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Effect of 2.45 GHz microwave radiation on permeability of unilamellar liposomes to 5(6)-carboxyfluorescein. Evidence of non-thermal leakage

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The influence of 2.45 GHz microwave radiation on the membrane permeability of unilamellar liposomes was studied using the marker 5(6)-carboxyfluorescein trapped in phosphatidylcholine liposomes. The release of the fluorescent marker was followed by spectrofluorimetry after an exposure of 10 minutes to either microwave radiation or to heat alone of the liposome solutions. A significant increase of the permeability of carboxyfluorescein through the membrane was observed for the microwave-exposed samples compared to those exposed to normal heating only. Exposure to 2.45 GHz microwave radiation of liposomes has been previously found to produce increased membrane permeability as compared with heating. However, in contrast to previous studies, the observations reported here were made above the phase transition temperature of the lipid membrane. The experimental setup included monitoring of the temperature during microwave exposure simultaneously at several points in the solution volume using a fiberoptic thermometer. Possible mechanisms to explain the observations are discussed.

Introduction

The potential health hazards associated with exposure to microwave radiation has received much attention in view of numerous documented observations confirming that microwave radiation can affect biological systems [1]. The simplest and most widely accepted explanation for such effects has been given in terms of macroscopic heating of the tissues due to the absorption and consequent thermal dissipation of the radiation. However, recent studies showing biological effects of low-level microwave radiation raise the question whether or not non-thermal effects could be involved as well [2]. It has been suggested that, under such circumstances, biological membranes and, more specifically, phospholipids in natural membranes may represent, the major sites of interaction with microwave radiation [3]. Accordingly, in our studies concerned with addressing this problem we have chosen the essentially spherical lipid bilayer boundaries of phospholipid liposomes as a simple model system representing cell membranes.

It is well known that leakage from the interior of liposomes and natural membranes of entrapped substances may take place [4–10]. More recently, an increase in such leakage upon microwave exposure of these systems has been reported [3,11–15], although the observation of the effect is not unequivocal [16,17]. In the work to be described here, we have studied the release of the fluorescent marker 5(6)-carboxyfluorescein from liposomes made from L-α-phosphatidylcholine. Particular attention has been directed towards ensuring that the liposomes were of uniform size, of narrow size-distribution, and possessing pure lipid membranes.

The microwave frequency of 2.45 GHz has been chosen for use in this study due to its technological predominance. This frequency is used extensively in domestic, industrial, and therapeutic applications and is, in addition, the proposed transmission frequency for future power satellites [18].

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On the basis of our results, we confirm that microwave exposure enhances the release of the entrapped dye from the liposomes. Although no definite mechanism for the effect emerges, we shall be able to exclude some potential mechanisms for the phenomena observed.

Materials and Methods

Preparation of unilamellar liposomes

The lipid used was L-α-phosphatidylcholine (PC) from soybean in chloroform solution (Sigma Cat. No. P6263) and the dye to be entrapped was 5(6)-carboxyfluorescein (CF) also obtained from Sigma (Cat. No. C 7153). The method involves adding the dye during the liposome preparation process which results in its incorporation into the liposome interior. The complete preparation is carried out in two major steps. First multilamellar liposomes are produced which may be freeze-dried and stored ready for the next stage of the preparation of unilamellar liposomes through extrusion. The preparative procedures are described in more detail as follows.

250 mg of PC in chloroform solution was evaporated to dryness in a rotary evaporator and the dry PC film redissolved first in 2 cm^3 and then an additional 8 cm^3 of a 0.017 mol dm^{-3} CF solution in 0.05 mol dm^{-3} Tris-saline buffer of pH 7.4. In order to assist dispersion of the PC a bath sonicator (Branson Sonifier B-12) at a power setting near 65 W was used. Vortexing of the solution produced multilamellar vesicles which were then seven or eight times in succession frozen in liquid nitrogen and thawed in order to ensure equal distribution of the solute between lamellae and adequate hydration of the lipid. Finally, the liposome solutions were freeze-dried and stored until the next stage of the preparative procedure; we note that freeze-drying has been reported to enhance the capacity of encapsulating solutes by the liposomes [10].

The preparation of the unilamellar liposomes proceeded from the freeze-dried PC/CF mixture; this was first rehydrated by dissolving it in 10 cm^3 of 0.05 mol dm^{-3} Tris-saline buffer. The solution was then passed through an extruder (Lipex Biomembranes Inc., Vancouver, Canada) equipped with a base filter and a 0.1 μm filter (Nucleopore, SN : 110605). The solution was extruded five times interspersed with freezing/thawing using liquid nitrogen as for the preparation of the multilamellar liposomes. Finally, the excess CF was separated from the liposomes by gel filtration using columns packed with Sephadex G-25. The resulting unilamellar liposomes are stable for at least 2–3 days at a storage temperature of 5 °C; however, all experiments were carried out as soon as possible, usually within 10 h, after completion of the preparation.

The phase transition temperature of the liposomes was found to be within the reported range of between −15 °C and +7 °C of phosphatidylcholine liposomes [19]. The size and polydispersity of the liposomes were determined by dynamic light scattering using an instrument consisting of a laser light source, a temperature-controlled cell holder and a MALVERN K7027 'LOGLIN' correlator. The apparatus was calibrated using a standard monodisperse sample of polystyrene latex spheres.

Spectrophotometric measurements

Absorbance and fluorescence of the solutions were measured using a Varian Cary 219 spectrophotometer and an Aminco SPF-500 spectrofluorimeter, respectively. For the purposes of the fluorescence measurement the liposome solutions were diluted with Tris-saline buffer to an absorbance near 0.25 in a 0.5 cm cuvette at 490 nm, the excitation wavelength to be used. The lipid concentration in the samples was approx. 0.1 mg/ml. Fluorescence emission was measured at 518 nm.

The liposomes were checked for the possibility of leakage of the dye through the membrane by measuring the fluorescence intensity immediately after dilution of the liposome sample and comparing it with that measured 3 h later. Only those sample solutions showing no change in fluorescence intensity were used for the subsequent experiments of microwave exposure.

All spectrofluorimetry was carried out at a constant room temperature of 21 °C; however, between measurements the samples were placed in an ice-bath and kept in the dark.

Microwave exposure experiments

Microwave radiation was generated by a continuous wave magnetron operating at 2.45 GHz. The magnetron was coupled to an IEC R32 waveguide (S-band) which was terminated with a horn antenna with aperture dimensions of 157 mm × 135 mm in the E and H planes, respectively. The output power was monitored with a power meter connected to a waveguide directional coupler situated between the magnetron and the antenna. All irradiations were performed at a distance of 0.4 m between the sample and the antenna aperture. The walls of the exposure room were lined with microwave absorbing material (Eccosorb, Emerson and Cumming Inc., Canton, MA, U.S.A.).

The sample container was made of polytetrafluoroethylene (Teflon^R) with a volume of 1 cm^3; it has been described in detail elsewhere [20]. The sample container was placed inside a poly(methyl methacrylate) (Perspex^R) trough, as shown in Fig. 1, through which water was circulated from a thermostatted bath placed well outside the radiation field. Stirring of the sample itself was achieved by a slow flow of air bubbles via a small plastic tube protruding through the lid of the sample container. The sample temperature was measured using

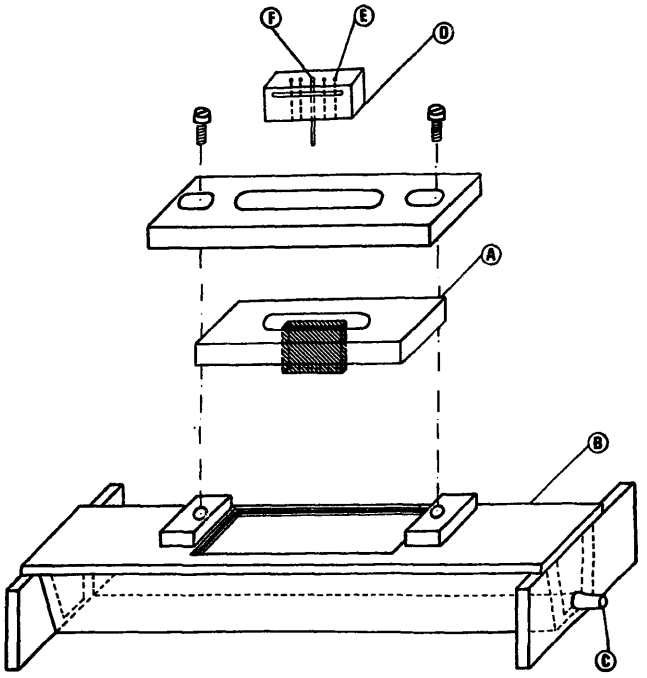


Fig. 1. Schematic picture of PTFE (Teflon) exposure cuvette (A). The cuvette is lowered into a water jacket (B), which is connected to a thermostated water bath by tubing (C). The lid (D) has holes (E) for optical fibre temperature probes. The sample is stirred by air, which is supplied via the tube (F).

a Luxtron 755 multichannel fluoroptic thermometer with four fiberoptic non-perturbing probes positioned at different depths in the sample. The thermometer and the power meter were connected to an HP 85 desk computer based measurement system programmed to register the temperature on all four probes every fourth second. We ascertained that the presence of the thermometer had no influence on the fluorescence results.

Experimental procedure

The experimental procedure was as follows. Liposome samples were removed from ice, left at room temperature for one minute and then placed in a water bath at 38.5 °C for another minute. One liposome sample (1 cm^3) was exposed to microwaves for 10 min and another identical sample was placed, without exposure to radiation, in a water bath kept at a temperature 1.0–1.5 C° higher than the final temperature of the microwave irradiated sample; the heating of this second sample was continued for 11 min compensating for the time taken to fill the sample container for microwave exposure. After the exposure period the temperature of both samples was rapidly decreased to 10 °C within about 30 s. The samples were then transferred to 0.5 cm^3 silica cuvettes and their fluorescence determined as soon as they warmed to room temperature of 21 °C. Finally, the average liposome size of the samples was measured by dynamic light scattering and compared with that prior to the microwave or heat exposure.

Dosimetry

Specific absorption rate (SAR) of the radiation was determined in a separate experiment. The test solution in the sample container and the stationary water in the Perspex^R trough were allowed to come to room temperature; without bubbling air through the sample the container was exposed to microwave radiation for about 10 min and the temperature of the sample was recorded as a function of time of exposure. If we write w for the absorbed power and M for the mass then the specific absorption rate (SAR), the absorbed power per unit mass is given by

$$\mathrm{SAR} = \frac{w}{M} = C_p \cdot \frac{\Delta T}{\Delta t} \tag{1}$$

where C_p is the heat capacity and $\Delta T/\Delta t$ corresponds to the initial slope of the temperature versus time function during irradiation.

Determination of complex permittivity

In order to determine the microwave absorption properties of the liposome solution compared to those of buffer solution the complex permittivity was measured. The complex permittivity is defined as

$$\epsilon^* = \epsilon' - j\epsilon'' \tag{2}$$

In this equation $j^2 = -1$, ϵ', the real permittivity, describes the wave propagation while ϵ'', the imaginary permittivity, describes the absorption properties of the medium. The complex permittivity of solutions of liposomes at a temperature of 21 °C and at various concentrations was determined at 2.9 GHz using the cavity resonance method [22]. The resonance curves were displayed and measured by means of a network analyzer connected to the cavity; the real permittivity is determined by the frequency of the resonance, f, and the loss by the sharpness of the resonance. The latter is expressed as the Q factor,

$$Q = f/\Delta f \tag{3}$$

where Δf is the bandwidth at an attenuation of 3 decibels of the maximum power [21,22]. The relevant formulae for a thin sample in a cylindrical TM_{010} cavity are given by [22].

$$\frac{\epsilon'}{\epsilon_0} = 1 + 0.539 \frac{V}{v} \frac{(f_0 - f_1)}{f_0} \tag{4}$$

$$\frac{\epsilon''}{\epsilon_0} = 0.269 \frac{V}{v} \left(\frac{1}{Q_1} - \frac{1}{Q_0} \right) \tag{5}$$

where v is the volume of the sample, V the volume of the cavity and the subscripts 1 and 0 refer to the results of the measurements with and without the sample in the cavity, respectively.

Results

From the dynamic light scattering measurements we obtained the average diameter of 185 ± 10 nm for the liposomes in the samples used. The standard deviation was estimated from an essentially monoexponential autocorrelation function [23]. Its value reflects low polydispersity of the liposomes in the preparations. Importantly, the same measurements showed no significant change in either the average size of the liposomes or its standard deviation for either the microwave irradiated samples or the control samples exposed to heat only.

In Fig. 2 are shown the results of the fiberoptic temperature measurements for both the microwave-exposed and the normally heated control samples. As already referred to in the previous section the temperature to which the control samples were exposed was somewhat in excess of that reached by the irradiated samples as a result of the absorption of microwave radiation. This was done to ensure that the observed effect could not be due to a macroscopic overheating of the microwave-exposed sample.

In the dosimetry determination the temperature increase as a function of time was registered when the liposome sample was exposed to high intensity microwave radiation. From the initial slopes of these curves, using, as an approximation, the heat capacity value of water at 20 °C and scaling to the output power used we obtain SAR values of 38 ± 1 W/kg for the liposome solution.

The results of the measurement of the complex permittivity are summarised in Fig. 3. When the liposome concentration increases there is a decreasing absorption of microwave radiation, as indicated by the trend shown by the imaginary permittivity, ε''. This is in accordance

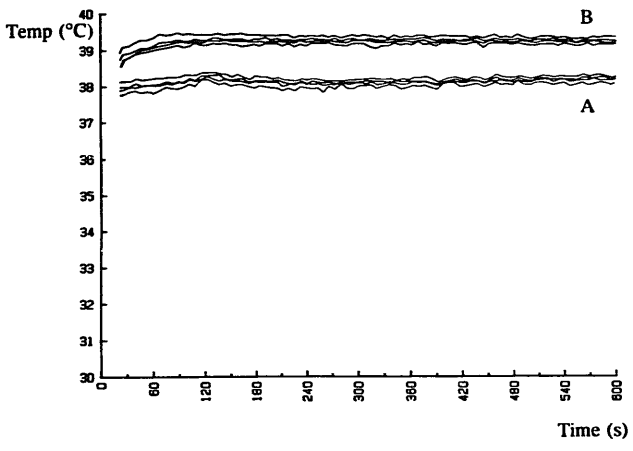
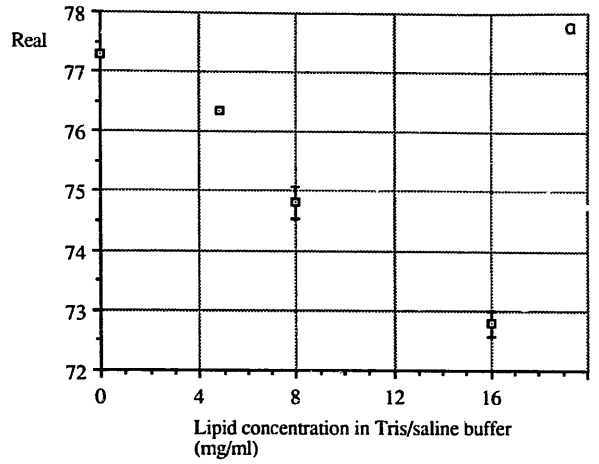


Fig. 2. Temperature as a function of time in a representative 2.45 GHz microwave-exposed sample (A) with temperature measurements in four points taken with a fiberoptic thermometer. Curves (B) shows temperature measurements in the control sample which is in the water bath.



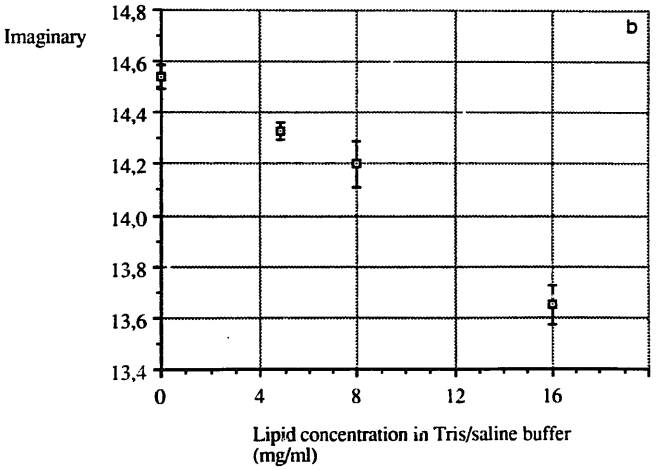


Fig. 3. Results of measurements of the complex permittivity as a function of the lipid concentration in Tris/saline buffer. (a) The real part of the complex permittivity. (b) The imaginary part of the complex permittivity. The error bars indicate standard error of mean.

with what could be expected, as lipids usually exhibit low microwave absorption compared to water.

The fluorescence spectra of four liposome solutions shown in Fig. 4 are typical of the results of many microwave irradiation experiments. Different preparation batches of liposomes were found to display somewhat different leakage rates, however, the leakage from the microwave exposed liposomes was always greater than that from the normally heated liposomes. The minimum fluorescence intensity is shown by a sample before exposure to either radiation or heat and the maximum intensity is achieved when the sample is heated to 90 °C when, due to rupture of the liposomes, all CF is released into free solution. The significant result is shown by the remaining two spectra in Fig. 4: the leakage of the dye as a result of microwave irradiation is greater (58% of maximal fluorescence) compared with the heated control (36% of maximal fluorescence) in spite of the fact that the control was exposed to higher temperatures.

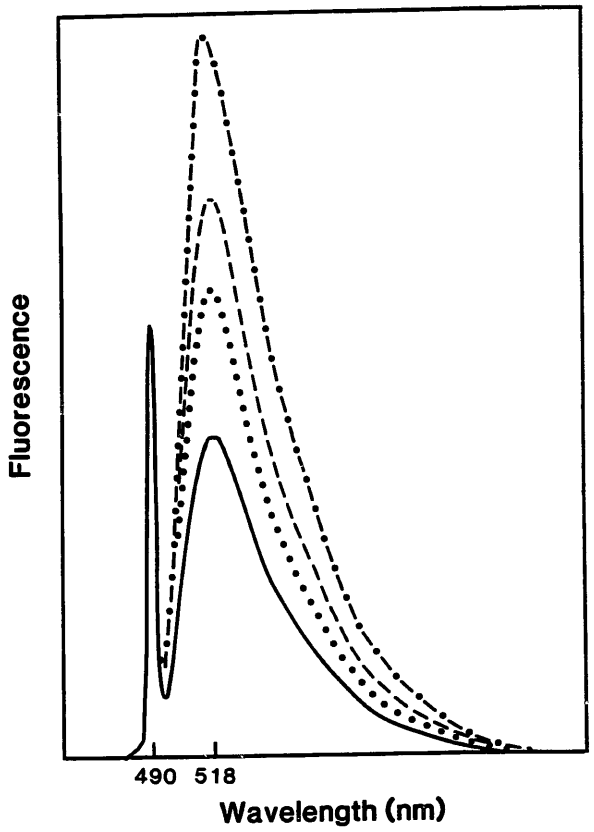


Fig. 4. Representative fluorescence, emission, spectra obtained on four differently treated liposome samples in a 0.5 cm quarts cuvette: — — —, microwave-exposed (2.45 GHz) sample (38 °C, 10 min); ·····, normally heated sample (39 °C, 11 min); ———, non-exposed sample; ·–·–·, sample fluorescence after total disintegration of liposomes (90 °C, 10 min) defines the upper limit of membrane leakage.

As an additional control experiment we observed that if a sample was heated to 45 °C, instead of 40 °C, the leakage of the dye increased by a further 7%. This would give a leakage of 43% of maximal release for the normally heated sample, which is still significantly less than the 58% resulting from microwave irradiation.

We note that at 21 °C, the temperature at which the fluorescence was measured, there is no detectable leakage of the dye from the liposomes.

Discussion

We first note that the method of preparation of the liposomes did not involve organic solvents, detergent or, except for aiding the dissolution of the *lipids*, the use of sonication; all of these are factors that make liposomes more complex and may affect their stability. In addition, the average size of the liposomes was reproducible from sample to sample showing relatively little polydispersity. Thus the quality of the liposome preparations adds confidence to the assertion that the results of microwave exposure experiments reflect a true property of the lipid bilayer forming the vesicle membranes.

Next we recall that we studied the liposomes, which were kinetically stable, well above their liquid-crystalline phase transition temperature. Liposomes are generally known to release encapsulated water soluble contents more readily near their phase transition temperature [3–10,14]. Our results extend this observation by demonstrating that liposomes display leakage that increases with temperature far above their lipid membrane phase transition temperature.

It has also been proposed [3] that increased liposome leakage as a result of microwave exposure should occur *only* near the phase transition temperature. However, the proposition that such changes in permeability are associated with the lipid liquid-crystalline transition is not substantiated by our results. We recorded a significant increase in leakage of liposomes above their phase transition temperature upon exposure to microwave radiation compared with the heated control samples.

The mechanism through which microwave radiation causes an increase in the membrane permeability is, of course, of primary interest. As a result of this study we may, at least, exclude some potential mechanisms.

First, the release of the dye upon microwave radiation increases without a corresponding release of membrane phospholipids, the average size of the liposomes remaining unchanged as measured by dynamic light scattering. We conclude, in agreement with a previous report [3], that the increase in permeability cannot be due to the disruption of the membranes to any significant extent.

Second, it is unlikely that the release of the dye from the liposome interior is due to dielectric breakdown of the phospholipid bilayer; pore formation has been reported to occur as a result of intense electric field exposure when the field strength exceeded 3000 kV m^{-1} [24], several orders of magnitude larger than the electric field strength of approximately 10 V m^{-1} used in this study.

Third, thermal effects deserve most serious consideration. We believe that since the samples were stirred the possibility of local temperature rises, on a macroscopic level, due to inhomogeneous heating may be excluded. Nevertheless, the dissipation can be inhomogeneous on a microscopic level. A larger absorption of microwave energy in the proximity of the liposomes might lead to local heat effects on the membranes. This hypothesis may seem contradictory to the results in Fig. 3, demonstrating a trend of decreasing absorption of microwave radiation with increasing liposome concentration. However, the permittivity measurements refer to relatively concentrated liposome solutions where the conditions may differ significantly from those of the leakage experiments. The results only indicate that the pure lipid cannot be responsible for any extra heat dissipation

compared to that of the buffer solution. However, this does not exclude the possibility that, for example, different ionic conditions in the dilute system, such as a relatively higher ion concentration at the liposome surface compared to the bulk solution, may result in local heating of the liposomes.

The largest localized temperature difference effect would occur if the energy was dissipated within the membrane rather than in the whole of the liposome cavity leading to a possible membrane disruption. However, dynamic light scattering does not show the presence of debris from ruptured liposomes after microwave irradiation; indeed, as shown in the Appendix, the diffusion of heat from the thin membrane to the surroundings is highly efficient and should prevent local temperature rises of sufficient magnitude to cause disruption of the membrane.

The increase in the leakage of the dye from the liposomes with increasing temperature without disruption of the membrane in the absence of microwave radiation implies that, at least for the particular liposomes studied here, thermal motion of the lipid molecules within the membrane results in the formation of transient pores through which entrapped molecules may be released from the interior of the liposomes. The additional leakage of the dye from the liposomes upon exposure to microwave radiation cannot be attributed, as we have shown, to the disruption of the liposome membrane. We conclude that microwave radiation is most likely to cause the formation of transient pores, additional to those due to thermal fluctuations, which then results in an increase in the release of the entrapped dye compared with the non-irradiated control samples. Accepting this explanation the next problem to be pursued becomes the elucidation of the particular structure or structural elements of the liposomes that are responsible for the formation of such transient pores due to some specific interaction with the radiation field.

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Appendix

The transfer rate of energy due to microwave absorption per unit volume of membranes, j in units of $J s^{-1} m^{-3}$, may be estimated from a hypothetical temperature rise, ΔT, for liposome solution, compared to buffer solution, during the exposure time Δt:

$$j = (C_p \cdot \rho \cdot \Delta T/\Delta t)(V/v) \quad \text{(A-1)}$$

where C_p and ρ are, respectively, the average heat capacity and density of the solution, V is the volume of the solution and v that of the membrane. Assume first that there is no conduction of heat from the membrane to the ambient solution; the corresponding temperature rise in the membrane, ΔT_m, is then obtained from:

$$j\Delta t = \Delta T_m \cdot C_{pm} \cdot \rho_m \quad \text{(A-2)}$$

where C_{pm} and ρ_m are, respectively, the heat capacity and density of the membrane. This may represent a very substantial rise in temperature; for example, $\Delta T_m = 1000$ K is obtained for a hypothetical bulk temperature rise of $\Delta T = 2$ K with the approximations $C_{pm} \equiv C_p$ and $\rho \equiv \rho_m$ when the lipid volume fraction is $v/V = 0.002$.

A membrane heated to such an extent is certainly expected to disintegrate; however, as evidenced by the dynamic light scattering results, this does not happen which leads to the conclusion that owing to the small thickness of the membrane the diffusion of heat from it to the surrounding medium is highly efficient. This proposition may be tested by approximate numerical estimates of the cooling of the membrane. Specifically, according to the Fourier law of thermal diffusion the thermal energy flux, ϵ in units of $J s^{-1} m^{-2}$, is proportional to the temperature gradient in the system; if we assume that the maximum temperature rise occurs in the centre of the bilayer of total thickness d, the heat transfer rate at the membrane boundary per unit area is

$$\epsilon = -\lambda \Delta T_m/(0.5 \cdot d) \quad \text{(A-3)}$$

where we may take the value of $\lambda = 0.3\ J s^{-1} m^{-1} K^{-1}$ for glycerol to be a reasonable approximation for the lipids and $d = 5.0$ nm. One now defines a characteristic cooling time, τ, which is the time required to transfer through the outer membrane boundary into bulk solution the heat generated inside the membrane due to the absorption of radiation, calculated form the equality

$$\Delta t \cdot j \cdot A \cdot d = \tau \cdot \varepsilon \cdot A \quad \text{(A-4)}$$

where A is the outer membrane surface area. Using the approximate values of $C_{pm} = 4200\ J kg^{-1}$ and $\rho_m = 1000\ kg m^{-3}$ one obtains $\tau \cong 10^{-10}$ s.

Next one needs some estimate of the time required for the disintegration of the membrane. This we take, tentatively, to be no less than the smallest relaxation time, the rotational correlation time, τ_{rot}, of a spherical membrane fragment whose radius, r, determined by the thickness of the membrane, is 2.5 nm. The Debye expression [25].

$$\tau_{rot} = 4\pi r^3 \eta/(3kT) \quad \text{(A-5)}$$

requires a value for η, the viscosity of the medium. Taking the viscosity to be that of water one calculates $\tau_{rot} \cong 10^{-8}$ s; this is the lower limit for τ_{rot} since the rest of the membrane, laterally joining the revolving part,

must have a damping effect increasing the effective value of the viscosity. Nevertheless, the results of these numerical estimates show that the cooling time is at least two orders of magnitude less than the minimum time required for the membrane to disintegrate.

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