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Rapid and sensitive ethidium bromide fluorescence quenching assay of polyamine conjugate–DNA interactions for the analysis of lipoplex formation in gene therapy

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Abstract

A rapid and sensitive fluorescent assay method is reported for assessing polyamine conjugate–calf thymus DNA binding affinity using cholesterol polyamine carbamates with ethidium bromide as a probe. A reproducible method has been developed with an optimal excitation wavelength. Salt concentration is shown to be a critical parameter for both the observed fluorescence intensity of ethidium intercalated in DNA, and also for the binding of positively charged polyammonium ions to DNA, effecting charge neutralisation. This charge neutralisation precedes DNA condensation, a key first step in gene therapy.

Keywords: Cholesterol polyamine carbamates; DNA binding affinity and condensation; Ethidium bromide; Fluorescence quenching; Intercalation; Lipoplexes in gene therapy

1. Introduction

Ethidium bromide (Eth Br) (Fig. 1) is a cationic dye and a trypanocidal and antiviral drug that interacts with both double stranded DNA and RNA by intercalation between the base pairs [1–9]. The fluorescent complex between Eth Br and polynucleic acids was first reported by Le-Pecq and Paoletti in 1967 [1]. A large increase in fluorescence is observed when the phenanthridium moiety of this molecule intercalates DNA making it a useful probe to measure drug–DNA interactions [1]. There are two binding sites: the primary site, which has been interpreted as intercalation between base pairs, and the secondary site, which is thought to be electrostatic between the cationic Eth Br and the anionic phosphate groups on the DNA surface [10]. The secondary mode of binding is most evident at low salt and high dye concentrations. Binding of dye is saturated when one dye molecule is bound for every four or five base pairs (bp) [10]. Analysis of binding using Manning's theory of counterion condensation of polyelectrolytes [11] indicates each intercalated Eth Br molecule lengthens the DNA by about 0.27 nm and outside binding only becomes signifi-

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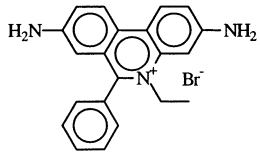


ethidium bromide

Fig. 1. Structure of ethidium bromide.

cant at low salt concentrations [10]. Detailed studies using X-ray diffraction [4,12,13], binding isotherms [3], ^{1}H and ^{31}P-NMR spectroscopy [14], and Sobell et al.'s molecular modelling [4] lead to the conclusion that intercalation follows a neighbour exclusion model and therefore this excludes occupancy of the neighbouring interbase pair sites. These molecular modelling studies of Eth Br intercalation into DNA have also shown that binding is accompanied by a helical screw axis displacement (or dislocation) in its structure [4]. The helical axes are displaced $\approx +1.0$ Å (for B DNA), base pairs in the immediate region are twisted by 10°, giving rise to an angular unwinding of $-26°$ and the intercalated base pairs are tilted relative to one another by 8°. These changes in DNA conformation mean that intercalation is limited to every other base pair at maximal drug–nucleic acid binding ratios, i.e. a neighbour exclusion model [4]. These modelling studies also indicate that the conformational flexibility of DNA allows intercalation of the Eth Br at kinked regions of the double helix. In summary, intercalation of Eth Br occurs at regions where the base pairs are unwound (kinked), which induces a conformational change in the double helix, restricting the total number of intercalation sites.

When studying conformational changes within DNA, it is important to ensure that binding takes place exclusively at intercalation sites [15]. Free Eth Br (in solution) is strongly quenched by aqueous solvent and therefore only exhibits weak fluorescence relative to that from intercalated dye. It has been proposed that the major pathway for deactivation of free Eth Br in aqueous solution involves proton transfer from the excited singlet state to water [9]. When Eth Br intercalates DNA, there is a significant fluorescence enhancement [1] due to the hydrophobic environment surrounding the Eth Br molecule allowing slow proton transfer to water molecules and leading to a longer lifetime for the excited state [9]. This enhancement of Eth Br fluorescence, observed on binding to DNA, is attributed to a reduction in the excited-state proton-transfer rate [9]. Indeed, Eth Br within the hydrophobic environment of the intercalation site is sterically protected from the aqueous solvent, allowing fluorescence. The fluorescence is not affected by the molecular weight of the DNA or the base composition [1]. A two stranded hydrogen-bonded structure, not simply a stacked structure, is required for strong binding of Eth Br [1,9]. Eth Br does not bind with equal affinity to all inter-base pair sites, purine–pyrimidine sequences bind more strongly than purine–purine and pyrimidine–pyrimidine sequences [2,12,16]. There are abrupt changes in the fluorescence intensity at $>$pH 11 and <3, these values represent the pH values of denaturation of DNA, further evidence that a double stranded structure is required [1].

At high salt (>0.5 M NaCl) concentrations, Eth Br binds almost exclusively to double-stranded polynucleotides by intercalation with a resulting enhanced fluorescence over that of non-intercalated dye [17]. The intensity of fluorescence shows the expected qualitative decrease with increasing salt concentration, but with some binding that is rather independent of changes in salt concentration [1]. The changes in binding are due to increases in the dissociation coefficient, with the total number of binding sites remaining constant [1]. However, at low salt concentrations (10 mM and below), Eth Br can bind to the outside of the helix where the fluorescence efficiency is low, but the absorption spectrum is the same as that obtained on binding in the intercalation sites of DNA [1].

Measurement of the ability of a drug to displace Eth Br from DNA is established as a valid measurement of DNA binding ability for both intercalative and non-intercalative drugs, but within certain limits (vide infra) [17–26]. Significantly, displacement of Eth Br from DNA provides an indirect method of measuring the binding affinity of drugs that lack a chromophore. However, it does not provide a direct measure of the

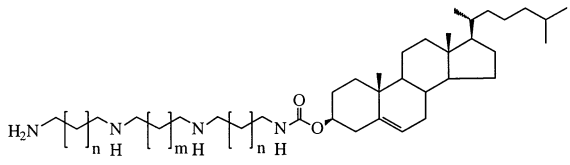
binding constant, but offers a qualitative comparison of binding affinities within a series of compounds with similar structures. Their assay uses direct excitement of the Eth Br (λexcit = 546 nm, λemiss = 595 nm), with no absorbance or fluorescence by the polyamine conjugate or drug at the critical wavelengths, and NaCl concentrations between 5 and 50 mM. Loss of Eth Br fluorescence has also been used to measure the alkylation of DNA, as methylated DNAs have an unaltered binding constant for Eth Br, but a reduction in the number of binding sites, hence the loss in fluorescence intensity is directly proportional to the extent of alkylation [27].

Non-viral gene therapy is a rapidly expanding area of pharmaceutical research that requires physicochemical methods of characterising the interactions of small molecules and polymers with DNA. Displacement or binding exclusion of Eth Br to DNA is one such technique that is employed to measure these interactions. In this paper, certain useful analytical techniques for this research area are evaluated and optimised for rapid, reproducible and efficient evaluation of lipoplex formation using Eth Br as a fluorescent probe. Thus, an aim is to quantify lipoplex formation, the first and a key step in non-viral gene therapy. Lipoplex formation (a lipoplex is defined as a cationic lipid–nucleic acid complex) is a new area of research [28] in which the displacement assay [24,25,29,30] and adaptations based on the exclusion of Eth Br binding to DNA [30–35] have also been used. DNA collapse, by charge neutralisation of cationic lipids, is thought to be a key step in lipoplex formation [28]. The fluorescent intensity of the intercalated Eth Br is not affected by increasing concentrations of cationic lipid until a specific lipid to DNA ratio is reached, upon which a large and sharp decrease of the intensity is observed. Härd et al. [36] have demonstrated that the binding constant of Eth Br is dependent on the molecular flexibility of DNA in linker regions of chromatin and that this flexibility is altered through cationic compaction. Thus, DNA condensation might be expected to lower the affinity of Eth Br for DNA and therefore its exclusion cannot be considered to be a direct measure of a drug's binding affinity. Basu et al. [17] also concluded from a study of (non-conjugated) polyamines binding to DNA that simple polyamine–DNA association was not entirely responsible for the release of Eth Br. DNA bending induced by the polyamine binding above a critical concentration caused conformational changes within the double helix that facilitated the release of bound Eth Br.

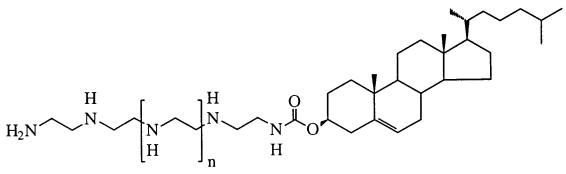
The model for Eth Br intercalation proposed by Sobell et al. [4] shows the need for flexibility within the double helix of DNA to allow intercalation. Eth Br exists in equilibrium between the intercalated sites and free in solution. Therefore, loss of flexibility in the double-stranded structure of DNA through condensation will result in a shift in the binding equilibrium of Eth Br into the solution phase, with the resultant loss in fluorescence.

In Minksy and co-workers' [31] adaptation of the displacement assay, Eth Br is indirectly excited by energy transfer from the DNA, and this produces a much greater fluorescent enhancement (unpublished data from this laboratory). The assay is based on exclusion, rather than displacement of Eth Br. This is achieved by preforming complexes of DNA and conjugate and then immediately prior to spectroscopic analysis, adding an excess of Eth Br. The fluorescence is independent of the size of DNA (100–23 000 bp), closed circular supercoiled plasmid DNA (defined sequence and mass) has a similar fluorescence to calf thymus DNA (random sequence and mass), and the value is not affected by the absolute concentrations of DNA and conjugate (or drug), a polyamine in our studies.

In this paper, the best conditions for rapid, reproducible and efficient evaluation of lipoplex formation using Eth Br as a fluorescent probe are established and reported. The excitation of Eth Br, both directly (546 nm) and by energy transfer via the DNA (260 nm), is evaluated, and also the addition of Eth Br before and after complex formation is compared. A series of cholesterol polyamine carbamates 1–6 (Fig. 2), whose synthesis we have previously described [34], is evaluated using the experimentally determined optimum conditions. Furthermore, changes in the salt concentration and their effect on fluorescence, bind-



1 m = 2, n = 1
2 m = 1, n = 1
3 m = 0, n = 1
4 m = 1, n = 0



5 n = 1
6 n = 2

Fig. 2. Structures of cholesterol polyamine carbamates.

ing affinity of Eth Br and of the cholesterol polyamine carbamates are evaluated using the assay.

2. Experimental

2.1. Materials

Calf thymus DNA and Eth Br were obtained from Sigma, the cholesterol polyamine carbamates were synthesised and their positive charges at pH 7.4 calculated as previously described [34]. Compounds were lyophilised as their poly-TFA salts, weighed, and dissolved in MilliQ water. Eth Br was weighed and a stock solution (0.5 mg/ml) made up in MilliQ water. Buffer and NaCl solutions were also made up in MilliQ water and buffers were pH adjusted to 7.4 with NaOH. A stock solution (2 ml) of calf thymus DNA (1 mg/ml) for the exclusion assay was dissolved in 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4 buffer and its concentrations determined spectroscopically [28]. For the displacement assay, a stock solution of approximately 20 μg/ml (3 ml) was made and its concentration was also accurately determined spectroscopically [28].

2.2. Apparatus

Fluorescence studies were carried out with a Perkin–Elmer LS 50B Luminescence Spectrometer (λexcit = 260 nm, λemiss = 600 nm; 1 cm path length 3 ml glass cuvette: slit width 5 nm). An IBM compatible personal computer was used for data collection, using FL WinLab (Perkin–Elmer) software. DNA concentration and purity were determined using triplicate spectrophotometric readings at 260 (for DNA concentration) and 280 nm (protein contamination) with a Milton Roy Spectronic 601 spectrometer [28].

2.3. Exclusion assay

Six μg (6 μl of 1 mg/ml solution, [DNA base pair] = 3.0 μM, [28]) of DNA was diluted to 250 μl with buffer (2 mM HEPES, 150 mM NaCl, 10 μm EDTA, pH 7.4). Varying masses of cholesterol carbamate (dependent on the charge ratio required) were diluted to a volume of 250 μl with buffer (2 mM HEPES, 150 mM NaCl, 10 μm EDTA, pH 7.4) and added to the DNA, mixed in a microcentrifuge and incubated for 30 min at ambient temperature. Each solution was then diluted to 3 ml with 20 mM NaCl. Immediately prior to analysis, 3 μl of Eth Br solution (0.5 mg/ml, 1.3 mM, effectively present in excess) was added, the sample was mixed on a bench top vortex and the fluorescence measured. The fluorescence was expressed as the percentage of the maximum fluorescence signal when Eth Br was bound to the DNA in the absence of competition for binding and was corrected for the background fluorescence of total Eth Br in buffer solution by subtraction.

2.4. Displacement assay

The concentration of the DNA stock solutions (approximately 20 μg/ml, 3 ml) was determined spectroscopically and 6 μg (approximately 300 μl) of DNA was diluted to 3 ml with buffer (20 mM

NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) in a glass cuvette stirred with a micro-flea. Immediately prior to analysis, Eth Br solution (3 μl, 0.5 mg/ml) was added to the stirring solution and allowed to equilibrate 1 min. Aliquots (5 μl) of the cholesterol carbamate (0.25 mg/ml) were then added to the stirring solution and the fluorescence measured after 1 min equilibration. The fluorescence was expressed as the percentage of the maximum fluorescence signal when Eth Br was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free Eth Br in solution. One hundred and fifty mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4 buffer was used for the experiments conducted at elevated salt concentration.

3. Results and discussion

3.1. Optimisation of the excitation wavelength

Both direct and indirect excitation of Eth Br have been used to determine the relative binding affinity of molecules for DNA. In this paper, we determine if changing the excitation wavelength from 546 (direct excitation) to 260 nm (indirect excitation through energy transfer) was valid. Therefore, carbamate 1 was tested in the displacement assay at low salt (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) using both excitation wavelengths and fluorescence emission was measured at 600 nm (slit width 5 nm; 1 cm path length). The results are detailed in Figs. 3 and 4, and are represented as a function of charge ratio [28], the positive charge equivalents of the polyamine conjugate to the negative charge equivalents of the DNA phosphate backbone. In Fig. 3, these data are represented as a function of the percentage fluorescence and they show no apparent difference between the two methods. However, when these data are represented as a function of absolute fluorescence, Fig. 4, it is apparent that indirect excitation of the Eth Br produces a more sensitive assay. Indirect excitation of Eth Br by irradiation at 260 nm was chosen as the method of inducing fluorescence of Eth Br.

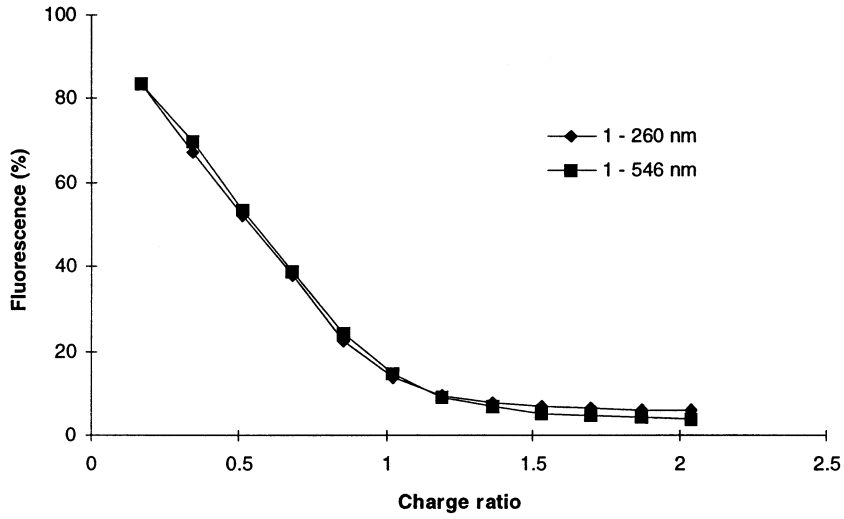


Fig. 3. Eth Br displacement assay of cholesterol polyamine carbamate 1. (◆) Displacement assay, 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined using an excitation wavelength of 260 nm. (■) Displacement assay using an excitation wavelength of 546 nm.

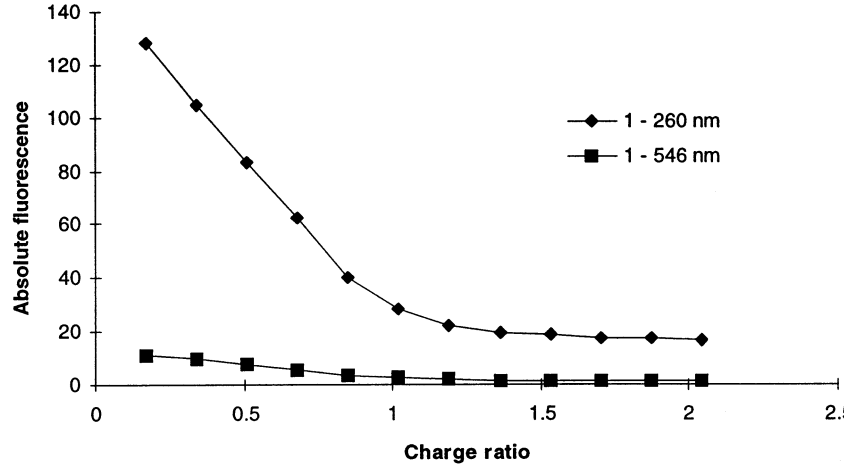


Fig. 4. Eth Br displacement assay of cholesterol polyamine carbamate 1. (◆) Displacement assay, 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the absolute fluorescence determined using an excitation wavelength of 260 nm. (■) Displacement assay using an excitation wavelength of 546 nm.

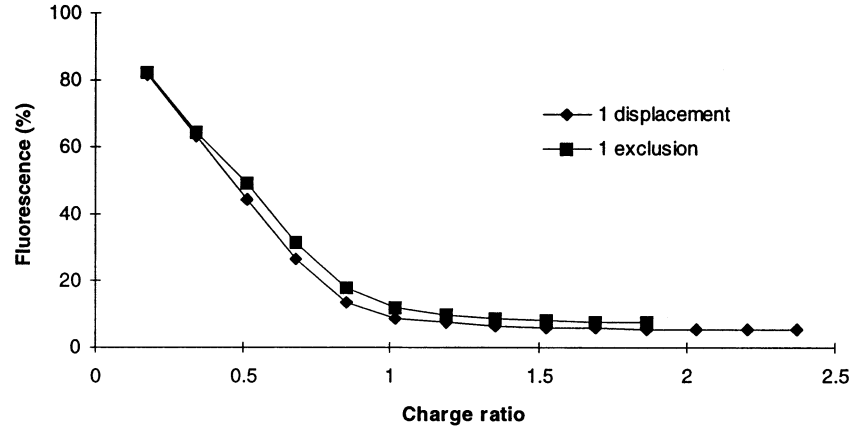


Fig. 5. Eth Br displacement and exclusion assays of cholesterol polyamine carbamate 1. (◆) Displacement assay, 6 μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence determined. (■) Exclusion assay, 6 μg of CT DNA was mixed with varying masses of cholesterol carbamate (dependent upon the charge ratio required) in buffer (500 μl, 2 mM HEPES, 150 mM NaCl, 10 μm EDTA, pH 7.4) and incubated for 30 min. Each solution was then diluted to 3 ml with 20 mM NaCl, prior to analysis, 3 μl of Eth Br solution (0.5 mg/ml) were added and the fluorescence measured ($n = 2$).

3.2. Comparison of the displacement and exclusion assay

In order to determine if excluding Eth Br from binding rather than displacing it from its intercalation sites would have any influence on the assay (λexcit = 260 nm; λemiss = 600 nm; slit width 5 nm), we compared the literature protocols of Cain [18] and Minsky [31] and their independent coworkers. We employed the conditions they reported, resulting in small, but reproducible final salt concentrations. Fig. 5 shows the results of carbamate 1 in both assays. The small difference between the curves is due to the slightly higher

salt concentration in the exclusion assay, as the binding of this type of compound is salt dependent [17,37,38]. The results from the displacement assay are from a single experiment and those from the exclusion assay are the mean of two experiments (Fig. 5).

The reproducibility of the assay was then verified by repeat experiments, Fig. 6 shows the results of six repeats of carbamate 1 in the displacement assay and Fig. 7 two repeats of carbamate 1 in the exclusion assay. It is apparent from Fig. 7 that there are large variations in the read-

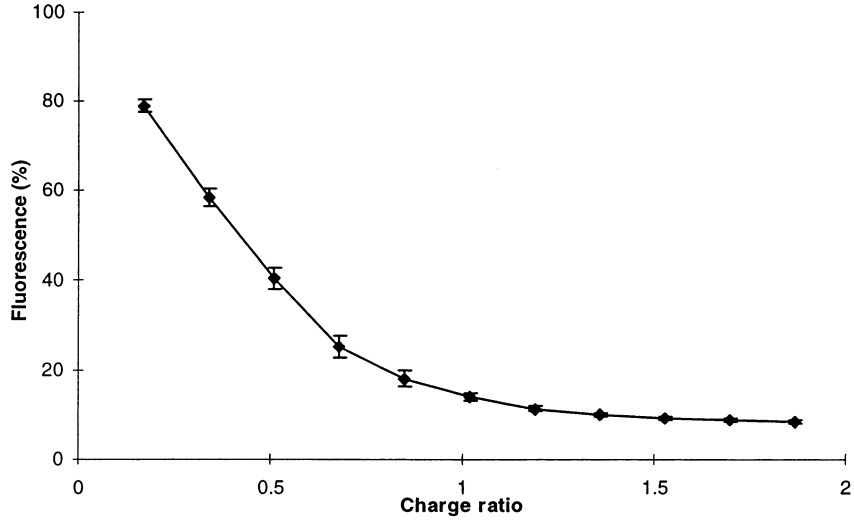


Fig. 6. Eth Br displacement assay of cholesterol polyamine carbamate 1. Six μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined ($n = 6$).

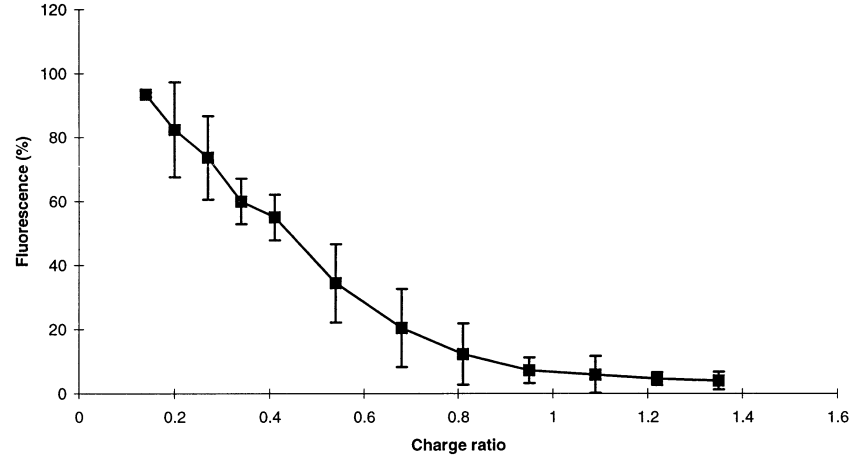


Fig. 7. Eth Br exclusion assay of cholesterol polyamine carbamate 1. Six μg of CT DNA was mixed with varying masses of cholesterol carbamate (dependent upon the charge ratio required) in buffer (500 μl, 2 mM HEPES, 150 mM NaCl, 10 μM EDTA, pH 7.4) and incubated for 30 min. Each solution was then diluted to 3 ml with 20 mM NaCl, prior to analysis, 3 μl of Eth Br solution (0.5 mg/ml) were added and the fluorescence (%) measured ($n = 2$).

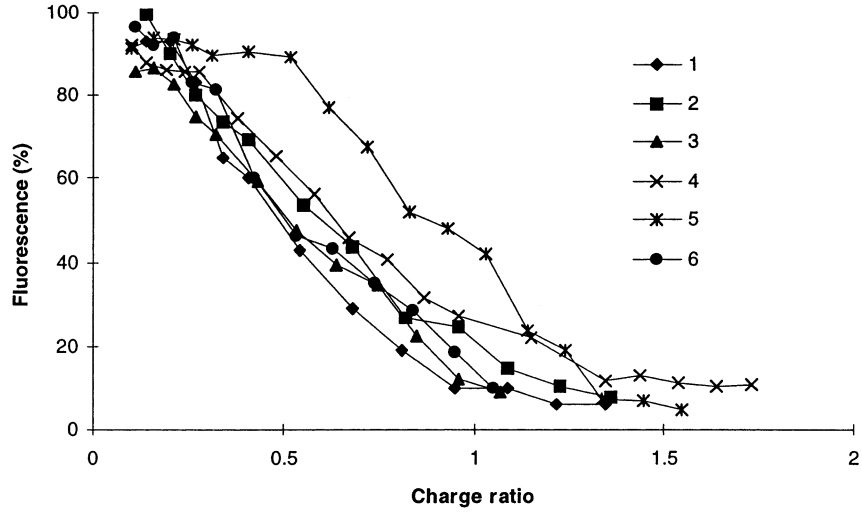


Fig. 8. Eth Br exclusion assay of cholesterol polyamine carbamates 1–6 at low salt concentration (20 mM NaCl). Exclusion assay, 6 μg of CT DNA was mixed with varying masses of cholesterol polyamine carbamate (dependent upon the charge ratio required) in buffer (500 μl, 2 mM HEPES, 150 mM NaCl, 10 μM EDTA, pH 7.4) and incubated for 30 min. Each solution was then diluted to 3 ml with 20 mM NaCl, prior to analysis, 3 μl of Eth Br solution (0.5 mg/ml) were added and the fluorescence measured.

ings for the exclusion assay when compared to the displacement assay. Gershon et al. [31] reported at intermediate ratios of binder to DNA clear fluctuations of the fluorescence intensity as a function of time were observed, culminating in background fluorescence values. At high and low ratios the fluorescence is not time dependent in its behaviour. The time dependent fluctuations were attributed to the large sensitivity of the DNA condensation process to minor changes in the environmental conditions. For the exclusion assay, a stock solution of DNA at 1 mg/ml (2 ml) was made and 6 μl aliquots (6 μg) used for each data point. For the displacement assay, stock solutions of approximately 20 μg/ml (3 ml) were made, the concentration determined spectroscopically, and the required volume to give 6 μg ($\approx$ 300 μl) of DNA diluted to 3 ml with buffer. Therefore, as the fluctuations in the exclusion assay are dependent on minor changes in the DNA concentration, the less accurate method of DNA sample preparation may account for some of the fluctuations.

To confirm the reproducibility of the displacement assay over the exclusion assay carbamates 1–6 were screened, Fig. 8 shows the exclusion assay of all six cholesterol polyamine carbamates 1–6 and Fig. 9 the displacement assay. These data demonstrate the problems of fluctuations in the fluorescence at intermediate values in the exclusion assay, that are absent in the displacement assay results (Fig. 9). In conclusion, both assays produce similar overall results, however, the displacement assay is much more rapid and without the fluctuations in fluorescence at intermediate values.

3.3. Low and high salt concentrations in the displacement assay

The binding affinity of spermine for DNA has been shown to be salt dependent [17,37,38], and variation of the salt concentration in the assay may provide important information with respect to lipoplex formation. Therefore, we have investigated the binding behaviour of cholesterol polyamine carbamates 1–6 at physiological salt concentrations (150 mM NaCl, Fig. 10). The fluorescent intensity of Eth Br is also salt dependent and therefore to increase the sensitivity of the assay the excitation and emission slit widths were increased from 5 to 10 nm. Fig. 10 shows a change in order of the relative binding affinity of each carbamate compared to the results at low

salt concentrations (20 mM NaCl, Fig. 9) and incomplete exclusion of all the Eth Br. Basu et al. [17] have previously shown, using pentaamines, the inability to displace completely Eth Br from DNA at elevated salt concentrations. This phenomenon was explained by aggregation of the polyamine–DNA complex before complete exclusion of the Eth Br had occurred. Carbamates 1–6 produced similar results. Thus, Eth Br fluorescence decreases at elevated salt concentrations

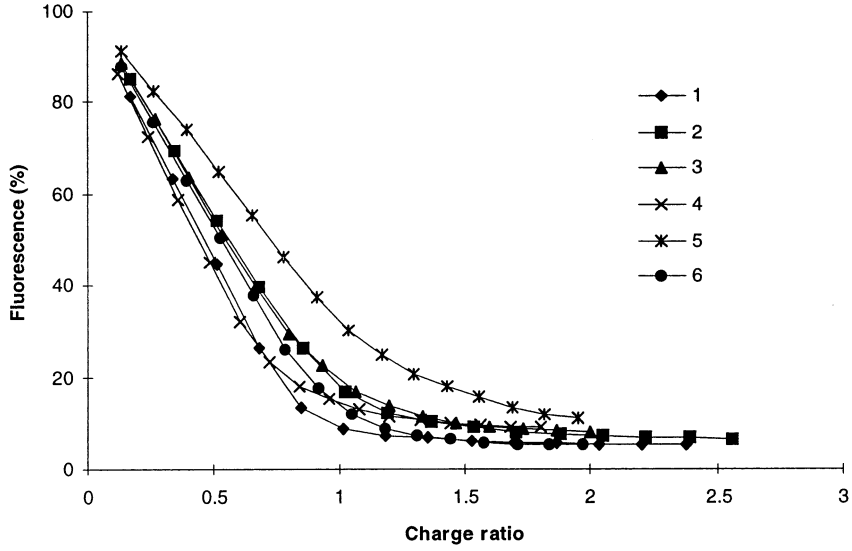


Fig. 9. Eth Br displacement assay of cholesterol polyamine carbamates 1–6 at low salt concentrations (20 mM NaCl). Six μg of CT DNA in buffer (3 ml, 20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined.

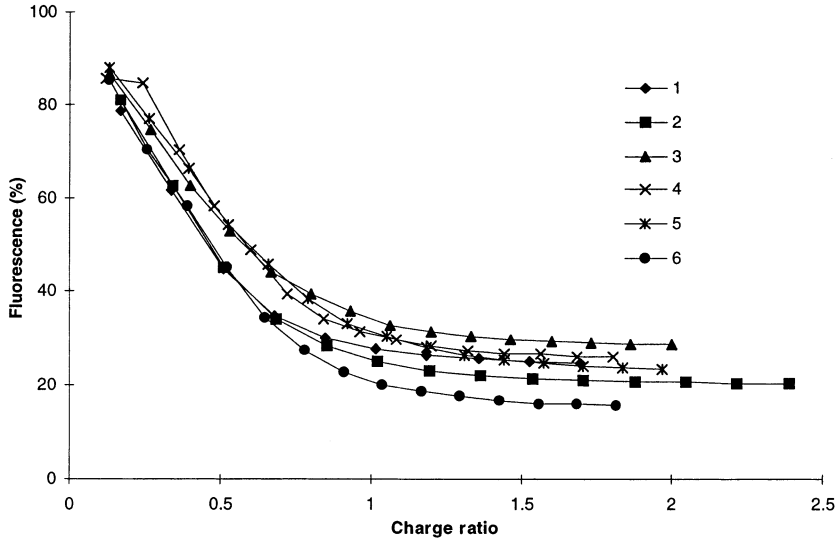


Fig. 10. Eth Br displacement assay of cholesterol polyamine carbamates 1–6 at high salt concentrations (150 mM NaCl). Six μg of CT DNA in buffer (3 ml, 150 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) was mixed with Eth Br (3 μl of 0.5 mg/ml) and aliquots of compound (5 μl of 0.25 mg/ml, 1 min equilibration time) were added and the fluorescence (%) determined.

resulting in a decrease in the sensitivity of the assay and incomplete displacement of Eth Br is apparent. Variations in salt concentration may provide useful information with respect to lipoplex formation and stability, a first key step in non-viral gene therapy.

4. Summary

In conclusion, in this assay we have shown the indirect excitation of Eth Br through energy transfer from the DNA provides a more sensitive assay than direct irradiation of Eth Br. Also, we have established that the optimised displacement assay is more reproducible than the exclusion assay, is more rapid and lacks the fluctuations in fluorescence found at intermediate values in the exclusion assay. At low salt concentrations, this Eth Br assay provides a sensitive measure of relative binding affinity across a series of similar compounds. The binding of lipopolyamines to DNA has been shown to be salt concentration dependent, direct comparison between low and high salt concentrations is complex as fluorescence is also salt dependent. However, high salt (150 mM NaCl) provides an important assessment of lipoplex integrity, a key measure in non-viral gene therapy, and also of DNA binding affinity under more physiological conditions where salt concentration affects lipoplex stability. This Eth Br assay has been previously used to provide relative binding constants [18,39,40], even with simple polyamines where the 50% fluorescence inhibition value is approximately inversely proportional to the binding constant [18,40]. In addition, measurement of the ability to displace the intercalator dye Eth Br has been shown to be valid for comparisons of DNA binding affinity within a series of intercalator or non-intercalator ligands [17–26,29–35,38–43]. The quantification of DNA condensation efficiency, lipoplex formation and stability are key parameters in this research area. Therefore we have optimised this useful, rapid, sensitive and reproducible fluorescence Eth Br assay. This displacement assay is robust and will provide rapid access to much needed quantitative data for the assessment of similar lipopolyamine structures.

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