

Lipidic Carriers of siRNA: Differences in the Formulation, Cellular Uptake, and Delivery with Plasmid DNA[†]

Sebastien Spagnou,[‡] Andrew D. Miller,^{‡,§} and Michael Keller^{*,‡}

IC-Vec Ltd., Flowers Building, Armstrong Road, London SW7 2AZ, United Kingdom, and
Imperial College Genetic Therapies Centre, Department of Chemistry, Flowers Building, Armstrong Road,
London SW7 2AZ, United Kingdom

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ABSTRACT: RNA interference (RNAi) has become a popular tool for downregulating specific gene expression in many species, including mammalian cells [Novina, C. D., and Sharp, P. A. (2004) The RNAi revolution, *Nature* 430, 161–164]. Synthetic double-stranded RNA sequences (siRNA) of 21–23 nucleotides have been shown in particular to have the potential to silence specifically gene function in cultured mammalian cells. As a result, there has been a significant surge of interest in the application of siRNA in functional genomics programs as a means of deciphering specific gene function. However, for siRNA functional genomics studies to be valuable and effective, specific silencing of any given target gene is essential, devoid of nonspecific knockdown and toxic side effects. For this reason, we became interested in investigating cationic liposome/lipid-mediated siRNA delivery (siFection) as a meaningful and potentially potent way to facilitate effective functional genomics studies. Accordingly, a number of cationic liposome/lipid-based systems were selected, and their formulation with siRNA was studied, with particular emphasis on formulation parameters most beneficial for siRNA use in functional genomics studies. Cationic liposome/lipid-based systems were selected from a number of commercially available products, including lipofectAMINE2000 and a range of CDAN/DOPE systems formulated from different molar ratios of the cationic cholesterol-based polyamine lipid *N*¹-cholesteryloxy carbonyl-3,7-diazanonane-1,9-diamine (CDAN) and the neutral helper lipid dioleoyl-L- α -phosphatidylethanolamine (DOPE). Parameters that were been investigated included the lipid:nucleic acid ratio of mixing, the extent of cationic liposome/lipid–nucleic acid complex (lipoplex) formation plus medium used, the lipoplex particle size, the mode of delivery, and dose–response effects. Results suggest that concentrations during siRNA lipoplex (LsiR) formation are crucial for maximum knockdown, but the efficacy of gene silencing is not influenced by the size of LsiR particles. Most significantly, results show that most commercially available cationic liposome/lipid-based systems investigated here mediate a significant nonspecific downregulation of the total cellular protein content at optimal doses for maximal specific gene silencing and knockdown. Furthermore, one pivotal aspect of using siRNA for functional genomics studies is the need for at least minimal cellular toxicity. Results demonstrate that CDAN and DOPE with and without siRNA confer low toxicity to mammalian cells, whereas lipofectAMINE2000 is clearly toxic both as a reagent and after formulation into LsiR particles. Interestingly, LsiR particles formulated from CDAN and DOPE (45:55, *m/m*; siFECTamine) seem to exhibit a slower cellular uptake than LsiR particles formulated from lipofectAMINE2000. Intracellularly, LsiR particles formulated from CDAN and DOPE systems also appear to behave differently, amassing in distinct but diffuse small nonlysosomal compartments for at least 5 h after siFection. By contrast, LsiR particles formulated from lipofectAMINE2000 accumulate in fewer larger intracellular vesicles.

RNA interference (RNAi) in animals and basal eukaryotes, quelling in fungi, and post-transcriptional gene silencing in plants are examples of a broad family of phenomena collectively called RNA silencing (2, 3). The phenomenon of specific RNA inactivation was first discovered in plants

as a defense mechanism against virus infection (4, 5), and later in *Caenorhabditis elegans* (6). The common features of RNA silencing are the production of small (21–23-nucleotide) double-stranded RNAs that act as specific determinants for downregulation of gene expression (6). The key enzyme in the intracellular production of small double-stranded RNAs is Dicer, a cytosolic ribonuclease III that digests long double-stranded RNA into 21–23-nucleotide units (7, 8). These short double-stranded RNAs are unwound, and one of the two strands becomes associated with a complex of proteins and the target transcript, designated as the RNA-induced silencing complex (RISC), that leads to

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^{*} To whom correspondence should be addressed: IC-Vec Ltd., Flowers Building, Armstrong Road, London SW7 2AZ, U.K. E-mail: m.keller@icvec.com. Telephone: ++44 207 594 3150. Fax: ++44 207 594 5803.

[‡] IC-Vec Ltd.

[§] Imperial College London.

target RNA destruction (9). The discovery that synthetic double-stranded RNA sequences (siRNA)¹ of 21–23 nucleotides can surrogate in this process and have the potential to specifically downregulate gene function in cultured mammalian cells (10) has now opened the gateway to applications of the RNAi concept in functional genomics programs and even in therapy. Research into applications of the RNAi concept is generally at a preliminary level. Early research *in vivo* has demonstrated the potential of synthetic siRNA and transgenic siRNA to downregulate both exogenous and endogenous gene expression in adult mice (11, 12). Thus, potential side effects caused by siRNA appear to be the stimulation of the interferon system but little more (13, 14). Research into delivery of siRNA is itself at a quite preliminary level. For instance, despite the widespread use of cationic liposome/lipid systems to deliver plasmid DNA (pDNA) and oligodeoxynucleotides (ODNs) to cells (15–20), there has been little reported in the literature concerning the formulation of siRNA with cationic liposomes and lipids and its delivery to cells (siFection) either *in vitro* or *in vivo*. Even basic studies concerning the formulation of cationic liposome/lipid systems with siRNA are yet to be reported. One reason for this may be the apparent misconception that all nucleic acids are much alike and should be delivered to cells in comparable ways using comparable delivery systems. Superficially this is true. Both pDNA and siRNA have anionic phosphodiester backbones with identical negative charge:nucleotide (nt) ratios and should therefore interact electrostatically with cationic liposome/lipid systems to form cationic liposome/lipid–nucleic acid (lipoplex) particles that are able to transfer the nucleic acids into cells. However, pDNA and siRNA are otherwise very different from each other in molecular weight and molecular topography with potentially important consequences.

All pDNA condenses into small nanoparticles of 60–100 nm after neutralization of 70–90% of its phosphodiester backbone charge with a cationic agent (18, 19, 21–24). Cationic agent-condensed pDNA can then exist in a variety of different morphologies depending upon the cationic condensing agent, such as spheres, toroids, and rods (18, 19). Irrespective of the agent, there is a minimal size for pDNA condensation corresponding to ~400 nucleotides (25). Such behavior ensures that pDNA is almost entirely encapsulated or encased by the cationic agent and protected from enzymatic or physical degradation within nanometric particles (17, 23, 26–32).

In contrast to pDNA, siRNA cannot condense into particles of nanometric dimensions, being already a small subnanometric nucleic acid. Therefore, electrostatic interactions between siRNA and a cationic liposome/lipid system pose two potential problems: (1) a relatively uncontrolled interaction process leading to siRNA–lipoplex (LsiR) particles of excessive size and poor stability and (2) incomplete encapsulation of siRNA molecules, which thereby exposes siRNA

to potential enzymatic or physical degradation prior to delivery to cells. Such considerations should make clear the fact that pDNA and siRNA are completely different kinds of nucleic acids and that LsiR particle formulation should be regarded as a problem distinct and different from LD particle formulation. Consequently, it cannot be assumed that what works for pDNA must work for siRNA as well. Therefore, in considering the best approach to studying cationic liposome/lipid-mediated siRNA delivery to cells, we elected to start from first principles with regard to LsiR formation and then proceed to siFection studies thereafter. Results documented in this paper suggest that an optimized siRNA formulation procedure is quite different from the pDNA formulation, and that parameters such as toxicity and efficacy can be controlled by the development of specific protocols for LsiR formation.

MATERIALS AND METHODS

Chemistry

*N*¹-Cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN) was synthesized as described previously (24). Dioleoyl-L- α -phosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids. All other chemicals were purchased from Sigma Aldrich (Dorset, U.K.) unless otherwise stated. Dried dichloromethane was distilled with phosphorus pentoxide; other solvents were purchased predried or as required from Sigma-Aldrich or BDH Laboratory Supplies (Poole, U.K.). HPLC-grade acetonitrile was purchased from Fisher Chemicals (Leicester, U.K.), and other HPLC-grade solvents were from BDH Laboratory Supplies. Analytical HPLC (Hitachi-LaChrom L-7150 pump system equipped with a Polymer Laboratories PL-ELS 1000 evaporative light scattering detector) was conducted on a Vydac C4 peptide column with a gradient from 0.1% aqueous TFA to 100% acetonitrile (0.1% TFA; 0–15 min), then 100% acetonitrile (0.1% TFA; 15–25 min), and then 100% methanol (25–45 min).

Nucleic Acids and Formulation

DNA. Plasmid DNA pUMVC1 (pDNA, 7528 bp) was obtained from the University of Michigan Vector Core (<http://www.med.umich.edu/vcore/Plasmids/>) and amplified and purified by Bayou Biolabs. The concentration of pDNA was determined spectrophotometrically ($A_{260} = 1 \approx 50 \mu\text{g}/\text{mL}$), and the pDNA molar concentration was determined using the average nucleotide base pair molecular mass of 660 Da.

siRNA. Anti- β -gal siRNA-1 (5'-CUA CAC AAA UCA GCG AUU UUU-3') was purchased from Dharmacon and stored as a 20 μM solution as indicated by the manufacturer. The nonspecific siRNA sequence (5'-UAG CGA CUA AAC ACA UCA AUU-3') was obtained from Dharmacon and stored at 20 μM as indicated by the manufacturer. The 3'-FITC-labeled anti-GFP siRNA sequence was a kind gift of Qiagen GmbH (Hilden, Germany).

Liposomes. CDAN/DOPE liposomes were prepared at a concentration of 3 mg of total lipid/mL by addition of ddH₂O (milliQ) to lyophilized CDAN/DOPE powder (molar ratios as indicated) under vortexing, to give multilamellar liposomes 300–500 nm in diameter (PCS) (pH \approx 3.5). These liposomes

¹ Abbreviations: CDAN, *N*¹-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine; FCS, fetal calf serum; FACS, fluorescently activated cell sorting; DOPE, dioleoyl-L- α -phosphatidylethanolamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; LsiR, cationic liposome/lipid–siRNA complex; pDNA, plasmid DNA; PCS, photon correlation spectroscopy; siRNA, synthetic (small) interfering RNA. Throughout this article, siRNA represents synthetic, 21–23-nucleotide RNA sequences.

were further diluted to give a final concentration of 0.3 mg/mL. Unilamellar liposomes were produced by sonication in a sonomatic water bath (Longford Ultrasonics) for 5–10 min.

Cells and Transfection Procedures

Cell Cultures. HeLa or IGROV-1 cells were seeded in a 48-well plate at a density of 40 000 cells/well 24 h before the experiment in growth medium (DMEM, 10% FCS, penicillin, and streptomycin) and cultured at 37 °C (10% CO₂). Prior to transfection, the medium was replaced with fresh growth medium. OptiMEM-1/DMEM was purchased from Invitrogen.

Transfections. The β -Gal reporter gene (pUMVC1- β -Gal, 7528 bp) was transfected with PRIMOfect (IC-Vec Ltd.) according to the manufacturer's instructions (32). Typically, 0.1 μ g (HeLa) or 0.25 μ g (IGROV-1) of pDNA was transfected per 48-well plate. The total nucleic acid:lipid ratio was 1:12 (w/w) as recommended in the instruction manual. After a pDNA transfection time of 3 h, the transfection medium was replaced with fresh growth medium (150 μ L), after which LsiR siFection experiments were performed using LsiR systems prepared in fresh OptiMEM (final volume of 100 μ L) just prior to siFection.

All siFection experiments described in this work were carried out on 48-well plates. For this purpose, the siRNA (0.1 μ g, 30 nM) was diluted with fresh OptiMEM to a final volume of 100 μ L. CDAN/DOPE liposomes (4.35 μ L, 0.3 mg/mL) were then added under vortex mixing [to give a lipid:siRNA ratio of 13:1 (w/w)], and the LsiR mixture was left to stand for 5 min before being used. Finally, the LsiR mixture was then introduced into the appropriate well of a given 48-well plate containing cells in complete growth medium (including FCS and antibiotics) (150 μ L) and incubated at 37 °C in 10% CO₂ for 3 h. The medium was then replaced with fresh growth medium, and cells were incubated for 16–72 h before the β -Gal reporter assay (Roche).

Apoptosis Assay. The APOPercentage assay kit (Biocolor Ltd.) detects and measures apoptosis in mammalian, anchorage-dependent cells, and was applied according to the manufacturer's instructions. The assay uses a dye that is selectively imported by cells that are committed to apoptosis due to a loss of the extracellular cell membrane asymmetry. The accumulation of dye in apoptotic cells was measured by a spectrophotometer ($\lambda_{\text{max}} = 570$ nm).

Lactate Dehydrogenase Assay. The CytoTox-96 assay (Promega) evaluates cytotoxicity by assessing the total release of cytoplasmic lactate dehydrogenase (LDH) into culture medium as a consequence of damaged cell membranes. Detecting medium via LDH enzyme activity is a classic marker for cellular cytotoxicity since the collapse of cellular integrity is assessed directly. The assay is based upon a coupled enzymatic assay involving the conversion of a tetrazolium salt, 2-*p*-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), into a formazan product. The reaction is catalyzed by LDH released from cells and diaphorase present in the assay substrate mixture.

Lysosomal Labeling. Sixteen hours prior to siFection, rhodamine dextrane ($M_w \approx 3000$, Molecular Probes) was added to the growth medium at a final concentration of 50 μ M/well. Thereafter, cells were thoroughly washed with PBS

(33) and then subjected to LsiR siFection using fluorescein-labeled siRNA.

FACS Analyses. FACS data were obtained on a Calibur (Beckton-Dickinson); 150 000 cells were seeded per well (six-well plate) and transfected with 1 μ g of siRNA/well 16–24 h postseeding. Fluorescein (FITC)–siRNA–CDAN/DOPE complexes were prepared in water, at an siRNA concentration of 0.05 mg/mL, as described above, and the fluorescent LsiR mixture was added to the wells to give a total volume of 1 mL. Three hours post-transfection, the cells were treated with trypsin to detach them from the plate surface and collected in complete medium by spinning at 1200 rpm before resuspension in 300 μ L of cold PBS; 50 000 cells were counted per FACS experiment.

RESULTS

To evaluate CDAN/DOPE and other cationic liposome/lipid systems for their ability to deliver siRNA to cells, we decided to employ a model gene knockdown assay system to establish delivery parameters cleanly. The model gene knockdown assay involved the introduction of an exogenous β -galactosidase (β -Gal) reporter gene (pUMVC1) introduced by cationic liposome-mediated pDNA transfection. Three hours post-transfection, a specific, commercially available anti- β -Gal-siRNA or a control siRNA-NS was then delivered by means of appropriate cationic liposomes and lipids in a process that we now call siFection. The extent of β -Gal gene knockdown was then assessed by a standard cellular β -Gal assay. Although we recognize that such a system is partially artificial due to the requirement of a pDNA transfection prior to the siRNA delivery, this model does not interfere with the evaluation of the optimization process of the siRNA delivery, which was the aim of this study.

Prior to attempting to use CDAN/DOPE cationic liposomes to deliver the siRNA to cells for functional gene knockdown experiments, we optimized the formulation of cationic liposome/lipid–siRNA (LsiR) systems using anti- β -Gal-siRNA. Different ratios of total lipid to nucleic acid were tested at fixed siRNA concentrations, and particle formation was judged to be most efficient at a ratio of 13:1 (w/w) due to the reproducibility of LsiR particle formation (Figure 1A). This particular formulation proved to be devoid of aggregation in a low-salt aqueous environment but exhibited a tendency of increased particle size in PBS at neutral pH (Figure 1B). By adding excess liposomes into diluted siRNA in OptiMEM, we achieved complete complexation as judged by gel retardation assays (data not shown). As a result, the lipid:nucleic acid ratio throughout this study was kept at this optimized ratio, a regime where aggregation is prevented. Following this, we performed our first set of exogenous β -Gal gene knockdown assays by delivering siRNA with CDAN/DOPE cationic liposomes prepared at different CDAN:DOPE stoichiometric ratios. Interestingly, while there was no difference in the capacity of any cationic liposome formulation to deliver anti- β -Gal-siRNA and achieve substantial specific β -Gal gene knockdown, there was a significant difference between the three formulations in their ability to deliver control siRNA-NS (0.2 μ g, 60 nM/well) without the appearance of nonspecific β -Gal gene knockdown. In short, a 60:40 (*m/m*) CDAN:DOPE formulation was found to cause substantial nonspecific β -Gal

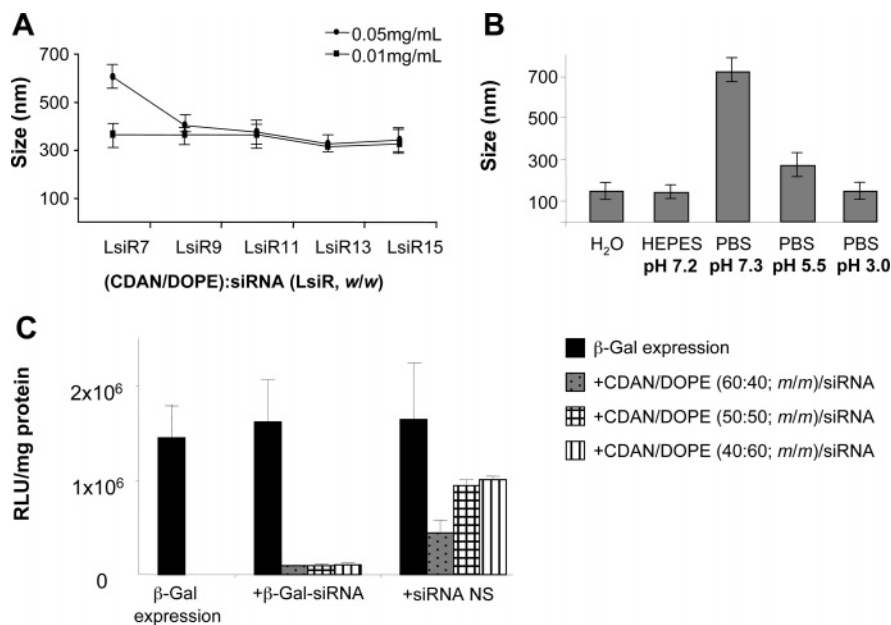


FIGURE 1: (A) Dynamic light scattering experiments for varying lipid:siRNA ratios (w/w, 7–15) for a siRNA concentration of 2 $\mu\text{g}/\text{mL}$ in a total volume of 250 μL of water. Values represent an average of three readings. (B) Influence of the buffer system and pH on LsiR (13:1 lipid:siRNA ratio, w/w) particle size. (C) Elucidation of the optimal CDAN:DOPE ratio for optimal siFection of HeLa cells: 48-well plate, 0.25 μg of pUMVC1/well (3 h), PRIMOfect as the transfection reagent for pDNA, and 0.2 μg (60 nM/well) of siRNA (3 h). All transfections and siFections were carried out in complete growth medium. The β -Gal activity and total protein content were assayed 24 h after pDNA transfection. Note that since the nonspecific control siRNA-NS is 50% homologous with the specific anti β -Gal siRNA, then semispecific effects from the control siRNA-NS may be observed at siRNA concentrations as low as even 60 nM.

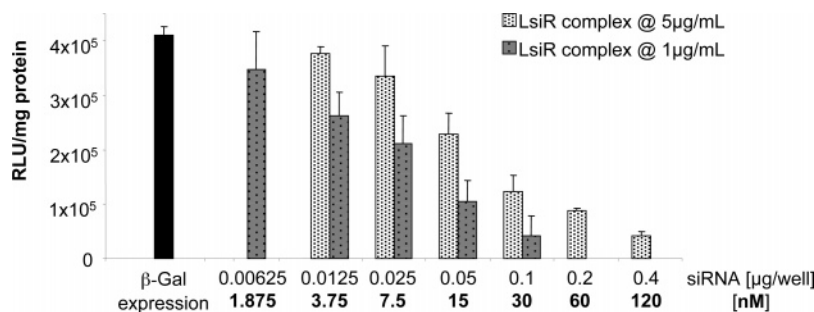


FIGURE 2: Influence of the formulation procedure on optimal β -galactosidase protein downregulation. LsiR (13:1 lipid:siRNA ratio, w/w) systems obtained from CDAN/DOPE (45:55, m/m; siFECTamine) liposomes were prepared in a total volume of 100 μL of OptiMEM at a concentration of 5 or 1 $\mu\text{g}/\text{mL}$. These LsiR systems were then pipetted into wells (48-well plate) containing cultured HeLa cells (40 000) in 150 μL of complete growth medium (10% serum, antibiotics), and cells were incubated at 37 $^{\circ}\text{C}$ in CO_2 for 3 h. The β -Gal activity and total protein content were assayed 24 h post-pDNA transfection. Note that LsiR systems generated at high dilution yield a consistently better profile of gene silencing.

gene knockdown, while the other formulations containing molar CDAN ratios of $>50\%$ were responsible for much less (Figure 1C). Accordingly, to minimize the possibility of nonspecific β -Gal gene knockdown and ensure maximum ease of cationic liposome preparation, a compromise 45:55 (m/m; siFECTamine) CDAN:DOPE formulation was prepared and was used throughout the remainder of the studies. As remaining data show, by adjusting the total amount of siRNA delivered per siFection experiment ($<0.2 \mu\text{g}$, 60 nM/well), we could reduce the level of nonspecific β -Gal gene knockdown caused by CDAN/DOPE (45:55, m/m) to essentially insignificant levels.

Once these parameters were set, we investigated the influence of dilution on LsiR particle formation and upon LsiR siFection. Aliquots of siRNA and CDAN/DOPE liposomes were combined under heavy vortex conditions, giving two LsiR samples with final siRNA concentrations of 1 and 5 $\mu\text{g}/\text{mL}$, respectively. Quite clearly, at equivalent doses of siRNA per well (0.00625–0.4 μg , 1.875–120 nM/

well), the more dilute sample of LsiR produced more efficient gene knockdown post-siFection than the more concentrated sample (Figure 2). In contrast, the lamellar state of the CDAN/DOPE liposomes (multilamellar vs unilamellar) did not influence the efficacy of gene silencing. Multilamellar liposomes with sizes between 300 and 600 nm and small unilamellar liposomes with a diameter of 30–50 nm resulted in equal β -Gal gene silencing (data not shown). However, we observed a strong impact on the efficacy of knockdown by the aqueous environment in which LsiR systems were produced. By using OptiMEM rather than water for the dilution of liposomes and siRNA and their mixing, we were able to generate significantly enhanced β -Gal gene knockdown and downregulation of the β -Gal protein.

We further investigated the optimal quantity of siRNA per well (48-well plate) to maximize β -Gal gene knockdown. A clear dose–response pattern emerged from these experiments (Figure 2). Upon closer examination, the optimal dose of siRNA per well (48-well plate) for maximizing β -Gal gene

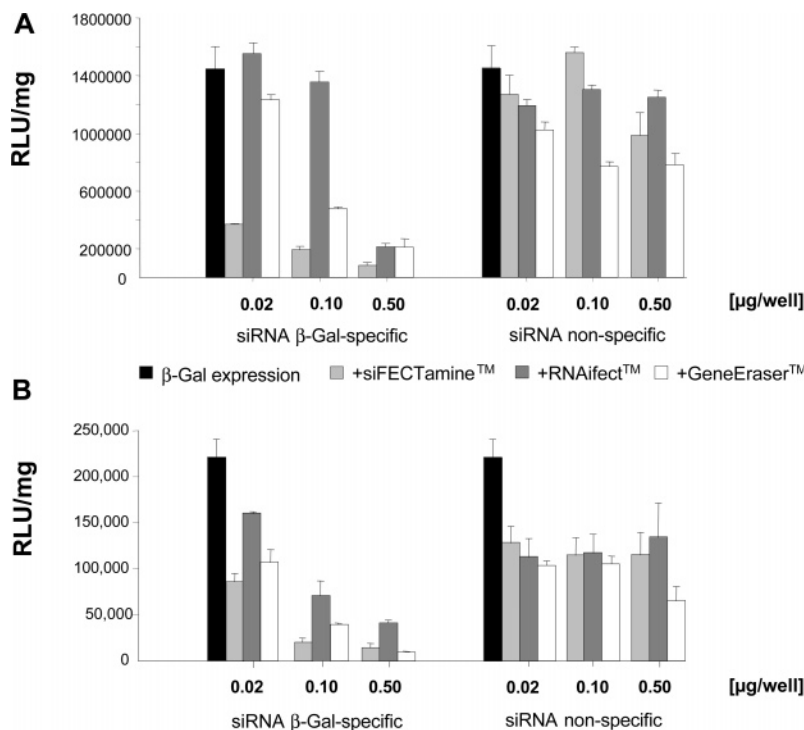


FIGURE 3: Comparison of CDAN/DOPE (45:55, *m/m*; siFECTamine) cationic liposomes with two commercial reagents (RNAiFect and GeneEraser) on two different cell lines: (A) HeLa and (B) IGROV-1. The siRNA doses that were used were 0.02, 0.1, and 0.5 μ g/well, which correspond to 6, 30, and 150 nM, respectively.

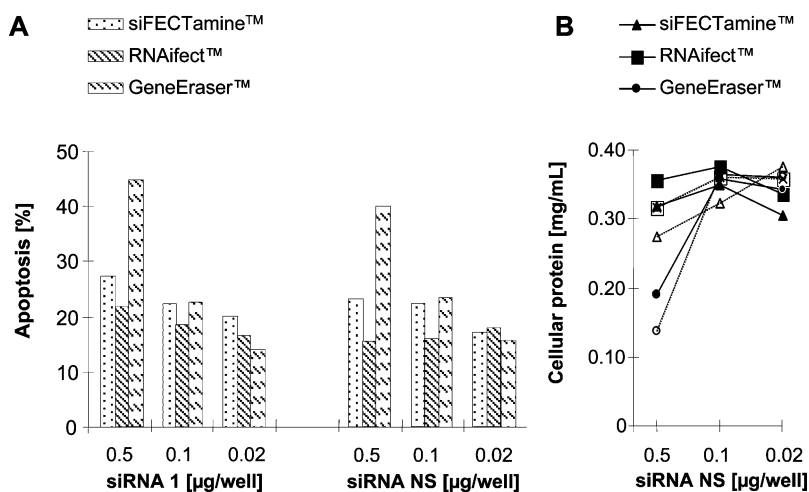


FIGURE 4: Induction of apoptosis by LsiR (A) and total protein content of cells for different amounts of siRNA per well (B). Note that in panel B, the dashed lines represent the nonspecific siRNA-NS, whereas the solid lines represent the specific anti- β -Gal siRNA formulation. Except for GeneEraser at doses of 0.5 μ g/well (150 nM), no difference in induction of apoptosis or general downregulation of total cellular protein content was observed between the different delivery reagents.

knockdown was found to be 0.1 μ g of siRNA/well (30 nM) in experiments with both HeLa and IGROV-1 cells (Figure 3). Increasing the dose of siRNA above these levels generated increasing toxicity that resulted in the detachment of the cells from the surface of the plate. Next, we compared CDAN/DOPE liposomes with different commercially available transfection reagents specifically designed for siRNA transfection (Figure 3) at three different doses. In general, CDAN/DOPE (45:55, *m/m*) liposome-mediated siFectation performed best, giving the most significant β -Gal gene knockdown using anti- β -Gal-siRNA at doses of \sim 0.1 μ g (30 nM) of siRNA/well (Figure 3A). On the more difficult to transfect cell line, IGROV-1, increasing quantities of siRNA for siFectation experiments were required with the undesired side effect that more than 50% nonspecific gene knockdown with certain

formulations was observed (Figure 3B).

In the case of all cationic liposome/lipid systems that were used, detachment of cells from the surface of wells was observed whenever overall larger doses of siRNA (\geq 0.5 μ g of siRNA/well) were used, suggesting the emergence of some toxicity effects. This effect is clearly undesirable and not compatible with functional genomics programs. Therefore, we investigated whether siRNA induced programmed cell death (apoptosis). Using an apoptosis assay that detects membrane asymmetry in apoptotic cells, we were unable to detect significant apoptosis at the siRNA doses that were investigated ($<$ 0.5 μ g/well, 48-well plate) except perhaps with GeneEraser at the highest siRNA dose (0.5 μ g/well, 150 nM) (Figure 4A). Similarly and in support, the total cellular protein content of cells post-siFectation was measured

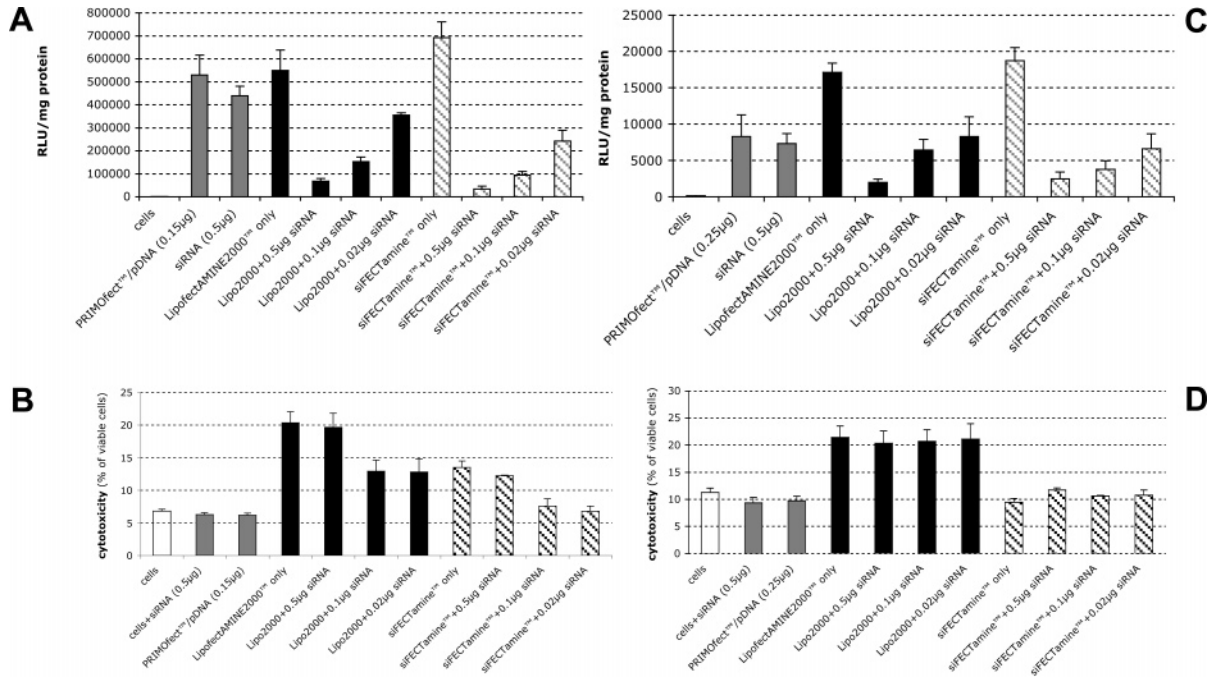


FIGURE 5: Comparison of CDAN/DOPE (45:55, *m/m*; siFECTamine) cationic liposomes and lipofectAMINE2000 for siRNA-mediated β -Gal gene knockdown in HeLa cells (A) and IGROV-1 cells (C): 0.15 μ g (HeLa) or 0.25 μ g (IGROV-1) of pDNA(lacZ) was transfected 3 h prior to siFection, and the β -Gal activity was assayed 24 h post-transfection. For both HeLa (B) and IGROV-1 (D) cells, the cellular mortality induced by toxic effects of siRNA delivery reagents siFECTamine and lipofectAMINE2000 was measured by the lactate dehydrogenase (LDH) assay, with and without the involvement of siRNA. LDH, a typical marker of cell toxicity, is quantified by the LDH-catalyzed conversion of tetrazolium salt, 2-*p*-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), into a formazan product ($I_{max} = 492$ nm). Note that lipofectAMINE2000 on its own is clearly toxic to both cell lines. Also note that the amount of “transfection reagent only” corresponds to a putative dose of 0.5 μ g of siRNA/well (150 nM).

in comparison with that of control cells, and little perturbation in total protein content was observed except with GeneEraser once again at the highest siRNA dose (0.5 μ g/well, 150 nM) (Figure 4B).

Given the current widespread use of lipofectAMINE2000 in the transfection market of nucleic acids, we compared the efficacy and toxicity of CDAN/DOPE liposomes with the efficacy and toxicity of that reagent. Both reagents mediated similar high levels of β -Gal gene knockdown in two independent cell lines (Figure 5A,C). The cellular mortality (toxicity) induced by the transfection reagents lipofectAMINE2000 and CDAN/DOPE liposomes both with and without siRNA was assessed using a lactate dehydrogenase (LDH) reporter assay. From these studies, it became clear that CDAN/DOPE liposomes both as a liposomal formulation and in combination with siRNA were essentially nontoxic on both HeLa and IGROV-1 cells. By contrast, lipofectAMINE2000 as a lipid formulation and as LsiR lipoplex was clearly toxic to both cell lines (Figure 5B,D). Interestingly, on the more difficult to transfect IGROV-1 cells, the addition of transfection reagent 3 h post-pDNA transfection boosted the production of β -Gal protein by a factor of 2 for both lipofectAMINE2000 and CDAN/DOPE liposomes, which was observed to a lesser extent on HeLa cells (Figure 5A,C). Mechanistic studies on the cellular uptake of fluorescently labeled siRNA revealed that CDAN/DOPE liposome-mediated siRNA delivery to the intracellular environment of cells was slower than lipofectAMINE2000-mediated delivery (Figure 6). In addition, CDAN/DOPE-LsiR particles were found to be located at or within small, dense intracellular vesicles different from lysosomes, which were labeled separately with rhodamine dextrane. Strikingly, each

cell contained numerous amounts of such vesicles, whereas for lipofectAMINE2000, there appeared to be fewer but much larger vesicles containing the fluorescent siRNA. FACS studies using a fluorescently labeled siRNA confirmed the high percentage of cells containing fluorescent siRNA post-siFection mediated by either lipofectAMINE2000 or CDAN/DOPE liposomes (>95%) (Figure 7).

DISCUSSION

The popularity of siRNA as a tool for functional genomics and gene target validation is growing. So also is the possibility that small synthetic RNA sequences (siRNA) could be a therapeutic agent of significant utility in emerging genetic therapies. We used the LacZ (β -Gal) reporter gene that was introduced by PRIMOfect-mediated transfection 3 h prior to cationic liposome/lipid-mediated siRNA delivery (siFection), and evaluated the influence of biophysical parameters on the siRNA delivery and gene knockdown process. We recognize that this system is somewhat remote from the downregulation of an endogenous gene, since the experimental setup, by virtue of using a reporter gene to quantify gene silencing, requires prior transfection of cells with that reporter gene. Such a step is clearly not necessary in the case of housekeeping gene knockdown. However, this fact does not interfere with the formulation parameters that were investigated in generating the most efficient possible CDAN/DOPE-LsiR system. As a matter of fact, we began our investigations with a stably transfected green fluorescent protein (GFP) cell line, seeking to knock down the GFP gene. However, in the course of that work, we realized that the intracellular half-life of the constitutively expressed GFP gene was too long to achieve a level of knockdown above

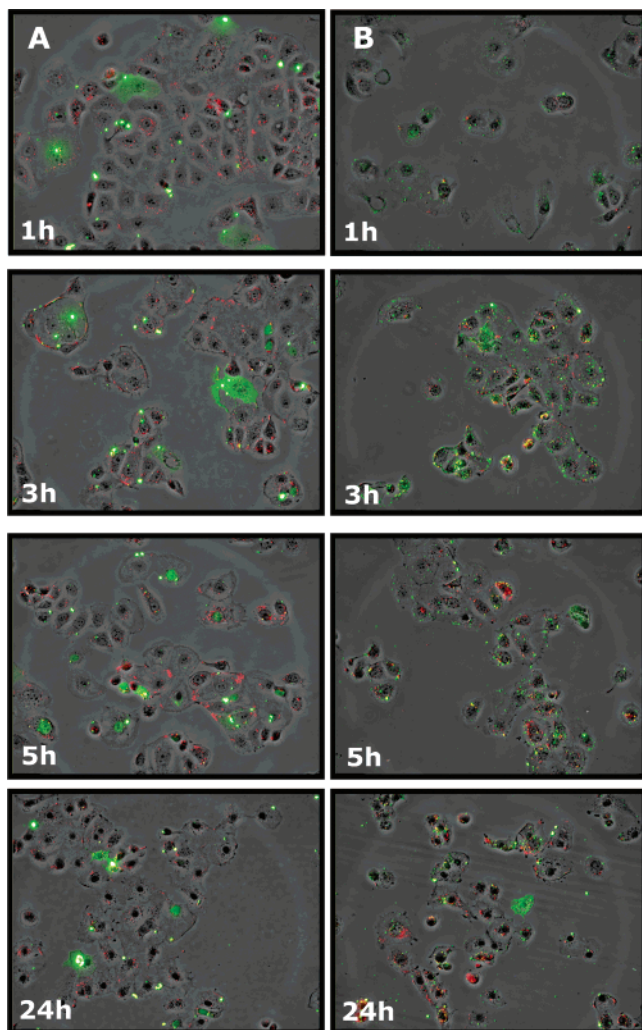


FIGURE 6: Mechanistic study of the kinetics of cellular uptake of siRNA mediated by lipofectAMINE2000 (A) and CDAN/DOPE (45:55, *m/m*; siFECTamine) liposomes (B). Cells were incubated with rhodamine dextran ($M_w = 3000$; $50 \mu\text{M}$) for 16 h. Subsequently, 3'-fluorescein-labeled siRNA was formulated with lipofectAMINE2000 and siFECTamine, and IGROV-1 cells were incubated with these LsiR systems for 1, 3, 5, or 24 h before fixing with 4% *p*-formaldehyde. The fluorescence of the siRNA (green) and dextran (red) was detected by epi-fluorescent microscopy. Note that in the case of siFECTamine-mediated delivery, significant green fluorescence intensity was observed after only 3 h, whereas for lipofectAMINE, high intensities of green fluorescence were observed after incubation of LsiR particles with cells for 1 h.

50% in the lifetime of adherent cells, which led us to switch to the β -Gal reporter gene system.

An interesting feature of LsiR formulation and use is the impact of the aqueous medium in which LsiR systems are formed on the efficacy of siFection. The increased salt concentration of OptiMEM-1 seems to be beneficial to the gene knockdown process. In addition, the dilution during LsiR formation appears to be pivotal to the efficiency of gene silencing as well (Figure 2). This is consistent with the formation of discrete LsiR particles which sediment with a broader surface coverage on cells. In contrast to plasmid DNA (pDNA) delivery, we were unable to observe a difference between the use of small LsiR particles (50–100 nm) and larger aggregates (200–600 nm) for gene knockdown. In the case of pDNA, larger cationic liposome/lipid-pDNA (LD) particles tend to yield better transfection results

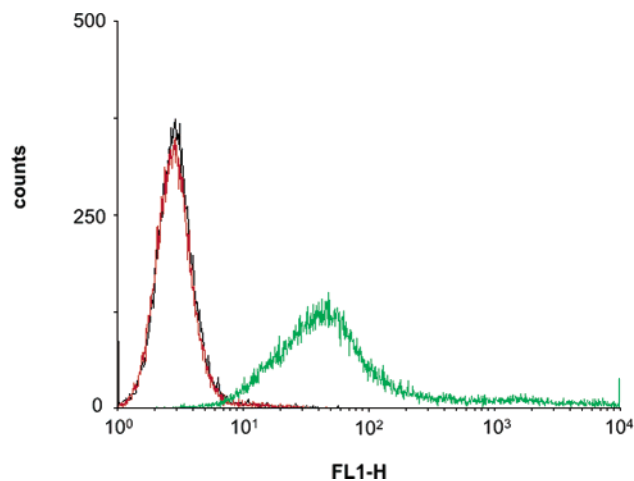


FIGURE 7: FACS analyses of HeLa cells (black line) following siFection with naked fluorescein-labeled siRNA (red line) and siFECTamine/3'-FITC-siRNA (green line). Note that the vast majority of all counted cells (50 000) post-siFection contained fluorescent siRNA.

than smaller particles due in part to a more efficient sedimentation of the LD particles onto cell surfaces (28). However, as interesting as these features of LsiR systems might be, the most important feature of siRNA is gene silencing and/or gene knockdown with high specificity (34–36). LsiR siFection must be guaranteed to render specific siRNA gene knockdown; otherwise, the value of this process is doubtful. Only cationic liposomes and lipids capable of generating LsiR systems capable of rendering unambiguous, specific gene knockdown are of value.

Before the discovery of RNA interference, antisense oligonucleotides were primary tools for targeted gene silencing; however, they have been shown to cause significant nonspecific effects (37–39). In particular, their affinity for cellular proteins has been shown to cause significant complications in interpreting antisense oligonucleotide-mediated gene silencing effects (40). Such a scenario might also be true for siRNA, although to date, no such observations have been reported in the literature. Using a number of cationic liposome/lipid-based siRNA delivery systems, we observed that at siRNA doses of $0.5 \mu\text{g}/\text{well}$ (150 nM) on a 48-well plate, the nonspecific siRNA (control siRNA-NS) was able to mediate significant β -Gal gene knockdown and downregulation of β -Gal protein (Figure 3), further suggesting that nonspecific downregulation of other cellular proteins was also taking place. Such a scenario would interfere greatly with functional studies of a cellular protein and therefore must be avoided. Hence, altogether, we would suggest that siRNA delivery reagents that are able to mediate optimal gene silencing at doses significantly lower than $0.5 \mu\text{g}$ (150 nM/well, 48-well plate) should always be selected to minimize the possibility of nonspecific gene knockdown effects in RNA interference (Figure 3). Furthermore, we note that the commercially available siRNA sequence specific for the β -Gal gene (anti- β -Gal siRNA) and applied throughout this study is not entirely devoid of off-target affinities either. For instance, potential interactions with the mRNA of the *Homo sapiens* translocase from the inner mitochondrial membrane 8 homologue A protein can be predicted or interactions with the mRNA of the *H. sapiens* ATPase family gene 3 (AFG3) protein. Fortunately, refined algorithms such

as siDIRECT (RNAi Co.) and SSearch (Qiagen) are now available, which allow for the unambiguous selection of siRNA sequences that minimize the likelihood of such off-target effects (41).

In the context of nonspecific gene knockdown, we thought it would be essential to investigate effects on the cellular viability caused by either siFection reagents themselves or LsiR particles. First, we applied a colorimetric assay that detects apoptosis in cells due to the loss of the asymmetric lipid distribution of the extracellular membrane (42). At lower doses, none of the investigated reagents appeared to induce apoptosis. However, at doses of 150 nM/well, GeneEraser clearly did induce apoptosis (Figure 4A). Total protein levels produced by the cells following siFection were also consistent with these observations. Twenty-four hours post-pDNA transfection (21 h post-siFection), a clear decrease in the total cellular protein content was observed in the case of GeneEraser-mediated siRNA delivery, whereas RNAiFect and CDAN/DOPE systems did not induce significant changes in total protein levels. Quantification of cellular mortality (toxicity) was achieved by using a lactate dehydrogenase (LDH) assay. The test is based on a fluorescent measure of the release of cytoplasmic lactate dehydrogenase (LDH), a classic marker of cytotoxicity, into culture medium through damaged cell membranes. This enzymatic assay results in the LDH-catalyzed conversion of a tetrazolium salt into a formazan product ($I_{\max} = 492$ nm) (Figure 5). Evidence suggested that CDAN/DOPE (45:55, *m/m*; siFECTamine) liposomes were neither toxic alone nor toxic in combination with siRNA, whereas lipofectAMINE2000 was clearly a toxic reagent even without siRNA at the doses required for optimal siFection. Such a high degree of cellular toxicity would certainly jeopardize the outcome of any functional study using this reagent to transfect siRNA. We would suggest that these cellular toxicity tests described in this study should be used as a matter of routine for judging the efficacy and suitability of siRNA delivery reagents. The appearance of cellular toxicity in any test should be taken seriously and cast into doubt the suitability of the reagent as a means of delivering siRNA for specific gene knockdown studies or indeed for any other more extended functional genomics studies leading to potential therapeutic applications.

A potential clue to the origins of toxic effects may come from detailed mechanistic studies of siRNA delivery. In our mechanistic studies, we formulated 3'-fluorescein-labeled siRNA with either lipofectAMINE2000 or CDAN/DOPE liposomes. IGROV-1 cells were incubated with LsiR systems for 1 or 3 h, respectively, and fixed after 1, 3, 5, or 24 h with *p*-formaldehyde according to established procedures (43). A striking feature of these experiments is the unexpectedly slow uptake of siRNA mediated by CDAN/DOPE liposomes. We had previously shown that pDNA uptake mediated by CDAN/DOPE liposomes can lead to the internalization of large amounts of pDNA even into nuclei within minutes (20). Hence, in terms of intracellular uptake and localization, siRNA and pDNA can differ substantially. LsiR systems prepared from CDAN/DOPE cationic liposomes required at least 1 h to be detected intracellularly by fluorescence microscopy and were localized in numerous small cytosolic vesicles (Figure 6). These vesicles were found to be different from lysosomes that were marked with rhodamine dextrane. By contrast, lipofectAMINE2000-

mediated delivery of siRNA to the intracellular environment was faster than CDAN/DOPE-mediated delivery, resulting in the detection of more green fluorescence intensity after incubation of LsiR particles with cells for 1 h, and fewer, larger cytosolic vesicles. The nature of these vesicles now needs to be identified. Studies in this vein are ongoing and will be reported in due course.

SUMMARY

A variety of CDAN/DOPE liposomal systems for siRNA formulation have been investigated with respect to optimized mediation of protein downregulation and gene knockdown and avoidance of nonspecific effects and toxicity. The formulation process is optimal only when siRNA and cationic liposomes and lipids are combined under a high-dilution regime (Figures 1 and 2). Interestingly, and contrary to observations with LD particles involving plasmid DNA, the size of the LsiR particles between 60 and 400 nm did not influence the efficacy of gene knockdown. Rather unusually, the intracellular uptake of LsiR particles prepared from CDAN/DOPE cationic liposomes (45:55, *m/m*) was slow. LsiR particles amassed in defined intracellular vesicles different from lysosomes where they remained detectable for at least 24 h post-siFection. This slow uptake of CDAN/DOPE (45:55, *m/m*)-LsiR particles might be one of the reasons that low toxicity is observed at low and high doses of siRNA (Figures 4 and 5). Clearly, the most important aspect of effective siFection is the specificity of siRNA-mediated gene knockdown and the surface coverage of siFection (35, 36), which becomes an important aspect at doses reaching 150 nM siRNA/well. Therefore, such a concentration of siRNA per well must be avoided, and delivery reagents performing best under such high-concentration regimes should be avoided (Figures 3 and 4).

Altogether, this study demonstrates that plasmid DNA and siRNA formulate very differently with cationic liposomes and lipids, and that, mechanistically, the two species of nucleic acids vary substantially in both the intracellular uptake and accumulation into defined intracellular vesicles. Therefore, prior to using a specific delivery system for any application of siRNA, research into optimized formulations and delivery conditions is pivotal so that the outcome of the study both *in vitro* (functional genomics) and *in vivo* (therapeutics) is not jeopardized (12).

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