

## Development of Signal Enhanced Dot Blot Immunoassay for Determination of Amyloid Beta

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### ABSTRACT

Alzheimer's disease (AD) is a primary common neurodegenerative disorder worldwide and increases prevalence in elder ages of life span. The critical detection of early diagnosis is the most important to support treatments to defence this incapacitating disease. Amyloid-beta proteins have various significant physiological or pathophysiological effects on AD progression. It was affirmed that amyloid-beta protein levels are detected for preclinical diagnosis and prognosis as a reliable biomarker for neurodegeneration. The available methods for detection of amyloid-beta proteins are time consuming and complicated operations. Herein, a silver-enhanced nanoparticle-labelled dot blot immunoassays had been developed for detection of A $\beta$  (1-42) providing high potential early diagnosis for AD. Under optimized condition, the limit of detection was 1.0  $\mu$ M by naked eyes. The sensitivity of the results were enhanced by using silver enhancer (Silver nitrate and hydroquinone in 1:1 ratio).

**Keywords:** Amyloid Beta, Dot blot assay, Silver enhancement

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## Introduction

Alzheimer's disease (AD) is the most common age related neurodegenerative disorder which pathologically characterized by modification of diffusible assemblies (oligomers) of amyloid-beta ( $A\beta$ ) protein that are dominant neurotoxic species and complex fibrillary amyloid plaques (Yan *et al.*, 2015). The previous study reported  $A\beta$  peptide comprising of 39 - 42 amino acids is the primary constituent of plaques are mostly found in cerebrospinal fluid (CSF) and brain of patients who suffered from AD (Hardy *et al.*, 1992). They can either associated to other events including inflammation and tau aggregation as neurofibrillary tangles (Hardy. *et al.*, 2010). Therefore,  $A\beta$  peptides have been considered important biomarkers to monitor Alzheimer's diseases (AD) for diagnostic purposes (Andreasen *et al.*, 1999)

Recent year, there are many of detection method in  $A\beta$  such as electrochemiluminescence immunoassay (Gravina *et al.*, 1995) capillary electrophoresis, immunoprecipitation-mass spectrometry (Picou *et al.*, 2010) flow cytometry-fluorescence resonance energy transfer assay (Wang *et al.*, 1996) surface plasma resonance-immunochip assay (Santos *et al.*, 2007) resonance light scattering assay (Wang *et al.*, 2011) and enzyme-linked immunosorbent assay (Wang *et al.*, 2012) which still have limitations due to need of sophisticated instruments, time consuming, need of skillful person for their applications (Gagni *et al.*, 2013) These complicated operations limited their applications to extended for rapid and routine analysis of  $A\beta$  protein levels in the clinical samples such as blood of AD patients.

By developing effective technologies in health-related diagnostics for developing countries and coupling these technologies to existing communication infrastructures, healthcare in areas without access to trained medical personnel may be possible. Dot blot immunoassay is one technique of immunoassay that can also be used to analyze the presence or identify of soluble antigens as known as a dot blot immunoassay. Samples are spotted onto the nitrocellulose membrane and let the dots are dried. Membrane is blocked and incubated with antibody detection system in this technique. It can be divided into direct and indirect immunoassay (Kavruk *et al.*, 2013). Accordingly, there is simple, rapid, highly sensitive and non-invasive detection of  $A\beta$  could be more interesting and high effectiveness of helping to the world.

In this study, gold nanoparticle- conjugated rabbit antibody  $A\beta_{1-42}$  immunoassays for detection of  $A\beta_{1-42}$  antigen have developed. The parameters including pH condition, antibody concentration, membrane substrate that influence dot-blot assay were investigated. In order to enhance the sensitivity of device, signal enhancement system by sandwich assay and silver enhancer was developed.

## Objective of study

This study is to develop dot blot immunoassay with signal enhancement system for the rapid screening of  $A\beta_{1-42}$  antigen. In order to hit the objective of this study, we are going to develop dot blot immunoassay method by using gold nanoparticles with silver enhancer to signal simplify. Then, developed assay will be applied to detect amyloid beta in real blood sample analysis.

## Materials and Methods

Gold nanoparticle-based immunoassays was developed for the detection of A $\beta$ <sub>1-42</sub> antigen. The prerequisite materials for this method are AE99, Fusion 5, Hybond ECL nitrocellulose (Lot.9549062, GE Healthcare, UK), Buffer solution:5% non-fat dry Milk in TBS-T (20 mM Tris-HCL, pH 7.5), 0.8% (W/V) NaCl (Lot.D00130978, CALBIOCHEM, Germany), 0.1% (V/V) Tween-20, Antibody A $\beta$  (1-421b1220015), Human Beta Amyloid 1-42 full length protein (ab82795). TMB as Substrate. Trisodium citrate, hydroquinone, silver nitrate (AgNO<sub>3</sub>) were used as an enhancing agents.

## Synthesis of Gold nano particles

100  $\mu$ l of an aqueous HAuCl<sub>4</sub> (5%) was added into a flask containing 80 ml deionized water, then the solution was brought to boil, with constant stirring. 3.5 ml of an aqueous sodium citrate (1%) solution was needed to add urgently. The solution was heated for another 20 min until a deep-red solution was occurred. That solution was centrifuged for 10 min at 3500 rpm and supernatant was collected (Turkevich *et al.*, 1951). The gold nanoparticles (AuNPs) were characterized by UV spetrometer (fig.1). The maximum wavelength was 520 nm.

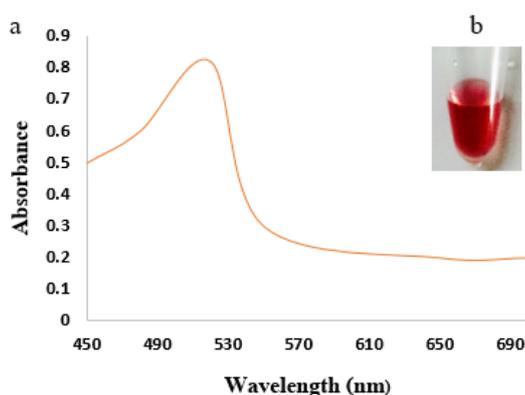


Figure 1. Figure 1 (a) a UV spectra of synthesised AuNPs (b) image of synthesized AuNPs solution

## Optimization of pH in AuNPs solution

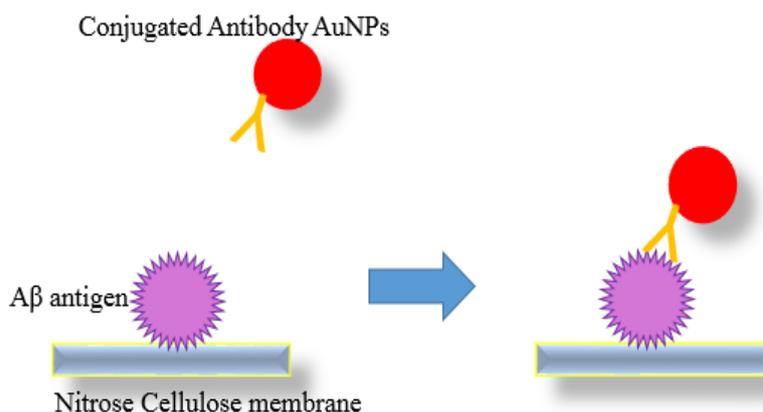
After determining the optimal concentration of bindin A $\beta$  oligomers antibody gold conjugated were prepared. An aliquot (50  $\mu$ l) of A $\beta$  oligomers antibody prepared in PBS (0.1 M, pH 7.4) was added slowly to 2 ml colloidal gold solution pH8 (adjusted by 0.5 M NaOH) followed by the addition of bovine serum albumin (BSA) (100  $\mu$ l, 10%) under gentle stirring after 45 min. The mixture was incubated for another 1 h at 4°C and then centrifuged (10 000 rpm for 15 min at 4°C) to remove supernatant unconjugated antibody. The pellet obtained was washed with PBS once again. The pellet was finally re-dispersed in 0.5 ml PBS (pH7.4) containing 2% BSA and stored at 4°C.

### Optimization of Ab for AuNPs-Ab conjugates

The optimal concentration of rabbit A $\beta$  oligomer antibody for conjugation was determined by titrating aliquots of diluted antibody with colloidal AuNPs. Rabbit A $\beta$  oligomer antibody was diluted to different concentration of ratio ( 1:5000 to 1:1) dil in PBS (0.1 M, pH 7.4). The pH of colloidal gold solution and the diluted rabbit A $\beta$  oligomer antibody was adjusted to pH 8.0 with 0.1 N NaOH of variable concentrations. Added separately to 50  $\mu$ L of AuNPs in 5  $\mu$ L of antibody incubate 15 mins at RT then follow 5  $\mu$ L of 10% NaCl additional. After that these solution were measure by UV-vis spectra of absorbance of wavelength at 520 are recorded high significant level.

### Dot blot immunoassay for amyloid beta detection

The Schematic diagram of direct dot blot immunoassay for amyloid beta detection was shown in Scheme 1.



Scheme 1. Schematic diagram of direct dot blot immunoassay for determination of A $\beta$ <sub>1-42</sub>

In this dot blot assay, 1  $\mu$ L of amyloid beta antigen aliquots are spotted three times on dry Hybond ECL nitrocellulose and allowed to dry 10 mins in room temperature. The blot is blocked with 5% non-fat dry milk in TBS-T (20 mM Tris-HCL, pH7.5, and 0.8% NaCl (W/V), and 0.1% Tween-20 (V/V) for one hour at room temperature. After that spotted the conjugated antibody and blots incubated for 15 min at 4°C. Blots are washed three times in 10 mins with TBS-T solution. Finally, read the results by naked eyes.

## Results

### Effect of pH condition for antibody-conjugated gold nanoparticle

The pH of 2 ml colloidal gold aliquots was adjusted from pH 5 to 11 by 0.5 M NaOH. The effect of pH value in AuNPs is significantly color change from red to blue occurred immediately causing the aggregation of the

nanoparticles in solution after adding 0.1 M NaCl when measured by UV-vis spectra in fig. 2(b) and optimal pH of AuNPs was assumed that the value of pH level 8 was given high absorbance in wavelength at 520 nm in fig. 2(b).

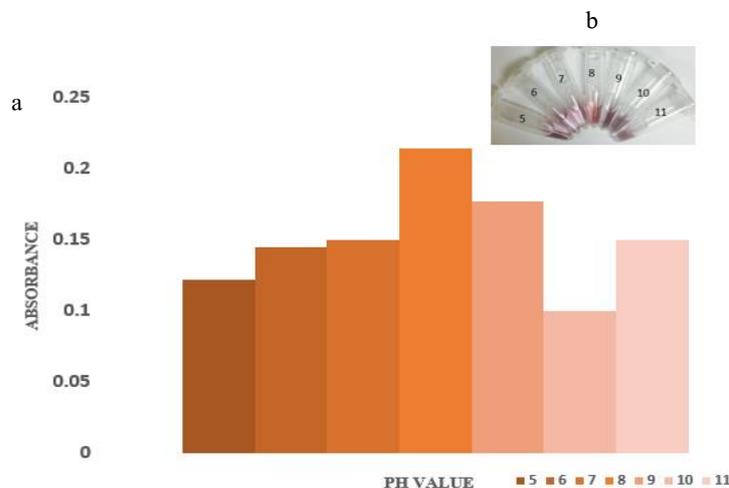


Figure 2. The optimal pH for binding antibody to AuNPs was determined between pH 5 and 11 in fig. 2(a). UV-vis spectra measured signal effect of AuNPs bond with antibody concentration 1:5000 dilution. Fig. 2(b) The color image of AuNPs solution in different pH was measured by UV-vis spectra wavelenth at 520 nm.

**Effect of Ab concentration on binding AuNPs**

The concentration of Aβ antibody was optimized for preparation of AuNPs conjugated Antibody. 5 μL of variable concentrations of antibody ratio from 1:5000 to 1:1 in 0.1 M PBS, was added separately to colloidal gold solution are appeared different color absorbance shown in figure 3. The significant absorbance of ratio of antibody and AuNPs solution was 1:5 dilution. Therefore, the ratio of (1:5 dil) AuNPs and antibody combined was selected to use in further experiment.

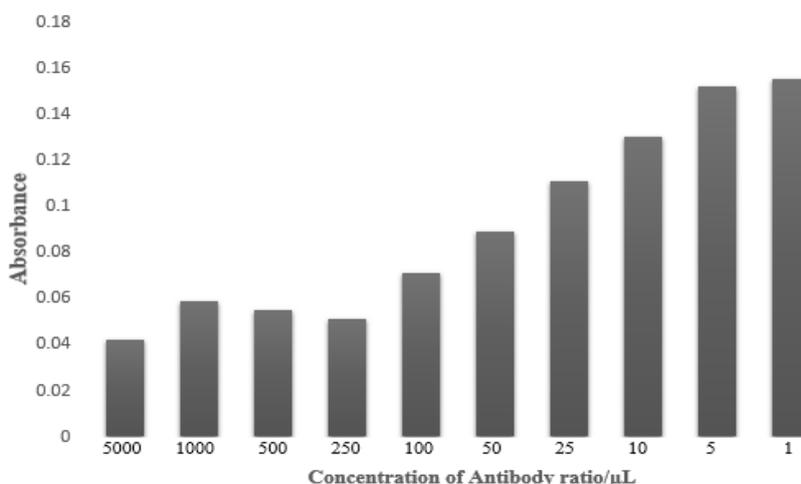


Figure 3. UV vis spectra of gold nanoparticle with various concentration (1:1 to 1:5000 (Ab: PBS buffer) measure at wavelength 520 nm.

**Effect of paper types**

Next, the effect of membrane properties on dot blot assay were investigated including fusion 5, AE99 and Hybond ECL. It was indicated the properties of three different type of filter paper in table 1. The result show that color intensity of AuNPs are significantly different in three types of paper fig. 4

Table 1. The properties of three different type of filter paper

Membranes	Pore Size	Thickness	Capillary Flow Rate	Properties
Whatman AE99	8.00 $\mu\text{m}$	1.5 mm	120 – 160 s/4cm	provides a good combination of sensitivity with fast wicking in a LFA, constructed of 100% nitrocellulose
Fusion 5	2.3 $\mu\text{m}$	370 $\mu\text{m}$	38 s/4cm	hydrophilic material, easy, fast, and efficient for traditional modular components from a lateral flow testing
Hybond ECL	0.45 $\mu\text{m}$	2.5 mm	No	provides excellent sensitivity, resolution, and low background in fluorescence blotting detection applications, High binding capacity

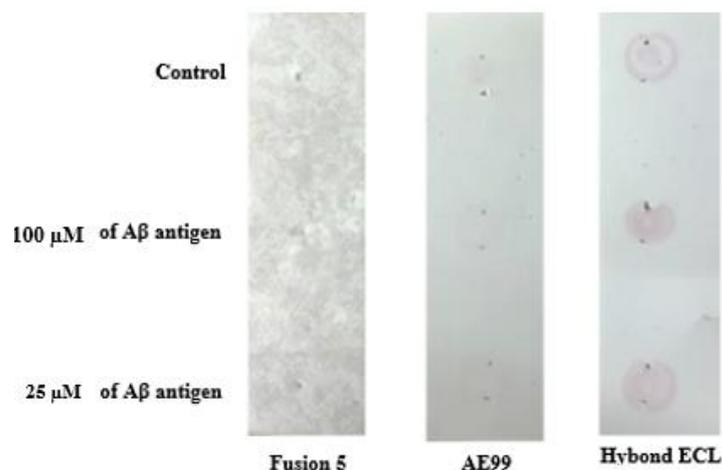


Figure 4. Effect of membrane materials on dot blot assay.

**Effect of incubation time**

There is the need of incubation time in antigen-antibody binding step to obtained high performance of colored signal in direct dot blot assay. It had left in three different incubation ( 10 min, 15 min, 30 min) times after combined with AuNPs conjugated antibody as shown in Fig. 5.

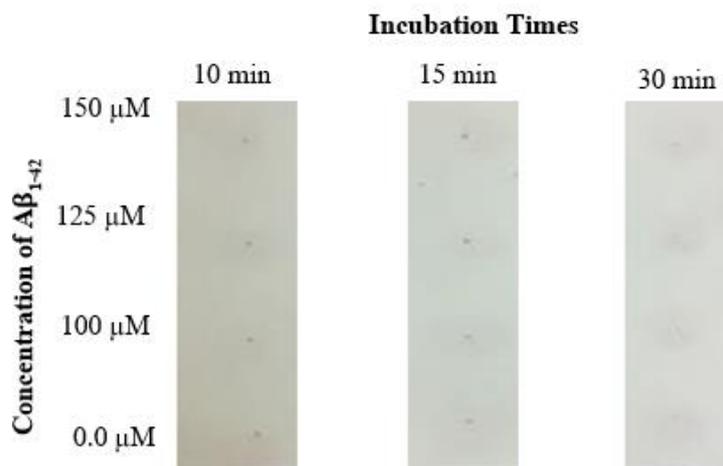


Figure 5. Effect of incubation time on dot-blot assay

Under optimized conditions of the dot blot immunoassay was performed for  $A\beta_{1-42}$  antigen detection. The results were shown in Fig. 6. The color intensity was increased with increasing of Ag concentration. The limit of detection was 1.0  $\mu\text{M}$  can be seen by naked eye.

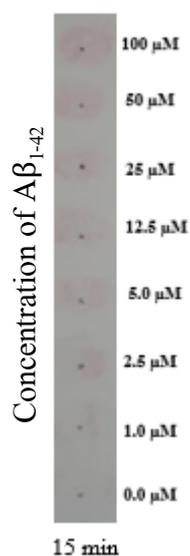


Figure 6. Optimized conditions of the dot-blot immunoassay was performed for  $A\beta_{1-42}$  antigen detection

### Signal enhancing system

In order to enhance signal binding antigen-antibody complex with AuNPs, It has been used silver-enhancer solution was composed of 1.0 g hydroquinone, 35 mg AgNO<sub>3</sub>, 50 ml citrate buffer and 50 ml distilled water (Chu *et al.*, 2005); stock solution is kept from light.

Firstly, Spots 1.0 ul of primary antibody ( goat anti A  $\beta$  oligomer) were immobilized in NC membrane in both controls and test strips and incubate at RT in 1 hr. Six micro wells were prepared different concentration of synthetic A  $\beta_{1-42}$  antigen (100  $\mu$ M, 50 $\mu$ M, 25 $\mu$ M, 5.0 $\mu$ M, 1.0  $\mu$ M, 0.0 $\mu$ M) with distilled water. Add with AuNPs conjugated antibody in each wells. After drying for 1 hr at RT, strips are dipped into the each wells including negative control as used D/W. Then wait until antigen attached with conjugated antibody flow to target primary antibody let them be antigen-antibody sandwich immunoassay reaction.

To get high sensitivity of the signal, 20 ul of silver enhancement solution were prepared in another six micro wells then strips are transferred into enhancing solutions.

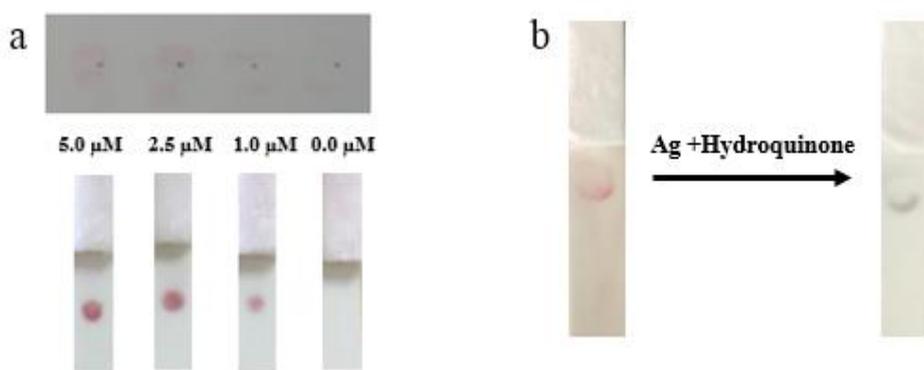


Figure 7. Comparison of Direct Dot blot immunoassay and sandwich immunoassay for determination of amyloid beta antigen in fig. 7(a), Silver-enhanced nanoparticle-labelled sandwich immunoassays for detection of A $\beta_{1-42}$  in fig. 7(b).

### Discussion

#### Optimization of pH

The effect of pH value in AuNPs is significantly color change from red to blue occurred immediately causing the aggregation of the nanoparticles in solution after adding 0.1 M NaCl when measured by UV-vis spectra in fig. 2 and optimal pH of AuNPs was assumed that the value of pH level 8 was given high absorbance in wavelength

at 520 nm. The colloidal AuNPs formed by citrate reduction were stable in a colloidal state and the presence of cations in salt solutions negated this charge repulsion and caused these particles to agglomerate and eventually precipitate (Yamamoto et al., 2004).

#### **Optimization of Ab concentration for AuNPs-Ab conjugate**

The best results for an optimal conjugation between antibodies and AuNPs was observed at antibody concentration ratio (1:5). The UV-vis spectra of antibody gold nanoparticles exhibited maximum absorbance at a wavelength of 520 nm as shown in figure 3. Demonstrate that antibody concentration ratio (1:5) enough to attach the surface of AuNPs.

#### **Optimization of type of membranes**

A $\beta$  oligomer antibodies labelled AuNPs specifically interacted with A $\beta$  surface antigen of to form a complex between conjugated antibody and antigen at the test zone. By the second state, the intensely red color which were produced by the accumulation of AuNPs in the dots are increasing of Ag concentration. As a result, demonstrates the evidence for specify detecting antigen. There are three different paper types including (whatman AE99, whatman G-5, nitrocellulose membrane) were investigated for providing high performance of dot-blot assay. The result in fig. 4 showed that the direct dot-blot assay cannot be performed on fusion 5 which is glass filter. While, Hybond ECL membrane which is standard NC membrane for dot-blot assay provide clearly results compared with AE 99. Therefore, Hybond ECL membrane was used in this experiment.

#### **Effect of incubation time**

The optimization of antigen-antibody binding step incubation was assumed that increase of incubation time, detection sensitivity increased significantly in fig.5. As the incubation time reached 15 min after binding with conjugated antibody, a simply color appeared highly. Therefore, 15 min was chosen in the further experiment as in fig.6.

#### **Signal enhancement system**

Although the dot blot immunoassay reached a sensitivity for amyloid beta antigen, a more sensitive to apply for detection of A $\beta$ <sub>1-42</sub> in real sample was needed. The comparison of results obtained by dot blot assay and sandwich

assay for beta amyloid detection were shown in fig. 7(a). Sandwich immunoassay is binding of antigen or antibody only occurs in the epitope of an antigen or antigen-binding site of an antibody. Since, there is a complementary relationship between epitope and antigen-binding site both in chemical structure and spatial configuration, therefore it provided reaction between antigen and antibody shows a strong specific higher sensitivity.

In order to enhance signal, the sandwich immunoassay with Signal-amplifying System using silver enhancer was developed binding antigen-antibody complex with AuNPs, Silver enhancement method was applied on final step by adding the mixing of Silver Nitrate and hydroquinone (v/v, 1:1) . The results were shown in fig. 7(b). The red color of AuNPs labels on test zone was change to blue color. This is due to the Ab-AuNPs conjugates reduce the silver ion to silver metal in the present of hydroquinone which absorbed at the surface of AuNPs.

### Conclusion

Dot blot immunoassay was successfully used for detection of  $A\beta_{1-42}$  antigen. In this technique, sample 1 $\mu$ L of three times are applied on the NC membrane followed by blocking , incubation and adding of antibody conjugate AuNPs. The color intensity of AuNPs was increased with increasing of Ag concentration. The limit of detection was 1.0  $\mu$ M, the sandwich immunoassay with Signal-amplifying System using silver enhancer has been developed that Silver nitrate used as a enhancing agent and hydroquinone to reduce formation of bulks in AuNPs attached sandwich immunocomplex and also gives high signal measured by naked eyes.

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