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# ASSOCIATING OLIGONUCLEOTIDES WITH POSITIVELY CHARGED LIPOSOMES

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**Abstract:** Oligonucleotides (ODNs) are short (up to 30 bases) fragments of single-stranded nucleic acids that are used as sequence specific regulators of gene expression and anti-sense based therapeutics. ODNs are frequently aggregated with particulates in order to improve their pharmacological characteristics. Complexes of ODN and lipid aggregates are among the most commonly mentioned in the literature. In order to control the formation and final properties of such aggregates, a detailed description of how ODN interacts with the lipid surface is needed. In this paper, we present the results of fluorescence measurements regarded an association of 20 base ODN, labelled with fluorescein, and a lipid surface containing various amount of positive charge. Unilamellar lipid vesicles were formed from egg phosphatidylcholine (PC) and various amounts of the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). It was found that about 20 mol% of DOTAP in the lipid bilayer suffices to obtain complete ODN association. This result was further confirmed via measurements performed by fluorescence correlation spectroscopy (FCS). These in turn showed that the diffusion time of labelled ODN in the presence of cationic liposomes decreases. Also, the particle number

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Abbreviations used: ODN – oligonucleotide; PC – phosphatidylcholine; DOTAP - 1,2-dioleoyl-3-trimethylammonium-propane; FCS – fluorescence correlation spectroscopy; ODN-FAM – oligonucleotide labeled with fluorescein (for details see Materials section); RNA – ribonucleic acid; DNA - deoxyribonucleic acid.

and count rate were reduced, concurring with conclusions derived from steady-state fluorescence spectroscopy results.

**Key Words:** Lipoplex, Cationic Lipid, Oligonucleotide, FCS

## INTRODUCTION

Antisense oligonucleotides have already found their place in the pharmacotherapy of various diseases [1-3]. They are applied in a variety of formulations, of which the particulate forms seem the most promising. Aggregates containing lipids, called lipoplexes, are among those under extensive study [4, 5]. Such aggregates are formed from nucleic acids and cationic lipids, which are thought to protect ODN from hydrolysis and provide some means of its distribution within the organism [6, 7]. Electrostatic interaction between negatively charged nucleic acid and the positively charged lipid surface are believed to stabilize the aggregate. Nucleic acid-lipid interactions are also an important factor in ODN association with biological membranes. Its penetration of the plasma membrane is a crucial step in internalisation, which is needed to associate it with intracellular targets (ribosomes and RNA in the cytoplasm or DNA in the nucleus [3]). Furthermore, studies on ODN association with lipid surfaces may provide valuable information on the effect of lipid composition on the strength and character of local interactions between lipid molecules and nucleic acid. When large negatively-charged molecules are combined with cationic liposomes, massive conformational changes of both DNA and the liposome take place. The procedure is very sensitive to a large number of parameters, thus the resulting aggregates are frequently not homogeneous [8, 9]. Small ODNs, on the other hand, are not expected to induce any global alteration in liposome topology, and their own secondary structure should remain preserved. Therefore, the association of ODNs with lipid surfaces is a useful model that may provide valuable information on local events and their dependence on the composition and conformational state of aggregate lipid components [10]. Such information is useful in identifying the processes that lead to lipid-nucleic acid aggregate formation, otherwise impossible to follow.

## MATERIALS AND METHODS

A 20 base long phosphorothioate DNA oligonucleotide labeled at the 5'-end with fluorescein (5'-FAM-ACTGACTGACTGACTGACTG), designated as ODN-FAM, was synthesized via the solid-support phosphoramidite method on an ABI 392 DNA synthesizer, with S-TETRA sulphurization [11]. The fluorescein residue was attached via a phosphorothioate linkage after final detritilation with a phosphoramidite FAM reagent (Applied Biosystems, Foster City, CA, USA). ODN-FAM was isolated using the RP HPLC technique on a Hamilton PRP1 column. The synthetic monovalent cationic lipids – 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and L-α-phosphatidylcholine (PC) –

were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Liposomes were prepared as described previously [12]. Briefly, chloroform solutions of DOTAP and egg-PC were mixed in the desired molar ratios, the organic solvent was evaporated and the resulting lipid film hydrated by adding a filtered buffer (a 10 mM phosphate buffer with 140 mM NaCl, pH 7.4). Large, unilamellar vesicles were obtained by extrusion through a polycarbonate filter with 100 nm pore size.

**Fluorescence measurements**

Steady-state measurements were performed on a spectrofluorimeter with a thermostatic cuvete holder (Perkin Elmer LS50, Wellesley, MA, USA). The absorption/emission wavelengths were set to 499/514nm. The thermally equilibrated vesicle suspension was titrated with the ODN-FAM solution at 20°C. The resulting plot of fluorescence intensity versus ODN concentration gives a straight line, the slope of which was used to evaluate ODN binding efficiency (Figure 1).

In the FCS measurements, a ConfoCor®1 (Carl Zeiss, Jena, Germany) with a water-immersion objective was used. The fluorescent probe, in a confocal volume element of approximately 1 fL, was excited by an $Ar^+$-laser with a wavelength of 488 nm. Fluorescence intensity signals, I(t), were collected from an SPCM-200-PQ avalanche photodiode and processed by a hardware correlator to yield the normalized autocorrelation function G(τ) ($G(\tau) = 1 + \langle \delta I(t)\delta I(t+\tau)\rangle / \langle I(t)\rangle^2$, where $\langle\ \rangle$ denotes the temporal average). The obtained curve was fitted to a theoretical model according to Evotec's FCS ACCESS evaluation software, that is, to the following equation:

$$G(\tau) = 1 + \frac{1}{N}\cdot\frac{1}{1+(\tau/\tau_D)}\left\{\frac{1}{1+(\tau/\tau_D)(\omega_1^2/\omega_2^2)}\right\}^{1/2}, \qquad (1)$$

where $\omega_1$ is the radius of the volume element in the xy plane, $\omega_2$ is its half-length in the z direction; $\tau_D$ is diffusion time and N is particle number. When the time of diffusion through the volume element is much longer than the time of fluorescence decay ($\tau_D >> \tau_f$), the diffusion coefficient D can be determined as $\tau_D=\omega_1^2/4D$.

Count rate (CR), a measure of sample average fluorescence intensity, was normalized according to the following formula:

$CR = \frac{CR' - CR_0}{CR_B - CR_{B0}}\cdot 100\%$, where CR' is the count rate before normalization; $CR_0$ represents the count rate for the first experimental point (without ODN-FAM added); $CR_B$ was obtained in the experiment where the buffer contained oligonucleotide alone; and $CR_{B0}$ is count rate for the first experimental point of the control experiment. A detailed description of FCS theory, its conceptual

basis and exact formulation in mathematical models can be found in several reviews [13, 14].

## RESULTS AND DISCUSSION

When ODN-FAMs were added to the buffer free of vesicles, the resulting fluorescence intensity increased linearly with the amount of added labelled oligonucleotide (correlation coefficients were always better than 0.99). This linear dependence indicates the absence of any processes in the bulk that could cause a fluorescence change (Figure 1b). The dependence of ODN-FAM fluorescence on its concentration in the buffer was a reference value for the subsequent experiments. Titration curve slopes, calculated for ten points, were used as a measure of fluorescence intensity in all the following measurements. In experiments involving liposomes, ODN-FAM concentration was limited to values at which titration curves remained linear, therefore avoiding possible interference from processes other than single molecule binding onto the liposome surface. When labelled oligonucleotides were added to a suspension of vesicles formed from DOTAP alone, the slope reached a minimal value which was used as a lower limit in the association scale (Figure 1a). This decreased value of fluorescence intensity was assumed to reflect the extent of oligonucleotide association with the lipid surface. Steady-state fluorescence results for ODN-FAM association with lipid surfaces containing various fractional amounts of DOTAP mixed with PC are shown in Figure 1.

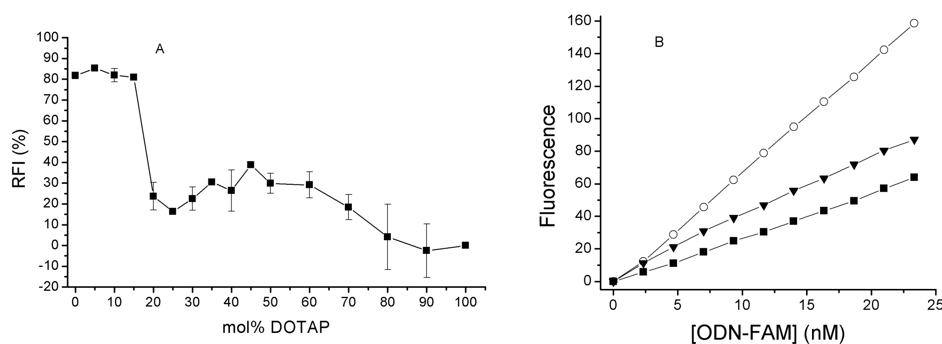


Fig. 1. Steady-state fluorescence results. A) The Rate of Fluorescence Increase (RFI) at various molar fractions of DOTAP in PC liposomes. The Rate of Fluorescence Increase was calculated according to the following formula: $RFI=(A-A_{DOTAP})/(A_{buf}-A_{DOTAP})\cdot 100\%$, where: $A=\tan(\alpha)$ – slope of the ODN-FAM fluorescence titration curve, $A_{buf}$ – slope of the ODN-FAM fluorescence titration curve in the absence of vesicles, $A_{DOTAP}$ – slope of the ODN-FAM fluorescence titration curve in the presence of the pure DOTAP vesicles. B) Typical ODN-FAM fluorescence titration curves for 0 mol% (open circles), 30 mol% (filled rectangles) and 100 mol% (filled squares) of DOTAP in PC liposomes. The lipid concentration in the suspension was 0.05 mg/ml. The concentration of ODN-FAM during titration varied from 0 to 23.3 nM.

The slope remains constant up to about 20 mol% DOTAP in the bilayer. Above this level, the fluorescence intensity drops by about 60% and stays at this low level throughout all the remaining higher DOTAP concentrations. The molecular justification for the appearance of a maximum at 50 mol% DOTAP remains to be elucidated. Results from steady-state fluorescence measurements show that at all DOTAP concentrations exceeding 20 mol%, ODN binding to the lipid surface is almost complete. Since fluorescein is known to be very sensitive to a variety of environmental conditions, including polarity and local proton concentration, changes in fluorescence intensity upon ODN-FAM binding to the membrane surface may reflect a combination of various influences. These can be a change in local polarity (lipid bilayer interface polarity is much lower than that in the bulk [15]), or an alteration of local proton concentration induced by the adsorption of highly charged nucleic acid or by a change in lipid headgroup dipole orientation [16]. A description of how these factors affect fluorescein associated with ODN remains to be given. Despite the lack of detail on the reasons for this change in fluorescence intensity, it seems to be a good indicator of ODN-FAM association with liposomes.

In order to confirm the assumption that the decreased fluorescence intensity of ODN-FAM results from oligonucleotide association with the lipid surface, experiments using Fluorescence Correlation Spectroscopy (FCS) were performed. FCS is a technique that allows the association of ODN with liposomes to be determined by measuring labelled molecule diffusion time, which is independent of fluorescence intensity. Figure 2 shows the correlation function obtained for oligonucleotides in the buffer alone and in the presence of positively charged vesicles containing 50 mol% DOTAP.

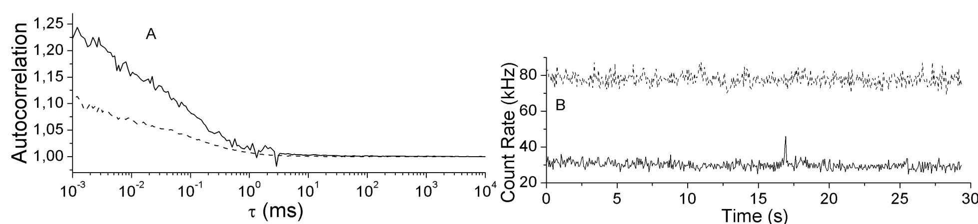


Fig. 2. Example FCS Results. A) Normalized autocorrelation functions obtained for 23.3nM ODN-FAM in buffer alone (dashed line) and in the presence of DOTAP/PC (1:1 mol:mol) vesicles (solid line). B) Corresponding fluorescence count rates. Lipid concentration 0.05mg/ml.

Diffusion time, count rate and particle number as retrieved from the correlation functions are presented in Figure 3. As expected from the steady-state fluorescence measurements (Figure 1), the count rate decreases upon the addition of positively charged vesicles. Simultaneous changes in particle number and diffusion time indicate that oligonucleotide adsorbs onto the vesicle surface, justifying our previous conclusions. Diffusion time for ODN alone is much

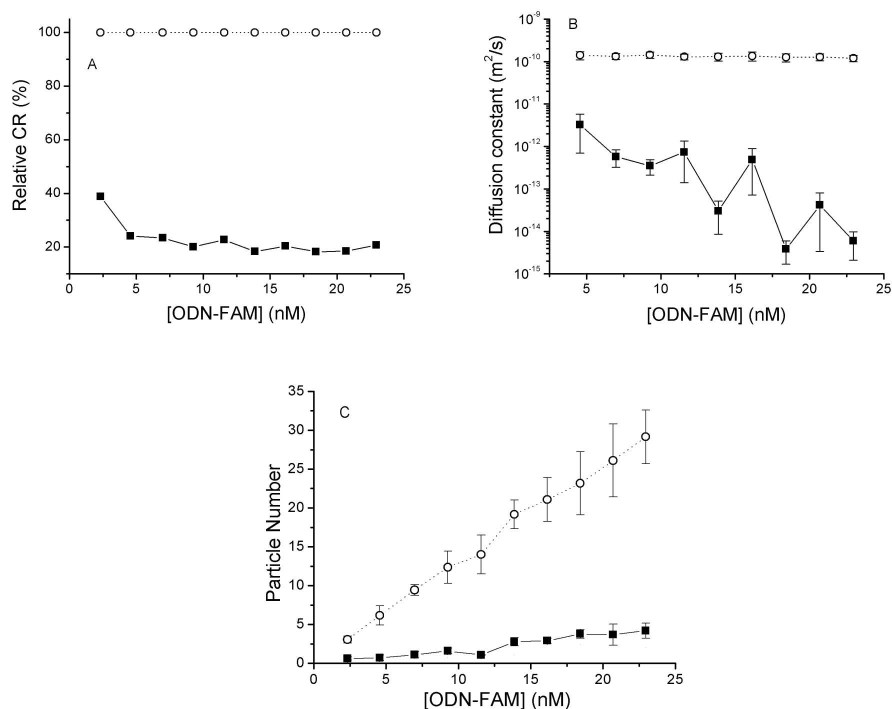


Fig. 3. Changes of the relative count rate (A), diffusion constant (B) and particle number (C) during FCS titration of liposomes with ODN-FAM. Open circles represent data points obtained for ODN in buffer alone, whereas filled squares are data obtained in the presence of cationic vesicles (DOTAP/PC 1:1 mol:mol). Lipid concentration was 0.05mg/ml.

higher than when it is in the presence of charged vesicles, proving oligonucleotide association. The diffusion constant for bound ODN is $5*10^{-12}$ $m^2/s$, similar to that of lipid vesicles ($4.5*10^{-12}$ $m^2/s$). The decreased particle number in the presence of positively charged vesicles indicates that more than one oligonucleotide is bound to a single vesicle or that vesicles aggregate upon oligonucleotide adsorption. The even lower count rate concurs with the fluorescence steady-state data. The decreasing fluorescence intensity indeed indicates ODN-FAM association with the lipid bilayer surface.

In summary, the data presented in this paper show that a 20 bp oligonucleotide labelled with fluorescein adsorbs onto the lipid surface in a manner that is dependent on positive charge concentration, i.e. 20 mol% DOTAP suffices for complete binding. The results obtained with steady-state fluorescence measurements were further confirmed by FCS measurements. Indeed, the observed decrease in fluorescein fluorescence intensity can be used as a measure of the extent of oligonucleotide adsorption onto the lipid surface.

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