Binding of Avidin Modified Antibody to Biotinylated Metal Supported Membranes and Liposomes Changes the Physical Properties of Lipid Bilayer

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Abstract. Various methods have been applied to study the physical properties of metal supported bilayer lipid membranes (s-BLM) and large unilamellar liposomes containing biotinylated phospholipids during the binding of IgG modified by avidin. Electrostriction method applied to s-BLM showed that addition of avidin-IgG (A-IgG) complex in a small concentration (0.2 μmol/l) resulted in an approximately twofold decrease of membrane capacitance, C, increase of elasticity modulus in direction perpendicular to the membrane plane, $E_\perp$, by 5–20 %, increase in intrinsic membrane potential, $\Delta\Phi_m$, by $\sim$ 30 mV, and an approximately 5–15 % increase of the coefficient of dynamic viscosity, η. The method of ultrasonic velocimetry showed that addition into the suspension of liposomes of unmodified IgG did not induce any changes in ultrasound velocity, while addition of A-IgG complex in a concentration range of 0–1 μmol/l led to an increase of the velocity number $[u]$. The plot of changes of $[u]$ versus A-IgG concentration has a tendency to saturate at concentrations above 0.5 μmol/l, which suggests long-range interactions in the membrane induced by strong binding of A-IgG to the biotin sites on the membrane. Probably, this binding leads to a decrease of the coefficient of adiabatic compressibility of liposomes.

Key words: Antibody — Avidin — Biotin — Lipid bilayer — Electrostriction — Ultrasonic velocimetry

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Introduction

Among a number of interesting properties of liposomes, their capability to be targeted by macrophages seems to be most exciting Liposome surface can be modified by antibodies and such a system can serve (for example in immunodeficiency) as a tool in therapy (for review see Claassen 1996) Another important application of antibody modified liposomes is their use in immunoanalysis (Durst and Reeves 1995) Convenient, free-standing bilayer lipid membranes (BLM) (Mueller et al 1962) and recently developed solid supported bilayer lipid membranes (s-BLM) (Tien and Salamon 1989) upon suitable modification by antibody can also be use as an immunosensors (Castillo et al 1966, Nikolelis et al 1993, 1995, Wang et al 1995) One of the most important questions in these works has been how to bind antibody (Ab) to the membrane surface and how this binding would influence the physical properties of lipid bilayer To bind antibody to the membrane surface, the powerful avidin-biotin technology can be used (Wilchek and Bayer 1990) Avidin is known to form strong complexes with bilayer containing biotinylated phospholipids (Noppl-Simson and Needham 1996) We can therefore expect avidin modified Ab to strongly interact with lipid bilayer of s-BLM and liposomes containing biotinylated phospholipids This method has been already successfully used for immobilization of glucose oxidase on s-BLM (Hianik et al 1996a)

In the present work, we studied the interaction of avidin modified human IgG (A-IgG) with s-BLM and with large unilamellar liposomes composed of crude cephalin fraction modified by biotin We applied the electrostriction method to s-BLM, which allowed to determine changes in elasticity modulus in direction perpendicular to the membrane surface, $E_{\perp}$, electrical capacitance, C, coefficient of dynamic viscosity, η, and changes in intrinsic membrane potential, $\Delta\Phi_m$, following addition of A-IgG An ultrasonic velocimetry method was used for liposome suspension This method has already proved as an effective tool to study the binding of various compounds with liposomes (for review see Hianik et al 1996b)

Materials and Methods

Preparation of s-BLM, liposomes, chemical modifications of lipids and antibodies

s-BLM were formed on freshly cut tip of Teflon coated stainless steel wire, diameter 0 33 mm (Leico Industries Inc , New York, USA), according to Tien and Salamon (1989) Contact to one side of s-BLM was maintained through the wire, while a saturated calomel electrode (SCE) was used to establish contact to the other side through the aqueous phase 0 1 mol/l KCl + 0 1 mol/l Tris-HCl (pH 7 4) s-BLM were formed from crude cephalin fraction isolated according to Folch et al (1957) Lipids were modified by D-biotin-N-hydroxy-succinimide ester (Merck, Darmstadt,

Germany) according to Rivnay et al. (1987). The degree of modification was controlled using TLC. Lyophilised human gamma globuline (IgG) (Imuna Šarišské Michaľany, Slovakia) was conjugated with avidin (Molecular Probes Inc., Eugene, USA) via glutaraldehyde (Sigma, St. Louis, USA) (see Rivnay et al. 1987; Wilchek and Bayer (1990) for description of avidin-biotin technology). Reaction products were checked using electrophoresis (Laemli 1970). Large unilamellar liposomes (of approximately 100 nm in diameter) were prepared by extrusion method according to MacDonald et al. (1991). Liposomes were formed from a mixture of crude cephaline fraction and that modified by biotin (1:1 w/w). The total concentration of lipids was 2.5 mg/ml in 10 mmol/l phosphate buffer (pH 7.4). All chemicals used were of analytical grade.

Detection of the binding of A-IgG was done by the method of electrostriction and ultrasonic velocimetry.

Electrostriction method

Electrostriction method gives the possibility to simultaneously determine the Young modulus of elasticity in direction perpendicular to the membrane plane, $E_\perp$, membrane capacitance, C, coefficient of dynamic viscosity, η, and intrinsic membrane potential, $\Delta\Phi_m$. Both $E_\perp$ and membrane capacitance (C) were measured by applying an ac voltage (amplitude $U_0 = 50$ mV and frequency $f = 1$ kHz) to the lipid bilayer. Membrane capacitance is given by

$$C = I_1/2\pi f U_0 \tag{1}$$

where I_1 is the amplitude of the 90 – degree component of the first current harmonic. The modulus of elasticity perpendicular to the membrane surface $E_\perp$ is given by

$$E_\perp = -p/(\Delta d/d) \tag{2}$$

where $\Delta d/d$ is the relative change in membrane thickness resulting from the application of pressure p. In our setup, this pressure results from the applied ac voltage (electrostriction), and induces a time dependent change of bilayer thickness reflected in a third harmonic component of the current (amplitude I_3, see Hianik and Passechnik 1995). Using $p = C_s U_0^2/2d$ for the electrostrictive pressure, where C_s is the specific BLM capacitance per unit area, the Young modulus can be calculated from

$$E_\perp = C_s U_0^2 I_1/4dI_3 \tag{3}$$

For this calculation we used $C_s = 4 \times 10^{-3}$ F/m^2 and $d = 4.6$ nm (average values of C_s and d from paper by Tien and Salamon 1989), representing values for a "final state" of the membrane. (This procedure to calculate the value of Young modulus

is valid only when all other factors which could affect $E_\perp$ (Hianik and Passechnik 1995), such as changes of membrane area, are excluded) An additional valuable parameter for such an analysis is phase shift φ, defined as phase difference between the measured third harmonic component and that expected for an ideally elastic body The phase shift can vary from 0 degrees for an ideal, loss-free membrane to 90 degrees for a purely viscous body The value of phase shift φ and the elasticity modulus $E_\perp$ determine the coefficient of dynamic viscosity η In the case of linear frequency-dependent modulus of elasticity $E_\perp$, (holds for $E_\perp(f)$ around $f = 1$ kHz, see Hianik and Passechnik 1995) the following relationship can be used to determine coefficient of dynamic viscosity

$$\eta = E_\perp \sin\varphi/2\pi f \tag{4}$$

The value of η can be interpreted as internal friction between hydrocarbon chains of phospholipids during periodical compression of the membrane by electrostriction pressure

Two different sources of potentials are present in the system electrode potentials, ΔU_{el}, and bilayer surface, or intrinsic potentials, $\Delta\Phi_m$ For a classical BLM separating two aqueous phases the electrode potentials can generally be closely matched, making their net contribution in the total circuit very small With supported membranes, however, there is an inherent asymmetry in the electrode materials (stainless steel wire vs calomel electrode) and environments (lipid vs aqueous phase) Thus, the electrode potentials on the two sides of the bilayer may be quite different, and cannot be determined easily Similarly, the different environments at the two faces of the BLM will introduce further asymmetry into the system Both the configurations of the head group layer in contact with the metal support, and the electrical interaction with the support (e g mirror charges) will differ from the situation at the aqueous boundary In general, there will be both surface charge potential and surface dipole potential at each BLM face Of these two components only the Gouy-Chapman surface charge potential (U_{GC}) at the aqueous boundary can be determined independently (Hianik and Passechnik 1995) The changes in intrinsic potential can be determined using equation

$$\Delta\Phi_m = -U_1 + U_0 I_2/4I_3 \tag{5}$$

where I_2 is the amplitude of the second harmonic of the membrane current with frequency $2f$, which is generated if the membrane is compressed simultaneously both by *ac* and *dc* voltage U_1 is *dc* voltage externally applied to the BLM The method of measurement of $\Delta\Phi_m$ is based on simultaneous determination of the amplitudes of the current harmonics I_2 and I_3 (see Hianik and Passechnik 1995)

Measurements of the electromechanical and electrical parameters were carried out under the control of an IBM PC/AT 286 computer, and were done at $T = 20$°C

Ultrasonic velocimetry

The method of ultrasonic velocimetry is based on the relation between velocity of ultrasound u, density ρ, and adiabatic compressibility β of the "solvent" (Stuehr and Yeager 1965)

$$u^2 = 1/\rho\beta \tag{6}$$

Both density and adiabatic compressibility can reflect the peculiarities of the structure of liposomes if they serve as the acoustic medium Density can be measured by an independent method, e g an oscillation densitometer (Kratky et al 1973), whereas adiabatic compressibility can be determined by means of ultrasonic velocimetry (Sarvazyan 1991) Parameter β is directly related to the volume compressibility of a given solution or dispersion, e g microemulsion particles such as li posomes Ultrasonic velocity was measured using a differential fixed path velocimeter with acoustic resonators (Sarvazyan 1982) Determination of the resonance parameters was based on measuring the midpoints and widths of the phase-frequency curves of the resonance peaks by means of a home-made electronic circuit based on phase-frequency feedback circuits that were computer-controlled The resonators contained a sample volume of 0 7 ml and were equipped with magnetic stirrers for continuous stirring of the solution during the measurements One resonator contained the lipoprotein solution, the reference resonator contained the blank (10 mmol/l phosphate buffer) The experiments were performed at 7 2 MHz The magnitude of the alternating pressure in the ultrasonic wave was less than 0 01 bar This allowed avoiding the influence of ultrasound on structural transition of biocolloids and also to exclude any inhomogeneous heating effects from absorption which could distort the standing wave field in the liquid, filling the velocimeter cavity, and shift the resonance peaks Frequency f of a resonance peak of the acoustic resonator is related to sound velocity, u (Sarvazyan 1982)

$$\Delta u/u = (\delta f/f)\,(1+\gamma) \tag{7}$$

where Δu is change in sound velocity caused by changes of physical properties of the measured liquid, γ is a correction term ($\gamma < 0\ 003$) which can be omitted from eq (7) for the type of resonator used in this work The relative precision of measurements of velocity at a single temperature was about 2×10^{-4} % The measurements were performed at $T = 25$°C Cells were thermostated with Lauda RK8CS ultrathermostat

The theory of ultrasonic velocimetry has been described in a number of reviews (see e g Sarvazyan 1991) Below listed are the basic equations referred to in this work for the so-called velocity number $[u]$

$$[u] = (u - u_0)/u_0 c \tag{8}$$

where c is the concentration of the solution expressed in mg/ml (in our experiments it was the concentration of lipids), and the index "0" refers to solvent (buffer). Velocity number $[u]$ is related to adiabatic compressibility of diluted particles. The compressibility of diluted solutions of proteins or liposomes is usually expressed using the value of the apparent compressibility Φ_k

$$\Phi_k = (\beta V - \beta_0 V_0)/CV \tag{9}$$

where β, β_0 are adiabatic compressibilities, V and V_0 are sample volumes, and C is the molar concentration. For very dilute solutions,

$$\Phi_k/\beta_0 \approx -2(u/u_0)/u_0 C - M/\rho_0 = 2\Phi_V \tag{10}$$

where M is molecular mass of the dispersed particles, ρ_0 is the density of the solvent, and Φ_V is the apparent molar volume of solution, or for specific values,

$$\varphi_k/\beta_0 = -[u] - 1/\rho_0 = 2\varphi_V \tag{11}$$

where φ_k/β_0, φ_V are the specific apparent compressibility and the volume of the solution, respectively.

Results and Discussion

1. Study of binding properties of A-IgG to s-BLM

Addition of A-IgG complex into the electrolyte at a final concentration of 0.2 μmol/l resulted in considerable changes of macroscopic parameters of the membrane. Elasticity modulus $E_\perp$, coefficient of dynamic viscosity η, phase shift φ and intrinsic membrane potential $\Delta\Phi_m$ increased, whereas membrane electrical capacitance, C, decreased (Fig. 1). The close contact of avidin modified IgG with biotin binding sites at the membrane surface resulted in immobilization of the polar part of the membrane. Probably, this chelation-like interaction decreases the specific area per phospholipid, and thus increases the ordering of the hydrophobic part of the membrane. As a result, an increase of $E_\perp$ occurs. The measured value of the phase shift φ of s-BLM was around 50°, i.e. evidencing the usual character of membrane deformation as a viscoelastic body (Hianik et al. 1996c). The increase of the coefficient of dynamic viscosity η upon the addition of A-IgG reflects the increase in the friction of the hydrocarbon chains due to an increased ordering of the membrane. The membrane capacitance decreased almost twofold. This suggests that the binding of A-IgG to the membrane led to the formation of an additional protein layer with a thickness comparable to that of the hydrophobic moiety of the membrane (i.e. 5

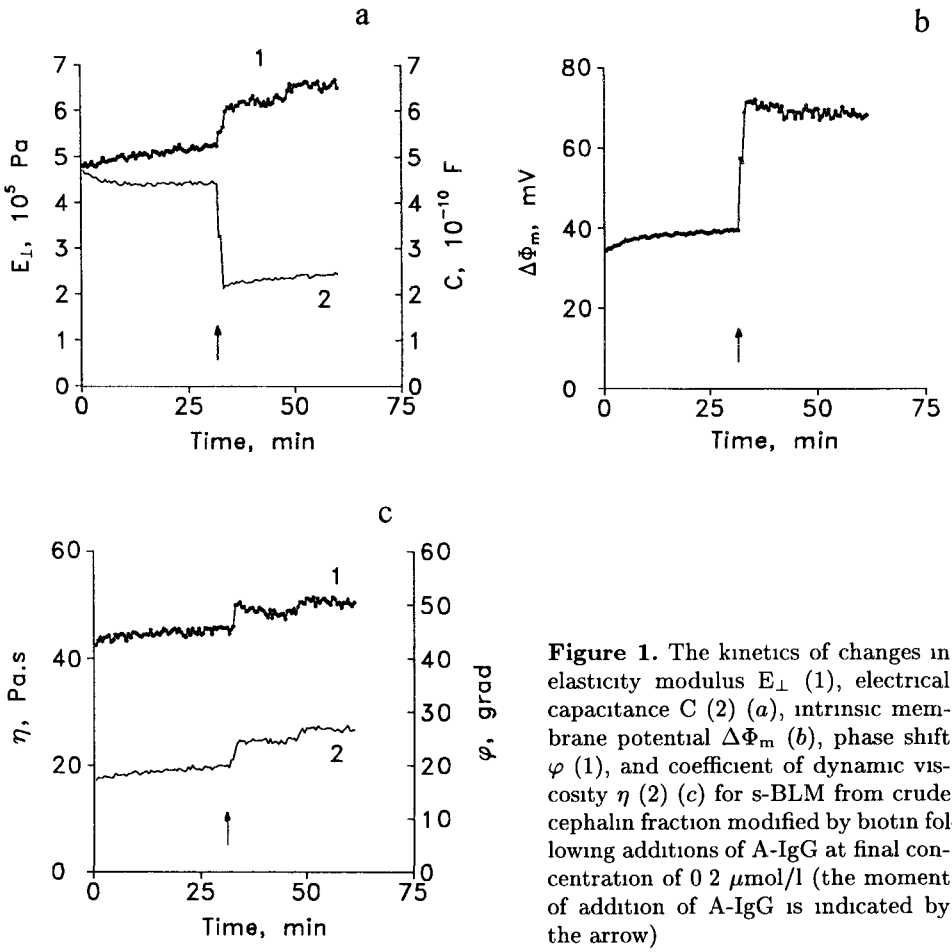

Figure 1. The kinetics of changes in elasticity modulus $E_\perp$ (1), electrical capacitance C (2) (*a*), intrinsic membrane potential $\Delta\Phi_m$ (*b*), phase shift φ (1), and coefficient of dynamic viscosity η (2) (*c*) for s-BLM from crude cephalin fraction modified by biotin following additions of A-IgG at final concentration of 0 2 μmol/l (the moment of addition of A-IgG is indicated by the arrow)

nm). Addition of unmodified IgG at a final concentration of up to 50 μmol/l did not change parameters of s-BLM.

We could show that addition of A-IgG to the electrolyte resulted in an increase of the intrinsic membrane potential $\Delta\Phi_m$. Unmodified membrane is characterized by intrinsic membrane potential $\Delta\Phi_m \sim$ 40 mV (negative end of the wire). The relatively large value of $\Delta\Phi_m$ is connected with s-BLM asymmetry (Hianik et al. 1996c). An increase of $\Delta\Phi_m$ is very probably due to neutralization of the negative charge of biotinylated phospholipids upon the binding of positively charged avidin (at neutral pH avidin bears positive, whereas biotin negative charge, see Leckband et al. 1994). Thus, the binding of A-IgG induces further asymmetry of surface potential, which is reflected in an increase of the intrinsic potential changes. If we

assume that the changes of the value of $\Delta\Phi_m$ are due to changes of the Gouy-Chapman potential (U_{GC}) then we can roughly estimate the changes of surface charge density σ of the monolayer due to binding of A-IgG Surface charge density is connected with the value of U_{GC} by the following relation (Sargent and Hianik 1994)

$$\sigma = [2\varepsilon_0\varepsilon RTc\,(\exp(-U_{GC}F/RT) - 1)]^{1/2} \qquad (12)$$

where c is the electrolyte concentration, ε_0 and ε are dielectric permittivity of free space and relative dielectric permittivity of the polar part of the membrane respectively, F is Faraday constant, R is gas constant, and T is absolute temperature Assuming of $\varepsilon \sim 10$ (typical for polymers, see e g Schoch and Sargent 1980), from equation (12) we receive changes of surface charge density due to binding of A-IgG complexes to s-BLM $\Delta\sigma \sim 3\,35 \times 10^{-2}$ elementary charges/nm^2 Having the exact number of elementary charges per binding site the number of complexes can be determined which are bound to s-BLM surface For example, if 1 binding site (one binding site of avidin per binding site of biotin) neutralizes one elementary charge, and if one A-IgG complex is connected with s-BLM by two binding sites (as revealed from the structure of the avidin, see Wilchek and Bayer 1990), then there is one A-IgG complex per $\sim$ 60 nm^2 Taking into account that the cross – section of the avidin molecule is approximately 30 nm^2, then our evaluation seems quite realistic

Fig 2 shows the dependence of relative changes of elasticity modulus on A-IgG concentration We can see that this dependence starts saturating beyond $c = 0\,2$ μmol/l A-IgG

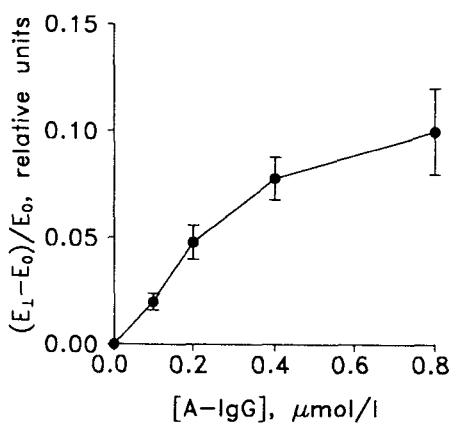

Figure 2. The dependence of relative changes of elasticity modulus ($E_\perp$-E_0)/E_0 (E_0 is the initial elasticity modulus of membrane, and $E_\perp$ corresponds to the equilibrated elasticity modulus of membrane after addition of A-IgG) on A-IgG concentration Results represent mean ± S E calculated for 10 membranes at $c = 0\,2$ μmol/l and 3 membranes for other concentations

The changes of membrane mechanical properties bring evidence for a considerable influence of A-IgG binding process on the membrane ordering We can expect

that the changes of membrane ordering at the A-IgG binding site might subsequently spread on a large membrane area. As a result, s-BLM can be characterized as a new structural state different from untreated membrane. This conclusion is supported by the recently obtained results of interaction of monoclonal antibodies with s-BLM formed on smooth gold layer covered by alkylthiols (Hianik et al. 1998a).

2. Application of ultrasonic velocimetry to the study of A-IgG interaction with liposomes composed of biotin-modified phospholipids

In order to check the possible changes of the physical parameters of membranes during interaction with A-IgG, we measured the changes of the velocity number $\delta[u] = \delta(\Delta u/u_0 c)$ (c is the concentration of the lipid). The addition of unmodified IgG into liposome suspension did not induce any changes in the parameter $[u]$ (Fig. 3*a*). We should note that unmodified IgG did not influence the parameters of s-BLM either. However, when A-IgG was added, $[u]$ started increasing at relatively low concentrations of this complex (Fig. 3*b*). We can see that parameter $[u]$ grows with the increasing concentration of A-IgG; however, already at $\sim$ 0.5 μmol/l, this dependence starts deviating from linearity. This result is in good coincidence with that obtained on s-BLM The maximal changes of $[u]$ occurring when A-IgG concentration changes from 0 to 1 μmol/l are 0.043 ml/g (or 0.06 ml per mole of lipid). The question arises what processes could lead to the changes of $[u]$. It is seen from equation (11) that two parameters can contribute to changes of $[u]$: change of the apparent specific volume φ_V, and changes of apparent specific compressibility φ_k.

The value of apparent specific volume for diluted solutions can be expressed as a sum of two terms,

$$\varphi_V = \varphi_m + \varphi_h \tag{13}$$

where φ_m is the intrinsic specific volume of the macromolecules which is inaccessible for the surrounding water molecules, and φ_h is the hydration term determined by the change of the water density in the hydration shell of the macromolecule. The change of φ_m as a result of the binding of A-IgG on the biotin binding sites at the liposome surface could be determined by the change of the intrinsic volume of the membrane $\delta(\varphi_m)_M$, hydration of the membrane $\delta(\varphi_h)_M$, intrinsic volume of A-IgG $\delta(\varphi_m)_A$ and its hydration $\delta(\varphi_h)_A$:

$$\delta\varphi_V = \delta(\varphi_m)_M + \delta(\varphi_h)_M + \delta(\varphi_m)_A + \delta(\varphi_h)_A \tag{14}$$

An analogous equation holds for partial specific compressibility

$$\delta\varphi_k = \delta(\varphi_{mk})_M + \delta(\varphi_{hk})_M + \delta(\varphi_{mk})_A + \delta(\varphi_{hk})_A \tag{15}$$

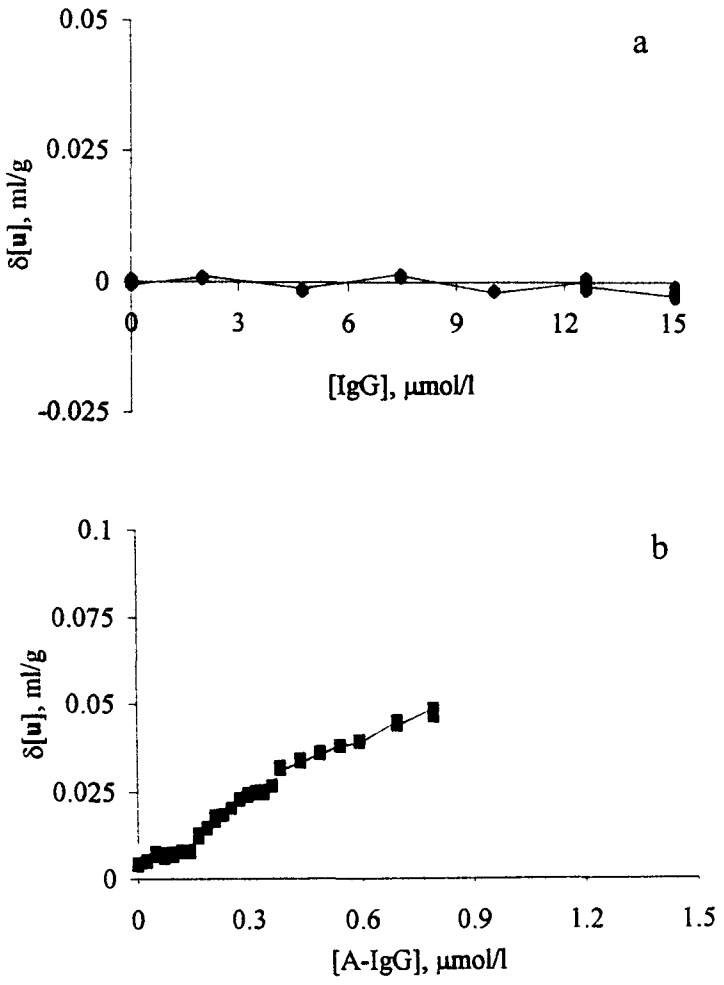

Figure 3. The dependence of changes of velocity number $\delta[u]$ on concentration of *a*) IgG and *b*) A-IgG in suspension of liposomes composed of a mixture of crude cephalin fraction and that modified by biotin (1 1 w/w) Electrolyte 10 mmol/l phosphate buffer, pH 7 4, $T = 25\,^{\circ}C$ The data are means from 5 independent measurements for one liposome sample The standard deviation of the $[u]$ was approximately 0 15%, which is smaller than the size of the symbols

where φ_{mk} is the intrinsic compressibility (compressibility of volume φ_m) and φ_{hk} is the hydration contribution resulting from the changes of compressibility of water in the hydration shell as a result of solute-solvent interaction. Let us consider all the four terms in equations (14, 15).

Changes of A-IgG and membrane hydration

There is no reason to expect sufficient changes in hydration of A-IgG molecules as a result of their binding to the membrane. Usually, in association processes hydration decreases. This leads to a negative value of $\delta[u]$ (Buckin 1988) as a result of the release of the hydration water which is normally less compressible than bulk water. In our case, we have positive value of $\delta[u]$.

Changes of internal volume and compressibility of A-IgG

The value of $\delta[u]$=0.043 ml/g recalculated per 1 g of A-IgG gives the value of the changes in the ultrasonic velocity increment of protein amounting to 0.64 ml/g. For most globular proteins, specific partial volume φ_V at 25°C is $\sim$ 0.71 ml/g in average (Zamyatnin 1973). If the whole value of $\delta[u]$ were determined by changes of protein volume, the volume of A-IgG would have to increase by 90% as a result of its binding to the liposome surface. Obviously, this is not realistic.

The coefficient of adiabatic compressibility of the protein globule β is about 1.5×10^{-11} Pa^{-1} (Sarvazyan and Kharakoz 1977). That gives the following contribution to the value of ultrasonic velocity increment of A-IgG: $\beta\varphi_V/2\beta_0 = 0.11$ ml/g ($\beta_0 = 4.45 \times 10^{-11}$ Pa^{-1} at 25°C). This is less than the measured effect related to 1 g of A-IgG complex (i.e. 0.64 ml/g). In addition, it does not seem realistic to assume the existence of considerable changes of volume compressibility of A-IgG due to the binding of this large macromolecular complex to relatively small biotin binding sites on the membrane. Even if some decrease of volume compressibility of A-IgG takes place due to restriction of conformational mobility of the groups of A-IgG at binding sites, such changes are at least not dominant.

Changes of internal volume and compressibility of membrane

The volume occupied by phospholipid molecules in the lipid membrane is about 700 ml/mol (Marsh 1990). This means that the measured value of $\delta[u]$ corresponds to an increase by $\sim 10^{-2}$% of the lipid bilayer volume as a result of A-IgG binding. These are, of course, very small changes, which can be realised without problems. For comparison, we may mention that changes of membrane volume in gel-liquid crystalline phase transition of DPPC are about 5.9% (Marsh 1990).

Let us evaluate the possible contribution of the A-IgG binding to the coefficient of adiabatic compressibility of liposomes. Being transformed into changes of coefficient of compressibility the measured $\delta[u]$ gives: $\delta\beta = -2\beta_0\delta\,[u]/\varphi_V = 0.39 \times 10^{-11}$ Pa^{-1}, where φ_V is the specific molar volume occupied by phospholipids in the membrane (We took $\varphi_V = 0.988$ ml/g measured for POPC liposomes. The values of φ_V only weakly depend on lipid composition (Hianik et al. 1998b)). This means that a decrease by $\sim$ 1% in membrane compressibility as a result of A-IgG binding is sufficient to explain the measured value of $\delta[u]$.

Thus, according to our analysis the main reason for changes of $\delta[u]$ in dependence on A-IgG concentration is the influence of A-IgG on the membrane volume compressibility This is supported by the above presented results on the increasing elasticity modulus $E_\perp$ of s-BLM due to A-IgG binding From the shape of the dependence of $\delta[u]$ on A-IgG concentration the following conclusions would be made

At relatively small concentrations of A IgG ($< 0\ 5$ μmol/l), $\delta[u]$ practically linearly depends on A-IgG concentration, i e each A-IgG complex gives an additive contribution to the changes of the overall volume compressibility of the membrane It can be imagined that each complex induces a distortion of the structure of certain area of the membrane (Hianik and Passechnik 1995) The point ($c \sim 0\ 5$ μmol/l) at which the dependence $\delta[u]$ starts shifting from linearity towards saturation can be considered as the concentration of A-IgG at which the regions of distorted membrane structure start overlapping The concentration of lipid used ($\sim 3\ 8$ mmol/l) corresponds to the concentration of liposomes of $\sim 10^{-8}$ mol/l ($c = 3\ 8$ mmol/lN, where N is number of lipid molecules in liposome, which is $\sim 4 \times 10^5$ for liposome with an average diameter of ~ 100 nm and an area per phospholipid molecule of $\sim 0\ 6$ nm^2) Thus, at the critical concentration of A-IgG $\sim 0\ 5$ μmol/l there are approximately 50 A-IgG complexes per 1 liposome, i e the area of distorted mem brane structure per 1 complex is approximately 630 nm^2 This area corresponds to a circle with a diameter of ~ 28 nm, i e six times larger than the thickness of the membrane This area is larger than that obtained from s-BLM experiments However, in the former case there is uncertainty in the evaluation of the relative dielectric permittivity of the polar part of the membrane as well as of the number of elementary charges per one binding site In addition, the changes of intrinsic potential may not only be connected with changes of Gouy-Chapman potential but also with dipole potential of the outer monolayer of s-BLM (in contact with the electrolyte) We can, however, conclude that the binding of the A-IgG complex to the membrane leads to changes in the properties of the lipid bilayer which are not only restricted to the dimensions of the immediate contact of A-IgG with biotin binding sites (i e the area, which corresponds to approximately 30 nm^2 – the cross-section of one avidin molecule – see Noppl-Simon and Needham 1996), but are spread over a considerably larger area

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