Published in final edited form as:

Biomaterials. 2010 April ; 31(11): 3079–3086. doi:10.1016/j.biomaterials.2010.01.006.

Mechanisms of Nucleotide Trafficking during siRNA Delivery to Endothelial Cells using Perfluorocarbon Nanoemulsions

Megan M. Kaneda,

Biomedical Engineering, Washington University School of Medicine, St. Louis, MO, 63108, USA

Yo Sasaki,

Genetics, Washington University School of Medicine, St. Louis, MO, 63108

Gregory M. Lanza,

Medicine, Biomedical Engineering, Washington University School of Medicine, St. Louis, MO 63108, USA

Jeffrey Milbrandt, and

Pathology and Immunology, Neurology, Washington University School of Medicine, St. Louis, MO, 63108, USA

Samuel A. Wickline*

Medicine, Biomedical Engineering, Physics, Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO, 63108, USA

Abstract

RNA interference (RNAi) is a useful *in vitro* research tool, but its application as a safe and effective therapeutic agent may benefit from improved understanding of mechanisms of exogenous siRNA delivery, including cell trafficking and sorting patterns. We report the development of a transfection reagent for siRNA delivery which employs a distinctive non-digestive mode of particle-cell membrane interaction through the formation of a hemifusion complex resulting in lipid-raft transport of cargo to the cytosol, bypassing the usual endosomal nanoparticle uptake pathway. We further demonstrate markedly enhanced efficacy over conventional transfection agents for suppressing endothelial cell expression of upregulated vascular adhesion molecules.

Introduction

RNA interference (RNAi) is a powerful basic research tool used for understanding how gene expression induces morphological cellular responses. However, despite much recent progress in the field[1-3], its therapeutic promise has been difficult to realize clinically in the face of challenges of efficient and specific cell delivery methods. RNAi is a post transcriptional gene silencing pathway induced by the presence of double stranded RNA[4]. In mammalian cells, RNAi can be induced by exogenous short interfering RNA (siRNA), which incorporates intracellularly into the RNA induced silencing complex (RISC) and mediates sequence specific mRNA degradation[5,6]. The introduction of exogenous siRNA into the cytosol is the focus

*Corresponding Author: Samuel A Wickline, Professor of Medicine, Biomedical Engineering, Physics. CTRAIN Group, Washington University School of Medicine, Campus Box 8215, 660 South Euclid Ave., St. Louis, MO 63110. Fax: 314-454-5265 saw@wuphys.wustl.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of many non-viral transfection methods, and while viral mediated transfection is typically more effective, reports of severe off-target effects including death suggest the need for caution in the design and use of viral based delivery vehicles and nucleotide cargo[7-10].

Standard liposomal transfection reagents undergo clathrin-mediated endocytosis in diverse cell types[11-14]. The specific internalization process may depend in part on particle size[15], composition[11], and can be influenced by varying membrane composition among cell types [16]. In this process, clathrin coated pits are pinched off from the plasma membrane to form intracellular clathrin coated vesicles. These vesicles eventually fuse inside the cell to form endosomes and ultimately lysosomes. The active cargo delivered through this form of endocytosis must then escape from these vesicles before unavoidable reductions in pH elicit cargo degradation. (For a review of all types of endocytosis see reference [17].)

We now report the development of a nanoparticle based siRNA delivery system, which is independent of clathrin trafficking but instead relies on lipid raft-mediated internalization. As compared to clathrin-dependent endocytosis in general, lipid raft transport is less well studied, yet appears to represent a non-digestive mode of internalization that is utilized extensively by endothelial cells for cargo transport[18-21]. Thus, if able to transport siRNA, lipid raft mediated endocytosis may represent an attractive delivery strategy for cytoplasmic deposition of siRNA without the need for an endosomal escape strategy.

Previous experiments in our lab have illustrated the use of molecularly targeted perfluorocarbon nanoparticles (PFC-NP) as a drug delivery vehicle both *in vitro*[22-24] and *in vivo*[25-28], but whether the same agent might be efficacious as a delivery system for nucleotides has not been shown. This nanoparticle platform is capable of multifunctional ligand/drug interactions including peptide[24], small molecule[25-28], biological[23] and antibody cargos[29,30] and thus may represent an economical platform for polyvalent targeting and multiplexed therapeutics. The unique mechanism governing interaction between the target cell and the nanoemulsion vehicle operates through the formation of a hemifusion complex that has been previously demonstrated[24,31,32], which initiates a generalized paradigm of cytoplasmic cargo delivery employing natural membrane lipid raft kinetics and trafficking. In this report, we propose to use this non-classical pathway for nanoparticle based delivery of siRNA. We anticipate that this non-classical method of internalization might aid in the rational design of future transfection reagents by taking advantage of the cells own natural cargo internalization and trafficking machinery for cytosolic nucleotide delivery.

Materials and Methods

Nanoparticle Formulation

Liquid perfluorocarbon nanoparticle emulsions were formulated using methods previously developed in our laboratory[22] with the cationic lipid 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP, Avanti Polar Lipids, Alabaster, AL) comprising 65% of the lipid mixture, 25% 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids) and cholesterol comprising the remaining surfactant. Briefly, the surfactant components, perfluorocarbon, and water were sonicated and emulsified at 20,000 PSI for 4 min in an ice bath (S110 Microfluidics emulsifier, Microfluidics, Newton, MA). For fluorescent detection of nanoparticles, 0.22% 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine-N-(Carboxyfluorescein) (Avanti Polar Lipids) was added to the surfactant layer.

Transfection Complex Sizing and siRNA Loading

To determine particles size and siRNA loading, transfection complexes were formed by incubating PFC-NP with siRNA (siGENOME VCAM-1 siRNA2, Dharmacon, Lafayette, CO)

for 40 min at room temperature in phosphate buffered saline. Particle size was measured using light scattering analysis on a Zeta Plus particle sizer(Brookhaven Instruments, Newton, MA). Unbound siRNA was removed from transfection complexes by centrifugation and run on a 5% agarose gel. siRNA quantity was measured by densitometry using the image analysis software ImageJ(NIH, Bethesda, MD). siRNA loading was calculated from the quantity of free siRNA and an siRNA standard curve. Loading charge ratio was calculated from bound siRNA with a negative charge of 42 per molecule and 304,421 positive charges per PFC-NP.

Cell Culture

Mouse endothelial 2F-2B cells (ATCC, Manassas, VA) were maintained under standard cell culture conditions at 37°C with 95% air and 5% CO₂ in a humidified chamber. 2F-2B cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1 mM sodium pyruvate (Washington University Tissue Culture Support Center, St Louis, MO). Endothelial cell VCAM-1 upregulation was achieved by the addition of 10 ng/ml TNF α (Sigma, St Louis, MO) to the cell culture media for 24h and confirmed by western blot analysis.

siRNA Transfection

Transfections were performed under standard cell culture conditions with passage 3 2F-2B cells. Transfection complexes were formed by incubating 10 pM PFC-NP with 5 nM siRNA (siGENOME VCAM-1 siRNA2 & siCONTROL Non-Targeting siRNA 4, Dharmacon) for 40 min at room temperature in Optimem I(Invitrogen). Transfection complexes were transferred to the cells at 70-80% confluence and incubated at 37°C with 5% CO₂ for 2h. Unbound complexes were removed by washing with PBS and cells were incubated in DMEM with 10% FBS and 1 mM sodium pyruvate for 48 h. mRNA and protein were harvested at 48h post transfection. Viability was assessed after transfection by flow cytometry with the addition of 0.5 uM TO-PRO3 (Invitrogen). Transfections using Lipofectamine2000(Invitrogen) were performed according to the manufactures protocol. Briefly, Lipofectamine2000 was diluted with Optimeml to a concentration of 8.4 μ g/ml and incubated at room temperature for 10 min. The diluted lipid was mixed with either 5 nM or 100 nM siRNA and incubated at room temperature for 40 min before addition to cells. Transfection complexes were added to cells as described for PFC-NP.

Reverse Transcription Quantitative Real-Time PCR

RNA levels were determined from total cell RNA isolated using an RNeasy kit(Qiagen, Valencia, CA) and cDNA was transcribed using MMLV reverse transcriptase (Invitrogen). Gene expression was quantified using SYBR green detection on a 7300 System (Applied Biosystems, Foster City, CA) with gene specific primers (Quantitect Primer Assay, Qiagen). VCAM-1 expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to obtain relative expression values. All data is reported as an average fold change from the control, untreated sample from three separate experiments.

Western Blot Analysis

For protein analysis, treated cells were lysed in RIPA buffer(10 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1.0% IgepalCA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 5% glycerol) with 1 mM PMSF and Complete Protease Inhibitor Cocktail (Roche). Proteins were resolved on a 4-15% linear gradient gel (Biorad) and transferred to a 0.45 uM nitrocellulose membrane (Invitrogen). Immunoblotting was performed with the following primary antibodies: anti-VCAM-1 (1:200 Santa Cruz Biotechnology, Santa Cruz California), anti-GAPDH (1:1500, Santa Cruz Biotechnology). The secondary antibody used was anti-rabbit HRP (1:5000, Santa Cruz Biotechnology) which was developed with ECL Western

Blotting Substrate (Pierce, Rockford IL). Percent knockdown of VCAM-1 was calculated using densitometry from the western blot image with ImageJ(NIH). All data is reported as an average fold change from the control, untreated sample from three separate experiments.

Mechanisms of siRNA Delivery

For confocal analysis of siRNA/nanoparticle trafficking, fluorescently labeled siRNA (siGLO mouse Lamin A/C siRNA, Dharmacon) and/or fluorescently labeled nanoparticles were used to form transfection complexes as described above with the following modifications. Cells were plated in delta T culture dishes (Bioprotech, Bultex, PA) and incubated with 25 pM nanoparticles plus 12.5 nM siRNA for 2 h. After the removal of transfection complexes from the plates, cells were cultured in 2F-2B media under standard conditions for 2h. Cells were fixed in 4% paraformaldehyde at 37°C for 10 min and visualized via confocal microscopy (Zeiss Meta 510, Thornwood, NY).

To determine the endocytic processes that may be involved in cytoplasmic siRNA delivery, experiments were performed at three temperatures (37°C, 4°C and 17°C). Co-localization of fluorescently labeled siRNA with common pathway markers of macropinocytosis (70 kDa dextran-FITC, 2.5 mg/ml, Sigma), clathrin-mediated endocytosis (transferrin-AlexaFluor488, 100 µg/ml, Invitrogen), and lipid raft/caveolae mediated endocytosis (cholera toxin subunit B-AlexaFluor488, 1 µg/ml, Invitrogen) were evaluated via confocal microscopy. Lipid raft staining was performed with cholera toxin subunit B according to the lipid raft labeling kit protocol (Invitrogen) which includes incubation at 4°C to maintain staining on the cell membrane and prevent internalization of the marker.

Results

Perfluorocarbon Vehicle Physical Characterization

To immobilize siRNA on PFC-NP, liquid perfluorocarbon nanoparticle emulsions were formulated by methods previously developed in our laboratory[22] with the use of the cationic lipid 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) comprising 65% of the lipid mixture, and with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 25%) and cholesterol comprising the remaining surfactant components of the lipid monolayer. PFC nanoparticles exhibited an average diameter of 296.9 ± 4.9 nm (average $\pm$ standard error), and an average of 304,421 positive charges per nanoparticle (DOTAP valence = +1). To visualize the lipid membrane of the perfluorocarbon vehicle, a lipid with a fluorescently labeled head group (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(carboxyfluorescein)) was incorporated into the surfactant layer (Figure 1).

Transfection complexes were formed through electrostatic interactions between the negatively charged phosphate backbone of the siRNA and the positive head-group of the DOTAP lipid within the surfactant layer of the nanoparticles (Table 1). Transfection complex diameter as measured by dynamic light scattering exhibited stable particle sizes at the lower bulk concentrations of siRNA (Table 1), while siRNA loading as measured by densitometry revealed increased siRNA loading with increasing bulk siRNA concentration. The optimization of particle loading versus stability also mandates consideration of the fact that a net positive particle charge would favor interactions with the negatively charged constituents of the cell membrane, including glycosaminoglycans and proteoglycans, which could promote cargo delivery[33-38]. Towards this end, a transfection complex “charge ratio” was calculated from the average number of positive charges per particle (valence = +304,421) and the siRNA loading of each condition. The transfection complex size appears reasonably stable for loading conditions which retain a net positive particle charge (Table 1, loading ratio > 1), so further experiments were carried out with 10 pM nanoparticles plus 5 nM siRNA.

Nanoparticle based siRNA delivery

A fluorescently labeled siRNA was incubated with carboxyfluorescein labeled cationic nanoparticles and the resulting transfection complexes were incubated in low serum media under standard cell culture conditions (37 °C/5% CO₂) for 2h to delineate the interaction of transfection complexes with mouse 2F-2B endothelial cells. Unbound particles were removed by vigorous washing, and media containing 10% serum was added to the cells. When observed immediately after the adding the serum containing media, both the siRNA (red) and nanoparticle (green) labels were co-localized at the cell membrane with little intracellular fluorescent signal apparent (2h, Figure 2). This behavior accords with the previously described spontaneous formation of cell-nanoparticle hemifusion complexes which occur without the expenditure of energy[31,32]. By 4h after transfection, intracellular colocalization of siRNA and nanoparticles was apparent (Figure 2), indicating internalization of the transfection complexes.

The delivery of functional siRNA was defined for both mRNA and protein levels using SYBR green real time PCR and western blot analysis respectively. In these experiments, 2F-2B cells were treated for 2h with 10 pM cationic nanoparticles complexed with 5 nM of either an siRNA to VCAM-1 or an irrelevant siRNA, followed by removal of the transfection reagents and subsequent incubation in cell culture media for 48 hours. Cells treated with VCAM-1 siRNA exhibited a significant decrease in VCAM-1 mRNA levels after 48h to 28.1 ± 6.7 % of the baseline level as compared with cells without treatment and for cells treated with the irrelevant siRNA (Figure 3). At 48h post-incubation, corresponding VCAM-1 protein levels also manifested a significant decrease to 14.8 ± 0.7 % of baseline versus untreated cells or irrelevant siRNA.

Cellular viability was defined by measurements of membrane permeability after the addition of the dye TOPRO3. Cationic nanoparticles loaded with 5 nM VCAM-1 siRNA demonstrate a small, but significant decrease in viability vs. no treatment ($87.9 \pm 1.9\%$ vs. $96.0 \pm 1.0\%$). However, this effect does not appear to reflect an off-target siRNA effect, but more likely the cationic composition of the nanoparticles because both VCAM-1 and irrelevant siRNA produced similar small changes in viability (Figure 3). We have previously tested non-cationic targeted nanoparticles that exhibit a net negative charge (zeta potential = -34 mV in water [27]) and observed no significant effect on viability[22]. Thus, we postulate that the cationic lipid composition of the siRNA binding vehicles elicits a mild effect on viability *in vitro* under conditions of nanoparticle excess, which is consistent with the prior reports of cationic lipid effects *in vitro*. Interestingly, a protective effect on cell viability for the siRNA-complexed cationic nanoparticles was observed versus the cationic particles alone (Figure 3).

Transfection reagent comparison

For comparative transfection analysis, the commercially available transfection reagent Lipofectamine2000 was used to transfect 2F-2B cells with VCAM-1 siRNA. The levels of VCAM-1 mRNA determined by real time PCR measured 48h after transfection indicate that two Lipofectamine2000 transfection conditions manifested significantly higher VCAM-1 mRNA levels than did cells treated with siRNA-nanoparticles (Figure 4, relative to baseline: $90.9 \pm 1.4\%$ with 5 nM siRNA + Lipofectamine2000, and $70.3 \pm 5.7\%$ with 100 nM siRNA + Lipofectamine2000, and $28.0 \pm 6.7\%$ with 5 nM siRNA + PFC-NP). However, the preparations of Lipofectamine2000 used in these experiments were sufficient to decrease the LaminAC mRNA levels of C32 (human melanoma) cells to 47.7% and 32.0% when complexed to 5 nM and 100 nM LaminAC mRNA respectively, which is consistent with other reports of Lipofectamine2000 efficacy in non-endothelial cell types (Kaneda et al. unpublished results). Accordingly, the PFC delivery system functions substantially better than does Lipofectamine2000 for endothelial cell transfection (Figure 4).

The diameters of the Lipofectamine2000 transfection complexes measured with dynamic light scattering revealed that under all of the experimental conditions the Lipofectamine2000 complexes were significantly larger ($p < 0.05$) than the nanoparticles alone, or nanoparticles with 5 nM siRNA (Figure 4). The nanoparticle based transfection complexes were on the scale of hundreds of nanometers, while the Lipofectamine2000 complexes were tens of micrometers. We note that the size of the Lipofectamine2000 transfection complexes is standard for these types of lipid systems, and that it has been shown that particle size can be sufficient to affect the eventual mode of endocytic uptake[15]. The difference in size of these complexes represents one source of the differing interactions with the cell membrane and subsequent downstream mechanisms of intracellular trafficking and transfection efficiency between the two systems.

Mechanisms of intracellular siRNA transport

To further elucidate the differences between PFC-NP mediated and Lipofectamine2000 mediated siRNA delivery, transfection complexes were added to 2F-2B cells under conditions of broad inhibition of energy requiring processes (4°C) and inhibition of endocytosis (17°C). As compared with standard cell culture conditions (37°C), where both siRNA and nanoparticle lipids were internalized by 4h post transfection, inhibition of endocytic and energy requiring processes inhibits internalization and leads to the accumulation of siRNA and nanoparticle components at the cell membrane (Figure 5). These observations confirm that while active cellular processes do not drive the initial interaction of transfection complexes with the cell membrane, the subsequent internalization of both the siRNA and lipid components of PFC-NP involves an energy dependent transport process.

To define the mechanism of internalization of siRNA, transfection complexes were prepared with fluorescently labeled siRNA (red – DY-547), and 2F2B cells were subjected to co-labeling with selected markers of endocytic pathways (green): macropinocytosis (70 kDa dextran), clathrin mediated endocytosis (transferrin), and lipid raft/caveolae mediated endocytosis (cholera toxin B). In contrast to cationic liposomes or particles, which typically enter cells through clathrin-mediated endocytosis, siRNA delivered with PFC-NP do not co-localize with transferrin (Figure 5), which serves as a common pathway marker for this classical mechanism of endocytosis. Additionally, internalization of PFC-NP/siRNA transfection complexes does not involve macropinocytosis in view of the lack of co-localization of the siRNA with dextran. However, siRNA co-localizes strongly with lipid rafts on the cell membrane, and to a lesser extent with intracellular caveolae, suggesting that PFC-NP mediated siRNA delivery occurs by an energy requiring lipid-raft associated process (Figure 5). This observation implies a distinctive initial lipid raft mediated entry process, although the subsequent downstream intracellular trafficking steps associated with such a mechanism remain to be defined[39]. Additionally, the size of PFC-NP/siRNA complexes employed in these experiments (292.8 nm, Table 1) is consistent with published data in which particles larger than 200 nm are internalized via caveolae[15].

Discussion

The materials and methods reported herein employ a synthetic perfluorocarbon nanoparticle approach to cellular delivery of nucleic acids. Previous experiments in our lab have illustrated the use of molecularly targeted PFC-NP as a drug delivery vehicle both *in vitro*[22-24] and *in vivo*[25-28], but whether the same agent might be efficacious as a delivery system for nucleotides has not been shown. The present data, in concert with other reports on peptide [24,40], small molecule[25-28], biological[23], and antibody[29,30] cargos, suggest that this system is capable of multifunctional ligand/drug interactions and thus may represent a economical platform for polyvalent targeting and multiplexed therapeutics. We speculate that

the unique mechanism governing interaction between the target cell and the vehicle through the formation of hemi-fusional complexes demonstrated previously[24,31,32], which initiates a generalized paradigm of cytoplasmic cargo delivery associated with natural lipid raft kinetics.

In these experiments, we modified the basic PFC-NP platform for delivery of siRNA by doping the lipid monolayer of the nanoparticle with a cationic lipid (Figure 1), which imparted an overall average positive valence of $\sim 300,000$ per particle. Spontaneous interactions between the negatively charged siRNA and the positively charged particles promoted the formation of stable transfection complexes. We evaluated optimizing conditions for loading of siRNA onto the particles by controlling the bulk siRNA concentration in solution (Table 1). The transfection complex appears to be more size stable while the overall charge of the transfection complex remains positive. This characterization is also advantageous for interaction with negatively charged components of the cell membrane[33-38].

The putative lipid vesicle/lipid membrane interaction that occurs when the particles come into contact with the targeted cells has been described for this class of vehicles previously[24,31, 32], although principally for molecularly targeted constructs where binding initiates the formation of a hemifusion stalk that is then driven to completion by the energy stored in the nanoparticle lipid monolayer. Regardless, once interactions with the cell membrane have been established, the siRNA complexed to the cationic lipid anchor is eventually internalized into the cytoplasm (Figure 2), which is necessary for siRNA to engage the RISC complex. Effective siRNA delivery was confirmed both at the mRNA and protein levels for the VCAM-1 siRNA/PFC-NP transfection complexes (Figure 3).

In contrast to the present PFC-NP delivery mechanism, cationic liposome mediated nucleotide delivery relies primarily on clathrin-mediated endocytosis as its main mode of internalization [11-14]. Clathrin-coated vesicles traffic to late endosomes where their contents are subject to lysosomal degradation. Thus, effective nucleotide delivery through this pathway requires an endosomal escape step which must take place before the nucleotides degrade under low pH conditions[20]. Lipid rafts are hydrophobic membrane domains present in large quantities on the plasma membrane of endothelial cells[16,41]. Their contents may not undergo transport to endosomes or lysosomes[19], but instead may be transported directly to the endoplasmic reticulum or golgi[42]. This distinction in vesicular trafficking makes lipid raft mediated endocytosis attractive as a possible non digestive mode of internalization[20].

This unique delivery mechanism far exceeds the capacity of Lipofectamine2000 to deliver siRNA to this specific cell type. This could be a result of the relatively high lipid raft content of endothelial cells resulting in increased transport of cargo through this pathway leading to greater cytoplasmic siRNA versus delivery methods employing clathrin mediated endocytosis. Whether specific molecular targeting would augment delivery efficacy is a subject of current interest.

Conclusions

These data accord with prior reports of negatively charged perfluorocarbon nanoparticle mediating drug delivery by association of lipid raft components on the cell membrane. Subsequent internalization and delivery to the cytosol may proceed without trafficking to the Golgi or endoplasmic reticulum due to minimal intracellular co-localization of transfection complexes that serve as standard markers for this pathway, indicative of the specialized sorting capability of this modified particle. Once interactions with the cell membrane have been established, the siRNA complexed to the cationic lipid anchor is subsequently internalized into the cytoplasm, which is necessary for siRNA functional interactions. Fortuitously, the cells that are often most difficult to transfect (endothelial cells) contain abundant endogenous

machinery for lipid-raft mediated transport, which could render this approach ideal for transfecting vascular targets.

Acknowledgments

We thank Huiying Zhang, MD for assistance with TEM and Jason Gustin, PhD for assistance with real time PCR. This work was supported in part but the US National Institutes of Health Grants (AG013730 to JM, R01 HL073646 and U54 CA119342 to SAW, and R01 NS059302 to GML) and an American Heart Association predoctoral fellowship to MMK (0810144Z).

References

1. Veisheh O, Kievit FM, Gunn JW, Ratner BD, Zhang M. A ligand-mediated nanovector for targeted gene delivery and transfection in cancer cells. *Biomaterials* 2009;30(4):649–657. [PubMed: 18990439]
2. Davis ME. The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. *Mol Pharm* 2009;6(3):659–668. [PubMed: 19267452]
3. Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, et al. RNAi-mediated gene silencing in non-human primates. *Nature* 2006;441(7089):111–114. [PubMed: 16565705]
4. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391(6669):806–811. [PubMed: 9486653]
5. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411(6836):494–498. [PubMed: 11373684]
6. Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* 2002;110(5):563–574. [PubMed: 12230974]
7. Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW, et al. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* 2007;317(5837):477. [PubMed: 17656716]
8. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 2003;302(5644):415–419. [PubMed: 14564000]
9. Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 2003;348(3):255–256. [PubMed: 12529469]
10. Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 2003;80(12):148–158. [PubMed: 14567964]
11. Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol Ther* 2005;12(3):468–474. [PubMed: 15963763]
12. Rejman J, Conese M, Hoekstra D. Gene transfer by means of lipo- and polyplexes: role of clathrin and caveolae-mediated endocytosis. *J Liposome Res* 2006;16(3):237–247. [PubMed: 16952878]
13. Zuhorn IS, Kalicharan R, Hoekstra D. Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. *J Biol Chem* 2002;277(20):18021–18028. [PubMed: 11875062]
14. Wong AW, Scales SJ, Reilly DE. DNA internalized via caveolae requires microtubule-dependent, Rab7-independent transport to the late endocytic pathway for delivery to the nucleus. *J Biol Chem* 2007;282(31):22953–22963. [PubMed: 17562704]
15. Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J* 2004;377(Pt 1):159–169. [PubMed: 14505488]
16. Fra AM, Williamson E, Simons K, Parton RG. Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J Biol Chem* 1994;269(49):30745–30748. [PubMed: 7982998]

17. Doherty GJ, McMahon HT. Mechanisms of endocytosis. *Annu Rev Biochem* 2009;78:857–902. [PubMed: 19317650]
18. Harris J, Werling D, Hope JC, Taylor G, Howard CJ. Caveolae and caveolin in immune cells: distribution and functions. *Trends Immunol* 2002;23(3):158–164. [PubMed: 11864845]
19. Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, Beltram F. Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. *Mol Ther* 2003;8(2):284–294. [PubMed: 12907151]
20. Khalil IA, Kogure K, Akita H, Harashima H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacol Rev* 2006;58(1):32–45. [PubMed: 16507881]
21. Schnitzer JE, Oh P, Pinney E, Allard J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol* 1994;127(5):1217–1232. [PubMed: 7525606]
22. Lanza GM, Yu X, Winter PM, Abendschein DR, Karukstis KK, Scott MJ, et al. Targeted antiproliferative drug delivery to vascular smooth muscle cells with a magnetic resonance imaging nanoparticle contrast agent: implications for rational therapy of restenosis. *Circulation* 2002;106(22):2842–2847. [PubMed: 12451012]
23. Marsh JN, Senpan A, Hu G, Scott MJ, Gaffney PJ, Wickline SA, et al. Fibrin-targeted perfluorocarbon nanoparticles for targeted thrombolysis. *Nanomed* 2007;2(4):533–543.
24. Soman NR, Lanza GM, Heuser JM, Schlesinger PH, Wickline SA. Synthesis and characterization of stable fluorocarbon nanostructures as drug delivery vehicles for cytolytic peptides. *Nano Lett* 2008;8(4):1131–1136. [PubMed: 18302330]
25. Winter PM, Neubauer AM, Caruthers SD, Harris TD, Robertson JD, Williams TA, et al. Endothelial $\alpha(v)\beta_3$ integrin-targeted fumagillin nanoparticles inhibit angiogenesis in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2006;26(9):2103–2109. [PubMed: 16825592]
26. Cyrus T, Zhang H, Allen JS, Williams TA, Hu G, Caruthers SD, et al. Intramural delivery of rapamycin with $\alpha v \beta_3$ -targeted paramagnetic nanoparticles inhibits stenosis after balloon injury. *Arterioscler Thromb Vasc Biol* 2008;28(5):820–826. [PubMed: 18292395]
27. Winter PM, Schmieder AH, Caruthers SD, Keene JL, Zhang H, Wickline SA, et al. Minute dosages of $\{\alpha\}\{\nu\}\{\beta\}_3$ -targeted fumagillin nanoparticles impair Vx-2 tumor angiogenesis and development in rabbits. *Faseb J* 2008;22(8):2758–2767. [PubMed: 18362202]
28. Zhou HF, Chan HW, Wickline SA, Lanza GM, Pham CT. $\{\alpha\}v\{\beta\}_3$ -Targeted nanotherapy suppresses inflammatory arthritis in mice. *Faseb J* 2009;23(9):2978–2985. [PubMed: 19376816]
29. Morawski AM, Winter PM, Crowder KC, Caruthers SD, Fuhrhop RW, Scott MJ, et al. Targeted nanoparticles for quantitative imaging of sparse molecular epitopes with MRI. *Magn Reson Med* 2004;51(3):480–486. [PubMed: 15004788]
30. Morawski AM, Winter PM, Yu X, Fuhrhop RW, Scott MJ, Hockett F, et al. Quantitative “magnetic resonance immunohistochemistry” with ligand-targeted (^{19}F) nanoparticles. *Magn Reson Med* 2004;52(6):1255–1262. [PubMed: 15562481]
31. Partlow KC, Lanza GM, Wickline SA. Exploiting lipid raft transport with membrane targeted nanoparticles: A strategy for cytosolic drug delivery. *Biomaterials* 2008;29(23):3367–3375. [PubMed: 18485474]
32. Soman NR, Baldwin SL, Hu G, Marsh JN, Lanza GM, Heuser JE, et al. Molecularly targeted nanocarriers deliver the cytolytic peptide melittin specifically to tumor cells in mice, reducing tumor growth. *J Clin Invest* 2009;119(9):2830–2842. [PubMed: 19726870]
33. Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc Natl Acad Sci U S A* 1989;86(18):6982–6986. [PubMed: 2780554]
34. Labat-Moleur F, Steffan AM, Brisson C, Perron H, Feugeas O, Furstenberger P, et al. An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Ther* 1996;3(11):1010–1017. [PubMed: 9044741]
35. Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc Natl Acad Sci U S A* 1996;93(22):12349–12354. [PubMed: 8901584]

36. Mounkes LC, Zhong W, Cipres-Palacin G, Heath TD, Debs RJ. Proteoglycans mediate cationic liposome-DNA complex-based gene delivery in vitro and in vivo. *J Biol Chem* 1998;273(40):26164–26170. [PubMed: 9748298]
37. Ruponen M, Ronkko S, Honkakoski P, Pelkonen J, Tammi M, Urtti A. Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes. *J Biol Chem* 2001;276(36):33875–33880. [PubMed: 11390375]
38. Kopatz I, Remy JS, Behr JP. A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. *J Gene Med* 2004;6(7):769–776. [PubMed: 15241784]
39. Kirkham M, Parton RG. Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim Biophys Acta* 2005;1745(3):273–286. [PubMed: 16046009]
40. Moulton KS, Heller E, Konerding MA, Flynn E, Palinski W, Folkman J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation* 1999;99(13):1726–1732. [PubMed: 10190883]
41. Stan RV. Structure and function of endothelial caveolae. *Microsc Res Tech* 2002;57(5):350–364. [PubMed: 12112442]
42. Nabi IR, Le PU. Caveolae/raft-dependent endocytosis. *J Cell Biol* 2003;161(4):673–677. [PubMed: 12771123]

Figure 1.

Cationic perfluorocarbon nanoparticles. Liquid perfluorocarbon based nanoparticles are formulated with the cationic lipid 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) and the fluorescent lipid 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine-N-(Carboxyfluorescein) within the surrounding phospholipid-surfactant monolayer (illustrated in the cartoon and TEM image). Electrostatic interactions between the charged lipids of the nanoparticles and the negatively charged phosphate backbone of siRNA lead to the formation of transfection complexes.

Figure 2. Delivery of fluorescently labeled transfection complexes to 2F-2B mouse endothelial cells. Mouse endothelial cells were treated with 12.5 nM DY-547 fluorescently labeled siRNA (red) and 25 pM cationic perfluorocarbon nanoparticles (green). Confocal micrographs illustrate the interaction of transfection complexes with cells directly after the removal of free transfection complexes (2h), and delivery of siRNA across the plasma membrane 4h after transfection. The white outline (4h) indicates the cell membrane. Scale bar, 10 μ m.

Condition	Live cells (%)
No treatment	96.0 ± 1.0
Cationic Nanoparticles	78.4 ± 2.2 *
Nanoparticles + 5 nM VCAM-1 siRNA	87.9 ± 1.9 *
Nanoparticles + 5 nM Irrelevant siRNA	84.5 ± 1.7 *
Positive control (0.05 % Triton-X100)	0.1 ± 0.001 *

Figure 3.

Effective siRNA mediated VCAM-1 knockdown with perfluorocarbon nanoparticles. Mouse endothelial cells were treated with transfection complexes (10 pM cationic nanoparticles + 5 nM siRNA) for 2h under standard cell culture conditions. 2F-2B cells transfected with 10 pM perfluorocarbon nanoparticles + 5 nM anti-VCAM-1 siRNA show a decrease in VCAM-1 mRNA level ($28.1 \pm 6.7\%$) vs. both irrelevant siRNA ($81.8 \pm 7.3\%$) and no treatment controls (100%). VCAM-1 protein levels at 48h post incubation follow the same trend (VCAM-1: $14.8 \pm 0.7\%$, irrelevant siRNA: $87.7 \pm 4.9\%$ and no treatment control: 100%) (A). Short term membrane permeability (viability) was measured after treatment via flow cytometry with TOPRO3. Loading of perfluorocarbon nanoparticles with siRNA exerts a protective effect on cell viability versus cationic particles alone. * denotes $p < 0.05$ vs. no treatment control using a two tailed student's t-test. Data are presented as average $\pm$ standard error.

Figure 4.

Perfluorocarbon nanoparticle-based siRNA transfection efficiency exceeds that of Lipofectamine2000. 2F-2B cells were transfected with anti-VCAM-1 siRNA under the following conditions: 10 pM perfluorocarbon nanoparticles + 5 nM siRNA (PFC-NP), Lipofectamine2000 + 5 nM siRNA, or Lipofectamine2000 + 100 nM siRNA. VCAM-1 mRNA levels (graphed as average + standard error) determined by real time PCR indicated that the decrease in VCAM-1 mRNA level 48h post incubation with PFC-NP is significantly lower than that under both Lipofectamine2000 conditions (3.3 fold decrease vs. Lipofectamine2000 + 5 nM siRNA and 2.5 fold decrease vs. Lipofectamine2000 + 100 nM siRNA). Transfection complex size for perfluorocarbon nanoparticles and complexes of nanoparticles and siRNA are significantly smaller than those for the Lipofectamine2000 conditions. * denotes $p < 0.05$ vs. perfluorocarbon nanoparticles + 5 nM siRNA using a two tailed students t-test. [†] denotes $p < 0.05$ vs. Lipofectamine2000 alone. Data are presented as average ± standard error.

Figure 5.

Mechanism of perfluorocarbon nanoparticle mediated siRNA transfection. 2F-2B cells were incubated with transfection complexes containing 12.5 nM DY-547 fluorescently labeled siRNA(red) and 25 pM cationic nanoparticles(green) for 2h at either 37°C, 17°C, or 4°C, showing that intracellular siRNA delivery is an energy requiring transport process. For co-localization studies, 2F-2B cells were treated with 12.5 nM DY-547 labeled siRNA(red) and with markers for macropinosomes (70kDa dextran), clathrin pits (transferrin), lipid rafts (CT-B, post stained), and caveosomes(CT-B). siRNA appears to interact with lipid rafts on the cell membrane, and to a more limited extent with caveosomes within the cells, but not with clathrin pits or macropinosomes.

Table 1

Characterization of transfection complexes. Transfection complexes were formed by adding 10 pM perfluorocarbon nanoparticles to the indicated amount of siRNA (siRNA Concentration). Particle diameter as measured by light scattering exhibits an increase in transfection complex size with increasing bulk siRNA concentration. siRNA loading onto nanoparticles is described as number of siRNA molecules per nanoparticle. Nanoparticle siRNA loading increases as the amount of siRNA in solution increases. Transfection complex charge ratio was calculated from the average number of DOTAP molecules per nanoparticle (304,421) and a negative charge of 42 per siRNA molecule.

siRNA Concentration (nM)	Particle Diameter (nm)	siRNA per nanoparticle	Transfection complex charge ratio(+/-)
Nanoparticles only	296.9 ± 4.9	—	—
1	294.2 ± 2.6	764	9.49
5	292.8 ± 2.9	1384	5.24
10	321.4 ± 2.2*	3105	2.33
25	343.8 ± 3.3*	9765	0.74
50	503.4 ± 8.8*	13351	0.54

* denotes $p < 0.05$ vs. nanoparticles alone using a two tailed student's t-test. Particle diameter is reported as average ± standard error.