RESEARCH ARTICLE

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Preparation of Stable and Optimized Antibody-gold Nanoparticle Conjugates for Point of Care Test Immunoassays

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Immunoassays are the most reliable andcost-effective for the diagnosis of various diseases also the common platform that helps us identify the unknown protein in a short period of time. The market is flooded with different types of diagnostic immunoassays but it is necessary to develop more cost-effective immunoassay with increased performance. The major cost factor in this regard is the quality and amount of biomolecules that are being utilized in the product. Optimization of biomolecules can aid us in developing cost-effective products without compromising its performance. This study aims to optimize the maximum amount of antibodies that can be conjugatedon commonly used 40 nm gold nanoparticles that are indicators for the conclusion of test results. The excess usage of antibody may increase unnecessary product cost as it may get eliminated during the washing steps, while the lower amount of antibody usage may reduce the product's performance in terms of sensitivity and stability. This study helps the manufacturer and researchers to find the optimum value of antibody molecules that can be conjugated on the surface of 40 nm gold nanoparticles. According to this study, ~3.4 µg of antibody molecules is sufficient to saturate the surface of 40 nm gold nanoparticles of optical density 1.0.

Introduction

The most common platform for the diagnosis of various diseases such as Malaria, Tuberculosis, Hepatitis, Syphilis, etc. has been shifted to Rapid Immunochromatographic assays such as Lateral Flow Test and Flow-Through (Vertical Flow) Test format [**1**]. The lateral flow test was introduced in the late 1980s. Since then, it has been developing and playing an important role in the POC test market. Most of the lateral flow or flow-through tests utilize colloidal gold conjugation with specific biomolecules as a detection system. These tests are more convenient to use due to their reliability and easy procedures with low cost and rapid results without a specialized person for performing the assay [**2,3**].

A typical lateral flow test comprises of a sample pad, absorbent pad, glass fiber as conjugation pad, nitrocellulose membrane, detecting biomolecules (antigen or antibodies) and colloidal gold conjugation with specific proteins. Among all the materials described above, the core and costliest part of technology is the detection system that is colloidal gold conjugation [**4**].

Several methods are available for the diagnosis of Malaria, each having its own advantages and disadvantages. The foremost method of diagnosis being Microscopy. It remains the "gold standard" for the laboratory confirmation of Malaria. The test can be performed in any lab facility that can perform routine hematology tests. It directly detects Malarial parasite in the thick or thin blood smear on a slide. However the main disadvantage of this test being reliability and proficiency of personnel performing the test. An average lab professional does not perform this test regularly thus optimal proficiency cannot be maintained [**5**]. Another method is in the form of rapid immunochromatographic assays (RDTs) or Lateral flow Immunoassays (LFIA). These are available in dipstick or cassette format and can provide results in 2-20 minutes of time. These tests provide a useful alternative where Microscopy cannot be performed. Malaria lateral flow tests have proven of great significance in resource-poor settings where lab facilities are unavailable and diagnosis needs to be performed. They have proven a reliable alternative to Microscopic determination of Malaria, detecting the antigen of the Malarial parasite. The Malaria lateral flow tests detect the HRP2 and/or pLDH antigen of Malarial parasite for the detection and diagnosis different type of infection from different species of Malarial parasite viz. plasmodium falciparum, plasmosium vivax, plasmodium ovale, and plasmodium malariae [**5**].

Alternatively, another method is also available like Molecular Diagnosis which detects parasite nucleic acids and is slightly more sensitive than Microscopy method. However, it requires very sophisticated instrument labs

and well-trained professionals to perform the test. The time of diagnosis also is increased due to the process involved [**5**]. Enzyme-linked immunosorbent assay (ELISA) and immune-fluorescence are also utilized for the diagnosis of Malaria,but it also requires sophisticated instruments and lab professionals to perform the test. Apart from it, the major disadvantage is that it measures past exposure instead of current infection. This may mislead with the diagnosis and prognosis of the disease.

Generally, the Malaria lateral flow tests or RDTs available in the market are the antigen detecting tests that utilize anti-HRP2 antibody and anti-PLDH antibody conjugation to colloidal gold to detect HRP2 and pLDH antigen of P. *falciparum*, P. *vivax*, P. *malariae*, P. *ovale* respectively [**6**]. Currently the conjugation of antibodies to gold nanoparticles has no specific concentration defined. The researchers vary the ratio as per required varying from 15-125 mg/mL [**7**]. The ratio of the amount of antibody used in the conjugation process to the gold nanoparticle concentration is very critical. Unoptimized antibody to colloidal gold ratio may lead to drawbacks viz. if lower concentration of antibody is conjugation with colloidal gold than the sensitivity of the test is compromised and also the chances of loss of stability over time; whereas higher concentration antibody conjugation colloidal gold may lead to wastage of excess antibody molecules during the washing and concentration step during the conjugation process. Also, if less washing steps are used then un-conjugated excess antibody molecules will not be removed from the conjugation system and may interfere in the assay by producing non-specific/undesired reactions. Therefore, it is mandatory to optimize this ratio of antibody to the colloidal gold to develop a cost-effective and reliable LFIA system. This research specifically states and analyze the amount of anti-malarial antibody required for gold conjugation and thus effectively aid in developing a better and cost-effective antigen detection system for the malaria lateral flow tests.

Materials

Chemicals required

All the chemicals purchased were of analytical grade and used as it is without any further purification.

Gold chloride, Trisodium citrate dihydrate, boric acid, disodium tetraborate decahydrate, sodium chloride, sodium azide, di-sodium hydrogen orthophosphate, mono-sodium di-hydrogen orthophosphate, triton x100 were obtained from SRL Chemicals, monoclonal anti-HRP2 antibody (Type: IgG) and bovine serum albumin (BSA) were procured from IRIS NANOTECH. All solutions were prepared in de-ionized and double distilled water.

Lateral flow test components

Nitrocellulose membrane (pore size: 10 microns), sample pad, absorbent pad, conjugation pad (glass fiber), plastic cassette (Top & Bottom) were all procured from IRIS NANOTECH.

Instruments

Magnetic stirrer cum Hot plate, pH meter, Centrifuge, Spectrophotometer, and the particle size analyzer.

Experiment

Preparation of 40 nm colloidal gold solution

The gold nanoparticles of 40 nm were prepared by a typical method mentioned in [**8**]. Briefly, a 100 mL of 0.01% gold chloride solution was heated till boiling with continuous stirring. To this solution, 1.0 mL of 1% aqueous solution of Trisodium citrate dihydrate was added rapidly. After 10 minutes of boiling a color change was observed from faint yellow to magenta red indicating the formation of gold nanoparticles. The solution was then kept to cool down at room temperature in an amber colored glass container. The prepared colloidal gold solution was then analyzed by UV Visible Spectrophoto-meter from the wavelength of 400-700 nm range. The maximum absorbance value of the sample was found at 526 nm and optical density was observed to be 0.936.

The particle size of the prepared colloidal 5 gold solution was measured by Malvern Zetasizer nano (model: ZS90), which was observed 39.18 nm with polydispersity index value (PDI) 0.187.

The colloidal gold solution was processed for the conjugation with the monoclonal anti-HRP2 antibody.

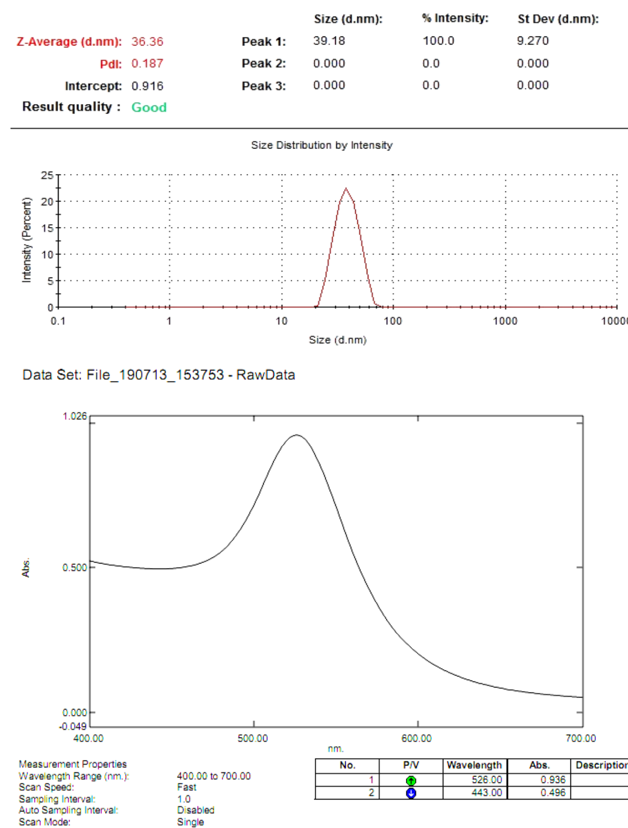

Fig. 1. Particle Size Analysis in Malvern Zetasizer and Spectrophotometric analysis from 400nm to 700nm of 40 nm Raw Gold Nanoparticles.

Gold to antibody binding titration

The procured anti-HRP2 antibody was dialyzed by 50kDa dialysis membrane in 2mM borate buffer of pH 8.00 to remove extra molecules such as preservatives and stabilizers to prevent their interference during conjugation.

To a series of 10 test tubes, 100uL of 2mM borate buffer of pH 8.00 was taken. To this buffer anti-HRP2 antibody of different concentration ranging from 1µg to 10 µg was added and mixed properly for 5 minutes to homogenize the solution. 1 mL of ph 8.00 adjusted raw colloidal gold solution was added in each tube with proper mixing and incubated for 4 hours at room temperature for effective binding of antibodies to gold particles [**9**]. The binding of the antibody to gold nanoparticles was confirmed by spectrophotometric and particle size analysis as shown in **Table 1**.

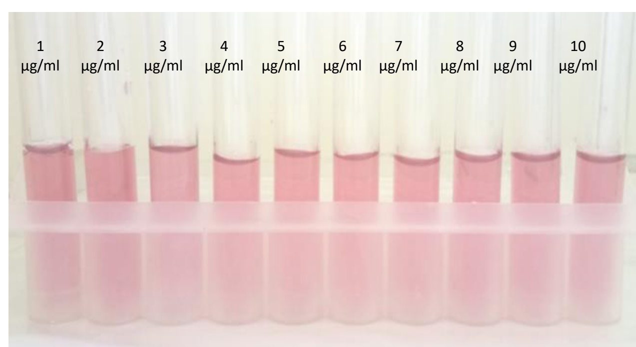

Fig. 2. 40 nm colloidal gold conjugated with different concentration of Anti Malarial Antibodies.

A Freshly prepared 10% solution of Sodium Chloride was added to all the test tubes with the final concentration of 1% w/v and color change was observed from magenta red to purple and light gray indicating the aggregation of un-conjugated or incomplete conjugation colloidal gold with antibody molecules. This happens as a result of the positive charge of electrolyte solution (1% NaCl Final Concentration) that destabilizes the negatively charged colloidal gold solution [**10**].

After the addition of sodium chloride, all the samples were again analyzed by spectrophotometer and particle size analyzer and their respective data are listed in **Table 2**.

Table 3. Optical Density, Absorbance max and particles size of colloidal gold conjugated with different concentration of anti-malarial antibodies after addition of NaCl. We can observe aggregation in Gold NPs with 1 µg and 2 µg antibody added conjugates, a minor aggregation in 3 µg antibody added conjugate and no aggregates were observed from 4-10 µg of gold conjugate solution.

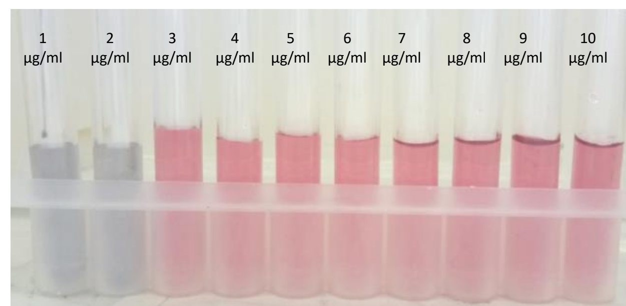

Fig. 3. Color change of 40 nm colloidal gold conjugated with different concentration of anti-malarial antibodies after addition of NaCl.

A further experiment was carried out, taking 1mg/mL solution of anti-HRP2 antibody in 2mM borate buffer of ph 8.00. The concentration of this solution was confirmed by measuring its absorbance at 280 nm in UV Visible spectrophotometer. To this solution, 9.00 mL of above prepared 40 nm colloidal gold solution ph 8.00 was added and mixed properly for 10 minutes for homogenizing and effective binding of antibody molecules to gold nanoparticles. This 10 ml final conjugation solution was then centrifuged at 7000 RPM for 4 hours to completely remove the conjugation particles from the solution. The supernatant was then again analyzed by UV Visible spectrophotometer to find the remaining protein concentration.

Table 1. Optical Density, Absorbance max and particles size of colloidal gold conjugated with different concentration of anti-malarial antibodies after addition of NaCl. We can observe aggregation in Gold NPs with 1 µg and 2 µg antibody added conjugates, a minor aggregation in 3 µg antibody added conjugate and no aggregates were observed from 4-10 µg of gold conjugate solution.

Concentration of antibodies in µg (after addition of 1.0 % NaCl)												
		Raw Gold NPs	1 µg	2 µg	3 µg	4 µg	5 µg	6 µg	7 µg	8 µg	9 µg	10 µg
Observed OD		0.936	0.591	0.535	0.930	1.027	1.037	1.050	1.054	1.056	1.059	1.062
Peak Max		526	541	537	528	528	529	529	529	529	529	529
Particle Size	Peak 1	39.18	666.9	529.1	51.10	52.29	57.16	53.80	51.75	61.25	57.57	55.51
	Peak 2	NA	267.6	148.6	144.6	0	0	0	0	0	0	0

Table 2. Optical Density, Absorbance maximum and Particle size of colloidal gold, conjugated with different concentration of antimalarial antibodies. It is clearly observed as the antibody molecules are binding with the gold particles, the particle size is increasing up to3 µg antibody conjugation while it remains nearly constant onwards 4-10 µ concentration of antibodies.

The concentration of antibodies in µg (before the addition of NaCl)											
	Raw Gold NPs	1 µg	2 µg	3 µg	4 µg	5 µg	6 µg	7 µg	8 µg	9 µg	10 µg
Observed OD	0.936	0.987	0.994	1.070	1.087	1.090	1.092	1.091	1.091	1.091	1.091
Peak Max (nm)	526	526	526	528	529	528	529	529	529	529	529
Particle Size(nm)	39.18	39.68	40.77	50.90	56.30	54.09	52.23	54.64	54.07	55.47	57.50

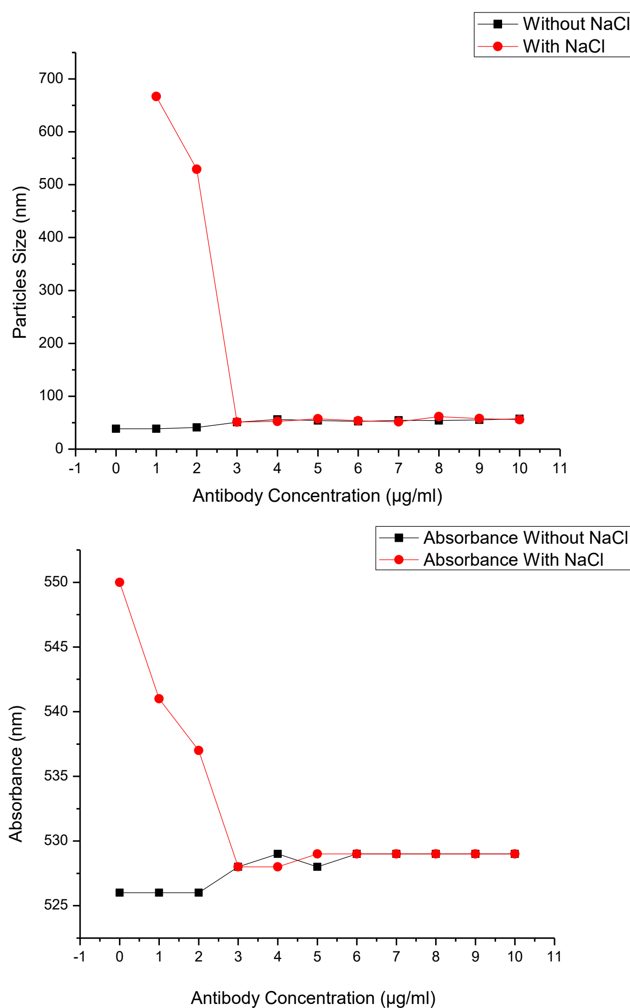

Fig. 4. Effect of Sodium Chloride (NaCl) on particle size and absorbance of gold nanoparticles conjugated with anti-malarial antibodies of different concentrations.

Conjugation

To identify the effect of gold to antibody conjugation with a different concentration on the performance of lateral flow test, six different concentrations were selected for colloidal gold conjugation and performed a real-time lateral flow test with confirmed malaria falciparum positive sample. To a series of six different test tubes, 1.0 ml of 2.0 mM borate buffer of pH 8.0 was taken. To this buffer solution, different antibody solution ranging from 20.0 µg, 30.0 µg, 40.0 µg, 60.0 µg, 80.0 µg& 100.0 µg were added respectively. The solution was mixed properly without forming the foam for 10 minutes to homogenize the solution. To this solution, 10.0 ml of 40 nm colloidal was added with proper mixing and incubated for 1.0 hour. A solution of freshly prepared 10.0 % W/V BSA was then added to the final concentration of 1.0 % to block the unoccupied site of colloidal gold to prevent nonspecific interactions. The solution was incubated for 4 hours and then centrifuged at 8000 rpm for 4 hours to remove the excess biomolecules and also to concentrate the gold conjugation [**9**]. The final OD of gold conjugation was adjusted to 20.0 by 2.0 mM borate buffer of pH 8.0 containing 0.1 % of Sodium Azide as a preservative and 0.1 % BSA as a stabilizer. The finally prepared gold conjugation was then sprayed on conjugation pad of glass fiber and dried for 30 minutes at 37 ºC. The final gold conjugation sprayed pad was then cut and assembled into a lateral flow test device format for the testing purpose.

Results and discussion

In case of titration experiment, it was observed that colloidal gold particles were binding with the antibody molecules by either electrostatic interaction, the dative hydrophobic bond formation of just by physical adsorption of molecules on the surface of gold nanoparticles. There are also chances of Gold – sulfur bond formation between gold to thiol group-containing amino acids of the antibody molecules. Furthermore, the maximum absorbance of the conjugation particles is shifting from 526 nm to 529 nm indicating the formation of gold-antibody complex and increasing the particle size of colloidal gold. The increase in the particle size was also confirmed by particle size analyzer and observed that the size was increased from 39.18 nm to 54.40 nm averagely for all concentration conjugation gold particles.

The addition of sodium chloride lead to the particle aggregation and the observed data clearly indicates that the colloidal gold conjugation at lower concentration then 3 µg/ml has more shifting of absorbance max value from 526 nm to 541 nm & 537 nm in 1µg and 2 µg gold conjugate respectively, indicating the bigger particles size of aggregates, whereas the colloidal gold conjugated at 3.0 µg/ml did not show any shift in absorbance max value but minor aggregation was observed in it. This indicates that the surface of colloidal gold of 1 O.D. is almost covered by the antibody molecules at the concentration of 3.0 µg/ml. As it is observed that in case of colloidal gold conjugated with antibodies with 3.0 µg/ml, there is no color change in absorbance value but in particle size analysis report, a secondary peak of 144.6 nm of aggregated gold nanoparticles is observed due to effect NaCl. Therefore, the optimum concentration of antibody molecules to saturate the surface area of 40 nm gold nanoparticles of 1 O.D is falling between 3.0 µg/ml to 4.0 µg/ml. Also, we can also observe that there is no change in particle size or absorbance max after the addition of sodium chloride to the colloidal gold conjugate at higher concentration than 4.0 µg/ml.

In the case of the second experiment, the concentration of antibody in the supernatant solution was estimated by using the same method and observed that nearly 30.6µg of antibody concentration has been decreased from its initial concentration. Therefore, 30.6µg/9.0 ml colloidal gold also indicates that 3.4µg/ml is the maximum concentration of antibody molecules that can be conjugation to 40 nm colloidal gold of OD 1.0.

Finally, the assay was performed by using malaria falciparum positive specimen using the same protocol

mentioned in the commercial kits with the manually assembled devices to check the intensity of different concentration conjugation and sprayed gold conjugation. It is clearly visible that device that contains the low concentration as 2.0 µg/ml antibodies to colloidal gold conjugation colloidal gold showed less intensity while 3.0 µg/ml antibodies to colloidal gold conjugation colloidal gold showed darker intensity. It was also observed that all the colloidal gold conjugation at 4.0 µg/ml or higher concentration also showed the same intensity as 3.0 µg/ml as shown in **Fig. 5**.

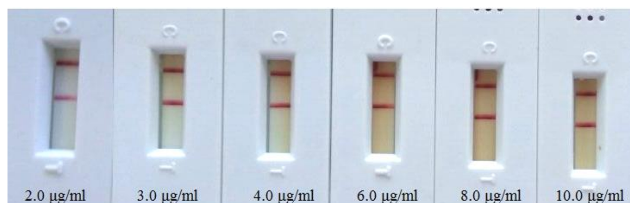

Fig. 5. Performance test of 40 nm colloidal gold conjugated with different concentration of anti-malarial antibodies from 2.0 µg/ml, 3.0 µg/ml, 4.0 µg/ml, 6.0 µg/ml, 8.0 µg/ml and 10.0 µg/ml with same malaria positive sample.

Protein Concentration before conjugation (Stock): 1.000 mg/ml

Protein Concentration after Conjugation (Supernatant): 0.964 mg/ml (Diluted 1:10 due to addition of Colloidal Gold)

Here the difference is 1.000mg/ml - 0.964mg/ml = 30.6µg

30.6 µg/9.0 ml colloidal gold = 3.4 µg/ml

Therefore we can consider that 3.4 µg/ml is the best concentration to completely conjugate the 1.0 O.D 40 nm colloidal gold with IgG antibodies.

Conclusion

As a conclusion, ~3.4µg/ml of IgG antibody to colloidal gold would be the best concentration to saturate the entire surface ratio of 40.0 nm gold nanoparticles having concentration of OD 1.0 of maximum absorbance i.e. 526 nm and is the best concentration to develop a reliable and cost-effective IgG antibody-based lateral flow test for the detection of various diseases.

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Keywords

40 nm Gold nanoparticles (Gold NPs), gold conjugate, anti-malarial antibody, lateral flow immunoassay.

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