Gold Nanoparticles: Synthesis Characterization and conjugation

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Abstract

Gold nanoparticles (Au NPs) with diameters ranging between 4-150 nm have been synthesized in water. The strong reducing agent sodium borohydride ($NaBH_4$) was used to produce small Au NPs with diameter about 4 ± 1 nm. 15 and 30 nm Au NPs were obtained by a slightly modified Turkevich and Frens method using sodium citrate as both reducing and stabilizing agent at high temperature. The attempt to produce Au NPs with diameter larger than 30-40 nm by the Turkevich method resulted in an increase in the polydispersity and the shape diversity of the final Au NPs, indicating the importance of the trial of new reducing agents in the production of Au NPs especially for diameters above 40 nm. Therefore, hydroxylamine-o-sulfonic acid (NH_2SO_4H) (HOS) was used here for the first time as a new reducing agent for $HAuCl_4$ at room temperature to produce Au NPs with diameter of about 60, 90 and 150 nm. This new method using HOS is an extension of the approaches described to produce Au NPs with diameter above 40 nm by direct reduction. The obtained nanoparticles were characterized using uv-visible spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM). Further biocojuguation on 15, 30 and 90 nm Au NPs were performed by grafting covalently Apolipoprotein E (ApoE) and Bovine Serum Albumin (BSA) through an ethylene glycol-N-hydroxysuccinimide linker (NHS-PEG-S-S-PEG-NHS) making them very attractive for drug delivery and cell targeting. Finally, functionalized polyethylene glycol-based thiol polymers were also used to stabilize the pre-synthesized Au NPs-PEG-Protein.

Keywords : Gold nanoparticles, hydroxylamine-o-sulfonic acid, polyethylene glycol, stabilization, portein, bioconjuguation, water.

I. Introduction

Gold nanoparticles (Au NPs) have a strong absorption band and high luminescent properties, due to the surface plasmon resonance (SPR), originated from the coherent oscillations of conduction-band electrons on nanoparticle surface upon interaction with an electromagnetic radiation of appropriate wavelength.[1] The SPR optical absorption and scattering properties of the Au NPs are also known to depend from the final size and shape of the nanoparticles, and to be very sensitive to the surrounding media and the aggregation state of the nanoparticles.[2-6] Therefore, Au NPs have attracted researcher's attention for their use as a very useful tool for a wide range of applications, including chemical sensing, optoelectronics, diagnostics, thermal therapy, drugs and gene delivery.[2,3,5,7-16] Furthermore, the low cytotoxicity of Au NPs and their ease of bioconjuguation have contributed to the boom of gold nanoparticles in bionanotechnology ,[17,18] many publications on the studies of the effect of nanoparticle size and shape on cytotoxicity, biodistribution, and interaction with biosystem have appeared recently.[19-23] Therefore, the synthesis of Au NPs of different sizes/shapes has attracted much interest in the last few decades. Different ways to synthesize gold nanoparticles are now available in the literature, among which Au NPs are mainly produced through chemical reduction of gold precursor (typically $HAuCl_4$). At first, Turkevish in 1951 reported on the formation of colloidal gold nanoparticles using trisodium citrate to reduce tetrachloroauric acid in water, later Frens published in 1971 an improved and slightly modified method still popular to days. Unfortunately, Au NPs obtained by the above methods are between 10 and 40 nm, with a larger size makes the Nps less monodisperse and irregular in shapes.[24,25]

Methods to improve monodispersity and shape of gold nanoparticles when their diameter exceeds 50 nm use a seeding growth strategy.[26-28] Large Au NPs were 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.[29,30] When CTAB is present various shapes such as nanorods nanoprism etc. can be obtained by varying the synthesis conditions and mainly the CTAB concentration.[30] However, Au NPs-CTAB are known to be toxic, and excess of CTAB in the media has to be removed prior to bological assys.[31] Quasisphercal gold nanoparticles of diameter

ranging from 15 to 300 nm were synthesized by different authors including us, through seeded growth methods using 2-mercaptosuccinic acid, hydroquinone, hydroxylamine hydrochloride and H_2O_2.[28,32-34] Moreover, seeding growth methods may not always give a homogeneous growth of all the seeds and some smaller nanoparticles can sometimes be found in solution (usually less than 5 %).

Nanotechnology and nanomedecine require improved synthetic methods for nearly monodisperse and stable colloidal solutions, avoiding the use of cytotoxic chemicals. Amine containing molecules are well known to be widely used as reducing agents for nanoaprticles synthesis and stabilization.[35] When polyelectrolytes such as chitosan, polyallylamine and polyethylenimine are present during the synthesis of nanoparticles, positively charged nanoparticles can be otained,[36] the presence of this positive charges on the surface of the nanoparticles maybe of benifits for electrostatic complexation for biomolecules such as the phosphate esters backbones of the nucleic acid,[37-39] while the more general process for the bioconjugation of nanoprticles is known to occur through formation of a biologically stable amide linkage between the NH_2 groups of the biomolecules and carboxylic acid at the surface of the nanoparticles or vice versa.[40-42] The attachment of biomolecules such as peptides, proteins and other targeted ligands containing specific molecules that recognize receptors on various cell lines on the surface of nanoparticles plays a key role not only for their interactions with biological tissues, but also in their biological functions and cellular uptake, that can facilitate their use in drug delivery, diagnosis and therapeutics.[43-48]

Here we report a fast and simple protocol for the synthesis of seedless gold nanoparticles colloidal solutions with size range between 4 and 150 nm in water.[49,50] We also show in this study the failure of the attempt to obtain Au NPs with diameter larger than 30-40 nm via the citrate reduction, due to an increase in the polydispersity and the shape diversity of the final Au NPs.[26] This result indicates the importance of the trial of new reducing agents in the production of Au NPs especially for diameters above 40 nm. Therefore, we show in this study for the first time that we can obtain Au NPs with diameters of about ~60, ~80 and ~150 nm Au NPs by using HOS as a new reducing agent for $HAuCl_4$, $3H_2O$ at room temperature. Our results from dynamic light scattering based on the Rayleigh scattering, known for spherical nanoparticles, show that the obtained nanoparticles were not polydisperse (PdI < 0.15), as further justified by electron microscopy and UV-visible spectroscopy.

This simple method can serve as a complement for the commonly used Turkevich and Frens method in the direct production of Au NPs. Further biocojuguation on 15, 30 and ~ 90 nm PEGylated Au NPs was performed by grafting Apolipoprotein E (ApoE) and Bovine Serum Albumin, via an amide linkage using an amine reactive functional group N-hydroxysuccinimide, that easily react with amino groups from the protein in aqueous media with a pH between 8 and 10, making the Au NPs-PEG-Protein more attractive for biomedical applications.[42] More works are now in progress in order to understand the effect of temperature and pH on the synthesis of Au NPs via hydroxyl-O-sulfonic acid reduction, and to graft other biomolecules such as transferrin etc, as well as on testing these AuNPs-biomolecules conjugates in biology.

II. Materials and methods

A. Chemicals and Materials:

Tetrachloroauric acid trihydrate ($HAuCl_4.3H_2O$), gold (III) chloride ($AuCl_3$), sodium citrate ($C_6H_5Na_3O_7.2H_2O$), sodium borohydride ($NaBH_4$), hydroxylamine-o-sulfonic acid (NH_2SO_4H), sodium hydroxide (NaOH), 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=7 Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich. Thiol terminated poly(ethylene glycol) methyl ether, M_w = 2100; 5400; and 10800 g mol^{-1} were purchased from Polymer Source. 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.

B. Preparation, Bioconjugation of Au NPs.

Diameter of 4 ± 1nm Au NPs: To an aqueous solution (150 mL) of $HAuCl_4.3H_2O$ (0.25 mmol L^{-1}) was added 0.22 mL of a 340 mmol L^{-1} sodium citrate aqueous solution and the mixture was stirred vigorously in an ice bath. To this solution was added 0.375 mL of an ice cold solution of $NaBH_4$ (100 mmol L^{-1}). An instantaneous colour change from pale yellow to deep red-orange was noted after addition of $NaBH_4$. The AuNPs obtained with this procedure were approximately 4 ± 1nm.

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 ± 2.5 nm.[28]

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.[50]

Diameters 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 in this study for the first time 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 HOS the colour of the solution changed from pale yellow to colourless in about 5 minutes, then to gray, blue, and then slight red-pink after several hours, the solution was kept under stirring for about 18 hours. The Au NPs 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.

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 between 7.5~9 by adding few drops of NaOH (0.1 M) solution, followed by the addition of 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.

PEGylation of Au NPs- Protein conjugate: A solution of mPEG-SH of the desired molecular weight was added to a solution of citrate-capped Au nanoparticles with stirring. The solution was stirred for ~1 h allowing mPEG-SH to be grafted on the Au NPs surface prior to DLS measurement.

III. 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.

IV. Results and Discussion

A. Chemical Synthesis and characterization of Au Nanoparticles .

Gold nanoparticles with size dependent optical properties are now very useful in different field of nanotechnology.[46,51,52] Different ways to produce gold nanoparticles can be found in the literature.[29,36,53-57] The most used techniques are based on the reduction of gold chloride precursor mainly of $HAuCL_4$ by different reducing agent.[24,25,28,29,36,53-58] However, no universal reducing agent in known yet in order to produce monodisperse gold nanoparticles of different sizes, instead different ways such as seeding growth methods and different reducing agents with variable strength are usually used.[26-28,32,49] Chemical reduction of $HAuCL_4$ with a strong reducing agent such as sodium borohydride or hydrazine occurs very

fast and in non controllable manner to produce gold nanoparticles usually with diameter below 5 nm.[49] While the reduction with sodium citrate produce Au NPs with diameters larger than 8-9 nm and is more widely used until to date for the synthesis of Au nanoparticles and further studied.[50,59,60] The mechanism of the formation of Au nanoparticles obtained by citrate reduction of $HAuCl_4$ was found to contain four steps as concluded Polte *et al.* from their study using in-situ nanoparticle growth monitoring via XANES and SAXS.[59] Moreover, Ji *et al.* have described two pH dependents mechanisms for the formation of Au nanoparticles with citrate, whereby Au nanoparticles were found to form by fast, random particle attachment and ripening for solutions with pH < 6.5, whilst slower nucleation and ripening was observed for synthesis solutions with pH 6.5-7.7.[50] In our study presented here, we have synthesized Au NPs with diameters ranging between 4-and 150 nm using three different reducing agents. The produced Au NPs have size dependent optical absorption as shown from the characterization by Uv−visible absorption spectroscopy (Figure 1), transmission electron microscopy TEM (Figure 2 and 3) and dynamic light scattering (Figure 4). The figure 1 show the Uv-visible spectra of the nearly monodisperse different diameters Au nanoparticles (~4, ~15, ~30, ~60, ~90 and ~150 nm) synthesized in this study, a clear red shift of the plasmon absorption maximum (λ_{max}) from 513 nm to 600 nm accompanied with a further broadening of the absorbance band from 50 nm to 380 nm with increasing Au nanoparticle diameter from ~4 ± 1 nm to ~ 148 ± 22 nm respectively is observed. The red shift and the SPR broadening is due to a higher oscillation modes (quadrupole, octopole absorption and scattering) that also affect the extinction cross section with increasing size.[61] The use of the strong reducing agent sodium borohydride in the presence of sodium citrate as stabilizing agent at room temperature allowed us to produce Au NPs with diameter of 4 ± 1 nm as resulted from TEM analysis (Figure 2 (a)), while the 15 nm Au nanoparticles were obtained by chemical reduction of $HAuCl_4$ with sodium citrate at pH < 6.5 and at a temperature of about 95 °C,

suggesting that Au NPs form by fast, random particle attachment and ripening as outlined by Ji *et al.* (Figure 2(b)).[50] Histograms of the nanoparticle diameters presented on the right of each TEM image are obtained from TEM image analysis using *Image J* software. An attempt to produce larger Au nanoparticles at pH < 6.5 was also performed through the decrease of both the citrate/$HAuCl_4$ ratio and the temperature. Our results here show clearly that when the nanoparticles increase above 35 nm for a ratio citrate/$HAuCl_4$ of 0.76 different shapes with mostly elongated particles were obtained in the final solution as shown in the figure 3(a). However, 30 nm Au nanoparticles with better circularity and polydispersity used in this study were obtained by a previously reported method by Ji *et al.* through the chemical reduction of $HAuCl_4$ with sodium citrate at pH > 6.5 (pH ~7 adjusted by addition of Na OH 1M to the solution mixture) and at a temperature of about 95 °C, the increase in the pH to about 7 results in a more controlled synthesis based on slower nucleation and ripening, the resulted nearly spherical Au nanoparticles with diameter 32 ± 4.5 nm are shown in figure 2 (c).[50] Further decreasing in the ratio of citrate/$HAuCl_4$ to 0.35 and 0.26 as well as the temperature from 100 to about 70 °C lead only to a slight increase in the size of the nanoparticles with a large increase polydispersity and shape diversity as shown in the TEM images presented in the figure 3 ((c), and (d)). In fact we show here that it is not possible to obtain large Au NPs by simply using the Turkevich and Frens method via a simultaneous decrease of the citrate/ $HAuCl_4$ and the temperature.

Large Gold nanoparticles are usually obtained through a seeding growth method.[26,32,34] We have reported recently on the synthesis of Au NPs with diameters ranging between 15 and 170 nm in aqueous solution using a seed-mediated growth method, employing hydroxylamine hydrochloride as a reducing agent.[28] Here we report on a new method for the synthesis of Au NPs of diameters above 50 nm using a simple and one step synthesis based on the use of hydroxylamine-o-sulfonic acid (HOS) as a new reducing agent of $HAuCl_4$ at room

temperature and in the presence of sodium citrate as reducing agent. In fact, Zou et al. reported a seed-mediated synthesis of branched gold nanoparticles with the assistance of citrate using hydroxylamine sulfate in presence of Au NPs-citrate seeds.[62] Rozenskranz found in 1973 that 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,[63] these results makes HOS use as a biologically friendly reducing agent and a candidate of choice. Nearly monodisperse Au NPs with diameter of about ~60, ~90 and ~150 nm with size dependent optical properties (figure 1) were obtained in this study as shown in the figure 2 (d), (e) and (f), from the electron micrographs of the Au nanoparticles obtained via the reduction of 0.1, 0.2 and 0.5 mmol L^{-1} $HAuCl_4$. $3H_2O$ with HOS at 0.155, 0.31 and 0.775 mmol L^{-1} respectively. Our results from TEM image analysis using *Image J* software show that the size was found to increase from 61 ± 6.5 nm to 92 ± 12 nm and 148 ± 22 nm when the concentration of both $HAuCl_4$ and HOS increases, while keeping the ratio HOS/ $HAuCl_4$ constant at 1.55. The circularity of the resultant nanoparticles was also not very affected and nearly spherical shapes were obtained. In fact HOS seems to be a very weak reducing agent, therefore allowing a slow reduction of $HAuCl_4$ (more than 18 hours) that lead to a nearly monodisperse colloidal solution. **Figure 4** shows the diameter distribution by intensity of the as synthesized Au nanoparticles with mean diameters of ~15, ~30, ~60, ~90, and ~150 nm before conjugation. The size distribution peak by intensity was found to shift from ~20 nm (Zav 17 nm) to ~ 170 nm (Zav = 161 nm) when the size increases from ~15 ± 2.5 nm to 148 ± 22 nm (results from TEM). The zeta potential of all the Au nanoparticles was in the range between -31 for 15 nm Au NPs to -40 mV for ~ 30 nm. As discussed previously, we note here that the final size of the obtained nanoparticles from HOS was found to depend on both the reducing agent and $HAuCl_4$ concentrations. This new method presented in this study using NH_2SO_4H (HOS) can be applied as an extension of the approaches described to produce Au NPs with diameter above 40 nm by direct reduction. However, since both the

gold precursor and the reducing agents are acidic, the pH is expected to decreases when their concentration increases.[14,50,64,65] More studies are now in progress to study the effect of pH and temperature on the final nanoparticles size distribution as well as the yield of this reduction. In fact, Au nanoparticles produced in this are electrostatically stabilized, and as such they are very sensitive to any change in the ionic strength and/or pH of the medium in which they are dispersed, which can induce nanoparticle aggregation. We have demonstrated previously that conjugation on the surface of AuNPs-citrate either through Au-SH chemical bonding with polyethylene glycol PEG, or by auto-assembly of triblock and diblock surfactant polymers based on polyethylene oxide and polypropylene oxide can improve the stability and biocompatibility of the nanoparticles.[6,28,60,66,67] Moreover, proteins such as BSA and other biomolecules are also known to increase the stability of nanoparticles under physiological condition.[45,68-71]

B. Bioconjugation of Au NPs

The presynthesized Au NPs in water mainly 15, 30 nm (obtained through the Turkevich/Frens method) and ~ 90 nm obtained by HOS reduction were used for further bioconjugation with different proteins attached through a biologically stable amide linkage in water. In this study ApoE protein, known to enhance the permeability of nanoparticles across the *"Blood Brain Barrier"* was attached on the 30 nm AuNPs **(Scheme1)**,[72,73] a similar method was also used to conjugate the 15 nm Au NPs BSA.[42,74] As we previously reported, the stability of the Au NPs under physiological conditions (0.157 mol.L^{-1} NaCl) can be well improved by grafting a polyethylene glycol-based thiol polymers shell on their surface.[28,67] We have 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.[28] In this study, we have chosen to graft first a functional oligomer NHS-PEG_{280}-S-S-PEG_{280}-NHS, based on fourteen 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 stabilizing agent and chemical linker able to react with amino group of the protein in water.[17,42,73,75,76] Therefore, the addition of NHS-PEG_{280}-S-S-PEG_{280}-NHS to the presynthesized Au

NPs colloidal solution resulted in the formation NHS functional polyethylene glycol layer grafted on the Au NPs surface, where NHS-PEG_{280}-S is attached through a gold thiol (Au—S) chemical bond as shown in **Scheme 1**. The successful PEGylation of the Au NPs was confirmed by DLS/Zeta potential (ξ) measurements, where a slight increase in the size of nanopartciles of about 2-3 nm was detected **(Figure 5)**, and ξ was found to decrease slightly from -40 to about -35 mV. After PEGylation, ApoE protein dispersed in a citric buffer solution (pH ~ 7) was added to the Au NPs-PEG-NHS solution (Scheme 1), the pH was adjusted to ~9, and the solution was left to react for about four hours under shaking. The protein was able to be grafted on the PEG layer of the Au NPs through 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 5)**. Figure 5 show a size increase of about 5 nm is after protein conjugation. Similarly, the Bovine serum Albumin was also grafted on the 15 nm AuNPs **(Figure 6)**, the size was found to increase by about 4 nm after PEG-NHS attachment, and 7 nm after protein attachment. However, we note here that the conjugation of the 15 seems to cause a slight flocculation as can be detected from DLS measurements; indeed, no change in the initial red colour of the colloidal solution was observed. The filtration of the Au NPs-PEG-NHCO-BSA colloidal solution through a 0.2 μm nylon filter was found to remove the larger sizes flocks observed in DLS **(Figure 6)**. Finally we note that number of protein used in this study was estimated to be 55 BSA proteins per 15 nm AuNPs and about 158 ApoE per 30 nm AuNPs.

Further stabilization of the AuNPs-Protein was also performed using a tiny amount of mPEG-SH 2000, 5000, and 10 000 **(Figure 7)**. PEGylation of nanoparticles is well known to increase the stability of nanoparticles in biological fluids and to increase the circulation in the blood stream. However, the addition of the SH-PEG may also remove some the protein coating from the surface of the nanoparticles by ligand exchange. Therefore the amount to SH-PEG added here was estimated in order to have a partial coverage of the AuNPs surface. **Figure 7** show the results from DLS on the PEGylation of the 30 nm AuNPs-PEG-ApoE, an increase in the size of the AuNPs-PEG-ApoE by ~2, 7 and 12 nm accompanied with an increase in the zeta potential from -34 mV, -28 and -20 mV (results not shown) with the increase of the PEG length from 47 to 122 and 245 EO units respectively. This

increase in the size and zeta potential indicate the successful attachment of the PEG on the Au NPs surface that might increase their stability.[67] We note that from our results obtained in our previous study on PEGylation with a maximum coverage of the Au NPs surface with mPEG-SH, a larger increase in the nanoparticles size by about, ~8, ~15 and ~23 nm with the increase of the PEG length from 47 to 122 and 245 EO units respectively was observed, the zeta potential was also found to increase to ~ -8 ± 2 mV for the PEG with 245 EO units. These results can confirm that only a partial coverage with mPEG-SH is performed here, therefore leaving the proteins grafted on the Au NPs surface. The effect of the PEGylation on biological applications (uptake/delivery protein stability and conformation etc…) will be evaluated in a future study.

In this study, DLS was found to be a very sensitive technique for the characterization of dispersions of nanoparticles and nanoparticle –protein/polymer hybrids. In fact, DLS is also known as a method of choice for protein (and other biomolecule) molecular weight distributions in solution.[77] Consequently, the conjugation of protein and PEG can dramatically affect the Brownian motion of particles by introducing additional frictional drag and thus reducing nanoparticle diffusivity that allowed us to follow each step of conjugation (PEGylation/protein attachment). As shown in Figures 5, 6 and 7, a shift in the size distribution of Au NPs to bigger sizes after each step can confirm the successful grafting of PEG-NHS and proteins on the Au NPs-PEG surface without any noticeable aggregation of the colloidal solution, further confirmed by TEM (results not shown). A similar chemistry approach was also applied to attach ApoE protein on 15 and 90 nm Au NPs as well as can be used for other types of proteins (*ie.* Transferrin, etc...). The herein presented method is successful for bioconjuguation of gold nanoparticles in water, and can also be applied to other biologically active molecules (Folic acid etc...).

Conclusions

The successful applications of nanoparticles in biomedical science require a stable colloidal solution in complex media with a high ionic strength, a very low or no cytotoxic effect as well as coating with biomolecules of interest. In this study, a new method for synthesizing big 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 obtained nanoparticles were not polydisperse (PdI <0.17) and have size dependent optical properties. In contrast, we show here that the citrate reduction method is not successful in producing Au NPs with sizes more than 40 -50 nm. Therefore, 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.

Table and Figure Captions

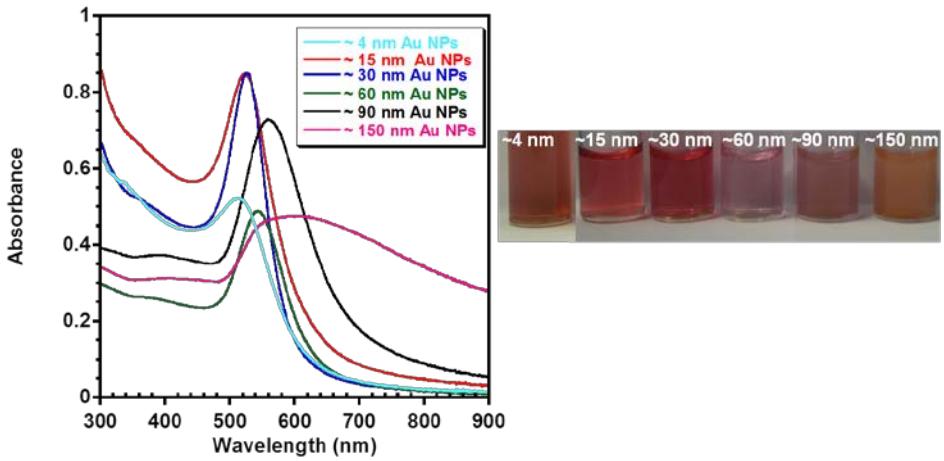


Figure 1. UV-visible spectra (left) of Au NPs used in this work, the corresponding colloidal solution (right).

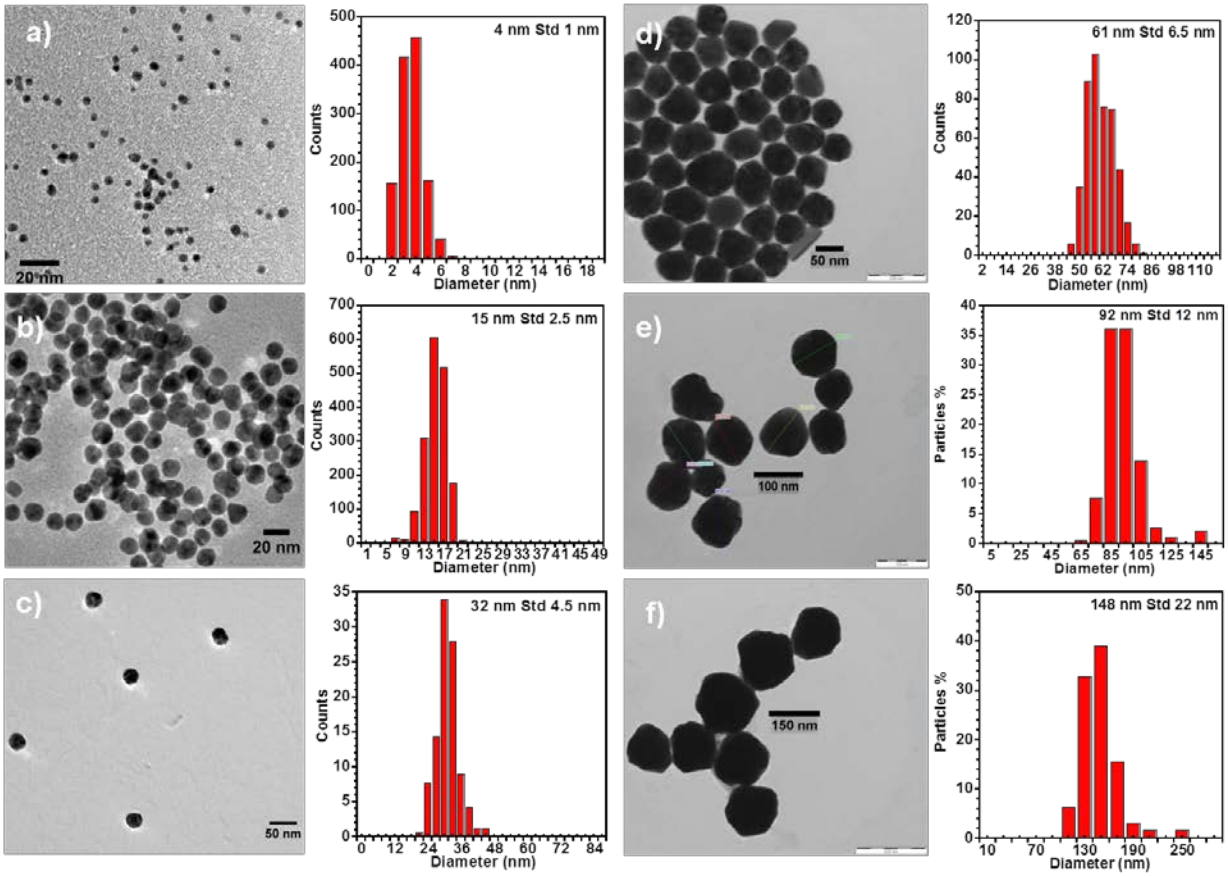


Figure 2. TEM micrographs of the Au NPs with diameter ~4 nm (a) obtained from $NaBH_4$, ~15 nm (b); ~30 nm (c) obtained from citrate, and ~60 nm (d); ~90 nm(e) and ~150 (f) nm obtained from HOS reduction. To the right of each picture a histogram showing the Au NPs diameters distribution obtained from TEM image analysis using *Image J* software.

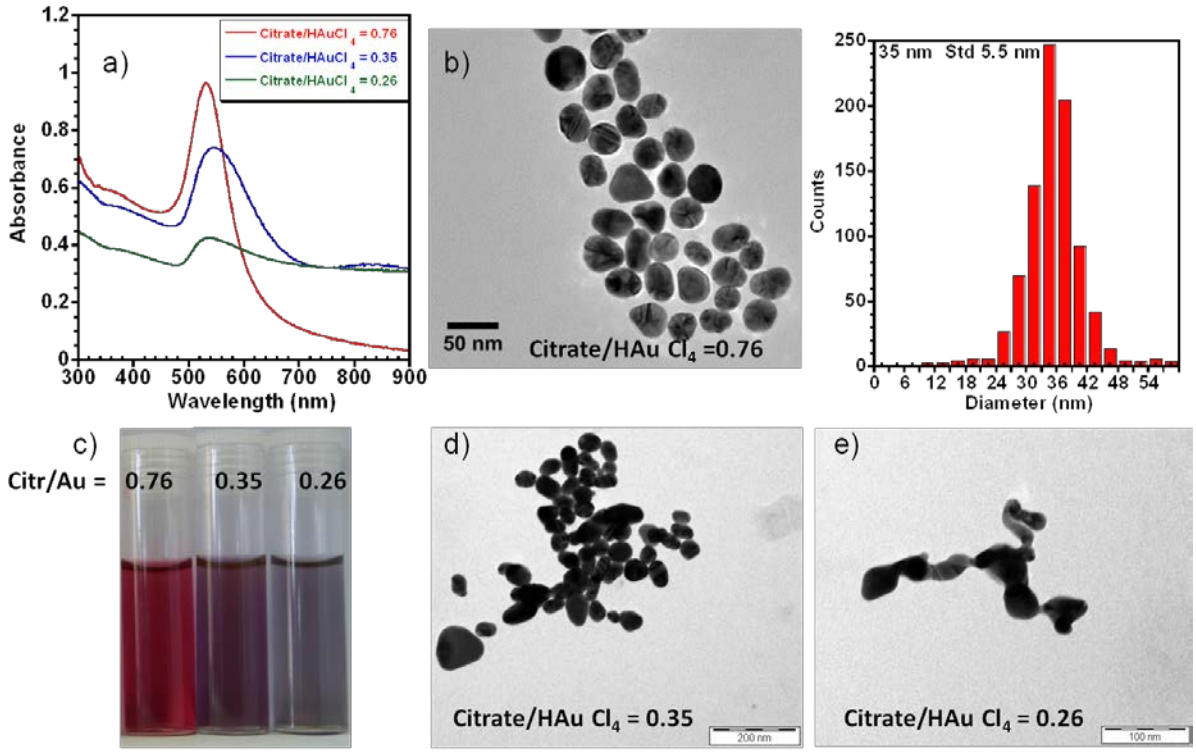


Figure 3. UV-visible spectra (a) of Au NPs obtained from the direct reduction with sodium citrate at different citrate/$HAuCl_4$ at ~ 80 °C. The corresponding TEM micrographs ((b), (d), (e)) and colloidal solution of each ratio is also presented (c). The histogram showing the nanoparticle diameters distribution obtained from TEM image analysis using *Image J* software for the ratio citrate/$HAuCl_4$ 0.76.

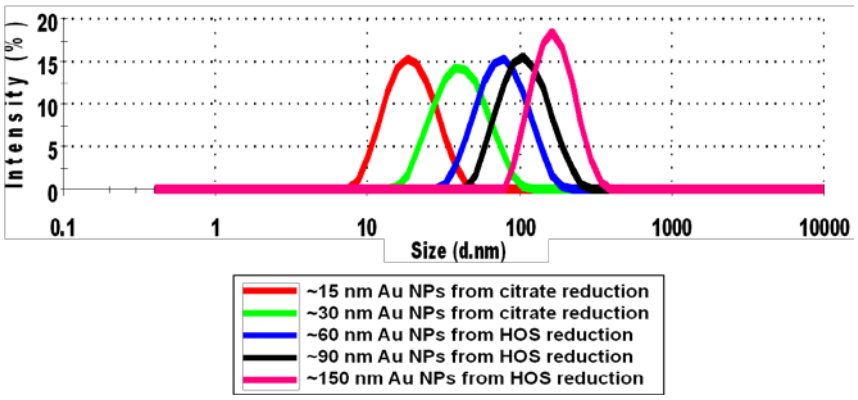
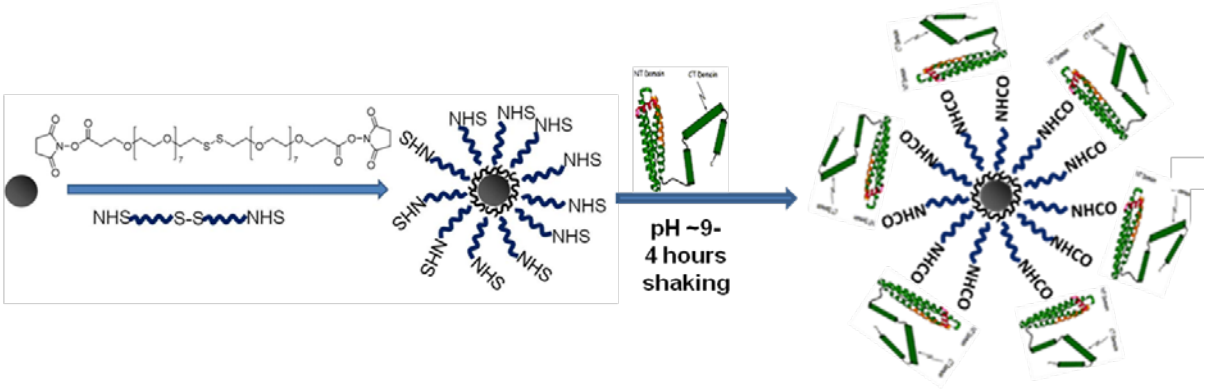


Figure 4. Size distribution by intensity obtained from DLS on the nearly monodisperse Au NPs obtained in this work, Au NPs with diameter ~60; ~90 and ~150 nm obtained by

reduction of $HAuCl_4$ (0.1, 0.2 and 0.5 mmol L^{-1} respectively) with HOS at room temperature (PdI < 0.17).



Scheme 1. formation Au NPs-PEG-NHS and grafting of ApoE protein on Au NPs-PEG-NHS in water, a similar synthesis was used to graft BSA on 15 nm Au NPs.

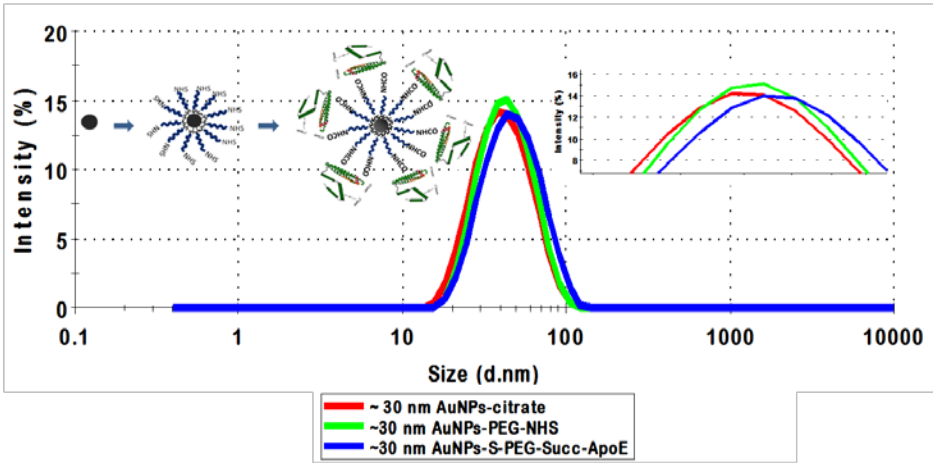


Figure 5. Size distribution by intensity obtained from DLS on 30 nm Au NPs-citrate before, and after PEGylation with NHS-PEG-S-S-PEG-NHS, and further attachment of ApoE protein through a biologically stable amide linkage; insert left (overall bioconjugation process Scheme 1 and 2), insert right show the Au NPs size increase after each step.

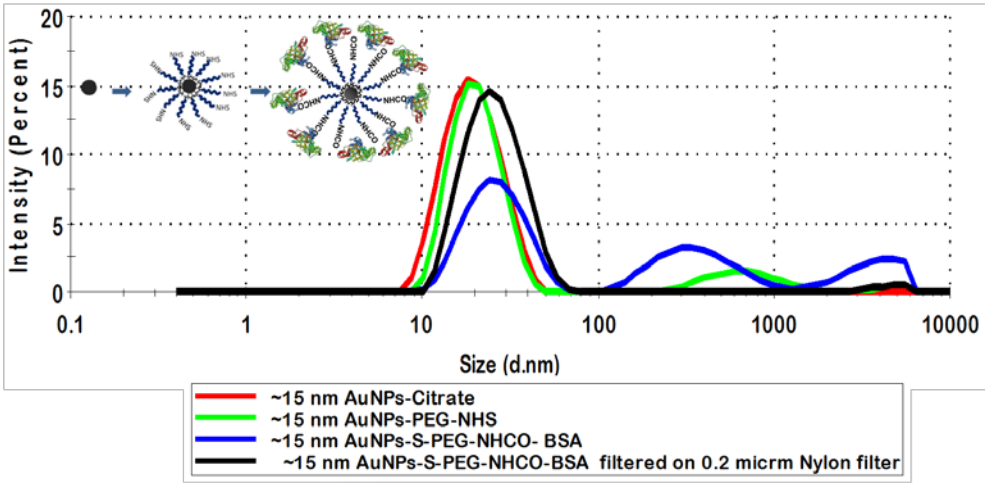
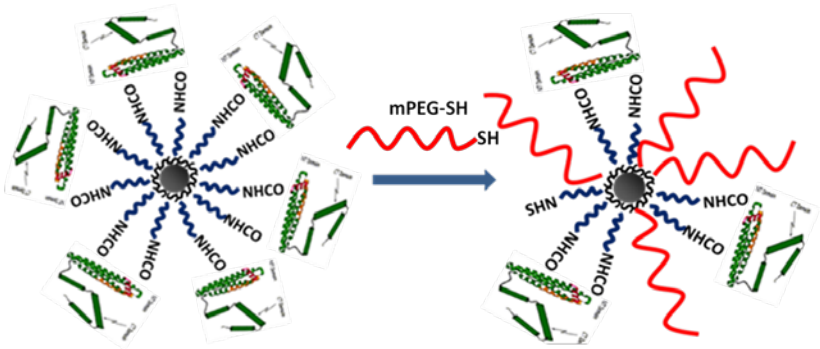


Figure 6. Size distribution by intensity obtained from DLS on 15 nm Au NPs-citrate before, and after PEGylation with NHS-PEG-S-S-PEG-NHS, and further attachment of BSA protein through a biologically stable amide linkage; insert left (overall bioconjugation process Scheme 1), conjugation on 15 nm Au NPs caused slight aggregation, the aggregated Au NPs were removed via filtration of the colloidal solution on 0.2 μm Nylon filter.



Scheme 2. Further PEGylation of ~ 30 nm Au NPs-PEG-NHCO-APoE in water (mPEG-SH with Mw of 2100, 5400 and 10800 g mol^{-1} were used.

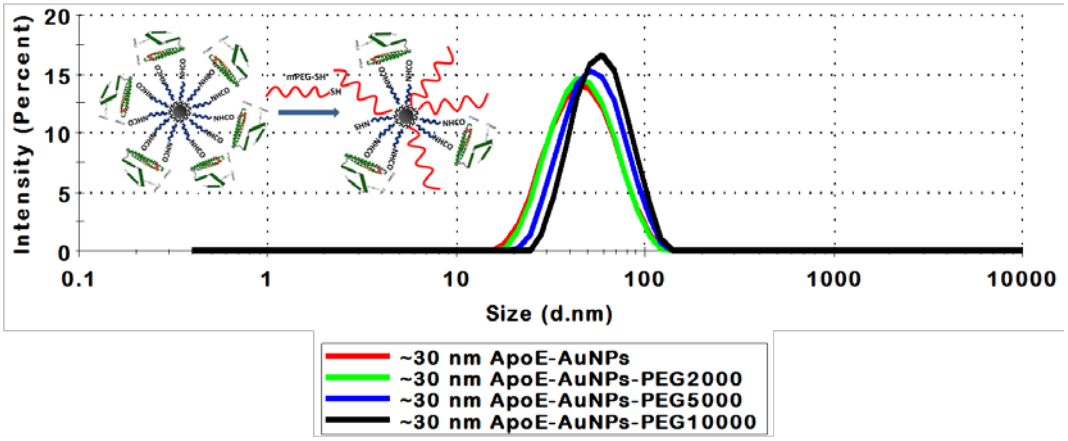


Figure 7. Size distribution by intensity obtained from DLS on 30 nm Au NPs-PEG-NHCO-ApoE (ApoE-AuNPs) before, and after PEGylation with mPEG-SH (Mw~2100, 5400 and 10000 g mol^{-1}) insert show the overall process Scheme 2).

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