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Structural Characterization of Photopolymerizable Binary Liposomes Containing Diacetylenic and Saturated Phospholipids

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The use of liposomes to encapsulate materials has received widespread attention for drug delivery, transfection, diagnostic reagent, and as immunoadjuvants. Phospholipid polymers form a new class of biomaterials with many potential applications in medicine and research. Of interest are polymeric phospholipids containing a diacetylene moiety along their acyl chain since these kinds of lipids can be polymerized by Ultra-Violet (UV) irradiation to form chains of covalently linked lipids in the bilayer. In particular the diacetylenic phosphatidylcholine *1,2-bis*(*10,12-tricosadiynoyl*)-*sn-glycero-3-phosphocholine* (DC8,9PC) can form intermolecular cross-linking through the diacetylenic group to produce a conjugated polymer within the hydrocarbon region of the bilayer. As knowledge of liposome structures is certainly fundamental for system design improvement for new and better applications, this work focuses on the structural properties of polymerized DC8,9PC:*1,2-dimyristoyl-sn-glycero-3-phosphocholine* (DMPC) liposomes. Liposomes containing mixtures of DC8,9PC and DMPC, at different molar ratios, and exposed to different polymerization cycles, were studied through the analysis of the electron spin resonance (ESR) spectra of a spin label incorporated into the bilayer, and the calorimetric data obtained from differential scanning calorimetry (DSC) studies. Upon irradiation, if all lipids had been polymerized, no gel−fluid transition would be expected. However, even samples that went through 20 cycles of UV irradiation presented a DSC band, showing that around 80% of the DC8,9PC molecules were not polymerized. Both DSC and ESR indicated that the two different lipids scarcely mix at low temperatures, however few molecules of DMPC are present in DC8,9PC rich domains and *vice versa*. UV irradiation was found to affect the gel−fluid transition of both DMPC and DC8,9PC rich regions, indicating the presence of polymeric units of DC8,9PC in both areas. A model explaining lipids rearrangement is proposed for this partially polymerized system.

Introduction

Liposomes have been widely used for many applications, from membrane models to drug delivery systems.[1−6] Possible improvement in therapeutic benefits of liposomal encapsulated drugs depends on their lifetime and distribution within the organism, both factors related to drug delivery system stability. Long-term physicochemical stability, both *in vitro* and *in vivo*, of this kind of system is crucial. This topic has always been a matter of interest, and therefore many particulate vehicles of different compositions were obtained using different strategies and methodologies.[7−12]

Literature also showed that biologically active synthetic polymers have received considerable scientific interest and attention in recent years for their potential use as promising novel delivery systems. Significant amount of research has also been carried out involving polymer-linked drugs as targeted and sustained release drug delivery systems.[13,14]

Interest in polymeric lipids arose as an option to combine in one system both liposomes and polymer characteristics. This kind of lipids has been used in many applications from membrane models to drug delivery systems, vaccines carriers, biosensors or coating materials.[7,15−21] Lipid polymerization leads to a covalent union between lipids chains improving the noncovalent interaction that keeps lipid lamellar phase formed. Thus an important impact on the stability of the polymerized system can be obtained.[8,15,17,20−23]

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(1) Samad, A.; Sultana, Y.; Aqil, M. *Curr. Drug Delivery* **2007**, *4*, 297–305.
(2) Ulrich, A. S. *Biosci. Rep.* **2002**, *22*(2), 129–150.
(3) Sharma, A.; Sharma, U. S. *Intern. J. Pharm.* **1997**, *154*, 123–140.
(4) Lundahl, P.; Beigi, F. *Adv. Drug Delivery Rev.* **1997**, *23*, 221–227.
(5) Gregoriadis, G. *Trends Biotechnol.* **1995**, *13*, 527–537.
(6) Felnerova, D.; Viret, J.-F.; Glück, R.; Moser, C. *Curr. Opin. Biotechnol.* **2004**, *15*, 518–529.
(7) Alonso-Romanowski, S.; Chiaramoni, N. S.; Lioy, V. S.; Gargini, R. A.; Viera, L. I.; Taira, M. C. *Chem. Phys. Lipids* **2003**, *122*, 191–203.
(8) Fabani, M. M.; Gargini, R. A.; Taira, M. C.; Iacono, R.; Alonso-Romanowski, S. *J. Liposome Res.* **2002**, *12*, 13–27.
(9) Gadras, C.; Santaella, C.; Vierling, P. *J. Controlled Release* **1999**, *57*, 29–34.
(10) Takeuchi, H.; Yamamoto, H.; Toyoda, T.; Toyobuku, H.; Hino, T.; Kawashima, Y. *Int. J. Pharm.* **1998**, *164*, 103–111.
(11) Mohammed, A. R.; Weston, N.; Coombes, A. G. A.; Fitzgerald, M.; Perrie, Y. *Int. J. Pharm.* **2004**, *285*, 23–34.
(12) Noble, C. O.; Guo1, Z.; Hayes, M. E.; Marks, J. D.; Park, J. W.; Benz, C. C.; Kirpotin, D. B.; Drummond, D. C. *Cancer Chemother. Pharmacol.* **2009**, *64*, 741–751.

(13) Vicent, M. J.; Dieudonné, L.; Carbajo, R. J.; Pineda-Lucena, A. *Expert Opin. Drug Delivery* **2008**, *5*, 593–614.
(14) Dhal, P. K.; Polomoscanik, S. C.; Avila, L. Z.; Holmes-Farley, S. R.; Miller, R. J. *Adv. Drug Delivery Rev.* **2009**, *61*, 1121–1130.
(15) Hayward, J. A.; Johnston, D. S.; Chapman, D. *Ann. N. Y. Acad. Sci.* **1985**, *446*, 267–281.
(16) Markowitz, M. A.; Chow, G.-M.; Singh, A. *Langmuir* **1994**, *10*, 4095–4102.
(17) Clark, M. A.; Blair, H.; Liang, L.; Brey, R. N.; Brayden, D.; Hirst, B. H. *Vaccine.* **2001**, *20*, 208–217.
(18) Daly, S. M.; Heffernan, L. A.; Barger, W. R.; Shenoy, D. K. *Langmuir* **2006**, *22*, 1215–1222.
(19) Morigaki, K.; Schönherr, H.; Okazaki, T. *Langmuir* **2007**, *23*, 12254–12260.
(20) Subramaniam, V.; D´Ambruoso, G. D.; Hall, H. K., Jr.; Wysocki, R. J., Jr.; Brown, M. F.; Saavedra, S. *Langmuir* **2008**, *24*, 11067–11075.
(21) Ahl, P. L.; Price, R.; Smuda, J.; Gaber, B. P.; Singh, A. *Biochim. Biophys. Acta* **1990**, *1028*, 141–153.
(22) Blume, A. *Chem. Phys. Lipids* **1991**, *57*, 253–273.

In previous work Alonso-Romanowski et al.,[7] showed that polymerized liposomes made of (*1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine*) (DC8,9PC) and *1,2-dimyristoyl-sn-glycero-3-phosphocholine* (DMPC) had a higher stability toward the digestive tract (including saliva): acidic solutions, bile and pancreatin, measuring the release of glucose-6-phosphate or bovine serum albumin. Considering the data mentioned above, polymerization is related to rigidity that confers membrane enhanced stability, which is a key matter when designing, for example, a drug delivery system. In this sense, leakage reduction due to polymer conjugation in the membrane might be dependent on liposomal formulation and structure.[24] Not much is known about the miscibility of binary mixtures of polymerizable with saturated lipids or about the obtained membrane structure before and after polymerization; what is known is that each system behaves differently, showing particular properties.[22] Although there are discussions in the literature about the miscibility of polymerizable lipid mixtures and also about phase behavior of lipid mixtures involving diacetylenic lipids,[15,21-23,25-27] little is discussed about the system's structure after UV irradiation. Specifically, little information is available about the rearrangements the membrane components adopt after UV irradiation and where the formed polymers allocate within the membrane in a partially polymerized system.

In order to improve the understanding of this particular system we have structurally characterized polymerized and nonpolymerized DC8,9PC:DMPC membranes. For that purpose, samples with different DC8,9PC:DMPC molar ratios and irradiation cycles were studied in deep by differential scanning calorimetry (DSC) since lipid phase transitions monitored by DSC are extremely sensitive to lipid−lipid interaction and packing, hence to the presence of impurities in the lipid domain.[28] They were also studied by electron spin resonance (ESR) of spin labels incorporated in the bilayer which provides independent information about the structure of the two bilayer phases, gel and fluid.[29-31]

Materials and Methods

Materials. The phospholipids: *1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine* (DC8,9PC - Figure 1) and *1,2-dimyristoyl-sn-glycero-3-phosphocholine* (DMPC - Figure 1) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and Lipoid GmbH (Ludwigshafen, Germany), respectively. The spin label *16-doxyl-stearic acid* (16-SASL - Figure 1) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lipids were used without further purification. All other reagents were analytical grade and used without further purification. Milli-Q water was used throughout.

Liposome Preparation. DC8,9PC:DMPC lipids mixtures, 40 μmol total lipids were dissolved in chloroform, and the solvent was removed under vacuum and flashed with nitrogen to obtain
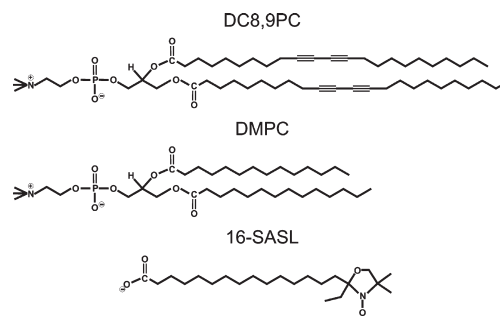

Figure 1. Chemical structure of the DC8,9PC and DMPC phospholipids, and 16-SASL spin label.

the lipid film. Liposomes were prepared according to Bangham et al.[32] Three different DC8,9PC:DMPC mixtures were prepared at [1:1], [1:0.5], and [1:0.25] molar ratios, suspended in buffer PBS (10 mM), and vortexed at 50 °C to obtain large multilamellar vesicles. Large unilamellar vesicles were obtained, at the same temperature, using a Mini Extruder from Avanti Polar Lipids with a 200 nm membrane pore.

A Stratalinker 1800 for cross-linking was used to irradiate the lipid samples in order to induce the diacetylenic extruded vesicles polymerization. For each irradiation cycle, a 254 nm UV light dose of 360 mJ/cm^2 was used during 71 s per cycle. Pure lipids and mixtures of them were prepared, and some of them exposed to 20 irradiation cycles. Furthermore, DC8,9PC:DMPC [1:1] lipid mixture was prepared and exposed to different numbers of irradiation cycles (5, 12, and 20). Temperature was maintained at 4 °C for 5 min in between cycles. The absence of absorbance at $\lambda \approx 610$ nm ensured there were vesicles and not tubules present in the suspension.[7,33]

Samples were freeze-dried overnight under reduced pressure (in the range of 33×10^{-3} to 65×10^{-3} mbar) in a LABCONCO lyophilizer (Kansas City, MO, U.S.A.) and stored at −20 °C until further use.

For DSC and ESR measurements, the freeze-dried samples were rehydrated, adding 10 mM PBS buffer, pH 7.4, up to the total lipid concentration of 10 mM.

For ESR measurements, lipid dispersions were added to dried films of 16-SASL (0.6 mol % of the total lipid concentration), heated up to 50 °C, and vortexed for 5 min, so spin labels would incorporate into the lipid bilayers.

DSC Measurements. Differential scanning calorimetry data were obtained with a MicroCal MC-2 instrument. Temperature was varied from 5 up to 55 °C, at a scan rate of 20 °C/h. Total lipid concentration was 10 mM for all samples. Baseline subtractions and peak integrals were done with the MicroCal Origin software provided by MicroCal. All DSC data were obtained in triplicate, the numerical values are average, and the uncertainties are standard deviations. When not shown, the uncertainties are smaller than the size of the symbols.

ESR Spectroscopy. ESR measurements at X band were performed with a Bruker EMX spectrometer. The sample temperature was controlled within 0.1 °C by a Bruker BVT-2000 variable-temperature device, and varied from 5 to 60 °C. To ensure thermal equilibrium, before each scan the sample was left at the desired temperature for at least 10 min. The ESR data were acquired immediately after sample preparation. Field-modulation amplitude of 1 G and microwave power of 10 mW were used.

All data shown are means of at least three experiments, and the uncertainties are the standard deviations. When not shown, the uncertainties are smaller than the size of the symbols.

In this work, 16-SASL was used, as this probe is very sensitive to bilayer packing due to the presence of the nitroxide group at the bilayer core, and it could be incorporated into DC8,9PC

(23) Freeman, F. J.; Hayward, J. A.; Chapman, D. *Biochim. Biophys. Acta* **1987**, *924*, 341–351.

(24) Calvagno, M. G.; Celia, C.; Paolino, D.; Cosco, D.; Iannone, M.; Castelli, F.; Doldo, P.; Frest, M. *Curr. Drug Delivery* **2007**, *4*, 89–101.

(25) Lopez, E.; ÓBrien, D. F.; Whitesides, T. H. *Biochim. Biophys. Acta* **1982**, *693*, 437–443.

(26) Armitage, B.; Bennett, D. E.; Lamparski, H. G.; O'Brien, D. F. *Adv. Polym. Sci.* **1996**, *126*, 53–84.

(27) Takeoka, S.; Sakai, H.; Ohno, H.; Tsuchida, E. *Macromolecules* **1991**, *24*, 1279–1283.

(28) Heimburg, T. *Biophys. J.* **2000**, *78*, 1154–1165.

(29) Marsh, D. *CRC Handbook of Lipid Bilayers*, 1st ed.; CRC press: Boston, 1990; Section II.

(30) Riske, K. A.; Amaral, L. Q.; Lamy, M. T. *Langmuir* **2009**, *25*, 10083–10091.

(31) Riske, K. A.; Barroso, R. P.; Vequi-Suplicy, C. C.; Germano, R.; Henriques, V. B.; Lamy, M. T. *Biochim. Biophys. Acta* **2009**, *1788*, 954–963.

(32) Bangham, A. D.; Standish, M. M.; Watkins, J. C. *J. Mol. Biol.* **1965**, *13*, 238–252.

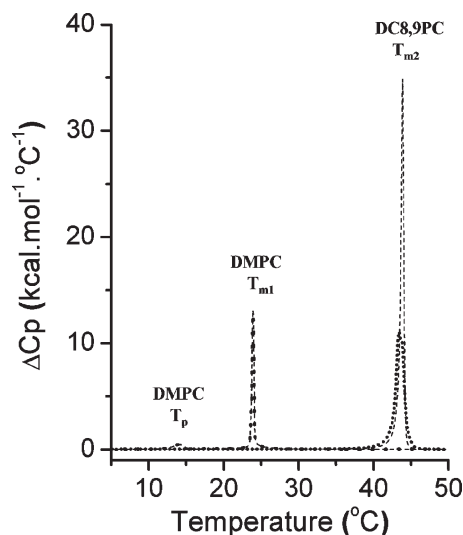(33) Svenson, S.; Messersmith, P. B. *Langmuir* **1999**, *15*, 4464–4471.

Figure 2. DSC heating scans of 10 mM DC8,9PC and DMPC dispersions in buffer, (- - - -) before and (· · · · ·) after 20 cycles of UV irradiation.

membranes (for instance, the phospholipid spin probe *1-palmitoyl-2-(16-doxylstearoyl)-sn-glycero-3- phosphocholine* (16-PCSL) could not be incorporated in DC8,9PC membranes). For the lipids in the gel phase, at low temperatures, the best parameter to be used is the direct measurement of the line width of the central field line, ΔH_0, this parameter being highly sensitive to chain order/mobility.[31] At high temperatures (35−60 °C), at the lipid fluid phase, the spin probe has a nearly isotropic movement, and rotational correlation times can be calculated from the peak-to-peak width of the ESR Lorentzian lines, according to the motional narrowing theory,:[34−36]

$$\Delta H_L(m) = A + Bm + Cm^2$$

where m is the mth component of the nitrogen nuclear spin ($m = 0$, 1, or −1), A is the Lorentzian line width of the central line ($\Delta H_L(0)$), and B and C are

$$B = \frac{1}{2}\Delta H_L(0)\left(\frac{\Delta H_L(+1)}{\Delta H_L(0)} - \frac{\Delta H_L(-1)}{\Delta H_L(0)}\right)$$

$$C = \frac{1}{2}\Delta H_L(0)\left(\frac{\Delta H_L(+1)}{\Delta H_L(0)} + \frac{\Delta H_L(-1)}{\Delta H_L(0)} - 2\right)$$

The correlation time for doxyl labels is either $\tau_B = -1.22B$ or $\tau_C = 1.19C$, ($\tau_B = \tau_C$ for isotropic movement). Lorentzian linewidths are calculated using a computer program, which performs nonlinear least-squares fitting of the experimental ESR spectrum using a model of a Lorentzian−Gaussian function for corrections for nonresolved hyperfine splitting.[37,38]

Results and Discussion

DSC Measurements. Figure 2 shows the DSC scans of pure DMPC and DC8,9PC dispersions, before and after 20 cycles of UV irradiation. They will be used here as control samples, to be compared with the mixed samples of DC8,9PC and DMPC. As expected, the well-known DSC profile of DMPC,[29,39] does not change with irradiation, presenting a pretransition around 13.8 °C, and a main gel−fluid transition at 23.8 °C. The thermal parameters for DMPC main gel−fluid transition are shown in Table 1: the transition temperature (T_{m1}), the transition enthalpy variation (ΔH_{m1}), and the half-maximum width of the transition peak ($\Delta T_{m1}^{1/2}$). The same parameters are listed in Table 1 for pure DC8,9PC bilayers, which do not exhibit a pretransition, and present a sharp thermal transition at 43.8 °C (T_{m2}). After irradiation, which is known to cause lipid polymerization,[7] the DC8,9PC transition temperature does not change much, but the transition was found to be less cooperative ($\Delta T_{m2}^{1/2}$ increases from 0.4 to 1.2 °C; see Figure 2 and Table 1).

Considering that polymeric units do not display a gel−fluid transition,[15,22] an estimation of the amount of DC8,9PC molecules involved in the polymeric units were obtained by:

$$\% \text{ of polymerized DC8,9PC molecules}$$
$$= [1 - (\Delta H_{m2 \text{ irradiated sample}}/\Delta H_{m2 \text{ nonirradiated sample}})]100$$

According to data in Table 1 the relatively small decrease found for DC8,9PC vesicles on the transition enthalpy after irradiation (from 21 to 17 kcal/mol, see Table 1), is indicative that only a small percentage of the lipids are polymerized after the sample irradiation procedure, around 20% of DC8,9PC molecules only. Hence, in DC8,9PC UV-irradiated bilayers there is a coexistence of large nonpolymerized lipid regions with small polymerized domains.

It has been previously described that addition of short phosphatidylcholines can improve polymerization efficiencies of diacetylenic lipids.[40] Ahl et al.,[21] worked with the same mixture used in this study but at a different ratio (DC8,9PC:DMPC [1:2]) and comparing polymerization efficiencies when *dinonanoylphosphatidylcholine* was added to the binary mixture instead of DMPC. Their results, with UV spectroscopy, showed that the best polymerization efficiency was achieved when using in the binary mixture *dinonanoylphosphatidylcholine* followed by DMPC and finally with DC8,9PC alone. Our findings showed no polymerization efficiency improvement of DC8,9PC when DMPC is added in a [1:1] molar ratio. On the contrary, DC8,9PC:DMPC [1:0.5] and [1:0.25] ratios significantly reduced the polymerization efficiency. Nonetheless, according to Alonso-Romanowski et al.,[7] the mixture of DC8,9PC and DMPC [1:1] molar ratio had an improved stability for oral vaccines. Particularly, polymerized DC8,9PC:DMPC [1:1] had a higher stability in saliva, buffer pH 2, bile, and pancreatin when compared to the same nonpolymerized formulation. Special interest in this mixture therefore arose to gain further insight into the structural characteristics of this system.

Hence, vesicles made of three different mixtures of DC8,9PC and DMPC were analyzed, before and after UV irradiation: DC8,9PC:DMPC molar ratios of 1.00:0.25, 1.00:0.50, and 1.00:1.00. These samples will be called here [1:0.25], [1:0.5], and [1:1], respectively. Their DSC profiles are shown in Figure 3, together with those of the controls, pure DMPC and DC8,9PC bilayers, before (Figure 3a) and after (Figure 3b) UV irradiation. First, it is interesting to point out that the DMPC pretransition disappears in the presence of DC8,9PC (Figure 3a and b). Bilayer structural changes related to this transition are still the subject of

(34) Hubbell, W. L.; McConnell, H. M. *J. Am. Chem. Soc.* **1971**, *93*, 314–326.
(35) Freed, J. H.; Fraenkel, G. K. *J. Chem. Phys.* **1963**, *39*, 326–348.
(36) Schreier, S.; Polnaszek, C. F.; Smith, I. C. *Biochim. Biophys. Acta* **1978**, *515*, 395–436.
(37) Bales, B. L. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum Publishing Corporation: New York, 1989; Vol. 8, p 77.
(38) Halpern, H. J.; Peric, M.; Yu, C.; Bales, B. L. *J. Magn. Reson.* **1993**, *103*, 13–22.

(39) Bayerl, T. M.; Köchy, T.; Bruckner, S. *Biophys. J.* **1990**, *57*, 675–680.
(40) Sing, A.; Gaber, B. P. In *Applied Bioactive Polymeric Materials*; Gebelein, C. G., Carraher, C. E., Foster, V. R., Eds.; Plenum Press: New York, 1988; p 239.

Table 1. Thermal Parameters Calculated from Figures 2 and 3, for Pure DC8,9PC and DMPC Dispersions, and Mixtures of DC8,9PC:DMPC: Transition Temperatures (T_{m1} and T_{m2}), Half Maximum Width of the Transition Peaks ($\Delta T_{m1}^{1/2}$, $\Delta T_{m2}^{1/2}$) and Enthalpy Variations (ΔH_{m1} and ΔH_{m2})[a]

DC8,9PC:DMPC [molar ratio]	T_{m1} (°C)	$\Delta T_{m1}^{1/2}$ (°C)	ΔH_{m1} (kcal/mol of DMPC)	T_{m2} (°C)	$\Delta T_{m2}^{1/2}$ (°C)	ΔH_{m2} (kcal/mol of DC8,9PC)
[1:0] nonirrad.	-	-	-	43.8 ± 0.1	0.4 ± 0.1	21 ± 1
[1:0] irrad.	-	-	-	43.2 ± 0.3	1.2 ± 0.1	17 ± 2
[1:0.25] nonirrad.	23.0 ± 0.3	1.9 ± 0.2	6 ± 1	42.3 ± 0.1	1.6 ± 0.1	23 ± 1
[1:0.25] irrad.	22.4 ± 0.3	5.5 ± 0.5	7 ± 1	41.5 ± 0.1	3.0 ± 0.1	21 ± 1
[1:0.5] nonirrad.	23.0 ± 0.2	1.1 ± 0.1	6 ± 1	41.3 ± 0.1	2.5 ± 0.1	24 ± 1
[1:0.5] irrad.	22.7 ± 0.5	1.8 ± 0.5	6 ± 1	40.3 ± 0.1	4.2 ± 0.1	24 ± 1
[1:1] nonirrad.	22.8 ± 0.1	1.1 ± 0.1	5 ± 1	39.2 ± 0.1	3.3 ± 0.1	22 ± 1
[1:1] irrad.	22.5 ± 0.1	3.5 ± 0.1	4 ± 1	38.9 ± 0.3	7.0 ± 0.3	18 ± 1
[0:1] nonirrad.	23.9 ± 0.1	0.3 ± 0.1	5 ± 1	-	-	-
[0:1] irrad.	23.8 ± 0.1	0.3 ± 0.1	5 ± 1	-	-	-

[a] T_{m1}, $\Delta T_{m1}^{1/2}$, and ΔH_{m1} refer to DMPC or DMPC-rich regions, and T_{m2}, $\Delta T_{m2}^{1/2}$, and ΔH_{m2} refer to DC8,9PC or DC8,9PC-rich regions.

discussion in the literature,[28,31,41] and are out of the scope of the present paper.

It is remarkable that all samples maintain both the DMPC and the DC8,9PC gel−fluid transition temperature (T_{m1} and T_{m2}), indicating the presence of DMPC and DC8,9PC-rich domains. The considerable broadening of the half-maximum widths of the transition peaks ($\Delta T_{m1}^{1/2}$ and $\Delta T_{m2}^{1/2}$) in the mixtures, as compared to pure DMPC or DC8,9PC bilayers (Table 1), indicates the presence of the other lipid as an impurity in large DMPC or DC8,9PC domains. The shift of the transition temperatures to lower values (see T_{m1} and T_{m2} in Table 1) is in line with the coexistence of domains rich in DMPC and DC8,9PC, but containing small amounts of the other lipid as an impurity. This is different from what is observed, for instance, with mixtures of DMPC and *distearoylphosphatidylcholine* (DSPC): the presence of DSPC, which has a higher T_m, stabilizes the DMPC bilayer, increasing its main transition temperature.[42]

Irradiation leads to even broader phase transitions to all lipid mixtures studied. As pure DMPC is not affected by irradiation, polymer units are more likely distributed disaggregated throughout the membrane. This indicates the presence of polymerized DC8,9PC units in both DC8,9PC and DMPC-rich domains. On the other hand, the decrease on T_{m2} and the increase on $\Delta T_{m2}^{1/2}$, from [1:0.25] to [1:1] (Table 1) clearly indicates the increasing presence of DMPC molecules in DC8,9PC-rich regions as the relative DMPC concentration increases.

Though the DMPC gel−fluid transition is strongly disturbed by the presence of polymerized DC8,9PC (Figure 3b), the enthalpy (ΔH_{m1}) of the DMPC main transition is not much affected, either before or after irradiation (5 ± 1 kcal/mol, Table 1). Similarly, before irradiation, although the presence of DMPC molecules affects the position and the form of nonirradiated DC8,9PC main transition (Figure 3a and Table 1), the enthalpy of DC8,9PC transition (ΔH_{m2}) is not much affected (22 ± 1 kcal/mol, Table 1). However, after irradiation, a decrease in

ΔH_{m2} is observed. Interestingly, both pure DC8,9PC and DC8,9PC:DMPC [1:1] samples have a polymerization efficiency of around 20%, calculated from Table 1, as discussed above.

For a better analysis of the irradiation procedure used, DSC traces of [1:1] samples which underwent different UV irradiation cycles (5, 12, and 20 cycles) were obtained (Figure 4 and Table 2). Clearly, both DMPC and DC8,9PC main transitions are sensitive to the number of UV irradiation cycles (See Figure 4). As seen in Table 2, for DMPC-rich domains, T_{m1} does not change much, but $\Delta T_{m1}^{1/2}$ increases with the number of cycles, indicating the presence of increasing polymerized DC8,9PC units. The two calorimetric parameters of the DC8,9PC gel−fluid transition, $\Delta T_{m2}^{1/2}$ and ΔH_{m2}, indicate that the lipid is getting more polymerized as the number of cycles increases: $\Delta T_{m2}^{1/2}$ increases, and ΔH_{m2} decreases.

ESR Measurements. The structure of the samples studied by DSC, irradiated and nonirradiated DMPC and DC8,9PC and the mixtures of DC8,9PC:DMPC, [1:0.25], [1:0.5], and [1:1], were analyzed via the ESR signal of a stearic acid spin-labeled at the 16th carbon atom, 16-SASL, incorporated in them. This spin probe was chosen because it is rather sensitive to bilayer packing and order, as it labels the bilayer core.[43,44]

Figure 5 shows the ESR spectra of 16-SASL incorporated in samples of pure DMPC and DC8,9PC, before and after 20 cycles of UV irradiation, at three different temperatures. The spectra are normalized by the maximum signal amplitude. If we first focus on the spectra at 5 °C, where DMPC and DC8,9PC are at the gel phase, for both lipids the spectra indicate the presence of two ESR signals, one rather broad, corresponding to the spin label incorporated in a gel bilayer, and another one very narrow (three narrow lines), due to the probe "free" in aqueous medium. This result indicates that the spin label is partitioned between the membrane and the bulk aqueous medium. However, whereas in DMPC the intensity of the "free" signal is rather low (see arrows in Figure 5, for the low- and high-field features of the signal), in

(41) Mannock, D. A.; Lee, M. Y. T.; Lewis, R. N. A. H.; McElhaney, R. N. *Biochim. Biophys. Acta* **2008**, *1778*, 2191–2202.
(42) Heimburg, T. *Thermal Biophysics of Membranes*, 1st ed.; Wiley-VCH - Verlag GmbH & Co. KGaA: Weinheim, 2007; Chapter 7.

(43) Griffith, O. H.; Jost, P. C. In *Spin Labelling. Theory and Applications*; Berliner, L. J., Ed.; Academic Press: New York, 1976; Vol. *1*, p 453.
(44) Benatti, C. R.; Feitosa, E.; Fernandez, R. M.; Lamy-Freund, M. T. *Chem. Phys. Lipids* **2001**, *111*, 93–104.
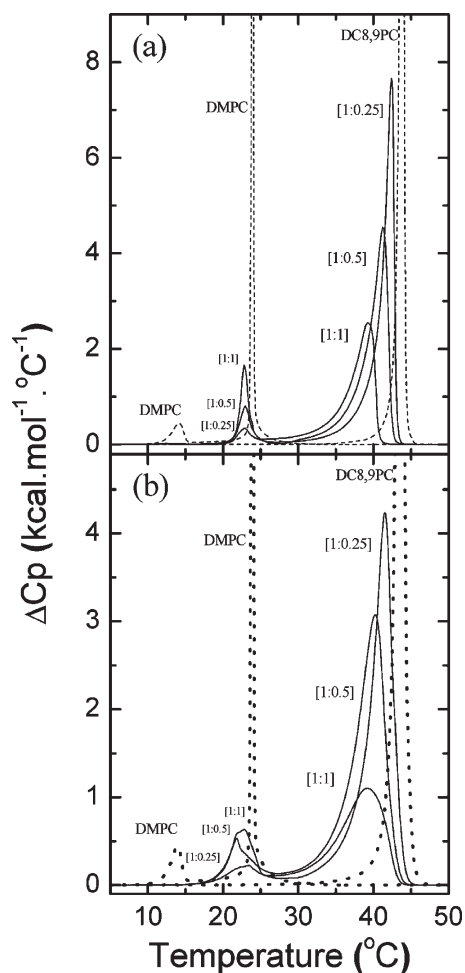
Figure 3. DSC heating scans of mixtures of DC8,9PC:DMPC dispersions in buffer (10 mM total lipid). The different molar ratios are indicated. Results (reproduced from Figure 2) of pure 10 mM DC8,9PC and DMPC dispersions are shown for comparison: (a) before and (b) after 20 cycles of UV irradiation.
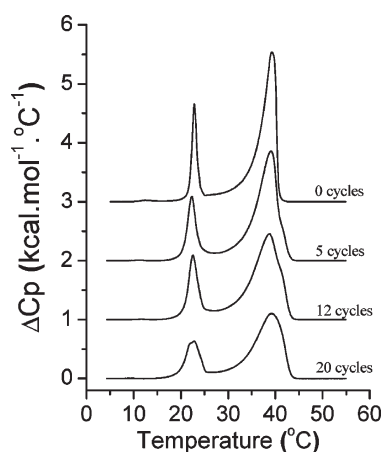

Figure 4. DSC heating scans of a molar mixture [1:1] of DC8,9PC: DMPC dispersion in buffer (10 mM total lipid) after different cycles of UV irradiation, as indicated. The traces are shifted for clarity.

DC8,9PC the "free" signal is very intense, at 5 and 30 °C where DC8,9PC bilayers are in the gel phase.

To analyze such composite spectra it is necessary to subtract the "free" signal from the composite one, making possible the

study of the ESR signal due to the spin label incorporated in the membrane. This procedure is shown in Figure 6; Figure 6a reproduces the composite spectra of 16-SASL incorporated in nonirradiated DC8,9PC at 30 °C. Figure 6b shows the "free" signal, yielded by 16-SASL in buffer, and Figure 6c shows the spectra resulting from the subtraction of the "free" signal (b) from the composite one (a). The free signal (b) is subtracted from the composite experimental signal (a) until the latter does not present the narrow "free" signal features. Normalizing the two ESR spectra ((a) and (b) in Figure 6) for the area of their second integral (proportional to the spin-label concentration), it is possible to calculate the percentage of "free" label for each experimental ESR spectrum. As indicated by the experimental spectra (Figure 5), 16-SASL partitions well into gel DMPC bilayers, and only ∼2 mol % was found to be "free" in solution. However, around 25 mol % of 16-SASL was found to be "free" in solution in the presence of DC8,9PC gel bilayers, hence not incorporated in the membrane, either before or after irradiation. Moreover, the resulting ESR signal (Figure 6c) is distorted by spin exchange, indicating spin−spin interaction in the membrane.[45] As the spin label concentration is rather low, compared to the total lipid concentration, 0.6 mol % only, this is a clear indication that 16-SASL is not uniformly distributed in the gel membrane, but is mostly located in spin-label-rich regions. This is completely different from what was found for DMPC in the gel phase, or for most of the gel membranes studied so far,[44,46] and is certainly related to the highly rigid membrane formed by DC8,9PC in the gel phase, either polymerized or not. (A phospholipid, spin-labeled at the 16th carbon atom, 16-PCSL, was also tried, but it was found to be even less incorporated in DC8,9PC bilayers at low temperatures.) For fluid DMPC (above 25 °C), or DC8,9PC (above 45 °C), 16-SASL is totally incorporated in the bilayer (see Figure 5), and an ESR signal typical of fluid membrane is observed.

Figure 7 presents the spectra of Figure 5 corrected by the subtraction procedure described above (Figure 6). As known for DMPC, the gel and fluid lipid phases are well characterized by a rather anisotropic and a much more isotropic ESR signal, respectively.

As discussed above, the gel phase of DC8,9PC cannot be properly analyzed, as all obtained spectra indicate spin−spin interaction (see Figure 7). However, for higher temperatures, the packing of fluid DMPC and DC8,9PC can be compared by the rotational correlation time of 16-SASL incorporated in the bilayers. As discussed in Material and Methods, two correlation times were calculated, τ_B and τ_C, and were found to be rather similar for temperatures above 35 °C, indicating a nearly isotropic movement for the probe in these bilayers.[47] Figure 8 shows the values of τ_C obtained for DMPC and DC8,9PC, before and after irradiation. As expected, irradiation does not change DMPC bilayer packing (similar τ_C values), but it was interesting to find that 16-SASL presents similar correlation time both in irradiated and nonirradiated DC8,9PC membrane. Considering that the polymerized membranes were shown to be more packed than nonpolymerized ones,[7] this result strongly suggests that 16-SASL incorporates in nonpolymerized lipid regions only. This is probably related to the high percentage of nonpolymerized lipids in the irradiated sample (around 80% according to DSC traces,

(45) Jost, P. C.; Griffith, O. H. In *Spin Labelling. Theory and Applications*; Berliner, L. J., Ed.; Academic Press: New York, 1976; Vol. *1*, p 251.

(46) Turchiello, R. F.; Juliano, L.; Ito, A. S.; Lamy-Freund, M. T. *Biopolymers* **2000**, *54*, 211–221.

(47) Marsh, D. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum Publishing Corporation: New York, 1989; Vol. *8*, p 255.

Table 2. Thermal Parameters Calculated from Figure 3, for a Molar Mixture [1:1] of DC8,9PC:DMPC: Transition Temperatures (T_{m1} and T_{m2}), Half Maximum Width of the Transition Peaks ($\Delta T_{m1}^{1/2}$, $\Delta T_{m2}^{1/2}$), and Enthalpy Variations (ΔH_{m1} and ΔH_{m2})[a]

Number of irradiation cycles	T_{m1} (°C)	$\Delta T_{m1}^{1/2}$ (°C)	ΔH_{m1} (kcal/mol of DMPC)	T_{m2} (°C)	$\Delta T_{m2}^{1/2}$ (°C)	ΔH_{m2} (kcal/mol of DC8,9PC)
0	22.8 ± 0.1	1.1 ± 0.1	5 ± 1	39.2 ± 0.1	3.3 ± 0.1	22 ± 1
5	22.6 ± 0.3	2.1 ± 0.1	6 ± 1	38.8 ± 0.3	4.3 ± 0.3	20 ± 2
12	22.5 ± 0.1	2.5 ± 0.1	6 ± 1	38.5 ± 0.2	6.6 ± 0.1	21 ± 1
20	22.5 ± 0.1	3.5 ± 0.1	4 ± 1	38.9 ± 0.3	7.0 ± 0.3	18 ± 1

[a] T_{m1}, $\Delta T_{m1}^{1/2}$, and ΔH_{m1} refer to DMPC-rich regions, and T_{m2}, $\Delta T_{m2}^{1/2}$, and ΔH_{m2} refer to DC8,9PC-rich regions.
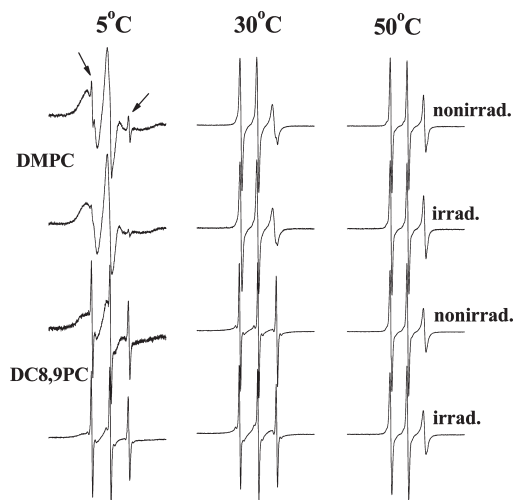
Figure 5. X-band ESR spectra of 16-SASL in DMPC and DC8,9PC dispersions (10 mM), before (nonirrad.) and after (irrad.) 20 cycles of UV irradiation, at different temperatures. The arrows indicate the low- and high-field peaks corresponding to the ESR signal of 16-SASL "free" in solution. Total spectra width 100 G. Spectra are normalized by the maximum signal amplitude.

as discussed above), and the high rigidity of polymer units. The higher τ_C values obtained for 16-SASL incorporated in fluid DC8,9PC membranes (Figure 8) indicate that they are more tightly packed than fluid DMPC bilayers at the same temperature.

Figure 9 presents the ESR spectra (normalized by the maximum signal amplitude) of 16-SASL incorporated in the three different mixtures of DC8,9PC:DMPC studied by DSC, [1:0.25], [1:0.5], and [1:1], before and after 20 cycles of UV irradiation, at three different temperatures.

The ESR signals are presented after the subtraction of the "free" signal, as discussed above (Figure 5). Different from pure DC8,9PC, and similar to pure DMPC, the percentage of "free" signal was found to be rather small (around 3%) for all mixed samples in the gel phase. Moreover, if one compares the ESR signal yielded by 16-SASL incorporated in the mixtures at 5 °C (left column in Figure 9) with those of 16-SASL in pure DMPC bilayers (left column, first and second spectra in Figure 7), it can be seen that they are strikingly similar. This is in accord with the coexistence of DMPC and DC8,9PC-rich regions in the mixture of lipids, in the gel phase, as indicated by DSC data (Figure 3). Moreover, in accord with the discussion made above, relative to Figure 7, it was pointed out that 16-SASL is hardly incorporated
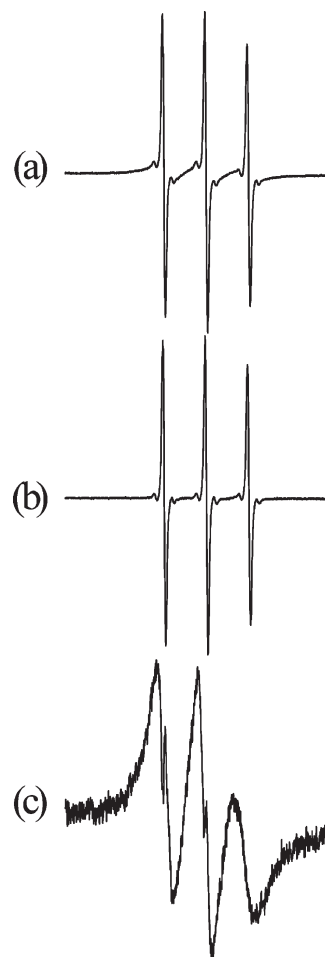

Figure 6. X-band ESR spectra of (a) 16-SASL incorporated in 10 mM DC8,9PC nonirradiated dispersion, at 30 °C, (b) 16-SASL "free" in buffer, at 30 °C. (c) Subtraction of the "free" signal (b) from the composite spectra (a), corresponding to 65% of (a). Spectra are shown normalized by the maximum signal amplitude.

in gel DC8,9PC bilayers. Hence, in mixtures of DC8,9PC and DMPC, at low temperatures (below DC8,9PC gel−fluid T_m), 16-SASL monitors DMPC-rich regions only.

The gel-phase ESR signals can be compared by the central field line width, ΔH_0 (see Figure 9), which gets smaller as the microenvironment monitored by the spin label gets less packed.[34] As expected, ΔH_0 decreases as temperature increases (Figure 10). ΔH_0 values measured on the spectra yielded by 16-SASL in the gel phase of pure DMPC are slightly larger than those measured with the label incorporated in the mixtures (Figure 10), indicating
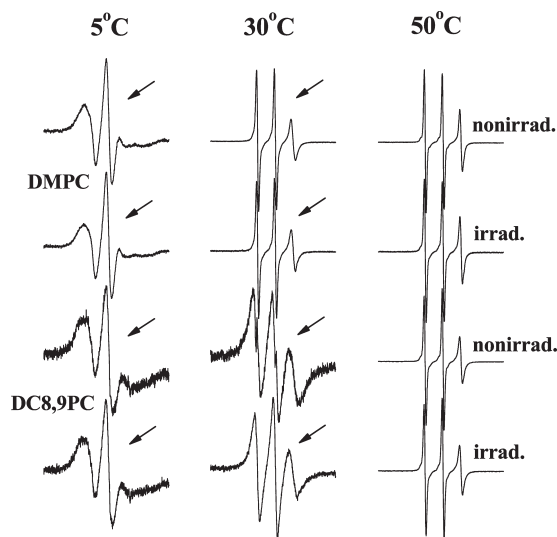
Figure 7. Spectra indicated by arrows are the ESR spectra shown in Figure 5, after the subtracting procedure described in Figure 6.
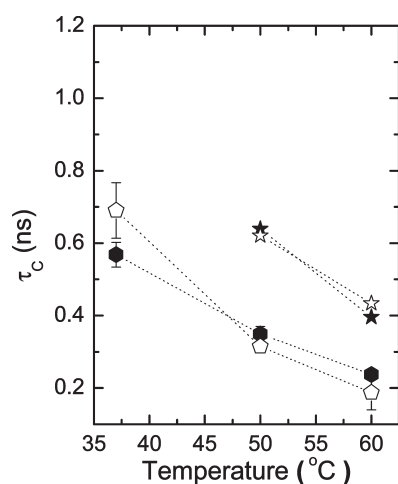

Figure 8. Temperature dependence of the rotational correlation time (τ_C) of 16-SASL incorporated in fluid DMPC (pentagon) and DC8,9PC (star) bilayers (spectra in Figures 5 or 7). Full and open symbols correspond to nonirradiated and irradiated samples, respectively.

the presence of impurities (DC8,9PC molecules) in the DMPC-rich domains, making them less packed than those of pure DMPC. This is entirely in accord with the shift to lower temperatures and the broadening of the DMPC gel–fluid transition monitored by DSC, observed for the DMPC: DC8,9PC mixtures (Figure 3). It is interesting to point out that a stronger effect was observed for the irradiated [1:1] mixture (Figure 10b) which correlates perfectly with the 18% of polymerized DC8,9PC molecules, calculated from de DSC data for the DMPC:DC8,9PC [1:1] mixtures.

As discussed before, the fluid phase of the lipids can be well analyzed by the calculation of the rotational correlation time of 16-SASL incorporated in the bilayers. Correlation times obtained with irradiated and nonirradiated mixtures are rather similar (Figure 11), and they were found to be in between the values obtained with pure DMPC and DC8,9PC (values of Figure 8 are repeated in Figure 11 for comparison). Hence, either DMPC and DC8,9PC get mixed up when both lipids are in the fluid phase, or the spin label moves relative quickly between DMPC and
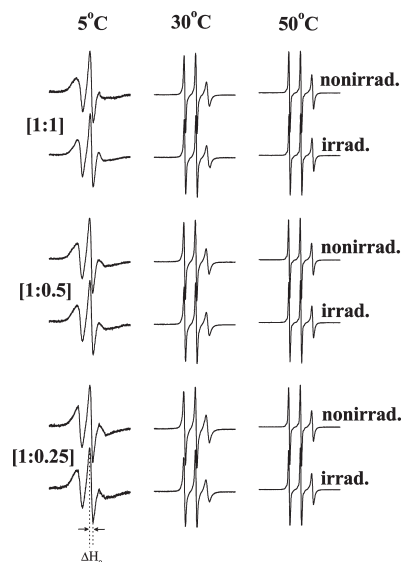

Figure 9. X-band ESR spectra of 16-SASL in three different molar mixtures of DC8,9PC:DMPC, [1:0.25], [1:0.5], and [1:1], before and after 20 cycles of UV irradiation, at three different temperatures. Total lipid concentration was 10 mM. Total spectra width 100 G. Spectra are normalized by the maximum signal amplitude and, when necessary, were subtracted from the signal of 16-SASL "free" in solution.
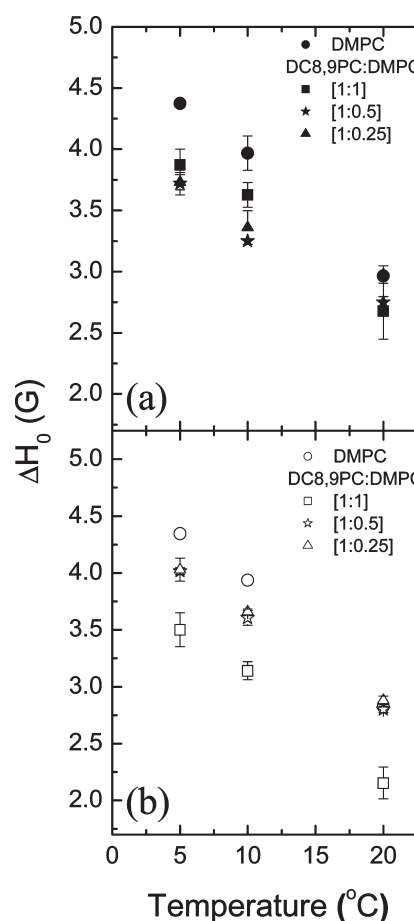

Figure 10. Temperature dependence of the central field line width, ΔH_0, directly measured on the ESR spectra (Figure 9) of 16-SASL incorporated in DC8,9PC:DMPC molar mixtures [1:0.25], [1:0.5], and [1:1], before (a) and after (b) 20 cycles of UV irradiation.
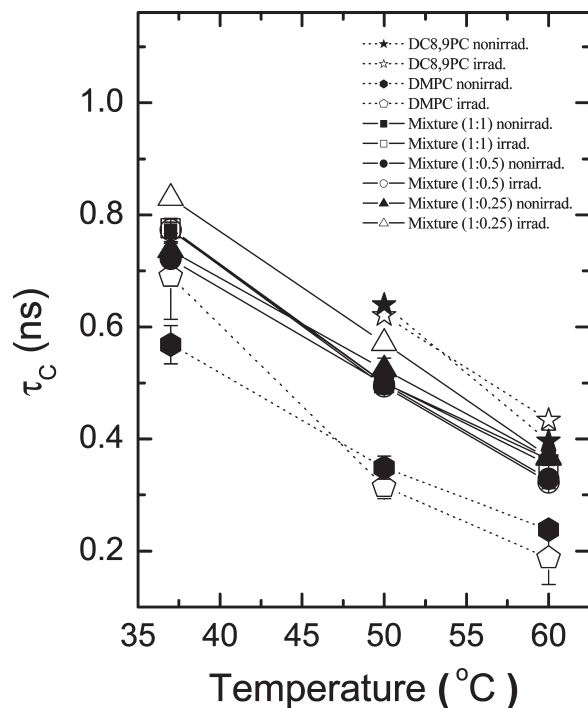
Figure 11. Temperature dependence of rotational correlation times (τ_C) of 16-SASL incorporated in DC8,9PC:DMPC molar mixtures [1:0.25], [1:0.5], and [1:1], before and after 20 cycles of UV irradiation (spectra in Figure 9).
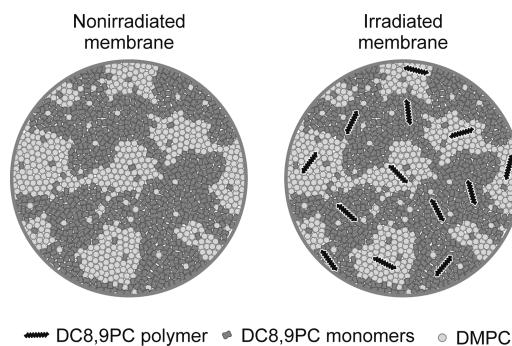

Figure 12. Proposed model to describe the membranes of the mixtures in the gel phase.

DC8,9PC-rich domains (compared to the microwave frequency), yielding an average ESR signal.

The same analysis was performed for the DC8,9PC:DMPC mixture [1:1] with samples which underwent different numbers of UV irradiation cycles (5, 12, and 20 cycles). Similar to the DSC result (Figure 4), 16-SASL incorporated in the [1:1] sample after 20 cycles of irradiation was found to be the most affected, yielding the lower ΔH_0 value for lower temperatures, though the calculated correlation times for the three samples in the fluid phase were found to be rather similar (results not shown).

Conclusions

We have studied in detail mixtures of the photopolymerizable lipid DC8,9PC with the saturated lipid DMPC, prepared at different lipid ratios, and some subjected to different irradiation cycles in order to study the membrane structure, specially after polymerization. Specifically, it was important to elucidate the rearrangements

membrane components adopt after irradiation, and where the formed polymeric units were allocated within the membrane.

As expected, our findings show that DMPC is not affected by UV irradiation. Moreover, DSC clearly indicate that most of DC8,9PC molecules are not affected by the UV irradiation procedure used here, and only a maximum of 20% of the monomers are polymerized after 20 irradiation cycles in the DC8,9PC and DC8,9PC:DMPC [1:1] mixture. Besides, no further polymerization can be achieved after 20 irradiation cycles, according to results obtained previously,[7] and it has also been suggested that polymer degradation occurs upon excessive UV irradiation.[18,48,49] Regarding this issue, our findings and those obtained by Ahl[21] conveyed that, for polymerization efficiency, not only the nature of the lipids is important but also the ratio used in the mixtures.

ESR structural studies indicate that in the case of DC8,9PC, the gel phase is found to be rather rigidly packed, not quite allowing the insertion of a spin-labeled stearic acid, and its fluid phase is more rigid than that of DMPC.

Both DSC and the ESR signal of 16-SASL incorporated in the bilayers clearly indicate that the two different lipids scarcely mix at low temperatures, when both DMPC and DC8,9PC are in the gel phase. Data obtained in the present work indicate the presence of few molecules of DMPC in DC8,9PC-rich domains, and *vice versa*, enough to act as impurities, decreasing the lipid gel−fluid temperature transition, broadening the transition, and changing the 16-SASL ESR signal. At high temperatures, where both lipids are in the fluid phase, they seem to be blended.

Finally, it has been revealed in this work that the binary system DC8,9PC:DMPC should be considered as a ternary one after UV irradiation, conformed by DMPC and monomeric and polymeric units of DC8,9PC. As UV irradiation was found to affect the gel−fluid transition of both DMPC and DC8,9PC-rich regions, polymeric units of DC8,9PC are present in both areas. Derived from these results, a proposed model for membranes of DC8,9PC:DMPC mixture [1:1], before and after UV irradiation in the gel state, can be seen in Figure 12. In this working model DC8,9PC polymers represent most likely small segregated domains, that can be found in DMPC and DC8,9PC-rich regions.

Data reported previously by Alonso-Romanowski et al.,[7] showed an improved stability at 37 °C in saliva, buffer of pH 2, bile, and pancreatin for polymerized (irradiated) DC8,9PC:DMPC [1:1] vesicles compared to the nonpolymerized (nonirradiated), despite the low amount of polymeric units formed after irradiation (around 20% of the DC8,9PC molecules). Thus, since the regions rearrangement adopted by lipids in the membrane seem not to change after UV irradiation, the mere presence of polymeric units in both DMPC and DC8,9PC-rich domains is responsible for the improved stability observed in the irradiated system.

To the best of our knowledge, this is the first thorough study on a binary system converted into a complex, partially polymerized ternary one with the assistance of UV irradiation. This strategy may open a new discussion angle for complex versatile systems with partial miscibility assembly of monomers of different saturated and nonsaturated and polymeric units rendering more stable membranes. We hope present discussions on structural characteristics and system understanding will have implications in promoting usage of mixtures containing polymerizable lipids when designing new systems with potential applications as carriers, microarrays, membrane-based microbiosensors,

(48) Alekseev, A. S.; Vitala, T.; Domnin, I. N.; Koshkina, I. M.; Nikitenko, A. A.; Peltonen, J. *Langmuir* **2000**, *16*, 3337–3344.
(49) Ogawa, K. *J. Phys. Chem.* **1989**, *93*, 5305–5310.

reconstituted transmembrane proteins assemblies, together with biophysical theoretical studies.