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Pentacyclic triterpenes modulate liposome membrane fluidity and permeability depending on membrane cholesterol content

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ABSTRACT

Since the membrane-related processes represent an integral part of the biological activities of drugs, their effect on the membrane dynamics is actually considered. In this study, we investigated the effect of pentacyclic triterpenes (TTPs), oleanolic acid (OA) and erythrodiol (ER), on the fluidity and permeability of liposomes membranes differing by their cholesterol content. All liposomes were prepared by reverse phase evaporation technique (REV). Spin-labeled liposomes exposed or not to TTPs were used for fluidity studies by using 5- and 16-doxy stearic acids (DSA). TTPs-loaded liposomes (phospholipid:cholesterol of 1:1), and preformed vesicles exposed to TTPs were used for permeability studies by monitoring the release of sulforhodamine B (SRB) at 37°C. The apparent release constants of SRB were determined by Higuchi model based on a biphasic curve shape (0-10 h; 10-48 h). TTPs-loaded liposomes were characterized for their size and homogeneity. Results showed that ER increased the membrane fluidity at the upper region of the membrane while the both TTPs produced a condensing effect at the deeper region of the membrane. The membrane composition was a critical parameter modulating the effect of TTPs on the membrane permeability. Also, this study consolidated the fact that a fluidizing membrane agent is not necessarily a permeabilizing-membrane compound.

Keywords: cholesterol, erythrodiol, fluidity, liposomes, oleanolic acid, permeability

1. Introduction

Nowadays, much attention is devoted to plant-derived molecules as a cost-effective therapeutic strategy for prevention of different diseases (Žiberna et al., 2017). In particular, pentacyclic triterpenes (TTPs) are marketed as therapeutic agents or dietary supplements (Ayeleso and Matumba, 2017; Furtado et al., 2017). They are widely-spread in several medicinal plants and are found in a great variety of fruits, vegetable oils extractable from olives (*Olea europaea*), and cereals (Dopierała et al., 2020). Oleanolic acid (OA) and erythrodiol (ER) are the two natural pentacyclic TTPs considered in our study (Fig. 1).

OA (Fig. 1(a)) exists in nature in free acid form or as an aglycone precursor for TTP saponins (Lopez et al., 2014). Literature data highlight the prophylactic and therapeutic role of OA including anti-inflammatory (Bednarczyk-Cwynar et al., 2015), antioxidant (Ayeleso and Matumba, 2017), hypoglycemic (Alqahtani et al., 2013), cardioprotective (Marica Bakovic, 2015), and anti-cancer activities (Sen, 2020).

ER (Fig. 1(b)), the precursor of OA (Martín et al., 2012), is a natural dihydroxy TTP differing from OA by the presence of a hydroxyl group at C28. As for OA, ER showed vasorelaxant, cardiotonic and antidysrhythmic activities in animal models (Rodríguez-Rodríguez et al., 2004) as well as antioxidant and antithrombotic properties (Allouche et al., 2010).

As far as the multiple biological targets of pentacyclic TTPs are widely explored, it is rather surprising the lack of knowledge linking their biological effects to the membrane-related processes (Flasiński et al., 2014; Prades et al., 2011). This fact arises from their structural similarities with cholesterol (CHOL) which is a modulator of membrane rigidity (Najafinobar et al., 2016), thickness (Cathcart et al., 2015), fluidity (Redondo-Morata et al., 2012), permeability (Kaddah et al., 2018a), stability (Toppozini et al., 2014), and of the encapsulation efficiency of drugs in liposomes (Haeri et al., 2014). Among the very few studies, it has been reported that pentacyclic TTPs affect the membrane phase properties, alter the CHOL-rich domains, and may affect the function of membrane-associated proteins (Prades et al., 2011). Also, it has been demonstrated that the interaction of ER (Abboud et al., 2016; Habib et al., 2015) and the tetracyclic TTPs like cucurbitacin E (Habib et al., 2013) and corticoids (Abboud et al., 2016, 2015; Kaddah et al., 2018b) affect the membrane dynamics.

The objective of this work was to determine the effect of pentacyclic TTPs on the fluidity and permeability of dipalmitoylphosphatidylcholine (DPPC) liposomes membranes presenting various CHOL contents. In this context, permeability studies were carried out by monitoring the release kinetics of a fluorescent dye, sulforhodamine B (SRB) at 37°C from TTPs-loaded liposomes (CHOL 100%) at 2.5 and 10% over 10 days as well as from preformed DPPC liposomes (CHOL 0, 2.5, 5, 10, 20, 25, 30, 50 and 100%) followed by the exposure to TTPs at 2.5% over 48h. The apparent release constants of SRB were then determined using the Higuchi model. Also, the TTPs-loaded liposomes were characterized for their size and homogeneity by laser granulometry. For fluidity studies, selected preformed DPPC liposomes (CHOL 0, 10, 20, 30 and 100%) were investigated in the

absence and presence of TTPs by electron paramagnetic (EPR) spectroscopy using 5- and 16-doxyl stearic acids (DSA) as spin labels where the TTP to DPPC molar ratio was of 2.5%.

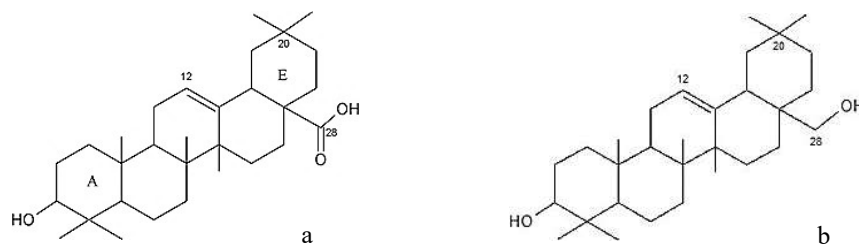


Fig.1. Structure of oleanolic acid (a) and erythrodiol (b)

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) (purity $\geq 99\%$) was purchased from Lipoid GmbH, Germany. Oleanolic acid (purity $\geq 97\%$) was purchased from Sigma-Aldrich, China. Erythrodiol (purity 97%) was furnished from Extrasynthèse, Genay, France. CHOL (purity 99%), SRB (95% content dye) and gel sephadex G25 were all purchased from Sigma-Aldrich, Germany. 5- and 16-DSA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triton X-100 and Trizma Base were purchased from Sigma-Aldrich, Switzerland. The organic solvents chloroform and methanol were from Sigma-Aldrich, France, and diethyl-ether was from VWR Chemicals Prolabo, France.

2.2. Preparation of liposomes for permeability and fluidity studies

All liposomes suspensions were prepared by reverse phase evaporation (REV) technique. Two protocols were used to study the effect of TTPs on liposome membrane fluidity and permeability following a previously reported procedure (Kaddah et al., 2018b).

Briefly, blank and SRB-loaded liposomes at various cholesterol contents (CHOL to DPPC molar percentage of 2.5, 5, 10, 20, 25, 30, 50 and 100%) were prepared, and followed by exposure to TTPs; the TTP to DPPC molar percentage was of 2.5%. Three batches were prepared for each formulation, and were used for monitoring the released SRB during 48 h. Also, TTPs-loaded liposomes composed of an equimolar mixture of DPPC and CHOL (CHOL100%) encapsulating SRB were prepared; the TTP percentage was of 2.5% and 10%. Three batches were prepared for each formulation, and used for monitoring the release of SRB during 10 days.

For fluidity studies, spin-labeled liposomes containing various CHOL to DPPC molar percentage (CHOL 0, 10, 20, 30 and 100%) and a TTP to DPPC molar percentage of 2.5 were prepared. 5- and 16-DSA were used as spin probes. Three batches were prepared for each spin-labeled formulation. All the liposome suspensions used for fluidity and permeability studies were extruded five times through a 1 μm polycarbonate membrane, followed by five times through a 0.4 μm membrane in order to ensure uniform liposome size distribution. The SRB-loaded liposomes were then purified from unloaded molecules by molecular sieve chromatography using sephadex G25 column, and stored at 4°C until use for membrane permeability study.

2.3. Vesicle size analysis of blank and TTPs-loaded liposomes

The liposomes particle size was analyzed by laser granulometry (Partica-LA-950V2 Laser Diffraction Particle Size Distribution Analyzer, Horiba, Japan) that allowed the detection of particles from 0.01 μm to 3000 μm . In addition, the polydispersity index (PDI) was calculated as being the span factor. This parameter is an indicator of the width of particle size distribution, and the level of homogeneity and uniformity of the liposome preparation (Mehanna et al., 2017; Ong et al., 2016). The span factor was calculated as follows (Nasr et al., 2013) :

$$\text{Span} = \frac{d_{90} - d_{10}}{d_{50}} \quad \text{Eq. (1)}$$

where d_{10} , d_{50} and d_{90} were the diameters at 10%, 50% and 90% of the cumulative intensity, respectively. If the span factor < 0.4 , the size distribution was considered monodispersed (Hamishehkar et al., 2015). The size particle and span values were expressed as the means of three different preparations \pm standard deviation.

2.4. Fluidity study based on electron paramagnetic resonance spectroscopy measurements

EPR experiments were carried out on a Bruker 500E spectrometer coupled with a nitrogen gas temperature controller accessory according to the method described in our previous work (Kaddah et al., 2018b). The membrane fluidity of DPPC liposomes (CHOL 0, 10, 20, 30 and 100%) exposed or not to TTPs (2.5%) was determined at 30, 40 and 50°C using 5- and 16-DSA as spin labels. The membrane dynamics were characterized by the EPR parameters through the maximum hyperfine splitting ($2A_{max}$) (Rajan et al., 2016), the order parameter “S” (Barbosa et al., 2008), and the rotational correlation time (τ_c) (Sgherri et al., 2014). The EPR parameters are calculated according to the explanation given in the appendix A.

2.5. Permeability study based on SRB release kinetics

Permeability study was carried out at 37°C by monitoring the release of SRB from blank and TTPs-loaded liposomes (2.5 or 10% of DPPC) over time as well as from preformed liposomes exposed or not to TTPs (2.5% of DPPC) using a spectrophotometer (Nicolet evolution 300 Thermoelectron, England). The method was described previously (Kaddah et al., 2018a). The release of SRB from vesicles was calculated according to the formula:

$$\text{Release SRB (\%)} = \frac{F_t - F_0}{F_{max}} \times 100 \quad \text{Eq. (2)}$$

where F_t is the fluorescence of the suspension at time t , F_0 is the initial fluorescence measured at time t_0 , and F_{max} is the maximal fluorescence achieved by the addition of buffer containing Triton X-100 (1%) leading to the total release of SRB from vesicles.

2.6. Kinetic modeling of SRB release profiles

The apparent release constants were determined by fitting the SRB release kinetics data into the Higuchi diffusion-controlled model expressed by the following equation (Higuchi, 1963) :

$$Q = K_H \times t^{1/2} \quad \text{Eq. (3)}$$

where Q corresponded to the percentage of released SRB in the medium at time t , and K_H was the Higuchi release constant.

2.7. Statistics

Results are expressed as the mean \pm standard error of the mean (S.E.M.). Data were analyzed by a one-way analysis of variance. The differences between means were analyzed using a Student's t-test. The criterion of significance was taken as $p < 0.05$.

3. Results and discussion

3.1. Liposome size and span analysis

The mean size distribution of blank and TTPs-loaded liposomes was determined by laser granulometry. The liposome size distribution for the TTPs-loaded liposomes was presented in Fig.2.

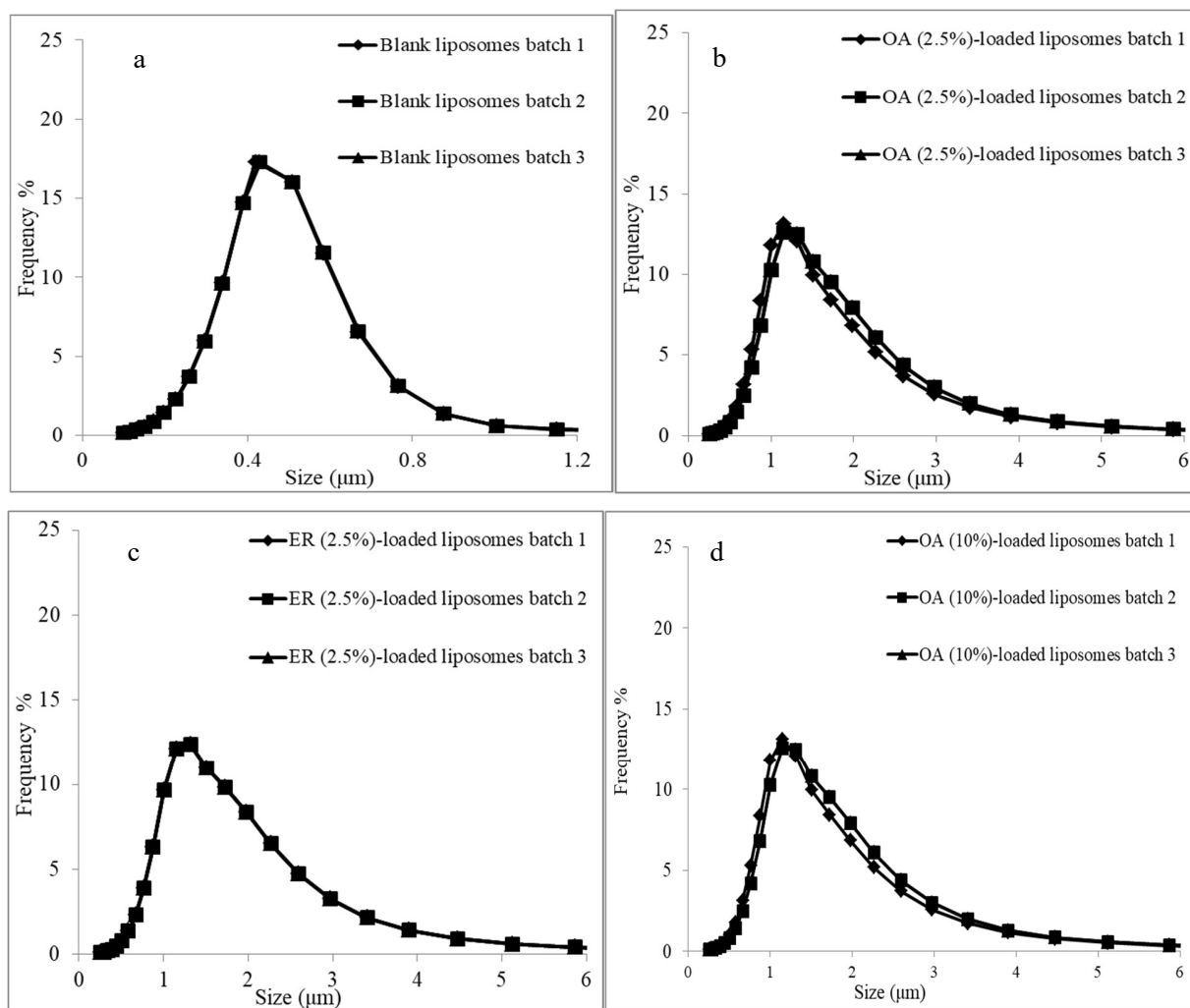


Fig.2. Liposome size distribution determined by laser granulometry for blank and TTPs-loaded liposomes at 2.5 and 10%. Blank liposomes (a), OA-(b), ER (c)-loaded liposomes at 2.5%; OA (d)-loaded liposomes at 10%. Measurements were performed on three different batches.

Blank liposomes and TTPs-loaded liposomes showed a unimodal-Gaussian size distribution (Fig. 2); overlaid curves were obtained for figures 2a and c. Blank liposomes had a mean diameter equal to 0.42 μm . Compared to blank liposomes, the mean particle size of TTPs-loaded liposomes (Figs. 2b, c and d) was about three times larger than the control ($P < 0.05$). The mean size values were 1.26 and 1.32 μm for OA-(Figs. 2(b) and (d)) and ER-(Fig. 2(c)) loaded liposomes, respectively. Results showed that the size distribution of liposomes did not change with increasing the OA content to 10% (Fig. 2(d)). The liposome mean size values were outlined in Table 1. According to literature, the increased size of liposomes may be assigned to the aggregation of vesicles (Biruss et al., 2007). Using X-ray diffraction and infrared spectroscopy, Lorincz et al. (2015) showed that ursolic acid, a derivative of OA, promoted the formation of non-layered (e.g. cubic or hexagonal) structures for DPPC liposomes containing 10% of ursolic acid, and this was accompanied with a significant expansion in the acyl chain cross-sectional area of a lipid molecule (Lórinicz et al., 2015). Given that OA and ER shared the same TTP

backbone with ursolic acid, we suppose that TTPs may promote structural defects leading to the expansion of the lipid acyl chains thus increasing the liposome size distribution.

Table 1

Mean size distribution and PDI values of TTPs-loaded liposomes determined by laser granulometry.

Liposomes (DPPC:CHOL:TTP)	Size (μm)	PDI
Blank liposomes (100:100:0)	0.42 ± 0.00	0.40 ± 0.00
ER-loaded liposomes (100:100:2.5)	$1.32 \pm 0.00^*$	0.34 ± 0.05
OA-loaded liposome (100:100:2.5)	$1.26 \pm 0.04^*$	0.34 ± 0.04
OA-loaded liposome (100:100:10)	$1.26 \pm 0.05^*$	0.34 ± 0.05

Values are expressed as the mean of three different measurements \pm SD

*P < 0.05 compared with the blank liposomes value

In addition, the width of liposome size distribution expressed by the “span factor” was calculated according to Eq. (1), and the PDI values were outlined in Table 1. Span factor expressed by PDI values reflects the uniformity of the vesicle size within the formulation (Abd-Elal et al., 2016). Blank liposomes have a span value of 0.40. Compared to blank liposomes, there was no significant effect of TTPs (2.5 and 10%) on the width of the liposome size distribution ($P > 0.05$), and the span values were of 0.34. According to literature, the size distribution was considered as narrow or monodispersed for span values < 0.4 (Guo et al., 2015; Zhou et al., 2012). TTPs loaded at 2.5 and 10% did not alter the homogeneity of the liposomes suspensions thus generating vesicles with uniform size distribution.

3.2. Electron paramagnetic resonance study

EPR spin labeling spectroscopy has emerged as a powerful tool to study the structural and dynamic changes of lipid membranes (Sahu and Lorigan, 2018; Stepien et al., 2015). In the present study, the fluidity of DPPC membrane (CHOL 0, 10, 20, 30 and 100%) exposed or not to TTPs (2.5%) was determined at 30, 40 and 50°C. 5- and 16-DSA were used as spin labels where their paramagnetic doxyl groups were linked to C5 (close to the bilayer interface region) and C16 (hydrophobic end) positions of the hydrocarbon chain (Kornmueller et al., 2016; Nusair et al., 2012). Therefore, the fluidity of the membrane can be estimated from the maximum hyperfine splitting ($2A_{max}$) (Fig. 3) and the order parameter “S” (Table 2) both obtained from 5-DSA spectrum in addition to the rotational correlation time (τ_c) (Fig. 4) obtained from 16-DSA spectrum.

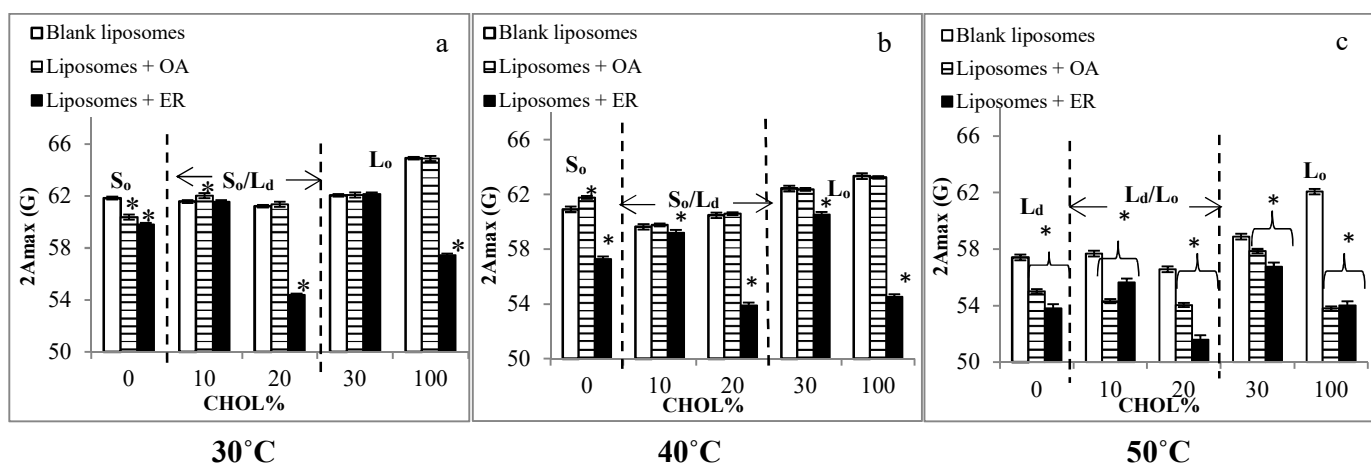


Fig.3. Effect of temperature and CHOL content on the maximum hyperfine splitting ($2A_{max}$) for spin-labeled blank liposomes (CHOL 0, 10, 20, 30 and 100%) exposed or not to TTPs at 2.5%. (a) $2A_{max}$ at 30°C, (b) $2A_{max}$ at 40°C, (c) $2A_{max}$ at 50°C. S_0 : solid-ordered phase, S_0/L_d : solid-ordered/liquid-disordered phase, L_d/L_o : liquid-disordered/liquid-ordered, L_o : liquid-ordered phase. Values are expressed as the mean of three independent measurements \pm SD.

* $P < 0.05$ compared with the control value

For blank cholesterol-free liposomes (CHOL 0%), DPPC exists in a gel or solid-ordered (S_0) state ($\sim 30^\circ\text{C}$) (Attwood et al., 2013). It undergoes a gel to liquid crystalline phase transition at the main phase temperature (T_m) of 41°C (Perez-Camacho and Ruiz-Suarez, 2017) which is close to the studied temperature of 40°C . This phase transition is accompanied with an increase in the membrane fluidity (Heerklotz, 2004). At 50°C , DPPC membrane exists in a liquid-disordered (L_d) phase (Nagarajan et al., 2012). A decrease in the $2A_{max}$ values (Fig.3) is observed with the raise of temperature, and this is accompanied with an increase in the membrane fluidity (Clark et al., 2016). This is in agreement with previous findings (Coderch et al., 2000; Kaddah et al., 2018b; Zhao et al., 2007).

Compared to the control (CHOL 0%) at 30, 40 and 50°C , OA and ER increased the membrane fluidity of the upper region of the membrane except for formulations exposed to OA at 40°C (near T_m) where a decrease in the membrane fluidity was observed (Fig. 3b). The fluidizing effect of TTPs (2.5%) is concomitant with the fluidizing effect of CHOL at low content (2.5%) in the gel state (Han et al., 1997). Our findings were in agreement with a recent study conducted by Abboud et al. (2016) who demonstrated by calorimetric and spectroscopic techniques that ER (2.5 %) fluidizes the CHOL-free DPPC liposome membranes (Abboud et al., 2016). However, it is worth to mention that the formulations exposed to OA showed unstable variations of the $2A_{max}$ values. Prades et al. (2011) demonstrated by X-ray diffraction that OA when loaded at 2% into CHOL-free DPPC liposomes promoted the occurrence of domains with different thickness (Prades et al., 2011). This may explain the variations in OA-modulating membrane fluidity following the temperature raise (phase transition).

At intermediate CHOL concentrations (CHOL 10 and 20%), DPPC membrane undergoes a S_o/L_d phase transition at 30 and 40°C (Aguilar et al., 2012) and a L_d/L_o phase transition at 50°C (Almeida, 2011). This is accompanied with the condensing and ordering effect of CHOL (Decker et al., 2012; Waheed et al., 2012) leading to a decrease in the membrane fluidity (Mills et al., 2008), and the organization of CHOL into CHOL-poor and -rich domains at these contents (Javanainen et al., 2017; Magarkar et al., 2014). Compared to the controls (CHOL 10 and 20%), ER increased the membrane fluidity of the upper region of the membrane at all the studied temperatures same as for OA at 50°C. In contrast, variations in OA-modulating membrane fluidity with respect to the controls were observed: a decrease of the membrane fluidity (CHOL 10%) at 30°C while no major changes of the membrane dynamics at 40°C were noticeable. This variation could be due to the heterogeneous distribution of CHOL affecting the relative position of OA within the lipid membrane.

Above CHOL 30% and at temperature ranging between 10 and 50°C, the onset of the L_o state occurs (Topozini et al., 2014). The lipid chains are tightly packed, and this is associated with the formation of nanodomains enriched in CHOL known as raft domains (Gulshan and Smith, 2014; Javanainen et al., 2017). Compared to CHOL 0%, the $2A_{max}$ values of blank liposomes (CHOL 30 and 100%) increased, meaning a decrease in the membrane fluidity. These findings were in agreement with the data previously published (Kaddah et al., 2018a; Tierney et al., 2005). Compared to the controls (CHOL 30 and 100%), ER increased the membrane fluidity of the upper region of the membrane at all the studied temperatures while OA increased the membrane fluidity only at 50°C.

Results showed that ER fluidizes the lipid membrane of the upper region of the membrane (CHOL 0-100%) at all the studied temperatures, and whatever was the CHOL content. The presence of a free hydroxyl group in the D-ring at the C28 position oriented the ER molecule towards the polar headgroups of the membrane. Similarly, using structural analogues of 17-estradiol, it has been reported that the fluidizing effect of 17-estradiol on the natural and synthetic membranes was due to the free hydroxyl group at the C17 position (Whiting et al., 2000). However, minor effects on the membrane fluidity were observed in the presence of OA (30 and 40°C). OA may be displaced to deeper regions of the membrane above CHOL 20% at 30 and 40°C. Teixeira et al. (2010) demonstrated by infrared spectroscopy that the H-bonding between the OA carboxylic groups leads to the dimerization of OA molecules (Teixeira et al., 2010) leading them to displace toward the hydrophobic core of the membrane (Lőrincz et al., 2015). Similarly, Prades et al., 2011 suggested that OA penetrates the deeper regions of DPPC membrane.

In parallel, the order parameter “S” was determined from 5-DSA spectra as an alternative indication of the effect of TTPs on the membrane fluidity of the upper region of the membrane. Results were outlined in Table 2.

Table 2

Order parameter “S” values for spin-labeled DPPC liposomes (CHOL 0, 10, 20, 30 and 100%) exposed or not to TTPs (2.5%) determined from 5-DSA spectrum at 30, 40 and 50°C

T (°C)	30			40			50		
	Blank liposomes	Liposomes + OA	Liposomes + ER	Blank liposomes	Liposomes + OA	Liposomes + ER	Blank liposomes	Liposomes + OA	Liposomes + ER
0	0.27 ± 0.05	0.20 ± 0.02*	0.18 ± 0.07*	0.19 ± 0.00	0.05 ± 0.20*	0.05 ± 0.09*	0.15 ± 0.11	0.07 ± 0.23*	0.06 ± 0.25*
10	0.79 ± 0.12	0.85 ± 0.19*	0.80 ± 0.21	0.29 ± 0.09	0.28 ± 0.10	0.20 ± 0.11*	0.24 ± 0.23	0.13 ± 0.11*	0.14 ± 0.07*
20	0.54 ± 0.07	0.53 ± 0.07	0.37 ± 0.12*	0.21 ± 0.11	0.28 ± 0.27	0.09 ± 0.07*	0.21 ± 0.14	0.13 ± 0.00*	0.12 ± 0.00*
30	0.65 ± 0.11	0.64 ± 0.05	0.65 ± 0.22	0.25 ± 0.14	0.23 ± 0.11	0.11 ± 0.22*	0.22 ± 0.07	0.15 ± 0.09*	0.14 ± 0.17*
100	0.94 ± 0.05	0.94 ± 0.01	0.25 ± 0.04*	0.29 ± 0.22	0.27 ± 0.15	0.09 ± 0.01*	0.28 ± 0.18	0.14 ± 0.22*	0.14 ± 0.22*

Values are expressed as the mean of three different preparations ± SD.

*P < 0.05 compared with the control value.

According to Table 2, results obtained from the order parameter “S” were compatible with those obtained from the $2A_{max}$ parameter.

In addition, the effect of TTPs on the membrane fluidity was also investigated at the deeper region of the membrane by the rotational correlation time (τ_c) at 30, 40 and 50°C obtained from 16-DSA spectra. Results are presented in Fig.4.

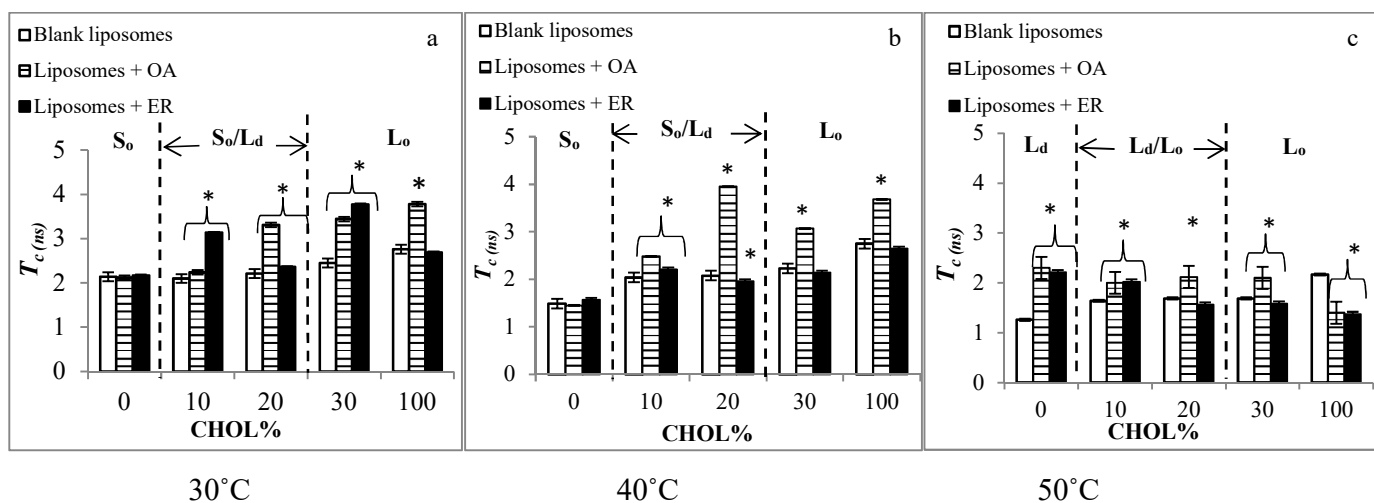


Fig.4. Effect of temperature and CHOL content on the rotational correlation time (τ_c) for spin-labeled blank liposomes (CHOL 0, 10, 20, 30 and 100%) exposed or not to TTPs at 2.5 mol%. (a) τ_c at 30°C, (b) τ_c at 40°C, (c) τ_c at 50°C. S_0 : solid-ordered phase, S_0/L_d : solid-ordered/liquid-disordered phase, L_d/L_0 : liquid-disordered/liquid-ordered phase, L_0 : liquid-ordered phase. Values are expressed as the mean of three independent measurements ± SD.

*P < 0.05 compared with the control value.

The τ_c values of formulations exposed or not to TTPs decreased with the raise of temperature; this means an increase in the membrane fluidity which is in accordance with previous findings (Kaddah et al., 2018b).

At CHOL 0% (S_o state), OA and ER both decreased the membrane fluidity at the deeper region of the membrane at 50°C, and this is in accordance with the condensing effect of CHOL above the main phase transition temperature (Han et al., 1997). However, no noticeable changes were observed at 30 and 40°C. This means that TTPs were exclusively located near the interface region of the membrane at CHOL 0%.

At intermediate CHOL concentrations (10 and 20%) and compared to the controls, OA and ER tend to decrease the membrane fluidity at the deeper region of the membrane. Above CHOL 30% (L_o state), TTPs decreased the membrane fluidity, and this effect was noticeable for OA. This means that TTPs produced a condensing effect at the deeper region of the membrane (CHOL 0-100%), and this effect was more pronounced for OA. These findings were in agreement with a recent study conducted by Lopez et al. (2014) which demonstrated that the presence of a carboxylic group at the C17, which is near to two methyl groups at C20, promoted the lipid raft stabilization of OA with sphingolipids in a manner resembling to that of CHOL (Lopez et al., 2014).

This means that the structural features of TTPs, rather than the membrane composition, control the effect of TTP on the membrane fluidity.

In addition, the hydrophobicity of the molecules is not the main factor controlling the TTPs-modulating membrane fluidity effects. While the “log P” value of ER (7.6, PubChem) is higher than that of OA (6.12, PubChem), ER affected the membrane fluidity at the upper region of the membrane, while the effect of OA was rather in the deepest membrane region.

3.3. Permeability study

The SRB release kinetics were studied over 10 days for blank liposomes and TTPs (2.5 and 10%)-loaded liposomes. Also, the release kinetics of SRB from preformed liposomes containing various CHOL content (CHOL 0-100%) and exposed to TTPs 2.5% were studied over 48 h. The procedure was described in our previous article (Kaddah et al., 2018a).

3.3.1. Release kinetics of SRB from TTPs-loaded liposomes

Although the phospholipid:cholesterol stoichiometry has been a matter of debate (Castanho et al., 1999), 1:1 or 2:1 molar ratios were considered to be the most widely used for DPPC:CHOL binary mixture (Bruglia et al., 2015). Previous data showed that the DPPC:CHOL molar ratio of 1:1 is stable in water (Collins and Phillips, 1982), and was considered as a “long-lived complex” (Phillips and Finer, 1974). For this reason, the DPPC:CHOL molar ratio of 1:1 was primarily chosen, and the permeability study of TTPs-loaded liposomes

was carried out at 37°C over 10 days. The SRB release kinetics from blank and TTPs-loaded liposomes were presented in Fig. 5.

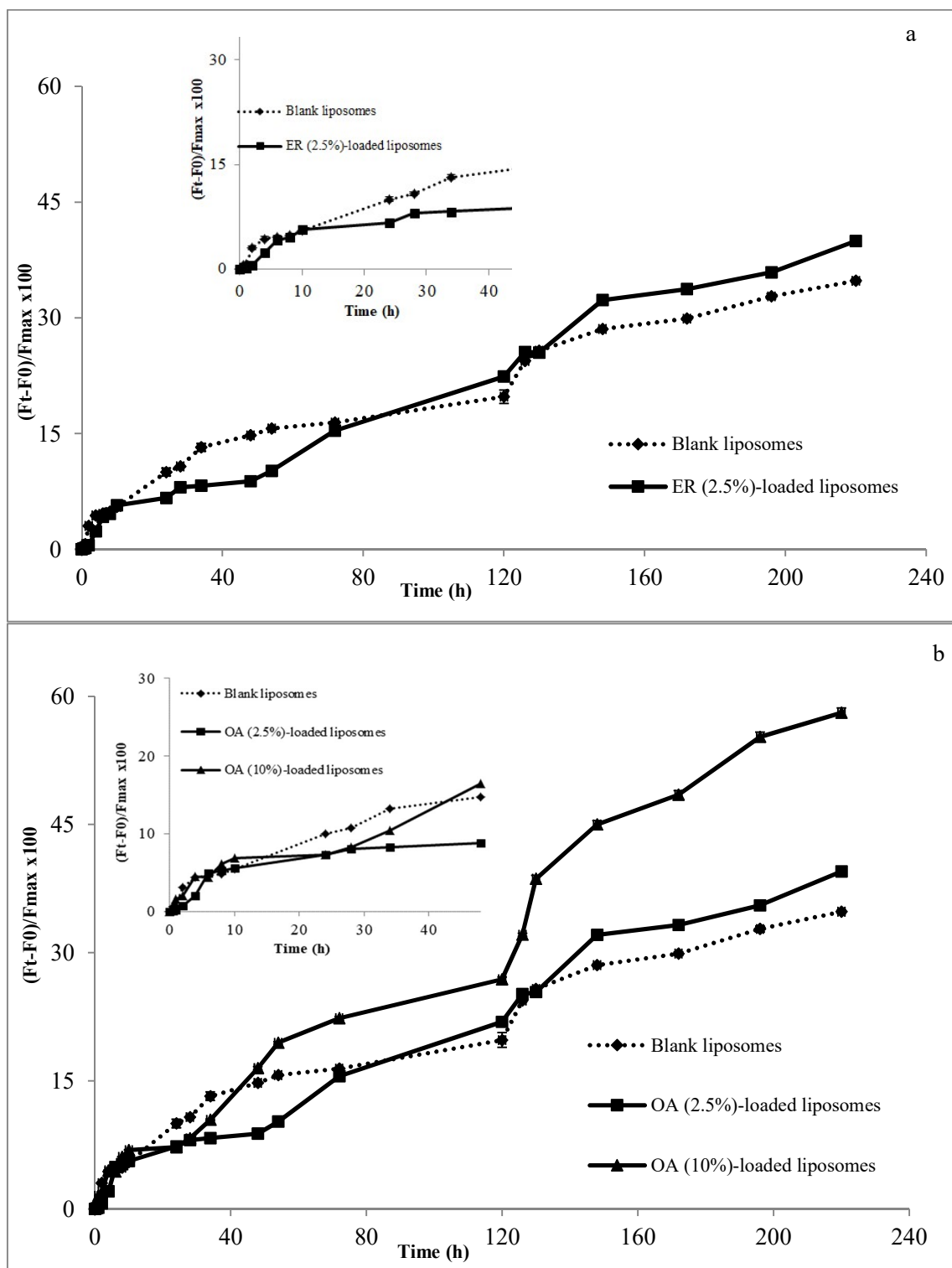


Fig. 5. The $(F_t - F_0)/F_{max} \times 100$ variation with time at 37°C for TTPs-loaded liposomes, DPPC:CHOL molar ratio was of 1:1. (a) ER-loaded liposomes at 2.5 %, (b) OA-loaded liposomes at 2.5 and 10%. Values are expressed as the mean of three independent release kinetics \pm SD.

Between 0 and 48 hours, the percentage of released SRB from TTPs-loaded liposomes at 2.5% was below that of blank liposomes (Figs. 5 (a) et (b)) which lines with the condensing effect of TTPs proved by EPR measurements. After a longer period of incubation, TTPs showed rather a minor permeabilizing effect. The latter was more noticeable at high OA content (10%) (Fig. 5 (b)). Prades et al. (2011) reported that the limit of TTP miscibility in the phospholipid membrane was of 20:1. In our study, the DPPC:TTP molar ratio of 100:5 which is higher than the physiological conditions. Lorincz et al. (2015) demonstrated that the high content of TTP promotes the presence of aggregates, and the distortion of the lipid membrane through the formation of the non-layered structures (Lőrincz et al., 2015).

3.3.2. Release kinetics of SRB from DPPC liposomes differing by CHOL content and exposed to TTPs

The SRB release from the preformed DPPC vesicles prepared at various CHOL content (0-100%) and exposed or not to TTPs 2.5%, were monitored over 48 h. The kinetic release curves were presented in the appendix B (Fig. B). The kinetics from blank formulations showed a biphasic pattern except for formulations incorporating high CHOL content (50 and 100%), and results were in accordance with our previous data (Kaddah et al., 2018b). The SRB release data for formulations exposed or not to TTPs were then treated by Higuchi model based on the Fick's law where the drug release is a diffusion process (Refai et al., 2017; Romero et al., 2018). Also, Higuchi model is among the most common models used to study the release of drugs through the lipid bilayers (Barzegar-Jalali et al., 2008; Javadzadeh et al., 2010). Hence, the Higuchi release constants of SRB (K_H) were determined based on the biphasic curve shape (K_1 : 0-10 h; K_2 : 10-48 h). We propose that the first release phase K_1 is linked to the adsorbed SRB molecules at the lipid membrane surface while the second release phase K_2 is assigned to the release of SRB from the intra-liposomal aqueous core to the extra-liposomal medium. Figs. 6 (a) and (b) represent the Higuchi release constants K_1 and K_2 , respectively for blank liposomes (CHOL 0-100%) exposed or not to TTPs at 2.5 %.

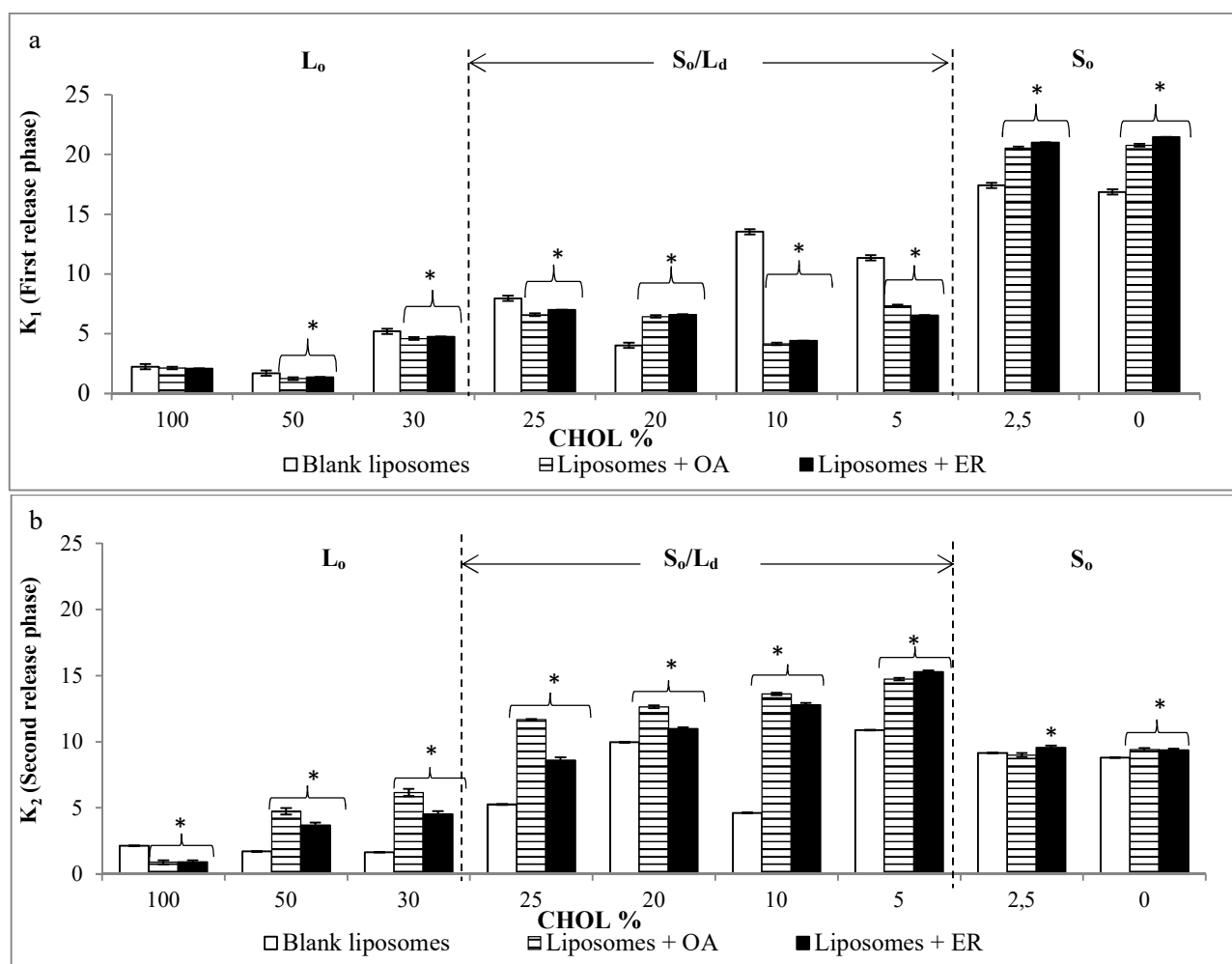


Fig.6. Higuchi SRB release constants determined for the two release phases for blank liposomes (CHOL% 0-100) exposed or not to TTPs at 2.5 %. (a) K_1 values: first phase, from 0 to 10 h; (b) K_2 values: second phase, from 10 to 48 h. Values are expressed as the mean of three different measurements \pm SD. S_0 : solid-ordered phase, S_0/L_d : solid-ordered/liquid-disordered phase, L_0 : liquid-ordered phase.

* $P < 0.05$ compared with the control value

Blank liposomes showed a decrease in the release of SRB with increasing the CHOL content at 37°C (Kaddah et al., 2018a). Compared to controls (CHOL 0 and 2.5%) (S_0 state), OA and ER both increased the membrane permeability in the first and second release phases. This observation is concomitant with the fluidizing effect of ER demonstrated by EPR measurements at 40°C while it was inconsistent with the condensing effect of OA.

At intermediate CHOL contents (CHOL 5-25%) (S_0/L_d state) and with respect to the controls, TTPs decreased the membrane permeability in the first release phase for formulations (CHOL 5, 10 and 25%) while the contrary effect was only observed at CHOL 20%. In the second release phase, TTPs increased the membrane permeability whatever was the CHOL content. Interestingly, similar tendencies were obtained when analyzing the effect of tetracyclic TTPs on membranes containing intermediate CHOL contents (5-25%) (Kaddah et al., 2018b).

The results obtained at CHOL 30 and 50% lines with those obtained at intermediate CHOL content in the first and second phases. At CHOL 100%, TTPs decreased the membrane permeability in the second release phase while no noticeable changes were observed in the first release phase.

Finally, the Higuchi release constants (K_1 and K_2) for TTPs-loaded liposomes were determined and compared to those obtained from liposomes exposed to TTPs. Results showed similar release constants values in both release phases (data not shown). This means that the two different approaches adopted in exposing liposomes to TTP did not alter the TTPs-modulating membrane properties.

4. Conclusion

This paper provides compelling evidence regarding how the membrane composition, the structural features of drugs, and the experimental protocol design affect the TTPs-modulating membrane properties. Based on our results, the following outcomes were proposed: (i) the structural feature of TTP was considered as a main factor affecting the membrane fluidity, (ii) the CHOL content was a critical factor controlling the effect of TTPs on the membrane permeability, (iii) the modification in the experimental protocol design did not influence the TTPs-modulating membrane properties. Although TTP promoted the membrane condensation, their permeabilizing effect on DPPC membrane was observed at CHOL content higher than 2.5%. This study strengthens the concept that a condensing-membrane agent did not necessarily produce a decrease of membrane permeability.

Appendices

Appendix A

Appendix B

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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