ORIGINAL ARTICLE

Gold-nanoparticles antibody conjugates optimization in a lateral flow immunoassay for multivariants SARS-CoV-2 biosensor

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ABSTRACT

Introduction: The optimization of multivariate detection systems in lateral flow assays using gold nanoparticles (AuNPs) conjugates represents a pivotal advancement in the field of point-of-care diagnostics. Lateral flow immunoassays (LFIA) are widely employed due to their simplicity, rapidity, and cost-effectiveness; however, enhancing their sensitivity and specificity remains a challenge. The research involves the investigation of key parameters influencing the performance of antibody conjugation through physical adsorption methods, such as pH value of AuNPs, concentration of antibody (Ab) as well as incubation time. **Materials and Methods:** S1 Delta and Omicron antibody of SARS-CoV-2 were used as the recognition element. The LFIA platform was developed through a series of optimization steps, including the selection and conjugation of appropriate pH for AuNPs, optimal concentration specific S1 Antibody (Ab-S1) and optimal incubation time for AuNPs conjugates. The performance of the LFIA was evaluated using specific S1 Delta and Omicron antigens. **Results:** The findings underscore the significance of optimum pH value which are pH 8.0 and concentration of Ab which are 8 µg/mL as well as 1 hour of specific incubation time to be optimal in multivariate lateral flow detection that able to maximize conjugation efficiency providing valuable insights for diagnostic assays. **Conclusion:** The results from this project highlight the complexity and sensitivity of LFIA creation as well as the significance of thorough validation and optimization methods respectively.
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INTRODUCTION

On 30th January 2020, the World Health Organization (WHO) declared the Severe Acute Respiratory Syndrome Coronavirus Type 2 (SARS-CoV-2) epidemic a public health emergency of international concern (1). SARS-CoV-2 is a single-stranded RNA virus belonging to the betacoronavirus 2B lineage (1,2), with four structural proteins: nucleocapsid (N), spike (S), membrane (M), and envelope (E) (3). Since the outbreak, various "variants of concern" have emerged, such as the Omicron variant, which has 30 mutations in its spike protein, including 15 in the receptor-binding domain (RBD) (4). Omicron's spike protein demonstrates a higher binding affinity to the angiotensin-converting enzyme 2 (ACE2) receptor, contributing to its enhanced transmissibility compared to other variants like Delta (5).

Lateral flow immunoassays (LFIAs), widely used for point-of-care (POC) diagnostics, detect specific viral proteins, such as spike, membrane, or nucleocapsid proteins (6). Unlike antibody-based tests that rely on the hosts' immune response, antigen LFIAs directly detect viral proteins. A key component of these tests is nanoparticle-antibody conjugates, which allow for colorimetric detection (7). Among various nanoparticles, gold nanoparticles (AuNPs) are favoured due to their unique optical properties, biocompatibility, and stability (8). The characteristic red hue of AuNP suspensions, due to localized surface plasmon resonance (LSPR), makes them effective in biosensing applications (9). Moreover, the electrostatic and physical adsorption between AuNPs and antibodies is most stable when the pH of the reaction solution is near the antibody's isoelectric point (13). Optimizing pH minimizes charge repulsion and enhances the efficiency of conjugation (14). Similarly,

determining the optimal antibody concentration is essential for achieving efficient and specific binding. Low antibody concentrations may result in weak interactions, while excessive concentrations could lead to non-specific binding and reduced assay accuracy (15). Additionally, the study investigates incubation times to improve conjugation efficiency. Previous research suggests that a 30-minute incubation period provides the best results for sensitivity (16). The antibody-nanoparticle conjugate formation can be optimised throughout the incubation process, which also improves the assay's specificity. Antibodies need at least 30 to 2 hours of incubation time at room temperature to conjugate with nanoparticles (2).

Despite the wide use of Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) for SARS-CoV-2 detection, the method is time-consuming and costly, requiring specialized equipment and expertise (3, 18). RT-PCR, though considered the "gold standard," is not well-suited for distinguishing between SARS-CoV-2 variants like Delta and Omicron. Current diagnostic techniques do not typically differentiate between these variants without advanced viral sequencing or multiplex PCR genotyping, which are not commonly used in clinical settings (19). There is an urgent need for accurate, affordable, and rapid diagnostic tests to quickly identify infections and improve SARS-CoV-2 control (20). Given the need for fast, accurate, and cost-effective diagnostic tools, this study aimed to optimize LFIA for detecting both Delta and Omicron variants. While LFIAs have been essential in controlling the spread of COVID-19, they cannot distinguish between variants (21). Developing a multiplex LFIA, capable of detecting multiple targets simultaneously, could enhance POC testing by improving sensitivity and reducing costs (15).

The optimization of AuNPs for conjugation with SARS-CoV-2 S1 specific antibody presents a critical challenge due to the complex interplay of variables, including pH, antibody concentration, and incubation time. To maximize conjugation efficiency and AuNPs stability, a comprehensive investigation into the synergistic effects and optimal conditions for pH, antibody concentration, and incubation time is required. Therefore, this research aims to determine the ideal pH range, the optimal antibody concentration and establish the most effective incubation time, providing valuable insights for diagnostic assays as well as fabricate a multiplex lateral flow immunoassay (LFIA) biosensor that able to detect both the Delta and Omicron variants of SARS-CoV-2.

MATERIALS AND METHODS

Samples

Forty nm of Gold Nanoparticle supplied in 0.1 mM PBS, 95%, ODI with 526 absorption was acquired from Fisher Scientific (M) Sdn Bhd (Canada), the SARS-CoV-2 Omicron (BA.4/BA.5/BA.5.2) Spike S1+S2 trimer Protein and SARS-CoV-2 Delta (B.1.617.2) Spike S1+S2 trimer Protein was obtained from Sino Biological Inc. (Beijing, China). For antigen, SARS-CoV-2 Omicron (BA.4/BA.5/BA.5.2) Spike RBD Protein and SARS-CoV-2 Delta Spike RBD (L452R, T478K) was obtained from Sino Biological Inc. (Beijing, China). Nitrocellulose (NC) membrane was obtained from Fisher Scientific, with a pore size of 0.4 µm and 0.2 µm while the glass fibre with pore size of 0.45 µm for the conjugate pad was obtained from Bioflow Lifescience Sdn Bhd (Selangor, Malaysia). Polyvinyl chloride (PVC) backing card was purchased from EBIZ2U Stationery & Printing Sdn Bhd (Malaysia). Tween-20 solution obtained from Lab Sciences Engineering Sdn Bhd (Selangor, Malaysia) 10x Phosphate Buffer Saline (PBS) solution of pH 7.4 and Potassium Carbonate (K_2CO_3) was obtained from Chemiz (M) Sdn. Bhd. (Selangor, Malaysia). Bovine Serum Albumin (BSA) of pH 7.0 was obtained from Nacalai Tesque, Inc (Kyoto, Japan). Sodium Chloride and Sodium Hydroxide was supplied by EMD Millipore Corporation (Denmark). Skim Milk was purchased from a local store. All distilled water used was purified using an ultra-pure water system which was readily available in the laboratory.

pH Optimization of Gold Nanoparticles (AuNPs)

In order to identify the optimal pH of AuNPs, method from previous study was referred (23). Firstly, 150 µL of gold nanoparticle solution was added into five 0.5 mL tubes. Different amount of 0.1 M K_2CO_3 was added into each tube containing the gold nanoparticles followed by gently vortexed the tubes. 20 µL of each vortex mixture was dropped onto litmus paper to determine its pH value. Then, 100 µL of each mixture containing AuNPs + 0.1 M K_2CO_3 was pipetted into each tube containing 30 µL 10% of NaCl and the mixture was vortexed. The colour changes of each mixture were observed and recorded to choose the optimum pH of conjugation. After 10 minutes incubation period, 100 µL of each mixture was transferred to a 96-well plate reader to measure absorbance at 520 nm. These steps were repeated thrice to ensure reproducibility, with Table I indicated different volumes of 0.1 M K_2CO_3 while others are constant.

Table I: Different volumes of 0.1M K_2CO_3

Tube No.	Volume of 0.1M K_2CO_3 (µl)
1	0
2	1
3	5
4	15
5	25
6	50

SARS-CoV-2 S1 Specific Antibody (Ab) Concentration Optimization for AuNP Conjugates

In this section, to identify the optimal pH of AuNPs, method from previous study was referred with slight adjustment where the step-by-step procedure for optimizing SARS-CoV-2 S1 specific antibody (Ab) concentration for AuNPs conjugates was detailed (23). First, identical amount of 150 µL of AuNP solutions was added into five 0.5 mL tubes. Then, to obtain different concentrations of 8 µg/mL, 9 µg/mL, 10 µg/mL, 11 µg/mL and 12 µg/mL, different amounts of antibody were added into the tube. The mixture was then mixed using a vortex for about 10 minutes at room temperature. Following that, 10% NaCl was added into the tubes. After 5 minutes, 100 µL of each mixture containing AuNPs + 0.1 M K_2CO_3 +10% of NaCl + Ab was pipetted to transfer to a 96-well plate reader to measure the absorbance wavelength at 520 nm. The experiment was repeated twice to obtain the average value. Table II shows the summarize amount of Ab-S1 concentration and the pH of AuNPs in each tube.

Incubation Time Optimization for AuNPs Conjugates

This section outlines the optimized procedure for incubating antibodies with AuNPs conjugates, based on methods referenced in a previous study (9). Initially, 150 µL of AuNP solution was added to four 0.5 mL tubes. The pH of the AuNP solution was adjusted to 8.0 using 5 µL of 0.1 M K_2CO_3. After a 5-minute incubation period, a specific amount of antibody (Ab) was added to achieve a concentration of 8 µg/mL. Each tube was then incubated at 37°C for 15 minutes, 30 minutes, 1 hour, and 3 hours. Subsequently, 30 µL of 5% BSA solution was added to each tube, followed by an additional 15 minutes of incubation at 37°C. The mixture in each tube was centrifuged at 3000 rpm for 10 minutes to remove large impurities and obtain the gold pellet. After centrifugation, the supernatant was discarded, and the gold pellet was resuspended in 0.01 M PBS pH 8.4 with 1% skim milk, then stored in refrigerator with temperature of 4°C until further use.

Next, each conjugated solution was soaked onto a conjugate pad for 15 minutes to ensure even distribution. For test strip preparation, 1.25 µL of Delta antibody with a concentration of 1.0 µg/µL was pipetted onto the membrane. Omicron antibody and Anti-mouse IgG were dispensed onto the test line and control line of the membrane with identical volumes and concentrations, spaced 3 mm apart. The membrane was left to dry for 1 hour, then immersed in 0.01 M PBS solution with 1% skim milk for 30 minutes to minimize non-specific binding of additional sample elements to the NC membrane, thus reducing the risk of false-positive results. After drying for 1 hour at room temperature, the NC membrane was cut to final dimensions of 4 mm x 22 mm and affixed to a polyvinyl chloride (PVC) backing card. The sample pad, absorbance pad, conjugate pad, and NC membrane were mounted sequentially on the PVC backing card, ensuring overlaps of 2 mm between the conjugate pad and NC membrane, and 6 mm between the sample pad and conjugate pad.

Four test strips, each corresponding to a different antibody incubation time, were prepared. To determine the optimal antibody incubation time for AuNPs conjugates, a sample containing Spike Delta Antigen protein and sample buffer was applied to the sample pad of each test strip, and the visible results were recorded.

Testing of SARS-CoV-2 Antigen Sample on the strip

In this section, two detection strips were prepared to validate the efficiency of the final detection strips, each optimized with the ideal pH, antibody concentration, and incubation time for AuNPs conjugates. Detection strip (a) was tested with a sample containing Spike Delta Antigen protein and sample buffer applied to the sample pad. Detection strip (b) was used to test S1 Omicron antibody, with a sample containing Spike Omicron Antigen and sample buffer pipetted onto the sample pad. Visible results from each detection strip were recorded accordingly.

RESULTS

Optimization of AuNPs stability with different pH values

The absorbance for AuNPs was measured at a wavelength of 520 nm. The pH level for AuNPs without adding K_2CO_3 solution is known to be pH 6.0. However, the absorbance test demonstrates a lower absorbance value which indicates decreased stability of AuNPs. According to previous studies, the optimal pH for stability of gold nanoparticles conjugates was pH 8.0 (24). From this study, the pH level of AuNPs was adjusted from the range of 6.0 - 10.0 by adding different volume of K_2CO_3. Based on Table II, the pH level and absorbance measurement of the gold nanoparticle solution are shown, together with the volume of 0.1M K_2CO_3 that was added to each tube, which are 0, 5, 15, 25, and 50 µL. Based on Fig. 1 which shows the qualitative results with different pH values, tube with pH 8.0 offers a vibrant red colour and remains red for a longer time of AuNPs compared to other tubes.

Table II: Amount of Ab-S1 concentration (µg/mL) and pH of Gold nanoparticles in each tube

Tube	Amount of Ab Concentration(µg/ml)	Average absorbance (nm)	Standard deviation	Average pH	Average absorbance (nm)	Standard deviation
1	8	0.148	0.01273	6	0.113	0.00058
2	9	0.152	0.152	8	0.13	0.00115
3	10	0.164	0.164	9	0.128	0.00608
4	11	0.177	0.177	10	0.125	0.00702
5	12	0.162	0.162	10	0.106	0.00306

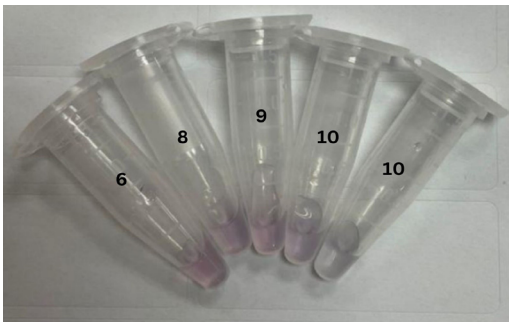

Fig. 1: Qualitative results show tubes with different colour intensity of AuNPs. 30 µL of each tube was taken to determine pH of each tube and was recorded. The optimal pH of AuNPs was determined based on the colour intensity of the AuNPs exhibited, values of absorbance and stability.

Optimization concentration of SARS-CoV-2 S1 Specific Antibody (Ab) for AuNPs

To determine the optimal concentration of S1 antibody, the absorbance value of each tube with different concentration has to be obtained. The measurements and results of the absorbance value are listed in Table II. Based on Fig. 2 which shows the qualitative results with different concentrations of antibodies, tube with concentration of 8 µg/mL offers a vibrant red colour and remains red for a longer time of AuNPs compared to other tubes. Tube with concentrations of 12 µg/mL also offers a red vibrant colour however, the red colour fading over 5 minutes indicating the lower stability of conjugation. Based on Table II, the qualitative results have been supported by the quantitative results where concentration of 8 µg/mL exhibits the lowest number of absorbance values.

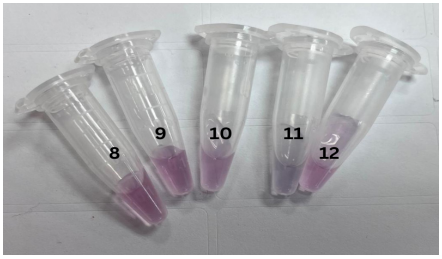

Fig. 2: Qualitative results show tubes with different colour intensity of AuNPs due to different concentration of antibody. The concentration of antibodies needed to be at least as high as that which would allow gold colloids to stay stable even in the presence of 10% sodium chloride. Thereafter, a series of optimization processes were conducted using optimal pH which was determined as pH 8.0 with different concentration value based on previous reported studies.

Antibody incubation time affect the stability of antibody and gold nanoparticles (Ab-AuNPs) conjugates

Based on Table III, the qualitative results with 1 hour of incubation time for antibody nanoparticles conjugate to form demonstrated an optimized incubation time which a red visible line has formed at the control line. Incubation time with lower or more than 60 minutes are unable to obtain a positive results as previous studies had suggested that non-optimal incubation may lead to aggregation of AuNPs that able to change the optical properties of the nanoparticles, affecting their performance in detection assays and potentially altering the specificity and sensitivity of the assay (25).

Table III: Qualitative results of various incubation time for antibody nanoparticles conjugate.

Tube	Incubation time (minutes)	[a]Results
1	15	No line formed
2	30	No line formed
3	60	Red visible line formed
4	180	No line formed

[a] The results were demonstrated within a time range of 15 to 30 minutes.

Testing the efficiency of multiplex LFIA for multivariants of SARS-CoV-2

Testing of the efficiency of each LFIA detection strip was determined by the colour changes and visible red line on the test lines and control line. The control line serves as an internal control that helps to validate the efficiency of the LFIA strip where visible red control line indicates the test strip runs correctly. The results collection for testing SARS-CoV-2 antigen protein specific to its respective antibody to obtain positive results was demonstrated in Fig. 3. Based on the results, detection strip (a) shows a red visible migration flow of AuNPs along the membrane to the absorbance pad and no visible line recorded on each DL and CL indicate an invalid result for detection strip for validation of Delta variants SARS-CoV-2. For detection strip (b), the results show a red visible migration flow of AuNPs along the membrane to the absorbance pad and no visible line recorded on OL meanwhile there was visible red line recorded on CL indicate an invalid result for detection strip for validation of Omicron variants SARS-CoV-2. The absence of CL on strip (a) experiment indicate that the detection strips are not functioning properly whereas strip (b) consider functioning properly due to visible CL.

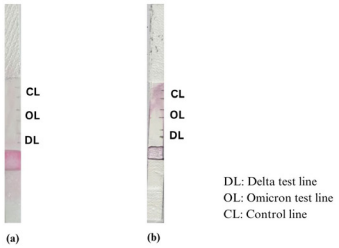

Fig. 3: Qualitative results of (a) Validation results for Delta variants and (b) Validation results for Omicron variants. The CL indicates as Control line, DL as Delta test line and OL indicates as Omicron test line. The results were demonstrated within a time range of 15 to 30 minutes.

DISCUSSION

Optimization of AuNPs stability with different pH values

The absorbance test is used to gauge how much light of a certain wavelength is absorbed by a substance. The stability of AuNPs can be impacted by changes in pH or external factors like temperature or ionic strength.

Alterations in these conditions may lead to aggregation and lower absorbance values. The surface charge of particles engaged in conjugation, such as nanoparticles, can be affected by the pH of the reaction environment. The effectiveness of conjugation can be impacted by variations in surface charge by the electrostatic interactions between particles and molecules (26). Optimizing the pH value around the isoelectric point of an antibody can thereby increase the efficiency and specificity of the conjugation reaction as it helps to reduce charge repulsion between the molecules, increasing their contact and efficient conjugation (14). Gold nanoparticles have an approximate 520 nm surface plasmon resonance (SPR) wavelength, depending on their size. As particle size and intensity increase, red shifts the SPR wavelength (27). When the mixture's pH value is close to or slightly higher than the antibody's isoelectric point, the electrostatic and physical adsorption between the AuNPs and antibodies is most stable. Based on Table II, the qualitative results have been supported by the quantitative results where pH 8.0 with 5 µL of 0.1 M K_2CO_3 exhibit highest number of absorbance values indicates lower aggregation and higher stability of AuNPs. From the results, we can conclude that the optimal pH for conjugation is 8.0 which helps to prevent electrostatic repulsion between AuNPs and antibodies.

Optimization concentration of SARS-CoV-2 S1 Specific Antibody (Ab) for AuNPs

The affinity of the antibody for the target molecule or surface is influenced by its concentration. Lower sensitivity and specificity of the conjugate may be caused by incomplete binding or weak interactions at too low a concentration. On the other hand, a concentration that is too high can cause steric hindrance and non-specific binding, which will affect the assay's accuracy. The performance of the conjugate is maximised by optimising the antibody concentration, which provides effective and targeted binding (28). For this project, a minimum amount of antibodies is required to prevent aggregation that may lead to false-positive results (29).

By adjusting the antibody concentration, non-specific binding is reduced while ensuring effective binding to the analyte. The concentration of antibodies needed to be at least as high as that which would allow gold colloids to stay stable even in the presence of 10% sodium chloride (30). According to the results, Tube 1 had the most appropriate antibody concentration for conjugation with AuNP, which was 8µg/mL, as the colour persisted for a considerable amount of time without aggregation (30). This indicates that the stability and effectiveness are maintained, non-specific interaction with molecules other than the target molecules is minimised, and the antibody concentration is below the level at which aggregation begins to occur. Additionally, the amount of antibody used can be decreased while maintaining the desired performance of the conjugates, which helps minimise costs, by selecting the minimal absorbance as the appropriate concentration (31).

Antibody incubation time affect the stability of Ab-AuNPs conjugates

Incubation time affect the stability of antibody and gold nanoparticle conjugates as the stability of such conjugates is influenced by several factors, including the interaction between the antibody and AuNPs, as well as the conditions under which the conjugation reaction occurs. The effectiveness of the antibody binding to the AuNPs can be impacted by the incubation period during the conjugation procedure (25). Stronger binding may be facilitated by longer incubation durations. However, a prolonged incubation period may result in non-specific binding, antibody aggregation, or possibly denaturation, which would lower the stability of the conjugate (32). The specific incubation time for Ab-AuNP conjugate has been discovered through series of qualitative analysis assessments with different incubation time which demonstrated in Table III. Therefore, the optimal incubation of antibody for conjugation with AuNPs is 60 minutes which indicates minimum aggregation of AuNPs in the conjugation that offers stability of AuNPs conjugate.

Testing the efficiency of multiplex LFIA for multivariants of SARS-CoV-2

The attempt to develop an LFIA for SARS-CoV-2 detection did not yield the anticipated results due to challenge related to the efficiency of LFIA strips. For the potential cause of failure, the factor that contribute to the failure of the rapid LFIA is the concentration of antibodies on the NC membrane. LFIA may perform less effectively if the antibody concentration is lower because the control line may not be present or may be weak. By binding with the probe regardless of whether the target is present, the control line assures that the test will run properly (6). A stronger and more observable signal is produced when there are more antibody binding sites available on the nitrocellulose membrane due to increase of antibody concentration. On the other hand, a lower antibody concentration will produce a weaker signal, making it more difficult to precisely detect and understand the test results (28). The concentration of antibody used in this project was 1.0 µg/µL, lower than the optimal concentration reported in the previous study which is 2.0 mg/mL (23). The consideration of increasing the concentration of antibody was considered, however due to limited resources of antibody, the approach of increasing the concentration up to 1.0 mg/mL was hindered. This factor may result in absence of visible lines at the DL, OL and CL.

CONCLUSION

In the present study, the conjugation of SARS-CoV-2 antibody with gold nanoparticles have been synthesized. S1 subunits of SARS-CoV-2 were utilized

as the targeted antibody in the conjugation of antibody with nanoparticles due to their high sensitivity and significant function in replication in the host cell. The conjugation of Delta Ab-S1 and Omicron Ab-S1 with AuNPs via physical adsorption method has been optimized involving the aspects such as the pH value of nanoparticles and the antibody concentration needed for the conjugation. The determination of optimum pH of nanoparticles (pH 8.0) and the optimum concentration of antibody for the conjugation (8 µg/mL) were conducted through the absorbance test. Moreover, it is evident from the experimental findings that the incubation time significantly influences the sensitivity of the conjugate in the detection system. Through systematic exploration and analysis, the study has established a clear relationship between the duration of incubation and the resulting sensitivity of the assay. The study had identified that there exists an optimal range of incubation time within which the sensitivity of the conjugate is maximized. Deviating from this range, either by excessively prolonging or abbreviating the incubation, leads to a decline in the assay's sensitivity. However, this study encountered several obstacles that prevented the study from obtaining desired results that related to sensitivity, specificity, and reproducibility of the LFIA results. The failure of the multiplex LFIA highlights the complexities and challenges associated with diagnostic assay development, and it underscores the need for continued research and collaboration in this critical area. The study about the influence of gold nanoparticle size and shape optimization can be recommended for future study since the size and shape of nanoparticle play important role in the assay's sensitivity and specificity. Smaller nanoparticles may improve sensitivity, while differently shaped particles might enhance stability or binding efficiency. Additionally, the low specificity or cross-reactivity of the utilized antibodies may be the cause of multiplex LFIA failure. Future research should focus on identifying highly specific antibodies for each variant of concern, such as Delta and Omicron. This would strengthen the test's ability to differentiate between variants. While AuNPs are commonly used due to their optical properties, alternative nanoparticles like quantum dots, magnetic nanoparticles, or silver nanoparticles may offer better sensitivity and multiplexing capabilities.

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