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Interactions of fenspiride- and azithromycin-loaded liposomes with model lipid membranes: calorimetric studies in kinetic regime

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Differential scanning calorimetry (DSC) technique was applied to study interactions of drug-loaded liposomes with model lipid membrane. Two types of drugs were studied, namely, water-soluble one (anti-inflammatory drug fenspiride, FS) and lipid-soluble one (antibiotic azithromycin, AZ) with different mechanisms of drug-membrane interactions. Drug transfer from the liposomes based on L-α-dipalmitoylphosphatidylcholine to L-α-dimirystoylphosphatidylcholine membranes was monitored by analyzing DSC profiles of the systems obtained in consecutive scans. It was shown that FS-loaded liposomes have weaker membranotropic effect as compared to FS water solution of equal FS concentrations. For both FS and AZ, relatively fast drug transfer from liposomes into model membrane was accompanied by slower lipid exchange between these structures. Similar transfer rates were shown for both drugs despite the fact that AZ transfer is mainly membrane-mediated, whereas FS transfer is primarily water-mediated.

Keywords: fenspiride, azithromycin, liposomes, model lipid membranes, drug-membrane interactions, differential scanning calorimetry.

Взаємодія ліпосом, що містять фенспірид та азитроміцин, з модельними ліпідними мембранами: калориметричні дослідження у кінетичному режимі. *О.В.Ващенко, Н.О.Касян, Л.В.Будянська, Л.М.Лисецький*

Методом диференціальної скануючої калориметрії (ДСК) досліджено взаємодію ліпосом, що містять лікарську речовину, з модельними ліпідними мембранами. Обрано лікарські речовини двох типів, які мають різні механізми взаємодії з мембраною: водорозчинна (протизапальна речовина фенспірид) та жиророзчинна (антибіотик азитроміцин). Використано ліпосоми на основі L-α-дипальмітоїлфосфатидилхоліну та мультибішарові модельні мембрани L-α-димиристоїлфосфатидилхоліну. Процес переходу лікарської речовини з ліпосом до мембрани відстежували за змінами ДСК-профілей системи ув послідовних циклах сканування. Показано, що ліпосоми з фенспіридом мають слабкішу мембранотропну дію порівняно з водним розчином такої ж концентрації. Процес відносно швидкого вивільнення лікарських речовин із ліпосом і зв'язування з ліпідною мембраною відбувався на тлі більш повільного обміну ліпідами між цими структурами. Для обох лікарських речовин спостерігалися близькі швидкості переходу, незважаючи на те, що для азитроміцину цей процес опосередкований, головним чином, ліпідним середовищем, а для фенспіриду – і водним середовищем.

Методом дифференциальной сканирующей калориметрии (ДСК) исследовано взаимодействие липосом, содержащих лекарственное вещество, с модельными липидными мембранами. Выбраны лекарственные вещества двух типов, имеющие различные механизмы взаимодействия с мембраной: водорастворимое (противовоспалительное вещество

фенспирид) и жирорастворимое (антибиотик азитромицин). Использованы липосомы на основе L-α-дипальмитоилфосфатидилхолина и мультибислойная модельная мембрана L-α-димиристоилфосфатидилхолина. Процесс перехода лекарственного вещества из липосом в мембрану отслеживали на основании изменения ДСК-профилей системы в последовательных циклах сканирования. Показано, что липосомы с фенспиридом обладают более слабым мембранотропным действием по сравнению с водным раствором такой же концентрации. Процесс относительно быстрого выхода лекарственных веществ из липосом и связывания с липидной мембраной происходил на фоне более медленного обмена липидами между этими структурами. Для обоих лекарственных веществ наблюдались близкие скорости перехода, несмотря на то, что для азитромицина этот процесс опосредован, главным образом, липидной средой, а для фенспирида – водной средой.

1. Introduction

Differential scanning calorimetry (DSC) is considered as a powerful tool for biomedical investigation of biological and model lipid membranes [1–6]. Alongside with numerous studies of drug-membrane interactions [7–12] and characterization of drug-delivery systems [13, 14], kinetics of drug-membrane interactions could be also explored by DSC technique [9, 15–20]. Moreover, correlations are searched and attempts are launched to use certain DSC parameters (e.g. phase transitions temperatures and enthalpies) for prediction of drug permeation [9, 21]. This would reduce the number of time-consuming and expensive permeation experiments for development of the optimal drug-carrier systems.

In [9, 15, 21] a detailed procedure of application of DSC technique for evaluation of the drug release from delivery systems to model biomembranes was described. Briefly, various drug-loaded delivery systems (solid lipid nanoparticles, polymer micelles, liposomes, etc.) were incubated with model lipid membranes (unilamellar or multilamellar liposomes). Corresponding changes in DSC profiles were considered and interpreted as kinetics of drug transfer into biomembrane models. The effects were compared to those of unloaded carriers and of "free" drugs.

In accordance with the described approach, in this work we studied interactions between drug-loaded unilamellar vesicles (liposomes) and multilamellar model membrane. Two types of drugs were studied, namely, water-soluble one (anti-inflammatory drug fenspiride, FS) and lipid-soluble one (antibiotic azithromycin, AZ) with different mechanisms of drug-membrane interactions. For lipid-soluble drugs, the transfer process is mainly membrane-mediated, whereas for water-soluble ones, both membrane-mediated and water-mediated mechanisms could occur. Membranotropic effects of FS and AZ were probed earlier in equilibrium studies [22–24], where it was shown that both drugs decreased phase transitions temperatures of model biomembranes.

In order to monitor changes both in liposomes and in model membranes, we used different types of lipids. Liposomes were based on L-α-dipalmitoylphosphatidylcholine (DPPC), whereas L-α-dimirystoylphosphatidylcholine (DMPC) was used for membrane preparation. These two phospholipids are structurally rather similar: they have the same polar heads and differ in the hydrocarbon chain length by two methylene groups. The melting temperatures of these lipid structures differ by ~ 18°C (42.0°C for DPPC and 24.2°C for DMPC), allowing us to distinguish the phase transitions peaks for liposomes and for model membranes on DSC thermograms. This makes it possible to discern between two processes: (1) drug release from the liposomes and (2) drug accumulation into the model membrane. The composition of the liposomes used was developed to provide opposite membranotropic effects of the drug and the lipid constituents. This gives us an opportunity to trace transfer of each constituent into the membrane. Such approach could allow us to reveal some new features of drug-membrane interactions which are significant in drug delivery process.

2. Experimental

2.1. Samples preparation

Multilamellar model lipid membranes were obtained from hydrated L-α-dimirystoylphosphatidylcholine (DMPC). DMPC powder was put in a glass vial and mixed with bidistilled water in proportion 2:3 w/w. The samples were stored in a cool place for 2–3 days, with periodical heating and intensive stirring. Completeness of hydration was verified by correspondence of the DMPC membrane phase transition temperatures to literature data.

Unilamellar liposomes were prepared from hydrated L-α-dipalmitoylphosphatidylcholine (DPPC) or DPPC mixture with bovine cardiolipin sodium salt (CL) in proportion 19:1 w/w. Lipids were placed in a glass

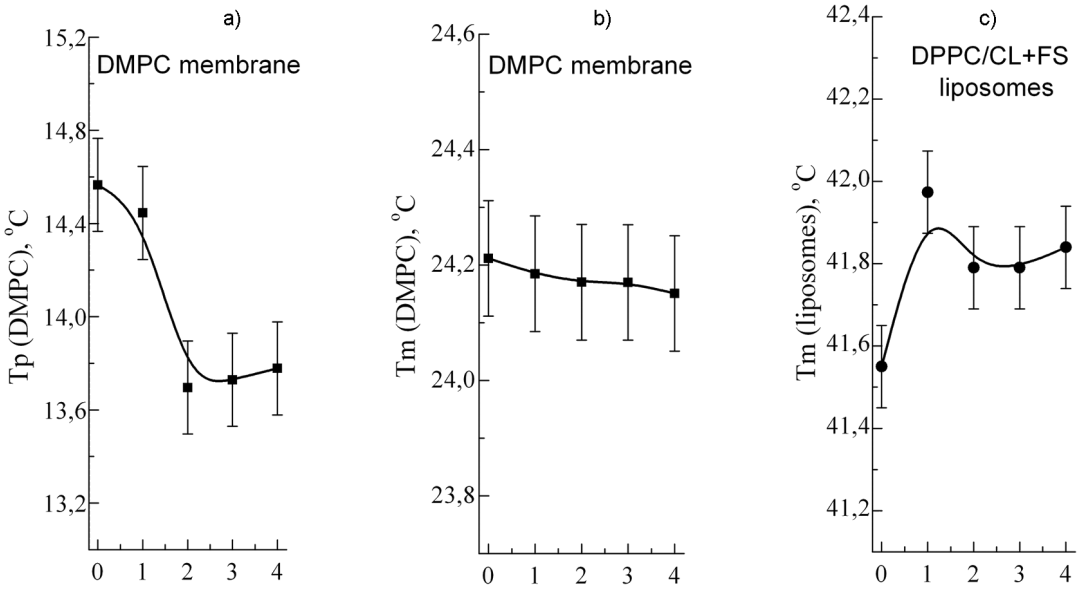

Fig. 1. Changes of the phase transitions temperatures after addition of DPPC/CL + FS liposomes to DMPC membranes: a) — pre-transition of DMPC membrane, b) — main phase transition of DMPC membrane; c) — main phase transition of DPPC/CL + FS liposomes. Numbers of DSC scans are marked on the abscissa axis (0 — corresponding phase transition temperatures before mixing).

vial and dissolved in ethanol. Solvent evaporation was performed with a concentrator "Concentrator Plus" (Eppendorf). Dry lipid films were hydrated with bidistilled water in lipid : water proportion 1:99 w/w. Than the systems were incubated at room temperature for 2–3 h, with periodical heating and intense shaking. The hydration process was verified by DSC technique, similarly to multilamellar membranes. A mini-extruder (Avanti Polar Lipids, USA) with 100 nm pores polycarbonate membrane was used to obtain unilamellar liposomes.

Fenspiride-loaded liposomes (DPPC/CL+FS liposomes) were prepared by passive loading technique [25] using FS water solution (0.05 % w/w). The resulting lipid:FS ratio was 19:1 w/w.

Azithromycin-loaded liposomes (DPPC+AZ liposomes) were prepared by passive loading technique [25]. Dry DPPC powder was mixed with AZ powder in weight proportion DPPC:AZ 19:1 and dissolved in excess of ethanol. Further procedure was analogous to unilamellar liposomes preparation.

Reference system (DMPC membrane with DPPC+AZ liposomes) was prepared in "equilibrium" regime. DMPC to DPPC ratio was 4:1 w/w. DPPC+AZ liposomes were incubated at room temperature together with DMPC membranes for 1 day with periodic heating to ~ 55°C and intense stirring. It is assumed that this procedure increases probability and intensity of interactions of the liposomes with the model lipid membranes and therefore the reference system mimics equilibrium conditions.

2.2 Kinetic study of the drug-membrane interactions using DSC technique

Differential scanning calorimetry (DSC) studies were performed using a microcalorimeter Mettler DSC 1 (Mettler Toledo). DMPC model membrane and the liposomes (drug-loaded or blank) were placed into an aluminum crucible and sealed without sample stirring. The weight ratios of DMPC to DPPC (or DPPC/CL) were 16:1 in the systems with FS and 4:1 in the systems with AZ. The resulting drug contents were 0.3 % w/w for FS and 1.25 % w/w for AZ with respect to the lipid content. DSC thermograms were recorded in consecutive scans in heating mode (scanning rate 1 K/min, temperature range 0–70°C). The samples were cooled to 0°C between the scans. Time interval between two adjacent scans was ~ 75 min. The procedure was repeated 4–5 times. Kinetics of drug-membrane interactions was monitored *via* changes of DSC peaks of the main phase transition and pre-transition for both the membranes and the liposomes. Experimental error was ±0.1°C for the main phase transition temperature and ±0.2°C for the pre-transition temperature.

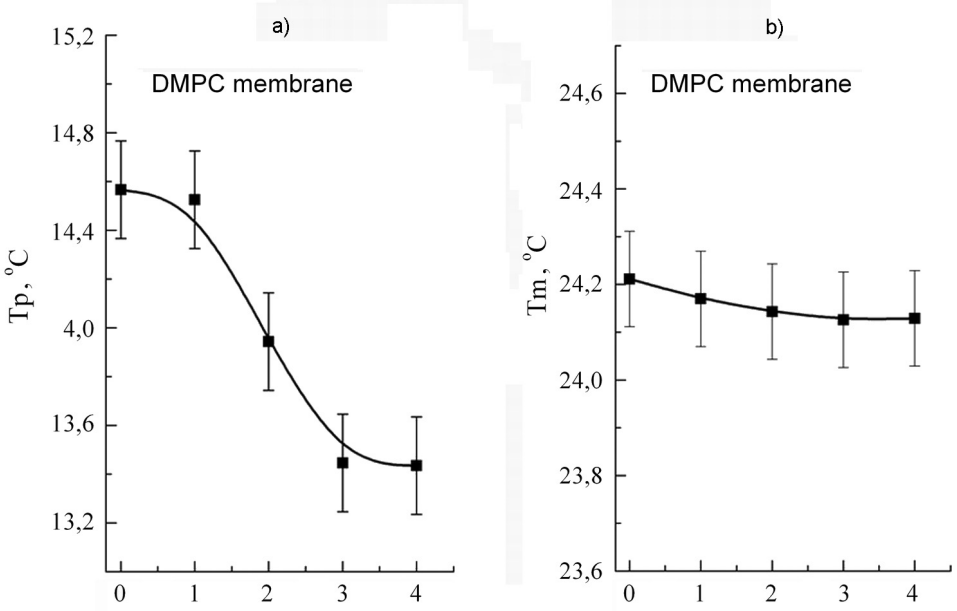

Fig. 2. Changes of the phase transitions temperatures after addition of FS water solution (0.05 % w/w) to DMPC membranes: a) — pre-transition of DMPC membrane, b) — main phase transition of DMPC membrane. Numbers of DSC scans are marked on the abscissa axis (0 — corresponding phase transition temperatures before mixing).

3. Results and discussion

3.1 Fenspiride-loaded liposomes

Interaction of the dispersion of DPPC/CL + FS liposomes with multilamellar DMPC membranes was studied by monitoring changes of their DSC profiles in real time regime. The DSC profiles contained three peaks of thermotropic phase transitions: 1) pre-transition of DMPC membrane (T_p = 14.6°C), 2) main phase transition, or melting, of DMPC membrane (T_m = 24.2°C) and 3) main phase transition of DPPC/CL + FS liposomes (T_m = 41.6°C). After liposomes addition, T_p of DMPC membrane drops by 0.8–0.9°C in two first scans and then remains almost unchanged, Fig. 1a; T_m has subtle tendency to decrease with time within the experimental error, Fig. 1b. Opposite changes are observed for DPPC/CL + FS liposomes: T_m sharply increases on ~ 0.4°C in the first scan, slightly decreases (on ~ 0.2°C) in the second one and than remains almost unchanged, Fig. 1c.

It should be noted that there are two forms of the drug in DPPC/CL + FS liposomes: "entrapped" FS (enveloped in the inner water environment and associated with liposomal membrane) and "free" FS (in water solution). In the resulting system, FS redistributes between DMPC, DPPC/CL and water phase.

To get more insight into the processes, simpler cases were considered:

– changes in DSC profiles upon addition of FS water solution (without liposomes) to DMPC membrane, Fig. 2;

– changes in DSC profiles upon addition of DPPC/CL liposomes (without FS) to DMPC membrane, Fig. 3.

Upon addition of FS water solution to DMPC membrane, decrease of T_p up to 1.1°C are observed in the second and the third scans, Fig. 2a. The values of T_m of DMPC membrane remain almost unchanged within the experimental error, Fig. 2b. Thus, changes of the phase transitions of the DMPC membranes have the same character upon addition either FS water solution or FS-loaded liposomes, Fig. 4. Obviously, the total FS concentration in the systems is too low to cause substantial T_m changes of DMPC membrane, Fig. 1b, Fig. 2b, but it is sufficient enough to decrease T_p, Fig. 1a, Fig. 2a.

Introduction of blank DPPC/CL liposomes (without FS) caused negligible effects both on T_p and T_m of DMPC membrane, Fig. 3. Therefore T_p changes (Fig. 1, Fig. 2) take place only due to the effect of "free" FS. Gradual decrease in T_m of DPPC/CL liposomes is observed under subsequent scans, Fig. 3c. This could result from immediate liposomes-membrane interaction and diffusion of DMPC molecules from the membrane to DPPC/CL liposomes.

Comparative changes of T_p of DMPC membrane upon addition of FS water solution ("free" FS) and FS-loaded liposomes

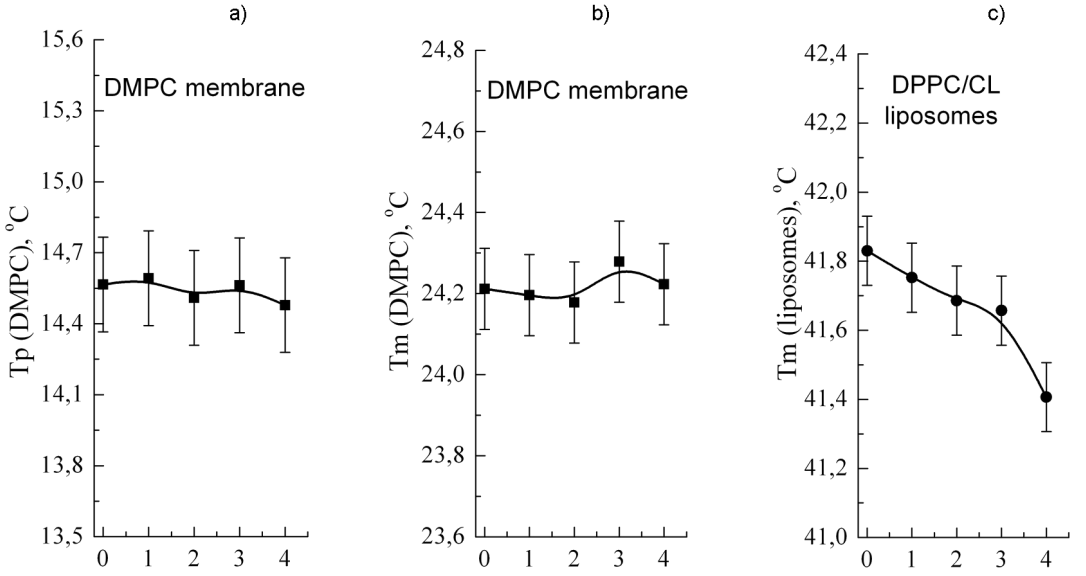

Fig. 3. Changes of the phase transitions temperatures after addition of DPPC/CL liposomes to DMPC membranes: a) — pre-transition of DMPC membrane, b) — main phase transition of DMPC membrane; c) — main phase transition of DPPC/CL liposomes. Numbers of DSC scans are marked on the abscissa axis (0 — corresponding phase transition temperatures before mixing).

("free+entrapped" FS) containing identical amount of the drug are shown in Fig. 4. The membranotropic effect for "free" FS, as compared to "free+entrapped" FS, becomes more pronounced with time. This may indicate that certain amount of FS remains associated with liposomal membrane or enveloped in liposomal water interior, thus it does not interact with DMPC membrane. Noticeable elevation of T_m of FS-loaded liposomes immediately after their addition to DMPC membrane, Fig. 1c, is caused due to decrease of total FS concentration in the system (dilution). Further decrease in T_m value of DPPC/CL + FS liposomes could be related with diffusion of DMPC molecules into the liposomes which bears witness to immediate interaction of these structures [18–20]. Thus, FS transfer from the liposomes to the membrane is coupled with diffusion of lipid molecules.

3.2 Membranotropic action of liposomes loaded with azithromycin (AZ)

Because of substantial lipophylicity of AZ (log$P$ = 4.0), it has preferential localization in the membranes hydrophobic core, so the majority of the drug molecules are "liposome-entrapped", with negligible portion of "free" AZ.

Kinetic study of the DSC profiles for DMPC membrane and DPPC+AZ liposomes were performed as in section 3.1. Fig. 5 demonstrates phase transitions tempera-

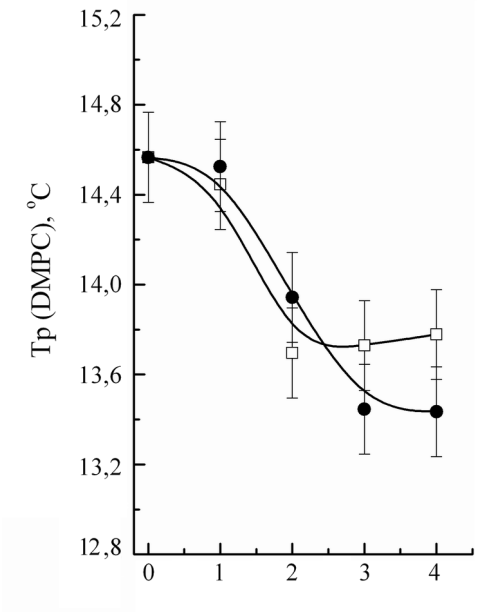

Fig. 4. Comparative changes of T_p of DMPC membrane upon addition of FS water solution (filled circles) and DPPC/CL + FS liposomes (open squares). Numbers of DSC scans are marked on the abscissa axis (0 — corresponding phase transition temperatures before mixing).

tures of DPPC + AZ liposomes and DMPC membranes obtained in subsequent scans after mixing. Data for the reference ("equilibrium") system are plotted (points "R" in Fig. 5), which reflect further trends of T_p and T_m changes after a long period of time.

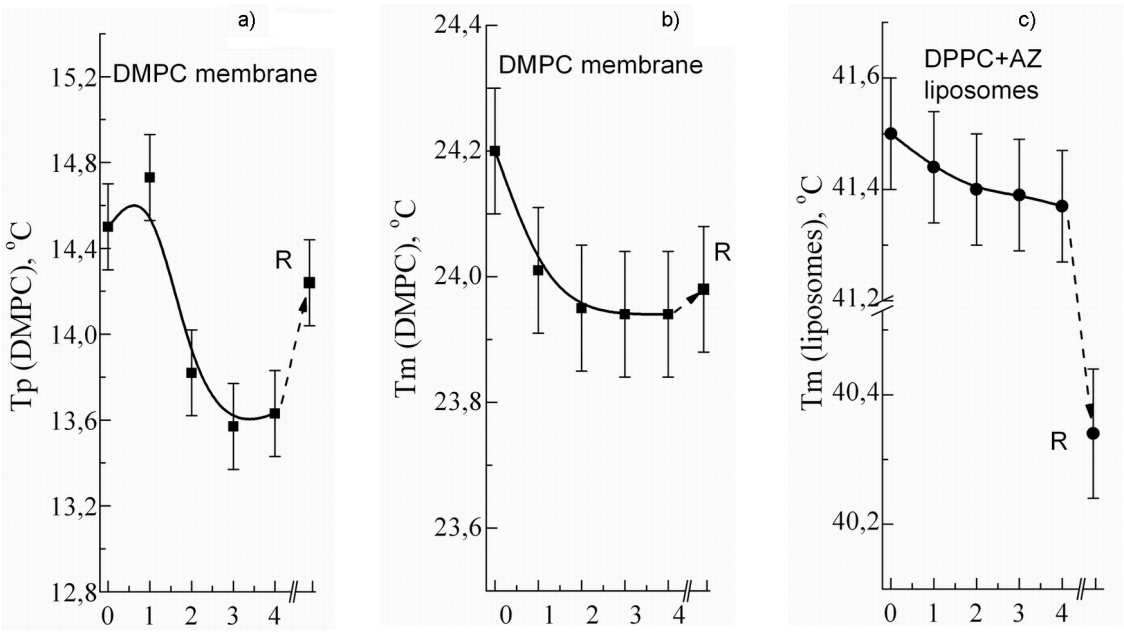

Fig. 5. Changes of the phase transitions temperatures after addition of DPPC + AZ liposomes to DMPC membranes: a) — pre-transition of DMPC membrane, b) — main phase transition of DMPC membrane; c) — main phase transition of DPPC + AZ liposomes. Numbers of DSC scans are marked on the abscissa axis (0 — corresponding phase transition temperatures before mixing). R — corresponding phase transition temperatures for the reference system.

Changes of the phase transition temperatures after mixing indicate interactions between the lipid structures. Simultaneous decrease of T_m and T_p of DMPC membrane obviously reflects AZ inclusion into DMPC membrane. Shift of T_p is more pronounced and reaches ~ 1°C after 3 scans. Total AZ concentration after mixing is ~ 1 % w/w with respect to the lipid content (DMPC + DPPC). The observed shifts are in qualitative and quantitative agreement with membranotropic action of AZ obtained in equilibrium conditions [22, 23]. This indicates that AZ readily and uniformly distributes between DMPC and DPPC lipids. The maximal AZ effect is observed already in 2–3 scans, similarly to FS (see Fig. 1a), that evidences similar transfer rates for these drugs. This finding is rather intriguing taking into account that AZ transfer process is mainly membrane-mediated, whereas for FS, water-mediated mechanism primarily takes place.

At the same time, T_m of DPPC liposomes remains almost unchanged, with slight tendency to decrease, Fig. 5c, coupled with pronounced broadening of the peak. This result is unexpected because AZ release from DPPC liposomes would cause rise of their T_m. So, we suppose that the membranotropic effect of AZ is overlapped by diffusion of DMPC molecules into the liposomes. Further trends of changes of phase transition temperatures can be predicted by considering the reference system (R). For the liposomes, T_m continues to decrease, Fig. 5c, whereas for DMPC membrane, T_m remains almost unchanged, Fig. 5b. Increase of T_p by ~ 0.6°C is observed for DMPC membrane, but it doesn't reach the corresponding value of pure DMPC membrane, Fig. 5a. Thus, the observed trends indicate mutual diffusion of DMPC and DPPC lipids. Calorimetric evidences for this process are (1) broadening of the corresponding DSC peaks and (2) their approach on the temperature scale. On the background of mutual lipids diffusion, the membranotropic effect of AZ is poorly noticeable. Thus, together with relatively fast drug release from liposomes into model lipid membrane, slower diffusion-driven lipids exchange between these structures can be noted.

4. Conclusions

Interactions of FS- or AZ-loaded liposomes with model lipid membranes were studied by DSC technique in real time regime. Analysis of DSC profiles of both model lipid membranes and liposomes allowed us to reveal important features of drug-membrane interactions which could be significant in drug delivery processes:

Membranotropic effect of FS in water solution ("free") appeared to be stronger than that for FS in liposomal dispersion ("free+entrapped") for equal FS concentrations indicating that certain amount of FS remains bounded to liposomes, thus not interacts with DMPC membrane.

Similar transfer rates were shown for lipid-soluble AZ and water-soluble FS despite the fact that AZ transfer is mainly membrane-mediated, whereas FS transfer is primarily water-mediated.

Relatively fast drug release from liposomes into model membrane has been observed accompanied by slower diffusion of lipids between these lipid structures.

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