after passing the temperature-dependent threshold pressure value, which varies from 8 mN m^{-1} at 20°C to 13 mN m^{-1} at 11 °C. This could be attributed to the effect of mutual shielding of hydrophobic sites of the molecules. Being compressed up to the state of dense packing, the molecules are in contact with each other by hydrophobic sites and tend to form aggregates, the solubility of which is higher than that of a single molecule. The dependence of the solubilities of the aggregates and of single molecules on temperature is quite similar since the solubility grows with an increase in temperature, which can be easily seen from the Figure.

The same behaviour is seen in the dependence of the thickness of the films on the surface pressure (Fig. 4). The deposition was carried out at a temperature of 15 °C. The thickness reaches a plateau at the same value of surface pressure, about 11 mN m^{-1}. The value of the thickness, 4 nm, corresponds to the diameter of the sphere approximating the protein A molecule [18], thus proving that the film is composed of a monolayer. The decrease of the effective thickness at lower pressures is due to the decrease in density of the film, since it is well known that the two parameters (refractive index, which is proportional to the density and effective thickness), both obtained from ellipsometry, are strongly coupled in the case of thin films. Thus, the film becomes bidimensionally densely packed at the same pressure at which the solubility changes, a fact that can hardly be ascribed to simple coincidence of events.

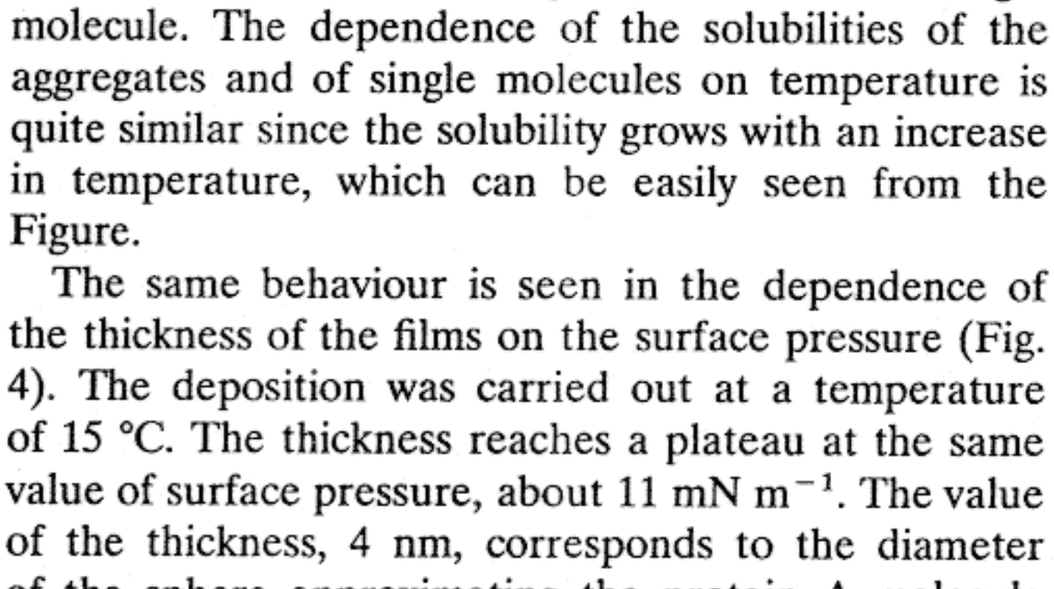

Fig. 3. Protein A compression isotherms at different subphase temperatures. The saturation of the curves is evident.

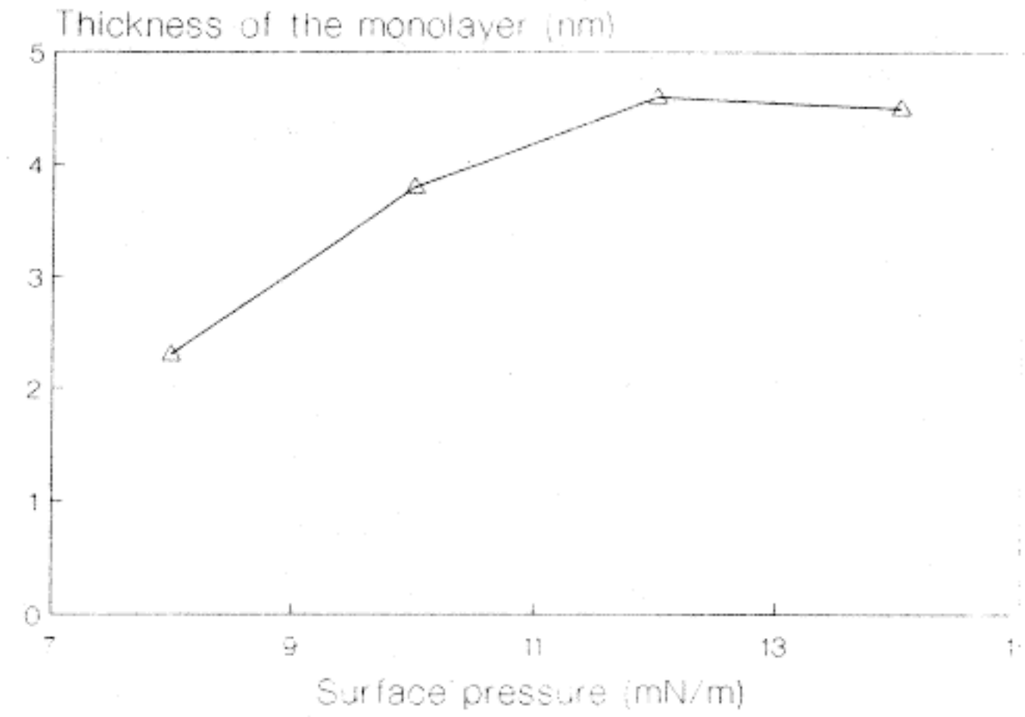

Fig. 4. Dependence of the thickness of deposited layer of protei A on deposition pressure. Ellipsometry data.

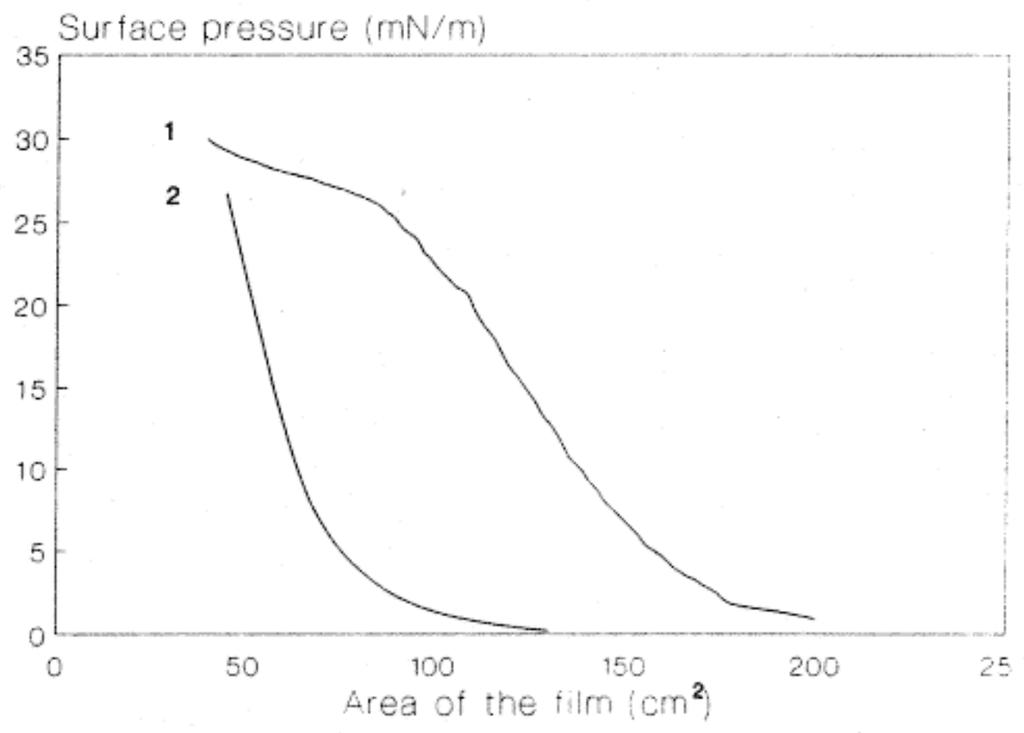

Fig. 5. Compression isotherms of rabbit antimouse IgG and ovalbumin Curve 1, ovalbumin monolayer; curve 2, IgG monolayer; subphase 100 mM carbonate buffer pH 8.6; subphase temperature, 20 °C.

For further treatment protein A monolayers were deposited onto the supports at a pressure of 10 mN m^{-1}. After deposition the samples were washed as described above. Microgravimetric measurements show that after washing, 35% of the molecules remain on the surface in the bound form, resulting in a monolayer surface density of 8.29 pM cm^{-2}. The last figure corresponds to an area per molecule value of 20 nm^2.

Ovalbumin

The compression isotherm of an ovalbumin monolayer is shown in Fig. 5 (curve 2). The deposition was carried

out at a surface pressure of 25 mN m^{-1}. After washing, 60% of the molecules remain in the bound state, i.e., ovalbumin binds with a GOPTS-activated surface approximately twice as well as protein A. The surface density of the ovalbumin film achieves the value of 40.15 pM cm^{-2}, which corresponds to an area per molecule of about 5 nm^2. This value is low with respect to the theoretical value of 35–40 nm^2, calculated from the diameter of the ovalbumin globule being approximately 6 nm [19]. The discrepancy may be accounted for by the roughness of the electrode surface.

Immunoglobulins

The IgG monolayers were deposited as described above, on three types of substrates: GOPTS-activated surfaces (a), sublayers of ovalbumin treated with glutaraldehyde (b) and sublayers of protein A (c). Onto the substrates (c) the films were deposited both by the Langmuir–Schaefer technique and by simple adsorption from solution. The pressure of the IgG monolayer during deposition was 25 mN m^{-1}, since this value gives the highest immunological activity of the film [4,5].

The results of microgravimetric measurements of the deposition on different substrates are summarized in Table 1, from which it is evident that the binding efficiency of the GOPTS-activated surface is the highest. The binding efficiency with ovalbumin–GA and protein A is approximately 1.7 times lower. The resulting molecular density of the films deposited onto the protein A sublayer is higher when the Langmuir–Schaefer technique is used. Once again it should be noted that the given density of the IgG films is higher as compared to the data obtained by means of fluorescent assays [4], which gave a typical value of 1–2 pM cm^{-2}. Such a difference is due to a significant error in determining the real area of the rough resonator surface.

3.2. Immunological activity of the films

Two factors that govern the immunological sensitivity of the films are specific and non-specific reactive abilities. The higher the former and the lower the latter, the more sensitive towards specific antigens is the film of antibodies. In order to estimate the specific activity of the films obtained by various techniques, the kinetics of the binding of the antigen from the pure solution were measured without addition of any substance that could be involved in non-specific reactions. The corresponding curves of frequency shift are shown in Fig. 6, where IgG films deposited on ovalbumin (2), GOPTS (3) and protein A (4,5) sublayers are presented. The film corresponding to curve 4 was obtained by adsorption of IgG molecules from the solution onto the protein A sublayer. Curve 1 corresponds to the silanized clean resonator and serves as the baseline. The slight increase of the frequency is due to the adsorption of water on the resonator. The best results are obtained in the case of deposition onto the protein A sublayer and the use of the Langmuir technique increased the activity of the film. The comparison of curves 2 and 3, where two concurring effects are presented,is rather interesting. On the one hand, the IgG molecules deposited onto the ovalbumin sublayer should better preserve their native structure; on the other hand, the films deposited on the GOPTS-activated surface have a higher packing density. It is evident that the density contribution is the prevailing one. The activity of the films deposited on the protein A layer is higher due to the combined effect of the higher molecular density and orientation.

In order to check the antigen recognition ability of the IgG films, we measured the same kinetic curves in the binding buffer containing BSA (10 mM Tris–HCl, 100 mM NaCl, 0.5% BSA, 0.02% NaN_3, pH 7.4). The total exposure time was 40 min, during which several measurements were made. Washing with water and drying in nitrogen flux were performed before every measurement.

Fig. 7 illustrates the antigen binding by a RAM IgG film oriented on the protein A sublayer. Different curves correspond to different values of antigen concentration. Even a concentration of 100 pM is detectable. The detection thresholds of antigen are 10 and 100 times less for the GOPTS–IgG and the ovalbumin–IgG films,

Table 1
Surface density and immunological activity of the RAM IgG Langmuir–Blodgett films immobilized on the aluminium surface by different methods

Method of surface modification	Fraction of antibodies bound at the surface (%)	Surface density of antibody-immobilized film (pmol cm^{-2})	Area per IgG molecule in the immobilized film ($Å^2$)	Antigen detection limit (pmol l^{-1})
GOPTS-activated surface	83	9.83	1700	100
Ovalbumin sublayer	50	3.14	5300	1000
Protein A sublayer (LB deposition)	45	11.21	1500	10
Protein A sublayer (adsorption)		6.8	2500	100

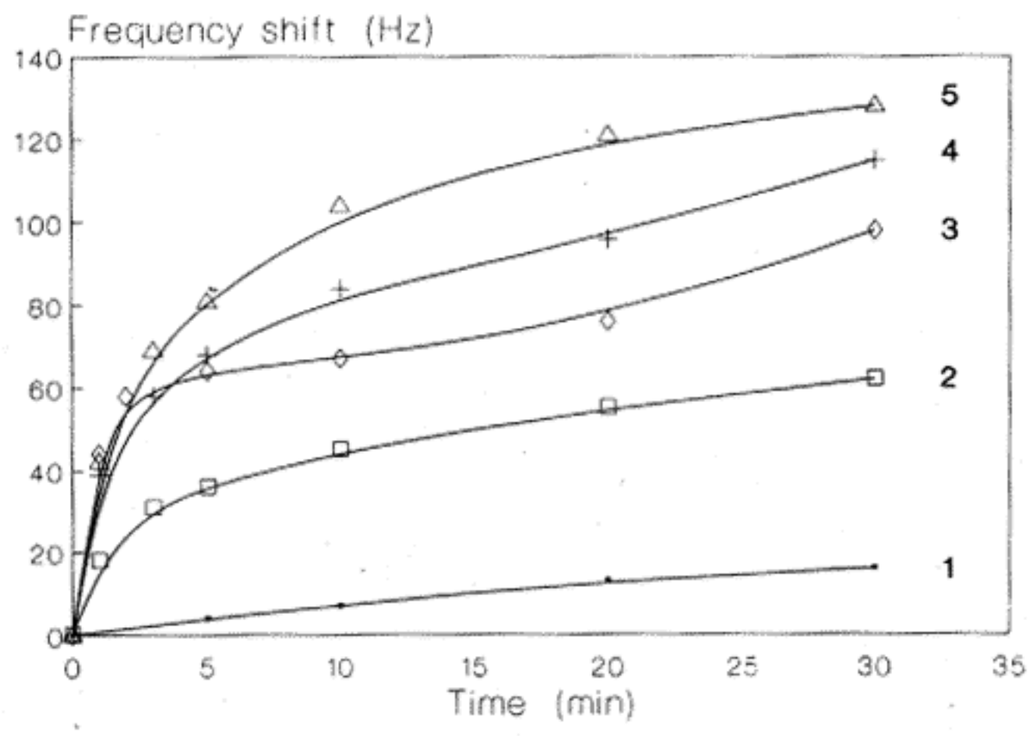

Fig. 6. Kinetics of specific binding of mouse IgG by RAM antibody films, deposited onto different structures. Test solution contains 1 nM of antigen (mouse IgG): 1, resonator frequency shift in water (baseline); 2, RAM IgG films deposited by Langmuir-Schaefer technique onto the ovalbumin sublayer; 3, RAM IgG films deposited by Langmuir–Schaefer technique onto the GOPTS-silanized aluminium substrate; 4, RAM IgG films absorbed onto the protein A sublayer from solution; 5, RAM IgG films deposited by Langmuir-Schaefer technique onto the protein A sublayer.

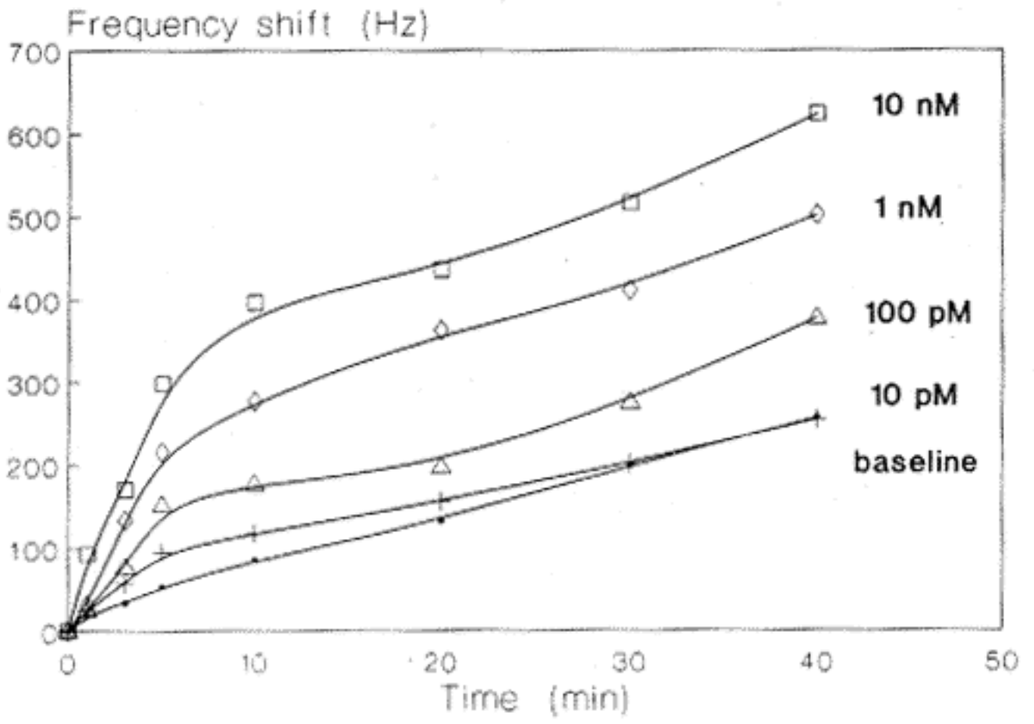

Fig. 7. Kinetics of specific binding of mouse IgG by Langmuir films of RAM IgG deposited onto the protein A sublayer. Test solution: 30 mM Tris–HCl, 100 mM NaCl pH 7.4, containing 1% of BSA. Curves correspond to different antigen concentrations; baseline is the frequency shift without antigen.

respectively (Figs. 8 and 9), since the corresponding kinetic curves almost coincide with the baselines. It should be noted that in the case of protein A not only the specific sensitivity of the IgG film is higher but the non-specific binding is lower as well (the baseline almost concides with the binding activity of the antigen from a 10 pM solution).

Acknowledgements

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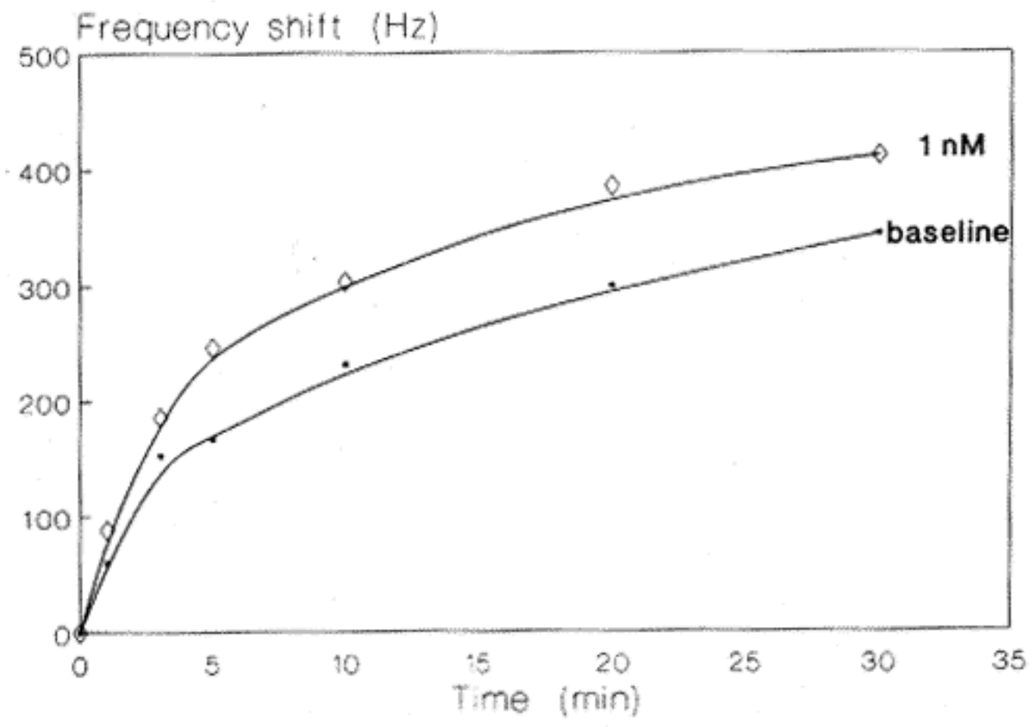

Fig. 8. Specific binding of antigen by Langmuir films of RAM IgG deposited onto the GOPTS-silanized aluminium substrate. Test solution: 30 mM Tris–HCl, 100 mM NaCl pH 7.4, containing 1% BSA. Baseline corresponds to frequency shift without antigen.

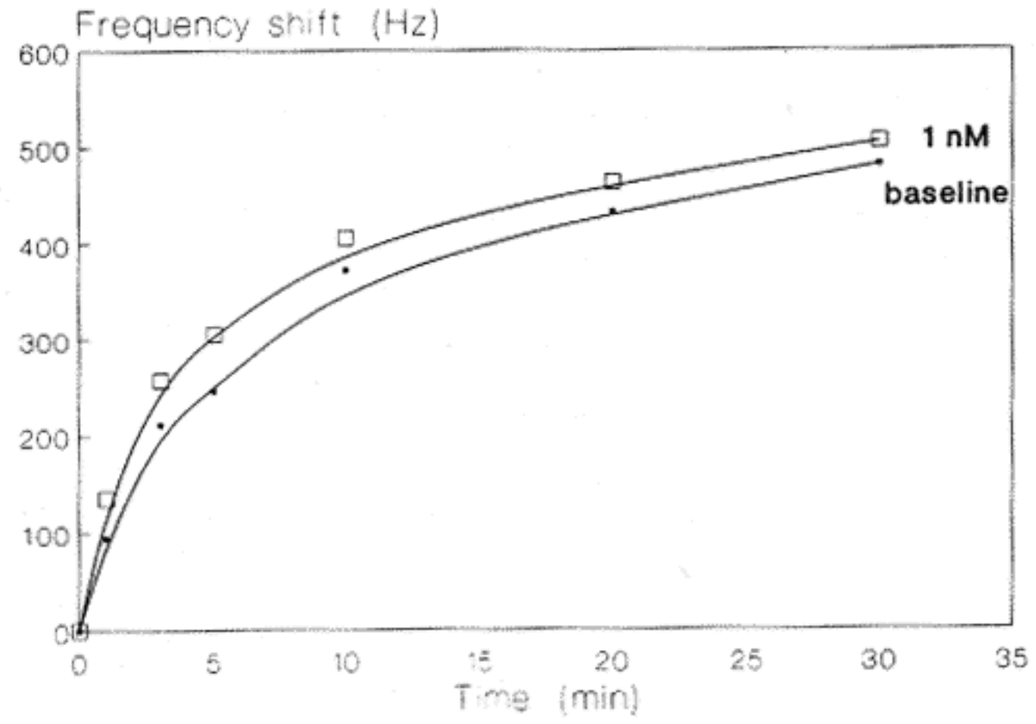

Fig. 9. Specific binding of antigen by Langmuir films of RAM IgG deposited onto the ovalbumin sublayer. Test solution: 30 mM Tris–HCl, 100 mM NaCl pH 7.4, containing 1% BSA. Baseline corresponds to frequency shift without antigen.

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Biographies

Timothy Dubrovsky was born in Moscow, Russia, on September 29, 1964. After receiving his M.Sc. degree in chemistry in 1987 from the Moscow Institute of Fine Chemical Technology, he worked as a junior research scientist at Bakh Institute of Biochemistry, Russian Academy of Sciences. Since 1993 he has been working as a research scientist at the Institute of Biophysics, University of Genoa. His research interests include fluorescent immunoassay, structure and immunological activity of the Langmuir–Blodgett films of immunoglobulins. He received a Ph.D. in biochemistry from the Institute of Biochemistry, Russian Academy of Sciences, in 1994.

Andrey Tronin was born in Moscow, Russia, on January 8, 1954. He received his M.Sc. degree in physics from Moscow Physical Engineering Institute in 1979 and his Ph.D. degree in crystallography at the Institute of Crystallography of the Academy of Sciences of the USSR in 1989. He is now working at the Institute of Crystallography. His research interests include ellipsometry and protein-containing Langmuir–Blodgett films.

Svetlana Dubrovskaya was born in Moscow, Russia, on December 11, 1962. She received her M.Sc. degree in chemistry in 1987 from the Moscow Institute of Fine Chemical Technology. Since 1993 she has worked as a research scientist at the Institute of Biophysics, University of Genoa. Her research insterests include Langmuir–Blodgett films of proteins.

Sergei Vakula was born in Moscow, Russia, on May 12, 1966. He received his M.Sc. degree in chemistry from the Moscow State University in 1988, and his Ph.D. in chemical enzymology and catalysis from the Division of Chemical Enzymology of the Moscow State University in 1991. Since 1991 he has worked at the Institute of Biophysics, University of Genoa, and is presently head of laboratory of Polo Nazionale Bioelettronica. His research interests include biosensors, protein and lipid thin films and the protein structure–function relationship.

Claudio Nicolini was born in Udine, Italy, on April 4, 1942. After receiving his doctoral degree in physics in 1966 from the University of Padua, Italy, he worked as a research associate at Brown University, MIT and BNL, then as associate professor at Bari University. In 1972 he changed field, moving to Temple Medical School, first as fellow then as associate professor of pathology, becoming full professor and director of the division of biophysics in 1975. He was science and technology advisor to the Italian Prime Minister during 1984–86, is scientific director of several academic and industrial organizations and vice-president of the National Program on 'Technologies for Bioelectronics'. He was appointed in 1990 by the Italian Parliament as a member of the National Science and Technology Council. Among his scientific interests are biophysics, both molecular and cellular, and bioelectronics. He has over 200 publications cited by ISI, and is author of four patents and 10 books. He is presently the Director of the Institute of Biophysics at the University of Genoa and President of Polo Nazionale Bioelettronica.