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Dendrigraft Poly-L-lysine (d-PLL) Coated Gold Nanoparticles in Water for siRNA Delivery to Prostate Cancer Cells

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ABSTRACT

Herein we describe a simple method for the synthesis of dendrigraft poly-L-lysine (d-PLL) coated different sizes gold nanoparticles in water (AuNPs-d-PLL) as potential to delivery vehicles of siRNA to PC-3 prostate cancer cells. AuNPs-d-PLL with diameters ranging between 50−120 nm have been synthesised in aqueous solutions using d-PLL (7 KD) as a capping ligand, and ascorbic acid as a reducing agent. The size of the resulting AuNPs was found to depend on several parameters (*i.e.* the concentrations of the gold salt, ascorbic d-PLL and temperature). The obtained AuNPs-d-PLL were characterized using UV-visible spectroscopy (UV-vis), Scanning Electron Microscopy (SEM), and Dynamic Light Scattering (DLS). The ability to PEGylate the AuNPs-d-PLL with SH-PEG-OCH_3 and SH-PEG-Folate was demonstrated via DLS and zeta potential mesurements, their capacity to complex siRNA was verified (DLS, gel electrophoresis), and transfer of AuNPs-d-PLL-PEG-FA.siRNA to PC-3 cells was investigated.

Keywords: gold nanoparticles, polymers, stabilization, siRNA delivery, cancer.

1 INTRODUCTION

Prostate cancer is the second leading cause of men death, therefore cancer research diagnosis for early detection and treatment are of high importance [1]. siRNA is known to selectively down-regulate genes implicated in the pathology of a disease by inhibiting cancer cells prolifiration, suggesting their potential use in cancer treatments [2]. Unprotected siRNA may suffer from short plasma half-life due to their enzymatic degradation, renal clearance, as well as their electrostatic repulsion by the negatively charged phospholipidic cell membrane that might also lead to a very low uptake by cells [3, 4]. Therefore, using nanoparticles (NPs) for small siRNA delivery may be a good way to overcome these obstacles, and help in their shuttling to cells improving siRNA-based therapeutic agents in cancer treatment [4, 5]. In fact, it has been demonstrated that NPs-siRNA cargos not only shield naked siRNA from degradation, but also have the ability to be further modified with a targeting ligand that can specifically recognize a tumor cell [6, 7]. NPs capped with positively charged molecules, show an increase of interactions with cell membranes, enhancing their endocytosis [8,9]. Moreover, AuNPs, beside their particular size and shape dependent optical properties, proved a low cytotoxicity, and a high affinity to thiols and amines that facilitate their modification with biomolecules containig such groups (*i.e.* antibodies, proteins, and thiol terminated ligands) [10, 11]. AuNPs are now useful candidates in many biomedical application such as biomaging, photothermal therapy, and drug delivery [12]. For successful use in siRNA delivery, AuNPs should exhibit a positively charged surface in order to complex the negatively charged siRNA. We have previously demonstrated that non toxic positively charged AuNPs could be obtained using L-cysteine and polyethylenimine (PEI) as stabilizers [10, 13]. The latter (PEI) is now widely used in the synthesis of NPs and the delivery of siRNA, since it was shown that a decrease of the pH in cells allows PEI to become protonated, hence facilitating endosomal escape and destabilization of endosomal membranes [14-16].

Previously, we reported that AuNPs-PEI 2KD and AuNP-PEI 25KD could both succesfuly complex siRNA, and the latter (25 KD) only could deliver siRNA into PC3 cancer cells (~98 %) without further modification with a targeting ligand [9]. Moreover, we have also demonstrated that the very low uptake of AuNPs-PEI 2KD could be enhanced by modification with a targeting ligand (such as Anisic Acid (AA) or Folic acid (FA)) [10,14]. Au-PEI-AA mediated

efficient siRNA uptake into PC3 prostate cancer cells via binding to the sigma receptor, and resulted in highly efficient knockdown of the RelA gene (B70%) when cells were transfected in serum-free medium [14]. Similarly, the AuNPs-PEI-FA.siRNA specifically delivered 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, and produced an enhanced endogenous gene silencing compared to the non-targeted AuNPs-PEI [10]. However, we have noticed that AuNPs-PEI-FA size increased by threefold after siRNA complexation, mainly due to some flocculation upon siRNA addition. Therefore, we believe that PEGylation might be of interest to enhance their stability.

In this study, we used dendrigraft poly-L-lysine (d-PLL) as a stabilising ligand for gold AuNPs in water. The d-PLL generation two (P_2) with narrow molecular weight distribution of about 7 KD was obtained by a previously reported method [17]. AuNPs-d-PLL with diameters ranging between 50–120 nm were obtained by chemical reduction of tetrachloroauric acid ($HAuCl_4$) with $_L$-ascorbic acid in the presence of d-PLL. They were further modified with SH-PEG_{5000}-OCH_3 (Mw 5400 $g.mol^{-1}$) or SH-PEG_{5000}-FA. Succesful PEGyltion was confirmed by DLS and zeta potential measurements. The resulting PEGylated AuNPs-d-PLL were shown to complex siRNA, and folate modified ones could deliver siRNA to PC-3 cells.

2 PREPARATION AND CHARACTERIZATION D-PLL CAPPED GOLD NANOPARTICLES IN WATER

AuNP-d-PLL with different diameters (50–120 nm) were obtained by chemical reduction of $HAuCl_4.3H_2O$ with ascorbic acid in the presence of d-PLL. The resulting AuNP-d-PLL were fully characterized using UV-vis, SEM, and DLS. Figure 1 represents the UV-vis spectra of selected AuNPs-d-PLL synthesised at room temperature. The effect of different concentrations of $HAuCl_4.3H_2O$, while fixing the concentration of d-PLL (6 μM) and Ascobic acid excess to three on the final AuNPs is evaluated. It is clearly seen from UV-vis (spectra were recorded at same dilution *i.e* 0.25 mM) that the variation in $HAuCl_4$ concentration affect the position of the palsmon resonance band of the resulting AuNPs, whith a band shift of about 64 nm from 544 nm to 608 nm when the concentration of $HAuCl_4$ was increased from 0.25 mM to 1 mM. The plasmon shift was also accompanied with a band broadening, as well as a decrease in the absorption band intensity, and an increase in the baseline mainly due to light scattering by larger AuNPs [11, 13]. The colour change of the colloidal AuNPs solution was also recorded. We can conclude that the AuNPs-d-PLL offer size dependent optical properties with a plasmon band shift to longer wavelengths related to an increase in the size of the AuNPs with increasing $HAuCl_4$ concentration as verified by DLS measurements (Figure 2). Similarly, the variation of the excess of ascorbic acid from 1 to 5, and the increase in temperature were translated into a blue shift and a decrease in the AuNPs size (data not shown).

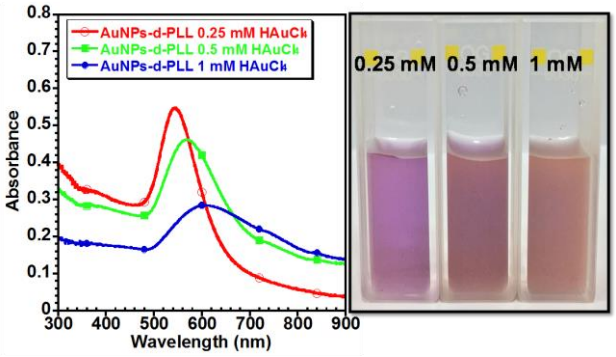


Figure 1: UV-vis spectra of AuNPs-d-PLL obtained with 0.25, 0.5 and 1 mM $HAuCl_4$ (spectra were recorded at same dilution 0.25 mM). Right, as-obtained colloidal solution.

Figure 2 shows the size distribution by intensity from DLS analysis on AuNPs-d-PLL colloidal solutions of figure 1.

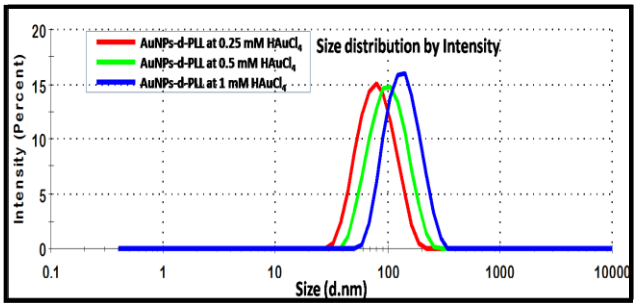


Figure 2: size distribution by intensity on AuNPs-d-PLL obtained with 0.25, 0.5 and 1 mM $HAuCl_4$ (spectra were recorded at same dilution *i.e* 0.25 mM). Curves demonstrate an increase in the NPs sizes with $HAuCl_4$ concentrations.

The hydrodynamic diameter (Dh) obtained from DLS measurements on AuNPs-d-PLL indicated that all samples were nearly monodispersed with one size distribution and a polydispersity index (PDI) varying from 0.18, 0.14 and 0.09 for 0.25, 0.5 and 1 mM $HAuCL_4$, respectively [15]. Moreover, it is clearly seen that Dh increases from 85 ± 2 nm for 0.25 mM $HAuCL_4$ to 139 ± 2 nm when 1 mM $HAuCL_4$ was used, further confirming that the size of the AuNPs-d-PLL increase with gold salt precursor concentration. We should note that the size distribution by intensity from DLS was also found to be much larger than the AuNPs core size measured by SEM (data not shown), indicating that the AuNPs were successfully coated with d-PLL. Zeta potential measurements have shown that AuNPs-d-PLL obtained in this study were positively charged with a zeta potential (ζ) of + 25, + 31 and + 40 mV for 0.25, 0.5 and 1 mM $HAuCL_4$ respectively due to d-PLL attachment.

Furthermore, the resulting colloidal solutions of AuNPs-d-PLL were stable for several months when stored at 4 °C.

3 SYNTHESIS OF SH-PEG5000-FA, PEGYLATION AUNPS-D-PLL AND COMPLEXATION OF SIRNA

The carboxyl group of folic acid was first activated with N-hydroxysuccinimde via dicyclohexylcarbodiimide (DCC) coupling to obtain Folate N-hydroxysuccinimidyl ester [10]. Thiolated polyethylene glycol folate (SH-PEG_{5000}-FA) was synthesised by coupling (amide linkage) between SH-PEG-NH_2 (Mn 5,000) and Folate N-hydroxysuccinimidyl ester [18]. The obtained Folic-NHS ester and SH-PEG_{5000}-FA were characterized by FTIR (data not shown) and the latter was used to conjugate the positively charged AuNPs-d-PLL. PEGylation was performed by adding dropwise a certain volume of a solution of SH-PEG-OCH_3 (185 µmol L^{-1}) and SH-PEG-FA (185 µmol L^{-1}) under stirring. Stirring was maintained for few hours (1 to 24 hours), and the final colloidal solutions were purified and further concentrated (500 µg mL^{-1}) by centrifugation. The ability of PEGylated AuNPs-d-PLL to complex fluorescent siRNA (Mw 13,800 g mol^{-1}) was also tested. The AuNPs-d-PLL-PEG-OCH_3 and AuNPs-d-PLL-PEG-FA were incubated with siRNA for 1 hour with constant shaking. The results from DLS and Zeta potential measurements of PEGylation and siRNA complexation are shown in Figure 3.

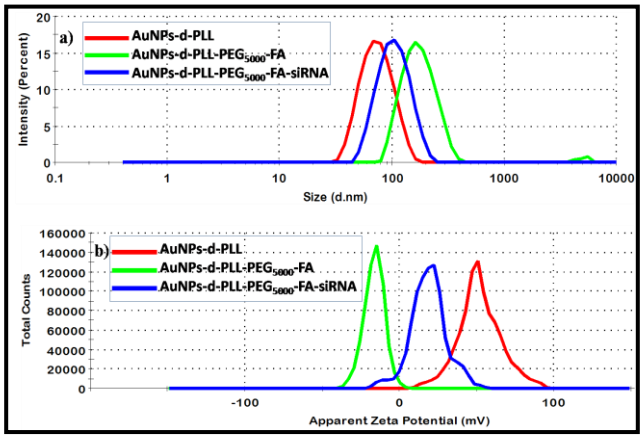


Figure 3: a) size distribution by intensity of AuNP-d-PLL from DLS measurements, showing the size increase from 79 nm to 112 nm, and to 180 nm after SH-PEG-FA attachment and siRNA complexation, respectivly. (b) Zeta potential (ζ) for the corresponding colloidal solutions decreases from +33 mV to -16 mV indicating succesful PEGylation and siRNA complexation.

Figure 3a show the size distribution by intensity from DLS measurements on AuNPs-d-PLL before and after PEGylation with SH-PEG_{5000}-FA, and complexation with siRNA. It is clearly seen that the AuNPs-d-PLL size increased by about 30 nm (from 79 ± 3 nm to 112 ± 8 nm) after conjugation with SH-PEG_{5000}-FA, while after 1 hour soaking with siRNA, the corresponding AuNPs-d-PLL-SH-PG_{5000}-FA increased by about 50 nm (from 112 ± 8 nm to 180 ± 10 nm), conserving their polydispersity index (PDI 0.18 ± 0.03) [3, 4]. Furthermore, the zeta potential of the colloidal solution was found to decrease from 33 ± 6 mV for AuNPs-d-PLL to +18 ± 3 mV after SH-PEG-FA attachment, and to about -16 ± 1 mV after soaking with siRNA as clearly seen in Figure 3b. These results demonstrate the successful PEG attachment and complexes formation of AuNPs-d-PLL-SH-PG_{5000}-FA.siRNA. AuNPs-PEG.siRNA complex formation was also demonstrated by gel electrophoresis and found to be enhanced when the AuNPs-PEG size increases (data not shown). We should note here that similar results were also observed when SH-PEG-OCH_3 (Mw 5400 g mol^{-1}) were implicated.

4 APPLICATION OF AUNPS-D-PLL-PEG-FA IN SIRNA DELIVERY

Cellular uptake was also performed on PC3-PSMA cancer cells following 24 h incubation. Uptake studies shown in Figure 4 indicated that AuNPs-d-PLL-PEG-FA could achieve significantly higher fluorescein-positive cells (~ 30%) relative to that of AuNPs-d-PLL-PEG-OCH_3 (~ 3%), suggesting that it could deliver siRNA into cells. We should also note that the cellular uptake was also verified using laser scaning microscopy (data not shown).

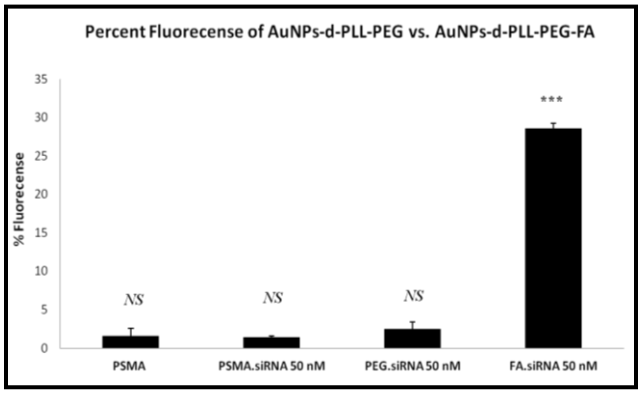


Figure 4: Figure 4: Fluorescein-positive PC-3 cells (%, mean ± SD) Cells were transfected by 50 nM 6-FAM siRNA complexed with AuNP-d-PLL-PEG-FA or AuNPs-d-PLL-PEG-OCH_3 (MR25) and incubated for 24 h in normal growth medium. After 24 h transfection, 5×10^4 cells per sample were measured using Partec Cube 8 Flow Cytometer and analyzed using FlowJo.

5 MATERIALS AND METHODS

4.1 Chemicals and Materials:

Purified H_2O (resistivity ≈ 18.2 MΩ cm) was used as a solvent for AuNPs synthesis. All glassware were cleaned

with aqua regia (concentrated HCl/concentrated HNO_3 3/1), rinsed with distilled water, ethanol and acetone, and oven-dried prior to use. Tetrachloroauric acid trihydrate ($HAuCl_4.3H_2O$), $_L$-ascorbic acid ($C_6H_8O_6$) MISSION® siRNA Universal Negative Control #1 6-FAM (M_W = 13 .853 KD), Folic Acid, N-hydroxysuccinimde (NHS) dicyclohexylcarbodiimide (DCC), Thiol-PEG-Amine (Mn 5000) were purchased from Sigma-Aldrich. Thiol terminated poly(ethylene glycol) methyl ether, M_w = 5400 g mol^{-1} was purchased from Polymer Source. Poly($_L$-lysine) dendrigraft (PLL M_w = 7 KD) was synthesized and characterized and previously reported [14].

4.2 Preparation of AuNPs-d-PLL

In a 50 mL round flask containing 24.82 mL aqueous solution $HAuCl_4$ (1 mM) and PLL (6 μM), 0.188 mL of ascorbic acid (0.1 M) as reducing agent. After addition of ascorbic acid the colour of the solution change from pale yellow to red violet. The solution was kept under stirring overnight. The obtained nanoparticles have a hydrodynamic diameter of about 85 ± 2 nm (size by intensity) and a zeta potential +25 ± 0.2 mV. Bigger AuNPs were obtained by fixing the d-PLL concentration at 6 μM and doubling both $HAuCl_4$ concentration (*i.e* 0.5 mM and 1 mM) and ascorbic acid (1.5 mM and 3 mM).

4.3 PEGylation of AuNPs-dPLL

To 12.5 mL of AuNPs-d-PLL (50 μg mL^{-1}) 0.1 mL of SH-PEG_{5000}-OCH_3 (185μM) or 0.1mL of SH-PEG_{5000}-FA (185μM) M_W =5400) were added dropwise (FA sample in dark). Samples were then left under stirring for few hours. DLS measurement confirmed succesful PEGylation. Samples were purified by centrifugation at 8500 rpm for 10 mins, the supernatant was discarded, and the particles were concentrated ten times for siRNA complexation.

4.3 Preparation of AuNPs-d-PLL-PEG.siRNA complexes

AuNPs-d-PLL-PEG (500 μg mL^{-1}) were added to the siRNA solutions at different mass ratios (MRs) of AuNPs to siRNA, followed by 1 h incubation in dark with slow shaking at RT. The ability of AuNPs-d-PLL-PEG to complex siRNA was confirmed by DLS and gel retardation.

4.4 Fluorescence activated cell sorting

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 fluorescent siRNA complexed with AuNPs-D-PLL-PEG-FA and (PEG-OCH_3) (MR25) and incubated for 24 h in 300 μL 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 FACS Buffer in cuvettes labeled accordingly on ice. Gates were set at 50,000 cells per sample, and siRNA-FITC fluorescence was detected using Partec Cube 8 Flow Cytometer and analyzed using FlowJo.

6 INSTRUMENTATION

Optical spectra were obtained on a UV/Vis Analytikjena SPECORD® 250 PLUS spectrophotometer (300−900-nm range, 0.5 nm resolution).

Dynamic Light Scattering (DLS) and Zeta Potential: Measurements were carried out with the Malvern instrument (Zeta sizer Nano series) at 25°C. Measurements on each sample were performed in triplicate.

FACS Analysis was performed using a Partec Cube 8 Flow Cytometer, and data were analyzed using FlowJo.

7 CONCLUSION

Different sizes of AuNPs-d-PLL were synthesized in water and characterized, using UV-vis, DLS and Zeta potentilal. The $HAuCl_4$ concentration was found to affect the final AuNPs size. AuNPs-d-PLL were PEGylated with SH-PEG-OCH3 and SH-PEG-Folate, and were shown to complex siRNA without any sign of aggregation. Finally, AuNP-d-PLL-PEG-FA showed a successful transfer of siRNA into PC-3-PSMA cells paving their use as potential shuttle agents in siRNA/drug delivery and diagnostic applications.

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