# TEMPERATURE DEPENDENCE OF BILAYER PROPERTIES IN LIPOSOMES AND THE USE OF FLUORESCENT PROBES AS A TOOL TO ELUCIDATE THE PERMEATION MECHANISM OF HYDROPHILIC SOLUTES

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## ABSTRACT

Solute transport across lipidic membranes is a fundamental process for both living organisms and drug delivery. In order to establish the mechanism of solute passive transport through lipidic membranes, we determined the effect of temperature upon the rate of glucose transport (as hydrophilic solute model) through dipalmitoylphosphatidylcholine large unilamellar liposomes and compare the results to those obtained for a variety of fluorescent probes (pyrene, PRODAN, diphenylhexatriene, diphenylhexatriene-TMA). All these probes, independent of their localization in the liposomes, report a monotonous change in the micro-properties sensed with temperature, with **a maximum rate of change at** the main transition temperature of the bilayer. These results contrast with those obtained for the rate of glucose influx, were it is observed a clear maximum rate of intake **at** the transition temperature. These contrasting results imply that the microviscosity (common factor for used probes) is not the property of the bilayer that controls the rate off solute transport which, at least in the vicinity of the main transition temperature, is regulated by formation of transient pores, particularly at the transition temperature ($T_m$). This would indicate that, at least near $T_m$, glucose and other hydrophilic solutes diffuses through transient pores formed in the bilayer. Interestingly, the presence of these pores does not affect the fluorescence characteristics of the probes.

**Keywords:**liposome, permeation, glucose, glucose oxidase, fluorescent probe

**Abbreviations**

| | |
|---|---|
| LUV | Large unilamellar vesicles |
| GOx | Glucose Oxidase |
| DPPC | 1,2-Dipalmitoyl-sn-glycero-3-phosphatydylcholine |
| $T_m$ | Main phase transition temperature |

## 1. INTRODUCTION

Diffusion of small solutes across lipidic membranes is a fundamental process whose occurrence and control sustain the existence of living organisms[1]. The transport process can proceed by two main paths. In biological membranes, it takes place mediated by an active transport of solutes through selective protein carriers[2]. The second path corresponds to passive transport[3,4]. Regarding this last pathway, and dependent on the solute and the bilayer characteristics, permeation can follow two main mechanisms[5]:

i) A solubilization/diffusion process which takes place by the adsorption of the solute in the bilayer followed by a passive diffusion through the membrane and, finally, a desorption into the liposome inner pool or

ii) permeation through transient defects and/or well defined pores formed in the bilayer

In this type of transport, the rate of diffusion of a given solute is modulated by a variety of factors such as membrane characteristics (type of lipid, composition), nature of the diffusing solute and the experimental conditions (pressure and temperature)[6]. Regarding this last parameter, the response of the permeation rate to temperature changes can provide insights about the main mechanism of the passive solute permeation since it can be expected that mechanism i) will depend upon the local viscosity of the bilayer, a parameter that can be modulated by the temperature and estimated employing an array of fluorescent probes whose photophysics is determined by the local microviscosity of the liposomal bilayer. On other hand, a diffusive behavior that does not correlate with the response of the fluorescent probes could provide evidence of the occurrence of a more complex mechanism, as described in point ii). In the present work we report a comparative study on the effect of the temperature upon membrane micro properties (microviscosity and micropolarity) sensed by a variety of fluorescent probes, with the permeation rate of glucose (as hydrophilic solute model) through dipalmitoyl phosphatidylcholine bilayers in large unilamellar vesicles (LUVs) in order to get insight of the solute permeation mechanism.[6]

### 2. Material and methods

Glucose oxidase (GOx; from *Aspergillus Niger*); 1,2-dipalmitoyl-sn-glycero-3- phosphatydylcholine (DPPC), Pyrene, 1,6-diphenyl-1,3,5-hexatriene (DPH), N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatrien)phenylammonium p-toluenesulfonate (TMA-DPH), and Sepharose-CL4B from Sigma, glucose from Merck, and PRODAN from Molecular Probes were employed as received. Experiments were carried out in Hepes buffer (10 mM, pH 7.4). Ultra-pure water, employed to prepare the solutions, was obtained from a Modulab Type II equipment. The consumption of oxygen as a function of time was followed with an Oxygraph System (Hansatech Instruments). Fluorescence spectra were recorded in a Shimadzu spectrofluorophotometer (RF-5301 PC). Anisotropy measurements were carried out in an I.S.S. K2 spectrofluorometer.

#### 2.1. Liposome preparation and GOx encapsulation

Liposomes were prepared by adding into a round-bottom flask a known DPPC amount dissolved in chloroform. The solvent was removed by slow evaporation at 40 °C on a thermostated bath. Re-suspension of the film was made using an appropriate volume of a GOx solution, in order to reach the desired working concentration of lipids. The suspensions were subjected to ten freezing–thawing cycles, transferred into a nitrogen pressurized homemade extruder and extruded 10 times through polycarbonate filters (400 nm nominal pore size, Nucleopore, Corning Costar) at 52 °C. This procedure maximizes the formation of unilamellar liposomes[7, 8].

Non-encapsulated GOx was removed by eluting the solution through a column containing Sepharose-CL4B. This procedure did not affect the activity of the entrapped enzyme (data not shown).

#### 2.2. Determination of glucose diffusion in liposomes

Glucose (10 mM) was externally added to liposomes containing GOx entrapped in their inner pool. The rate of the process was followed by registering the consumption of oxygen as a function of time on an Oxygraph system, according to the procedure described by Ahumada et al. (2014)[6].

#### 2.3. Incorporation of Prodan and Pyrene into DPPC liposomes

Fluorescent probes (Prodan or Pyrene), dissolved in ethanol, were mixed with a stock solution of lipids in chloroform in order to obtain a lipid to probe ratio of 500:1. After this, liposomes preparation followed the same procedure than that described above (Section 2.1)..

#### 2.4. Fluorescence measurements

Fluorescences were measured with a Shimadzu spectrofluorometer. Probes incorporated into liposomes samples were measured at different temperatures in the 20° to 60° C range. Excitation wavelengths were 360 nm and 345 nm for Prodan and Pyrene, respectively. Emission from Prodan was characterized by the ratio of the fluorescence intensities measured at wavelengths of 486 and 436 nm. Monomeric Pyrene fluorescence was characterized by the intensity measured at 375 nm, while excimer formation was quantified by the intensity of the band centred at 445 nm.


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**2.5. Incorporation of DPH and TMA-DPH into DPPC liposomes**

Fluorescent probes, dissolved in dimethyl sulfoxide, were incorporated from a concentrated stock solution after the liposomal suspension preparation. After this addition, the suspensions were incubated for one hour at 10 °C above the main transition temperature of the bilayer. Added solvent constituted less than 0.5% of the total volume of the solution. Lipid to probe ratio was 500:1. The same procedure was followed for both DPH and TMA-DPH incorporation.

**2.6. Anisotropy measurement**

Fluorescence anisotropy measurements were performed in a phase shift and modulation I.S.S. K2 spectrofluorometer interfaced to personal computers employing I.S.S. software. All measurements were carried out in 1 cm path-length quartz cuvettes whose temperature was controlled by an external Cole Palmer bath circulator. The temperature was measured in the sample cell before and after each determination using an Omega digital thermometer. Polarization measurements were done in the "L" configuration using Glam-Thompson prism polarizers in both excitation and emission beams. Excitation wavelength was set at 360 nm. The emission was filtered through a WG-420 Schott high pass filter of negligible fluorescence.

## 3. RESULTS AND DISCUSSION

The main characteristics of the fluorescent probes employed in the present work are shown in Table 1.

**Table 1.** Main characteristics of employed fluorescent probes.

| Fluorescent Probe | Measured property | Location | Reported membrane property |
|---|---|---|---|
| **Prodan (a)** | Ratio between maximum fluorescent intensities at 486 y 436 nm | Interface | Water mobility in the lipid heads zones |
| **Pyrene (b)** | Band I vibrational structure | Lipid domains | Micropolarity |
| **Pyrene ( c )** | Excimer formation | Lipid domains | Lateral diffusion and local concentration of pyrene in the bilayer |
| **Pyrene (d)** | Fluorescent intensities in air saturated and nitrogen purged solutions | Whole bilayer | $O_2$ concentration/mobility |
| **DPH (e)** | Fluorescent anisotropy | Lipid domains | Order and microviscosity |
| **TMA-DPH (f)** | Fluorescent anisotropy | Interface | Order and/or microviscosity of the polar region |

**Letters between parentheses correspond to those of Fig. 1**

Results obtained regarding the change of the measured properties with temperature in the 20 to 60º C range are given in Figure 1. It is remarkable the similarity of the results in spite of the differences in the location and characteristics of the different probes. In fact, all probes show, at the vicinity of the temperature of the main phase transition (ca. 42ºC). A faster rate of change of the measured property, and values of the measured property between those observed prior and after the thermal provoked main phase transition. It is interesting to note that all the systems shown in Fig. 1, including that shown in Fig 1b, present a very similar behavior, in spite of the fact that the measured property is the fluorescence intensity of the 0-0 vibronic band, a property determined by the micropolarity of pyrene surroundings inside the liposomes[9].

The similarities in the results presented in Fig. 1 are a clear indication that fluorescent probes response are dependent of the microproperties of the co-existing microphases. In particular for properties that depend upon the probes displacement, they are determined by the membrane microviscosity of the liposome. It can then be expected that the permeation rate of a solute that takes place by mechanism i) (see section 1) would show a similar dependence with the temperature. In order to test this possibility we measure the rate of glucose diffusion across the bilayer

In order to evaluate the diffusion of glucose across lipidic membranes in liposomes, we measured the rate of oxygen consumption in the oxidation of glucose catalyzed by encapsulated GOx when glucose was externally added to a liposomal dispersion. The entrance of glucose to the vesicles is the rate limiting step of the catalyzed reaction[6,10], so the rate of oxygen (a solute that readily diffuses across lipidic membranes) consumption gives a direct measure of the diffusion of glucose in the liposomes[6]. This is supported by results showing that the rate of oxygen consumption is independent of the local GOx concentration.[6,10] Figure 2 shows the change in the rate of oxygen consumption with the temperature when the enzyme is encapsulated into the liposomes and is completely different than that obtained in absence of the aggregates (Figure 3) Furthermore, the resulting profile of the encapsulated enzyme activity is also completely different from that obtained employing the fluorescent probes (Fig. 1), with a **maximum rate** at the transition temperature. This is a clear indication that the factors that modulate the permeation of glucose are fundamentally different from those that govern the behavior of the fluorescent probes on the bilayer. Regarding the behavior of the rate of permeation of glucose, it is interesting to note that for others solutes diffusing through pure and/or mixed bilayers, it has been reported that the rate of solute diffusion reaches a **maximum value** at the main transition temperature[6,10,11]. This maximum diffusion rate has been related to the presence of rafts in the bilayer that would favour the formation of transient holes and/or packing defects throughout which takes place the permeation of solutes [6,10].

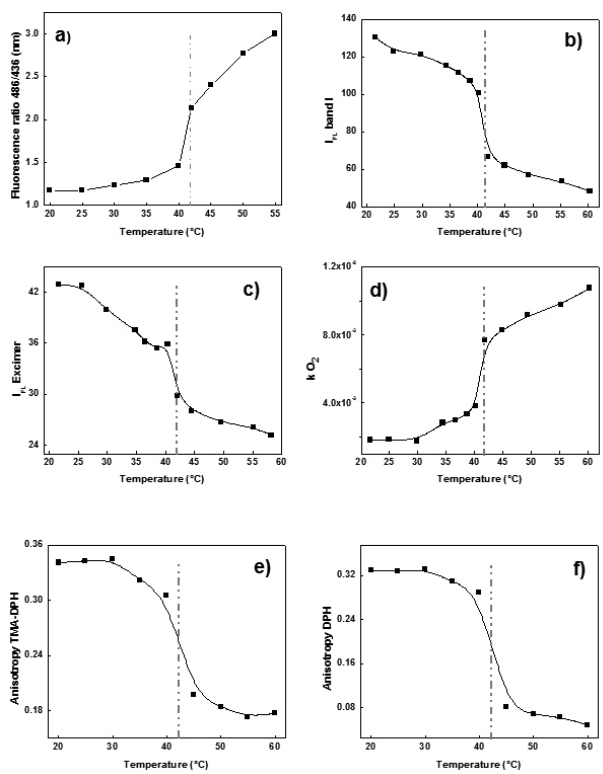


**Fig 1.** Temperature profiles for different properties of a lipid bilayer. a) Ratio between maximum fluorescent intensities of PRODAN (486/436 nm); b) Pyrene Band I Fluorescence intensity measured under nitrogen at 375 nm; c) Fluorescence intensity of pyrene excimer measured at 477 nm; d) quenching of pyrene fluorescence by molecular $O_2$ (k $O_2$); Fluorescence anisotropy of TMA-DPH (360 nm); f) Fluorescence anisotropy of DPH (360 nm). Melting phase transition ($T_m$) of DPPC bilayer at 42 °C (**---**). Vertical lines drawn at 42 ºC.

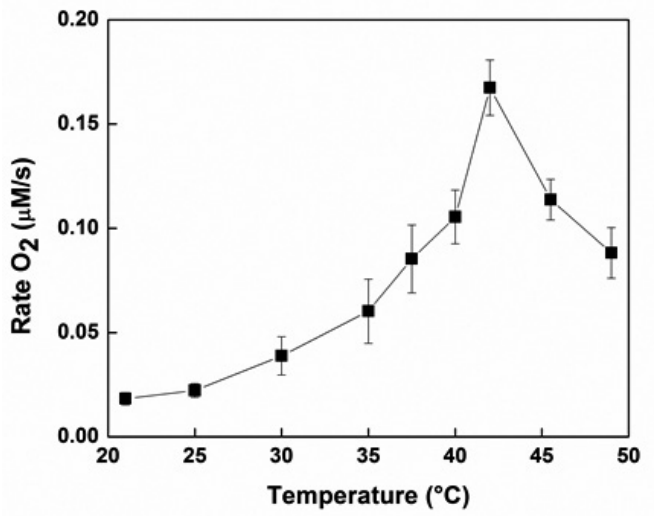


**Fig 2.** Rate of $O_2$ consumption by encapsulated glucose oxidase plotted as a function of temperature. Data obtained for the initial reaction rate following the addition of glucose (10 mM) to a suspension of DPPC (1,36mM) liposomes containing encapsulated glucose oxidase (GOx; 6,6 μM) (Ahumada et al. 2014).

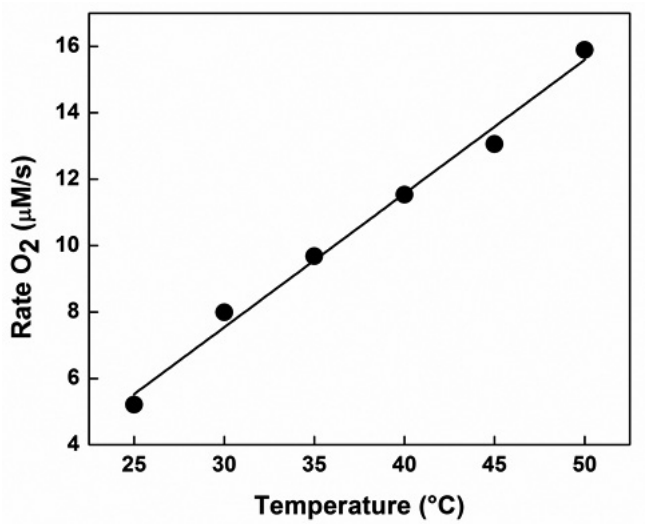


**Fig.3.** Dependence of Gox activity with temperature in homogeneous solution. [Glucose] = 10 mM; [Gox] = 0.05 mg/mL.

The differences observed in Figs.1 and 2 can be attributed to the fact that lipid bilayers are systems that have micro-domains, i.e., zones that have similarities to the gel state or to the liquid-crystalline state that coexist, particularly at temperatures near that of the phase transition. The micro-domains formation at $T_m$ and the maximum fluctuation between them does not affect in a significant manner the fluorescent behavior of the probes employed, which will report an average value corresponding to the presence of both micro-domains. On the other hand, the transport rate of hydrophilic solutes (i.e. glucose[6] or hydrogen peroxide[10]) will be fundamentally governed by processes that present its maximum occurrence at $T_m$, processes such as the apparition of defects on the bilayer in zones where micro domains with different characteristics interact with each other.This allows us to conclude that, at least in this system and a temperatures close to $T_m$, permeation takes place through transient pores and defects (mechanism ii) see section 1) with maximum rates at (or near) the main transition temperature of the bilayers.

## 4. CONCLUSIONS

A comparison of the effect of temperature near the phase transition temperature ($T_m$) of DPPC liposomes on the fluorescent behavior of different probes and on the rate of glucose transport across the bilayer, allows us to conclude that the last process takes place, at least near $T_m$, by a mechanism involving the formation of transient pores and or packing defects. A comparison between the temperatures profiles generated by fluorescent probes located in the microdomains with those observed in the rates of solute permeation can be proposed as an easy but conclusive assay to determine the mechanism of solute diffusion through bilayers.

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