Published in final edited form as:

Methods Mol Biol. 2020 January 01; 2059: 155–166. doi:10.1007/978-1-4939-9798-5_7.

A method for targeted non-viral siRNA delivery in cancer and inflammatory diseases

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Summary

Small interfering RNA (siRNA) based therapy has been subject of intense research since the discovery of RNA interference (RNAi), providing a tool to potentially silence any chosen gene. Nevertheless, efficient delivery still presents a major hurdle to translating this promising technology into medical practice. Here, we describe a straightforward method to prepare and characterize an effective delivery system consisting of low molecular weight polyethylenimine (PEI) and transferrin (Tf). Tf-PEI polyplexes are not only able to successfully transport and protect the sensitive nucleic acid payload from degradation, but also to selectively deliver the siRNA to transferrin receptor (TfR) overexpressing cells, playing key roles in the pathology of numerous cancer types as well as inflammatory diseases.

Keywords

siRNA; polyplexes; gene delivery; T cell targeting; transferrin; receptor expression; knockdown; inflammation; asthma; cancer

1 Introduction

RNA interference (RNAi) offers the potential to selectively target and functionally inhibit disease-related genes [1], paving the way for developing novel treatment options to solve unmet medical needs. Small interfering RNA (siRNA), as the most intensively studied RNAi mediator for therapeutic application in recent studies, [2] arouses significant interest in modern research. Despite numerous studies aiming to translate this revolutionary technology into an applicable medication, its way into clinical routine is still hampered, and so far only one siRNA drug product has been clinically approved. [3] Naked siRNA displays several unfavorable characteristics such as its strong charge and susceptibility to nuclease degradation. [4] In order to avoid rapid clearance and facilitate transport across extra- and intracellular barriers it is therefore imperative to employ a suitable delivery system for the delicate payload. Although viral vectors may offer high transfection efficiencies, due to their

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debatable safety profiles regarding immunogenicity and tumorigenicity, non-viral vectors are usually the preferred option. [5]

Cancerous and inflammatory tissue share several common features, both exhibiting enhanced vascular permeability orchestrated by mediators such as nitric oxide (NO), bradykinin, and prostaglandins. [6] The vascular anatomy of tumors is, moreover, characterized by reduced lymphatic drainage. Nanosized siRNA systems are, therefore, not only preferentially taken up into tumor tissue via the leaky vasculature, but subsequently also captured due to poor clearance effects. [7] Although this phenomenon, known as the enhanced permeability and retention (EPR) effect, is expected to improve delivery of nanoparticles into tumors, it actually only results in a rather modest 2-fold increase compared to critical normal organs in most tumor models but often is not reflected in the clinical picture. [8] The main difference between tumor and inflammatory tissue is the latter having a functional lymphatic system in place. It does, however, release various mediators inducing the EPR effect and additionally allows enhanced uptake of nanomedicines by dilation of vessels and contraction of endothelial cells. [9] Despite the preferential passive accumulation of siRNA in pathological tissues, delivery can be further improved by precise and active targeting of desired tissues and cell types. Targeted nanoparticles are generally designed to interact with specific cell subtypes via certain ligands in order to pointedly deliver their payload as well as increase retention in target cells. [10] This strategy is often achieved by aiming at receptors overexpressed on disease-related cells and eventuates in improved efficacy and reduced toxicity, resulting in an overall improved therapeutic outcome. Therefore, nanoparticles can be functionalized with targeting moieties that are recognized by specific cell types including peptides, oligosaccharides, antibodies, and proteins, of which transferrin is a promising example. Transferrin is a native circulating glycoprotein, responsible for controlling iron levels in biological fluids. Transferrin receptors (TfR) are transmembrane proteins which bind plasma iron complexes with transferrin, enabling iron internalization into cells via receptor-mediated endocytosis. [11] While TfR 1 is ubiquitously expressed on almost all generic cell surfaces at low levels, its expression is upregulated in highly proliferating cells such as in various cancer types and inflammatory tissues. [12] Enhanced TfR 1 expression has been reported in a wide range of different cancers, including breast, liver, colon, ovarian, and lung cancer, as well as leukemia and even glioblastoma. [13] This expression profile can not only be exploited to target cancer specific cells, but furthermore can facilitate cerebral delivery of siRNA by overcoming the otherwise hard to conquer blood brain barrier, since brain endothelial cells also express TfR. [14] In a similar approach, siRNA can successfully be delivered to activated T cells via transferrin targeting, presenting a way to precisely reach disease-related cells in inflammatory diseases such as asthma. [15] Transfection of T cells is a challenging task in general, as they are devoid of caveolae, and therefore do not actively endocytose nanoparticles. [16] Hence, the employment of transferrin as a targeting ligand again fulfills two purposes by offering a gate to enter the difficult to reach T cells and selectively targeting activated TfR overexpressing T cells while leaving naïve ones unaffected. This chapter describes a novel approach to generate a versatile siRNA delivery system composed of low molecular weight polyethylenimine (PEI) and transferrin (Tf) suitable for targeting of disease-related cells in cancer [17] as well as inflammatory diseases [15]. It is moreover

depicted how the Tf-PEI conjugate and resulting siRNA polyplexes can be characterized and how respective cell lines are tested for sufficient TfR expression. Finally, a transferrin competition assay is presented as a method to verify ligand-mediated uptake of the targeted polyplexes *in vitro*.

2 Materials

2.1 Conjugate Synthesis

1. Buffers: (see Note 1)
Buffer 1 (B1): 20 mM HEPES, 150 mM NaCl, pH 7.5.
Buffer 2 (B2): 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.1.
Buffer 3 (B3): 20 mM HEPES, 150 mM NaCl, 20 mM EDTA, pH 7.1.
Buffer A: 20 mM HEPES, 0.5 M NaCl.
Buffer B: 20 mM HEPES, 3 M NaCl.
2. 5 kDa PEI (Lupasol® G100, BASF, Ludwigshafen, Germany).
3. Human holo-transferrin (Sigma-Aldrich, St. Louis, USA).
4. Succinimidyl 3-(2-pyridyldithio) propionate (SPDP, ThermoFisher Scientific, Waltham, USA).
5. Dithiothreitol (DTT, ThermoFisher Scientific, Waltham, USA).
6. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA).
7. Centrifugal filters:
MWCO 3.000: Amicon® Ultra-4 (Merck Millipore, Burlington, USA)
MWCO 10.000: VIVASPIN 6 (Sartorius, Göttingen, Germany)
8. HiTrap™ SP HP columns, 1ml (GE Healthcare, Chicago, USA).
9. Centrifugal tubes, 1.5 ml.
10. Glass vials, 4 ml.

2.2 TfR1 Expression

1. Phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, USA).
2. Human FcR binding inhibitor (eBioscience, Frankfurt, Germany).
3. Anti-human CD71PE (eBioscience, Frankfurt, Germany).
4. Mouse anti-human IgG1-PE (ThermoFisher Scientific, Waltham, USA).
5. Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, St. Louis, USA).

2.3 Polyplex Preparation and Particle Characterization

1. HEPES buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.5).

2. Disposable micro-cuvette (Malvern Instruments, Malvern, UK).
3. Folded capillary cells (Malvern Instruments, Malvern, UK).

2.4 siRNA Encapsulation

1. 5 % glucose solution.
2. FluoroNunc 96-well white plate (Thermo Fisher Scientific).
3. 4x SYBR® Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific).

2.5 Competition Assay

1. Fluorescently labeled siRNA (here: amine modified siRNA (Integrated DNA Technologies, Coralville, USA) was labeled with succinimidyl ester (NHS) modified AF 488 (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol).
2. 96-well plates (Thermo Fisher Scientific).
3. Human holo-transferrin (Sigma-Aldrich, St. Louis, USA).
4. Phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, USA).
5. Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, St. Louis, USA).

3 Methods

3.1 Conjugate Synthesis

1. Add 940 µl of a 5 kDa PEI solution (1 mg/mL in B1) in a 4 ml glass vial and drop-wise add 94 µl of SPDP solution (20 mM in DMSO) (*see Note 2*) while stirring, resulting in PEI-SPDP.
2. Wrap vial with parafilm and stir overnight at room temperature.
3. Add 10 mg of transferrin (*see Note 3*) to 1 ml B1 in a 4 ml glass vial and let stir until completely dissolved. Slowly add 31 µl of SPDP solution (20 mM in DMSO), wrap with parafilm and stir for 2 h at room temperature, resulting in Tf-SPDP.
4. In the mean-time, purify PEI-SPDP with a centrifugal filter (MWCO 3.000). Put 1 ml B2 in the column and add PEI-SPDP. Wash the used glass vial with more B2 and add to the column up to a total volume of 4 ml. Centrifuge at 4.000 g for 40 min at 4 °C. Discard the flow-through and mix the remaining solution well by pipetting up and down. Add B2 up to a column volume of 4 ml and centrifuge again at 4.000 g for 45 min at 4 °C. Transfer the remaining volume (*see Note 4*) to a 1.5 ml tube and wash the column 2 x with 100 µl B2. Store in the refrigerator (2-8 °C) for later use.
5. Purify Tf-SPDP with a centrifugal filter (MWCO 10.000). Put 1 ml B2 in the column and add Tf-SPDP. Wash the used glass vial with more B2 and add to the column up to a volume of 6 ml. Centrifuge at 4.000 g for 20 min at 4 °C. Discard

the flow-through and mix the remaining solution well. Add B2 up to a column volume of 6 ml and centrifuge again at 4.000 g for 25 min. (*see Note 5*) Transfer to a 1.5 ml tube and wash column with 100 µl B2. Store in the refrigerator (2-8 °C) for later use.

6. Measure the SPDP amount in purified PEI-SPDP (from step 4) by Pyridine-2-thione assay. (*see Note 6*) Prepare a 50 x dilution of PEI-SPDP in B1 (8 µl PEI-SPDP + 394 µl of B1). Add triplicates of 100 µl each in a 96 well plate and measure absorbance at 342 nm against B1 as blank. Subsequently, add 1 µl of 150 mM DTT solution to each well, mix and incubate for 15 min at room temperature. Measure absorbance again and calculate moles of SPDP per mole of PEI with the following equation:

$$\frac{\text{Change of absorbance}}{8080} = \frac{\text{molecular weight of protein}}{\text{concentration of protein} \left(\frac{\text{mg}}{\text{ml}} \right)}$$

7. Based on the measured amount of SPDP in PEI-SPDP, add a 10 molar excess of DTT solution and let react under an inert atmosphere of nitrogen for 2 h at room temperature.
8. Purify activated PEI-SPDP with a centrifugal filter (MWCO 3.000). Put 1 ml of B3 in the column, add PEI-SPDP and wash used vial with B3 until a column volume of 4 ml. Centrifuge at 4.000 g for 40 min at 4 °C.
9. Mix activated PEI-SPDP with purified Tf-SPDP in a 4 ml glass vial and add 10 µl of DMSO to aid formation of the disulfide bonds. Wrap with parafilm and stir at 4 °C overnight.
10. Filter the Tf-PEI conjugate with a 13 mm, 0.22 µm syringe filter and purify with 2 connected HiTrap SP HP 1 ml columns using an FPLC system, such as the ÄKTA pure protein purification system. (*see Note 7*) After removing the storage solutions from the columns with buffer A, slowly (1 ml/min) let the conjugate run through and collect 0.5 ml fractions. Wash the vial used for the conjugate with 3 ml buffer A, then switch to buffer B and rinse with 9 ml, collecting 0.5 ml fractions. (*see Note 8*)
11. To check which buffer B fractions contain the purified conjugate, measure absorbance at 280 nm with buffer B as blank.
12. Combine those fractions with the highest absorbance values and purify with centrifugal filters (MWCO 10.000) (*see Note 9*). Put 1 ml B1 in the column and add the conjugate fractions, fill with B1 up to a volume of 6 ml. Centrifuge at 4.000 g for 35 min at 4 °C. (*see Note 10*)
13. Quantify the final conjugate via 2,4,6-trinitrobenzenesulfonic acid (TNBS) (*see Note 11*) assay. It is advisable to 20 x dilute the conjugate for this step. First, determine the transferrin concentration by absorption measurement at 280 nm with the help of a standard curve starting with 1 mg/ml transferrin in B1, serially

diluted to 0.03125 mg/ml. (see Note 12) Based on the standard curve, calculate the transferrin concentration in the conjugate.

Dilute the TNBS stock solution to obtain a 3 mM working solution in B1.

Prepare a PEI standard curve starting with 400 µg/ml PEI in B1, serially diluted to 3.125 µg/ml. (see Note 13) Based on the result of the transferrin concentration, prepare a solution with 2 x the calculated transferrin concentration in the diluted conjugate. Dilute each point of the standard curve 1:1 with this transferrin solution in order to account for the transferrin amount in the conjugate.

Distribute 100 µl of each point of the standard curve in a 96 well plate and fill 3 wells with the 20 x diluted conjugate as well as 3 wells with only B1 as blank samples. Add 30 µl TNBS per well and cover with aluminum foil, incubate 5 min at room temperature and measure absorbance at 405 nm. Based on the PEI standard curve, calculate the PEI concentration in the conjugate.

14. If appropriate, filter the final conjugate with a syringe filter to make it sterile and store at 4 °C. The coupling reaction is shown in Figure 1.

3.2 TfR1 Expression

To validate whether your cell line is suitable for transfection with the Tf-PEI conjugate, it is advised to determine endogenous TfR1 expression levels.

1. Harvest cells following trypsinization (or other appropriate methods). Count viable cells and resuspend in PBS at 10×10^6 cells/ml and distribute 100 µl/tube of cell suspension (100.000 cells/tube) in 1.5 ml tubes in triplicates each for TfR1 (also known as CD71), isotype control and unstained blank. If T cells are present, add 10 µl of diluted FcR binding inhibitor (1:10 in PBS) to each sample, mix and incubate for 5 min in the refrigerator (2-8 °C).
2. Add 1 µl undiluted antibody to appropriate samples, triplicates for CD71 and isotype control. Leave 3 samples unstained. Vortex and incubate 30 min in the refrigerator (2-8 °C).
3. Centrifuge at 350 g for 5 min, discard supernatant and add 500 µl of PBS/2 mM EDTA. Centrifuge again and discard supernatant, repeat this washing step 2 times. Resuspend cells in 400 µl PBS/2 mM EDTA.
4. Adjust the laser power at the flow cytometer using the unstained blank samples.
5. Gate the cells to exclude debris and cell clumps and count at least 10.000 events per sample.
6. Determine the % of positive cells in the PE channel for each sample as depicted in Figure 2.

3.3 Polyplex Preparation and Particle Characterization

1. For polyplex preparation, dilute appropriate amounts of Tf-PEI in HEPES buffered saline (HBS) or 5 % glucose. Add defined amounts of siRNA solution

and mix well by pipetting. (see Note 14) Allow the polyplexes to form via electrostatic interactions during 20 min incubation at room temperature.

2. To measure hydrodynamic diameter, PDI and zeta potential of prepared polyplexes, prepare 100 μ l of Tf-PEI/siRNA formulations with different N/P ratios in HBS and add into disposable micro-cuvettes. Determine sizes with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 173° backscatter angle with 3 times 15 runs per sample. Use 0.88 mPa*s for viscosity and 1.33 for refractive index for data analysis with the Zetasizer software. Subsequently, dilute all samples with 900 μ l of nanopure water and transfer to a folded capillary cell. Perform three zeta potential measurements per sample using the same device. Typical results compared to unmodified PEI polyplexes are shown in Figure 3.

3.4 siRNA Encapsulation

1. Determine siRNA condensation efficiencies via SYBR gold assay. Therefore, prepare polyplexes with 50 pmol siRNA at rising N/P ratios in 5 % glucose, distribute in a FluoroNunc 96-well white plate and incubate for 20 min. Use controls containing only siRNA (N/P=0) to account for 100 % free siRNA.
2. Subsequently, add 30 μ l of 4x SYBR gold nucleic acid gel stain and incubate for another 10 min in the dark. (see Note 15)
3. Conduct fluorescence measurements at excitation wavelength of 485/20 nm and emission wavelength of 520/20 nm as illustrated in Figure 4.

3.5 Transferrin Competition Assay

1. Seed desired cells in an appropriate culture plate (here: 400.000 Jurkat cells per well in a 96 well plate) either in regular culture medium, or in medium containing defined concentrations of free transferrin (here: 0.1 and 2 mg/ml).
2. Prepare polyplexes with 50 pmol of fluorescently labeled siRNA (here: AF 488 labeled siRNA) in 5 % glucose and add to triplicates of appropriate wells. Leave 3 wells of cells untreated as blank controls.
3. Incubate the transfected cells for 24 h at a humidified atmosphere with 5 % CO₂ at 37 °C.
4. Harvest the cells and centrifuge at 350 g for 5 min. Discard the supernatant, wash the cells with PBS/2 mM EDTA, centrifuge again and discard the supernatant. Repeat this washing step 2 times.
5. Resuspend the cells in 400 μ l PBS/2 mM EDTA per sample.
6. Adjust the laser power at the flow cytometer using the unstained blank samples.
7. Gate the cells to exclude debris and cell clumps and count at least 10.000 events per sample.

8. Determine the median fluorescence intensity in the respective channel for each sample as depicted in Figure 5.

4 Notes

1. Prepare all buffers in purified water and ideally filter them with a 0.33 μm bottle top filter to get rid of any airborne particles, they do not have to be sterile.
2. The SPDP solution should always be prepared freshly. Yellow coloring is a sign for oxidation, indicating the solution should not be used any longer.
3. Ideally, remove the transferrin from the refrigerator and allow adjusting to room temperature before use.
4. The remaining volume should be $\sim 300 \mu\text{l}$.
5. The remaining volume should be $\sim 200 \mu\text{l}$.
6. DTT dissolves the disulfide bonds, resulting in pyridine-2-thione as a free moiety absorbing at 343 nm. Since this is emerging in a 1:1 ratio, it can be accounted for the amount of SPDP in PEI-SPDP. In the next step of the conjugation, the whole amount of SPDP can therefore be reduced with DTT.
7. If no FPLC system is available, the purification can also be performed manually with a syringe. Therefore, fill a 6 ml luer lock syringe with the appropriate buffers or the conjugate sample, respectively. Connect the syringe to the HiTrap column and slowly (max. 1 ml/min) push the solutions through. It is advised to always add a bit of fluid to the column before connecting the syringe to avoid formation of air bubbles.
8. Rinsing with buffer A, containing a low amount of salt, results in removing of free unconjugated transferrin, while Tf-PEI and free PEI bind to the negatively charged groups of the HiTrap column. Rinsing with buffer A as a high salt buffer, consequently leads to replacement of PEI from the binding sites with Na^+ , eluding purified Tf-PEI as well as free PEI.
9. This purification step is performed to remove any unconjugated free PEI polymer.
10. The remaining volume should be $\sim 500 \mu\text{l}$.
11. The TNBS assay measures the primary amine groups of PEI by reacting to a chromogenic product.
12. It is necessary to determine this concentration, as the transferrin in the conjugate might interfere with the PEI concentration measurement. Therefore, a transferrin solution with a respective concentration has to be added to the standard curve in order to account for any effects influencing the measurement of the conjugate.
13. Please be aware that each point is diluted 1:1 with the transferrin solution in the next step, meaning the 400 $\mu\text{g/ml}$ will only account for 200 $\mu\text{g/ml}$ PEI in the final assay analysis.

14. Calculate the mass of polymer required for 50 pmol siRNA to yield a certain amine to phosphate ratio (N/P ratio) according to the following equation: $m(\text{PEI in pg}) = 50 \text{ pmol} \times 43.1 \text{ g/mol} \times \text{N/P} \times 52$ (protonable unit of PEI = 43.1 g/mol, number of nucleotides of 25/27mer siRNA = 52).
15. SYBR gold is a nucleic acid staining dye only fluorescing when intercalating with free siRNA. If no fluorescent signal can be detected, it can be expected that the siRNA is fully protected inside of coherent polyplexes and not accessible to the dye.

Acknowledgement

This work was supported by the ERC Starting Grant ERC-2014-StG – 637830 “Novel Asthma Therapy”.

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Figure 1.

Schematic representation of the conjugation process of transferrin (Tf) and polyethylenimine (PEI), resulting in the Tf-PEI conjugate. Both components are first activated with succinimidyl 3-(2-pyridyldithio) propionate (SPDP). PEI-SPDP is subsequently reduced with dithiothreitol (DTT), before reacting with Tf-SPDP to the final conjugate. Adapted from [15].

Figure 2.

Levels of transferrin receptor 1 (TfR1, also known as CD71) on Jurkat cells as quantified by CD71-PE antibody staining and determination of median fluorescence intensity (MFI) by flow cytometry. Results are presented as means of triplicates $\pm$ SD. Blank samples were left unstained, while isotype controls were stained with an IgG antibody to exclude unspecific binding. The high TfR1 expression makes Jurkat cells a suitable model cell line for Tf-PEI experiments.

Figure 3. Hydrodynamic diameters, PDI, and zeta potentials of PEI and Tf-PEI polyplexes at different N/P ratios as measured by dynamic light scattering (DLS) and laser Doppler anemometry (LDA), respectively. Data points indicate means $\pm$ SD. Due to the shielding effect of the negatively charged transferrin, Tf-PEI polyplexes are distinctly smaller than those made of unmodified PEI and hold slightly negative zeta potentials, exhibiting improved characteristics for siRNA delivery.

Figure 4.

SiRNA encapsulation efficiencies of Tf-PEI and PEI polyplexes at increasing N/P ratios determined by SYBR gold assay. Tf-PEI polyplexes only show slightly enhanced amounts of non-encapsulated siRNA compared to regular PEI, potentially caused by steric hindrance of transferrin interfering with the interaction between PEI and siRNA.

Figure 5. Cellular uptake of PEI and Tf-PEI polyplexes containing fluorescently labeled (AF488) siRNA in Jurkat cells with or without the presence of free transferrin in the culture medium. Results are depicted as means of triplicates $\pm$ SD, blank samples represent non-treated cells. While the concentration of free transferrin does not affect uptake of PEI polyplexes, uptake of Tf-PEI is clearly diminished by the addition of free transferrin in a concentration-dependent manner, indicating a transferrin mediated uptake mechanism.