Coupling Optical and Electrical Measurements in Artificial Membranes: Lateral Diffusion of Lipids and Channel Forming Peptides in Planar Bilayers

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ABSTRACT

Planar lipid bilayers (PLB) were prepared by the Montal-Mueller technique in a FRAP system designed to simultaneously measure conductivity across, and lateral diffusion of, the bilayer. In the first stage of the project the FRAP system was used to characterise the lateral dynamics of bilayer lipids with regards to phospholipid composition (headgroup, chain unsaturation etc.), presence of cholesterol and the effect of divalent cations on negatively-charged bilayers. In the second stage of the project, lateral diffusion of two fluorescently-labelled voltage-dependent pore-forming peptides (alamethicin and S4s from Shaker K^+ channel) was determined at rest and in the conducting state. This study demonstrates the feasibility of such experiments with PLBs, amenable to physical constraints, and thus offers new opportunities for systematic studies of structure-function relationships in membrane-associating molecules.

INTRODUCTION

The need to couple functional measurements (nearly exclusively electrical measurements for channels) with other physical methods informing about membrane dynamics or structure (with respect to both lipid matrix and protein or peptide effectors) is a long-standing issue in Biophysics. It requires ingenious experimental set-ups that often compromise between the high sensitivity and rapid kinetics allowed by electrophysiological recordings (at the single-channel level, 1-10 molecules can be functionally monitored in a membrane area of, say, 1 mm^2 on a millisecond timescale) and the usually low signal to noise ratio associated with optical or spectroscopic methods. Refer to the review by MacDonald and Wraight (1). Nevertheless, beginning with the classical papers on natural excitable membranes of the sixties, eg. (2), successful and meaningful studies have been reported ever since, perhaps with fewer such attempts reported recently despite technological improvements. Some of these

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technologies are still in development, for example, near-field optical microscopy and fluorescence correlation spectroscopy, among others.

In spite of the fact that planar lipid bilayers are still the best-suited artificial membrane system for the study of reconstituted ion channels and receptors, data dealing with their physical characterisation, especially as regards dynamics are scant. The dynamics of molecules in membranes (order parameter, degree of freedom of lipid aliphatic chains, lateral and rotational diffusions), often referred to as membrane fluidity, have been associated with modulation of the activity of many important functions of proteins in biological membranes (for a review, see 3). Following cold or heat adaptation and diet changes, the membrane lipid composition can readjust via metabolic pathways so as to provide an optimal lipid environment (the "homeoviscous theory", see e.g. 4-5). Alterations in the microviscosity (and presumably the lateral mobility) can also be strongly correlated to some pathologies (6). Transient fluidity changes were also shown, through monitoring of the rate of excimer formation, to occur in pyrene labeled nerve fibers during action potentials (7). Lateral diffusion within natural or artificial membranes is one of the most well-characterised parameters related to the dynamic state of the membrane (see review 8). This diffusion has fundamental implications in functional coupling between membrane components through collisional mechanisms as, for example, in the photosynthetic electron chains (9), in visual transduction (10) as well as in receptor-mediated endocytosis (11) and in intercellular adhesion (12). Recently, new techniques such as "single particle tracking" demonstrate that lateral diffusion is not homogeneous throughout the plasma membrane of most cells (13-15). Specific examples include cell junctions of vascular endothelium (16) and neurons (17).

Over the last fifteen years, fluorescence recovery after photobleaching (FRAP) has become the most direct and elegant way of measuring the rate of lipid and protein lateral diffusion, mostly within biological membranes such as red blood cells (18). Significant theoretical and methodological improvements such as the "fringe or periodic pattern bleaching" (19), later improved (20) and more recently "scanning microphotolysis" (21) complement the basic technique.

Although most spectroscopic investigations dealing with membrane models have been carried out on populations of lipid vesicles, planar lipid bilayers have become popular model systems for characterising the function of purified voltage-gated channels, receptors and their peptide models (see e.g. 22-24). Pioneering work on direct measurement of the lateral diffusion coefficient (D) of fluorescent probes in lipid bilayers was carried out with bilayers supported on a circular platinum loop (25) and on electron microscopy grids (26). However, little effort has since been devoted to characterising D in more "realistic" model membranes used in reconstitution studies. The present work gives the first determinations of D on "virtually solvent- free" Montal-Mueller bilayers (referred hereafter as PLB for planar lipid bilayers) formed in a conventional manner (27), and which are amenable to simultaneous fluorescence recovery after photobleaching (FRAP) as well as conductance experiments.

MATERIALS AND METHODS

Materials

The lipids were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Cholesterol (CHOL) was purchased from Sigma Chemical Company (St Louis, MI). The fluorophore N-(7-nitrobenzoyl-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phospho-ethanolamine (NBD-PE) was purchased from Molecular Probes (Eugene, OR). Fluorescently labelled alamethicin (Alm-Gly-FITC) was prepared as previously described (28). Fluorescently labelled S4 Shaker K^+ (N-(7-nitrobenzoyl-2-oxa-1,3-diazol-4-yl) -S4 Shaker (NBD-S4 Shaker) was a gift from Dr Y. Shai (29).

Virtually solvent-free planar lipid bilayer formation

A 25μm thick PTFE septum (Goodfellow, Cambridge, UK) with a hole of 200-300 μm diameter (made by an electric arc discharge) in the centre was clamped into a specially designed chamber allowing simultaneous electrical (conductance and capacitance) and FRAP measurements (Figure 1).

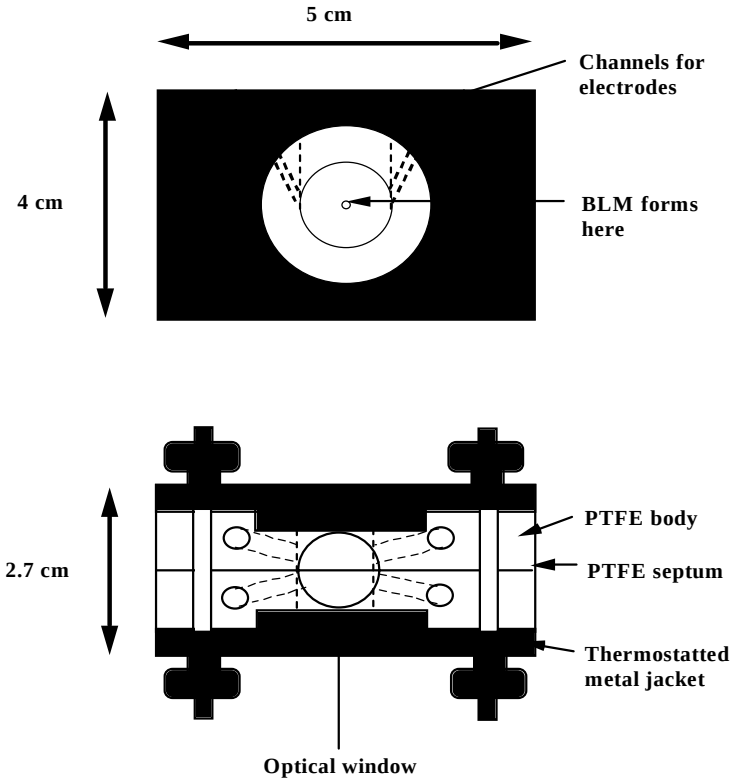

Figure 1. A Schematic diagram of the cell developed for formation of planar lipid bilayers (PLBs) for combined optical and electrical measurements

The body of the cell was machined from two blocks of PTFE. The cell was assembled by carefully positioning the PTFE septum between the cell halves with the hole located centrally. Optical windows were fitted in the recesses of the outer faces of the cell. These in turn were held in position by a temperature controlled brass housing which was clamped against each face of the cell. Prior to membrane formation, the hole in the septum was coated with 1 µl of 1% (v/v) hexadecane in hexane on each side. The hexane was allowed to evaporate. To form Montal and Mueller bilayers, buffer was added to each side of the chamber such that the level was above the hole in the septum. Lipid containing 1 mole% NBD-PE was spread from a hexane-ethanol (9:1 v/v) solution on the buffer surface in the chambers and allowed to stand for a few minutes to allow evaporation of the solvent. The buffer level on the *trans*-side was lowered below the hole in the PTFE septum and then raised back to its original level. PLB formation was monitored visually and by capacitance measurements.

Capacitance and conductance measurements.

Conductance and capacitance measurements were performed from the same experimental set-up. A DC voltage or a mixed DC-AC voltage from a signal generator (20 mHz Pulse/Function generator, model 628, Dynatech, Nevada) is applied in the *cis* side of the bilayer via Ag/AgCl electrode. A second Ag/AgCl *trans* electrode is connected to an I-V converter (RAP Montgomery, model HAMK2, London, UK) with a 1 GΩ feedback resistor. The output signal is then filtered by a dual variable filter (Kemo, model VBF4, Beckenham, UK) and sent to an oscilloscope, a X-Y recorder or a microcomputer. For the conductance measurements, a DC or a low frequency (0.01 Hz) triangular voltage is applied to ensure steady-state instantaneous current responses. For capacitance measurements, a triangular wave of high frequency (amplitude 10 mV, frequency 100Hz) was applied to the bilayer. If the conductance of the bilayer remains low, the capacitance of the system could be directly obtained from the rms value of the squared output ($I = C \cdot dV/dt$) , capacitance being equal to V/K, with K =10 $mV \cdot pF^{-1}$ The system was calibrated with capacitors of known values and the contribution of the line (cables, connections ... outside the bilayer) was substracted.

Fluorescence recovery after photobleaching (FRAP)

Measurement of the lateral diffusion coefficient of the fluorophore in planar lipid bilayers was achieved using the FRAP method. A schematic diagram of the apparatus (Figure 2) was developed from our conventional spot photobleaching FRAP set-ups that are based on upright (30) and inverted (31) microscopes.

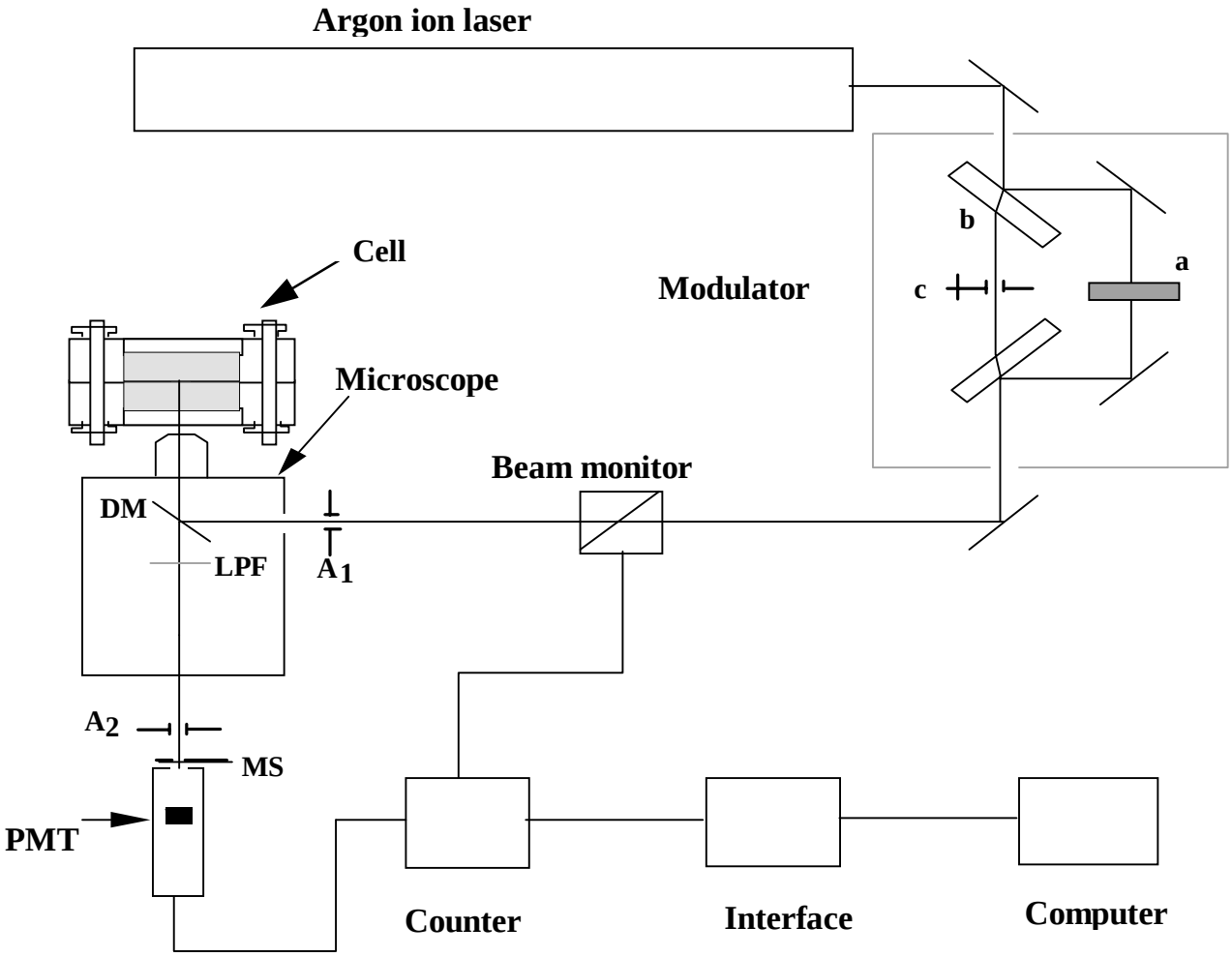

Figure 2. Schematic diagram of the principal components of the Fluorescence Photobleaching (FRAP) apparatus

Essentially the apparatus was constructed using components from a Nikon Optiphot microscope mounted on its side (i.e. with a horizontal optical axis). The nosepiece and trinocular eyepiece of the microscope were mounted on a special Nikon bracket which was bolted to an optical rail (Newport, Microcontrole) on the laser table (Photon Control, Cambridge). The PLB cell was mounted on a separate bracket attached to a micrometer-controlled translation stage to allow focusing, and the stage was again bolted to the optical rail.

The intensity of the beam of a 10W argon ion laser (Innova 100-10) was attenuated by reflection off glass flats and by passing through a neutral density filter as shown in Figure 2. When the fast electronic

shutter (Uniblitz) (c) is closed, only the monitoring laser beam (a) illuminates the sample. When the shutter is open the intense, bleaching beam (b) which is transmitted through two of the glass flats passes through to the sample. The beam provided by the modulator (Coherent Innova, model 304A) passed through a beam monitor (beam splitter and photodiode), the signal from which is used to electronically compensate for minor fluctuations in laser beam intensity. The laser beam then passed through a pinhole aperture (A_1) located at the image plane, at the entrance port of the epi-illumination attachment of the fluorescence microscope. A dichroic mirror (DM) and long pass filter (LPF) were used to ensure only emitted light reached the photon counting photomultiplier tube (PMT; Thorn-EMI 9816B) positioned at the camera port of the trinocular eyepiece. The PMT was protected during the bleaching pulse by an electronic gating circuit and a mechanical shutter (MS). Prior to entering the detector, the emitted light beam passed through a second aperture (A_2) again positioned at the image plane. The laser beam profile and spot radius at the point of focus were determined using a beam scanner (Photon Inc, BeamScan model 2180, California, USA). The laser beam was of Gaussian cross sectional intensity with half-width at $1/e^2$ height of the laser beam at its point of focus equal to 3.3 μm (spot radius). System timing and control, data acquisition and data analysis are performed using a VME microcomputer system (Motorola 68020). The acquisition software was developed in house. The microscope and cell were enclosed with an aluminium box which acted as a Faraday cage and minimised noise in the conductance measurements.

All FRAP experiments were performed at a controlled room temperature of 23°C. The laser spot (spot radius 3.3 μm) was directed at the centre of the PLB and was sufficiently small for the surrounding bilayer to act as a "near infinite reservoir". In the experiments described below, generally ten FRAP curves were collected for each set of conditions and averaged before analysis. FRAP data were analysed by non-linear least squares fitting to an expression defining the time dependence of fluorescence recovery observed with a circular beam of Gaussian cross sectional intensity and had the form (32):

$$F(t) = \frac{F(0) + F(\infty)\left(t/\beta t_D\right)}{1 + \left(t/\beta t_D\right)},$$

where F(t) is the observed fluorescence as a function of time, F(0) is the intensity of the fluorescence immediately after the bleach pulse, F(∞) is the fluorescence at infinite time after the bleach pulse, β is the depth of bleach parameter and t_D is the characteristic diffusion time. The lateral diffusion coefficient, D, is given by $D = w^2/4t_D$, where w (spot radius) is the half-width at $1/e^2$ height of the laser beam at its point of focus on the membrane. The percentage mobile fraction (%R; %recovery) is given by:

$$\%R = \frac{F(\infty) - F(0)}{F(t < 0) - F(0)}$$

where F(t<0) is the prebleach fluorescence (33).

RESULTS AND DISCUSSION

At the initial stages of discussing the concept of combining the FRAP and bilayer set-up, two configurations were discussed. Firstly, having the FRAP system on the vertical axis and the bilayer set-

up on the horizontal axis. The second configuration involved developing a novel FRAP system on the horizontal axis and forming the bilayer in the conventional manner in the vertical axis. The first configuration was attractive because at the Institute of Food Research (IFR) we already had well established FRAP systems using the conventional inverted (31) and upright (30) microscopes. Therefore, we had to design a novel bilayer system which would allow formation of PLBs on the horizontal axis and use it like a “specimen slide” to interrogate the bilayer with the FRAP system. However, attempts at Rouen University to develop a system which would allow the formation of the PLBs on the horizontal axis showed that this approach was problematical. Therefore, we decided to proceed with the second configuration and mounted the microscope horizontally on an optical rail and then connected the necessary electronics to carry out FRAP.

Initial tests of the horizontal FRAP layout, indicated that problems with the alignment of the optics had to be solved. Briefly, in the FRAP set-up the bleach laser beam, the monitoring laser beam and the detection of fluorescence by the PMT have to be coincident at the point focus on the planar bilayer. At the IFR we had considerable expertise in aligning the optics of the inverted (31) and upright (30) microscopes to successfully carry out FRAP experiments. However, with the microscope essential dismantled and mounted horizontally all the usual reference points, used to align the optics, were no longer present. To overcome this difficulty a special viewer was designed to allow the fluorescent spot to be monitored at the exit pinhole and to ensure that all the optics were in register to give optimal FRAP.

Introduction of the bilayer set-up involved the designing and construction of a bilayer chamber which was compatible with the FRAP set-up. The design shown in Figure 1 has proved to be excellent. It has to noted that the distance from the centre of the chamber where the hole in the septum is located to the optical window is 1cm and therefore requires the use of long working distance objectives in order to observe the formation of the PLBs. To minimise the electrical interference from the laser equipment, the current-voltage converter used to measure capacitance and conductance of the PLBs was installed as close as possible to the chamber between the incident and collecting parts of the microscope with the whole being enclosed in a Faraday box. Special software was also developed to link the bilayer and FRAP detection and analysis systems in order to synchronise the data collection.

In conjunction with establishing the equipment we also transferred the techniques necessary to make PLBs from Rouen and Essex Universities to IFR. Three techniques commonly used to form bilayers were tested for the value to this project:

1) The liposome method (34) proved the most reliable way to form a bilayer. Although conductivity measurements could be performed with this membrane, an excess of liposomes in the light path made FRAP measurements impossible.
2) The "painting" method (35) formed bilayers which allowed both conductivity and FRAP measurements. The main disadvantage of the bilayers formed by this technique was that they contained large amounts of spreading solvent and the physical properties of such bilayers were not constant.
3) The Montal-Mueller method (27) of forming bilayers was ideal for both FRAP and conductivity measurements.

The bilayers were usually prepared with the fluorophore incorporated into the lipid phase. However, some fluorescently labelled peptides were added directly into the bulk phase and incorporation into the bilayer was monitored by conductance and fluorescence measurements. The fluorophores were chosen such that there was little or no fluorescence from the free fluorophore in bulk aqueous phase, but, when incorporated into the lipid phase, there was a significant enhancement in fluorescence. This assisted in minimising the interference from the fluorescence of the fluorophore in the bulk aqueous phase and ensured that only fluorescence from the fluorophore incorporated into the bilayer was being monitored. However, if the choice of fluorophore was restricted such that there were appreciable amounts of fluorescence in the bulk aqueous phase, then we used a rapid perfusion system to exchange the aqueous phase in the chamber and then proceeded with the experiment. This procedure would eliminate interference from the fluorescence of the fluorophore from the bulk aqueous phase and allow the measurement of fluorescence of the fluorophore associated with the bilayer.

The new experimental set-up was tested for standard lipid diffusion regimes in nude bilayers (without "membrane effectors") with different lipids, cholesterol, and added calcium (36). We then proceeded to illustrate how important information about the pore forming mechanism of alamethicin could be derived from the approach described in this article. Alamethicin, a natural peptaibol (rich in the non-coded alpha-aminoisobutyric acid), being still the prototype (the most extensively-studied) of highly voltage-dependent pore-formers is useful in modelling aspects of physiologically-important ion channels and receptors (i.e. the action potential Na^+, K^+ and Ca^{2+} channels). As a result, the "barrel-stave" model (popular for describing the pore forming mechanisms of alamethicin and by later extension of a number of natural or designed amphipathic peptides) for dynamic intramembrane conducting aggregates applies strictly to channel- and pore-formers whose open state (or active conformation) is driven by voltage. In this model, the central conducting part of the pore is made up by the juxtaposition of the hydrophilic sectors of neighbouring helices forming a transmembrane bundle. The uptake and release of alamethicin monomers by this bundle, via lateral diffusion in the bilayer, is the simplest explanation accounting for the non-integral conductance increments. There is general agreement between conductance values and a geometric model for a pore of varying size, depending upon the number of monomers or helical rods. In this scheme, it is obvious that the lateral diffusion of monomers within the lipid bilayer is an important process in regulating the pore structure. To this end, we prepared fluorescently labelled pore forming peptides and determined their lateral diffusion properties at rest and in the conducting state.

Results show that the voltage-induced "building up" of peptide conducting aggregates (whether Alamethicin (28) or S4s, the gating element of Shaker potassium channels) leads to a slight but significant reduction in the lateral diffusion of the peptide. The voltage-dependent process can be divided into i) a voltage-driven partition of the peptide from the aqueous bulk and/or insertion into the bilayer, and ii) an intrinsic voltage-sensitivity (gating proper) of the embedded aggregates. The two do not match exactly and the apparent high voltage-dependence observed in macroscopic conductance experiments (see below) mainly reflects the first process.

$$G = I / V = \langle N \rangle(V) \cdot \gamma \cdot Po(V)$$

where G and I are macroscopic conductance and current, V the applied voltage, $\langle N \rangle(V)$ the number of channels expressing or opened at a given voltage, γ the average single-channel conductance—or the

conductance of the most probable substate—and Po(V) the probability of opening at the specified voltage. Thus the reduced lateral diffusion coefficients observed with these two different peptides (quite unrelated in their sequence and amphipathy) most probably result from the further membrane-embedment of the pore-formers under the influence of voltage (on the helical dipoles), the first step leading to “peptide channels”.

This study illustrates the feasibility of coupled optical and electrical measurements on reconstituted systems, i.e. ion channels or peptide models interacting with planar lipid bilayers. This is an exquisite experimental/biophysical configuration which allows precise control of the chemical species involved (lipids, peptides etc) and of electrolytes on both sides of the bilayer, and it is unique in maintaining gradients (electrical and chemical). These parameters are much to harder to control in the conventional lipid vesicles (or proteolipid vesicles). As recently suggested (1), combining spectroscopic and electrical recording techniques offers real opportunities in the investigation of structure-function relationships of membrane interacting peptides.

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