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RESEARCH ARTICLE

Ultraviolet irradiation of diacetylenic liposomes as a strategy to improve size stability and to alter protein binding without cytotoxicity enhancement

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Abstract

Membrane-modification effects, induced by ultraviolet (UV) irradiation in diacetylenic liposomes, were analyzed upon contact with cells, biological membranes, and proteins. Liposomes formulated with mixtures of unsaturated 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine and saturated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, in a 1:1 molar ratio, were compared with those that were UV-irradiated and analyzed in several aspects. Membrane polymerization inherence on size stability was studied as well as its impact on mitochondrial and microsomal membrane peroxidation induction, hemolytic activity, and cell viability. Moreover, in order to gain insight about the possible irradiation effect on interfacial membrane properties, interaction with bovine serum albumin (BSA), lysozyme (Lyso), and apolipoprotein (apoA-I) was studied. Improved size stability was found for polymerized liposomes after a period of 30 days at 4°C. In addition, membrane irradiation had no marked effect on cell viability, hemolysis, or induction of microsomal and mitochondrial membrane peroxidation. Interfacial membrane characteristics were found to be altered after polymerization, since a differential protein binding for polymerized or nonpolymerized membranes was observed for BSA and Lyso, but not for apoA-I. The substantial contribution of this work is the finding that even when maintaining the same lipid composition, changes induced by UV irradiation are sufficient to increase size stability and establish differences in protein binding, in particular, reducing the amount of bound Lyso and BSA, without increasing formulation cytotoxicity. This work aimed at showing that the usage of diacetylenic lipids and UV modification of membrane interfacial properties should be strategies to be taken into consideration when designing new delivery systems.

Keywords: *Diacetylenic lipids; lipid peroxidation; drug delivery; cytotoxicity; lipid-protein binding*

Introduction

Liposomes are one of the most extensively studied particulate drug delivery systems. They have been used as targeted delivery and controlled release systems, particularly to avoid fluctuations in plasma drug levels and, in recent years, to overcome cellular barriers and enzymatic degradation. These systems have generated a great interest due to their versatility, in terms of fluidity, size, charge, and number of lamellae, and also their ability to incorporate both lipophilic and lipophobic compounds (Rawat et al., 2008; Kshirsagar et al., 2005; Mozafari, 2005; Felnerova et al., 2004; Moses et al., 2003; Sharma and Sharma, 1997; Gregoriadis, 1995; Riaz, 1995; Poste et al., 1984).

The *in vivo* application of liposomes as a drug delivery system is conditioned mainly by the system-releasing properties, rapid removal from circulation, and system stability, particularly due to membrane rupture by the interaction with serum components. One possible approach to avoid this problem is the use of polymerizable lipids, which have been shown to improve membrane stability after polymerization

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(Blume, 1991; Ahl et al., 1990; Freeman et al., 1987; Hayward et al., 1985).

Diacetylenic lipids are part of the family of polymerizable lipids that can be polymerized by ultraviolet (UV) irradiation to form chains of covalently linked lipids in the bilayers (O'Brien et al., 1998; Ahl et al., 1990; Freeman et al., 1987; Lopez et al., 1982; Regen et al., 1982). They can aggregate into a variety of vesicular and nonvesicular assemblies, depending on lipid concentration, structure, and processing conditions (Svenson and Messersmith, 1999; Spector et al., 1998; Yager et al., 1988; Georger et al., 1987).

Polymerizable lipids can be useful to form membranes with enhanced physical and chemical stability, and their potential applications have been widely studied, in terms of drug encapsulation, model membranes, biosensors, and vaccines (Guo et al., 2010; Subramaniam et al., 2008; Daly et al., 2006; Alonso-Romanowski et al., 2003; Fabani et al., 2002; Jelinek and Kolusheva, 2001; Chen et al., 1996a, 1996b; Okada et al., 1995; Rhodes et al., 1994; Hayward and Chapman, 1984). Alonso-Romanowski et al. (2003) showed that membranes composed with the polymerizable lipid, 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$), and the saturated lipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), are more stable in buffer and plasma after polymerization, when compared with nonpolymerized ones. Thus, UV irradiation, in this mixture, reduces leakage, increases rigidity, and contributes to the hydrophilicity-hydrophobicity balance, according to the lipid composition, when compared with the nonirradiated one (Alonso-Romanowski et al., 2003). Recently, Guo et al. (2010) proposed a novel anticancer drug carrier, based on the mixture of the polymerizable lipids, 10,12-pentacosadiynoic acid and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, which also showed enhanced stability after polymerization.

As discussed above, formulating liposomes containing polymerizable diacetylenic lipids would help to obtain higher system stability after polymerization. However, to our knowledge, there have been no reports about the incidence of UV irradiation in the development of formulation toxicity as well as its effects on liposome size and protein binding. Thus the main aim of this study is to analyze the effects of UV irradiation on DMPC:$DC_{8,9}PC$ 1:1 liposomes relative to size stability, system toxicity and protein binding of model proteins such as bovine serum albumin (BSA), lysozyme (Lyso), and apolipoprotein (apoA-I), in order to allow further advances in improving drug delivery system performance by means of polymerizable diacetylenic lipids.

Methods

Materials

Polymerizable lipid ($DC_{8,9}PC$) was from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA), and phospholipid DMPC was from Lipoid GmbH (Ludwigshafen am Rhein, Germany). TBA (2-thiobarbituric acid) was from Merck (Darmstadt, Germany), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was from USB Corporation (Lincoln, Massachusetts, USA). Cell culture MEM/EBSS NEAA modified medium was from HyClone (Logan, Utah, USA), antibiotic-antimycotic was from Gibco (Grand Island, New York, USA), and fetal bovine serum (FBS) was from Bioser (Barcelona, Spain). Lyso and BSA were from Sigma Co. (St. Louis, Missouri, USA), and apoA-I was a generous gift from Alejandra Tricerri and Horacio Garda from the Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Universidad Nacional de La Plata (UNLP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina. All other reagents were of analytical grade and were used without further purification.

Liposome preparation

Liposomes were prepared according to Bangham et al. (1965). Briefly, lipids were dissolved in chloroform, and the solvent was evaporated until a thin, dry film was obtained. The film was flushed with nitrogen and then suspended in TBS buffer (10 mM of Tris-HCl, 154 mM of NaCl, pH 7.4), in general, to a final 5 mg/mL concentration. In a typical formulation, lipids were used in a 1:1 molar ratio (DMPC:$DC_{8,9}PC$). Then, the suspension was extruded at 50°C 15 times through 0.2 μm pore polycarbonate membranes, using a miniextruder from Avanti Polar Lipids. Lipid concentration was determined by a phosphate assay, according to Bartlett's method (1959). Scanning electron microscopy (SEM) images were obtained from lyophilized liposomes, which were prepared from a frozen suspension at –80°C overnight (O.N.) and then taken to a Freezone 4.5 Labconco lyophilizer (Kansas City, Missouri, USA), precooled at –50°C. The lyophilization process pressure was maintained within the range of 33×10^{-3}–65×10^{-3} mbar for 24 hours. After the vacuum was removed, tubes were closed and stored at 4°C until further use.

Extruded vesicle polymerization

Diacetylenic vesicles were polymerized under 254 nm of UV light (20 cycles of 360 mJ/cm^2 each), using a UV-Stratalinker 1800 (Stratagene, La Jolla, California).

Temperature was maintained at 4°C for 5 minutes in between cycles. Spectra were recorded with a Shimadzu 160-A double-beam spectrophotometer (Kyoto, Japan), between 400 and 650 nm at room temperature (Alonso-Romanowski et al., 2003).

Size stability

Vesicle size was determined at 25°C by measuring the autocorrelation function at a 90-degree scattering angle in a 90Plus/Bi-MAS particle size analyzer (Brookhaven Instruments Corporation, Holtsville, New York, USA), with a light source of 632.8 nm and a 10-mW laser. Each result is the average of three measurements. Samples were kept at 4°C until analyzed, and measurements were carried out on days 1 and 30 after sample preparation. Data acquisition and analysis were conducted from using the software package (Brookhaven Instruments 90Plus particle-sizing software) supplied by the manufacturer.

Lipid peroxidation

A lipid peroxidation (LPO) assay was conducted, as described previously by Buege and Aust (1997). Briefly, a 100 µL sample aliquot (25 mM total lipid concentration) was incubated with 25 µL of 3 M of NaCl, 50 µL of rat mitochondrial or microsomal membranes (20 mg of protein/mL), and 10 mM of Tris-maleate buffer (pH 7.0), up to a final 1 mL volume, for 20 minutes at 30°C. Peroxidation preinduction was performed by adding to the previous mixture 10 µL of a 1 mM Fe^{+2} solution and 20 µL of 1 mM of ascorbic acid with a 30-minute incubation at 30°C. Finally, peroxidation was induced by adding 10 µL of a 1 mM Fe^{+2} solution and 80 µL of 1 mM of ascorbic acid, and peroxidation was allowed to occur for 15 minutes at 30°C.

The reaction was stopped by mixing a 250 µL aliquot with 1 mL of TBA reagent (Buege and Aust, 1997), followed by a 15-minute incubation at 100°C. Samples were centrifuged for 5 minutes at 20,800 × *g*, and supernatant absorbance, at 535 nm, was measured with a Shimadzu UV-1603, UV-visible spectrophotometer. The peroxidation index refers to nanomoles of formed malondialdehyde (MDA) per milligram of protein of microsomal or mitochondrial membrane. MDA concentration was calculated from using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$, according to Buege and Aust (1997).

Mitochondrial and microsomal membranes were obtained from rat liver homogenate in 0.9% NaCl (w/v) by differential centrifugation (Baudhuin, 1997).

Hemolysis

Bovine erythrocytes were obtained from healthy animals (Slaughterhouse-Calchaquí SRL, Quilmes, Argentina), following Servicio Nacional de Sanidad y Calidad Agropecuaria (SENASA) Argentinean regulations, and were separated from 20 mM of ethylene diamine tetraacetic acid (EDTA) noncoagulated blood by centrifugation at 400 × *g* for 10 minutes. Phosphate-buffered saline (PBS)-washed erythrocytes obtained from 750 µL of blood were incubated with an aliquot of 350 µL of liposomes (3 mg/mL, in PBS) for 30 minutes at 37°C in a Ferca TT400 orbital shaker at 200 rpm. After incubation, samples were centrifuged for 10 minutes at 400 × *g* to separate nonhemolyzed cells. Supernatant absorbance, at 530 nm, was measured by using a microplate reader (MRXTC; Dynex Technologies, Chantilly, Virginia).

Hemolysis percentage (%H) was calculated from using Equation 1:

$$\%H = A_{sample}/A_{100\%} \times 100 \quad (1)$$

where A_{sample} corresponds to sample absorbance at 530 nm and $A_{100\%}$ corresponds to supernatant absorbance after two cycles of freezing and thawing (100% hemolysis). Control experiments were performed from measuring the supernatant absorbance of erythrocytes incubated with 350 µL of PBS buffer, instead of liposomes (Fujisawa et al., 2000).

Viability evaluation in L929 cell line

L929 cells were seeded in a 96-well plate at 2×10^4 cells/well cell density. Cells were cultured with 150 µL of MEM/EBSS NEAA (HyClone) modified media, prepared according to the manufacturer's instructions, and supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (0.25 µg/mL), and 10% (v/v) FBS. The plate was incubated in a 5% CO_2 atmosphere for 24 hours at 37°C. After, at 90% cell confluence, the medium was removed and cells were cultured in the presence of different concentrations of polymerized and nonpolymerized DMPC:$DC_{8,9}$PC (1:1) liposome formulations (5.00, 3.00, 2.00, 1.00, 0.75, 0.50, 0.25, and 0.10 mM) diluted in maintenance media (same media described above, but supplemented with 1% FBS, instead of 10%). After a 13-hour incubation, culture media were removed and cells were washed twice with PBS and incubated for 2 hours in a 5% CO_2 atmosphere at 37°C with 0.5 mg/mL of MTT solution prepared in maintenance media (Mosmann, 1983). Supernatants were discarded and cells were homogenized with 200 µL of ethanol 95% (v/v). Absorbance at 595 nm was determined from using a microplate reader (MRXTC; Dynex Technologies). Cells incubated only with maintenance media were used as a control, obtained absorbance was taken as 100% cell viability, and sample data were adjusted to this value.

SEM

Polymerized or nonpolymerized liposomes (5 mM total lipid concentration) were mixed with FBS (1:4 dilution) or the equivalent volume of distilled water in a 3:1 (v/v) ratio. The FBS dilution was optimized to reduce liposome-protein binding artifacts caused by a high amount of proteins present in undiluted serum. After a 30-minute incubation at 37°C in a Thermo Formo Orbital shaker (Thermo Scientific, Waltham, Massachusetts, USA) at 200 rpm, liposomes were centrifuged for 10 minutes at 10,000 × *g* in a bench-top centrifuge. The supernatant was removed, and the pellet was washed once with distilled water. After, the pellet was lyophilized, as described above. It is worth mentioning that all the samples were lyophilized at the same time and with using a unique vacuum chamber under the same experimental conditions. Thus, any morphology difference we observed would not be attributed to changes resulting from the lyophilization process. SEM images were obtained with a Philips Quanta 200 microscope at Comisión Nacional de Energía Atómica, Centro Atómico Constituyentes (CNEA-CAC; Buenos Aires, Argentina). The vacuum applied was 0.6 torr in an environmental mode.

Model protein adsorption

Liposome-protein interaction was tested by using three different model proteins: BSA, Lyso, and apoA-I. A 100 µL aliquot of polymerized and nonpolymerized liposomes (12 mg/mL) in Tris-buffered saline (TBS) buffer (pH 8.0) were mixed with a 100 µL protein solution (1.5 mg/mL). "Incubation was performed for 30 minutes at 37°C. Samples were centrifuged for 20 minutes at 20,800 × *g*. Supernatant was removed, pellets were washed with 100 µL of buffer, and, after a new 20-minute centrifugation at 20,800 × *g*, protein concentration was determined in the supernatant by the bicinchoninic-acid method (Smith et al., 1985). More protein present in supernatant is indicative of less protein bound to the liposome membrane.

Statistical analysis

Results are expressed as mean ± standard error (SE). Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by Tukey's test, with a significance level of 0.05 (MicroCal Origin software; MicroCal Software, Inc., Piscataway, New Jersey, USA).

Results

Polymerization process

The polymerization process was followed by measuring the absorbance in the UV-visible region after each polymerization cycle (Alonso-Romanowski et al., 2003; Johnston et al., 1983; Albrecht et al., 1982). Figure 1 shows the absorbance at 520 nm, which is indicative of a polymer formation, for each cycle from 0 to 20. As seen in Figure 1, a plateau is reached after the 15th cycle, indicating no further polymerization of the DMPC:$DC_{8,9}PC$ 1:1 mixture, can be obtained by the methodology used. Thus, all the results presented in this work for polymerized membranes correspond to samples that underwent 20-UV irradiation cycles to ensure a total possible polymerization—for this mixture and with this methodology—was achieved.

Size stability

Size distribution analysis of polymerized or nonpolymerized vesicles was performed on days 1 and 30 after liposome preparation. As can be seen in Figure 2, the size distributions for polymerized or nonpolymerized vesicles were bimodal, indicating that two main liposome populations were present for both formulations, whether analyzed on days 1 (white square or triangle, for polymerized or nonpolymerized vesicles, respectively) or 30 (black square or gray triangle, for polymerized or nonpolymerized vesicles, respectively). As can be seen in Table 1, on day 1, polymerized vesicles had a higher mean diameter, when compared with nonpolymerized vesicles (effective diameter for polymerized samples: 897.0 ± 41.2 nm; for nonpolymerized: 206.1 ± 3.2 nm). After 30 days at 4°C incubation, vesicle diameters changed for both conditions: Effective diameter for polymerized samples was 1,320.5 ± 50.1 nm and

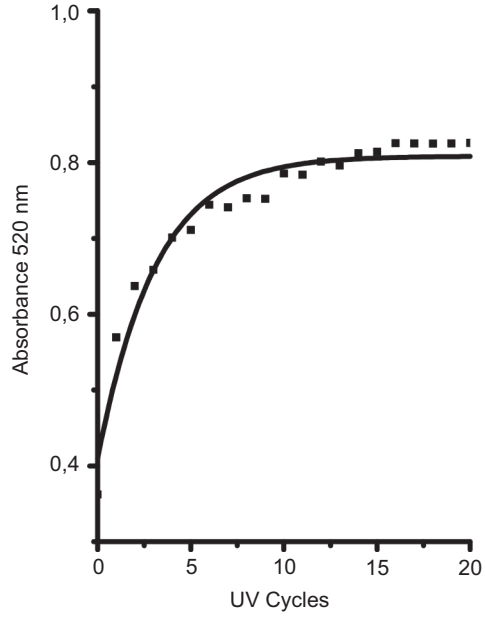

Figure 1. Absorbance at 520 nm plotted against the number of UV-irradiation cycles.

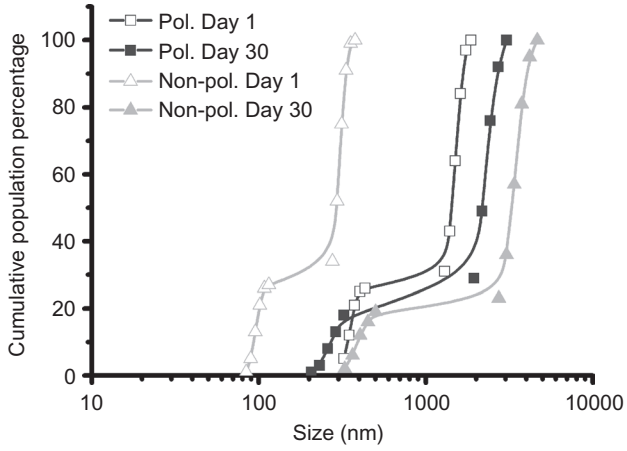

Figure 2. Cumulative liposome population percentage as a function of size of polymerized (Pol) and nonpolymerized (Nonpol) DMPC:$DC_{8,9}$PC 1:1 vesicles on days 1 (white square or triangle for polymerized or nonpolymerized vesicles, respectively) or 30 (black square or gray triangle for polymerized or nonpolymerized vesicles, respectively) after preparation. Size is presented as a logarithmical scale, and each symbol represents the obtained size for the denoted liposomal population percentage.

Table 1. Light-scattering measurements.

Group	Mean size (nm) (day 1)	Mean size (nm) (day 30)
Nonpolymerized DMPC:$DC_{8,9}$PC (1:1)	206.1 ± 3.2	1,836.4 ± 52.5
Polymerized DMPC:$DC_{8,9}$PC (1:1)	897.0 ± 41.2	1,320.5 ± 50.1

Liposome diameter results, measured by light scattering, after a 1- or 30-day incubation period at 4°C. Values are the means of three determinations ± standard error (SE); all values are statistically different.

nonpolymerized was 1,836.4 ± 52.5 nm. Note that these diameter values represent a size change of around 47% for polymerized samples and 790% for nonpolymerized ones.

LPO, hemolysis, and cell viability determination

To investigate possible effects of UV irradiation on the development of the system's cytotoxicity, polymerized or nonpolymerized membranes were analyzed for their capacity to induce LPO on microsomal and mitochondrial membranes, to induce bovine red-blood-cell hemolysis, and to affect cell viability of the L929 cell line.

LPO results showed no significant differences with respect to control experiments, where mitochondrial or microsomal membranes were incubated with the same volume of buffer (10 mM of Tris-maleate; pH 7.0), instead of liposomes, for nonpolymerized samples (see Figure 3). In the case of polymerized diacetylenic liposomes, a slight, but significant, peroxidation, if compared to control samples, was induced in microsomal membranes

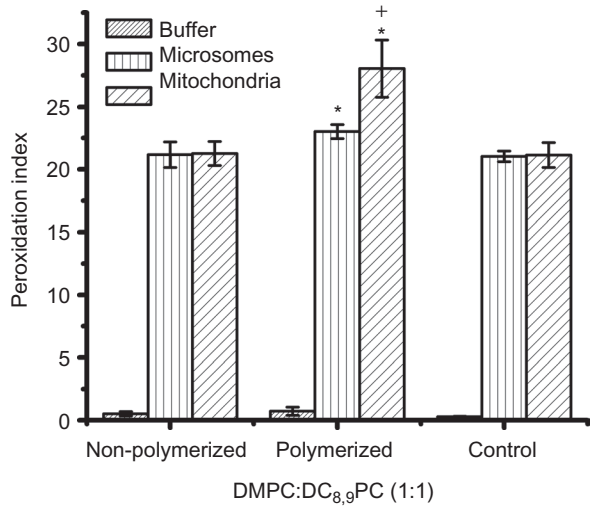

Figure 3. Peroxidation index of nonpolymerized and polymerized DMPC:$DC_{8,9}$PC 1:1 liposomes. Bars correspond to buffer (bars filled with tight oblique lines), microsomes (bars filled with vertical lines), and mitochondria (bars filled with spaced oblique lines) membranes. Microsomal and mitochondrial membranes and buffer incubated with buffer instead of liposomes were used as a control. *Significantly different with respect to the same control membrane; +significantly different with respect to nonpolymerized with mitochondrial membrane.

and was higher in mitochondrial ones (see Figure 3). No significant peroxidation-induction difference was found between polymerized or nonpolymerized samples for microsomal membranes, though that was not the case for mitochondrial ones, in which polymerized samples induced higher peroxidation, when compared to nonpolymerized. It is important to remark that buffer bars correspond to samples that underwent the same incubation period and protocol, as previously described, but without microsomal or mitochondrial membranes. This experiment indicated if the formulations could peroxidate *per se*.

Hemolysis results, presented in Table 2, showed no significant differences between polymerized or nonpolymerized DMPC:$DC_{8,9}$PC 1:1 mixtures, when compared to a control, for incubation periods of 30 and 60 minutes. After a 90-minute incubation, there was a significant difference with respect to the control sample for both conditions (polymerized or not); however, no difference between them arose. It is worth mentioning that hemolysis values obtained after 90 minutes were less than 2% for both conditions.

The cell line, L929, was used as a model to test DMPC:$DC_{8,9}$PC 1:1 toxicity, especially after being UV-irradiated for polymerization. If UV irradiation has an effect on altering the formulation toxicity, then a reduction on cell viability should be observed, when comparing polymerized or nonpolymerized membranes at the same concentration under the same experimental conditions. Figure 4 shows no viability reduction (i.e., less

Table 2. Hemolysis percentage (%H).

Group	%H (30 minutes)	%H (60 minutes)	%H (90 minutes)
Control	1.18 ± 0.46	0.942 ± 0.17	0.93 ± 0.08
Nonpolymerized DMPC:$DC_{8,9}$PC (1:1)	0.68 ± 0.01	1.00 ± 0.06	1.43 ± 0.18*
Polymerized DMPC:$DC_{8,9}$PC (1:1)	0.77 ± 0.05	1.24 ± 0.19	1.47 ± 0.06*

Hemolysis results after incubation periods of 30, 60, or 90 minutes. Values are the means of five determinations ± standard error (SE).
*Significantly different, with respect to the control.

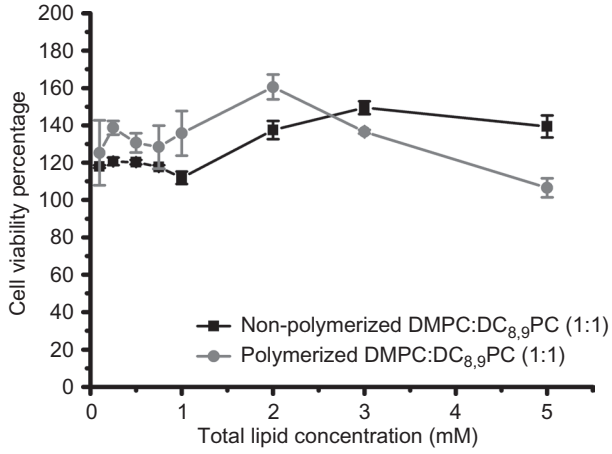

Figure 4. Graph of L929 cell viability percentage as a function of total lipid concentration (range, 0–5 mM) for nonpolymerized (black squares) and polymerized (gray circles) liposomes.

than 100%) after a 13-hour incubation period for polymerized or nonpolymerized membranes at the assayed concentrations.

SEM

In order to detect possible differences in the final lyophilized structure, due to differences in membranes after UV irradiation, of polymerized or nonpolymerized liposomes with or without prior incubation with FBS, SEM images were obtained for all conditions. As all the samples followed the same lyophilization process, differences detected in the obtained structure should be attributable to the different conditions assayed. Figure 5 shows the images obtained at a 1200X magnification for the lypophilized structures corresponding to nonpolymerized liposomes without prior FBS incubation (Figure 5 A) and with prior FBS incubation (Figure 5 B). Images obtained for polymerized samples without FBS incubation are shown in Figure 5 C and with FBS incubation in Figure 5 D.

As can be seen in Figure 5, no significant differences were found for any condition but those "regular holes" observed in nonpolymerized samples without prior FBS incubation (see white arrows in Figure 5A) that were

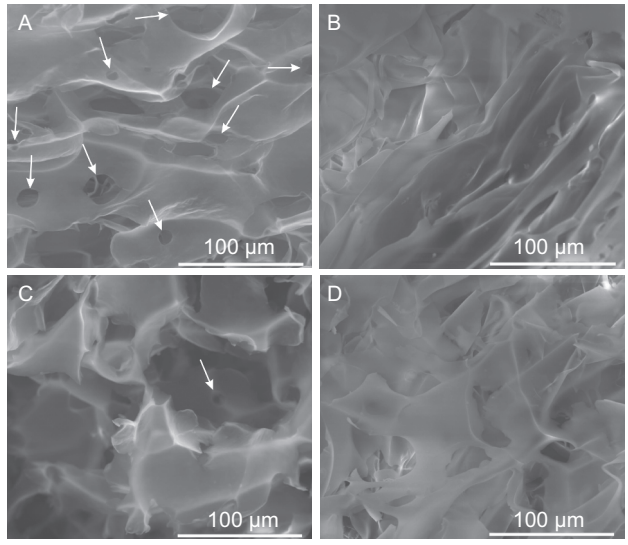

Figure 5. Pictures obtained by SEM microscopy at a 1200X magnification of lyophilized nonpolymerized DMPC:DC8,9PC 1:1 liposomes without prior FBS incubation (A) and with prior FBS incubation (B) and polymerized liposomes without (C) and with prior FBS incubation (D). White lines represent a size of 100 µm, and white arrows indicate the observed "regular holes" found mainly for the non-FBS-incubated nonpolymerized sample.

almost absent in nonserum-incubated polymerized ones (Figure 5C). Samples, whether polymerized or not, previously incubated with FBS, showed no significant differences between them (Figure 5D and B, respectively), but it is not so when comparing polymerized and nonpolymerized samples with and without prior incubation with FBS (Figure 5B and A, respectively, for nonpolymerized membranes, and Figure 5D and C, respectively, for polymerized ones).

Model protein interaction

A quantitative assay on lipid-protein interaction was carried out with using BSA, apoA-I, and Lyso as protein models. These proteins were selected by taking into account their differences in charge and molecular weight (BSA: 66 KDa and pI 4.8; Lyso: 14.3 KDa and pI 11; and apoA-I: 28 KDa and pI 5.27). BSA was also chosen as a model protein in the blood, since it accounts for around 50% of all serum proteins (Yokouchi et al, 2001). Lyso is one of the proteins that bears a net positive charge, so it could be used to assay differences in binding according to protein charges, when compared with BSA, and it has also been described in hydrophobic interaction with membranes (Gorbenko et al., 2007). On the other hand, apoA-I was chosen because of its well-known binding affinity for phospholipid membranes (Tricerri et al., 2005).

As can be seen in Figure 6, BSA bound more tightly (i.e., less protein in the supernatant) to nonpolymerized than to polymerized liposomes. Lyso showed a similar

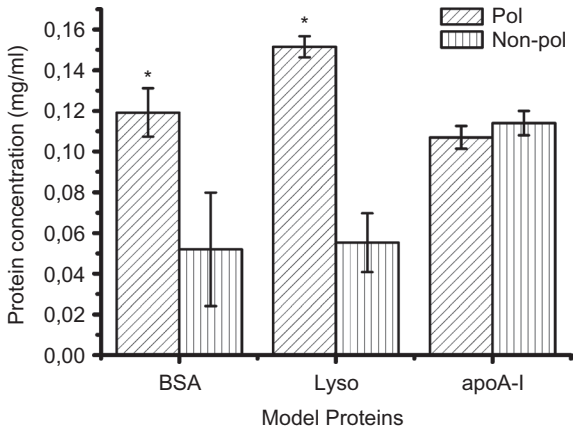

Figure 6. Protein concentration for unbound BSA, Lyso, and apoA-I found in supernatant, for nonpolymerized (Nonpol, bars filled with vertical lines) and polymerized (Pol, bars filled with oblique lines) DMPC:$DC_{8,9}$PC 1:1 liposomes after a 30-minute incubation at 37°C. *Significantly different with respect to Nonpol membranes.

behavior, but that was not the case for apoA-I, which interacted equally with polymerized or nonpolymerized membranes.

Discussion

Polymerization process

After polymerization, DMPC:$DC_{8,9}$PC 1:1 liposomes form conjugated polymers, containing, alternatively, triple, single, and double bonds. The polymer backbone shows a strong absorbance in the visible region of the spectrum, and the degree of membrane polymerization is related to both the magnitude and the wavelength of visible absorbance peaks and depends on the number of polymer units electronically coupled (Alonso-Romanowski et al., 2003; Johnston et al., 1983; Albrecht et al., 1982). Absorbance at 520 nm corresponds to a long conjugation of cross-bridges formed between linear polymers through intra- and intermolecularly carbon chains and was chosen to follow the polymerization process (Johnston et al., 1983).

Our results suggest that after the 15th UV-irradiation cycle, no further polymerization could be obtained in this formulation by the methodology used in this work. This situation has been previously described by Hayward et al. (1985) and Ahl et al. (1990), in which a maximum of polymerization was reached according to the lipid-mixture formulation, the polymerization process employed, and the type of polymerizable lipid used. Taking this result into consideration, all samples used in this work went through 20-UV irradiation cycles to ensure the same polymerization degree for all samples, so no differences due to this variable would account.

Size stability

One of the aims of this study was to know whether polymerization had an effect on the size of polymerized and nonpolymerized liposomes. Two-sided liposome populations were observed for polymerized or nonpolymerized membranes, and the comparison between them clearly demonstrated that nonpolymerized liposomes had a smaller size population distribution than polymerized ones on day 1 (effective diameter for nonpolymerized ones: 206.1 ± 3.2 nm; for polymerized samples: 897.0 ± 41.2 nm), even though both samples were extruded through a 0.2 μm pore membrane. This size difference for polymerized samples with respect to membrane pore diameter might be attributed to the polymerization process, in which samples were subjected to alternate cycles of cooling and energy irradiation (that induce local heating), as stated above. This process of cooling and local heating could lead to membrane fusion and/or aggregation, rendering higher diameters. After 30 days, it was observed that size had changed drastically in nonpolymerized liposomes, whereas less change was observed in polymerized ones. Polymerized samples' effective diameter changed from 897.0 ± 41.2 to 1,320.5 ± 50.1 nm (around a 47% change) and in nonpolymerized ones from 206.1 ± 3.2 to 1,836.4 ± 52.5 nm (around a 790% change). Thus, our data are indicative that even though a higher diameter is obtained after irradiation, UV-exposed membranes gain on size stability (i.e., less fusion or aggregation), when compared with nonirradiated ones. In this sense, irradiation of DMPC:$DC_{8,9}$PC 1:1 mixture leads not only to a higher stability against aggressive conditions, such as those found in the digestive tract, as stated by Alonso-Romanowski et al. (2003) for the same mixture, but also to a higher system resistance to fusion or aggregation in a 4°C storage condition during 30 days, as found in this work.

LPO, hemolysis, and cell viability determination

In this article, the other focus was whether UV-light–induced polymerization could develop the toxicity of the DMPC:$DC_{8,9}$PC 1:1 mixture. As the UV-irradiation process could trigger the development of free radical species, and since LPO is a complex process that occurs as a consequence of free radicals and leads to different product formations that may react with protein and or DNA having toxic or mutagenic effect (Marnett, 1999; Albert and Girotti, 1998), it was of interest to evaluate whether polymerized formulations were able to peroxidate *per se* or to induce peroxidation in natural membranes, such as microsomal or mitochondrial ones.

Results for the ability to induce peroxidation on microsomal membranes showed no significant differences with respect to control experiments for nonpolymerized

samples. This was not the case when polymerized liposomes were used, since they induced peroxidation, when compared with a control. No significant difference was found when comparing the ability to induce microsomal peroxidation between polymerized or nonpolymerized membranes, though it was found when comparing this effect on mitochondrial ones.

However, though it was statistically different, there was not a big difference for the peroxidation index and the same effect was not seen in microsomal membranes, concluding that the UV irradiation of DMPC:$DC_{8,9}$PC 1:1 mixture might lead to the development of some free radicals present in a very low concentration that is not able to induce high peroxidation on these biological model membranes. It is important to highlight that no *per se* peroxidation was found for either irradiated or nonirradiated membranes.

Red blood cells are one of the first cells that can interact with a drug delivery system after an intravenous inoculation. That is why we used a model of bovine red blood cells to determine the ability of the sample to induce their lysis, especially after polymerization. The highest percentage of hemolysis was reached after a 90-minute incubation and it was only 2%, without significant differences between polymerized or nonpolymerized membranes.

Obtained results can also be reflected in cell viability determination, since no viability reduction was detected in the L929 cell line with polymerized and nonpolymerized vesicles at the assayed lipid concentration. It is worth mentioning that the cytotoxic effect of polymeric liposomes might be dependent on the overall formulation toxicity, whether polymerized or not. For example, Chiaramoni et al. (2009) determined cell toxicity in the Vero cell line of polymerized $DC_{8,9}$PC:1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine:cholesterol 2:2:1, which presented a dose-independent toxicity; however, no information was available for nonpolymerized liposomes so that an adequate comparison could be made. The researchers pointed out that the observed toxicity could have been due to cholesterol or DMPE presence in the formulation. Our data are indicative that polymerization of the diacetylenic lipid, $DC_{8,9}$PC, in the presence of DMPC does not lead to toxicity development. So, formulation toxicity should be more related to lipids involved in the system, instead of a polymerization process of $DC_{8,9}$PC molecules. This should not be the case if polyunsaturated fatty acids are included in the formulation, since LPO could be drastically enhanced during the polymerization process due to the presence of multiple double bonds, with the consequent possible effect on formulation toxicity (Dix and Aikens, 1993). In line with these idea, the incorporation of cationic lipids, which are generally toxic, may also have an impact on cytotoxicity (Lv et al., 2006).

SEM

Polymerized and nonpolymerized liposomes were incubated with and without FBS, and the obtained lyophilized structure was analyzed under a SEM microscopy in order to detect surface differences between irradiated or nonirradiated membranes. Our results show similar SEM images to those obtained by Lu and Hickey (2005) for lyophilized liposomes in the presence of mannitol with or without protein encapsulation, though our samples were not coated with a gold-palladium alloy. When incubated with FBS, both irradiated and nonirradiated membranes showed "compact" structures, probably due to liposome/serum components/liposome interactions, if compared with those obtained for samples without FBS incubation. We cannot point to a difference, taking into account SEM resolution, between polymerized or nonpolymerized liposomes in the presence of FBS. UV irradiation has an effect on DMPC:$DC_{8,9}$PC 1:1 membranes, since differences arose between the final lypophilized structure of polymerized or nonpolymerized membranes without FBS incubation. This also supports previous findings by Alonso-Romanowski et al. (2003), that DMPC:$DC_{8,9}$PC 1:1 polymerization induced by UV light does have consequences on membrane behavior. At present, it is not possible to explain properly why these lyophilized structures are different after irradiation, and an extensive investigation in the structural properties of these membranes is needed to be done, though it exceeds the scope of the present article.

Model protein interaction

It is well known that protein adsorption depends on liposome-surface properties, such as phospholipid type, surface charge, liposome size, and membrane rigidity (Mönkkönen et al., 1994; Allen et al., 1991 Patel and Moghimi, 1989; Bonté et al., 1987).

Assays on lipid-protein interaction for polymerized or nonpolymerized DMPC:$DC_{8,9}$PC 1:1 membranes were carried out by using BSA, apoA-I, and Lyso as protein models. Results showed significant differences between polymerized and nonpolymerized liposomes for BSA and Lyso interaction. Both proteins bound more tightly to nonpolymerized liposomes, independently of their net charge and size. As reported by Yokouchi et al. (2001), BSA protein adsorption is related to hydrophobic, rather than to electrostatic, interactions. Following this idea, in the present work, even though liposomal surface charges had not been changed (i.e., lipid ratio and formulation is the same), a difference between polymerized

or nonpolymerized membranes upon BSA interaction was observed, suggesting that UV irradiation has consequences on membrane hydrophobicity, enough to alter BSA binding. In the case of Lyso, according to Gorbenko et al. (2007), there are two types of interactions with membranes: First, it is electrostatic, and, when closeness is important, hydrophobic forces prevail. As BSA, Lyso showed a differential interaction between polymerized or nonpolymerized membranes, interacting more with nonpolymerized ones and leaving less protein in solution, which supports what was found in the case of BSA.

Conversely, apoA-I showed no differences whatsoever, suggesting that an UV-induced surface modification has no significant effect on this particular protein. A possible explanation for this finding is that at 25°C (incubation with proteins was performed at 37°C), the two formulations, whether polymerized or not, have fluid DMPC, as shown with the hydrophobicity-absorbance ratio, according to Alonso-Romanowski et al. (2003), and it has been discussed by Tricerri et al. (2005) that apoA-I has the ability to capture fluid DMPC from phospholipids membranes. As fluid DMPC can be found in both conditions (polymerized or not), membrane irradiation would not make a difference for this particular protein, since its interaction would be mainly focused on fluid DMPC.

Conclusions

In conclusion, polymerizable diacetylenic lipids may provide novel constituents for the development of new liposomal drug-carrier systems, since polymerization induced by UV irradiation affects membrane interfacial characteristics, enhancing storage-size stability, with no effect on formulation toxicity, but with the potential to reduce the adsorption of one of the major serum components, as it is BSA, or Lyso that its known to cause membrane disruption.

This work aimed at promoting further research focused on the toxicity and UV-irradiation effect on different formulations, containing other polymerizable lipids, to gain insight about the potentiality of these kinds of lipids to not only enhance membrane stability, but also to alter protein binding for a rational design of novel carriers.

Declaration of interest

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References

Ahl, P. L., Price, R., Smuda, J., Gaber, B. P., Singh, A. (1990). Insertion of bacteriorhodopsin into polymerized diacetylenic phosphatidylcholine bilayers. Biochim Biophys Acta 1028:141–153.

Albert, C., Girotti, W. (1998). Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res 39:1529–1542.

Albrecht, O., Johnston, D. S., Villaverde, C., Chapman, D. S. (1982). Stable biomembrane surfaces formed by phospholipids polymers. Biochim Biophys Acta 687:165–169.

Allen, T. M., Austin, G. A., Chonn, A., Lin, L., Lee, K. C. (1991). Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size. Biochim Biophys Acta 1061:56–64.

Alonso-Romanowski, S., Chiaramoni, N. S., Lioy, V. S., Gargini, R. A., Viera, L. I., Taira, M. C. (2003). Characterization of diacetylenic liposomes as carriers for oral vaccines. Chem Phys Lipids 122:191–203.

Bangham, A. D., Standish, M. M., Watkins, J. C. (1965). Diffusion of univalent ions across lamellae of swollen phospholipids. J Mol Biol 13:238–252.

Bartlett, G. R. (1959). Phosphorus assay in column chromatography. J Biol Chem 234:466–468.

Baudhuin, P. (1997). Section I. In: Packer, L., Fleischer, S. (Eds.), Biomembranes, selected methods in enzymology (pp 91–103). San Diego, California, USA: Academic Press.

Blume, A. (1991). Phase transition of polymerizable phospholipids. Chem Phys Lipids 57:253–273.

Bonté, F., Hsu, M. J., Papp, A., Wu, K., Regen, S. L., Juliano, R. L. (1987). Interactions of polymerizable phosphatidylcholine vesicles with blood components: relevance to biocompatibility. Biochim Biophys Acta 900:1–9.

Buege, J. A., Aust, S. D. (1997). Section III. In: Packer, L., Fleischer, S. (Eds.), Biomembranes, selected methods in enzymology (pp 413–421). San Diego, California, USA: Academic Press.

Chen, H., Torchilin, V. P., Langer, R. (1996a). Lectin-bearing polymerized liposomes as potential oral vaccine carriers. Pharm Res 13:1378–1383.

Chen, H., Torchilin, V. P., Langer, R. (1996b). Polymerized liposomes as potential oral vaccine carriers: stability and bioavailability. J Contr Rel 42:263–272.

Chiaramoni, N. S., Gasparri, J., Speroni, L., Taira, M. C., Alonso, S., del, V. (2009). Biodistribution of liposome/DNA systems after subcutaneous and intraperitoneal inoculation. J Liposome Res [Online] Available at: http://informahealthcare.com/doi/abs/10.3109/08982100903244518.

Daly, S. M., Heffernan, L. A., Barger, W. R., Shenoy, D. K. (2006). Photopolymerization of mixed monolayers and black lipid membranes containing gramicidin A and diacetylenic phospholipids. Langmuir 22:1215–1222.

Dix, T. A., Aikens, J. (1993). Mechanisms and biological relevance of lipid peroxidation initiation. Chem Res Toxicol 6:2–18.

Fabani, M. M., Gargini, R. A., Taira, M. C., Iacono, R., Alonso-Romanowski, S. (2002). Study of *in vitro* stability of liposomes and *in vivo* antibody response to antigen associated with liposomes containing GM1 after oral and subcutaneous immunization. J Liposome Res 12:13–27.

Felnerova, D., Viret, J-F., Glück, R., Moser, C. (2004). Liposomes and virosomes as delivery systems for antigens, nucleic acids, and drugs. Curr Opin Biotechnol 15:518–529.

Freeman, F. J., Hayward, J. A., Chapman, D. (1987). Permeability studies on liposomes formed from polymerisable diacetylenic phospholipids and their potential applications as drug delivery systems. Biochim Biophys Acta 924:341–351.

Fujisawa, S., Atsumi, T., Kadoma, Y. (2000). Cytotoxicity of methyl methacrylate (MMA) and related compounds and their interaction with dipalmitoylphosphatidylcholine (DPPC) liposomes as a model for biomembranes. Oral Dis 6:215–221.

Georger, J. H., Singh, A., Price, R. R., Schnur, J. M, Yager, P., Schoen, P. E. (1987). Helical and tubular microstructures formed by polymerizable phosphatidylcholines. J Am Chem Soc 109: 6169–6175.

Gregoriadis, G. (1995). Engineering liposomes for drug delivery: progress and problems. Trends Biotechnol 13:527–537.

Gorbenko, G. P., Ioffe, V. M., Kinnunen, P. K. J. (2007). Binding of lysozyme to phospholipids bilayers: evidence for protein aggregation upon membrane association. Biophys J 93:140–153.

Guo, C., Liu, S., Dai, Z., Jiang, C., Li, W. (2010). Polydiacetylene vesicles as a novel drug sustained-release system. Coll Surf B Biointerf 76:362–365.

Hayward, J. A., Chapman, D. (1984). Biomembrane surfaces as models for polymer design: the potential for haemocompatibility. Biomaterials 5:135–142.

Hayward, J. A., Johnston, D. S., Chapman, D. (1985). Polymeric phospholipids as new biomaterials. Ann N Y Acad Sci 446:267–281.

Jelinek, R., Kolusheva, S. (2001). Polymerized lipid vesicles as colorimetric biosensors for biotechnological applications. Biotechnol Adv 19:109–118.

Johnston, D. S., McLean, L. R., Whittam, M. A., Clarke, A. D., Chapman, D. (1983). Spectra and physical properties of liposomes and monolayers of polymerizable phospholipids containing diacetylene groups in one or both acyl chains. Biochemistry 22: 3194–3202.

Kshirsagar, N. A., Pandya, S. K., Kirodian, G. B., Sanath, S. (2005). Liposomal drug delivery system from laboratory to clinic. J Postgrad Med 51:5–15.

Lopez, E., O'Brien, D. F., Whitesides, T. H. (1982). Effects of membrane composition and lipid structure on the photopolymerization of lipid diacetylenes in bilayer membranes. Biochim Biophys Acta 693:437–443.

Lu, D., Hickey, A. J. (2005). Liposomal dry powders as aerosols for pulmonary delivery of proteins. AAPS PharmSciTech Available at: www.aapspharmscitech.org/view.asp?art=pt060480. Accessed on January 29, 2010.

Lv, H., Zhang, S., Wang, B., Cui, S., Yan, J. (2006). Toxicity of cationic lipids and cationic polymers in gene delivery. J Contr Rel 114:100–109.

Marnett, L. J. (1999). Lipid peroxidation-DNA damage by malondialdehyde. Mutat Res 424:83–95.

Mönkkönen, J., Valjakka, R., Hakasalo, M., Urti, A. (1994). The effect of liposome surface charge and size on the intracellular delivery of clodronate and gallium *in vitro*. Int J Pharm 107:189–197.

Moses, M. A., Brem, H., Langer, R. (2003). Advancing the field of drug delivery: taking aim at cancer. Cancer Cell 4: 337–341.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Inmunol Meth 65:55–63.

Mozafari, M. R. (2005). Liposomes: an overview of manufacturing techniques. Cell Mol Biol Lett 10:711–719.

O'Brien, D. F., Armitage, B., Benedicto, A., Bennett, D., Lamparski, H. G., Lee, Y. S., et al. (1998). Polymerization of preformed self-organized assemblies. Acc Chem Res 31:861–868.

Okada, J., Cohen, S., Langer, R. (1995). *In vitro* evaluation of polymerized liposomes as an oral drug delivery system. Pharm Res 12:576–582.

Patel, H. M., Moghimi, S. M. (1989). Serum opsonins and phagocytosis of saturated and unsaturated phospholipid liposomes. Biochim Biophys Acta 984:384–387.

Poste, G., Kirsh, R., Koestler, T. (1984). The challenge of liposome targeting *in vivo*. In: Gregoriadis, G. (Ed.), Liposome technology, vol III (pp 1–28). Boca Raton, Florida, USA: CRC Press.

Rawat, M., Singh, D., Saraf, S., Saraf, S. (2008). Lipid carriers: a versatile delivery vehicle for proteins and petides. J Pharm Soc Jpn 128:269–280.

Riaz, M. (1995). Stability and uses of liposomes. Pak J Pharm Sci 8:69–79.

Regen, S. L., Singh, A., Oehme, M., Singh, M. (1982). Polymerized phosphatidylcholine vesicles: synthesis and characterization. J Am Chem Soc 104:791–795.

Rhodes, D. G., Hui, S. W., Xu, Y. H., Byun, H. S., Singh, M., Bittman, R. (1994). Structure of polymerizable lipid bilayers. VII: Lateral organization of diacetylenic phosphatidylcholines with short proximal acyl chains. Biochim Biophys Acta 1215: 237–244.

Sharma, A., Sharma, U. S. (1997). Liposomes in drug delivery: progress and limitations. Int J Pharm 154:123–140.

Spector, M. S., Selinger, J. V., Singh, A., Rodríguez, J. M., Price, R. R., Schnur J. M. (1998). Controlling the morphology of chiral lipid tubules. Langmuir 14:3493–3500.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985). Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85.

Subramaniam, V., D' Ambruoso, G. D., Hall, H. K., Jr., Wysocki, R. J., Jr., Brown, M. F., Saavedra, S. (2008). Reconstitution of rhodopsin into polymerizable planar supported lipid bilayers: influence of dienoyl monomer structure on photoactivation. Langmuir 24:11067–11075.

Svenson, S., Messersmith, P. B. (1999). Formation of polymerizable phospholipid nanotubules and their transformation into a network gel. Langmuir 15:4464–4471.

Tricerri, M. A., Toledo, J. D., Sanchez, S. A., Hazlett, T. L., Gratton, E., Jonas, A., et al. (2005). Visualization and analysis of apolipoprotein A-I interaction with binary phospholipids bilayers. J Lipid Res 46:669–678.

Yager, P., Price, R. R., Schnur, J. M., Schoen, P. E., Singh, A., Rhodes, D. G. (1988). The mechanism of formation of lipid tubules from liposomes. Chem Phys Lipids 46:171–179.

Yokouchi, Y., Tsunoda, T., Imura, T., Yamauchi, H., Yokoyama, S., Sakai, H., et al. (2001). Effect of adsorption of bovine serum albumin on liposomal membrane characteristics. Coll Surf B Biointerf 20:95–103.