Chapter 6

RNAi Using a Chitosan/siRNA Nanoparticle System: In Vitro and In Vivo Applications

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Abstract

Delivery is a key issue in development of clinically relevant RNAi therapeutics. Polymeric nanoparticles formed by self-assembly of polycations with siRNA can be used for extracellular delivery, cellular uptake and intracellular trafficking as a strategy to improve the therapeutic potential of siRNA. This chapter describes a chitosan-based nanoparticle system for in vitro and in vivo transfection of siRNA into cells. The method exploits the mucoadhesive and mucopermeable properties of this cationic polysaccharide to deliver siRNA across mucosal epithelium and provides a platform for targeting human diseases with RNAi therapeutics.

Key words: siRNA, Chitosan, Nanoparticles, Macrophages, Nasal Delivery, Intraperitoneal Delivery, TNFα, Freeze Drying.

1. Introduction

RNA interference (RNAi) has proven to be an effective method to knockdown expression of individual genes by the action of double-stranded RNA *(1, 2)* adding to renewed optimism that it will be possible in the foreseeable future to use gene inhibitory medicine to treat human disease. The most prominent obstacle is delivery of the large siRNA molecule into diseased cells of the patient. Polycationic polymer-based nanoparticle (or polyplex) systems used for site-specific delivery, cellular uptake and intracellular trafficking of plasmid DNA *(3, 4)* can be adopted to improve the therapeutic potential of siRNA. This chapter describes a chitosan-based nanoparticle system for in vitro and in vivo

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silencing applications *(5, 6)*. The mucoadhesive *(7)* and mucopermeable *(8)* properties of this cationic polysaccharide are exploited for siRNA delivery across mucosal epithelium as an approach to target diseases of the mucosa and overcome serum-induced breakdown and first-pass clearance associated with intravenous administration of nanoparticles. Furthermore, chitosan-mediated transfection by a mannose-like lectin receptor *(9)* interaction facilitates delivery into macrophages and silencing of cytokines such as tumour necrosis factor alpha (TNFα) that are involved in inflammatory and infectious diseases. Intraperitoneal administration into a serum-free macrophage-rich environment can be used as a strategy to utilize this property for in vivo applications *(10)*.

2. Materials

2.1. Nanoparticle Preparation, Storage and Drying

1. 60% sucrose solution.
2. Filter (0.2 μm) (Sartorius, Goettingen, Germany).
3. siRNA, including fluorescently labelled siRNA (Dharmacon, Co, USA, DNA Technology, Aarhus, Denmark and Integrated DNA Technologies (IDT), IA, USA).
4. Solubilization buffer: 0.2 M NaAc, pH 4.3. Filter through a 0.2-μm filter, make with nuclease-free water.
5. Nanoparticle Formation Buffer: 0.2 M NaAc, pH 5.5 pH adjusted with 1 M NaOH, make with nuclease-free water.
6. Chitosan Solution: <5 mg/ml Chitosan (Bioneer, Hørsholm, Denmark or Novomatrix, Sandvika, Norway) in 0.2 M NaAc, pH 5.5. Dissolve chitosan in solubilization buffer overnight, filter through a 0.2-μm filter and adjust pH to 5.5 with 1 M NaOH (*see* **Notes 1** and **2**).

2.2. Transfection and Reverse Transfection

1. Complete media: RPMI Medium (RPMI) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Add 0.5% G418 if cells are expressing a plasmid with a neomycin resistance gene.
2. Phosphate-buffered saline (PBS): Prepare 10X stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4. Adjust to pH 7.4 with 1 M HCl and autoclave before storage at room temperature.

2.3. Flow Cytometry

1. PBS (prepared as described above).
2. Trypsin-EDTA.
3. 1% Paraformaldehyde in PBS.

2.4. Cytotoxicity

1. CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Wisconsin, USA).

2.5. Tumour Necrosis Factor Alpha (TNFα) ELISA

1. Goat anti-mouse TNFα capture antibody, recombinant murine TNFα, goat anti-mouse TNFα detection antibody, streptavidin-horseradish peroxidase, tetramethylbenzidine (R&D Systems).
2. Lipopolysaccharide (LPS) (InvivoGen, Ca, USA).
3. Bovine serum albumin (BSA)
4. Coating buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3 (pH 9.6).
5. Blocking buffer: PBS with 5% sucrose and 0.05% NaN_3 (pH 7.4).

2.6. Extraction and Isolation of Primary Macrophages

1. MEM Media.

2.7. Pulmonary Gene Silencing by Nasal Administration

1. Isoflurane.
2. Zoletilmix/Torbugesic mix: 0.5 ml zoletilmix, 1.3 ml sterile water, 0.7 ml torbugesic (diluted prior 1:100 in sterile water), dosage is ~0.14 ml for an animal weighing 22 g.
3. DAPI (Sigma, St. Louis, MO, USA).
4. VivaSpin20 centrifugal concentrators (MW cut-off 100 kDa) (Vivascience, USA)

2.8. Intraperitoneal Transfection of Peritoneal Macrophages

1. Syringes (1 ml) (Terumo, NJ, USA).
2. Needles 25G, 0.5 × 25 mm (BD, NJ, USA).

3. Methods

3.1. Nanoparticle Preparation

The formation of interpolyelectrolyte complexes (nanoparticles or polyplexes) occurs between anionic siRNA duplexes and cationic chitosan polymer *(5, 6)*.

1. Dilute chitosan solution to 0.8 mg/ml with particle formation buffer.
2. Stir 1 ml in a tube (internal diameter = 13 mm, internal length = 96 mm) containing a small magnetic fly (length = 7.5 mm, width/height = 1.5 mm).
3. Add 20 μl of 100 μM siRNA in distilled water slowly in one slow continual action from a pipette tip that has been inserted into the liquid while stirring at medium speed on a bench stirrer (*see* **Note 3**). Using the aforementioned amounts of chitosan (84%DD) and siRNA will result in a N:P ratio of ~63 (*see* **Note 4**).
4. Leave the solution to stir for an hour after which it is ready for use.

3.2. Storage and Drying

After nanoparticle formation it is possible to store the particle at 4°C for 1 week. But in that case, the particle solution should be mixed gently prior to use.

If an aqueous lyoprotectant such as sucrose is added to the nanoparticles at a final concentration of 5% or more (e.g. by adding 200 μl 60% sucrose to a 1 ml particle solution) the particles can be frozen and thawed later without loss of activity; they can also be vacuum dried and freeze dried *(11)*. If a lyoprotectant is not added the nanoparticles cannot be dried nor frozen without a complete loss of activity. If freeze drying is desired the particles are frozen to –20°C and freeze dried at –20°C and under 200 mT (Duradry/Durastop freezer dryer system, FTS Systems, NY, USA). The nanoparticles can be vacuum dried without freezing but a small loss (10–20%) in transfection efficiency will then be observed. After drying the nanoparticle/sucrose solution it should become transparent, flat and highly viscous. After drying, the nanoparticles are best stored dry, in the dark at 4°C or at –20°C for optimal storage, but the nanoparticles are still capable of 40% knockdown after 2 weeks, if stored at 25°C.

3.3. Transfection

1. Seed the cells the day before transfection at a concentration of 25,000–55,000 cells/cm^2 in complete media (500 μl for 24-well plates, 100 μl for 96-well plates).
2. On the day of transfection remove the media and add fresh complete media. The volume of complete media added should be 250 μl for 24-well plates and 100 μl for 96-well plates minus the volume of transfection agent added.
3. Add the transfection agent to give a 50 nM siRNA concentration in the wells (for batch of nanoparticles made with 20 μl 100 μM siRNA in 1 ml 0.8 mg/ml chitosan one should add 6.4 μl nanoparticle solution to 244 μl media) (*see* **Note 5**).
4. Remove the transfection media from the cells after at least 4 h and within 24 h (*see* **Note 6**).
5. Wash the wells with PBS (500 μl or more for 24-well plates, 100 μl or more for 96-well plates).
6. Replace the PBS with fresh complete media (500 μl or more for 24-well plates, 100 μl or more for 96-well plates).

3.4. Reverse Transfection

It is an efficient gene silencing method with potential applications for high throughput gene screening tool and longer shelf-life use *(11)*.

1. Add complete media containing 25,000–100,000 cells/cm^2 to the wells. The amount of nanoparticles dried in each well and the volume of media added should be set so the final concentration in each well equals 50 nM (e.g. adding 250 μl media with 100,000 cells to each well on

a freeze dried 24-well plate where in each well was added 7.6 μl chitosan/siRNA/sucrose solution (composed of 1 ml 0.8 mg/ml chitosan, 20 μl 100 μM siRNA and 200 μl 60% sucrose)).
2. Mix the wells gently.
3. After 24 h remove the media and replace it with new growth media (500 μl or more for 24-well plates, 100 μl or more for 96-well plates) (*see* **Note 7**).

3.5. Flow Cytometry

To determine and quantify the transfection and knockdown efficiency in a cell line flow cytometry can be performed; this either requires that the cell line expresses a fluorescent protein (such as enhanced green fluorescent protein (eGFP) endogenously, or that the cell line has been transfected with a plasmid expressing such a protein prior to or at the same time as the knockdown study (known as a co-transfection, in which case a plasmid transfection agent is also needed). Alternatively the cells can be transfected with siRNAs that have been fluorescently labelled with dyes such as Cy3 or Cy5 (Dharmacon, Co, USA). In this case flow cytometry measures the cellular binding and uptake of the siRNA molecules.

To perform the flow cytometry the transfected cells are typically harvested after 48 or 72 h, suspension cells can be harvested directly (jump to Step 5), adherent cells are harvested using the following protocol.

1. Remove media.
2. Wash each well with 500 μl PBS (24-well plates) or 100 μl PBS (96-well plates).
3. Add 250 μl trypsin-EDTA and incubate for ~5 min at 37°C.
4. Remove trypsin-EDTA and add of 500 μl complete media (for 24-well plates) or 100 μl complete media (for 96-well plates).
5. Transfer the media to 1.5-ml tubes and centrifuge for 5 min at 1500 rpm.
6. Remove media and wash the cells with 500 μl PBS.
7. Centrifuge for 5 min at 1500 rpm.
8. Remove PBS and add 500 μl PBS with 1% paraformaldehyde or formaldehyde.

The cells are then stored at 4°C in the dark between 1 and 7 days before flow cytometry is performed. Flow cytometry is carried out by first selecting the cell population in a dot plot of side scatter vs. forward scatter and then measuring the geometric mean of the fluorescence in a histogram of this cell population. The transfection efficiency of the chitosan/siRNA nanoparticles can then be calculated as 1 minus the geographical mean of match-transfected cells divided by the geographical mean of mismatch-transfected cells (*see* **Note 8**).

3.6. Cytotoxicity

To investigate whether the chitosan/siRNA nanoparticles induce toxicity in a specific cell line, a cytotoxicity assay can be carried out.

1. After 48 h of transfection, remove media and add 100 μl complete media (96-well plate).
2. Add 20 μl CellTiter 96® AQueous One Solution Cell Proliferation Assay to wells containing cells and a "no cells" control.
3. Incubated the cell for 1.5 h at 37°C and 5% CO_2 (*see* **Note 9**).
4. Read absorbance at 490 nm and 600 nm (*see* **Note 10**).

The $absorbance_{490\ nm} - absorbance_{600\ nm}$ is directly correlated to the viability of the cells, and the toxicity of the particles can be inferred by comparing this value for transfected cells and non-transfected control cells.

3.7. TNFα ELISA

The determination of TNFα secretion can be used as a measure of downregulation of inflammatory responses in primary macrophages.

1. Harvest the cells after transfection is ended as if for flow cytometry.
2. Add 100 μl of the cell suspension to each well on a 96-well plate for ELISA and another 100 μl cell suspension to a 96-well plate for a cytotoxicity assay.
3. Add 10 μl of 1 mg/ml LPS to the wells and incubate the plate at 37°C and 5% CO_2 for 5 h.
4. Meanwhile coat maxisorp plates overnight at 25°C with 100 μl of goat anti-mouse TNFα capture antibody in a concentration of 2 mg/ml in coating buffer.
5. Block the wells for 1 h with 300 μl of 1% BSA in a blocking buffer.
6. Add 100 μl of supernatants from the cells incubated with LPS to the maxisorp.
7. Add control recombinant murine TNFα to the coated maxisorp plate wells (100 μl in each well).
8. Incubated overnight at 4°C.
9. Subsequently, incubate the wells 25°C for 2 h with 100 μl biotinylated goat anti-mouse TNFα detection antibody (150 ng/ml).
10. Dilute and add streptavidin-horseradish peroxidase 1:200 in TBS with 0.1% BSA.
11. Incubate the mixture for 20 min.
12. Add H_2O_2 and tetramethylbenzidine for colour development.
13. Incubate the plates in the dark for 10–20 min.
14. Stop the reaction by addition of 50 μl 5% H_2SO_4.
15. Read the absorbance at 450 and 570 nm; the latter wavelength is used as a reference. Between each step the plates

are washed three times with PBS-0.05% Tween 20, pH 7.4. As mentioned earlier the measured quantities of TNFα should be normalized to the viability of each sample.

3.8. Extraction and Isolation of Primary Macrophages

For evaluation of particle transfection and gene silencing in "*difficult-to-silence*" primary cells:

1. Kill adult mice by cervical dislocation.
2. Inject 5 ml of MEM containing 20% FBS intraperitoneally with a 25G needle.
3. Agitate the abdomen gently while the peritoneum is exposed and breached.
4. Remove the medium using a syringe.
5. Centrifuge the medium for 10 min at 2500 rpm.
6. Resuspend the pellet resuspended in MEM containing 50% FBS.
7. Plate the suspension on a multiwell 12-well plate.
8. Allow the macrophages to adhere for 2 h before medium (containing non-adherent cells) is removed.
9. Add fresh medium containing 5% penicillin/streptomycin to the cells.
10. After 40 h remove the medium: the macrophages are now isolated and ready for transfection (same particle system 20 μl 100 μm siRNA described earlier).

3.9. Pulmonary Gene Silencing by Nasal Administration

The route of administration is an important determinant for successful RNA-based silencing therapeutics. Local administration to the mucosal surfaces lining the respiratory tract is an attractive alternative to the intravenous route for the treatment of pulmonary diseases.

1. Anaesthetize the mice with isoflurane.
2. Place the mice on their back and administer 30 μl particles (made as above but with 250 μM siRNA, concentrate to 1 mg/ml siRNA using VivaSpin centrifugal concentrator) intranasally (15 μl in each nostril) each day for 5 days (*see* **Note 11**).
3. On day 6 the mice are anaesthetized with an injection of 0.14 ml zoletilmix/torbugesic mix.
4. Perfusion fixate manually with 4% formaldehyde phosphate-buffered solution.
5. Harvest lungs.
6. Paraffin-embed the lungs.
7. Cut exhaustively in 3-μm sections.
8. Sample every 100th section together with the next.
9. Transfer sections into DAPI solution for counterstaining.
10. Wet mount on a Super Frost slide.
11. Analyse the slides in a fluorescence microscope with a UV/GFP filter, a 20x objective, a mounted digital

camera and a motorized stage in conjunction with CAST software.

12. Count the number of EGFP-expressing epithelial bronchial cells by a physical fractionator.

3.10. Intraperitoneal Transfection and TNFα knockdown in Peritoneal Macrophages

Investigation of transfection and gene silencing in systemic macrophages by the intraperitoneal injection. The intraperitoneal route facilitates uptake of particles into systemic macrophages in a serum-free environment.

1. Inject 200 μl of particles containing fluorescently labelled siRNA (N:P ~ 63 prepared as above) into the peritoneal cavity of mice with a 25G needle.
2. Massage the abdominal area to facilitate the distribution of the particles within the peritoneum.
3. Peritoneal macrophages are isolated, according to the procedure described above, 2 h post-injection and plated in 96-well plates or mounted with DAPI nuclear stain onto glass-bottom dishes (MatTek Corp. Ma, USA) or glass coverslips in 12-well plates.
4. Uptake of fluorescently labelled siRNA is monitored by a Zeiss semi-confocal epifluorescence microscope (or equivalent).
5. TNFα levels in the harvested supernatants of LPS-stimulated macrophages are measured 24 h post-injection according to the above protocol.

4. Notes

1. If a higher concentration of chitosan or a buffer with a higher pH is used when dissolving the chitosan it has a tendency to dissolve incompletely.
2. We suggest using a chitosan with a molecular weight over 100 kDa and with a degree of deacetylation (DD) of at least 80%. DD refers to percentage of free amino groups (cationic charges) on the polymer chain after the process of deacetylation of chitin.
3. It is very important that the whole volume of the liquid is being stirred. The volume of the reagents can also be scaled down to 500 μl or 250 μl; in this case, it is, however, even more important to ensure that the liquid is being stirred during the entire process and that continual addition of siRNA is not compromised by hitting the flea with the pipette tip as this can affect particle formation.
4. The N:P ratio refers to the ratio of positive charges on the chitosan amines to the negative charges on the phosphates in the siRNA backbone. To calculate N:P ratios

a mass-per-charge of 325 Da was used for RNA and mass-per-charge of 168 Da was used for chitosan (at 84% deacetylation). The concentration of the chitosan and siRNA can be scaled down and the N:P ratio changed slightly to 1 ml 0.2 mg/ml chitosan and 20 μl 20 μM siRNA; however, particles produced by the aforementioned concentrations show high transfection and silencing efficiency.

5. The complete media can be mixed with the particles prior to addition to the wells rather than adding the media first and then the particles separately to each well.
6. The transfection efficiency and toxicity of the particles appear to vary between different cell types; we suggest limiting the transfection time to 4 h if toxicity is observed.
7. The 24-h transfection period is necessary as the cells need time to attach before the media is changed.
8. If flow cytometry is to be carried out on the same day as cell harvesting, the fixation agent should not be included. Instead it is a good idea to include 2% serum in the PBS to stabilize the cells until and during the flow cytometry. Store the fixed cells in darkness at 4°C. It is always best to include two additional controls containing non-transfected fluorescent and non-fluorescent cells. The geographical mean of the non-fluorescent cells should then be subtracted from the geographical means of the other samples before further calculations are carried out. Non-specific knockdown induced by the particles can be inferred by comparing the geographical mean of the mismatch-transfected cells with that of the non-transfected control cells. If using fluorescently labelled siRNA it is recommended to confirm that the siRNA is taken up by the cells using fluorescent microscopy.
9. Increasing the incubation time will increase absorption time and may be necessary if few cell are present.
10. Other wavelengths can be used but will reduce the sensitivity.
11. Higher N:P with more excess chitosan at this concentration can be used to improve mucoadhesion and mucopermeation in vivo properties but should be tested in an epithelial cell line as the N:P ratio has been shown to affect silencing efficiency *(3)*.

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