Labile Catalytic Packaging and Delivering of Short Interference RNA to Cancer Cells: Control of Gold Nanoparticles “Out” of siRNA Complexes

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ABSTRACT

We report a novel approach to efficiently package and deliver siRNAs with low generation dendrimers by using Au nanoparticles as a “labile catalytic” packaging agent. The Au nanoparticles helped low generation dendrimers to package siRNA but are not included in the final siRNA complexes. Therefore, the potential toxic problem accompanied with the Au nanoparticles can be easily solved by selectively separating the gold nanoparticles before the siRNA nanoparticles are delivered. This is a new concept in using inorganic engineered nanoparticles in nucleic acid packaging and delivery applications.

Keywords: gene therapy, siRNA, plasmid DNA, non-viral gene delivery, Au nanoparticles

INTRODUCTION

The inefficient transport of nucleic acids through the cell membrane is a major limiting factor in clinical application of therapeutic nucleic acids, including antisense, anti-gene, and short interference RNA (siRNA) strategies. It has been recognized that a prerequisite for the facile transport of DNA/RNA through the cell membrane is packaging of the nucleic acid to nanoparticles of ~100 nm size.¹ Viral vectors are efficient of accomplishing this. However, the immune response elicited by viral proteins has posed a major challenge to this approach.² Hence, there is much interest in developing nonviral gene delivery vehicles.

Dendrimers are highly branched three-dimensional polymers with large number of controllable peripheral functionalities, useful as gene delivery agents, drug delivery vehicles, and magnetic resonance imaging agents. Protonated amino groups of dendrimers appear to buffer acidic endosomal compartment and release DNA to the cytoplasm. While higher generation dendrimers show higher cytotoxicity³,⁴ and their synthesis and purification are usually tedious with low yield, low generation dendrimers are nontoxic and easy to synthesize. However, the limited surface charge of low generation dendrimers leads to their inefficient complexation with DNA and low cellular uptake efficacy. In our recent studies we found that only higher generations of PPI dendrimers could enhance oligodeoxynucleotide (ODN) uptake to breast cancer cells as demonstrated by confocal microscopy.⁵

There is a surge of interest in using inorganic engineered nanoparticles for medical and biological applications. They are expected to solve some difficult human health problems due to their unique properties and their remarkably large surface area. Studies using inorganic engineered nanoparticles modified with cationic molecules, including dendrimers have demonstrated enhancement in DNA condensation, delivery and transfection in mammalian cells.

However, the inorganic nanoparticles were encapsulated inside the resulting DNA nanoparticles. Toxicity of the inorganic nanoparticles in human body is a major concern. Currently the toxicology of most inorganic engineered nanomaterials is not available and is in the process of assessment. A new field of nanotoxicology was just born to study the effects of engineered nanodevices and nanostructures in living organisms. Conflicting data have emerged and long-term fate of the nanomaterials in human body is not known yet.

In this work, we report a new usage of Au nanoparticles to help low generation dendrimers to effectively package and deliver siRNA but the Au nanoparticles are not included in the final siRNA nanoparticles. Therefore the potential toxic problem accompanied with the Au nanoparticles can be solved by selectively removing the gold nanoparticles before the siRNA nanoparticles are delivered. More importantly, the siRNA nanoparticles packaged by this novel approach can be internalized by cancer cells and efficiently silence their target mRNA. The efficiency is even superior to higher generation dendrimers.

MATERIALS AND METHODS

Chemicals. Polypropyleneimine hexadecaamine dendrimer (PPI dendrimer) generation-3 (G3) and generation -5 (G5) (Figure 1) and other chemicals used in this study were purchased from Aldrich (Milwaukee, WI), and used without further purification. siRNA that are sequence specific for human bcl-2 mRNA was custom synthesized by Ambion (Austin, TX). The sequence of the siRNA used as follows:
sense strand, 5'-GUGAAGUCAACAUGCCUGC-dTdT-3'; antisense strand, 5'-GCAGGCAUUGACUUCAC-dTdT-3'.

Packaging siRNA with G5 PPI dendrimers and with Au nanoparticles modified with G3 PPI dendrimers for cell uptake experiment. The condensed siRNA complexes were prepared at 2.4 amine/phosphate (N/P) ratio either in DI water or 10 mM Hepes buffer (pH 7.2) by adding stock solution of PPI G5 dendrimer or stock solution of Au nanoparticles modified with G3 PPI dendrimers into prepared siRNA solution. The samples were vortexed briefly, and the solutions were then incubated at room temperature for 30 min to ensure complex formation.

Cell Lines. Human lung carcinoma cell line A549 was obtained from the ATCC (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 37°C in a humidified atmosphere of 5% CO2 (v/v) in air. All of the experiments were performed on the cells in exponential growth phase.

Cellular Internalization. Cellular internalization of FITC-labeled siRNA nanoparticles packaged with Au nanoparticles modified with PPI G3 and PPI G3 alone were analyzed by fluorescence microscope (Olympus America Inc., Melville, NY). Prior to the visualization A549 cells were plated (20,000 cells/well) in 6-well tissue culture plate. The cells were treated with siRNA nanoparticles packaged with Au nanoparticles modified with PPI G3 and PPI G3 alone for 24 hrs. The concentration of PPI G3 dendrimer for both cases was 1.57 μM and the concentration of siRNA was 0.25 μM. After 24 hrs of treatment, cells were washed three times with phosphate buffered saline (PBS) and 1 mL of media was added to each well.

Gene Knockdown. The ability of the siRNA nanoparticles formed from Au nanoparticles modified with PPI-G3 to silence the target mRNA expression were studied with quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The siRNA nanoparticles packaged with PPI G5 dendrimer alone were used as a positive control. After 24hrs of incubation of A-549 lung cancer cells with siRNA nanoparticles, the total cellular RNA was isolated using an RNase kit (Qiagen, Valencia, CA). First strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ) with 4mg of total cellular RNA and 100 ng of random hexadeoxynucleotide primer (Amersham Bioscience). After synthesis, the reaction mixture was immediately subjected to polymerase chain reaction, which was carried out using GenAmp PCR System 2400. The pairs of BCL2 and β2-m primers used to amplify each type of cDNA. PCR products were seperated in 4% NuSieve 3:1 Reliant agarose gels in 1×TBE buffer (0.089 M Tris/Borate, 0.002 M EDTA, pH 8.3; Research Organic Inc., Cleveland OH) by submarine electrophoresis. The gels were stained with ethidium bromide, digitally photographed and scanned using Gel Documentation System 920 (NucleoTech, San Mateo, CA). Gene expression was calculated as the ratio of mean band density of analyzed RT-PCR product to that of the internal standard (β2-m).

RESULTS AND DISCUSSION

Our approach relies on the competing affinities of gold and nucleic acids for the amine sites of low generation polypropyleneimine (PPI) dendrimers. In neutral pH solutions, the primary amines of the dendrimers are protonated (pKa about 9.8), while most of their tertiary amines (pKa about 5.9–7.0) are not, which provide strong Au/amine interactions, so that each gold nanoparticle anchors several low generation dendrimers through multiple gold/amine interactions. The largely increased positive charges on each of the delivery vehicles (here gold nanoparticles modified with low-generation dendrimers) resemble higher generation dendrimers (Scheme 1) and enable effective packaging of nucleic acids into nanostructures. However, when multiple nucleic acids interact with the delivery vehicle, the local pH effects caused by the presence of nucleic acids increase the local acidity of the dendrimers. As a result, the number of unprotonated tertiary amine sites on dendrimer is decreased due to protonation, which in turn decreases the affinity of dendrimer to gold while increasing the affinity to nucleic acids. The large local pH effect has been used to extend the conductivity of polyaniline to neutral or slightly basic solutions, including our previous work.

To examine the feasibility of this hypothesis, we prepared Au nanoparticles modified with generation 3 (G3) PPI dendrimer according to a protocol described by Wang and his colleagues. Briefly, 32.8 mg of G3 dendrimer was mixed with 8.8 ml of 2.45 mM HAuCl4 solution at a

Scheme 1. A gold nanoparticle anchored with several low generation dendrimers through Au-amine bonds. Addition of nucleic acids leads to an increase of local acidity, which protonates the tertiary amines, weakening the Au-amine interactions. As a result, the gold nanoparticles are released from the dendrimers and are not included in the final siRNA nanoparticles.
molar ratio of 0.9:1 and then heated at 80°C for one hour with continuous stirring. As a result, a clear and reddish gold nanoparticle solution was obtained with each gold nanoparticle physically anchoring 6-8 G3 dendrimers. The largely increased positive charges on each of the condensing agents (here gold nanoparticles modified with G3 dendrimers) enabled the low generation dendrimers to effectively condense DNA as higher generation dendrimers do. To remove the un-reacted HAuCl₄, the solution thus obtained was cooled to 22°C and dialyzed (3.5k-Da cutoff) against 200 ml of double-distilled water twice before it was used to condense nucleic acids. UV-visible spectroscopy was used to characterize the formed nanoparticle solution. A strong absorption was observed at ~523 nm, which was due to the surface plasmon resonance of the gold nanoparticles. The size of the nanoparticles was ~8.3 ± 2 nm as determined from electron microscopy measurements.

Condensation of PGL3 Plasmid DNA. We first tested the ability of the Au nanoparticles to condense PGL3 plasmid DNA. We found that gold nanoparticles modified with PPI G3 dendrimer can efficiently condense DNA into nanoparticles. Furthermore, by AFM phase imaging and TEM imaging of the condensates, we confirmed that the gold nanoparticles left the dendrimers upon DNA condensation. Our finding is very different from previous reported DNA condensation by using Au and other nanoparticles as condensing agents, which were encapsulated in the final DNA nanoparticles.

Packaging of 21-bp siRNAs to Nanoparticles. There is an increasing enthusiasm for developing therapies based on RNA interference (RNAi), a post-transcriptional gene silencing method, mediated by small duplex RNAs of 19-23 base pairs. The advantage of RNAi compared to other gene therapies is in delivering it across the cell membrane to the cytoplasm where it can enter the RNAi pathway and guide the sequence-specific mRNA degradation. It is reported that the short oligonucleotides are more difficult to package into well-defined particles than the long plasmid DNA.

To demonstrate the wide application of this new packaging approach in gene therapy, we next tested whether the gold nanoparticles modified with G3 PPI dendrimer could catalytically provoke siRNA nanoparticles formation. The siRNA nanoparticles were prepared by mixing the dialyzed and appropriately diluted G3 PPI dendrimer-modified Au nanoparticles solution and siRNA solution in physiological buffer. The nanoparticles were imaged using AFM and TEM.

Figure 1A shows that siRNA nanoparticles with an average height of 10 ± 3 nm and diameter of 70 ± 10 nm were formed after 30 min of condensation. siRNA nanoparticles were also observed in TEM images (Figure 1C), indicated by green arrows. Remarkably, both AFM and TEM images clearly show that the Au nanoparticles were not included inside the siRNA nanostructures.

Cellular Uptake. We next studied the ability of the siRNA nanoparticles to undergo facile cellular uptake in A549 human lung cancer cell line. The cells were treated with fluorescein isothiocyanate (FITC)-labeled 21-bp siRNA nanoparticles fabricated from G3 PPI dendrimer alone and G3 PPI dendrimer-modified Au nanoparticles for 24 hours. The siRNA is designed to silence BCL2 mRNA to suppress non-pump drug resistance. The concentration of G3 PPI for both cases was 1.56 µM and the concentration of siRNA was 0.25 µM. Cells dosed with 0.25 µM siRNA alone did not show any FITC-labeled siRNA after 24 hours treatment (data not shown). Cells dosed with siRNA nanoparticles fabricated by G3 PPI alone showed very mild fluorescein staining on the surface and inside of the cells (Figure 2A), indicating a low level of siRNA uptake. Similar result was obtained when G3 PPI dendrimer was used to deliver antisense oligodeoxynucleic acids in our previous study. In contrast, the siRNA nanoparticles fabricated by Au nanoparticles modified with G3 PPI dendrimers was highly effective in transporting siRNA to the cell, as shown by the green fluorescence in the cells (Figure 2B). This result clearly demonstrated that the Au nanoparticles can help the low generation dendrimers effectively deliver siRNA into cancer cells. The reason why siRNA nanoparticles can be internalized is still under investigation. We hypothesize that the siRNA nanoparticles fabricated by Au nanoparticles modified with G3 PPI dendrimers may include more G3 dendrimers, which results in larger positive surface charges, which are essential for nonspecific delivery.

The ability of the siRNA nanoparticles formed from Au nanoparticles modified with G3 PPI to silence the target
mRNA expression were studied with quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The siRNA of same sequence without FITC label was used for this study. For comparison, we also performed the same experiment on the siRNA nanoparticles formed from higher generation dendrimers (G5 PPI), which has been demonstrated much more efficient in packaging and delivering antisense ODNs into breast cancer cells compared to G3 PPI.\[5\] The siRNA nanoparticles packaged by G5 PPI and Au nanoparticles modified with G3 dendrimers were incubated with A-549 cancer cells for 24hrs. Gene expression was calculated as the ratio of mean band density of analyzed RT-PCR product to that of the internal standard (\(\beta\)-m). As shown in Figure 2C, a decrease in the expression of BCL2 gene was observed after incubation of cancer cells with siRNA-G5 PPI nanoparticles (Fig. 2C, lane 3). However, the siRNA nanoparticles fabricated by Au nanoparticles modified with G3 PPI are even more efficient than those fabricated by G5 PPI in inhibiting the BCL2 mRNA expression. This is possibly due to the strong interaction between G5 PPI and the siRNAs that prevents the release of siRNAs from the condensates for efficient silencing. It has been reported that strong binding and efficient DNA condensation do not correlate directly with gene-transcription efficiency, probably because tight binding prevents transcription. Nevertheless, these results are striking, which soundly demonstrated that the Au nanoparticles can help the low generation dendrimers effectively deliver siRNAs into cancer cells and efficiently inhibit their target mRNA expression.

In summary, we report a novel approach to efficiently package and deliver siRNAs with low generation dendrimers by using Au nanoparticles as a “labile catalytic” packaging agent. The Au nanoparticles helped low generation dendrimers to package siRNAs but are not included in the final siRNA complexes. Compared to the siRNA particles fabricated by low generation dendrimer alone (G3 PPI), the siRNA nanoparticles packaged through this novel approach (by Au nanoparticles modified with G3 PPI) can be internalized by cancer cells and the internalized siRNAs can efficiently silence their target mRNA. The efficiency is even superior to higher generation dendrimers (G5 PPI). More importantly, this approach provides a possibility to remove the gold nanoparticles before the siRNA nanoparticles are delivered, therefore the possible toxic problem accompanied with the Au nanoparticles can be solved. This is a new concept in using inorganic engineered nanoparticles in nucleic acid packaging and delivery applications. Currently, detailed studies on selectively removing the Au nanoparticles from the nucleic acid complex solution, and the toxicity of the nucleic acid complex after the removal are under investigation.

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