Chapter 3

PepFect6 Mediated SiRNA Delivery into Organotypic Cultures

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Abstract

Gene silencing by small interfering RNA (SiRNA) is an attractive therapeutic approach for pathological disorders that targets a specific gene. However, its applications are limited, as naked RNA is rapidly degraded by RNases and is inadequately internalized by the target cells in the body. Several viral and non-viral vectors have been described to improve the delivery of SiRNAs both in cultured cells as well as in vivo. Increasing evidence suggests that cell-penetrating peptides (CPPs) are an efficient, non-cytotoxic tool for intracellular delivery of SiRNA. Recently, a new peptide, PepFect6 (PF6), based system has been described for efficient SiRNA delivery in various cell types. PF6 is an amphipathic stearyl-TP10 peptide carrying a pH titratable trifluoromethylquinoline moiety that facilitate endosomal release. PF6 forms stable non-covalent complexes with SiRNA. Upon internalization, the complexes rapidly escape the endosomal compartment, resulting in robust RNA interference (RNAi) responses. This chapter describes a protocol to use the PF6-nanoparticle technology for SiRNA delivery into organotypic cultures of the inner ear i.e., cochlea. We also highlight different critical points in the peptide/SiRNA complex preparation, transfection and in analyzing the efficacy of PF6-SiRNA associated RNAi response.

Key words Cell-penetrating peptides (CPPs), PepFect6 (PF6), SiRNA, Inner ear, Cochlea, Organotypic cultures, Fluorescence recovery after photobleaching (FRAP)

1 Introduction

Small interfering RNAs (SiRNA) have emerged as a new class of therapeutics with a great potential for treating genetic disorders. However, when introduced into the tissue, naked SiRNAs in general are degraded by nucleases, which make the SiRNA delivery more complicated and challenging. In addition, unaided double-stranded RNA can induce innate immune response via interaction with RNA-binding proteins such as Toll-like receptors and protein kinase receptors [1]. The major hurdle in the development of RNAi therapies is the intracellular delivery of SiRNAs due to their negative charge and large size [2]. Various delivery strategies have been developed using both viral and nonviral vectors. In recent years, cell-penetrating peptides (CPPs) have emerged as potential

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nonviral vectors for delivery of nucleic acids and other bioactive molecules, both in vitro and in vivo [3–5]. CPPs are short cationic and/or amphipathic peptides that can be covalently or non-covalently combined with negatively charged cargo, e.g., SiRNA. The major route of cellular entry for most of CPPs is via endocytosis. They are trafficked through the early endosomes to late endosomes, which are acidified to pH 5–6. Subsequently, the late endosomes fuse with lysosomes where the pH further drops to 4.5 and various degradative enzymes are present. The CPP cargo that fails to escape from these acidic vesicles is ultimately degraded [6]. Therefore, for efficient SiRNA delivery, CPP technology must be improved for higher endosomal escape.

Modification of the existing CPPs has helped to overcome this endosomal entrapment [3, 7, 8]. A novel CPP, called PepFect6 (PF6), has been recently developed that exhibits increased release of SiRNA molecules from the acidic endosomal compartments [3]. It is created by introducing a stearyl and trifluoromethyl quinoline (QN) moieties into Transportan 10 (TP10). Earlier studies show that stearylation of TP10 improves the activity and reduces cytotoxic effects of the peptide in serum, while addition of lysosomotropic agent chloroquine (CQ) derivative QN enhances the endosomal release of peptide/SiRNA complex [3, 8–10].

PF6 can be non-covalently complexed with SiRNAs. It forms homogenous, unimodal 70–100 nm nanoparticles with SiRNA, which are stable at 4 °C in water for at least 4 weeks. A molar excess of CPP over SiRNA neutralizes the negative charges of SiRNA and thus protects it from serum enzymes [11]. However, in the presence of serum, the nanoparticles are larger (125–200 nm) with wider distribution and are relatively less stable, especially at 37 °C [3, 11]. Nevertheless, the PF6-SiRNA nanoparticles are endocytosed within an hour by the cells, resulting in downregulation of the target gene without cytotoxicity or immunogenicity in vitro [3]. When injected systemically, PF6-SiRNA nanoparticles elicit an RNAi response without any acute toxicity to the vital organs [3, 10] suggesting promising perspectives for their future therapeutic applications without any risks of inflammation.

This chapter describes a protocol for the use of the non-covalent PF6 based intracellular delivery of SiRNA into the organotypic cultures of the cochlea. SiRNA targeting a gap junction protein, connexin 26, is used to test the functional efficacy of PF6-SiRNA complexes. Connexin 26 knockdown results in the interrupted intercellular communication among the supporting cells of the cochlea [12, 13]. Fluorescence recovery after photobleaching (FRAP) assay is a useful tool to study the gap junction functions. Rate of diffusion of fluorescent dye after FRAP displays functional effects of downregulation of connexin 26 expression by PF6-SiRNA complexes.

2 Materials

2.1 Cochlear Organotypic Cultures

1. Cell-Tak, used according to the manufacturer's protocol.
2. Phosphate-buffered saline (PBS).
3. 35 mm disposable dishes.
4. Forceps # 5 and #55 for fine dissections of cochleae.
5. 70 % ethanol.
   (a) Cochlear medium: Advanced reduced Dulbecco's modified Eagle's medium with phenol red (DMEM), 1 % N1 supplement, 1.65 % Glucose, 1 % Penicillin.
6. 4 well plate (3–4 explant/well; for RNA extraction) or Center-Well Organ Culture Dish for FRAP assay (1 explant/dish).

2.2 Peptide, Oligonucleotide and Transfection Reagents

1. PF6 is developed as a peptide-based carrier for SiRNA delivery by introducing modifications into the well-characterized TP10 peptide. Protocols for the synthesis and purification of PF6 are described by Andaloussi et al. [3, 9]. PF6 and derivatives are stable for at least 1 year when stored at –20 °C in a lyophilized form.
2. SiRNA targeting the hypoxanthine phosphoribosyltransferase (HPRT) 1 gene sense 5′-GCCAGACUUUGUUGGAUUU GAA ATT-3′ and antisense 5′-AAUUUCAAAUCCAACA AAGUCUGG CUU-3′ (biotin conjugated).
3. SiRNAs targeting the connexin 26 gene (NM_001004099) conjugated with biotin sense 5′-AAGUUCAUGAAGGG AGAGAUATT-3′- Cy5/biotin and antisense 5′-UAUCUCU CCCUUCAUGAACUU-3′.
4. Salt-free water.
5. Cochlear medium (as above).
6. 1.5 ml Eppendorf tubes.

2.3 Fluorescence Recovery After Photobleaching (FRAP) Assay

1. Cochlear cultures in the center-well culture dishes.
2. Calcein, AM.
3. PBS.
4. Laser confocal microscope (LSM 510, Zeiss).

3 Methods

3.1 Organotypic Cultures of the Cochleae

The dissection of the inner ear cochlea is described elsewhere [14]. In brief, the explants are cultured as follows:

1. Coat the dishes with Cell-Tak (135 μg/ml) for 1 h and wash with water, 3 times at room temperature.

2. Clean all instruments with 70 % ethanol or autoclave before dissection.
3. Decapitate the pups and dip the heads in 70 % alcohol for disinfection.
4. Split the skull in two and using #4 jeweler's forceps cut out the cochlea.
5. Immerse cochleae in PBS maintained on ice.
6. Dissect away the bony capsule using a #5 jeweler's forceps and transfer the soft tissue to PBS supplemented with 5.5 μl/ml of 30 % glucose.
7. Carefully identify the stria vascularis, spiral ganglion, and modiolus under high power and pull away from the explant one at time (*see* **Note 1**).
8. Place one explant per coated center-well dishes with 1 ml cochlear culture medium.
9. Check under microscope and adjust so the hair cells face upward (*see* **Note 2**).

3.2 Formation of PF6/siRNA Nanocomplexes and Delivery of PF6-siRNA Nanocomplexes into Organotypic Cochlear Cultures

The procedure for formation of PF6/SiRNA nanocomplexes constitutes an important factor in the successful transfection and should be followed carefully.

1. Take the vial containing the PF6 lyophilized powder out of the freezer and equilibrate for 30 min at room temperature without opening the vial, to avoid exposure to humidity. Dilute PF6 in salt-free water to a final storage concentration of 1 mM in a 1.5 ml tube (*see* **Note 3**). Aliquot the peptides in 20 μl aliquots to avoid several cycles of freeze-thawing, which will decrease the efficacy of the peptide. This stock solution is stable for about 2–3 months when stored at −20 °C.
2. When running an experiment, thaw one tube and add 180 μl of water to obtain a 100 μM peptide solution.
3. Mix gently by tapping the tube or by vortexing at low speed for a few seconds.
4. Prepare the nanocomplexes for experiments in duplicates in RNase-free environment (*see* **Note 4**). The final treatment volume is 1 ml per well, i.e., 2 ml for duplicate. Prepare the complexes in 1/10th of the final volume in salt-free water (*see* **Note 5**), i.e., 200 μl complexes. Do not exceed the volume required for four transfections, as this might cause aggregation.
5. Start by adding the appropriate volume of SiRNA and then water and finally the PF6 for each reaction (at a molar ratio of 1:20 or 1:40 of SiRNA over peptide) (*see* **Note 6**). Conduct a dose response assessment at each molar ratio (Fig. 1).

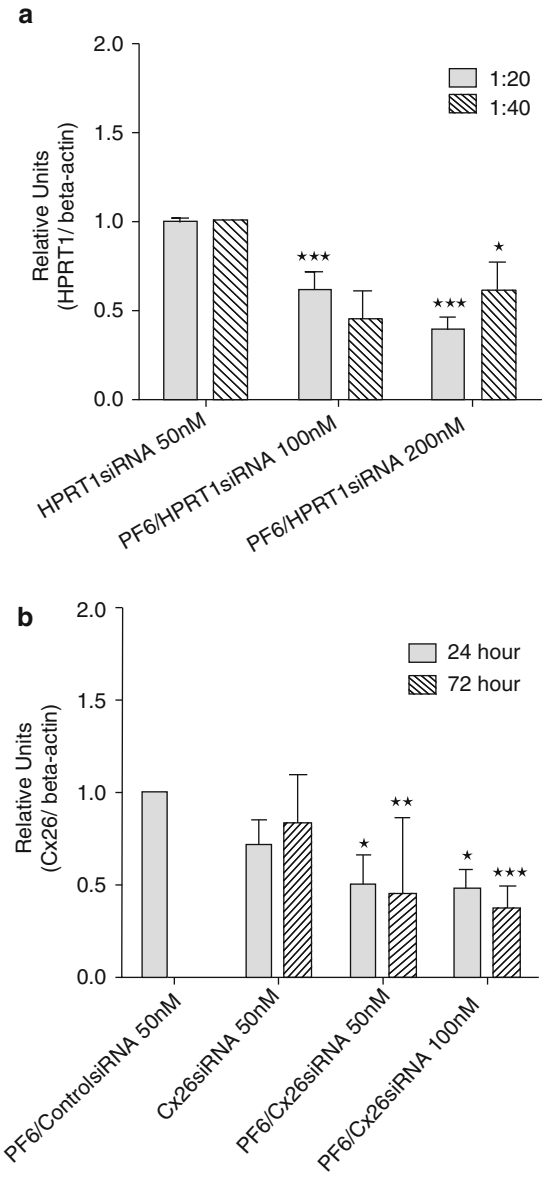

Fig. 1 PF6-mediated delivery of siRNA targeting of HPRT1 and Connexin (Cx) 26 genes in the organotypic cochlear cultures. (**a**) Efficiency of PF6 was analyzed by comparing the HPRT1 RNAi responses of PF6/HPRT1-siRNA and HPRT1-siRNA alone in the cultures by RT-qPCR following 24 h of incubation ($n=4$). (**b**) The cultures were incubated either with Cx26-siRNA alone (50 nM), or PF6/control-siRNA (50 nmol/L) or PF6/Cx26-siRNA nanoparticles in two different concentrations (50 and 100 nM). Quantitative analysis of transcript levels of Cx26, relative to beta-actin mRNA, was determined by RT-qPCR and compared with PF6/control-siRNA cultures after 24 ($n=6$) and 72 ($n=4$) hours. Actin was used as internal standard. *Error bars* indicate mean ± SEM, ***$P<0.001$, **$P<0.01$, *$P<0.05$ (Mann–Whitney *U*-test). *HPRT* hypoxanthine phosphoribosyl transferase, *RNAi* RNA interference, *RT-qPCR* reverse transcription-quantitative PCR, *siRNA* short interfering RNA. (Reproduced from ref. 15 with permission from Nature Publication Group)

Nevertheless, do not exceed the final concentration of PF6 over 2 μM in serum-free media and 5 μM in serum containing media to avoid peptide associated toxicity.

6. For control, prepare only SiRNA sample in similar manner (PF6 is replaced with water).
7. Incubate for the tubes for 30–60 min at RT.
8. After incubation, prepare a serial dilution from prepared complex solution in fresh tubes with 100 μl water (*see* **Notes 6** and **7**).

3.3 FRAP Assay: Addressing the PF6-Based siRNA Delivery Efficacy

1. Check if all the cultures are attached and growing on the plate. Randomly divide the dishes into three groups; control, only SiRNA (50 nM) and PF6-SiRNA complex (SiRNA 100 nM).
2. Replace the media in wells with fresh media, 900 μl in each well.
3. Add 100 μl of prepared complex to each well and shake the plate slightly. Incubate the plates at 37 °C, 5 % CO_2 for 24 or 72 h (*see* **Note 8**).
4. Take out one plate at a time from the incubator, at the specific incubation time point. Add 2.5 μmol/L calcein to the medium, incubate for another 10 min at 37 °C.
5. Wash the culture thoroughly with warm PBS to remove free calcein and image immediately using a confocal laser scanning microscope equipped with a 40× water immersion objective at room temperature (*see* **Note 9**).
6. Select a cell in the outer sulcus that is surrounded by other cells from all sides. Acquire images of the size of 256 × 256 pixels, one every second. Photobleach the selected cell with 100 % laser power of 488 nm between image 15th and 16th and image further (Fig. 2). Analyze the cells with bleaching efficiencies larger than 20 % for FRAP as reported earlier in ref. [15]. The mean fluorescence intensity of the bleached region is corrected for the background variations.
7. Image only two or three cells are per culture, to avoid calcium responses (*see* **Note 10**). Analyze five to six different cultures per condition from three to four independent experiments for calculating the significant differences.

4 Notes

1. Dissection of cochleae should be done very carefully. The hair cells are very sensitive to mechanical damages. While dissecting, hold the organ of Corti only in the hook region or at the extreme distal part.

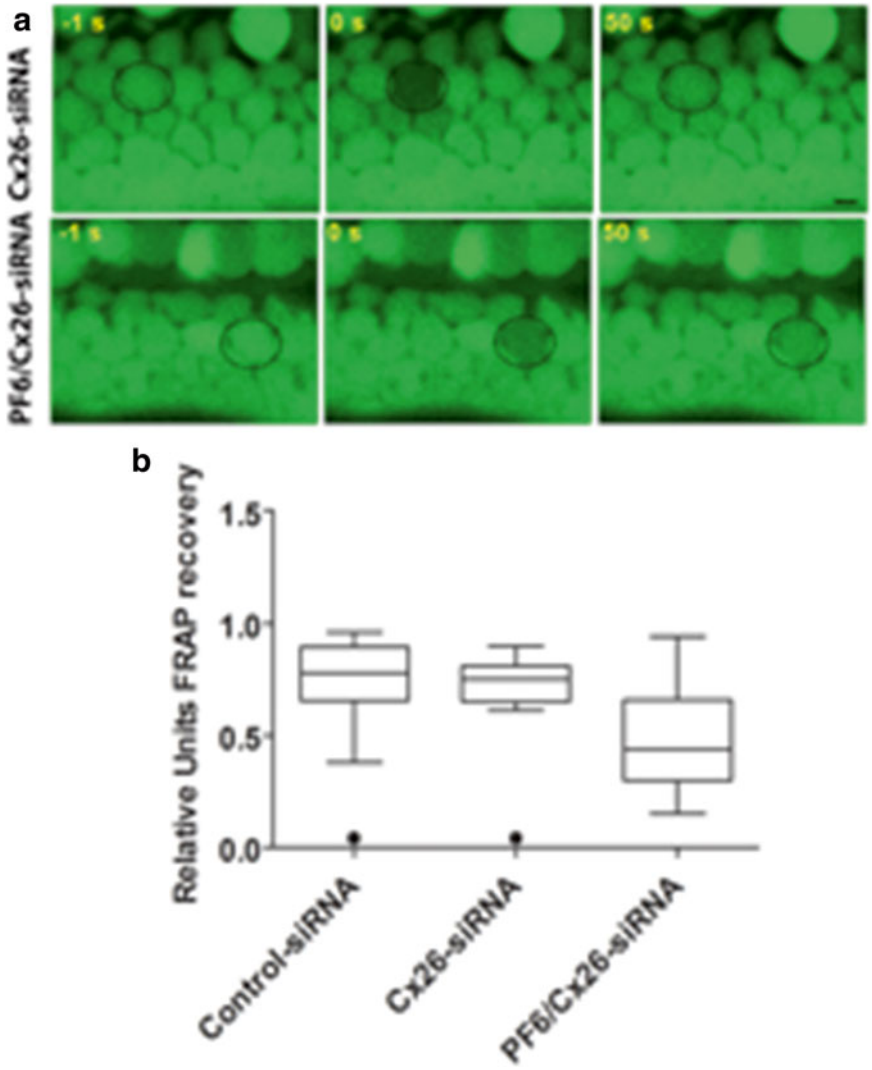

Fig. 2 Reduced gap junctional communication in PF6/Cx26-siRNA-treated cochlear cultures. (**a**) Representative images of the Cx26-siRNA-treated supporting cells (*upper panel*) and PF6/Cx26-siRNA-treated supporting cells (*lower panel*) before (−1 s, image 15), immediately (0 s, between image 15 and 16), and 50 s after photobleaching. The bleached cell (marked with a *circle*) recovered after 50 s in the Cx26-siRNA-treated cultures, while the PF6/Cx26-siRNA-treated cells showed reduced recovery. Bar = 5 μm. (**b**) The quantitative measurement of the fluorescence recovery revealed significantly reduced recovery of PF6/Cx26-siRNA-treated cells in comparison with the cells from the cochlear cultures treated with Cx26-siRNA alone or PF6/control-siRNA for 72 h. $^{**}P < 0.01$, $^{*}P < 0.05$ (Mann–Whitney *U*-test). *FRAP* fluorescence recovery after photobleaching, *siRNA* short interfering RNA. (Reproduced from ref. 15 with permission from Nature Publication Group)

2. Make sure that the hair cells in the cochlea faces upwards. The cochlea should be pushed down so it attaches the bottom of the plate and over time the cells can spread otherwise the cochlea will float away during the washing.
3. It is advisable to resuspend the peptide in salt-free water rather than buffers.
4. RNases can degrade the SiRNA so clean the laminar hood and tube stands with ethanol and RNase-free solutions.
5. Excess salt may hinder with the homogeneous complex formation. Hence, SiRNA and PF6 should be mixed in salt-free water.
6. For optimal transfection result, molar ratio of SiRNA: PF6 should be between 1:10–1:40 depending upon tissue/cell types and amount of SiRNA required for efficient downregulation of target gene expression.
7. It is advisable to include a dose–response curve for each SiRNA.
8. If the medium contains serum, it is suggested to incubate the cells with complexes for 4 h in serum-free medium and then add serum containing medium. However, cochlear culture medium is serum-free medium.
9. The cultures should be thoroughly washed with warm PBS in order to remove all possible traces of calcein from the solution, otherwise free calcein in solution will interfere with the FRAP occurring through gap junctions.
10. Calcein fluorescence is sensitive to the changes in the intracellular calcium concentration. Exposure to laser sometimes leads calcium influx. Hence, cells showing calcium response (grater than 5 %) should be excluded from analysis.

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