A method for synthesis of gold nanoparticles using 1-amino-2-naphthol-4-sulphonic acid as reducing agent

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The present communication describes the synthesis of gold nanoparticles (AuNPs) using 1-amino-2-naphthol-4-sulphonic acid (ANSA) with enhanced stability. The method of preparation is similar to the conventional method using sodium citrate as reducing agent. Briefly, ANSA was quickly added to a boiled solution of hydrogen tetrachloroaurate under stirring, resulting in the synthesis of deep red-coloured AuNPs. The AuNPs prepared by ANSA (AuNPs–ANSA) exhibit twofold increase in stability towards sodium chloride over those prepared by sodium citrate. AuNPs–ANSA are 35.1 nm in size and particles has various shapes, viz. hexagonal, pentagonal, spherical, etc. AuNPs–ANSA can be functionalized with immunoglobulins and functionalized nanoparticles exhibit flow properties in lateral-flow assay.

Keywords: Gold nanoparticles, immunoglobulins, lateral flow assay, reducing agent.

GOLD nanoparticles (AuNPs) offer unique physical, electronic, magnetic, thermal, optical and biomedical properties. High absorption coefficient, large surface area, ease of conjugation to various biomolecules and excellent biocompatibility make them suitable for several applications. These particles have found applications in sensing, diagnostics, treatment and catalysis[1–12]. Conventionally, AuNPs were prepared by reduction of tetrachloroauric acid with sodium citrate in aqueous environment[13–15]. The reduction essentially requires addition of sodium citrate to boiled solution of tetrachloroauric acid under constant stirring. Citrate ions provide negative surface charge to AuNPs by adhering to their surface, and this results in the stabilization of these particles. A number of other reducing and stabilizing agents such as EDTA, amines, glycerol, appin and hydroquinone have been employed for gold colloid preparation[16–20]. These reducing agents result in 10–50 nm sized AuNPs. Smaller sized (1.2–2.8 nm) AuNPs can also be prepared in organic phase by reduction of chloro (triethylphosphine) gold (Et_3PAuCl) with 9-borabicyco[3,3,1]nonane (9-BBN)[21]. Binding of electron-donating end group of a ligand such as poly (allylamine) hydrochloride or triphenylphosphine to the inorganic nanoparticle core has also been reported[17,21–23]. AuNPs are destabilized by cations, and this restricts their application within threshold limits of ion concentration. The reducing agent used in the present study is 1-amino-2-naphthol-4-sulphonic acid (ANSA), which may reduce tetrachloroauric acid as well as impart negative charge on the colloid surface (Figure 1). The high ionic stability of nanoparticles prepared by this method offers added advantage in comparison to gold colloids prepared by citrate reduction method. Size appropriateness is essential for *in vivo* applications. Particles smaller than 10 nm are likely to diffuse non-specifically into non-target tissues and organs, whereas particles larger than 100 nm are likely to get trapped in the liver and lung[24–26]. In comparison to citrate reduction, the method presented here provides nanoparticles of large diameter (35 nm) which is also advantageous in lateral flow techniques due to their better visibility without compromising on flow property.

Hydrogen tetrachloroauarate (III) ($HAuCl_4$) and sodium citrate tribasic dihydrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). ANSA was obtained from Sisco Research Laboratories Pvt Ltd, Mumbai, India. Rabbit immunoglobulin G (IgG) and goat anti-rabbit IgG (affinity-purified) were from Merck Millipore (Bengaluru). All other reagents were of analytical grade. The materials required for the fabrication of lateral flow strip (backing card glued with nitrocellulose membrane in the mid portion, sample pad and adsorbent pad) were procured from Advanced Microdevices Pvt Ltd (Ambala). All the glassware used for nanoparticles synthesis was treated overnight with aqua regia, rinsed with generous amounts of double-distilled water and then MilliQ water before use. The water employed in the study was MilliQ (18.2 MΩ). Particle size analysis was performed using Malvern Zetasizer ZS90, UK. Spectral analysis was performed using a UV-visible spectrophotometer (Shimadzu Analytical Pvt Ltd, Mumbai). TEM images were acquired using a transmission electron microscope (FEI TECNAI G2 F20, Oregon, USA).
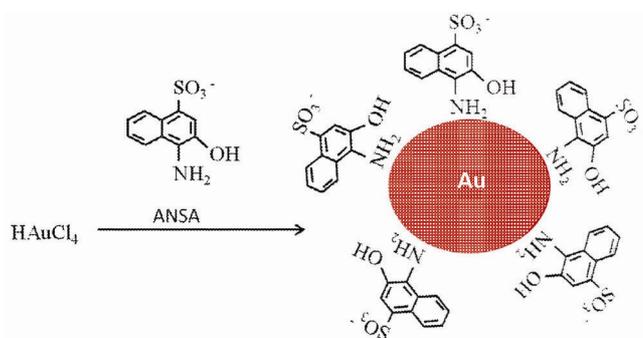

Figure 1. Depiction of synthesis and stabilization of gold nanoparticles (AuNPs) by 1-amino-2-naphthol-4-sulphonic acid (ANSA) from $HAuCl_4$.

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For the synthesis of AuNPs, 100 ml of 1 mM HAuCl$_4$ aqueous solution was added to a 250 ml flask and boiled. Under rapid stirring at 600–650 rpm, 10 ml of 38.8 mM ANSA was added quickly in a single step. Before adding, pH of ANSA solution was adjusted to 8.5 with 0.5 M NaOH. The colour of HAuCl$_4$ solution immediately changed from pale yellow to deep red. Boiling was continued for another 10 min. Then, the colloid solution was allowed to cool to room temperature (25–27°C) under stirring. Shape and size distribution of synthesized AuNPs–ANSA were characterized by TEM and particle size analyser respectively. For the calculation of concentration, extinction coefficient of nanoparticles was used as described by Liu et al.[27]. For comparison, nanoparticles were also synthesized using sodium citrate tribasic dihydrate as a reducing agent (AuNPs-citrate).

To check salt stability, 250 ml of AuNPs–ANSA synthesized as above was diluted with water to a concentration of 4 nM. Sodium chloride solution (25 μl) of appropriate molarity was added to obtain a final concentration of 30, 40, 50, 60, 70, 80 and 90 mM. AuNPs were photographed after 2 min. Salt stability of AuNPs–citrate was also checked under similar conditions.

For conjugation of rabbit IgG to AuNPs–ANSA, 5 μg rabbit IgG (dissolved in 5 μl MilliQ water) was added to 40 ml 1 nM AuNPs–ANSA and incubated for 1 h at room temperature (25–27°C) with continuous mixing on a rota-mixer.

For the construction of lateral flow strip, protective sheets at both ends of the backing card were peeled-off. Sample pad of size 26 mm × 6 mm and absorbent pad of size 26 mm × 6 mm were cut and pasted on backing sheet, such that one end of the sample pad overrides 5 mm from one end of the nitrocellulose membrane (30 mm × 6 mm), while one end of the absorbent pad overrides 5 mm on the other end of the nitrocellulose membrane. Then 2 μg of goat anti-rabbit IgG dissolved in water to a volume of 1 μl was applied as a streak on nitrocellulose membrane at the indicated point (Figure 2) and left for drying at room temperature for 30 min.
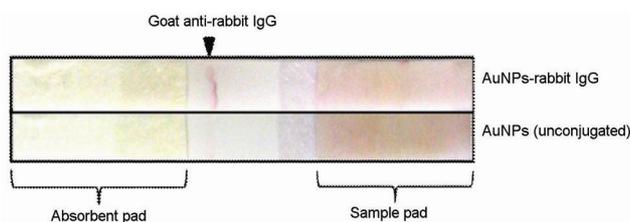
For the detection of anti-rabbit IgG on lateral-flow strip using functionalized AuNPs–ANSA, 500 μl of rabbit-IgG functionalized AuNPs suspension was added to 2.0 ml microcentrifuge tube. The lateral-flow strip was then placed in this tube vertically in such way that the lower end of the sample pad dips in AuNPs solution. Nanoparticles migrated on the strip towards the absorbent pad. Binding of functionalized AuNPs–ANSA with goat anti-rabbit IgG immobilized on the strip was then checked. Non-functionalized AuNPs–ANSA were also used for checking specificity of detection of interaction.

ANSA is a well-known reducing agent which carries negative charge on sulphonate group. Thus, this reagent has the potential to serve dual purpose of reducing gold chloride and imparting negative charge to AuNPs. These properties of ANSA are similar to citrate, which is the most widely used reducing agent for the preparation of AuNPs. Gold can bind to deprotonated nitrogen and thus ANSA can form a surface layer on AuNPs. The method of preparation of AuNPs using ANSA is similar to that using citrate. Both require addition of reducing agent to a boiled solution of HAuCl$_4$, under stirring. Colour change from yellow to deep red is immediate with both the reagents. This suggests that AuNPs are formed immediately. Negative charge on sulphonate group of ANSA imparts negative surface charge to AuNPs (Figure 1). AuNPS–ANSA exhibits plasmon resonance peak at 521 nm, a characteristic of AuNPs. ANSA is poorly soluble at acidic pH and therefore it needs to be solublized at alkaline pH prior to use.
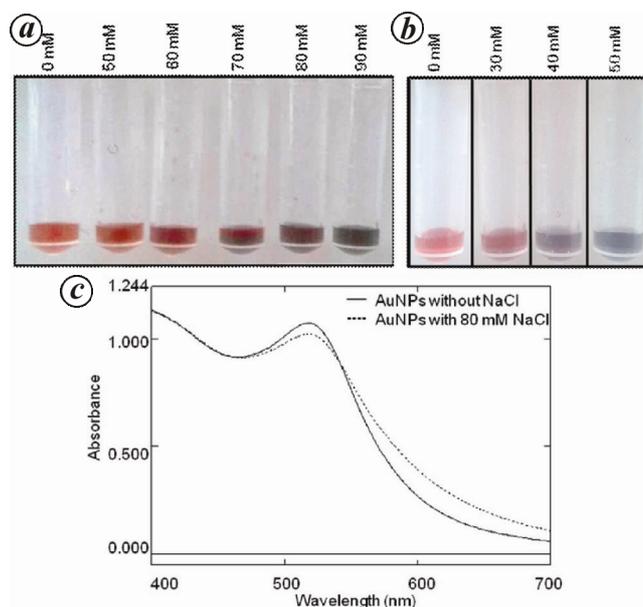

Figure 2. Flow of immunoglobulin G (IgG) conjugated AuNPs–ANSA through lateral flow strip and their binding to anti-IgG antibodies on the strip. Goat anti-rabbit IgG was immobilized on nitrocellulose membrane at the point indicated. Nanoparticle migrate in the direction from sample pad to absorbent pad. Deep red colour band was visible at the point of application of goat anti-rabbit IgG in lateral flow using IgG-conjugated AuNPs.

Figure 3. Effect of NaCl on colour and spectrum changes in AuNPs. *a*, Colour of AuNPs–ANSA in the presence of indicated NaCl concentration. *b*, Colour of AuNPs–citrate in the presence of indicated NaCl concentration. *c*, Visible spectra of AuNPs–ANSA in the presence (dashed line) or absence (solid line) of 80 mM NaCl.
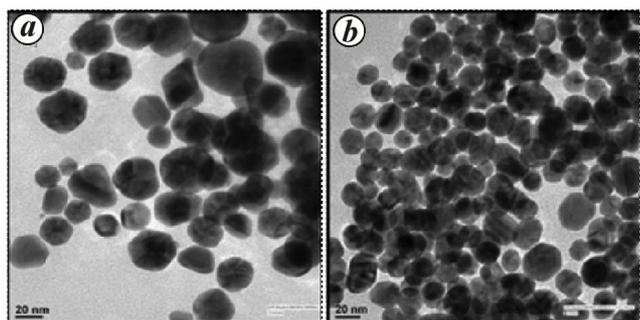
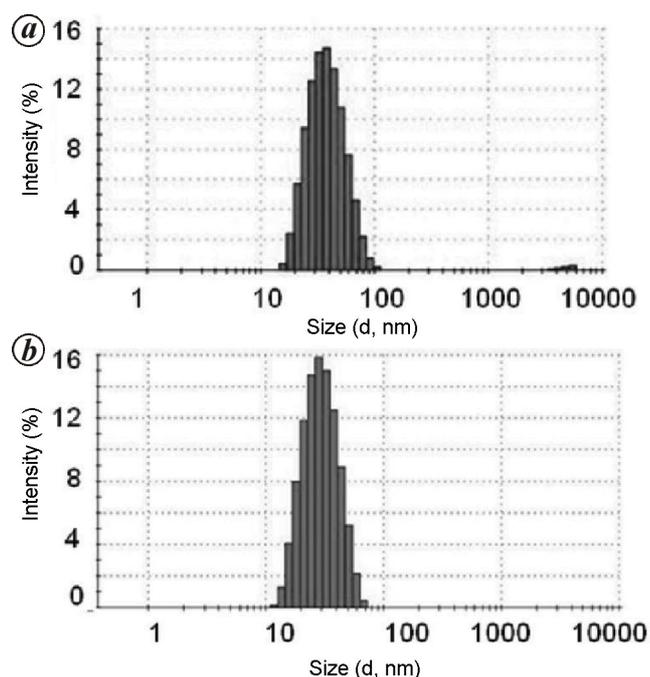**Figure 4.** Transmission electron microscopy of AuNP preparations: (*a*) AuNPs–ANSA and (*b*) AuNPs-citrate.

Figure 5. Size distribution of AuNPs (*a*) AuNPs–ANSA and (*b*) AuNPs–citrate.

AuNPs carry surface negative charge and therefore cations such as Na^+ can neutralize the charge. This results in aggregation of AuNPs which is reflected in colour transition from deep red to violet/blue/black. Poor ionic stability of AuNPs limits its use for *in vitro* and *in vivo* applications. Figure 3 *a* shows the stability of AuNPs–ANSA in the presence of variable concentration of NaCl. Although colour change is visible at 80 mM NaCl, it is distinct at 90 mM NaCl. Comparatively, AuNPs prepared by citrate can withstand 30 mM NaCl and particles aggregate at 40 mM NaCl (Figure 3 *b*). Thus, there is about twofold higher ionic stability of AuNPs–ANSA over AuNPs–citrate. Stability of AuNPs–ANSA in 80 mM NaCl can also be judged by visible spectra (Figure 3 *c*). Small drop in absorbance at 521 nm in 80 mM NaCl suggests that particles remain largely stable. Thus, AuNPs–

ANSA can withstand higher ionic concentration when in use for *in vivo* and *in vitro* applications. Nanoparticles synthesized by this method are stable even up to 30 days at 4°C, when stored in clean amber glass containers. The shape of AuNPs–ANSA as imaged using TEM is varied, viz. hexagonal, pentagonal, spherical, etc. while AuNPs–citrate are nearly spherical (Figure 4). The shape of particles influences their circulation in blood. More hexagonal nanoparticles remain in the blood than spherical particles (14% versus 2%) after 10 h (ref. 28), and therefore such nanoparticles are likely to work better for drug delivery. Figure 5 shows the typical particle size distribution of AuNPs–ANSA and AuNPs–citrate. Mean particle size of AuNPs–ANSA and AuNPs–citrate is 35.1 and 22.3 nm respectively. Standard deviation in size of AuNPs–ANSA and AuNPs–citrate prepared in three different batches varies from 15.04 to 16.55 nm, and 10.2 to 11.15 nm respectively. Larger-sized AuNPs provide better visibility in comparison to smaller sized particles and therefore, AuNPs–ANSA may be advantageous for visual detection in lateral flow-based diagnostic methods. Size of AuNPs–citrate can also be tuned by varying relative ratio of $HAuCl_4$ and citrate, and colloidal gold particles of 40 nm are better suited in lateral flow[29]. AuNPs–ANSA can be functionalized by coating with rabbit IgG and these particles interact with goat anti-rabbit IgG on lateral flow strip (Figure 2). AuNPs–ANSA after conjugation with IgG move freely through the pores of nitrocellulose membrane of strip, indicating that these particles do not aggregate on conjugation. Thus, AuNPs–ANSA can be functionalized with ligands for various applications.

Hydrogen tetrachloroaurate(III) on reduction with citrate leads to the formation of AuNPs, wherein citrate ion is present on the surface and imparts stability to the particles. Strong reducing agents result in precipitation of gold in solution. We have attempted controlled reduction of $HAuCl_4$ with ANSA, wherein amino nitrogen may result in Au(III) reduction and sulphonate is exposed imparting negative charge to the nanoparticles. This can be an alternate to the citrate method and due to high ionic stability, AuNPs–ANSA have the potential to even work better for certain applications.

The reduction of $HAuCl_4$ with ANSA could not be achieved in aqueous solution at room temperature. It appears that reduction by ANSA occurs only at high temperature. The possibility of reduction by intermediates formed from degradation of ANSA at elevated temperature cannot be ruled out.

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