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# Probing the dynamics of planar supported membranes by Nile red fluorescence lifetime distribution

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**Abstract**

The structure and dynamics of planar supported membranes were studied by using the fluorescence probe Nile red. The width of fluorescence lifetime distribution of Nile red was used to infer the heterogeneity of membranes. The width of fluorescence lifetime was larger and the lifetime was shorter in supported membranes when compared to vesicle membranes. This was interpreted as due to the presence of water-filled membrane discontinuity leading to a heterogeneous surface in supported membranes. Microdomain causing agents such as cholesterol, sphingomyelin, etc. caused a larger level of heterogeneity in supported membranes when compared to vesicle membranes. 

*Keywords:* Supported membrane; Fluorescence lifetime distribution; Nile red; Fluorescence probe

Recent years have witnessed advances in membrane studies that have led to the revision of the opinion on the conventional fluid mosaic model of the membrane structure [1]. The idea that membranes merely provide the membrane proteins with an appropriate solvent is no longer valid. We now know that different species of lipids are asymmetrically distributed over the cytoplasmic and exoplasmic leaflets of the cell membranes [2]. The lipids are also organized in the lateral dimension due to preferential packing of sphingolipids and cholesterol into microdomains [3–7]. These lipid domains play an important role in the various biological functions associated with the cell membrane [3,5]. Moreover, the physical state of the cell membranes is important in controlling many processes in the living cell [8,9]. Knowledge of membrane heterogeneity and dynamics is thus necessary for a complete description of cell physiology.

Several model membrane systems have been used to study the various physical properties and the physiology of cell membranes. Vesicles have been used to study membrane transport and black lipid films have proved useful for carrying out electrical measurements on lipid bilayers. Planar supported bilayers have been developed more recently [10–12]. They are self-assembling two-dimensional systems consisting of a single phospholipid bilayer either attached to a solid substrate or separated from the solid support by an ultrathin film of water/polymer. They were originally developed to study cellular interactions in the immune system where they have proved to be very useful [11,12]. A major advantage of planar supported membranes is that they are geo-

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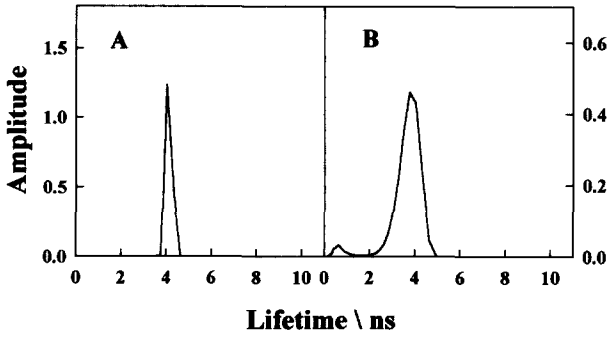


Fig. 1. Typical fluorescence lifetime distribution of Nile red in DMPC vesicles (A) and in DMPC membrane supported on a microscope coverglass (B). The peak position and width were 4.13 ns and 0.34 ns for A and 3.8 ns and 1.43 ns for B respectively.

metrically well defined which enables extensive spectroscopic studies on a microscopic scale. This is not easily achievable with other model membrane systems. The structure of supported membranes has been characterized by various spectroscopic techniques such as NMR [13], total internal reflection fluorescence [14,15], surface scattering of neutrons [16,17] and lateral diffusion measurements [14,15].

Here we report a study on the dynamics of planar supported bilayers and its comparison with that of vesicle membranes using time-resolved fluorescence microscopy. We have used the fluorescence lifetime distribution of the membrane probe Nile red [18] to characterize membrane dynamics, through the heterogeneity of the population of the probe. Analysis of fluorescence decay kinetics as a distribution of lifetimes by the maximum entropy method (MEM) has been validated in complex systems [19–21]. The width of lifetime distribution of Nile red has been used to extract information on the dynamics of various lipid bilayers [22,23]. Our results indicate that the planar supported bilayers are less fluid, more polar and heterogeneous when compared to vesicle membranes.

Planar supported membranes were prepared by fusion of sonicated vesicles [24] with either glass coverslips [25] or freshly cleaved mica surfaces. The lipid vesicles had the membrane probe Nile red in the ratio of 1:100–200 (Nile red/lipid mol/mol). Bilayer membrane was allowed to self-assemble by placing a coverslip/mica sheet over a drop of vesicle suspension in a Petri dish for about 45 min. The dish was then carefully filled with the buffer (20 mM $KH_2PO_4$, 150 mM KCl, pH 7.4) and the supported membrane rinsed several times by shaking gently and decanting the buffer. The bilayer thus formed was sandwiched between two coverslips, taking care not to expose the bilayer to air.

Table 1
Comparison of fluorescence lifetime distribution of Nile red obtained by MEM analysis for vesicles and planar supported membranes

| Lipids | Vesicle membrane | | Supported bilayer membrane | | | |
|---|---|---|---|---|---|---|
| | Peak position (ns) | Width of lifetime distribution (ns) | Glass support | | Mica support | |
| | | | Peak position (ns) | Width of lifetime distribution (ns) | Peak position (ns) | Width of lifetime distribution (ns) |
| 1. SBPL | 3.93 ± 0.03 | 0.73 ± 0.20 | 3.66 ± 0.16 | 1.30 ± 0.29 | 3.93 ± 0.11 | 1.14 ± 0.12 |
| 2. SBPL+15% sphingomyelin | 4.02 ± 0.02 | 0.77 ± 0.28 | 3.47 ± 0.01 | 2.01 ± 0.13 | | |
| 3. SBPL+15% phosphatidic acid | 3.90 ± 0.02 | 1.14 ± 0.41 | 3.36 ± 0.04 | 1.35 ± 0.21 | | |
| 4. SBPL+30 μM gramicidin | 3.90 ± 0.03 | 0.83 ± 0.17 | 3.30 ± 0.10 | 1.47 ± 0.13 | | |
| 5. SBPL+60 μM gramicidin | 3.74 ± 0.01 | 0.71 ± 0.16 | 3.66 ± 0.11 | 2.04 ± 0.21 | | |
| 6. SBPL+20% cholesterol | 5.03 ± 0.08 | 1.74 ± 0.47 | 3.45 ± 0.10 | 1.76 ± 0.10 | 4.39 ± 0.50 | 1.91 ± 0.73 |
| 7. DMPC | 4.17 ± 0.05 | 0.32 ± 0.03 | 3.82 ± 0.03 | 1.08 ± 0.27 | 3.32 ± 0.19 | 1.67 ± 0.30 |
| 8. DMPC+15% cholesterol | 5.46 ± 0.01 | 0.93 ± 0.08 | 3.96 ± 0.08 | 1.91 ± 0.13 | | |
| 9. DPPC | 4.18 ± 0.09 | 1.44 ± 0.17 | 3.98 ± 0.04 | 1.53 ± 0.25 | | |
| 10. Egg PC | 3.77 ± 0.03 | 0.57 ± 0.07 | 3.42 ± 0.05 | 1.12 ± 0.22 | 3.28 ± 0.09 | 1.43 ± 0.35 |
| 11. Egg PC+10% cholesterol | 4.06 ± 0.02 | 0.58 ± 0.07 | 3.60 ± 0.09 | 1.19 ± 0.11 | | |
| 12. Egg PC+20% cholesterol | 4.31 ± 0.13 | 1.07 ± 0.44 | 3.58 ± 0.06 | 1.94 ± 0.54 | 3.43 ± 0.18 | 1.39 ± 0.14 |

SBPL, soybean phospholipid; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; egg PC, egg lecithin phosphatidylcholine.
All the measurements were carried out at approx. 25°C.
The % concentrations are all w/w.

Fluorescence lifetime measurements were carried out using the time-resolved fluorescence microscope described elsewhere [23,26]. The fluorescence from Nile red was discriminated against the excitation laser pulses (575 nm, 7 ps) by the use of a dichroic mirror (610 nm, XF43 of Omega Optical). Although spatial resolution was not required in these experiments, the use of fluorescence microscope (with a high numerical aperture objective lens) was essential for efficient collection of fluorescence photons from the single bilayer membranes. Fluorescence decay curves were analyzed by the MEM [19] to obtain a distribution of lifetimes.

Fig. 1 shows typical MEM distributions of fluorescence lifetime of Nile red in supported and in vesicle membranes. The following two points are clearly discernible from these distributions: (i) the position of the main peak is shifted to lower values of the lifetime in the case of supported membranes when compared to vesicle membranes and (ii) the width of the distribution is significantly higher in the case of supported membranes. Table 1 lists these parameters obtained in a large number of membranes. A shift in the peak position towards the lower lifetime values and an increase in the width of the distribution in the case of supported membranes were consistently observed in all the cases. Further, the differences observed in the case of supported membranes when compared to vesicle membranes were not dependent on the type of solid support media. Supported membranes showed similar characteristics when the support used was either microscope coverglass or freshly cleaved mica (Table 1).

The fluorescence lifetime of Nile red is very sensitive to the polarity of the solvent [18]. Comparison with the solvent polarity dependence of fluorescence lifetime [18] suggests that the decrease in the peak position of the lifetime distribution observed in supported membranes could be due to an increase in the water content of supported membranes when compared to vesicle membranes. Variation in the fluorescence lifetime and intensity of diphenylhexatriene [27,28] has been used earlier in assessing the water content in membranes. It is interesting to note that the decrease in the peak position is accompanied by an increase in the width of lifetime distribution in all the cases. This behavior is in contrast to the observations in vesicle membranes where a decrease in the peak lifetime was generally associated with a decrease in the width of lifetime distribution [22,23]. This correlation in vesicle membranes was prominent especially during gel–liquid crystal phase transition in dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) vesicles [22,23]. In this system the shorter lifetime observed in the liquid crystalline phase (when compared to the gel phase) could be associated with the increase in water content. The narrower distribution of lifetime in the liquid crystalline phase was hypothesized to be due to rapid reorientation of the probe leading to averaging of both the radiative ($k_r$) and the non-radiative ($k_{nr}$) decay rates.

The current observations in supported bilayers could be explained by the following model: supported membranes could be more heterogeneous when compared to vesicle membranes. This heterogeneity could have been the result of water-filled pores interrupting the continuity of the bilayer. The presence of water-filled pores is expected to increase the effective polarity of the membrane resulting in a decrease in the fluorescence lifetime of Nile red. Concomitantly, the heterogeneity of the environment induced by the water-filled pores would have caused the observed increase in the width of lifetime distribution.

Two-dimensional diffusion measurements by fluorescence recovery after photobleaching (FRAP) have shown that supported membranes are continuous structures [14,29]. It should be noted that the timescale of FRAP measurements in membranes is more than seven orders of magnitude smaller when compared to fluorescence lifetime measurements. Hence, the heterogeneity of the supported membrane inferred from lifetime distribution is likely to have been homogenized by the dynamics of structural fluctuations [30]. This would have resulted in the inference of a homogeneous and continuous membrane from FRAP experiments. This model demands that the timescale of structural fluctuations would be in the broad range of $10^{-2}$–$10^{-8}$ s. The use of suitable probes having either longer fluorescence lifetimes [31] or measurable phosphorescence signals [32] would be useful in identifying the timescale of structural fluctuations.

The dynamics of planar supported membranes also differ from that of vesicle membranes in another

aspect. The DMPC vesicle membrane (which is in the liquid crystalline state) showed a nearly 5-fold narrower width of lifetime distribution when compared to the DPPC vesicle membrane (which is in the gel state) (Table 1). This is similar to our earlier observations [22,23]. However, this 5-fold difference was reduced to a 1.5-fold difference in supported membranes (Table 1). While the distribution width associated with DPPC was similar in both vesicle and supported membranes, DMPC showed a significantly larger width in supported membranes when compared to vesicles. This suggests that the physical state of DMPC (which is in the liquid crystalline phase at 25°C) is closer to the gel state on solid supports. The observed temperature invariance (in the temperature range of 14–40°C) of lifetime distribution width of Nile red in DMPC (gel to liquid crystalline phase transition temperature approx. 22°C) on solid supports (data not shown) also supports the proposed transformation of the liquid crystalline membrane into a gel-like state by solid supports. Hence, the observed increase in the width of lifetime distribution in all the supported membranes studied could also have been caused by a transformation to a gel-like state apart from an increase in the heterogeneity of the membrane proposed above.

There is growing evidence to suggest that cell membranes are inhomogeneous and that the inhomogeneity is mainly due to the presence of microdomains [3–7]. A variety of membrane components such as cholesterol, sphingomyelin, and other lipids have been implicated in the formation of microdomain structures. Our present data show that the width of lifetime distribution increases in the presence of additives such as cholesterol, sphingomyelin, phosphatidic acid and gramicidin (Table 1). The increase in width was observed in the vesicle as well as supported membranes. The increase seen in the case of vesicle membranes is similar to our earlier observations [22,23]. The striking feature of these data is the significantly larger increase in the width in supported membranes when compared to vesicle membranes. This behavior was consistently observed with all the membrane additives used in this work. These results would imply that the domain causing agents induce a greater level of heterogeneity in supported membranes when compared to vesicle membranes having the same chemical composition. This need not be surprising when one realizes that the forces that stabilize the vesicle and supported membranes are not identical. The presence of an ultrathin (approx. 18 Å) layer of water sandwiched between the support and the bilayer in the supported membranes [13,16] could have resulted in a change in the nature and the level of forces which stabilize the membrane. It is likely that this layer of water could be more ordered (when compared to bulk water) leading to confinement of the bilayer to the solid support. Such a confinement to the solid support could have resulted in an increase in the level of heterogeneity caused by domain forming agents such as cholesterol and sphingomyelin. Further, the presence of an ultrathin layer of water on one side and bulk water on the other side of the membrane could also be the cause of increased heterogeneity of supported membranes when compared to vesicle membranes.

The implications of our results and the model presented above are as follows. Although planar supported bilayers are well suited for modeling cell surface characteristics and for the use as two-dimensional nano-structures, their limitation with regard to their homogeneity should be borne in mind. Our observation that the width of the lifetime distribution in supported membranes is independent of the position of observation and the fact that the area of observation is approx. 1.5 μm in diameter [26] shows that the microdomains inferred from our studies should be sub-micrometer in size.

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