A Simple Synthesis of Polymer Coated Gold and Silver Nanoparticles in Water for Potential Use in Biomedical Applications

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ABSTRACT

In this study Silver (Ag) and Gold (Au) nanoparticles (NPs) of different surface charges (positively charged, negatively charged, or nearly neutral) and sizes were obtained using ascorbic acid as a reducing agent and multiple polymers such as Chitosan (Chit), Poly-L-Lysine (PLL), Polyvinyl alcohol (PVA), Polyethylene glycol (PEG) or sodium citrate as stabilizing ligands. The obtained NPs were characterized using UV-visible spectroscopy (UV-vis), Dynamic Light Scattering (DLS), and Zeta Potential (ζ) measurements. On the other hand, AuNPs with different coating (PLL, PVA and PEG) and charges were tested on whole human blood samples, specifically on neutrophils using a Neutrophil Function Test (NFT). Our results demonstrated that AuNPs-PVA with a slight negative charge (ζ = -15 mV) and PEgylated AuNPs-PLL (AuNPs-PLL-PEG) with a slight positive charge (ζ = + 9 mV) were found to be the least detected by neutrophils making them suitable for biomedical applications.

Keywords: nanoparticles, cancer, cytotoxixity, polymers, neutrophils.

1 INTRODUCTION

Engineered nanoparticles with multiple properties have been widely used during the last few decades in sevral biomedical applications (*i.e.* imaging, sensing, drug delivry and therapy) [1]. Moreover, gold (Au) and silver (Ag) nanoparticles (NPs) have recently gained considerable attention for a wide range of applications in theranostics [2]. AuNPs were known to possess size and shape dependent optical properties due to surface plasmon resonance band, a low cytotoxicity and a high affinity to biomolecules containing thiols and amino groups, facilitating their wide use in diagnostics, thermal therapy and drug/gene delivery [3, 4]. AgNPs also offer size and shape dependent optical properties, but they suffer from the release of silver ions if oxidizing species (such as molecular oxygen or hydrogen peroxide) are present. Therefore, AgNPs are well known to be extensively used in food consumer products industry and in medicine due to their antibacterial action [5]. Many studies on testing NPs delivery to cells use *in vitro* and *in vivo* cytotoxicity assays [6]. For effective *in vivo* applications, NPs are usually coated with Polyethylene glycol (PEG) which is known to offer a stealth character and long circulation time in the blood compartments [7]. NPs should also to be very stable, functionalized and well characterized in order to ensure optimum trafficking to the targeted site [8]. It has been shown that NPs' physicochemical characteristics, mainly size and charge, modulate their biodistribution, cytotoxicity, and immunorecognition as reported from *in vitro* and *in vivo* studies [9]. To date, safety concern is elevated due to the possible cytotoxicity and immunogenicity that the charged nanoparticles may display. However, most studies fail to assess the immunogenicity of the engineered NPs under normal physiological conditions. Such assessment is necessary to examine how players of the immune system, particularly phagocytes (neutrophils and macrophages), which form the first line of defense that encounter and interact with NPs [10, 11]. Therefore, a growing interest in nanotoxicology is lately emerging mainly by studying the interaction of a given NP with isolated polymorphonuclear cells (PMNs) [12, 13]. Whilst data from *in vitro* studies could be biased by several factors including activation of cells upon isolation and lack of sera proteins in the microenvironment of primary generated cell lines, *in vivo* studies are costly, time consuming, and require ethics consideration.

Here, we developed a simple and novel *in vivo-like* method to test for NP-immunorecognition from a freshly withdrawn human blood sample. Briefly, we synthesized highly stable Ag and AuNPs in water, using sodium citrate and multiple polymers such as chitosan, (Chit) Poly-$_L$-Lysine (PLL), Polyethylene glycol (PEG) and Polyvinyl alcohol (PVA) as stabilizing agents and ascobic acid as a reducing agent. The obtained nanoparticles were

characterized using Uv-vis, DLS, and ζ measurements. The AuNPs with different coating/charges (PLL, PVP and PEG) were tested on whole human blood samples, specifically on neutrophils using neutrophil function test (NFT), to determine whether differently charged AuNPs might result in a various activation efficiency of neutrophils. Neutrophil activation was tracked using Nitrotetrazolium Blue (NBT) dye reduction and formazan granule formation upon ROS release [10].

2 SYNTHESIS AND CHARACTERIZATION OF NANOPARTICLES IN WATER

AgNPs-citrate with a diameter about 74 ± 0.6 nm (Z_{av} from DLS measurements) were synthesised by chemical reduction of silver nitrate ($AgNO_3$) in the presence of sodium citrate as stabiliser and ascobic acid as a reducing agent at 70 °C for 50 mins. In fact, we first have tested the reduction of $AgNO_3$ by sodium citrate at 70 °C, however no colour change was observed in the first few hours, and a heating overnight was required to partially reduce the $AgNO_3$ confirming the role of citrate as stabilising agent only. UV-vis spectroscopy on the obtained AgNPs-Citrate colloidal solution show a single absorption band centered round 420 nm, this is the plasmon resonance band of silver nanoparticles. DLS analysis show that the AgNPs-citrate were nearly monodispersed with one size distribution and a polydispersity index (PdI) about 0.15, and a ζ of -25 ± 3 mV. To further improve the stability of AgNPs-citrate, Pluronic® F127 a tribloc copolymer PEO-PPO-PEO was added to the colloidal solution. The latter was found to succesfully adsorb onto AgNPs surface (UV-vis spectra shown in figure 1), therefore increasing their Zav by about 15 ± 3 nm and decreasing their ζ to about -8 ± 2 mV. However, AgNPs were not used here for biological tests.

On the other hand, AuNPs with different capping polymers were obtained by an in situ reduction of $HAuCl_4.3H_2O$ in the presence of polymers, and ascorbic acid at room temperature. Mainly PVA, Chit and PLL were used as capping polymers. The PVA and Chit were commercially available from sigma aldrich while the PLL was a synthetic dendrigraft polymer of generation two with a molecular weight of 7 KD [14]. The obtained AuNPs were also charcterized by UV-vis DLS, Zeta potential measurements and scanning electron microscopy (SEM). Figure 1 shows the UV-vis spectra of AuNPs capped with the above stated polymers as synthesized in this study. It is clearly seen from UV-vis that AuNPs possessed a single absorption band centered around 524 nm for AuNPs-PVA and 526 nm for AuNPs-PLL, indicating that they were very close in size (30 ± 5 nm) as confirmed from SEM analysis (data not shown). However, AuNPs-Chit showed a band shifted to higher wavelength (549 nm) indicating that they were bigger in size [15]. Therefore, in the continuity of this study the AuNPs-PVA and AuNPs-PLL will be further chracterized and compared in biological assay.

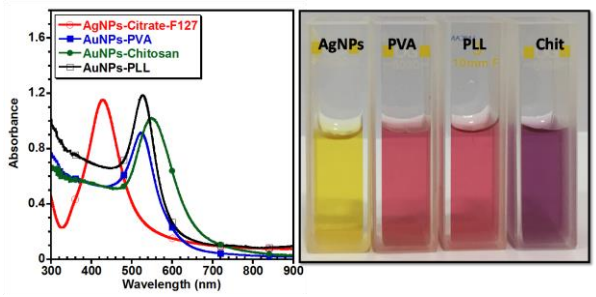

Figure 1: UV-vis spectra of Ag and AuNPs samples with different sizes/coating ligands depict a plasmon band shift due to their size/element-dependent optical properties.

The AuNPs-PLL were further coated with thiolated polyethylene glycol ($SH\text{-}PEG\text{-}OCH_3$, Mw 5400 g mol^{-1}). The high affinity of SH group to Au is known to displace ligands from AuNPs surface (ligand exchange) via a stronger S-Au bond formation [16]. Successful PEGylation of AuNPs-PLL with thiolated PEG was confirmed by DLS and ζ measurements. Figure 2 shows the size distribution by intensity of the AuNPs-PVA, AuNPs-PLL and AuNPs-PLL-PEG.

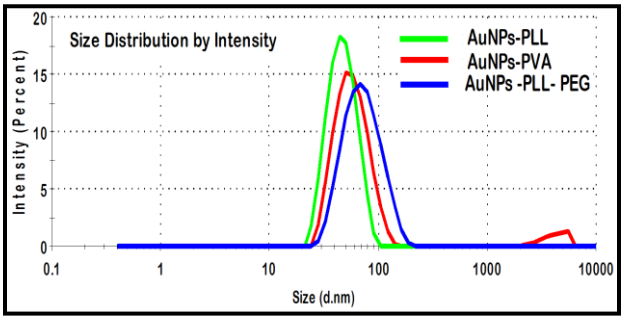

Figure 2: size distribution by intensity of AuNP-PVA AuNPs-PLL and AuNPs-PLL-PEG from DLS. AuNPs-PLL size increases from 47 nm to 75 nm after PEGylation.

DLS analysis proved that all the AuNPs colloidal solutions were nearly monodispersed with a single size distribution and a PDI varying from 0.115 ± 0.003 for AuNPs-PLL with a size by intensity of 47.5 ± 0.3 nm, to 0.288 ± 0.011 with a size 59 ± 6 nm for AuNPs-PVA. We noticed that PVA coated nanoparticles had a diameter and PDI slightly larger than AuNPs-PLL, indeed their core sizes were not very different by SEM. This is not surprising as the PVA molecular weight used in this study was larger, and more polydisperse (13 to 30 KD) than PLL (7KD). As shown in figure 2 PEGylation of the AuNPs-PLL caused an increase in the diameter by about 28 nm (75.26 ± 0.26 nm). The successful PEGylation of AuNPs-PLL was further proven by zeta potential analysis. Figure 3 represents the ζ

potential measurements on AuNPs-PVA, AuNPs-PLL and AuNPs-PVA-PEG.

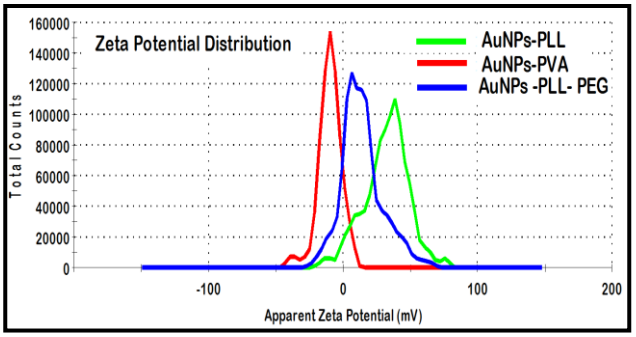

Figure 3: zeta potential for the AuNPs-Polymers used in this study for NFT test. As shown AuNPs-PVA were slighltly negative (-10 mV), while PEGylation decreased the zeta potential of the positively charged AuNPs-PLL from +34 mV to +12 mV.

It is clearly observed that AuNPs-PVA pocess a ζ of - 9.72 ± 1.33 mV, while the AuNP-PLL showed a positive ζ of +33.8 ± 2.5 mV indicating that they were successfully coated with polymers. However, PEGylation of the AuNPs-PLL was shown to decrease the zeta potential by about 21 mV (+12 ± 3 mV) indicating attachment of PEG onto the AuNPs-PLL.

3 NEUTROPHIL FUNCTION TEST (NFT) AND MICROSCOPIC EXAMINATION

On the other hand, immunogenicity of the above synthesised AuNPs with different coats (PLL, PVA and PEG) were tested on whole human blood samples, specifically on neutrophils using NFT [10]. We assumed that such an environment mimics *in vivo* physiological conditions, rather than working with isolated neutrophils. NFT test is used in this study to determine whether differently charged AuNPs might result in variant activation efficiency of neutrophils. Neutrophil activation was tracked using Nitrotetrazolium Blue (NBT) dye reduction and formazan granule formation upon reactive oxygen species release [10]. As a positive control, phorbol myristate acetate (PMA) was used as stimulant. Interestingly, as shown in Figure 4, AuNPs-PVA with a slight negative charge (ξ = -10 mV) and PEGylate AuNPs (AuNPs-PLL-PEG) with a slight positive charge (ξ = +12 mV) were found to be the least detected by neutrophils. However, positively charged AuNPs-PLL (ξ = +34 mV) induced a significant activation of ~70 % of the neutrophil pool relative to PBS (negative control). Furthermore, we should note that AuNPs-PLL resulted in increasing cell size population that was detected by flow cytometry. Finally, cytotoxicity assay of positively charged AuNPs-PLL was performed on PC3 cell lines, and did not show any signs of toxicity (data not shown).

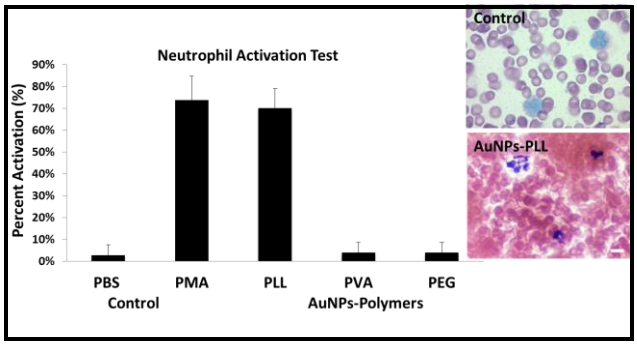

Figure 4: Neutrophil activation by positively charged AuNPs-PLL showed similar activation efficacy of neutrophils as compared to the control stimulant PMA with ~70% of neutrophil pool forming formazan deposits. PVA and PEG coated NPs only exhibited less than 5 % activation level with the majority of neutrophils being inactive (n=4). On the right (upper panel) a micrograph showing a negative control with two non-activated neutrophils, (lower panel) shows three activated neutrophils upon addition of AuNPs-PLL (scale bar 10 μm).

4 MATERIALS AND METHODS

4.1 Chemicals and Materials:

Purified H_2O (resistivity ≈18.2 MΩ.cm) was used as solvent. All glassware was cleaned with aqua regia (3 parts of concentrated HCl and 1 part of concentrated HNO_3), rinsed with distilled water, ethanol, and acetone and oven-dried before use. Silver nitrate ($AgNO_3$), Tetrachloroauric acid trihydrate ($HAuCl_4$, $3H_2O$), $_L$-Ascorbic Acid, Phorbolmyristate acetate (PMA), Nitrotetrazolium Blue (NBT), Chitosan (Chit), Poly (vinyl alcohol) (PVA), thiolated Pyridine, were purchased from Sigma Aldrich. Thiol terminated poly(ethylene glycol) methyl ether, M_w = 5400 was purchased from Polymer Source. Poly($_L$-lysine) dendrigraft (PLL M_w = 7 KD) was synthesized and characterized and previously reported [14]. Red Blood Cell Lysis Buffer was purchased from Partec. All chemicals were used as received without further purification.

4.2 Preparation of Ag and AuNPs-Polymers:

Synthesis of AgNPs-Citrate-F127: In a three neck round flask 50 mL of 3 mM sodium citrate was heated. When the temperature reached 70°C, 0.25 mL $AgNO_3$ (50 mM) were added into the solution, followed by the fast injection 0.5 mL of freshly prepared ascorbic acid (100 mM). The color of the solution instantly changes from colorless to yellowish orange upon ascorbic acid injection. The reaction was kept at 70 °C for around 50 minutes. The DLS results shows an average size of 74 ± 0.6 nm with a PDI = 0.151 and zeta potential of about -28 mV. While the UV-vis spectrum show an absorption band with maximum wavelength (λ_{max}) centered at 419 nm. The obtained AgNPs-Citrate were

coated with F127 a tribloc copolymer (PEO-PPO-PPO) by simply adding few micrL of F127 (0.8 mM) and stirring overnight. The succesful adsorption of F127 onto AgNPs-Citrate lead to a slight red shift of few nm (3 to 5 nm) in UV-vis, an increase in the size by DLS (~15 nm) with a decrease in the zeta potential from -28 mV to -10 mV.

Synthesis of AuNPs-Chitosan: To a 25 mL solution of $HAuCl_4$ (0.25 mM) and 0.1 % (w/v) of Chitosan 0.097 mL of ascorbic acid (0.1 M) as a reducing agent was added. After addition of ascorbic acid the colour of the solution changed from pale yellow to red. The solution was kept under stirring for 23 hours. The obtained nanoparticles have a hydronamic diameter of about 121 ± 2 nm (size by intensity) and a zeta potential +46 ± 1 mV.

Synthesis of AuNPs-PLL: In a 50 mL round flask containing 22.07 mL of deionized water, 0.336 mL $HAuCl_4$ (18.6 mM) was added under stiring, followed by the fast addition of 2.5 mL of PLL (100 µM) stabilising ligand and 0.097 mL of ascorbic acid (0.1 M) as a reducing agent. After addition of ascorbic acid the colour of the solution changed from pale yellow to red. The solution was kept under stirring for 24 hours. The obtained nanoparticles have a hydronamic diameter of about 47.5 ± 0.3 nm (size by intensity) and a zeta potential +33 ± 2.5 mV.

Synthesis of AuNPs-PVA: In a 50 mL round flask containing 24.07 mL of deionized water, 0.336 mL $HAuCl_4$ (18.6 mM) was added under stiring, followed by the fast addition of 0.5 mL of PVA (0.091 % w/v) stabilising ligand and 0.097 mL of ascorbic acid (0.1 M) as a reducing agent. After addition of ascorbic acid the colour of the solution changed from pale yellow to deep red. The solution was kept under stirring for 24 hours. The obtained nanoparticles had a hydronamic diameter of about 59.0 ± 6.0 nm (size by intensity) and a zeta potential -9 ± 1 mV.

Synthesis of AuNPs-PLL-PEG: To 5 mL of AuNPs-PLL colloidal solution under stirring, 0.27 mL of SH-PEG5000 (111 µM) was added drop wise during 3 minutes, the solution was kept under stirring for two hours. The succesful PEGylation was confirmed by DLS were the size of the AuNPs-PLL was found to increse from 47.5 ± 0.3 nm (size by intensity) to 75.26 ± 0.76 nm while the zeta potential was found to decrease from +33 ± 2.5 mV + 12.0 ± 3 mV confirming the attachment of PEG onto AuNPs-PLL surface.

4.3 *Neutrophil Function Test and Microscopic examination*

1 mL of whole human blood was withdrawn via intravenous injection and stored in EDTA tubes. 100 µL of blood was transferred to an eppendorf tube containing 50 µL of 1 mg/mL NBT (Nitrotetrazolium Blue), and 50 µL of differently charged AuNPs (50 µg/mL) or with PMA as a positive control. The mixture was then incubated at 37 °C for 15 min. Smears where then fixed using 1 mL ice cold methanol and left to dry at room temperature. Finally, smears were stained using the Wright's stain technique (Eosin-Methylene Blue) and observed under a light microscope at 40 x and 100 x.

5 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.

6 CONCLUSION

Highly stable and nearly monodisperse AgNPs and AuNPs capped with different polymers could be easily synthesized in water. NFT assay and flow cytometry results on AuNPs –polymers, indicate the absence of a significant inflammatory activity associated with AuNP-PVA and AuNP-PLL-PEG. Moreover, AuNPs-PLL were also found to be non cytotoxic to PC3 cancer cells. Therefore, supporting the use of PEGylated AuNPs-PLL as suitable shuttle vectors for *in vitro* and *in vivo* delivery to cancer cells and cancer theranostics.

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