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Dendronized gold nanoparticles for siRNA delivery

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Small interfering RNA (siRNA) is a versatile tool for regulation of gene expression.^[1] The ability of siRNA to silence translation and protein expression of cognate cellular mRNA provides the potential to regulate a wide array of therapeutically-relevant processes.^[2] Despite the therapeutic promise of siRNA, there are challenges to its effective clinical and laboratory use.^[3] Naked siRNA has low stability against nuclease activity and exhibits poor transfection efficacy due to its relatively large molecular weight and anionic character.^[4] These challenges have been addressed using viral and synthetic delivery vehicles.^[5] Viral delivery systems feature high transfection efficiencies,^[6] however, these vectors can face challenges arising from immunogenicity^[7] and mutagenicity.^[8]

Non-viral siRNA delivery systems have the potential to provide improved safety and predictability relative to viral vectors,^[9] and can be broken down into two major groups: covalently conjugated particles^[10] and assemblies formed through electrostatic assembly with cationic materials.^[11] Both strategies are effective, with the supramolecular strategy allowing the release of genetic material from the surface of the delivery vehicle into the cytosol following uptake, an important requirement for some applications.^[12] A variety of cationic materials have been used to condense siRNA for delivery, including cationic lipids^[13] and polymers.^[14, 15]

Functionalized inorganic nanomaterials including carbon nanotubes,^[16] iron oxide nanoparticles,^[17] quantum dots,^[18] and gold nanoparticles^[19] provide alternative platforms for siRNA delivery, featuring diverse structures, sizes, core properties and ease of functionalization.^[20] Additionally, these materials produce the structural properties of high molecular weight polymers using low molecular weight ligands, with the inorganic core functioning as a space-filling structural element for the presentation of the surface monolayer.^[21] We hypothesized that reducing the bulk of the collapsible structure of a polymer to an inorganic nanoparticle would reduce the toxicity of the vehicle (by eliminating unnecessary exposable functionalities that are hidden during complexation), while retaining the favorable properties of polymer delivery vectors through proper functionalization of the surface monolayer. This is an important feature for siRNA delivery. Due to the small size of siRNA, the molecule experiences less efficient interactions with

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Supporting Information

Supporting information is available on the Wiley Online Library or from the authors.

cationic delivery materials than plasmid-sized DNA. siRNA therefore requires a greater number of vehicles per molecule, or a vehicle with a greater amount of cationic character. Both cases potentially lead to greater toxicity issues. We report here the use of gold nanoparticles featuring dendritic polyethylenimine-like ligands^[22] to generate a supramolecular siRNA delivery vehicle featuring high knockdown efficiency and low toxicity.

Three different particles featuring gold cores (2 nm diameter) and triethylenetetramine (TETA) terminated dendron ligands were generated for this study *via* Murray place-exchange reaction (**Scheme 1a**).^[23] The dendronized ligands feature biodegradable glutamic acid scaffolds and cationic TETA moieties that interact electrostatically with negatively charged siRNA (**Scheme 1b**). All three particles are cationic and resist aggregation as indicated by their zeta potentials. (**Figure 1 and Figure S1**).

The molar ratio of dendronized AuNPs to anionic siRNA required for efficient formation of condensed complexes was determined through an agarose gel retardation assay (**Figure 1**). All of the particles appeared to interact with siRNA, as band intensities associated with free siRNA fell with increasing concentrations of each. The G2 particle, however, appeared to be most efficient at retarding the electrophoretic mobility of siRNA, showing complete retardation at a NP/siRNA molar ratio of 2. Both the **G0-AuNP** and **G1-AuNP** still showed free siRNA bands at a NP/ β -gal-siRNA ratio of 4 (**Figure 1**). In further experiments, we found that, when allowed to fully complex with siRNA, **G0-AuNP**/ β -gal-siRNA and **G1-AuNP**/ β -gal-siRNA complexes precipitated at these higher NP/ β -gal-siRNA ratios, thus limiting their utility. These results indicate that tuning the degree of multivalency is important for generating AuNP/siRNA complexes that are useful for delivery applications. For this reason, **G2-AuNP**, capable of generating the most stable self-assembled nanoplexes with siRNA, was used for all subsequent studies. To confirm the condensation ratio between **G2-AuNP** and siRNA, an ethidium bromide (EtBr) fluorescence exclusion assay, in which the quenching of EtBr upon displacement by **G2-AuNP**, was performed (**Figure S2**). The corrected fluorescence curve likewise demonstrated the minimum binding ratio for **G2-AuNP**/ β -gal-siRNA complexation was 2:1.

The **G2-AuNP**/ β -gal-siRNA complexes were characterized by dynamic light scattering (DLS). Nanoplexes in ddH₂O (double distilled water, MilliQ) were ~110 nm diameter (**Figure S3**). The assembly size increased to ~700 nm in a serum-containing media (OptiMEM®). The size (~825 nm) did not respond to significant changes even in the presence of media composed of 40% serum (**Figure S4**). Such changes are typical of supramolecular systems, which are easily affected by changes in pH, ionic strength (which can shield charges and alter the protonation state of surface ligands) and proteins (which can adsorb to the surfaces of nanoparticles and form supramolecular “coronas”).^[24]

siRNA mediated knockdown of β -galactosidase (β -gal) expression was studied using an enzyme activity assay in SVR-bag4 endothelial cells. To determine the optimal concentration of siRNA, SVR-bag4 cells were treated with **G2-AuNP**/ β -gal siRNA at different concentrations (NP/siRNA = 2). β -gal silencing appeared to be saturated at **G2-AuNP**/ β -gal-siRNA complex concentrations above 0.042 μ M (**Figure 2a**). To investigate the correlation between gene silencing efficiency and NP/siRNA ratios, SVR-bag4 cells were also treated with a series of **G2-AuNP**/ β -gal-siRNA complexes with molar ratios of 0.5, 1, and 2 (**Figure 2b**). Gene silencing was found to increase with NP/siRNA ratio, with maximum gene silencing (48%) observed in the case of completely complexed siRNA (NP/siRNA = 2). The knockdown efficiency observed for **G2-AuNP**/ β -gal-siRNA at NP/siRNA 2 was similar to Lipofectamine™ (Invitrogen), a commercially available vehicle with a

validated protocol. (**Figure 2b**). In the absence of either AuNP, siRNA, or a correct siRNA sequence, no significant suppression of β -gal was observed (**Figure 2b**).

Cytotoxicity of both **G2-AuNP** and **G2-AuNP**/ β -gal-siRNA complexes was evaluated by Alamar blue® assay. Again, SVR bag4 cells were used. Exposure of cells to the **G2-AuNP** alone for 2 days led to a slight decrease in cell viability (**Figure 3a**). Exposure of cells to **G2-AuNP**/ β -gal-siRNA complexes for an identical period of time resulted in no significant toxicity (**Figure 3b**), likely through the charge-quenching effects of siRNA in the nanoparticle complex.

In summary, we have demonstrated the utility of dendronized AuNPs as a vector for siRNA delivery. In these studies, **G2-AuNP** suppressed β -gal expression by ~ 50% with minimal toxicity. These nanoparticle vectors possess the benefits of polymeric delivery vehicles such as PEI, while minimizing toxicity through the use of non-toxic core functionality. The application of these systems in biotechnology and biomedicine are currently being explored and will be reported in due course.

Experimental Section

Chemicals

All chemicals used for the fabrication of gold nanoparticle (AuNP) were purchased from Sigma-Aldrich, Fisher Scientific or Strem, unless otherwise specified.

Dichloromethane (DCM) and tetrahydrofuran (THF) used as solvents for chemical synthesis were dried according to standard procedure. ^1H NMR spectra were recorded at 400 MHz on a Bruker AVANCE 400. Hewlett-Packard 8452A and Cary 100 Scan UV-Vis spectrophotometers were used to record and analyze spectra. Fluorescence from the Alamar blue® assay was detected by SpectraMax M5 microplate reader (Molecular Device Inc., USA).

Design of genetic materials

Sequence of β -gal siRNA was designed (5'-CA GUU GCG CAG CCU GAA UGU U3', 5'AA CAU UCA GGC UGC GCA ACU G-3') and nonsense siRNA (*Silencer*® negative control siRNA) was purchased from Applied Biosystems (Ambion Inc. TX). Each sequence was electrostatically condensed with dendronized AuNPs for the fabrication of nanocomplexes.

Fabrication of dendronized AuNPs

Dendronized AuNPs were prepared by place-exchange reaction with a TETA terminated thiol ligand following the general procedure described by Murray *et al* (**Supporting Information**).^[22,24] First, 1-pentanethiol protected gold nanoparticles (AuNP-C5, 2.5 mg) were dissolved in 1.5 mL of DCM/methanol(MeOH) (2:1, v/v) solution. Then, equal volume of DCM/MeOH solution (2:1, v:v) containing 10.5 mmol of TETA terminated thiol ligands was added to the AuNP-C5 solution for fabrication of dendronized AuNPs. After being stirred at room temperature for 2 days, the solvents were evaporated under reduced pressure. The dark color nanoparticle residues were purified three times by DCM. Immediately following this, nanoparticles were dissolved in ddH₂O and allowed to stay at room temperature for 2 days to remove the volatile components. These functionalized AuNPs were stable in solution for months and were stored in ddH₂O at 4 °C.

Size and zeta potentials

Dynamic light scattering (DLS) and zeta potential (ζ) measurements were performed using a Malvern Zetasizer (Nano Series, Malvern Instruments Inc., USA), and the data analysis was done using Malvern PCS software.

Gel electrophoresis

The condensation of β -gal siRNA with dendronized AuNPs was determined by agarose gel retardation assay.^[25] To prepare each mixture, 75 pmol of siRNA was incubated with the required amount of dendronized AuNPs at room temperature for 30 min. Aliquots of samples were loaded onto a 1% agarose gel, pre-stained with ethidium bromide (EtBr) and visualized with a UV transilluminator after electrophoresis at 100 V for 60 min.

EtBr quenching assay

siRNA binding was determined by EtBr exclusion assay. All samples were prepared in a phosphate buffered saline solution (PBS, pH 7.4). Fluorescence spectra were recorded on a SpectroMax M5 microplate reader (Molecular Devices) with excitation at 545 nm. During the titration, a mixture (2 mL) of siRNA (0.1 μ M) and ethidium bromide (EtBr, 1 M) was placed in the cuvette and the initial emission spectrum was recorded. Aliquots (10 μ L) of a solution of siRNA (0.1 μ M), EtBr (1.0 μ M), and **G2-AuNP** (3.5 μ M) were subsequently added to the solution while in the cuvette. After each addition, a fluorescence spectrum was recorded. The fluorescence intensities were normalized and calibrated at a wavelength of 589 nm. The graph of fluorescence intensities (FI) was plotted against the ratio [**G2-AuNP**]/[siRNA] to obtain the optimum binding.

Cell culture

Pancreatic endothelial cells (SVR bag4, CRL-2387TM) were purchased from American Type Culture Collection (ATCC, VA) and cultured at 37 °C, 5% CO₂ in a humidified atmosphere. Cells were grown in the low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin).^[26] For siRNA transfection and cytotoxicity studies, 3×10^4 cells/well were transferred to a 24-well plate and incubated 24 hours prior to the experiment.

Cellular transfection and β -gal assay

G2-AuNP/ β -gal-siRNA (75 pmol siRNA, 150 pmol AuNP) complexes and **G2-AuNP**/nonsense-siRNA (75 pmol siRNA, 150 pmol) complexes were formed by mixing their components in 300 μ L of reduced serum media (OptiMEM® (Gibco, NY)) prior to cell treatment. After a 20 min incubation, 1.5 mL of cell culture media (10% FBS-DMEM) was added to the mixture. A β -gal producing cell line (SVR bag4) (3×10^4 cells/well) was treated with the condensed complexes at a concentration of 0.042 μ M for 6 hours. Cells were similarly treated with β -gal siRNA (all 0.042 μ M), nonsense naked siRNA, **G2-AuNP**/ β -gal-siRNA complex, Lipofectamine™/ β -gal-siRNA or **G2-AuNP**/nonsense-siRNA complex. The final volume used for the cell experiment was 570 μ L and the concentration of siRNA was from 0.021 to 0.084 μ M in media. Then, cells were washed with PBS to remove the untransfected material and further incubated in 10% FBS-DMEM medium for 2 days to observe the gene silencing effect. Cells were then washed 3 times with PBS and then disrupted by a cell lysis buffer (Genlantis, CA). To measure β -gal activity, cells were first incubated for 1 hour at room temperature with lysis buffer (Gene Therapy Systems Inc., CA). After centrifugation of lysates, β -gal levels were determined by a β -gal kit (Genlantis, CA). In brief, chlorophenol red-D-galactopyranoside (CPRG) was added as a substrate and then the absorbance was measured at 570 nm on a SpectroMax M5 microplate reader.

Cytotoxicity study

Cytotoxicity was determined by an Alamar blue® assay (Invitrogen Biosource, USA), which is based on the conversion of resazurin to resorufin *via* a reduction reaction dependent on cellular metabolic activity.^[27] Briefly, SVR-bag4 cells previously incubated with either **G2-AuNP** (<1 nmol) or **G2-AuNP/β-gal-siRNA** (β-gal siRNA=75 pmol) at various molar ratios for 2 days. Following incubation, cells were washed with PBS and treated with 10% Alamar blue® dye in low glucose DMEM (500 μL/well). After incubation, the fluorescence intensity was recorded (E_x : 560 nm, E_m : 590 nm) using a SpectroMax M5 microplate reader to determine cell viability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

a) Chemical structures of **G0-AuNP**, **G1-AuNP**, and **G2-AuNP**. b) Schematic illustration of AuNP/ β -gal-siRNA complexation and transfection into cells.

Figure 1.

Hydrodynamic diameter and zeta potential of **G0-AuNP**, **G1-AuNP**, and **G2-AuNP**. Gel retardation assay of AuNP/ β -gal-siRNA complexation at different molar ratios showed a decrease in band intensity due to the fluorescence quenching by complexation with AuNPs.

Figure 2. β -gal gene silencing in SVR-bag4 cell. a) Gene silencing effect of G2-AuNP/ β -gal-siRNA with different concentration from 0.021 to 0.084 μ M (NP/siRNA=2). b) Gene silencing effect of naked β -gal siRNA, naked nonsense siRNA, **G2-AuNP**, **G2-AuNP**/ β -gal-siRNA complex at various molar ratios (0.5, 1 and 2), and **G2-AuNP**/nonsense-siRNA complex (NP/siRNA=2). 0.021 μ M of siRNA was used in each experiment.

Figure 3. Cell viability determined by Alamar blue® assay. Cell viability was determined after the treatment of a) **G2-AuNP** and b) **G2-AuNP/β-gal-siRNA** complex at various molar ratios (β-gal siRNA=75 pmol).