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Physico-chemical characterization of a double long-chain cationic amphiphile (Vectamidine) by microelectrophoresis

Véronique Pector [a,*], Jacques Caspers [a], Sunanda Banerjee [a], Luc Deriemaeker [b], Robert Fuks [c], Abdel El Ouahabi [a], Michel Vandenbranden [a], Robert Finsy [b], Jean-Marie Ruysschaert [a]

[a] *Laboratoire de Chimie Physique des Macromolecules aux Interfaces (LCPMI), Université Libre de Bruxelles, Campus Plaine CP 206/2, B-1050 Brussels, Belgium*

[b] *Fysische en Colloïd Chemie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium*

[c] *Laboratoire de Chimie Générale, Institut de Pharmacie, Université Libre de Bruxelles, Campus de la Plaine, CP 206/4 B-1050 Brussels, Belgium*

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Abstract

We recently synthesized a novel cationic amphiphile (*N*-*t*-butyl-*N*′-tetradecyl-3-tetradecylaminopropionamidine or Vectamidine (previously described as diC_{14}-amidine)) that associates with DNA and RNA and facilitates their entry and expression into eukaryotic cells. Among several parameters that have been shown to influence the transfection process, the surface charge density plays a key role. Quantitative information about that charge density associated to the cationic amphiphiles organized in liposomal structure is not yet available. We provide here evidence by titration and microelectrophoresis measurements that an evaluation of the intrinsic acidity constants, the surface pH and the counterion binding constants allows to determine the charge density at physiological pH of Vectamidine liposomes. The knowledge of this superficial charge is a prerequisite to a molecular understanding of the DNA-cationic amphiphile complex formation. The method described could be extended to any kind of cationic amphiphile.

Keywords: Cationic liposome; Microelectrophoresis; Zeta potential

1. Introduction

Cationic liposomes have been largely described to associate with genetic material [1–11] and to facilitate their entry and expression into eukaryotic cells. Many laboratories [3, 12–15] are studying the mechanism underlying the formation and the penetration of such complexes into cells. Several parameters have been shown to enhance the cationic amphiphile transfection efficiency (phosphatidylethanolamine [16], lipid:DNA ratio [17], cholesterol [18, 19], Zeta potential [20], etc.). Among them, the surface charge density has been suggested to play a key role: the positive charge of the complexes would facilitate the interaction with a largely negatively charged plasma membrane.

Evaluation of this charge density implies that the

* Corresponding author. Fax: +32 (2) 6505382; E-mail: vpector@ulb.ac.be

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intrinsic acidity constants ($pK_{a\,int}$), the surface pH and the counterion binding constants are known.

In this work, we determined the apparent pK_a values ($pK_{a\,app}$) by titration and the bulk counterion binding constants by microelectrophoresis of *N-t*-butyl-*N′*-tetradecyl-3-tetradecylaminopropionamidine (Vectamidine) (Fig. 1-I,II,III) liposomes to calculate the intrinsic pK_a and to evaluate the real charge density of the liposomal surface at physiological pH.

2. Material and methods

Vectamidine (previously described as diC_{14}-amidine) (Fig. 1-I,II,III) has been synthesized by aminolysis of ethyl *N-t*-butylacrylimidate with tetradecylamine as described [21] (BiotechTools, Belgium). 'Diisopropylamidine' or *N-t*-butyl-*N′*-isopropyl-3-isopropylaminopropionamidine (Fig. 1-IV) has been synthesized according to a method described earlier [22]. It is a colorless liquid purified by horizontal distillation at 80°C/0.1 Torr. HEPES was purchased from Sigma and NaCl from Merck.

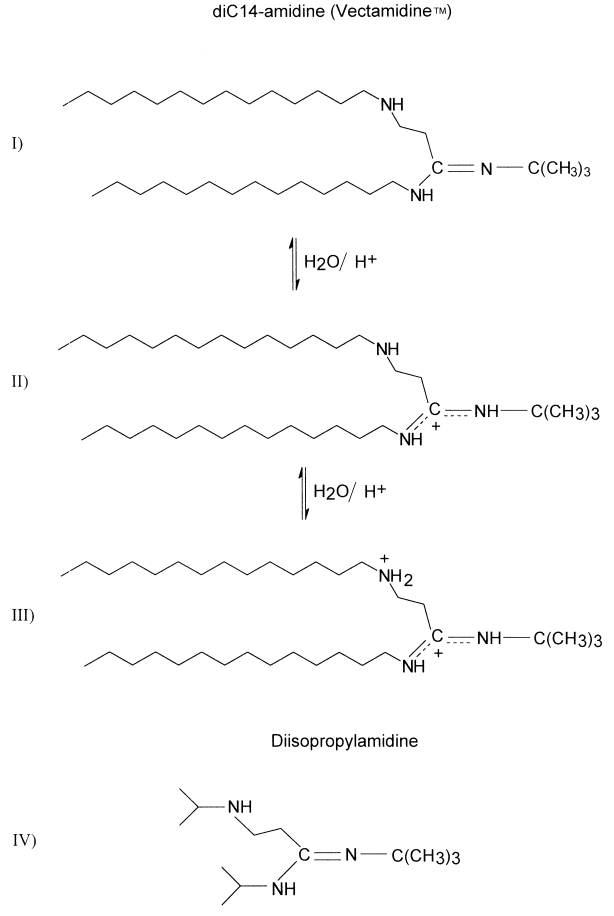

Fig. 1. Structure of *N-t*-butyl-*N′*-tetradecyl-3-tetradecylaminopropionamidine (or Vectamidine) (I, II, III) and of *N-t*-butyl-*N′*-isopropyl-3-isopropylaminopropionamidine (IV) (or diisopropylamidine).

2.1. Preparation of Vectamidine liposomes

Vectamidine was dissolved in chloroform, dried under a stream of nitrogen and left in a desiccating vacuum overnight. The liposomes were formed after buffer addition (HEPES alone or HEPES-NaCl at different concentrations; pH 7.3, buffered with NaOH) to the lipid film and mechanical mixing above the transition temperature (23°C) [21].

2.2. Acid-base titration of Vectamidine liposomes

Fourteen milligram of Vectamidine liposomes (4.4 mM final) were titrated in 6 ml of decarbonated water at various saline concentrations (0.030, 0.100 and 0.130 M NaCl) under a nitrogen stream and magnetic stirring, with a standard solution of 0.1 M HCl in a micrometric syringe.

2.3. Acid-base titration of diisopropylamidine

Ten milligram of diisopropylamidine (or *N-t*-butyl-*N′*-isopropyl-3-isopropylaminopropionamidine) (4.4 mM final) were titrated in 6 ml of decarbonated water at various saline concentrations (0.030, 0.100 and 0.130 M NaCl) under a nitrogen stream and magnetic stirring, with a standard solution of 0.1 M HCl in a micrometric syringe.

2.4. Laser Doppler velocimetry

Electrophoretic mobility measurements by laser Doppler velocimetry of the Vectamidine liposomes were performed at 25°C on a Coulter DELSA 440 SX at the stationary level in a capillary cylindrical cell (length 0.5 cm; diameter 2 mm; height 1 mm) with an applied voltage of 10 V. The vesicle concentrations were 3.74 mM in HEPES buffer without NaCl and 2.8 μM in NaCl-HEPES buffer.

Table 1
Apparent pK_a values (p$K_{a\,app}$) of the amine and amidine functions on the Vectamidine liposomes and diisopropylamidine, obtained at the half-equivalence by titration of the liposomes by HCl 0.1 M at increasing NaCl concentrations, under a nitrogen stream

	Conc. NaCl (M)	p$K_{a\,app}$(1) (amine)	p$K_{a\,app}$(2) (amidine)
Vectamidine liposomes	0.030	4.6 ± 0.1	8.8 ± 0.1
	0.100	5.05 ± 0.1	9.2 ± 0.1
	0.130	5.2 ± 0.1	9.4 ± 0.1
Diisopropylamidine	0.030	7.7 ± 0.1	10.2 ± 0.1
	0.100	7.5 ± 0.1	10.3 ± 0.1
	0.130	7.6 ± 0.1	10.3 ± 0.1

2.5. Dynamic laser light scattering

Vesicle sizes were determined using a set-up composed of a Lexel ^{95}Ar-iron Laser (λ 488 nm), an ALV goniometer, an EMI 6893A with Selfoc pigtailed collimator and a Brookhaven BI9000AT correlator. The scattering intensities were recorded at a 90° angle. The vesicle concentrations were 3.74 mM in HEPES buffer without NaCl and 1.87 mM in NaCl-HEPES buffer.

3. Results and discussion

3.1. Determination of apparent acidity constants ($pK_{a\,app}$) of Vectamidine liposomes by titration

To evaluate the residual charge surface density, Vectamidine liposomes were titrated by HCl 0.1 M at various saline concentrations: 0.030 M, 0.100 M and 0.130 M NaCl[1].

The acidity constant pK_a values are significantly affected by the ionic strength of the medium (Table 1), suggesting a modification of the surface potential [23].

This surface effect is not observed with diisopropylamidine (or *N-t*-butyl-*N′*-isopropyl-3-isopropylaminopropionamidine) which contains the polar head of the Vectamidine and two isopropyl 'chains' (Fig. 1-IV). The hydrocarbon chains are likely to be too short to allow the formation of a liposomal structure. The titration curve exhibits two jumps but the pK_a values (7.5 and 10.3) are not significantly affected by the ionic strength (Table 1) and are intrinsic pK_a values.

3.2. Evaluation of the bulk counterion association constants by microelectrophoresis at pH 7.3

As shown above, pK_a values associated to the Vectamidine liposomes are significantly affected by the ionic strength and an evaluation of the bulk counterion association constants is a prerequisite to any kind of evaluation of the surface charge. As described below, measurements of microelectrophoretic mobility give access to these constants and finally to the intrinsic pK_a values.

The electrophoretic mobility μ is related to the Zeta potential ξ by Henry's law [24]:

$$\xi = \frac{3\eta\mu}{2\varepsilon_0\varepsilon_r}[f(\kappa a)]^{-1} \quad (1)$$

where η is the viscosity of the medium, ε_0 the permittivity of the vacuum, ε_r the relative permittivity of the medium, κ^{-1} the Debye-Huckel length and a the radius of the liposome. The Henry's function f(κa) varies from 1.0 to 1.5 for a values between 1 and $a \geq 200$ nm, at high ionic strength [25, 26]. In such conditions, Eq. 1 becomes (Smoluchowski relation) [27–29]:

$$\xi = \frac{\eta\mu}{\varepsilon_0\varepsilon_r} \quad (2)$$

[1] The volume of the HCl solution required to observe each jump corresponds to the required volume to titrate 95% of each titratable group (amidine and amine). The addition of a protonophore (CCCP or carbonyl cyanide *m*-chlorophenylhydrazone) did not affect significantly the position of the observed jumps, suggesting that the proton can access freely to the titratable groups located in the inner membrane of the liposomes. Identical jumps were observed by reverse titration by NaOH 0.1 M, demonstrating that the liposomal structure is maintained throughout the titration.

The Zeta potential ξ is the potential Ψ at the hydrodynamic plane of shear, assumed to be located [27, 30, 31] at 0.2 nm from the charged surface of the liposomes and, in this case, the electrical potential ψ_0, at the liposome-water interface, is derived from the Zeta potential ξ [30, 31].

ψ_0 is related to the surface charge density σ through the Gouy-Chapman equation [30–33]:

$$\psi_0 = \frac{2RT}{F} sh^{-1}\left\{\frac{\sigma}{(8RT\varepsilon_0\varepsilon_r C)^{1/2}}\right\} \quad (3)$$

R is the gas constant, T the absolute temperature, F the Faraday constant and C the ionic concentration (monovalent ions).

The surface charge density is given by [34]:

$$\sigma = \frac{N^* e}{AN} \quad (4)$$

e is the absolute value of the electronic charge, A the area of the polar group of Vectamidine (50 Å^2 corresponds to the area occupied by a Vectamidine molecule in a closely packed monolayer (surface pressure isotherms: unpublished results)), N^* the number of positively charged groups in the outer layer of the liposome and N the total number of groups in that layer.

3.2.1. HEPES anion association constant (K_{HEPES}) evaluation

Without NaCl in the medium, at pH 7.3, we assume that only ($HEPES^-$) anions interact with the single positively charged amidino groups.

Solutions were prepared at pH 7.3 and at increasing concentrations of [HEPES] and [$HEPES^-$]. At pH 7.3, 40% of the HEPES molecules are anionic (HEPES acidity constant $pK_a = 7.5$). Fig. 2 shows that the electrophoretic mobility of the Vectamidine liposomes decreases with increasing [$HEPES^-$] concentration (μ increases when $-\log$[$HEPES^-$] increases).

This decrease in mobility cannot be entirely correlated to a 'screening' effect due to the presence of ions in the aqueous medium. Indeed, a 3×10^{-8} m^2 V^{-1} s^{-1} mobility on Fig. 2 corresponds (for a particle with a diameter of 100 nm) to a Zeta potential (Eq. 1) of 44 mV. Therefore, the anionic adsorption of the buffer on the positively charged liposomes can not be neglected [34–36].

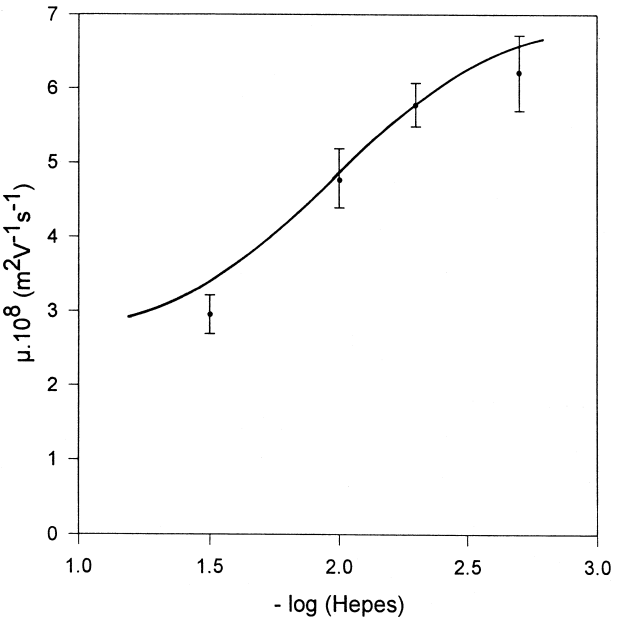

Fig. 2. Electrophoretic mobility (μ) of the diC_{14}-amidine liposomes in terms of HEPES concentration at pH 7.3. $T° = 20°C$. ——, computed curve $\mu = f(HEPES)_\infty$; $K_{HEPES} = 100$ l mole^{-1}. ●, experimental points. Error bars represent standard deviation.

Denoting the fraction of sites associated with $HEPES^-$ by γ, the ratio N^*/N will be equal to $1-\gamma$. Denoting the ratio σ/e by σ', Eq. 4 becomes:

$$A\sigma' = 1-\gamma \quad (5)$$

The 'interfacial' association constant (Langmuir adsorption) of the HEPES anion is given by [33, 34]:

$$K_{HEPES} = \frac{\gamma}{(1-\gamma)_0[HEPES^-]_0} = \frac{1-A\sigma'}{A\sigma'[HEPES^-]_0} \quad (6)$$

where [$HEPES^-$]$_0$ is the surface concentration of $HEPES^-$, related to the bulk concentration [$HEPES^-$]$_\infty$ [27, 34] by:

$$[HEPES^-]_0 = [HEPES^-]_\infty \cdot e^{F\psi_0/RT} \quad (7)$$

From the electrophoretic mobility, the Zeta potential ξ can be calculated, depending upon the liposome size, from Eq. 1 in HEPES buffer in which the Vectamidine liposomes were shown to have a diameter of 60 ± 3 nm (polydispersity: 0.2) or from Eq. 2 in HEPES-NaCl buffer, in which the diameter of the liposome was 300 ± 15 nm (polydispersity: 0.2). The electrical potential prevailing at the liposome-water interface (ψ_0) is derived from the Zeta potential (ξ) [30, 31] and the surface charge density (σ) from the electrical potential ψ_0 (Eq. 3). The in-

terfacial association constant K_{HEPES} is determined from Eqs. 6 and 7. Conversely, the mobility μ is calculated, for one bulk HEPES anion concentration $[HEPES^-]_\infty$ and one interfacial association constant K_{HEPES}.

A good agreement between the experimental and the theoretical curves was observed for an HEPES association constant $K_{HEPES} = 10^2 \pm 20$ l mol^{-1} (Fig. 2).

3.2.2. Chloride association constant (K_{Cl}) evaluation

Fig. 3 shows the evolution of the electrophoretic mobility (μ) of the Vectamidine liposomes in terms of NaCl concentrations in a HEPES 0.01 M pH 7.3 buffer.

We assume that at pH 7.3 both Cl^- and $HEPES^-$ interact with the positively charged amidine groups.

Denoting as β the fraction of sites associated with Cl^-, the ratio N^*/N is equal to $1-\beta-\gamma$. Eq. 5 can be written as

$$A\sigma' = 1-(\beta+\gamma) \quad (8)$$

An equilibrium relation similar to Eq. 6 describes the Cl^- adsorption:

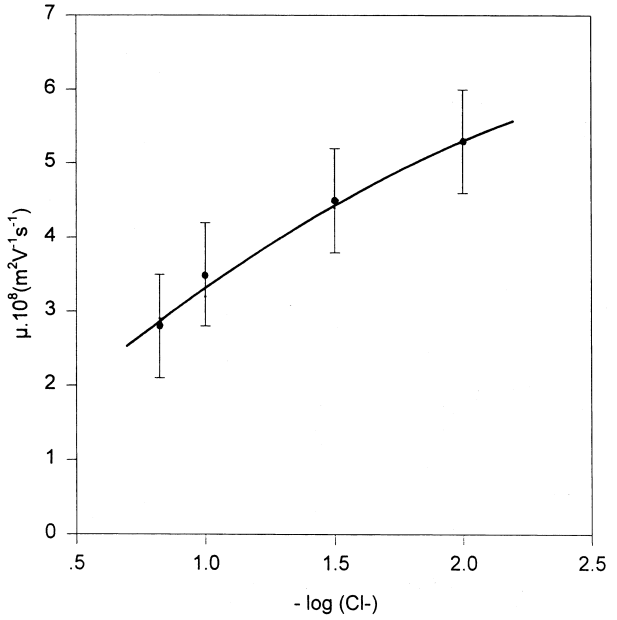

Fig. 3. Electrophoretic mobility (μ) of the diC_{14}-amidine liposomes in terms of Cl^- concentration at pH 7.3. Buffer: HEPES 0.01 M buffered to pH 7.3 with NaOH. $T° = 20°C$. ——, computed curve $\mu = f(Cl^-)_\infty$; $K_{Cl} = 3$ l mole^{-1}. ●, experimental points. Error bars represent standard deviation.

$$\beta = K_{Cl} A\sigma' [Cl^-]_0 \quad (9a)$$

$$\gamma = K_{HEPES} A\sigma' [HEPES^-]_0 \quad (9b)$$

$[Cl^-]_0$ is related to $[Cl^-]_\infty$ by a relation similar to Eq. 7:

$$[Cl^-]_0 = [Cl^-]_\infty \cdot e^{F\psi_0/RT} \quad (10)$$

Eqs. 9a and 9b can be combined in a unique relation:

$$K_{HEPES} = \frac{1-A\sigma'(1+K_{Cl}[Cl^-]_0)}{A\sigma'[HEPES^-]_0} \quad (11)$$

K_{HEPES} is determined for a given value of K_{Cl} (or vice versa K_{Cl}). These equations are used the other way round to calculate the mobility as a function of the bulk Cl^- for imposed values of K_{HEPES} and K_{Cl}. A good fitting between experimental and theoretical curves is observed for $K_{Cl} = 3 \pm 0.5$ l mol^{-1} and $K_{HEPES} = 10^2 \pm 20$ l mol^{-1} (Fig. 3).

In the conditions used in the transfection experiments (i.e. 0.15 M NaCl and 10^{-2} M HEPES at pH 7.3) (Eqs. 9a and 9b, the remaining surface charge density (σ′) is 3×10^{-3} charge per Å^2 and represents only 18% of the value in the absence of any counterion adsorption.

3.3. Intrinsic pK_a values ($pK_{a\,int}$) determination of the Vectamidine liposomes: comparison of the calculated and experimental titration curves

The intrinsic pK_a values ($pK_{a\,int}$) of the Vectamidine titratable groups is evaluated from the surface pH and the association constants of the buffer counterions.

If we define N_1 sites 1 as the amine titratable groups and N_2 sites 2 as the amidine titratable groups, four site equilibrium constants can be defined [37]:

$$Ka_{int}(1) = \frac{(1-\alpha_1-\beta_1)[H^+]_0}{\alpha_1} \quad (12)$$

$$Ka_{int}(2) = \frac{(1-\alpha_2-\beta_2)[H^+]_0}{\alpha_2} \quad (13)$$

$$K_{Cl}(1) = \frac{\beta_1}{\alpha_1 [Cl^-]_0} \quad (14)$$

$$K_{Cl}(2) = \frac{\beta_2}{\alpha_2[Cl^-]_0} \quad (15)$$

α_1 and α_2 are respectively the fraction of positively charged sites 1 and 2; β_1 and β_2 respectively the fractions associated with Cl^-; $[H^+]_0$ and $[Cl^-]_0$ the ionic concentrations at the interface; $K_{Cl}(1)$ and $K_{Cl}(2)$ the association constants characterizing the interactions of the charged sites with Cl^-. It is assumed that $K_{Cl}(1) = K_{Cl}(2) = 3 \pm 0.5$ l mol^{-1} and that Cl^- does not interact with the uncharged sites.

The positive electrostatic contribution to the surface potential (ψ_0) at the liposome-water interface is given by the Gouy-Chapman equation (Eq. 3). The surface charge density σ is given by:

$$\sigma = \frac{(\alpha_1 + \alpha_2)e}{A} \quad (16)$$

$[Cl^-]_0$ is given by Eq. 10 and $[H^+]_0$ by [27, 34]:

$$[H^+]_0 = [H^+]_\infty \cdot e^{-F\psi_0/RT} \quad (17)$$

where the subscript ∞ refers to the bulk concentration of the proton.

From the $Ka(1)/Ka(2)$ ratio, $K_{Cl}(1)$ and $K_{Cl}(2)$, the parameters α_1, α_2, β_1 and β_2 are calculated for different ψ_0 values (see Appendix) from Eqs. 3,10,12–16.

$[H^+]_0$ is calculated from Eqs. 12 and 13 (for given values to $Ka(1)_{int}$ and $Ka(2)_{int}$). The calculated value of $[H^+]_\infty$ corresponding to a given ψ_0 (for given α_1, α_2, β_1, β_2) is determined from Eq. 17.

The $[H^+]_\infty$ value can be correlated to the required volume of added HCl in the initial medium through Eqs. 18 and 19.

The volumes $V_{HCl}(1)$ and $V_{HCl}(2)$ of HCl solution required to titrate the first and second classes of sites are calculated assuming that the amidine sites are titrated first:

$$V_{HCl}(1) = \frac{n^\circ_B(\alpha_2 + \beta_2) - [OH^-]_\infty V_s}{C_{HCl} + [OH^-]_\infty} \quad (18)$$

$$V_{HCl}(2) = \frac{n^\circ_B}{C_{HCl}}(1 + \alpha_1 + \beta_1) \quad (19)$$

n°_B is the initial number of moles of Vectamidine in the suspension (2.6×10^{-5} moles), V_s is the volume of the suspension (6 ml) and C_{HCl}, the HCl concentration (0.1 M).

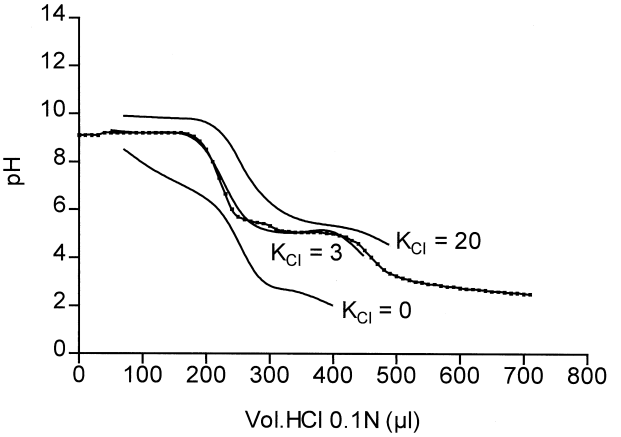

Fig. 4. Titration of the diC_{14}-amidine vesicles (4.4 mM) by HCl 0.1 M in the presence of 0.1 M NaCl under a nitrogen stream and with magnetic agitation. $T° = 20°C$. ——, theoretical curve for $K_{Cl} = 0$, 3 or 20 ($pK_a(1) = 5.5$ and $pK_a(2) = 9.7$). ■, experimental curve.

At a 0.100 M ionic strength, the calculated curve fits the experimental data for a $pK_a(1)$ value of 5.5 ± 0.5 and a $pK_a(2)$ of 9.7 ± 0.5 (Fig. 4).

The fact that this fitting was verified at 0.030 M and 0.130 M provides strong evidence that the calculations described above give access to the intrinsic pK_a determination.

Theoretical curves obtained with chloride binding constants K_{Cl} other than 3 (data not shown) do not fit the experimental one (Fig. 4).

The second titratable group (amino group) of Vectamidine is shifted of 2 units as compared to the diisopropylamidine. Since pK_a values are intrinsic parameters, sensitive to the surface effects (surface pH), that ΔpK_a must be correlated to other factors [38, 39]. In a 'biphasic medium', such as a suspension of liposomes in water, the dielectric constant at the lipid-water interface is lower than in the bulk [40]. Moreover, computer modeling of the orientation of Vectamidine molecules in a liposome structure has revealed (unpublished data) that this amine function is partly embedded in the lipid layer core. This hydrophobic surrounding of the titratable groups in the liposomes could be responsible for the observed intrinsic pK_a shift [41].

4. Conclusion

Endocytosis has been proposed as a possible path-

way for the entry of cationic liposomes-DNA complexes into cells [3, 12–15]. The stability of these complexes at acidic endosomal pH will depend upon the intrinsic acidity constants of the cationic amphiphile. The ionization states of most cationic molecules do not depend on the pH and one can expect that the endosomal pH will not affect the stability of the complex. On the opposite, both amine and amidine groups are titratable in the Vectamidine molecule.

Since the amine acidic constant is 4 pH units lower as compared to the amidine one, the Vectamidine is more positively charged at endosomal pH than at pH 7.

It has been suggested that the ability of the Vectamidine-DNA complexes to destabilize and to escape from endosomes [10] could be correlated with this modification of the ionization state of the cationic molecule, even if the contribution of the short saturated alkyl chains should not be minimized.

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Appendix

From Eqs. 14–16, the parameters a and b can be defined as follows:

$$K_{Cl}[Cl^-]_0 = b = \frac{\beta_1}{\alpha_1} = \frac{\beta_2}{\alpha_2}$$

$$\frac{\sigma A}{e} = a = \alpha_1 + \alpha_2$$

Or, equivalently:

$$\begin{aligned} \alpha_2 &= a - \alpha_1 \\ \beta_1 &= b\alpha_1 \\ \beta_2 &= b\alpha_2 \end{aligned} \qquad \text{(A1)}$$

The ratio $Ka(1)_{app}/Ka(2)_{app}$ is practically constant and equal to 1.5×10^4 despite the fact that $Ka(1)_{app}$ and $Ka(2)_{app}$ depend upon the ionic strength. We assume that the ratio $Ka(1)_{int}/Ka(2)_{int}$ will be equal to 1.5×10^4.

Combining the relations of Eq. A1 with

$$K = \frac{Ka(1)_{int}}{Ka(2)_{int}}$$

and from Eqs. 12 and 13, one obtains after some simplifications (since $K \gg 1$):

$$A\alpha_2^2 + B\alpha_2 + C = 0 \qquad \text{(A2)}$$

where $A = K(1+b)$, $B = -K[1+a(1+b)]$, $C = Ka$.

The solution of the quadratic Eq. A2 for a given value of Ψ_0 (Eq. 3) (for given values of a and b, Eq. 10) allows to determine α_2 (with the conditions $0 < \alpha_2 < 1$ and $0 < \beta_2 < 1$)). Knowing α_2, the parameters α_1, β_1 and β_2 are determined from the relations of Eq. A1.

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