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Evaluation of liposomal delivery of antisense oligonucleotide by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

Two widely used commercial cationic liposome formulations, Lipofectamine and Escort, were evaluated for drug delivery efficacy with capillary electrophoresis coupled with laser-induced fluorescence detection using a fluorescein conjugated 2′-*O*-methyl-phosphorothioate (Me-PS) antisense oligonucleotide and the HeLa cell line. Binding constants were estimated by monitoring changes in the electrophoretic mobility of the oligonucleotide with the liposome solution in the running buffer. From these changes in mobility, the binding constants for Lipofectamine and Escort liposomes with Me-PS oligomer were estimated to be 1139 and 590 M^{-1}, respectively. Additionally, intracellular concentrations and gene expression were quantified for the liposome formulations.
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1. Introduction

Capillary electrophoresis (CE) has been used for numerous biological applications [1–4]. Reasons for its popularity as an analytical separation technique include selectivity, sub-nanoliter (nl–pl) injection volumes, and fast analysis times. Coupled with laser-induced fluorescence detection (LIF) CE becomes extremely sensitive with limits of detection approaching the single molecule level [5]. For these reasons, CE–LIF has been used to analyze either naturally fluorescent or fluorescently labeled compounds at the cellular level [6–8].

One area of biological interest is the use of antisense drugs, single-stranded oligonucleotides targeted to a specific sequence of RNA, to combat various diseases including certain cancers and acquired immunodeficiency syndrome (AIDS) [9,10]. One of the main obstacles in realizing the potential of these drugs is effective and efficient delivery to specific cells. In vitro techniques for delivery have included electroporation, scrape-load and spontaneous uptake [11–13]. However, these methods are diagnostic tools and do not translate well in vivo. Cationic liposomes have shown some promise for in vivo use since they readily form a polyelectrolyte complex with anionic oligonucleotides and because the lipid bilayer carries a positive charge [14]. These features, combined with the positively charged lipids that compose the liposomes, lend them well to

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favorable electrostatic interactions with the negatively charged cell membrane [15].

A limited amount of research has been done investigating the cellular uptake of liposome–oligonucleotide complexes. Cell association was either determined by autoradiography using a radiolabeled oligomer or by confocal microscopy when the oligomer was fluorescently labeled [16,17]. The disadvantage of both techniques lies in the fact that they are semi-quantitative and give a limited amount of information. A quantitative analytical technique such as CE–LIF would give accurate determination of the intracellular concentration of fluorescently labeled oligomer following liposomal delivery.

Numerous commercially available liposome products exist but direct quantitative comparisons between different formulations for variance in uptake or gene expression has not been done. In the case of Lipofectamine, the liposome formulation consists of a polycationic lipid whereas the Escort liposome is composed of a lipid with a single cationic amine group. Both formulations contain a zwitterionic lipid giving the liposome additional stability in solution. Therefore it is useful to compare these two liposomes in order to estimate the effect of cationic lipid structure.

The goal of this research was to use CE–LIF to evaluate the efficiency of these liposome formulations based on binding constants for liposome–oligonucleotide interaction, intracellular concentration of the antisense and luciferase gene expression. CE–LIF was used to evaluate the binding between the liposome and 2′-*O*-methyl-phosphorothioate (Me-PS) in addition to monitoring intracellular concentration while varying oligonucleotide and liposome concentrations. Luciferase activity was measured in order to evaluate the gene expression. Additionally, a kinetic study of intracellular Me-PS and gene expression was done over the 6-h incubation period.

2. Experimental

2.1. Materials

Tris base, sodium hydroxide, Triton X-100 and sodium dodecyl sulfate (SDS) were from Aldrich (Milwaukee, WI, USA). EDTA was obtained from Fisher Scientific (Pittsburgh, PA, USA). Cell Scrub buffer was purchased from Gene Therapy Systems (San Diego, CA, USA) minimum essential medium (MEM), penicillin-G, phosphate-buffered saline (PBS) and fungizone were purchased from Gibco BRL (Gaithersburg, MD, USA). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). The 18-mer 2′-*O*-methyl-phosphorothioate oligoribonucleoside, of which the 5′-end was covalently labeled fluorescein, was custom-synthesized at Midland Certified Reagent (Midland, TX, USA) and used as received: the oligomer, which is referred to as Me-PS (Fig. 1), CCU–CUU–ACC–UCA–GUU–ACA, was targeted to a sequence in the intron 2 of human β-globin gene. HeLa cells stably transfected with the recombinant plasmid (pLuc/705) carrying the luciferase gene that is interrupted by a mutated β-globin intron 2 (IVS2-705) were described elsewhere [18]. The mutation in the intron causes aberrant splicing of the luciferase pre-mRNA, preventing translation of the luciferase. If the cells are treated with the Me-PS targeted to the aberrant splice site then splicing is corrected, restoring luciferase activity. Lipofectamine reagent {2:1 molar cationic 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)-ethyl]-*N*,*N*-dimethyl-1-propanamonium trifluoroacetate (DOSPA) and zwitterionic dioleoyl phosphatidylethanolamine (DOPE)} was purchased from Gibco BRL (Gaithersburg, MD, USA) and Escort reagent {2:1 molar cationic *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-triethylammonium chloride (DOTAP) and zwitterionic (DOPE)} was obtained from Sigma (St. Louis, MO, USA). Albumin standards and bicinchoninic acid (BCA) reagents for the protein assay were purchased from Pierce (Rockland, IL, USA), while the luciferase assay reagents were purchased from Boeringer Mannheim (Indianapolis, IN, USA). Tris-containing buffer solutions were prepared by dissolving Tris base in Milli-Q water and adjusting to pH 7.6 with 1 *M* HCl.

2.2. Cell culture and preparation

Stably transfected HeLa cells were cultured in 75-mm T-flasks in complete MEM, which contained 10% fetal bovine serum, 1% gentamicin solution and 1% penicillin-fungizone solution. The first passage cells were then aliquoted into cryogenic vials and

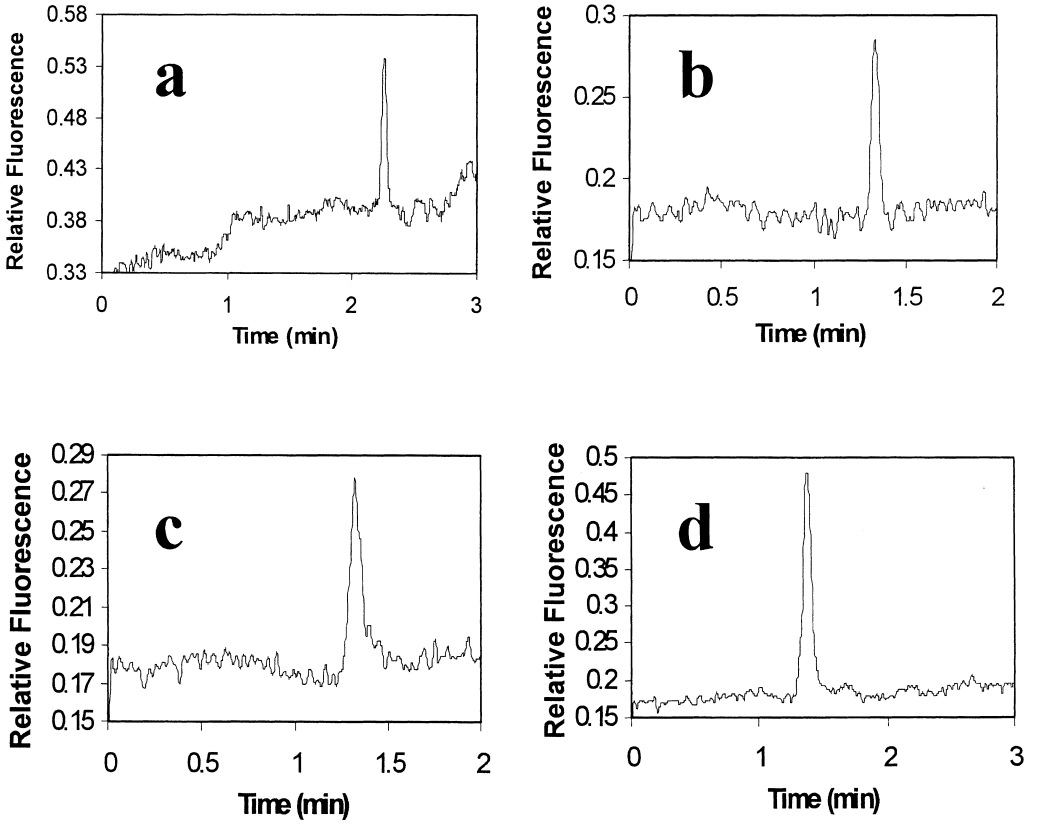

Fig. 1. Direct detection with reverse polarity following treatment of capillary with $1.34 \cdot 10^{-5}$ *M* Lipofectamine liposome solution of (a) acetone neutral marker, (b) Me-PS oligomer ($1.2 \cdot 10^{-8}$ *M*), (c) fluorescein isothiocyanate (FITC)-dextran ($2 \cdot 10^{-8}$ *M*) and (d) mixture of Me-PS and FITC-dextran. Buffer, 10 m*M* Tris–HCl (pH 7.6); voltage, −40 kV.

stored in liquid nitrogen for individual experiments at which time cells were thawed and grown using complete MEM in 35-mm multiwell dishes in a CO_2 cell culture incubator at 37 °C and 5% CO_2 until reaching 70–85% confluency. Liposome delivery experiments were done on cells in individual 35-mm wells.

2.3. Oligonucleotide delivery and analysis

All oligonucleotide–liposome solutions were prepared by adding appropriate volumes of oligonucleotide in 10 m*M* Tris–HCl buffer, pH 7.6 and liposome reagent to Opti-MEM for a volume of 250 μl. This solution was allowed to sit at room temperature for 10–15 min before adding 2.75 ml for a final volume of 3 ml. Confluent HeLa cells were prepared by removal of growth medium and washing with phosphate buffered saline. To these cells were then added 1-ml aliquots of the oligomer–liposome solution. The cells were returned to the incubator at 37 °C under CO_2.

Following a 6-h incubation period, the solution was removed and the cells were washed with Cell Scrub solution and 3×cold PBS. Lysing buffer, 100 μl (10 m*M* Tris–HCl, pH 8, 0.5 m*M* EDTA, 0.05% Triton X-100), was added and the cells were scraped into the buffer using a plastic scraper. The cell suspension was pipetted into a 1.5-ml centrifuge tube, placed on ice for 10 min and gently vortexed to disrupt the plasma membrane. It was then centrifuged at 500 *g*. The supernatant (cell extract) was transferred to a small centrifuge tube for further analysis.

CE–LIF analysis of the extract involved gravity injection for 5 s onto a capillary, which had been conditioned with Milli-Q water, methanol and 1 *M* sodium hydroxide. The running buffer for these experiments was 1 *M* Tris–HCl (pH 7.6), 15 m*M* SDS. Additionally, BCA protein assay and luciferase reporter gene assays were performed on the cell extracts as directed [19,20].

2.4. Luciferase gene expression assay

Luciferase gene expression was monitored by combining 20 μl of crude cell extract with 100 μl of luciferase assay reagent and measuring the relative light units produced on the Monolight 2010 luminometer over a period of 20 s.

2.5. Apparatus

The CE–LIF system consisted of an argon ion laser (Omni Chrome, CA, USA) modulated by a chopper controlled by a lock-in amplifier (EG&G, Trenton, NJ, USA). The 488 nm modulated laser line was focused onto the 50 μm I.D.×360 μm O.D. bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) through an upright microscope with a 40× fluorite objective which both sent the excited fluorescent beam and collected the emitted beam. The laser excitation beam would pass through a dichroic filter (495 nm) and the subsequent emission beam would pass through a long pass (500 nm) and short pass (515 nm) filters and the signal was picked up by the photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA). The signal was then sent to the A/D converter and the lock-in amplifier. All data acquisition and data analysis was performed using Labview software on a personal computer. A separate microscope stage was set up at the injection end where the capillary was held in place with a micromanipulator (not shown).

For all luminescence experiments a Monolight 2010 luminometer (Analytical Luminescence Lab., Spaarks, MD, USA) was used and for all spectroscopy experiments to determine protein concentration a Spectronic 20D spectrometer (Milton Roy, Ivyland, PA, USA). Spectrofluorometry analysis was done on a RF-5000 spectrofluorometer (Shimadzu, Kyoto, Japan).

3. Results and discussion

3.1. Mobility change analysis of the liposome–oligonucleotide complex

The interaction between the liposome and the oligonucleotide was investigated by monitoring changes in electrophoretic mobility of the oligomer in the presence of increasing concentrations of either Lipofectamine or Escort. In this case the liposome was added to the 10 m*M* Tris–HCl, pH 7.6 buffer solution in the untreated silica capillary and the fluorescently labeled Me-PS was injected and directly detected with LIF. The cationic liposomes modify the negatively charged fused-silica capillary wall. After a certain concentration, the capillary wall becomes positively charged and the direction of the electroosmotic flow (EOF) is reversed. It would then be necessary to reverse the polarity of the CE system power supply in order to detect the Me-PS at the anodic end.

In order to monitor reversal in EOF the polarity was kept normal (EOF from positive to negative electrodes) and varying Lipofectamine concentrations prepared from stock reagent diluted with the 10 m*M* Tris–HCl, pH 7.6 buffer were flushed through the conditioned capillary for 5 min and allowed to settle out for another 5 min. At this point the neutral acetone marker and the Me-PS were injected individually and their respective migration times were observed (Fig. 1). At concentrations of $3.9 \cdot 10^{-2}$ *M* Lipofectamine and Escort the EOF was reversed. It was found that this method of flushing the capillary with Lipofectamine or Escort was very reproducible and stable at low concentrations of Me-PS injected onto the system. The polarity for the CE system could then also be reversed allowing injection of the Me-PS oligomer from the same end of the capillary.

Changes in the migration of the antisense with concentration of the liposomes that were added to the background buffer solution were monitored. The electrophoretic mobility of the oligomer was calculated using Eq. (1):

$$\mu = \frac{L_t L_s}{V} \cdot \left(\frac{1}{t_R} - \frac{1}{t_{eo}} \right) \quad (1)$$

where L_t and L_s are the total and separation lengths of the capillary, respectively, t_R is the migration time of the oligomer, t_{eo} is the migration time of the neutral acetone marker and V is the applied voltage. The binding constants of the oligomer to the liposomes, K, were determined from the nonlinear regression of Eq. (2):

$$\mu = \frac{\mu^f + \mu^c K[L]}{1 + K[L]} \quad (2)$$

where μ^f and μ^c are the electrophoretic mobilities of free and complex forms at the liposome concentrations of 0 and ∞, respectively, and [L] is the cationic lipid concentration. The variations of the oligomer mobility with the cationic lipid concentration are summarized in Fig. 2. The binding constants, K, were determined to be 1139 and 570 M^{-1} for Lipofectamine and Escort, respectively.

3.2. Quantitation of delivered oligomer and gene expression with varying concentrations of liposome

To obtain a quantitative measure of antisense oligonucleotide uptake, cell extracts were prepared as described in the Experimental section and analyzed using CE–LIF for intracellular concentration of Me-PS. Gene expression was measured using a novel splicing modification assay [18]. In this assay, the 2′-*O*-methyloligoribonucleoside phosphorothioate targeted to the aberrant 5′ splice site in Luc-705 pre-mRNA was delivered to the HeLa Luc-705 cell line in a complex with the appropriate carrier. The assay measured the activity of the luciferase enzyme translated on the correctly spliced mRNA, which had been generated by the antisense action of the oligonucleotide.

Concentrations of Lipofectamine and Escort were varied keeping the concentration of Me-PS constant in order to evaluate a possible correlation of binding constant with uptake (Fig. 3). Intracellular concentration was highest with Lipofectamine for liposome concentrations up to $1 \cdot 10^{-5}$ *M*. This would correspond to the larger binding constant associated with the Lipofectamine liposomes. Neither Lipofectamine nor Escort shows efficient uptake at the lower

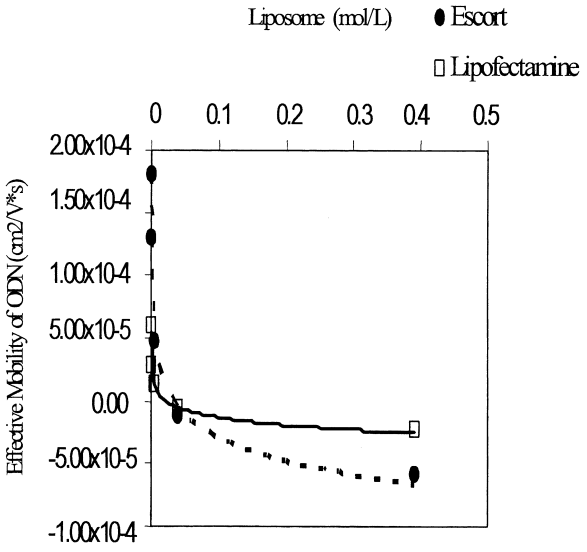

Fig. 2. Plot of calculated effective mobility versus concentration of Lipofectamine or Escort added to the background, running buffer. Concentration of Me-PS oligomer was $1.2 \cdot 10^{-8}$ *M* and the concentrations of Lipofectamine or Escort added to the running buffer were $3.9 \cdot 10^{-5}$, $3.9 \cdot 10^{-4}$, $3.9 \cdot 10^{-3}$, $3.9 \cdot 10^{-2}$ and 0.39 *M*.

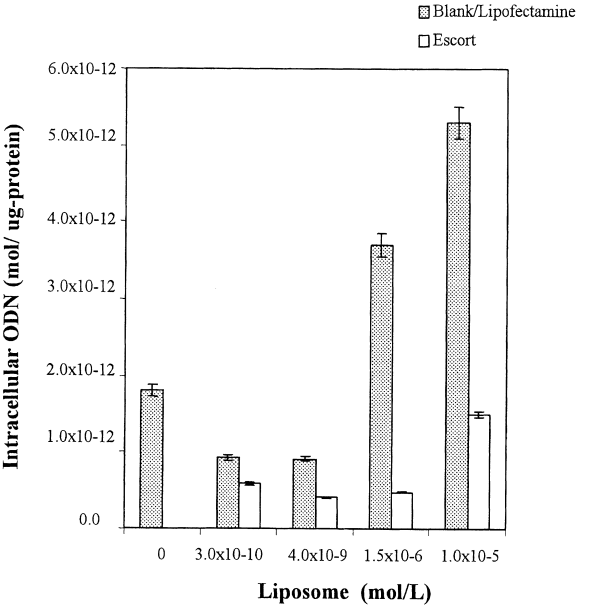

Fig. 3. Intracellular concentrations of Me-PS oligomer (mol Me-PS/μg protein) with varying concentrations of Lipofectamine and Escort ($3 \cdot 10^{-10}$, $4 \cdot 10^{-9}$, $1.5 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ *M*). Concentration of oligomer was relative to measured total cellular protein. Buffer, 10 m*M* Tris–HCl (pH 7.6), 15 m*M* SDS; −40 kV.

concentrations. There seems to be a critical liposome concentration, which is necessary for an increase in oligomer uptake into the HeLa cell. Additionally, cells treated with Me-PS without either liposome reagent spontaneously took up concentrations similar to those for the highest concentration of Escort. Monitoring luciferase activity showed that gene expression increased in a similar manner for both reagents with the exception of $1.48 \cdot 10^{-6}$ *M* (Fig. 4). It appears that lower intracellular concentrations of Me-PS using Escort does not necessarily correlate with lower gene expression, at least in the case of increased liposome concentration with constant concentration of oligomer. In terms of the blank Me-PS without liposome reagent, a cellular uptake concentration similar to Escort does not correlate with similar gene expression. This can be explained by the fact that spontaneous uptake through endocytosis traps the oligomer in discrete cytoplasmic vesicles and does not allow them to pass into the nucleus [21]. These results show quantitative evidence that liposome delivery at an optimized concentration is necessary for efficient gene expression. Additionally, there is not necessarily a direct correlation between cellular uptake of an oligomer and subsequent gene expression.

3.3. Quantitation of intracellular concentration and gene expression with varying concentrations of delivered oligomer

Oligonucleotide concentrations were then varied keeping the Lipofectamine and Escort concentrations constant. Intracellular concentration increased proportionately for Lipofectamine while the effectiveness of Escort liposome uptake was consistently lower (Fig. 5). Again this can be attributed to the larger binding constant of the Lipofectamine liposomes. However, as demonstrated with the previous results varying liposome concentration, higher uptake does not always mean increased gene expression. Results summarized in Fig. 6 confirmed this conclusion as shown by Escort gene expression over an order of magnitude higher than Lipofectamine. It has been shown that the antisense oligomer needs to be released from the bulky liposome complex before being allowed entrance into the small nuclear membrane pores. Since the Escort shows weaker binding to the oligomer this would explain why the Escort delivery generally gives less intracellular concen-

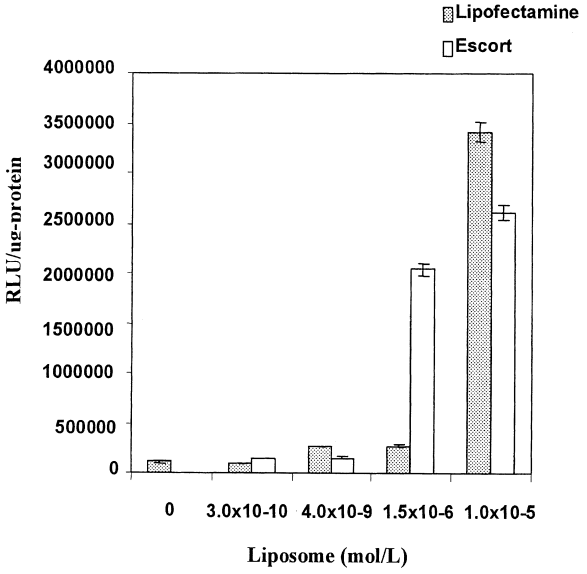

Fig. 4. Luciferase activity (gene expression) in RLU/μg protein with varying concentrations of Lipofectamine and Escort ($3 \cdot 10^{-10}$, $4 \cdot 10^{-9}$, $1.5 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ *M*). A 10-μl volume of cellular extract was added to 100 μl of luciferase reagent.

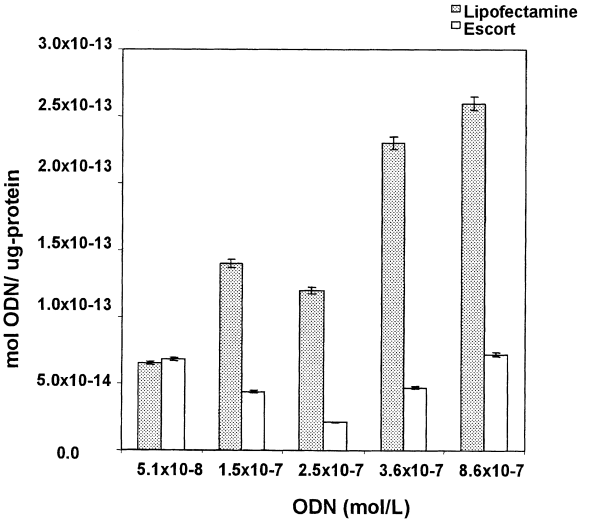

Fig. 5. Intracellular concentrations of Me-PS oligomer, with varying concentrations of Me-PS ($5.1 \cdot 10^{-8}$, $1.52 \cdot 10^{-7}$, $2.5 \cdot 10^{-7}$, $3.6 \cdot 10^{-7}$ and $8.6 \cdot 10^{-7}$ *M*), keeping the concentration of liposome reagent constant (0.025 μg/ml). ODN: oligonucleotide.

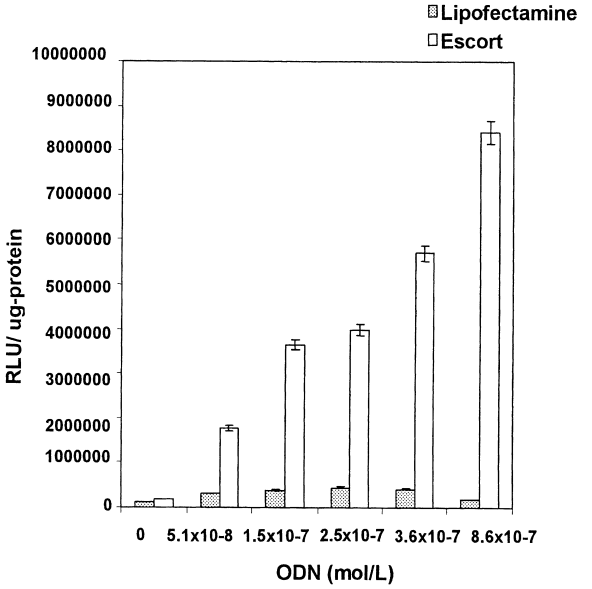

Fig. 6. Luciferase activity of extracts, with varying concentrations of Me-PS ($5.1\cdot10^{-8}$, $1.52\cdot10^{-7}$, $2.5\cdot10^{-7}$, $3.6\cdot10^{-7}$ and $8.6\cdot10^{-7}$ *M*), keeping liposome reagent concentration constant (0.025 μg/ml).

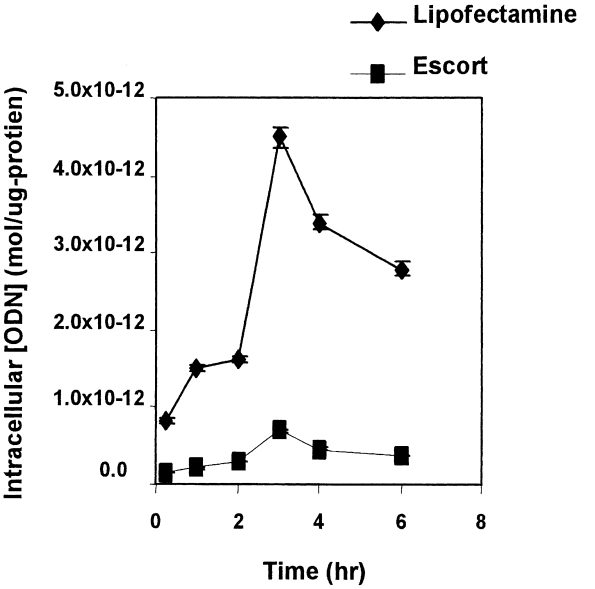

Fig. 7. Intracellular concentration of Me-PS oligomer over the course of a 6-h incubation period. Concentration of Me-PS, 0.3 μ*M* and concentration of Lipofectamine and Escort, 0.025 μg/ml.

tration but greater antisense activity. Additionally, the oligonucleotide and liposome concentrations used in these experiments were not arbitrary. In order to take into account a possible influence by liposome fusion, oligonucleotide concentrations were varied with a constant liposome concentration to give negative to positive ratios ranging from 0.2 to 3.5. Typically maximum liposome fusion occurs at a ratio of 1:1 (−/+) for previously studied liposome interaction with oligomers [22]. It is possible to attribute the distribution of gene expression for Lipofectamine in Fig. 7 to fusion effects where activity increases up to a 1:1 (−/+) ratio and then decreases at higher concentrations of oligomer.

3.4. Comparison of delivery kinetics between liposome formulations

An important aspect of studying the antisense oligonucleotide drug efficacy is the kinetics of delivery. For this reason HeLa cells treated with constant concentrations of Me-PS and liposome reagents were analyzed during the 6-h incubation period. Again, Lipofectamine delivery gave intracellular concentrations an order of magnitude higher than Escort (Fig. 8). Also apparent in these results is that for both liposome reagents cellular uptake

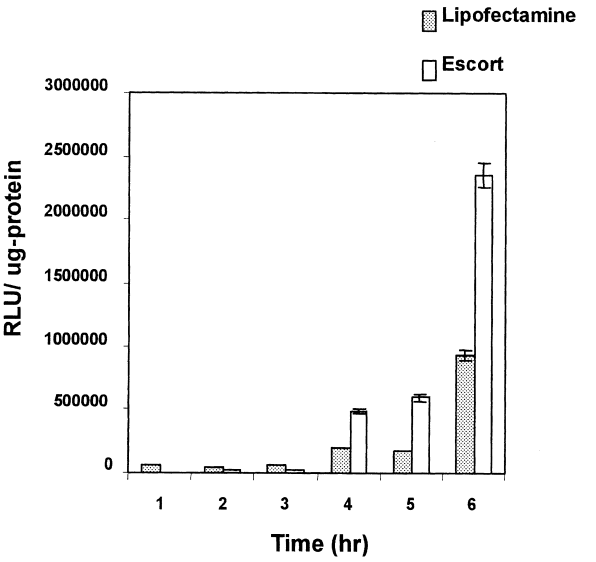

Fig. 8. Luciferase activity of extracts delivered with 0.3 μ*M* Me-PS and either Lipofectamine or Escort, 0.025 μg/μl over a 6-h incubation period.

reaches a maximum around 3 h and then steadily decreases for the remainder of the incubation time. The summary of gene expression for both Lipofectamine and Escort in Fig. 8 indicates that, although uptake may have peaked at 3 h, gene expression continues to increase up to the end. Again, Escort gives the higher gene expression even with much lower observed intracellular concentrations of Me-PS.

4. Conclusions

Affinity capillary electrophoresis (ACE) was applied to quantitatively evaluate the interaction between a 2′-*O*-methyl-phosphorothioate antisense oligonucleotide and two different liposome formulations. The binding constants were obtained easily using ACE and corresponding differences in mobility of the oligomer. Our results showed that ACE can be used as a powerful tool for investigating the interactions between oligonucleotides and liposomes. Additionally, intracellular concentrations of delivered antisense oligomer and the corresponding gene expression was evaluated for both commercially available liposome formulations, thereby studying any possible connections between strength of binding and drug efficacy at the cellular level.

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