Lateral Diffusion in Model Membranes Is Independent of the Size of the Hydrophobic Region of Molecules

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ABSTRACT We have systematically investigated the probe size and shape dependence of lateral diffusion in model dimyristoyl phosphatidylcholine membranes. Linear hydrophobic polymers, which differ in length by an order of magnitude, were used to explore the effect on the lateral diffusion coefficient of hydrodynamic restrictions in the bilayer interior. The polymers employed are isoprenoid alcohols—citronellol, solanesol, and dolichol. Tracer lateral diffusion coefficients were measured by fluorescence photobleaching recovery. Despite the large difference in lengths, the nitrobenzoxadiazole labelled alcohols all diffuse at the rate of lipid self-diffusion (5.0×10^{-12} m^2 s^{-1}, 29°C) in the liquid crystal phase. Companion measurements in isotropic polymer solution, in gel phase lipid membranes and with nonpolar fluorescent polyaromatic hydrocarbons, show a marked dependence of the lateral diffusion coefficient on the probe molecule size. Our results in the liquid crystal phase are in accord with free area theory which asserts that lateral diffusion in the membrane is restricted by the surface-free area. Probe molecules which are significantly longer than the host phospholipid, seven times longer in the case of dolichol, are still restricted in their lateral motion by the surface properties of the bilayer in the liquid crystal phase. Fluorescence quenching experiments indicate that the nitrobenzoxadiazole label does not reside at the aqueous interface, although it must reside in close proximity according to the diffusion measurements.

INTRODUCTION

The fluid mosaic model of Singer and Nicolson (1) has emphasized the dynamic nature of biological membranes. The fluid nature of this model means the constituent lipids are free to diffuse in the plane of the membrane. Experimental evidence accumulated over the last 20 years suggests that lateral diffusion in the bilayer membrane does occur and is governed by the two-dimensional solution to the diffusion equation (2–7). The dynamic structure of the phospholipid bilayer has important implications for its function as a reaction environment. Many biochemical processes require lateral mobility of membrane proteins to trigger cellular responses and to form specialized structures at the cell surface. Receptor clustering (8), ligand-receptor interactions (9), and conventional chemical reactions (10) have the potential to be rate-limited by the two-dimensional dynamics of the cell surface. Electron transfer reactions with their low activation energies are good candidates for diffusion controlled kinetics. Two important redox reactions in the cell membrane have been postulated to be diffusion controlled reactions (11, 12).

While intuitively one expects a dependence of the rate of diffusion on molecular size, the nature of this dependence is uncertain. Aside from a long-standing theoretical interest in the size dependence of the lateral diffusion coefficient, there is an obvious interest from the point of view of biochemical kinetics. In model membranes consisting primarily of phospholipids, simple models exist which predict the size dependence of the lateral diffusion coefficient in two extremes of diffusant size (7, 13). The extremes are 1) diffusants which are much larger than the solvent lipids and 2) diffusants which are the same size as the host lipid molecules. The first size regime is most appropriate for the diffusion of large proteins in model membranes composed of very much smaller phospholipids. The theory, introduced by Saffman and Delbruck (13), treats the bilayer as a thin sheet of fluid, characterized by a certain viscosity, through which model cylinders diffuse. The second regime is treated by what has become known as free area theory. Free area theory is a semi-empirical theory based on statistical mechanical considerations of density fluctuations in the lipid bilayer. Voids which are opened by such density fluctuations are filled by the movement of neighboring molecules into the void. Since lipids in the free area model are considered to be hard rods and such motion, by the nature of the membrane, is restricted to a plane, this is a two-dimensional problem. Within the framework of this two-dimensional system we seek to explore the effect on the probe diffusion coefficient of large systematic alterations in the third dimension. We have examined a series of molecules with cross-sectional areas roughly comparable to phospholipids but which are much longer in length. Vaz and coworkers (14, 15) have examined the dependence of the diffusion coefficient on phospholipid chain length. No significant differences were observed, although the scale of the variation was very much less than undertaken in this work.

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EXPERIMENTAL

Probe molecules/fluorescence labeling

A series of naturally occurring hydrocarbon polymers, polyisoprenoid alcohols, were fluorescently labeled to permit diffusion measurements by FPR. The isoprenoid alcohols, Fig.

1, are citronellol (Aldrich, Milwaukee, WI), solanesol (Aldrich), and dolichol. Both citronellol and dolichol have a saturated α-subunit. Dolichol, isolated from human liver upon autopsy, is ω-(tri-*trans*)poly-*cis* and a mixture of homologs between 16 and 22 isoprenes in length (average of 19). Solanesol has an all-*trans* stereochemistry about the isoprene double bonds. Labeled citronellol has approximately the same length as the host phospholipid used in our studies.

The fluorophore employed was a carboxylic acid derivative of the common nitrobenzoxadiazole fluorophore, *N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-6-aminohexanoic acid (NBD-acid, Fig. 2 *a*). NBD-acid was prepared according to a standard procedure (16). The coupling reaction, an esterification, was a dicyclohexyl carbodiimide (Aldrich) mediated acid condensation reaction with an alkyl aminopyridine catalyst (17) in methylene chloride (Caledon, Toronto, Canada). This reaction has a significant by-product, *N*-acylurea, production of which is very solvent-dependent (18). The synthesis, separation, isolation, and characterization of the NBD conjugates is the subject of a separate communication (B. J. Balcom and N. O. Petersen, submitted for publication). Prior to use, stock solutions of the NBD-labeled alcohols were stored at 4°C, in the dark, as ethanol solutions. Under these conditions, the labeled alcohols are stable for months or years.

Lipid self-diffusion coefficients were measured using an NBD headgroup labeled dipalmitoyl phosphatidylethanolamine (NBD-PE) purchased from Avanti Polar Lipids (Alabaster, AL). Diffusion measurements were also performed with the fluorescent polyaromatic hydrocarbons tetracene and rubrene (Aldrich) (Figs. 2, *b* and *c*).

Sample preparation and diffusion measurements: membranes

Diffusion measurements were performed with a home-built spot photobleaching fluorescence photobleaching recovery (FPR) instrument (19). The illumination source was a Coherent Inc. (Toronto, Canada) Innova 70 4-watt argon ion laser. The laser, 476.5 nm, was operated in light regulation mode at 100 mW. Timing of the bleach and monitor beams was controlled by a Digital Equipment Corporation, Kanata, Canada, Minc 23 minicomputer with a VT 105 display terminal. The fluorescence microscope used was a Zeiss Universal model fitted for epi-illumination. An RCA 31034A

$$H(CH_2C(CH_3)=CHCH_2)_nCH_2C(CH_3)=CHCH_2OH$$

$$H(CH_2C(CH_3)=CHCH_2)_nCH_2CH(CH_3)CH_2CH_2OH$$

FIGURE 1 The isoprenoid alcohols employed. Citronellol has $n = 1$ corresponding to the top structure. Solanesol has $n = 8$ corresponding to the bottom structure. Dolichols have a range of n values from 15 to 21, corresponding to the top structure, with the three terminal double bonds being *trans*, the others *cis*. Solanesol is poly-*trans*.

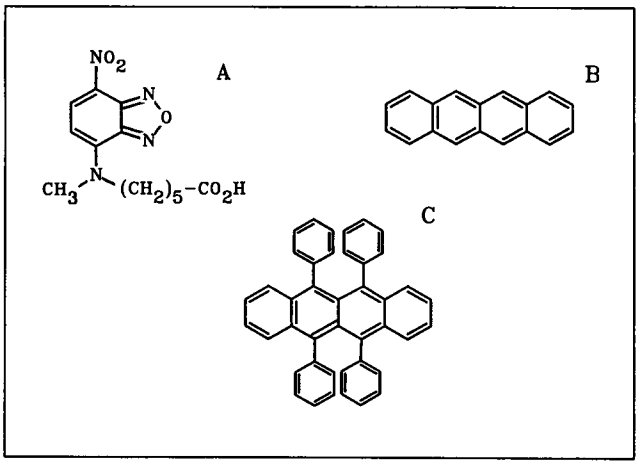

FIGURE 2 (*a*) *N*-Methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-6-aminohexanoic acid, the NBD label; (*b*) tetracene, a fluorescent, polyaromatic hydrocarbon; (*c*) rubrene, a tetraphenyl derivative of tetracene.

photo multiplier tube, cooled with dry ice, was fitted to the top of the optical column. A 140-mm lens, installed on the optical bench behind the microscope, focused the laser beam onto the image plane of the microscope. The objective lens was a ×40 water immersion lens with a numerical aperture of 0.75. The combination of the 140-mm focusing lens and a ×40 objective lens produced a 1.1-μm diameter beam in the focal plane of the microscope.

The bulk of the diffusion measurements were made with dimyristoyl phosphatidylcholine (DMPC, Fluka, Ronkonkoma, NY) as the host lipid in model membranes. Vesicles formed in the absence of labeled fluorophore are not fluorescent. Samples were prepared using the conventional technique of Kapitza et al. (20) Generally, 0.5 μmol of lipid and a small amount of NBD-labeled material (less than 0.1% of the lipid on a molar basis) were applied as a 50-μl droplet in a 2:1 chloroform:methanol solution to the surface of a clear, dry, circular glass coverslip of 18-mm diameter. The slide was heated to 40°C and the lipid film dried under a weak flow of nitrogen gas. The coverslip and film were put under high vacuum for 8 h, then lowered slowly (lipid film down) onto a clear 22-mm diameter glass coverslip with a 300- or 400-μl droplet of doubly distilled deionized water on its surface. The two slides, forming a lipid/water sandwich, were sealed together with hot wax and stored overnight, in the dark, at 40°C. Samples were inverted and mounted in a hollow copper disk prior to measurement. An axially symmetric hole in the disk was covered with a glass coverslip. The disk was filled with water, covering the sample, and a light film of thermally conducting grease was applied to the bottom. The temperature was regulated with a Cambion Bipolar temperature controller (Cambion Division of Midland Ross, Brampton, Ontario) with a microscope stage subassembly. Temperature measurements were made by dipping a small 100-kΩ thermistor into the water near the focal point of the microscope. Translation of the resistance via the manufacturers calibration yielded the temperature. Measurements were performed at 29 ± 1 and 19 ± 1°C.

Diffusion measurements were made on the upper surface of the large multibilayers formed by the above technique. Vesicles chosen for measurements were typically 60 μm or larger in diameter. The microscope's phase contrast optics permitted facile inspection and selection of vesicles. The laser beam was focused on the upper surface by adjusting the microscope's fine focus until a bright spot of fluorescence was observed through the microscope eyepiece. Samples were, in all cases, handled in diminished light prior to experiment.

Diffusion measurements on vesicles in the liquid crystal phase were made at 29 ± 1°C. For any one sample typically five measurements on each of four or five vesicles were made. Each of the measurements was an average of three bleach/recovery sequences. Control experiments showed that individual measurements, in one spot, gave the same recovery curve with a mobile fraction of essentially one. Multiple bleach recovery cycles were therefore employed to improve the signal-to-noise ratio. Standard deviations were calculated based on the discrete diffusion measurements assuming a normally distributed population. Vesicles under study were checked for movement in the eyepiece of the microscope before and after measurement. Results were disregarded on the rare occasion when the sample moved during measurement since the rate of fluorescence recovery is artificially high.

Measurements in the lipid gel phase (19 ± 1°C) usually followed measurements on the same sample in the liquid crystal phase. Samples were cooled thermoelectrically on the microscope stage subassembly under control of the Cambion temperature controller. Diffusion measurements were generally delayed by 1 h from the start of the cooling cycle, although cooling from 29°C took only several minutes. Slow diffusion in this temperature regime, and mobile fractions less than one, required substantial attenuation of the bleach and monitor beams, longer observation times and single bleach/recovery sequences. The long time required for these measurements usually meant only one experiment per vesicle although frequently more than five vesicles were measured.

Sample preparation and measurements in model polymer systems

Diffusion measurements of the NBD-labeled isoprenoid alcohols were made in a series of poly(propylene glycol) ethers (PPG). These polymers are linear chain molecules commercially available in a variety of average chain lengths. The variable chain lengths results in a series of solvents with different viscosities at room temperature. Polymers (Aldrich) of average gram molecular weights 425, 1000, 2000, 3000, and 4000 were employed. These polymers have viscosities, reported by Aldrich, of 80, 150, 300, 600, and 930 centiPoise at 25°C.

Samples for diffusion measurements in PPG matrices were prepared as follows. Small aliquots of the stock NBD alcohol solutions were added to small glass 1-dram sample vials. The solvent was removed under high vacuum yielding 1.4 nmol of the labeled alcohol as a dry film. To these vials, 400 μl of warm (40°C) neat PPG was added. The labeled alcohols readily dissolved in all the polymers. Portions of the NBD-containing polymer solutions (concentrations of about 10^{-6} M) were drawn into small rectangular microslides by dipping one end into the warm solution. The microslides (5 cm × 2 mm × 100 μm; Vitro Dynamics Inc., Rockaway, NJ) were sealed on filling by hot wax at each end. The microslides took from 2 to 15 min to fill, by capillary action, depending on the PPG viscosity. The sample holder was a thin hollow disk of copper with three shallow parallel grooves 2.5 mm in width extending from one side of the disk surface to the other. A glass coverslip fit into the bottom of the disk. Once again thermally conductive grease was applied to the bottom of the disk, and the assembly was maintained at 25 ± 1°C with the Cambion temperature controller. Diffusion measurements on each of the three microslides (different samples) in the sample holder were made at two discrete locations where five measurements were undertaken. Each of the five measurements was an average of three individual bleach/recovery sequences. As before, control experiments showed that individual measurements in one spot had the same recovery curve and constant mobile fractions of essentially one. Multiple bleach/recovery sequences co-added summed to increase the signal-to-noise ratio. The laser beam was focused at the midpoint of the capillary microslide in both depth and width.

Steady state fluorescence measurements

Steady state fluorescence measurements in vesicles were made with an LS-1 fluorimeter manufactured by Photon Technology International (London, Ontario, Canada). Fluorescence anisotropy measurements were performed with the addition of the proper polarizing optics. Fluorescence emission measurements were performed by exciting the NBD chromophore at 480 nm and measuring the emission intensity at 2-nm intervals between 500 and 650 nm. Individual intensities at each wavelength were the average of 50 discrete lamp flashes. The entire emission spectrum was measured and averaged 25 times. The excitation, emission, and PMT monochromator slits were set for 4-nm bandwidth. The sample chamber of the fluorometer was temperature-controlled (31 ± 1°C) with a circulating, constant temperature bath.

Steady state fluorescence measurements were performed using the NBD adducts of solanesol and citronellol as well as cholesterol (NBD-cholesterol; Molecular Probes, Eugene, OR), dipalmitoyl phosphatidylethanolamine (NBD-PE; Avanti Polar Lipids), and palmitoyl phosphatidylcholine (NBD-PC; Avanti Polar Lipids) labeled in the number two fatty acid position. Small unilamellar vesicles were formed by the ethanol injection technique (21). Samples containing 0.5 μmol of DMPC and less than 2.5 nmol of NBD-labeled material in 40 μl of ethanol were injected into 2 ml of rapidly stirred 60°C aqueous solution. The aqueous solution was pH 7 Tris buffer with 150 mM sodium chloride. Stern-Volmer quenching experiments were performed with aqueous Co^{2+}

(Cobalt chloride hexahydrate, Fluka) as the quencher (22). Vesicles for quenching experiments were formed by injection into solutions containing the appropriate cobalt ion concentration. Correction was made for the internal filter effect of the cobalt ion in solution.

RESULTS

Measurements in the liquid crystal phase

The NBD-labeled isoprenoid alcohols comprise an homologous series of labeled hydrocarbon polymers which, since they will incorporate into a bilayer, should reside with their alkyl tails in the bilayer interior. Tracer diffusion coefficients were measured for NBD-citronellol, NBD-solanesol, and NBD-dolichol sequestered in liquid crystal phase (29°C) DMPC bilayers. The NBD-PE tracer diffusion coefficient was measured to provide a constant point of reference since, presumably the diffusion coefficient of NBD-PE reflects the self-diffusion coefficient of the host lipid.

The results of these measurements are found in Table 1. Surprisingly, all the labeled alcohols diffuse at the same rate. This rate, in fact, is equal to that of lipid self-diffusion as measured by NBD-PE. The means are equal according to Student's *t* test at a 95% confidence level. The diffusion coefficient of NBD-PE is 5.0×10^{-12} m^2 s^{-1}. Essentially all the incorporated fluorophore is mobile, as evidenced by mobile fractions which approach one. The equivalence for these probes is very surprising since the largest probe, NBD-dolichol, has a hydrocarbon tail which is an order of magnitude larger than NBD-citronellol, the smallest probe.

The lack of size discrimination observed for the NBD-labeled isoprenoids was unexpected. To further explore the size effect in membranes, we measured the diffusion coefficient of the fluorescent polyaromatic hydrocarbons tetracene and rubrene in the same bilayer system. Both these molecules have a significant absorption at 476.5 nm which makes them amenable to diffusion measurements by FPR (23, 24). They have, however, only recently been exploited for FPR diffusion measurements. These molecules were not employed as labeling agents, but as intrinsically fluorescent nonpolar probes. Their lack of polarity suggests they should reside in the hydrocarbon interior of the bilayer. Results of our diffusion measurements with rubrene and tetracene are also shown in Table 1. Rubrene, which is approximately twice as large as tetracene moves substantially slower. Both move faster than NBD-PE as evidenced by Student's *t* test at a 95% confidence level. Control experiments without the bleach pulse show that the monitor beam itself is sufficiently intense to bleach fluorophore in these systems. This manifests itself in the FPR experiment, Table 1, as an apparent decrease in the mobile fraction.

TABLE 1 Probe diffusion in DMPC multibilayers

Probe	Lipid/label	D (10^{13} m^{-2} s)*	Xm‡	N§
Liquid crystal phase 29°C				
NBD-PE‖	1200¶	50 ± 2	0.97	38
NBD-citronellol	1500	48 ± 3	1.01†	24
NBD-solanesol	2000	53 ± 2	0.97	24
NBD-dolichol‖	2000	46 ± 2	0.98	39
Tetracene	1200	110 ± 9	0.86	15
Rubrene	3300	58 ± 3	0.89	20
Gel phase 19°C				
NBD-PE	1500	0.32 ± 0.01	0.66	20
NBD-citronellol	1500	1.00 ± 0.05	0.83	18
NBD-solanesol‖	2000	0.14 ± 0.02	0.40	22
NBD-dolichol	2000	0.08 ± 0.01	0.70	5
Tetracene**	1200			
Rubrene**	3300			

*Diffusion coefficient, standard error of the mean.
‡Mobile fraction.
§Number of measurements.
‖Aggregate of two sample preparations.
¶Second preparation has lipid-label = 1500.
†Mobile fractions larger than 1.0 are physically impossible. This is the fitted value.
**Diffusion measurements proved impossible due to extreme monitor beam photobleaching.

Measurements in the gel phase

The liquid crystal to gel phase transition in a phospholipid bilayer is characterized by the formation of ordered, rigid arrays of hydrocarbon chains (25, 26). The rigid interior in this phase suggests that a probe's tail length should have a significant effect on lateral diffusion. Not surprisingly, diffusion in the gel phase of the membrane (phase transition, 23°C) does depend on the size of the probe. Our results, Table 1, show that NBD-citronellol diffuses faster than NBD-PE which diffuses faster than NBD-solanesol. NBD-PE moves in the gel phase at a rate of 3.2×10^{-14} m^2 s^{-1}. The Student *t* test, 95% confidence level, shows that all three labeled alcohols diffuse at rates different from that of NBD-PE and each other. The diffusion coefficients measured in the gel state were lower by factors of 50 for NBD-citronellol, 400 for NBD-solanesol, and 500 for NBD-dolichol versus their diffusion rates in the liquid crystal membranes at 29°C. Observation of diffusion coefficients which depend on size, in the expected manner, suggest that we are measuring motion of the labeled probes properly incorporated in the membrane.

Our diffusion results in the gel phase are characterized by mobile fractions of less than one. This probably relates to the mechanism of diffusion in this phase. This mechanism is uncertain but has been attributed to probe movement along cracks or defects in the bilayer assembly (27). Probes which are not accessible to these defect structures will not appear mobile on the time scale of the experiment. Alternative explanations are of course possible. Different states of probe aggregation or differences in probe partitioning between multiple domains in the gel state could explain the size dependence of the diffusion coefficient in the gel state. For these reasons the results in the gel state should not be overinterpreted. The important feature of these experiments is that differences in hydrocarbon chain length result in different measured diffusion coefficients and these diffusion coefficients decrease with size.

Curiously, the diffusion coefficient of the polyaromatic hydrocarbons rubrene and tetracene could not be measured

below the main phase transition. Monitor beam photobleaching was so extreme for these probes that it proved impossible to focus the laser on the surface of the bilayer. Alteration of the microscopes plane of focus produced a brief flare of fluorescence which was extinguished in less than 1 s. Trissl (28) has reported that rubrene will not incorporate into black lipid membranes. Based on our results, it seems likely that rubrene did incorporate but was bleached to such an extent that minimal fluorescence was observed in their experiment.

Diffusion in linear chain polymer media

Our measurements in gel state model membranes were intended to represent a limiting case where one might expect to see a strong dependence of the diffusion coefficient upon molecular size. This limit of course is close packing of the alkyl chains in the membrane interior. As discussed above, interpretation of our results in this phase is somewhat clouded by the uncertainties of probe partitioning or aggregation in this phase. For this reason we undertook further measurements in a model system which expresses a different limiting behavior. In three-dimensional polymer melts effects related to the membrane surface are removed but we retain the disorganized random chain environment of a liquid crystal phase model membrane. In an effort to better understand the diffusive behavior of the NBD-labeled isoprenoid alcohols in general, a series of studies were therefore undertaken in three-dimensional viscous polymer melts. A range of polymers were utilized in order to observe the trend in diffusion coefficient since a three-dimensional tensor solution viscosity is not directly translatable to a membrane viscosity. We performed these diffusion experiments to answer a simple question: Does the diffusion coefficient depend on probe length in simple random chain media? Experimental results are shown in Table 2 and in an abridged form in Fig. 3. The approximate viscosity range of the bilayer interior is shown by the boxed region of Fig. 3. Once again there is a measurable, systematic, difference between diffusion coefficients of the various labeled alcohols. As one might expect, the measured diffusion coefficient decreases with increasing chain length and increasing viscosity. Simple models of the diffusion process suggest that the diffusion coefficient should be inversely proportional to both the frictional coefficient of the diffusant and the viscosity. The frictional coefficient will increase with the length of the molecule. A similar length dependence has been observed in glycerol/water mixtures and paraffin oil (B. J. Balcom, unpublished work). The fluorophores tetracene and rubrene (primarily rubrene) displayed significant monitor beam bleaching in PPG solution. As with the results in liquid crystal phase model membranes, monitor beam bleaching decreases the apparent mobile fraction.

TABLE 2 Probe diffusion in poly(propylene glycol) at 25°C

Solvent	μ	D	X_m‡	N§
mol/g	cP^{-1}	10^{13} m^{-2} s*		
NBD-methanol‖				
425	80	85 ± 10	0.99	10
1000	150	64 ± 8	1.00	18
2000	300	63 ± 7	0.98	9
3000	600	53 ± 5	0.98	10
4000	930	59 ± 5	0.86	8
NBD-citronellol‖				
425	80	70 ± 7	0.99	19
1000	150	44 ± 3	1.00	18
2000	300	38 ± 2	0.97	17
3000	600	36 ± 2	0.98	16
4000	930	39 ± 3	0.98	10
NBD-solanesol‖				
425	80	41 ± 2	0.97	10
1000	150	34 ± 2	0.96	10
2000	300	20 ± 1	0.95	10
3000	600	16 ± 1	0.95	10
4000	930	14 ± 1	0.93	10
NBD-dolichol‖				
425	80	33 ± 4	0.99	10
1000	150	24 ± 2	0.95	9
2000	300	14 ± 1	0.93	10
3000	600	12 ± 1	0.92	10
4000	930	11 ± 1	0.93	10
Tetracene				
2000¶	300	53	0.94	1
4000†	930	54 ± 2	0.93	4
Rubrene				
2000**	300	26 ± 2	0.81	10
4000‡‡	930	22 ± 2	0.78	7

*Diffusion coefficient, standard error of the mean.
‡Mobile fraction.
§Number of measurements.
‖Probe concentration = 3.5×10^{-6} mol liter^{-1}.
¶[Tetracene] = 9.6×10^{-6} mol liter^{-1}.
†[Tetracene] = 5.7×10^{-5} mol liter^{-1}.
**[Rubrene] = 6.8×10^{-5} mol liter^{-1}.
‡‡[Rubrene] = 4.7×10^{-5} mol liter^{-1}.

Steady state fluorescence quenching experiments

Steady state fluorescence quenching experiments were performed in an effort to localize the NBD moiety sequestered in our bilayers. The set of labels investigated comprised three NBD-labeled molecules (NBD-PE, NBD-cholesterol, and NBD-PC) for which the probe location is known and two, NBD-citronellol and NBD-solanesol, which have an uncertain probe location. NBD-PE is known to localize the NBD label near the aqueous surface (22, 29), while NBD-cholesterol, due to the rigid steroid ring structure, submerges the NBD label in the membrane interior (22, 29). The NBD label in NBD-PC has been shown to loop back toward the aqueous interface (22, 29). An important distinction between the two sets of molecules described above should be noted. The first group are known surfactants to which a fluorescent probe has been added. Whatever the orientational tendency of the NBD-labeled alcohols, however, their hydrophilic contribution to the orienting force is provided solely by the NBD label and its associated ester linkage.

The results of the steady state quenching experiments are shown in Fig. 4 as a Stern-Volmer plot. The slope of such plots cannot be interpreted as true quenching rate constants, since the system is anisotropic and the quencher and fluorophore are physically separated. The slope, however, may be interpreted as an indication of the degree of exposure of

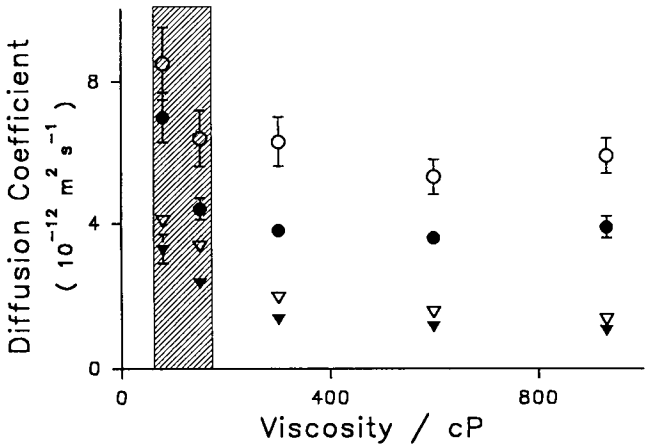

FIGURE 3 Lateral diffusion coefficients of trace amounts of NBD-labeled isoprenoid alcohols in neat PPG polymer solutions. The larger PPG polymers are more viscous. The probes employed were NBD-methanol (○), NBD-citronellol (●), NBD-solanesol (△), and NBD-dolichol (▼). The approximate viscosity range of the bilayer interior is shown by the shaded region. Error bars represent standard errors of the mean.

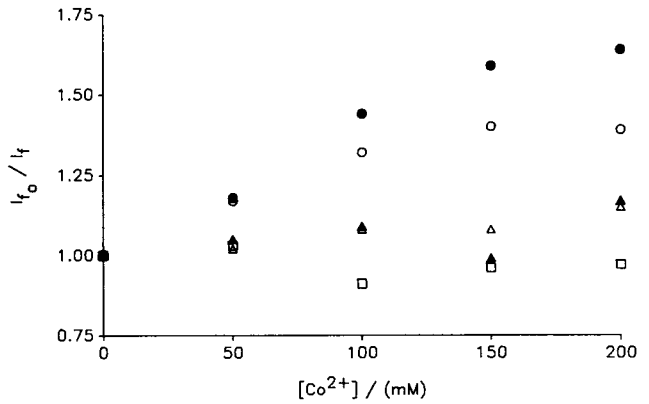

FIGURE 4 Stern-Volmer plot of fluorescence quenching of NBD-labeled molecules in DMPC bilayers by aqueous Co^{2+}. The labeled probes are NBD-PE (●), NBD-PC (○), NBD-cholesterol (▲), NBD-citronellol (□), and NBD-solanesol (△).

the NBD group to the membrane surface (22). Fluorophores sequestered in the interior of the membrane will be quenched less effectively than those at the surface. NBD-PE as expected was well quenched and NBD-PC was partially quenched. NBD-cholesterol was not quenched. These results are in accord with our prior knowledge of the location of these probes, more properly their fluorescent labels, in the membrane. Because NBD-citronellol and NBD-solanesol are not quenched, the fluorophore in each is not located at the surface of the membrane. We cannot say how deep in the membrane they reside, because no quenching only indicates the NBD label is not within the collision (30) quenching distance of the cobalt ion.

Steady state fluorescence anisotropy measurements

Steady state fluorescence anisotropy measurements in small unilamellar vesicles were performed to examine the short time scale "mobility" of the various NBD-labeled species. Our results, Table 3, show that the NBD-labeled alcohols reorient to a lesser extent than the controls during the lifetime of the excited probe. The structural feature orienting the labeled alcohols is the NBD label itself, and its associated ester linkage. This portion of the molecule is of course its only hydrophillic moiety. Interaction with the surface, in some manner, therefore likely provides sufficient resistance to free motion of these probes to increase the measured anisotropy. The lengthy "tails" of these molecules will also act to reduce the tumbling/twisting motion of these molecules.

DISCUSSION

We have found that the diffusion coefficient of our NBD-labeled isoprenoid alcohols is independent of the length of these molecules in liquid crystal phase model membranes. The measured diffusion coefficients are equivalent to the lipid self-diffusion coefficient. In gel phase model membranes the diffusion coefficient depends on the probe size, as it does in three-dimensional polymer melts.

The invariance of the measured diffusion coefficient in the liquid crystal phase for the labeled alcohols does not result from systematic error in performance of the experiments. The vesicles under study are in mechanical and thermal equilibrium and are also free of fluorescent impurities. Control experiments show bleaching of fluorophore is irreversible, so recovery of fluorescence does not reflect the kinetics of the reverse reaction but true diffusive motion of labeled molecules. Microscopic observation of our multibilayers revealed no anomalous structure in the presence of fluorophore. Furthermore, the labeled alcohols are not water soluble and fluorescence originates only from the lamella of the bilayer structure. Diffusive motion is therefore restricted to the bilayer.

Our FPR measurements are a true measure of the lateral diffusion coefficient of our labeled probes in DMPC membranes. This is supported by the close agreement found between our measurements of the rate of motion of NBD-PE in DMPC multibilayers and a measurement on the same system by Vaz et al. (15). We measure the diffusion coefficient as 5.0×10^{-12} m^2 s^{-1} at 29°C, while Vaz et al. (15) measure 5.7×10^{-12} m^2 s^{-1} at 30°C. In the gel phase at 19°C, our measured diffusion coefficient for NBD-PE, 3.2×10^{-14} m^2 s^{-1} is in qualitative agreement with the results of Derzko and

TABLE 3 Fluorescence anisotropy measurements in DMPC bilayers

Probe	Lipid/label	r*
Liquid crystal phase 31°C		
NBD-PE	576	0.165
NBD-PC	392	0.129
NBD-cholesterol	569	0.118
NBD-citronellol	440	0.218
NBD-solanesol	373	0.183

*The anisotropy was calculated according to the equation $r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$. I refers to the fluorescence intensity detected by the fluorimeter. Subscripts vv and vh refer to the polarization of the excitation and emission optics. G is an instrumental correction factor.

Jacobson (27). Our results show that differential hydrodynamic interactions in the membrane interior have no effect on lateral diffusion in the liquid crystal phase. The consistency of the results suggests a common diffusive mechanism for both labeled phospholipids and our series of labeled isoprenoid alcohols. The common feature in the isoprenoid alcohol series, other than the fundamental isoprene unit, is the NBD-acid group. Fluorescence quenching studies have previously suggested that NBD, and its associated ester linkage, has sufficient polarity to localize in the vicinity of the aqueous interface. The restricted short time scale mobility of these same labels suggests the NBD label provides the orienting moiety for these molecules. If the NBD label, and associated ester, is sufficiently polar to reside somewhere in the vicinity of the bilayer/water interface, the rate of lateral motion of the labeled alcohols may be limited by the free area fluctuations at the surface. NBD need not reside at the interface to experience this effect. The alkyl chains of the host phospholipid are quite ordered near the aqueous surface and become very random in their motion only near the center of the membrane (31). If the surface interaction predominates and the bulk of the probe chain resides in the fluid interior of the membrane leaflet, one might anticipate little or no dependence of chain length on the diffusion coefficient. Companion measurements with nonpolar fluorophores support this interpretation. Tetracene and rubrene have no tendency to reside near the aqueous surface because they are nonpolar. They diffuse faster than NBD-PE and the labeled isoprenoid alcohols. Free area theory suggests that diffusion faster than the host lipid is not possible (7). Violation of this basic tenet of the theory suggests that the rate of diffusive motion for tetracene and rubrene is not controlled by the surface behavior of the bilayer. Rather they must move in the hydrocarbon interior of the membrane, although there will be some coupling to the rate of host lipid diffusion. The lack of a common surface restriction explains the variation of the diffusion coefficient with size for these molecules. Diffusion measurements of the labeled alcohols in the gel phase also support this interpretation. As discussed previously, the hydrocarbon chains in the gel phase are packed in a crystalline or semicrystalline state. In this case the alkyl chains of the labeled alcohols should have an effect on the diffusion coefficient as the membrane interior becomes more restrictive. This is precisely our observation. Diffusion in the gel phase is slower and is dependent on probe length in the expected way. Experiments in this phase are not, however, definitive.

Experiments in three-dimensional polymer solutions were intended to explore the chain length effect in a system lacking the surface interaction of a bilayer but mimicking the bilayer interior. Our experiments show a finite chain length effect. The model systems are not a true rendering of the membrane interior since unquestionably the membrane interior must retain some residual order perpendicular to the aqueous surface. The PPG matrices, by contrast, are random media with all manner of interacting chains. Nevertheless, since we do not see the chain interaction effect in membranes one must conclude this is because the surface interaction is not only critical but in fact overwhelms any hydrodynamic effects in the bilayer interior.

Not only do the labeled isoprenoid alcohols span an order of magnitude in length but they vary by approximately a factor of four in mass. Clearly this variation in mass is unimportant, as is the variation in size. Molecular dynamics studies of impurity diffusion in two-dimensional hard disk systems (32) suggest that there should be a mass dependence of the diffusion coefficients. We do not observe this behavior presumably because the restricting feature of NBD-labeled alcohol diffusion is the NBD headgroup itself and the alkyl chains are unimportant. This model views the labeled alcohols as a ball and chain system. The ball, localized near the surface, restricts the long range motion, while the chain is permitted a great deal of dynamic freedom. Increasing the length of the chain in this analogy does not alter the long range motion but does, of course, increase the overall dimensions of the molecule.

Recently Rajarathnan et al. (33) measured the rate of lateral diffusion of headgroup labeled ubiquinone in model membranes and mitochondria membranes. Their diffusion measurements, with relatively large probe concentrations, using a variant of the basic FPR technique, agreed with our results on the structurally similar isoprenoid alcohols. They found that the labeled ubiquinone diffused, in both types of membranes, at the same rate as NBD-PE. They suggest, as do we, that the NBD label is sufficiently polar to penetrate to some degree into the region of the more rigid phospholipid acyl chains but does not reside at the surface.

CONCLUSION

We have demonstrated that valid diffusion measurements may be performed with a series of NBD-labeled alcohols in DMPC model membranes. FPR diffusion measurements in liquid crystal bilayers revealed a surprising lack of dependence on the depth of probe penetration in the bilayer. Statistically insignificant variations were observed, even for probes which were an order of magnitude longer than the smallest probe used. The labeled isoprenoid alcohols citronellol, solanesol, and dolichol diffuse at the rate of lipid self diffusion which is 5.0×10^{-12} m^2 s^{-1}. Companion experiments with model polymer systems, with nonpolar fluorescent polyaromatic molecules and measurements in the membrane gel phase show that surface interactions control the rate of diffusion in the liquid crystal phase and free area theory may be used to treat the results.

The remarkable independence of the diffusion coefficient with increasing molecular length, and results in a different system which shows a weak dependence of the diffusion coefficient on probe radii at the surface, for molecules larger than the host lipid (B. J. Balcom and N. O. Petersen, manuscript in preparation), permit us to make a few generalizations about lateral diffusion of biological molecules in model membranes. The key structural feature, controlling the rate of diffusion, appears to be that at the surface of the membrane, not the details of molecular architecture else-

where. Surfactant biological molecules such as vitamin A, α-tocopherol, vitamin K, and ubiquinone should all diffuse at the rate of lipid self-diffusion in the liquid crystal phase. A large number of related molecules, one may also predict, will diffuse at this rate. Thus in biophysical calculations involving long range diffusive transport of nonprotein biomolecules, the lipid self-diffusion coefficient is likely an excellent estimate of the molecules' rate of motion.

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