LIPOSOMES BEHAVIOR IN ANTIBIOTICS AND RADIOFREQUENCY FIELD ENVIRONMENT

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Abstract. Liposomes are largely used today as drug carriers. Their behavior in various chemical and physical environments should be well known in view of a rational design of vesicles of appropriate formulations. We investigated the liposomal membrane behavior under different physical and chemical conditions such as exposure to antibiotics, radiofrequecy fields and thermal changes. Measuring the Generalized Polarization of laurdan-labeled liposomes in the presence of the charged aminoglycoside gentamicin, under exposure to 2.45 GHz and under cyclic variation of temperature, the phase transition behavior of the phospholipids was characterized.

Key words: liposomes, gentamicin, microwaves, fluorescence, generalized polarization, phase transition.

INTRODUCTION

Liposomes are known to be reliable drug carriers which enhance efficiency, reduce toxicity and modulate pharmacokinetics of the administered drugs. As the stability and biological fate of lipid vesicles is closely related to their characteristics [6, 10], it is important to know how their properties are influenced by different factors such as: heating, presence of charged compounds as well as irradiation by electromagnetic fields (e.g., radiofrequency domain, RF) [1, 2, 8, 11]. These factors may be encountered in patients who are under treatment or clinical investigation [4, 7]. An *in vitro* study of the impact of these factors on the liposomal membrane could be a useful simulation in view of designing reliable drug carrier vesicles in such environment.

In our study we exposed dimiristoyl-phosphatidylcholine (DMPC) liposomes, prepared with and without a negatively charged lipid (cardiolipin, CL) to the action

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of a polycationic antibiotic (gentamicin, GT) and to the action of 2.45 GHz microwave field at different incident powers. Freshly prepared vesicles as well as liposomes which underwent 4 heating-cooling cycles were examined.

Fluorescence emission spectra from laurdan-labeled DMPC liposomes and laurdan-labeled DMPC + CL liposomes were recorded in the situations described above and the generalized polarization of the lipid vesicles was computed. The variation of phase transition temperature of the lipid membrane with the number of heating-cooling cycles was determined at different power levels of the applied microwave field.

MATERIALS AND METHODS

LIPOSOMES PREPARATION AND LABELING

All lipids were purchased from Sigma Aldrich and used without further purification.

Liposomes with two different compositions were prepared using:

1. a neutral phospholipid alone (DMPC) and
2. DMPC + CL, molar ratio of 9:1, in order to produce negatively charged vesicles which better mimic the living cell membrane.

The preparation procedure was the following: an appropriate amount of lipids (either DMPC alone or a mixture of DMPC and CL at molar ratio 9:1) was dissolved in chloroform (at a concentration of 5 mg lipids/mL) and dried at 40 ºC in a round bottom flask, under nitrogen stream and continuous rotation for 45 minutes. Thus, a uniform film of lipids was formed and kept further for 6 hours under vacuum. The lipids were then resuspended in a previously de-aerated phosphate buffered saline solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH = 7.4) at a final lipid concentration of around 10 mM. The milky suspension was sonicated until it became clear. The concentration of the liposomes suspension was adjusted to have an optical density of 0.1 at 350 nm. The liposomes were stable if kept under nitrogen at −20 ºC, for five weeks in case of DMPC vesicles, and for 2 weeks, in case of DMPC + CL vesicles. Liposome suspensions were thus conserved by freezing being thawed at room temperature and sonicated before each experiment.

For the experiments with liposomes incubated with gentamicin, an aliquote of a stock solution of antibiotic (Fluka, 50 mg/mL in water) was added to the liposomal suspension at a final concentration of 357 μg/mL.

Labeling with laurdan (0.1 mM stock solution in DMSO) was done in the spectrofluorometric cuvette, 7 minutes before each experiment, at 1 μM final dye concentration.

RADIOFREQUENCY FIELD EXPOSURE AND THERMAL CYCLING

The power of the microwave field (2.45 GHz, SAIREM solid state generator) injected in the sample was measured by a digital power meter (HEWLETT PACKARD, EPM SERIES, equipped with a variable attenuator – PASTERNAK, 0–20 dB). The microwave antenna is a coaxial copper wire (0.5 mm thick) immersed in the spectrofluorometric cuvette containing liposome suspension. During the microwave exposure the temperature was monitored using a Luxtron optical fiber thermometer. Both coaxial antenna and Luxtron probe were immersed in the liposomal suspension at a level slightly above the optical beam of the fluorometer (FLUOROLOG, HORIBA JOBIN-YVON equipped with a Peltier thermostating system, TLC50, QUANTUM NORTHWEST INC.). Applied field power, temperature and fluorescence were measured simultaneously. During the whole experiment the suspension was agitated by a magnetic stirrer. The system was characterized in a previous work [3].

In each experiment, the sample was initially equilibrated at low temperature (either 10 or 13 ºC) and a first Laurdan emission spectrum was taken. The temperature was then raised by 2 ºC, the system was allowed to equilibrate and a new emission spectrum was taken. The procedure was repeated until a temperature of 38 or 40 ºC was reached, which is above the phase transition temperature of the liposome phospholipids. Then, the sample was cooled back to the initial temperature and measurements were repeated several times.

Same experiments were performed with samples exposed to microwaves at different applied powers: 213, 411, 611 and 800 mW. In all these cases the temperature inside the sample was left to reach a stationary value which was above the temperature of the thermostat (due to microwave energy absorption by the sample).

LAURDAN SPECTROSCOPY OF DMPC VESICLES

Laurdan emission spectra (λ_{ex} = 364 nm and λ_{em} = 400–550 nm) were recorded in different temperature ranges covering an interval of 10–40 ºC, as shown above.

Laurdan is known to be a fluorophore sensitive to the polarity characteristics of the environment [5, 9]. In apolar media its emission spectrum is characterized by an emission peak at around 430–440 nm (called "blue emission", I_B), while in polar media the emission spectrum presents a maximum at 480–500 nm ("red emission", I_R). In intermediate situations, the overall recorded spectra will be a mixture of the two components.

A typical example of laurdan emission spectra recorded at different temperatures is given in Fig. 1:

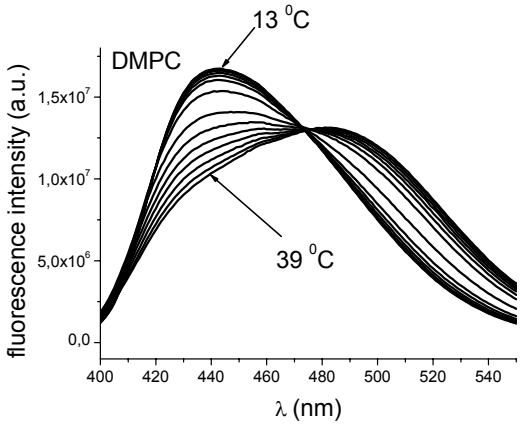


Fig. 1. Evolution of laurdan emission spectra with temperature increases in DMPC liposomes suspension.

The so-called "Generalized Polarization" (*GP*) is defined as:

$$GP = \frac{I_B - I_R}{I_B + I_R} \tag{1}$$

where I_B and I_R are the fluorescence intensities emitted by laurdan at the specific wavelengths for the gel phase (less polar) and liquid crystalline phase of the membrane respectively; the liquid crystalline phase is more polar due mainly to water penetration in the polar part of the lipid bilayer. According to this definition, a higher *GP* value corresponds to the prevalence of the gel phase, while a lower *GP* corresponds to the predominantly liquid crystalline phase. *GP* may vary between +1 and –1.

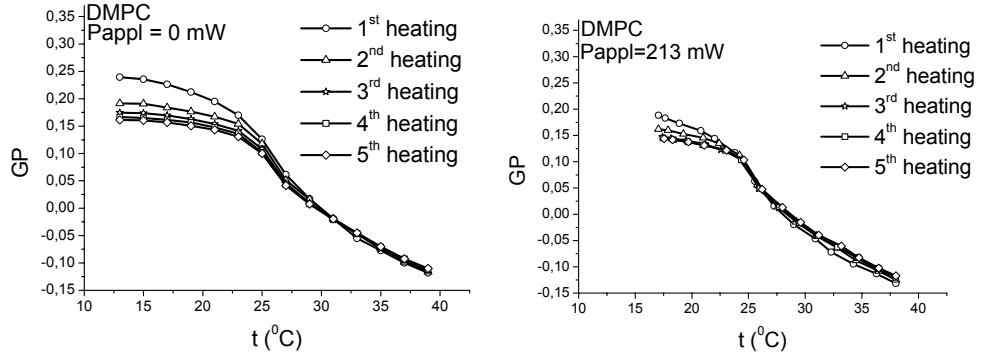
The plot of *GP versus* temperature gives usually an S-shaped curve with an inflexion point corresponding to the critical phase transition temperature (T_c) of the lipids within the membrane.

RESULTS AND DISCUSSION

EFFECT OF TEMPERATURE CYCLIC VARIATION ON GENERALIZED POLARIZATION OF NEUTRAL AND CHARGED LIPOSOMES

DMPC liposomes

GP evolution *vs*. temperature for a different number of heating-cooling cycles is shown in Fig. 2.



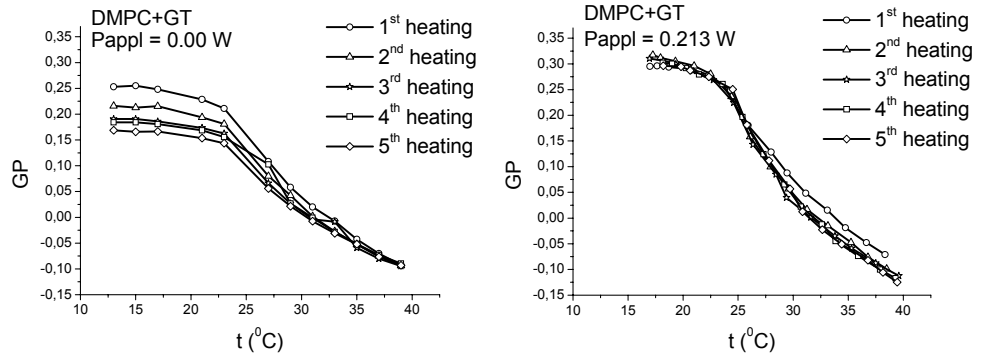
a) b)

Fig. 2. Evolution of GP thermal behavior of DMPC liposomes with the number of heating-cooling cycles; a) no RF field applied, b) under 213 mW RF field exposure.

It may be observed that at temperatures lower than the phase transition temperature, corresponding to the gel-phase of the membrane (left side of curves in Fig. 2a), the *GP* decreases with the number of heating-cooling cycles, while for temperatures higher than T_c (liquid crystalline state), the *GP* at given temperature does not vary with the number of cycles (right side of same curves).

This result may be explained by the membrane disorganization during consecutive heating and cooling, which become visible in the more structured, "gel" phase, but is not seen in the "liquid" crystalline state, in which the membrane is more fluid anyway.

Exposing DMPC liposomes to microwave fields does not change the described trend of vesicles behavior, but the *GP* variation with the number of thermal cycles becomes smaller (Fig. 2b, left side of curves).



a) b)

Fig. 3. Evolution of GP thermal behavior of DMPC liposomes with the number of heating-cooling cycles in presence of gentamicin: a) no RF field applied, b) under 213 mW RF field exposure.

Adding the polycationic molecules of gentamicin to the DMPC liposome suspension keeps the same *GP* trend with the number of heating-cooling cycles (Fig. 3a, left side of curves), but when the RF field is applied, the liposomes become apparently stabilized and there is no more variation of *GP* with the number of thermal cycles (Fig. 3b), at least in the gel phase of the membrane.

There is obviously an interaction of the RF field with the charged antibiotic molecules which, in some way, prevent the thermal disordering of the DMPC bilayer.

DMPC + CL liposomes

The negatively charged DMPC + CL liposomes show the same thermal evolution of *GP* with the number of cycles as the neutral vesicles (Fig. 4a) when no field is applied. However, the application of microwave fields leads to a pronounced variation of *GP* with the number of cycles in the $t < T_c$ temperature domain (Fig. 4b), which was not the case of pure DMPC vesicles (Fig. 2b).

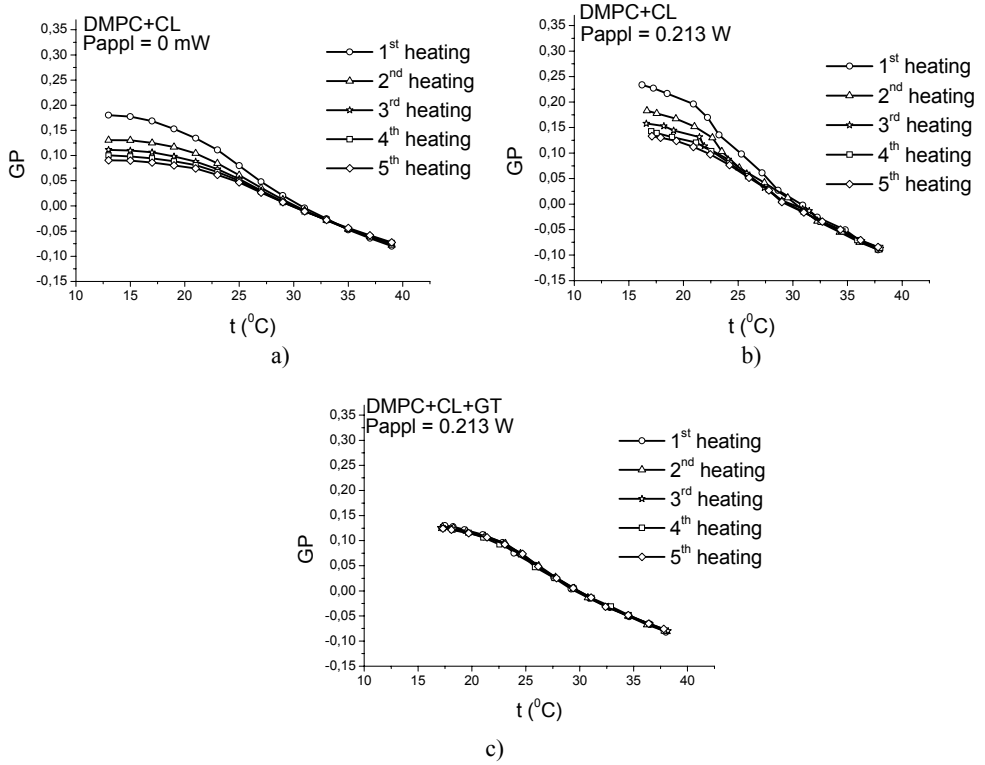


Fig. 4. GP variation of DMPC + CL liposomes with temperature for five heating-cooling cycles: a) no RF field applied, b) exposure to 213 mW RF field, and c) gentamicin incubated liposomes exposed to 213 mW RF field.

This may be explained by a strong interaction of the RF field with the negatively charged molecules of CL which leads to a lipid bilayer disorganization

accompanied by water penetration (hydration). When gentamicin is added, any difference in *GP* profiles between thermal cycles disappears (Fig. 4c). The hydration effect due to the negative charges of CL is compensated by the action of the RF field and the charged antibiotic is acting as on a "neutral" vesicle membrane (similar to the behavior of pure DMPC liposomes shown in Fig. 3b).

VARIATION OF T_C WITH THERMAL CYCLING IN PRESENCE OF GENTAMICIN AND RADIOFREQUENCY FIELD

The critical transition temperatures (T_c) were determined for each experiment as inflexion points of the *GP versus* temperature curves (fitted with a sigmoid Boltzmann type function).

Figure 5a shows that at all powers of RF field applied, the neutral (DMPC) liposomes are stable (in the sense that there is no variation of critical transition temperature with five heating-cooling cycles).

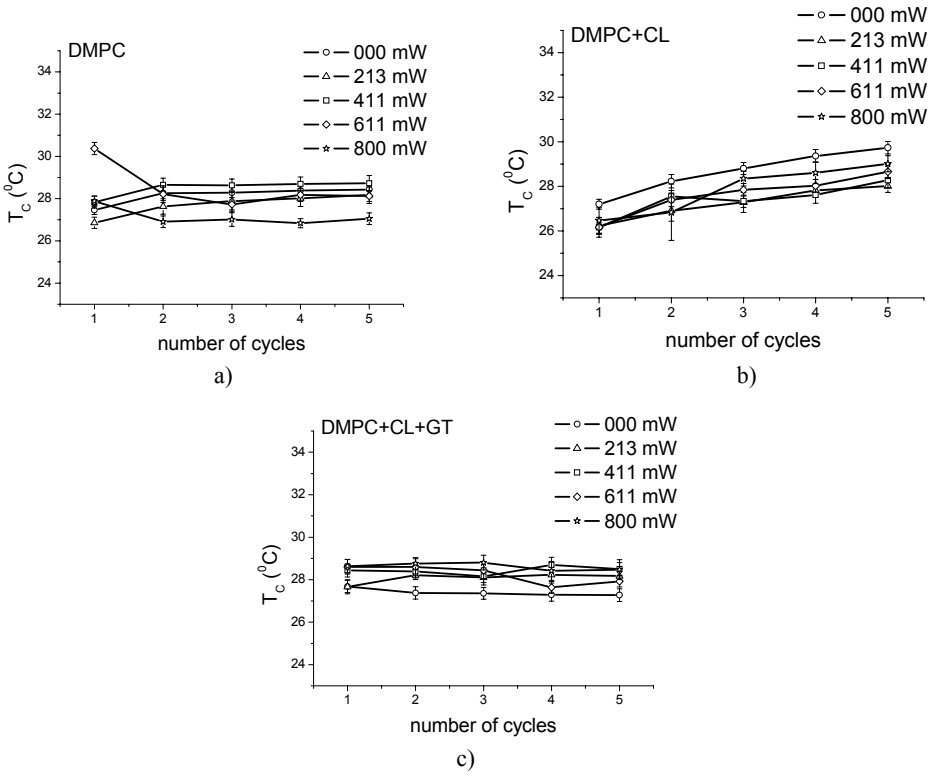


Fig. 5. Variation of critical transition temperature (T_c) of: a) pure DMPC liposomes, b) DMPC + CL liposomes, and c) DMPC + CL liposomes in the presence of gentamicin. Variation of T_c with the number of heating-cooling cycles is plotted for applied RF powers of: 0 (control), 213, 411, 611 and 800 mW.

In the negatively charged (DMPC + CL) liposomes, T_c increases with thermal cycling, suggesting a progressive raise of the vesicles' thermal resistance with the number of heating and cooling cycles (Fig. 5b) at all intensities of the applied RF field.

The presence of the charged antibiotic seems to compensate the cardiolipin charges, annihilating the increase of T_c with the number of thermal cycles, and bringing the behavior of the liposomes suspension in microwaves field to the picture observed in neutral (DMPC) liposomes (Fig. 5c).

CONCLUSIONS

Liposomes change their properties depending on the chemical and physical environment. One of the most important properties of lipid vesicles used as drug carriers is their stability. In our investigation, we evaluated the membrane stability of neutral and charged DMPC liposomes by computing the membrane *GP*, based on laurdan emission spectra, recorded in the presence of a charged antibiotic (gentamicin) and under the action of microwaves field of various powers.

It appeared that the presence of the charged antibiotic (gentamicin polycation) modulates dramatically the thermal resistance of the DMPC liposomes, especially in the presence of cardiolipin. While in the negatively charged liposomes (DMPC + CL), the T_c increases regularly with progressive thermal cycling, pointing to a membrane reorganization which leads to the bilayer stability; the addition of gentamicin annihilates this trend, keeping the T_c at constant level. This trend is also present if radiofrequency fields of various powers were applied.

These findings may serve as a guide for a rational design of lipid vesicles as drug carriers in the special environment of the patients who undergo clinical investigation and therapy.

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