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Encapsulation of DNA in negatively charged liposomes and inhibition of bacterial gene expression with fluid liposome-encapsulated antisense oligonucleotides

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Abstract

Antisense therapy for the treatment of bacterial infections is a very attractive alternative to overcome drug resistance problems. However, the penetration of antisense oligonucleotides into bacterial cells is a major huddle that has delayed research and application in this field. In the first part of this study, we defined efficient conditions to encapsulate plasmid DNA and antisense oligonucleotides in a fluid negatively charged liposome. Subsequently, we evaluated the potential of liposome-encapsulated antisense oligonucleotides to penetrate the bacterial outer membrane and to inhibit gene expression in bacteria. It was found that $48.9 \pm 12\%$ and $43.5 \pm 4\%$ of the purified plasmid DNA and antisense oligonucleotides were respectively encapsulated in the liposomes. Using fluorescence-activated cell sorting analysis, it was shown, after subtraction of the fluorescence values due to the aggregation phenomenon measured at 4°C, that about 57% of bacterial cells had integrated the encapsulated antisense oligonucleotides whereas values for free antisenses were negligible. The uptake of the encapsulated anti-*lacZ* antisense oligonucleotides resulted in a 42% reduction of β-galactosidase compared to 9% and 6% for the encapsulated mismatch antisense oligonucleotides and the free antisense oligonucleotides respectively. This work shows that it is possible to encapsulate relatively large quantities of negatively charged molecules in negative fluid liposomes and suggests that fluid liposomes could be used to deliver nucleic acids in bacteria to inhibit essential bacterial genes.

Keywords: Fluid anionic liposome; Antisense oligonucleotide; Phosphorothioate antisense; Bacterium; Gene inhibition; DNA encapsulation

1. Introduction

The increase of bacterial resistance to conventional antibiotics has become a major problem giving rise to an urgent need for new approaches to treat bacterial infections. In cystic fibrosis patients, pathogens like *Pseudomonas aeruginosa* show high levels of resistance and lead to progressive lung deterioration and premature death [1–4]. To overcome antibiotic resistance, fluid liposomes named Fluidosomes, a negatively charged liposomal formulation without cholesterol and made of a combination of dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylglycerol (DMPG) phospholipids (at a molar ratio from 5:1 to 18:1) were developed. These liposomes with an overall low gel–liquid crystalline

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transition temperature (T_C) ≤37°C [5] were conceived as a delivery vehicle to enhance drug penetration through bacterial membranes [6,7]. These fluid liposome-encapsulated antibiotics succeed in eradicating *P. aeruginosa* in an animal model of chronic pulmonary infection [6]. Recently, the superior efficiency of fluid liposome-encapsulated aminoglycoside over rigid liposomes in an animal model of bacterial infection was supported by an independent group [8]. The enhanced bactericidal activity of fluid liposomes would result from a mechanism of fusion between the liposomes and the bacterial membranes as demonstrated by our group [9]. Previous studies analyzing the innocuousness of fluid liposomes have demonstrated that they do not induce an immune response following repeated intraperitoneal and intratracheal administrations to mice [10] and cannot fuse with human lung epithelial cells [11].

Antisenses can regulate gene expression by binding to a complementary mRNA strand by conventional Watson–Crick hydrogen bonds, thereby providing specific targeting to the mRNA [12]. Because antisense oligonucleotides are highly susceptible to intracellular endonucleases, considerable efforts have been made to develop chemical modifications to improve their stability. Phosphorothioate oligonucleotides are the most commonly used and widely commercially available chemically modified oligonucleotides, in which the non-bridging oxygen atoms are replaced by sulfur in the DNA backbone of the molecule. This modification increases resistance to nuclease degradation [13]. Selective inhibition of bacterial genes to kill bacteria or to alter their resistance offers many advantages over conventional chemotherapies. Antisense oligonucleotides can be specifically designed to target only bacterial sequences thus not affecting eukaryotic cells. Also, there are no known mechanisms by which bacteria could expulse antisense oligonucleotides outside their cytoplasm like they do with common antibiotics using efflux pumps [14–16]. Moreover, a recent study demonstrated that the activities of drug efflux pumps do not affect antisense nucleic acid susceptibility [17].

In this study, we first tested different types of solutions (NaCl, KCl, $NaHCO_3$, $MgCl_2$, $CaCl_2$ and phosphate-buffered saline (PBS)) for the encapsulation of plasmid DNA and antisense oligonucleotides in negatively charged Fluidosomes. The second part of this study consisted in the analysis of the uptake of the encapsulated antisense oligonucleotides by *Escherichia coli* CSH36 expressing a normal outer membrane which was evaluated by fluorescence-activated cell sorting (FACS). The functional transfer of DNA in an *E. coli* strain which constitutively expresses the *lacZ* (β-galactosidase) gene due to a mutation in the regulatory *lacI* gene, was assessed by the reduction of β-galactosidase activity after the incubation of *E. coli* CSH36 with the encapsulated phosphorothioate anti-*lacZ* antisense oligonucleotide.

2. Materials and methods

2.1. Bacterial strain and antisenses

The pUC 18 plasmid (New England BioLabs, Beverly, MA, USA) was used for the study of DNA encapsulation. *E. coli* CSH36 (alternate name E7074), a laboratory strain which constitutively expresses the *lacZ* gene due to a mutation in the regulatory *lacI* gene, was used [18]. Bacteria were grown in LB broth (Gibco BRL, Life Technologies, ON, Canada). An anti-β-galactosidase antisense oligonucleotide was designed to target the start codon region of the *E. coli* β-galactosidase gene: 5′-GGT CAT AGC TGT TTC-3′ [19]. A mismatch antisense oligonucleotide was also designed and used as a negative control: 5′-TAC TGT GAT CTG TGC-3′. The antisense oligonucleotides used in the present study had a phosphorothioate backbone. This modification was obtained by a substitution of a non-bridging oxygen for a sulfur in the oligonucleotide backbone in order to render the antisenses more stable and less subject to degradation by endonucleases [19]. These phosphorothioate antisense oligonucleotides were ordered from Alpha DNA (Montreal, QC, Canada). After salt removal, the lyophilized oligonucleotides were rehydrated with PBS 1X. FITC-labeled antisense oligonucleotides used in uptake measurements were obtained from Synthegen (Houston, TX, USA). Unless otherwise indicated, encapsulated oligonucleotide antisenses were added at a concentration of 10 μM.

2.2. Fluid liposomes

Fluid liposomes were prepared from synthetic

phospholipids DPPC and DMPG (Avanti Polar Lipids, Alabaster, AL, USA) at a molar ratio of 10:1. Liposomes were prepared by a dehydration–rehydration vesicle (DRV) method as previously described [6,9]. Briefly, appropriate amounts of both phospholipids were dissolved in chloroform in a round-bottomed flask and dried to a lipid film by rotoevaporation (Büchi Rotavapor) at 65°C under vacuum (Büchi 168-Vacuum/Distillation Controller). The lipids were then redissolved in PBS 1:20 and transferred to screw neck diagnostic bottles with thin flange lyophilization stoppers designed for lyophilization and hypodermic applications (Wheaton, NJ, USA). The liposomes were then freeze-dried (Virtis Genesis 12 EL Console freeze dryer, Canberra Parkard, Toronto, ON, Canada) at 4°C. At the end of the lyophilization process, the bottles were mechanically closed under vacuum. Finally, the stoppers were sealed with aluminum caps and the bottles were kept at −70°C until use.

2.3. Encapsulation and quantification of the enclosed DNA

The pUC 18 plasmid was purified using the Concert Plasmid Maxiprep System (Life Technologies, Gibco BRL, USA) and quantified by OD_{260} using an UV-visible spectrophotometer (Milton Roy Spectronic 1001 plus, USA). To encapsulate DNA, a volume of 0,5 ml of purified plasmid DNA (0.2 mg/ml of encapsulation solution) or of antisense oligonucleotides (7.4 mg/ml of PBS) was injected into the diagnostic bottles containing the lyophilized phospholipids (15 mg of phospholipids) which were kept under vacuum during the procedure. The hydrated phospholipids were vortexed until the powder was completely dissolved in the DNA solution. The bottles, still under vacuum, were incubated at 65°C with agitation (100 rpm) for 60 min. Vortexing was performed every 10 min. After the incubation period, the liposome–DNA solution was diluted in 9.5 ml of encapsulation solutions. These solutions contained either NaCl (50 mM and 150 mM), KCl (50 mM), $NaHCO_3$ (50 mM), $CaCl_2$ (50 mM), or $MgCl_2$ (50 mM) prepared in sterile distilled water and PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, K_2HPO_4 1.8 mM). The diluted liposomes were centrifuged at $6000 \times g$ for 40 min in order to remove the DNA present in the supernatant. Plasmid DNA (250 μg) was resuspended in 1000 μl of encapsulation solution. Each encapsulation solution was tested at least three times. The liposome diameters were heterogenous: about 63% of the size distribution measurements of liposomes was shifted towards the diameter range comprised between 316.2 and 562.3 nm whereas about 46% consisted in large heterogeneous vesicles as determined by an electronic particle size analyzer (Coulters Electronics, Miami, FL, USA).

The antisense oligonucleotides encapsulated in liposomes were quantified using Oligreen (Molecular Probes, Eugene, OR, USA) as described by the manufacturer, and a Perkin-Elmer LS-50B fluorescence spectrophotometer (excitation at 490 nm, emission at 520 nm). Before performing the cytometry studies, a sample of the FITC-labeled antisense oligonucleotides encapsulated in liposomes was submitted to DNA extraction for quantification using a methanol–chloroform (1:2) solution and a Perkin-Elmer LS-50B fluorescence spectrophotometer (excitation at 490 nm, emission at 520 nm).

2.4. Monitoring DNA localization

Two methods were used to confirm that DNA was present inside and not outside of the liposomes. The first method consisted in placing DNA-encapsulated liposomes and controls in an agarose gel (0.4%) containing ethidium bromide (0.5 μg/ml). The gel was submitted to an electric current of 7 V/cm to obtain free DNA migration. The second method to ascertain that the measured DNA was contained inside the liposomes consisted in treating the preparation with DNase in order to eliminate the DNA present outside of the liposomes. The DNase treatment for 15 mg of phospholipids rehydrated with 250 μg of DNA consisted in 250 DNase units, 5 mM $MgCl_2$, 0.1 M sodium acetate and lasted for 3 h at room temperature. The reaction was stopped with 20 mM EDTA. After the DNase treatment, liposomes were dissolved and separated from DNA as described below. The DNA was quantified and compared to untreated liposome-encapsulated DNA. The control for DNase activity consisted in adding an equivalent amount of DNA to empty liposomes (externally),

followed by treatment with DNase and quantification of the resulting DNA. All DNA quantifications in gels were determined visually.

2.5. UV absorbance measurements of DNA

To evaluate the efficiency of DNA encapsulation with the different encapsulation solutions, UV absorbance measurements were performed after liposomes were dissolved in a methanol–chloroform (1:2) solution. The lipids were present in the organic phase whereas DNA was found in the aqueous phase. The aqueous phase containing plasmid DNA and antisense oligonucleotides was diluted 1/100 in the different encapsulation solutions and analyzed on an Milton Roy Spectronic 1001 plus spectrophotometer. Samples were placed in quartz cuvettes and scanned at A_{260}. Standard curves were drawn in the different encapsulation solutions with concentrations of DNA varying from 0.001 mg/ml to 0.5 mg/ml.

2.6. Delivery of the encapsulated antisense oligonucleotides in E. coli

Bacteria were grown overnight in LB broth (Gibco BRL, Life Technologies, ON, Canada). From this culture, 300 μl were added to 10 ml of fresh LB broth. The culture was grown to mid-logarithmic phase then diluted to an optical density (OD) of 0.05 at 600 nm. Six tubes containing the same volume of this bacterial culture were prepared and different concentrations of free FITC-labeled antisense oligonucleotides, encapsulated FITC-labeled antisense oligonucleotides and PBS-containing liposomes were added. Bacteria were incubated for 4 h at 37°C with vigorous shaking. Samples from each tube were placed on the surface of a 24% sucrose (Sigma, ON, Canada) cushion and centrifuged at 4°C for 20 min (6000 rpm) in an Eppendorf Centrifuge 5402. Bacteria were washed once with LB broth, then placed on another sucrose cushion and centrifuged. The supernatant was discarded and the final cell pellet was fixed with 2% paraformaldehyde (Sigma) diluted in PBS. The solutions were kept at 4°C overnight. Bacteria were analyzed by means of a FACS Calibur flow cytometer (Becton Dickinson). Forward angle (FSC) and 90° light (side) scattering (SSC) measurements as well as green (FL1) fluorescence were collected, and 10 000 events were counted for each sample. The purity of the bacterial population sorted after electronic gating was closed to 100%.

2.7. Determination of β-galactosidase activity

Bacteria were prepared as described above. Free antisense oligonucleotides, encapsulated antisense oligonucleotides, encapsulated mismatch antisense oligonucleotides, free mismatch oligonucleotides and encapsulated PBS were added to bacteria and incubated for 4 h at 37°C with agitation. In this assay, a single data point of 4 h was selected based on the results obtained following the time course of the FACS analysis. Samples from each tube were placed on the surface of a 24% sucrose cushion and centrifuged for 20 min at 6000 rpm, 4°C. Supernatants were discarded and bacteria were resuspended in Z buffer (8.52 g Na_2HPO_4, 5.5 g $NaH_2PO_4{\cdot}H2O$, 0.75 g KCl, 0.246 g $MgSO_4{\cdot}7H_2O$, 2.7 ml β-mercaptoethanol per liter; pH 7). β-Galactosidase concentration was measured using the substrate *o*-nitrophenyl-β-galactoside (Sigma) as previously described [18] with minor modifications: after the incubation period with the substrate, the bacteria were treated with 20 μl of chloroform (EM Science, Gibbstown, NJ, USA) and 20 μl of 0.1% SDS (Bio-Rad, Hercules, CA, USA). The solution was vortexed for 10 s and placed at 30°C for 5 min. The absorbance was measured at 420 nm using a spectrophotometer (Milton Roy Spectronic 1001 Plus).

3. Results

3.1. DNA encapsulation with the different encapsulation solutions

The two encapsulation solutions containing $CaCl_2$ (50 mM, pH 7.0) or $MgCl_2$ (50 mM, pH 7.0) caused the DNA and liposomes to precipitate thus allowing visualization by ethidium bromide staining in the agarose gel where the DNA was visible in the wells at the end of the migration period (Fig. 1b, lanes 1, 4, 7, 10). Also, a precipitate was visible when the solution was observed in the tube. The encapsulation solutions composed of salts containing monovalent cations, either NaCl (50 mM, and 150 mM,

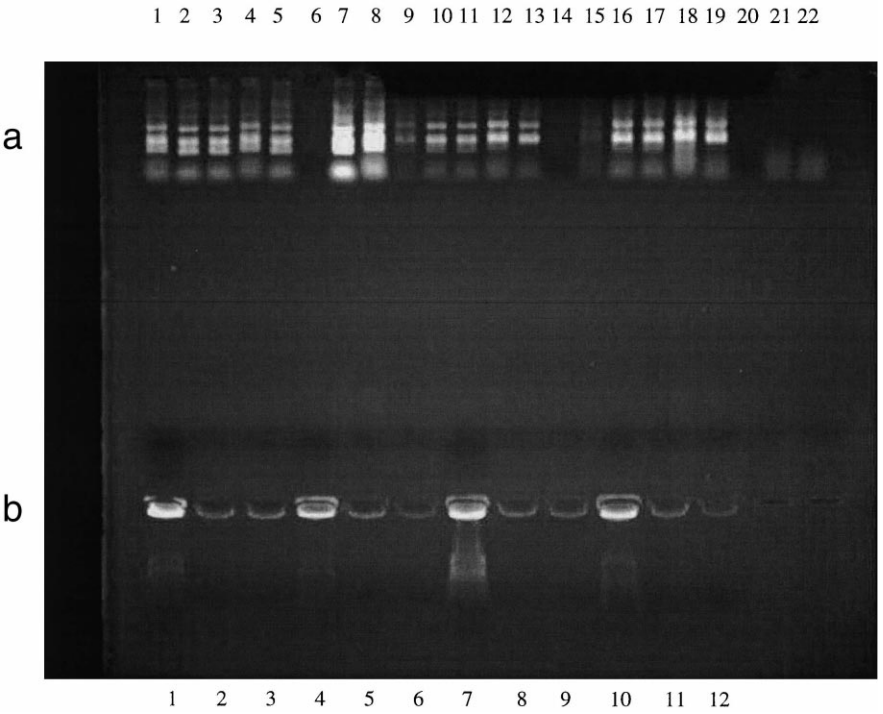

Fig. 1. (a, upper gel) Evaluation of DNA quantity before and after treatment of liposomes with DNase. Initial preparations of DNA in the different encapsulation solutions (no liposomes) (lanes 1–5), DNA after dissolving liposomes with methanol–chloroform (1:2) solution (lanes 15–19) and after DNase treatment of liposomes (external DNA) followed by dissolving liposomes (lanes 9–13). Encapsulation solutions: $CaCl_2$ (lanes 1, 9, 15), NaCl (lanes 2, 10, 16), KCl (lanes 3, 11, 17), $MgCl_2$ (lanes 4, 12, 18), and $NaHCO_3$ (lanes 5, 13, 19). NaCl solution controls without DNA (lanes 6, 14, 20); DNA controls in $CaCl_2$ and in NaCl without DNase treatment (lanes 7 and 8 respectively) and after DNase treatment (lanes 21 and 22 respectively). Electrophoresis was performed in a 0.4% agarose gel. (b, lower gel) Demonstration of the absence of migration with whole liposomes without DNase treatment (lanes 1–5); after DNase treatment (7–11). Control NaCl solution without DNA (lanes 6, 12). Encapsulation solutions: $CaCl_2$ (lanes 1, 7), NaCl (lanes 2, 8), KCl (lanes 3, 9), $MgCl_2$ (lanes 4, 10), and $NaHCO_3$ (lanes 5, 11).

pH 7.0), KCl (50 mM, pH 7.0) or $NaHCO_3$ (50 mM, pH 7.0) and PBS were efficient in promoting the encapsulation of DNA in liposomes. At first, when these liposomes were placed in agarose gels, encapsulated DNA could not migrate, nor could it be detected in the wells (Fig. 1b, lanes 2, 3, 5, 6, 8, 9). However, following the lysis of these liposomes, migrating DNA could be observed (Fig. 1a, lanes 10, 11, 13, 16, 17, 19). The quantities of DNA detected in the gels after DNase treatment (lanes 10, 11, 13) were approximately the same and corresponded to about 50% of the DNA signal given by the initial DNA preparation dissolved in the encapsulation solution before encapsulation (Fig. 1a, lanes 2, 3, 5). Negligible differences in DNA quantities were observed between untreated (lanes 16, 17, 19) and DNase-treated liposome-encapsulated DNA (lanes 10, 11, 13).

The UV absorbance measurements of extracted DNA from eight independent preparations of liposome-encapsulated plasmid DNA and three independent preparations of encapsulated antisense oligonucleotides in PBS gave a mean encapsulation of $48.87 \pm 11.65\%$ and $43.53 \pm 4.32\%$ respectively.

3.2. *Delivery of the encapsulated antisense oligonucleotides in bacteria*

FACS analysis was performed at 37°C to determine if encapsulated antisense oligonucleotides could efficiently be delivered to bacteria. To assure that liposomes do not sediment at all during centrifugation through the sucrose cushion, a preliminary test was performed that consisted in adding only liposomes on the surface of the sucrose cushion. The cytometry analysis of the lower part (no pellet was

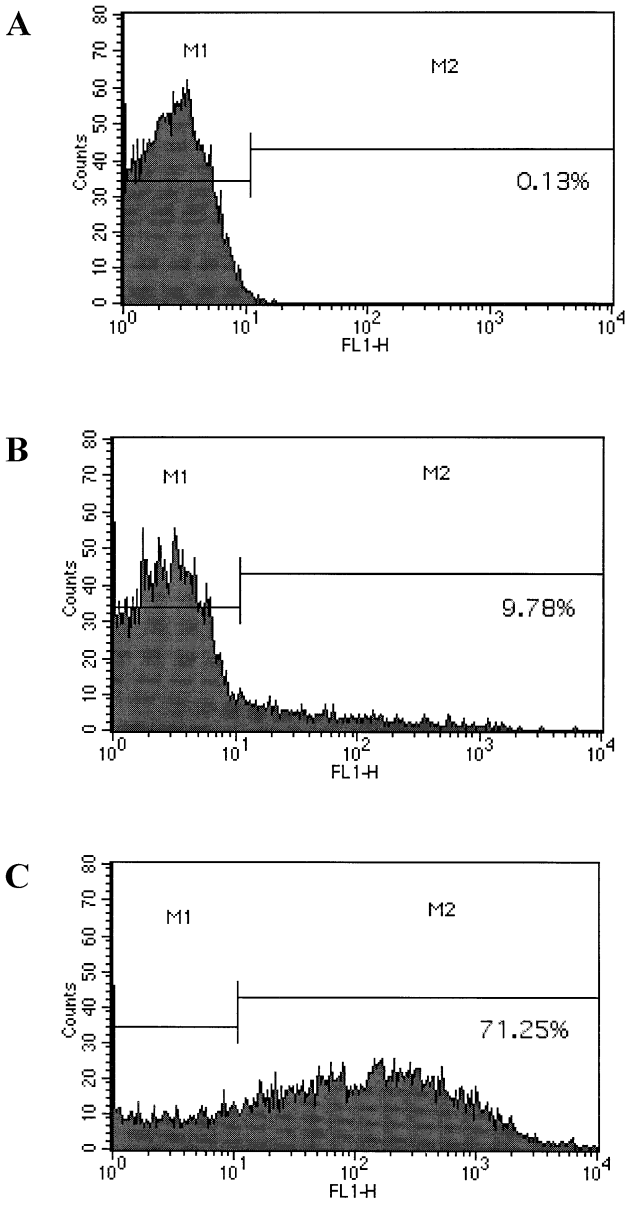

Fig. 2. Flow cytometry histograms of *E. coli* CSH36 after a 4 h incubation period at 37°C. (A) Control bacteria; (B) bacteria with free FITC-labeled antisense oligonucleotides; (C) bacteria with encapsulated FITC-labeled antisense oligonucleotides. The *x*-axis represents mean fluorescence intensity and the *y*-axis bacterial counts.

visible) of the gradient showed a total absence of liposomes in this fraction. Other controls indicated that bacteria did not emit intrinsic fluorescence (Fig. 2A) and no fluorescence was detected with fluid liposomes containing PBS used as negative control (data not shown). Bacteria treated with free antisense oligonucleotides showed very low levels of fluorescence (9.78%) (Fig. 2B). After a 4 h incubation period of the bacteria with encapsulated FITC-antisense oligonucleotides, 71.25% of bacteria showed FITC-fluorescence (Fig. 2C). To evaluate the extent of the aggregation phenomenon of liposomes and antisenses on the outer bacterial membrane versus the penetration of antisense in bacteria by liposome fusion, a kinetic study was performed at 4°C and 37°C. The results indicate that at time zero, fluorescence values were the same at 4°C and 37°C; after 4 h of incubation at 37°C, the bacterial fluorescence due to the liposome-encapsulated oligonucleotide transfer strongly increased with time (Fig. 3). After subtraction of values obtained at time zero, the fluorescence that may be caused by agglutination (observed at 4°C) counted for 17% and 20% after 120 and 240 min respectively for the encapsulated antisenses compared to 43% and 100% after 120 and 240 min respectively for the free antisenses (Fig. 3). This means that at the end of the experiment, aggregation only was observed between the free antisenses and the bacterial cells. On the other hand, 80% of the fluorescence observed with the encapsulated antisenses corresponded to a true antisense internalization which corresponded to 57%.

3.3. Determination of the β-galactosidase activity

The mismatch antisense oligonucleotide used as negative control contained the same amount of T, A, G and C deoxyribonucleotides as the anti-*lacZ* antisense oligonucleotide but in a different order. The addition of 25 μM of the encapsulated anti-*lacZ* antisense oligonucleotide to *E. coli*, with an incubation period of 4 h at 37°C, caused a significant reduction of β-galactosidase of 42% compared to 9% and 6% for the encapsulated mismatch antisense oligonucleotide and the free antisense oligonucleotide respectively (Fig. 4). This result demonstrates that the encapsulated antisenses not only enter the bacterial cells, but interact with the target mRNA. In the second β-galactosidase assay, a dose-dependent activity using 5, 10, and 25 μM encapsulated antisense oligonucleotides causes the inhibition of the β-galactosidase whereas a plateau was reached with 50 μM.

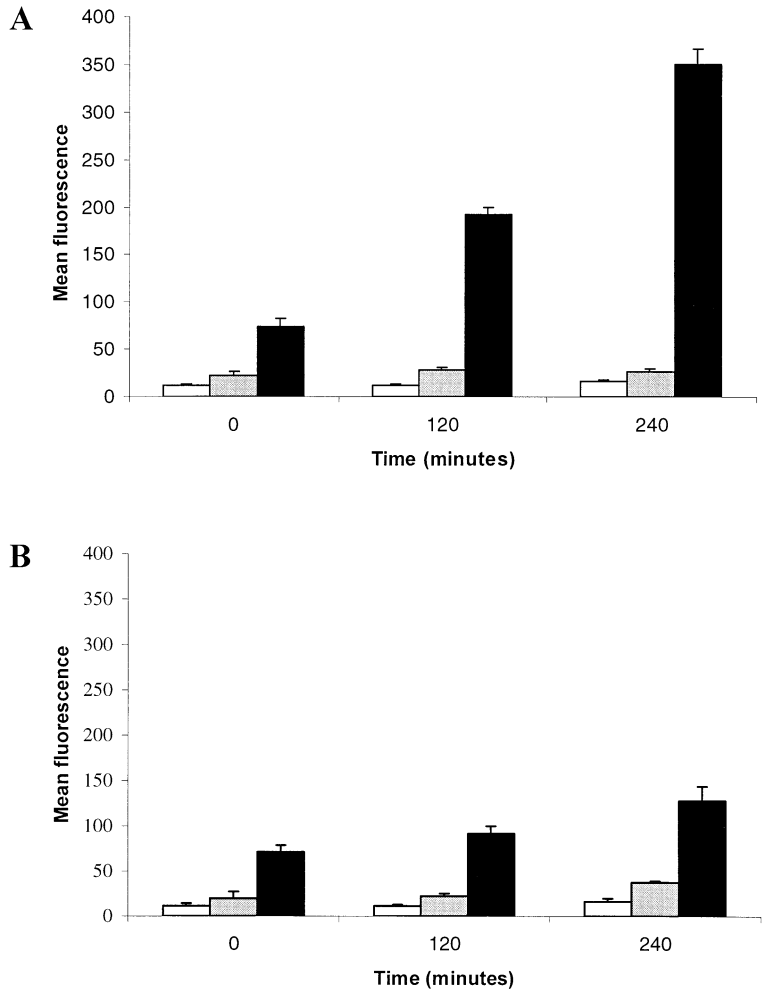

Fig. 3. Kinetics of antisense uptake in bacteria measured by FACS analysis as described in the text. □, control bacteria; ▨, free antisense oligonucleotides; ■, encapsulated antisense oligonucleotides. (A) Experiment conducted at 37°C; (B) experiment conducted at 4°C.

Gradual inhibitions of β-galactosidase activity of 4%, 26% and 42% were observed with the encapsulated antisense at 5, 10 and 25 μM respectively compared to ≅2–6% with free antisense and 0–9% with the encapsulated mismatch antisense (Fig. 5). The inhibition of the β-galactosidase gene expression was dose-dependent (Fig. 5) and was inhibited in a significant manner only with the encapsulated anti-*lacZ* antisense oligonucleotide (Fig. 5). Free mismatch antisense oligonucleotide showed no gene inhibition (data not shown). Finally, our results show that the phosphorothioate antisense oligonucleotides were not degraded during the 4 h period at 37°C since their activity was maintained over that period of time.

4. Discussion

Multiple modes of DNA delivery have been developed for gene therapy [20]. Among the problems associated with DNA transfer in cells are the stability of the carrier DNA complex, toxicity and the efficient delivery to and expression of the desired DNA in the cytoplasm. The use of viral particles, a very efficient method for gene delivery, involves potential risks of infection, immune reaction and may both slow the cell cycle and induce apoptosis [21,22]. Although cationic liposome–DNA complexes present advantages over viral vectors, they have also major limitations due to aggregation behavior [23–25], significant toxicity [26–29] and low transgene expression

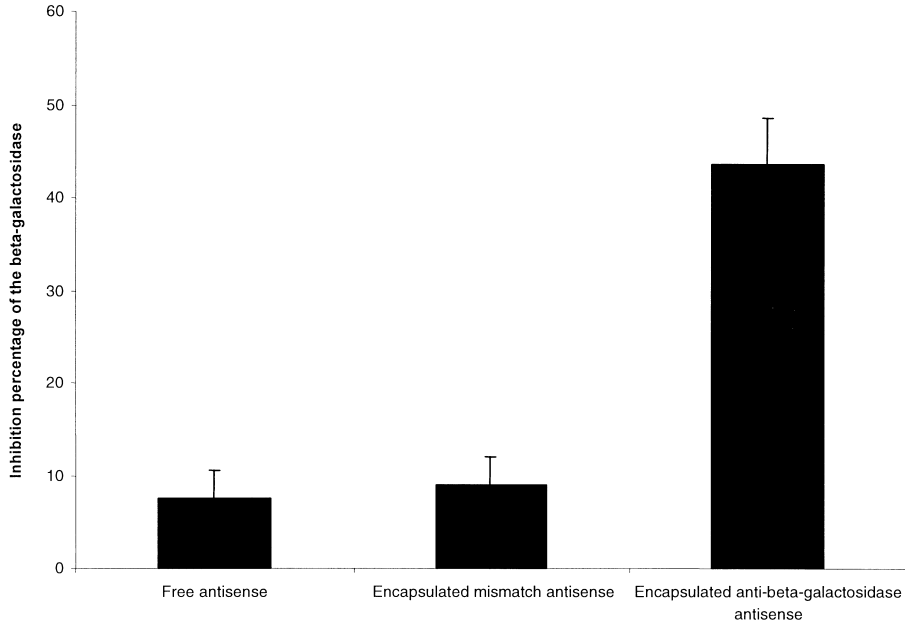

Fig. 4. Effect of the encapsulated anti-*lacZ* antisense oligonucleotides on the β-galactosidase expression in *E. coli* CSH38 after a 4 h incubation period at 37°C with 25 μM of antisense oligonucleotides.

[30,31]. Moreover, the problem of toxicity is a complex one since it would be independent of the cationic lipid:DNA ratio, the cationic lipid species and the level of transgene expression attained [27]. It has been suggested that the mechanism of toxicity of cationic lipids would depend on the alterations of the net charge of cell membranes and adverse effects on the activity of ion channels, membrane receptors, and enzymes [32]. Another problem met with cationic liposome-mediated transfection is the low release

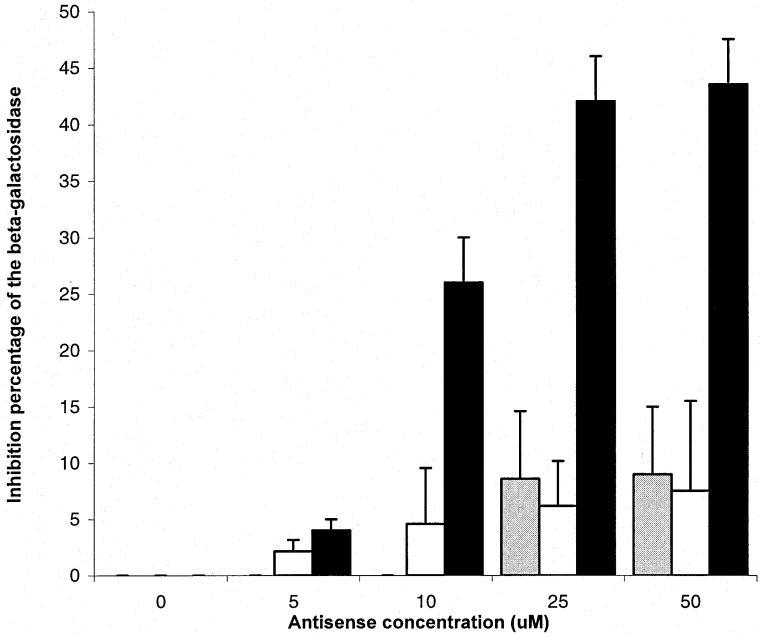

Fig. 5. Dose-dependent (μM) inhibition of the β-galactosidase gene in *E. coli* CSH38 after a 4 h incubation period at 37°C. □, free antisense oligonucleotides; ▨, encapsulated mismatch antisense oligonucleotides; ■, encapsulated anti-*lacZ* antisense oligonucleotides (data for free mismatch antisense oligonucleotides are not shown because all values were negative).

of plasmid DNA from the liposome–DNA complex for efficient gene expression [33,34].

In the present work, entrapment of DNA in anionic DPPC/DMPG liposomes prepared with the DRV method using encapsulation solutions made of monovalent salts was significant both with plasmid DNA (48.3%) and antisense oligonucleotides (43.5%). Similar plasmid DNA entrapment values were reported with anionic PC:CHOL:PG liposomes [35]. It also appears that in solutions made with monovalent salts, the DNA quantified was practically only within the liposome considering that: (i) following its encapsulation, the DNA was protected from DNase degradation as demonstrated by the fact that approximately the same amount of DNA was detected in untreated and treated liposome-encapsulated DNA; (ii) free DNA could not be observed in agarose gel following the DNase treatment; (iii) encapsulated DNA could not be observed with ethidium bromide in agarose gel; (iv) DNA could be observed in agarose gel after lysis of the DNA-encapsulated liposomes. It seems that, in contrast to DNA–liposome complexes where DNA is external, internalization of DNA would be favored in the multilamellar liposomes generated by the DRV process [35]. Moreover, in our hands, the addition of DNA preparation to the lyophilized liposomes under vacuum conditions during the whole process of rehydration contributes to the efficiency of the encapsulation procedure.

Liposomes are versatile drug carriers which can be associated with both hydrophilic and hydrophobic molecules. The finding that a plasmid DNA–cationic liposome complex microinjected directly into the nucleus showed low transfection efficiency supports the hypothesis that the release of plasmid DNA from the complex is necessary for efficient gene expression in liposome-mediated transfection [34]. Xu and coworkers demonstrated that the anionic liposomes with a composition that mimics the cytoplasmic-facing monolayer of the plasma membrane rapidly released plasmid DNA from the complex [36]. In the absence of calcium ions, DNA molecules have no effect on negatively charged liposomes because electrostatic repulsion prevents its interaction with anionic multilamellar vesicles [37]. Consequently, the use of anionic liposomes could help the release into cytoplasm of plasmid DNA from the liposome–DNA complex, a required step to the subsequent gene expression efficiency.

FACS analysis clearly shows that bacterial uptake of encapsulated antisense oligonucleotides is higher than the uptake of free antisense oligonucleotides. The difference in number of fluorescent bacteria between the two preparations is very significant since it reached 71.25% with the encapsulated antisense oligonucleotides compared to 9.78% for the free ones (Fig. 2). By running the same experiment at 4°C and 37°C (Fig. 3), the values of fluorescence corresponding to aggregation (values obtained at 4°C) could be excluded. The results indicate that after an incubation period of 4 h at 37°C with the encapsulated antisense, the true value of the fluorescent antisense in the bacterial cells was 57% instead of 71%. Moreover, the same operation performed on the fluorescence values due to free antisense indicates that the aggregation phenomenon represents the whole value of the observed fluorescence at the end of the experiment.

To demonstrate the activity of the anti-*lacZ* antisense oligonucleotides after their internalization in the bacterial cells, the inhibition of β-galactosidase production by *E. coli* CSH36, a bacterial strain that constitutively produces β-galactosidase, was measured. This strain was selected to avoid the addition of isopropyl β-D-thiogalactoside (IPTG) to the preparation since the latter could have been trapped in the fluid liposomes. In this case, IPTG would have been inaccessible to the surrounding bacteria and could have interfered with the normal induction of the targeted gene. To avoid the degradation of the antisense oligonucleotides by endonucleases, the antisenses used in the present study had a phosphorothioate backbone obtained by a substitution of a non-bridging oxygen by a sulfur in the nucleotide backbone. The stability of the antisenses used is well demonstrated by the results obtained after an incubation period of 4 h (Fig. 3). A control using a non-modified antisense showed no gene inhibition (data not shown). The β-galactosidase inhibition obtained with the encapsulated anti-*lacZ* antisense oligonucleotides was concentration-dependent and reached a maximum level of 45% (Figs. 4 and 5). This limit is close to the percentage of bacterial cells (57%) which apparently show a true internalization of the antisense oligonucleotides from the liposomes.

The limit can also be related to the non-toxic effect of the antisense for the bacteria. Thus, after the addition of the liposome-encapsulated anti-*lacZ* antisense oligonucleotides that combined with the bacteria, the sustained growing of new bacterial cells would continue to produce β-galactosidase during the 4 h incubation period. At concentrations of 25 and 50 μM, the encapsulated mismatch antisense oligonucleotides showed an inhibition of about 8%. This low level of inhibition could be attributed to a non-specific steric hindrance after the penetration of the mismatch antisense into the bacterial cells. Some may argue that the diameter range of the majority of the liposomes that shifts towards 316–562 nm seems too large to allow fusion with bacteria of 1–5 μm dimension. The fusion mechanism of the fluid liposomes with bacteria does not necessarily implicate the penetration of the whole liposome into the bacteria. It is possible that a partial fusion between bacterial and liposomal membranes occurs, allowing the penetration into the bacterial cytoplasm of liposome-containing molecules. The well known conjugation process that exports effector molecules into other cells demonstrates that such a transfer is possible. This hypothesis is not inconsistent with our previous work on the mechanism of fusion [9] and with the present results.

In conclusion, we showed that fluid anionic DRV liposomes can encapsulate, under vacuum condition, significant amounts of plasmid and oligonucleotide DNA using monovalent salt solutions. It is also demonstrated that DNA is entrapped within the multilamellar vesicles rather than being adsorbed to their surface. Because the toxicity problem of cationic liposomes may limit their clinical use, different types of anionic liposomes could be used to introduce genes both in bacterial and mammalian cells and enhance their expression. This study also suggests that anionic fluid liposomes directed at an essential gene start codon could be efficiently delivered inside bacteria and inhibit the gene's expression to eventually eliminate bacterial strains resistant to conventional antibiotics.

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