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Lipid transfer between cationic vesicles and lipid–DNA lipoplexes: Effect of serum

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Abstract

Differential scanning calorimetry was used to examine the lipid exchange between model lipid systems, including vesicles of the cationic lipoids ethyldimyristoylphosphatidylcholine (EDMPC), ethyldipalmitoylphosphatidylcholine (EDPPC) or their complexes with DNA (lipoplexes), and the zwitterionic lipids (DMPC, DPPC). The changes of the lipid phase transition parameters (temperature, enthalpy, and cooperativity) upon consecutive temperature scans was used as an indication of lipid mixing between aggregates. A selective lipid transfer of the shorter-chain cationic lipoid EDMPC into the longer-chain aggregates was inferred. In contrast, transfer was hindered when EDMPC (but not EDPPC) was bound to DNA in the lipoplexes. These data support a simple molecular lipid exchange mechanism, but not lipid bilayer fusion. Exchange via lipid monomers is considerably more facile for the cationic ethylphosphatidylcholines than for zwitterionic phosphatidylcholines, presumably due to the higher monomer solubility of the charged lipids. With the cationic liposomes, lipid transfer was strongly promoted by the presence of serum in the dispersing medium. Serum proteins are presumed to be responsible for the accelerated transfer, since the effect was strongly reduced upon heating the serum to 80 °C. The effect of serum indicates that even though much lipoplex lipid is inaccessible due to the multilayered structure, the barrier due to buried lipid can be easily overcome. Serum did not noticeably promote the lipid exchange of zwitterionic liposomes. The phenomenon is of potential importance for the application of cationic liposomes to nonviral gene delivery, which often involves the presence of serum in vitro, and necessarily involves serum contact in vivo.
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Keywords: Liposome; Lipid exchange; cmc; Monomer; Cationic lipid; Serum

1. Introduction

Lipid vesicles have been regularly and successfully used as experimental biomembrane models. The exchange of lipid molecules between membranes is a common process of significant importance to a proper functioning of cells and organisms. The mechanisms of intracellular lipid transfer are manifold, including membrane fusion, spontaneous redistribution between membranes by lipid monomer equilibration through aqueous phase, molecular transfer during collision, and redistribution controlled by lipid transfer proteins. Membrane fusion has been widely discussed and recognized as an important biological event for such key cell functions as compartmentalization, endocytosis, secretion, synaptic transmission, etc. [1]. Most membrane lipids, including phospholipids and sterols, have a finite, low monomer concentration in the aqueous phase. By being in equilibrium with the interface, monomer lipids have a tendency to redistribute spontaneously between membranes [2–10]. In general, spontaneous transfer of natural phospholipids by monomer diffusion proceeds very slowly and is expected to be of little consequence to intercellular lipid transfer [6].

The usual approach to lipid mixing includes preparation of molecular mixtures in organic solvent, with subsequent solvent removal and hydration. This procedure is believed to produce essentially equilibrium mixtures, but it is not quite biologically relevant, since in biological systems, lipid exchange can take place in aqueous medium.

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Because of their potential application in gene therapy and drug delivery applications [11,12], positively charged lipoids are now being intensively synthesized and studied (the term 'lipoid' is appropriate since 'lipid' refers to natural products, whereas 'lipoid' is used to molecules that are similar to lipids in structure and in properties but not natural products). However, comprehensive knowledge on the physico–chemical characteristics of their aggregates is still deficient. Among the numerous cationic lipoids synthesized and tested recently, phosphatidylcholine triesters (Fig. 1) [13–15] are the only membrane lipid derivatives shown to be metabolized by cells. They are thus considered promising nonviral transfection agents. These compounds are chemically stable, hydrate well and form uni- or oligolamellar liposomes. The saturated ethyl-phosphatidylcholines have been shown to exhibit gel–liquid crystalline phase transitions at temperatures close to those of the parent phosphatidylcholines [13,16–18].

Here, we describe lipid transfer in mixtures of cationic phospholipid (ethylphosphatidylcholine) dispersions. We found that monomer molecular exchange through the aqueous phase represents the major mechanism of transfer between aggregates; furthermore, lipid mixing was strongly promoted by the presence of serum in the dispersing medium.

2. Materials and methods

2.1. Lipids

The cationic phospholipids were the following: dimyristoyl- and dipalmitoyl-*O*-ethylphosphatidylcholine (EDMPC and EDPPC, respectively), as triflate salts [13] or chloride salts [Avanti Polar Lipids]. The zwitterionic phospholipids were the following: dimyristoyl- and dipalmitoylphosphatidylcholine (DMPC and DPPC, respectively) [Avanti Polar Lipids].

Dispersions of the pure cationic lipoids were prepared in water by vortexing at a temperature above their gel–liquid crystalline phase transition. Samples of 2–5 mM concentrations were routinely used; for some control measurements, 1 mM and 10 mM lipid concentrations were also tested. These lipoids spontaneously generate unilamellar (or oligolamellar) liposomes, with a mean diameter of ~200 nm, as estimated by dynamic light scattering [13]. Dispersions were optically clear; short sonication did not change their calorimetric behavior. In order to prepare similar unilamellar vesicle dispersions of the zwitterionic DMPC and DPPC, they were additionally subjected to bath-sonication in the liquid crystalline phase for 10 min (until optical clarity). To some samples, herring sperm DNA [Invitrogen] (at 1:1 charge ratio) and/or fetal bovine serum [Gibco] (20 vol.%) were added at a temperature above their gel–liquid crystalline phase transition 1 h prior to the measurements.

Dispersions of different lipids and/or lipoplexes were mixed at low temperatures (in the gel phase), at equimolar lipid ratio, and immediately loaded into the calorimeter cell, precooled to 5 °C.

Samples for the construction of EDMPC/EDPPC phase diagram were prepared from mixtures of EDMPC and EDPPC as chloroform solutions; after solvent removal with a stream of argon and subsequent vacuum treatment, the samples were hydrated at 50 °C.

2.2. DSC measurements

Repeated heating and cooling scans were performed with the samples mixed as aqueous dispersions, using a VP-DSC Microcalorimeter (MicroCal Inc., Northampton, MA) at 30 °C/h over the temperature interval 5–50 °C, unless

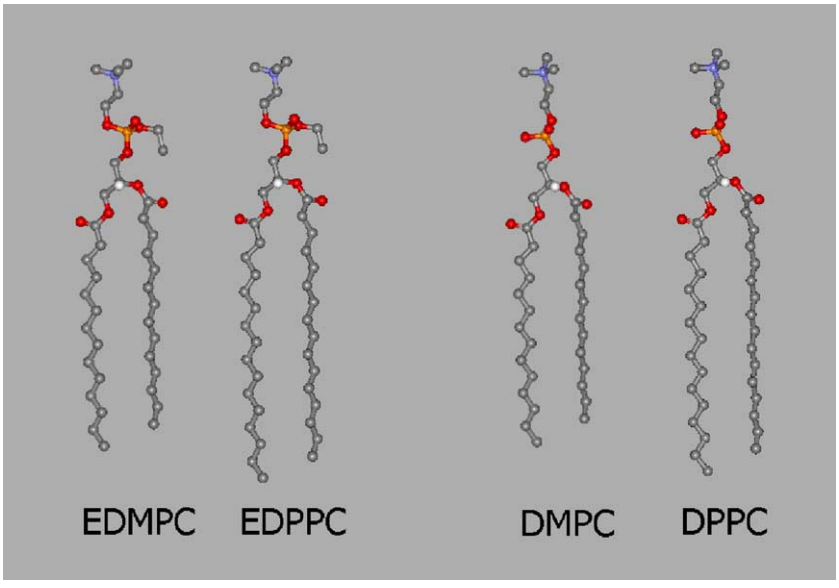

Fig. 1. Molecular structures of the cationic and zwitterionic phospholipids studied.

otherwise indicated (thus, ~3 h elapsed between the beginnings of sequential heating scans). Thermograms were analyzed using OriginLab (Northampton, MA) software. The onset and the completion temperatures of the phase transitions needed for the construction of the phase diagram were determined by the intersections of the peak slopes with the baseline on the thermograms. The maximums of the heat capacity curves were taken as the phase transition temperatures of the pure components. For asymmetric or split line shapes of the composite aggregates, the transition temperature was defined as that temperature at which the area of the endotherm was divided into equal halves.

3. Results and discussion

3.1. EDMPC/EDPPC phase diagram: equilibrium mixing

The ethylated cationic phosphatidylcholine derivatives, EDMPC and EDPPC (molecular structures shown in Fig. 1, left), exhibit virtually unchanged gel–liquid crystalline transition temperatures relative to the parent phosphatidylcholines, DMPC and DPPC—at 24 °C and 41 °C for EDMPC and EDPPC, respectively [13,16,18,19]. The presence of DNA does not significantly change these transition temperatures [13,16]; neither does the presence of 20 vol.% serum.

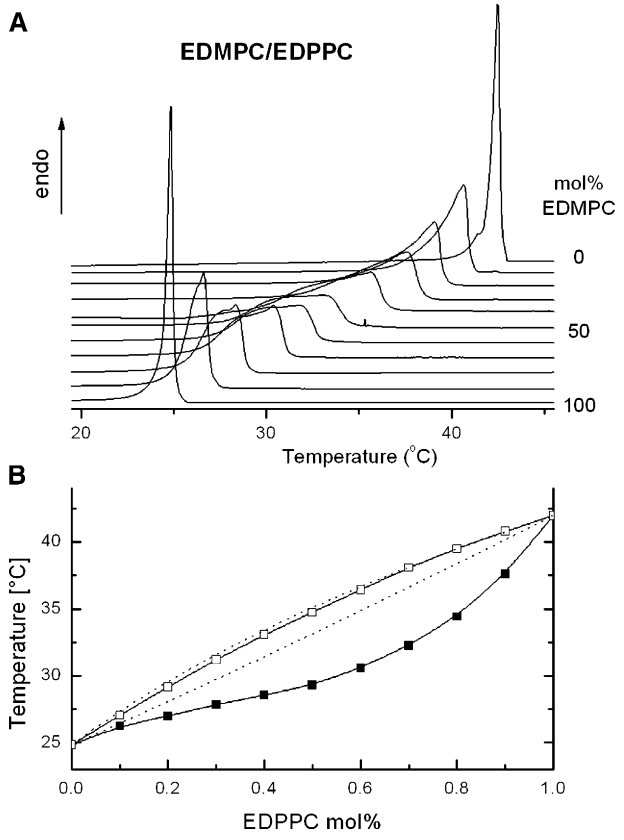

Fig. 2. (A) Heating thermograms of the dispersions of the EDMPC/EDPPC mixtures at different ratios, mixed as from chloroform solutions, subsequently dried and hydrated; heating rate, 30 °C/h. (B) Phase diagram constructed from the calorimetric data: full squares—transition onset; open squares—transition end; the phase diagram for the ideal mixture is shown with dotted line.

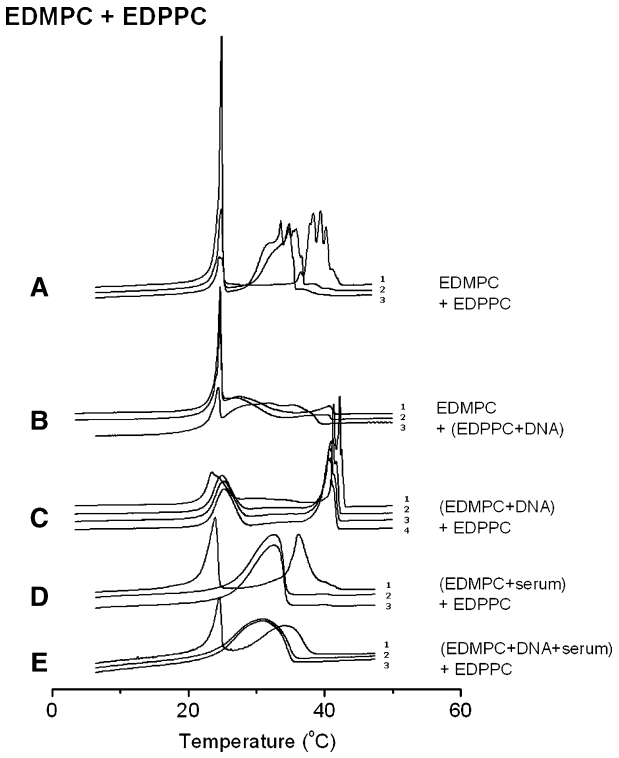

Fig. 3. Consecutive heating thermograms of mixtures of EDMPC and EDPPC dispersions, prepared separately, mixed extempore at 5 °C at equimolar lipid ratio, immediately filled into the calorimetric cell at that temperature and scanned at 30 °C/h between 5 and 50 °C (thus, 3 h elapse between the onset of the sequential heating scans): (A) dispersions of EDMPC and EDPPC; (B) dispersions of EDMPC and EDPPC/DNA lipoplexes; (C) dispersions of EDMPC/DNA lipoplexes and EDPPC; (D) dispersion of EDMPC with 20 vol% serum and EDPPC; (E) dispersion of EDMPC/DNA lipoplexes with 20 vol% serum and EDPPC.

In mixtures, transition temperatures are informative about the mode of mixing (i.e., the homogeneity of the mixture) and depend on composition. For miscible components, the transition temperature can be used as a measure of composition. We tested the miscibility of the cationic EDMPC and EDPPC by recording temperature scans of samples of different composition (premixed in organic solvent, dried, and subsequently hydrated) and constructing their phase diagram. Similarly to the parent phosphatidylcholines [20], they were found to exhibit near-ideal mixing (Fig. 2). With change of composition, the transition temperature of the mixed samples gradually shifted between the transition temperatures of the pure components. Thus, transition temperature is indicative of the composition of EDMPC/EDPPC samples, and we further used it to assess the composition of the lipid aggregates in the following experiments.

3.2. Mixtures of cationic lipoid dispersions

When EDMPC and EDPPC aqueous dispersions were mixed at 5–10 °C (in the lipid gel phase), and immediately

scanned in the calorimeter, an endotherm identical to that of the pure EDMPC was observed at 24 °C. Another endotherm is seen at higher temperature, which is considerably broadened and shifted to ~1–2 °C lower temperature relative to that of pure EDPPC (Fig. 3A). Upon successive heating scans, the low-temperature endotherm did not change in temperature and shape but only decreased in enthalpy, while the upper endotherm progressively shifted to lower temperature, broadened, and (slightly) increased in enthalpy. At the 4th scan (not shown), the temperature of the upper endotherm (32.7 °C) was already close to the temperature of the equimolar EDMPC/EPPC sample prepared by mixing in chloroform (32 °C), and the enthalpy of lower endotherm had decreased by ~80%. Thus, the major changes involve the temperature of the upper transition (originally EDPPC), T_{EDPPC}, and the enthalpy of the lower transition (originally EDMPC), ΔH_{EDMPC}. There is a linear relationship between the enthalpy decrease of the lower transition and the temperature decrease of the upper transition (Fig. 4A). These changes are compatible with EDPPC vesicles acquiring progressively more EDMPC molecules, while EDMPC vesicles decrease in size and/or number. Variations of the lipid concentration between 1 and 10 mM did not influence the rate of change of the transition parameters (not illustrated). These results are compatible with monomeric exchange through the aqueous medium and not upon vesicle collision.

3.3. Comparison with mixtures of zwitterionic lipid dispersions

Qualitatively similar behavior to that of the cationics was observed with dispersions of the zwitterionic phospholipids. When DMPC and DPPC were mixed at low temperature (in the gel phase) and cycled in the calorimeter, the predominant changes were a decrease of the lower transition enthalpy ΔH_{DMPC} and a decrease of the upper transition temperature T_{DPPC} as well as cooperativity (Fig. 5A), with a linear relationship between ΔH_{DMPC} and T_{DPPC} (Fig. 4B). However, these processes were considerably slower than in the case of the cationic derivatives (Fig. 4A). The initial rates of change of both ΔH_{DMPC} and T_{DPPC} were about 40–50 times lower than for ΔH_{EDMPC} and T_{EDPPC}, respectively.

A similar evolution of the phase transition characteristics upon mixing aqueous dispersions of phosphatidylcholines has been previously observed [2,3,8,21] and explained as a monomer lipid transfer through the aqueous phase. Indeed, a difference in the hydrocarbon chain lengths of two methylene groups in each chain, as with the dimyristoyl-

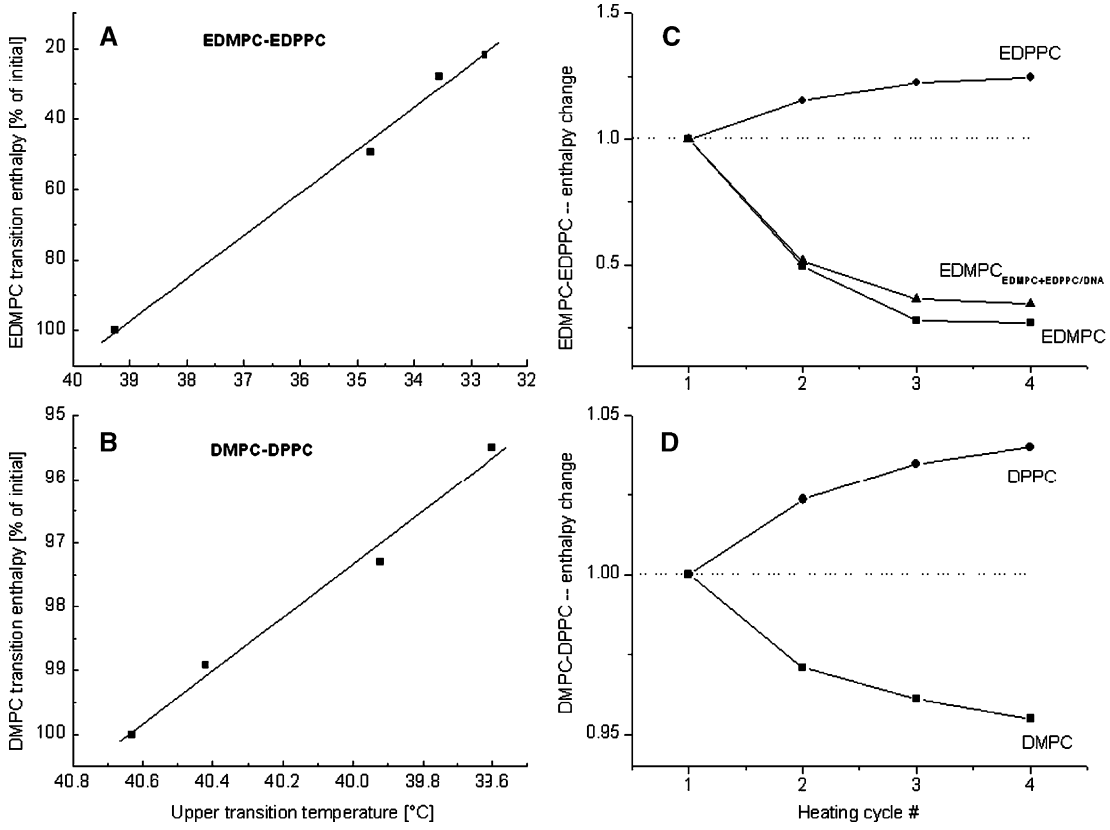

Fig. 4. (A) The EDMPC transition enthalpy (as percent of the initial) plotted vs. the EDPPC transition midpoint temperature for successive heating scans of mixed dispersions of EDMPC and EDPPC prepared as described in the legend of Fig. 3A. (B) Similar plot for mixed dispersions of DMPC and DPPC. (C) Relative enthalpy change of the lower (EDMPC) and higher (EDPPC) endotherms during successive heating scans of mixed dispersions of EDMPC+EDPPC, and EDMPC+EDPPC/DNA. (D) Relative enthalpy change of the lower (DMPC) and higher (DPPC) endotherms during successive heating scans of mixed dispersions of DMPC+DPPC.

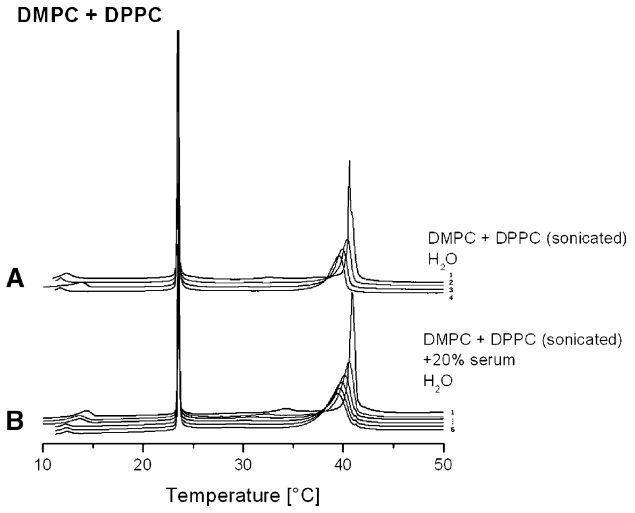

Fig. 5. Consecutive heating thermograms of mixtures of DMPC and DPPC sonicated dispersions, prepared separately, mixed extempore at 5 °C at equimolar lipid ratio, immediately filled into the calorimetric cell at that temperature and scanned at 30 °C/h between 5 and 50 °C (thus, 3 h elapse between the onset of the sequential heating scans): (A) dispersions of DMPC and DPPC; (B) dispersions of DMPC and DPPC with 20 vol.% serum.

and dipalmitoyl-derivatives, results in a ~30-fold change of the cmc [22], i.e., 30× more DMPC than DPPC monomers are available in the aqueous phase (here and further in the text, the term "cmc" is used to indicate the critical lipid concentration required for the transition of monomeric lipids to any supramolecular assembly, regardless of geometry). It is hence this difference that is responsible for the mostly unidirectional transfer to the DPPC aggregates. The difference in the initial rates of the transition temperature changes for DMPC and DPPC are of the same order of magnitude, according to our data, namely, 0.2 °C per initial heating cycle for DPPC vs. ≤0.01 °C for DMPC (Fig. 5A).

A similar argument could also account for the large difference in the rate of change of the transition parameters of cationic and zwitterionic phosphatidylcholines. Charged (anionic) lipids are known to exhibit generally higher cmc values than the electrically neutral compounds [23]. For example, the cmc of diC_{10}PC was reported to be ~20× lower than that of the anionic diC_{10}PS and 80–90× lower than that of the anionic diC_{10}PG [23]. In the study reported here, the 40–50× higher rate of transition parameter change for the cationic derivatives could be interpreted as an indication of higher cmc values of these derivatives relative to those of the source phosphatidylcholines. Monolayer data also indicate considerably higher surface activity of the cationic than the zwitterionic phospholipids [13].

The mechanism of transfer by aqueous diffusion is often hard to distinguish from an activation–collision mechanism, in which lipids are transferred during transient collisions between donor and acceptor vesicles; the difference is particularly indistinct when the collision is not rate-limiting [24]. In the case studied here, the strongly asymmetric transfer and the concentration independence of the transfer rate seem to be in support of the aqueous diffusion mechanism.

It has been noted previously that lipid exchange takes place in the liquid crystalline phase and it is largely absent in the solid phase [8]. We observed during the initial scan that, while the transition endotherm of the dimyristoyl compound is unchanged relative to the pure lipid, the dipalmitoyl lipid endotherm is already altered—shifted to lower temperature and with decreased cooperativity (Figs. 3A and 5A). This is hardly noticeable with the zwitterionic PCs, but is clearly expressed with the cationic EPCs, for which the transfer is much faster (Fig. 3A). Apparently, the transition to the liquid crystalline phase of just the dimyristoyl lipid at ~24 °C is sufficient for molecular transfer to be initiated, and it takes place while the sample is being heated to 41 °C. Thus, transfer evidently occurs even when EDPPC is in the gel phase.

The peculiar complex irregular shape of the upper peak on Fig. 3A (observed at 30 °C/h and 60 °C/h scan rate) could be due to auto-oscillations of the specific heat driven by the phase transition, taking place far from equilibrium, in a regime with continuing monomer inclusion (composition change) [25] (oscillating heat capacity patterns were observed reproducibly, although the exact shape of the peak differed for different experiments). Splitting of the upper transition endotherm at the early stages of mixing of DMPC and DPPC sonicated vesicles has been previously discussed as a likely consequence of the imbalance in distribution of DMPC between the inner and outer monolayers of the DPPC acceptor vesicles [8].

3.4. Mixtures of cationic lipoid dispersions with lipoplexes

Cationic lipoids have been developed and intensively studied mainly because of their application as carriers of DNA for transfection. From this viewpoint, their interaction with DNA and the properties of their lipoplexes are important. Thus, we examined the effect of DNA on lipid transfer in cationic lipoid dispersions.

The addition of DNA does not significantly change the transition temperatures of the pure EDMPC and EDPPC dispersions [13,16]. If the same protocol of mixing of lipid dispersions described above was followed, but with an isoelectric amount of DNA present in the EDPPC dispersion, the changes in the upper transition happened even faster than in the pure lipid dispersions: the upper transition was almost completely eliminated during the initial heating (Fig. 3B). This is expected, because (partial) neutralization of the positive charge of EDPPC by the DNA must reduce the electrostatic repulsion and, in this way, facilitate the incorporation of EDMPC monomers into the EDPPC bilayers.

If an isoelectric amount of DNA was present in the EDMPC dispersion, however, the consequences to the lipid redistribution were the opposite—exchange was considerably suppressed relative to that of the pure lipid

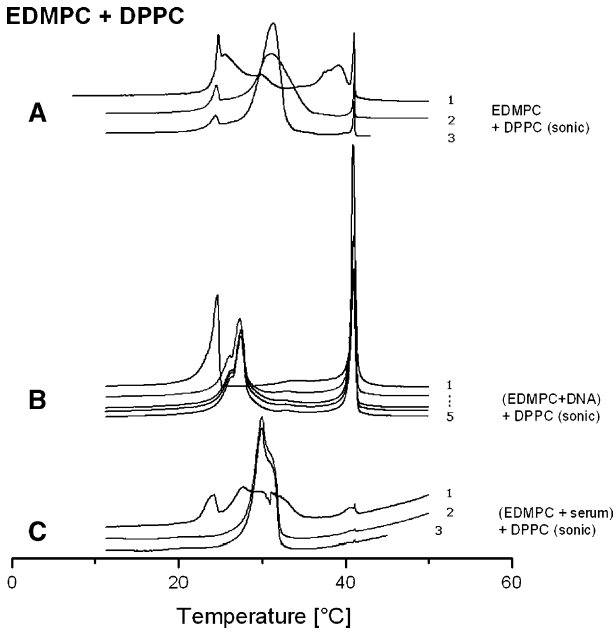

Fig. 6. Consecutive heating thermograms of mixtures of EDMPC dispersion and DPPC sonicated dispersion, prepared separately, mixed extempore at 5 °C at equimolar lipid ratio, immediately filled into the calorimetric cell at that temperature and scanned at 30 °C/h between 5 and 50 °C (thus, 3 h elapse between the onset of the sequential heating scans): (A) dispersions of EDMPC and DPPC; (B) dispersions of EDMPC/DNA lipoplexes and DPPC; (C) dispersions of EDMPC with 20 vol.% serum and DPPC.

dispersions (Fig. 3C). Presumably, DNA in the EDMPC dispersions binds the lipid surface and reduces the rate of release of monomers that are responsible for the transfer.

3.5. Mixtures of cationic and zwitterionic liposomes

When dispersions of the cationic EDMPC and the zwitterionic DPPC were mixed, lipid transfer was accelerated relative to EDMPC→EDPPC transfer (Fig. 6A). This is a predictable result of the reduction of electrostatic repulsion. Similarly to the case of the EDMPC/EDPPC mixture, the binding of EDMPC by DNA is assumed to hinder the transfer (Fig. 6B).

3.6. Effect of serum

Because transfection is usually carried out in the presence of serum, we also examined the effect of serum on lipid exchange. As may be seen from Fig. 3D, the presence of serum qualitatively changed the evolution of the transition endotherms that occurred upon mixing of the liposome or lipoplex dispersions: The endotherms were shifted symmetrically into a single broad band at an intermediate temperature after the first heating cycle. This evolution of endotherms was not appreciably altered by the presence of DNA (Fig. 3E).

A rapid, *symmetric* shift of the transition endotherms in the presence of serum was observed even if only one of the dispersions contained cationic lipid and the other was neutral—as was the case when DPPC was combined with the [EDMPC+serum] dispersion (Fig. 6C).

If serum was added to uncharged (zwitterionic) liposomes, however, the lipid exchange mechanism and rate were not altered—the evolution of the thermograms upon sequential heating scans was virtually the same as in the absence of serum (Fig. 5B).

In order to gain some insight into the nature of the serum effect, we preheated the serum to elevated temperatures for 15 min before adding it to the lipid dispersions. As illustrated in Fig. 7B, the enhanced mixing produced by serum was still observed after heating the serum to 55 °C; however, heating to 80 °C eliminated the effect (Fig. 7C).

These results suggest that the active serum factor is denaturated between 55 °C and 80 °C (at present, we did not make efforts to identify exactly which serum constituent(s) is specifically responsible for the effect). The factor is evidently rather stable, since many enzymes are inactivated below 55 °C (e.g., the enzymes from the complement pathway [26] and many others [27]). Structural proteins are generally stable at high temperatures [28] (albumin denaturates irreversibly at ~65–70 °C [29,30]), but clearly, the inactivation temperature of the lipid mixing (exchange or fusion) activities does not itself reveal much about its mechanism. The existence of such an activity in serum is, however, of considerable importance relative to in vivo transfection. Thus, lipoid transfer out of lipoplexes could be a factor that needs attention in the development of these kinds of agents for gene therapy.

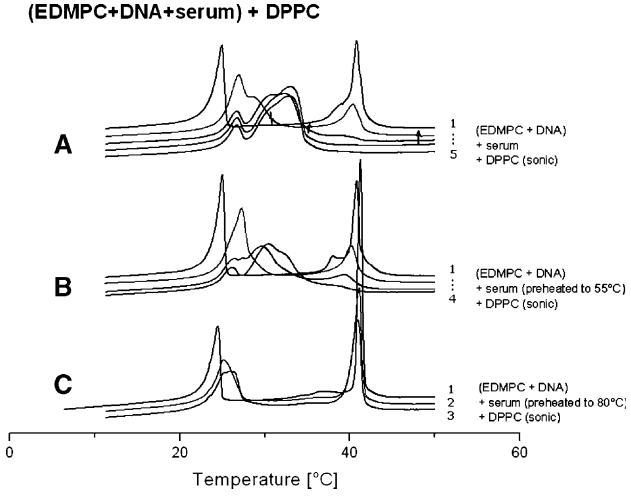

Fig. 7. Consecutive heating thermograms of mixtures of dispersion of EDMPC/DNA with 20 vol.% serum and DPPC sonicated dispersion, prepared separately, mixed extempore at 5 °C at equimolar lipid ratio, immediately filled into the calorimetric cell at that temperature and scanned at 30 °C/h between 5 and 50 °C (thus, 3 h elapse between the onset of the sequential heating scans): (A) untreated serum; (B) serum preheated to 55 °C for 15 min and added to the EDMPC lipoplexes at 50 °C; (C) serum preheated to 80 °C for 15 min and added to the EDMPC lipoplexes at 50 °C.

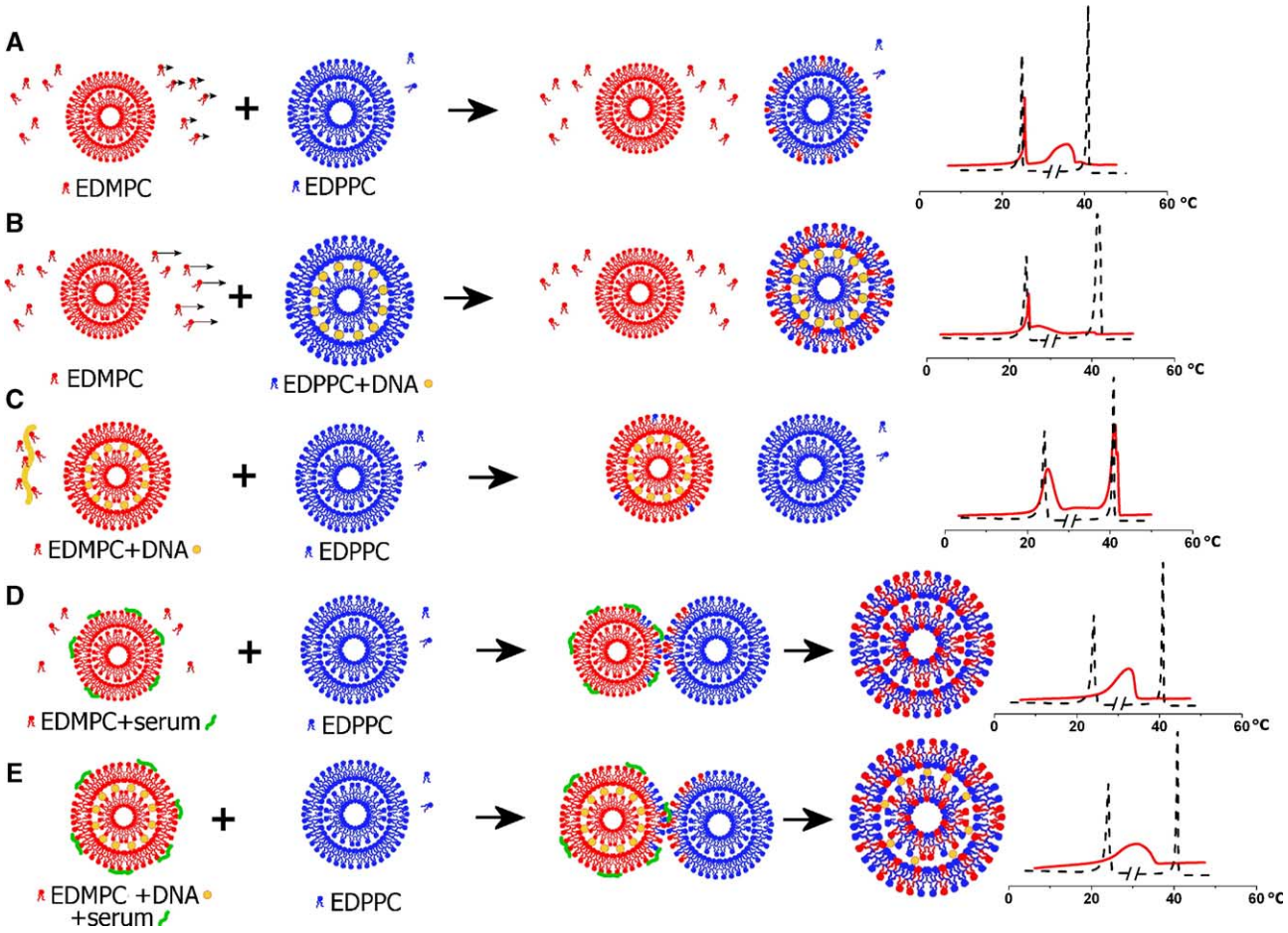

Fig. 8. Suggested scheme of lipid exchange in cationic phospholipid dispersions. Sample thermograms corresponding to the suggested mechanism are shown in red on the right; the dashed lines in the thermograms are for the pure EDMPC and EDPPC dispersions.

A plausible scheme of cationic lipid exchange mechanisms based on the data presented above is shown in Fig. 8:

(A) In mixtures of liposomes of dimyristoyl and dipalmitoyl compounds, the lipid transfer takes place mainly by means of monomer exchange. Since the cmc of the shorter-chain EDMPC is presumably more than an order of magnitude larger (~30 times, as estimated in [22]), the transfer is asymmetric, primarily from EDMPC to EDPPC—thus, the size and/or number of EDMPC liposomes decreases ($\Delta H_{\mathrm{EDMPC}}$ decreases), while EDPPC liposomes progressively incorporate EDMPC (T_{EDPPC} shifts to intermediate temperatures) (Fig. 8A).

(B) When DNA interacts with the EDPPC vesicles, it (partially) neutralizes the positive charge. Thus, the incorporation of EDMPC monomers is facilitated by the reduction of electrostatic repulsion (Fig. 8B).

(C) When DNA is present in the EDMPC dispersion (at 1:1 charge ratio), it hinders the transfer presumably by binding to the lipid surface, thus reducing the rate of release of monomers (Fig. 8C). The effect of DNA is ultimately an electrostatic effect, but much of the actual magnitude could be attributed to the fact that the lipoplexes are multilayered and most of the lipid is not accessible.

(D,E) Serum proteins (negatively charged) strongly promote a *symmetric* cationic lipoid mixing (both with and without DNA) (Figs. 8D, E). Even though much lipoplex lipid is inaccessible, the effect of serum shows that the barrier due to buried lipid can be easily overcome.

The observed sizable molecular exchange through the aqueous phase could be useful from fundamental viewpoint—it could be informative as a relative estimate of the cmc of amphiphiles. The process of extensive monomer exchange of cationic phospholipids could be of considerable importance in some aspects of their application as carriers for DNA delivery.

Acknowledgements

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