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Hydroxylamine-O-sulfonic acid as a New Reducing Agent for the Formation of Nearly Monodisperse Gold Nanoparticles in water: Synthesis Characterisation and Bioconjugation

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ABSTRACT

Gold nanoparticles (Au NPs), with diameters ranging between 15 and 150 nm have been synthesized in water. 15 and 30 nm Au NPs were obtained by the Turkevich and Frens method using sodium citrate as both a reducing and stabilising agent at high temperature (Au NPs-citrate), while 60, 90 and 150 nm were formed using hydroxylamine-o-sulfonic acid (HOS) as a reducing agent for $HAuCl_4$ at room temperature. This new method using HOS is an extension of approaches previously reported for producing Au NPs with mean diameters above 40 nm by direct reduction. Functionalised polyethylene glycol-based thiol polymers were used to stabilise the pre-synthesised Au NPs. The nanoparticles obtained were characterised using uv-visible spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM). Further biocojuguation on 15, 30 and 90 nm PEGylated Au NPs were performed by grafting Bovine Serum Albumin, Transferrin and Apolipoprotein E (ApoE).

Keywords: Gold nanoparticles, Hydroxylamine-O-sulfonic acid, Polyethylene glycol (PEG), Protein, Bioconjugation.

1 INTRODUCTION

Gold nanoparticles (Au NPs) with size and shape dependent optical absorption and scattering properties that originate from the localized surface plasmon resonance have received considerable attention for a wide range of applications, including chemical sensing, optoelectronics, diagnostics, thermal therapy, drug and gene delivery[1-3]. Furthermore, the low cytotoxicity of Au NPs and their ease of bioconjuguation have contributed to the application Au nanoparticles in bionanotechnology [4,5]. Many publications on the effect of nanoparticle size and shape on cytotoxicity, biodistribution, and interaction with biosystem have also appeared recently [6-9]. Au NPs are typically produced through the chemical reduction of a Au precursor, such as $HAuCl_4$. In 1951 Turkevish *et al.* [10] reported the formation of colloidal Au nanoparticles using trisodium citrate to reduce tetrachloroauric acid in water. In 1971 Frens *et al.* [11] published an improved and slightly 'modified' Turkevish for synthesizing Au NPs that is still popular to day. Unfortunately, the mean diameters of Au NPs obtained by the above methods typically range between 10 and 40 nm, with the larger diameter NPs often being size-polydisperse and of irregular shape [10,11]. Methods to improve the size-monodispersity and shape of Au nanoparticles when their diameter exceeds 50 nm rely on a 'seeded growth' strategy. Large Au NPs have been formed by reducing gold $HAuCl_4$ or A(III)-surfactants (*i.e* CTAB or Triton X-100) complexes in the presence of Au NPs seeds with hydroxylamine hydrochloride or ascorbic respectively [12-14]. When CTAB is present various shapes, such as nanorods nanoprism *etc*., can be obtained by varying the synthesis conditions and the CTAB concentration [13]. However, Au NP-CTAB aggregates are known to be toxic, and excess of CTAB in the media has to be removed prior to bological assys [15]. Quasi-sphercal Au nanoparticles with diameter ranging between 15 to 300 nm have been successfully synthesised by different authors through seeded growth methods, using 2-mercaptosuccinic acid, hydroquinone and H_2O_2 [16-18]. However, seeded-growth methods may not always give a homogeneous enlargement of all of the seeds and small diameter nanoparticles can sometimes usually found in solution (often less than 5 %). Nanotechnology and nanomedecine applications however require improved synthetic methods for generating size-monodisperse and stable colloidal solutions, avoiding the use of cytotoxic chemicals. Amine containing molecules are well konown to be widely used as reducing agents for nanoaprticles synthesis and stabilization [19]. Zou *et al.* [20] reported the seed-mediated synthesis of branched Au NPs with the assistance of citrate using hydroxylamine sulfate in presence of Au NPs-citrate seeds. In 1972 Rozenskans *et al.* [21] discovered that the exposure of DNA solutions to low levels (2 mmol L^{-1}) of hydroxylamine-O-sulfonic acid (HOS) resulted in limited degradation without significant change to the thermal helix-coil profile of the DNA. Here we report a fast and simple protocol for synthesizing 'seedless' Au NP colloidal

solutions with size ranges betweem 60 and 150 nm in water, using HOS as a reducing agent at room temperature. Dynamic light scattering, show that the nanoparticles obtained were not size-polydisperse (PdI < 0.15), as justified by electron microscopy and UV-visible spectroscopy. Our simple method can serve to complement the commonly used Turkevich and Frens methods. Furthermore, biocojuguation on 15, 30 and ~ 90 nm PEGylated Au NPs was performed by grafting Apolipoprotein E (ApoE) Bovine Serum Albumin and Transferrin, making the Au NPs-Protein more attractive for biomedical applications.

2 PREPARATION AND CHARACTERIZATION OF GOLD NANOPARTICLES IN WATER

Au NPs with mean diameters of 15 and 30 nm were synthesized in water using previously reported methods based on a controlled chemical reduction of a $HAuCl_4$ solution with sodium citrate at high temperature [11, 22, 23]. However, nanoparticles with dimaeter ~ 60, ~ 90 and ~ 150 nm were obtained using a new method based on the chemical reduction of $HAuCl_4$ solution with hydroxylamine-O-sulfonic acid at room temperature and in the presence of sodium citrate as stabilising agent (see Section 4). The Au NPs obtained were further characterised using UV-visible spectroscopy, transmission electron microscopy (TEM) and dynamic light scattering (Figure 2). UV-visible data in Figure 1 clearly shows the size dependent optical properties of the nanoparticles, with a shift of the plasmon band from 543 to 559 nm, accompanied with peak broadening measured at the base of the peak (from 116 nm to 158 nm), as the mean diameter of the Au NPs increased from ~ 60 to ~ 90 nm respectively. While the spectra for the nanoparticles of ~ 150 nm showed a very broad plasom peak and a higher base-line due to the light scattering by Au NPs. TEM analysis of the nanoparticles utilizing the *Image J* software, verified that the Au NPs obtianed by HOS reduction, see Figure 1, had diameters of 61 ± 6.5 nm, 91 ± 9 nm and 148 ± 22 nm.

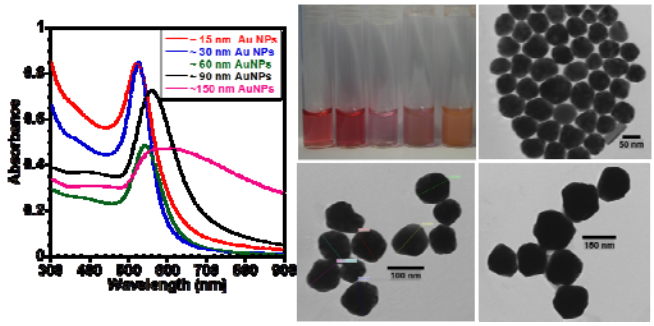


Figure 1. UV-visible spectra (left) of synthesized Au NPs. The corresponding colloidal solution (top left) and TEM micrographs of the Au NPs with diameter ~60 nm (top right); ~95 nm (bottom left) and ~150 nm (bottom right).

The final colloidal solutions were electrostatically stabilized due to the presence of sodium citrate, and had zeta potentials of approximately -35 ± 3 mV. However citrate-stabilized Au NPs are known to be very sensitive to slight changes in pH or ionic strength. Previously we have shown that after the addition of NaCl (0.157 $mol.L^{-1}$) to citrate-stabilized AuNPs, a change color from red to blue occurred immediately (aggregation), followed by the formation of a colorless solution after approximately 3-4 hr due to total precipitation of the NPs as aggregates [24]. Therefore, to maintain Au NPs stability in complex biological media, and prior to any bioconjugation, Au NPs had to be stabilized.

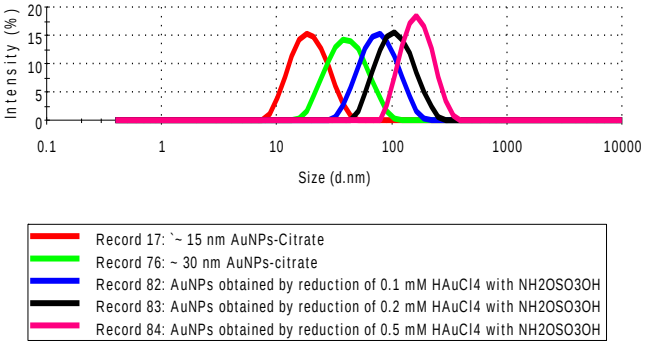
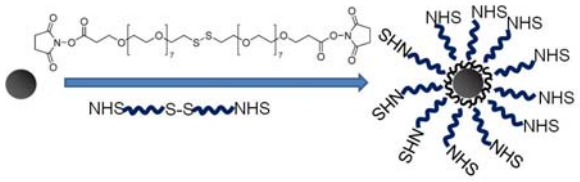


Figure 2. Size distribution by intensity obtained from DLS on synthesized Au NPs Au NPs with mean diameters of ~60, ~95 and ~150 nm were obtained by reduction of $HAuCl_4$ (0.1, 0.2 and 0.5 mmol L^{-1} respectively) with HOS at room temperature (PdI <0.17).

3 BIOCONJUGATION OF AUNPS

The pre-synthesized Au NPs in water, with mean diamters of 15 and 30 nm (obtained from the Turkevich/Frens methods) and ~ 90 nm obtained by HOS reduction, were used for bioconjugation experiments with different proteins attached through a biologically stable amide linkage in water. In this study ApoE protein, known to enhance the permeability of NPs across the "*Blood Brain Barrier* was attached onto the Au NPs [25]. As we previously reported, the stability of the Au NPs under physiological conditions (0.157 $mol.L^{-1}$ NaCl) can be significantly improved by grafting a polyethylene glycol-based thiol polymer shell onto their surface [24]. We also determined the number of PEG-SH ligands needed to coat the surface of a Au NP of a particular diameter and estimated the grafting density using thermal gravimetric analysis (TGA) and TEM [23]. In this study, we have grafted first a functional polymer NHS-PEG_{280}-S-S-PEG_{280}-NHS, based on forteen ethylene glycol units, thiol disulfide and N-hydroxy succinimide (NHS) reactive ester group as an active group on the Au NPs surface (Scheme 1) used as both a stabilising agent and chemical linker to react with amino group of the protein in water [4, 26-27]. The addition of NHS-PEG_{280}-S-S-PEG_{280}-NHS to a pre-synthesized Au

NP colloidal solution resulted in the formation of a NHS fuctional polyethylene glycol layer grafted onto the surface of the Au NPs surface, where NHS-PEG_{280}-S is attached through a gold thiol (Au—S) chemical bond as shown in Scheme 1.



Scheme 1. Formation of Au NPs-PEG-NHS in water.

The successful PEGylation of the Au NPs was confirmed by DLS/Zeta potential (ξ) measurements, where a slight increase in the size of the NPS, by approximately 2-3 nm, was detected (see Figure 3), and ξ was found to decrease from -35 to about -25 mV. After PEGylation, ApoE protein dispersed in a citric buffer solution (pH ~ 7) was added to the Au NPs-PEG-NHS solution (Scheme 2), the pH was adjusted to ~9 and the solution was left to react for about four hours under shaking. The protein was successfully grafted onto the PEG layer of the Au NPs through the formation of an amide linkage (NHCO) between the amino groups of the protein and the NHS at the surface of the Au NPs-PEG-NHS, as confirmed by DLS (Figure 3).

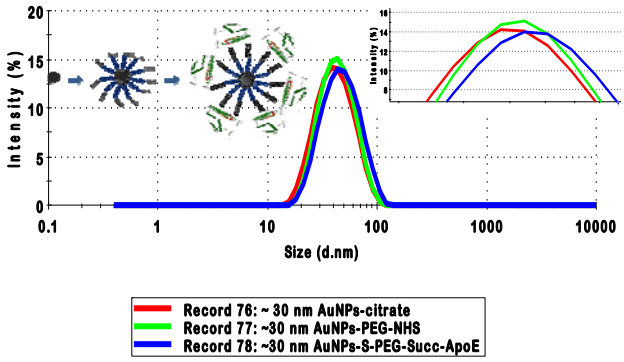
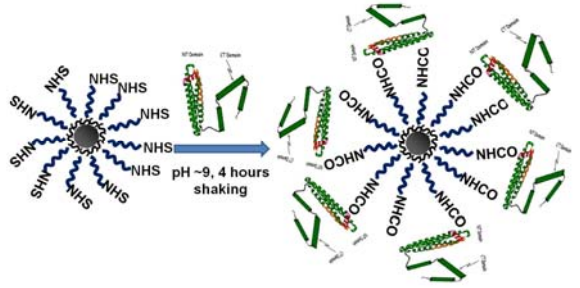


Figure 3. Size distribution by intensity obtained from DLS on 30 nm Au NPs-citrate before, and after PEGylation with NHS-PEG-S-S-PEG-NHS, and further attachment of ApoE protein through a biologically stable amide linkage; insert left (overall bioconjugation process Scheme 1 and 2), insert right show the Au NPs size increase after each step.



Scheme 2. Grafting of ApoE protein on Au NPs-PEG-NHS

In this study, DLS was found to be a very sensitive technique to follow each step (PEGylation/protein attachment). As shown in Figure 3, a shift in the diameter distribution of the 30 nm Au NPs to bigger diameters after each step confirmed the successful grafting of PEG-NHS and ApoE onto the Au NPs-PEG surface, without any noticeable aggregation of the colloidal solution, further confirmed by TEM (results not shown). A similar chemistry aproach was also applied to attach ApoE protein onto 15 and 90 nm Au NPs as well as other types of proteins (Transferrin, and Bovine Serum Albumin) on 15, 30 and 90 nm Au NPs. The herein presented method is succesful for bioconjuguation of Au NPs in water, and can also be applied to other biologically active molecules (Folic acid etc.).

4 MATERIALS AND METHODS

4.1 Chemicals and Materials:

Tetrachloroauric acid trihydrate ($HAuCl_4.3H_2O$), sodium citrate ($C_6H_5Na_3O_7.2H_2O$), hydroxylamine-o-sulfonic acid, sodium hydroxide, 4,7,10,13,16,19,22,25,32,-35,38,41,44,47,50,53Hexadecaoxa-28,29-dithiahexapenta-contanedioic acid di-*N*-succi-nimidyl ester, NHS-PEG-S-S-PEG-NHS with n=7were purchased from Sigma Aldrich. Apolipoprotein E (ApoE) Human Plasma. Very Low Density Lipoprotein was purchased from rpeptide Startech Scientific, the 50 mM NH_4HCO_3 medium was exchanged with citric buffer (pH ~7) by dialysis at 4°C during 24 hours prior to use.

4.2 Preparation, Bioconjugation of Au NPs.

Diameter of ~15 nm: 50 mL of an aqueous solution of $HAuCl_4.3H_2O$ (0.25 mmol L^{-1}) was heated to 95°C while stirring. 0.17 mL of a 340 mmol L^{-1} sodium citrate aqueous solution was rapidly added. The colour of the solution changed from pale yellow to dark blue, and then to deep red-burgundy within about 8 minutes. Stirring and heating was maintained during 1h after addition of sodium citrate. The heat was then removed and the solution was kept under stirring, until cooled to room temperature. The Au NPs obtained with this procedure were ~ 15 ± 1.5 nm [23].

Diameter of ~30 nm: A similar method used for the 15 nm Au NPs is used with a slight change were the pH of the solution was adjusted to pH ~ 7 with NaOH before heating and the heat was maintained for 4 hours after addition of sodium citrate. The AuNPs obtained with this procedure were approximately 30 ± 4 nm [22].

Preparation of 60 nm, 90 nm and 150 nm AuNPs: For Au NPs, larger than 30 nm, a very weak reducing agent hydroxylamine-o-sulfonic acid was used to reduce $HAuCl_4.3H_2O$ in the presence of sodium citrate at room. For ~60 nm AuNPs, to 150 mL of an aqueous solution of 0.1 mmol $L^{-1}HAuCl_4.3\ H_2O$ and 0.48 mmol L^{-1} sodium

citrate was added Hydroxylamine-o-sulfonic acid (final concentration 0.15 mmol L^{-1}). After addition of Hydroxylamine-o-sulfonic acid the colour of the solution changed from pale yellow to gray, blue, and then slight red-pink after few hours, the solution was kept under stirring for about 18 hours. The AuNPs obtained were 61 ± 6.5 nm. By increasing the concentration of $HAuCl_4$ from 0.1 to 0.2 and 0.5 mmol L^{-1} respectively, while keeping all the other conditions fixed, we found that the size of Au NPs increased from 61 ± 6.5 nm to ~91 ± 9 nm and ~148 ± 22 nm respectively. Finally, increasing the temperature to 60 °C was found to accelerate the reduction rate (~30 min instead of few hours), resulting in an increase in the mean diameter of the nanoparticles from ~60 nm to ~80 nm. While, increasing the pH of the solution from about 4 to 6 resulted in a decrease in the size of the final Au NPs size by 10 to 15 nm.

PEGylation of Gold Nanoparticles: PEGylation was performed by adding PEG_{280}-S-S-PEG_{280}-NHS to the Au NP solution, stirring was maintained for 2 hours.

Protein bioconjugation: The pH of the Au NPs-PEG-NHS solution was adjusted to pH~9 by adding few drops of NaOH (0.1 M) solution, followed by the protein solution (~ 50 protein/particle) in citric buffer addition to the Au NP colloidal solution. The solution was left to react under shaking for 4 hours prior to DLS measurement.

5 INSTRUMENTATION

Optical spectra were obtained on a CARY Uv−vis spectrophotometer with a Xenon lamp (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.

Transmission Electron Microscopy (TEM): A drop of nanoparticles dispersion was first placed on a carbon-coated TEM copper grid (Quantifoil, Germany) and left to air-dry, before being introduced into the sample chamber of the TEM. Samples were analysed using a JEOL JEM-2100 TEM operating at 200 kV. All images were recorded on a Gatan 1.35 K × 1.04 K × 12 bit ES500W CCD camera. TEM images were analysed using Image J software.

6 CONCLUSION

The successful applications of NPs in biomedical science requires a stable colloidal solution in complex media with a high ionic strentgth, a very low or no cytotoxic effect as well as coating with biomolecules of interest. In this study, a new method for synthesising bigh size Au NPs between 60 and 150 nm through direct reduction of gold precursor using a hydroxylamine-o-sulfonic acid as weak reducing agent at room temperature was performed. The NPs obtained were not size-polydisperse (PdI <0.17) and had size dependent optical properties. Furthermore, the above method represent an extension of the approaches described by Turkevich, Frens and others to produce Au NPs with a diameter above 50 nm without increasing polydispersity of the colloidal solution. Finally, bioconjugation of the obtained Au NPs with proteins make them very attractive for applications in biology, a concept to be investigated in future work.

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