Electric Field-Induced Concentration Gradients in Planar Supported Bilayers

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ABSTRACT A simple method of generating electric field-induced concentration gradients in planar supported bilayers has been developed. Gradients of charged, fluorescently labeled probes were visualized by epifluorescence microscopy and could be observed at field strengths as low as 1 V/cm. Steady-state concentration gradients can be described by a simple competition between random diffusion and electric field-induced drift. A model based on this principle has been used to determine the diffusion coefficient of the fluorescent probes. This technique achieves a degree of electrical manipulation of supported bilayers that offers a variety of possibilities for the development of new molecular architectures and the study of biological membranes.

INTRODUCTION

Poo and Robinson first demonstrated in 1977 that electric fields could induce redistribution of charged membrane components (Poo and Robinson, 1977). These researchers observed field-dependent redistribution of fluorescently labeled concanavalin A in the membranes of living muscle cells. More recently, controlled microelectrophoresis in supported bilayers has been demonstrated (Stelzle et al., 1992). In this work steady-state drift velocities of uniformly distributed, fluorescent probes were measured, allowing the authors to determine the mobility, μ, of the probes. In the present work, electric fields are employed to generate concentration gradients of charged probes in confined regions of a planar supported bilayer. This effect can be described by a simple competition between random diffusion and electric field-induced drift. Image analysis of these steady-state concentration profiles has been used to determine the diffusion coefficient of the fluorescent probes. The ability to electrically manipulate membrane composition will likely become a useful tool in the study of cell membranes and the principles of interfacing these membranes with solid supports. The simplicity of this method, requiring little more than a microscope, also makes it highly accessible.

MATERIALS AND METHODS

Planar supported bilayers were produced by fusion of small unilamellar vesicles (SUVs) with clean glass coverslips (Corning, Corning, NY). The SUVs were prepared roughly according to the Barenholz procedure (Barenholz et al., 1977): The lipid solution, generally in chloroform, was evaporated onto the walls of a round-bottom flask, which was then evacuated overnight. Lipids were resuspended in 10 mM Tris buffer at pH 8.0 with 100 mM NaCl by vortexing moderately for several minutes. The lipid concentration at this point was typically around 6 mg/ml. The lipid dispersion was then probe sonicated to clarity on ice under a steady flow of argon to minimize oxidation of the unsaturated lipids. The SUVs were separated from other lipid structures by ultracentrifugation for 5 h at 45,000 rpm. The supernatant contained the SUVs with typical yields of 50–75%. SUVs were stored at 4°C and used within 3 weeks. L-α-Phosphatidylcholine from egg (egg-PC) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine], sodium salt (DOPS) were obtained from Avanti Polar Lipids (Alabaster, AL). The fluorescent probes, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE), *N*-(Texas Red sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE), 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD cholesterol), and 4-(4-didecylaminostyryl)-*N*-methylpyridinium iodide (4-Di-10-ASP), were obtained from Molecular Probes (Eugene, OR). The glass surfaces were prepared by rinsing with purified water (Millipore system, 10 MΩ-cm), drying under a nitrogen stream, and heating at 400°C for 5 h and were used within 8 h. The bilayer was deposited by placing a coverslip over an 80-μl drop of SUV suspension in a petri dish for several minutes. The dish was then carefully filled with distilled water, taking care not to allow the concentrated vesicle dispersion to contact the top of the coverslip. The supported membrane was rinsed by shaking gently. Confined regions were created by scratching the membrane-coated surface with a pair of tweezers. The supported membrane was then assembled underwater into a sandwich with another coverslip.

The electrophoresis cell consisted of two 0.01-inch-diameter platinum wire electrodes on two glass plates (Fig. 1). The coverslip sandwich was arranged in such a way as to form a bridge between the two glass supports. Electrical connection was achieved through water contact. All glass was rinsed before use to remove any residual salt deposits. Fields up to 60 V/cm were applied with a standard power supply. Currents were monitored with a Keithley picoammeter (Cleveland, OH) and were typically around 0.5 μA for a single 18-mm square coverslip sandwich at 15 V/cm. This corresponds to a total power dissipation of 1.5×10^{-5} W, which should produce a negligible amount of Joule heating. Membranes were observed in a temperature-controlled room (21°C) with an epifluorescence microscope (Zeiss, Oberkochen, Germany) and a 10× objective. Images were monitored with a low-light-level video camera (Cohu, San Diego, CA) and recorded with an S-VHS VCR (JVC, Elmwood Park, NJ). The camera's gamma factor ($I_{out} = I^{\gamma}$) was set at $\gamma = 0.75$ and the image intensity data transformed accordingly. Image analysis was performed on a Macintosh computer.

THEORY

The concentration gradients in a confined region of supported bilayer can be described by a competition between

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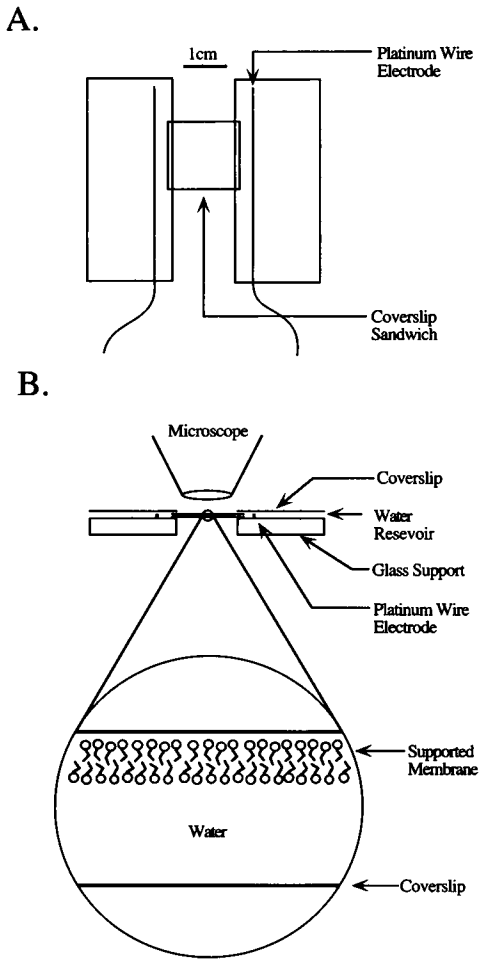

FIGURE 1 (*A*) Top view of the bilayer electrophoresis cell consisting of a coverslip sandwich forming a bridge between the platinum wire electrodes on two glass supports. The supported membrane coats the inside surface of the top coverslip in the sandwich. (*B*) Side view of the same arrangement showing an additional two coverslips used to contain the water reservoir serving to make electrical contact with the electrodes. The inset depicts a cross section of the coverslip sandwich illustrating the location of the supported membrane (not drawn to scale).

random diffusion and electric field-induced drift:

$$\frac{\partial C}{\partial t} = D\nabla^2 C - \mathbf{V} \cdot \nabla C$$

where C is the concentration, D is the diffusion coefficient, and $\mathbf{V}$ is the drift velocity. The concentration profile at steady state can be obtained from the following conditions:

$$\frac{\partial C}{\partial t} = 0 \qquad \text{and} \qquad \mathbf{J} = -D\nabla C + \mathbf{V}C = 0$$

where $\mathbf{J}$ is the flux. It can be shown that solutions satisfying these conditions have the form

$$C(\mathbf{r}) = C_o e^{\mathbf{V}\cdot\mathbf{r}/D}$$

for a confined region of membrane where $\mathbf{r}$ is the position vector. Hence, regardless of the shape of the region, steady-state concentration gradients will have a characteristic exponential profile determined by the ratio between the scalar magnitude of the drift velocity and the diffusion coefficient.

Another commonly occurring type of shape, relevant to results presented below, has straight line boundaries in one direction but is effectively infinite in the other. In this case, one must allow for a constant flux in the unbounded direction. If the coordinate system is defined with the y axis parallel to the boundary, the conditions on $\mathbf{J}$ are

$$J_x = 0 \qquad \text{and} \qquad J_y = CV_y.$$

(Strictly speaking, $J_y = CV_y + \textit{constant}$ also gives rise to steady-state solutions but with linear profiles in the y direction. These, however, are not realizable for systems starting from homogeneity.) The resulting steady-state concentration gradient will show the characteristic (V_x/D) exponential profile perpendicular to the boundary but will exhibit a constant profile in the parallel direction. For the experiments presented here it was more convenient to define the coordinate system with the x axis parallel to the field; the concentration profile in this direction is given by

$$C(x) = C_o e^{V(\cos^2\gamma)x/D}$$

where γ is the angle between the direction of the field and the normal to the boundary. The ratio V/D can be determined in a straightforward manner from the concentration profile along the direction of the field for a wide variety of bounded and semi-bounded shapes.

The drift velocity can be measured directly during the approach to steady state. The configuration most useful for this measurement consists of a boundary perpendicular to $\mathbf{V}$ with no other obstacles nearby. Upon application of the field, all probe molecules will begin to drift with the constant average drift velocity; thus an image of the boundary will drift along with this velocity. The time-dependent behavior of this image can be described by

$$C(x, t) = \frac{2h}{\pi}\sum_{n=0}^{\infty}\frac{e^{-(2n+1)^2Dt}\sin((2n+1)(x - Vt))}{(2n+1)}$$

where $\mathbf{V}$ is in the x direction. This is the Fourier series representation of a step function with a diffusive decay; the height, h, is taken to be much smaller than the period. Hence, the midpoint of the concentration profile, corresponding to $x - Vt = 0$, moves along with the drift velocity. This point can be located accurately with image analysis software, allowing it to be tracked for many minutes over distances of more than 100 μm (Fig. 2). Generally four or five measurements of the position were made at various times for the same membrane, showing the expected linear dependence (Fig. 3).

RESULTS AND DISCUSSION

A variety of membrane compositions and probes were observed to exhibit similar behavior. Membranes consisting of

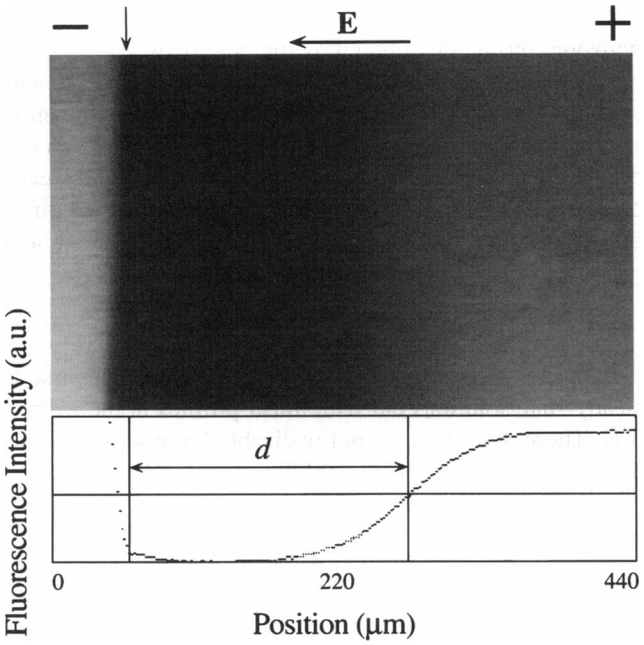

FIGURE 2 Unaltered video micrograph of the drifting image of a barrier during approach to steady state. The scratch boundary is marked with a vertical arrow and the field is in the x direction as indicated. The membrane consisted of pure egg-PC with 1% by mole Texas Red DHPE. The image was taken 600 s after a 20V/cm field was first applied. Below is a trace of the fluorescence intensity (vertical average) across the image. Location of the midpoint, d, of this intensity profile allowed determination of the drift velocity.

pure egg-PC or an 80:20 mixture of egg-PC and the net negatively charged DOPS were doped, 1% by mole, with either NBD-PE or Texas Red DHPE, both of which also bare a net negative charge. For all four combinations, similar results were observed: the probes built up concentration gradients toward the anode side of each bounded region. The positively charged 4-Di-10-ASP, incorporated at 1% by mole into either of these two membrane compositions, built up gradients toward the cathode. A membrane containing both 4-Di-10-ASP and NBD-PE showed build-up of fluorescence at both ends of the confined regions. Probe redistributions were observable for field strengths as low as 1 V/cm and were generally reversible. Concentration gradients could be switched back and forth numerous times by flipping the direction of the field and were observed to relax back to uniformity when the field was removed. However, at field strengths greater than 25 V/cm it was possible to induce irreversible changes in the membrane when the field was run for extended periods of time (>30 min). In particular, a substantial reduction of the probe diffusion in the concentrated regions was common. It is likely that the unusually high concentration of charged components in these regions causes the membrane to interact differently with the solid support, thus altering the diffusion. This irreversible behavior was avoided for the results presented here.

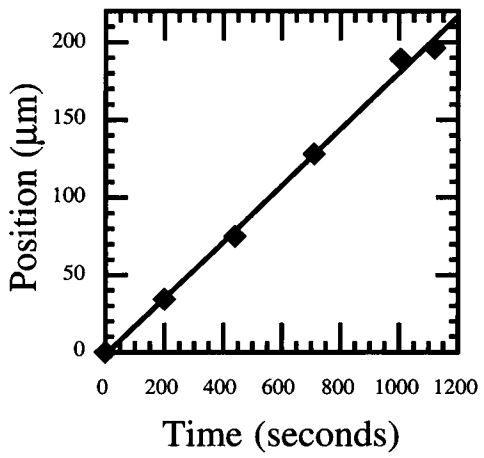

FIGURE 3 Plot of midpoint measurements at several times for a pure egg-PC, 1% by mole Texas Red DHPE membrane run at 15 V/cm showing the linear dependence of the image position with time.

It is known that electroosmosis can play a significant role in the field-induced motion of charged particles in membranes (McLaughlin and Poo, 1981). For the experiments described here, there generally was a bulk electroosmotic flow of solution toward the cathode on the order of 100 μm/s for field strengths of 15 V/cm, as estimated from the motion of stray vesicles. The purely electrophoretic contribution to the drift velocity is related to the applied field, $\mathbf{E}$, the charge of the probe, ze, and the mobility, μ, as

$$\mathbf{V} = \mu ze\mathbf{E}.$$

In general, frictional coupling to the bulk electroosmotic flow will also contribute to the drift velocity. The magnitude of this effect can be estimated by comparison of the measured drift velocity to values predicted from the above equation with μ obtained from the Einstein relation:

$$\mu = D/kT$$

where k is the Boltzmann constant and T is the temperature; the diffusion coefficient was determined as described below. For the probes studied here, measured drift velocities were typically 55–65% of the predicted magnitude for purely electrophoretic drift. Stelzle et al. have observed similar electroosmotic contributions to the mobility of the probes they studied (Stelzle et al., 1992). To further characterize the effect of electroosmosis, membranes containing the neutral probe, NBD cholesterol, were examined.

Membranes consisting of egg-PC, DOPS, NBD cholesterol, and Texas Red DHPE in a 78:18:3:1 ratio were observed. After 10 min at 50 V/cm (within the range of reversible behavior), the Texas Red DHPE was distributed in a typical profile, whereas the NBD cholesterol showed uniform fluorescence everywhere that was depleted of Texas Red DHPE. However, in the regions where the Texas Red probe was concentrated, the NBD fluorescence was substantially reduced. To confirm that this effect was due to fluorescence quenching, NBD cholesterol and Texas Red DHPE were observed in separate membranes run in parallel

on the same electrophoresis device. After 10 min at 50 V/cm large concentration gradients were observed for the Texas Red containing membrane, whereas the NBD cholesterol membrane appeared uniform. These results indicate that electroosmosis does not have a major effect on the large-scale structure of the membrane over the time scales of these experiments.

Quantitative information on these concentration profiles reveals that the simple model describing them as a competition between diffusion and field-induced drift is reasonably accurate. For the quantitative study, pure egg-PC membranes containing 1% by mole of either Texas Red DHPE or NBD-PE were examined under field strengths ranging from 15 to 25 V/cm. Exponential fits were obtained for a number of traces across the midsection of a region like that depicted in Fig. 4. No attempt was made to fit the clearly nonexponential roll-off at the peak of the concentration profile, which may be due to fluorescence self-quenching or other effects related to the substantially altered membrane composition. Ratios of *V/D* obtained from the exponential fits were combined with measurements of *V* for each membrane to get an estimate of the diffusion coefficient. The value of

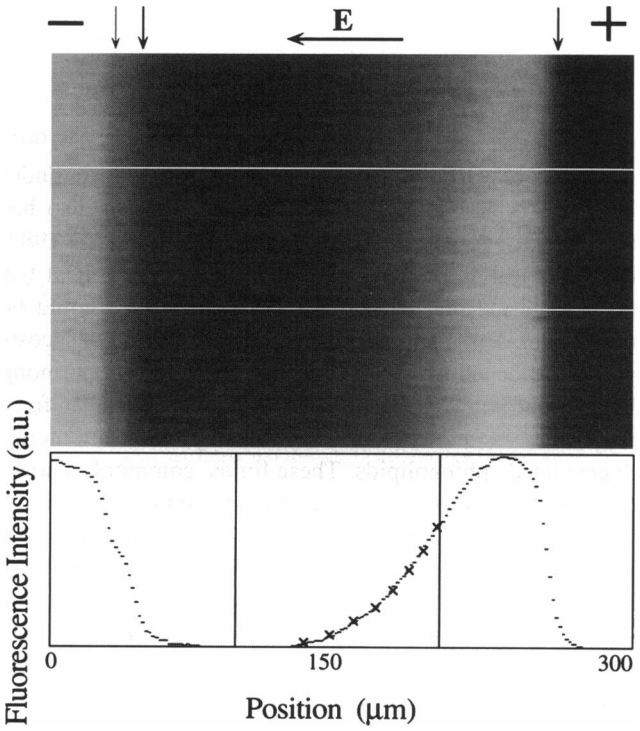

FIGURE 4 Unaltered video micrograph of a steady-state concentration profile in a semi-bounded region of membrane. The scratch boundaries are marked with vertical arrows (gray arrow indicates a weak secondary scratch), and the field is in the *x* direction as indicated. The pure egg-PC, 1% by mole Texas Red DHPE membrane, had been held at an electric field strength of 15 V/cm for more than 20 min when this image was taken. Below is a fluorescence intensity trace of the region between the two white lines. The vertical bars depict typical limits of the regions that were fit to exponentials to obtain the *V/D* ratio. ×, trace of an exponential fit to the data.

D obtained was 4.6 ± 1.2 $\mu m^2/s$, which is comparable to values obtained from fluorescence recovery after photobleaching studies (4.4 ± 0.5 $\mu m^2/s$) (Stelzle et al., 1992) for similar systems.

CONCLUSION

This work introduces a simple and accessible method of generating concentration gradients in planar supported membranes. A significant point is that these concentration gradients are easily observable when the membrane is carved into confined regions and that the concentration profiles do not show a sensitive dependence on shape. Reasonable estimates of the diffusion coefficient from image analysis indicate that a model of this phenomenon as a competition between electric field-induced drift and diffusion is accurate. Hence this technique provides a simple means of determining the diffusion coefficient of species in a supported membrane. Measurement of the drift velocity as the system approaches steady state alleviates the need to precisely control the field at all points in the membrane and allows estimation of electroosmotic effects. The behavior of the neutral, negatively charged, and positively charged probes examined was dominated by electrophoretic influences. Preliminary results indicate that a glycan-phosphatidyl inositol-linked form of the major histocompatibility complex can be manipulated in a similar way. Such electrical concentration of membrane-associated proteins may assist the crystallization process. It has been demonstrated that electric fields can induce phase separation in lipid monolayers at the air-water interface (Lee et al., 1994). The technique introduced here is currently being applied to the study of electric field-induced phase separations and chemical gradient-induced motion in the more biologically relevant bilayer membranes.

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