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DOTAP cationic liposomes prefer relaxed over supercoiled plasmids

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Abstract

Cationic liposomes and DNA interact electrostatically to form complexes called lipoplexes. The amounts of unbound (free) DNA in a mixture of cationic liposomes and DNA at different cationic lipid:DNA molar ratios can be used to describe DNA binding isotherms; these provide a measure of the binding efficiency of DNA to different cationic lipid formulations at various medium conditions. In order to quantify the ratio between the various forms of naked DNA and supercoiled, relaxed and single-stranded DNA, and the ratio between cationic lipid bound and unbound DNA of various forms we developed a simple, sensitive quantitative assay using agarose gel electrophoresis, followed by staining with the fluorescent cyanine DNA dyes SYBR Green I or SYBR Gold. This assay was compared with that based on the use of ethidium bromide (the most commonly used nucleic acid stain). Unlike ethidium bromide, SYBR Green I DNA sensitivity and concentration-dependent fluorescence intensity were identical for supercoiled and nicked-relaxed forms. DNA detection by SYBR Green I in solution is ~40-fold more sensitive than by ethidium bromide for double-stranded DNA and ~10-fold for single-stranded DNA, and in agarose gel it is 16-fold more sensitive for double-stranded DNA compared with ethidium bromide. SYBR Gold performs similarly to SYBR Green I. This study shows that: (a) there is no significant difference in DNA binding isotherms to the monocationic DOTAP (DOTAP/DOPE) liposomes and to the polycationic DOSPA (DOSPA/DOPE) liposomes, even when four DOSPA positive charges are involved in the electrostatic interaction with DNA; (b) the helper lipids affect DNA binding, as DOTAP/DOPE liposomes bind more DNA than DOTAP/cholesterol; (c) in the process of lipoplex formation, when the DNA is a mixture of two forms, supercoiled and nicked-relaxed (open circular), there is a preference for the binding to the cationic liposomes of plasmid DNA in the nicked-relaxed over the supercoiled form. This preference is much more pronounced when the cationic liposome formulation is based on the monocationic lipid DOTAP than on the polycationic lipid DOSPA. The preference of DOTAP formulations to bind to the relaxed DNA plasmid suggests that the binding of supercoiled DNA is weaker and easier to dissociate from the complex.

Keywords: Cationic liposome; Supercoiled DNA; Relaxed DNA; Ethidium bromide; SYBR Green I; SYBR Gold; Lipoplex

Abbreviations: DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethylammonium chloride; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DOSPA, 2′-(1″,2″-dioleoyloxypropyldimethyl-ammonium bromide)-*N*-ethyl-6-amidospermine tetratrifluoroacetic acid salt; PC, phosphatidylcholine; CHOL, cholesterol; EtBr, ethidium bromide; TB, Tris–borate; TBE, Tris–borate EDTA; LUV, large unilamellar vesicles; MLV, multilamellar large vesicles; ssDNA, single-stranded DNA

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1. Introduction

Addition of DNA to a preformed cationic liposome leads to formation of a complex (termed a lipoplex) in which structural changes at the level of both the DNA and the liposome occur [1–3]. These changes are dependent on the composition of the lipid mixture (type of cationic lipid, L^+, and associated 'helper' lipid), its concentration in the lipoplex, L^+/DNA^- ratio, and medium composition. In most cases the lipoplexes formed are heterogeneous in their physical and chemical characteristics [1,4].

The finding that DNA complexed to cationic liposomes can transfect cells efficiently in vitro [2,5] and in vivo [6–11] has resulted in the evaluation of cationic lipids for gene therapy application. Cationic liposome-mediated transfection of many cell types has been demonstrated [12–16], employing many different cationic lipids [5,12,13,17–20].

Despite their efficacy, the biophysical characteristics of lipoplexes are just beginning to be understood. Several recent reports have described electron micrographs of metal-shadowed [21], negatively stained [22,23], freeze-fractured [1], and cryo-TEM [24] cationic lipid–DNA complexes, all of which indicate that lipids served to condense DNA. These studies illustrate the high degree of variability in lipoplex structures and the need to understand and control the parameters that govern the organization of these structures.

Gradient centrifugation techniques have been used to physically separate free DNA from the lipoplexes prepared under different formulation conditions [25,26]. Additional methods have been used in order to indirectly quantify the binding of DNA to cationic lipids; these include: (a) intercalating dyes such as ethidium bromide [21,25,26], TO-PRO-1 [27], Hoechst dye 33258 [25], and PicoGreen [28]; (b) agarose gel electrophoresis [21,25,26,29]; and (c) DNase protection assays [21,25,27,29]. The amount of free DNA provides a measure of the binding efficiency of different cationic liposome formulations; therefore it is a useful parameter in comparing complexation efficiencies.

This paper describes an assay to quantify DNA type (supercoiled or relaxed) and amount of DNA complexed to the cationic lipids. The assay is based on conventional agarose gel electrophoresis, followed by staining with the fluorescent cyanine DNA dyes, SYBR Green I or SYBR Gold. We demonstrated this quantitative assay for characterizing different lipoplex formulations (DOTAP/DOPE, DOTAP/CHOL, and DOSPA/DOPE) complexed with three forms of DNA: supercoiled or nicked-relaxed plasmids, or single-stranded DNA (ssDNA).

The assay has proven to be reproducible, sensitive, and simple to perform.

2. Materials and methods

2.1. Lipids

N-(1-(2,3-Dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), and egg phosphatidylcholine (PC) were obtained from Avanti Polar Lipids (Alabaster, AL). 2′-(1″,2″-dioleoyloxypropyldimethyl-ammonium bromide)-*N*-ethyl-6-amidospermine tetratrifluoroacetic acid salt (DOSPA) was a gift from Dr Marilyn Ferrari (Vical, San Diego, CA). Cholesterol was obtained from Sigma (St. Louis, MO). Based on TLC analysis [30,31] all lipids were $\geq 98.0\%$ purity.

2.2. Fluorescent DNA probes

SYBR Green I and SYBR Gold dyes were obtained from Molecular Probes (Eugene, OR) and ethidium bromide (EtBr) from Sigma.

2.3. General

All other chemicals were analytical grade or better. All solutions were prepared with water purified using the WaterPro PS HPLC/Ultrafilter Hybrid (Labconco, Kansas City, MO), which provides low levels of total organic carbon and inorganic ions in sterile pyrogen-free water.

2.4. DNA samples

Plasmid pCi/sayw, expressing sequences coding for the S (small) protein of hepatitis B virus (HBsAg, subtype ayw), and plasmid pCi/LS, expressing sequences coding for both L (large) and S (small) pro-

teins of hepatitis B virus, were received (in Tris–borate EDTA buffer (TBE), pH 8.0) from Professor Jörg Reimann (University of Ulm, Germany). The plasmids were purified using the QIAGEN plasmid Mega kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Both plasmid preparations contained the supercoiled and the nicked relaxed forms (see Section 2.8).

Part of the DNA (pCi/LS) (which was mainly in the supercoiled form) was converted to DNA in the relaxed form by nicking with topoisomerase I (EC5.99.1.2) from wheat germ (Sigma). Following the product information accompanying topoisomerase I, 10 μg of DNA (pCi/LS) was incubated for 3 h at 37°C with 15 units of topoisomerase I in a 50-μl reaction mixture containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT), and 20% (v/v) glycerol. Evaluation by gel electrophoresis showed a complete conversion to the relaxed form.

M13 ssDNA was a gift from Professor Hillel Bercovier (Hebrew University – Hadassah Medical School). M13 ssDNA was purified employing the Wizard M13 DNA purification system from Promega (Madison, WI).

2.5. Determination of DNA purity

Plasmid purity was assessed using agarose gel electrophoresis and A_{260}/A_{280} ratios. Qualitative agarose gel (1%) electrophoresis of both plasmids and M13 ssDNA showed that pCi/sayw has a relatively high level of plasmid DNA in the relaxed form, while pCi/LS was mostly in the supercoiled form. M13 ssDNA was found to be mostly in the relaxed form. In both plasmids and M13 ssDNA batches, no chromosomal DNA or RNA was present. In pCi/sayw the ratio of A_{260}/A_{280} was 1.93, and in pCi/LS it was 1.88, indicating absence of protein contamination [32].

2.6. Quantification of DNA by organic phosphate determination

The absolute concentration of DNA was quantified by determination of organic phosphate [30,33] and is expressed as equivalent concentration of organic phosphate, which represents DNA negative charges [3].

2.7. DNA determination by ethidium bromide

We based our determination on the method of Eastman et al. [26], which demonstrates that at 2.5 nmol EtBr, increases in fluorescence were linear up to 15 nmol DNA. Therefore 2.5 nmol EtBr in H_2O was added to DNA in the range of 3–15 nmol of the three forms, supercoiled and relaxed and M13 ssDNA. Samples were incubated for 10 min at room temperature. The fluorescence (excitation 260 nm and emission 591 nm, using a cutoff filter of 515 nm) of each sample was corrected for the background fluorescence of EtBr in the absence of DNA.

2.8. Characterization of plasmid DNA topology

For plasmid DNA one can distinguish between three types of extreme topological states:

1. Relaxed covalently sealed (sealed-relaxed);
2. Relaxed due to the interruption of a phosphodiester bond (nicked-relaxed), also referred to as open circular;
3. Supercoiled.

In order to distinguish between sealed-relaxed (a) and nicked-relaxed (b) we ran agarose gel electrophoresis (see Section 2.14) twice, once without ethidium bromide in the electrophoresis medium, followed by post-staining with EtBr after the electrophoresis was performed ([34,35] and references therein). A second electrophoresis was done while EtBr was present in the electrophoresis medium (pre-staining). It was found that the time of staining did not affect the mobility of the pDNA, and for both pre- and post-staining by EtBr identical patterns of the DNA electrophoreses were obtained, with the same proportions of stained bands for the supercoiled (high mobility) and relaxed (low mobility), indicating lack of unwinding of the relaxed pDNA. As such unwinding is typical of sealed-relaxed DNA, our results indicate that in all preparations of plasmid DNA used in this study the relaxed plasmid was a nicked-relaxed form, hereafter referred to as just 'relaxed'.

As expected, this was also found for the 100% nicked pDNA prepared using topoisomerase I.

2.9. DNA determination by SYBR Green I

Different amounts of DNA (pCi/LS) in the supercoiled and the relaxed forms and M13 ssDNA, in the range of 0.077–1.232 nmol (25–400 ng), were incubated at room temperature for 10 min with SYBR Green I (diluted 1:5000 in Tris–borate buffer (TB), pH 8.0). The resulting fluorescence was measured using an excitation wavelength of 497 nm and emission wavelength of 520 nm.

All fluorescence measurements in solutions or dispersions were done on an LS50B luminescence spectrometer (Perkin Elmer, Norwalk, CT), using excitation and emission slits of 5 nm.

2.10. Liposome preparation

Neutral multilamellar large vesicles (MLV) were prepared by freeze-drying overnight mixtures of egg PC (16 μmol) and DOPE (8 μmol) dissolved in *tert*-butanol. The lyophilized cake was hydrated with 1 ml phosphate-buffered saline (PBS) (pH 7.4) and vortexed for several minutes to form MLV (size 2.0–2.5 μm) [36].

Large unilamellar vesicles (LUV) were prepared by mixing DOTAP and DOPE (mole ratio 1:1), DOTAP and CHOL (mole ratio 1:1) or DOSPA and DOPE (mole ratio 1:1) in *tert*-butanol and freeze-drying overnight. The lyophilized cake was hydrated with 20 mM Hepes (pH 7.4) and vortexed for several minutes to form cationic MLV.

LUV were downsized from the MLV, using the extrusion system LipoSofast [37] (Avestin, Ottawa, Canada), 11 times through 0.4-μm and 11 times through 0.1-μm pore-size filters (Poretics, Livermore, CA), successively. In all cationic liposome batches the concentration of each type of lipid was 20 mM [33].

2.11. Particle size measurement

The particle size distribution of liposome dispersions was determined at 25°C by dynamic light scattering (DLS) with the Coulter model N4 SD (Coulter Electronics, Hialeah, FL). See Barenholz and Amselem [30] for further details.

2.12. Lipid determinations

DOPE was quantified by organic phosphate determination [30]. Cholesterol was determined by cholesterol oxidase [38]. TLC using copper sulfate staining was used to quantify cationic lipids. Ester lipid stability was quantified by measuring the level of released non-esterified fatty acids using the NEFA kit no. 994-75409 of Wako Chemicals, Neuss, Germany [31,38].

2.13. Lipoplex preparation

Cationic LUV were mixed (on the day of the experiment) with DNA at various specified cationic lipid/DNA (L^+/DNA) ratios and incubated at room temperature for 5–10 min before use [33].

2.14. Agarose gel electrophoresis

For the standard curve, different amounts of free DNA in the range of 1–64 ng (1, 4, 16, 32, 64 ng) were loaded on 1% agarose (Sigma) gel in TB, pH 8.0. Cationic lipid–DNA complexes were prepared at the desired lipid/DNA ratio. An amount of lipid–DNA complex containing 64 ng DNA was loaded on 1% agarose gel in TB, pH 8.0. Samples were loaded on the gel with 2 μl of loading dye (0.25% bromophenol blue and 40% sucrose (w/v) in H_2O).

Electrophoresis was carried out in TB according to the standard procedure [32] under a constant electric field of 100 V for 1 h at room temperature. The gels were post-stained with SYBR Green I or SYBR Gold (diluted 1:5000 in TB) for 30 min at room temperature. The gel fluorescence intensity was scanned and measured by a computerized Fujfilm Fluorescent Image Analyzer FLA-2000 (Fuji Photo Film, Japan) using an excitation wavelength of 473 nm and an emission wavelength of 520 nm. The 473 nm excitation wavelength used is the closest one to the optimal available on this instrument.

Quantification of DNA from the gel image is expressed as emission intensity/area. The fluorescence intensity of SYBR Green I (or of SYBR Gold) in each DNA band in the gel was adjusted to the background intensity of the probe, in the absence of

DNA, and then normalized to dye fluorescence of 64 ng free DNA. Similar results were obtained with the two DNA stains: slopes were almost identical ($y = 19.425x + 63.719$, $R^2 = 0.971$ for SYBR Green I and $y = 22.078x + 8.2198$, $R^2 = 0.9857$ for SYBR Gold).

3. Results

3.1. Quantification of DNA in solution by SYBR Green I: effect of DNA form

The quantification of DNA by SYBR Green I is well established [39,40]. Calibration curves for DNA quantification by SYBR Green I for three forms of DNA (supercoiled plasmid (pCi/LS) and relaxed plasmid (pCi/LS), and M13 ssDNA) (see Materials) are shown in Fig. 1A. DNA amounts for all three were in the range 0.077–1.232 nmol (25–400 ng). The results shown in Fig. 1A demonstrate clearly that there is a linear relationship between SYBR Green I fluorescence intensity and DNA concentration. However, while the absolute values and the slopes of the curves were nearly identical for the supercoiled and the relaxed plasmids, these are much lower for M13 ssDNA.

The limit of detection of DNA by SYBR Green I was ≥0.077 nmol (DNA phosphate) for the supercoiled and the relaxed plasmids and ≥0.616 nmol (DNA phosphate) for the M13 ssDNA.

The linearity of DNA quantification by SYBR Green I requires that the dye be in excess. As the molecular mass of SYBR Green I is proprietary and was not disclosed by its producer (Molecular Probes), the concentration of the dye was described

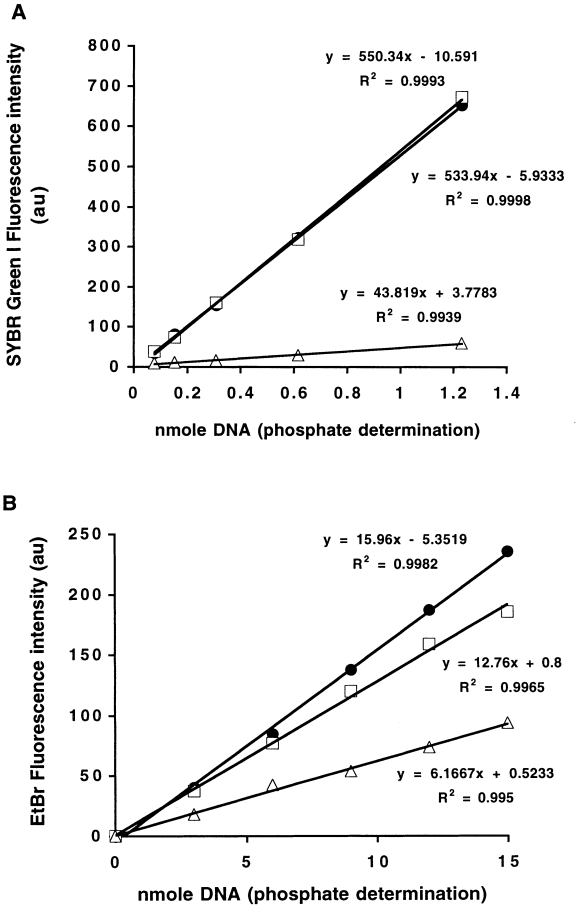

Fig. 1. Quantification of DNA in solution by 1:5000 dilution SYBR Green I (A) and EtBr (B). Fluorescence intensity was measured after incubation for 10 min at room temperature with supercoiled plasmid (pCi/LS) (●), relaxed plasmid (pCi/LS) (□), and M13 ssDNA (△). Fluorescence intensity was measured with slits of excitation and emission of 5 nm.

Table 1
Comparison between EtBr–DNA and SYBR Green I–DNA interactions in naked DNA and in lipoplexes

Sample	EtBr[a] (% unquenched fluorescence)	SYBR Green I[b] (% unquenched fluorescence)
DNA[c]	100.0	100.0
DOTAP/DOPE–DNA[d]	5.2	51.0
DOTAP/CHOL–DNA[d]	1.7	24.0
DOSPA/DOPE–DNA[d]	3.0	31.4

[a]2.5 nmol EtBr in H_2O was added to 15 nmol DNA.
[b]SYBR Green I (diluted 1:500 in 20 mM Hepes, pH 7.4) was added to 15 nmol DNA.
[c]EtBr or SYBR Green I fluorescence intensity obtained upon DNA interaction represents 100%.
[d]Cationic lipid:DNA mole ratio = 2.0.

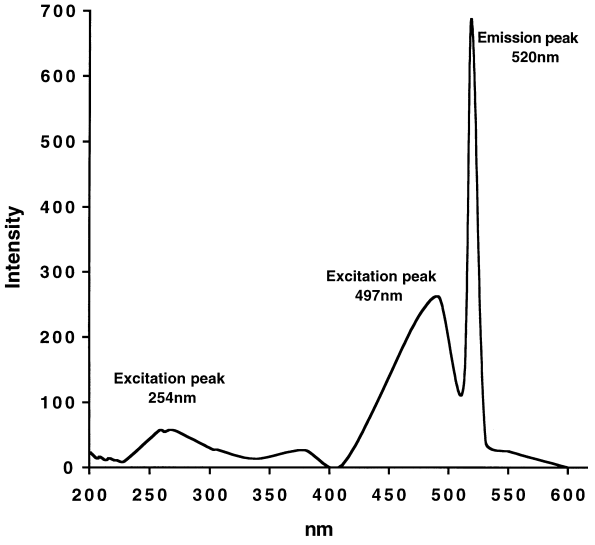

Fig. 2. Fluorescence excitation and emission spectra of SYBR Green I bound to double-stranded DNA.

as a dilution factor. It was found that for up to 1.5 nmol DNA a dilution of 1:5000 gave good linearity. In order to retain this linearity for higher DNA amounts (up to 15 nmol) a SYBR Green I dilution of 1:500 was used (Table 1).

A DNA quantification using EtBr for DNA quantification (Fig. 1B) reveals that the sensitivity of the EtBr assay is much lower (sensitivity limit is ≥ 3 nmol DNA). This value is ~ 40-fold lower than with SYBR Green I for the supercoiled and the relaxed plasmids, and ~ 10-fold lower for the M13 ssDNA. Unlike with SYBR Green I, the absolute values and the slope of the curves with EtBr for the supercoiled and the relaxed plasmids were not identical. For M13 ssDNA slope and absolute values were lower.

3.2. *Quantification of DNA forms (supercoiled and relaxed) by separation on gel electrophoresis*

Fig. 2 shows that SYBR Green I has two peaks in its excitation spectra: one in the visible range (497 nm), which appears only when SYBR Green I interacts with DNA, and the second in the UV range (254 nm).

First, we studied which of these two peaks is preferable for excitation in the gels.

Different amounts of DNA (pCi/sayw) in the range 1–258 ng (0.00308–0.795 nmol DNA phosphate) were loaded on the gels. Panels A and B in Fig. 3 were post-stained with SYBR Green I. The gel in A was excited at the visible range (473 nm was used instead of 497 nm due to the technical limitation of the Fluorescent Image Analyzer), while the

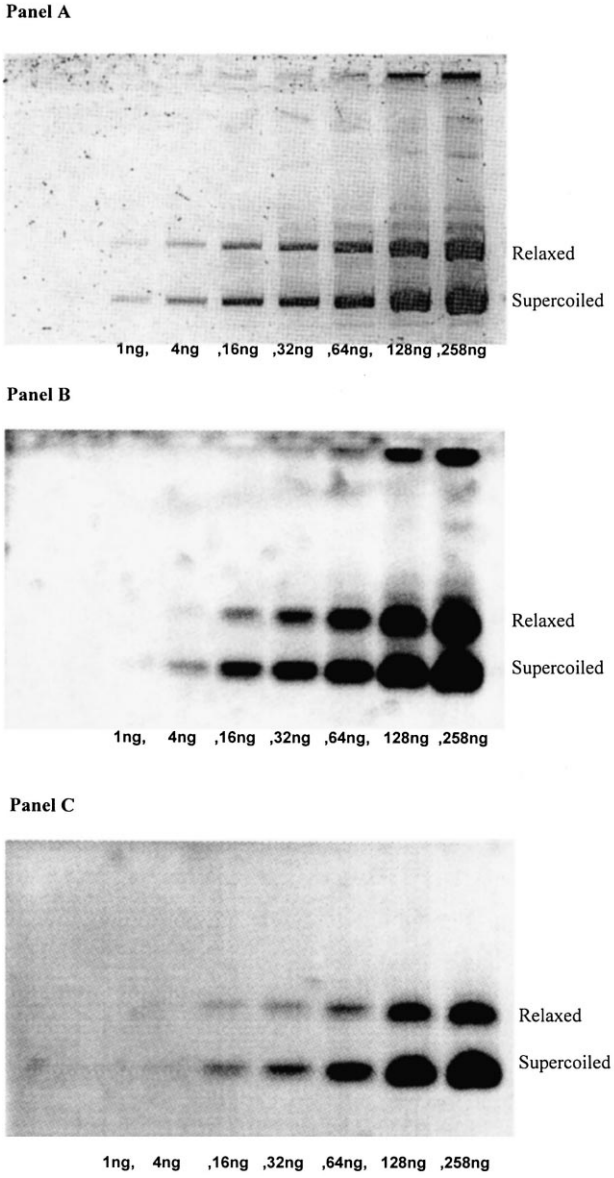

Fig. 3. Quantification of DNA forms (supercoiled, nicked-relaxed) after separation by 1% agarose gel electrophoresis. Different amounts of DNA (pCi/sayw 53% supercoiled, 47% nicked-relaxed) in the range 1–258 ng were loaded on the gels. The gels shown in panels A and B were post-stained with SYBR Green I. The gel in panel C was post-stained with 5 μg/ml EtBr. Panel A was excited at 473 nm, while panels B and C were visualized using UV light in the range 260–365 nm.

gel shown in B was visualized using UV light at (260–365 nm). Fig. 3 shows clearly that when the excitation was done in the visible range (473 nm) the sensitivity of the probe was higher than with UV visualization. It is expected that sensitivity could be improved 1.17-fold by excitation at the visible peak (497 nm).

Comparison between SYBR Green I to post-staining with 5 μg/ml EtBr (the optimal condition for EtBr staining), as shown in panel C of Fig. 3, demonstrates that the limit of DNA detection was 1 ng when excitation of SYBR Green I was done at 473 nm, compared with 16 ng with EtBr. When UV visualization was used, the limit of DNA detection for SYBR Green I was only 4-fold higher than for EtBr.

3.3. *DNA characterization by agarose gel electrophoresis followed by SYBR Green I staining*

We also wanted to explore the possibility that the presence of non-cationic liposomes affects the DNA migration in agarose gel electrophoresis.

The DNA pCi/sayw was applied in two forms, free DNA (Fig. 4A) and DNA that was mixed with neutral MLV composed of egg PC/DOPE (2:1 mol/mol), in large lipid excess (lipid/DNA mole ratio ~800 (Fig. 4B)).

Different amounts of DNA in the range of 4–64 ng were loaded on agarose gel. As shown in Fig. 4, in both cases the data fit linear regression. Analysis of Fig. 4 shows that both slopes (of supercoiled and relaxed DNA forms), which relate fluorescence intensities and DNA amounts, are almost identical, as was demonstrated before for quantification in solution (Fig. 1A). Also, comparing Fig. 4A and B shows clearly that neutral MLV, which lack interaction with DNA [41,42], do not have a retardation effect on the migration of free DNA, and do not have any effect on the quantification of DNA by SYBR Green I in the agarose gel electrophoresis system.

DNA quantification by agarose gel electrophoresis followed by SYBR Green I staining was similar when we used the loading dye that contained 40% (w/v) sucrose instead of 50% glycerol, and without EDTA, and the running buffer was TB instead of TBE (data not shown).

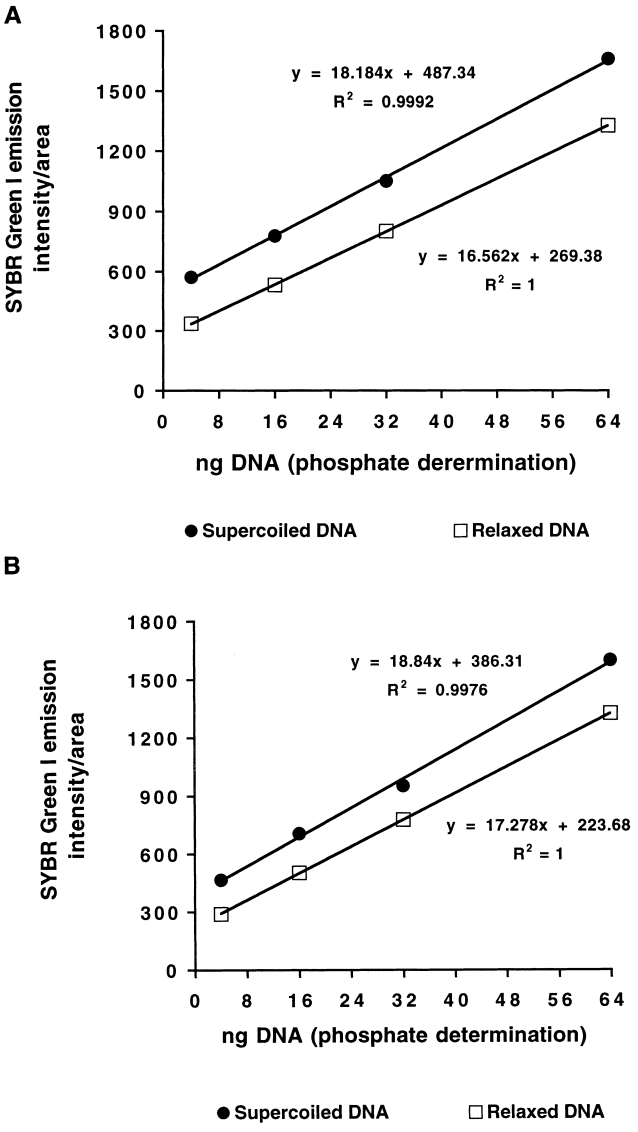

Fig. 4. DNA characterization by gel electrophoresis, followed by SYBR Green I staining. The retardation effect of MLV non-cationic liposomes on DNA migration in agarose gel electrophoresis was determined by loading different amounts of DNA in the range 4–64 ng on 1% agarose gel. The DNA (pCi/sayw) was applied in two forms, free DNA (A) and DNA that was mixed with MLV non-cationic liposome (B).

3.4. *Quantification of 'free' DNA in mixtures of cationic liposomes and DNA: Comparing binding of supercoiled and relaxed DNA forms*

After establishing that neutral liposomes do not interfere with DNA quantification (see Section 3.3), the next step was application of the above assay to

quantify binding of DNA to cationic lipids. The amount of DNA bound is calculated using gel electrophoresis retardation as total DNA input minus free DNA (DNA lipoplex = total DNA−free DNA). For this the level of unbound DNA (free DNA) in coexistence with the lipoplexes was determined by gel electrophoresis, followed by SYBR Green I staining. DNA complexed with cationic lipid was fully retarded and did not migrate into the gel under these conditions (Fig. 5).

The DNA (pCi/sayw) used was 53% in the supercoiled form and 47% in the relaxed form (see Sections 2.4 and 2.8). Three formulations of cationic liposomes were prepared, two based on the monocationic lipid DOTAP and one based on the polycationic lipid (up to five positive charges per molecule) DOSPA. For DOTAP formulations, four mole ratios of cationic lipid to DNA (0.25, 0.5, 1.0, 2.0) were studied, and for the DOSPA formulation five mole ratios of cationic lipid to DNA (0.125, 0.25, 0.5, 1.0, 2.0) were studied. The amount of free DNA was determined directly after post-staining of gel with SYBR Green I. This permits calculating the level of DNA in the lipoplexes as total DNA minus free DNA.

In Fig. 5 we can see that for all three cationic liposomes used the amount of free DNA decreased as the ratio of cationic lipid to DNA increased. The supercoiled DNA form was compared with the relaxed form. For DOTAP/DOPE at DOTAP:DNA molar ratio 2.0, most of the DNA (96%) (the supercoiled and the relaxed forms) was complexed, while at DOTAP:DNA molar ratio 0.5, 48% of the DNA in the supercoiled form and 74% in the relaxed form were complexed.

In DOTAP/CHOL at DOTAP:DNA molar ratio of 2.0 there is no preference, and 14% of each of the supercoiled and relaxed forms of the DNA remained free. At DOTAP:DNA mole ratio 0.5 there is a large preference, as only 30% of the DNA in the supercoiled form was complexed, compared with 69% of the relaxed form.

In DOSPA/DOPE at DOSPA:DNA molar ratio range of 2.0–0.5, all the DNA was complexed with the lipids, while at DOSPA:DNA mole ratio of 0.25, ~30% of the DNA remained free and of the ~70% bound DNA, 47% was supercoiled and 53% was the relaxed form. At DOSPA:DNA mole ratio of 0.125,

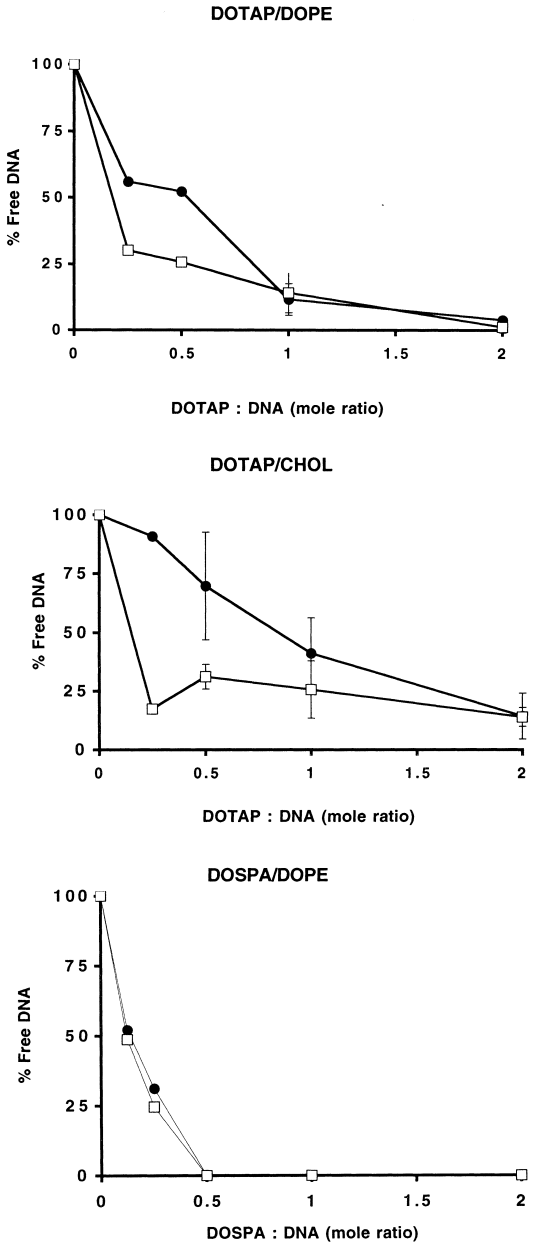

Fig. 5. Quantification of 'free' DNA in lipoplexes: comparing binding of supercoiled and relaxed DNA forms. Three different formulations of prepared LUV cationic liposomes DOTAP/DOPE, DOTAP/CHOL, and DOSPA/DOPE were mixed with DNA (pCi/sayw) at different cationic lipid:DNA mole ratios. An amount of lipid–DNA complex containing 64 ng DNA was loaded on 1% agarose gel. The amount of free DNA was determined directly after post-staining of gel with SYBR Green I. Supercoiled (●) DNA form was compared to the relaxed (□) form. Each point represents the mean of three experiments (S.E. shown as bars).

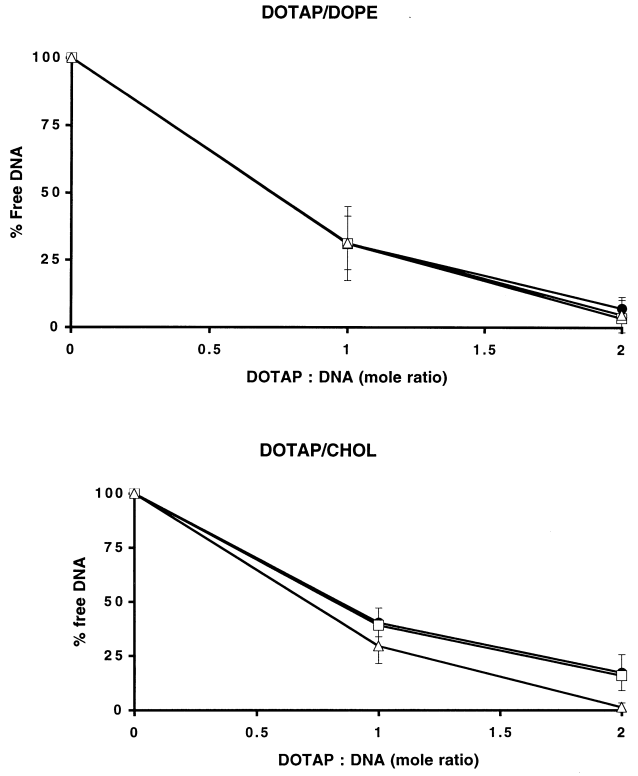

Fig. 6. DOTAP/DOPE and DOTAP/CHOL LUV cationic liposomes were mixed with DNA (pCi/LS) supercoiled plasmid (●) or with DNA (pCi/LS) relaxed plasmid (□) or with M13 ssDNA (△) at cationic lipid:DNA molar ratios 1 and 2. An amount of lipid–DNA complex containing 64 ng DNA was loaded on 1% agarose gel. The amount of free DNA was determined directly after post-staining of gel with SYBR Green I. Each point represents the mean of three experiments (S.E. shown as bars).

~50% of the DNA was in the lipoplex, and of this, 47.5% was supercoiled and 52.5% was relaxed. The results in the two formulations based on the monocationic lipid DOTAP indicate that when the DNA is in excess over the cationic lipid ($L^+/DNA^- < 1.0$) and the plasmid contains a mixture of supercoiled and relaxed DNA forms (like pCi/sawy) (Fig. 5), there is a preference in the binding of the relaxed form of DNA over the supercoiled form. However, this phenomenon was less prominent when we used the polycationic lipid DOSPA, where the relaxed DNA binding to DOSPA was only slightly higher than that of the supercoiled DNA.

To further study this preferential binding we studied the three forms of DNA (supercoiled plasmid, relaxed plasmid, and M13 ssDNA) (see Section 2.4) separately. DOTAP/DOPE and DOTAP/CHOL cationic LUV liposomes were mixed with DNA (pCi/LS) (supercoiled plasmid), or with DNA (pCi/LS) (relaxed plasmid), or with M13 ssDNA (which was 90% in the relaxed form), at DOTAP:DNA mole ratios of 1.0 and 2.0. The reaction mixtures were analyzed by agarose gel electrophoresis, followed by staining with SYBR Green I and the percent of free DNA was determined as described in Section 2.

The results in Fig. 6 demonstrate that for DOTAP/DOPE at DOTAP:DNA molar ratio 2.0, 97% of relaxed plasmid, 95% of M13 ssDNA, and 93% of supercoiled plasmid was complexed. At DOTAP:DNA mole ratio of 1.0, both plasmids (supercoiled and relaxed), and M13 ssDNA had similar binding (69%). The relaxed DNA and M13 ssDNA binding was approximately equal to that of the supercoiled form.

For DOTAP/CHOL at DOTAP:DNA molar ratio 2.0, each plasmid (supercoiled and relaxed), when interacting separately, had similar binding (~84%), while almost 100% of M13 ssDNA was complexed. Supercoiled and relaxed plasmids also had similar binding at DOTAP:DNA mole ratio 1.0 (~60%). M13 ssDNA binding was 10% higher than both plasmid forms.

Therefore, we can conclude that the binding isotherms of each of the three forms of DNA are similar (Fig. 6). When the cationic liposomes are complexed with mixtures of supercoiled and relaxed forms of DNA there is a preferential binding of the relaxed form (Fig. 5). In addition, it seems that for DOTAP the helper lipid affects the level of binding, which is higher for DOTAP/DOPE (1:1) than for DOTAP/CHOL (1:1) (Figs. 5 and 6).

4. Discussion

4.1. SYBR Green I is a preferred dye for DNA quantification

Currently, ethidium bromide (EtBr) is the most commonly used DNA stain for gel electrophoresis [32]. EtBr is an aromatic planar cationic fluorophore. Its fluorescence intensity (excitation at 260 nm and emission at 591 nm) increases upon its intercalation

between base pairs of double-stranded DNA. This is explained by its steric protection from molecular oxygen, thereby inducing dequenching that is proportional to the level of fluorophore intercalation [43]. Therefore measurements of EtBr fluorescence intensity can be used to quantify the amount of DNA on a gel, or its concentration in solution [32]. When EtBr intercalation is prevented by DNA condensation or by DNA encapsulation, EtBr fluorescence intensity will be quenched, as EtBr will remain fully accessible to quenching by molecular oxygen, and therefore no increase of fluorescence intensity due to dequenching will occur. Thus the change in EtBr fluorescence intensity can also be used as a means to assess level of condensation or encapsulation of DNA [21,26]. As shown in Table 1, condensing DNA in lipoplexes prevents intercalation of EtBr, leading to very efficient (95–98%) quenching of EtBr fluorescence.

SYBR Green I and SYBR Gold are new nucleic acid stains which can also be used to detect DNA on a gel and quantify DNA concentration in solution [44]. Both dyes are proprietary products of Molecular Probes (Eugene, OR). The only structural information given describes them as unsymmetrical cyanine dyes developed for sensitive detection of nucleic acids in electrophoretic gels [44]. A major advantage of these dyes is that they do not fluoresce significantly when not bound to DNA. For example, SYBR Green I fluorescence intensity at 520 nm upon excitation at the visible range (497 or 473 nm) is dependent on the interaction of fluorophore with the DNA [44]. The mechanism of binding of these dyes to DNA is not known [44]. The fact that SYBR Green I is less toxic [44] and less mutagenic than ethidium bromide [45], suggests that SYBR Green I probably does not intercalate, or intercalates weakly [45].

As with ethidium bromide, SYBR Green I affinity to double-stranded DNA was higher than to single-stranded DNA. Unlike ethidium bromide, SYBR Green I DNA sensitivity was not affected by the change of DNA conformation (topology) from the supercoiled to the nicked-relaxed form (Fig. 1A,B). As shown in Fig. 2, SYBR Green I has two peaks in its excitation spectra, one in the visible range (497 nm) which is dependent on the interaction with DNA, and the second in the UV range (254 nm). The sensitivity of DNA detection is higher for excitation at the visible range. A linear relationship was shown between SYBR Green I fluorescence intensity and DNA concentration in solution (Fig. 1A) and in agarose gel electrophoresis (Fig. 4). DNA detection by SYBR Green I in solution is ~40-fold more sensitive than with ethidium bromide for double-stranded DNA and ~10-fold for ssDNA.

Another major difference between SYBR Green I and EtBr is that even when all the DNA is present in the lipoplexes (under conditions that EtBr is almost completely quenched), the SYBR Green I fluorescence intensity is only partially quenched (49–76% (Table 1)). The level of the quenching is dependent on the composition of the cationic lipid formulation. The quenching in the case of SYBR Green I is probably related to self-quenching as a result of increase in fluorophore local concentration upon DNA condensation.

It was found that SYBR Gold performs similarly to SYBR Green I for DNA determination in post-stained agarose gel electrophoresis. The lower price of SYBR Gold compared with that of SYBR Green I makes SYBR Gold use attractive.

4.2. *SYBR Green I is a suitable fluorophore to determine level of DNA complexation*

Agarose gel electrophoresis, followed by SYBR Green I staining, and excitation at 473 nm, is a sensitive and convenient assay, utilizing the advantage of SYBR Green I for measuring free or uncomplexed DNA present in cationic lipid gene delivery systems. This assay eliminated the need for more labor-intense gradient centrifugation techniques [25,26] to determine the level of free DNA. Different intercalating dyes, such as ethidium bromide [21,25,26], TO-PRO-1 [27], Hoechst dye 33258 [25], and PicoGreen [28], have also been used to determine the free DNA in lipoplex formulations. The limit of DNA detection using, gel electrophoresis, followed by SYBR Green I staining is 1 ng, which is 16-fold higher than that of ethidium bromide. Also very important is the complete lack of interaction between SYBR Green I and lipids, including cationic lipids (data not shown). We found that such interaction exists in cases of some other DNA stains, such as YOYO-1 (Even-Chen and Barenholz, unpublished). Thus, every DNA stain

used to characterize lipoplexes has to be tested for its interaction with lipids.

Three formulations of cationic liposomes were used in our study: two based on the monocationic lipid DOTAP (DOTAP/DOPE, DOTAP/CHOL) and one based on the polycationic lipid DOSPA (DOSPA/DOPE). The agarose gel electrophoresis data indicate that a DNA molecule either migrates as a free molecule or it interacts with a liposome in such a way that it is fully retarded due to charge neutralization by complexation with cationic liposomes. The fact that the presence of neutral liposomes, which do not bind DNA, has no effect on DNA electrophoretic mobility, while cationic liposomes induce a complete retardation of all DNA in the lipoplex, suggests that the DNA retardation is only electrostatic in nature and that this method can be applied to determine binding levels of DNA to lipids.

After finding that neutral liposomes in the form of MLV or LUV in large excess do not interfere with DNA quantification of unbound DNA by SYBR Green I, we applied the assay of gel electrophoresis to determine the percentage of free DNA in lipoplex preparations. As expected, the amount of free DNA was found to be dependent on the cationic lipid/DNA ratio, and on the type of lipids used in the preparation. Similar results were obtained by us for 'polyplexes', which are complexes of DNA and cationic polymers (S. Sinai, T. Hazzan, A. Domb, Y. Barenholz, in preparation).

4.3. *Effect of lipid composition on DNA binding to cationic liposomes*

Cationic liposomes of three lipid compositions were evaluated in their capacity to bind DNA in three forms. Two of the lipid compositions used contained the monocationic lipid DOTAP, being different in their helper lipid (DOPE versus cholesterol). The third formulation (DOSPA/DOPE (1:1)) is based on the polycationic lipid DOSPA. DOTAP has a single quaternary amine that is fully charged at all pHs [46] and therefore moles of cationic lipid and mole equivalent positive charges are identical. These DOTAP single positive charges are neutralized by the DNA phosphates [47]. For DOSPA-based lipoplexes the electrostatics is more complicated. DOSPA has a maximum of five positive charges: one quaternary, two primary, and two secondary amines. While the quaternary amine is charged at all pHs, in the other four charges, the level of charge is pH- and medium-dependent so DOSPA charge can be anywhere between +5 (at low pH) to +1. This is the reason why it is simpler to express DOSPA in moles and not in mole equivalent charges. Assuming that in our experimental system DOSPA possesses four positive charges per DOSPA, then the DOSPA:DNA molar ratio of 0.125–2.0 is translated into a 0.5–8.0 charge ratio. Comparing the relationship between cationic lipid charges per DNA charge (L^+/DNA^-) and DNA binding (Figs. 5 and 6) indicates that when DOSPA is calculated as having four positive charges per molecule there is not much difference between DOSPA and DOTAP (both in the presence of DOPE as helper lipid) in their capacity to bind DNA. Differences between DOTAP and DOSPA do exist when the form of DNA is taken into consideration (see Section 4.4). However, at least in the case of DOTAP, the helper lipid (comparing cholesterol and DOPE) affects level of DNA binding. Higher levels of binding were observed with DOPE as helper lipid (Figs. 5 and 6). This is somewhat surprising since DOTAP/CHOL is more strongly charged at pH 7.4, being closer to 100% DOTAP [46] than DOTAP/DOPE. This better binding of DNA to DOTAP/DOPE can be explained by the need for removal of small counterions as the first step in the complexation [47,48], which is easier for DOTAP/DOPE than for DOTAP/CHOL [47]. An alternative explanation is that the primary amino group of DOPE is also involved in the binding of DNA [46,47].

4.4. *Effect of DNA structure and liposome composition on DNA binding to cationic liposomes*

In the process of lipoplex formation, when DNA contains the two topological forms, supercoiled and relaxed, and the L^+/DNA^- charge ratio ≤ 2.0, there is a preference for binding to the relaxed form of DNA. This preference is striking when the cationic liposome formulations are based on the monocationic lipid DOTAP, while it appears to a lesser degree for the polycationic lipid DOSPA (Fig. 5). Our suggestion for explaining this preference is that the

negative charges of the relaxed form of DNA are more available and can better adapt to the cationic lipid than those of the supercoiled form, especially for lipid molecules which have one positive charge per molecule of cationic lipid, and therefore rearrangements of the positive charges on the liposome surface are easier. Relaxed, but not supercoiled, DNA can induce change in positive charge distribution, and therefore it competes favorably with the supercoiled DNA when both coexist. This is not the case for the polycationic lipid DOSPA. However, when only one DNA form is present there is no competition, and binding isotherms of all DNA forms are similar (Fig. 6). It was demonstrated that the supercoiled pDNA is the preferred topology for transfection mediated via calcium phosphate, DEAE dextran ([34] and references listed therein) and lipoplexes ([22], and our unpublished work on the same hGH lipoplexes used in that study).

However, the answer to the question of whether this superiority in transfection is related to the difference in binding of the plasmid forms to cationic liposomes requires more research. The results shown in Fig. 5 demonstrate that the preferential binding is reduced or even nonexistent (depending on lipoplex helper lipid) at $L^+/DNA^- > 1.0$, which is the preferred condition for transfection (as exemplified in [1,2,20,33]). This argues against the relevance of the preferential binding to transfection efficiency. This leaves us with the explanations which suggest that the supercoiled topology results in acceleration of the transition to an 'open' transcription complex or that it allows (or improves) binding of factor(s) which enhance transcription ([34] and references therein).

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