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How to make siRNA lipoplexes efficient? Add a DNA cargo

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ABSTRACT

Background: We recently reported an efficient formulation of siRNA targeting TNF-α, that was able to restore immunological balance in a mouse arthritis model following intravenous injection.
Method: Since this efficient formulation included the pre association of siRNA with a DNA cargo, we decided to extensively characterise siRNA lipoplexes with or without DNA cargo, in order to better understand the DNA cargo enhancing effect.
Results: We showed that addition of DNA cargo to siRNA lipoplexes led to specific gene extinction *in vitro*, using reduced siRNA concentration. This procedure is also applicable to other lipid vectors, like Lipofectamine or DMRIE-C. No structural modification could be observed in siRNA lipoplexes upon addition of DNA cargo using dynamic light scattering or transmission electronic microscopy. Nevertheless, we observed some slight differences, in the amount of lipid required to obtain neutrality of the complex and in stability of the complex towards incubation with heparan sulfate.
Conclusions: These results suggest that the addition of DNA cargo to siRNA complexes is an easy procedure that leads to more efficient complexes to transfer siRNA at low concentration and in the presence of serum.

1. Introduction

RNA interference (RNAi) has recently emerged as a powerful tool to specifically reduce the expression of any chosen gene, hence offering new promising developments of nucleic acid based therapeutics. RNAi was first described [1] in the nematode worm *Caenorhabditis elegans* as a multi-step post-transcriptional mechanism of gene silencing produced by long double-strand RNAs, which involves the generation of 21–23 nt small interfering RNA (siRNA) and results in the degradation of the homologous RNA. Subsequently, this sequence-specific gene extinction by dsRNA was observed and experimentally demonstrated in mammalian cells [2]. Two main strategies coexist to generate and administrate these short dsRNAs. First, short hairpin RNAs (shRNAs) (reviewed in [3]) can be endogenously expressed using DNA expression cassettes delivered to cells, using the same vectors as the one used for gene transfer, including viruses. This approach allows for stable intracellular expression of shRNAs, which are then processed into active siRNAs by the host cell and is particularly indicated for applications that require a gene to be silenced for a prolonged period of time. Precise control of the intracellular level of siRNA, as well as capacity to turn off its production when treatment is no longer necessary, represent two major challenges to this use of shRNA. The second strategy is the direct delivery of synthetic siRNAs. As gene silencing induced by synthetic siRNAs is limited by the number of RNA molecules present into a cell, siRNA-induced gene silencing is a transitory phenomenon. Gene silencing with siRNA has become the method of choice for mammalian cell genetic analysis and has strong potential for the therapeutic treatment for a variety of diseases (reviewed in [4]). Indeed, the intrinsically transient nature of siRNAs makes them more amenable to disease treatments in which the treatment is given over a period of time and then stopped once the desired therapeutic outcome is achieved, such as regression of a tumour or inhibition of viral growth, making its use relatively similar to more classical drugs.

The main obstacle for developing siRNA as a small-molecule drug is to deliver it *in vivo* to tissues and across the cell membrane to the cytoplasm, where it can enter the RNAi pathway and guide the sequence-specific mRNA degradation. In the absence of transfection reagents or high pressure that may damage the plasma membrane, most cells do not spontaneously take up siRNAs. Currently, chemically synthesized siRNA is most effectively introduced into cells using electroporation or commercially available cationic lipid vectors, but

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these methods are not widely used for *in vivo* delivery, due to a restricted local application for electroporation and a lack of efficiency or significant non specific effects for cationic lipid vectors. So far, the vast majority of *in vivo* studies employing RNA interference technology have used high doses of non-modified naked siRNAs, usually delivered through hydrodynamic injection [5]. The high siRNA dose needed to achieve *in vivo* efficient silencing raises several concerns related to siRNA non-specific effects on non-targeted genes [6], triggering of immune response ([7] for a review) or anti-angiogenic effect independent of siRNA sequences [8].

Cationic liposome carriers can significantly reduce the necessary siRNA doses for efficient gene silencing by enhancing siRNA stability in the serum and improving cellular uptake [9]. Lipoplexes (cationic lipid/nucleic acid complexes) have been developed to deliver plasmid DNA or RNA through the cellular membrane but these agents allow for little control over the process of their interaction with siRNA, leading to final products of excessive size and low stability [10]. While exhibiting some similarities, plasmid DNA and siRNA present several important differences that may prove crucial in terms of complex formation upon interaction with cationic lipids [11]. Due to their small size, synthetic siRNA molecules are not necessarily subject to the same process of condensation that leads to the collapse of plasmid DNA into nanostructures in the presence of a cationic agent. Instead, siRNA molecules may retain their initial structure after interaction with the positively charged lipid head groups. It is therefore not possible to predict siRNA/lipid interactions based on the previous knowledge of plasmid DNA behaviour. The siRNA-specific properties may lead to the formation of particles that do not allow complete nucleic acid condensation, which in turn can result in degradation by macromolecules present in the extracellular environment and consequently in low silencing efficiency.

We recently reported an efficient formulation of siRNA into lipoplexes that was able to deliver a siRNA targeting TNF-α following intravenous injection, thereby restoring immunological balance in the mouse collagen-induced arthritis model [12]. This formulation included the pre association of siRNA with a plasmid DNA cargo. Whereas TNF-α siRNA injected alone or complexed alone in a lipoplex confer mild or intermediate protection against arthritis, protection was complete with the cargo formulation. This DNA cargo enhancing effect was later confirmed in cellular assays [13]. In the current study, we extensively characterised siRNA lipoplexes with or without DNA cargo, in order to achieve a better understanding of the DNA cargo enhancing effect.

2. Materials and methods

2.1. Cell culture

Mouse melanoma (B16-F0, ATCC Number: CRL-6322) cells from LGC Promochem were grown in DMEM with Glutamax (Fisher Bioblock), 10% foetal calf serum, streptomycin (100 μg/ml) and penicillin (100 U/ml), and passaged twice weekly by trypsinization. B16-Luc and B16-GFP were obtained by stably incorporation into the genome of B16-F0 cells the firefly luciferase gene (Luc+, pGL3, Promega) or the jellyfish *Aequorea victoria* GFP gene (EGFP, Clontech) respectively, under the control of a cytomegalovirus or a simian virus promoter, respectively. Cells were modified by electroporation and selected by their resistance to geneticine (2 mg/ml, Invitrogen). A clonal cell line with stable constitutive expression of luciferase or GFP was obtained by limit dilution of geneticine resistant cells.

2.2. siRNAs and plasmids

siRNAs were purchased from Qiagen or Eurogentec (see Table 1 for details). siRNAs from Eurogentec were already hybridized, while siRNAs from Qiagen were hybridized following supplier's recommendations. Plasmids used as cargo contained no eukaryotic expression cassette and were purified on Qiagen columns.

2.3. Preparation of lipoplexes

Cationic lipid RPR209120 was synthesized as described [14]. RPR 209120 2-{3-[Bis-(3-amino-propyl)-amino]-propylamino}-N-ditetradecyl carbamoyl methyl-acetamide (Fig. 1) and DOPE (dioleyl phosphatidyl ethanolamine; Avanti Polar Lipids), were dissolved in chloroform and mixed in equimolar amounts. The organic solvent was evaporated under vacuum at 20 °C using a Heidolph rotating-dessicator to form a thin film. The dried film was then hydrated for 24 h with ppi water at 20 °C to produce large multilamellar vesicles. The suspension was finally sonicated (115 V, 80 W, 50–60 Hz) with a G112SP1G model sonicator (Laboratory Supplies Co. Hicksville, N.Y.) to obtain a homogeneous suspension of liposomes with a diameter of 80–100 nm as measured by light scattering (Zetasizer, Nanoseries, Malvern) as described [15]. Fluorescent lipoplexes were obtained by incorporating Rhodamine-labelled DOPE in liposomes (5 mol% of total lipid). Lipofectamine and DMRIE-C were from GibcoBRL. RPR120535

Table 1
Sequences of siRNAs directed against various genes

Gene target	siRNA		Supplier	Sequence of primers used for q-PCR
	Name	Sequence (sense strand)		
MMP9	MMP9-1	ACCGAGCTATCCACTCATCAA	Qiagen	Up CCCTCTGAATAAAGACGACATAG Low GGTATAGTGGGACACATAGTGG
	MMP9-2	CCCACTTACTATGGAAACTCA	Qiagen	
IGFR2	IGFR2-1	GCACCAGGAUCCAUCUUGU	Eurogentec	Up TGAAGATCGTGGGTGTGAGG Low GTGAGGGATGAGAGCAGTCG
	IGFR2-2	GAGCUAUGAUGAAUGUGUA	Eurogentec	
	IGFR2-3	GGAAACUCGUACUUUAUCA	Eurogentec	
	IGFR2-4	GGAUGUCUGCGAUCAUAUU	Qiagen	
	IGFR2-5	GCAUGGUGUCAGAAGACAA	Qiagen	
M6PR	M6PR-1	GACACAUACAGCUACAUAU	Eurogentec	Up TCTGAGGAACGAGGCAAAGTC Low AACCCCCAATGATATAGACAGCA
	M6PR-2	GGAUCUUGGCAACCUAGUA	Eurogentec	
	M6PR-3	CGAGGCAAAGUCCAGGAUU	Eurogentec	
	M6PR-4	CACUGGAUACUCUAAUUAA	Qiagen	
	M6PR-5	GGAUCAUGCUGAUAUAUAA	Qiagen	
Luc	Luc	CUUACGCUGAGUACUUCGA	Qiagen	
MMP12	MMP12	CTGCAGCATTCCAATAATCCA	Qiagen	
	CTLE	UUCUCCGAACGUGUCACGU	Qiagen	

siRNAs are composed of two complementary strands, 19 unmodified RNA bases plus 2×3′-DNA overhang bases, usually dTdT, except for siRNA IGFR2-4, antisense strand dAdG and siRNA M6PR-5 antisense strand dAdA.