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Kinetics of spreading of photodamaged DOPC liposomes at the air-water interface

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Abstract The kinetics of surface film formation from DOPC small unilamellar liposomes spread at the air-water interface was studied. A simple theoretical and experimental approach was used.

It was found that the surface transformation process was accelerated under photodynamic lipid peroxidation in presence of methylene blue and red light illumination. A theoretical approach, coupling the photodynamic action and the process of spreading of liposomes was developed. The mechanisms of photomodification and destabilization of the liposomal bilayer structures was analyzed.

Key words Photodamaged liposomes – spreading – surface film

Introduction

It is well established now that cell membranes may be targets for photomodification. The mechanisms of various membrane photosensitization reactions, as well as the chemical nature of the modified sites, have been intensively studied in connection with the fundamental and practical importance of this phenomena [1, 2]. An important case is the photosensitized lipid peroxidation requiring molecular oxygen, a sensitizing dye and exciting light [3]. A suitable model liposome membrane system has been used to study the photodynamic lipid peroxidation. The process of photodynamic lipid peroxidation leads to damage of the membrane structure and lysis of liposomes [4–7]. The individual liposomes, containing marker molecules like glucose, are progressively lysed during illumination, releasing their contents [2, 4, 5]. Release of glucose under these conditions is accompanied by a photodynamic decrease in light scattering of the liposomal suspensions, due to damage and disruption of the membrane structure. Upon stopping the illumination, no further lysis occurs [5].

It is interesting to detect the disruption stage of the membrane structure due to the photodynamic lipid peroxidation. A recently developed approach to study the kinetics of spreading of liposomes at the air–water interface [8–14] was used. The behavior of the liposomes spread at the air–water interface is essentially governed by the following processes: liposome diffusion from the interface into the bulk phase and the more complex liposome-surface interaction, resulting in a slow transformation of

closed bilayer structures into a surface film. To approach this phenomenon quantitatively, Schindler [9] has proposed a complex scheme with five parameters. In a first approximation (Fig. 1A), the diffusion process with diffusion coefficient (D) was kinetically distinguished from the other processes, summarized in a single irreversible transformation of perfectly closed vesicles into surface tensioactive structures of aggregated lipid molecules. This simple theoretical scheme allows one to obtain a global kinetic constant (K) for the surface transformation step [11–14]. As a result of formation of membrane defects, caused by lipid peroxidation, the surface transformation of perfectly closed vesicles into open structures is expected to be accelerated.

The purpose of this paper is to study the influence of the photodynamic lipid peroxidation on the kinetics of surface film formation from spread DOPC liposomes irradiated by red light in the presence of methylene blue (MB).

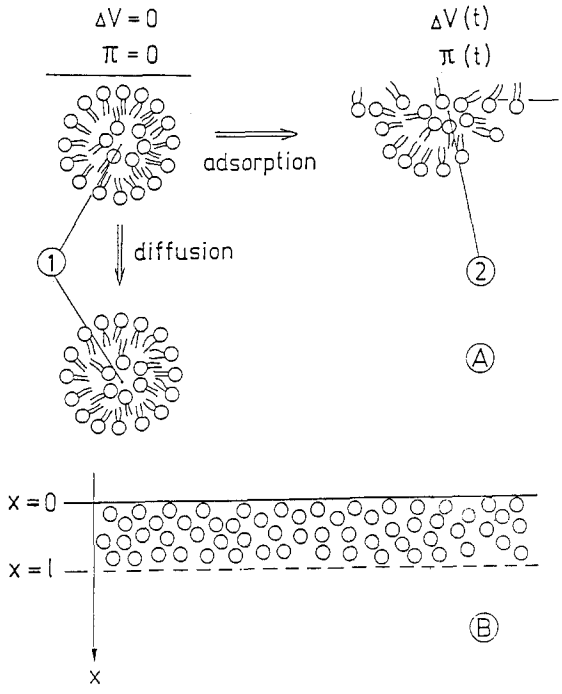

Fig. 1 Panel A: Schematic picture describing the two processes involved in the formation of the surface film from a spread liposomal suspension. Type 1 structures = intact, closed liposomes; Type 2 = partly open liposomes. Panel B: Initial distribution of liposomes (type 1) after the spreading of a given volume of liposomal suspension at the interface; L is the thickness of the liposome layer

Materials and methods

Preparation of liposomes

Dioleoylphosphatidylcholine (DOPC) was purchased from the Sigma Chemical Company. Analytical grade chloroform was purchased from Merck. The 0.15 M sodium chloride (NaCl) solutions were made with doubly-distilled water and Merck purest quality NaCl heated at 600 °C for 90 min.

Small unilamellar liposomes (S.U.V) with a mean diameter of 50 nm and liposomal concentration $C_0 = 2.4 \times 10^{14}$ liposomes/ml were prepared by the Bangham method [15] following sonication as previously described [13].

Photodynamic lipid peroxidation

Methylene blue (MB) was purchased from Fluka AG. The photodynamic reaction was performed in Eppendorf plastic test tubes as follows. A volume of 5 μl of an aqueous solution of MB was mixed in the dark, at room temperature, with 100 μl of the liposome suspension. Afterwards, the DOPC liposome suspension was stirred for 30 s and was incubated for 30 min in the dark.

The DOPC liposome suspensions, incubated with MB ($C_{MB} = 0.01$ mg/ml of liposomal suspension) were irradiated over different times periods T($T = 5, 6, 7$ or 30 min) while being exposed to air, without oxygen bubbling. The light source was a He-Ne laser (LHN 101 P–Bulgaria), 2mW, $\lambda = 633$ nm. The distance between the laser and the test tube was 10 cm. DOPC liposomes incubated with MB, but not irradiated ($T = 0$) were used as controls.

Measurement of superficial parameters

Volumes of 100 μl of MB free DOPC liposomal suspensions ($C_{MB} = 0$) and similar suspensions, preincubated for 30 min with MB ($C_{MB} = 0.01$ mg/ml of liposomal suspension), non-irradiated ($T = 0$) or irradiated during various times of irradiation ($T = 5, 6, 7$ or 30 min) were spread at a constant surface area (186 cm^2) in a rectangular Teflon trough 7 mm deep. Spreading was performed over a period of about 1 min using an Agla micro syringe. The subphase was 0.15 M NaCl and the temperature of the subphase was 20–22 °C. The kinetics of surface film formation were recorded by following the variations of the surface pressure (π) and surface potential (ΔV) with respect to time. The zero time corresponds to the beginning of the spreading procedure. The surface pressure (π) was measured by the Whilhelmy plate method by using a platinum plate and electronic balance (KSV 2200, Finland) with an accuracy of 0.02 dyn/cm.

The surface potential (ΔV) was measured by using a gold-coated Am^{241} ionizing electrode and a reference calomel electrode with an electrometer RFT-6350 (Germany) connected to a chart recorder SERVOGOR S (Hungary). The accuracy was $\pm$ 15 mV for the initial surface potential (V_0) of the clear air-water interface. However, the accuracy of the rate of surface potential variation $d\Delta V/dt$ was $\pm$ 0.01 mV/s.

Theoretical approach

In the simple theoretical approach [11–14], one considers the presence of only two types of phospholipid structures at the air-water interface. On the one hand, perfectly closed structures (type 1 structures in Fig. 1A) showing no surface active properties and without any effect on the surface potential; on the other hand, a partly open structure (type 2 structures in Fig. 1A) whose effect on both surface pressure and surface potential increase with time. The kinetics of surface film formation after the spreading of the liposomal suspension at the air-water interface can be described by two simultaneous competitive processes (Fig. 1A): i) an irreversible diffusion process of type 1 structures into the liquid bulk subphase, ii) an irreversible transformation process at the interface, changing the type 1 structures into a surface film of type 2 structures.

In the general case, when the rates of two simultaneous parallel processes are of the same order of magnitude, both processes influence the overall phenomenon. When this is not the case, the phenomenon is governed by the faster process.

Diffusion-controlled kinetics measurements are determined for the spreading of small amounts of liposomes. Supposing that all the liposome suspension, initially spread at the interface, forms a layer with thickness L and initial liposomal concentration C_0 (Fig. 1B), Fick's equation must be solved.

An expression for evaluating the liposome concentration $C(0,t)$ for $x = 0$ in the subsurface first liposome layer (solution zone underlying the surface) can be obtained [13]:

$$C(0,t) = C_0 erf\frac{L}{2\sqrt{Dt}}, \tag{1}$$

where D is the bulk diffusion coefficient.

The limiting case for the transformation-controlled process is determined by spreading large amounts of liposomes and large L. The diffusion of liposomes towards the liquid bulk phase does not affect the liposomal concentration $C(0,t)$ at the subsurface. The irreversible transformation of liposomes type 1 into a surface structure type 2 can be described by an appropriate adsorption kinetic equation like the Langmuir equation, neglecting the desorption term [11, 14]:

$$\frac{dn^*}{dt} = KC(0,t)d\left(1 - \frac{n^*}{n^*_\infty}\right), \tag{2}$$

where $n^*(t)$ is the number of type 2 structures adsorbed on a 1 cm^2 area at time t; n^*_∞ is the maximal number of type 2 structures in a close packed layer; $(1 - n^*/n^*_\infty)$ is the available surface area at time t; d is the liposome diameter; $C(0,t)$ is the concentration of type 1 liposomes in the first liposome layer at $x = 0$ able to be transformed; $C(0,t)d$ is the same quantity related to a 1 cm^2 area and K is the transformation constant.

The desorption term in the Langmuir equation is neglected because destroyed liposomes are, for all purposes, totally insoluble and the adsorption process is practically irreversible.

The general case can be described by Fick's equation with boundary conditions taking into account the transformation process. The general solution is very complicated. The following approximation is valid for an important diffusion flow [13]

$$\frac{dn^*}{dt} = KC_0 derf\frac{L}{2\sqrt{Dt}}\left(1 - \frac{n^*}{C_0 d}\right). \tag{3}$$

After a serial development and integration from $t = 0$ and $n^* = 0$ to t and n^*, respectively, one obtains:

$$\ln\left(1 - \frac{n^*}{C_0 d}\right) = -\frac{2KL}{\sqrt{D\Pi}}\sqrt{t}. \tag{4}$$

Surface transformation of spread liposomes subjected to photodynamic lipid peroxidation

The process of photodynamic lipid peroxidation leads to damage of part of liposomes. The formation of defects with a critical size is due to a complex series of possible peroxidation reactions [3, 4] followed by a slow reorganization of reaction products in the liposomal lipid bilayer:

$$L \xrightarrow{\text{peroxidation}} L_1 \xrightarrow{\text{reorientation}} L_2$$

In accordance with the results obtained from monolayer studies [16–18], we assume that the process of reorganization of lipid molecules, with a characteristic time of several hundred seconds, is much slower than the photochemical step. Therefore, the characteristic time of defect formation should be identified with a characteristic time for the molecular reorganization process $L_1 \rightarrow L_2$ described by a first order kineic equation. The probability

(P_d) of the formation of a defect with critical size is related to the time of its formation by the following general equation [19]:

$$P_d = 1 - e^{-t/\tau} . \quad (5)$$

Equation (5) can be used to find the part of defected vesicles in the liposomal suspension during both irradiation and spreading experimental phases. Thus, after an irradiation time T, two populations of intact and damaged liposomes with concentration $C_0 e^{-T/\tau}$ and $C_0(1 - e^{-T/\tau})$ exist respectively. After spreading an amount of this liposomal suspension, the interfacial transformation of the two populations of liposomes can be described by a general kinetic model, based on Eqs. (3) and (5) and taking into account the surface transformation, liposome diffusion, and defect formation processes. The rate constant K corresponds to the surface transformation of closed intact liposomes. The surface transformation rate constant $\tilde{K}$ of liposomes with critical defects, resulting from the photodynamic effect, is expected to be larger than K for intact liposomes. Hence, the overall transformation rate dn^*/dt is given by the sum of the rates of intact and defect-containing liposomes:

$$\frac{dn^*}{dt} = KC_0 derf\frac{L}{2\sqrt{Dt}}\left(1 - \frac{n^*}{C_0 d}\right)e^{-(T+t)/\tau} + \tilde{K}C_0 derf\frac{L}{2\sqrt{Dt}}\left(1 - \frac{n^*}{C_0 d}\right)(1 - e^{-(T+t)/\tau}) . \quad (6)$$

In order to compare the theoretical model with the experimental results, the following approximations of the general Eq. (6) can be useful.

For an irradiation time T sufficiently large ($T \gg \tau$) from the general equation (6), we obtain the limiting case. The limiting case corresponds to damage of all liposomes and saturation of the photodynamic action before spreading:

$$\frac{dn^*}{dt} = \tilde{K}C_0 derf\frac{L}{2\sqrt{Dt}}\left(1 - \frac{n^*}{C_0 d}\right) . \quad (7)$$

After a serial development of $erf\, L/2\sqrt{Dt}$ for small values of argument and integration from $t = 0$ and $n^* = 0$ to t and n^*, respectively, one obtains:

$$\ln\left(1 - \frac{n^*}{C_0 d}\right) = -\frac{2\tilde{K}L}{\sqrt{D}}t^{1/2} . \quad (8)$$

For an irradiation time T comeasurable with the defect formation time $\tau (T \approx \tau)$, we can neglect, as a first approximation, the photodynamic action during the spreading kinetics (the large part of process of defect formation occurs during the irradiation). Then, from Eq. (6), we obtain the following simplified equation:

$$\frac{dn^*}{dt} = KC_0 derf\frac{L}{2\sqrt{Dt}}\left(1 - \frac{n^*}{C_0 d}\right)e^{-T/\tau} + \tilde{K}C_0 derf\frac{L}{2\sqrt{Dt}}\left(1 - \frac{n^*}{C_0 d}\right)(1 - e^{-T/\tau}) . \quad (9)$$

and after integration

$$\ln\left(1 - \frac{n^*}{C_0 d}\right) = -\left[\frac{2KL}{\sqrt{D\Pi}}e^{-T/\tau} + \frac{2\tilde{K}L}{\sqrt{D\Pi}}(1 - e^{-T/\tau})\right]t^{1/2} . \quad (10)$$

For an irradiation time T much shorter than time of defect formation τ ($T \ll \tau$) from Eq. (6) we obtain for the beginning ($t \ll \tau$):

$$\ln\left(1 - \frac{n^*}{C_0 d}\right) = -\frac{2KL}{\sqrt{D\Pi}}t^{1/2} - \frac{2}{3}\frac{(\tilde{K} - K)}{\tau\sqrt{D\Pi}}t^{3/2} , \quad (11)$$

and towards the end of spreading kinetics ($t \gg \tau$) the Eq. (8), predicting the saturation.

Results and discussion

As an example, Fig. 2 shows the variation with time of the relative change of surface potential ($\Delta V/\Delta V_\infty$) after the spreading at constant surface area of two different volumes of DOPC liposome suspension: 1600 μl (curve 1) and

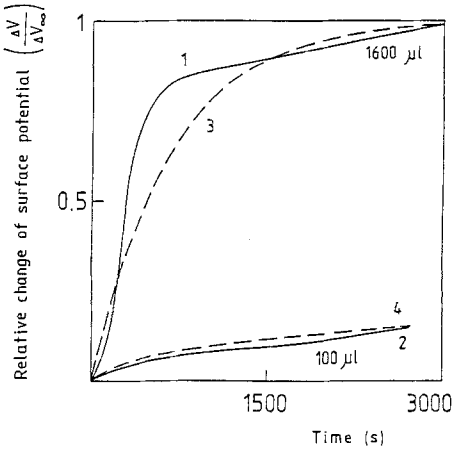

Fig. 2 Variation of the relative change of surface potential ($\Delta V/\Delta V_\infty$) with time for two spread volumes of DOPC liposomal suspensions: curve 1–1600 μl and curve 2–100 μl (spreading surface: 186 cm^2; ΔV_∞ = 440 mV is the value of surface potential at saturation, corresponding to closely packed surface film). Curve 3 represents the theoretical prediction of Eq. (2) ($K = 1.5 \times 10^{-3}\,s^{-1}$); curve 4 is the theoretical prediction of Eq. (3) ($D = 8.6 \times 10^{-8}\,cm^2\,s^{-1}$; $K = 1.5 \times 10^{-3}\,s^{-1}$)

100 μl (curve 2). As has been shown in [13] for large amounts of spread liposomes (above a critical spreading volume of 1600 μl), the process is determined by the surface transformation 1 → 2. This process is not influenced by the diffusion of liposomes to the bulk, while for spreading volumes of 100 μl, kinetics are governed by both diffusion and transformation processes.

By way of illustration, experimental results for $(\Delta V/\Delta V_\infty)$ for 1600 μl (curve 1) and 100 μl (curve 2) liposome volumes are compared in the same Fig. 2 with the theoretical predictions from Eq. (2) (curve 3) and Eq. (3) (curve 4). The adsorption kinetic equation (2), rougly describes the transformation 1 → 2 controlled kinetics following experimental curve 1, and nevertheless predicts acceptable values for a half- and a full-saturation. The value obtained for the kinetic constant of surface transformation is $K = 1.5 \times 10^{-3}\,s^{-1}$. The theoretical prediction from Eq. (3) describing the general case, when both diffusion and transformation process 1 → 2 determine the rate of the phenomenon, is in qualitative agreement with the experimental curve 2. The values of $K = 1.5 \times 10^{-3}\,s^{-1}$ and $D = 8.6 \times 10^{-8}\,cm^2/s$, calculated from the classical Einstein formula are used.

Figure 3 shows typical results for the kinetics $\pi(t)$ and $\Delta V(t)$ after spreading of 100 μl methylene blue-free DOPC liposomal suspension ($C_{MB} = 0$) and the suspension, preincubated during 30 min with MB ($C_{MB} = 0.01$ mg/ml), non irradiated ($T = 0$) or irradiated over increasing time periods ($T = 5$, 6, 7 and 30 min). The curves obtained for irradiation times greater than 30 min ($T = 60$, 120 and 180 min) are practically identical (data not shown).

The control suspension, preincubated with MB and non-irradiated ($T = 0$), shows an acceleration effect on the spreading kinetics with respect to MB-free suspensions. This effect can be explained by a modification of the liposomal surface structure and composition as a result of adsorption of MB molecules. In fact, the ratio of MB molecules to liposomes is very large ($C_{MB}/C_0 \approx 10^2$ MB molecules per liposome). We may assume that after 30 min preincubation in the dark, an adsorption equilibrium is reached and a number of MB molecules is adsorbed on each of the liposomes. As a result of the adsorption process, the surface structure and composition of all liposomes are modified and the apparent rate of surface transformation K of liposomes into a mixed DOPC/MB surface film increases. By plotting $\ln(1 - n^*/C_0 d)$ as a function of $t^{1/2}$ in accordance with Eq. (4), after appropriate statistical procedure, we obtain from the slopes a value of $K = 2.5 \times 10^{-3}\,s^{-1}$ greater than $K = 1.5 \times 10^{-3}\,s^{-1}$ for MB-free suspension. A typical result for $T = 0$ is presented in Fig. 4. It can also be noted that the accelerated spreading kinetics may be party due to the adsorption of MB-free molecules at the air-water interface.

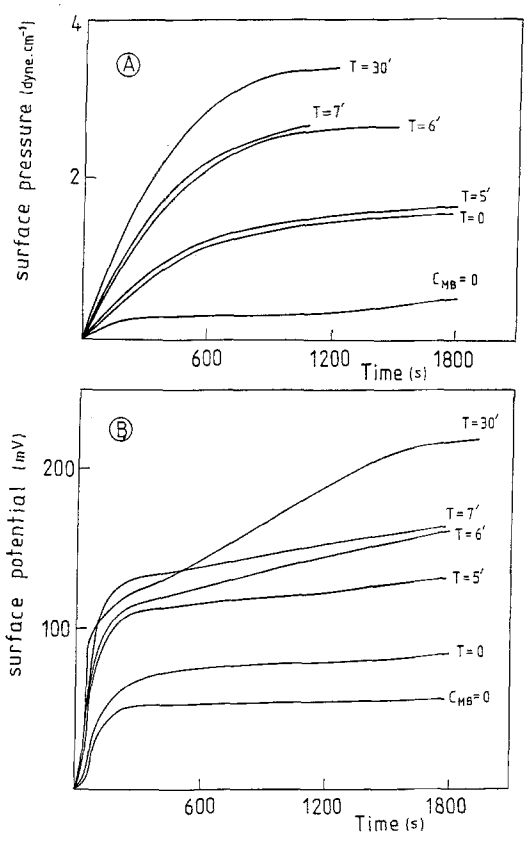

Fig. 3 Variation with time of surface pressure (π) (panel A) and surface potential (ΔV) (panel B) for spreading volume of 100 μl of MB-free liposomal suspension ($C_{MB} = 0$) or the same suspension preincubated during 30 min in the dark with MB ($C_{MB} = 0.01$ mg/ml f liposomal suspension), non-irradiated ($T = 0$) or irradiated with red light over various times ($T = 5$, 6, 7 and 30 min)

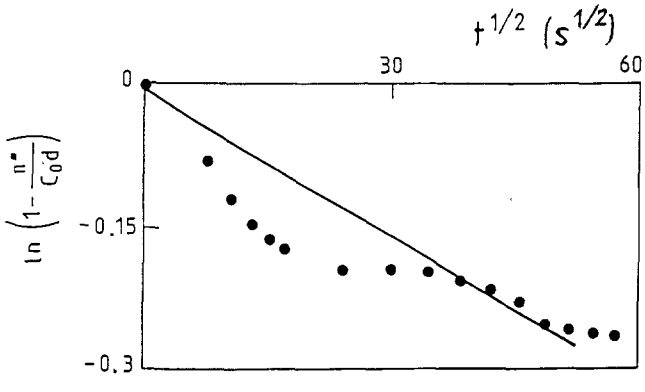

Fig. 4 Plot of $\ln(1 - n^*/C_0 d)$ as a function of $t^{1/2}$ for experimental data from Fig. 3B (curve $T = 0$)

As far as the photodynamic action on spreading kinetics of liposomes is concerned, the observed effect increases with the irradiation time T and a saturation at $T = 30$ min is reached. When the characteristic time τ is

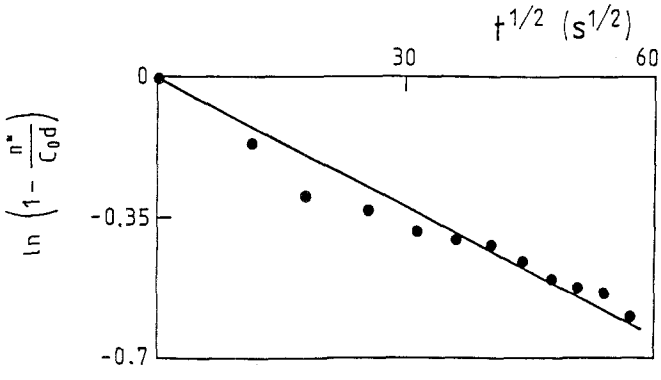

Fig. 5 Plot of $\ln(1 - n^*/C_0 d)$ as a function of $t^{1/2}$ for experimental data from Fig. 3B (curve $T = 6$ min)

smaller than $T = 30$ min, a value of the surface transformation rate constant of defected liposomes $\tilde{K} = (5.5 \pm 1.2) \times 10^{-3}\,s^{-1}$ can be obtained from the $\Delta V(t)$ curve of saturation at $T = 30$ min in accordance with Eq. (8).

The experimental results, obtained at $T = 5$, 6, and 7 min may be in principle described using the general kinetic equation (6). As a first approximation, we use the simplified Eq. (10). A typical result in scale $\ln(1 - n^*/C_0 d)$ as a function of $t^{1/2}$ obtained from $\Delta V(t)$ at $T = 6$ min is shown in Fig. 5. From the slope, an estimation of the characteristic time of the molecular reorganization process $\tau = (2.73 \pm 1.57) \times 10^2$ s is obtained. One must note certain significant dispersions in the experimental results. The above-mentioned values of $\tilde{K}$ and τ are mean values obtained after appropriate statistical procedures. Because of large dispersions in the experimental results, it is impossible to obtain accurate results for small irradiation times on the order of several seconds ($T \ll \tau$) or to verify Eq. (11).

The value obtained for the surface transformation rate constant $\tilde{K}$ of defected liposomes is 2.2 times larger than K of intact liposomes modified by MB.

The estimated value for τ also seems reasonable in light of the results obtained for the characteristic time for orientation processes in monolayer. In fact, the values of τ of a few hundred seconds for pentadecanoic acid, palmitic acid [17] and lipid monolayer [18, 19] are obtained.

In conclusion, the new approach developed in the present study gives additional information about the mechanism of alteration of liposomal bilayer structures as a result of the photodynamic lipid peroxidation. Our results suggest a photodynamic action on the liposomes over an irradiation time of 30 min. This result seems to be in agreement with the experimental observation of liposomal suspensions by light scattering, showing that the photodynamic action occurs over approximately 30 min Figs. 5 and 6 in [6]. The observed characteristic time of defect formation is related to a slow reorganization of reaction products in the liposomal lipid surrounding.

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