AuAg-alloy-nanoprobes for Specific Nucleic Acid Detection

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ABSTRACT

The derivatization of gold-silver alloy nanoparticles with thiol-ssDNA oligonucleotides (AuAg-alloy-nanoprobes) and their use in nucleic acid detection is presented. A non-cross-linking method has been previously developed by our group using gold nanoparticles, which is based on the colorimetric comparison of solutions before and after salt-induced nanoprobe aggregation. Only the presence of a complementary target stabilizes the nanoprobe, preventing aggregation and colorimetric change after salt addition. Through this approach, the AuAg-alloy-nanoprobes allowed to specifically detect a sequence derived from the RNA polymerase β-subunit gene of *Mycobacterium tuberculosis*, the etiologic agent of human tuberculosis, with a 2.5-fold enhanced sensitivity (0.3 µg of total DNA) when compared to their gold counterparts.

Keywords: gold, silver, nanoparticles, diagnostic, nucleic acid

1 INTRODUCTION

Gold nanoparticles (NPs) derivatized with thiol-modified ssDNA oligonucleotides (Au-nanoprobes) have been extensively used for the development of new highly sensitive and selective molecular diagnostic assays for DNA/RNA, mainly due to their unique optical properties [1,2]. These optical properties derive from the characteristic surface plasmon resonance (SPR) band that can be easily tailored through the synthesis of NPs with different metal composition, either in an alloy or core-shell structure, e.g. different gold:silver ratios [3]. Silver NPs also present excellent proprieties for biodetection, allowing for an enhanced sensitivity in DNA detection when compared to their gold analogues [4]. However, existing protocols for silver NPs functionalization with thiol-modified oligonucleotides are cumbersome and time consuming, resulting in nanoprobes with limited stability [4-7]. To circumvent this limitation, Cao and co-workers used Ag-Au core-shell NPs, which present the advantage of ease of derivatization of the gold surface and the enhanced SPR extinction coefficient of silver, allowing the application of common strategies used in Au-nanoprobe assembly [8,9]. Nonetheless, AuAg-alloy NPs can be more easily synthesized than their core-shell counterparts, via a simple citrate co-reduction method [10], while still exhibiting the remarkable functional properties of core-shell NPs. Additionally, alloy NPs are not prone to inter-layer core-shell metal diffusion that can alter the optical properties of the NPs [11].

Here we report the synthesis of AuAg-alloy NPs and their derivatization with thiol-modified ssDNA oligonucleotides (AuAg-alloy-nanoprobes) for application in molecular diagnostics. The AuAg-alloy-nanoprobes were used to specifically detect a sequence derived from the RNA polymerase β-subunit gene of *Mycobacterium tuberculosis*, the etiologic agent of human tuberculosis. Complementary targets were detected by means of a non-cross-linking method that has been previously developed by our group using Au-nanoprobes [12]. The assay consists of the spectrophotometric comparison between solutions before and after salt induced nanoprobe aggregation, where only the presence of a complementary target stabilizes the nanoprobe, thus preventing aggregation and colorimetric changes.

2 MATERIALS AND METHODS

All chemicals were from Sigma-Aldrich and of the highest purity available. All thiol-modified oligonucleotides were purchased from STAB Vida, Lda (Portugal).

2.1 AuAg-alloy nanoparticles synthesis

Gold-silver alloy nanoparticles (Au:Ag molar ratio of 0.5:0.5) were prepared by a citrate reduction method adapted from Link *et al* [10]. Briefly, 25 mg of $HAuCl_4 \cdot 3H_2O$ and 10.8 mg of $AgNO_3$ were dissolved in 250 mL of milli-Q H_2O and brought to a boil while stirring in a 500 ml round-bottom flask. Twenty-five milliliters of 1% sodium citrate were quickly added and the mixture was refluxed for 15 min with continuous stirring. The flask was left to cool down to room temperature and the solution was centrifuged at 233 g for 20 min to remove any silver precipitate and further filtered through a 0.2 µm nylon filter (Whatman, USA). Gold-silver alloy nanoparticles were

characterized by Transmission Electron Microscopy (TEM) and UV-visible spectroscopy. The elemental composition (i.e. Au:Ag ratio) was determined by Inductively Coupled Plasma (ICP) upon dissolving the nanoparticles in freshly prepared *acqua regia*.

2.2 AuAg-alloy-nanoprobes synthesis

The AuAg-alloy NPs were functionalized with a thiol-modified 16mer oligonucleotide [5'-thiol-$(CH_2)_6$-GGA CGT GGA GGC GAT C-'3] derived from the *M. tuberculosis* RNA polymerase β-subunit gene sequence (GenBank accession no. BX842574), following a previously described protocol for gold NPs [13]. Briefly, 10μM thiol-modified oligonucleotide was added to AuAg-alloy NPs in a ratio of 1:3500 NPs per oligonucleotide, in phosphate buffer 10 mM (pH8) with 0.01% sodium dodecyl sulfate (SDS). The solution was incubated at room temperature for 20 min and the concentration of NaCl increased to 0.05 M using phosphate buffer 10 mM (pH8), 1.5 M NaCl, 0.01% SDS. The solution was then submitted to ultrasounds for 10 sec. This salt aging process was repeated three times with increments of 0.1 M NaCl until a concentration of 0.3 M NaCl was reached. After a final incubation over-night at room temperature, the solution was washed three times and ressuspended in phosphate buffer 10 mM (pH8), 0.1 M NaCl. Functionalization was assessed by evaluation of the UV spectra upon increasing concentrations of NaCl or $MgCl_2$, and further confirmed using an oligonucleotide intercalating dye – Oligreen (Invitrogen, USA), after displacement of the immobilized thiolated-oligonucleotides with 100mM dithiotreitol (DTT).

2.3 Non-cross-linking assay

A set of two ssDNA oligonucleotides were used as a complementary and non-complementary targets (MycPOS, 5'-GGC CGC TGC GGC GGG GCT CAG ATC GCC TCC ACG TCC-3' and MycNEG, 5'- TGG ATT TAA GCA GAG TTC AAA TCT GTA CTG CAC CCT GGA G -3', respectively). Additionally, total DNA samples retrieved from clinical isolates previously tested positive or negative for *M. tuberculosis* with the INNO-LiPA-Rif-TB kit were prepared as described for the Au-nanoprobe assay [12].

Assay solutions containing the AuAg-alloy-nanoprobe and target DNA were prepared by mixing an appropriate amount of target DNA (up to 100 nM or 36 μg/mL of ssDNA oligonucleotide or total DNA, respectively) with AuAg-alloy-nanoprobe (final concentration 15 pM) in 10 mM phosphate buffer (pH8). After 10 min of denaturation at 95ºC, the mixtures were allowed to stand for 30 min at room temperature and $MgCl_2$ was added to a final concentration of 140 mM. Blank measurements were made in exactly the same conditions but replacing the target with an equivalent volume of 10 mM phosphate buffer (pH8). UV-visible spectra of solutions were registered 15 minutes upon salt addition using a UV-Vis Spectrophotometer Cary 50 (Varian, USA) and an Ultra-Micro quartz cell (Hëllma, Germany). Digital photographs were also taken using a Canon 450D SLR digital camera (Canon, USA). Each assay was repeated at least three times.

3 RESULTS AND DISCUSSION

Gold-silver alloy NPs can be synthesized with different ratios of each metal, where increasing molar fractions of silver leads to a typical blue-shift of the SPR absorption band peak [10]. Previous attempts on surface modification of ~20 nm AuAg-alloy NPs (16% Au:84% Ag) with thiolated oligonucleotides have not been able to deliver stable conjugates that could be used for biodetection [8]. To try enhance the functionalization of the alloy NPs, we have increased the gold fraction to 50%, while still holding enough silver to increase the nanoparticles' extinction coefficient by five-fold when compared to the gold analogues with approximately the same diameter [10]. Nonetheless, the resulting 25 nm AuAg-alloy NPs (47% Au:53% Ag) were also unable to be successfully functionalized with thiol-modified oligonucleotides, and aggregated during the salt aging procedure.

We then hypothesized that decreasing the citrate/metal ratio, while maintaining the 50% Au: 50% Ag ratio could enlarge the resulting NPs and increase the gold availability at the surface, thus increasing the available surface for easy derivatization with the thiol-modified oligonucleotides.

This approach yielded stable 42 nm AuAg-alloy NPs (49% Au: 51% Ag) – see Figure 1 – that could be successfully functionalized with thiol-modified oligonucleotides via a similar procedure of that described for the synthesis of Au-nanoprobes [13]. These AuAg-alloy-nanoprobes presented an increased stability to salt-induced aggregation when compared to the AuAg-alloy NPs alone, withstanding a saline concentration up to 2 M NaCl (Figure 2). In the presence of 2 M NaCl, AuAg-alloy NPs aggregated and their initial yellow color changed to blue due to a red-shift from ~460 nm to >600 nm of the SPR absorbance band.

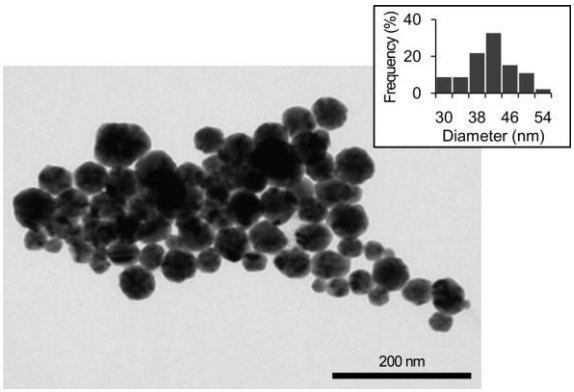

Figure 1. TEM image and size histogram (inset) of AuAg-alloy NPs with an average diameter of 42 nm.

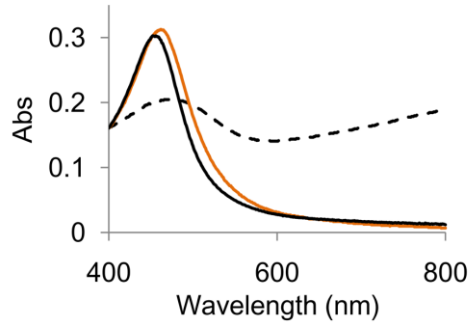

Figure 2. UV-visible absorption spectrum of AuAg-alloy NPs alone (black full line) and AuAg-alloy NPs (black broken line) or AuAg-alloy-nanoprobes (orange full line) in presence of 2 M NaCl.

This increase of resistance to salt induced aggregation constitutes an indication of the success of functionalization, as it has been previously demonstrated for pure gold NPs functionalization [14]. The yield of thiol-ssDNA immobilization at the NPs surface was determined to be 14%, as evaluated by the Oligreen Fluorescent dye.

To further confirm the functionalization of the AuAg-alloy NPs and test their capability to detect a specific nucleic acid sequence, the AuAg-alloy-nanoprobes were used in hybridization reactions with ssDNA oligonucleotide targets (MycPOS and MycNEG) following a non-cross-linking method previously described for Au-nanoprobes [12,14]. After salt addition, only the AuAg-alloy-nanoprobes in presence of a complementary target (MycPOS) retained their initial yellow color, while the nanoprobes alone or in presence of a non-complementary target (MycNEG) aggregated and changed color from yellow to blue – see Figure 3. Moreover, the AuAg-alloy-nanoprobes remained unaltered when in presence of up to 70 fmol complementary ssDNA target.

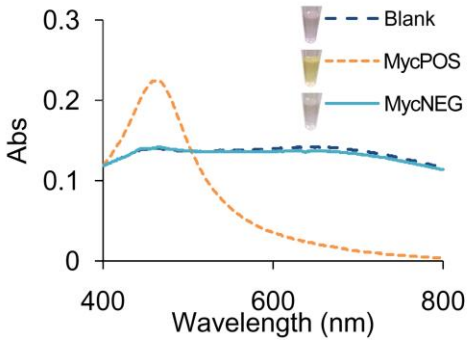

Figure 3. UV-visible absorbance spectrum and digital photographs of AuAg-alloy-nanoprobes in the absence (Blank) or presence of complementary (MycPOS) and non-complementary (MycNEG) DNA targets, registered 15 min after salt addition.

To test the AuAg-alloy nanoprobe robustness and compare their sensitivity with the previously described Au-nanoprobe for *M. tuberculosis* detection [12], a set of clinical DNA samples were tested. As it has been observed for the ssDNA oligonucleotide targets, only the samples that were tested positive for *M. tuberculosis* (as determined by the INNO-LiPA-Rif-TB kit) remained unaltered upon salt addition, for a total DNA sample as low as 0.3 µg. The nanoprobe alone and in presence of samples that tested negative for *M. tuberculosis* aggregated and changed color due to the concomitant red-shift in their SPR absorption peak. These results indicate that AuAg-alloy nanoprobes behave similarly to Au-nanoprobes, but potentiate a 2.5-fold increase of sensitivity - 0.3 and 0.75 µg of total DNA for AuAg-alloy and Au-nanoprobes, respectively.

4 CONCLUSIONS

A simple method for synthesis of gold-silver alloy nanoparticles and their functionalization with thiol-modified oligonucleotides was presented. The resulting AuAg-alloy-nanoprobes demonstrated a combination of the optical properties of Ag nanoparticles (i.e. high extinction coefficient) with the ease of functionalization via a thiol bond provided by the gold, allowing to increase the sensitivity of the non-cross-linking method previously developed for Au-nanoprobes. These AuAg-alloy-nanoprobes can in principle be used in a plethora of other methods and applications towards nanodiagnostics and, combined with other nanoprobes (e.g. Au-nanoprobes), may allow the development of a multiplex method for the specific detection of multiple DNA sequences in a one pot reaction.

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