ORIGINAL ARTICLE

Reconstituted influenza virus envelopes as an efficient carrier system for cellular delivery of small-interfering RNAs

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Application of RNA interference for *in vivo* evaluation of gene function or for therapeutic interventions has been hampered by a lack of suitable delivery methods for small interfering RNA (siRNA). Here, we present reconstituted viral envelopes (virosomes) derived from influenza virus as suitable vehicles for *in vitro* as well as *in vivo* delivery of siRNAs. Virosomes are vesicles that bear in their membrane the influenza virus spike protein hemagglutinin (HA). This protein mediates binding of native virus to and fusion with cellular target membranes. Accordingly, virosomes with membrane-incorporated HA bind to cells, are taken up by receptor-mediated endocytosis, and fuse with the endosomal membrane to release their contents into the cytoplasm. When complexed to cationic lipids, siRNA was successfully

encapsulated in virosomes. Virosomes with encapsulated siRNA fused with target membranes in a pH-dependent manner and delivered the encapsulated siRNA to several cell lines *in vitro*. Virosome-delivered siRNA markedly downregulated the synthesis of newly induced and constitutively expressed green fluorescent protein. Moreover, intraperitoneal injection of siRNA-loaded virosomes resulted in delivery of the nucleotides to cells in the peritoneal cavity. Our results indicate that virosomes are a promising delivery device for *in vivo* application, especially where topical administration of siRNA, for example, to the respiratory tract is envisaged.

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Introduction

RNA interference is a mechanism by which short stretches of double-stranded RNA (dsRNA), the so-called small-interfering RNAs (siRNAs), downregulate gene expression in a highly sequence-specific manner (for recent reviews see Dykxhoorn *et al.*,¹ Agrawal *et al.*,² Novina and Sharp,³ Meister and Tusch⁴). siRNAs have a length of 19–21 bp and are usually processed from longer strands of dsRNA. The presence of siRNAs in the cytoplasm triggers the formation of the so-called RNA-induced silencing complexes (RISC) that consist of several proteins and one of the strands of the siRNA molecule. By virtue of the RNA moiety, the RISC binds to mRNAs in a sequence-specific manner and induces their degradation. The phenomenon was originally described for the nematode *Caenorhabditis elegans*, but has later been found in a variety of other organisms, including plants, protozoa, insects, parasites and mammals.^{4–6}

Initial attempts to apply RNA interference for selective downregulation of gene expression in mammalian cells

failed, since long strands of dsRNA induce the synthesis of type I interferons. These cytokines lead to a general downregulation of protein synthesis, which ultimately results in apoptosis.⁷ However, small stretches of dsRNA (<30 bp) are well tolerated by cells and efficiently and selectively downregulate gene expression.⁸ This observation opened the gateway for the application of RNA interference also in mammalian systems. Since then, the technique has found wide application for the elucidation of gene function *in vitro* and *in vivo*.^{9,10} Moreover, RNA interference is being developed as a therapeutic strategy for diseases that are associated with overexpression of (mutant forms of) proteins like infectious diseases, cancer, neurological disorders and sepsis.^{11–15}

A major obstacle for the use of siRNAs *in vivo* has been the absence of efficient delivery systems suitable for transporting the molecules to the cytosol of appropriate target cells (for reviews see Lieberman *et al.*,¹¹ Wall and Shi,¹⁶ Sioud¹⁷ and Downward¹⁸). Cellular uptake of free siRNA is rather inefficient. Moreover, free siRNA is considered to be vulnerable to degradation under *in vivo* conditions.^{19,20} Attempts to improve delivery of siRNA *in vivo* include complexation of siRNA to polyethylenimine, formulation of siRNA with cationic lipids or collagen derivatives, and entrapment of siRNA in biodegradable microspheres.^{19,21–25} Most of these approaches aim primarily at improving the protection of siRNA from degradation and prolonging the circulation time. While

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this is indeed a crucial issue, efficient translocation of siRNA into the cytosol of the target cells is also of utmost importance.

Viral membranes have evolved for millions of years to protect the viral genetic material and to deliver it faithfully to the cytoplasm and the nucleus of cells in order to allow for replication of the virus. In an attempt to exploit these properties for siRNA therapy, we have encapsulated siRNA into reconstituted membrane vesicles derived from the influenza virus, the so-called virosomes.

Influenza virus is a membrane-enveloped virus with two major envelope glycoproteins: hemagglutinin (HA) and neuraminidase (NA). During the infection process, influenza virus binds to sialic acid residues on glycolipids and glycoproteins of the target cell membrane by virtue of the viral HA and is taken up by endocytosis. Under the low pH conditions in the endosome, the HA acquires a fusion-active conformation and subsequently mediates fusion of the viral and the endosomal membrane. Thereby, the viral nucleocapsids get access to the cytosol and virus replication can proceed.²⁶

Virosomes are prepared from membrane-enveloped viruses, in particular influenza virus, by solubilization of the viral membrane with a suitable detergent, removal of the nucleocapsids by ultracentrifugation and reconstitution of the viral envelope through extraction of the detergent.^{27–29} Vesicles reconstituted in this manner consist of the viral lipids and the viral glycoproteins HA and NA and resemble the native virus particles in size and morphology. Moreover, influenza virosomes bind to sialic acid residues on the cell membrane and fuse with artificial and cellular target membranes in a strictly pH-dependent manner.^{27,28}

Owing to their fusogenic activity, influenza virosomes are suitable carriers for the delivery of encapsulated substances into the cytosol of target cells. Membrane-impermeable drugs like geldanamycin and the A chain of diphtheria toxin can be passively encapsulated into the virosome lumen and are successfully transported to the cytosol.^{30,31} Immunogenic peptides and proteins encapsulated in virosomes are delivered to antigen-presenting cells *in vitro* and *in vivo*. They get access to the MHC class II as well as the MHC class I presentation route indicating uptake of the virosomes by endocytosis and fusion of the virosomal and the endosomal membrane.^{32–34} In order to allow for delivery of plasmid DNA, cationic lipids can be added to the solubilized membrane components prior to reconstitution and are incorporated into the virosomal membrane. Virosomes modified in this manner bind exogenously added DNA with high affinity and transfect cells efficiently.³⁵ In all cases, delivery of encapsulated or associated material to the cytosol is strictly dependent on the fusogenic properties of the viral HA: inactivation of the fusion activity of HA by low pH pretreatment in the absence of a target membrane completely inhibits subsequent delivery of proteins or DNA.

Here, we investigate the potential capacity of virosomes to deliver siRNAs to the cytosol of target cells. We show that siRNA duplexes can be encapsulated in virosomes prepared with cationic lipids where they are protected from nuclease activity. These virosomes fuse with target membranes and efficiently deliver the encapsulated siRNAs to a variety of cells *in vitro*

inducing silencing of the target gene. Initial results indicate that these virosomes are also suitable for delivery of siRNA *in vivo*.

Results

Generation of virosomes

Influenza virus (A/Panama/2007/99 (H3N2)) was solubilized using 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine (DCPC). This short-chain phosphatidylcholine has detergent-like properties and has a critical micelle concentration of 14 mM.³⁶ Ultracentrifugation of the solubilized virus resulted in a clear supernatant that contained approximately 35–40% of the total viral protein. SDS-PAGE revealed that the solubilized protein fraction consisted mainly of HA and some NA. The nonstructural protein 2 was present in small amounts, while other viral proteins were absent (de Jonge *et al.*, manuscript in preparation). These results indicate that DCPC treatment solubilizes the viral membrane almost completely, but leaves the nucleocapsids intact. The supernatant containing the viral membrane components was used for reconstitution of virosomes.

Earlier experiments had shown that peptides, proteins, or DNA oligonucleotides can be encapsulated in virosomes when they are added to the solubilized viral lipids and membrane proteins prior to detergent removal.^{32,33} (A Huckriede, unpublished observations). However, this process of passive encapsulation is rather inefficient as the encapsulated volume is small as compared to the total volume of the suspension. In order to allow more efficient encapsulation, siRNA was therefore first complexed with the cationic lipid *N,N*-dioleoyl-*N,N*-dimethylammoniumchloride (DODAC) and this suspension was added to the solubilized viral membrane prior to reconstitution.

Amounts of DODAC equalling 10–34% of the viral phospholipid were complexed with a fixed amount of siRNA and then added to the solubilized viral membrane components. Formation of virosomes was achieved by removal of DCPC by dialysis. After reconstitution, the preparations were purified on a discontinuous sucrose gradient to remove any material that had not been encapsulated in or was not tightly associated with the reconstituted vesicles. As shown in Figure 1, the virosomes concentrated as an opaque band on the 10/50% sucrose interface. When virosomes were prepared with 10 or 22 mol% DODAC (corresponding to $\pm$ charge ratios of 0.73 and 1.6, respectively), little siRNA was encapsulated and the bulk of the indocarbocyanin (Cy5)-labeled siRNA (appearing blue in visible light) was found in the buffer fraction. In contrast, siRNA accumulated in the virosome band when the virosomes were prepared with 34 mol% DODAC ($\pm$ charge ratio 2.48). Since free siRNA–DODAC complexes spread over the entire gradient, the accumulation of siRNA in the 10/50% sucrose interface indicates its binding to or encapsulation in the virosomes.

Quantification of the encapsulated siRNA revealed that use of 10, 22, or 28% of DODAC resulted in the encapsulation of 1.5, 2, and 5% of the added siRNA, respectively. In contrast, more than 35% ($36.7 \pm 14.2\%$) of the siRNA could be recovered when 34% DODAC was included in the preparation. Based on these results,

% DODAC	10	22	34
charge ratio	0.73	1.6	2.48
% recovery	1.5	2	40

Figure 1 Characterization of siRNA encapsulation into virosomes. Virosomes were prepared with 3.2 pmol Cy5-labeled siRNA/nmol of viral phospholipid and the indicated amounts of DODAC (in mol% of phospholipids). After reconstitution of the viral membranes, the preparations were analyzed on a discontinuous sucrose gradient (10/50% sucrose). Results of a typical experiment are shown.

virosomes prepared with 34% DODAC were used in the following experiments.

Characterization of virosome composition and morphology

The virosome preparation recovered from the discontinuous gradient was further analyzed by equilibrium density centrifugation on a linear sucrose gradient to obtain more insight into its composition and homogeneity (Figure 2). Protein, phospholipid, and siRNA all peaked at a sucrose concentration of app. 30% (density 1.113 g/l) and were virtually absent from the other fractions. This result indicates a tight association of the three components and the absence of free lipid, protein, or siRNA that would have migrated to the top (protein) and the bottom (lipid) of the gradient, respectively. The protein:phospholipid ratio in the 30% sucrose fraction was 0.94 and the virosomes contained 2.75 pmol siRNA/ μ g protein. The peak showed a shoulder for protein and siRNA, but not for phospholipid. This shoulder indicates the presence of another type of particles in which protein and siRNA molecules are enriched as compared to phospholipid.

Electron microscopy confirmed the presence of virosomes, which were mainly spherical and covered with prominent spikes (Figure 3a and b). The mean size of the virosomes was found to be about 60 nm and was considerably smaller than that of native virus (Figure 3c; ± 100 nm). Some particles resembled HA rosettes and may account for the shoulder fraction observed after equilibrium density centrifugation.

Figure 2 Equilibrium density analysis of siRNA virosomes. Virosomes prepared with 34 mol% DODAC and 3.2 pmol Cy5-labeled siRNA/nmol phospholipid were centrifuged to equilibrium on a continuous sucrose gradient (10–60% sucrose). Fractions were analyzed for protein (filled circles), phospholipids (open circles), and siRNA (triangles) as indicated in Material and methods.

Analysis of siRNA encapsulation

In order to quantify the degree of encapsulation of siRNA in virosomes, the preparations were subjected to treatment with benzonase, an enzyme with nuclease activity (Figure 4). Nonencapsulated siRNA was completely degraded by the enzyme. In contrast, siRNA encapsulated into virosomes was protected from degradation and formed a prominent and distinct band after electrophoresis on a polyacrylamide gel. Only when the virosomes were solubilized with detergent prior to benzonase treatment, the siRNA became accessible for benzonase and was degraded. Quantification of RNA recovered from untreated virosomes and from intact virosomes that were exposed to benzonase revealed that virosome-encapsulated siRNA was protected to >80%. Thus, siRNA encapsulated in virosomes is well shielded from extravirosomal proteins and therefore very stable even in the presence of nucleases.

Evaluation of fusogenic properties

To allow the evaluation of their fusogenic characteristics, virosomes with and without encapsulated siRNA were prepared with 10 mol% 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphatidylcholine (pyrPC) incorporated into the membrane. When present in a membrane in high concentration, pyrene molecules form excimers (excited dimers) with a characteristic emission maximum at 480 nm. Upon dilution of the probe (e.g. after fusion with an unlabeled target membrane), the excimers fall apart. The concurrent drop in excimer fluorescence is therefore a direct measure for fusion. When empty virosomes were incubated with erythrocyte ghosts serving as target membranes and the pH was adjusted to 5.5 to bring HA in its fusion-active conformation, rapid fusion occurred to a final extent of 50–60% (Figure 5). Fusion of virosomes with encapsulated siRNA proceeded equally rapidly and reached the same maximal extent as that of empty virosomes when measured at pH 5.5. At neutral pH (pH 7.4) no fusion of siRNA virosomes could be observed. Exposure of the

Figure 3 Morphological analysis. Virosomes with encapsulated siRNA (a and b) and native influenza virus (c) were contrasted with 3% ammonium molybdate and analyzed by electron microscopy. The arrow in (b) points to a virosome, and the arrowhead to a rosette-like particle. Bars = 100 nm.

virosomes to low pH in the absence of target membranes followed by neutralization completely inhibited subsequent fusion. These results indicate that the presence of substantial amounts of siRNA does not interfere with fusion activity. Fusion is mediated by the viral HA in the virosomal membrane, which adopts its fusion-active conformation only under low pH conditions, but is irreversibly inhibited when exposed to low pH in the absence of target membrane.

Figure 4 Nuclease protection assay. Free siRNA or siRNA encapsulated in virosomes was treated with benzonase in the presence or absence of C12E8 as indicated. After inhibition of the nuclease activity by chelation of Mg^{2+} , the preparations were solubilized and analyzed on a 15% polyacrylamide gel. Results of a typical experiment are shown.

Figure 5 Fusion characteristics of siRNA virosomes. Pyrene-labeled virosomes were mixed with human erythrocyte ghosts in fusion buffer. The fusion buffer was adjusted to the indicated pH and pyrene excimer fluorescence was followed online for 4 min. Dotted line: empty virosomes pH 5.5; black line: siRNA virosomes pH 5.5; dashed line: siRNA virosomes pH 7.4; and dotted line: fusion-inactivated siRNA virosomes pH 5.5. Results shown are representative for typical virosome preparations.

Delivery of siRNA to cultured cells

Before setting out to evaluate siRNA delivery by virosomes, we first tested whether the virosome preparations had any toxic effects on cells. Baby hamster kidney cells (BHK21) and A2780 cells were incubated with various amounts of virosomes for up to 72 h. The cells were visually inspected on a regular basis and in some cases tested in a viability assay using WST reagent. In general, virosomes were found to be well tolerated, the percentage of surviving cells being between 80 and 100% at the concentrations used in the delivery and

inhibition assays. Cell viability did not deteriorate during the 72 h period.

In order to determine whether virosomes are suitable for siRNA delivery, BHK21 cells were incubated with virosomes containing siRNA labeled with fluorescein (6-carboxy fluorescein, FAM) for 90 min, washed extensively, and subsequently analyzed by confocal laser scanning microscopy (Figure 6). Most cells were fluorescent due to binding or internalization of siRNA-loaded virosomes. Optical sectioning in Z-direction revealed that some of the virosomes were located on the cell surface (Figure 6a), while others were present in the cell interior close to the nucleus (Figure 6b). These locations presumably reflect binding of the virosomes to their cellular receptor and internalization via endosomes. Pre-exposure of the virosomes to low pH, a treatment that inhibits virosomal fusion activity, had no effect on the binding of virosomes to the cell surface and uptake into endosomes (not shown).

Delivery of siRNA by virosomes was further analyzed and quantified by flow cytometry (Figure 7). BHK21 cells and A2780 expressing enhanced green fluorescent protein (EGFP) cells were incubated for 24 h with increasing amounts of Cy5-labeled siRNA encapsulated in virosomes. After extensive washing, cell-associated fluorescence, caused by bound or internalized siRNA-containing virosomes, was measured. Both cell lines exhibited strong fluorescence indicating binding and/or uptake of virosomes (Figure 7a). All cells were found to be positive for siRNA fluorescence as could be deduced from a general shift of the fluorescence distribution curves of virosome-treated cells as compared to control cells. The amount of cell-associated fluorescence was dependent on the amount of siRNA virosomes added and was roughly the same for the two cell lines tested. Cells incubated with empty virosomes or virosomes containing nonlabeled siRNA did not show any fluorescence, indicating that incubation with virosomes did not alter the autofluorescent properties of the cells (data not shown).

Comparison of siRNA delivery by virosomes with transfection of siRNA by Lipofectamine 2000 (L2000)

Figure 7 Quantification of siRNA delivery by flow cytometry. (a) BHK21 cells (white; 2×10^4 /well) and A2780-EGFP cells (hatched; 6×10^4 /well) were incubated for 24 h with virosomes containing the given amounts of Cy5-labeled siRNA. Cell-associated fluorescence was then measured by flow cytometry. (b) A2780-EGFP cells (6×10^4 /well) were transfected with the indicated amounts of siRNA using L2000 (white), or fusion-active (hatched) or fusion-inactivated virosomes (crossed), respectively. Cell-associated fluorescence was determined as in (a).

Figure 6 Microscopical analysis of siRNA delivery. BHK21 cells (1×10^4 /well) were incubated with 10 pmol of FAM-labeled siRNA encapsulated in virosomes for 90 min. Cells were then fixed and stained with rhodamine-phalloidin to visualize stress fibers. Specimens were analyzed by confocal microscopy. Focus was on the cell surface (a) or on the cell interior (b). Bar = 10 μ m.

revealed that the amount of cell-associated fluorescence was of the same order of magnitude for both methods with virosomes being slightly more efficient at low siRNA concentrations (Figure 7b). Binding and/or uptake into endosomes of fusion-inactivated virosomes was as effective as that of fusion-active virosomes consistent with the results obtained by confocal microscopy (see above).

Inhibition of EGFP neosynthesis by virosome-delivered siRNA

Next, we investigated whether virosome-encapsulated siRNA was not only delivered to cells but did indeed reach the cytoplasm and was functionally active. For this purpose, Madin Darbin canine kidney (MDCK) cells were transfected with virosomes containing siRNA directed against the mRNA of the green fluorescent protein (siGFP). After 24 h, the cells were transfected with a plasmid encoding the EGFP. Expression of the protein was investigated 1 day later by fluorescence microscopy (Figure 8). Transfection of the plasmid in control cells resulted in high expression of EGFP in the vast majority of cells. In contrast, after cotransfection of siGFP and plasmid DNA, EGFP expression was hardly detectable (not shown). Exposure of the cells to virosome-encapsulated siGFP prior to plasmid transfection markedly reduced EGFP expression (Figure 8, left column). The effect was strictly dose-dependent.

Figure 8 Effect of virosome-delivered siGFP on EGFP neosynthesis. MDCK cells (4×10^4 /well) were treated with fusion-active virosomes containing the indicated amounts of siGFP (left column) or siHA (middle column) or with fusion-inactivated virosomes containing siGFP (right column). One day after siRNA transfection, the cells were transfected with pCMV-EGFP. After 24 h the cells were inspected under an invert fluorescence microscope. Results of a typical experiment are shown. Bar = 250 μ m.

Whereas 1 and 2.5 pmol virosome-delivered siGFP had little effect, larger amounts were found to be very effective and 15 pmol almost completely inhibited EGFP expression. In contrast, virosome-mediated delivery of control siRNA (siHA) did not have a significant effect on EGFP expression, except for the highest amount studied (Figure 8, middle column). Some nonspecific effects of siRNA at high concentrations were also observed for L2000-delivered control siRNA and have been described by others.^{37,38} Results obtained with BHK21 cells were very similar to those shown for MDCK cells in Figure 8 (not shown).

In order to elucidate the mechanism of virosome-mediated siRNA delivery fusion activity of the virosomes was destroyed by incubation in low pH buffer and subsequent neutralization. As shown above (Figure 7b), this treatment does not affect binding of the virosomes to the cell surface and uptake into endosomes, but inhibits HA-mediated membrane fusion. Control experiments verified that low pH treatment as such had no effect on siRNA. In cells treated with fusion-inactivated siRNA virosomes EGFP expression was not affected even at the highest concentration used (Figure 8, right column), indicating that siRNA did not get access to the cytoplasm. This observation strongly emphasizes that transfection of siRNA is mediated by the fusogenic properties of the viral HA, which enable merging of the virosomal and the endosomal membrane and release of the encapsulated siRNA into the cytoplasm. Moreover, it further underlines that reduction in EGFP expression is not an unspecific effect due to incubation of the cells with virosomes as such.

Depletion of constitutively expressed proteins by siRNA virosomes

The above experiments show that virosome-mediated siRNA delivery can efficiently inhibit protein expression in a situation where the siRNA is present before mRNA transcription and protein synthesis begin. However, in a therapeutic situation, it will be important that the delivered siRNA can induce depletion of constitutively expressed proteins. In order to mimic such a situation, we used cells that constitutively express EGFP. The cells, of which app. 90% were positive for EGFP, were incubated with increasing amounts of siGFP encapsulated in virosomes. EGFP fluorescence was measured by flow cytometry 24, 48, and 72 h later (Figure 9). Already 24 h after transfection, a dose-dependent effect of virosome-delivered siGFP on the amount of EGFP protein was observed (Figure 9a). EGFP fluorescence further decreased over time, the effect being maximal at 72 h after siRNA transfection. Importantly, all cells had been successfully transfected since the fluorescence of the entire EGFP-positive cell population shifted to lower mean fluorescence levels (Figure 9b). Cell viability did not deteriorate over the 72 h period, indicating that the decrease in EGFP fluorescence was not due to toxic effects of the virosomes. This result shows that virosome-delivered siRNA successfully inhibited neosynthesis of EGFP, thereby inducing a gradual decrease in the steady-state level of the protein. Transfection of 0.3 pmol of siRNA had little effect, whereas transfection of 10 pmol was already found to be optimal in reducing EGFP fluorescence to 23% after 72 h. Fusion-inactivated

Figure 9 Effect of virosome-delivered siGFP on constitutive EGFP expression. (a) A2780-EGFP cells (6×10^4 /well) were transfected with the indicated amounts of siGFP encapsulated in virosomes. EGFP fluorescence was measured by flow cytometry 24, 48, and 72 h later. (b) Fluorescence distribution curve of cells transfected for 72 h as in (a). Shaded: control; bold line: 1 pmol; medium line: 3 pmol; and light line: 10 pmol siRNA. (c) A2780-EGFP cells were incubated with the indicated amounts of siGFP using fusion-active virosomes, fusion-inactivated virosomes, or L2000, respectively, or received empty virosomes. EGFP expression as the percentage of untreated control cells 72 h after transfection is shown. Results are representative for three experiments performed.

virosomes were not able to deliver siRNA to the cytoplasm and consequently had no effect on EGFP fluorescence (Figure 9c). Similarly, empty virosomes

were ineffective, further underlining that EGFP reduction was not due to aspecific effects of virosomes on cellular protein synthesis. Transfection of siGFP with L2000 was slightly more efficient than virosome-mediated transfection. However, A2780 cells, used in this experiment, were very vulnerable for toxic effects of the transfection agent and tolerated only low concentrations of the drug, which limited the amount of siGFP that could be used to 3 pmol at maximum.

The results described demonstrate that virosomes successfully deliver siRNA to the cytosol such that it can degrade (pre-existing) mRNA leading to gradual depletion of the target protein.

Virosome-mediated siRNA delivery in vivo

In order to obtain insight into the suitability of virosomes for use *in vivo*, mice were injected intraperitoneally (i.p.) with 200 pmol FAM-labeled siRNA encapsulated in virosomes. After 2 h, the mice were killed and cells present in the peritoneal cavity were harvested and analyzed by flow cytometry (Figure 10). Based on the forward/sideward scatter, two major cell populations could be distinguished. The larger, more granulous cells presumably represent granulocytes and macrophages, while the small cell population most probably represents the lymphocytes (Figure 10a and b). Together, these cell populations comprise $\pm 80\%$ of the total number of cells harvested.³⁹ Upon injection of siRNA virosomes, $85.2 \pm 1.1\%$ ($n=3$) of the granulocyte/macrophage population (Figure 10c and d) and $24.5 \pm 2.4\%$ ($n=3$) of the lymphocytes (Figure 10e and f) were associated with FAM-labeled siRNA, indicating binding and/or uptake of siRNA virosomes by various cell types.

Discussion

In this paper, we show that virosomes, reconstituted from the influenza virus membrane envelope, are suitable carriers for the delivery of encapsulated siRNA to target cells *in vitro* as well as *in vivo*. Inhibition of target protein synthesis indicating successful transport of siRNA to the cytosol is strictly dependent on the fusion activity of the virosomes, which is mediated by HA incorporated in the virosomal membrane. Thus, by using reconstituted viral envelopes, the typical properties of viral membranes, namely efficient cell binding and fusion with cellular membranes, can be exploited for delivery of small nucleic acids directly into the cytoplasm.

Preparation of virosomes with encapsulated siRNA is straightforward with routine recoveries of app. 40% of the viral membrane proteins and the added siRNA in the final preparation. Virosomes prepared under the conditions described contain about 2.75 pmol siRNA/ μ g of virosomal protein. Based on the number of HA molecules in the viral membrane and the size of the virosomes this equals roughly 60–70 siRNA molecules/virosome.⁴⁰

Inclusion of cationic lipids was found to be essential for efficient loading of the virosomes with siRNA. Interestingly, there was no linear relationship between the amount of cationic lipids present and the amount of siRNA encapsulated. Encapsulation efficiency was equally low ($\leq 5\%$) for charge ratios of 0.73, 1.6, and 2, but mounted to about 40% when the charge ratio was

Figure 10 Virosome-mediated siRNA delivery *in vivo*. Mice were injected i.p. with 200 μ l of buffer (left) or the same amount of buffer containing 200 pmol FAM-labeled siRNA encapsulated in virosomes (right). After 2 h, the mice were killed. Cells were harvested from the peritoneal cavity and were analyzed by flow cytometry. (a and b): Forward/sideward scatter plots of control (a) and siRNA-injected mice (b). (c and d): Analysis of gated granulocytes/macrophages (R1) from control (c) and siRNA-injected animals (d). (e and f): Analysis of gated lymphocytes (R2) from control (e) and siRNA-injected mice (f).

increased to 2.48. The association of nucleotides and cationic lipids to multimolecular structures is a highly complex process that is poorly understood so far.^{41,42} The specific nature of the cationic lipid involved, the presence and sort of helper lipids, the salt conditions and even the sequence of addition of lipids and nucleotides appear to influence the nature of the nucleotide–lipid complexes. Obviously, the charge ratio is also of major importance. Complexes of oligonucleotides and cationic lipids are large and unstable at $\pm$ charge ratios of 0.5–2, while small stable particles are formed at higher charge ratios.⁴³ It is tempting to speculate that only the small size aggregates obtained at high charge ratios allow further coating of the aggregates by membrane proteins and lipids such that well-defined virosomes are formed. However, we cannot exclude that other phenomena also play a role.

The functional integrity of HA, in particular its property to mediate membrane fusion, was found to be crucial for siRNA delivery (Figures 8 and 9c). *In vitro* virosomes prepared with DODAC and siRNA fused with erythrocyte ghosts to an extent of 55% (Figure 5). This fusion activity is substantial, although somewhat lower

than that of virosomes prepared without DODAC ($\pm 70\%$; de Jonge *et al.*, manuscript in preparation). As depicted in Figure 5, fusion of DODAC-containing virosomes proceeded rapidly and was strictly dependent on low pH. The same characteristics are shown by native influenza virus.²⁷ Although inactivation of the fusion activity of the virosomal HA had no effect on binding of siRNA virosomes to cells and internalization into endosomes, it completely abrogated their capacity to deliver siRNA into the cytoplasm. Together, these observations strongly support our concept that virosomal delivery relies on the natural function of the viral envelope to fuse with cellular membranes.

Virosome-delivered siGFP inhibited neosynthesis of EGFP in plasmid-transfected cells in a dose-dependent manner (Figure 8). In cells that express EGFP constitutively virosome-delivered siGFP led to gradual depletion of the target protein. Obviously, the time course and the maximal extent of depletion depend on the half-life of the protein investigated. The half-life of (E)GFP has been determined in several cell lines to be about 26–54 h.^{44,45} Thus, if EGFP synthesis was inhibited completely, at 72 h the amount of residual protein should theoretically be 25% of the initial amount. We found a residual amount of 23% EGFP, indicating that EGFP synthesis was blocked very efficiently in virtually all cells by the virosome-delivered siRNA.

Owing to its excellent effectiveness and specificity, RNA interference is considered to have a very high potential for therapeutic intervention. The rapidly growing list of diseases for which siRNA-mediated therapy is under development includes infectious diseases, neurological disorders, acute liver failure, cancer, sepsis, inflammation and others (for reviews see Lieberman *et al.*,¹¹ Ryther *et al.*,¹⁵ Wall and Shi,¹⁶ Sioud¹⁷ and Stevenson⁴⁶). Three basic approaches have been followed for the application of RNA interference *in situ*: delivery of small siRNA molecules, delivery of plasmid vectors encoding small hairpin (sh) RNAs, which are processed to siRNA molecules within the target cell, and use of viral vectors that encode shRNA or both strands of siRNA.^{47–52}

As shown here, virosomes are highly suitable for delivery of siRNA molecules. Moreover, their capacity to transfer plasmid DNA into the cytosol opens the possibility to use virosomes for the delivery of plasmids encoding shRNAs.³⁵ As compared to other nonviral approaches for siRNA delivery including hydrodynamic injection of free siRNA molecules and liposome-mediated transfection, virosomes offer a number of advantages:^{13,25,47,48,53}

- (1) Virosomes bind to the cellular receptor of influenza virus, thereby facilitating internalization of siRNA. The receptor, sialic acid, is present on most cells. Consequently, virosomes have a broad target cell range.
- (2) Owing to HA-mediated fusion of the virosomal and the endosomal membrane, virosomes actively deliver their content to the cytoplasm. In contrast, the escape of free or liposome-encapsulated siRNA from the endosome is generally inefficient and so far poorly understood.^{54,55}
- (3) Virosome-mediated transfection is not affected by serum components in contrast to transfection with cationic liposomes.⁴²
- (4) Virosomes have been shown to be safe when injected intramuscularly, i.p., subcutaneously, or intranasally.^{32,34}

In contrast, hydrodynamic injection uses large volumes of buffer ($\pm 10\%$ of the total body weight) and cationic lipids are often toxic.^{55–57}

Possible drawbacks of influenza virosomes lay in their immunogenic properties. Most people have undergone influenza infections and will possess circulating antibodies that might interfere with virosome treatment. It is therefore desirable to use for the generation of siRNA-containing virosomes influenza strains that have not circulated recently. Another point of concern is that injection of virosomes itself will probably induce an HA-specific immune response that might impair siRNA delivery when repeated injections are required. The presence of HA-specific antibodies at various time points after virosome administration and the relevance of these antibodies for repeated virosome injections are currently under investigation. If the antibodies pose a problem, virosomes generated from non-crossreactive influenza strains can be used to allow repeated administration.

While under certain conditions pre-existing immunity and the generation of antibody responses are disadvantageous, they can be highly beneficial in other cases. Opsonization of virosomes with antibodies will specifically target them to cells with antibody receptors like natural killer cells, macrophages, and dendritic cells. Fusion activity is most probably not affected by opsonization as indicated by reports on antibody-mediated enhancement of influenza infection of macrophages.^{58,59} Virosomes might therefore be an ideal device to specifically deliver siRNA to cells that carry Fc receptors. Dendritic cells are attractive targets and approaches for the elucidation of gene function and the regulation of immune responses by siRNA-mediated interference in dendritic cells have already been described.^{60–63}

The most natural target for virosome-delivered siRNA would be the respiratory tract, the common site of infection by influenza virus. Nonadjuvanted virosomes administered intranasally are poorly immunogenic, probably due to strong tolerance mechanisms at mucosal sites.⁶⁴ Repeated administration should therefore be possible. In the respiratory tract, the virosomes will bind to and enter the respiratory epithelium cells with high efficiency. Possible target diseases include respiratory infections like influenza infection itself and the severe acute respiratory syndrome for which siRNA-based therapeutic approaches are already under development.^{21,65–71}

In summary, virosomes combine the advantages of synthetic delivery systems (well defined, safe) with the desired properties of viral delivery systems (targeting to cells, active translocation of siRNA into the cytoplasm). They may therefore be an attractive addition to the methods for *in vivo* administration of siRNA, especially where delivery of siRNA to the respiratory epithelium or to cells with Fc receptors like dendritic cells, macrophages, and NK cells is envisaged. The results presented form a promising foundation for future experiments that will be devoted to detailed *in vivo* studies addressing the biodistribution and stability of siRNA virosomes as well as the functional activity of virosome-delivered siRNA.

Materials and methods

Materials

DCPC was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). DODAC was obtained from Inex Pharmaceuticals Inc. (Vancouver, BC, Canada). pyrPC was purchased from Molecular Probes (Eugene, OR, USA). Influenza virus (A/Panama/2007/99 (H3N2)) was kindly provided by Solvay Pharmaceuticals B.V. (Weesp, The Netherlands).

SiRNA directed against GFP (siGFP; sense 5'-CAAG CUGACCCUGAAGUUC-3', antisense 5'-GAACUUCAG GGUCAGCUUG-3') and influenza HA H3 (siHA; sense 5'-UAUGCGACAGUCCUCCUACCAdTdT-3', anti-sense 5'-dTdTUGGGAGGACUGUCGCAUAdTdT-3') were purchased from Eurogentec (Seraing, Belgium). The siRNAs were unmodified or labeled at the 3'-end with either FAM or Cy5.

Cells

BHK21 were grown in GMEM (Life Technologies, Breda, The Netherlands) containing 2 mM glutamine, 5% fetal calf serum (FCS) and 10% tryptose phosphate broth. MDCK cells were grown in Iscove's medium (IMDM, Life Technologies), 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The human ovary carcinoma cell line A2780 was grown in RPMI1640 (Life Technologies) supplemented with 10% FCS and 100 U/ml penicillin, and 100 μ g/ml streptomycin. A derivative of this cell line transfected with pEGFP-N1 (Clontech, Palo Alto, CA, USA) to express EGFP was kindly provided by Ina Hummel (Department of Cell Biology, University of Groningen, The Netherlands). This cell line was grown under selective pressure in RPMI medium containing 500 μ g/ml geneticine.

Preparation of siRNA virosomes

Influenza virus was inactivated by the addition of β -propiolactone (BPL; Acros Organics, Geel, Belgium) to a final dilution of 1:1000. The virus suspension was incubated for 24 h with gentle mixing in the dark at room temperature. BPL was removed by dialysis against Hepes-buffered saline (HBS; 5 mM Hepes, 0.15 M NaCl, pH 7.4) overnight at 4°C.

Influenza virus (typically 1.5 μ mol phospholipid) was sedimented by ultracentrifugation (100 000 g, 1 h at 4°C) and the viral pellet was resuspended in 375 μ l HBS with a 1 ml syringe equipped with a 25-G needle. The viral membrane was dissolved by the addition of 375 μ l 200 mM DCPC in HBS followed by incubation on ice for 30 min. The nucleocapsids consisting of the viral RNA and internal viral proteins were pelleted by ultracentrifugation (100 000 g, 30 min at 4°C) and the supernatant containing the dissolved membrane components was used for the reconstitution of the viral membrane (see below).

DCPC, DODAC, and pyrPC were dissolved in chloroform and the required amounts (typically 24, 0.51, and 0.15 μ mol, respectively) were mixed in a test tube. The lipid mixture was dried under a stream of nitrogen and kept under vacuum for 2 h to remove remaining traces of chloroform. Sense and antisense siRNA were annealed according to the manufacturer's instructions. Annealed siRNA (typically 4.8 nmol in 240 μ l annealing buffer) was added to the dried lipids and the lipids were dissolved

by vortexing and subsequent incubation at room temperature for 15 min. This suspension was mixed with the supernatant containing the solubilized viral membrane components followed by incubation at room temperature for 30 min. Subsequently, the mixture was transferred to a 0.5–3 ml Slide-A-Lyzer with an MWCO of 10 kDa. Removal of DCPC allowing reconstitution of the viral membrane was performed by dialysis against 2 l of HBS overnight at 4°C, followed by another 4 h of dialysis after buffer refreshment.

The reconstituted virosomes were purified on a discontinuous sucrose gradient (10/50% w/v sucrose in HBS) by ultracentrifugation (100 000 g, 1.5 h at 4°C). The virosomes were recovered from the interface of the two sucrose layers, transferred to a 0.1–0.5 ml Slide-A-Lyzer with an MWCO of 10 kDa, and dialyzed against 2 l HBS overnight at 4°C.

For determination of the siRNA content, the virosomes were solubilized by the addition of the detergent C12E8 (octaethyleneglycol monododecyl ether; Fluka Chemie, Buchs, Switzerland) in HBS to a final concentration of 9 mM. The emission of Cy5- or FAM-labeled siRNA was measured on a fluorimeter (SLM-AMINCO Bowman Series 2) using excitation and emission wavelength of 633 and 667 nm for Cy5 and 497 and 514 nm for FAM, respectively. The protein content of the virosomes was determined by a micro-Lowry assay.⁷²

To study the fusion characteristics of the siRNA virosomes, fusion assays and fusion inactivation were performed as described previously.³³

Analytical sucrose gradient

Equilibrium density-gradient centrifugation was performed on 10–60% sucrose gradients in HBS. Gradients were centrifuged for 62 h at 300 000 g. Fractions were analyzed for protein and siRNA content as mentioned above. Phosphate was determined by the method of Böttcher and colleagues and values were corrected for phosphate present in siRNA nucleotides in order to estimate the content of phospholipids.⁷³

Electron microscopy

For negative staining, influenza virus or virosomes were contrasted with 3% ammonium molybdate pH 7.2 on carbon-coated Formvar films and analyzed on a Philips CM10 electron microscope.

Benzonase assay

Virosomes containing 12 pmol encapsulated siRNA were treated for 30 min at 37°C with 1 U benzonase (Merck, Darmstadt, Germany) in HBS containing 6 mM MgCl₂. The reaction was stopped by the addition of EDTA (final concentration 6 mM). Subsequently, the virosomes were solubilized with 5 mM C12E8. Alternatively, virosomes were solubilized in C12E8 prior to benzonase treatment. Control samples contained solubilized virosomes that were not treated with benzonase. All samples were run on a 15% polyacrylamide gel in 1 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and were visualized by staining with ethidium bromide.

Transfection of siRNA

For transfection experiments BHK21 cells, A2780-EGFP cells or MDCK cells were plated into 24- or 96-well trays. After 24 h, virosomes were added to the medium.

Parallel wells were transfected with the help of Lipofectamine 2000 (L2000; Life Technologies). For this purpose, siRNA was coincubated with L2000 (1.2 µl/24-well, 0.3 µl/96-well) for 20 min in OptiMem medium (Life Technologies). The mixtures were diluted with the respective growth media without penicillin and streptomycin and added to the cells. After 24 h, cells were washed, trypsinized, and analyzed for binding/uptake of labeled siRNA by flow cytometry (Epics Elite, Coulter, Miami, IL, USA).

For visualization of endocytosed siRNA, BHK cells were plated on eight-well Labtek chamber slides (1 × 10⁴/well; Nalge, Naperville, USA). After 1 day, virosomes with 10 pmol encapsulated FAM-labeled siRNA were added per well. After 90 min, the cells were washed with PBS (Life Technologies) and fixed for 10 min with 4% paraformaldehyde in PBS. Cells were washed again and then stained with a 1:200 dilution of phalloidin labeled with tetramethylrhodamine isothiocyanate (Sigma, St Louis, MO, USA) for 10 min. Cells were washed, mounted, and analyzed using an LEICA TCS SP2 3-channel confocal laser scanning microscope equipped with argon and helium lasers.

Evaluation of EGFP expression

For visual evaluation of EGFP expression, BHK or MDCK cells were transfected with siRNA as described above. After 24 h, the cells were transfected with pCMV-EGFP with the help of L2000 (0.15 µg DNA, 0.3 µl L2000). The next day, the cells were inspected with the help of a Zeiss Axiovert microscope equipped with epifluorescence.

For quantification of EGFP expression, A2780-EGFP cells were transfected with virosome-encapsulated siRNA or with siRNA–L2000 complexes as described above. EGFP expression was measured by flow cytometry 24, 48, and 72 h after transfection.

WST assay

BHK cells or A2780-EGFP cells were plated into a 96-well plate. The next day virosomes were added in concentrations of 0.5–5 pmol/well. 24, 48, or 72 h, virosomes were removed and 100 µl fresh medium and 10 µl WST reagent (Roche Molecular Biochemicals, Mannheim, Germany) were added. After 1 h, the extinction at 440 nm was measured using a Powerwave X microtiter plate reader (Bio-Tek Inc., Winooski, USA).

In vivo experiments

Specified-pathogen-free female C57BL/6 mice were purchased from Harlan CPB (Zeist, The Netherlands) and used at 6–10 weeks of age. The protocol for the animal experiments described in this paper was approved by the Animal Experimentation Ethical Committee of the University of Groningen. Mice (*n* = 3) were injected i.p. with 200 pmol FAM-labeled siRNA encapsulated into virosomes in a total volume of 200 µl HBS. Control mice received 200 µl buffer i.p. and 2 h after virosome injection, the mice were killed. The peritoneal cavity was flushed with 2 ml IMDM supplemented with 10% FCS and 50 µM β-mercaptoethanol. The cells were pelleted, treated with ACK buffer (150 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.2) to remove erythrocytes, washed, and analyzed by flow cytometry as described above.

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