Massive and Selective Delivery of Lipid-Coated Cationic Lipoplexes of Oligonucleotides Targeted *in Vivo* to Hepatic Endothelial Cells

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Purpose. Previously we reported on massive uptake of liposomes surface-modified with negatively charged aconitylated albumin (Aco-HSA) by liver sinusoidal endothelial cells (EC) *in vivo*. In the present work we applied this principle for the *in vivo* delivery of antisense oligonucleotides (ODN) to these cells.

Methods. Anti ICAM-1 ODN was complexed with the cationic lipid DOTAP and the complex was coated by an excess of neutral lipids including a lipid-anchored poly(ethylene glycol). Aco-HSA was coupled to the coated cationic lipoplexes (CCLs). Plasma disappearance, organ and intrahepatic distribution of Aco-HSA modified CCLs were determined in rats, using [^{3}H]-cholesteryl oleyl ether and ^{32}P-labeled ODN as markers.

Results. The Aco-HSA coupled CCLs were <160 nm in size, contained 1.03 ± 0.35 nmol ODN and 54 ± 18 μg Aco-HSA per μmol total lipid. These CCLs were rapidly eliminated from plasma, about 60% the injected dose of ^{3}H- or ^{32}P-label being recovered in the liver after 30 min. Within the liver, the EC accounted for two thirds of total liver uptake. Control non-targeted CCLs were eliminated very slowly: after 30 min still >90% of the particles was in the blood.

Conclusions. Our results demonstrate efficient targeting of antisense ODN to EC *in vivo*, employing plasma-stable coated cationic lipoplexes, surface modified with negatively charged albumin. 40% of the injected ODN was delivered to the target cells within 30 min.

KEY WORDS: stabilized cationic lipoplexes; ISIS 9125; rat liver endothelial cells; aconitylated human serum albumin; endothelial targeting; scavenger receptor.

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ABBREVIATIONS: 3′ UTR, 3′ untranslated region; ^{3}H-COE, [^{3}H]cholesteryl oleyl ether; Aco-HAS, *cis*-aconitic anhydride modified human serum albumin; CCLs, coated cationic lipoplexes; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; PEG_{2000}–DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; EC, Endothelial cells; HEPES, N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; HNE Buffer, 5 mM N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid], 150 mM NaCl and 0.1 mM EDTA containing Buffer; ICAM-1, intercellular adhesion molecule 1; KC, Kupffer cells; MPB-PE, 1,2-Dioleoyl-*sn*-glycerol-3-phosphoethanolamine-N-[4-(*p*-maleimidophenyl)butyramide]; PC, Parenchymal cells; poly I, polyinosinic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SATA, N-succinimidyl-S-acethylthioacetate; TE-PS/OG, Tris/EDTA buffer containing bovine brain phosphatidylserine and octylglucoside; TL, total lipid; mAB, monoclonal antibody; MDR1, Multidrug restistance gene; P-gp, P-glycoprotein.

INTRODUCTION

Because of their potential to modulate gene expression with high specificity, antisense oligodeoxynucleotides (ODN) represent a promising tool in gene therapeutic strategies (1,2). Despite their potentially powerful action *in vitro*, *in vivo* application of ODN is cumbersome due to a variety of complications or shortcomings, such as plasma instability, poor cellular uptake, hemodynamic toxicity, and a lack of target specificity (3). Nonetheless, several clinical strategies using ODN are currently under investigation. Such ODN are directed toward targets playing a role in cancer, viral diseases, or inflammatory disorders (4). In most studies phosphorothioate-modified ODN are administered, either by repeated injection or continuous infusion (5). Although degradation by plasma nucleases is substantially reduced with phoshorothioate-modified ODN, the use of unformulated ODN leaves many of the complications of *in vivo* use of ODN unresolved (6). It is recognized that there is a need for improved plasma stability and target-specific delivery (7,8). Lipoplexes, formed by complexation of ODN with cationic lipids, were shown to allow greatly enhanced uptake by cells and increased antisense activity *in vitro* (9). However, due to their positive surface charge, lipoplexes *in vivo* tend to interact with plasma components, leading to aggregation and disintegration of the particles. (10,11).

Recently, methods have been described to conceal the positive charges of the lipoplexes by means of a (phospho-) lipid coating (12,13). We adopted one of such methods to construct targeted plasma-stable lipid-coated cationic lipoplexes (CCLs) of a model antisense ODN directed against rat ICAM-1. These CCLs were targeted to scavenger receptors on hepatic sinusoidal endothelial cells by means of covalently coupled aconitylated albumin (Aco-HSA) (14). The particles were intravenously administered to rats and their elimination from the blood compartment, organ distribution and intrahepatic cellular distribution were determined.

MATERIAL AND METHODS

Chemicals

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(*p*-maleimidophenyl)butyramide] (MPB-PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (PEG_{2000}-DSPE), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster AL, USA). [1α,2α(n)-^{3}H] cholesteryl oleyl ether (^{3}H-COE), [γ-^{32}P] ATP and ready-to-go™ T4 polynucleotide kinase were from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Cholesterol (Chol), N-succinimidyl-S-acetylthioacetate (SATA) and *cis*-aconitic anhydride were from Sigma (St. Louis, Missouri). 20-mer phosporothioate oligodeoxynucleotide (ISIS 9125), targeted against 3′UTR rat ICAM-1 mRNA, was a generous gift from ISIS Pharmaceuticals (Carlsbad, California) through Dr. C. F. Bennett. Human serum albumin fraction V (HSA) was ob-

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tained from the Central Laboratory of the Red Cross (Amsterdam, The Netherlands). OliGreen® reagent was purchased from Molecular Probes, Inc. (Leiden, The Netherlands). Pronase (from *Streptomyces griseus*) and Collagenase A were obtained from Roche Diagnostics (Mannheim, Germany). All other chemicals were analytical grade or the highest purified grade available.

Animals

Specified pathogen free (SPF) male Wag/Rij rats (Harlan, Horst, The Netherlands) were kept under standard animal laboratory conditions, and had free access to standard lab chow and water. The studies as presented were consistent with the guidelines set by the National Institutes of Health (NIH publication #85-23, revised 1985) and approved by the local committee for care and use of laboratory animals.

Coated Cationic Lipoplex Preparation

CCLs were prepared according to Stuart and Allen (12) with slight modifications. To 8 μmol of DOTAP in 1250 μl chloroform 2620 μl methanol was added. 400 nmol ISIS 9125 dissolved in 1250 μl H_2O was added to the $CHCl_3$:MeOH mixture forming a monophasic system after vortexing. When appropiate, trace amounts of ISIS 9125 5′end labeled with γ-^{32}P were included. Following 30 min of incubation (room temperature), 1250 μl CHCl3 and 1250 μl H_2O were added. The mixture was vortexed briefly and centrifuged at 900 × g for 7 min to yield a biphasic system from which the upper, aqueous, phase was removed. POPC (24 μmol), Chol (16 μmol), MPB-PE (1.28 μmol) and PEG_{2000}-DSPE (1.6 μmol) were added to the organic phase from $CHCL_3$:MeOH (9:1) stock solutions. When appropriate, trace amounts of ^{3}H-COE were added. Subsequently, 2 ml H_2O was added resulting in a total lipid concentration of 25 mM in H_2O and this mixture was vortexed and sonicated (60 sec) to form a water-in-oil emulsion. The organic phase was concentrated by rotary evaporation under reduced pressure (≥0.5 bar). The initially formed gel phase collapsed, and ultimately reverted into an aqueous phase, which was briefly vortexed (after addition of glass beads). Residual $CHCl_3$ was eliminated by continued evaporation. The particles formed were extruded 41 times through polycarbonate membranes with a pore size of 400, 200, and 100 nm, respectively, using a hand extruder (Avanti Polar Lipids, Alabaster, Alabama).

Coupling of Aco-HSA

Cis-aconitylated human serum albumin (Aco-HSA), with an average of 53 of the 60 available ε-amino groups aconitylated, was prepared as described (15). The aconitylated protein was thiolated by means of N-succinimidyl-S-acetylthioacetate (SATA) and coupled to the MPB-PE in the outer surface of the CCLs by a sulfhydryl-maleimide coupling technique during an overnight incubation at 4ºC (15,16). N-ethylmaleimide, 80 mM in buffer, containing 5 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), 150 mM NaCl and 0.1 mM EDTA (HNE buffer, pH 7.4) was added to cap unreacted sulfhydryl groups. Protein-coupled CCLs were separated from unconjugated protein by OptiPrep® gradient ultracentrifugation. The Aco-HSA coupled CCLs were extensively dialyzed against HNE Buffer, pH 7.4. Control CCLs were prepared similarly but, instead of with Aco-HSA, they were incubated with cysteine in a molar amount twice that of MPB-PE to block reactive maleimido groups on the liposome surface. The Aco-HSA CCLs were characterized by determining protein (17), phospholipid phosphorus and ODN phosphorus content (18) and ODN content by OliGreen® assay. Particle size was determined by dynamic light scattering (Nicomp model 370 submicron particle sizer, NICOMP particle sizing systems, Santa Barbara, California) in the volume weighting mode.

Animal Studies

For *in vivo* studies, pentobarbital anesthetized Wag/Rij rats (200–250 g), were injected with 2 μmol (total lipid) of CCLs labeled either with 0.5 μCi ^{3}H-COE or ^{32}P-ODN via the penile vein. Blood samples were taken from the inferior vena cava. After 30 min, the liver was perfused and processed for measurement of radioactivity as described before (15). Total radioactivity in plasma was calculated according the equation: plasma volume (ml) = [0.0219 × body weight (g)] + 2.66 (19). When indicated, rats were injected with polyinosinic acid (poly I) in the indicated amount 2 min prior to injection with CCLs.

Isolation of Liver Cells

Liver endothelial cells (EC) and Kupffer cells (KC) were isolated after pronase perfusion and digestion of the organ, followed by gradient centrifugation and counterflow centrifugal elutriation as described (20). In addition, parenchymal cells (PC), EC and KC were isolated after collagenase perfusion of the liver (20). Cell numbers in liver suspension and in the cell fractions were determined microscopically and radioactivity in each cell fraction was determined. To calculate the specific radioactivity of each cell type, total cell numbers in each cell population were as follows: PC = (4.50×10^6 / bodyweight [g]), non-parenchymal cells = (1.94×10^6 / bodyweight [g]), EC = ($0.75 \times (1.94 \times 10^6$ / bodyweight [g])) and KC = ($0.25 \times (1.94 \times 10^6$ / body weight (g)), multiplied by cell-associated radioactivity and expressed as ratio = (total liver uptake/cell population specific uptake) × 100 (21).

Oligodeoxynucleotide Quantitation

ODN entrapped in CCLs was quantified by OliGreen® ssDNA assay (Molecular Probes, Leiden, The Netherlands) according to the manufacturers protocol with minor modifications and measured in a microtiterplate fluorescent reader (Bio-Tek Instruments, Winooski, Vermont). To facilitate accessibility of the ODN, CCLs samples were diluted into 10 mM Tris-HCl, 1 mM EDTA buffer (pH 7.5) containing 40 μM PS and 165 nM octylglucoside (TE-PS/OG). To correct for possible effects of phosphatidylserine and octylglucoside added to the analysis buffer, the ODN calibration standards were diluted accordingly in TE-PS/OG buffer. The calibration curve, constructed from 10 concentrations in triplicate, was always linear between 0.09 and 1.8 μg ODN per ml ($r \geq 0.98$).

Statistical Analysis

Statistical significance of differences was evaluated by two-tailed unpaired Student's *t* test. Differences were considered to be significant when $P < 0.05$.

RESULTS

The used Aco-HSA CCLs preparations (n = 5) had a mean size of 154.9 ± 11.9 nm and on average 54.0 ± 18.0 μg Aco-HSA per μmol total lipid was coupled. This corresponds with 0.7 nmol of Aco-HSA per μmol of total lipid and taking the particle size into account it can be estimated that one CCL is covered by approximately 100–200 molecules of the protein. Control CCLs without coupled protein had a mean size of 125 nm. The ODN encapsulation was on average 1.03 ± 0.35 nmol/μmol total lipid.

Figure 1 shows blood elimination of ^{3}H-COE and ^{32}P-ODN labeled CCLs, surface modified with Aco-HSA on intravenous injection. More than 90% of the injected dose of both the ^{3}H and the ^{32}P was eliminated within 30 min. The virtually identical behavior of the two labels indicates that the ODN remain firmly associated with the lipid particles while in blood circulation. Control CCLs, lacking the homing device Aco-HSA, were cleared much more slowly: only about 5% of the untargeted CCLs was eliminated from the bloodstream after 30 min. Injection of polyinosinic acid (5 mg/rat), a known inhibitor of scavenger receptor (ScR) mediated endocytosis, inhibited plasma disappearance and liver uptake by over 50%, confirming the involvement of the ScR in the uptake process (Table I). For both labels the liver accounted for more than 60% of the Aco-HSA-CCLs eliminated after 30 min (Fig. 2). With an uptake of 4% of the total dose, the spleen was the only other organ showing significant uptake after 30 min. Lungs, heart, and kidneys each were found to take up <1% of the injected dose. When rats were injected with untargeted ^{3}H-COE CCLs, liver uptake was about 5% of injected dose after 30 min; spleen uptake (3%) was not very different from that of the targeted preparation.

Table I. Effect of Polyinosinic Acid on Biodistribution of Aco-HSA CCLs

	Aco-HSA CCLs[a]	Aco-HSA CCL + 5 mg poly I[a,b]
Blood	8.1 ± 2.5	50.0
Liver	58.3 ± 19.3	26.1
Spleen	4.2 ± 1.5	3.9

[a] The amount of ^{3}H-Aco-HSA CCLs present in the indicated organ or blood 30 min after injection. Data are presented as % of injected dose, n = 3 ± SD.

[b] 5 mg Poly I was injected 2 min before the Aco-HSA CCLs. Data from a representative experiment.

The uptake of Aco-HSA CCLs by the hepatic endothelial, parenchymal and Kupffer cells was determined after isolation of these cell fractions (Fig. 3). For both labels the endothelial cell population accounted for about two thirds of total liver uptake. Uptake by the Kupffer cells amounted to approximately 40%, while the fraction of the parenchymal cells only accounted for 1.3% (^{3}H-COE label) of total liver uptake.

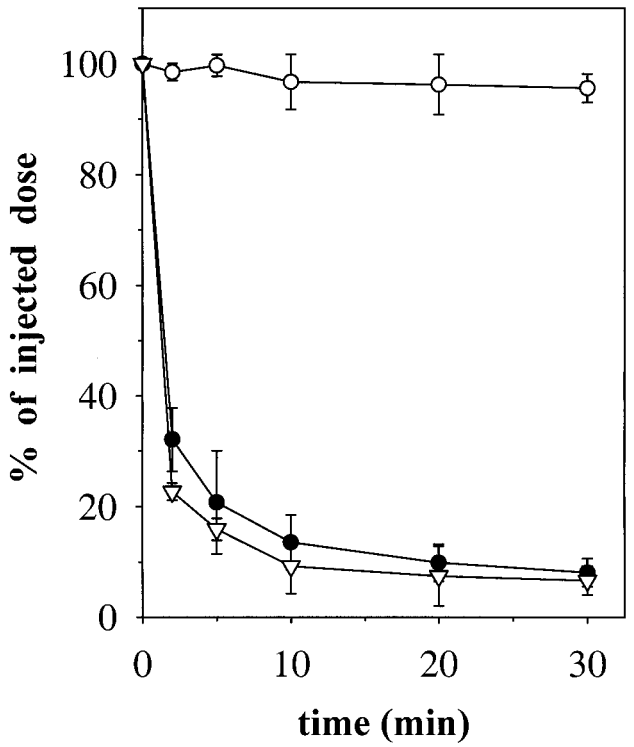

Fig. 1. Plasma disappearance of Aco-HSA CCLs and control CCLs. ^{3}H-COE-Aco-HSA CCLs (filled circles, n = 6 (SD) or ^{32}P-ODN Aco-HSA CCLs (2 μmol TL/rat) (open triangles, n = 6 (SD) or untargeted ^{3}H-COE CCLs devoid of Aco-HSA (open circles, n = 2 error bars indicate range) were injected into anaesthetized rats in a dose of 2 μmol TL/rat. Blood samples were taken at indicated time points and radioactivity was determined as described in Material and Methods.

DISCUSSION

Recently reports were published on plasma-stable cationic lipid based oligonucleotide carrier systems (12,13) in the framework of the development of clinically applicable ODN delivery systems. Little or no attention was paid so far to the

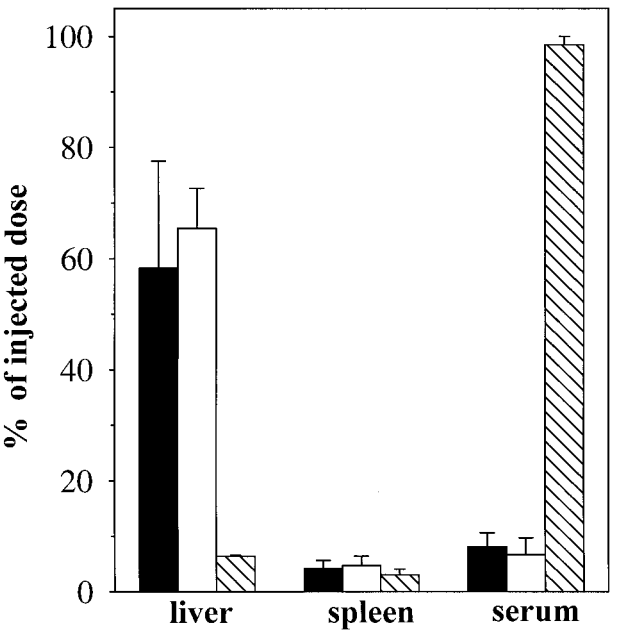

Fig. 2. Tissue distribution of ^{3}H-COE Aco-HSA CCLs (filled bars), ^{32}P-ODN Aco-HSA CCLs (open bars) and untargeted CCLs (hatched bars) 30 min after intravenous injection into anaesthetized rats (2μmol TL/ rat). Data are means ± SD of 6 independent experiments (CCLs n= 2 error bars indicate range).

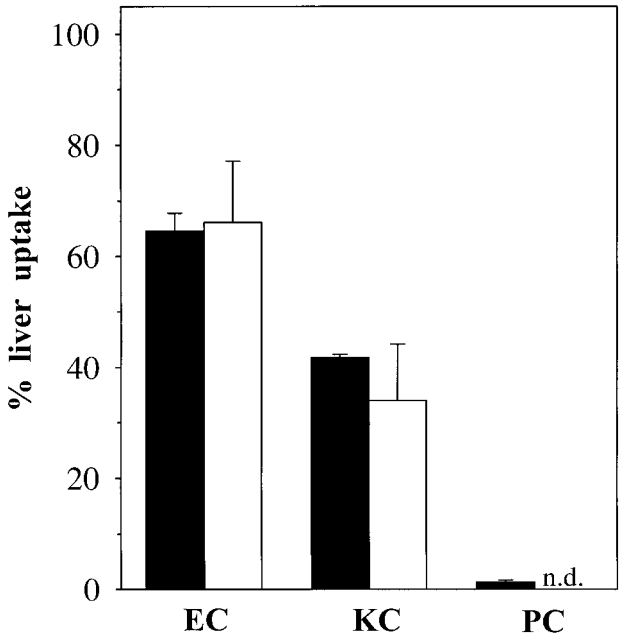

Fig. 3. Intrahepatic distribution of Aco-HSA CCLs 30 min after intravenous injection. ^{3}H-COE Aco-HSA (filled bars) and ^{32}P-ODN Aco-HSA CCLs (open bars) were injected into anaesthetized rats (2μmol TL/ rat). Data are calculated as percentage of total liver uptake, representing means ± SD of 3 independent experiments. Hepatic cell isolation and radioactivity determination were performed as described in Material and Methods.

in vivo targeting of such systems to defined tissues or cell populations. To minimize side effects and to achieve maximal efficacy, target-specific delivery of ODN preparations will be a necessity. In the present work, we present the rapid and efficient targeting of ODN formulated in lipid-coated cationic lipoplexes the hepatic sinusoidal endothelial cells. This was achieved by means of aconitylated human serum albumin as the targeting ligand. It was demonstrated before that Aco-HSA liposomes were preferentially taken up by liver endothelial cells via scavenger receptors (14,22). Pretreatment of rats with a known inhibitor of ScR-mediated uptake, polyinosinic acid, reveals that analogous to the Aco-HSA liposomes, also Aco-HSA CCLs are endocytosed by hepatic sinusoidal endothelial cells through ScR. The endothelial cell targeting is not fully exclusive; the Kupffer cells accumulate only half as much as EC while parenchymal cells hardly participate in the elimination process.

In our previous work on Aco-HSA-mediated targeting of liposomes, we observed a strong dependence of the endothelial cell uptake on liposomal size. In contrast to the 90 nm Aco-HSA liposomes that are mainly taken up by the EC, Kupffer cell uptake was dramatically increased when using liposomes of 150 nm, at the expense of endothelial cell uptake (14). However, in the case of Aco-HSA CCLs of 154 nm size, uptake occurred mainly by the endothelial cell population. This may be explained by the presence of the PEG chains on the CCLs preparations. PEG chains affect protein adsorption to the particles and thus may diminish, Aco-HSA independent, phagocytotic uptake by the Kupffer cells (23).

Unformulated ODN injected into rats showed high uptake by liver endothelial cells (24), a process that was also mediated by the scavenger receptors. The advantage of the lipid-coated formulation over unformulated ODN is thought to be a significantly increased escape of ODN from the endosomal into the cytosolic compartment, where it should display its biological effect. The presence of cationic lipids facilitates that process: antisense activity of ODN increased by 3 orders of magnitude as a result of the combination with cationic lipids (25). In addition, the phospholipid coating of the particles can be adjusted to compositions more likely to promote destabilization of the endosomal membrane (22).

Intravenous administration of unformulated ODN may elicit unwanted systemic side effects such as thrombocytopenia, complement activation and clotting abnormalities (4). It would seem likely that the neutral lipid-coated complexes as used in the present study will not exhibit these effects since their surface is similar to that of liposomes, which also do not show these effects. Yet, whether the ODN content of Aco-HSA CCLs is liberated from the endosomal pathway in a functional way after ScR-mediated uptake remains to be established. Recent findings on the efficacy of antisense material delivered by CCLs strongly suggest, that CCLs formulations result in a substantially increased activity as compared to unformulated ODN. Stuart and coworkers demonstrated that ODN can be functionally delivered intracellularly by anti-CD-19 monoclonal antibody (mAB) CCLs. With this preparation an effective downregulation of MDR-1 expression was achieved, in a human B-lymphoma cell line *in vitro* (26). Moreover, Pagnan et al. (27) reported inhibition of c-Myb protein expression and neuroblastoma cell proliferation inhibition *in vitro* with CCLs targeted by mAB against the disialoganglioside GD2.

A possible application of the anti-ICAM-1-specific ODN containing CCLs used in this study may be the inhibition of the expression of ICAM-1 on liver endothelial cells. This and other adhesion factors not only play a role in leukocyte recruitment but are also involved in the adherence and early arrest of metastasing colon carcinoma cells infiltrating the liver (28,29). Modulation of the expression of adhesion proteins on liver endothelial cells could thus be of therapeutic benefit in anti-inflammatory treatment strategies, or in the prevention of liver metastasis following resection of colon tumors (30).

CONCLUSIONS

We have shown that antisense oligonucleotides, encapsulated in lipid coated cationic lipoplexes, and thereby protected from plasma interference, can be efficiently targeted to liver endothelial cells in rats. By means of surface modification with negatively charged albumin as much as 40% of an injected CCLs dose is delivered to the liver endothelial cells within 30 min after intravenous administration.

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