Bioconjugated gold nanoparticles enhance siRNA delivery in prostate cancer cells

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Abstract

Here we describe a simple way to create a gold nanoparticle (AuNP) based non-viral delivery system to deliver siRNA into prostate cancer cells. Therefore, positively charged polyethylenimine (PEI) capped AuNPs were synthesized in water and further conjugated with the targeting ligand (folic acid) for folate receptors (AuNPs-PEI-FA). The AuNPs-PEI-FA could effectively complex small interfering RNA (siRNA) through electrostatic interaction. Flow cytometry displayed that AuNPs-PEI-FA could specifically deliver siRNA into LNCaP cells, a prostate cancer cell line overexpressing prostate specific membrane antigen (PSMA), that exhibits a hydrolase enzymic activity with a folate substrate. In contrast, internalization of siRNA into PC-3 cells, a prostate cancer cell line not expressing PSMA or folate receptors, was not achieved using AuNPs-PEI-FA.siRNA. Following endolysosomal escape the AuNPs-PEI-FA-.siRNA formulation resulted in significant endogenous gene silencing when compared to the non-targeted formulation, suggesting the potential of AuNPs-PEI-FA for targeted delivery of therapeutic siRNAs in the treatment of prostate cancer.

Key words: Gold nanoparticles, Targeting ligands, Receptor-mediated internalization, Non-viral siRNA delivery, Prostate cancer, Gene therapy

1. Introduction

Prostate cancer is one of the most leading causes of morbidity and mortality in men worldwide [1]. Gold nanoparticles (AuNPs) with size/shape dependent optical properties offered by the surface plasmon resonance (SPR) are now widely used in biomedical applications, including the contrast agents, photothermal agents and radiosensitizers for cancer diagnosis and therapy [2, 3]. Furthermore, the affinity of AuNPs to strongly bind biomolecules containing thiol and/or amino groups makes them suitable as bioconjugated vehicles for selective drug delivery [4]. Small interfering RNA (siRNA) is known to selectively downregulate any gene implicated in the pathology of disease [5, 6]. Recent knowledge of molecular genetic data discovered by sequencing the whole genome of cancer cells has resulted in significant changes in our understanding of the molecular pathogenesis of cancer and has facilitated the identification of a number of molecular targets against which RNAi has been developed [7, 8]. Indeed, delivery of siRNA using multifunctional nanoparticles (NPs) has been recently investigated to treat solid tumours and leukaemia [9-11].

In this study cationic bioconjugated AuNPs namely AuNPs-PEI-FA were obtained using $HAuCl_4.3H_2O$, ascorbic acid, polyethylenimine (PEI) and folic acids [folate, FA; in order to target the folate receptor (FR) overexpressed in prostate cancer cells]. Gel retardation showed that AuNP-PEI-FA could effectively complex siRNA through electrostatic interaction. Flow cytometry displayed that AuNPs-PEI-FA could specifically deliver siRNA into LNCaP cells. Consequently, the presence of the FA facilitated cell-specific receptor-mediated uptake and the presence of PEI (a polycation with dramatic buffering capacity for ‘proton sponge’ effect) induced endolysosomal escape of the siRNA subsequently achieving endogenous gene silencing in LNCaP cells.

2. Materials

Prepare all solutions using purified H_2O (resistivity ≈ 18.2 MΩ cm) as a solvent for the AuNPs synthesis. Clean all glassware used in the synthesis of AuNPs with aqua regia (3 parts of concentrated HCl and 1 part of concentrated HNO_3), rinse with distilled water, ethanol and acetone, and dry in oven before use. Freshly prepared solutions were used (unless specified a stock solution). All siRNA solutions were prepared in RNase-free water following Sigma-Aldrich recommendations.

2.1 Nanoparticles synthesis and bioconjugation with folic acid

1. 1 mM Gold(III) chloride trihydrate ($HAuCl_4.3H_2O$) ≥99.9% metal basis aqueous solution.
2. 100 mM L-ascorbic acid BioXtra, ≥99.0%, crystalline aqueous solution.
3. 2.3 mM PEI 2KD, average M_n ~1,800 by GPC, average M_w ~2,000 by LS aqueous solution stock at 4 °C.
4. Glass round flasks.
5. Magnetic stirrer.
6. Sonicator.
7. Folic Acid BioReagent, suitable for cell culture, suitable for insect cell culture, suitable for plant cell culture, ≥97%.
8. Dimethyl Sulfoxide (DMSO) Anhydrous ≥99.9%.
9. Trimethylamine ≥99.5%.
10. *N*-hydroxysuccinimide (NHS) 98%.
11. N,N'-Dicyclohexylcarbodiimide (DCC) for synthesis.
12. Whatman filter papers.

13. Schlenk line connected to Argon gas flow to operate under inert atmosphere.
14. Microbalance.
15. Centrifugator.

2.2 Preparation of AuNP.siRNA complexes

1. AuNPs-PEI and AuNPs-PEI-FA were concentrated to 500 μg mL^{-1} by centrifugation (5000 rpm, 1677 × g for 15 min at RT).
2. Negative Control siRNA; sense sequence 5′-UUC UCC GAA CGU GUC ACG U-3′; desalting purification.
3. Fluorescein amidite (FAM)-labeled siRNA; sense sequence 5′-UUC UCC GAA CGU GUC ACG U-3′, modified by 6-FAM on 5′ of sense sequence; desalting purification.
4. RelA siRNA; sense sequence 5′-CCA UCA ACU AUG AUG AGU U-3′; desalting purification.
5. Agarose (low gelling temperature, for molecular biology).
6. Tris/Borate/EDTA (TBE) buffer (BioReagent, suitable for electrophoresis, 10x concentrate).
7. SafeView.

2.3 Cell culture

1. RPMI-1640 medium for LNCaP cells (with sodium bicarbonate, liquid, without L-glutamine, sterile-filtered, suitable for cell culture).
2. RPMI-1640 medium for PC-3 cells (with L-glutamine and sodium bicarbonate, liquid, sterile-filtered, suitable for cell culture).
3. LNCaP cells, a human prostate cancer cell line PC-3 cells, a human prostate cancer cell line.

4. 12- and 24-well cell culture plates.
5. Cell incubator.

2.4 Fluorescence activated cell sorting, confocal microscopy and *in vitro* gene knockdown

1. Polystyrene Round-Bottom Tubes.
2. 6-well culture plates with glass bottom.
3. LysoTracker® Deep Red.
4. GenElute™ Mammalian Total RNA Miniprep Kit.
5. High-Capacity cDNA Reverse Transcription Kits.
6. Primers for RelA and GAPDH.

2.5 Characterization equipments

1. UV-visible Spectroscopy: CARY UV−visible spectrophotometer with a Xenon lamp (190−900 nm range, 0.5 nm resolution) was used to carry out Optical absorption spectra.
2. Scanning Electron Microscopy (SEM): FEI 630NanoSEM equipped with an Oxford INCA energy dispersive X-ray (EDX) detector operating at 5 kV was used to image the AuNPs-PEI deposited from solution onto a Si wafer and air-dried prior to analysis.
3. Dynamic Light Scattering (DLS) and Zeta Potential Measurements: Malvern Zetasizer Nano ZS was used to determine the size and charge distributions via dynamic light scattering (DLS) and electrophoretic mobility measurements respectively.
4. Confocal Microscopy: Olympus FV 1000 microscope was used and images were acquired and analysed using Olympus FV10-ASW software.
5. Real-time quantitative PCR (qPCR): Gene expression was assessed by real-time quantitative PCR (qPCR) using the Applied Biosystems Real Time PCR System (model 7300).

3. Methods

3.1 Synthesis of folic acid-conjugated gold nanoparticles

3.1.1 Synthesis of PEI capped gold nanoparticles (AuNPs-PEI)

1. Add 1.42 mL of 2.3 mM PEI to 32.6 mL of 1 mM $HAuCl_4.3H_2O$ solution with stirring at RT; the colour of the solution was changed from pale yellow to deep orange.
2. Add to the above solution (after about 1 minute) 0.5 mL of a freshly prepared 100 mM L-ascorbic acid aqueous solution, the colour change immediately (~ 5 s) to red brownish (*see* **Note 1**).
3. Keep the solution stirring gently overnight at RT achieving AuNP-PEI (*see* **Note 2**) as shown by UV-visible spectroscopy and SEM shown in Fig. **1** (*see* **Note 3**).

3.1.2 Synthesis of Folic acid activated ester (folic acid-NHS)

1. Dissolve 0.25 g (5.663×10^{-4} mol) of folic acid in 10 mL of dry DMSO.
2. Add to the above solution 0.125 mL (8.960×10^{-4} mol 1.5 equivalents) of triethylamine.
3. Sonicate the above solution for about 10 min, and further stir under Argon until folic acid is totally dissolved (*see* **Note 4**).
4. Add to the above homogeneous solution 0.13 g (11.295×10^{-4} mol, ~ 2 equivalents) of NHS and 0.235 g (11.38×10^{-4} mol, ~ 2 equivalents) of DCC.
5. Keep the mixture under stirring overnight in dark at RT, to allow the formation of folic acid-NHS.
6. Filter the resulting solution to remove the dicyclohexylurea byproduct (filtration is performed twice), and stock the final solution in dark at room T (*see* **Note 5**).

3.1.3 Synthesis of AuNP-PEI-FA

1. Take 3 mL of AuNP-PEI and add 30 μL of 25 mg mL^{-1} folic acid-NHS in DMSO. Keep the AuNP-PEI, folic acid-NHS under stirring in dark at RT for 10 min.
2. Add 50 μL of 0.1 M NaOH to the solution and leave it under stirring at RT for 48 h in dark, achieving AuNP-PEI-folic acid (thereafter referred as AuNP-PEI-FA).
3. To purify the AuNP-PEI-FA solution centrifuge at 12,000 rpm for 5 min remove the supernatant containing unreacted folic acid-NHS
4. The unreacted folic acid-NHS can be quantified by UV-vis (*see* **Note 6**).
5. The size and zeta potential distribution were determined by DLS and Zeta potential measurements as shown in Fig **2** (*see* **Note 7**)

3.2 Preparation of AuNP.siRNA complexes

1. Solutions of 500 μg mL^{-1}AuNP-PEI and 500 μg mL^{-1} AuNP-PEI-FA were added to 0.264 μg μL^{-1} siRNA solutions at the mass ratio (MR) 20 of AuNPs to siRNA, followed by 1 h incubation with 400 rpm shaking at RT, in order to achieve the AuNP-PEI.siRNA and AuNP-PEI-FA.siRNA formulations.
2. The ability of AuNPs to complex siRNA was analysed by gel retardation. Complexes of AuNPs and siRNA at different mass ratios (μg AuNPs to μg siRNA, siRNA was fixed to 0.25 μg siRNA) were loaded onto 1% (w/v) agarose gels in 100 mL TBE buffer containing 6 μL SafeView.
3. Electrophoresis was performed at 120 V for 30 min and the resulting gels were photographed under UV light (Fig. **3**).

3.3 Cell culture

1. LNCaP cells were maintained in RPMI-1640 medium supplemented with 10% FBS 50 units mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 2 mM $_L$-Glutamine, 10 mM HEPES, 7.2 mM d-glucose and 1 mM Sodium Pyruvate.
2. PC-3 cells were maintained in RPMI-1640 medium supplemented with 10% FBS.
3. All cells were grown in the incubator at 37 °C with 5% CO_2 and 95% relative humidity.

3.4 Fluorescence activated cell sorting

1. 100,000 LNCaP cells and 100,000 PC-3 cells were respectively seeded per well in 24-well plates and incubated for 24 h under the growth conditions mentioned above.
2. Cells were transfected with AuNP-PEI.siRNA (25 and 50 nM FAM siRNA) and AuNP-PEI-FA.sRNA (25 and 50 nM FAM siRNA) and incubated for 24 h in the growth medium mentioned above.
3. Following incubation cells were washed twice with PBS and trypsinised with Trypsin-EDTA.
4. Cells were subsequently centrifuged (1000 rpm for 5 min) and re-suspended in 1000 μL ice-cold PBS in Polystyrene Round-Bottom Tubes.
5. Ten thousands cells per sample were measured following the procedure outlined in Becton Dickinson FACScalibur manual. Fluorescein-positive cells (%) were displayed by Dot Plot (Fig. **4**).

3.5 Confocal microscopy

1. 300,000 LNCaP cells were seeded per well in 6-well culture plates with glass bottom.. Cells were treated with the AuNP-PEI-FA formulation containing 50 nM FAM siRNA and incubated under the growth conditions (mentioned above) for 24 h.
2. In order to label late endosomes and lysosomes, 75 nM LysoTracker® Deep Red were added to cells for 30 min at 37 °C.
3. The medium was then replaced with fresh growth medium. The intracellular route was analyzed using the confocoal microscopy (Fig. **5**).

3.6 *In vitro* gene silencing

1. 200,000 LNCaP cells were seeded per well in 12-well plates and incubated for 24 h under the growth conditions mentioned above.
2. Cells were then transfected by the AuNP-PEI-FA formulation (MR20) containing 50 nM RelA siRNA for 24 h. 50 nM naked RelA siRNA and the AuNPs-PEI-FA formulation containing 50 nM Negative Control siRNA were used as the negative controls.
3. Following incubation total RNA was isolated from the cells using the GenElute™ Mammalian Total RNA Miniprep Kit, according to the instructions supplied.
4. First-strand cDNA was generated from total RNA samples using High-Capacity cDNA Reverse Transcription Kits.
5. Gene expression was assessed by real-time quantitative PCR (qPCR) using the Applied Biosystems Real Time PCR System. Assays were performed using appropriate primers for RelA and GAPDH. Amplification was carried out by 40 cycles of denaturation at 95 °C (15 s) and annealing at 60 °C (1 min). The quantitative level of each RelA mRNA

was measured as a fluorescent signal corrected according to the signal for GAPDH mRNA using $2^{-\Delta\Delta}$ Ct method [13] (Fig **6**).

4. Notes

1. Addition of PEI 2KD to 1 mM $HAuCl_4.3H_2O$ form a complex between the $AuCl^{4-}$ ions and the amino groups in PEI detected by a change of colour from pale yellow to orange. To reduce the complex, immediately inject an excess of ~ 1.55 eq of Ascorbic Acid. The AuNPs formation can be easily observed by a colour change from yellow to red. Keep the stirring overnight to make sure that all gold is reduced and to stabilize the final AuNPs-PEI size.
2. Stirring a high concentration of 1 mM AuNPs overnight can cause some adsorption of the resulting AuNPs-PEI onto the wall of the round flask. Therefore, the adsorbed AuNPs might be recollected by sonication about 30 seconds.
3. The obtained AuNPs-PEI can be characterised by UV-vis spectroscopy (Fig **1**), the obtained AuNPs-PEI by the above mentioned method shows an absorption band at λ_{max} 560 nm at the origin of the red colloidal solution. This is the surface Plasmon resonance band characteristic of AuNPs colloidal solutions. Furthermore, SEM analysis (Fig **1**) proves the formation of AuNPs with a diameter of ~110 nm as determined by *Image J* software.
4. To dissolve the 0.25 g of Folic acid in DMSO, it was necessary to sonicate the solution for about 10 mins followed by stirring for another 30 min. Folic acid need to be manipulated in dark as it is light sensitive.
5. The above mentioned synthesis of folic acid-NHS is known to yield approximately 80% γ-carboxyl group of folic acid, preserving the binding of activated folate to folate

receptors [14]. The byproduct dicyclohexylurea is insoluble in water and is separated by simple filtration. The solution of 25 mg mL^{-1} folic acid-NHS in DMSO can be used without further purification.

6. Folic acid activated with N-hydroxysuccinimide ester becomes ready to react with free amino group in PEI at a pH (7 to 8), to produce an amide linkage between the Folic Acid and AuNPs-PEI leading to AuNPs-PEI-FA. To purify the final AuNPs-PEI-FA solution centrifuge at 12,000 rpm for 5 min remove carefully the supernatant containing the unreacted folic acid-NHS and run a UV-vis spectrum to quantify the Folic acid attached to the AuNPs.
7. To confirm the formation of AuNP-PEI-FA perform DLS/Zeta Potential analysis, the later show a very slight increase in the size distribution of AuNPs-PEI and a decrease in the value of the Zeta potential confirming the successful attachment of FA (Fig. **2**).

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Fig. 1 UV-visible spectra of AuNPs-PEI (left) and SEM image (right) of AuNPs-PEI (bar = 5 μm)

Fig. 2 Size distribution by intensity (left) of AuNPs-PEI (113 ± 1 nm, PDI = 0.07, n = 3) and AuNPs-PEI-FA (118 ± 1 nm, PDI = 0.09, n = 3); insert show that the size is slightly increased; Zeta potentials (right) of AuNPs-PEI (53 ± 1 mV, n = 3) and AuNPs-PEI-FA (46± 1 mV, n = 3) as obtained from DLS analysis. (Reproduced with permission from the Elsevier Copyright Clearance Center; J. Guo et al. *International Journal of Pharmaceutics 509 (2016) 16–27*)

Fig. 3 Complexation of siRNA (0.25 μg) with (A)AuNPs-PEI and (B) AuNPs-PEI-FA at different mass ratios (MRs) using gel retardation (1% agarose gel at 120 mV for 30 min). (Reproduced with permission from the Elsevier Copyright Clearance Center; J. Guo et al. *International Journal of Pharmaceutics 509 (2016) 16–27*)

Fig. 4 Fluorescein-positive PC-3 and LNCaP cells (%, mean ± SD) after 24 h transfection of AuNPs-PEI-FA.FAMsiRNA (MR20, 50 nM siRNA) analysed using flow cytometry (PC-3 = receptor negative cells and LNCaP cells = receptor positive cells). (**$P < 0.01$ and ***$P < 0.001$ compared to the untreated control). (Reproduced with permission from the Elsevier Copyright Clearance Center; J. Guo et al. *International Journal of Pharmaceutics 509 (2016) 16–27*)

Fig. 5 Arepresentative image showing intracellular distribution of AuNPs-PEI-FA formulation (MR20) containing FAM siRNA (50 nM) in LNCaP cells at 24 h post-transfection by confocal microscope. Late endosomes and lysosomes were labelled with 75 nMLysoTracker® Deep Red (Green = FAM siRNA, Red = LysoTracker® Deep Red). Most siRNAs were found in the cytoplasm. It is interesting to note that some FAM siRNAs were also found inside nucleus as indicated by arrows.

Fig. 6 Endogenous gene silencing in LNCaP cells by RelA siRNA (50 nM) formulated with AuNPs-PEI-FA (MR20) at 24 h post transfection.The naked RelA siRNA (50 nM) and Negative Control siRNA (50 nM) formulated with AuNPs-PEI-FA (MR20) were used as controls (NS = no significance, *$P < 0.05$ compared to formulation containing Negative Control siRNA). (Mean ± SD, $n = 3$). (Reproduced with permission from the Elsevier Copyright Clearance Center; J. Guo et al. *International Journal of Pharmaceutics 509 (2016) 16–27*)