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Gene transfer by cationic surfactants is essentially limited by the trapping of the surfactant/DNA complexes onto the cell membrane: a fluorescence investigation

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Abstract

The interaction between complexes of plasmid DNA with cetyltrimethylammonium bromide (CTAB) and L929 fibroblasts was first examined using confocal microscopy. The complexes labeled with the DNA intercalator, YOYO-1, were found to be trapped onto the external face of the plasma membrane; a feature that may constitute a major limiting step in transfection. Moreover, since no cytotoxic effect appeared in these conditions, we further inferred that the CTAB molecules remained bound to the DNA. The interaction of the complexes with the membranes was best modeled with neutral vesicles. From anisotropy thermotropic curves of DPHpPC-labeled vesicles and fluorescence resonance energy transfer measurements between these vesicles and YOYO-labeled complexes, we evidenced that the binding of the complexes to the vesicle surface opened the micelle-like domains and unwound DNA. However, DNA was not released but remained stably bound via electrostatic interactions to the CTAB molecules incorporated in the external liposome leaflet. Consequently, the large diameter of the unwound plasmid DNA is likely the major factor that precludes its internalization into the cells by endocytosis. In contrast, anionic vesicles that mimic the cytoplasmic facing monolayer of the plasma membrane rapidly released DNA from the complex. This may explain the previously reported high transfection efficiency of DNA complexed with liposomes composed of neutral lipids and cationic surfactants, since the latter may destabilize the endosomal membrane and induce the release of DNA in the cytoplasm. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Gene therapy; Surfactant; Membrane; Phospholipid; Fluorescence

1. Introduction

Gene therapy represents a promising way for the treatment of both genetic and acquired diseases. However, gene therapy is limited by the absence of efficient and harmless vectors, that are able to transport the gene into the target cells. Thus, many efforts have been done to develop these vectors. Viral vectors, such as retroviruses and adenoviruses, are very

Abbreviations: CTAB, cetyltrimethylammonium bromide; DPPC, L-α-phosphatidylcholine, dipalmitoyl; DPPG, L-α-phosphatidyl-DL-glycerol, dipalmitoyl; EYPC, egg yolk phosphatidylcholine; EtBr, ethidium bromide; DPH, 1,6-diphenyl hexatriene; DPHpPC, 2-(3-diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; FRET, fluorescence resonance energy transfer; CMC, critical micellar concentration

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efficient and able to target a wide range of cells [1,2]. However, they can only package DNA of limited size and they may suffer from the drawbacks of immunogenicity and potential mutagenicity [3]. As a result of such limitations, there have been substantial efforts to develop synthetic vectors [3,4]. However, the use of these non-viral vectors is still limited due to their low efficiency. This is essentially due to the limited understanding of the cellular and molecular mechanisms involved in non-viral vector-mediated transfection [5]. Thus, straightforward improvement of these compounds is somewhat difficult to achieve and as a consequence, these compounds are actually far to mimic the numerous specialized functions that viruses acquired during evolution.

Among the obvious parameters needed for achieving efficient transfection is the requirement that the interaction of DNA with the vectors should yield complexes of small size, close to that of viruses [6]. The requirement of a strict control of complex size stems largely from the 150 nm size limit for endocytosis and escape from blood vessel [7]. This factor partly limits the efficiency of cationic polymers [4] and cationic lipids [5,8] since DNA condensation by these compounds generally leads to large and polydisperse aggregates. In sharp contrast, cationic detergents have been shown to condense DNA into discrete particles containing a single nucleic acid molecule [9–11]. This is notably the case for cetyltrimethylammonium bromide (CTAB), which has been shown to induce a large discrete first-order phase transition of individual DNA molecules between elongated coil and collapsed globule [9,10]. This collapse transition is exhibited at a very low CTAB concentration of about 10^{-5} M, well below the critical micellar concentration (CMC) (mM range), and is related to the formation of micelle-like aggregates of CTAB on the polyanion [10]. This peculiar structure of CTAB/DNA complexes is thought to result from the interaction of the negatively charged DNA phosphate groups with surfactant counterions and further stabilization by hydrophobic interactions of the hydrocarbon tails of the CTAB molecules. Despite their unique and interesting features, detergents seem to be poorly efficient in vitro in gene transfer [12,13]. Moreover, due to their high water solubility, the addition of CTAB/DNA complexes into cells is thought to induce the fast release of CTAB into the environment that leads to detergent-related cytotoxicity.

In this context, since the exact cellular and molecular mechanisms that underlay the poor efficiency of DNA/CTAB complexes in gene transfer are still largely unknown, our objective was to further analyze these mechanisms in order to identify the limiting step(s) in gene transfer mediated by CTAB/DNA complexes. Towards this end, we investigated by confocal microscopy the interaction of CTAB/DNA complexes with L929 fibroblasts and found out that the interaction of these complexes with the membrane cells was the main limiting step. To further analyze the molecular mechanism of this limiting step, we then investigated by fluorescence spectroscopy the interaction of these complexes with neutral and anionic vesicles considered as membrane models. Moreover, since the interaction of the complexes with the membrane is essentially linked to the CTAB-containing hydrophobic domains of these complexes, we investigated the physicochemical properties of these domains by using hydrophobic fluorescent markers.

2. Materials and methods

2.1. Materials

Calf thymus DNA, L-α-phosphatidylcholine, dipalmitoyl (DPPC), L-α-phosphatidyl-DL-glycerol, dipalmitoyl (DPPG), egg yolk phosphatidylcholine (EYPC) and egg yolk phosphatidyl glycerol were purchased from Sigma. pCMV-luc plasmid was propagated and purified as described [14]. The DNA concentration expressed as mol of bases was determined spectroscopically by using $\varepsilon^{260} = 6600$ M^{-1} cm^{-1}. Ethidium bromide (EtBr), YOYO-1, 1,6-diphenyl hexatriene (DPH) and 2-(3-diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPHpPC) were from Molecular Probes.

2.2. Preparation of large unilamellar vesicles (LUV)

Phospholipids and probe (DPHpPC) stock solutions in chloroform–methanol (9:1) were mixed in a

molar ratio of 100:1 and dried under vacuum by rotary evaporation onto the wall of a round-bottom flask. The lipid film was maintained under vacuum for a least 0.5 h to remove any residual solvent, and then hydrated with Tris 15 mM, pH 8.4 buffer for 0.5 h. Multilamellar vesicles (MLV) were first obtained by vigorous vortex shaking for 2 min. LUV were then prepared by extrusion of the MLV suspension through polycarbonate filters (Nucleopore), using a thermostated extruder (Lipex Biomembranes). The size of the filter pores was first 0.2 μm (seven passages) and thereafter 0.1 μm (ten passages). These preparation steps were performed at ambient temperatures in the case of egg yolk lipids and at 50°C in the case of dipalmitoyl lipids. A homogeneous population of vesicles was obtained (0.11–0.12 μm diameter), as checked by quasi-elastic light scattering measurements with a N4SD Coultronics Nanosizer.

2.3. Steady-state fluorescence measurements

Steady-state fluorescence measurements were performed on a thermostated SLM 48000 spectrofluorometer. The binding of CTAB to DNA was examined using the fluorescence signal of two intercalating agents, EtBr and YOYO-1. Both agents were added at a 1:50 molar ratio to a 10 or 50 μM DNA concentration (as expressed in mol of phosphate) in Tris 15 mM, pH 8.4. CTAB was then added to the EtBr–DNA and YOYO–DNA complexes up to a ratio of CTAB/DNA of five; each ratio being prepared in a separate solution. No higher ratios or DNA concentrations were used since it has been shown that at CTAB concentrations above the CMC, large structures with multiple DNA molecules embedded within a micellar scaffold are formed [15]. The excitation wavelengths were 510 and 470 nm for EtBr and YOYO-1, respectively. For each addition, the full emission spectra were recorded and corrected for dilution, inner filter effect and buffer fluorescence.

The interaction of YOYO-labeled CTAB/DNA complexes with DPHpPC-labeled liposomes was monitored by fluorescence resonance energy transfer (FRET). The average distance, R, between the donor (DPHpPC) and the acceptor (YOYO-1) was computed from both the quenching of the donor and the sensitized emission of the acceptor. In the first case, the efficiency of transfer was calculated by:

$$E_D = 1 - \frac{\phi_{DA}}{\phi_D} \quad (1)$$

where ϕ_D and ϕ_{DA} correspond to the fluorescence quantum yield of the donor in the absence and in the presence, respectively, of the acceptor. The quantum yields were calculated using quinine sulfate in 0.05 M H_2SO_4 as a reference ($Q = 0.508$, [16]).

Using the sensitized fluorescence emission of the acceptor, the transfer efficiency is calculated by:

$$E_A = \frac{A_A}{A_D}\left(\frac{F_{AD}}{F_A} - 1\right) \quad (2)$$

where F_{AD} and F_A are the emission intensities in the presence and in the absence of the donor, respectively, while A_A and A_D are the absorbances at the donor excitation wavelength of the acceptor and the donor, respectively [17]. The emission intensities were corrected from screening effects at both excitation and emission wavelength according to Pigault et al. [18].

The Förster critical distance R_0 was calculated according to:

$$R_0^6 = 8.79 \cdot 10^{-25} n^{-4} \kappa^2 \phi_D J_{AD} \ (\mathrm{cm}^6) \quad (3)$$

where n designates the refractive index of the medium (usually taken as 1.333), ϕ_D the quantum yield of the donor, J_{AD} the overlap integral calculated from the overlap between the emission spectrum of the donor and the absorbance spectrum of the acceptor [17], and κ^2 the orientational factor.

Finally, using R_0 and either E_A or E_D, the distance, R, between the acceptor and the donor is calculated using:

$$R = R_0\left(\frac{1}{E} - 1\right)^{1/6} \quad (4)$$

2.4. Steady-state fluorescence anisotropy

A SLM 8000 spectrofluorimeter in the T-format configuration was used to measure steady-state fluorescence anisotropy, a. The excitation wavelength (for DPH and derivatives) was set at 360 nm and the emitted light was monitored with 435 nm interference filters (Schott). A home-built device ensured

the automatic rotation of the excitation polarizer, allowing continuous measurement of the anisotropy. The cuvette temperature was regulated with a circulating water bath (Haake F3), itself piloted by a temperature programmer (Haake PG10) to obtain the thermotropic profiles. The heating rate was 1.5°C/min and the temperature was continuously monitored with a thermocouple inserted into the cuvette just above the light beam. Pairs of data (a and T °C) were registered on a PC computer through an interface and processed with a program (Biokine) developed by Biologic.

2.5. Fluorescence lifetime measurements

Fluorescence lifetime measurements were performed with a time-correlated, single-photon counting technique using a pulse-picked frequency doubled Ti-sapphire laser pumped by a continuous wave argon laser (Spectra Physics). The excitation wavelength was set at 443 nm for both EtBr and YOYO-1. The emission wavelengths were set at 620 and 520 nm, for EtBr and YOYO-1, respectively. The single-photon pulses were detected with a microchannel plate Hamamatsu R3809U photomultiplier and recorded on a multichannel analyzer (Ortec 921) calibrated at 25.5 ps/channel. The instrumental response function was recorded with a polished aluminum reflector, and its full width at half maximum was 40 ps. The decay data were analyzed as a sum of exponentials using the maximum entropy method (MEM) and the Pulse5 software [19]. A lifetime domain spanning 200 equally spaced values on a logarithmic scale between 0.01 and 30 ns was routinely used.

In the case of DPH, fluorescence lifetimes were measured by the phase and modulation method using the SLM 48000 spectrofluorimeter in its lifetime configuration. The excitation was set at 360 nm, and the whole fluorescence spectrum was taken through high pass filters (Schott, KV408). The modulation frequencies of the exciting light ranged from 5 to 90 MHz. DPH in heptane was used as a lifetime reference, with a single exponential decay of 6.75 ns [20]. Data analysis was performed using the SLM software.

2.6. Confocal microscopy study

Mouse fibroblasts from the L929 strain were cultured as monolayers in 75 cm^2 culture flasks (Costar) in DMEM (4.5 g/l glucose) (Seromed) with 10% fetal calf serum (Biowhittaker), 1 mM sodium pyruvate, 2 mM L-glutamine and antibiotics (penicillin 50 U/ml, streptomycin 50 μg/ml) (Seromed) in an 8% CO_2 atmosphere, at 37°C. Twelve hours before incubation with CTAB/DNA complexes, cells were transferred into chambered coverglass (Lab-Tek) with 1 ml of the same medium. CTAB/DNA complexes were preformed in Tris 15 mM, pH 7.5 with 12 μM DNA (expressed as nucleotides) and 12–36 μM CTAB. The complexes were labeled with 0.16 μM YOYO-1 and incubated with the cells in an 8% CO_2 atmosphere, at 37°C. The final concentration of DNA was 6 μM. After incubation, cells were directly observed with an MRC-1024 (Bio-Rad) confocal laser scanning imaging system equipped with an inverted microscope and an $\times 60$ water immersion objective (Nikon).

3. Results

3.1. Labeling of CTAB/DNA complexes

To monitor the fate of CTAB/DNA complexes added to cell cultures, the complexes may be labeled with fluorescent markers. Since it has been shown that the CMC and the binding parameters of cationic detergents with DNA are exquisitely sensitive to the primary structure of the detergent [21], it was not possible to covalently label the detergent. Accordingly, our strategy was to label DNA by using fluorescent intercalating agents. As a first choice, EtBr was used. However, since the binding of CTAB has been shown to strongly modify the conformation of DNA [9,10], we first checked if EtBr remained intercalated after CTAB binding to a 10 μM pCMV-luc plasmid concentration. No EtBr fluorescence decrease was observed for [CTAB]/[CTDNA] ratios, r, lower than 0.8 (Fig. 1A). In contrast, the fluorescence sharply decreased at ratios $r \geq 1$ and reached, at $r = 4.5$, the fluorescence level of EtBr in the absence of DNA. This sharp fluorescence quenching is reminiscent of that observed when cationic polymers

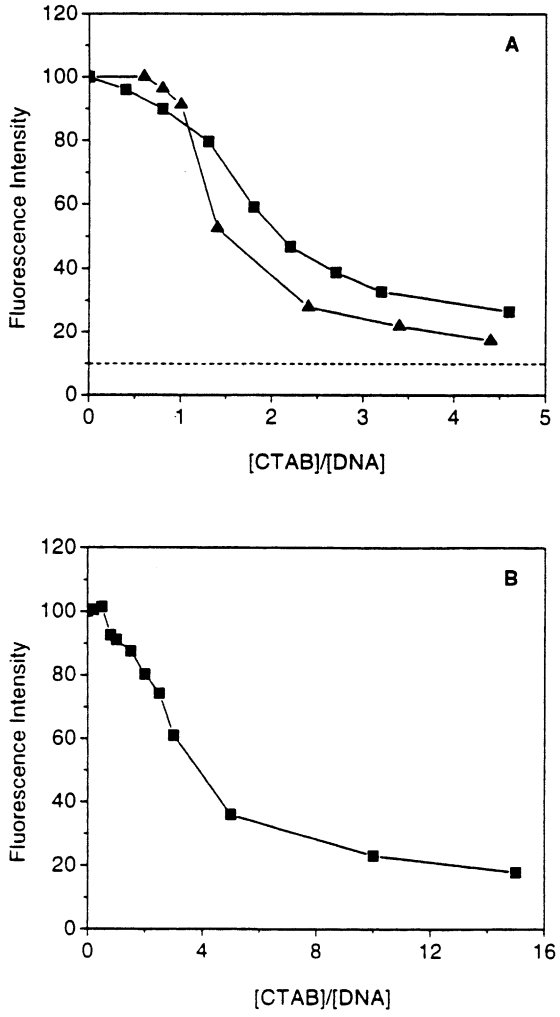

Fig. 1. Binding of CTAB to DNA. The binding was followed with the DNA intercalating agents EtBr (A) and YOYO-1 (B), added at a 1:50 molar ratio of probe to nucleotides. The concentration (expressed as nucleotides) of either pCMV-luc plasmid (squares) or calf thymus DNA (triangles) was 10 μM. The excitation wavelengths were 510 and 470 nm for EtBr and YOYO-1, respectively. The dotted line in (A) corresponds to the fluorescence of free EtBr.

are added to DNA [4], and thus suggests that EtBr is displaced upon binding of CTAB to DNA. However, we cannot exclude that the binding of CTAB changes the environment of EtBr without displacing it. To discriminate between these two hypotheses, we performed a time-resolved fluorescence investigation (Table 1). In keeping with the data of Le Pecq and Paoletti [22], free and DNA-bound EtBr were characterized by lifetimes of 1.6–1.8 and 22–23 ns, respectively. In the absence of CTAB, about 28% of EtBr was free, as expected from the reported binding parameters of EtBr to DNA [23] and their respective concentrations. Incremental addition of CTAB did not affect the fluorescence lifetimes of bound and free species but drastically reduced the relative amplitude of the long-lived lifetime to the benefit of the short-lived one (Table 1). This clearly indicates that EtBr is displaced upon binding of CTAB, a feature that may result from the condensation of DNA [24]. In fact, the displacement curve of EtBr clearly parallels the binding isotherms determined from potentio-

Table 1
Effect of CTAB binding on the time-resolved fluorescence parameters of EtBr- and YOYO-labeled DNA[a]

	[CTAB]/[DNA]	τ_i (ns)	α_i (%)	$\langle\tau\rangle$ (ns)
EtBr	0	1.8(±0.3)	28(±2)	17.2
		23.2(±0.2)	72(±2)	
	0.5	1.6(±0.3)	37(±6)	14.9
		22.7(±0.5)	63(±6)	
	1	1.6(±0.3)	57(±8)	10.6
		22.5(±0.6)	43(±8)	
	1.5	1.6(±0.1)	76(±8)	6.4
		21.7(±0.7)	24(±8)	
	2.5	1.7(±0.1)	93(±3)	2.9
		20.1(±0.9)	7(±4)	
	5	1.8(±0.2)	97(±1)	2.3
		18.7(±0.1)	3(±1)	
YOYO-1	0	0.19(±0.02)	19(±1)	2.4
		2.6(±0.1)	69(±6)	
		4.5(±0.4)	12(±4)	
	0.5	0.28(±0.03)	15(±1)	2.5
		2.6(±0.1)	71(±8)	
		4.2(±0.4)	14(±4)	
	1	0.24(±0.02)	15(±1)	2.4
		2.58(±0.08)	73(±6)	
		4.3(±0.3)	12(±5)	
	3	0.26(±0.04)	37(±2)	1.5
		0.7(±0.1)	5(±1)	
		1.8(±0.2)	44(±6)	
		3.9(±0.3)	14(±4)	
	6	0.23(±0.01)	66(±1)	0.5
		0.71(±0.06)	27(±2)	
		1.8(±0.3)	6(±2)	
		5.6(±0.6)	1(±1)	

[a]pCMV-luc plasmid DNA and dye concentrations were 50 and 0.2 μM, respectively. The excitation wavelength was 443 nm. The emission wavelengths were 620 and 520 nm, for EtBr and YOYO-1, respectively. The fluorescence lifetimes, τ_i, and the relative amplitudes, α_i, were obtained from MEM analysis, as described under Section 2. The mean fluorescence lifetime is given by $\langle\tau\rangle = \Sigma\alpha_i\tau_i$.

metric studies [10], suggesting that EtBr could be faithfully used to determine the binding extent of CTAB to DNA. Moreover, similar displacement curves were obtained when pCMV-luc plasmid was replaced by calf thymus DNA (Fig. 1A), suggesting that the binding of CTAB to DNA is only poorly dependent on DNA sequence and length. As a consequence, EtBr may be useful to assess if any interacting process implying the complexes releases the DNA but it cannot be used to monitor the interaction of CTAB/DNA complexes with cells.

In a second step, YOYO-1, a bis-intercalating agent, characterized by a much higher affinity [25] than EtBr for DNA, was tested. The fluorescence of the free YOYO-1 is 3200 times lower than that of the bound one and could thus be neglected. The fluorescence decrease of YOYO-1 induced by the binding of CTAB to DNA (Fig. 1B) was qualitatively similar to the EtBr one. However, about 20% of the initial fluorescence could not be quenched even in the presence of an excess of CTAB, suggesting that YOYO-1 molecules remain partly bound to DNA. To get further information, time-resolved experiments were performed. The fluorescence intensity decay of YOYO-labeled DNA in the absence of CTAB was characterized by a trimodal distribution that is dominated by a 2.6 ns lifetime class (Table 1). No change in either fluorescence lifetime or relative amplitude was observed for [CTAB]/[DNA] ratios below 1. In contrast, at higher ratios, both the fluorescence lifetimes and relative intensities were markedly affected. The major lifetime class decreased to 1.8 ns and its relative amplitude dramatically dropped from 70 to 6%. Furthermore, an additional 0.7 ns lifetime class appeared. Since the fluorescence of the free YOYO-1 molecules is negligible and since the parallel decrease of the mean fluorescence lifetime and steady-state fluorescence intensity excludes static quenching, we may reasonably conclude that YOYO-1 molecules were still bound to DNA but

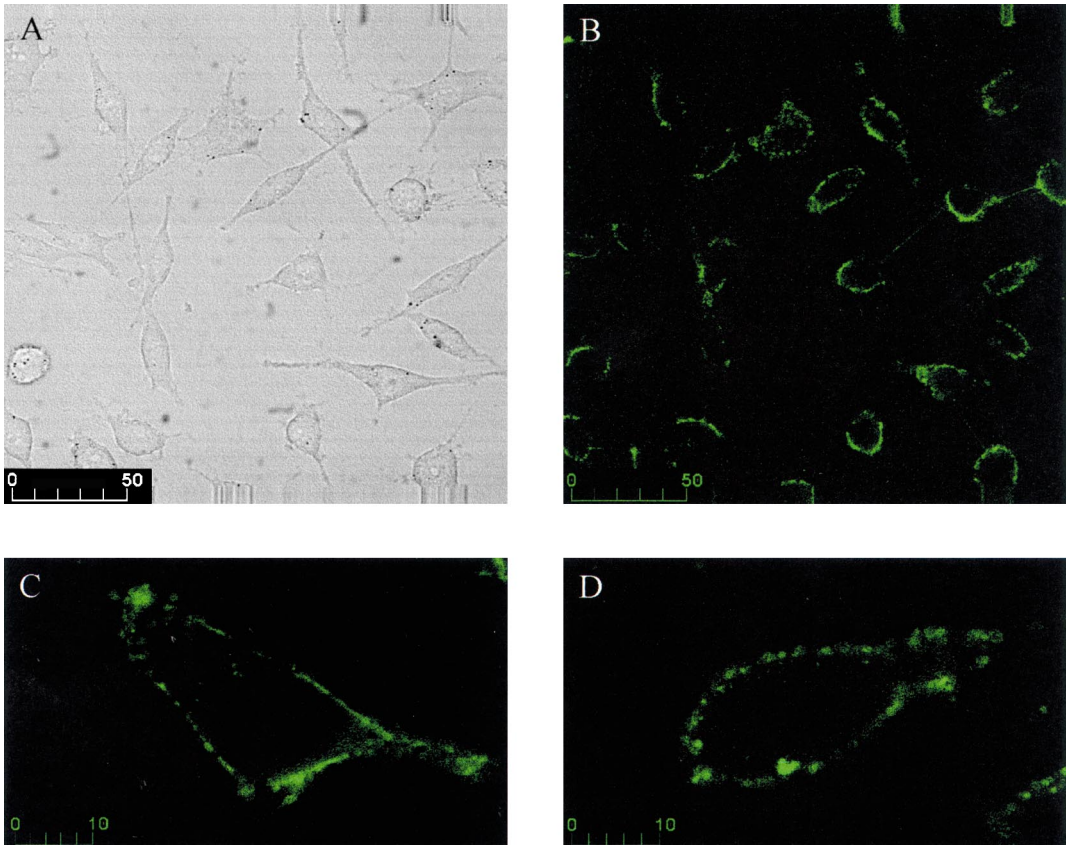

Fig. 2. Confocal images of L929 fibroblasts in the presence of YOYO-labeled CTAB/DNA complexes. The complexes formed at a [CTAB]/[DNA] ratio of 1 were incubated with L929 cells for either 0.5 (A–C) or 5 h (D). The final concentration of DNA is 6 μM. The light transmission image in (A) corresponds to the fluorescence image in (B).

their environment was markedly affected by the CTAB-induced collapse of DNA. As it has been reported that the bis-intercalation of the space-demanding YOYO-1 molecules locally distorts DNA molecules by unwinding the helix by 106° [26], we speculate that the CTAB-induced condensation opposes to the YOYO-1 induced conformational changes on DNA. This may in turn increase the solvent accessibility of YOYO-1 and thus decrease its fluorescence lifetime [27].

Nevertheless, since YOYO-1 is still bound in the presence of CTAB and since its fluorescence remains rather high, it may be readily used to follow the interaction of CTAB/DNA complexes with cells.

3.2. Interaction of CTAB/DNA complexes with L929 fibroblasts

CTAB/DNA complexes labeled with YOYO-1 were incubated with L929 cells and the pathway of the labeled DNA was followed by confocal microscopy at various time points. The final concentration of DNA was 6 μM and the ratio of [CTAB]/[DNA], r, was set to 1 or 3. At all the observed time points (from 1 to 24 h) and at both $r = 1$ and 3, it clearly appeared that all the DNA was attached to the surface of the membrane cells (Fig. 2B–D). However, no significant fluorescence could be perceived in the cytoplasm or the nucleus (Fig. 2C,D) even 24 h after addition of the complexes. Moreover, only a small fraction of the DNA attached to the surface cells could be removed by a washing step, suggesting that the complexes were firmly bound (data not shown). Importantly, the viability of the cells was unaffected by the CTAB/DNA complexes at least with the CTAB concentrations used in this assay. Indeed, even after 24 h, no sign of cellular death (usually detected by a change of cellular shape and cell detachment from the coverslip) could be observed on our samples. This is in sharp contrast to the observations made at the same concentrations of free CTAB, where nearly all the cells have a rounded shape and are detached from the coverslip, after a 5 h incubation (data not shown). The absence of cytotoxicity of CTAB/DNA complexes was further confirmed by trypan blue exclusion tests (data not shown). In conclusion, our data clearly indicated that cell membranes act as a trap for CTAB/DNA complexes. As for the interaction of DNA/lipid complexes with additional lipids [28], it is likely that the interaction of CTAB/DNA complexes with membrane cells is essentially mediated by hydrophobic interactions involving the micelle-like domains. Accordingly, to further understand the molecular events that sustained this limiting step, we further investigated the physicochemical properties of the micelle-like domains and analyze the interaction of CTAB/DNA complexes with phospholipid vesicles that constitute membrane models.

3.3. Physicochemical properties of the micelle-like domains of CTAB/DNA complexes

Upon addition of increasing amounts of CTAB to DNA, the formation of hydrophobic micelle-like CTAB domains along the DNA chain was followed by the increase of fluorescence intensity of DPH, a fluorescent probe that is non-fluorescent in water and commonly used in the characterization of the hydrophobic core of lipid vesicles or biomembranes [29]. Without CTAB, a weak and smooth DPH fluorescence spectrum is observed (Fig. 3), indicating that DNA itself could capture and protect some DPH

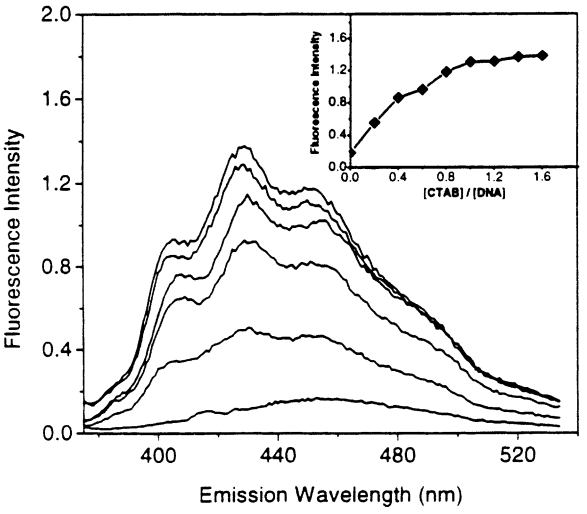

Fig. 3. Formation of CTAB micelle-like domains in CTAB/DNA complexes. The formation of these domains was followed through the fluorescence of 0.5 μM DPH. The complexes were formed with 50 μM DNA and CTAB added to a molar ratio (from bottom to top) of 0, 0.2, 0.6, 0.8, 1 and 1.6, respectively. Excitation wavelength was 360 nm. The insert describes the DPH fluorescence intensity at 430 nm as a function of the [CTAB]/[DNA] ratio.

from surrounding water, probably by localization in one of the grooves. Addition of CTAB induced a strong increase of DPH fluorescence intensity, confirming that hydrophobic domains readily formed. A plateau was reached for a ratio r of 1 (Fig. 3, insert) with an intensity close to that obtained with liposomes, suggesting that all DPH molecules are bound in the hydrophobic micelle-like domains. The shape of the curve in Fig. 3, insert, is typical of a cooperative phenomenon and is thus in full keeping with the model of discrete coil–globule transition [9,10]. Noticeably, using DPH as a label [30], the CMC of CTAB was found to be 420 μM in our conditions, a concentration that is at least one order of magnitude higher than the CTAB concentrations used to form the CTAB/DNA complexes.

The hydrophobic sites were further characterized by the mobility of DPH, that was estimated through the rotational correlation time, ρ. The parameter, ρ, is related both to the average fluorescence lifetime, τ, the DPH fundamental anisotropy, $a_0 = 0.362$ [31], and the steady-state fluorescence anisotropy, a, by Perrin-Weber's equation:

$$\left(\frac{a_0}{a} - 1\right) = 3\frac{\tau}{\rho} \quad (5)$$

The rotational correlation time, ρ, of DPH in the hydrophobic domains (Table 2) of the CTAB/DNA complexes was intermediate to the 4.8 and 14.5 ns correlation times measured for CTAB micelles and EYPC vesicles, respectively. This suggests that the binding of CTAB to the DNA lattice rigidifies to some extent the micelle-like domains. Moreover, since the time-resolved fluorescence parameters at low r (e.g. 0.2) were close to those at high r (e.g. 1 or 3), this confirms that at low ratios, fully condensed globules are already formed but their number is less than at high r. Identical results were obtained with calf thymus DNA (data not shown), suggesting that the formation of the CTAB micelle-like domain does not depend on the DNA sequence and length.

To get additional information, the environment sensitive dye Nile red was used [32]. The 653 nm maximum emission wavelength of this dye in CTAB/DNA complexes was identical to that in CTAB micelles and intermediate to the 635 and 665 nm values in liposomes and water, respectively (data not shown). This suggests that the polarity in the micelle-like domains was very similar to that in CTAB micelles obtained with a two orders of magnitude higher CTAB concentration.

3.4. Interaction of DNA/CTAB complexes with neutral LUV

The interactions of DNA/CTAB complexes with lipid bilayers were studied using LUV composed of DPPC or EYPC, which are dipolar zwitterions with no net charge.

As a first step to reveal the interactions between the phospholipid bilayers and the CTAB/DNA complexes, the phase transitions of DPPC vesicles were followed by recording the thermotropic profiles of fluorescence anisotropy, by labeling the vesicles with a DPH-labeled phospholipid, DPHpPC, that presents the advantage of being an integral part of the vesicle bilayer. DPPC was chosen in order to obtain model vesicles presenting a clear phase transition between an L_β gel phase and an L_α liquid

Table 2
Fluorescence parameters of DPH in CTAB/DNA complexes[a]

[CTAB]/[DNA]	a	τ (ns)	ρ (ns)
0.2	0.121(±0.006)	5.6(±0.4)	8.5(±0.8)
0.4	0.134(±0.005)	5.1(±0.2)	8.6(±0.6)
0.6	0.134(±0.004)	5.6(±0.3)	9.6(±0.5)
0.8	0.138(±0.005)	5.6(±0.2)	10.4(±0.4)
1	0.145(±0.004)	5.0(±0.2)	10.4(±0.5)
1.4	0.147(±0.003)	5.3(±0.2)	10.1(±0.5)

[a]pCMV-luc plasmid DNA and DPH concentrations were 50 and 0.5 μM, respectively. Excitation and emission wavelengths were 360 and 435 nm, respectively. The steady-state anisotropy, a, the fluorescence lifetime, τ, and the rotational correlation time, ρ, were expressed as the means ± S.E.M. for three experiments.

crystalline phase, with a phase transition temperature (T_m) of about 41°C.

The experiments were performed at a single DNA concentration (10 μM) and a vesicle concentration (expressed in mol of total phospholipids) comprised between 30 and 80 μM. The addition of DNA alone was found to not affect the profile of the phase transition (data not shown). In contrast, the addition of CTAB at the concentrations used to form the complexes induced a small but significant decrease in T_m (Fig. 4A) that is at variance with the slight T_m increase observed on the same system by Inoue et al. [33]. This discrepancy may tentatively be explained by our experimental conditions that may favor the partitioning of the surfactant in the liquid crystal as compared to the gel phase. This hypothesis is strengthened by the significantly higher decrease of DPH anisotropy induced by the addition of CTAB in the liquid crystal (L_α) as compared to the gel (L_β) phase. As expected, this destabilization was the highest at the lowest concentration of vesicles and decreased with increasing vesicle concentrations. Addition of CTAB/DNA complexes to a 30 μM concentration of vesicles modified the phase transition in an opposite direction (Fig. 4B). Indeed, with increasing r from 0.1 to 1, a progressive and positive shift of T_m was observed. The T_m shift reached a maximum for r between 0.7 and 1 ($\Delta T_m = 3.5$°C) and then decreased at higher r. Moreover, the addition of the complexes induced a significant increase of fluorescence anisotropy both in L_α and L_β phases, suggesting that the interaction rigidifies both phases. A very similar behavior was obtained by using calf thymus DNA instead of plasmid DNA. Furthermore, at higher lipid concentration (60 and 80 μM), the same general trends were observed, except that T_m increments were somewhat lower (data not shown). These results strongly suggest that the CTAB/DNA complexes strongly interact with the vesicles and stabilize them.

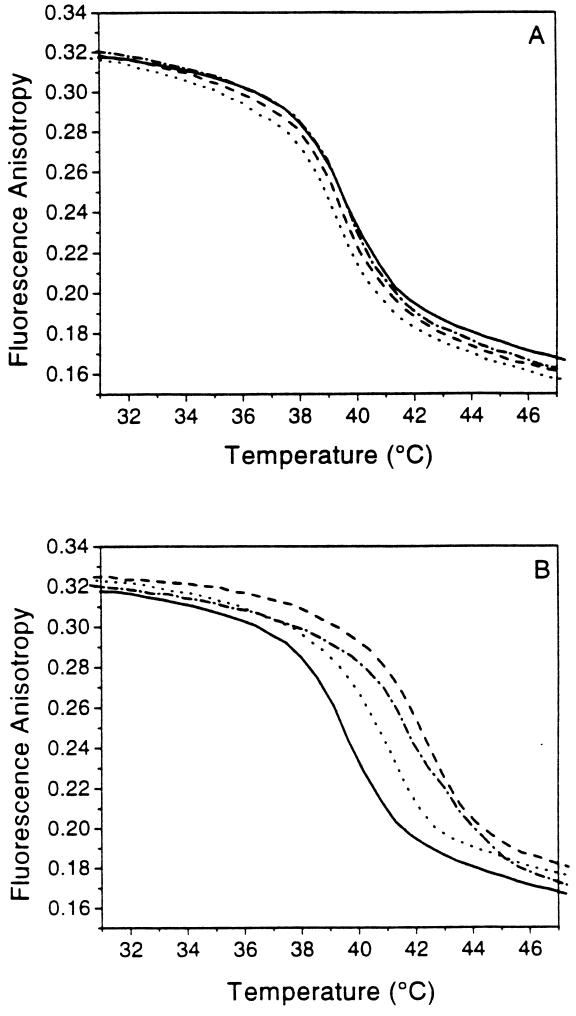

Fig. 4. Interaction of CTAB/DNA complexes with DPPC vesicles. The interaction with either CTAB alone (A) or CTAB/DNA complexes (B) was monitored by performing fluorescence anisotropy thermotropic curves, using DPHpPC as a dye. The DPPC concentration was 30 μM. The concentration of DNA was 10 μM. The concentration of CTAB was 0 (—), 4 (–·–), 10 (– –) or 20 (···) μM, respectively.

To further characterize these interactions, we measured by FRET the average distance, R, between the YOYO molecules bound to CTAB/DNA complexes and the fluorescent moiety of the DPHpPC labels. The experiments were performed with egg yolk lipids presenting a liquid crystalline phase at all temperatures. R was computed from both the quenching of the donor (DPHpPC) and the sensitized emission of the acceptor (YOYO). A favorable circumstance in this system is that at the excitation wavelength of the donor ($\lambda_{exc} = 360$ nm), only a very limited direct excitation of the acceptor occurs. The efficiencies obtained from the donor and the acceptor were highly consistent and reached a maximum at r between 1 and 2 (Table 3).

To calculate the average distance between DPHpPC and YOYO, the Förster critical distance was further needed and calculated using Eq. 3. To this end, the overlap integral, $J_{AD} = 1.44 \times 10^{-13}$ M^{-1}

Table 3
Steady-state FRET parameters of the interaction of CTAB/DNA complexes with neutral vesicles[a]

[CTAB]/[DNA]	E_D	E_A	R (Å)
0.5	0.03(±0.02)	0.03(±0.02)	84(±2)
1	0.29(±0.04)	0.30(±0.06)	54(±2)
1.5	0.26(±0.05)	0.31(±0.05)	55(±3)
2	0.24(±0.07)	0.23(±0.06)	57(±2)
3	0.14(±0.06)	0.19(±0.03)	62(±1)

[a]Plasmid and YOYO concentrations were as in Fig. 1. The concentrations of EYPC and DPHpPC were 80 and 0.8 μM, respectively The energy transfer efficiencies E_D and E_A, calculated from the donor fluorescence quenching and the acceptor fluorescence enhancement, respectively, were given as the mean (±S.E.M.) for at least three experiments. The interchromophore distance, R, was calculated from both donor and acceptor.

cm^3, was calculated from the emission spectrum of DPHpPC-labeled vesicles and the absorption spectrum of YOYO-labeled DNA. Moreover, a 0.42 quantum yield was measured for DPHpPC. The orientational factor κ^2 was taken as 2/3, assuming that the donor and the acceptor undergo a complete dynamic isotropic orientational averaging. The Förster distance, calculated using Eq. 3, was 47 Å. Finally, using R_0 and the efficiency of transfer calculated from the acceptor, E_A, or the donor, E_D, the interchromophore distance, R, was calculated at various r values (Table 3). The interchromophore distances were found to be rather large at substoichiometric ratios of CTAB to DNA, reached a minimum value ($R = 54$ Å) at $r = 1$–1.5 and slightly increased at higher ratios. Noticeably, a significant turbidity was observed at $r = 1$, suggesting that some vesicle aggregation occurred at this ratio.

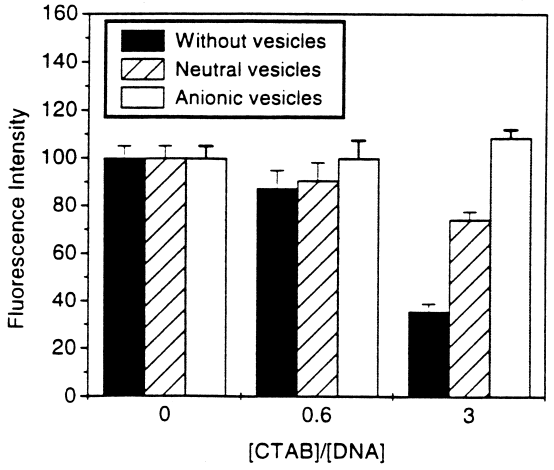

Fig. 5. EtBr intercalation. The DNA and EtBr concentrations were 10 and 0.2 μM, respectively. The concentration of phospholipids was 80 μM for either neutral or anionic vesicles.

In a final step, EtBr intercalation was used to investigate the condensation state of DNA in the interaction of CTAB/DNA complexes with neutral liposomes. We first checked that the addition of neutral vesicles to EtBr-labeled DNA in the absence of CTAB did not affect the fluorescence of EtBr (Fig. 5), in keeping with the aforementioned absence of interaction of DNA with neutral vesicles. Similarly, no fluorescence change was observed when neutral vesicles were added to CTAB/DNA complexes at $r = 0.6$, suggesting that neutral vesicles were unable to displace bound EtBr.

In sharp contrast, the addition of neutral vesicles to CTAB/DNA complexes at $r = 3$, a ratio where EtBr was largely displaced due to DNA condensation, induced a significant recovering of EtBr fluorescence. This suggests that the interaction induces a large re-exposure of EtBr intercalation sites that may be consecutive of DNA unwinding.

3.5. Interaction of DNA/CTAB complexes with anionic LUV

These experiments were performed with an equimolar mixture of DPPC and DPPG. The presence of the negatively charged DPPG renders the vesicles anionic, like are most cytoplasmic leaflets of plasma membranes [34].

In contrast to the mild effect of CTAB alone on DPPC vesicles, an important stabilization of the anionic vesicles (at 60 or 80 μM) was induced by the addition of CTAB. As shown in Fig. 6A, the phase transition temperature, T_m, was significantly

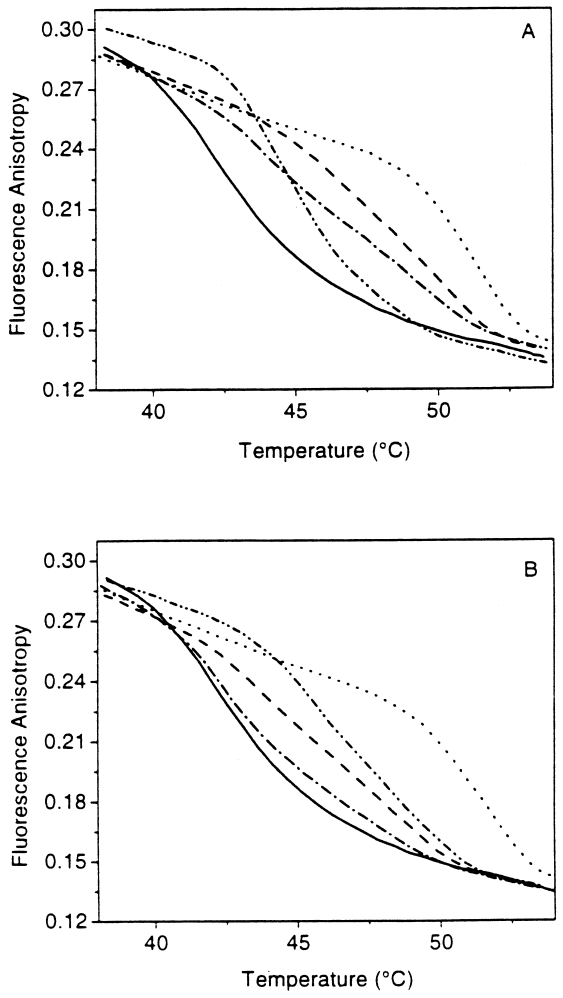

Fig. 6. Interaction of CTAB/DNA complexes with DPPC/DPPG vesicles. The interaction of the vesicles with either CTAB alone (A) or with CTAB/DNA complexes (B) was monitored as in Fig. 4. The anionic vesicles were formed with an equimolar concentration of DPPC and DPPG. The final phospholipid concentration was 60 μM. The concentration of DNA was 10 μM. The concentration of CTAB was 0 (—), 4 (–·–), 8 (– –), 10 (···) or 15 (–··–) μM, respectively.

and progressively increased up to $r=1$. At r values greater than 1, a reversal of this T_m increase was observed, and the phase transition appeared to be more cooperative, with a higher fluorescence anisotropy in the gel phase. In contrast, at lower vesicle concentration (30 μM), the addition of CTAB at concentrations above 10 μM induced a slight decrease in T_m (data not shown). This complex response of T_m to cationic surfactants has been suggested to result from the opposite effects of lipid–surfactant interaction (that is stronger in the gel than in the liquid crystal phase) and the free energy of CTAB (that is lower in the crystal liquid than gel phase); the balance between these two effects depending on the molar ratio of surfactant to anionic phospholipid [35].

The corresponding thermotropic profiles obtained in the presence of DNA/CTAB complexes were qualitatively similar to those with CTAB alone, but with somewhat smaller amplitudes especially at $r>1$ (Fig. 6B). This suggests that a large fraction of CTAB molecules is captured by the anionic vesicles and that the effects of CTAB/DNA complexes on the anionic vesicles are only due to this captured CTAB.

Furthermore, in sharp contrast to the neutral vesicles, no energy transfer could be evidenced between YOYO-labeled CTAB/DNA complexes and anionic vesicles labeled with DPHpPC. This conclusion holds true for all the CTAB to DNA ratios that have been tested. This strongly suggests that DNA did not stably bind to the anionic vesicles. Consequently, as CTAB is incorporated into the vesicles, DNA is probably released in a more or less unbound and thus unwound form into the bulk solution. This feature was confirmed by the complete recovering of EtBr fluorescence that was observed when anionic vesicles were added to CTAB/DNA complexes formed at $r=3$ (Fig. 5).

4. Discussion

In this report, we have investigated the interaction of CTAB/DNA complexes with L929 fibroblasts. To follow the intracellular fate of these complexes by confocal microscopy, the DNA intercalator YOYO was found to be an adequate tool, since in contrast to EtBr, the condensation of DNA induced by CTAB did not eject this dye. We find out that the complexes strongly interact with the cell membrane which acts as a trap for these complexes. Indeed, the complexes remain bound to the cell surface and no internalization of the complexes can be observed even 24 h after incubation of the complexes with the cells. This behavior is in sharp contrast to that observed with most other non-viral vectors like lipid particles, polypeptides or polycations where internalization of the DNA/vector complexes proceeds by endocytosis [5,36–38]. Furthermore, our observations

may well explain the inefficiency of CTAB for DNA transfection in L929 cells [12]. An alternate explanation would be linked to the cytotoxicity of CTAB which acts as a detergent. However, while a cytotoxic effect could be clearly evidenced for free CTAB, such an effect was not observed for CTAB/DNA complexes. Accordingly, since a similar cytotoxicity of free CTAB in the same concentration range has been previously shown on the same cells [12], this suggests that CTAB molecules must remain linked to DNA after the interaction with the cell membranes preventing their cytotoxic effect.

The interaction of CTAB/DNA complexes with cell membranes could be adequately modeled by the interaction of these complexes with neutral liposomes. This was not unexpected since zwitterionic neutral lipids are the major constituents of the external monolayer of the plasma membrane [34]. The existence of a stable interaction has been demonstrated by the rather short distances measured by FRET between the YOYO-labeled complexes and the DPHpPC-labeled liposomes. These distances were then compared to the theoretical distance computed by assuming that the DNA/CTAB complexes randomly bind as linear lattices to the vesicle surfaces. As the DPHpPC molecules represent 1% of the total lipids in the vesicle and as the surface occupied by one lipid at the vesicle surface is about 58 $Å^2$ [39], we deduced that the mean distance between two DPHpPC molecules is about 76 Å. Moreover, since the concentration of DPHpPC in our conditions is four times that of YOYO, and since DPHpPC is equally distributed in the internal and external leaflets of the liposome, we infer that there is statistically one YOYO molecule for two DPHpPC molecules in both leaflets. Since the center of the DPH dye is located at 14 and 26 nm from the outer liposome surface in the case of the external and internal leaflets, respectively, we calculate that the mean distance between one DPHpPC and one YOYO molecule is about 43 Å. The reasonable similarity between this calculated value and the measured distance at $r = 1$–1.5 strongly suggest that the DNA/CTAB complexes may be more or less unfolded and bind randomly in a relaxed form to the vesicle surface. The slight increase in interchromophore distance at higher CTAB to DNA ratios may be linked to a weaker ability of the complexes to unfold, suggesting that the complexes may be bound to the vesicles in a slightly more compact form. In contrast, a much higher increase of R was observed at CTAB to DNA ratios lower than 1. This may be due to the fact that the YOYO-labeled DNA regions not coated by CTAB are not in close contact with the vesicle surface. Alternatively, the affinity of the partially coated DNA molecule for the liposome may be significantly lower than the fully coated one and thus some DNA/CTAB complexes may be free in solution, decreasing the possibilities of energy transfer for DPHpPC.

The unfolding of the complexes and the resulting unwinding of DNA at CTAB to DNA ratios larger than 1 are confirmed by the ability of DNA to bind EtBr at a similar level than free DNA. Such an unfolding of DNA/CTAB complexes has already been reported in the interaction with giant vesicles formed with soybean phosphatidylcholine lipids [11]. The micelle-like domains of the DNA/CTAB complexes are supposed to play a central role in the interaction. Using Nile red and DPH, these domains are found similar to CTAB micelles with regard to their polarity. However, they differ to some extent from the CTAB micelles by a somewhat higher order parameter that is undoubtedly linked to the constraints brought about by the binding of the CTAB molecules to DNA. This may reduce the electrostatic repulsion between the CTAB molecules and stick them closer together.

Since surfactant molecules have been shown to be incorporated into the phospholipid bilayer due to the partition equilibrium between bilayer and aqueous phase [40], we infer that the binding of the CTAB/DNA complexes to the vesicle surface through hydrophobic forces results in the opening of the micelle-like domains of the CTAB/DNA complexes and the incorporation of the surfactant into the lipid bilayer (Fig. 7). Since in our conditions free DNA is unable to strongly interact with neutral vesicles, our data suggest that DNA molecules bind to the vesicle surface via electrostatic interactions with the CTAB molecules incorporated in the external vesicle layer. This hypothesis is further assessed by the thermotropic curves showing that both the gel and crystal liquid phases of DPPC vesicles are rigidified by the interaction with CTAB/DNA complexes. This may be explained by the strengthening of the hydrophobic interactions between the lipids and surfactants that is

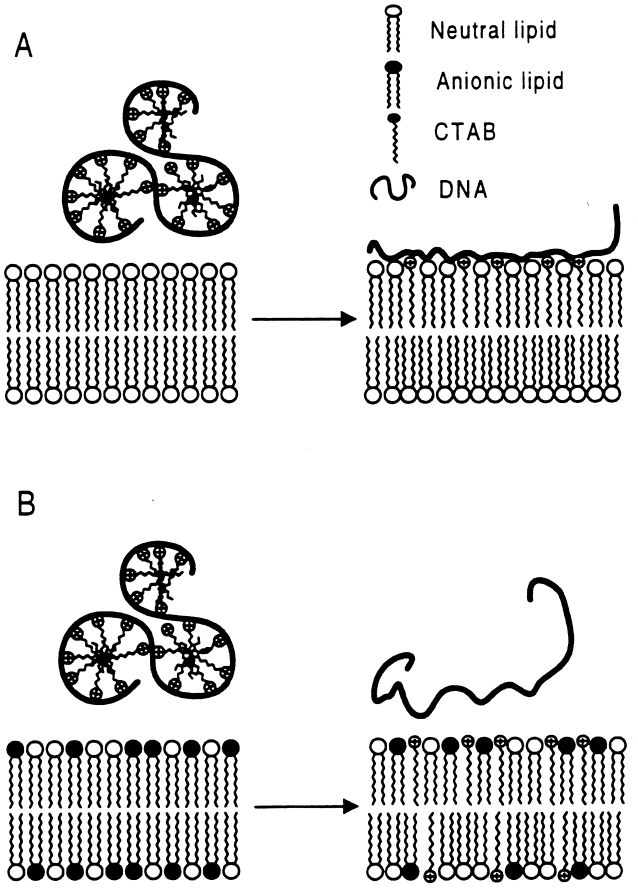

Fig. 7. Proposed mechanism for the interaction of CTAB/DNA complexes with neutral (A) and anionic (B) vesicles. The interaction with neutral vesicles induces an opening of the micelle-like domains and an insertion of CTAB in the external layer. After interaction, the unfolded DNA remains bound to the CTAB molecules. In the case of anionic vesicles, the CTAB molecules are transferred in the lipid bilayer and form neutral ion pairs with anionic lipids. Since no positive charge is then available, the unfolded DNA is released into the bulk solution.

induced by the external bridging of the surfactants with the DNA molecule. According to the increase in T_m, this stabilization is, as expected, higher in the ordered gel phase than in the disordered liquid crystal phase. Moreover, the bridging of CTAB molecules on the external bilayer face prevents them to be homogeneously dispersed in the vesicle bilayer and thus to exert their detergent effects. A similar effect in the cell may explain the absence of cytotoxicity.

The melting of CTAB/DNA complexes and the resulting unfolding of DNA is at variance with the behavior of complexes of DNA with liposomes or polycations like polyethyleneimine since in both cases it has been shown that the complexes are not dissociated by their interaction with plasma membranes [41,42]. Moreover, if we assume that the circular 5500 bp plasmid DNA bound to the cell surface is in a fully relaxed form, its calculated diameter may be as high as 600 nm, a feature that may readily explain the inability of the cell to internalize this DNA by endocytosis [43].

In contrast to neutral vesicles, anionic vesicles were found to displace bound DNA. The combination of FRET measurements, thermotropic curves and EtBr intercalation strongly suggest that CTAB molecules transfer from the CTAB/DNA complex into the hydrophobic part of the vesicle. The driving forces of this transfer may be similar to those observed with neutral vesicles except that in addition, the cationic detergents probably form ion pairs with the negatively charged DPPG phospholipids. This ion pair formation (that does not exist in neutral vesicles) probably prevents electrostatic interactions between CTAB and DNA and thus induces the release of DNA into the bulk solution (Fig. 7). A similar DNA release by anionic vesicles has been observed with cationic liposomes/DNA complexes [41]. According to the primary location of anionic lipids on the cytoplasmic facing monolayer of the plasma and endosomal membranes [44], Xu and Szoka [41] have proposed a mechanism by which the cationic liposomes/DNA complexes destabilize the endosomal membrane, resulting in displacement of the DNA from the cationic lipid and release of the DNA into cytoplasm. CTAB and other cationic surfactants of the same class are expected to promote a similar mechanism in ternary complexes which are able to be internalized. This may probably explain the transfection efficiency of liposomes composed of a neutral lipid, dioleylphosphatidylethanolamine and CTAB or a related cationic detergent [12,13]. Assuming that the liposome structure of these ternary complexes enables their internalization by endocytosis, it may be speculated that the cationic detergents destabilize the endosomal membrane and induce the release of DNA in the cytoplasm. Alternatively, if some complexes are able to enter by direct fusion with the cell membrane [45], the interaction of the cationic detergents with the cytoplasmic facing monolayer of this membrane may induce the release

of DNA in the cytoplasm. The major drawback in both hypotheses is that the detergents are released either in the endosomal or plasma membrane. This may thus explain that the cytotoxic effects of the ternary complexes are observed with CTAB concentrations only slightly higher than those with free CTAB [12,13].

In conclusion, CTAB and more generally the cationic detergents are highly promising since they are to our knowledge the only one that collapse individual DNA molecules. This leads to small particles, an ideal property for allowing efficient internalization of the complexes by endocytosis [4]. Moreover, the cationic detergents are likely to promote the release of DNA from the endosomal vesicle into cytoplasm. However, the two major drawbacks of CTAB are its cytotoxicity and its inability to allow the internalization of DNA into the cell. The knowledge of the cellular barriers of CTAB/DNA complexes should allow now to imagine rational strategies in order to suppress the deleterious effects of CTAB. An interesting clue has recently been proposed by Blessing et al. [46] with the synthesis of a cysteine-based detergent since after the collapse of individual DNA molecules, an aerobic oxidation of this compound leads to a cystine–lipid that gives stable complexes with DNA.

Acknowledgements

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