The Development of Lateral Flow Immunoassay for Hepatitis B Detection in Saliva

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Abstract

This study addresses the conventional blood-based approach for hepatitis B testing, aiming to enhance accessibility by developing a lateral flow sensor for hepatitis B virus (HBV) detection in saliva samples. Saliva collection offers a less invasive and more convenient alternative. Employing a lateral flow immunoassay with gold nanoparticles (AuNPs) conjugated with antibodies (Ab-AuNPs), the sensor visually displays results based on hepatitis B surface antigen (HBsAg) detection. The sensor's performance was comprehensively assessed, considering the limit of detection, sensitivity, and specificity. Artificial saliva containing varying concentrations of HBsAg was tested, revealing the sensor's capability to detect concentrations as low as 0.025 µg/mL with the naked eye. The lateral flow demonstrated a sensitivity of 83% (10/12) in positive controls and a specificity of 100% (8/8) in negative controls. Evaluation of the lateral flow immunoassay's validity and utility indices underscores its potential for further development and future application in real patient testing.

Keywords: Lateral flow immunoassay (LFIA), Hepatitis B surface antigen (HBsAg), Labeled gold nanoparticles (Ab-AuNPs)

I. INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health concern. According to the World Health Organization (WHO), it is estimated that approximately 300 million people worldwide are chronic carriers of the virus, with at least 1 million ultimately developing severe liver disease and, in some cases, liver cancer (World Health Organization, 2023). In Thailand, there are approximately 3.5 million people suffering from chronic HBV infection (Piratvisuth, 2020).

HBV can be transmitted through blood, body fluids, needle sharing, unprotected sexual contact, and from mother to child during childbirth due to the presence of the virus in blood, semen, and bodily fluids. The clinical spectrum of HBV infection ranges from subclinical to acute symptomatic hepatitis or, rarely, fulminant hepatitis during the acute phase and from the inactive hepatitis B surface antigen (HBsAg) carrier state to chronic hepatitis, cirrhosis, and its complications during the chronic phase (Sharma *et al*., 2005). Diagnosis of HBV currently involves blood tests to check liver function, HBV-specific blood tests for HBsAg and Anti-HBs, or liver biopsy, which is a specialized procedure performed by experts.

In addition to HBsAg, other antigens and antibodies associated with hepatitis B include hepatitis B e antigen (HBeAg), hepatitis B core antigen (HBcAg), and various antibodies such as anti-HBs, anti-HBe, and anti-HBc. While these markers provide comprehensive insights into the different stages of Hepatitis B infection, HBsAg stands out as a primary diagnostic target due to being the earliest detectable antigen during infection and signifies active viral replication. Its presence indicates both acute and chronic phases of Hepatitis B, making it a reliable marker for infection status (Kramvis *et al*., 2022). Finally, HBsAg is highly stable, facilitating accurate detection in various sample types, including saliva, which offers a non-invasive and easily collectible medium for diagnostic purposes. Its prominence in diagnostic assays is attributed to its sustained presence during the infectious period, allowing for early and accurate detection, critical for timely intervention and disease management (Cruz *et al*, 2011).

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The advantages of detecting HBsAg in saliva are noteworthy. Saliva, being easily and non-invasively collectible, provides a patient-friendly alternative to blood sampling. The detection of HBsAg in saliva offers a practical and accessible means of diagnosing Hepatitis B, especially in settings where traditional blood tests may pose challenges. This method not only reduces discomfort for individuals undergoing testing but also facilitates widespread screening efforts. Moreover, the non-invasive nature of saliva collection may encourage more individuals to participate in regular screenings, ultimately contributing to early detection and timely management of hepatitis B infections.

For these reasons the development of a sensor for screening hepatitis B Virus from saliva has been undertaken, aiming to facilitate quick, convenient, and accessible testing for HBV, and enabling individuals to perform self-checks with ease.

II. METHODS

1: Synthesis of gold nanoparticles (AuNPs)

A solution of trisodium citrate (1%, w/v) was prepared with a volume of 0.1 mL, and a solution of $K(AuCl_4)\cdot nH_2O$ (1%, w/v) was prepared with a volume of 0.25 mL. In a flask sealed with aluminum foil, 25 mL of DI water was added and heated on a hot plate at 100°C, stirring at 5000 rpm for 10 minutes. $K(AuCl_4)\cdot nH_2O$ solution (1%, w/v) was incrementally added along the edges of the flask. After reaching the boiling point, 0.1 mL of trisodium citrate solution (1%, w/v) was added, and the solution was allowed to sit until it turned dark purple. The pH of the AuNPs solution was adjusted to 8.1 using $NaHCO_3$ solution. The AuNPs solution was transferred into sterilized plastic bottles and stored at 4°C. The process was repeated with volumes of trisodium citrate solution (1%, w/v) at 0.25 mL, 0.5 mL, 1 mL, and 2 mL, respectively.

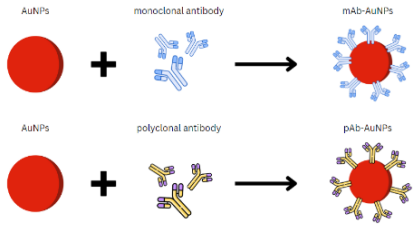

Figure 1. Antibodies conjugated to AuNPs

Light absorption testing was conducted in the wavelength range of 350-700 nm. The obtained values were analyzed by graphing the relationship between the wavelength spectrum and the light absorption of the AuNPs solution.

2: Sensor optimization

Testing of antibody concentrations

Polyclonal antibody (Rabbit anti-Surface Ag pAb: Ab68519) was diluted to six concentrations of 6.25, 12.5, 25, 50, 100, and 200 µg/mL, each with a volume of 25 µL. Subsequently, 125 µL of AuNPs solution was added and stirred at room temperature for 15 minutes. Following this, 125 µL of NaCl solution (10%, w/v) was added. The resulting solution was then analysed by using light absorption spectroscopy in the range of 400-700 nm. The process was repeated, with the variation of antibody type to monoclonal antibody (Mouse anti-Surface Ag mAb: Ab252692) (Figure 1).

Testing of binding interaction

The anti-mouse antibody, with a concentration of 1.5 mg/mL, was affixed onto nitrocellulose membrane strips in the control line (C-line) using Biodot XYZ32100047. The strips were then left to dry for one hour at 37°C. Subsequently, a lateral flow test strip was assembled, comprising a sample pad, conjugate pad, nitrocellulose membrane, absorbent pad, and backing card (Figure 2).

To create sensor strips, polyclonal antibody labeled with AuNPs (pAb-AuNPs) was dispensed onto the conjugate pad at a volume of 8 µL per strip and then dried at 37°C for 20 minutes. Monoclonal antibody, at a concentration of 1 mg/mL, was then dropped onto the test line (T-line) at a volume of 0.5 µL per strip. Testing was conducted by dropping HBsAg solution (1 µg/mL) mixed with PBT buffer in a 2:1 ratio onto the sample pad of positive control strips (100 µL each) and PBT buffer onto negative control strips (100 µL each). The light intensity of the test line was measured using ImageJ. The process was repeated, but with the substitution of mAb-AuNPs for pAb-AuNPs and exchanging the monoclonal antibody on the test line for polyclonal antibody.

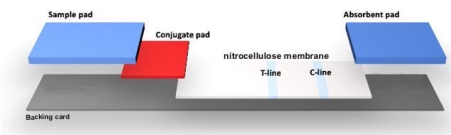

Figure 2. The test strip comprised a sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad affixed to a backing card.

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Concentration of polyclonal antibodies
Polyclonal antibody was dispensed onto the test line at concentrations ranging from 0.5 to 3.0 mg/mL in increments of 0.5 mg/mL, each at a volume of 0.5 µL, and this process was repeated three times. mAb-AuNPs was dropped onto the conjugate pad at a volume of 8 µL per strip and then dried at 37°C for 20 minutes. Testing was carried out by dropping HBsAg solution (1 µg/mL) mixed with PBT buffer in a 2:1 ratio onto the sample pad of positive control strips (100 µL each) and PBT buffer onto negative control strips (100 µL each). The light intensity of the test line was subsequently measured using ImageJ.

Testing mAb-AuNPs on the conjugate pad
mAb-AuNPs were dispensed onto the conjugate pad at volumes ranging from 1 to 8 µL, followed by drying at 37°C for 20 minutes. Testing was conducted by dropping HBsAg solution (1 µg/mL) mixed with PBT buffer in a 2:1 ratio onto the positive control strips (100 µL each) and PBT buffer onto the negative control strips (100 µL each). The light intensity of the test line was measured using ImageJ.

Study of buffer type
Buffer solutions (PBS, PBS + 0.5% Tween20 (PBST0.5), PBS + 1% BSA and PBT + 0.5% Tween20 (PBTT0.5)) were dispensed at a volume of 400 µL onto the sample area of the test strip. The strips were dried at 37°C for 60 minutes. Artificial saliva was dropped onto the sample area, with a volume of 100 µL per strip. The flow of mAb-AuNPs and the display of the test line were observed.

3: Testing the efficiency of the sensor
To test the efficiency of the sensor, artificial saliva was prepared following Engelhart *et al*. (2016), and the limit of detection was tested among different HBsAg concentrations. The sensor was tested with HBsAg solutions in artificial saliva, ranging in concentrations from 6 µg/mL to 0.01 µg/mL, each with a volume of 100 µL. The light intensity of the test line was measured using ImageJ. The intensity values for each HBsAg concentration were recorded. The limit of detection for the sensor was calculated based on the obtained results. Sensitivity and specificity were tested by using positive artificial saliva samples with different concentrations for 12 samples and negative samples for 8 samples.

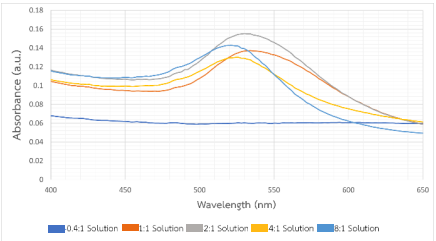


Figure 3. The relationship between the concentration of trisodium citrate solution and AuNPs absorbance

III. RESULTS AND DISCUSSION

1: Synthesis of gold nanoparticles (AuNPs)
In general, the most commonly used sizes for AuNPs in lateral flow immunoassays are between 20-40 nm and λ_{max} typically falls within the range of 520-540 nm. When considering the amount of substance used, the ratio of trisodium citrate solution (1%, w/v) to potassium tetrachloro-aurate(III) solution (1%, w/v) were tested using 0.4:1, 1:1, 2:1, 4:1, and 8:1 solutions (Figure 3). However, for the ratio of 0.4:1, AuNPs could not be synthesized. At the same time, AuNPs obtained from trisodium citrate solutions (1%, w/v) using the ratio of 1:1, 2:1, 4:1, and 8:1 can exhibit suspension in the solution, resulting in a pronounced dark red color. Through the study, it was observed that AuNPs of different sizes have varying λ_{max} values. Specifically, it was found that the AuNPs solutions obtained from 1:1, 2:1, 4:1, and 8:1 solution all have λ_{max} values within the range of 520-540 nm. And based on the absorbance wavelength values, we conclude that the optimal solution is the 1:1 ratio, as it provides the highest absorbance wavelength at 534 nm.

2: Sensor fabrication
From the study of the absorbance wavelength values of antibodies labeled with AuNPs in the range of 450-700 nm, aimed at finding the most suitable intensity for labeling, it was found that when analyzing the absorbance value at a wavelength of 538 nm, which is the λ_{max} of the

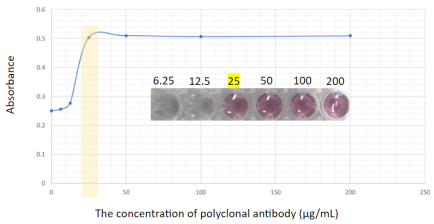


Figure 4. The relationship between the concentration of polyclonal antibody and the absorbance values of pAb-AuNPs at a wavelength of 538 nm

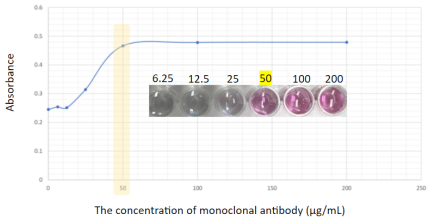


Figure 5. The relationship between the concentration of monoclonal antibody and the absorbance values of mAb-AuNPs at a wavelength of 538 nm

AuNPs solution. It was found that pAb-AuNPs obtained from polyclonal antibody with a concentration of 25 µg/mL has the optimal absorbance (Figure 4). Similarly, it was found that mAb-AuNPs obtained from monoclonal antibody with a concentration of 50 µg/mL (Figure 5) provided the optimal concentration for this assay.

Identifying suitable antibody pairs was done by comparing two methods: 1. pAb-AuNPs on the conjugate pad with monoclonal antibody on the test line and 2. mAb-AuNPs on the conjugate pad with polyclonal antibody on the test line. It was observed that both methods produced strong color on the test line (pointed by arrow) when tested with positive samples (Figure 6). When tested with negative samples, both methods resulted in faint color change on the test line (pointed by arrow), similar to each other. However, it was found that the mAb-AuNPs on the conjugate pad with polyclonal antibodies on the test line exhibited a higher light intensity on the T-line compared to the other method when tested with positive samples. Therefore, this method is suitable for this sensor.

Upon investigating the optimal conditions for the sensor, considering factors including mAb-AuNPs volume, polyclonal antibody concentration on the T-line, and buffer type, optimal parameters were established. An mAb-AuNPs volume of 4 µL (Figure 7) and a polyclonal antibody concentration of 1.5 mg/mL (Figure 8) proved optimal based on T-line light intensity, balancing effective detection without overwhelming the chemicals. The choice of $PBTS_{0.5}$ as the buffer was supported by its ability to expedite mAb-AuNPs movement toward the absorbent pad (Table 1).

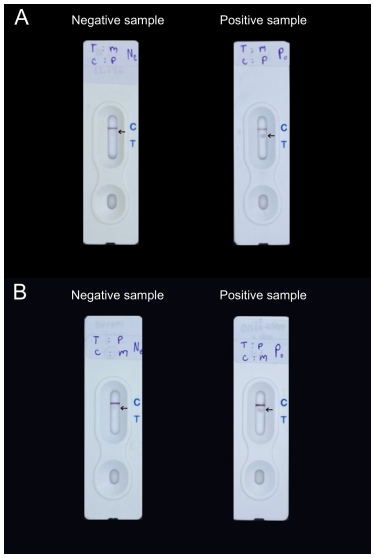
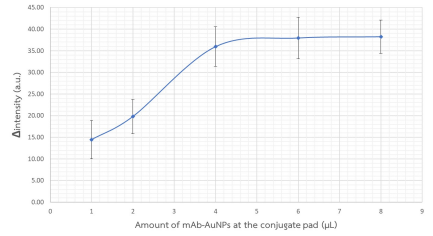


Figure 6. (A) The test strip where pAb-AuNPs are located on the conjugate pad, and monoclonal antibodies are situated on the test line. (B) The test strip with mAb-AuNPs on the conjugate pad and polyclonal antibodies on the T-line.



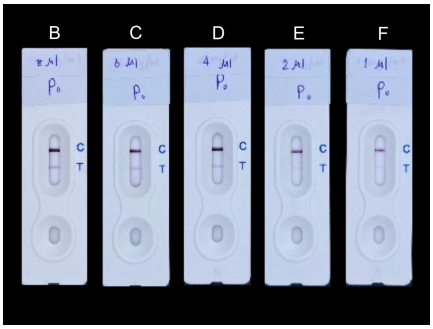


Figure 7. (A) the relationship between the light intensity of the T-line and the volume of mAb-AuNPs on the conjugate pad for positive samples. Sample test strips with variations at (B) 8 µL, (C) 6 µL, (D) 4 µL, (E) 2 µL, and (F) 1 µL.

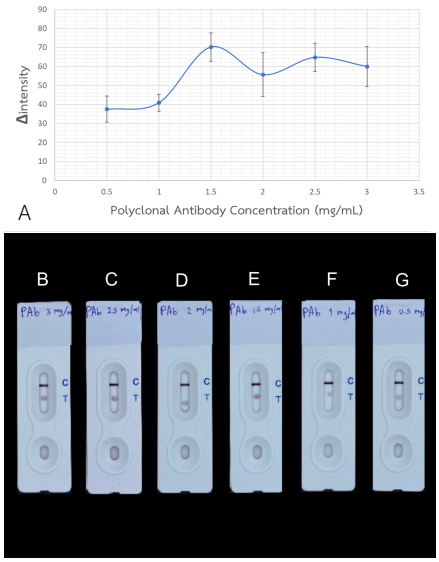


Figure 8. (A) the relationship between the light intensity of the T-line and the concentration of polyclonal antibody. Sample test strips with variations at (B) 3 mg/mL, (C) 2.5 mg/mL, (D) 2 mg/mL, (E) 1.5 mg/mL, (F) 1 mg/mL, and (G) 0.5 mg/mL.

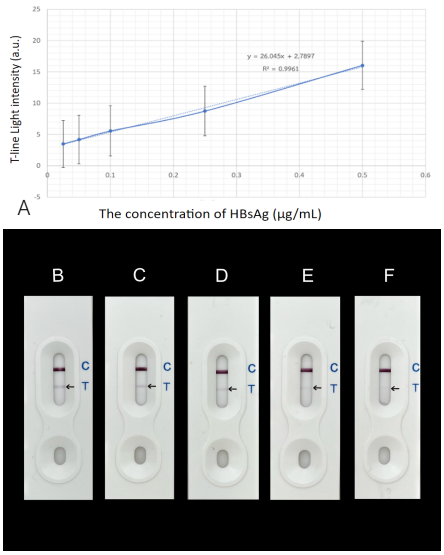


Figure 9. (A) The relationship between the intensity of the T-line and the concentration of HBsAg in the artificial saliva. Sample test strips with variations at (B) 0.5 µL/mL, (C) 0.25 µL/mL, (D) 0.1 µL/mL, (E) 0.05 µL/mL, and (F) 0.025 µL/mL.

3: Testing the efficiency of the sensor

From testing the sensor with an HBsAg solution in artificial saliva and gradually reducing the concentration of the HBsAg solution, observations were made regarding the performance of the test line and measurements of light intensity. It was found that this test line can detect HBsAg in artificial saliva visually at concentrations as low as 0.025 µg/mL (Figure 9). To assess the real-life display performance of the sensor, it is essential to conduct tests with actual saliva samples from patients at various stages of hepatitis B virus infection. Testing of the sensor with HBsAg solution in artificial saliva, using both positive and negative samples, revealed that the sensor achieved a sensitivity of 83.33% and a specificity of 100% (Table 2).

Buffer type	Migration time (min)
PBS	10.19
$PBST_{0.5}$	8.25
PBS + 1%BSA	14.30
$PBTT_{0.5}$	12.05

Table 1. Time taken for mAb-AuNPs to migrate to the absorbent pad for test strips composed of various buffer types on the sample support pad.

IV. CONCUSION

In evaluating the performance of the developed sensor on artificial saliva samples containing the hepatitis B virus antigen, optimal conditions for the test strip were determined, including the use of 1.5 mg/mL of polyclonal antibody on the T-line, 4 µL of mAb-AuNPs on the conjugate pad, and $PBST_{0.5}$ as a buffer. The test strip demonstrated detection of hepatitis B virus antigen in artificial saliva samples at concentrations as low as 0.025 µg/mL, discernible with the naked eye. It achieved a sensitivity of 83.33% and a specificity of 100%. This underscores the potential of the sensor as a user-friendly and uncomplicated prototype for detecting the hepatitis B virus antigen in saliva samples. The promising outcomes position it as a viable candidate for further development and practical application in the future.

	HBsAg present	HBsAg absent
Positive	10 (True positive)	0 (False positive)
Negative	2 (False negative)	8 (True negative)

Table 2. Sensor testing results for positive and negative samples.

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