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Assembly of Antibodies in Lipid Membranes for Biosensor Development

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ABSTRACT

An investigation of the incorporation of antibody in lipid films of a composition that has been used for biosensor preparation is reported. IgG that is incorporated into lipid monolayers prepared from 7:3 mixtures of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidic acid is edge-active, and enters and penetrates the fluid region of the mixed-phase system when monolayers are held at low pressure (< 20 mN/m). It was found that there is an "exclusion pressure" observed in pressure-area (π-A) curves that are collected for monolayers that contain antibody. This term refers to a specific threshold of lateral pressure (which is reached by monolayer compression) that can cause explusion of antibody from the interior of a membrane. Microscopic images of monolayers containing the fluorescent phospholipid nitrobenzoxadiazole dipalmitoyl phosphatidylethanolamine (NBD-PE), or antibody labeled with tetramethylrhodamine isothiocyanate (TRITC), were used to determine the structure of membranes, and the location of effects on structure caused by IgG. Ellipsometric measurements of lipid monolayers that were cast onto silicon wafers by the Langmuir-Blodgett method were used to study the thickness of monolayers and to investigate the structural changes that occurred at the "exclusion

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pressure." Both the use of fluorescent antigen and ellipsometry indicated that antibody binding activity was present and was dependent on compression pressure. The effects of pH and ionic strength of subphase, antibody concentration, incubation time, and lateral pressure have been examined. The results may indicate the conditions that can be used to improve the incorporation of active IgG for preparation of biosensors that are based on lipid membranes.

Index Entries: Monolayer; antibody; biosensor; lipid; fluorescence; ellipsometry.

INTRODUCTION

Numerous investigations of the use of lipid membranes as transducers of selective binding interactions have been reported (*1–4*). One form of selective binding interaction that has received a great deal of attention is that associated with antibody–antigen complexation owing to the availability of a wide range of chemical selectivities and to the spontaneous incorporation of antibodies into organized lipid membranes. A desirable feature in the development of stable and active biosensors that are based on lipid membranes is the penetration of antibodies into the lipid matrix, so that they are firmly anchored and so that the binding sites are exposed at the surface of the membrane. The purpose of this investigation is to define the relationship between lateral pressure within lipid membranes, and the extent of incorporation and retention of activity of antibodies.

Many water-soluble proteins spontaneously bind with and insert into biological membranes (*5,6*). The thermodynamics and mechanistic features of these associative processes continue to be studied by use of model biomembrane systems. In particular, studies of the interactions of enzymes, antibodies, lectins, and drugs with phospholipid membranes, such as liposomes and monolayers, are important because they provide an understanding of the mode of action of these compounds in biomembranes. A number of reports concerning the specificity of lipid–protein interactions and methods for protein incorporation into membranes have appeared (*7,8*).

A lipid monolayer that is floated at an air–water interface often provides an excellent matrix for investigation of lipid–protein association. The primary advantage of the use of such Langmuir monolayers is that the physical structure of the monolayer can be easily and reproducibly altered by adjustment of chemical composition, temperature, lateral pressure, phospholipid speciation, and interfacial electrostatics (*9*).

The fact that monolayers may contain a number of coexisting phases and that these coexisting phases are related to the location of proteins in lipid membranes has been confirmed by fluorescence microscopy (*10–13*). The fluorescent probe nitrobenzoxadiazole dipalmitoyl phosphatidyl-

ethanolamine (NBD-PE) has been widely used to study the microscopic structure of lipid membranes. NBD-PE preferentially concentrates in areas of low density in lipid monolayers, and spatial aspects of monolayer structure can be imaged with a resolution of ca. 500 nm (*12*), based on the concentration and self-quenching of the dye (*14*). Proteins that contain extrinsic fluorescent labels can also provide information about structural and distribution effects caused by protein incorporation (*15,16*).

A number of experimental studies of monolayer and bilayer lipid membranes (BLMs) have been undertaken using binary mixtures of egg or dipalmitoyl phosphatidylcholine (DPPC)/dipalmitoyl phosphatidic acid (DPPA). Ion conductivities of BLMs were evaluated to determine the effect of surface charge and phase-domain formation on the process of ion translocation (*17,18*). Ion conductivity was controlled by the surface distribution of ions at the membrane-solution interface as predicted from electrical double-layer theory. It was found that the conductivity of the membranes could be approximated as a linear function of the weight percentage composition of the charged lipid. The conductivity was observed to alter drastically at a lipid composition containing a minimum of 25% phosphatidic acid as this component within the membrane was increased. This was attributed to the presence of a phase transition induced by the phosphatidic acid. At concentrations of the acid $<25\%$, ion conduction occurred through zones that were enriched in the charged lipid. At higher concentrations of the acid, the average surface charge was the predominant factor that determined the magnitude of ion conductivity. The adjustment of pH to control the degree of ionization of the phosphatidic acid had a similar effect to the variation of the amount of the acidic phospholipid within the membrane for experiments done at fixed pH.

Fluorescence images of monolayers showed the general trend of increased contrast between domains as the ionization of the monolayer decreased, which was consistent with the formation of a denser phase that excluded the probe to a greater extent. These results indicated that the ratio and state of ionization of DPPA in a monolayer can alter the phase structure, and may therefore cause the same phenomenon for egg PC-DPPA bilayers.

Recent reports have indicated that some antibody-antigen and hydrolytic enzyme reactions at BLMs formed from a mixture of egg PC and DPPA can provide an electrochemical response in the form of a single transient of current (*1,2*). The mechanism responsible for the evolution of a transient signal can be activated by rapid addition of acid or base to BLMs that do not contain proteins. The appearance of a transient signal is therefore a feature associated with electrostatic changes at the surface of charged BLMs that are prepared from PC/DPPA (not observed for PC alone). The time-dependent reproducible burst of current associated with antibody-antigen interactions (*1*) confirms that the F_{ab} sites are available and remain active when IgG is in a BLM. Of analytical importance is that the

transient signal can be readily detected above baseline noise and offers a pulse profile that could be used to develop a chemical "switch." The practical success of a 7/3 proportion of PC/DPPA to achieve and control phase separation (*17,18*), and to achieve optimization of transduction of both antibody–antigen and enzyme–substrate interactions (*1,2*) suggested that this mixture should be used to study the association of IgG with lipid monolayers.

The γ-immunoglobulin IgG consists of an assembly of four chains: two heavy chains and two light chains, with a total mol wt of approx 160,000 Dalton (*19*). These peptide chains form a single "Y"-shaped unit, with the analytically useful antigen binding areas (F_{ab}) located near the termini of the two arms of the "Y." Ideally, IgG would associate with a membrane in a manner that would permit orientation of the F_{ab} segments to expose these selective binding sites to analyte in solution. The distribution of packing densities of lipid molecules (phases in coexistence) would determine whether protein could penetrate or remain in different areas of a membrane. This article reports the results of an investigation to establish the conditions for incorporation of active antibody into lipid membranes consisting of DPPC/DPPA.

MATERIALS AND METHODS

Materials

DPPC, DPPA, octadecyltrichlorosilane (OTS), rabbit IgG, antirabbit IgG, and goat IgG were obtained from Sigma Chemical Company (St. Louis, MO). IgG and anti-IgG were obtained in both labeled (tetramethylrhodamine isothiocyanate, TRITC) and unlabeled forms for studies of incorporation of fluorescent protein into lipid membranes and to observe selective binding. The fluorescent lipid probe NBD-PE was purchased from Avanti Polar Lipids (Birmingham, AL). All water was obtained from a Milli-Q five-stage cartridge purification system (Millipore, Mississauga, Canada) and had a specific resistance of not < 18 MΩ·cm. All other chemicals were of analytical reagent grade. All experiments were done at 21 ± 1°C. Silicon wafers (*p*-doped) were from International Wafer Service (Potola Valley, CA).

Apparatus

The output from an argon ion laser (Coherent Innova 70 cw, Coherent Laser Products, Palo Alto, CA), operated at 488 nm and 10 mW power, was directed into a Zeiss IM inverted microscope (Carl Zeiss, Oberkochem, Germany) situated above a monolayer as shown in Fig. 1. The laser beam was reflected down to the monolayer by a dichroic mirror. The fluorescence emitted from the monolayer was transmitted through the dichroic mirror to a low light level Dage-MTI SIT 66 video camera

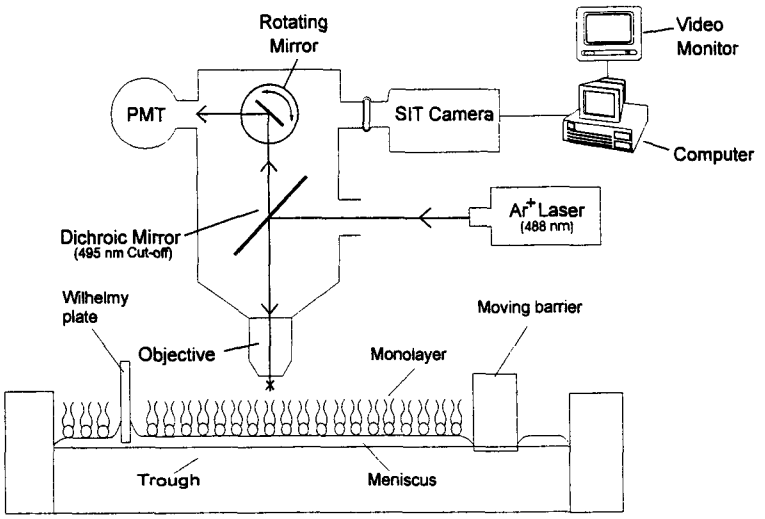

Fig. 1. Schematic diagram of the fluorescence microscope used for monolayer investigations.

(Dage-MTI, Michigan City, IN), which generated a video image. The camera was linked to a Data Translation video grabber card that digitized the image for storage and processing using an IBM PC-AT computer.

The film balance used in the monolayer investigations was a Lauda model 1974 (Sybron-Brinkmann, Toronto, Canada), with dimensions of 15 × 65 × 0.5 cm. The system was modified so that a Wilhelmy plate could be used for transduction of pressure. The Wilhelmy plate was calibrated using a stearic acid monolayer on an aqueous subphase containing 0.1*M* KCl at pH 7.4. Compression curves for stearic acid that were obtained using the Wilhelmy plate pressure transducer were assigned a collapse pressure of 52 m*M*/m, based on the collapse pressure obtained using the floating barrier that is available as standard equipment with the Lauda trough. The microscope, film balance, and camera were mounted on a gas-damped vibration isolation table.

An Auto EL II null reflection ellipsometer (Rudolph Research, Flanders, NJ) was used to determine the thickness of lipid films that were deposited onto silicon wafers. The optical source was a 1-mW continuous-wave helium-neon laser (632.8 nm), and all work was done at a fixed angle of incidence of 70.00°.

Methods

The phospholipid solution (DPPC/DPPA, 7/3 weight ratio) used for monolayer formation was prepared by placing 0.22 mg/mL of lipid mixture in a solvent of 3/1 (v/v) chloroform/methanol. Lipid solutions that contained fluorescent phospholipid were prepared by addition of an appropriate volume of a stock solution of 1 mg/mL NBD-PE in ethanol so as to achieve a 1 mol% composition of NBD-PE in the DPPC/DPPA mixture.

The phospholipid solutions were stored at −20°C. IgG and anti-IgG stock solutions were prepared as 0.2 mg/mL of protein in 10 m*M* phosphate buffer (pH 7.4) solution.

The subphase solutions used different concentrations of KCl, and were adjusted to different pH values by addition of 0.1*M* NaOH or 0.1*M* HCl. A total of 435 μL of phospholipid solution was deposited onto the subphase of a fully expanded trough (area 1000 cm^2, subphase volume 1000 mL) with a microsyringe, allowing each drop to spread before the next was added. A period of 30 min elapsed for solvent evaporation before compression of a monolayer commenced. Monolayers were compressed or expanded at a rate of 15 cm^2/min, and the pressure-area (π-A) isotherm was obtained. Concurrently, images of the microstructure of monolayers were collected for DPPC/DPPA monolayers containing 1 mol% NBD-PE.

After reproducibly compressing and expanding the monolayer, the monolayer was held at approx 2 m*N*/m. Different volumes of IgG solution were then spread onto the lipid monolayer surface to obtain different protein/lipid ratios. The IgG solution was spread on the monolayer over the course of 10 min by microsyringe. The π-A isotherm was obtained after an incubation period of 25 min.

Three types of membrane were studied by fluorescence microscopy to determine the location of protein in the monolayer:

1. Unlabeled protein was added to lipid monolayers that contained fluorescent NBD-PE;
2. Protein that was labeled with TRITC was added to lipid monolayers that did not contain NBD-PE; and
3. Labeled protein and NBD-PE were used in combination, and emission was observed by the placement and removal of band-pass filters centered at 550 and 600 nm.

Further fluorescence experiments were done to determine relative binding activities for rabbit-IgG incorporated into lipid monolayers. Imaging was done after fluorescent antirabbit IgG was injected into the subphase and was allowed to react for 30 min with unlabeled IgG that was in membranes at the air–water interface. The addition of fluorescent goat IgG was used as a control to determine nonselective reaction and further incorporation of protein into membranes.

Silicon wafers were cleaned by placement in a solution of ammonia: hydrogen peroxide:water (1:1:5) at 80°C for 5 min, followed by immersion in a solution of concentrated hydrochloric acid:hydrogen peroxide:water (1:1:6) for 5 min, followed by rinsing with water. Some wafers were coated with a dense hydrophobic C_{18} layer by reaction with OTS for 30 min.

Deposition of a DPPC/DPPA monolayer onto cleaned or OTS-coated silicon wafers was done using the Langmuir-Blodgett dipcasting technique (*21*). Wafers were mounted onto a vertical arm of a film lift and were oriented perpendicular to the air–water interface. Monolayers were cast at

a rate of 2.0 mm·min^{-1} at surface pressures of 25 and 30 m*N*/m. Barrier movement was constant for each monolayer deposition and allowed a transfer ratio of 1.0 to be established.

The thickness values of monolayers in air were determined using an Auto EL II ellipsometer. Data were analyzed using the "film 85" software package, version 3.0, programs 10, 13, and 70. Calibration was done using a standard oxidized silicon wafer, and program 70 was used to determine the n_s and k_s values of bare silicon wafers, whereas program 13 was used to determine the thicknesses of monolayers of lipid and lipid/protein (assuming a refractive index of 1.5 for the organic films). Each silicon wafer (in air) was investigated at eight spots so that average values for n_s, k_s and thickness could be established. Thickness determinations made use of the experimentally measured values of Δ and Ψ after monolayers had been deposited. Measurements were also collected from lipid membranes that contained IgG complexed with anti-IgG that were coated onto OTS-overcoated wafers. Nonselective interaction with goat IgG (as used in the fluorescent experiments) was used to prepare control samples.

RESULTS AND DISCUSSION

Monolayer Compression and Determination of Thickness

IgG was allowed to interact with monolayers of DPPC/DPPA to determine the effects of incorporation of the protein on the structure of the lipid membrane. The experimental results based on π-A curves and ellipsometry provided measurements of average changes of membrane structure. Fluorescence microscopy provided details about the microscopic structure of the membranes and indicated whether the interface between two phases was significant to the association of IgG with DPPC/DPPA monolayers.

The π-A curves shown in Fig. 2 provide an indication of the effects caused by the incorporation of IgG, and demonstrate that the pressure of monolayers that are held below 25 m*N*/m will increase by up to 3–5 m*N*/m when protein is added to a monolayer. The small increase in the surface pressure of the monolayer implies that the IgG was not interspersed deeply within the monolayer. Previous studies have suggested that IgG is localized at the polar surface of lipid membranes (*20*). Note that curve "b" eventually overlaps and follows the track of curve "a" at higher lateral pressures. Protein was incorporated into the membrane at low lateral pressure and caused an increase in pressure on association of the membrane with protein. High lateral pressure can cause a reversible exclusion of protein from a monolayer, so that the compression curve of the membrane that contained protein becomes identical to a membrane composed only of phospholipid. We have termed the point of overlap of curves

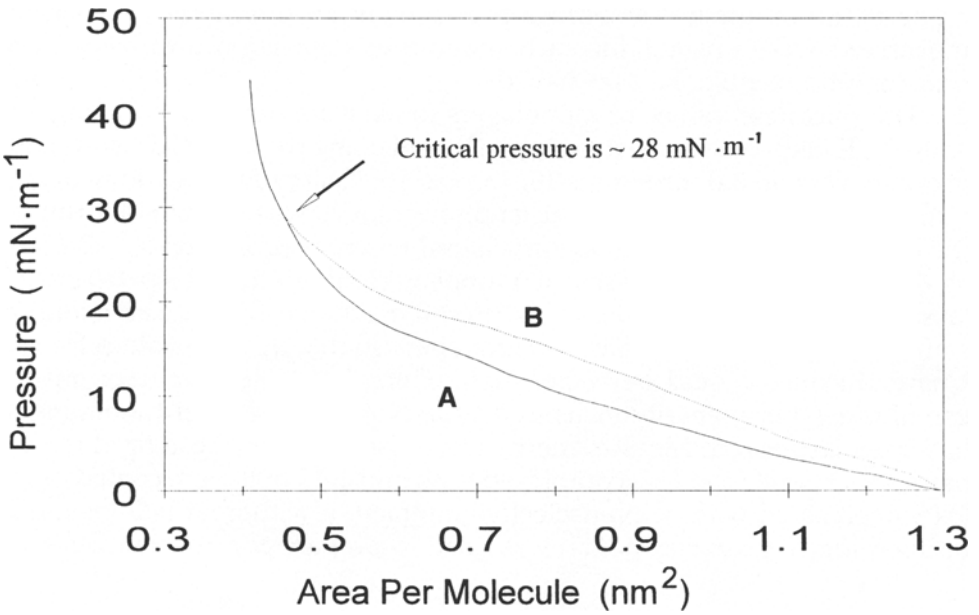

Fig. 2. Pressure-area isotherms of a DPPC/DPPA monolayer containing 1 mol% NBD-PE at pH 6.03 and ionic strength 0.1 before (**A**) and after (**B**) addition of protein (protein:lipid ratio 1:100).

"a" and "b" the "exclusion pressure," corresponding to the pressure at which the protein is excluded from the interior of the monolayer.

Ellipsometry is a technique that is used to measure the thickness and refractive index of overlayers that are deposited onto smooth, reflective substrates. The differences between the states of polarization of an incident and reflected beam of optical radiation can be determined by measuring the electric fields of the incident and the reflected beams of light. Each of these electric fields can be resolved into two orthogonal linearly polarized components, one (the "*p*" component) with its electric field vector parallel to the plane of incidence and the other (the "*s*" component) with its electric field vector normal to the plane of incidence. The state of polarization of a beam is determined by the relative amplitude and relative phase of the *p* and *s* components. Complex reflection coefficients are not separately measurable, but the ratio ρ can be measured by ellipsometric methods. This ratio can be defined as (22):

$$\rho = \tan \Psi \, exp\Delta i \tag{1}$$

The angular values of P and A are converted to the ellipsometric parameters Δ and Ψ by the relationships:

$$\Delta = 2P - 90° \quad \text{and} \quad \Psi = A \tag{2}$$

for the Auto EL II ellipsometer, which operates with the following polarizer settings: Polarizer, $-45° \leq P \leq 135°$; analyzer, $0° \leq A \leq 90°$;

compensator, C = 45°. The value of Δ is representative of the change in the phase of *p*- and *s*-polarized light on reflection, whereas Ψ is representative of the change in amplitude of *p*- and *s*-polarized light after reflection from the sample.

The values of Δ and Ψ are first determined for a bare substrate to give the real (n_s) and imaginary (k_s) components of the refractive index of the substrate. The values of Δ and Ψ are then measured for the substrate, which is coated with an overlayer. These values can be used to calculate the real (n_2) and imaginary (k_2) components of the refractive index of the film and the thickness (d) of the film if the incident angle (Φ), wavelength of radiation (λ_e), and index of refraction (n_o) of the ambient (air) are known. If the film is transparent (nonabsorbing), then $k_2 = 0$ and only n_2 and thickness are unknown. These values are determined using an iterative calculation based on the Drude equations (22).

The assumptions of the Drude equations are:

1. The samples are homogeneous, isotropic, nonabsorbing, and nonamplifying;
2. The films have uniform thickness and refractive index;
3. The sample and substrate are smooth;
4. The area of the film is many times its thickness; and
5. The boundaries among sample, substrate, and ambient are discrete and plane-parallel.

Under optimum conditions in which all assumptions are valid, changes in thickness on the order of 0.1 nm can be detected, whereas changes in refractive index on the order of 0.002 can be measured.

It should be noted that values that are determined by ellipsometric measurements are relative thickness values that were obtained by comparison of thickness values for bare silicon substrates and substrates that were covered with an immobilized membrane. It is extremely difficult, if not impossible, to calculate absolute thickness values using ellipsometry owing to the difficulty in establishing the real and imaginary refractive indices and the thickness of the film using a single measurement.

The thickness values are made less reliable owing to the fact that the immobilized membrane system is not ideal for ellipsometry in that it violates several of the assumptions of the Drude equations. For example, the film is oriented with acyl chains perpendicular to the air–film boundary and is therefore uniaxially anisotropic. In addition, it is possible that the surfaces are not smooth and that the boundaries among ambient, film, and substrate are not plane-parallel, especially in the case where proteins are bound to the membrane. Finally, it is possible that the films are discontinuous and are present in the form of patchy overlayers.

Uniaxially anisotropic films transmit *p*- and *s*-polarized light at different speeds and thus the "simple" equations for isotropic films on isotropic substrates do not hold. Azzam and Bashara (22) describe the corrections that must be applied in the case of uniaxially anisotropic films

Table 1
Ellipsometric Thickness Determinations of Lipid, and Protein:Lipid (1:100) Monolayers on Clean Silicon Wafers

Sample	Thickness, nm	Increase owing to IgG, nm
7:3 DPPC:DPPA at 25 m*N*/m	3.8 ± 0.4	~
7:3 DPPC:DPPA + IgG at 25 m*N*/m	8.1 ± 0.4	4.3
7:3 DPPC:DPPA at 30 m*N*/m	4.2 ± 0.2	~
7:3 DPPC:DPPA + IgG at 30 m*N*/m	6.6 ± 0.2	2.4

on isotropic substrates. The corrections involve recalculation of the reflection coefficients and phase thickness values to account for differences in transmission of *p*- and *s*-polarized light. In this work, the simple expressions for isotropic films on isotropic substrates were used to calculate thickness values. The failure to account for the anisotropic nature of the film would not produce relative errors in thickness of more than 10%.

A second set of corrections must be applied in the case where the film is not uniform. If the film exhibits patchy coverage or islands of film surrounded by bare substrate, then the assumption that the film has a refractive index of 1.50 is not valid. This can be accounted for by using Maxwell-Garnett theory (22). In this work, the films generally had surface coverage values that were close to 100% of the value expected for a close packed monolayer, and therefore this correction was not applied.

Calculation of thickness values used the following values for the various optical constants of the ambient-film-substrate system: λ_e = 632.8 nm, Φ = 70°, k_0 = 0, n_0 = 1.0003, k_2 = 0 (assumes film is nonabsorbing), n_2 = 1.50, k_s = 0.2182, and n_s = 3.8396 for the silicon substrate. The values of n_s and k_s were determined for each silicon wafer before immobilization of membranes.

Results from ellipsometric investigations of the effective thickness of phospholipid membranes and the change of thickness caused by the presence of IgG at different pressures (both just before and just after the "exclusion pressure") are presented in Tables 1 and 2. Casting of films onto the hydrophilic supports (clean silicon wafers) occurred only on withdrawal of the substrate from the solution in the trough. This would suggest that the polar head groups were oriented facing the substrate. Casting onto hydrophobic supports (OTS-coated silicon wafers) occurred on immersion of the substrate into the trough and provided lipid monolayer oriented with the polar head groups exposed to solution. Removal of the floating lipid monolayer at the air-water interface after immersion

Table 2
Ellipsometric Thickness Determinations of Lipid, and Protein:Lipid (1:100) Monolayers on Clean Silicon Wafers[a]

Sample	Thickness, nm	Increase owing to IgG, nm	Increase owing to reaction with anti-IgG, nm
7:3 DPPC:DPPA at 25 m*N*/m	3.5 ± 0.5	~	~
7:3 DPPC:DPPA + IgG at 25 m*N*/m	7.9 ± 0.4	4.4	~
7:3 DPPC:DPPA[b] + IgG + anti-IgG at 25 m*N*/m	10.4 ± 0.6	~	2.5
7:3 DPPC:DPPA at 30 m*N*/m	4.0 ± 0.4	~	~
7:3 DPPC:DPPA + IgG at 30 m*N*/m	6.2 ± 0.4	2.2	~
7:3 DPPC:DPPA[b] + IgG + anti-IgG at 30 m*N*/m	7.2 ± 0.6	~	1.0

[a] All thicknesses are corrected for OTS contribution.
[b] Controls using nonselective interactions of rabbit IgG with goat IgG provided insignificant increases of thickness (within SD) at protein:lipid ratios of 1:100. Significant but small increases of thickness were observed at lower protein:lipid ratios, presumably because of further protein incorporation into lipid membranes (*see* Fig. 4 and *text*).

of the substrate permitted retrieval of the coated wafer into air for ellipsometric studies. It is clear that the thickness values for the phospholipid membranes (no protein) are in the range associated with a monolayer (DPPC monolayer ~3 nm) and that these thicknesses are relatively invariant over the pressure range used in this work. Quantitative data about the effects on refractive index caused by the presence of protein or trapped water are not known. A comparison of the relative thickness values for membranes that contain IgG reveals that substantial adsorption/absorption of proteins occurs at both low and high compression pressures (also confirmed by the presence of TRITC-labeled IgG in fluorescence microscopy images in both cases). The dimensions of unhydrated IgG are approx 3.5 × 3.5 × 14.0 nm (*19*). Presuming that the loading of protein: lipid remains at the ratio of 1:100 as initially prepared, and presuming similar optical properties for protein and lipid, then rough estimates of membrane thickness can be calculated. The introduction of protein into a membrane at the air–water interface causes only small increases in pressure, and we will therefore assume that the protein largely lies on the

polar membrane surface. This then permits calculation of the relative average surface area contributions from lipids (based on average molecular area per lipid as determined from compression curves) and a range of area calculations for IgG bounded by two extremes: the protein lies on the surface with a cross-sectional area of 3.5 × 14.0 nm or the protein is upright (long axis perpendicular to the plane of the membrane) with an average molecular area of 3.5 × 3.5 nm. At 25 m*N*/m for membranes that contain IgG, the compression curves indicate an approximate area per lipid of 0.5 nm^2. For the protein:lipid ratio of 1:100, this then suggests two extremes of average surface thickness. One protein molecule that lays flat on a lipid surface would occupy about 50 nm^2 (at 3.5 nm + 3.8 nm total thickness), which is an area similar to that occupied by 100 lipid molecules (measured 3.8 nm thickness) at a 1:100 ratio. The resulting average thickness of a lipid membrane containing IgG would be in the range of 7.0–7.5 nm. At the other extreme, one protein molecule standing upright would occupy approx 13 nm^2 (at 14.0 nm + 3.8 nm total thickness), but would occupy only 13 nm^2 of a total of each 50 nm^2 area. The resulting average thickness of a lipid membrane containing IgG would be in the range of 8.0–8.5 nm. These thickness estimates must be reduced in magnitude to account for the unknown distance of penetration of protein into the lipid membrane. The results of Tables 1 and 2 indicate that the protein may be oriented upright in lipid membranes at pressures below the "exclusion pressure," whereas above the "exclusion pressure," the protein may lay flat on the surface of the lipid membrane. Note that there is no evidence from compression curves, the transfer ratios, or fluorescence images that would suggest the presence of multilayers. The constant intensities of fluorescence images of lipid membranes that contain TRITC-labeled IgG at both high and low pressures indicate that the protein remains on the membrane above the "exclusion pressure." Ellipsometry indicates that the IgG is adsorbed onto the membrane, but the π-A curves indicate that the protein does not penetrate into the lipid structure at high compression pressure. These results further suggest that at high compression pressure, the antibody lies along the surface.

Fluorescence Microscopy

DPPC/DPPA monolayers at the air–water interface undergo a phase transition from a low density phase to a higher density phase when compressed isothermally. A π-A curve of a DPPC/DPPA monolayer exhibits a two-phase coexistence region at pressures over 5 m*N*/m (*17*), and two types of domains were observed by fluorescence microscopy using NBD-PE. One of these phases had a negative head-group region of low charge density, and the other had a relatively high proportion of negatively charged head groups at pH 7.4 (*18*).

The NBD-PE probe was used at a low concentration of 1 mol% so as not to interfere significantly with the phase structure distribution caused

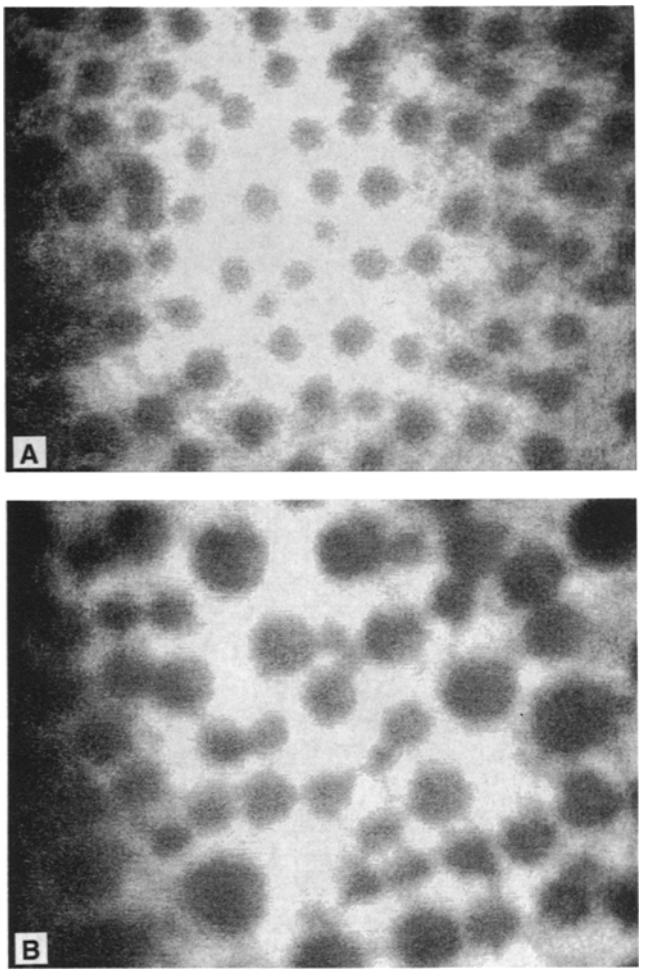

Fig. 3. Fluorescence microscope image of DPPC/DPPA monolayer containing 1 mol% NBD-PE at 5.0 m*N*/m at pH 8.06 before (**A**) addition of IgG and (**B**) at 5.2 m*N*/m after addition of IgG (protein:lipid ratio was 1:100). Width of image was 40 μm.

by interactions of DPPA, DPPC, and IgG, and was chosen for a number of reasons. The adsorption maximum for NBD-PE is 480 nm, which is close to the output of 488 nm from the argon laser. The emission is at 535 nm, so that there is a sufficient wavelength shift for fluorescence to be separated by a dichroic mirror. The NBD-PE probe does not respond directly to changes in pH and polarity when in the monolayer (*14*), leaving changes of membrane structure as the major cause of visual changes in images. Dense domains appear as dark regions in a bright (less dense) background when a low concentration of NBD-PE is included in the monolayer (*23*).

Figure 3 presents images of monolayers containing NBD-PE both with and without protein that were collected at pressures 5 m*N*/m (pH 8.06).

Incorporation of protein resulted in changes in the microscopic phase-domain structure. Figure 3A depicts a relatively regular shape and small size of dense phase domains (dark) within a bright fluid phase. The addition of IgG results in an enhancement of the relative size and clustering of domains (Fig. 3B). This effect can be attributed to a reduction of edge-free energy on accumulation of protein at the interface between liquid crystalline domains and fluid areas. Support for this argument can be derived from images of TRITC-labeled IgG that showed the appearance of halos at the domain boundaries of monolayers (which did not contain NBD-PE).

The addition of TRITC-labeled IgG to the unlabeled membrane produced images similar to Fig. 3B, indicating that the changes in structure were the result of the presence of the protein and that the protein preferentially partitioned into domains that were of greater disorder. This interpretation was confirmed by the concurrent use of NBD-PE and labeled IgG. The use of band-pass filters allowed spectroscopic identification of the sources of emission, since rhodamine had a maximum emission at 595 nm whereas that of NBD-PE was 535 nm. The similarity in images confirmed that the location of the protein was in the fluid domains.

Selective Binding

Selective binding activity was initially investigated for membranes that contained IgG (protein:lipid ratio of 1:100) at the air–water interface by use of fluorescent antigen and a nonselective fluorescent protein as a control sample. Membranes that were held at pressures below the "exclusion pressure" showed substantial fluorescence intensity increase when fluorescent antigen was added. Membranes held at pressures above the "exclusion pressure" showed some increase in fluorescence intensity, but this was about fivefold less than observed for membranes that were held below the exclusion pressure. Control samples showed insignificant fluorescence increases (note that the 1:100 ratio of protein:lipid seems to saturate the monolayer with adsorbed protein; *see* Fig. 4). Membranes held at high pressure could be brought below the "exclusion pressure," resulting in a substantial increase in fluorescence intensity. This suggested that denaturation of protein resulting in massive structural changes was not caused by transition through the "exclusion pressure." Ellipsometry confirmed this result, and it was determined that "exclusion" of protein was a slow, but reversible process in terms of effective thickness as pressure was cycled and membranes were subsequently coated onto silicon wafers for study. Investigation of long-term stability of proteins in membranes on silicon wafers to follow changes of binding activity were not possible because of gradual creep of deposited membranes beyond the coating area over periods of hours (i.e., deposited membranes were not structurally stable over periods of hours). Table 2 provides ellipsometric evidence for retention of binding activity for membranes that

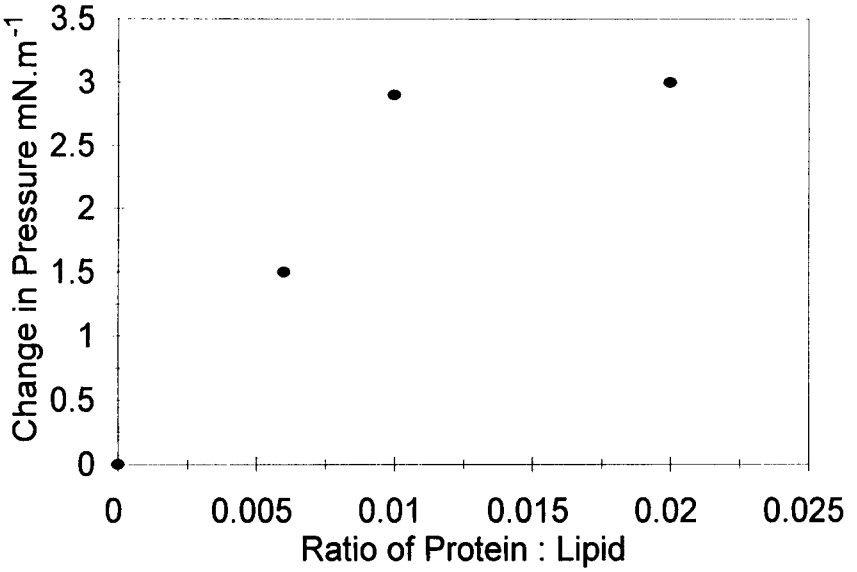

Fig. 4. Effect of protein concentration on the change in pressure. Measurement of change in pressure was at constant area per molecule area of 0.65 nm^2.

contain IgG (coatings provide an orientation that places the protein in solution). The fluorescence and ellipsometric results confirm that the F_{ab} sites were available for binding. OTS-treated wafers that were coated with monolayers containing IgG were also placed in 0.02 mg/mL anti-IgG solution for 30 min. Membranes containing fresh IgG were subsequently found to retain binding activity to anti-IgG, whereas IgG membranes that contained denatured (aged) IgG showed insignificant selective binding. The greater increases of effective thickness of membranes prepared below the "exclusion pressure" are consistent with the fluorescence results that indicate greater binding activity at low pressure. However, ellipsometric results taken alone do not conclusively prove this fact, because orientation of binding will have a significant impact on the measured effective thickness.

Effect of pH and Ionic Strength

The results of Table 3 show that pH affects the "exclusion pressure," whereas ionic strength has little impact unless there are large changes in relative surface potential at low ionic strength. This is consistent with the understanding of the role of electrostatics in determination of intermolecular interactions in DPPC/DPPA membranes (*18*). The pK_a values for phosphatidic acid were approx 3.5–4.0 and 8.5–9.0 in these monolayers and the pI of the protein was about 5–6 (*1*). A greater change of relative ionization of the phosphatidic acid head groups occurred at pH 4–5 than over the remainder of the pH range that was studied because of

Table 3
"Exclusion Pressure" in mN/m (SD Of 10%)
Observed at Different Values of pH and Ionic Strength
for Monolayers Composed of IgG:Lipid in a Ratio 1:100

	Ionic strength		
pH	0.001	0.050	0.150
4.00	43.0	30.5	28.0
5.01	24.0	26.0	26.5
6.03	26.5	25.0	25.0
7.05	23.5	21.0	22.5
8.06	22.5	24.8	22.0

the values of pK_a. The protein would experience a shift from positive to neutral charge over this pH range. Double-layer screening effects associated with ionic strength have little effect on intermolecular charge repulsions within the head-group region of the membranes. This means that other than in relatively extreme cases, the ionic strength should not cause substantial changes in surface pressure and the "exclusion pressure." However, electrostatic attractions of lipid head groups to protein (in this case, the protein may be associated with the head-group region [*20*]), head group–head group repulsions and protein–protein repulsions do have a substantial effect on molecular mobility and packing (*24*). The electrostatic attractions would be expected to produce the most change in "exclusion pressure" near pH 4–5 (the first pK_a, and transition of protein from positive to neutral change) where the relative changes in surface charge density would be greatest for changes in pH. This is observed in Table 3, where the largest changes in "exclusion pressure" occur in the range of pH 4–5 and indicate stabilization of the membrane owing to interactions of positively charged protein with negatively charged lipids. Only at the extreme of pH 4 does an increase from very low to higher ionic strength have a significant impact on the "exclusion pressure."

Effect of Protein Concentration

Interaction between a lipid monolayer that is held at low pressure and IgG resulted in a variable increase of pressure, which was dependent on the protein:lipid ratio. An increase in the relative change of pressure with increased protein availability can be seen in Fig. 4, which also shows that a surface saturation is eventually achieved (adsorption isotherm). The results of the experiments shown in Fig. 4 indicate that a protein: lipid of ratio of 1:100 would optimize the loading of IgG in DPPC/DPPA membranes for biosensor preparation. The "exclusion pressure" was dependent on the protein:lipid ratio. Increased loadings of IgG caused an increase in the "exclusion pressure" (35 mN/m for 1:100 ratio), whereas

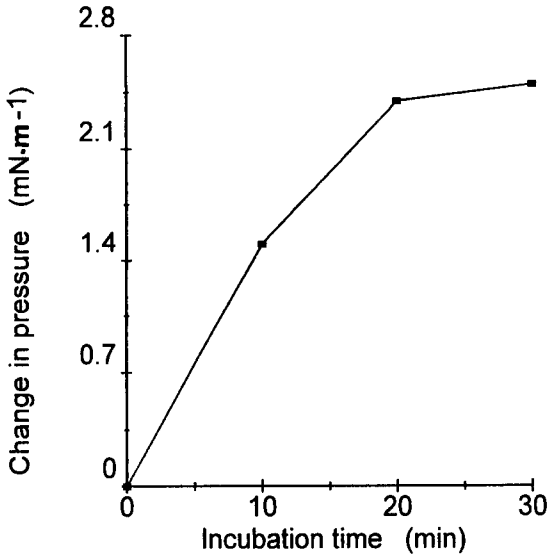

Fig. 5. Effect of incubation time on the change in pressure. pH 7.4, ionic strength 0.1, protein:lipid ratio 1:100. Measurement of change in pressure was at constant area per molecule area of 0.65 nm^2.

at lower concentrations, a lower pressure (25 m*N*/m for 1:150 ratio) was required for the same process. Note that in each experiment, a compression curve is collected from effectively 0 m*N*/m to high pressure to find the "exclusion pressure," so that the presence of more molecules at the surface is irrelevant to the measured "exclusion pressure" (i.e., not a constant area experiment). This result indicates that IgG may provide physical stabilization of the membrane, which is an important attribute for preparation of lipid-based biosensors. Furthermore, this physical association is likely responsible for the adherence of IgG to PC/DPPA BLMs in flowthrough experiments, and the stability of IgG in BLMs under flowing solution conditions (similar to flow injection analysis) suggests that the protein is relatively firmly anchored to membranes (25).

Effect of Incubation Time

Changes of lateral pressure caused by insertion of IgG into a lipid monolayer at various incubation times for a protein:lipid ratio of 1:100 are shown in Fig. 5. Equilibration of the absorption process requires a time of approx 25 min for the conditions stated. These monolayers were then stable for periods of a few hours.

Both fresh IgG and aged denatured (inactive as determined spectroscopically) IgG were deposited in separate experiments at a protein:lipid ratio of 1:100 into monolayers that contained NBD-PE and were then held at a surface pressure of 25 m*N*/m. After a period of 12 h, the surface pressure was observed to increase to a final value of 30 m*N*/m. The images

collected after this 12-h period showed a marked reduction in contrast between bright and dark regions, and showed a significantly different distribution of domains in terms of size and shape from those of Fig. 3B. The results indicate that the phase structure present at the air–water interface is in a metastable state over a time of a few hours, that it can take days to achieve equilibration, and that the phenomenon is not directly related to denaturation of the binding sites on the IgG. The time dependence does not necessarily detract from the use of lipid membrane transducers. The lipid membranes are easy to fabricate and are reproducible in the fabrication process. Freshly prepared membranes are preferred in any case to limit problems, such as chemical oxidation, accumulation of dust and impurities, loss of biochemical activity of selective binding reagents, and now the problem of equilibration of structure to a final stable state.

CONCLUSIONS

IgG can spontaneously integrate with a lipid monolayer composed of DPPC/DPPA when the membrane is held at low pressure. Incorporation requires about 25 min to reach equilibrium, and maximum loadings of protein require a protein:lipid ratio of approx 1:100. If the lateral pressure of a monolayer is maintained in the range of 25 m*N*/m or less, then the IgG may associate with the longitudinal axis relatively perpendicular to the membrane and with retention of binding affinity. Cautions associated with the preparation and study of biosensors that are based on lipid membranes include recognition that external forces or chemical interactions that cause pressure increases within lipid membranes can force the exclusion of IgG from the lipid matrix, and that lipid monolayers prepared by the methods described herein exist in a metastable state that relaxes over a period of many hours.

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