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Gold nanoparticle-assisted delivery of small, highly structured RNA into the nuclei of human cells

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ABSTRACT

Previous studies have shown that functionalized gold nanoparticles (AuNPs) can be used as a general platform for loading and delivering DNA oligonucleotides and short hairpin RNA to living systems. Here, we report the ability of functionalized AuNP to deliver RNA aptamers into the nuclei of human cells. An *in vitro*-synthesized RNA aptamer specific to the β-catenin protein was delivered into the HepG2 human cell line more efficiently via functionalized AuNP than liposome-based delivery, and resulted in nearly complete inhibition of β-catenin binding to the p50 subunit of NF-κB in the nucleus. This inhibition led to repression of NF-κB p50-dependent transcription of *CRP*. Also, the β-catenin aptamer in the nucleus led to down-regulation of β-catenin-mediated transcriptional activity through the TCF complex and resulted in decrease in the levels of *cyclin D*, and *c-myc* mRNA by ~47% and ~57%, respectively. In addition, we used functionalized AuNP to deliver another RNA aptamer targeted to the p50 subunit of NF-κB into the A549 human cell line, and this was sufficient to induce apoptosis of the cells. Our findings demonstrate that AuNP GDS can be used to deliver small, highly structured RNA aptamers into the nucleus of human cells where they modulate the activity of transactivators by interacting with target proteins.

1. Introduction

Aptamers are single-stranded DNA or RNA oligonucleotides that fold into stable three-dimensional structures and bind tightly to a specific molecular target [1–4]. Using the SELEX (systematic evolution of ligands by exponential enrichment) technique [4], aptamers have been developed against a variety of cancer targets, including extracellular ligands and cell surface proteins [5]. Aptamers have been used for therapy and diagnostics because they are small in size, nontoxic, minimally or not immunogenic and highly specific to target molecules [6–9].

Aptamers have been delivered and expressed primarily in living systems via viral vector-based systems because of their high transfection efficiency. One of disadvantages of these systems is that they inevitably generate flanking sequences in expressed aptamers and these vector-derived sequences sometimes hinder native structure and function of aptamers *in vivo* [10,11]. In addition, viral systems have limitations as gene delivery systems due to the strong immune response that is triggered by the expression of viral genes in the host cell. To overcome these problems, numerous nonviral gene delivery systems such as cationic lipids, polymers, dendrimers, and peptides have been developed [12–14]. While nonviral gene delivery systems are non-pathogenic, they exhibit significantly reduced transfection efficiencies compared to viral systems due to numerous extra- and intra-cellular obstacles. The induction of inflammatory toxicity and the rapid clearance of non-viral systems have further hindered the development of successful gene delivery systems [15–19].

Among the different approaches that have been explored to address these limitations, gold nanoparticles in particular have several properties that make them attractive candidates for the creation of gene delivery systems. Gold nanoparticles are especially easy to modify and are not toxic to mammalian cells [20]. However, there still are several obstacles for using gold nanoparticle-based gene delivery systems as a general method because construction of these systems for a specific target gene is both time-consuming and inconvenient [21].

In previous studies, our research group has developed a system that utilizes single-stranded DNA functionalized gold nanoparticles (AuNP GDS) as a general platform for loading and delivering DNA oligonucleotides and shRNA into mammalian cells [22–25].

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It is easy to load short nucleic acids into AuNP GDS by simple hybridization between the cargo DNA that is covalently attached to the AuNP and short nucleic acids that are designed for a specific purpose. This system is capable of delivering short DNA oligonucleotides into the nucleus and consequently modulating biological processes such as alternative splicing and signal transduction pathways in the nucleus [26].

In this study, in order to demonstrate the ability of the AuNP GDS to deliver short, highly structured RNA aptamers into the nucleus of human cells, functionalized gold nanoparticles hybridized with RNA aptamers were directly applied to human cell lines and the effects on target protein function were analyzed. Our results showed that this system can be used for efficient delivery of highly structured RNA aptamers into the nucleus of human cells where they function as repressors by interacting with their target proteins.

2. Materials and methods

2.1. Production of functionalized AuNP GDS conjugates

AuNP-αRNA I (10 nM) and aptamer were annealed for 10 min at 55 °C in reaction buffer (1× phosphate-buffered saline (PBS) containing 0.3 M NaCl) and incubated at 4 °C for an hour. The resulting conjugates were spun down at 13,000 rpm for 10 min, the supernatant was removed, and the conjugate pellet was resuspended in PBS. This precipitation and resuspension step was conducted three times. Citrate-stabilized gold nanoparticles (13 nm) and oligonucleotide-functionalized particles were prepared according to previously described procedures [21,22,24].

2.2. Mammalian cell culture and delivery of functionalized AuNP-αRNA I-RNA aptamers

HepG2 (human hepatocellular liver carcinoma) and A549 (human lung adenocarcinoma epithelial cells; NSCLC) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin (Welgene, Seoul, Korea). HepG2 (2.5×10^5) and A549 (1×10^6) cells were grown in six-well plates, incubated with AuNP GDS–RNA aptamer conjugates (1 nM) in the culture media for 12 h and then harvested for experiments.

2.3. In vitro synthesis of RNA aptamers

The RNA aptamers were synthesized from synthetic oligonucleotides containing a T7 promoter followed by a sequence of the β-catenin aptamer or p50 aptamer using a MEGAshortscript™ kit (Ambion, USA), according to the manufacturer's instructions. DNA templates were prepared by amplifying the template oligonucelotides using PCR. The β-catenin aptamer sequence was kindly provided by Dr. Sunjoo Jeong (Dankook Univ. Korea) and constructed as pU6-β-cat-Apt. The sequence of the β-catenin aptamer was 5′-AGGCCGAUCUAUGGACGCUAGGCACACCGGAUACUUUAACGAUUGGCU-3′ [27]. For the template synthesis of the β-catenin aptamer, pU6-β-cat-Apt was amplified with primers containing the T7 promoter (5′-TAATACGACTCACTATAGGG CGCTAGCAGAG CCGAGATAGGCCGATCTATGGACGCTAT-3′; T7 promoter is underlined) and the β-catenin aptamer-R (5′-AGCCAATCGTTAAAGTA TCCG-3′). We used the p50 aptamer template oligonucleotides for the synthesis of the p50 aptamer (5′-TAATACGACTCACTAT AGGGCAGAGCCGAGATATCTTGAAACTGTTTT AAGGTTGGCCGATC-3′; T7 promoter is underlined). The PCR primers used were T7-1 (5′-TTAATACGACTCACTATAGG-3′) and p50 aptamer-R (5′-GATCGGCCAACCTTAG-3′). pUC6-NC-Apt [28] was used as a negative control and was amplified with primers containing the T7 promoter (5′-TAATACGACTCCTATAGGGCGCTAGCAGAGCCGAGATTATAGTGT GTTCAACGCTTAG-3′) and NC aptamer-R (5′-CTCGTACAACGGACAAAA-3′). This construct was also provided by Dr. Sunjoo Jeong (Dankook Univ. Korea). All RNA aptamers have a sequence complementary to the RNA I oligo, which hybridized to AuNP-αRNA I. The synthesized RNA aptamers were purified using Illustra™ MicroSpin™ G-50 columns (GE; Little Chalfont, Buckinghamshire, UK).

2.4. MTT assay for cell viability

A549 cells were seeded in 96-well plates and cultured overnight to allow cell attachment. After that, cells were incubated with AuNP-αRNA I-p50 aptamer conjugates for 6 h followed by a 3-h incubation in 200 μl of medium contining doxorubicin (Dox) (10 μmol/l). A 0.5 mg/ml aliquot of MTT reagent (Sigma, St. Louis, MO) was then added to the media and the cells were incubated at 37 °C for 2 h. At the end of incubation, the MTT solution was removed, and DMSO was added and incubated at 37 °C for 10 min to stop the reaction, after which the sample absorbance at 540 nm was read using a Perkin–Elmer 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). The culture media was used as a negative control, and the values were normalized against those for the negative control.

2.5. Measurement of the RNA aptamer loading capacity on AuNP-αRNA I

AuNP-αRNA I (1 nM) was hybridized with increasing concentrations (0.1–1.0 μM) of RNA aptamer and the resulting conjugates were analyzed in a 15% polyacrylamide gel containing 8 M urea. The bands were visualized with ethidium bromide and quantitated using the Quantity One Image program (Bio-Rad Laboratories Inc.).

2.6. Detection of RNA aptamer delivery into cells

To detect the delivery of aptamer into HepG2 cells or A549 cells, the RNA aptamer was labeled with cy3 using the Silencer®siRNA Labeling Kit (Ambion, USA) according to the manufacturer's instructions. The AuNP-αRNA I-cy3 labeled-RNA aptamer was incubated with cells for 12 h, after which the resulting samples were fixed with 4% paraformaldehyde (Sigma). Fluorescence was detected using a Leica TCS SP5 II confocal microscope (Leica Microsystems, Germany).

2.7. Immunoprecipitation and Western blot analysis

The HepG2 cells (2.5×10^5) were incubated with AuNP-αRNA I-β-catenin aptamer conjugates in six-well plates. Cell lysates were prepared with Nonidet P-40 (NP-40) lysis buffer (50 mM Tris–HCl, pH 8.0; 0.15 mM NaCl; 1% NP-40) containing a protease inhibitor cocktail. The lysates were centrifuged, and the supernatants were precleared with normal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and protein G-agarose (Upstate, Charlottesville, VA, USA) for 2 h at 4 °C. Precleared lysates were incubated with β-catenin antibodies for 12 h followed by incubation with protein G-agarose for an additional 2 h at 4 °C. Immune complexes were centrifuged and washed three times with NP-40 lysis buffer and were subjected to SDS–PAGE. After transferring the proteins to nitrocellulose membranes, the membranes were blocked with 5% nonfat milk. Anti-β-catenin and anti-p50 polyclonal antibodies (Santa Cruz Biotechnology Inc.) were used for the detection of endogenous β-catenin and p50 proteins, respectively. As loading controls, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected using anti-GAPDH antibody (AbFrontier).