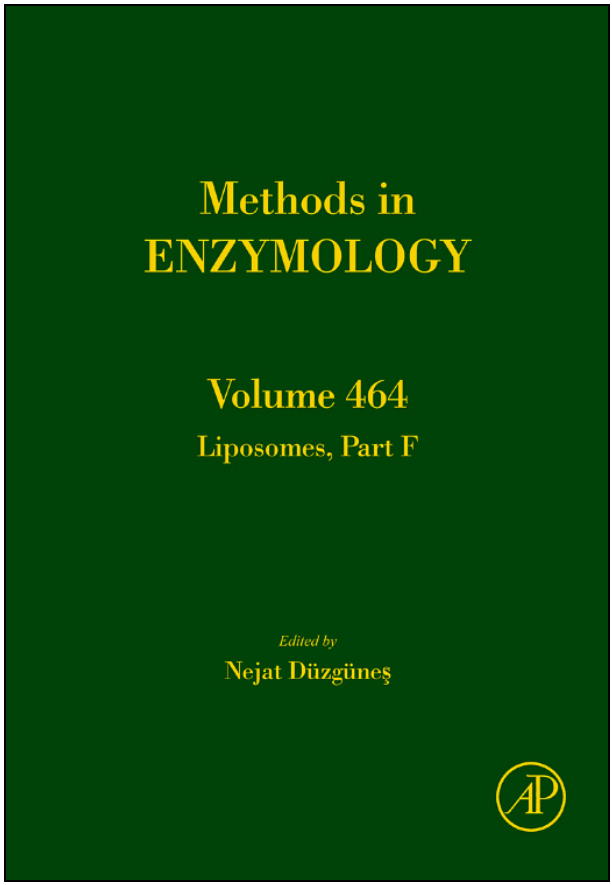

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CHAPTER SEVENTEEN

Engineering Cationic Liposome: siRNA Complexes for *In Vitro* and *In Vivo* Delivery

Jennifer E. Podesta *and* Kostas Kostarelos

Contents

Abstract

RNA interference, the sequence-specific silencing of gene expression by introduction of short interfering RNA (siRNA) is a powerful tool that that the potential to act as a therapeutic agent and the advantage of decreasing toxic effects on normal tissue sometimes seen with conventional treatments i.e. small molecule inhibitors. Naked, unmodified siRNA is poorly taken up by cells and is subject to degradation when exposed to blood proteins during systemic administration. It has also been shown to produce non-specific immune response as well as

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having the potential to generate 'off-target' effects. Therefore there is a requirement for a delivery system to not only protect the siRNA and facilitate its uptake, but additionally to offer the potential for targeted delivery with an aim of exploiting the high specificity afforded by RNA interference.

Cationic liposomes are the most studied, non-viral delivery system used for nucleic acid delivery. As such, the use of cationic liposomes is promising for siRNA for delivery. Furthermore, polyethylene glycol (PEG) can be incorporated into the liposome formulation to create sterically stabilized or 'stealth' liposomes. Addition of PEG can reduce recognition by the reticuloendothelial system (RES) thereby prolonging circulation time. Here we describe a methodology for the complexation of siRNA with cationic liposomes and PEGylated liposomes using two protocols: mixing and encapsulation. Moreover, the different formulations are compared head to head to demonstrate their efficacy for gene silencing.

1. Introduction

RNA interference using short interfering siRNA offers the potential for specific gene silencing, and as such, has great promise for gene function studies as well as a therapeutic agent (Castanotto and Rossi, 2009; de Fougerolles *et al.*, 2007). RNAi is initiated by short, double-stranded RNA 19–23 nucleotides (nt) in length complementary to the gene of interest, and blocks protein expression by targeting the mRNA for nuclease degradation before translation occurs (Elbashir *et al.*, 2001). By exploiting the RNAi pathway, synthetic siRNA can be designed to specifically silence genes for the treatment of diseases such as cancer or viral infections, for example, HIV/AIDS.

Nucleic acid molecules do not readily cross the cell membrane, and therefore require a delivery vehicle to facilitate cellular uptake. During the last 20 years much has been learned from gene therapy using plasmid DNA as well as antisense oligonucleotide studies about the interaction between the delivery agents and nucleic acids (Mintzer and Simanek, 2009). Cationic liposomes have been the most studied delivery systems for nucleic acids, due to the simplicity of the electrostatic interaction between cationic liposomes and negatively charged nucleic acids to form the vector complex. Cationic liposomes have been used successfully for the intracellular delivery of siRNA.

RNA interference mediated by siRNA requires delivery of siRNA into the cytoplasm of the cell, unlike plasmid DNA or plasmid- or virus-encoded short hairpin RNA that requires nuclear localization (Meister and Tuschl, 2004). Delivery to the cytoplasm removes one of the delivery hurdles by not having to cross the nuclear membrane. Previous reports utilizing naked siRNA *in vivo* have often relied on hydrodynamic injection (Lewis and Wolff, 2007). This technique is useful for generating liver uptake; however, this is done by inducing microperforations into the hepatocyte membrane to facilitate uptake. Moreover, the rapid injection of a large volume under high pressure is not clinically viable. Clinical siRNA therapy so far has primarily been limited to local administration of naked siRNA. This,

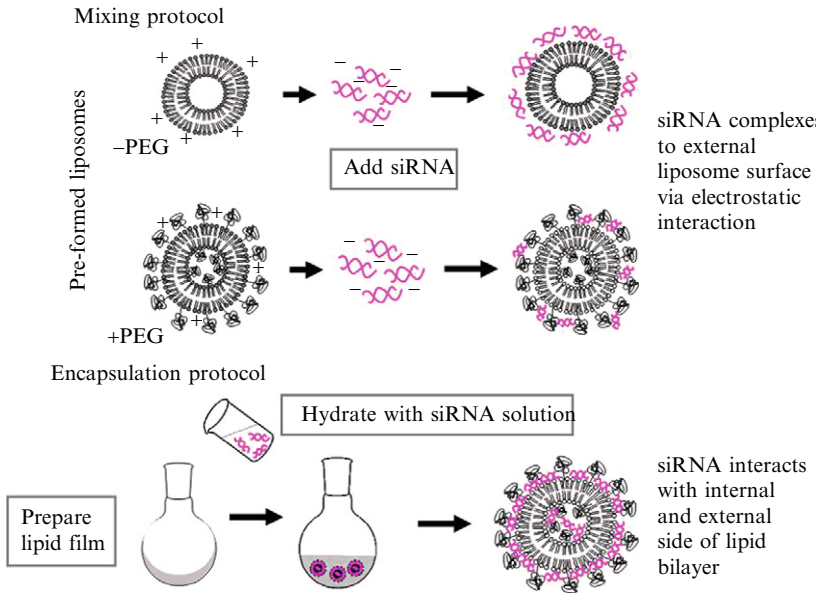
however, has led to a series of concerns over safety, efficacy, and specificity of the therapy (Cho *et al.*, 2009).

Activation of the innate immune system and generation of off-target effects are two serious side effects observed with siRNA. Reduction of the siRNA concentration is one way to reduce off-target effects. Shielding of siRNA from recognition by the reticuloendothelial system (RES) has the potential to prevent or limit immune activation. Furthermore, chemical modifications to the siRNA molecules have also led to more stable, less immunoreactive RNA species (Morrissey *et al.*, 2005).

For siRNA to progress as a viable therapeutic agent it requires a delivery vehicle. The vehicle should have the ability to: (a) target siRNA to the region of interest (be it a tumor or particular tissue within the body); (b) provide protection from nuclease degradation while in the blood; (c) limit excretion through the kidneys (Whitehead *et al.*, 2009); (d) allow use of minimal siRNA doses to reduce off-target effects; and (e) shield the siRNA from the immune system. Ultimately, it should achieve enough active copies of siRNA at the target site to elicit a therapeutic effect with limited toxicity.

To address all these requirements and specifications for an siRNA delivery system that can be used both *in vitro* and *in vivo,* we compared three systems based on a well-characterized cationic liposome (DOTAP:cholesterol) system and utilized two protocols for generating the liposome:siRNA complexes.

2. Cationic Liposome Systems for siRNA Delivery



2.1. DOTAP/Cholesterol

Liposomes are the most studied, nonviral delivery vehicle for gene therapy, and cationic liposomes in particular have been shown to have an affinity for tumor vasculature preferentially over zwitterionic and anionic liposomes (Thurston *et al.*, 1998). Also, the growth of blood vessels within a solid tumor is rapid and disorganized, resulting in leaky vasculature that may enable liposomes to extravasate through the gaps in the endothelium into the tumor. Cationic liposomes have already been extensively studied as carriers of anticancer drugs such as paclitaxel (Endo-TAG) and doxorubicin (Campbell *et al.*, 2009).

Liposomes formed from the cationic lipid DOTAP and cholesterol have been well characterized as a delivery system. Numerous preclinical studies utilize DOTAP/Chol liposomes for delivery of plasmid DNA and antisense oligonucleotides, and more recently, siRNA (Kim *et al.*, 2007; Ramesh *et al.*, 2001; Zhang *et al.*, 2008). We selected this liposome composition formulated at a 2:1 (DOTAP/Chol) molar ratio as the basis for designing and optimizing an siRNA delivery system.

Cationic liposomes are able to form complexes with negatively charged siRNA via electrostatic interactions. The charge ratio (N/P: positively charged nitrogen (DOTAP) to negatively charged phosphate (siRNA)) at which the liposomes and siRNA are mixed has a measurable effect on the physicochemical properties of the resulting complex. The size differences between plasmid DNA (several kb; supercoiled configuration) and siRNA (double-stranded RNA 19–23 nt in length) suggest that they will interact differently with the liposome. Modification from existing commercial liposome transfection reagents and development of novel vector systems specifically for siRNA transfer requires characterization of these systems. For this reason, complexes generated by mixing of liposomes and siRNA are characterized over a range of charge ratios to determine the optimum N/P ratio for complex formation and subsequent transfection studies. Although useful *in vitro*, cationic liposomes are not a perfect delivery system *in vivo*. When injected systemically, they are cleared rapidly from the blood, limiting their capacity to target tissues. Incorporation of a flexible polymer such as PEG onto the liposome can significantly prolong circulation time (Allen, 1994).[1]

[1] *Size and charge measurements*: The particle diameter and surface charge are measured using PCS in a nanosizer (Malvern). Liposomes are diluted 1:4 into filtered 5% dextrose to generate 500 μM (DOTAP) in 1 ml final volume. Higher dilutions may be used for size measurement; however, a higher concentration of the cationic component is required for zeta potential measurements. Diluted liposomes are pipetted into a clean (dust-free) cuvette and a series of measurements are recorded. The mean diameter and surface charge are calculated from 30 readings for both size and surface charge. The polydispersity index gives an indication of the homogeneity of the liposome population.

2.2. DOTAP:Cholesterol:DSPE-PEG2000

The addition of poly(ethylene glycol) (PEG) to create sterically stabilized liposomes (SSL), also termed "stealth" liposomes, has been used successfully to increase the circulation time after systemic administration. PEG has many properties that make it compatible with clinical use, such as low immunogenicity and toxicity (Allen, 1994). Moreover, it is soluble in aqueous and organic solvents, making it compatible for use in liposome formulations. Conjugation of PEG to a phospholipid, for example, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) enables PEG to be anchored in the lipid bilayer. DSPE-PEG is used as a means of shielding the liposome from interaction with serum proteins and subsequent uptake by the mononuclear phagocyte system (MPS). Coating the surface of the liposome with a polymer provides a physical barrier that decreases binding of opsonins in the blood. Macrophage recognition and internalization of opsonins is responsible for the rapid clearance of conventional liposomes (Immordino *et al.*, 2006). Additionally, the incorporation of PEG into the lipid bilayer offers a platform on which to add targeting moieties. Monoclonal antibodies, Fab' fragments and peptides have all been successfully used for active targeting of liposomes (Janssen *et al.*, 2003; Stephenson *et al.*, 2004).

Here, we describe a protocol for complexation between cationic liposomes and siRNA, with particular emphasis on the differences between PEGylated and non-PEGylated liposomes, and subsequent evaluation of the siRNA transfection efficiency. There are several reports of cationic liposome:siRNA complexes for silencing experiments in the literature; however, the effect of PEGylation on the physicochemical properties as well as biological functionality of the siRNA has not been addressed systematically. We propose utilizing the encapsulation method when complexing siRNA with PEGylated liposomes for the design and engineering of vectors for systemic *in vivo* administration.

3. Experimental Methods

3.1. General considerations

Preparation of liposomes includes a filtration step used to reduce the vesicle size. This also has the benefit of filter sterilizing the solution for use in cell culture.[2] Once the liposomes have been sonicated, filtration and all

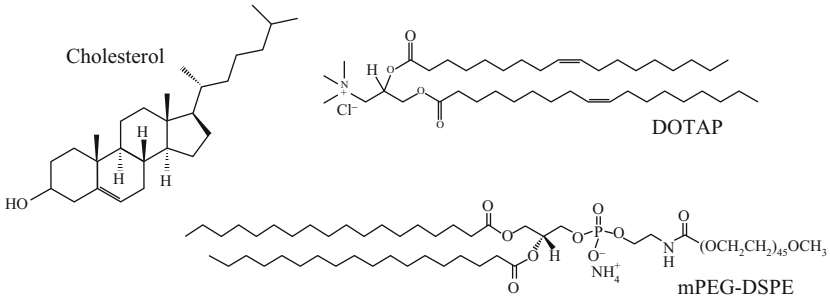
[2] *Cell culture*: Human cervical carcinoma HeLa cells (ATCC #CCL-2™, Manassas, VA, USA) are cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% (v/v) fetal bovine serum; 1 m*M* sodium pyruvate; 2 m*M* L-glutamine; 1500 mg/l sodium bicarbonate; 50 U/ml penicillin and 50 μg/ml streptomycin at 37 °C and 5% CO_2 in a humidified atmosphere. (All cell culture media and supplements are purchased from Invitrogen, UK.) HeLa cells are passaged 3 times weekly at 1:8; cell seeding density for silencing is determined empirically for each multiwell plate to yield 40–60% confluence at the time of siRNA transfection.

subsequent steps are carried out in a Class 2 laminar flow cabinet under sterile conditions. Aliquots for measurement can be removed aseptically and transferred to a sterile cuvette enabling the sample to be recovered for downstream applications, if necessary.

All solutions and glassware used are RNase-free; sterile, RNase- and DNase-free plasticware is used for all biological applications. Dextrose solution is prepared using RNase-free dH_2O. RNase-free water is prepared by treatment with 0.1% (v/v) diethylpyrocarbonate (DEPC) for 4 h overnight, followed by autoclaving at 121 °C for 20 min.

3.2. Materials

1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) from Avanti Polar Lipids, USA.



3.3. Cationic liposome preparation using the lipid film hydration method

1. DOTAP and cholesterol (2:1) are dissolved in chloroform:methanol (4:1 v/v) and the organic solvent is evaporated under pressure for 30 min at 40 °C using a rotoevaporator. The resulting thin lipid film is flushed with a stream of N_2 to remove any trace of the organic solvent. The lipid film may be stored under nitrogen at 4 °C overnight, if required.
2. The lipid film is hydrated in 5% (w/v) sterile filtered dextrose by rapid pipetting to produce large, multilamellar liposomes (MLVs). The MLVs are reduced to small, unilamellar vesicles (SUVs) by sonicating in a waterbath sonicator for 1 min followed extrusion through a 0.2 μm Anotop 10 filter (Whatman, UK). The liposome solution is then incubated at room temperature for a minimum 30 min to allow stabilization.
3. Liposome size (*z*-average diameter) and surface charge (ζ potential) are measured using laser Doppler velocimetry and photon correlation spectroscopy (PCS)/dynamic light scattering, respectively, in a Zetasizer Nano ZS (Malvern, UK), or similar instrument, at 25 °C.

3.4. PEGylated cationic liposome preparation using the lipid film hydration method

PEGylated liposomes are prepared using the same protocol as conventional liposomes. Briefly, DSPE-PEG2000 (5 mol%) is dissolved in the organic solvent with DOTAP and cholesterol. The PEGylated liposome is hydrated, reduced in size, and measured in the same way as the conventional, non-PEGylated liposome.

3.5. Liposome/siRNA complex formation

1. Liposomes and siRNA are diluted separately into 50% final volume.
2. The siRNA is added to the liposome by rapid pipetting to prevent localized high siRNA:liposome concentrations. This is mixed thoroughly by pipetting and brief vortexing.
3. The mixture is then incubated at room temperature for 20 min to allow complexation to occur.

Complexes are formed by mixing the liposomes and siRNA at a charge ratio where there is an excess of positive charge. We prepared complexes over a range of charge ratios (1:1-6:1, N/P) to investigate the extent of association as measured by the presence of free siRNA when the L/siRNA is run on a 1% agarose/Tris-borate-EDTA (TBE) gel (Fig. 17.1). Incorporation of PEG into the liposome prevents complete association of siRNA when used at comparable charge ratios as clearly seen by the presence of free siRNA at the 3:1 charge ratio. When complexed at a 4:1 charge ratio, siRNA is completely associated with the liposomal surface in the absence of PEG, however, where PEG is present there is varying amounts of detectable siRNA.

The volume of the complex will depend on the downstream application. Samples prepared for electrophoresis should have a sufficiently high siRNA concentration in a volume compatible with gel loading to ensure siRNA detection.[3]

3.6. PEGylated liposome preparation for encapsulation of siRNA using the lipid film hydration method

1. DOTAP, cholesterol, and DSPE-PEG2000 (2:1:0.1) are dissolved in chloroform:methanol (4:1, v/v). The organic solvent is evaporated

[3] *Agarose gel electrophoresis*: To visualize the siRNA component of the complex, in addition to any free, noncomplexed siRNA, complexes are subjected to agarose gel electrophoresis. Agarose gels are prepared using 1% (w/v) agarose in TBE buffer. Samples are prepared such that they have the same final siRNA concentration of at least 500 ng to facilitate visualization by ethidium bromide staining. Ethidium bromide (EtBr) intercalates the minor groove of nucleic acids and fluoresces when exposed to UV light. EtBr is added to the gel prior to casting at a final concentration of 0.5 μg/ml. Samples are mixed with the appropriate volume of loading dye immediately prior to loading into the well. The gel is then run for 45 min at 70 V. Images are captured using the G:BOX system with Gene Snap software.

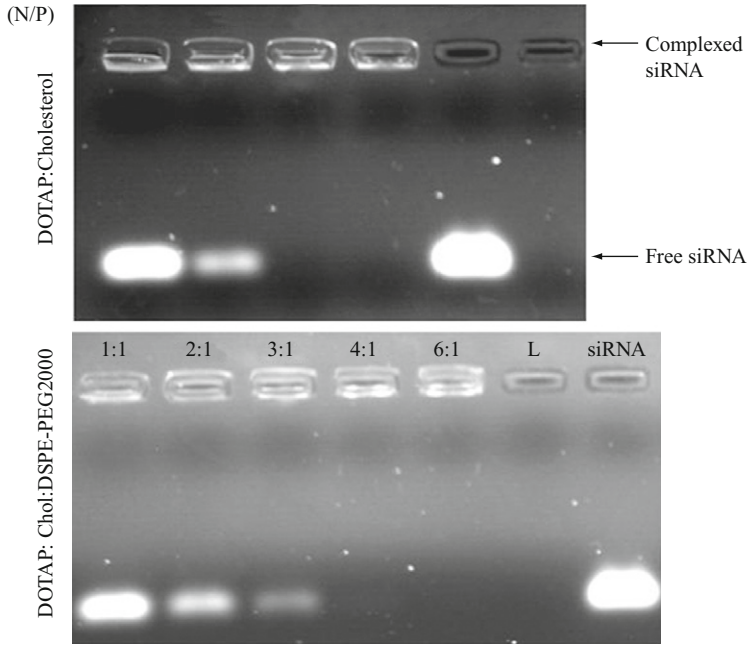


Figure 17.1 Complexation studies of DOTAP:Cholesterol/siRNA (top) and DOTAP: Chol:DSPE-PEG2000/siRNA (bottom). Liposome/siRNA complexes are formed at the charge ratio (N/P) indicated and incubated for 30 min at room temperature before loading onto 1% agarose/TBE gel. Charge ratios are calculated for fixed siRNA concentration of 2 μg. L, liposome alone. The presence of PEG on the liposome interferes with siRNA complexation.

under pressure at 40 °C for 30 min and the lipid film is flushed with N_2 to remove residual solvent.

2. The lipid film is hydrated using a solution of siRNA in 5% dextrose (w/v) prepared using RNase-free dH_2O. The amount of siRNA used to hydrate the film is calculated from the charge ratio.
3. Size reduction is performed as with conventional liposomes: sonication in a waterbath sonicator for 1 min, followed by extrusion through a 0.2 μm Anotop 10 filter (Whatman, UK). The PEGylated liposome/siRNA solution is then incubated at room temperature for a minimum of 30 min to allow stabilization. The complex should be maintained in a sterile environment for subsequent gene silencing experiments.
4. Liposome size (z-average diameter) and surface charge (zeta potential) are measured as before (Table 17.1).

Table 17.1 Size and surface charge of liposomes (DOTAP:Chol and DOTAP:Chol: DSPE-PEG) and liposome/siRNA complexes formed at 2:1 and 4:1 charge ratios using the mixing protocol and at 4:1 for the PEGylated liposome/siRNA complexes formed using the encapsulation protocol

Mixing protocol	N/P	Mean diameter (nm)	PdI	Zeta potential (mV)
DOTAP:Chol (2:1)	n/a	128.00	0.22	46.00
DOTAP:Chol/siRNA	2:1	832.33	0.28	38.00
DOTAP:Chol/siRNA	4:1	255.67	0.19	44.50
Mixing protocol				
DOTAP:Chol:DSPE-PEG2000 (2:1:0.1)	n/a	146.67	0.20	43.70
DOTAP:Chol:DSPE-PEG2000/siRNA	2:1	1946.67	0.56	9.19
DOTAP:Chol:DSPE-PEG2000/siRNA	4:1	203.33	0.14	17.10
Encapsulation protocol				
DOTAP:Chol:DSPE-PEG2000 (2:1:0.1)	4:1	247.67	0.44	25.63

Size is given as the mean diameter (nm) of at least 30 measurements, and the polydispersity index (PdI). Surface charge is the mean zeta potential (mV) for at least 45 measurements.

3.7. *In vitro* gene silencing

3.7.1. Day 0

- Cells are seeded in a multiwell plate in antibiotic-free media.
- The plate is then incubated overnight at 37 °C, 5% CO_2, in a humidified chamber.

3.7.2. Day 1

- Cells should be 30–50% confluent at the time of transfection.
- Cells are transferred to 2% serum-containing media without antibiotics and then incubated.

3.7.3. Mixing protocol

Fresh complexes are prepared for cell transfection studies using aseptic techniques throughout.

- Final siRNA concentration following dilution onto the cells needs to be determined empirically for each siRNA sequence. We suggest starting in the range 20–100 n*M*.
- Liposomes are diluted into prewarmed (25 °C) serum-free medium, such as Opti-MEM or Advanced RPMI (Invitrogen, UK) that are specifically

formulated for use without serum or with low serum concentrations, and equilibrated for 5 min.
- siRNA is thawed on ice and then diluted using RNase-free water.
- This is then mixed by adding the siRNA to the liposome by rapid pipetting and the mixture is incubated for 20 min at room temperature.

3.7.4. Mixing protocol and encapsulation protocol

- The complex is added dropwise to cells.
- Four to six hours after the addition of the complex, an equal volume of fresh media, which is supplemented with 18% serum, is added to the cells. The final serum concentration achieved is 10%. The mixture is then incubated overnight.

3.7.5. Day 2

- The medium is replaced by fresh growth medium (10% serum).

3.7.6. Assessing gene silencing

RNA interference is a posttranscriptional gene silencing phenomenon, and as such, knockdown of mRNA transcripts should be assessed by real time RT-PCR. Detection of the targeted protein by Western blot or immunohistochemistry can indicate gene silencing; however, factors such as the stability of the protein and the lack of direct correlation between mRNA and protein abundance could lead to false-negative results. Silencing of a gene with a quantifiable function is much more informative than measuring protein alone. The extent and duration of silencing are variable, therefore a high degree of mRNA degradation may need to occur before a biological effect is evident, and conversely a transient reduction of only a small amount of mRNA may have a profound downstream effect (Fig. 17.2). In this example (Fig. 17.2), the siRNA used is targeted to the Polo-like kinase 1 (Plk1) gene. Plk1 is a regulator of the cell cycle and regulated mitotic progression and as such has become an interesting target for anti-cancer therapy using small molecule inhibitors, antisense and siRNA (Judge *et al.*, 2009). Furthermore, previous comparison with non-targeting or negative siRNA has validated the specificity of the Plk1 siRNA for inducing cell death.

4. Troubleshooting

Preparation of complexes between liposomes and siRNA can result in localized precipitation, whereby the solution becomes turbid and the particles come out of solution. To prevent this, the siRNA solution is added to

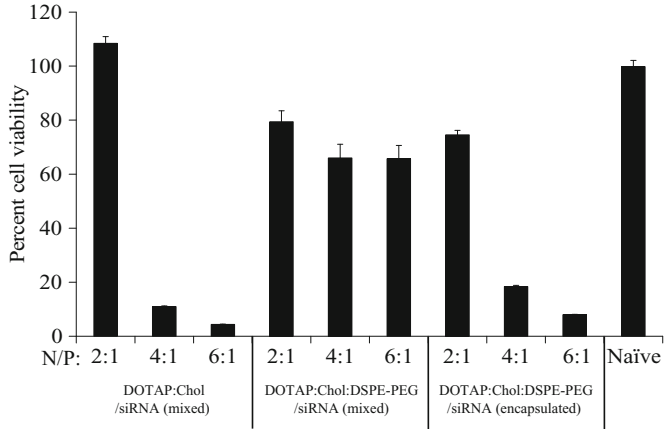


Figure 17.2 **Cell viability of HeLa cells transfected with siPlk1 using: DOTAP:Chol liposomes mixed with siRNA; DOTAP:Chol:DSPE-PEG2000 (5 mol%) liposomes mixed with siRNA; or DOTAP:Chol:DSPE-PEG2000 (5 mol%) liposomes hydrated with siRNA using the encapsulation protocol. Liposome/siRNA complexes are prepared at N/P 2:1, 4:1, 6:1 with a final siRNA concentration of 50 n*M*. Cell viability is assessed by the MTT assay 48 h posttransfection.**[4]

the liposomes, not vice versa and rapid mixing is used. Moreover, the presence of particulate complexes can have a cytotoxic effect independent of specific siRNA activity.

5. Concluding Remarks

Cationic liposomes are able to act as delivery systems for siRNA in much the same way as they have been used for plasmid DNA in gene therapy studies. As the field of RNAi expands, new synthetic siRNA molecules are being designed to improve their efficacy and stability as well as reduce off-target effects and immune stimulation. Incorporation of PEG into the lipid bilayer offers several advantages to the vector as discussed here. The problem of reduced siRNA affinity is easily overcome when using the encapsulation method for lipid film hydration without

[4] *MTT assay*: The colorimetric MTT assay is used to measure cell viability. Yellow (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced by mitochondrial reductase in metabolically active cells to produce purple formazan. Samples can then be measured spectrophotometrically at wavelengths of 550–600 nm, and reduction of cell viability caused by apoptosis or necrosis can be measured by comparison to the control. Stock MTT solution is prepared at 5 mg/ml in PBS, sterile filtered and stored at − 20 °C. MTT is diluted 1:6 (v/v) into the cell culture medium before addition to cells in 96-well plates at a final volume of 120 μl. Cells are incubated for up to 4 h at 37 °C, 5% CO_2. The MTT solution is removed and 200 μl DMSO is added to each well to solubilize the formazan before absorbance at 560 nm is read on a microtiter plate reader.

compromising biological activity. Moreover, this method provides a platform for developing actively targeted liposomes by addition of targeting sequences to PEG.

REFERENCES

Allen, T. M. (1994). Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *Trends Pharmacol. Sci.* **15,** 215–220.

Campbell, R. B., *et al.* (2009). Fighting cancer: From the bench to bedside using second generation cationic liposomal therapeutics. *J. Pharm. Sci.* **98,** 411–429.

Castanotto, D., and Rossi, J. J. (2009). The promises and pitfalls of RNA-interference-based therapeutics. *Nature* **457,** 426–433.

Cho, W. G., *et al.* (2009). Small interfering RNA-induced TLR3 activation inhibits blood and lymphatic vessel growth. *Proc. Natl. Acad. Sci. USA* **106,** 7137–7142.

de Fougerolles, A., *et al.* (2007). Interfering with disease: A progress report on siRNA-based therapeutics. *Nat. Rev. Drug Discov.* **6,** 443–453.

Elbashir, S. M., *et al.* (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411,** 494–498.

Immordino, M. L., *et al.* (2006). Stealth liposomes: Review of the basic science, rationale, and clinical applications, existing and potential. *Int. J. Nanomed.* **1,** 297–315.

Janssen, A. P., *et al.* (2003). Peptide-targeted PEG-liposomes in anti-angiogenic therapy. *Int. J. Pharm.* **254,** 55–58.

Judge, A. D., Robbins, M., Tavakoli, I., Levi, J., Hu, L., Fronda, A., Ambegia, E., McClintock, K., and MacLachlan, I. (2009). Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J Clin. Invest.* **119,** 661–673.

Kim, S. I., *et al.* (2007). Systemic and specific delivery of small interfering RNAs to the liver mediated by apolipoprotein A-I. *Mol. Ther.* **15,** 1145–1152.

Lewis, D. L., and Wolff, J. A. (2007). Systemic siRNA delivery via hydrodynamic intravascular injection. *Adv. Drug Deliv. Rev.* **59,** 115–123.

Meister, G., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* **431,** 343–349.

Mintzer, M. A., and Simanek, E. E. (2009). Nonviral vectors for gene delivery. *Chem. Rev.* **109,** 259–302.

Morrissey, D. V., *et al.* (2005). Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs. *Nat. Biotechnol.* **23,** 1002–1007.

Ramesh, R., *et al.* (2001). Successful treatment of primary and disseminated human lung cancers by systemic delivery of tumor suppressor genes using an improved liposome vector. *Mol. Ther.* **3,** 337–350.

Stephenson, S. M., *et al.* (2004). Folate receptor-mediated targeting of liposomal drugs to cancer cells. *Methods Enzymol.* **387,** 33–50.

Thurston, G., *et al.* (1998). Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. *J. Clin. Invest.* **101,** 1401–1413.

Whitehead, K. A., *et al.* (2009). Knocking down barriers: Advances in siRNA delivery. *Nat. Rev. Drug Discov.* **8,** 129–138.

Zhang, Y., *et al.* (2008). *In vivo* comparative study of lipid/DNA complexes with different *in vitro* serum stability: Effects on biodistribution and tumor accumulation. *J. Pharm. Sci.* **97,** 237–250.