Biochimica et Biophysica Acta 1848 (2015) 127–133

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Structural characterization of novel cationic diC16-amidine bilayers: Evidence for partial interdigitation

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ARTICLE INFO

Article history:
Received 25 July 2014
Received in revised form 25 September 2014
Accepted 2 October 2014
Available online 12 October 2014

Keywords:
Partial interdigitation
Electron spin resonance
Differential scanning calorimetry
diC16-amidine
Membrane fusion
Fluorescence resonance energy transfer

ABSTRACT

In this work, the bilayer structure of novel cationic lipid diC16-amidine was compared to the one of zwitterionic dipalmitoyl phosphatidylcholine (DPPC), which shares the same hydrophobic domain.
Differential scanning calorimetry shows that DPPC and diC16-amidine bilayers have similar phase transition temperatures, but diC16-amidine membranes display a less cooperative phase transition and an absence of pretransition.
Both bilayers were analyzed from surface to core, using 5-, 7-, 10-, 12-, 14-, and 16-PCSL spin labels. As expected, electron spin resonance (ESR) spectra show that the gel phase of DPPC presents a flexibility gradient toward the core. In contrast, this gradient exists in the gel phase of diC16-amidine bilayers but only down to the 12th lipid tail carbon. The 14th and 16th carbons of the cationic lipid are in a very rigid environment, similar to the one observed at the bilayer surface. These data suggest that diC16-amidine molecules are organized in a partially interdigitated gel phase. ESR spectroscopy also shows that the lamellar fluid phase of diC16-amidine is more rigid than the one of DPPC.
Fluorescence resonance energy transfer assays reveal that diC16-amidine displays a more efficient fusogenic activity in the gel phase than in the fluid one, suggesting that the partial interdigitation of the gel phase is important for the fusion process to occur. Since the gel–fluid transition temperature is 42 °C, diC16-amidine is fusogenic at the physiological temperature and is therefore a promising lipid for delivery applications without the need of helper lipids.

1. Introduction

Liposomes have become of huge importance in medicine and healthcare [1]. Over a dozen of liposome formulations have been approved for human use [2], offering suitable treatment for a series of diseases such as fungal infections [3,4], leishmaniasis [5], macular degeneration [6] and cancer [7–9]. They have also been employed in vaccine [10–12] and imaging [13,14] applications. This has prompted the development and characterization of new synthetic lipids, like cationic lipids [15,16].

A family of cationic lipids containing the amidine group was synthesized some 25 years ago [17] to deliver nucleic acids intracellularly [18,19]. Studies carried out with 14-carbon lipid tailed diC14-amidine demonstrated its capacity to enhance lipid mixing [20], and also to activate the innate immune system [21,22].

The high fusogenic efficiency of diC14-amidine bilayers was correlated with the presence of an interdigitated gel phase [20], in which lipid molecules were packed side by side and flipped horizontally from one to the other [23,24].

It was previously shown that diC16-amidine, a 16-carbon lipid tail derivative of diC14-amidine, fuses more efficiently than diC14-amidine with cell membranes at 37 °C [25]. Hence, characterizing the structural and biological properties of diC16-amidine could expand the applications of the amidine lipid family.

In this work, the structure of cationic diC16-amidine bilayers was characterized and compared to the structure of bilayers formed by dipalmitoyl phosphatidylcholine (DPPC), a zwitterionic lipid that has the same hydrophobic domain. The fusogenic activity of diC16-amidine was also assessed as a function of temperature. Differential scanning calorimetry (DSC), electron spin resonance (ESR) spectroscopy and fluorescence resonance energy transfer (FRET) were employed in this study.

2. Materials and methods

2.1. Reagents

Texas Red®-DHPE (Texas Red® 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine, triethylammonium salt) and NBD-PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2- dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine, triethylammonium salt) are Invitrogen

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http://dx.doi.org/10.1016/j.bbamem.2014.10.013
0005-2736/

(Molecular Probes) products. Hepes (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid)) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DPPC (dipalmitoyl phosphatidylcholine), asolectin (L-α-phosphatidylcholine (Soy-20%)) and spin labels 1-palmitoyl-2-(*n*-doxylsearoyl)-*sn*-glycero-3-phosphocholine (*n*-PCSL, $n = 5, 7, 10, 12, 14$, or 16) were supplied by Avanti Polar Lipids (Birmingham, AL, USA). diC16-amidine (3-tetradecylamino-*N*-*tert*-butyl-*N*′-hexadecyl-propionamidine) was synthesized as described [17] and stored as a powder at −20 °C. The chemical structures of DPPC, diC16-amidine and 10-PCSL are shown in Fig. 1. Ultrapure water of Milli-Q-Plus quality was used throughout.

2.2. Liposome preparation

A lipid film was formed from a chloroform solution, dried under a stream of N_2, and left under reduced pressure for a minimum of 2 h, to remove all traces of organic solvent. Dispersions were prepared by addition of Hepes buffer (10 mM, pH 7.4) followed by vortexing for about 5 min at 50 °C. For ESR experiments, 0.8 mol% 5-PCSL, 0.6 mol% 7-PCSL, 0.5 mol% 10-PCSL, 0.4 mol% 12-PCSL, 0.3 mol% 14-PCSL, or 0.3 mol% 16-PCSL were added to the lipid chloroform solutions when preparing the lipid films. No spin–spin interaction was observed at such small label concentrations. For fluorescence measurements, NBD-PE and Texas Red-DHPE® (at 0.8 mol% each) were dissolved with diC16-amidine in chloroform before lipid film formation. Final diC16-amidine concentrations were 0.85 μM, 2 mM and 10 mM for FRET, DSC, and ESR experiments, respectively. All samples were used right after preparation.

2.3. Differential scanning calorimetry (DSC)

DSC scans were performed in a Microcal VP-DSC Microcalorimeter (Microcal Inc., Northampton, MA, USA) equipped with 0.5 mL twin total-fill cells. Heating rates were 20 °C/h. Scans were performed at least in duplicate. The enthalpy of transition ΔH was obtained by integrating the area under the thermograms.

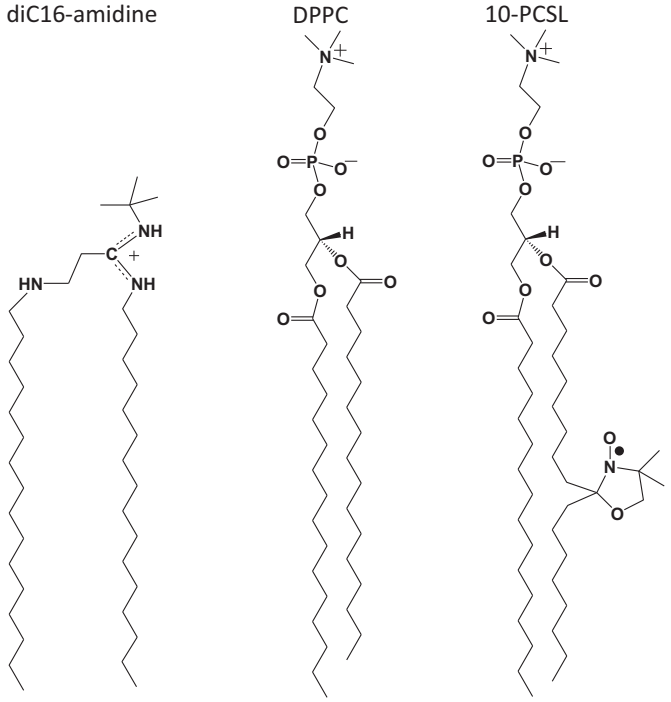

Fig. 1. Chemical structures of diC16-amidine, DPPC and 10-PCSL.

2.4. Electron spin resonance (ESR) spectroscopy

ESR measurements at X-band were performed with a Bruker EMX spectrometer. Field-modulation amplitude of 1G and microwave power of 5 mW were used. The temperature was controlled to about 0.2 °C with a Bruker BVT-2000 variable temperature device, and monitored with a Fluke 51 K/J thermometer with a probe placed just above the cavity. A high sensitivity ER4119HS cavity was used. All ESR data shown are means of the results of at least two experiments, and the uncertainties are the standard deviations. When not shown, the uncertainty was found to be smaller than the symbol in the graph.

The effective order parameter, S_{eff}, was calculated from the expression [26]

$$S_{eff} = \frac{A_{//} - A_{\perp}}{A_{ZZ} - (1/2)\left(A_{xx} + A_{yy}\right)} \frac{a_o'}{a_o}$$

where $a_o' = (1/3)(A_{xx} + A_{yy} + A_{zz})$, $a_o = (1/3)(A_{//} + 2A_{\perp})$, $A_{//}$ $(= A_{max})$ is the maximum hyperfine splitting directly measured in the spectrum (see Fig. 6), $A_{\perp} = A_{min} + 1.4\left[1 - \frac{A_{//} - A_{min}}{A_{zz} - (1/2)(A_{xx} + A_{yy})}\right]$, A_{min} is the measured minimum hyperfine splitting (see Fig. 6) and A_{xx}, A_{yy} and A_{zz} are the principal values of the hyperfine tensor for doxylpropane [27].

Rotational correlation times for isotropic motion, according to the motional narrowing theory, can be calculated from the peak-to-peak width of the ESR Lorentzian lines [28,29]:

$$\Delta H_L(m) = A + Bm + Cm^2$$

where m is the m-th component of the nitrogen nuclear spin, A is the Lorentzian linewidth of the central line, $\Delta H_L(0)$, and B and C are

$$B = \frac{1}{2}\Delta H_L(0)\left(\frac{\Delta H_L(+1)}{\Delta H_L(0)} - \frac{\Delta H_L(-1)}{\Delta H_L(0)}\right)$$

$$C = \frac{1}{2}\Delta H_L(0)\left(\frac{\Delta H_L(+1)}{\Delta H_L(0)} + \frac{\Delta H_L(-1)}{\Delta H_L(0)} - 2\right).$$

The correlation time for doxyl labels can be calculated using both B and C parameters: $\tau_B = -1.22\ B$ or $\tau_C = 1.19\ C$, ($\tau_B = \tau_C$ for isotropic movement). Lorentzian linewidths are calculated using a computer program, which performs nonlinear least-square fitting of the experimental ESR spectrum using a model of a Lorentzian–Gaussian function for corrections of non-resolved hyperfine splitting [30,31]. This methodology can only be applied to ESR spectra yielded by probes in the motional narrowing regime [30]. In the present work, it could only be applied to 16-PCSL. The average rotational correlation time $\overline{\tau}$ corresponds to the arithmetic mean of τ_B and τ_C.

2.5. Lipid mixing assay

Lipid mixing between cationic diC16-amidine liposomes and asolectin liposomes was monitored using Fluorescence Resonance Energy Transfer (FRET) assay. Cationic liposomes were labeled with NBD-PE and Texas Red®-DHPE as described above. Asolectin liposomes were added to labeled diC16-amidine liposomes at a mass ratio of 10:1 and loaded in a quartz thermostatized fluorescence cell. The samples were gently stirred throughout the experiment. The fluorescence was monitored using an SLM-8000 spectrofluorometer with excitation and emission slits of 4 nm. Generally, samples were excited at 470 nm and emission spectra were recorded between 500 nm and 625 nm. Control emission spectra were performed in parallel before and after lipid mixing. For each temperature, the percentage of fusion was calculated as the ratio between the NBD donor emission fluorescence (532–