Probing the influence of *cis–trans* isomers on model lipid membrane fluidity using *cis*-parinaric acid and a stop-flow technique

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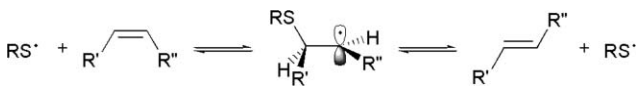
Stop-flow experiments exploiting the fluorescence of *cis*-parinaric acid in monounsaturated lipid vesicles allow the model membrane behaviour, notably the membrane fluidity, to be correlated to the *cis* : *trans* lipid ratios

Membrane properties are affected by the nature of phospholipids and some recent studies have addressed the influence of *cis* or *trans* isomers of unsaturated fatty acid residues.[1,2] The issue is of considerable importance given the possible consequences for different cell functions and protein activities.[3] To illustrate the point, one may consider that nutritional studies have evidenced the intake of *trans* fatty acids from food whereas natural lipids contain unsaturated fatty acid residues with the *cis* configuration.[4] Notably, under certain conditions, *trans* isomers can accumulate in tissues, which may result in various health complications.[5]

The formation of *trans* phospholipid isomers from the *cis* counterparts by a thiyl radical-catalysed process has been shown to occur under physiologically related conditions, both in lipid vesicles[6] and in cells.[7] This process is based on the mechanism of Scheme 1, with the equilibrium being shifted to the right, toward the more stable *trans* configuration.[6,8]

Along this line, we have previously shown that 1-palmitoyl-2-oleoyl L-α-phosphatidylcholine (POPC) undergoes a thiyl radical-catalysed isomerization, to afford a *cis* : *trans* mixture in the ratio of 17 : 83 (PEPC-83), where the *trans* isomer is 1-palmitoyl-2-elaidoyl L-α-phosphatidylcholine (PEPC). POPC and PEPC-83 were used to perform a study of the influence of lipid geometry on model unilamellar vesicles, concerning physical properties, such as "fluidity" and permeability, as well as dimensions.[9] We were further interested in examining the behaviour of this model, in particular in determining the effect of different *cis* : *trans* ratios on membrane fluidity. It is worth pointing out that previous models examined the effect of double bond geometry in vesicles considering either *cis* or *trans* isomers,[1] although 100% *trans* phospholipid content can never correspond to thermodynamically equilibrated situations, as well as to naturally occurring systems. In fact, a natural phenomenon of adaptation strategy in some bacteria is based on a regulation of the monounsaturated C16:1 fatty acid composition in membranes up to *cis* : *trans* = 32 : 68, in order to resist unfavorable environmental conditions.[10] Here we describe an approach for examining the effect of *cis* : *trans* ratios as derived from the use of a fluorescence-based stop-flow technique.†



Scheme 1 Mechanism of *cis–trans* isomerization of double bonds catalysed by thiyl radicals.

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The fluorescent lipophilic probe is *cis*-parinaric acid, c-PnA (fluorescence quantum yield and lifetime, $\phi = 0.020$ and $\tau = 1.9$ ns, respectively, in ethanol)[11] whose partition in natural lipid vesicles is well known and has the following features:[12,13] (i) negligible fluorescence emission in water, (ii) strong emission upon incorporation into phospholipid bilayers, and (iii) diffusion-controlled, time-dependent photodimerization with loss of luminescence under constant illumination. On this basis, by monitoring the luminescence changes *vs.* time and temperature of the c-PnA probe incorporated within POPC, PEPC-83, and POPC : PEPC-83 mixtures, one can gain a real-time picture of membrane properties as affected by their isomeric composition. All experiments were run in the temperature range 10–35 °C. The chain melting temperatures (T_m) for POPC, PEPC and PEPC-83 are −3, 35 and 22.7 °C, respectively.[9]

The stop-flow fluorescence experiments were conducted by employing constant illumination of a 1 cm^2 area of the reactor cell with light at 305 nm. Fig. 1 illustrates the results from vesicles made of the naturally occurring POPC lipid. As one can see, the behaviour registered at all investigated temperatures is quite

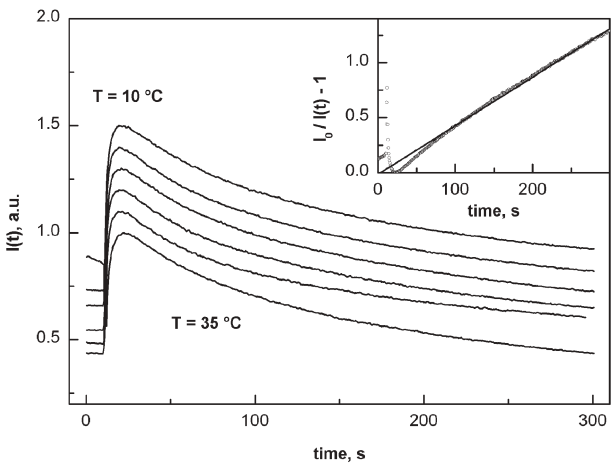

Fig. 1 Observation of the time-dependent luminescence intensity for vesicles obtained from POPC; temperatures are 35, 30, 25, 20, 15, and 10 °C. Inset shows data points as $I_0/I(t) - 1$, see eqn. (1), for the curve at 35 °C. From linear fitting (inset, full line), the derived slope is $S = 0.062$ s^{-1}. According to eqn. (1), and based on $I_0 = 6 \times 10^{11}$ photons cm^2 s^{-1} (as evaluated for our experimental set up), the dimerisation rate constant (35 °C) is $k_d = 1 \times 10^{-13}$ cm^2 s^{-1}, *cf.* ref. 13.

similar and is as follows. Immediately after the starting time and because of fast injection of the starting c-PnA solution in the POPC suspension, a rapid rise of the fluorescence intensity signals the insertion of the probe into the vesicle (this step is complete within a few seconds). Subsequently, loss of fluorescence takes place, owing to the photodimerization step of the probe.[13] This latter process is diffusion-controlled by the hosting bilayer[14,15] and is second order in the initial c-PnA concentration (proportional to the initial luminescence intensity I_0). This can be checked from use of eqn. (1a), the integrated form of eqn. (1b), that results in a straight line for $[I_0/I(t) - 1]$ *vs.* time (see inset of Fig. 1). In eqn. (1), I_0 and $I(t)$ are the fluorescence intensities immediately after the mixing and at time t, respectively, and k_d is the dimerization rate constant.[13]

$$\frac{I_0}{I(t)} - 1 = I_0 k_d t \tag{1a}$$

$$-\frac{dI(t)}{dt} = k_d I^2(t) \tag{1b}$$

Fig. 2 shows the fluorescence-based stop-flow results for PEPC-83 within the temperature interval 35 to 10 °C. It can be seen that the profiles for the time-dependent luminescence intensity at the lower temperatures are qualitatively different from those for 35 or 30 °C (which are in turn similar to those reported in Fig. 1 for POPC). In fact, for PEPC-83 (Fig. 2) the rise in luminescence intensity and, to a larger extent, the subsequent decay, is substantially slowed at $T \leqslant 25$ °C. This is expected from the PEPC-83 T_m value of 22.7 °C, due to the change from a disordered gel to a more ordered liquid crystalline phase of the lipid, which reduces the interaction of the probe with the bilayer and with itself.[9]

The time-dependent fluorescence intensities of Fig. 1 and Fig. 2, as well as that of a 1 : 1 mixture of POPC : PEPC (data not shown), could be analysed in terms of a three exponential decay, eqn. (2), where A and τ are a pre-exponential factor and a decay time, respectively. Here, (i) A_1 and τ_1 account for the rise portion of $I(t)$, see Fig. 1 and Fig. 2, where A_1 is a negative value and τ_1 is in the range 1 to 4 s, and (ii) the components 2 and 3 of $I(t)$ are due to loss of fluorescence caused by dimerization of the probe. This loss can be described by use of a single parameter, an average time of loss, τ_a, eqn. (3).[13]

$$I(t) = A_1 \exp\left(-\frac{t}{\tau_1}\right) + A_2 \exp\left(-\frac{t}{\tau_2}\right) + A_3 \exp\left(-\frac{t}{\tau_3}\right) \tag{2}$$

$$\tau_a = \frac{A_2}{A_2 + A_3} \times \tau_2 + \frac{A_3}{A_2 + A_3} \times \tau_3 \tag{3}$$

According to this approach, τ_a is therefore related to the diffusion process of the c-PnA probe within the host, with larger and larger values of τ_a indicating that diffusion becomes slower and slower. In particular, the results of Fig. 2 show that the *trans* lipid isomer yields vesicles wherein diffusion of the fluorescent probe is substantially reduced below a certain temperature.

The point is further addressed by comparing results of the analysis of a three exponential decay for vesicles formed by POPC, PEPC-83 and a 1 : 1 mixture of POPC : PEPC, as shown in Fig. 3. In the investigated temperature interval, the τ_a value variations in the three systems showed the most relevant changes between 100% *cis* and 83% *trans* content in vesicles. In particular, POPC did not show any change in the whole temperature range, whereas in PEPC-83 vesicles, diffusion and reactivity of the fluorescent probe were strongly influenced at temperatures below 25 °C. The comparison between the vesicles containing 83% and 50% *trans* monounsaturated fatty acids is worthy of further comment. Based on the T_m of POPC (−3 °C), and PEPC-83 (22.7 °C), an intermediate value is expected for a 1 : 1 mixture of POPC : PEPC. In Fig. 3, it is clear that the values of τ_a obtained below 25 °C are increasing on going from 50% to 83% *trans* content, as expected from the corresponding different lipid phases. More interestingly, the temperature at which a "critical" fluorescence change can be seen is 25 °C, similar for both *trans*-containing vesicle systems.

This indicates that monounsaturated *trans*-containing vesicles are more sensitive to temperature, that at a threshold value acts as a switch for the diffusibility and reactivity of the lipophilic fluorescent probe. By further reducing the percentage of *trans* isomer, the response to the temperature factor is less and less, and in fact it disappeared around 5% content (data not shown).

In conclusion, the stop-flow luminescence approach allowed the *trans* and *cis* isomer influence to be studied in model membranes,

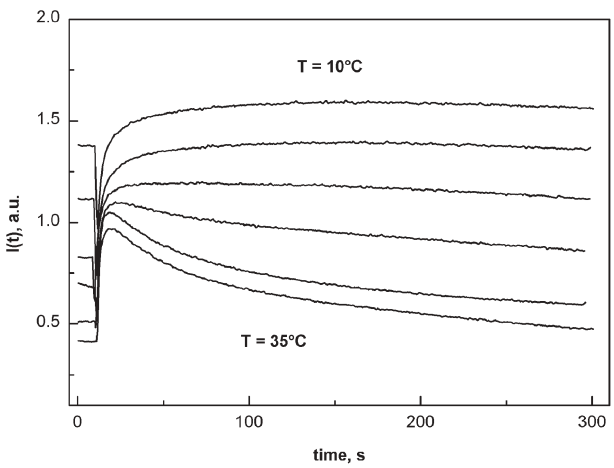

Fig. 2 Observation of the time-dependent luminescence intensity for the case of vesicles obtained from PEPC-83; temperatures are 35, 30, 25, 20, 15, and 10 °C.

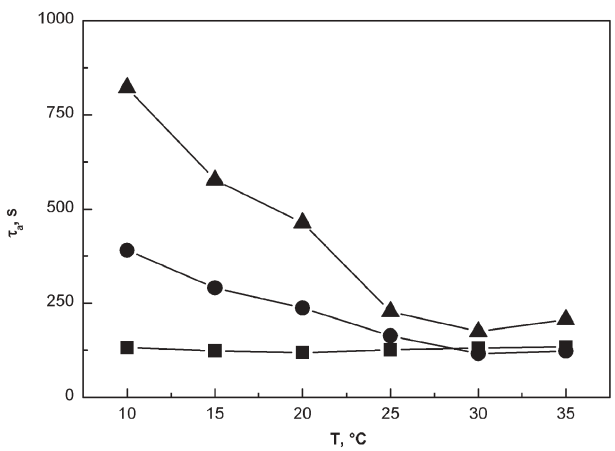

Fig. 3 Temporal decay of the luminescence intensity of c-PnA for vesicles obtained from POPC (■), PEPC-83 (▲) and a 1 : 1 POPC : PEPC mixture (●).

using the simplest monounsaturated system of POPC and PEPC. From experiments with different isomeric percentages, an important indication of the temperature effect was obtained, thus suggesting that a temperature threshold is obtained with *trans* isomers, which give more sensitive molecular interactions with the permeating species. Thus it appears that lowering the temperature is the switch that activates the response only in *trans*-containing systems. Further work is in progress for investigating complex systems, made of mono- and polyunsaturated fatty acid residues of different chain lengths, which more closely resemble cell membranes, and to determine the type and extent of fatty acid structures able to give the temperature response.

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Notes and references

† *POPC vesicle preparation.* The synthesis of PEPC-83 and vesicle formation in 10 mM phosphate buffer, pH 7.4 were performed as already described.[9] Briefly, POPC or PEPC-83 was first dissolved in methanol at room temperature. An aqueous 1 mM lipid suspension was then prepared by injecting the methanolic solution by a Hamilton syringe into 10 mL of a slowly stirred buffer solution. From this first suspension, different lipid suspensions could be obtained by serial dilution. *c-PnA solution preparation.* Fresh c-PnA solutions were prepared each working day by dissolving a small amount of crystals in absolute ethanol previously degassed with argon. Because of large undissolved particles, the c-PnA stock solution was then filtered and the probe concentration was determined by measuring the UV absorption at λ_{max} = 304.2 nm (ε = 77 300 cm^{-1} M^{-1}). The resulting c-PnA alcoholic solution was then diluted in phosphate buffer so that its final concentration reached 0.002 mM. The total alcohol never exceeded 2% in volume. *Fluorescence measurements with the stop-flow equipment.* Fluorescence measurements were performed on a Perkin Elmer Luminescence Spectrometer LS 50 B, in combination with a RX.2000 Rapid Mixing Accessory by Applied Photophysics. The excitation wavelength was set at 304 nm, while the emission monochromator was at 412 nm. The spectrometer is equipped with a repetition Xe discharge lamp yielding 20 kW for 8 μs pulse duration; this corresponds to a spectral radiance of *ca.* 0.8 × 10^{-4} W cm^{-2} sr^{-1} nm^{-1} at 300 nm. We adjusted the repetition rate of the source so that the starting rate of photon absorption by c-PnA for our samples was *ca.* 0.1 photons $molecule^{-1}$ s^{-1}. Under constant illumination, this allowed the occurrence of photochemical decay of c-PnA fluorescence within a few minutes.

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