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Electrostatic parameters of cationic liposomes commonly used for gene delivery as determined by 4-heptadecyl-7-hydroxycoumarin [1]

Nicolaas J. Zuidam, Yechezkel Barenholz *

Department of Biochemistry, The Hebrew University, Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel

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Abstract

Cationic liposomes are used to deliver genes into cells in vitro and in vivo. The present study is aimed to characterize the electrostatic parameters of cationic, large unilamellar vesicles, 110 ± 20 nm in size, composed of DOTAP/DOPE (mole ratio 1/1), DOTAP/DOPC (mole ratio 1/1), 100% DOTAP, DMRIE/DOPE 1/1, or DC–CHOL/DOPE (mole ratio 1/1). {Abbreviations: DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide; DC–CHOL, 3β[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol}. The cationic liposomes had a large positive surface potential and a high pH at the liposomal surface in 20 mM Hepes buffer (pH 7.4) as monitored by the pH-sensitive fluorophore 4-heptadecyl-7-hydroxycoumarin. In contrast to DOTAP and DMRIE which were 100% charged, DC–CHOL in DC–CHOL/DOPE (1/1) liposomes was only about 50% charged in 20 mM Hepes buffer (pH 7.4). This might result in an easier dissociation of bilayers containing DC–CHOL from the plasmid DNA (which is necessary to enable transcription), in a decrease of the charge on the external surfaces of the liposomes or DNA–lipid complexes, and in an increase in release of the DNA–lipid complex into the cytosol from the endosomes. Other electrostatic characteristics found were that the primary amine group of DOPE in cationic liposomes dissociated at high (> 7.9) pH_{bulk} and that a salt bridge was likely between the quaternary amine of DOTAP or DMRIE and the phosphate group of DOPE or DOPC, but not between the tertiary amine of DC–CHOL and the phosphate group of DOPE. The liposomes containing DOTAP were unstable upon dilution, probably due to the high critical aggregation concentration of DOTAP, 7×10^{-5} M. This might also be a mechanism of the dissociation of bilayers containing DOTAP from the plasmid DNA. © 1997 Elsevier Science B.V.

Keywords: Fluorescence; Surface potential; Gene delivery; Cationic liposome

Abbreviations: CAC: Critical aggregation concentration; DC–CHOL: 3β[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol; DMRIE: 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide; DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPE: 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride; DOTMA, *N*-(1-(2,3-dioleyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride; *e*, Electron charge; ϵ_0, Relative permittivity of free space; ϵ_r, Dielectric constant of the location of HC; HC, 4-heptadecyl-7-hydroxycoumarin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); LUV, Large unilamellar vesicles; MU, Methylumbelliferone

* Corresponding author. Fax: +972 2 6411663. E-mail: yb@cc.huji.ac.il

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1. Introduction

In the last decade, cationic liposomes have been widely used for the delivery of DNA into mammalian cells and currently they are also being tested in several clinical trials [1,2]. The cationic liposomes are composed of an amphipathic mono- or polycationic carrier and a 'helper lipid'. Examples of such cationic carriers are the monocationic lipids DOTMA [3,4], DOTAP [5], DC–CHOL [6], DMRIE [4], and the polycationic lipid lipopoly (L-lysine) [7,8]. The cationic carriers interact spontaneously with the negatively charged DNA. DNA–lipid complexes enter cells mainly by adsorptive endocytosis [9,10]. The success of the transfection is determined by the intracellular fate; most of the complexes are degraded by the lysosomes, but a small fraction apparently escapes into the cytosol and is able to enter the nucleus. In most experiments all the amphipathic, cationic lipids showed optimal expression of the tested genes when mixed with an equimolar amount of the helper lipid DOPE [4,8]. Replacement of DOPE by other lipids differing in headgroup and/or fatty acyl chain composition generally reduces the transfection efficiency of DNA into cells. In spite of extensive efforts, most of the observations are hard to explain. Fundamental knowledge is lacking and most of the achievements reached are based on trial and error [11].

It is obvious that electrostatic interactions will dominate the formation of complexes of the negatively charged DNA and positively charged liposomes, which is the first step in the transfection process. Surprisingly, to our knowledge, no study has been performed to characterize the electrostatic parameters of cationic liposomes in detail. In the present study, we characterized these parameters by the use of the lipophilic, pH-sensitive fluorophore 4-heptadecyl-7-hydroxycoumarin (HC). This fluorophore was also used (together with other techniques) to determine physical and chemical stability of the cationic liposomes.

2. Materials and methods

2.1. Materials

DOTAP, DOPE, and DOPC were obtained from Avanti Polar Lipids (Alabaster, AL). These lipids showed a single peak upon HPLC analysis (see below). DC–CHOL was a generous gift of Dr. L. Huang (Department of Pharmacology and Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA). Dry DMRIE/DOPE (mole ratio 1/1) was kindly supplied by Dr. P.L. Felgner (Vical, San Diego, CA). HC was purchased from Molecular Probes (Eugene, OR). MU was obtained from Sigma (St. Louis, MO). These and all other chemicals were of analytical grade. Double-distilled water was used.

2.2. Liposome preparation

Appropriate mixtures of lipids were dissolved in chloroform/methanol (1:1 v/v) in a round-bottom flask. An appropriate amount of the fluorescent probe HC dissolved in tetrahydrofuran was also added to this mixture. In all batches, the mole ratio of lipid to fluorophore was 200 or 400 to 1. The organic solvent was removed under vacuum by rotary evaporation. The thin film obtained was dissolved in *tert*-butanol, and the mixture was freeze-dried for at least 3 h under reduced pressure. The lyophilized cake was hydrated with 20 mM Hepes (pH 7.4) and vortexed for several minutes. Hydration of DC–CHOL/DOPE (1/1) liposomes was enhanced by sonication in a water bath for several seconds. The liposomes were downsized using the extrusion system Liposofast [12] (Avestin, Ottawa), 11 times through 0.4-μm- and 11 times through 0.1-μm-pore-size filters (Poretics, Livermore, CA), successively. In all batches, the concentration of each type of lipid was 20 mM and the final concentration of HC was 1×10^{-4} M.

2.3. Particle size measurements

The particle size distribution of a liposome dispersion was determined at 25°C by dynamic light-scattering (DLS) with a Coulter model N4 SD (Coulter Electronics, Hialeah, FL). See Barenholz and Amselem [13] for further details.

2.4. Fluorescence measurements

Cationic liposomes were diluted in 3 ml of 20 mM Hepes buffer (pH 7.4) to a concentration of 4×10^{-5} M of the cationic lipid. If necessary, the pH of the

medium was adjusted by addition of an appropriate amount of concentrated sodium hydroxide or hydrochloric acid. To abolish any possible pH gradient, the samples were sonicated for about 5 s in a water bath. The fluorescence measurements were performed under stirring conditions and at ambient temperature on an LS50B luminescence spectrometer (Perkin Elmer, Norwalk, CT). Fluorescence of HC was measured by scanning the excitation wavelength between 300 and 400 nm at an emission wavelength of 450 nm (bandwidths 5 nm) while using an emission filter at 430 nm.

2.5. Determination of DOTAP critical aggregation concentration (CAC)

The critical DOTAP concentration at which aggregation of monomers to amphiphile assemblies (CAC) occurred was determined by measuring surface tension as a function of the concentration of DOTAP in 20 mM Hepes (pH 7.4). The surface tension of a 2 cm-diameter du Nouy platinum ring was measured using a semi-automatic tensiometer, Fisher Surface Tensiomat (Fischer Scientific, Pittsburgh, PA). The measurements at each concentration were repeated until a constant value of the surface tension was reached. See Martin [14] for further details.

2.6. HPLC analysis of lipids

Lipids were analyzed by HPLC as described earlier by Jääskeläinen and Urtti [15]. Samples for HPLC analysis were prepared by Bligh and Dyer extraction [16]. The lipids were collected in the lower, chloroform phase. After addition of an equal volume of methanol to the chloroform phase, 20-μl aliquots were injected directly into the column. The HPLC system consisted of a Kontron HPLC autosampler 460, a Kontron HPLC pump 420, a Kontron HPLC gradient former 425, a computer-controlled, integrator-based Kontron Data system 450 (Kontron, Zürich), and a Sedex 55 evaporative light scattering detector (Sedere, Alfortville, France). The separation of the lipids was carried out on a Zorbax aminophase column (Whatman, Clifton, NJ) using acetonitrile/methanol/0.1 M ammonium acetate (pH 4.8) 70/20/8 (v/v) as the mobile phase. The flow rate was 2 ml min^{-1} and the column temperature was ambient. Evaporative light scattering detection was carried out at 40°C and 2.2 bar. Air was used to evaporate the eluent.

3. Results

3.1. Determination of electrostatic parameters of cationic liposomes

Large unilamellar vesicles (LUV) were composed of DOTAP/DOPE (mole ratio 1/1), DOTAP/DOPC (mole ratio 1/1), 100% DOTAP, DMRIE/DOPE (mole ratio 1/1), DC–CHOL/DOPE (mole ratio 1/1), 100% DOPC, or DOPC/DOPE (mole ratio 1/1). All LUV had an average size of about 110 ± 20 nm and contained the pH-sensitive, fluorescent probe HC. Its fluorophore is the hydroxycoumarin moiety, which is a weak acid. At $pH < pK_a$ the maximal fluorescence intensity is found at an excitation wavelength of about 320 nm, and at $pH > pK_a$ the excitation maximum is shifted to the wavelength of about 380 nm. The fluorescence intensity at the excitation wavelength of 330 nm is the pH-independent isosbestic point, which reflects the actual level of the fluorophore present in the lipid assembly. Therefore, the dissociation degree of HC incorporated into the liposomes of the above compositions can be monitored by the ratio of the excitation fluorescence intensities at 380 and 330 nm (380 nm/330 nm) against the pH (see Fig. 1A). Basically all titration curves in Fig. 1 (except that of DC–CHOL/DOPE) consist of a single sigmoid, although the sigmoids differ from each other in their pH dependency (lower pH range for most cationic LUV, and much higher pH range for the neutral LUV) and in their exact shape. The titration curve of DC–CHOL/DOPE LUV consists of two (lower, in the range of the cationic LUV, and upper, overlapping with the neutral LUV) combined sigmoids (Fig. 1). The fine details of Fig. 1A reflect the exact vesicle lipid composition (which is discussed in detail below) and to some extent the ionic strength of the medium, especially in relationship to the high concentration of sodium hydroxide used to achieve the higher pH values). The latter explains the differences in the maximal value of 380 nm/330 nm; as expected, the higher the pH the lower is the maximum. To simplify the comparison between the

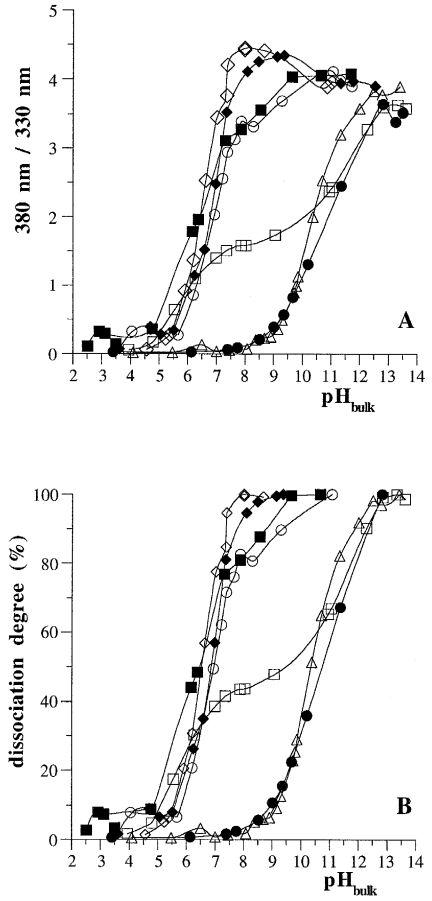

Fig. 1. The dissociation degree of HC in liposomes as monitored by the ratio of the excitation fluorescence intensities at 380 nm and at the isosbestic point 330 nm (A) or translated into percentages of the maximum value (B) against the pH_{bulk}. The liposomes were composed of DOTAP/DOPE (1/1) (○), DOTAP/DOPC (1/1) (♦), DOTAP (◇), DMRIE/DOPE (1/1) (■), DC–CHOL/DOPE (1/1) (□), DOPC (△), and DOPC/DOPE (1/1) (●). The curves shown are not fitted to Eq. (1).

different curves, the dissociation degree of HC in the liposomes was calculated assuming that 100% dissociation corresponds to the maximum value of 380 nm/330 nm (see Fig. 1B). When the maximum value of 380 nm/330 nm was reached, data points at higher pH values were neglected in the fit of these dissociation degree curves. The curves in Fig. 1B were fitted to a modified Henderson–Hasselbach Eq. [17]:

$$pH_{bulk} = pK_a + A\log\left(\frac{D - D_{min}}{D_{max} - D}\right) + \log\left(\frac{I_a}{I_b}\right) \qquad (1)$$

which can be converted to the equation

$$D = D_{max} + \frac{D_{min} - D_{max}}{1 + \exp\left\{\left[pH_{bulk} - pK_a + \log\left(\frac{I_a}{I_b}\right)\right]\ln(10)/A\right\}}$$

where the constant A is ideally 1 since the protonation of HC is a one-to-one event, (normally we found values between 0.9 and 1.2, but when HC was incorporated into DMRIE/DOPE (1/1) liposomes we found a value for A of 1.7); pK_a is the apparent proton binding constant of HC; D is the dissociation degree; D_{min} and D_{max} are the minimum and maximum value of D, respectively. I_a/I_b is the ratio of the fluorescence intensity of the pH-independent isosbestic point of HC (excitation at 330 nm) and of the unprotonated charged HC (excitation at 380 nm). $\mathrm{Log}(I_a/I_b)$ is 0 at an ideal isosbestic point; we found values between -0.1 and 0.1).

Proton binding to a molecule which is present at the water/lipid interface, such as HC, can be described by [18]:

$$pK_a = pK_H + \Delta pK_{pol} + \Delta pK_{el} \qquad (2)$$

where pK_H is the intrinsic proton binding constant, ΔpK_{pol} is the shift in pK_a due to a change in surface polarity (dielectric constant), and ΔpK_{el} is the shift in pK_a due to a change in surface potential. In 20 mM Hepes, 4-methylumbelliferone (MU, a water-soluble fluorophore, which is HC lacking the acyl chain) had a pK_a of 7.9, and HC in neutral micelles composed of 1 mM non-fluorescent, hydrogenated Triton X-100 had a pK_a of 9.0 (see Table 1). Similar results were reported earlier by Fernández and Fromherz [19]. By measuring the pK_a of HC in water/dioxane mixtures, they showed that the difference in these pK_a's ($=\Delta pK_{pol}$) was caused by a change in ϵ_r, the dielectric constant of the location of the probe (ϵ_r decreased from 78 to 32). The pK_a of HC in zwitterionic, non-charged DOPC liposomes was 10.5 (see Table 1). The ΔpK_{pol} of 2.6 ($=10.5 - 7.9$, see Eq. (2)) indicates that the ϵ_r of the location of HC in DOPC liposomes had a value of 8 as derived from Ref. [19]. DOPC liposomes are neutral throughout the pH range used in this study [18]. Therefore, ΔpK_{el} of HC in charged liposomal mem-

Table 1
The pK_a of MU or HC in micelles or liposomes and the electric properties of the lipid surfaces in 20 mM Hepes buffer ($pH_{bulk} = 7.4$) [a]

Probe	Micellar or liposome dispersion	pK_a	Ψ_0^{HC} (mV)	Ψ_0^{GC} (mV)	$pH^{HC}_{surface}$, measured	$pH^{HC}_{surface}$, calculated	$pH^{GC}_{surface}$, calculated
MU		7.9					
HC	Hydrogenated Triton X-100	9.0	0	0	7.4 [b]	7.4 [b]	7.4 [b]
HC	DOPC [b]	10.5	0	0	7.4 [b]	7.4 [b]	7.4 [b]
HC	DOPC/DOPE 1/1 [b]	10.7	0	0	7.4 [b]	7.4 [b]	7.4 [b]
HC	DOTAP/DOPE 1/1	6.8	217	200	10.9	11.1	10.8
HC	DOTAP/DOPC 1/1	6.7	222	193	11.2	11.2	10.7
HC	DOTAP	6.3	240	235	11.6	11.5	11.4
HC	DMRIE/DOPE 1/1	6.3	240	200	11.1	11.5	10.8
HC	DC–CHOL/DOPE 1/1 [c]						
	50% charged	– [c]	– [c]	180	10.2	– [c]	10.5
	100% charged	6.8	217	216	– [c]	11.1	11.1

[a] See text for symbols and determination of values. The pK_a, Ψ_0^{HC} and Ψ_0^{GC} are determined using Eqs. (1), (3) and (4), respectively. The $pH^{HC}_{surface}$ was measured by comparing the value of the dissociation degree of HC in the cationic liposomes with the value of the dissociation degree of HC in DOPC–liposomes (using Eq. (1)). The $pH^{HC}_{surface}$ was calculated using Eqs. (3) and (6). The $pH^{GC}_{surface}$ was calculated using Eqs. (4) and (6). Note that both $pH_{surface}$ and Ψ_0 also depend on the distance from the boundary of the membrane (which is λ – in the present study, 0.74 nm). Gouy–Chapman calculations with ϵ_r of 78 (as done by many others) will increase λ and decrease the values for both $pH^{GC}_{surface}$ and Ψ_0^{GC}. Also note that $pH_{surface}$ is actually an apparent quantity because it is also affected by both a degeneration of the proton activity due to the low ϵ_r of the medium close to the liposomal bilayer [19,20] and by a decrease in the proton concentration due to non-electrostatic effects [21]. Theoretical correction for the latter effect is poorly understood (Cevc, personal communication). We assume that these two effects are equal for both neutral and charged liposomes (no change in surface polarity), so it will not influence the electrostatic calculations.

[b] The $pH^{HC}_{surface}$ cannot be measured here because the measurement of pH values by use of a pH-sensitive probe is limited to the range where the fluorescence is sensitive to changes in pH. It has been assumed that Triton X-100-micelles and DOPC and DOPC/DOPE (1/1) liposomes are neutral at pH 7.4.

[c] The determination of the pK_a of HC in DC–CHOL/DOPE (1/1)-liposomes is interfering with the pK_a of DC–CHOL (see text). The correct pK_a of HC was found by overlaying the lower part of its dissociation curve with the dissociation curve of DOPC liposomes and adding the shift in pH units to the pK_a of DOPC liposomes. The Ψ_0^{GC} and $pH^{GC}_{surface}$ were calculated under the conditions that 100% or only 50% of the DC–CHOL was protonated.

branes can be estimated by taking the pK_a of HC in DOPC liposomes as a neutral reference, assuming that there is no change in surface polarity (see Eq. (2)).

Four interesting phenomena are observed in Fig. 1:

(i) Shift in pK_a (the apparent pK_a): The pK_a's of HC in all liposomes containing DOTAP or in liposomes composed of DMRIE/DOPE (1/1) were 3.8–4.2 units lower than the pK_a of HC in DOPC liposomes (see Table 1), indicating a negative ΔpK_{el} (positive electrical surface potential) and high $pH_{surface}$ for these cationic liposomes.

(ii) Deprotonation of DC–CHOL: The curve, which describes the pH-dependent dissociation degree of HC in DC–CHOL/DOPE (1/1) liposomes, shows that DC–CHOL in these bilayers had a pK_a of 8.0 (see Fig. 1). Comparing the value of the dissociation degree of HC in the DC–CHOL/DOPE (1/1) liposomes with the value of the dissociation degree of HC in DOPC liposomes (using Eq. (1)) showed that the sum of pK_H and pK_{pol} (which describes the intrinsic proton binding constant in the bilayer) of DC–CHOL is 10.2. At pH_{bulk} 7.4, the $pH_{surface}$ was also 10.2, indicating that only about 50% of the DC–CHOL in DC–CHOL/DOPE (1/1) liposomes was charged at pH_{bulk} 7.4. The correct pK_a of HC in DC–CHOL/DOPE (1/1) liposomes, if DC–CHOL would have been 100% protonated, was found by overlapping the lower part of the dissociation curve of HC in DC–CHOL/DOPE (1/1) liposomes with the dissociation curve of HC in DOPC liposomes. This curve showed that the pH of 50% HC ionization in DC–CHOL/DOPE (1/1) LUV shifted −3.7 pH units compared with DOPC LUV; thus the correct pK_a of HC in DC–CHOL/DOPE (1/1) LUV was 6.8 (see Table 1). Also in the biphasic pH-dependent

dissociation curve of HC in DC–CHOL/DOPE (1/1) liposomes (Fig. 1), the first (lower) sigmoid (pH_{bulk} range of 3–9) was related mainly to the ionization of DC–CHOL, while the upper sigmoid at higher pHs expressed mainly the level of protonation of the primary amino group of DOPE.

(iii) Dissociation of DOPE: The dissociation curve of HC in DOPC/DOPE (1/1) LUV was slightly shifted to higher pH values (relative to HC in DOPC liposomes), possibly due to partial dissociation of the primary amino group of DOPE at high pH_{bulk} compared with the complete dissociation of the choline quaternary amine moiety of the PC (see Section 4). A similar phenomenon is probably responsible for the deviation in the curve of the pH-dependent dissociation degree of HC in DOTAP/DOPE (1/1) and DMRIE/DOPE (1/1) liposomes above pH_{bulk} 7.9, when compared to the dissociation curves of HC in DOTAP and DOTAP/DOPC (1/1) liposomes. This part of the curve was omitted when the curves were fitted to Eq. (1).

(iv) Salt bridge between DOTAP or DMRIE and DOPE or DOPC: The dissociation degree of HC in DOTAP/DOPE (1/1), DOTAP/DOPC (1/1), and DMRIE/DOPE (1/1) liposomes showed a plateau (dissociation degree about 8%) between about pH_{bulk} 4.5 and 5.5, indicating the presence of a molecule that could bind a proton at about pH_{bulk} 5. This phenomenon was not found with HC in DOTAP and DC–CHOL/DOPE (1/1) liposomes; it is probably caused by a salt bridge between the quaternary amine of DOTAP or DMRIE and the phosphate group of DOPE or DOPC (see Section 4). The data points of this deviation were also omitted when the curves of the dissociation degree of HC were fitted to Eq. (1) (Fig. 1B).

The values of ΔpK_{el} were used to calculate the electrical surface potential at the location of the chromophore in the charged membranes, Ψ_0^{HC} (V), by conversion and rearrangement of the Boltzmann equation [18,21,22]

$$\Psi_0^{HC} = -\frac{\Delta pK_{el} kT}{e \ln 10} = \frac{\left(pK_a^{charged} - pK_a^{neutral}\right) kT}{e \ln 10} \quad (3)$$

where k is the Boltzmann constant (1.38×10^{-23} J K^{-1}), T is the absolute temperature (here 295 K), e is the electron charge (1.6×10^{-19} C) and $pK_a^{charged}$ and $pK_a^{neutral}$ are the pK_a's in charged bilayers and in neutral DOPC bilayers, respectively. Ψ_0 was also calculated according to the Gouy–Chapman approximation [18,21,22], Ψ_0^{GC}, given by:

$$\Psi_0^{GC} = \left(\frac{2kT}{ze}\right) \sinh^{-1}\left(\frac{ze\sigma\lambda}{2\epsilon_0 \epsilon_r kT}\right) \quad (4)$$

where z is the valency of the counterions (in the present study, 1), σ is the surface charge density (C m^{-2}), λ is the Debye screening length (m), ϵ_0 is the relative permittivity of free space (8.85×10^{-12} C^2 J^{-1} m^{-1}), and ϵ_r is the dielectric constant at the location of the fluorophore moiety, with a value of 8 (see above). The values of σ were calculated taking a molecular surface area of 0.82 nm^2 for DOPC [23], 0.65 nm^2 for DOPE [24], 0.30 nm^2 for DC–CHOL (assumption, deduced from the molecular areas of phospholipid/cholesterol layers [23]), 0.65 nm^2 for DMRIE (assumption), and 0.65 nm^2 for DOTAP (assumption, also based on the molecular area of DOPE and on molecular modelling (see Fig. 4). λ is given by [18,21,22]:

$$\lambda = \sqrt{\frac{\epsilon_0 \epsilon_r kT}{Ne^2 \sum c_i z_i}} \quad (5)$$

where N is the Avogadro constant and c_i is the concentration of all individual ions (here, 0.017 mol m^{-3}). In the present study, a value of 0.74 nm for λ was calculated. In Table 1, the values of Ψ_0 determined by both methods mentioned above are shown. Also shown in Table 1 are the values for $pH_{surface}$ as determined by comparing the value of the dissociation degree of HC in each of the liposomes used in the present study to the value of the dissociation degree of HC in the DOPC liposomes and as determined by using the Boltzmann equation for a given potential [22]:

$$pH_{surface} = 7.4 + \frac{\Psi_0 e}{kT \ln 10} \quad (6)$$

Only small discrepancies were found between the estimated values of Ψ_0 and $pH_{surface}$ by the different methods, which indicates good agreement between the experimental data and the theory.

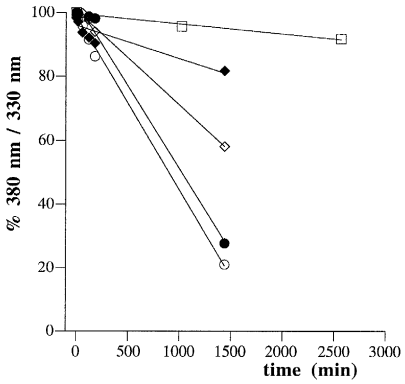

Fig. 2. The effect of liposome storage time on the dissociation degree of HC in cationic liposomes ($n = 2$). The concentration and composition of the cationic liposome dispersions during storage were: ○, 8×10^{-5} M total lipids of DOTAP/DOPE (1/1); ●, 8×10^{-5} M total lipids of DOTAP/DOPC (1/1); ◇, 4×10^{-5} M DOTAP; ◆, 8×10^{-5} M total lipids DC−CHOL/DOPE (1/1); □, 40 mM total lipids of DOTAP/DOPE (1/1) (stored at 40 mM and at each time point measured immediately after dilution to 4×10^{-5} M of DOTAP LUV, and to 8×10^{-5} M for LUV of all other compositions).

3.2. Stability of cationic liposomes

3.2.1. Dilution and storage-time effect on the degree of HC dissociation

Minor increases in the dissociation degree of HC with storage time were probably caused by formation of anionic fatty acids due to chemical hydrolysis of the lipids (see Section 4). Hydrolysis of phospholipids in buffered dispersions follows pseudo-first-order kinetics [25–27]. Upon storage of 40 mM DOTAP/DOPE (1/1) liposomes in 20 mM Hepes buffer (pH 7.4) at 25°C, HPLC analysis of the (phospho)lipids showed that the hydrolysis rate constants of DOTAP and DOPE were 2×10^{-7} s^{-1} and 4×10^{-7} s^{-1}, respectively. This indicates that 2% of DOTAP and 3% of DOPE were degraded after 24 h. However, when the LUV were diluted so that the cationic lipid reached a concentration of 40 μM, dispersions of liposomes containing DOTAP, [but not these containing DC–CHOL/DOPE (1/1)] show large changes in the dissociation degree of HC with time. These changes were much larger than expected based on hydrolysis rates (Fig. 2). A linear ($R \geq 0.98$) increase in the dissociation degree of the diluted liposomes containing DOTAP was found with time. The same observation for liposomes containing DOTAP was made in the presence of plasmid DNA (data not shown). HC is almost nonfluorescent in an aqueous medium, so only HC remaining in lipid assembly was observed. The amount of fluorescence at 330 nm (the isosbestic point, which reflects the amount of HC) hardly changed upon dilution, indicating that the major changes in the dissociation degree of HC cannot be explained by 'leaking' of the probe into the aqueous bulk or by degradation of the probe.

3.2.2. Dilution and storage-time effect on static light-scattering

Static light-scattering at 90° (in arbitrary units) of the diluted and undiluted LUV was followed for 24 h using the spectrofluorometer under the conditions that both excitation and emission wavelength were at 600 ± 2.5 nm. No precipitation was observed. Minimal changes (for cationic liposomes), or no changes (for neutral liposomes) in static light-scattering were observed for LUV of all compositions used in this study at the millimolar lipid concentration range. However, upon dilution to 40 μM DOTAP (80 μM total lipids for DOTAP/helper lipid 1/1 LUV) after 24 h, the following changes in the light-scattering occurred: For neutral liposomes almost no change. For DOTAP/DOPC (1/1) a continuous reduction with time from 1970 to 1261; and with 100% DOTAP a very dramatic reduction from 779 to 177; while for DOTAP/DOPE static light-scattering was reduced during the first 3 h of storage (from 1745 to 1302), followed by a large increase to 2724 at 24 h.

All the above (Sections 3.2.1 and 3.2.2) indicates that major changes in the overall composition and structure of the assembly occurred (see Section 4).

A likely explanation is desorption of the cationic lipid from the lipid assembly upon dilution. The rate of desorption is defined as k_{off}. For DOTAP in 80 μM DOTAP/DOPE LUV the first-order k_{off} of 1.45×10^{-5} s^{-1} was found, while for 500-fold higher lipid concentration, k_{off} was much slower (Fig. 2). This relationship between DOTAP/DOPE concentration and k_{off} suggests that DOTAP desorption is related to the high critical aggregation concentration (CAC) in which the transformation form monomers to amphiphile assemblies occurs (see Discussion). The CAC of DOTAP in 20 mM Hepes buffer (pH 7.4) was determined by measuring the surface tension

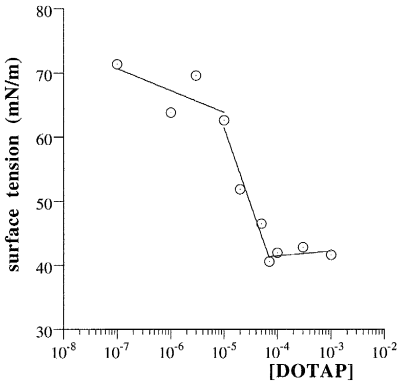

Fig. 3. Surface tension against [DOTAP] in 20 mM Hepes buffer (pH 7.4) at ambient temperature.

of the cationic lipid as a function of the concentration. Fig. 3 shows that above 5×10^{-6} M DOTAP, a linear decrease in surface tension was found with increasing logarithmic values of the concentration of DOTAP, until a limiting value was reached at the concentration of DOTAP corresponding to its CAC of 7×10^{-5} M.

4. Discussion

4.1. Characteristics of cationic liposomes

In the present study, we used the membrane-associated, pH-sensitive fluorophore HC to study the electrostatic parameters of cationic liposomes at the water–lipid interface. HC has the unique property that its fluorescence lifetime is unaffected by temperature or by the physical state of lipids [28]. This single-chain fluorophore has a very low CAC and it practically does not dissolve in the aqueous phase in a monomeric form. Also, HC aggregates in the aqueous phase form fully-quenched assemblies which do not contribute to the fluorescence intensity. The loss of HC from the amphiphile assembly can therefore be followed from the decrease in the fluorescence intensity as a result of excitation at 330 nm (pH independent isosbestic wavelength, see Section 3). Thus, alterations in the ratio of the excitation fluorescence intensities at 380 and 330 nm (380 nm/330 nm fluorescence) will reflect mainly changes in the electrical properties of the water–lipid interface, which is the plane of interaction between the negatively-charged DNA and cationic lipid assemblies. The following phenomena of cationic LUV were observed by the use of HC:

4.1.1. Effect of cationic lipid

All cationic liposomes used in the present study had a large positive Ψ_0 and, as a result, a high $pH_{surface}$ in 20 mM Hepes buffer at pH_{bulk} 7.4 (see Table 1). Although DOTAP and DMRIE are both quaternary amines, the curve of the dissociation degree of HC in DMRIE/DOPE (1/1) liposomes differs from the one of HC in DOTAP/DOPE (1/1) liposomes, the latter being less positively charged at pH_{bulk} 5–7 (see Fig. 1). This difference is probably related to the presence of the hydroxyl group in the headgroup of DMRIE. The consequences for transfection efficiency of this phenomenon are yet unclear, though it may make a difference in the behaviour of lipid–DNA complexes in acidic compartments such as endosomes and lysosomes. DC–CHOL in the DC–CHOL/DOPE (1/1) liposomes at pH_{bulk} 7.4 was only about 50% protonated, in contrast to liposomal DOTAP and DMRIE which were 100% ionized (see Fig. 1). To our knowledge, this has not been reported before. The pK_a and the sum of pK_H and pK_{pol} (which describe the intrinsic proton-binding constant in the bilayer) of DC–CHOL were found to be 8.0 and 10.2, respectively. This can be explained by the fact that DC–CHOL is a tertiary amine and DOTAP and DMRIE are quaternary amines. We assume that interaction of cationic DC–CHOL/DOPE (1/1) bilayers with negatively charged plasmid DNA will lead to further protonation of the DC–CHOL, due to lowering of the $pH_{surface}$ of the bilayers by the polynucleotide charged phosphates. However, this cannot be measured with the system used in the present study because the negative charge of the DNA and the induced positive charge of the DC–CHOL counteract each other (no net change in $pH_{surface}$). The fact that DC–CHOL in DC–CHOL/DOPE (1/1) bilayers is only partially charged might have three consequences for DNA–lipid interactions:

(a) It is likely that dissociation of lipid assemblies containing DC–CHOL from the plasmid DNA will require less energy than dissociation of assemblies containing fully-charged lipids from the plasmid DNA. To enable transcription, the DNA–lipid com-

plex should dissociate and release lipid-free (or lipid-poor) DNA [10].

(b) The charge at the outside of DC–CHOL-containing liposomes and the DNA–lipid interaction will be pH dependent. Indeed, Sorgi and Huang [29] found by dynamic light-scattering measurements that the sizes of DNA–DC–CHOL/DOPE (3/2) complexes in 20 mM Hepes buffer were pH dependent. They did not explain this phenomenon. At pH 9.5, no size changes of the 200-nm DC–CHOL/DOPE (3/2) liposomes were observed upon addition of different amounts of plasmid DNA, suggesting that no interaction between liposomes and plasmid DNA occurred. At pH 5.5 and 7.5, large complexes with a heterogeneous size up to 1–2 μm were observed in the range of DNA/DC–CHOL of 0.3–0.6. The largest complexes at pH 5.5 were found at a somewhat lower DNA/DC–CHOL ratio than at pH 7.5. These results can be explained by the pK_a of DC–CHOL of 8.0 found in the present study: at pH 9.5, DC–CHOL/DOPE (3/2) liposomes were approximately neutral and could not interact with plasmid DNA, and at pH 5.5 and pH 7.5, these liposomes were 100% and about 50% charged, respectively.

(c) When complexes containing DC–CHOL will be endocytosed and end up in the endosomes, the pH of the endosomes might increase by proton transfer from the bulk of the endosomes to the not-fully-protonated DC–CHOL. This phenomenon might increase the release of the DNA–lipid complexes into the cytosol by destabilization of the endosomal membrane and/or by preventing degradation of DNA. An increase in pH inside the endosomes was previously suggested to explain the much higher transfection efficiency observed with the tertiary lipopolyamines as compared to quaternary cationic lipids [1].

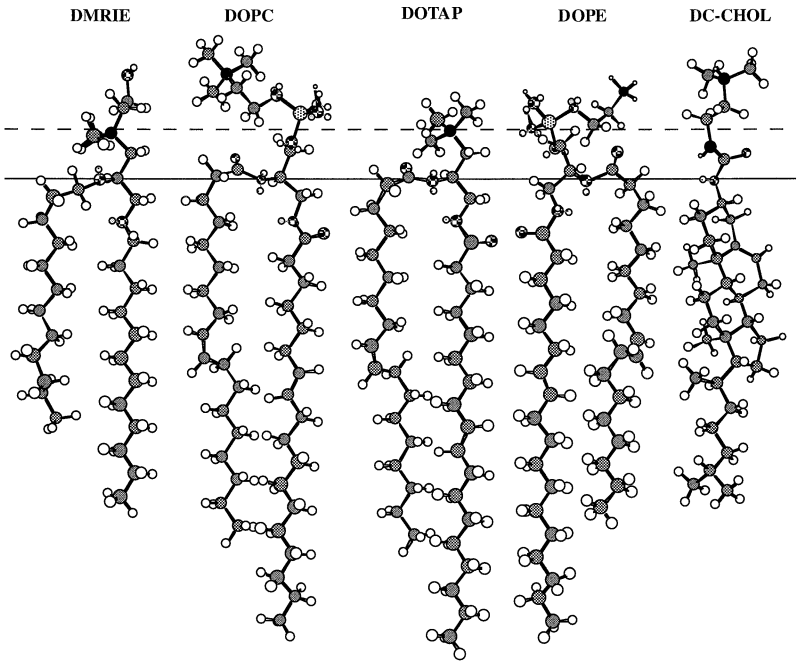

Fig. 4. Models of the molecules DMRIE, DOPC, DOTAP, DOPE, and protonated DC–CHOL obtained after free energy minimization using the software CSC Chem3DPlus™, version 3.1.1 (Cambridge Scientific Computing, Cambridge, MA). To facilitate comparison between the positions of the different molecules in a membrane, the oxygens at the sn2 position of DMRIE, DOPC, DOTAP, and DOPE were placed on the same plane relative to the membrane surface (unbroken line) as the oxygen of DC–CHOL that links the carbamoyl with the cholesterol moiety. These atoms are in a similar position in a membrane and there are discussions in the literature about whether or not the hydroxyl group of cholesterol interacts with the carbonyl group at the sn2-position of a phospholipid. The broken line is drawn through the quaternary amine group of DOTAP to facilitate estimation of the location of the charges in the headgroups. Also clearly shown are the differences in headgroup cross sections. However, more important is the effective size of the headgroup, which also includes bound water molecules and intermolecular hydrogen interactions. Symbols: ◦, lone electron pair; ○, H-atom; ; C-atom; ●, N-atom; , O-atom; , P-atom.

4.1.2. Effect of helper lipid

The primary amine group of DOPE in DOTAP/DOPE (1/1) and DOPC/DOPE (1/1) liposomes can dissociate at high (> 7.9) pH_{bulk} (see Fig. 1B). The pK_a and pK_H of this dissociation process are not clear from our data, shown in Fig. 1B because the pK_a of HC in liposomes (10.5 for neutral DOPC liposomes, see Table 1) is only slightly lower than the pK_a of the liposomal DOPE. In the literature, values of the sum of pK_H and pK_{pol} of 9–11.3 have been reported, depending on the method of determination and the hydration medium [18,21,22]. When the liposomes containing DOPE have a higher mole ratio of monocationic lipids or contain polycationic lipids and/or when the pH_{bulk} of the incubation medium is increased, a decrease in Ψ_0 and $pH_{surface}$ can be expected due to the dissociation of the primary amine group of DOPE (DOPE becomes negatively charged). The dissociation degree of HC in DOTAP/DOPE (1/1), DOTAP/DOPC (1/1), and DMRIE/DOPE (1/1) liposomes showed a plateau (dissociation degree about 8%) between about pH_{bulk} 4.0 and pH_{bulk} 5.5, indicating the presence of an ionizable group at pH_{bulk} 5, most likely the phosphate group of the phospholipids DOPE or DOPC. The pK_a of the phosphate group of PC or PE is between about 1 and 3.5, depending on method of determination and hydration medium [18,21,22]. It is possible that a salt bridge (or ion pair) between the quaternary amine of DOTAP or DMRIE and the phosphate group of the phospholipids DOPE or DOPC is responsible for this shift in pK_a of the phosphate group. Molecular modelling demonstrated that these two moieties are in a similar plane relative to the glycerol backbone (see Fig. 4). If such salt bridge occurs, then the positively charged moieties of DOPE or DOPC will also contribute to the overall positive charge, and potential, of the liposomal surface through their primary or quaternary amines, respectively. Therefore, the net charge of the bilayers does not change by this interaction, although the exact location of the positive charge may be modified. The plateau was not seen when HC was incorporated into 100% DOTAP or DC–CHOL/DOPE (1/1) bilayers. Spatial considerations (see Fig. 4) suggest that salt bridges may be more effective between the quaternary amine of DOTAP or DMRIE and the phosphate group of DOPE or DOPC than between the tertiary amine of DC–CHOL and the phosphate group of DOPE. DOPE, however, is essential to form vesicles with DC–CHOL [11].

4.1.3. Stability of liposomes

We found two different, relatively slow processes which modify the LUV electrostatic properties upon storage in aqueous dispersion. The slower of the two processes is independent of lipid concentration, and is probably caused by a chemical hydrolysis. In an aqueous dispersion, liposomal ester (phospho)lipids are hydrolysed to free fatty acids and 2-acyl- and 1-acyl-lyso(phospho)lipids and/or deacylated water-soluble glyco derivatives [25]. Free fatty acids are negatively charged at the high $pH_{surface}$ of the cationic liposomes used in the present study and will probably reduce the liposomal surface potential upon formation. Based on previously reported first-order rate constants for the hydrolysis of liposomal phospholipids [26,27], one would expect that hydrolysis rate constants of lipids in DOTAP/DOPE (1/1) liposomes at 25°C in an aqueous medium similar to that used here would be 2×10^{-7} and 5×10^{-7} s^{-1} at pH_{bulk} 7.0 and 8.0, respectively. These theoretical hydrolysis rate constants correspond well with the experimental hydrolysis rate constants found in the present study at pH 7.4 and at 25°C for DOTAP and DOPE, 2×10^{-7} and 4×10^{-7} s^{-1}, respectively, which indicates that 2% of DOTAP and 3% of DOPE were degraded upon storage for one day at 25°C. The decrease in the percent dissociation degree of 40 mM DOTAP/DOPE (1/1) liposomes of about 8% after 1 day storage (see Fig. 2) supports the hypothesis made above that the hydrolysis product free fatty acids can change the surface potential of cationic bilayers.

The second process which introduces instability is faster, and unrelated to the chemical stability of the ester lipids. It is dependent on the type of cationic lipid in the assembly, and on its concentration. This unique instability was found to be related to the cationic lipid CAC and was much faster below the CAC than above it (Figs. 2 and 3). DOTAP has a relatively high CAC (7×10^{-5} M; see Fig. 3) and dilution to concentrations below its CAC (to 4×10^{-5} M) induced instability, probably due to desorption of DOTAP from the assembly, as was also supported by reduction in static light-scattering. The light-scatter-

ing data (Section 3.2.2) suggest that the number and/or the size of DOTAP LUV was dramatically reduced in 24 h upon dilution to 40 μM DOTAP. The reduction in light scattering for the DOTAP/DOPC was much smaller, probably due to the DOPC, which has a much lower CAC than DOTAP, so it remained in the vesicular phase of LUV poor in DOTAP; for DOTAP/DOPE LUV, DOTAP was desorbed, leaving behind LUV poorer in DOTAP until the DOTAP/DOPE in the liposomes reached a critical low level; then a transformation from lamellar to hexagonal type II phase occurred, as suggested by the increase in light scattering, in agreement with Sternberg [30]. Combining the data on time-dependent changes in 380/330 HC fluorescence, and the static light-scattering of LUV in which the lipid was diluted 500–1000-fold, suggests that no compositional or structural changes occurred upon dilution of neutral LUV, while all LUV containing DOTAP went through time-dependent compositional and/or structural changes. The DC–CHOL is expected to have a lower CAC than DOTAP, which is reflected in the better stability of DC–CHOL/DOPE (1/1) liposomes upon dilution (see Fig. 2). Most phospholipids with two long acyl chains have a CAC of about 10^{-10} M, but the high CAC of DOTAP is not uncommon for other types of two-hydrocarbon chain cationic (quaternary amine) surfactants [31]. It may be related to the weaker cohesiveness between the amphiphiles in the cationic (quaternary amine) liposomes as assessed form their much higher leakiness compared with neutral (DOPC, or DOPC/DOPE) liposomes (Zuidam and Barenholz, unpublished). It is unclear yet how the high CAC of DOTAP affects the outcome of the transfection process. From the stability point of view, the properties of the complexes should not change upon dilution, as was the case for DC–CHOL/DOPE (1/1) liposomes. However, to enable transcription of the plasmid DNA, the lipids should be dissociated from the plasmid DNA at a certain stage of the transfection process [10,32]. It is possible that the difference in the transgene efficacy of various counter anions [33] may be related to their effect on the cationic lipid CAC and k_{off}. It seems that negatively charged lipid/water interfaces facilitate the release of nucleic acids from their complexes with cationic lipids [32]. The exact mechanism by which it occurred and its relevance to the transfection process remain to be clarified, as is the involvement of cationic lipid k_{off}.

The present article describes the electrostatic parameters of the lipid–water interface of cationic LUV which are similar in their size, and are commonly used to deliver DNA into cells, but varied in their cationic lipid and their helper lipid. The same HC-approach should be applied to try to relate the electrostatics, the physical stability, and other variables of the system which affect transgene efficacy, such as the cationic lipid counter anion [33]. In a following paper we will demonstrate that monitoring DNA–lipid interactions by determining how negatively charged plasmid DNA changes these electrostatic parameters, concomitant with time-dependent alteration in size, is a powerful tool for characterizing DNA–lipid complexes.

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References

[1] J.S. Remy, C. Silin, J.P. Behr, In: J.R.S. Phillippol, F. Schuber (Eds.), Liposomes as Tools in Basic Research and Industry, CRC Press, Boca Raton, FL, 1995, pp. 159–170.
[2] P.L. Felgner, Y.J. Tsai, J.H. Felgner, In: D.D. Lasic, Y. Barenholz (Eds.), Handbook of Nonmedical Applications of Liposomes, Vol. 4, CRC Press, Boca Raton, FL, 1996, pp. 43–56.
[3] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Proc. Natl. Acad. Sci. USA 84 (1987) 7413–7417.
[4] J.H. Felgner, R. Kumar, S.H. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, J. Biol. Chem. 269 (1994) 2550–2561.
[5] R. Leventis, J.R. Silvius, Biochim. Biophys. Acta 1023 (1990) 124–132.

[6] X. Gao, L. Huang, Biochem. Biophys. Res. Commun. 179 (1991) 280–285.
[7] X. Zhou, A.L. Klibanov, L. Huang, Biochim. Biophys. Acta 1065 (1991) 8–14.
[8] X. Zhou, L. Huang, Biochim. Biophys. Acta 1189 (1994) 195–200.
[9] K. Goyal, L. Huang, J. Liposome Res. 5 (1995) 49–60.
[10] J. Zabner, A.J. Fasbender, T. Moninger, K.A. Poellinger, M. Welsh, J. Biol. Chem. 270 (1995) 18997–19007.
[11] X. Gao, L. Huang, Biochemistry 35 (1996) 1027–1036.
[12] R.C. MacDonald, R.I. MacDonald, B.P.M. Menco, K. Takeshita, N.K. Subbarao, L. Hu, Biochim. Biophys. Acta 1061 (1991) 297–303.
[13] Y. Barenholz, S. Amselem, In: G. Gregoriadis (Ed.), Liposome Technology, Vol. 1, 2nd ed., CRC Press, Boca Raton, FL, 1993, pp. 527–616.
[14] A. Martin, Physical Pharmacy, 4th ed., Lea and Febiger, Philadelphia, PA, 1993.
[15] I. Jääskeläinen, A. Urtti, J. Pharm. Biomed. Anal. 12 (1994) 977–982.
[16] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[17] J.E. Whitaker, R.P. Haugland, D. Ryan, P.C. Hewitt, R.P. Haugland, F.G. Prendergast, Anal. Biochem. 207 (1992) 267–279.
[18] S.A. Tatulian, In: G. Cevc (Ed.), Phospholipids Handbook, Marcel Dekker, New York, 1993, pp. 511–552.
[19] M.S. Fernández, P. Fromherz, J. Phys. Chem. 81 (1977) 1755–1761.
[20] H.S. Harned, B.B. Owen, The Physical Chemistry of Electrolytic Solutions, Reinhold, New York, 1958.
[21] G. Cevc, Biochim. Biophys. Acta 1031 (1990) 311–382.
[22] J.F. Tocanne, J. Teissié, Biochim. Biophys. Acta 1031 (1990) 111–142.
[23] L.J. Lis, M. McAlister, N. Fuller, R.P. Rand, Biophys. J. 37 (1982) 657–665.
[24] S.M. Gruner, M.W. Tate, G.L. Kirk, P.T.C. So, D.C. Turner, D.T. Keane, C.P.S. Tilcock, P.R. Cullis, Biochemistry 27 (1988) 2853–2866.
[25] M. Grit, N.J. Zuidam, D.J.A. Crommelin, In: G. Gregoriadis (Ed.), Liposome Technology, 2nd ed., CRC Press, Boca Raton, FL, 1993, pp. 455–486.
[26] M. Grit, N.J. Zuidam, W.J.M. Underberg, D.J.A. Crommelin, J. Pharm. Pharmacol. 45 (1993) 490–495.
[27] N.J. Zuidam, D.J.A. Crommelin, J. Pharm. Sci. 84 (1995) 1113–1119.
[28] R. Pal, W.A. Petri, V. Ben-Yashar, R.R. Wagner, Y. Barenholz, Biochemistry 24 (1985) 573–581.
[29] F.L. Sorgi, L. Huang, Proc. Int. Symp. Control. Rel. Bioact. Mater. 22, Proc. of a symp. of the Controlled Release Soc., Seattle, 1995, pp. 460–461.
[30] B. Sternberg, J. Liposome Res. 6 (1996) 515–533.
[31] J. Israelachvili, Intermolecular and Surface Forces, 2nd Ed. Academic Press, London, 1991, p. 357.
[32] F.C. Szoka, Y. Xu, O. Zelphati, J. Liposome Res. 6 (1996) 567–587.
[33] M.J. Bennet, A.M. Aberle, R.P. Balasubramaniam, J.G. Malone, M.H. Nantz, R.W. Malone, J. Liposome Res. 6 (1996) 545–565.