Toward *in vivo* Targeting Delivery of siRNA for Efficient Cancer Therapy

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ABSTRACT

The main obstacle in siRNA therapy is RNA delivery to the cytoplasm, where it can guide sequence-specific mRNA degradation. Attempts to develop effective nonviral vectors for *in vivo* delivery of nucleic acids through a systemic route are hampered by difficulties of combining high extracellular stability with ready availability of the nucleic acids following entry into cells. Other challenges with non-viral gene delivery include limitations in target-cell specificity. Here we report a targeted siRNA delivery vector that displays good extracellular stability and intracellular bioavailability to permit efficient gene silencing.

Keywords: siRNA, poly(propyleneimine), PPI, dendrimers, targeted delivery.

1 INTRODUCTION

There is an increasing enthusiasm for developing therapies based on RNA interference (RNAi), a post-transcriptional gene silencing method, mediated by small duplex RNAs (siRNA). The advantage of RNAi compared to other gene therapeutic strategies lies in its high specificity and potency of gene silencing, coupled with the fact that it can target every gene. However, just like other gene therapy strategies, the main obstacle to the success of siRNA therapy is delivering RNA across the cell membrane to the cytoplasm, where it can enter the RNAi pathway and guide sequence-specific mRNA degradation [1]. An efficient, nontoxic delivery strategy has not been developed yet. It has been recognized that a prerequisite for the facile transport of DNA/RNA through the cell membrane is the condensation of the nucleic acid to nanoparticles of ~100 nm size. Viral vectors are efficient in accomplishing this; however, the immune response elicited by viral proteins has posed a major challenge in this approach [2]. Hence, there is much interest in developing nonviral gene delivery vehicles. Attempts to develop effective nonviral vectors for *in vivo* delivery of nucleic acids through a systemic route are hampered by difficulties of combining of high extracellular stability with ready availability of the nucleic acids following entry into cells. Extracellular stability is essential as the delivery system should be capable of withstanding the aggressive biological environment en-route to the target site, while availability of the nucleic acids permits efficient therapeutic effects within the cells. Here we report a targeted siRNA delivery vector that displays good extracellular stability and intracellular bioavailability for efficient gene silencing.

2 EXPERIMENTAL SECTION

2.1 Materials

Dimethyl-3-3'-Dithiobispropionimidate-HCl (DTBP) was obtained from Pierce (Rockford, IL). DAB-Am-64, polypropylenimine tetrahaxacontaamine Dendrimer G5 (PPI G5) was obtained from Sigma-Aldrich and used without further purification. Ethidium Bromide solution (EtBr) was obtained from Promega (Madison, WI) and Glutathione Reduced from AMRESCO (Solon, Ohio). α-maleimide-ω-N-hydroxysuccinimide ester poly(ethylene glycol) (MAL-PEG-NHS) was purchased from NOF Corporation. The sequence of siRNA targeted to *BCL2* mRNA and native LHRH peptide were custom synthesized by Ambion (Austin, TX) and American Peptide (Sunnyvale, CA), respectively.

2.2 Preparation of siRNA/PPI G5 complexes

The condensed siRNA complexes were prepared at the desired 2.4 amine/phosphate (N/P) ratio in either DI water or 10 mM HEPES buffer (pH 7.2) by adding stock solution of PPI G5 dendrimer (typically, 500 μM) into earlier prepared siRNA solution. The samples were vortexed briefly, and the solutions were then incubated at room temperature for 30 min to ensure complex formation.

2.3 Modification of siRNA/PPI G5 complexes

In order to crosslink individual complexes, DTBP dissolved in HEPES buffer immediately prior using (2.5 mg/mL), was added to prepared siRNA/PPI G5 complexes solution at various concentrations depending on the desired crosslinking ratio. For example DTBP: NH_2 = 3.2 indicates siRNA/PPI G5 complex crosslinked with DTBP using molar ratio of 3.2 between DTBP and total amino groups of PPI G5 available after the condensation reaction. After 3 hrs of crosslinking reaction, MAL-PEG-NHS (35mg/mL)

was added to the solution and the NHS groups on the distal ends of the PEG reacted with amine groups on the periphery of siRNA/PPI G5 complex for 1hr at room temperature. Afterwards, 12.5 mg/mL of LHRH peptide was added, and mixed overnight to covalently conjugate the peptide on the distal end of the PEG layer on the siRNA/PPI G5 complex through the maleimide groups on the PEG and the thiol groups in the peptide. siRNA/PPI G5 modified complexes were then purified by dialysis (MW cut-off 10 kDa) against water for 1 day and used for further studies.

2.4 Atomic Force Microscopy (AFM)

The formulated siRNA condensates were imaged with tapping mode atomic force microscope in ambient air (Nanoscope III A, Digital Instruments).

2.5 Cell Lines

Human lung carcinoma cells A549 and LHRH negative ovarian SKOV-3 cancer cells were obtained from ATTC (Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Sigma Chemical Co., Louis, MO) supplemented with 10% fetal bovine serum (Fisher Chemicals, Fairlawn, NJ). Cells were grown at 37ºC in a humidified atmosphere of 5% CO_2 (v/v) in air. All of the experiments were performed on the cells in the exponential growth phase.

2.6 Cellular Internalization

Cellular internalization of fluorescein (FITC) labeled siRNA condensates were analyzed by fluorescence and confocal microscopes. Prior the visualization, the cells were plated (20, 000 cells/well) in 6-well tissue culture plate and treated with the formulated condensates for 24 hrs.

2.7 Gene Expression

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used for the analysis of the suppression of *BCL2* gene expression in the A549 and A2780 cancer cells treated with the complexes of *BCL2* targeted siRNA. PCR products were separated by submarine electrophoresis. The gels were stained with EtBr, digitally photographed, and scanned using Gel Documentation System 920. Gene expression was calculated as the ratio of mean band density of analyzed reverse transcriptase-PCR product to that of the internal standard (β*2-m*).

3 RESULTS AND DISCUSSION

3.1 Packaging of siRNA into nanoparticles with PPI dendrimers

We used AFM tapping mode operating in ambient air to determine the size and shape of the siRNA nanoparticles formed in the presence of PPI G5 dendrimer. As shown in Figure 1A, uniformly distributed spheroid-like nanoparticles with an average diameter of 150 nm were the predominant products on the mica surface.

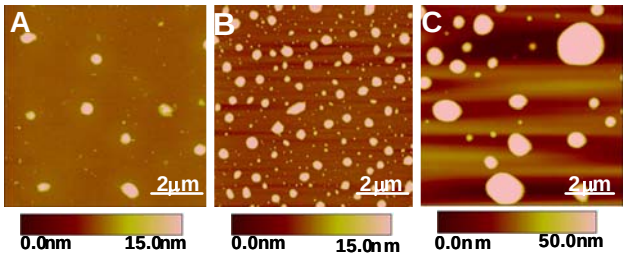

Figure 1. AFM images of siRNA nanoparticles packaged by PPI G5 dendrimer (A) after 30min of condensation. (B) siRNA nanoparticles modified with DTBP, NHS-PEG-MAL and LHRH, were stored for 48 hrs at room temperature. (C) Nonmodified siRNA nanoparticles stored for 48 hrs at room temperature.

3.2 Stabilization of siRNA nanoparticles by DTBP crosslinking and PEGylation

In order to provide lateral stabilization, the formulated siRNA nanoparticles were modified with an intercellular cleavable dithiol containing cross linker, such as DTBP. From TNBSA assays [3] we estimate that 60% of free amine groups on the siRNA nanoparticles were crosslinked. To extend the circulation time of the siRNA nanoparticles in blood stream before they are delivered into tumors, we further modified the siRNA nanoparticles with a hydroxysuccinimide ester functionalized polyethylene glycol. As shown in Figure 1B, the siRNA nanoparticles modified with DTBP and PEG were stored for 48 hrs at room temperature and their uniform size distribution was retained. In contrast, non-modified siRNA nanoparticles after the same period of time experienced an aggregation and partial decondensation (Figure 1C).

On the other hand, the stability of the siRNA nanoparticles with or without stabilization by DTBP and/or the PEG layer was evaluated by the ability of the siRNA nanoparticles against polyanion disruption. This method is commonly used to study the stability of DNA nanoparticles [4]. The experiments were performed by measuring the ability of a polyanion, poly(methacrylic acid) (PMAA) to restore siRNA access to EtBr binding. To evaluate if the siRNA could be released after delivery to the cytoplasm, glutathione, a reductive agent present in cytoplasm [5], was added to the crosslinked samples with and without PEGylation.

The EtBr fluorescence was dramatically increased after its incubation with free siRNA (from pink curve to red curve in Figure 2A). Compaction of the siRNA with PPI G5

dendrimers causes the EtBr fluorescence to decrease to the level of free EtBr (Figure 2A, black curve). Crosslinking the siRNA nanoparticles with DTBP did not introduce any change on the fluorescence, indicating that the siRNA nanoparticles remain compacted during the crosslinking process. PMAA was then added to the siRNA nanoparticle solution. As the concentration of PMAA was increased progressively, higher fluorescence readings were observed, which demonstrates that siRNA release from condensates, or at least local de-condensation of the siRNA nanoparticles takes place. For the siRNA nanoparticles without DTBP and PEG layers, 5 μM PMAA was able to release 85 % of the siRNA for EtBr binding (Figure 2B, black curve). For the siRNA nanoparticles caged with DTBP, 5 μM PMAA released only 14% of siRNA. Addition of PMAA up to 100 μM did not cause further release of siRNA, indicating that siRNA nanoparticles caged by DTBP are stable against PMAA disruption (Figure 2B, green curve). When 25 mM glutathione was introduced to promote reduction of the intramolecular disulfide bond in DTBP, the stabilization of siRNA reversed. 85% siRNA was released from the nanoparticles, estimated from the EtBr fluorescence increase.

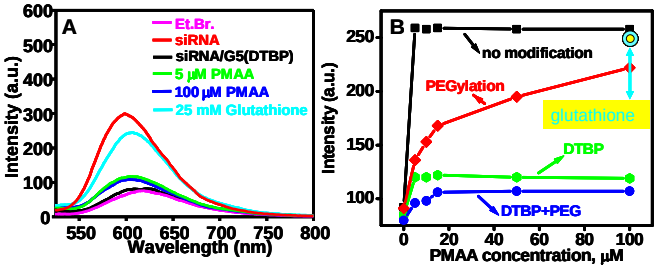

Figure 2. EtBr displacement assay to study the stability of siRNA nanoparticles against PMAA disruption. (A) Evolution of EtBr fluorescence spectra in the presence of free siRNA; siRNA nanoparticles formed from G5 dendrimers and crosslinked with DTBP in the presence of different concentration of PMAA and 25 mM reduced glutathione. (B) EtBr fluorescence from interactions with siRNA nanoparticles without and with DTBP crosslinker, with PEGylation, and with combination of DTBP and PEGylation in the presence of PMAA and 25 mM reduced glutathione.

For the PEG- protected siRNA nanoparticles, as the PMAA concentration increased, the fluorescence signal gradually reached 70% of the fluorescence, suggesting that PEG layer alone could not protect siRNA nanoparticles from the polyanion disruption (Figure 2B, red curve). With the combination of the DTBP crosslinking and PEGylation on the siRNA nanoparticles, addition of 5 μM PMAA released 10% of siRNA and no further release was observed at higher PMAA concentrations tested (blue curve in Figure 2B). Addition of 25 mM reduced glutathione into the solution led to 80% of siRNA being released from siRNA nanoparticles (Light blue curve in Figure 2A).

3.3 PEGylation prevents the siRNA nanoparticles from aggregation and eliminates nonspecific delivery of the siRNA nanoparticle to cells.

To demonstrate if the PEG layer can prevent siRNA nanoparticles from aggregation, we have compared the ability of the siRNA nanoparticles with different modification to undergo cellular uptake in A549 cancer cell line.

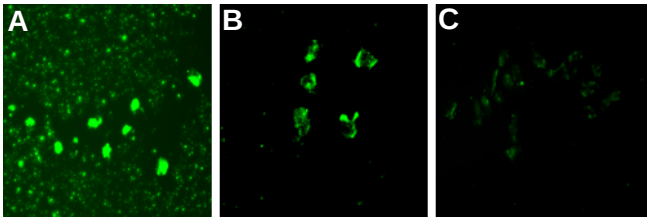

Figure 3. Fluorescence microscopy images of (A) siRNA/PPI G5 complexes, (B) siRNA/PPI G5-DTBP-PEG (NH_2 : DTBP = 1: 15.9) and (C) siRNA-PPI G5-DTBP-PEG (NH_2: DTBP = 1: 3.2) after 24 hrs of incubation with A549 cancer cells

Nonmodified condensed siRNA nanoparticles show serious aggregation in the cell medium (Figure 3A). In case of stabilization, one could barely see any aggregation of the siRNA nanoparticles (Figure 3B, C), indicating the PEG layer can remarkably prevent aggregation induced by physiological salts and serum proteins. Furthermore, the modified siRNA nanoparticles at lower ratio (DTBP: NH2=3.2:1 in the conjugation solution) have a tendency to decrease for siRNAs internalization in the cells compared to the one with higher ratio (DTBP: NH2=15.9:1). This is due to the special molecular structure of DTBP. The crosslinked siRNA nanoparticles increase the stability against polyelectrolyte exchange, while retaining the positive surface charges on the siRNA nanoparticle surface, which are essential for nonspecific internalization of nucleic acid nanoparticles. It was reported that modification of the DNA nanoparticles with hydrophilic polymers such as PEG could stabilize the polyplexes against salt-, and protein-induced aggregation. The increased stability against aggregation was attributed to the steric effects of the PEG layer that lead to decreased particle–particle and particle–protein interactions. In addition, PEGylation has been shown to reduce internalization of nontargeted nanoparticles [6]. The effect depends on the molecular weight of the PEG and the grafting density on the particles. The higher molecular ratio (DTBP: NH2=15.9:1) during the caging process results in a decrease of NH2 groups on the siRNA particles' surface for PEG conjugation. Even though the PEG layer prevents aggregation of the siRNA nanoparticles, large amounts of siRNAs were still internalized by the cells nonspecifically. On the other hand, with a lower molecular ratio (DTBP: NH2=3.2:1) during the caging process, more NH2 groups were left on the

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siRNA particles surface for PEG conjugation. Thus, the density of the positive charges on the siRNA nanoparticle surfaces was largely decreased after further PEGylation of the DTBP caged siRNA nanoparticles. Therefore, they more effectively prevent nanoparticles aggregation and eliminate nonspecific delivery of the siRNA nanoparticles to the cells.

3.4 Peptide conjugation for targeted delivery

Nonspecific delivery of genes toward both cancerous and normal tissues can result in serious side effects, thereby limiting their clinical applications [7]. To deliver the siRNA nanoparticles selectively to the distant tumor metastases after injection into the blood stream, the particles not only need to be protected from dissociation and captured by the liver macrophages, but they also have to be equipped with specific ligands to be delivered specifically into cancer cells. Recently, a modified peptide synthetic analog of luteinizing hormone-releasing hormone (LHRH) has successfully been used as a targeting moiety to tumors overexpressing LHRH receptors [8]. The use of this peptide prevented the accumulation of anticancer drugs in healthy organs, and enhanced both drug accumulation in tumors and its internalization by cancer cells. To specifically deliver the caged siRNA nanoparticles to cancer cells, we applied a heterobifunctional MAL-PEG-NHS, which was used as a linker between siRNA condensed nanoparticles and LHRH. It contains an amine reactive NHS ester and maleimide reactive group on the opposite side and allows us to further modify siRNA nanoparticles in a layer-by-layer fashion. We followed the well-documented coupling and separation procedures to covalently link PEG with amine groups on the caged siRNA nanoparticles (NH2:DTBP=1:3.2), and then conjugated the LHRH peptides to the distal end of PEG layer through the maleimide group and the cystein in the LHRH peptides.

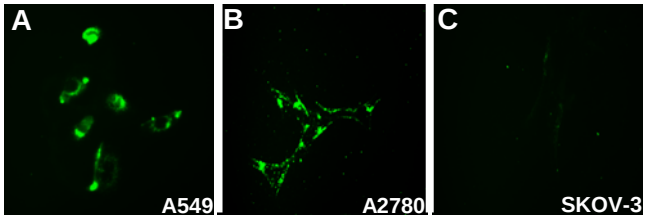

Figure 4. Representative fluorescence microscopic images of cellular uptake of the LHRH-PEG-DTBP-modified FITC-siRNA nanoparticles by LHRH positive, (A) A549 cells, (B) A2780 cells, and LHRH negative (C) SKOV-3 cancer cells.

Fluorescent microscopic study demonstrated the LHRH-siRNA nanoparticles can be specifically internalized by LHRH positive A549 cells and A2780 cells but not SKOV-3 cells, which expressed a low level of LHRH receptors (Figure 4).

RT-PCR was also used to study the ability of the peptide conjugated siRNA nanoparticles to silence *BCL2* targeted mRNA expression. The results demonstrate that the siRNAs can effectively knock down their target mRNA, despite variations in the efficiency in different cell lines, which may be related to the uptake and the reductive environment of the cells (Figure 5). It was reported that more LHRH receptors are expressed on A2780 cells, which facilitate the internalization of the siRNAs.

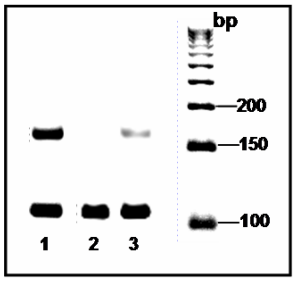

Figure 5. RT-PCR results show the effect of treatment on different cell lines. Lane 1, no treatment; Lanes 2 and 3 were treated by the engineered siRNA nanoparticles (LHRH-PEG-DTBP-siRNA-PPI G5) on the expression of BCL2 mRNA; Lane 2 for A2870 cells and Lane 3 for A549 cancer cells.

In summary, crosslinking and PEGylation confer the siRNA nanoparticles with high extracellular stability and ready availability of the nucleic acids following entry into cells. This reversible stability combined with the effective targeted delivery specifically to cancer tumor cells will ultimately improve siRNA therapeutic effects and minimize side effects caused by non-specific delivery. We envision this approach can be used *in vivo* systematic delivery of siRNAs for efficient cancer therapy.

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REFERENCES

[1] Dykxhoorn, D. M.; Lieberman, J. *Annu. Rev. Med.* **2005**, 56, 401-423.
[2] Bessis, N.; GarciaCozar, F .J.; Boissier, M.C. *Gene Ther.* **2004**, 11, S10-17.
[3] Oupicky, D.; Carlisle, R. C. and Seymour, L. Y. *Gene Ther.*, **2001**, 8**,** 713-724.
[4] Gosselin, M. A.; Guo, W. and Lee, R. J. *Bioconjugate Chem.* **2002,** 13**,** 1044-1053.
[5] Schmitz, T.; Bravo-Osuna, I.; Vauthier, C.; Ponchel, G.; Loretz, B. and Bernkop-Schnürch, A. *Biomaterials*, **2007,** 28**,** 524-531.
[6] Pack, D. W.; Hoffman, A. S.; Pun, S. and Stayton, P. S. *Nat. Rev. Drug Discovery*, **2005,** 4**,** 581-593.
[7] Langerak, A. D. (2001) *Chemotherapy Regimens and Cancer Care*. Landes Bioscience:, Georgetown, TX.
[8] Pakunlua, R. I.; Wang, Y.; Tsao, W.; Pozharov, V.; Cook, T. J. and Minko, T. *Cancer Reseach,* **2004**, 64**,** 6214-6224.