The Effect of the Lipid Bilayer State on Fluorescence Intensity of Fluorescein-PE in a Saturated Lipid Bilayer

Marek Langner[a], Hanna Pruchnik[b] and Krystian Kubica[b,*]

[a] Institute of Physics, Wrocław University of Technology, ul. Wybrzeże Wyspiańskiego 27, Wrocław, Poland

[b] Department of Physics and Biophysics, Agricultural University, ul. Norwida 25, 50–375 Wrocław, Poland. Fax: +48-71-3205172. E-mail: Kubica@OZI.AR.WROC.PL

* Author for correspondence and reprint requests

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Fluorescein-PE, Lipid Bilayer, Phase Transition

Fluorescein-PE is a fluorescence probe that is used as a membrane label or a sensor of surface associated processes. Fluorescein-PE fluorescence intensity depends not only on bulk pH, but also on the local electrostatic potential, which affects the local membrane interface proton concentration. The pH sensitivity and hydrophilic character of the fluorescein moiety was used to detect conformational changes at the lipid bilayer surface. When located in the dipalmitoylphosphatidylcholine (DPPC) bilayer, probe fluorescence depends on conformational changes that occur during phase transitions. Relative fluorescence intensity changes more at pretransition than at the main phase transition temperature, indicating that interface conformation affects the condition in the vicinity of the membrane. Local electrostatic potential depends on surface charge density, the local dielectric constant, salt concentration and water organisation. Initial increase in fluorescence intensity at temperatures preceding that of pretransition can be explained by the decreased value of the dielectric constant in the lipid polar headgroups region related in turn to decreased water organisation within the membrane interface. The abrupt decrease in fluorescence intensity at temperatures between 25 °C and 35 °C (DPPC pretransition) is likely to be caused by an increased value of the electrostatic potential, induced by an elevated value of the dielectric constant within the phosphate group region. Further increase in the fluorescence intensity at temperatures above that of the gel-liquid phase transition correlates with the calculated decreased surface electrostatic potential. Above the main phase transition temperature, fluorescence intensity increase at a salt concentration of 140 mM is larger than with 14 mM. This results from a sharp decline of the electrostatic potential induced by the phosphocholine dipole as a function of distance from the membrane surface.

Introduction

Fluorescence labelling is a research technique widely used in fluorescence microscopy and its sophisticated variation, confocal microscopy to study intracellular structures and the distribution of labelled macromolecules within the cell. Studies on macromolecules and their aggregates are particularly suitable for fluorescence studies. Biological membranes, which are ultimately multiphase systems composed of a variety of components, were studied using many fluorescence techniques that produce data regarding both the organisation and topological properties of their molecules (Epand *et al.*, 1996; Davenport, 1997). Such a diversity of applications requires that the fluorescence probe be well characterised. Only a small number of fluorescence probes have been thoroughly described (diphenylhexatriene (Kaiser and London, 1998), ANS (Slavik, 1982), NBD (Mazeres *et al.*, 1996), Fluoresceine (Fromherz and Masters, 1974; Soucaille *et al.*, 1988)). Fluorescence probes can be covalently attached to a carrier molecule such as DNA, a protein, or lipid (Epand, 1995). Such attachment affects the fluorescence properties of the probe. Covalent binding ensures its location within the aggregate, enabling independent investigation of various aggregate regions. Furthermore, the interaction of such a probe with other dyes or quenchers helps to establish its location.

Fluorescein is an example of a fluorescence probe frequently used in a variety of applications. In an aqueous solution, this hydrophilic dye occurs in a number of charged forms, making its adsorption and fluorescent properties

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strongly pH-dependent (Sjoback *et al.*, 1995). Its derivatives are convenient lipids, DNA and protein labels (Sjoback *et al.*, 1998). The fluorescence of the probe is then stable and its quantum efficiency is high. Hydrophilic fluorescein is suitable for leakage experiments. When covalently attached to a lipid molecule, fluorophore become a sensitive device for probing the membrane surface aqueous phase and its sensitivity to proton concentration enables the estimation of surface charge density, which may then be used as an indicator of amphiphilic compound adsorption (Langner and Kleszczynska, 1997; Langner *et al.*, 1998).

When the probe is located at the membrane surface, it should detect conformational changes in the lipid bilayer. In this paper, we correlate fluorescein-PE fluorescence intensity with lipid bilayer state. Fluorescein, being hydrophilic, will not penetrate the interfacial region, therefore its fluorescence reflects changes occurring only within the aqueous phase adjacent to the membrane surface. These changes in turn may depend on membrane conformational state. Fluorescein-PE fluorescence intensity depends on local pH, which is sensitive to electrostatic potential in the probe's vicinity (Tocanne and Teissie, 1990). This ability to detect changes in local electrostatic potential caused by charged amphiphilic molecules has been utilised previously (Langner and Kleszczynska, 1997; Langner *et al.*, 1998).

The DPPC lipid bilayer is a convenient membrane model, because it indicates conformational changes induced by temperature. A DPPC membrane has two phase transitions: pretransition at 35 °C and main phase transition at 42 °C (Marsh, 1990; Koynova and Caffrey, 1998). Below pretransition temperature, the lipid bilayer is in the gel phase. At temperatures above that of main phase transition, the membrane is in the liquid-crystal phase; at temperatures between that of phase transitions, the ripple phase is formed. Each one of these three phases (fluid, ripple and liquid crystal) has different conformation, lipid mobility and surface properties, hence the DPPC lipid bilayer serves as a convenient model to study the effect of lipid membrane organisation on Fluorescein-PE fluorescence intensity.

Materials and Methods

Materials

Dipalmitoylphosphatidylcholine (DPPC), dimirystylphosphatidylcholine (DMPC) and disterylophosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids (Alabaster, GA, USA), and the fluorescence probe N-(5-fluoresceinthiobarbamoyl)dipalmitoyl-L-a-phosphatidylethanolamine (Fluorescein-PE) from Molecular Probes (Eugene, OR, USA).

Vesicle preparation and fluorescence measurements

Multilamellar vesicles (MLV) were prepared according to Langner *et al.* (1998); Gennis (1989) and Gregoriadis (1995). In short, the lipid was mixed in chloroform with an appropriate amount of fluorescein-PE. The chloroform was then removed under vacuum, a phosphate buffer added and the sample vortexed (at a temperature above that of main phase transition). This produced a milky MLV suspension. Before each fluorescence measurement, a DPPC vesicle suspension was thermally equilibrated (about 15 min.) in a thermo-regulated cuvette holder. The excitation (λ_{EX}) and emission (λ_{EM}) wavelengths for Fluorescein-PE were λ_{EX} = 480 and λ_{EM} = 530 nm, respectively. Fluorescence intensities presented in this paper are expressed as relative changes. They were calculated according to the following formula: $(F_i - F_o)/F_o$, where F_o is probe fluorescence intensity when the membrane is in the gel phase, and F_i is measured fluorescence intensity measured at a certain temperature. Measurements were carried out on a Kontron fluorometer (Kontron Instruments, Zürich Switzerland), and fluorescence intensities were corrected for the inner filter and dilution effects (Lakowicz, 1988).

Differential scanning calorimetry

Multilamellar vesicles (MLV) for differential scanning calorimetry (DSC) were prepared from DPPC and an appropriate amount of fluorescein-PE. The compounds previously dissolved in chloroform were mixed and the solvent evaporated under vacuum. Afterwards, distilled water was added and the flask content was heated to 60 °C in a water bath. The lipid film was then dispersed by agitating the flask on a vortex mixer, giving a

milky liposome suspension. Final lipid concentration was 25 mg/ml. The lipid suspension was next loaded into the sample cell of a DSC microcalorimeter Metler Toledo Thermal Analysis System D. S. C. 821^e. A scan rate of 0.5 °C/min and 2 °C/min were employed but because no differences appeared between obtained results, we used the latter speed.

Main phase transition peak width and enthalpy were calculated using a Metler Toledo STAR^e software thermal analysis package.

Theoretical model

Fluorescein is covalently attached to the phosphatidylcholine headgroup, ensuring its location in the aqueous phase adjacent to the membrane surface (dye hydrophilicity prevents the penetration of the membrane interface). The probe is pH sensitive, hence it detects any changes in local charge distribution at the membrane interface.

According to the Goy-Chapman theory, surface pH ($pH_{surface}$) can be expressed by the formula (Westman *et al.*, 1982):

$$\mathrm{pH(surface)} = \mathrm{pH(bulk)} + \frac{F\Psi}{2.303RT}$$

where: Ψ – surface electrostatic potential,
F – Faraday constant,
R – gas constant,
T – temperature.

This shows that the fluorescein-PE fluorescence intensity changes as a result of either variation in bulk pH or changes in electrostatic potential (generated by charges at the membrane interface). Surface electrostatic potential (Ψ) depends on the type and amount of ions adsorbed onto the surface, charges associated with membrane-forming lipids, the lipid headgroup's dipole moment and water molecule organisation within the interface (water molecules in the immediate vicinity of the membrane are ordered differently than those in bulk aqueous solution) (Klosgen *et al.*, 1996; Cevc, 1991; Marrink *et al.*, 1996; Jendrasiak, 1996). The dipole moment that results from the combined effect of lipid headgroup residual charge and immobilised water may influence Fluorescein-PE fluorescence intensity. Such an effect, if present, should be evident especially at pretransition temperature, when conformational changes at the membrane interface are most expected (Seddon and Tampler, 1995; Seddon, 1996). Hence, the pH dependence of the electrostatic potential can be used to estimate changes in surface dipole potential associated with the phosphocholine headgroup. F_or calculation purposes, the angle between the phosphocholine headgroup and the membrane surface was assumed to depend on lipid bilayer state. In addition, average surface area per lipid molecule was assumed to be 50 A^2 in the gel phase and 70 A^2 in the liquid-crystal phase. Dipole potential was calculated according to the following formula (Makino *et al.*, 1991):

$$\psi(x) = \psi(0)\exp(-\varkappa x)$$

where

$$\varkappa^2 = \frac{2z^2F^2c,}{\varepsilon_0\varepsilon RT}$$

$$\psi(0) = \frac{1}{\varepsilon_0\varepsilon_1\varkappa_1}\sigma_1\exp(-\varkappa_1 d) + \frac{1}{2\varepsilon_0\varepsilon_2\varkappa_2}\sigma_2(1+\exp(-2\varkappa_2 d).$$

In the later equation, σ_1, σ_2 – average surface charge densities for the phosphate and ammonium groups of the phosphocholine headgroup, d – the normal component of the distance between phosphocholine residual charges, when P-N distance was assumed 5×10^{-10} m, ε_1, ε_2 – respective dielectric constants in the vicinity of the phosphate and ammonium atoms, $\varkappa_1$, $\varkappa_2$ – Debye lengths for the respective dielectric constants ε_1 and ε_2. The local pH was calculated as a function of the distance from the N^+ group (ranging form 0 nm to 10 nm) with different phosphocholine headgroup orientation with respect to the membrane surface.

In considering the equation describing dipole potential, the dependence of electrostatic potential on ionic strength, temperature and the dielectric constant can be calculated using the Monte-Carlo method (Mouritsen and Jorgensen, 1998). A molecular dynamic simulation shows that P and N groups in the phosphocholine moiety of DPPC have a different number of associated water molecules, validating the assumption that the dielectric constants in the vicinity of P and N groups are different (Flewelling and Hubbell, 1986; Tieleman *et al.*, 1997).

Results and Discussion

Figure 1A shows that fluorescein-PE fluorescence intensity does not depend on temperature when the probe is in an egg-PC membrane formed, because this lipid lacks phase transitions. This means that there is no intrinsic dependence of probe fluorescence intensity on temperature. When the probe is in the lipid bilayer, whose organisation depends on temperature, the fluorescence intensity temperature characteristic reflects such changes. Phosphatidylcholines with saturated hydrocarbon chains have a complex phase behaviour. Major organisational rearrangements occur at pretransition – associated with headgroup region transformation and main phase transition – when hydrocarbon chain organisation changes (Marsh, 1990; Koynova and Caffrey, 1998; Seddon and Tampler 1995; Seddon, 1996). When Fluorescein-PE is incorporated into such a membrane, its fluorescence intensity becomes a complex function of temperature (Fig. 1B). The dependence of fluorescence intensity on temperature for three selected phosphatidylcholines with saturated alkyl chains (DMPC, DPPC and DSPC) is qualitatively similar (Fig. 1B). When temperature approaches that of

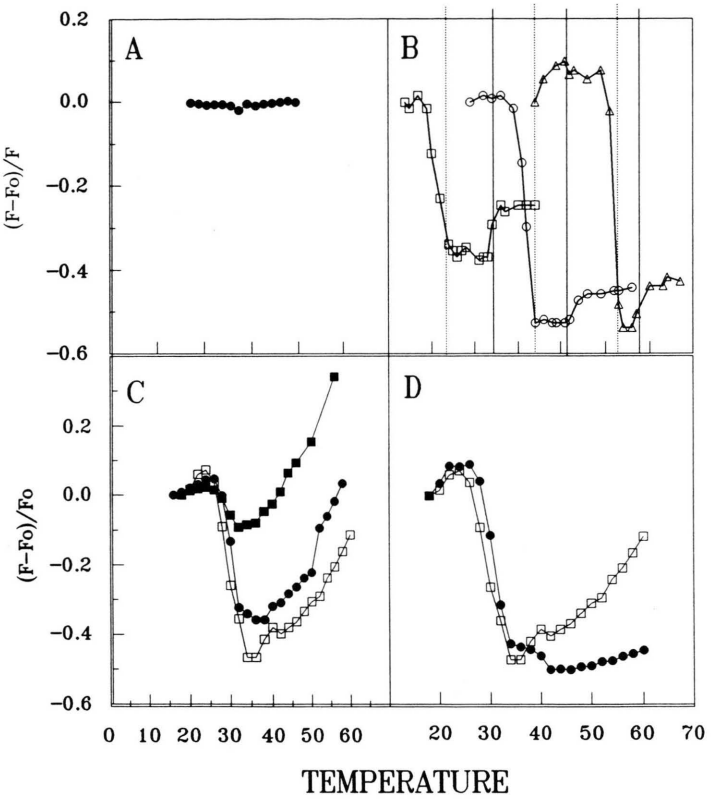

Fig. 1. A. The dependence of fluorescein-PE fluorescence intensity on temperature when the probe is incorporated into Egg-PC (filled circles).
B. The dependence of fluorescein-PE fluorescence intensity on temperature when the probe is incorporated into disteroylphosphatidylcholine (DMPC) – open squares, dipalmitoylphosphatidylcholine (DPPC) – open circles and disterylophosphatidylcholine (DSPC) open triangles, lipid bilayers. Pretransition (dashed lines) and main phase transition (solid lines) temperatures are indicated.
C. The effect of the amount of fluorescein-PE in the DPPC lipid bilayer on the dependence of relative fluorescence intensity on temperature characteristics. Open squares, filled circles and filled squares represent data obtained when the membrane contains 0.08 mol%, 0.15 mol% and 0.31 mol% of fluorescein-PE, respectively.
D. The effect of ionic strength on the dependence of fluorescein-PE fluorescence intensity on temperature when 0.08 mol% of the probe was in the DPPC lipid bilayer. Open squares and filled circles represent data obtained when the buffer solution contains 140 mM and 14 mM of NaCl, respectively.

pretransition, fluorescence intensity increases slightly. At temperatures around pretransition, fluorescence intensity falls sharply. This sharp decrease in fluorescence intensity is followed by a modest rise at temperatures above that of main phase transition. The complex dependence of the fluorescence intensity on temperature may be caused by membrane conformational changes that alter proton concentration at the surface or by lateral probe redistribution. It is known that fluorescein fluorescence intensity is concentration dependent; at high probe concentrations, self-quenching causes fluorescence intensity to decrease. If this is the case, then the effect should be more pronounced when the probe's mole-fraction in the membrane is elevated. To test this possibility, the dependence of fluorescence intensity as a function of temperature was measured for various probe concentrations (Fig. 1C). Obtained results are opposite to that caused by the self-quenching effect: the smaller the quantity of probe in the membrane, the bigger the relative change in fluorescence intensity. The effect of probe concentration is eminent for temperatures at which pretransition is expected, whereas at higher temperatures, plots are qualitatively similar. The elevated amount of the probe in the membrane seems to disrupt pretransition more than main phase transition. Additional information can be obtained from the dependence of dipole potential on ionic strength. Fig. 1D shows the temperature characteristics of relative change in fluorescence intensity for two salt concentrations (0.14 mM and 140 mM). Increased ionic strength does not affect Fluorescein-PE fluorescence up to main phase transition. When the membrane becomes fluid, fluorescence increase is larger with higher ionic strength. In theoretical analysis, it was assumed that at main phase transition, besides an increase in surface area per lipid molecule, the dielectric constant may change as a result of the altered P-N dipole orientation. At higher temperatures, above main phase transition (gel-liquid crystal phase transition) average surface area per lipid molecule increases. Hence, lipid headgroups are able to move more freely and are easily accessible to ions in the aqueous phase. Calorimetric measurements confirm results obtained using the fluorescence method, namely that increased amount of probe shifts pretransition temperature by 1.5 °C when it is highest (0.38 mol%), whereas main phase transition temperature is hardly affected (Fig. 2).

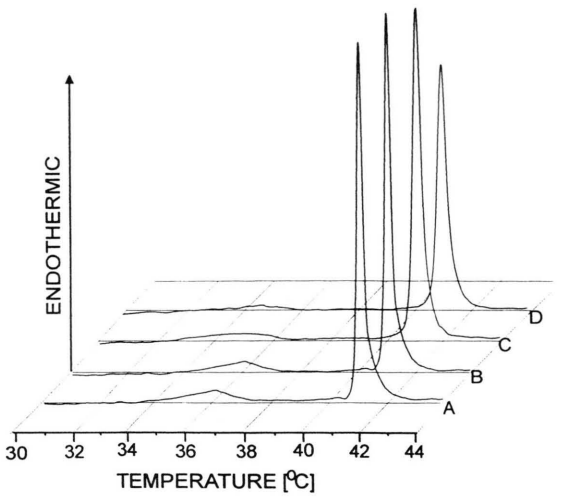

Fig. 2. Thermograms of DPPC vesicles with different amounts of probe. From top to bottom: 0 mol%, (A) 0.08 mol%, (B) 0.15 mol% and (C) 0.31 mol% (D) of fluorescein-PE.

Obviously the fluorescence intensity of the probe responds to changes in membrane surface conformational changes, which in turn depend on the lipid bilayer state. The dependence of pH sensitive probe fluorescence intensity on the conformational state of the membrane should be mediated by variation in local proton concentration. Surface proton concentration is affected by both bulk pH and surface electrostatic potential (Cevc, 1990). Bulk pH was maintained constant at 7.4, at which phosphocholine headgroups do not carry any net charge. Hence, local electrostatic potential reflects changes in phosphocholine dipole orientation. Fluorophore located near the choline ammonium group may sense the electrostatic potential of the P-N dipoles (Cevc, 1990).

Dipole potential depends on a number of factors: headgroup orientation, distance between residual charges and local dielectric permitivity (Brockman, 1994). Fluorescence data show that membrane interface properties change at pretransition temperature. Altered headgroup orientation and water organisation imply that dipole potential may be affected by a change in dipole orientation and/or local dielectric permitivity (Belaya *et al.*, 1994). The trimethyl ammonium group, carrying positive charge, does not form hydrogen bonds

with the surrounding water molecules. The phosphate group, on the other hand, forms hydrogen bonds, thus changing its headgroup orientation may alter its affinity to water or change water activity (Tieleman *et al.*, 1997). We assume that dielectric constant in the vicinity of the ammonium group does not change and that its value is equal to that in the bulk aqueous phase, whereas it can be altered at the phosphate group. Consequently, when the temperature of the liposome suspension is raised, dipole potential changes twice: first at pretransition, when head-group orientation elevates the ammonium group towards the aqueous phase causing the probe's fluorescence intensity to drop; a second time at main phase transition, where dipole density is lowered due to increased surface area per lipid molecule.

Figure 3 shows the dependence of calculated dipole potential as a function of the dielectric constant in the vicinity of the phosphate group. The dielectric constant was calculated when temperature was that at which the membrane is in the fluid phase (330 K) and for two ionic strengths (14 mM and 140 mM). When ionic strength is high (140 mM), the electrostatic potential in the vicinity of the probe is positive regardless of the value of the dielectric constant at the phosphate group (from 50 to 80), whereas at lower ionic strength, the bigger the difference between the dielectric constant at the phosphate and ammonium groups, the lower the electrostatic potential.

The dependence of dipole potential on temperature for two values of the dielectric constant at the phosphate group is presented on Fig. 4. Calculations show that when the values of the dielectric constant in the vicinity of the phosphate and ammonium groups are equal, electrostatic potential does not depend on ionic strength. On the other hand, when the value of the dielectric constant in the vicinity of the phosphate group is lower than that at the ammonium group, dipole potential is negative in the whole temperature range (290 K to 330 K). However, there is a distinct step-like change in electrostatic potential at main phase transition temperature for lower ionic strength (14 mM). When ionic strength is high (140 mM), the dipole potential decreases with rising temperature, and at temperatures below that of the main phase transition its value is positive, and is negative above. In addition, calculations show that a change in electrostatic potential at main phase transition temperature is larger when ionic strength is low. The magnitude of the calculated local pH change at distances from 5 nm to 10 nm within the membrane surface decreases by 30% and 70% when ionic strength is 14 mM and 140 mM, respectively.

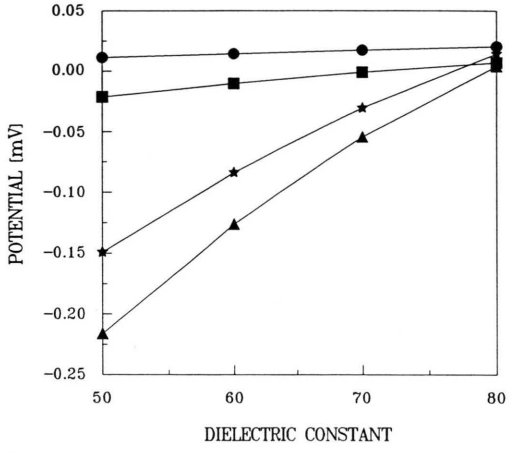

Fig. 3. The dependence of calculated electrostatic potential in the vicinity of the probe on the value of the dielectric constant at the phosphate group. The value of the dielectric constant at the ammonium group was assumed 80. Filled circles represent data obtained for a temperature of 300 K and 140 mM of NaCl, filled squares 300 K and 14 mM of NaCl, stars 330 K and 140 mM of NaCl, and triangles 330 K and 14 mM of NaCl.

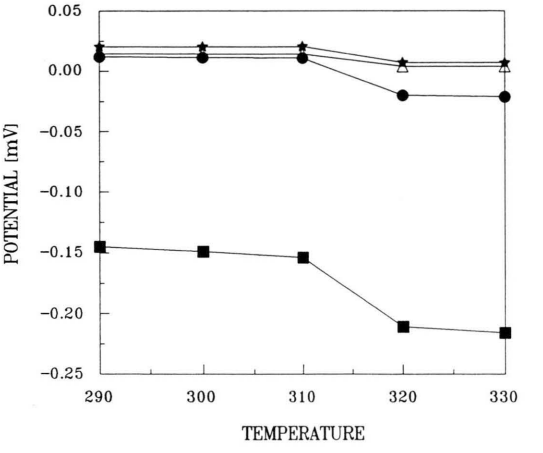

Fig. 4. Dependence of the electrostatic potential at the vicinity of the probe on temperature [K]. The dielectric constant in the vicinity of the ammonium group was assumed 80. Data are plotted for different ionic strengths and values of the dielectric constant in the vicinity of the phosphate group (circles stand for ε_1 = 50, C = 140 mM NaCl; triangles ε_1 = 80, C = 14 mM NaCl, squares ε_1= 50, 14 mM NaCl and asterisks ε_1 = 50, C = 140 mM).

Theoretical considerations lead to the conclusion that a change in the dielectric constant in the vicinity of the phosphate group is accompanied by a change of dipole potential sign at temperatures above that of main phase transition, which correspond to measured changes in fluorescein-PE fluorescence intensity.

Acknowledgements

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