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Binding and mobility of anti-dinitrophenyl monoclonal antibodies on fluid-like, Langmuir-Blodgett phospholipid monolayers containing dinitrophenyl-conjugated phospholipids

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The association of a fluorescently labelled anti-dinitrophenyl monoclonal antibody (ANO2) with Langmuir-Blodgett monolayers composed of three different binary mixtures of phosphatidylcholine and dinitrophenyl-conjugated phosphatidylethanolamine has been characterized. Quantitative fluorescence microscopy measurements demonstrated that measurable amounts of antibodies bound to the monolayers only at high molar fractions of dinitrophenyl-conjugated lipid ($\geq$ 5 mol%). Fluorescence pattern photobleaching recovery measurements showed that the apparent translational diffusion coefficients and mobile fractions of a fluorescent lipid were high for all monolayer compositions and that the antibody translational mobility was measurable but slow and depended on the two-dimensional antibody density. The results demonstrate that the ANO2-binding characteristics of Langmuir-Blodgett monolayers containing dinitrophenyl-conjugated phospholipids are substantially different from those of similar model systems but that the ANO2 antibodies, when bound, display similar diffusive behavior.

Introduction

Protein and lipid diffusion in and on biological cell membranes has been the subject of extensive investigation in recent years (reviewed in Refs. 1–3). Although numerous measurements of the rates of protein diffusion in and on cell membranes have been reported, the physical and chemical principles that govern these rates are not yet well understood. Defining the factors that influence molecular mobility, such as membrane composition, protein structure, protein–lipid interactions, and protein–protein interactions, is important in understanding the dynamics of cell surface processes.

One promising approach towards a better understanding of protein and lipid diffusion in and on two-dimensional fluid phospholipid membranes is to use phospholipid Langmuir-Blodgett films or similar types of phospholipid model membranes deposited on planar substrates (reviewed in Refs. 4–6). The physical properties of these planar membranes have been well-characterized (see, for example, Refs. 7–10), and the specific binding of a wide range of soluble proteins to molecular sites in the membranes has been demonstrated (see, for example, Refs. 11–17). The planar geometry is well-suited for measurements of protein and lipid translational mobility made with fluorescence pattern photobleaching recovery [18] and to measurements of the mobilities of loosely bound proteins made with fluorescence photobleaching recovery using total internal reflection illumination [19,20]. The use of planar membranes with these dynamic techniques in fluorescence microscopy provides a systematic method of investigating the effects of membrane structure and composition on protein and lipid translational mobility.

One model system of general and immunologic interest is that of antibodies associated with 'antigenic' supported planar membranes. Some desirable characteristics of these model immunologic systems for fluorescence microscopy are that the antibodies specifically bind to incorporated antigenic or haptenic molecules and that the translational mobilities of the membrane constituents and bound antibodies be measurable by fluorescence photobleaching recovery for a variety of experimental conditions.

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Previous work has shown that anti-hapten antibodies specifically bind to phosphatidylcholine Langmuir-Blodgett films that contain phosphatidylethanolamine conjugated with haptens such as dinitrophenyl (DNP) [21–27], trinitrophenyl (TNP) [28–32], nitroxide (NO) [7,22,33], and dinitropnenylnitroxide (DNP-NO) [34,35]. Measurements of the translational mobilities of antibodies bound to supported monolayers and bilayers containing NO- [33], DNP-NO- [34,35], and TNP-conjugated [32] phosphatidylethanolamine have shown that the bound antibodies are usually mobile on fluid-like films and immobile on solid-like films. On fluid-like films, the average apparent antibody translational diffusion coefficients are approximately equal to those of the lipids at low antibody densities but at higher antibody densities the translational mobility can be considerably slower and/or inhomogeneous. Film-bound antibodies may also spontaneously arrange into two-dimensional ordered arrays under some conditions [23–25].

Characterization of the mobility of antibodies bound to Langmuir-Blodgett films [32–35] has previously been restricted to planar membranes deposited on optically nontransparent substrates [32,35] which are not suitable for total internal reflection fluorescence microscopy, or to planar membranes that contain NO- or (DNP-NO)-conjugated phospholipids [33–35] where the effect of the spin label on lipid and antibody fluorescence intensities [35] complicates data analysis. Other studies have been on solid-phase lipid systems [26] in which antibody and lipid translational mobility is negligible over the distances (μm) and times (seconds to minutes) accessible to conventional fluorescence photobleaching techniques.

This work describes the association of the anti-DNP mouse IgG1 monoclonal antibody ANO2 with supported phospholipid monolayers deposited on tetradecyltrichlorosilane-treated fused quartz. The translational mobility of fluorescently labelled, bound ANO2 is characterized for three different fluid-like binary mixtures of phosphatidylcholine (PC) and DNP-conjugated phosphatidylethanolamine (DNP-PE). The results suggest that the ANO2-binding characteristics of these Langmuir-Blodgett monolayers are substantially different from those of other model systems but that the ANO2, when bound, displays diffusive behavior which is approximately equivalent to that previously observed.

Materials and Methods

Antibodies. The mouse monoclonal IgG1 antibody ANO2 [36] was purified from hybridoma supernatants by affinity chromatography with DNP-conjugated human serum albumin (Sigma Chemical Co., St. Louis, MO) [27]. Although raised against a DNP-NO conjugate, ANO2 antibodies bind with signiticant affinity to DNP [37]. Polyclonal sheep IgG (Sigma) was obtained commercially. ANO2 antibodies were labelled with fluorescein isothiocyanate (Molecular Probes, Inc., Eugene, OR) (F-ANO2) as described [34]. All antibodies were dialyzed extensively against phosphate-buffered saline (PBS, 0.05 M sodium phosphate, 0.15 M sodium chloride, 0.01% sodium azide, pH 7.4), passed through a 0.22 μm filter, and clarified at 100000 × g at 4°C for 2 h. Antibodies were judged to be pure by SDS-PAGE with Coomassie blue staining.

Supported phospholipid monolayers. Dimyristoylphosphatidylcholine (DMPC) (Calbiochem Corp., San Diego, CA), egg phosphatidylcholine (egg PC) (Sigma Chem. Co. St. Louis, MO), and *N*-dinitrophenyl-(egg phosphatidylethanolamine) (DNP-(egg PE)), *N*-dinitrophenylaminocaproyl-(egg phosphatidylethanolamine) (DNP-cap-(egg PE)), *N*-dinitrophenyldipalmitoylphosphatidylethanolamine (DNP-DPPE), and 1-acyl-2-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]phosphatidylcholine [NBD-PC] (Avanti Polar Lipids, Inc., Pelham, AL) were judged pure by thin-layer chromatography and used without further purification. Phospholipid concentrations were determined by assaying for phosphorus (DMPC and egg PC) [38] or spectrophotometrically (DNP- and NBD-conjugated phospholipids) by using DNP and NBD molar absorptivities equal to 17 mM^{-1} cm^{-1} at 362 nm [39] and 24 mM^{-1} cm^{-1} at 464 nm [40].

Substrate-supported phospholipid monolayers were constructed as previously described [7,26,27]. Known amounts of phospholipids in hexane/ethanol (9 : 1, v/v) were spread at room temperature on water (18 MΩ-cm) in a Langmuir trough (Vickers Model 4) at 150 $Å^2$/molecule and were compressed to 30 dyn/cm at 1 to 3 $Å^2$/molecule per min. Monolayers were deposited at 5 mm/min and at constant pressure on 3 to 6 fused quartz slides (Quartz Scientific, Fairport Harbor, OH; 1 inch × 1 inch × 1 mm) that had been treated with tetradecyltrichlorosilane (Lancaster Synthesis, Windham, NH) [35].

In some control measurements, monolayers containing DNP-PE were washed from substrates with 3 ml methanol. The eluent from two substrates was combined, the solvent was removed by evaporation with N_2, the lipids were redissolved in 0.5 ml chloroform/methanol (1 : 1, v/v), and the DNP concentrations were determined by absorption spectroscopy at 362 nm.

Fluorescence microscopy. For measurements in the absence of antibodies, supported monolayers were transferred from the Langmuir trough to a microscopy sample holder containing PBS [34]. For measurements in the presence of antibodies, supported monolayers were washed with 1 ml PBS, treated with sheep IgG in PBS (200 μl, 200 μg/ml, 10 min), treated with ANO2 or F-ANO2 in PBS (200 μl, 100 μg/ml, 30 min), washed with 3 ml PBS, and then mounted in PBS for microscopy. In some cases (only as specified), monolayers

were not pretreated with sheep IgG, were treated with higher (200 μg/ml) or lower (10 μg/ml) concentrations of F-ANO2, were treated with F-ANO2 for longer times (1 h) or were treated with F-ANO2 solutions containing 3 mM DNP-glycine (Sigma).

The average fluorescence intensities of NBD-PC and F-ANO2 associated with supported monolayers were measured at 30°C and room temperature, respectively. The translational mobility of NBD-PC and F-ANO2 was examined by fluorescence pattern photobleaching recovery (FPPR) [18] at 30°C as described [34]. Parameters for intensity measurements were as follows: laser wavelength, 488.0 nm; objective, 40 × 0.75 N.A.; laser power, 5–50 μW; radius of illuminated and observed area, 75 μm. Additional parameters for FPPR were as follows: bleaching laser power, 0.5 W; bleaching pulse duration, 100–500 ms; depth of bleaching, 30–80%; ruling periodicity in the sample plane, 16 μm; radii of illuminated and observed areas ≥ 150 μm and 38 μm, respectively. FPPR data were curve-fit to the functional forms of Eqn. 1 in Ref. 34 * to obtain best values of apparent diffusion coefficients and mobile fractions using a nonlinear curve-fitting routine.

Fluorescence intensities and recovery curves were recorded from 6 to 12 different positions on each of several different supported monolayers. Since the main source of variability in measurements was between different supported monolayers, experimental uncertainties were calculated as the standard errors in the means of the sets of numbers in which each element was an average over all measurements taken on a single coated substrate.

Results and Discussion

The association of ANO2 antibodies with supported phospholipid monolayers and the translational diffusion of fluorescent lipids and bound ANO2 were examined for three different fluid-like binary mixtures of dinitrophenyl-conjugated phosphatidylethanolamine (DNP-PE) and phosphatidylcholine (PC): *N*-dinitrophenyldipalmitoylphosphatidylethanolamine (DNP-DPPE) and dimyristoylphosphatidylcholine (DMPC); *N*-dinitrophenyl-(egg phosphatidylethanolamine) (DNP-(egg PE)) and egg phosphatidylcholine (egg PC); and *N*-dinitrophenylaminocaproyl-(egg phosphatidylethanolamine) (DNP-cap-(egg PE)) and egg PC.

Monolayers at the air / water interface

The surface pressures of all DNP-DPPE/DMPC, DNP-(egg PE)/egg PC and DNP-cap-(egg PE)/egg PC monolayers at the air/water interface monotonically increased during compression to smaller average molecular areas (Fig. 1a). The first derivatives of the curves slowly increased with compression so that the pressure-area curves were qualitatively similar to those observed for fluid-like monolayers. The pressure-area curves for DMPC agreed with previously published data [10,41]. None of the air/water interface pressure-area curves were changed by the presence of 5 mol% NBD-PC, consistent with results for similar systems [7].

At a given molar fraction of DNP-PE, the pressure-area curves of DNP-(egg PE)/egg PC and DNP-cap-(egg PE)/egg PC monolayers were equivalent within experimental error but differed significantly from those of DNP-DPPE/DMPC monolayers (Fig. 1b). At low molar fractions of DNP-PE, the average molecular areas at 5 and 30 dyn/cm were greater for DNP-(egg PE)/egg PC and DNP-cap-(egg PE)/egg PC monolayers than for DNP-DPPE/DMPC monolayers. A factor that may contribute to the difference is the heterogeneous and unsaturated nature of egg PC acyl chains. With increasing molar fractions of DNP-PE, the average molecular areas increased slightly in DNP-DPPE/DMPC monolayers but decreased slightly in DNP-(egg PE)/egg PC and DNP-cap-(egg PE)/egg PC monolayers. At high DNP-PE molar fractions, the average molecular areas were approximately equivalent for all three monolayer types, suggesting that the negative charge of DNP-PE was a dominant factor in determining molecular area.

At higher surface pressures (> 25 dyn/cm), monolayers containing large molar fractions of DNP-PE (> 0.75) were occasionally observed to undergo apparent slow collapse. Possible explanations for this behavior are loss of lipid to the subphase or formation of non-monolayer structures during compression, which could give rise to artifactually low calculated average molecular areas at high surface pressures. Therefore, the lipid surface densities in DNP-PE supported monolayers were independently estimated by washing monolayers from their substrates and measuring the DNP concentrations in the eluent. Control measurements showed that the fluorescence intensities of substrates containing DNP-PE monolayers doped with NBD-PC decreased after washing with 3 ml methanol by an average of 80%. Absorption spectroscopy measurements, averaged over several independent sample sets and corrected for DNP-PE losses due to incomplete washing, indicated that the amount of DNP-PE recovered in the methanol wash was approximately 1.2-fold higher than the amount calculated from the molecular density at the air/water interface and the substrate area. These results indicate that significant amounts of deposited lipids were not lost during air/water interface compression.

The ratios of the monolayer areas at the air/water interface that were removed during substrate coating to the known substrate areas ranged from 0.9 to 1. ,

* A typographical error in Eqn. 1 of Ref. 34 reads "$-\exp(-36\pi^2 Dt/a^2)$" rather than "$+\exp(-36\pi^2 Dt/a^2)$". All FPPR data in the work described herein and in Refs. 11, 26, 27 and 34 were analyzed with equations containing the correct sign (+).

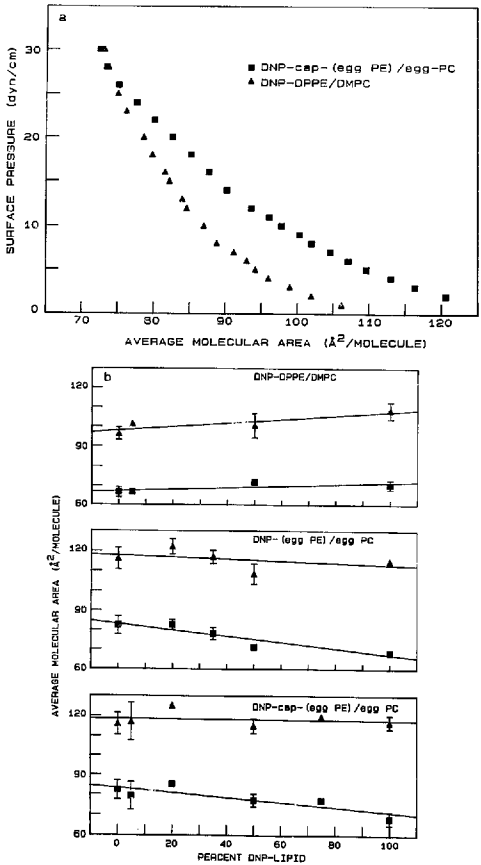


Fig. 1. Air/water interface pressure-area curves. Shown are (a) typical single compressions of equimolar mixtures of DNP-DPPE/DMPC (▲) and DNP-cap-(egg PE)/egg PC (■) and (b) the average molecular areas at 5 (▲) and 30 (■) dyn/cm obtained from three to eight independent air/water interface compressions.

consistent with previous measurements in similar phosphatidylcholine systems [7,10]. Together with other results reported herein, these ratios demonstrate that effective deposition occurs when a significant fraction or all of the phospholipids are conjugated with DNP. No difference in the coating ratio was observed for different lipid compositions.

F-ANO2 binding to supported monolayers

DNP-PE/PC supported monolayers were tested for

their ability to bind F-ANO2 antibodies. The surface fluorescence was assumed to be proportional to the density of bound F-ANO2. Previous work on similar systems has demonstrated linearity up to at least 14000 molecules/μm^2 [42] and that the saturating densities of ANO2 antibodies bound to phospholipid Langmuir-Blodgett films of similar phospholipid compositions are between 5000 and 10000 molecules/μm^2 [23,24,27,34].

DNP-(egg PE)/egg PC and DNP-cap-(egg PE)/egg PC monolayers treated with 100 μg/ml F-ANO2 for 30 min bound measurable * densities of F-ANO2 only when the amount of DNP-PE was ≥ 5 mol%, and DNP-DPPE/DMPC monolayers bound F-ANO2 only when the amount of DNP-PE was ≥ 20 mol% (Fig. 2). For all three binary phospholipid compositions, the density of bound F-ANO2 dramatically increased with the DNP-PE molar fraction above the critical DNP-PE density, and all three 100 mol% DNP-PE monolayers bound approximately equivalent densities of F-ANO2. F-ANO2 binding was judged to be specific in that negligible amounts of F-ANO2 were bound to pure PC monolayers or to DNP-PE/PC monolayers simultaneously treated with DNP-glycine at concentrations high enough to saturate the F-ANO2 binding sites. Monolayers containing bound F-ANO2 were uniformly fluorescent within optical resolution.

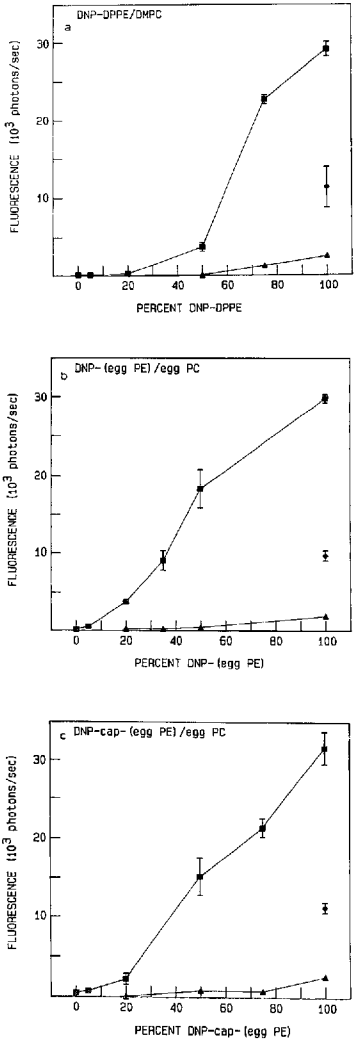
The fluorescence intensities of monolayers treated with 200 μg/ml F-ANO2 were 10–15% greater than those of monolayers treated with 100 μg/ml F-ANO2. This increase was independent of the monolayer composition within experimental uncertainty (data not shown) and is consistent with 100 μg/ml F-ANO2 representing a near-saturating solution concentration and with an apparent membrane association constant equal to that previously measured for ANO2 antibodies on supported monolayers composed of dimyristoylphosphatidylethanolamine conjugated with DNP-NO (DNP-NO-DMPE) and DMPC (32:68, mol%/mol%) ($8 \cdot 10^6$ M^{-1}) (Fig. 3, Ref. 34), DNP-cap-DPPE and DPPC (25:75, mol%/mol%) ($3 \cdot 10^6$ M^{-1}) (Fig. 6, Ref. 27), or DNP-dioleoylphosphatidylethanolamine (DNP-DOPE) and distearoylphosphatidylethanolamine (DSPC) (30:70, mol%/mol%) ($3 \cdot 10^6$ M^{-1}) (Fig. 6, Ref. 26). The ratios of the fluorescence intensities of 100 mol% DNP-DPPE, DNP-(egg PE) and DNP-cap-(egg PE) monolayers treated with 10 μg/ml relative to those of monolayers treated with 100 μg/ml F-ANO2 were 0.39, 0.33, and 0.36, respectively (Fig. 2). These values are also consistent with the previously measured ANO2 membrane association constants. Thus, for monolayers containing high amounts of DNP-PE (20–100 mol%), the apparent membrane association constant does not appear to dramatically depend on the chemical structure of the DNP-PE (e.g., DNP-DPPE, DNP-(egg PE), DNP-cap-(egg PE), DNP-cap-DPPE, (DNP NO)-DMPE, DNP-DOPE), on the chemical structure of the host phosphatidylcholine (egg PC, DMPC, DPPC or DSPC), or on the molar fraction of DNP-PE.

The fluorescence intensities arising from monolayer-bound F-ANO2 did not significantly change when monolayers were treated with 100 μg/ml F-ANO2 for one hour rather than 30 min. This result demonstrates that the interactions between F-ANO2 and the supported monolayers rapidly approached a steady-state if not equilibrium, and is consistent with reports for similar model systems where the density of anti-hapten antibodies bound to hapten-conjugated model membranes was approximately constant after several minutes [27,31].

The fluorescence intensities of bound F-ANO2 (Fig. 2), which corresponded to steady-state and saturating densities, were not measurable * for monolayers containing low molar fractions of DNP-PE (≤ 5 mol%) but increased dramatically at higher DNP-PE densities. The relative size of antibodies and lipids implies that if all of the DNP-PE was accessible to F-ANO2 binding, the saturating density should approach a maximum around 1 mol% DNP-PE and remain approximately constant for monolayers containing higher DNP-PE densities. One explanation for the observation that the monolayers do not saturate at low hapten densities (Fig. 2) is that the DNP exists in both antibody-accessible and antibody-inaccessible states [43–46]. For this phenomena to explain the low amount of bound ANO2 at low molar fractions of DNP-PE, the kinetic rates of transition between the putative accessible and inaccessible states would have to be very slow (i.e., hours) [45]. In previous work where fluorescently labelled antibodies bound to planar model membranes containing low molar fractions of hapten-conjugated lipids were detected, the antibody-hapten solution association constants were $\geq 10^7$ M^{-1} [29,31,32], whereas the solution association constant of ANO2 with DNP-conjugated haptens is somewhat lower ($3 \cdot 10^6$ M^{-1}) [27,37].

In several studies, supported monolayers have been pretreated with unlabelled, nonspecific proteins such as polyclonal IgG or bovine serum albumin to block potential nonspecific binding sites [22,26–28,33,34]. To determine if this type of pretreatment had a large effect on F-ANO2 binding to DNP-conjugated monolayers, fluorescence intensities were measured in both the presence and absence of unlabelled sheep IgG. The measured fluorescence intensities were higher by an average of 500 photons/s, averaged over all monolayer compositions, when monolayers were not pretreated with unlabelled sheep IgG (data not shown). Comparison of

* The term 'measurable' was defined as $\geq 50\%$ of the background fluorescence measured for an uncoated fused quartz slide mounted identically to those slides containing phospholipid monolayers and bound F-ANO2.



this number with the data in Fig. 2 shows that the increase in fluorescence intensity was significant only for monolayers that bound little or immeasurable amounts of F-ANO2, i.e., for DMPC monolayers with ≤ 20 mol% DNP-DPPE and for egg PC monolayers with ≤ 5 mol% DNP-(egg PE) or DNP-cap-(egg PE). Thus, for low amounts of bound F-ANO2, the majority of the measured fluorescence arises from specifically bound F-ANO2 only if monolayers are pretreated with a nonspecific, unlabelled protein, but for high amounts of bound F-ANO2 such pretreatment may not be necessary.

Fluorescent lipids in supported monolayers

Earlier work on similar systems has shown that anti-hapten antibodies bound to hapten-conjugated planar membranes are translationally mobile with diffusion coefficients equal to or less than the translational diffusion coefficients of fluorescent lipid probes in the membranes [32,34,35]. Therefore, to examine the suitability of DNP-conjugated Langmuir-Blodgett monolayers for studies of antibody translational diffusion, the translational mobility of the fluorescent lipid NBD-PC in the Langmuir-Blodgett monolayers was first investigated.

FPPR measurements indicated that NBD-PC was translationally mobile with apparent diffusion coefficients and mobile fractions indicative of fluid-like phospholipid membranes for all monolayer compositions (Fig. 3). The NBD-PC diffusion coefficient decreased slightly with increasing amounts of DNP-PE and was lower at a given DNP-PE molar fraction for DNP-(egg PE)/egg PC and DNP-cap-(egg PE)/egg PC monolayers than for DNP-DPPE/DMPC monolayers. The NBD-PC mobility is thus similar to F-ANO2 binding in that the results for DNP-(egg PE)/egg PC and DNP-cap-(egg PE)/egg PC monolayers are equivalent within experimental uncertainty but different from those for DNP-DPPE/DMPC monolayers.

Although all monolayers containing NBD-PC appeared uniformly fluorescent, the measured NBD-PC fluorescence intensities decreased significantly with increasing molar fractions of DNP-PE. The fluorescence intensities were equivalent at a given molar fraction of DNP-PE for the three sets of phospholipid compositions, except for 100 mol% DNP-PE monolayers in which the NBD-PC fluorescence was significantly higher in DNP-(egg PE) monolayers than in DNP-cap-(egg

Fig. 2. F-ANO2 fluorescence intensities on supported monolayers. The fluorescence intensities of (a) DNP-DPPE/DMPC, (b) DNP-(egg PE)/egg PC and (c) DNP-cap-(egg PE)/egg PC monolayers treated with 100 μg/ml F-ANO2 (■) or 10 μg/ml F-ANO2 (◆) increased with the molar fraction of DNP-PE and were negligible in the presence of 3 mM DNP-glycine (▲). Values are averaged for at least four samples obtained from two independent air/water interface compressions.

PE) and DNP-DPPE monolayers (Fig. 4). The bleaching efficiency also decreased with increasing amounts of DNP-PE, consistent with a decrease in the mean fluorescence intensity and the fluorescence lifetime. Possible explanations for these effects are that the DNP groups quench NBD (see, for example, Ref. 47), that the NBD groups are more exposed to the aqueous environment [40] in monolayers containing DNP-PE, or that mono-

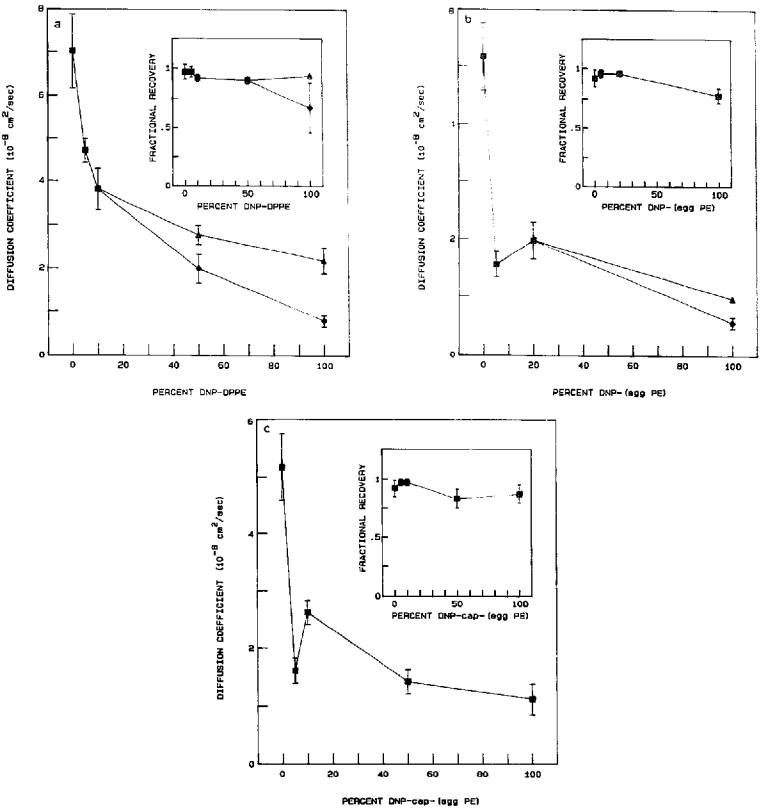


Fig. 3. NBD-PC translational mobility in supported monolayers. The apparent diffusion coefficient of NBD-PC in (a) DNP-DPPE/DMPC, (b) DNP-(egg PE)/egg PC and (c) DNP-cap-(egg PE)/egg PC monolayers was high and decreased with the molar fraction of DNP-PE. The mobile fractions (insets) are extrapolated values obtained from the best fits to the appropriate functional form [34] (See also footnote on p. 221). When monolayers containing low and intermediate molar fractions of DNP-PE were treated with 100 μg/ml unlabelled ANO2, the diffusion coefficients and mobile fractions were equivalent within experimental uncertainty (■). At higher DNP-PE molar fractions, values were lower in treated (◆) relative to untreated (▲) monolayers. Values are averages for two to eight samples obtained from at least two independent air/water interface compressions.

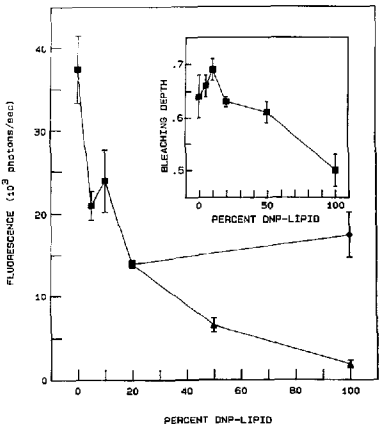


Fig. 4. NBD-PC fluorescence intensities and bleaching efficiencies in supported monolayers. The average fluorescence intensities and bleaching efficiencies (inset) of NBD-PC decreased with the molar fraction of DNP-PE. When statistically equivalent, values were averaged for all three sets of binary monolayer compositions (■), and when not statistically equivalent, values were averaged separately for DNP-DPPE/DMPC and DNP-cap-(egg PE)/egg PC monolayers (▲) and DNP-(egg PE)/egg PC monolayers (◆). For each DNP-PE molar fraction, measurements were made on 4 to 9 samples obtained from 2–6 independent air/water interface compressions. The bleaching duration for the data in the inset was 250 ms.

layers containing DNP-PE have regions of high NBD-PC densities in which self-quenching occurs. Previous work has demonstrated the presence of distinct and coexistent fluid-like phases in some types of phospholipid monolayers and bilayers [48,49].

The diffusion coefficient and mobile fraction of NBD-PC decreased when monolayers containing high molar fractions of DNP-PE were treated with unlabelled polyclonal sheep IgG and ANO2 antibodies (Fig. 3), but did not change for any phospholipid composition when monolayers were treated with only sheep IgG (data not shown). This result demonstrates that proteins bound to phospholipid surfaces can reduce lipid diffusion rates in a manner similar to bilayer-spanning membrane proteins (see, for example, Ref. 50). In addition, the NBD-PC fluorescence intensities and bleaching efficiencies of monolayers containing high molar fractions of DNP-PE increased (by 10–25%) when these monolayers were treated with sheep IgG and ANO2 antibodies but not with sheep IgG alone (data not shown). These observations suggest that bound ANO2 antibodies alter the structure of monolayers containing high amounts of DNP-PE, and may be related to the observation that antibodies specifically bound to vesicles containing hapten-conjugated lipids can decrease the hydrocarbon chain flexibility [51]. Bound antibodies might also change the monolayer structure by altering the effective viscosity of the aqueous phase adjacent to the monolayer [52] or by directly interacting with the NBD groups [53].

F-ANO2 mobility on supported monolayers

NBD-PC was translationally mobile for all monolayer compositions, indicating that all monolayers that bound significant amounts of F-ANO2 were candidates for model systems in which F-ANO2 translational mobility might be significant. Thus, the translational mobility of bound F-ANO2 was examined for DNP-DPPE/DMPC monolayers containing ≥ 50 mol% DNP-PE and for DNP-(egg PE)/egg PC and DNP-cap-(egg PE)/egg PC monolayers containing ≥ 35 mol% DNP-PE (Table I; see also Fig. 2).

When 100 mol% DNP-DPPE, DNP-(egg PE) and DNP-cap-(egg PE) monolayers were treated with 100 μg/ml F-ANO2, the fluorescence recovery after photobleaching was small or insignificant after 5 min for a ruling periodicity of 16 μm. Thus, using the data in Table I and the functional form for fluorescence pattern photobleaching data [34] (see footnote on p. 221), the majority of the bound F-ANO2 (> 80%) had an apparent translational diffusion coefficient lower than 10^{-11} cm^2/s. In contrast, when 100 mol% DNP-PE monolayers were treated with only 10 μg/ml F-ANO2,

TABLE I

Translational mobility of F-ANO2 on supported monolayers

Fluorescence recovery was monitored for 5 min after bleaching with a ruling periodicity of 16 μm. Shown below are the values of the diffusion coefficients obtained from non-linear curve-fitting and the *minimum* mobile fraction obtained from the fractional recovery at 5 min. Values are averages for three or four samples obtained from two independent air/water interface compressions.

Composition (mol%/mol%)	F-ANO2 (μg/ml)	Diffusion coefficient (10^{-10} cm^2/s)	Mobile fraction
DNP-DPPE	100	–	0.18 ± 0.05
DNP-(egg PE)	100	–	< 0.02
DNP-cap-(egg PE)	100	–	0.11 ± 0.02
DNP-DPPE	10	4.8 ± 0.8	0.72 ± 0.17
DNP-(egg PE)	10	6.2 ± 2.6	0.66 ± 0.24
DNP-cap-(egg PE)	10	7.9 ± 1.4	0.68 ± 0.17
DNP-DPPE/ DMPC (50:50)	100	15 ± 4	0.71 ± 0.04
DNP-(egg PE)/ egg PC (35:65)	100	4.0 ± 0.5	0.47 ± 0.11
DNP-cap-(egg PE)/ egg PC (35:65)	100	6.8 ± 2.0	0.78 ± 0.12

or when monolayers composed of DNP-(egg PE)/egg PC or DNP-cap-(egg PE)/egg PC (35 : 65, mol%/mol%) or of DNP-DPPE/DMPC (50 : 50, mol%/mol%) were treated with 100 μg/ml F-ANO2, the bound antibodies displayed significant translational mobility, with apparent diffusion coefficients between $3 \cdot 10^{-10}$ cm^2/s and $2 \cdot 10^{-9}$ cm^2/s and with mobile fractions between 0.3 and 0.9.

Although bound F-ANO2 had a measurable translational mobility at lower F-ANO2 surface densities, the apparent diffusion coefficient was in some cases a factor of 50 slower than the NBD-PC diffusion coefficient. In similar systems, antibodies bound at low densities are mobile with diffusion coefficients nearly equal to those of the lipids [32,34,35]. However, the result that the F-ANO2 translational mobility depends on the density of bound F-ANO2 is consistent with previous measurements in similar systems [32,34,35]. Of particular interest in this work is the result that the parameters of F-ANO2 translational mobility appear to depend *only* on the density of bound F-ANO2 and not on the chemical composition of the model membrane. Comparison of the data in Table I and Fig. 2 shows that the density and mobility of bound F-ANO2 is approximately equivalent for the six samples consisting of 100 mol% DNP-PE treated with 10 μg/ml F-ANO2 or of PC with 35–50 mol% DNP-PE but treated with 100 μg/ml F-ANO2. This result suggests that in-depth studies of the dependence of the two-dimensional diffusion of bound antibodies on the antibody density should be possible without accounting for the chemical structure of the model membrane to which the antibodies are bound.

Summary

The application of dynamic techniques in fluorescence microscopy to supported planar membranes, which are chemically, physically and geometrically well-defined, has significant potential for elucidating the ways in which protein and membrane structure and solution properties affect the thermodynamics and kinetics of protein–membrane interactions, the translational and rotational mobilities of membrane proteins and lipids, the interactions between membrane components, the conformation and flexibility of membrane-bound molecules, and the ways in which these different phenomena are coupled. In the present work, the association of anti-DNP monoclonal antibodies with phospholipid Langmuir-Blodgett monolayers containing DNP-conjugated lipids and the translational mobilities of the lipids and antibodies in these monolayers were characterized. The results show, for at least three binary mixtures of phosphatidylcholine and DNP-PE, that some but not most of the DNP groups are accessible to the monoclonal antibody ANO2; that the fluorescent lipid probe NBD-PC is highly mobile over a wide range of lipid compositions; that at high enough DNP-PE densities where significant amounts of ANO2 are bound to monolayers, the bound ANO2 can be translationally mobile; and that the ANO2 mobility is strongly dependent on the two-dimensional ANO2 surface density.

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