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# Bioconjugated Gold Nanoparticles Enhance Cellular Uptake: A Proof of Concept Study for siRNA Delivery in Prostate Cancer Cells

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## ABSTRACT

The chemistry of gold nanoparticles (AuNPs) facilitates surface modifications and thus these bioengineered NPs have been investigated as a means of delivering a variety of therapeutic cargos to treat cancer. In this study we have developed AuNPs conjugated with targeting ligands to enhance cell-specific uptake in prostate cancer cells. First, negatively charged AuNPs-citrate were synthesised in water by reducing hydrochloroauric acid ( $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ ) with sodium citrate, subsequently an ethylene glycol based cross-linker (NHS-PEG-S-S-PEG-NHS, the capping agent) was used to attach transferrin (Tf, the targeting ligand) onto the AuNPs-citrate producing transferrin-conjugated AuNPs (AuNPs-PEG-Tf). Secondly, positively charged AuNPs-PEI were obtained in water by reducing  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  using ascorbic acid in the presence of polyethylenimine (PEI, the capping agent), and folic acid (FA, the targeting ligand) was attached onto the AuNPs-PEI to produce folic acid-conjugated AuNPs (AuNPs-PEI-FA). Both bioconjugated AuNPs demonstrated low cytotoxicity in prostate cancer cells. The attachment of the targeting ligand Tf to AuNPs successfully achieved receptor-mediated cellular uptake in PC-3 cells, a prostate cancer cell line highly expressing Tf receptors. The AuNPs-PEI-FA effectively complexed small interfering RNA (siRNA) through electrostatic interaction. At the cellular level the AuNPs-PEI-FA specifically delivered siRNA into LNCaP cells, a prostate cancer cell line overexpressing prostate specific membrane antigen (PSMA, exhibits a hydrolase enzymic activity with a folate substrate). Following endolysosomal escape the AuNPs-PEI-FA.siRNA formulation produced enhanced endogenous gene silencing compared to the non-targeted formulation. Our results suggest both formulations have potential as non-viral gene delivery vectors in the treatment of prostate cancer.

## 1. Introduction

Prostate cancer is among the leading causes of morbidity and mortality in men worldwide, with approximately 220,800 new cases diagnosed and 27,540 deaths in the United States in 2015 [1]. Although current therapies using chemotherapeutics and small-molecule inhibitors have made significant progress, these strategies are limited by two primary issues: toxicity and lack of efficacy [2, 3]. Targeted delivery of therapeutics may help reduce toxicity and increase efficacy. Recently, novel nanomaterials tagged with relevant targeting ligands have been developed to interact with receptors highly expressed in tumour tissues for safe and efficient delivery of therapeutic cargos in the treatment of prostate cancer [4] [5] [6] [7].

Advances in the genetic basis of diseases including cancer have helped identify gene targets associated with disease progression. The discovery that small interfering RNA (siRNA), one of RNA interference (RNAi) effectors could selectively downregulate any gene opened up the possibility of developing RNAi therapeutics [8]. However in order to translate RNAi into clinical use effective non-toxic delivery systems are essential. The development of novel biomaterials has facilitated the formulation of multifunctional nanoparticles (NPs) to deliver siRNA and these have been investigated in the treatment of both solid tumours and leukaemia [9] [10] [11].

Gold is a versatile material which can be processed into different shapes (*i.e.* sphere, rod etc.) and sizes, including particles in the nanometer size range (1~100 nm) [12]. Due to unique physicochemical properties gold nanoparticles (AuNPs) have been widely used as contrast agents, photothermal agents and radiosensitisers for cancer diagnosis and therapy [13]. In addition AuNPs exhibit the ability to bind amine and thiol groups allowing surface modification with functional moieties (*i.e.* stabilising groups, bioactive polymers and

targeting ligands) [14], this strategy has been utilised to facilitate targeted drug delivery for cancer therapy [15].

In this study, two different bioconjugated AuNPs were synthesised as follows, 1) an anionic transferrin (Tf) tagged AuNP, AuNPs-PEG-Tf, and 2) a cationic folic acid (FA) tagged AuNP, AuNPs-PEI-FA, both designed to target cell surface receptors [transferrin receptor (TfR) and folate receptor (FR), respectively] overexpressed in prostate cancer cells. These AuNPs were fully characterised and their ability to achieve cell specific uptake was monitored in order to assess their potential as nontoxic gene delivery vectors.

## **2. Experimental**

### **2.1 Materials and Chemicals**

Purified H<sub>2</sub>O (resistivity  $\approx 18.2 \text{ M}\Omega \text{ cm}$ ) was used as a solvent for the synthesis of the AuNPs. All glassware was cleaned with aqua regia (3 parts of concentrated HCl and 1 part of concentrated HNO<sub>3</sub>), rinsed with distilled water, ethanol and acetone, and oven-dried before use. Tetrachloroauric acid trihydrate (HAuCl<sub>4</sub>·3H<sub>2</sub>O), L-ascorbic acid (C<sub>6</sub>H<sub>8</sub>O<sub>6</sub>), sodium citrate (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>·2H<sub>2</sub>O), sodium hydroxide (NaOH), 4,7,10,13,16,19,22,25,32,35,38,41,44,47,50,53-Hexadeca-28,29-dithiahexapenta-contanedioic acid di-N-succinimidyl ester (NHS-PEG<sub>280</sub>-S-S-PEG<sub>280</sub>-NHS), transferrin, fluorescein isothiocyanate isomer I, folic acid, dry dimethyl sulfoxide (DMSO), triethylamine, branched PEI (molecular weight,  $M_w \sim 2000$ ), dicyclohexylcarbodiimide and N-hydroxysuccinimide, were purchased from Sigma-Aldrich.

### **2.2 Synthesis of transferrin-conjugated gold nanoparticles**

An aqueous solution of  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  (100 mL,  $0.25 \text{ mmol L}^{-1}$ ) was heated to  $80^\circ\text{C}$  while stirring, 0.08 mL of 1 M NaOH were added, followed by rapid injection of 0.34 mL sodium citrate ( $340 \text{ mmol L}^{-1}$ ) (Fig. 1). The colour of the solution changed from pale yellow to colourless instantly after addition of the sodium citrate; at  $\sim 5$  min the colour changed to gray and then red at  $\sim 12$  min; heating was stopped once the solution became deep red, and no further colour change was observed upto  $\sim 4.5$  h after addition of sodium citrate. The solution was cooled to room temperature (RT) while stirring [16].

A freshly prepared solution (1 mL,  $0.5 \text{ mmol L}^{-1}$ ) of NHS-PEG<sub>280</sub>-S-S-PEG<sub>280</sub>-NHS was added to 25 mL of AuNPs-citrate solution under stirring for 10 min to produce AuNPs-PEG<sub>280</sub>-S-S-PEG<sub>280</sub>-NHS (thereafter referred as AuNPs-PEG-NHS) (Fig. 1).

The pH of the AuNPs-PEG-NHS solution was adjusted to  $\sim 7.5$ -8. 0.4 mL transferrin ( $8.8 \mu\text{mol L}^{-1}$ ) in citric buffer was added to 12.5 mL of AuNPs-PEG-NHS and incubated at RT under stirring for 4 h. The resultant AuNPs-PEG<sub>280</sub>-S-S-PEG<sub>280</sub>-transferrin (thereafter referred as AuNPs-PEG-Tf) (Fig. 1) were purified by centrifugation at 14,000 rpm for 20 min and resuspended in deionised water (DIW).

In addition, a freshly made solution (400  $\mu\text{L}$ , 1.5 mg for fluorescein isothiocyanate in 15 mL PBS buffer at pH 9) was added to 4 mL of the AuNPs-PEG-Tf solution and incubated with stirring at  $4^\circ\text{C}$  in the dark overnight to produce fluorescein-labelled AuNPs-PEG-Tf. Fluorescein-labelled AuNPs-PEG-Tf were purified using centrifugation at 12,000 rpm for 5 min at RT.

### **2.3 Synthesis of folic acid-conjugated gold nanoparticles**

1.42 mL of PEI ( $2.3 \text{ mmol L}^{-1}$ ) was added to 32.6 mL of  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  ( $1 \text{ mmol L}^{-1}$ ) with stirring at RT; the colour of the solution changed from pale yellow to deep orange. When 0.5

mL of L-ascorbic acid ( $100 \text{ mmol L}^{-1}$ ) was added the colour of the solution changed to red brownish at  $\sim 5 \text{ s}$ . The solution was incubated at RT with stirring gently overnight to produce AuNPs-PEI (Fig. 1).

Folic acid (0.25 g) was dissolved in 10 mL of dry DMSO ( $5.663 \times 10^{-4} \text{ mol}$ ), followed by the addition of 0.125 mL of triethylamine ( $8.960 \times 10^{-4} \text{ mol}$ , 1.5 equivalents). The solution was sonicated for 10 min and stirred under Argon until the folic acid was dissolved. Subsequently, 0.13 g of N-hydroxysuccinimide ( $11.295 \times 10^{-4} \text{ mol}$ ,  $\sim 2$  equivalents) and 0.235 g of dicyclohexylcarbodiimide ( $11.38 \times 10^{-4} \text{ mol}$ ,  $\sim 2$  equivalents) were added to the solution and the reaction mixture was left under stirring overnight at RT to produce the folic acid-NHS (Fig. 1). The byproduct, dicyclohexylurea, was removed by filtration. The solution of folic acid-NHS in DMSO ( $25 \text{ mg mL}^{-1}$ ) was used without further purification.

30  $\mu\text{L}$  of folic acid-NHS ( $25 \text{ mg mL}^{-1}$ ) was added to 3 mL of AuNPs-PEI and incubated with stirring at RT for 10 min. Subsequently 50  $\mu\text{L}$  of 0.1 M NaOH was added to the solution and left under stirring at RT for 48 h in dark to produce the AuNPs-PEI-folic acid (thereafter referred as AuNPs-PEI-FA) (Fig. 1). The resultant AuNPs-PEI-FA solution was purified by centrifugation at 12,000 rpm for 5 min and the free folic acid-NHS in the supernatant was quantified using a CARY UV-Vis spectrophotometer with a Xenon lamp (240-900 nm range, 0.5 nm resolution).

## 2.4 Dynamic light scattering (DLS)

Measurements of particle size and zeta potential were carried out using a Malvern Nano-ZS (Malvern Instruments, UK) at  $25^\circ\text{C}$  using the default non-invasive back scattering (NIBS) technique with a detection angle of  $173^\circ$ . Measurement for each sample was performed in DIW.

## 2.5 Transmission electron microscopy (TEM)

A drop of AuNP solution was placed on a carbon-coated TEM copper grid (Quantifoil, Germany) and air-dried prior to analysis using a JEOL JEM-2100 TEM operated at 200 kV. All images were recorded on a Gatan 1.35 K  $\times$  1.04 K  $\times$  12 bit ES500W CCD camera. TEM images were analysed using *Image J* software.

## 2.6 Scanning electron microscopy (SEM)

AuNPs were deposited from solution onto a Si wafer and air-dried prior to analysis using a FEI 630 NanoSEM equipped with an Oxford INCA energy dispersive X-ray (EDX) detector operated at 5 kV.

## 2.7 Cell culture and cytotoxicity

PC-3 cells [a human prostate cancer cell line, European Collection of Cell Cultures (ECACC), UK] were maintained in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS). LNCaP cells [a human prostate cancer cell line, American Tissue Culture Collection (ATCC), USA] were maintained in RPMI-1640 medium supplemented with 10% FBS, 50 units mL<sup>-1</sup> penicillin, 50  $\mu$ g mL<sup>-1</sup> streptomycin, 2 mM L-Glutamine, 10 mM HEPES, 7.2 mM d-glucose and 1 mM Sodium Pyruvate. LNCaP cells were cultured and seeded in Poly-d-Lysine (1 $\times$ ) coated tissue culture flasks and wells [17]. All cells were grown in the incubator (ThermoForma) at 37 °C with 5 % CO<sub>2</sub> and 95 % relative humidity.

The cytotoxicity of AuNPs was estimated using the MTT assay with 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Sigma) [18]. PC-3 (5,000 per well) and LNCaP (10,000 per well) cells were seeded within 200  $\mu$ L growth media in 96-well plates one day before transfection. AuNPs were incubated with cells for 48 h under normal growth conditions. Following incubation the solutions were replaced with 200  $\mu$ L fresh growth

medium, and 20  $\mu\text{L}$  MTT stock (5  $\text{mg mL}^{-1}$  in PBS) was added and incubated with cells for 4 h at 37  $^{\circ}\text{C}$ . The contents were then removed and 100  $\mu\text{L}$  DMSO was added to dissolve the purple formazan products. The data were obtained at 590 nm using a microplate reader. The concentration of AuNPs leading to 50 % cell growth inhibition ( $\text{IC}_{50}$ ) was estimated from the plot of the percentage of viable cells versus the concentration of AuNPs for each treatment.

## **2.8 Preparation and characterisation of AuNP.siRNA complexes**

Solutions of AuNPs-PEI and AuNPs-PEI-FA (500  $\mu\text{g mL}^{-1}$ ) were added to the siRNA solutions (Negative Control siRNA, sense sequence 5'-UUC UCC GAA CGU GUC ACG U-3', prepared in RNase-free water following Sigma-Aldrich recommendations), at different mass ratios (MRs) of AuNPs to siRNA, followed by 1 h incubation with slightly shaking at RT. The ability of AuNPs to complex siRNA was analysed by gel retardation. Briefly, complexes of AuNPs and siRNA (containing 0.25  $\mu\text{g}$  siRNA) at different MRs were loaded onto 1 % (w/v) agarose gels in Tris/Borate/EDTA (TBE) buffer containing SafeView (NBS Biologicals, UK). Electrophoresis was performed at 120 V for 30 min and the resulting gels were photographed under UV.

Particle sizes and zeta potentials of AuNP.siRNA complexes were measured as described in Section 2.4. DIW (0.2  $\mu\text{m}$  membrane-filtered) was added to the complexes and made up to 1 mL, before measurement. The concentration of siRNA was fixed at 1  $\mu\text{g mL}^{-1}$ .

## **2.9 Confocal Microscopy**

PC-3 cells (100,000 per well) were seeded in 6-well culture plates with glass bottoms (MatTek™). Cells were treated with 5  $\mu\text{g mL}^{-1}$  AuNPs-citrate, AuNPs-PEG-NHS, AuNPs-PEG-Tf and fluorescein-labelled AuNPs-PEG-Tf and incubated under the normal growth conditions for 24 h. Images were acquired on an Olympus FV 1000 microscope equipped

with differential interference contrast (DIC) and analysed using Olympus FV10-ASW software.

LNCaP cells (300,000 per well) were seeded in 6-well culture plates with glass bottoms. Cells were treated with AuNPs-PEI-FA formulations (MR20) containing 50 nM FAM siRNA (sense sequence 5'-UUC UCC GAA CGU GUC ACG U-3', modified by 6-FAM on 5' of sense sequence, prepared in RNase-free water following Sigma-Aldrich recommendations) and incubated under the normal growth conditions for 24 h. In order to label late endosomes and lysosomes, 75 nM LysoTracker® Deep Red (Molecular Probes, Invitrogen) were added to cells for 30 min at 37 °C. The medium was then replaced with fresh growth medium. Images were acquired on an Olympus FV 1000 microscope and analysed using Olympus FV10-ASW software.

## **2.10 Fluorescence activated cell sorting**

LNCaP (100,000 per well) and PC-3 (100,000 per well) cells were seeded in 24-well plates and incubated for 24 h under normal growth conditions. Cells were transfected by 25 and 50 nM FAM siRNA complexed with AuNPs-PEI (MR20) and AuNPs-PEI-FA (MR20) and incubated for 24 h in normal growth medium. Following incubation cells were washed twice with PBS and trypsinised. Cells were subsequently centrifuged (1,000 rpm for 5 min) and re-suspended in 1000 µL ice-cold PBS in Polystyrene Round-Bottom Tubes (Becton Dickinson). Ten thousands cells per sample were measured following the procedure outlined in Becton Dickinson FACScalibur manual. Fluorescein-positive cells (%) were displayed by Dot Plot.

## **2.11 *In vitro* gene silencing**

LNCaP cells (200,000 per well) were seeded in 12-well plates and incubated for 24 h under normal growth conditions. Cells were then transfected by the AuNPs-PEI-FA formulation

(MR20) containing 50 nM RelA siRNA (sense sequence 5'-CCA UCA ACU AUG AUG AGU U-3', prepared in RNase-free water; Integrated DNA Technologies, USA) 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.

Following incubation total RNA was isolated from the cells using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), according to the instructions supplied. First-strand cDNA was generated from total RNA samples using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Gene expression was assessed by real-time quantitative PCR (qPCR) using the Applied Biosystems Real Time PCR System (model 7300). Assays were performed using appropriate primers for RelA and GAPDH (TaqMan, Applied Biosystems). 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 [19].

## **2.12 Statistics**

The difference between two mean values was analysed using Student's *t* test. One-way analysis of variance (ANOVA) was used to compare multiple groups followed by Bonferroni's post hoc test. Statistical significance was set at  $*p < 0.05$ .

## **3. Results and discussion**

### **3.1 Synthesis of bioconjugated gold nanoparticles**

The starting materials AuNPs-citrate and AuNPs-PEI were synthesised in water using sodium citrate and ascorbic acid as reducing agents respectively [20, 21] (Fig. 1). The resulting AuNPs were characterised by UV-visible absorption spectroscopy, electron microscopy (TEM or SEM) and DLS (Fig. 2). For UV-visible measurements the AuNPs-PEI solution was diluted four times prior to measurement and readjusted to the same absorption as AuNPs-citrate. In Fig. 2A the red shift of the plasmon absorption maximum ( $\lambda_{\text{max}}$ ) from 526 nm to 560 nm was clearly observed; this shift was also accompanied with a further broadening of the absorbance band, which is usually attributed to higher oscillation modes (quadrupole, octupole absorption and scattering) that might also affect the extinction cross section with increasing size [22]. Therefore, the use of sodium citrate as both a reducing and stabilising agent at high temperature (80°C) allowed the production of AuNPs-citrate with diameter of ~ 33 nm (measured using TEM, Figs. 2B and 2D). In contrast, the use of ascorbic acid as a reducing agent for the reduction of HAuCl<sub>4</sub> in the presence of PEI as the stabilising agent led to the production of AuNPs with diameter of ~ 105 nm (measured using SEM, Figs. 2C and 2E). In addition, results in Fig. 2F show that AuNPs-citrate had a negative surface charge of ~ -45 mV and AuNP-PEI had a positive zeta potential of ~ +53 mV, indicating the high electrostatic stability afforded to the AuNPs by the citrate ions and PEI. The AuNPs-citrate and AuNPs-PEI are stable for more than one year when stored in the dark at 4°C.

For the synthesis of the AuNPs-PEG-Tf (Fig. 1) AuNPs-citrate were first conjugated with an ethylene glycol linker oligomer NHS-PEG<sub>280</sub>-S-S-PEG<sub>280</sub>-NHS with fourteen ethylene glycol units, a disulfide (-S-S-) and an N-hydroxysuccinimide (NHS) reactive ester. The PEG oligomer has a dual role as a stabilising agent and as a chemical linker to enable reaction with the amino groups of the protein in water [23-27], therefore leading to a stable and active colloidal solution. AuNPs-PEG-NHS showed a significant increase (~ 4 nm,  $p < 0.01$ ) in particle size (Fig. 3A) and a slight increase (~ 3 mV,  $p > 0.05$ ) in zeta potential (Fig. 3B)

when compared to AuNPs-citrate, indicating successful PEGylation. The addition of NHS-PEG<sub>280</sub>-S-S-PEG<sub>280</sub>-NHS resulted in the formation of an NHS functional PEG layer grafted on the AuNP surface, where NHS-PEG<sub>280</sub>-S is attached through a gold thiol (Au-S) bond and the NHS is available to the surface to react with the primary amine groups of the protein (Fig. 1). The Tf was attached on the PEG layer through formation of an amide linkage (NHCO) between the amino groups of the protein and the NHS of the AuNPs-PEG-NHS (Fig.1). Although DLS results showed no significant difference in particle sizes between AuNPs-PEG-NHS and AuNPs-PEG-Tf (Fig. 3A), the slower migration in gel electrophoresis indicated the successful attachment of Tf to AuNPs-PEG-NHS (Fig. S1).

The synthesis of AuNPs-PEI-FA (Fig. 1) was adapted from a method previously described [28], where fluorescein was attached onto gadolinium oxide NPs capped with PEI. In this work the amines of PEI are reacted with the NHS of activated folic acid. The resulting AuNPs-PEI-FA showed a significant increase in particle size ( $\sim 5$  nm,  $p < 0.01$ ) following attachment of folic acid-NHS onto AuNPs-PEI surface (Fig. 3C), while the zeta potential was found to decrease by  $\sim 7$  mV ( $p < 0.01$ ) (Fig. 3D). The attachment of folic acid onto AuNPs-PEI was further confirmed using UV-visible spectroscopy, which allowed the quantification of the folic acid-NHS left in solution after centrifugation of the AuNPs-PEI-FA; the results indicate that  $\sim 50$  % of the initial FA was grafted onto the AuNPs-PEI surface (Fig. S2).

### 3.2 Cytotoxicity

The cytotoxicity of the bioconjugated AuNPs was assessed in two human prostate cancer cell lines (LNCaP and PC-3) using an MTT assay (Fig. 4). In the LNCaP cell line, AuNPs-PEG-Tf, AuNPs-PEI and AuNPs-PEI-FA displayed IC<sub>50</sub> values of 103, 118 and 110  $\mu\text{g mL}^{-1}$  respectively. Similar data were also observed in PC-3 cells, with IC<sub>50</sub> values of 107, 135 and 120  $\mu\text{g mL}^{-1}$  for AuNPs-PEG-Tf, AuNPs-PEI and AuNPs-PEI-FA respectively. It

has been reported that toxicity of AuNPs is dependent on particle size, surface charge, morphology, surface chemistry, ligand types and the mechanisms of cellular uptake [29]. Previously, positively charged, surfactant-free AuNPs were synthesised in our laboratory by the reduction of AuCl<sub>3</sub> using hydroxylamine hydrochloride (NH<sub>2</sub>OH·HCl) in the presence of L-cysteine methyl ester hydrochloride [HSCH<sub>2</sub>CH(NH<sub>2</sub>)COOCH<sub>3</sub>·HCl] as a capping agent [30]. These L-cystein capped AuNPs demonstrated IC<sub>50</sub> values  $\approx$  7 to 9  $\mu\text{g mL}^{-1}$ . When L-cystein capped AuNPs were modified using PEG with different  $M_w$ , IC<sub>50</sub> values were slightly increased up to 15  $\mu\text{g mL}^{-1}$  [31]. Here we have demonstrated that the conjugation of Tf, FA or PEI ( $M_w$  2,000) onto the AuNPs may significantly improve biocompatibility relative to our previously synthesised L-cysteine capped AuNPs. Based on cytotoxicity data in Fig. 4, the concentrations of bioconjugated AuNPs used for subsequent *in vitro* studies ensured cell viability of > 80%.

### 3.3 Cellular uptake of transferrin-conjugated gold nanoparticles

Transferrin receptors (TfR), a transmembrane glycoprotein responsible for cellular iron transport, are found at low levels on the surface of many normal cell types; however, elevated expression has been reported in a wide range of cancer cells, and is often associated with poor prognosis [32]. Recently, Tf-targeted drug delivery systems have been investigated in cancer therapy [33] [34] [35]. In this study the cellular uptake of AuNPs-PEG-Tf using PC-3 cells (a human prostate cancer cell line known as overexpressing TfR [36]) was investigated.

AuNPs are a very useful contrast agent as they can be visualised using a large variety of techniques, including differential interference contrast (DIC) microscopy [37]. In this study, DIC images indicated that grafting of Tf facilitated the uptake of AuNPs into PC-3 cells relative to controls including untreated cells and cells treated with untargeted AuNPs-citrate and AuNPs-PEG-NHS (Fig. 5).

In addition, fluorescein-labelled AuNPs-PEG-Tf were synthesised via the interaction between the amino groups of Tf and the reactive  $-N=C=S$  groups of the fluorescein isothiocyanate isomer I, known to form a stable thiourea bond [38]. It is important to note that the fluorescein isothiocyanate was used at  $\sim 7.5$  equivalents, in order to ensure that fluorescein-labelled AuNPs-PEG-Tf have free transferrin to target TfR. When PC-3 cells were incubated with fluorescein-labelled AuNPs-PEG-Tf for 24 h, confocal microscopy images indicated a definite fraction of AuNPs-PEG-Tf (green) inside PC-3 cells (Fig. S3) further confirming the cellular uptake of AuNPs-PEG-Tf. Unlike cationic NPs, which bind to negatively charged cell surfaces, the negatively charged AuNPs-PEG-Tf ( $\sim -43$  mV) are unlikely to undergo non-specific cell uptake. In contrast, the uptake of these Tf-targeted NPs is most probably via receptor-specific uptake [39].

The anionic nature of the AuNPs-citrate ( $\sim -45$  mV), used to synthesise the Tf-targeted NPs was not influenced by the conjugation of PEG-NHS (AuNPs-PEG-NHS  $\sim -42$  mV) or further conjugation with Tf (AuNPs-PEG-Tf  $\sim -43$  mV). Due to the anionic nature of the AuNPs-PEG-Tf, negatively charged siRNA will not readily bind to the NPs. However, complexation of siRNA with protamine (a positively charged peptide) has been shown to produce complexes with an overall positive charge that can then interact electrostatically with negatively charged AuNPs [21]. The complexation of 'protamine.siRNA' using AuNPs-PEG-Tf and the subsequent biological activity of these formulations will be investigated in future studies.

### **3.4 Complexation of siRNA with folic acid-conjugated gold nanoparticles**

Folate receptors (FR) are known as a cellular membrane glycoprotein, consisting of three isoforms; being FR $\alpha$ , FR $\beta$  and FR $\gamma$  [40]. FR binds folic acid and structurally related folic acid derivatives and mediates cellular delivery of these compounds. It is now known that FR is

overexpressed on the vast majority of cancer tissues including prostate cancer, while its expression is limited in healthy tissues [40]. Targeted delivery via FR can be employed to increase the efficacy and reduce the toxicity of therapeutic agents for cancer therapy [35] [41]. In this study FA was chosen as a second targeting ligand, and the ability to attach FA to the AuNPs as a delivery vector for siRNA was investigated.

The formation of AuNPs (AuNPs-PEI and AuNPs-PEI-FA) and siRNA complexes via electrostatic interaction (positively charged amine groups on PEI and negatively charged siRNA) was examined by gel retardation (Fig. 6). The results indicate that complete complexation of siRNA with AuNPs-PEI took place from MR of 5 onwards (Fig. 6A); in contrast, the targeted NPs, AuNPs-PEI-FA did not achieve complete complexation of siRNA until MR of 20 onwards (Fig. 6B), suggesting that conjugation of folic acid onto AuNPs-PEI causes some degree of steric interference significantly reducing the siRNA binding capacity.

Using DLS at MR20, the diameter of AuNPs-PEI-FA.siRNA complexes in DIW was  $375 \pm 25$  nm (PDI = 0.4), which was significantly larger than that of the AuNPs-PEI-FA alone ( $118 \pm 1$  nm, PDI = 0.09) ( $p < 0.001$ ). In addition, the surface charge of AuNPs-PEI-FA complexes ( $\sim +20$  mV) was significantly lower than that of AuNPs-PEI-FA alone ( $\sim +46$  mV) ( $p < 0.001$ ). These results further confirm the successful complexation of siRNA by the targeted AuNPs-PEI-FA.

### **3.5 Receptor-mediated uptake of folic acid-conjugated nanoparticles**

LNCaP cells highly express the prostate-specific membrane antigen (PSMA) which exhibits folate hydrolase activity [42], while PC-3 cells do not express PSMA [43] or FR [44]. In this study, FAM siRNA was used to investigate the association and cellular uptake of AuNPs-PEI.siRNA (MR20) and AuNPs-PEI-FA.siRNA complexes (MR20) in both LNCaP (receptor positive) and PC-3 (receptor negative) cells. As shown in Fig. 7, AuNPs-PEI failed to induce

cellular association of siRNA in LNCaP or PC-3 cells, this is most likely due to the fact that lower  $M_w$  PEI (*i.e.* 600, 1,200 and 1,800 kDa) is known to display poor transfection efficiency [45]. In contrast, the results of flow cytometry displayed a significantly ( $p < 0.001$ ) greater cellular association of AuNPs-PEI-FA.siRNA formulation (up to 30%, at 50 nM siRNA) with LNCaP cells, while the number of fluorescein-positive PC-3 cells detected with the folic acid-targeted formulation was negligible (Fig. 7). These flow cytometry results imply that grafting of folic acid can enhance targeting of AuNPs to LNCaP cells through the FR-mediated pathway.

### **3.6 Intracellular trafficking of folic acid-conjugated nanoparticles**

PEI promotes endosomal/lysosomal escape of nucleic acids via the proton sponge effect by which the  $H^+$  buffering capacity of polyamines triggers endosomal  $Cl^-$  accumulation during acidification resulting in osmotic endosome swelling/disruption [46]. The intracellular trafficking of the AuNPs-PEI-FA.siRNA complex (MR20) was studied in LNCaP cells using FAM siRNA (Fig. 8). At 24 h incubation, FAM siRNA (green) was detected inside the cells and the absence of colocalisation with LysoTracker® Deep Red (red, a marker for late endosomes and lysosomes) (Fig. 8) suggests that the AuNPs-PEI-FA facilitated siRNA escape from the endolysosomes thus avoiding degradation of siRNA by endolysosomal enzymes [47] [48].

### **3.7 Endogenous gene silencing of folic acid-conjugated nanoparticles**

Nuclear factor  $\kappa$ -B (NF- $\kappa$ B) is a widely studied transcription factor found in many cancers including prostate cancer, it is associated with expression of a number of genes associated with cell proliferation, angiogenesis, metastases, tumour promotion and evasion of apoptosis [49]. NF- $\kappa$ B has five gene products: RelA, RelB, c-Rel, NF- $\kappa$ B1 and NF- $\kappa$ B2. The ability of AuNPs-PEI-FA to deliver functional siRNA was studied using RelA siRNA to silence RelA

expression in LNCaP cells [50]. After 24 h incubation, RelA siRNA formulated with AuNPs-PEI-FA (MR20) significantly downregulated RelA mRNA expression ( $p < 0.05$ ) compared to naked RelA siRNA and the Negative Control siRNA (non-silencing) formulated with AuNPs-PEI-FA (MR20) (Fig. 9), suggesting that silencing of RelA is related to RNAi activity rather than cytotoxicity.

It was noted however that the AuNP-PEI-FA.siRNA formulation (MR20) caused aggregation ( $> 1 \mu\text{m}$ ) when incubated within 10% FBS containing growth medium for 24 h (data not shown). This implies that following systemic administration non-specific binding of serum proteins onto AuNP formulations may occur causing particle aggregation and subsequent clearance by the mononuclear phagocytic system [MPS, also known as the reticuloendothelial system (RES)]. This phenomenon known as opsonisation results in a short *in vivo* half-life [51]. One of the most widely studied approaches to avoid RES uptake is the addition of a PEG moiety to form so called ‘stealth’ particles [52]. The conjugation of PEG (*i.e.* SH-PEG) onto the AuNPs-PEI-FA is chemically feasible and will simultaneously improve the *in vivo* stability [53] [54] while maintaining the ability to complex siRNA and to target receptor-positive prostate cancer cells.

#### 4. Conclusions

This proof of concept study has shown that the synthesis of gold nanoparticles tagged with two different ligands namely transferrin and folic acid is feasible. In both cases receptor-mediated cellular uptake occurred. The anionic AuNPs-PEG-Tf has potential to deliver cationic ‘protamine.siRNA’ to prostate cancer cells via TfR-specific uptake. The cationic AuNPs-PEI-FA were capable of complexing siRNA to form targeted NPs. This targeted formulation achieved FR-specific uptake of siRNA and facilitated successful gene

knockdown in prostate cancer cells without toxicity. These initial results highlight the potential to utilise AuNPs as targeted non-viral gene delivery vectors. Future modifications, including: 1) gold cores with different sizes; 2) PEG chains with various  $M_w$  and grafting density; and 3) varying the ratios of PEG and targeting ligands on the AuNPs, to optimise *in vivo* efficacy for prostate cancer therapy are possible.

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**Figure 1.** A schematic representation of (A) transferrin-conjugated AuNPs (AuNPs-PEG-Tf) and (B) folic acid-conjugated AuNPs (AuNPs-PEI-FA).

**Figure 2.** (A) UV-visible spectra of AuNPs-citrate and AuNPs-PEI; (B) TEM image of AuNPs-citrate (bar = 100 nm); (C) SEM image of AuNPs-PEI (bar = 5  $\mu$ m); (D) Size of AuNPs-citrate ( $33 \pm 5$  nm) and (E) size of AuNPs-PEI ( $105 \pm 15$  nm) analysed using TEM and SEM, respectively; (F) Zeta potentials of AuNPs-citrate ( $-45 \pm 2$  mV,  $n = 3$ ) and AuNPs-PEI ( $53 \pm 1$  mV,  $n = 3$ ) analysed using DLS.

**Figure 3.** Measurements of particle size and zeta potential of AuNPs using DLS. (A) Particle sizes of AuNPs-citrate ( $33 \pm 1$  nm, PDI = 0.2,  $n = 3$ ), AuNPs-PEG-NHS ( $37 \pm 1$  nm, PDI = 0.2,  $n = 3$ ) and AuNPs-PEG-Tf ( $38 \pm 1$  nm, PDI = 0.2,  $n = 3$ ); (B) Zeta potentials of AuNPs-citrate ( $-45 \pm 2$  mV,  $n = 3$ ), AuNPs-PEG-NHS ( $-42 \pm 1$  mV,  $n = 3$ ) and AuNPs-PEG-Tf ( $-43 \pm 2$  mV,  $n = 3$ ); (C) Particle sizes of AuNPs-PEI ( $113 \pm 1$  nm, PDI = 0.07,  $n = 3$ ) and AuNPs-PEI-FA ( $118 \pm 1$  nm, PDI = 0.09,  $n = 3$ ); (D) Zeta potentials of AuNPs-PEI ( $53 \pm 1$  mV,  $n = 3$ ) and AuNPs-PEI-FA ( $46 \pm 1$  mV,  $n = 3$ ). (The particle distributions shown as inserts in A and C)

**Figure 4.** Viability (MTT) of LNCaP and PC-3 cells treated with AuNPs (mean  $\pm$  SD,  $n = 3$ ).

**Figure 5.** Cellular uptake of Transferrin-conjugated AuNPs in PC-3 cells. PC-3 cells were treated with  $5\ \mu\text{g mL}^{-1}$  of AuNPs-citrate, AuNPs-PEG-NHS and AuNPs-PEG-Tf and incubated for 24 h. Images were acquired using transmitted light differential interference contrast (DIC) model of Olympus FV 1000 microscope (100 X).

**Figure 6.** Complexation of siRNA ( $0.25\ \mu\text{g}$ ) 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).

**Figure 7.** Fluorescein-positive PC-3 and LNCaP cells (% , mean  $\pm$  SD) after 24 h exposure to AuNPs-PEI-FA.FAM siRNA (MR20, 25 and 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)

**Figure 8.** A confocal microscope image showing the intracellular distribution of the AuNPs-PEI-FA formulation (MR20) containing FAM siRNA (50 nM) in LNCaP cells at 24 h post-exposure (40 X). Late endosomes and lysosomes were labelled with 75 nM LysoTracker® Deep Red (Green = FAM siRNA, Red = LysoTracker® Deep Red).

**Figure 9.** Endogenous gene silencing in LNCaP cells by RelA siRNA (50 nM) formulated with AuNPs-PEI-FA (MR20) after 24 h exposure. Naked RelA siRNA (50 nM) and Negative Control (non-silencing) 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  $\pm$  SD,  $n = 3$ )

## References:

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2015*. CA Cancer J Clin, 2015. **65**(1): p. 5-29.
2. Gao, Y., et al., *Nanotechnology-based intelligent drug design for cancer metastasis treatment*. Biotechnol Adv, 2014. **32**(4): p. 761-77.
3. Chen, Z.G., *Small-molecule delivery by nanoparticles for anticancer therapy*. Trends Mol Med, 2010. **16**(12): p. 594-602.
4. Xu, W., et al., *Aptamer-conjugated and doxorubicin-loaded unimolecular micelles for targeted therapy of prostate cancer*. Biomaterials, 2013. **34**(21): p. 5244-53.
5. Guo, J., J.C. Evans, and C.M. O'Driscoll, *Delivering RNAi therapeutics with non-viral technology: a promising strategy for prostate cancer?* Trends Mol Med, 2013. **19**(4): p. 250-61.

6. Wang, F., et al., *RGD peptide conjugated liposomal drug delivery system for enhance therapeutic efficacy in treating bone metastasis from prostate cancer*. J Control Release, 2014. **196**: p. 222-33.
7. Yallapu, M.M., et al., *Anti-cancer activity of curcumin loaded nanoparticles in prostate cancer*. Biomaterials, 2014. **35**(30): p. 8635-48.
8. Wittrup, A. and J. Lieberman, *Knocking down disease: a progress report on siRNA therapeutics*. Nat Rev Genet, 2015. **16**(9): p. 543-52.
9. Strumberg, D., et al., *Phase I clinical development of Atu027, a siRNA formulation targeting PKN3 in patients with advanced solid tumors*. International Journal of Clinical Pharmacology and Therapeutics, 2012. **50**(1): p. 76-78.
10. Tabernero, J., et al., *First-in-Humans Trial of an RNA Interference Therapeutic Targeting VEGF and KSP in Cancer Patients with Liver Involvement*. Cancer Discovery, 2013. **3**(4): p. 406-417.
11. Guo, J., et al., *Biomimetic nanoparticles for siRNA delivery in the treatment of leukaemia*. Biotechnology Advances, 2014. **32**(8): p. 1396-1409.
12. Ng, V.W.K., et al., *Gold: a versatile tool for in vivo imaging*. Journal of Materials Chemistry B, 2013. **1**(1): p. 9-25.
13. Jain, S., D.G. Hirst, and J.M. O'Sullivan, *Gold nanoparticles as novel agents for cancer therapy*. British Journal of Radiology, 2012. **85**(1010): p. 101-113.
14. Guo, J.F., et al., *Biomimetic gold nanocomplexes for gene knockdown: Will gold deliver dividends for small interfering RNA nanomedicines?* Nano Research, 2015. **8**(10): p. 3111-3140.
15. Fratoddi, I., et al., *Gold nanoparticles and gold nanoparticle-conjugates for delivery of therapeutic molecules. Progress and challenges*. Journal of Materials Chemistry B, 2014. **2**(27): p. 4204-4220.
16. Ji, X.H., et al., *Size control of gold nanocrystals in citrate reduction: The third role of citrate*. Journal of the American Chemical Society, 2007. **129**(45): p. 13939-13948.
17. Fitzgerald, K.A., et al., *The use of collagen-based scaffolds to simulate prostate cancer bone metastases with potential for evaluating delivery of nanoparticulate gene therapeutics*. Biomaterials, 2015. **66**: p. 53-66.
18. Guo, J.F., et al., *Anisamide-targeted cyclodextrin nanoparticles for siRNA delivery to prostate tumours in mice*. Biomaterials, 2012. **33**(31): p. 7775-7784.
19. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method*. Nat Protoc, 2008. **3**(6): p. 1101-8.
20. Rahme, K., et al., *PEGylated gold nanoparticles: polymer quantification as a function of PEG lengths and nanoparticle dimensions*. Rsc Advances, 2013. **3**(17): p. 6085-6094.
21. Rahme, K., et al., *Highly stable PEGylated gold nanoparticles in water: applications in biology and catalysis*. Rsc Advances, 2013. **3**(43): p. 21016-21024.
22. Link, S. and M.A. El-Sayed, *Shape and size dependence of radiative, non-radiative and photothermal properties of gold nanocrystals*. International Reviews in Physical Chemistry, 2000. **19**(3): p. 409-453.
23. Sperling, R.A. and W.J. Parak, *Surface modification, functionalization and bioconjugation of colloidal inorganic nanoparticles*. Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences, 2010. **368**(1915): p. 1333-1383.
24. Björn Jung and P. Theato, *Chemical Strategies for the Synthesis of Protein–Polymer Conjugates*. Advances in Polymer Science, 2013. **253**: p. 37–70.

25. Vasudevanpillai Bijuab, *Chemical modifications and bioconjugate reactions of nanomaterials for sensing, imaging, drug delivery and therapy*. Chemical Society Reviews, 2014. **43**(3): p. 737–962.
26. Martin Schäfflera, et al., *Blood protein coating of gold nanoparticles as potential tool for organ targeting*. Biomaterials, 2014. **35**(10): p. 3455-3466.
27. Pawan Kumar, et al., *Synthesis and conjugation of ZnO nanoparticles with bovine serum albumin for biological applications*. Applied Nanoscience 2013. **3**(2): p. 141–144.
28. Xu, W., et al., *Fluorescein-polyethyleneimine coated gadolinium oxide nanoparticles as T-1 magnetic resonance imaging (MRI)-cell labeling (CL) dual agents*. Rsc Advances, 2012. **2**(29): p. 10907-10915.
29. Fratoddi, I., et al., *How toxic are gold nanoparticles? The state-of-the-art*. Nano Research, 2015. **8**(6): p. 1771-1799.
30. Guo, J.F., et al., *Positively charged, surfactant-free gold nanoparticles for nucleic acid delivery*. Rsc Advances, 2015. **5**(23): p. 17862-17871.
31. Rahme, K., et al., *Evaluation of the physicochemical properties and the biocompatibility of polyethylene glycol-conjugated gold nanoparticles: A formulation strategy for siRNA delivery*. Colloids and Surfaces B: Biointerfaces, 2015. **135**(2015): p. 604-612.
32. Daniels, T.R., et al., *The transferrin receptor and the targeted delivery of therapeutic agents against cancer*. Biochim Biophys Acta, 2012. **1820**(3): p. 291-317.
33. Cui, Y., et al., *Transferrin-conjugated magnetic silica PLGA nanoparticles loaded with doxorubicin and paclitaxel for brain glioma treatment*. Biomaterials, 2013. **34**(33): p. 8511-20.
34. Chiu, R.Y.T., et al., *Improving the systemic drug delivery efficacy of nanoparticles using a transferrin variant for targeting*. Journal of Controlled Release, 2014. **180**: p. 33-41.
35. Wang, J., et al., *Biomolecules-conjugated nanomaterials for targeted cancer therapy*. Journal of Materials Chemistry B, 2014. **2**(48): p. 8452-8465.
36. Keer, H.N., et al., *Elevated transferrin receptor content in human prostate cancer cell lines assessed in vitro and in vivo*. J Urol, 1990. **143**(2): p. 381-5.
37. Boyer, D., et al., *Photothermal imaging of nanometer-sized metal particles among scatterers*. Science, 2002. **297**(5584): p. 1160-1163.
38. Hungerford, G., et al., *Effect of the labelling ratio on the photophysics of fluorescein isothiocyanate (FITC) conjugated to bovine serum albumin*. Photochemical & Photobiological Sciences, 2007. **6**(2): p. 152-158.
39. Guo, J., et al., *Therapeutic targeting in the silent era: advances in non-viral siRNA delivery*. Mol Biosyst, 2010. **6**(7): p. 1143-61.
40. Zhao, X., H. Li, and R.J. Lee, *Targeted drug delivery via folate receptors*. Expert Opin Drug Deliv, 2008. **5**(3): p. 309-19.
41. Scomparin, A., et al., *A comparative study of folate receptor-targeted doxorubicin delivery systems: Dosing regimens and therapeutic index*. Journal of Controlled Release, 2015. **208**: p. 106-120.
42. Hattori, Y. and Y. Maitani, *Enhanced in vitro DNA transfection efficiency by novel folate-linked nanoparticles in human prostate cancer and oral cancer*. J Control Release, 2004. **97**(1): p. 173-83.
43. Ghosh, A., et al., *Novel role of prostate-specific membrane antigen in suppressing prostate cancer invasiveness*. Cancer Res, 2005. **65**(3): p. 727-31.

44. Hattori, Y. and Y. Maitani, *Folate-linked nanoparticle-mediated suicide gene therapy in human prostate cancer and nasopharyngeal cancer with herpes simplex virus thymidine kinase*. *Cancer Gene Ther*, 2005. **12**(10): p. 796-809.
45. Godbey, W.T., K.K. Wu, and A.G. Mikos, *Size matters: Molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle*. *Journal of Biomedical Materials Research*, 1999. **45**(3): p. 268-275.
46. Varkouhi, A.K., et al., *Endosomal escape pathways for delivery of biologicals*. *J Control Release*, 2011. **151**(3): p. 220-8.
47. Guo, J.F., et al., *Systemic delivery of therapeutic small interfering RNA using a pH-triggered amphiphilic poly-L-lysine nanocarrier to suppress prostate cancer growth in mice*. *European Journal of Pharmaceutical Sciences*, 2012. **45**(5): p. 521-532.
48. Guo, J.F., et al., *Amphiphilic polyallylamine based polymeric micelles for siRNA delivery to the gastrointestinal tract: In vitro investigations*. *International Journal of Pharmaceutics*, 2013. **447**(1-2): p. 150-157.
49. Fitzgerald, K.A., et al., *The role of transcription factors in prostate cancer and potential for future RNA interference therapy*. *Expert Opin Ther Targets*, 2014. **18**(6): p. 633-49.
50. Evans, J.C., et al., *Cyclodextrin mediated delivery of NF-kappaB and SRF siRNA reduces the invasion potential of prostate cancer cells in vitro*. *Gene Ther*, 2015. **22**(10): p. 802-10.
51. Guo, J.F., et al., *Can non-viral technologies knockdown the barriers to siRNA delivery and achieve the next generation of cancer therapeutics?* *Biotechnology Advances*, 2011. **29**(4): p. 402-417.
52. Jokerst, J.V., et al., *Nanoparticle PEGylation for imaging and therapy*. *Nanomedicine (Lond)*, 2011. **6**(4): p. 715-28.
53. Geng, F., et al., *Pegylated glucose gold nanoparticles for improved in-vivo bio-distribution and enhanced radiotherapy on cervical cancer*. *J Biomed Nanotechnol*, 2014. **10**(7): p. 1205-16.
54. Xu, Q., et al., *Impact of Surface Polyethylene Glycol (PEG) Density on Biodegradable Nanoparticle Transport in Mucus ex Vivo and Distribution in Vivo*. *ACS Nano*, 2015. **9**(9): p. 9217-27.