Porous Silicon−Cell Penetrating Peptide Hybrid Nanocarrier for Intracellular Delivery of Oligonucleotides

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ABSTRACT: The largest obstacle to the use of oligonucleotides as therapeutic agents is the delivery of these large and negatively charged biomolecules through cell membranes into intracellular space. Mesoporous silicon (PSi) is widely recognized as a potential material for drug delivery purposes due to its several beneficial features like large surface area and pore volume, high loading capacity, biocompatibility, and biodegradability. In the present study, PSi nanoparticles stabilized by thermal oxidation or thermal carbonization and subsequently modified by grafting aminosilanes on the surface are utilized as an oligonucleotide carrier. Splice correcting oligonucleotides (SCOs), a model oligonucleotide drug, were loaded into the positively charged PSi nanoparticles with a loading degree as high as 14.3% (w/w). Rapid loading was achieved by electrostatic interactions, with the loading efficiencies reaching 100% within 5 min. The nanoparticles were shown to deliver and release SCOs, in its biologically active form, inside cells when formulated together with cell penetrating peptides (CPP). The biological effect was monitored with splice correction assay and confocal microscopy utilizing HeLa pLuc 705 cells. Furthermore, the use of PSi carrier platform in oligonucleotide delivery did not reduce the cell viability. Additionally, the SCO−CPP complexes formed in the pores of the carrier were stabilized against proteolytic digestion. The advantageous properties of protecting and releasing the cargo and the possibility to further functionalize the carrier surface make the hybrid nanoparticles a potential system for oligonucleotide delivery.

KEYWORDS: porous silicon, nanoparticles, drug delivery, cell penetrating peptide, oligonucleotide, drug delivery

INTRODUCTION

Alternative splicing is a process by which multiple proteins can be synthesized from a single gene. The splicing takes place in the cell nucleus when precursor mRNA (pre-mRNA) is converted to mRNA by removal of the introns. Therefore, alternative splicing is one of the main sources of proteomic diversity in multicellular eukaryotes. However, up to 50% of genetic disorders are attributable to aberrant splicing of pre-mRNA. Nevertheless, splicing can be modified or aberrant splicing can be corrected to achieve the correct expression of protein with splice correcting oligonucleotides (SCO).

SCOs are a subcategory of antisense oligonucleotides, which are a larger group of oligonucleotide drugs that regulate protein expression. SCOs are chemically modified oligonucleotides with a length of 17−22 nucleotides. In general, antisense therapy is an attractive approach that could be used to treat many serious conditions, including cancer and neurodegenerative diseases. Currently several new drugs belonging to this category are undergoing clinical trials. The latest addition to antisense oligonucleotide drugs is Mipomersen (Kynamro), which was recently approved by FDA for the treatment of familial hypercholesterolemia. There are several mechanisms of antisense action, i.e., inhibition of transcription or translation and modulation of pre-mRNA splicing. Splice correcting antisense action differs from other antisense mechanisms, which usually bind to their mRNA target and activate RNase H enzyme, which then destroys the targeted mRNA sequence. SCOs do not activate RNase H but instead bind to their specific pre-mRNA sequence and sterically prevent the aberrant splicing and thus restores the protein synthesis of the normal product.

The problem with splice correcting and other antisense therapies is often encountered in finding an effective delivery method since pre-mRNA splicing takes place in the nucleus and translation occurs in the cytosol. Furthermore, unprotected and unmodified RNA is quickly degraded by plasma nucleases after its systemic administration, and therefore, there is a need...
to develop efficient delivery methods before there can be wider use of antisense and gene therapies. Several ways have been devised to deliver oligonucleotides through biological membranes, e.g., nonviral (synthetic) delivery vectors. Polycations, lipids based vectors, cell penetrating peptides (CPPs), etc., are able to utilize a variety of internalization mechanisms and are less immunogenic. Therefore, they are generally considered safer than viral vectors.

Transportan-based stearylated CPPs are promising vectors for gene delivery, as they display a high transfection efficacy and low cytotoxicity. CPPs and negatively charged oligonucleotides spontaneously form noncovalent complexes, which are readily taken up by cells. The stearyl moiety in the N-terminus of the peptide increases the hydrophobicity of the peptide, resulting in improved endosomal escape and a better biological effect, also in the presence of serum proteins in the transfection medium. NickFects are novel rationally designed stearyltransportan 10 analogues for the delivery of genetic material. NickFects (NPS1) has proved to be an effective and versatile vehicle for intracellular delivery of plasmid DNA, SCO, and small interfering RNA (siRNA) into various adherent and suspension cells, and it does not seem to exert cytotoxic side effects.

In recent years, new drug formulations, especially those utilizing nanotechnology, have been evaluated in drug delivery. Porous silicon (PSi) is a promising material for drug delivery because it is a biocompatible and easily tunable substance. For example, PSi can be bioinert, bioactive, or made resorbable by adjusting the porosity of the material. In vitro studies have shown PSi as a material of low immunogenicity. Intravenously administered PSi nanoparticles show dissolution and renal excretion of the dissolution products with no evidence of toxicity over a time span of weeks in mice. A top down manufacturing approach allows the modification of shape, size, surface chemistry, and drug loading properties of PSi particles. In recent years, PSi micro- and nanoparticles have been evaluated as a delivery platform for several types of drugs and imaging agents, e.g., low solubility small molecule drugs, peptides, siRNA loaded nanomicelles, and quantum dots. The passivated surfaces of PSi nanoparticles can be functionalized with aminosilanes, which enables easy and effective fluorescence labeling of the particles for imaging.

In the present work, we describe a highly promising platform for the delivery of SCOs, enjoying the advantages of both PSi and CPP. PSi nanoparticles were designed to carry high amounts of SCOs, and to have enhanced cell internalization and controlled release of their cargo by tuning their pore size and surface charge. Cell penetrating peptide (NFS1) was used to enhance endocytotic escape and to improve the transfection efficacy. The effects of the SCO loading and peptide functionalization on the nanoparticles were assessed with dynamic light scatter (DLS) measurements and transmission electron microscopy (TEM). The in vitro delivery of SCOs was studied with splice correction assay and with confocal microscopy. The cell viability was evaluated after transfection with PSi nanoparticles by monitoring the cell proliferation. Additionally, the stability of the SCO-NFS1 cargo toward protease degradation was assessed. To the best of our knowledge, this is the first report where this type of hybrid nanocarrier has been successfully utilized in oligonucleotide delivery.

**MATERIALS AND METHODS**

**Reagents.** Ultrapure water was used throughout the study, reagents as H2O. Ethanol was purchased from Alita Oy, Finland. The reagents used in cell culturing were purchased from Sigma, with the exception of Dulbecco’s Modified Eagle Medium (DMEM), which was purchased from Invitrogen. All chemicals for the production of the nanoparticles were purchased from Sigma-Aldrich and used without further purification. All plasticware in contact with the nanoparticles was rinsed with ethanol prior to use.

**Preparation of Amine Modified PSi Nanoparticles.** PSi nanoparticles were prepared according to the method described by Bimbo et al. PSi film was prepared through anodizing a silicon wafer (100) (p-type with the resistivity of 0.01−0.02 Ωcm) in an HF/ETOH mixture (1:1, 38% HF). After drying at 65 °C, the PSi films were ball milled in ethanol to produce PSi nanoparticles. The PSi nanoparticles with the diameter below 200 nm were recovered by centrifugation at the rate of 3980 rpm. Subsequently, the surface of PSi nanoparticles was passivated through thermal oxidation or carbonization processes, corresponding to the samples of TOPSi and TCPSi, respectively.

TOPSi nanoparticles were chemically oxidized by NH4H2O/H2O2/H2O (1:1:5, vol/vol) and HCl/H2O2/H2O (1:1:6, vol/vol). The sample was rinsed three times with H2O and dried at 65 °C overnight in air. To remove residual H2O, the TOPSi nanoparticles were further dried at 85 °C for 2 h under vacuum. The amino modification was carried out in anhydrous toluene under reflux. The mass concentration of aminosilane 3-(2-aminoethylamino)propyltrimethoxysilane (AEPMS) was controlled at 2.0%. The film samples were used in the N2 ad/desorption measurement because the big interparticle pores, which were formed during the agglomeration of the nanoparticles after drying, seriously disturbed the interpretation of N2 ad/desorption isotherms. The amine modified PSi nanoparticles were recovered by centrifugation after 4 h of refluxing. The sample was rinsed with ethanol three times to remove the residual aminosilane. The final obtained nanoparticles were designated as TOPSi-NH2.

TCPSi nanoparticles were first treated with the solution of HF/ETOH (1:1, 38% HF). The subsequent treatments of surface oxidation with H2O2 and amine modification were similar to those described for TOPSi. The final obtained amine modified TCPSi nanoparticles were designated as TCPSi-NH2.

Film samples of TOPSi-NH2 and TCPSi-NH2 were prepared for the convenience of contact angle measurement and the N2 ad/desorption measurements. The amine modification procedures of film samples were identical with those of nanoparticles.

**Nanoparticle Characterization.** N2 ad/desorption (Tristar II 3020, Micromeritics) was applied to determine pore parameters of amino modified PSi samples. The specific surface area was calculated using the multiple-point Brunauer—Emmett—Teller (BET) method. The pore size distribution was calculated from the desorption branch using the Barrett—Joyner—Halenda (BJH) theory. Water contact angle was measured by placing a drop of H2O on the surface of dried PSi film with identical surface chemistries to the nanoparticles. The images of the water drop on the film substrate were captured by a digital camera (Sony DSC-H20). The zeta potential and the hydrodynamic...
size of the nanoparticles were measured with Malvern Zetasizer Nano ZS (Malvern, Southborough MA, USA)

**Peptide Synthesis, Purification, and Characterization.**

NF51 was synthesized on an automated peptide synthesizer (Applied Biosystems, USA) using fluorenlymethoxyoxycarbonyl (Fmoc) solid-phase peptide synthesis strategy with Rink-amide methylbenzhydrylamine resin. The stearic acid was coupled manually to the N-terminus of the peptide overnight, at room temperature with S equiv of stearic acid. Hydroxysbenzotriazole/\(O\)-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HOBT/HBTU) were used as a coupling reagent in dimethylformamide (DMF) with N,N-disopropylethylamine (DIEA) as an activator base. The cleaving of the product was performed with trifluoroacetic acid (TFA), 2.5% triisopropylsilane, and 2.5% water for 2 h at room temperature (RT). Peptides were purified by reversed-phase high-performance liquid chromatography on C4 column (Phenomenex Jupiter C4, 5 μm, 300 Å, 250 × 10 mm) using a gradient of acetonitrile/water containing 0.1% TFA. The molecular weight of the peptides was analyzed by matrix-assisted laser desorption-ionization/time-of-flight mass spectrometry (MALDI-TOF) (The Voyager-DE PRO Biospectrometry System, USA). The molarity of the peptides was determined based on dilutions of accurately weighted substances.

**SCO Loading Capacity of the Nanoparticles.** To study the SCO loading of PSi nanoparticles, Cy5-labeled SCO (Cy5-SCO) (Microsynth AG, Balgach, Switzerland) was used. Prior to the SCO loading, TOPSi-NH₂ and TCPsi-NH₂ (0.9 mg/mL and 1.2 mg/mL in EtOH) were washed four times with \( \text{H}_2\text{O} \) by centrifuging and decanting the supernatant, followed by the addition of \( \text{H}_2\text{O} \) and dispersion with a sonic bath. Finally, the NPs were suspended in \( \text{H}_2\text{O} \) (0.2 mg/mL). Cy5-SCO was diluted into 1 \( \mu \)M solution in \( \text{H}_2\text{O} \), and the nanoparticle concentration was adjusted to 0.00; 0.02, 0.04, 0.06, 0.08, or 0.10 mg/mL maintaining the total volume as 10 μL. The suspension was vortexed, incubated for 5 min at RT, and centrifuged 15 min with 13 200 rpm (Eppendorf Centrifuge 5425, Eppendorf AG, Hamburg, Germany). To measure the nanoparticle bound fraction of Cy5-SCO, 5 μL of the supernatant was taken and diluted to 1:20 with \( \text{H}_2\text{O} \). The fluorescence of the samples was measured (Fluoroskan Ascent FL, Labsystems, Turku, Finland), using wavelengths for excitation 464 nm and emission 678 nm. The samples were measured as quadruplicate.

**CPP Functionalization of SCO Loaded Nanoparticles.** In order to load 1 \( \mu \)g SCO totally into the nanoparticles, SCO was mixed with 0.04 mg/mL TOPSi-NH₂ or 0.08 mg/mL TCPsi-NH₂ as described above. The CPP functionalization was done in suspension by adding different concentrations of NF51, using molar ratios (SCO/NF51) 1:0, 1:2, 1:4, 1:6, 1:8, and 1:10. The samples were vortexed and incubated for 1 h in RT before further usage. The hydrodynamic size, polydispersity index (PDI), and zeta potential of nanoparticles were measured with dynamic light scatter at 23 °C. The nanoparticle samples were diluted to 5.7 \( \mu \)g/mL in \( \text{H}_2\text{O} \) prior the measurements.

**Transmission Electron Microscopy.** Copper sample grids (mesh 200) were carbon coated prior to sample preparation. Nanoparticle sample was diluted 1:2 with 2% uranyl acetate in \( \text{H}_2\text{O} \) and dried to the grid. The images were taken with JEM-2100F (JEOL, Tokyo, Japan) using acceleration voltage of 200 kV.

**Cell Culture.** HeLa pLuc 705 cells, kindly provided by Prof. R. Kole, were cultured in DMEM with 10% Fetal bovine serum, 0.1% Pen/Strep, and 0.1% l-glutamine (remarked as serum containing medium), at +37 °C and 5% CO₂.

**Splice Correction Assay.** Splice correction assay, developed by Prof. R. Kole and his colleagues, is based on a permanently transfected HeLa cell line, which expresses luciferase gene with aberrant splicing (HeLa pLuc 705). SCO can prevent the aberrant splicing by binding to the mutated site of pre-mRNA. Therefore, translation ends into fully functional luciferase protein, which can be detected with luciferase assay. To improve the stability of SCO, this 18-mer RNA is a phosphorothioated and 2-O-methylated (sequence 5′′′-CUU ACC UCA GUU ACA-3′′′).

HeLa pLuc 705 cells were seeded 24 h before the transfection to 24-well plate (50 000 cells/well) containing medium (well). Cells were transfected with 100 nM SCO (Microsynth AG, Balgach, Switzerland) utilizing different molar ratios of NF51 in serum free medium. The concentrations of nanoparticles were 0.004 mg/mL (TOPSi-NH₂) and 0.008 mg/mL (TCPsi-NH₂). After 4 h, treatment transfection medium was replaced with fresh serum containing medium and incubated for 20 h. The cells were washed with cold phosphate buffered saline (PBS) and lysed with 100 μL of 0.1% triton X-100 (Sigma-Aldrich, Steinheim, Germany) for 30 min at +4 °C. Luciferase activity was measured using Promega’s luciferase assay system on GLOMAXTM 96 microplate luminometer (Promega, Sweden) and normalized to total protein content measured with DC protein determination assay (Bio-Rad, USA). Lipofectamine TM 2000 (Invitrogen, Sweden) was used as a positive control according to the manufacturer’s protocol. Untreated cells were used as a negative control. The experiment was done in triplicate and the values are represented as mean ± SD.

**Cell Proliferation Assay.** The effect of functionalized nanoparticles and SCO-NF51 complexes to the proliferation of HeLa pLuc 705 cells was assessed with (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) proliferation assay (Promega, Sweden) based on the activity of mitochondrial dehydrogenases to convert tetrazolium salts into formazan, which absorbs light at 450 nm. HeLa pLuc 705 cells (10 000/well) were seeded on a 96-well plate 24 h prior to the treatment. The similar samples and sample concentrations were used as in the splice correction assay with optimizing the amounts to fit with 96-well form. The assay was conducted according to the manufacturers protocol. The absorbance was measured with Tecan Sunrise microplate reader (Tecan Trading AG, Switzerland) using wavelength 450 nm. Untreated cells were normalized to 100% viability. Statistics were calculated with GraphPad Prism, v. 5.03 (GraphPad Software, Inc.), using one-way ANOVA and Dunnet’s test (n = 4 ± SD, untreated n = 20 ± SD).

**Fluorescent Labeling of the Nanoparticles for Confocal Microscopy.** To visualize the nanoparticles in confocal microscopy, the nanoparticles were labeled with fluorescein 5-isothiocyanate (FITC, Sigma Steinheim, Germany). TOPSi-NH₂ nanoparticles were suspended (0.5 mg/mL) into FITC-solution (0.001 mg/mL FITC in EtOH). The sample was let to react in the dark for 18 h at RT. Afterward, the FITC-labeled TOPSi-NH₂ nanoparticles (TOPSi-FITC) were washed three times with ethanol and five times with water by centrifuging and decanting the supernatant. Finally, the zeta potential and Cy5-SCO loading capacity were determined as previously described (Supporting Information S2).
Table 1. Physicochemical Properties of the Prepared Thermally Oxidized (TOPSi) and Thermally Carbonized Porous Silicon Samples (TCPsi); Amino Modification Is Represented as -NH₂

<table>
<thead>
<tr>
<th>sample</th>
<th>surface area (m⁡²/g)</th>
<th>pore size (nm)</th>
<th>pore volume (cm³/g)</th>
<th>contact angle (deg)</th>
<th>-NH₂ content (mmol/g)</th>
<th>zeta potential (mV)</th>
<th>hydrodynamic size (nm)</th>
<th>polydispersity (PDI)</th>
</tr>
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<tbody>
<tr>
<td>TOPSi</td>
<td>238.3</td>
<td>10.9</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOPSi-NH₂</td>
<td>137.2</td>
<td>8.4</td>
<td>0.40</td>
<td>37.2</td>
<td>0.57</td>
<td>55.6</td>
<td>189.9</td>
<td>0.09</td>
</tr>
<tr>
<td>TCPsi</td>
<td>240.6</td>
<td>11.0</td>
<td>0.70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCPsi-NH₂</td>
<td>156.8</td>
<td>9.2</td>
<td>0.54</td>
<td>65.4</td>
<td>0.63</td>
<td>49.3</td>
<td>173.9</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Confocal Microscopy. Cells were seeded 24 h before the transfection to Lab-TekTM 8-chambered cover glasses (Nunc; Thermo Fisher Scientific) (50 000 cells/0.4 mL of full growth medium/well). The transfection was done in a similar manner as in the splice correction assay with exception of the volume of medium per well, which was 0.2 and 0.4 mL. Additionally, Cy5-SCO was used with TOPSi-FITC nanoparticles (0.1 mg/mL). Images were captured 4 and 24 h after single treatment using LM 700 Confocal Laser Microscope (Zeiss, Germany) equipped with a live-cell incubation chamber maintaining humidified atmosphere of 5% CO₂ and 37 °C and processed with ZEN 2011 software (v. 1.0. Carl Zeiss MicroImaging GmbH). Lysosome staining was performed by adding 200 μL of serum free medium with 100 nM LysoTracker Red DND-99 (Life Technologies, Eugene, OR, USA) 1 h before the imaging. The final concentration of the stain was 50 nM, and the 1 h incubation was done at +37 °C. Nuclear staining was done after lysosome staining by removing the medium and adding 200 μL of Hanks’ balanced salt solution (HBSS, Sigma, Steinheim, Germany) with 10 μg/mL bisBenzamide H 33342 trihydrochloride (Sigma, Steinheim, Germany) and incubated for 10 min at +37 °C. Finally, the buffer solution was removed, and 200 μL of fresh HBSS was added to the cells.

Protease Stability Testing. The protease stability of SCO-NF51 complex formulated with TOPSi-NH₂ was tested with trypsin. The before mentioned SCO-NF51 complex (molar ratio 1:4) were prepared with or without TOPSi-NH₂ nanoparticles (0.04 mg/mL). Control samples were plain SCO and SCO loaded in the TOPSi-NH₂ nanoparticles. All the samples had 1 μM Cy5-SCO concentration. The prepared samples were incubated in 50 mM Tris-HCl (MP Biomedicals, Germany, pH 7.5) for one hour at 37 °C with or without 0.0025 mg/mL sequencing grade modified trypsin (Promega, Madison WI, USA). After the incubation, the samples were diluted 1:8 with sample buffer resulting in 15% (w/w) glycerol (Merck) in the samples. Thereafter, 10 μL of the samples were run in a 0.8% agarose slab gel (TopVision Agarose, Fermentas, Vilinus, Lithuania) in 1× TAE buffer with a steady voltage for one hour (100 V). Afterward the gel was imaged with Typhoon 9400 Variable mode imager (GE Healthcare, Piscataway NJ, USA) using excitation wavelength 633 nm and emission filter 670 nm. The free Cy5-SCO band intensities were measured with ImageQuant TL 1D version 7.0 (GE Healthcare). The data was normalized to SCO samples, which were used as a control column to determine significant differences with one-way ANOVA and Dunnett’s test (n = 3 ± SEM). The statistics were calculated with GraphPad Prism, v. 5.03.

RESULTS AND DISCUSSION

Nanoparticle Characterization. Psi nanoparticles with two different surface stabilizations for SCO delivery were prepared and characterized. The surface of PSI nanoparticles was passivated by thermal oxidation or carbonization processes, leading to the corresponding production of TOPSi and TCPsi samples. To optimize the oligonucleotide loading to the nanoparticles with electrostatic interactions, the stabilized PSI nanoparticles were modified with aminesilane (TOPSi-NH₂ and TCPsi-NH₂). The pore parameters, amine content, and surface hydrophilic properties of these PSI nanoparticles are presented in Table 1. The surface area of the PSI nanoparticles was reduced significantly after the amine surface modification, and the pore volumes were also reduced in a similar manner. The pore size, zeta potential, and surface area were similar with both particle types. However, the differences in contact angles revealed that TOPSi-NH₂ was substantially more hydrophilic than TCPsi-NH₂.

SCO Loading Capacity of the Nanoparticles. Varying concentrations of TOPSi-NH₂ and TCPsi-NH₂ nanoparticles were screened to determine the optimal way to obtain 100% loading efficiency for Cy5-SCO from 1 μM loading solution (Figure 1). To determine the loading efficiency, unbound Cy5-SCO was measured with a fluorometer from the supernatant of the nanoparticle suspension. TOPSi-NH₂ bound Cy5-SCO twice as effectively as TCPsi-NH₂. The minimum concentration of nanoparticles to bind 1 μM Cy5-SCO from solution was 0.04 mg/mL for TOPSi-NH₂ and 0.08 mg/mL for TCPsi-NH₂, respectively. The molecular weight of Cy5-SCO is 6691.5 g/mol. Therefore, the respective loading percentages (w/w) were 14.3% and 7.7%. The higher binding capacity of TOPSi-NH₂ is attributed to its more hydrophilic surface, which contributes to better SCO binding. Additionally, TOPSi-NH₂
has a slightly higher zeta potential, which further increases the loading capacity (Table 1).

CPP Functionalization of SCO Loaded Nanoparticles. This phase of the experiment was conducted to optimize the amount of CPP needed to complex the SCO loaded in the nanoparticles. The CPP of choice was recently discovered as NF51. Various molar ratios (SCO/NF51) were tested, and the corresponding hydrodynamic sizes (Figure 2A) and zeta potentials (Figure 2B) of the functionalized nanoparticles were measured. The surface charge of the CPP-functionalized nanoparticles was designed to be positive to ascertain the cell internalization of the particles and also to prevent any possible agglomeration of the particles. The DLS measurements showed that loading with SCO reduced slightly the size of the nanoparticles, due to the breakdown of some minor aggregates. The addition of NF51 increased the nanoparticle sizes back to their original values. Increasing the relative NF51 concentration did not affect the particle sizes to any significant extent. A similar trend was observed for both of the particle types, the overall changes being small. However, dramatic changes in zeta potentials were observed after SCO loading and NF51 functionalization. The SCO loading negated the zeta potential of the nanoparticles and increasing the amount of NF51 in the formulation changed the zeta potential to positive. The zeta potential did not return as high a level as the original particles. The size distributions as the hydrodynamic sizes are shown in Supporting Information S1.

Transmission Electron Microscopy. TEM images were examined to verify that the SCO complexation with NF51 was taking place on the surface and in the pores of the nanocarriers instead of occurring freely in the solution. The structure of TOPSi-NH2 nanoparticles was compared to plain SCO-NF51 complex and to SCO loaded TOPSi-NH2 nanoparticles with NF51 (Figure 3). A high molar ratio (1:10) of SCO-NF51 was used to ensure that the NF51s competes with the electrostatic interactions of SCO and the nanoparticles. The nanoparticles formed small

Figure 2. Hydrodynamic size (A) and the zeta potential (B) of the SCO loaded nanoparticles (TOPSi-NH2 and TCPSi-NH2) with varying amount of NF51. The concentration of SCO is 1 μM, whereas the respective TOPSi-NH2 and TCPSi-NH2 concentrations are 0.04 and 0.08 mg/mL in all the samples. The concentration of peptide is represented as a molar ratio (SCO/NF51). Polydispersity index (PDI) is <0.11 in all of the samples.

Figure 3. TEM images from TOPSi-NH2 nanoparticles (A,D), SCO-NF51 complex, (B,E), and SCO-loaded TOPSi-NH2 nanoparticles with NF51 (C,F). The molar ratio of SCO-NF51 was 1:10 (B,C,E,F). The nanoparticles form small clusters on the sample grid. The typical web-like structures associated with SCO-NF51 complexes are not present in the image of SCO-loaded TOPSi-NH2 nanoparticles with NF51.
clusters, where a few nanoparticles were bound together, during the sample preparation process. Nonetheless, the shape of the plain TOPSi-NH$_2$ nanoparticles (panels A and D) was readily distinguishable from SCO-NF51 complexes (molar ratio 1:10), which formed large web-like structures (panels B and E). The shape of SCO loaded TOPSi-NH$_2$ nanoparticles with NF51 peptide (molar ratio 1:10) was similar to the plain particles indicating that the majority of the complex was situated within the nanoparticles (panels C and F).

**Splice Correction Assay.** The splice correction assay is an excellent method for assessing SCO delivery as it is based on up-regulation of luciferase protein expression, which requires living and functional cells.$^{27}$ The biological effect of SCO was compared while being delivered with PSi nanoparticles, SCO-NF51 complex, or Lipofectamine 2000. The variable molar ratios of NF51-peptide compared to SCO (100 nM) were used (Figure 4). After 24 h of incubation, plain SCO did not have splice correcting activity nor did SCO loaded nanoparticles without NF51. An effect could be seen only when NF51 was incorporated into the formulation. However, increasing the SCO-NF51 molar ratio over 1:4 did not improve the biological effect any further. TOPSi-NH$_2$ nanoparticles displayed similar transfection efficiency as SCO-NF51 complex and three times better than with TCPSi-NH$_2$ nanoparticles. However, the

![Figure 4](image.png)

**Figure 4.** Biological effect of SCO delivery was assessed with the splice correction assay. The biological effect is presented as the fold increase observed in the untreated cells. Lipofectamine 2000 was used as positive control. $n = 3 \pm SD$; **$p < 0.01$; ***$p < 0.001$; ns = no significant difference to untreated cells.

![Figure 5](image.png)

**Figure 5.** The effect of oligonucleotide–peptide complexes and nanoparticles on the proliferation of HeLa pLuc 705 cells was assessed with the MTS-assay. The use of PSi nanoparticles in SCO delivery does not reduce the viability of the cells significantly. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns = no significant difference to the untreated cells.
transfection efficacy was still only moderate as compared to that achieved with Lipofectamine 2000.

**Cell Proliferation Assay.** The effect of functionalized nanoparticles and SCO-NF51 complexes to the proliferation of HeLa pLuc 705 cells was assessed with MTS assay (Figure 5). After 24 h, there was no statistical significant reduction in the proliferation of the cells. However, cell proliferation was reduced significantly (p < 0.001) with Lipofectamine 2000.

**Confocal Microscopy.** The SCO delivery and internalization into HeLa pLuc 705 cells was further studied with confocal microscopy (Figure 6). Since TOPSi-NH$_2$ nanoparticles showed more biological effect (Figure 4), we mainly focused on imaging the cellular uptake of these particles. To visualize both the nanoparticles and the SCO oligonucleotide, fluorescence labeled TOPSi-FITC nanoparticles and Cy5-SCO oligonucleotides were used, respectively. For localization of cellular compartments, the nucleus and lysosomes were stained. From the fluorescence microscopy images, the fast internalization of SCO-NF51 complexes was seen. Already after 4 h, the complexes have internalized to the cells and are mostly overlapping with the lysosomes. However, some exception can be seen where the SCO signal is not in the lysosome.

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**Figure 6.** Confocal microscopy images of HeLa pLuc 705 cells treated with Cy5-SCO for 4 h. The images were taken after 4 and 24 h. Cy5-SCO (red) was formulated either with NF51 peptide or loaded into FITC labeled TOPSi-NH$_2$ nanoparticles (green) without or with NF51. To visualize the intracellular compartments, the nucleus (blue) and lysosomes (white) were stained. The arrows point out Cy5-SCO not in lysosomes. Coalescent Cy5-SCO and TOPSi-FITC is visualized with yellow color. The scale bar is 20 μm.
(downward arrow). There was no difference in the accumulation after 24 h; majority of the oligonucleotide was still coalescent with lysosomes.

The SCO loaded TOPSi-FITC nanoparticles formed large aggregates attached to the cell membranes and are visible also in the brightfield image. After 4 h, the SCO signal is seen coalescent with the nanoparticles (yellow) and most of the particles are on the cell membranes. After 24 h, some of the nanoparticles have been taken inside the cells, but the signal is coalescent with the lysosomes. Also large aggregates are visible on the cell membranes.

When NF51 peptide is used to functionalize the SCO-loaded TOPSi-FITC nanoparticles, the particles showed improved suspension stability. The improved suspension properties are likely related to the steric stabilization of the nanocarriers by the NF51 peptide. Although the large aggregates are missing, the particles still form smaller aggregates that are visible in the brightfield image. After 4 h, the NF51 functionalized and SCO-loaded particles are mostly seen on the cell membranes and free in the cell culture medium, but few particles can be seen inside the cells but outside the lysosomes (upward arrow). The signal from SCO and nanoparticle is coalescent. After 24 h, the majority of the nanoparticles are internalized by the cells, and the signal is mostly overlapping with the lysosomes. noteworthy is that the signals from SCO and TOPSi-FITC is still mainly coalescent, which indicates only minor release of the cargo.

In both cases where NF51 was applied, the SCO signal is visible inside the cell separate from the lysosomes after 4 h. Even though it represents only a small portion of the total signal from SCO, it correlates with the biological activity seen in splice correction assay where NF51 in the formulation was crucial for the biological activity. After 24 h in all the samples, the main part of SCO signal is seen to be coalescent with the lysosomes. This is seen also with TOPSi-FITC nanoparticles although the fluorescence signal is weaker in liposomal pH due the pH-dependent fluorescence of FITC.

The nanoparticle internalization is slower than the uptake of SCO-NF51 complexes suggesting a different uptake mechanism for the functionalized nanocarrier. The mechanism how SCO reaches the nucleus remains unclear, but the biological data connects it to the NF51 peptide.

Stability against Protease. NF51 and SCO are known to form nanostructures when complexing. The TEM data suggested that, when NF51 is added to SCO loaded PSi nanoparticles, this complexing takes place mostly inside the porous material. The nanocarriers ability to protect its cargo from environment was studied, as both SCO and NF51 were crucial in the PSi nanoparticle formulation to achieve a biological effect in the splice correction assay (Figure 4). The stability of SCO-NF51 complexes, formulated with and without TOPSi-NH₂ nanocarriers, was assessed against trypsin protease (Figure 7). The columns in the figure represent the band intensities of free Cy5-SCO in agarose gel. SCO-NF51 complexes formulated with TOPSi-NH₂ nanoparticles resist trypsin digestion. The data was compared to SCO column (n = 3 ± SEM, ns = nonsignificant difference, ** = p < 0.01). The increased stability toward protease degradation was attributable to the decreased surface area of SCO-NF51 complex when it was located inside the pores.

**CONCLUSIONS**

The development of novel delivery platforms is crucial before there can be any widespread use of oligonucleotide drugs. In this respect, mesoporous silicon is a potential candidate due to its beneficial properties. Its modifiable surface chemistry, biodegradability, and imaging aspects are all attractive features for a delivery platform, which can protect the cargo from a harmful environment and release it to the target site. In this study, negatively charged SCOs could be loaded into amino modified mesoporous silicon nanoparticles in a fast and highly efficient manner without the use of harmful solvents. Both of the PSi nanoparticles studied were suitable for SCO delivery in vitro regardless of the surface stabilization mechanisms. However, NF51 cell penetrating peptide was required in the formulation to achieve the biological effect by endosomal escape of SCO. The transfection ability of PSi nanoparticles was lower than that of commercial Lipofectamine 2000. However, cell proliferation was not reduced when PSi nanoparticles were used in the splice correction assay, in contrast to the situation with Lipofectamine 2000. The in vivo oligonucleotide delivery is challenging as the body has several defense mechanisms, and there is a clear need to devise new delivery platforms. Fast and efficient loading, low cytotoxicity, the protection of the cargo against proteases, and the possibility to further functionalize the carrier mean that mesoporous silicon nanoparticles represent a very flexible drug delivery platform.

Figure 7. Stability of SCO-NF51 complexes formulated with or without TOPSi-NH₂ nanoparticles (NP) in the presence of protease (trypsin). The columns represent the band intensities of free Cy5-SCO in agarose gel. SCO-NF51 complexes formulated with TOPSi-NH₂ nanoparticles resist trypsin digestion. The data was compared to SCO column (n = 3 ± SEM, ns = nonsignificant difference, ** = p < 0.01).
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REFERENCES