Dot-blot sandwich immunoassay with silver signal enhancement for simple amyloid beta protein fragment 1-42 detection

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ABSTRACT: Amyloid beta 1-42 ($A\beta_{1-42}$) is a key pathophysiological hallmark of Alzheimer's disease (AD). Changes in biofluids of $A\beta$ have been associated with AD risk. Herein, a dot-blot sandwich immunoassay was developed for simple detection of the $A\beta_{1-42}$ using gold nanoparticles as signal label. The results were easily observed by naked eye, and an image processing program provided semi-quantitation. Furthermore, addition of silver enhancer to the assay further improved sensitivity of the measurement. The proposed assay for $A\beta_{1-42}$ had a linear range of 1–200 µg/ml, with a detection limit of 1 µg/ml and 0.3 µg/ml by naked eye and calculation, respectively. Performance of the developed assay was assessed in analyses of spiked plasma samples. The recoveries of $A\beta_{1-42}$ were in the range of 112–158%, indicating that the developed assay is practical and reliable. The proposed method provides a low cost and simple assay for $A\beta_{1-42}$ measurement.

KEYWORDS: Alzheimer's disease, amyloid beta, dot-blot sandwich immunoassay, silver signal enhancement

INTRODUCTION

Alzheimer's disease (AD)[1,2] is a primary and common neurodegenerative disorder. Early signs include alteration of cognition, memory loss and subtle behavioural changes. One of the pathological hallmarks of AD is the deposition in the brain of amyloid-beta peptides ($A\beta$), which are composed mainly of the $A\beta$ protein fragment 1-42 ($A\beta_{1-42}$). Recent studies in large cohorts demonstrate that the $A\beta_{1-42}$ levels in body fluids, including plasma and cerebrospinal fluid, are correlated with AD processes in patients[3–5]. Hence $A\beta_{1-42}$ is an important marker of AD. In current clinical practice, AD diagnostics are based on the detection of $A\beta_{1-42}$ using an enzyme-linked immunosorbent assay (ELISA)[6]. Although this method is reliable, it is time consuming and requires skilled personnel. Thus other immunoassays have been developed which combine different methods of signal labelling and detection for $A\beta$ measurement. These

methods include electrochemistry[2,7–9], fluorescence analysis[4,10,11], flow cytometry[12], resonance light scattering[13] and immunoprecipitation-HPLC mass spectrometry[14]. However, these have their own limiting requirements: external equipment, high costs, and well-trained personnel. Hence it remains a great challenge to develop a user-friendly method for $A\beta_{1-42}$ detection. We chose colorimetric detection as suitable for a self-screening test because these assays are rapid and simple. Antibody-modified silver nanoparticles (AgNPs)[15] were developed based on the interaction between amyloid and Cu^{2+}. Furthermore, the mixing of C-terminal and N-terminal antibodies for $A\beta(1-42)$, conjugated to gold nanoparticles (AuNPs), was reported to successfully detect $A\beta_{1-42}$[16]. These methods were based on a similar method of aggregation of AgNPs causing a colour change which can be detected using colorimetric methods. The specific reaction can be influenced by several conditions such as pH and

presence of electrolytes, and other compounds in the test sample. Alternatively, a dot-blot immunoassay[17] is rapid, easy to perform, and adaptable for use as a screening test. Gold nanoparticles[18] have been extensively used for signal labelling in immunoassays because of their optical properties, including the strong extinction of light. These AuNPs-based colorimetric assays provide a high sensitivity test with results that can be easily distinguished by the naked eye (without external equipment). Gold nanoparticle-based dot-blot immunoassays[19] have been reported for β-amyloid peptide 1-42. However, the signal sensing enhancement, biotin-streptavidin and a light scattering intensity method were used which still needed complex equipment. Recently, silver enhancement has been developed for increasing the signal of AuNPs[20]. The method is based on the catalytic reduction of silver ions by AuNPs in the presence of a reducing agent, resulting in precipitated silver ions on the AuNP surface. These enlarged particles become a darker colour which is more easily visualized. Here we describe a dot-blot immunoassay which used AuNPs, coupled with silver enhancement for signal labelling. Importantly this enhancement led to a 5-fold increase in sensitivity. The results were easily visible by the naked eye within 30 min. Thus we propose that this developed method be applied for the rapid measurement of $A\beta_{1-42}$.

MATERIALS AND METHODS

Chemicals and materials

Anti-$A\beta_{1-42}$ monoclonal antibody (mAb) (ab201061) was purchased from Abcam (Cambridge, MA, USA). Anti-$A\beta_{1-42}$ polyclonal antibody (pAb) (AB5078P) was obtained from Merck (Darmstadt, Germany). Aβ protein fragment 1-42 (A9810), 30 nm gold nanoparticles, NaOH, HCl, non-fat dry milk, $AgNO_3$, citric acid, tween-20, tris hydrochloride, and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate were obtained from Sigma-Aldrich (St. Louis, USA). Potassium dihydrogen phosphate, NaCl, and sodium dihydrogen phosphate were purchased from BDH Prolabo (Lutterworth, UK). Hydroquinone was provided by Tokyo Chemical Industry Co., Ltd. (Japan). Silver enhancer consisted of reagent A: 100 mg silver acetate in 50 ml of Milli-Q water, and reagent B: 250 mg of hydroquinone in 50 ml of 0.2 M citrate buffer pH 3.8. Equal volumes of solutions A and B were mixed before use. Analytical-grade reagents and 18 MΩ-cm water were used throughout this experiment. Whatman AE99 paper was obtained from GE Healthcare (UK) and Hybond ECL nitrocellulose membrane was purchased from Bio-Med (USA).

Preparation of $A\beta_{1-42}$ oligomer

The Aβ protein fragment 1-42 (Sigma-Aldrich) was prepared as an Aβ oligomer[21]. The lyophilized proteins were dissolved in Milli-Q water at a concentration of 1 mg/ml and incubated at 37 °C for 1 h. The $A\beta_{1-42}$ oligomer solution was aliquoted and stored at −80 °C until use. In addition, the comparison results of binding assay using $A\beta_{1-42}$ oligomer prepared by H_2O, standard $A\beta_{1-42}$ solution from commercial ELISA kit (Merck) and NaOH as protocol from Abcam company were performed by ELISA kit (Merck). The results showed that our $A\beta_{1-42}$ preparation with H_2O displayed a signal (binding) with both used antibodies (the pAb, AB5048P, and the mAb, ab201061) as good as standard $A\beta_{1-42}$ and better than $A\beta_{1-42}$ prepared with NaOH (data not shown).

Antibody and antigen binding assay

The specific binding of anti-$A\beta_{1-42}$ antibody and $A\beta_{1-42}$ oligomer was tested by using an ELISA. First, $A\beta_{1-42}$ oligomer antigen was coated overnight onto the micro-well. Then anti-$A\beta_{1-42}$ mAb and anti-$A\beta_{1-42}$ pAb were added to separate wells and incubated for 1 h at room temperature (RT). After washing with PBS-T, HRP-conjugated anti-IgG antibody (AP132, Merck, Darmstadt, Germany) was added and incubated for 1 h. The micro-wells were washed to remove excess antibody. The signal was developed using tetramethylbenzidine (TMB), a substrate solution, then 1 N HCl was applied to stop the reaction. The resulting absorbance was measured at 450 nm using the Synergy HTX Multi-Mode Reader (BioTek, Winooski, Vermont, USA).

Preparation of antibody-conjugated AuNPs

The optimization of pH and antibody concentration was performed for Ab-AuNPs conjugation. First, the effect of pH was investigated at pH 5, 6, 7, 8, 9, and 10. Then 11.5 µg/ml of anti-$A\beta_{1-42}$ mAb in 10 mM PBS pH 7.4 was mixed with 50 µl of the AuNP solution. The mixture was incubated for 15 min at RT followed by the adding of 10 µl of 10% NaCl for testing. After that, the results were measured by Nano-drop UV spectrometer (Thermo Fischer, Wilmington, DE, USA). Next, the optimal concentration of anti-$A\beta_{1-42}$ mAb for conjugation

was determined by titrating aliquots of diluted anti-Aβ$_{1-42}$ mAb at different concentrations (0, 1, 10, 75, 100, 150, 175, and 200 µg/ml 10 mM PBS pH 7.4) with AuNPs at pH 8. After adding 50 µl of AuNPs in 5 µl of anti-Aβ$_{1-42}$ mAb, the solution was incubated for 15 min at RT. Then 5 µl of 10% NaCl was added in the mixture. Finally, absorbance of the solution was measured by UV-Vis spectrophotometer at 520 nm.

Conjugation of antibody and AuNPs

Conjugation of anti-Aβ$_{1-42}$ mAb was performed by mixing 20 µl of 175 µg/ml anti-Aβ$_{1-42}$ mAb in 10 mM PBS pH 7.4 with 200 µl AuNPs solution at pH 8, and incubating under gentle stirring for 1 h at RT. After that 20 µl of 3% bovine serum albumin (BSA) was added to block the surfaces of the AuNPs to reduce non-specific binding. The mixture was incubated for 1 h at RT then centrifuged at 15 000 rpm for 30 min at 4 °C to remove supernatant with unconjugated antibody. The separated precipitate was finally re-suspended in 20 µl 3% BSA + PBS at pH 7.4 containing 4% sucrose, then stored at 4 °C. After that the synthesized antibody-labelled AuNPs were characterized. UV-Vis absorption of AuNPs before and after conjugation with antibody was obtained by UV-Vis Spectra 2450 (Shimadzu). A transmission electron microscopy (TEM) image was obtained by HITACHI H-8100EM (Hitachi, Tokyo).

Dot-blot sandwich immunoassay

1 µl of anti-Aβ$_{1-42}$ pAb was spotted on nitrocellulose membrane and allowed to dry 10 min at RT. The membrane was blocked with 5% non-fat dry milk in TBS-T [20 mM Tris-HCl, pH 7.5, and 0.8% NaCl (W/V), 0.1% Tween-20 (V/V)] for 1 h with a conditional shaker at RT. The membrane was washed three times using TBS-T + 0.1% Tween-20 as washing solution. For testing, a mixture comprising 2 µl of sample and 2 µl of antibody-labelled AuNPs were applied onto pre-immobilized anti-Aβ$_{1-42}$ pAb and incubated for 15 min at RT, followed by washing three times with washing buffer. A schematic representation of positive results was shown in Fig. 1a. Then 2 µl of the silver enhancer was applied to the blot. After 15 min, membrane was washed 3 times (Fig. 1b). A picture of dot-blot assay results was taken by high resolution photo camera (Canon EOS 1000, Japan). For analysis of data, an image processing program was utilized (Fig. 1c). The intensity of the signal colour in the test zone were analysed by PHOTOSHOP (Adobe PHOTOSHOP CS6). The mean intensity value of each test zone was used to obtain a calibration curve for interpreting the results.

Preparation of plasma sample

Human blood samples were collected from Alzheimer's patients. Participants were recruited at the Department of Neurology of Thammasat University Hospital (Pathum Thani, Thailand). Participants were diagnosed by the medical neurologist, volunteered for the study, and provided both written and oral informed consent. The study protocol was approved by Human Research Ethics Committee of Thammasat University (MTU-ECIM-4-045/59). Ten millilitres of blood samples were drawn and transferred into the ethylenediamine tetraacetic acid (EDTA)-coated blood collection tubes (BD Vacutainer, Franklin Lakes, NJ, USA), and immediately centrifuged at 2500 rpm for 15 min at 4 °C. The plasma was separated and stored at −80 °C until use.

RESULTS AND DISCUSSION

Specific binding of antibody and antigen

The specific binding of the two antibodies (the mAb AB5048P, and the pAb ab201061) with the Aβ$_{1-42}$ oligomer was investigated by ELISA (Fig. 2). The results showed that both antibodies reacted with the prepared Aβ$_{1-42}$ oligomer, giving a strong absorbance signals at 450 nm when compared to the no primary antibody control (conjugate ctrl.) and no antigen control (PBS). Hence both antibodies used in this study bound specifically to the Aβ$_{1-42}$ antigen and were used in further studies.

Preparation and characterization of antibody-conjugated AuNPs

Conjugation of antibody with AuNPs was influenced by pH and concentration of antibody. Hence these parameters were optimized. The effect of pH was evaluated by adjusting from pH 5–11. At pHs below 6 and above 8, the colour of AuNPs was significantly changed from red to blue violet, occurring immediately after adding 0.1% NaCl. This was due to the aggregation properties of the AuNPs. The AuNPs were found to be most stable at 520 nm at pH 8 (Fig. 3a). Thus this value was chosen for use in the study. Concentration of antibody was investigated at 0, 1, 10, 75, 100, 150, 175, and 200 µg/ml of anti-Aβ$_{1-42}$ mAb (Fig. 3b). After adding of 10% NaCl, the colour of the AuNP solution changed to blue due to aggregation of AuNPs. However, the anti-Aβ$_{1-42}$ mAb at 175 µg/ml prevented this
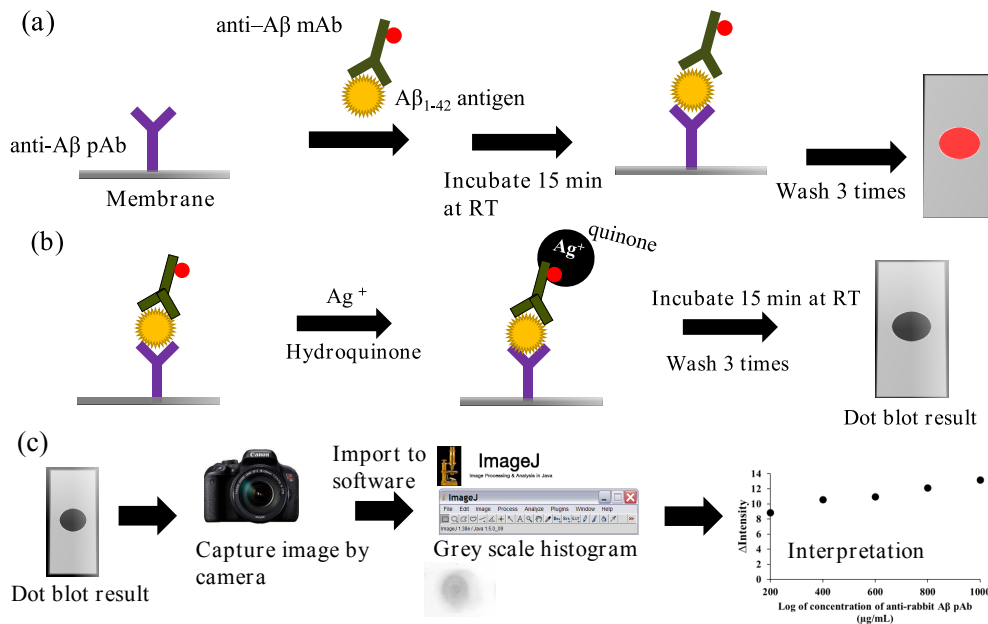
Fig. 1 Schematic drawings of dot-blot immunoassay with signal enhancement: (a) before and (b) after enhancement, and (c) the analysis of data.
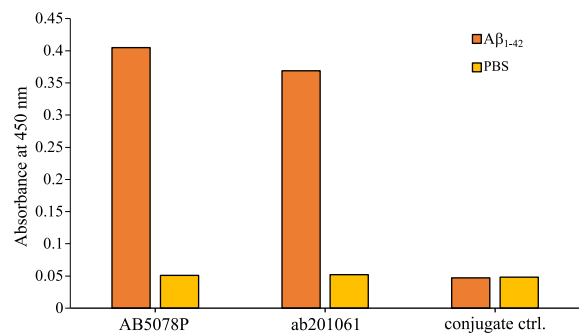
Fig. 2 The absorbance of antibodies AB5078P and ab201061 at 450 nm measured by indirect ELISA using HRP-conjugated anti-IgG antibody and TMB substrate. No primary anti-A$\beta_{1\text{-}42}$ antibody (conjugate ctrl.) and no A$\beta_{1\text{-}42}$ antigen (PBS) were used as controls.

aggregation (and colour change). This was because the amount of antibody was optimal enough for binding of AuNPs. Hence 175 µg/ml of anti-A$\beta_{1\text{-}42}$ mAb was selected to use for conjugation (at pH 8) with AuNPs. These antibody-labelled AuNPs were further characterized by UV-Vis spectrophotometer and TEM. The UV-Vis spectra of AuNPs showed a maximum wavelength at 520 nm. After conjugation, the UV-Vis absorbance increased slightly because of the antibody interaction with the AuNPs

(Fig. 3c). Furthermore, TEM images showed that the antibody-stabilized AuNPs remained as clusters of particles without aggregation (Fig. 3d).

Optimization of dot-blot immunoassay

Parameters potentially affecting the sensitivity of the assay for A$\beta_{1\text{-}42}$ detection, including paper-substrate, immobilized antibody concentration and incubation time of antigen/antibody binding were investigated.

Effect of paper type

To select optimal materials for use in the sandwich assay, the effect of membrane properties on the dot-blot sandwich immunoassay were investigated using Fusion 5, AE99 and Hybond ECL papers (Fig. 4a). The results showed that Hybond ECL, which has 0.45 µm pore size and a 100% pure nitrocellulose (NC) membrane, provided the best performance with clear A$\beta_{1\text{-}42}$ detection (Fig. 4a). Hence it was selected to use in this work.

Effect of capture antibody concentrations

The effects of varying concentrations of capture antibody (anti-A$\beta_{1\text{-}42}$ pAb) on NC membrane were investigated (Fig. 4b). The results showed that
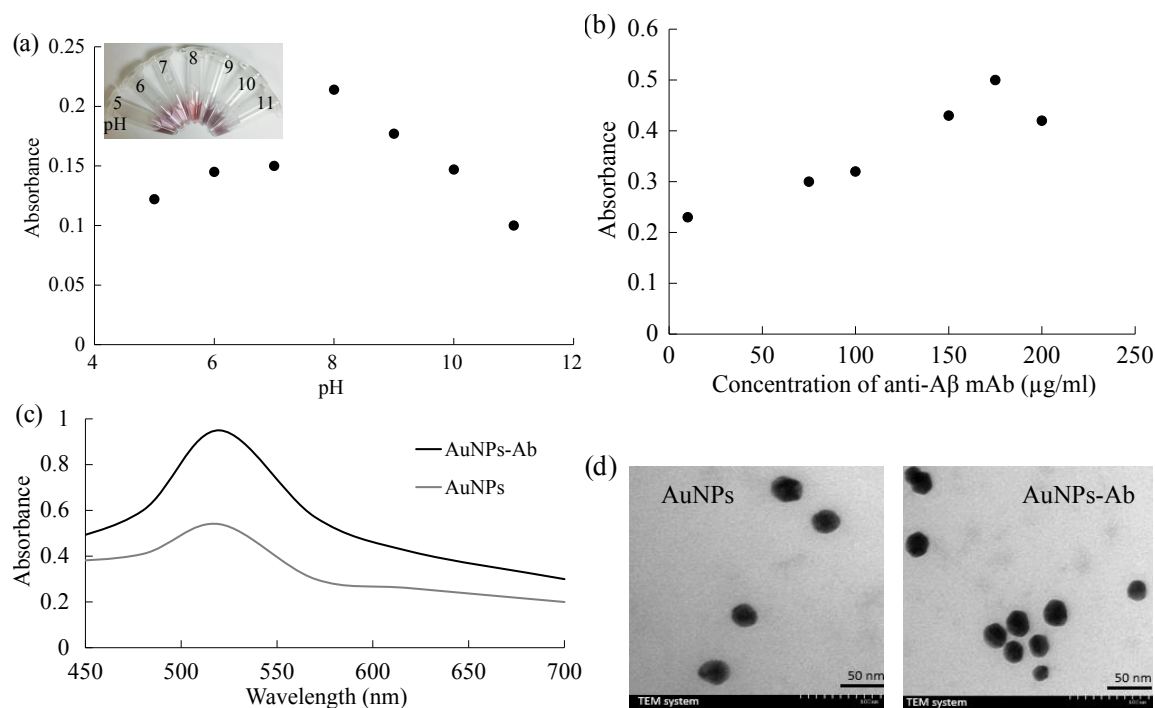
Fig. 3 Optimization of antibody and AuNP conjugation: (a) plot of absorbance observed at 520 nm versus pH value of the AuNP solution after adding of 10% NaCl (inset: the image of results at different pH): fixed anti-Aβ_{1-42} mAb concentration at 50 μg/ml, (b) plot of absorbance observed at 520 nm versus concentration of anti-Aβ_{1-42} mAb, (c) UV-Vis absorption spectra, and (d) TEM images of AuNPs and anti-Aβ_{1-42} mAb labelled AuNPs.

colour intensity of signals at the test zone was significantly increased with greater anti-Aβ_{1-42} pAb concentrations. Hence 1000 μg/ml of the polyclonal anti-Aβ_{1-42} antibody was selected for further experiments.

Effect of incubation time

The incubation time for the antigen and antibody binding step is very important to provide high performance of sandwich immunoassays. Hence the incubation time was investigated, assessing three different times (15 min, 30 min and 1 h). The plot of the intensity (results minus background) are shown in Fig. 4c. We found that an incubation time of 15 min provided the highest sensitivity of Aβ_{1-42} detection. Although the highest un-adjusted signal was obtained after 1 h, the background also increased strongly due to the membrane drying during the long incubation. Hence an incubation time of 15 min was selected.

Detection of Aβ_{1-42} using AuNPs signal labelling with silver enhancement

The sandwich immunoassay was performed to detect Aβ_{1-42} antigen at different concentrations (0–200 μg/ml). Presence of the target Aβ_{1-42} antigen was indicated with a red colour due to binding of the antibody-labelled AuNPs-Aβ_{1-42} antigen complex with immobilized pAb on the blot (Fig. 5a(i)). Intensity increased with concentration of Aβ_{1-42} antigen, plateauing with concentrations above 100 μg/ml. The limit of detection by naked eye was found to be 5 μg/ml. To enhance sensitivity, a silver enhancement system was added to the dot-blot sandwich immunoassay. The optical incubation time for enhancement was found to be 10 min and this was selected for use in the assay. After silver enhancement, the colour on the blotted membrane changed from red to a darker colour (Fig. 5a(ii)). This was because AuNPs reduce silver ions to silver metal in the presence of hydroquinone. The silver cover on the AuNP surface provided particle enlargement. Surface plasmon properties of red AuNPs were turned to a dark brown colour which was easily visualized and observed within 5 min.
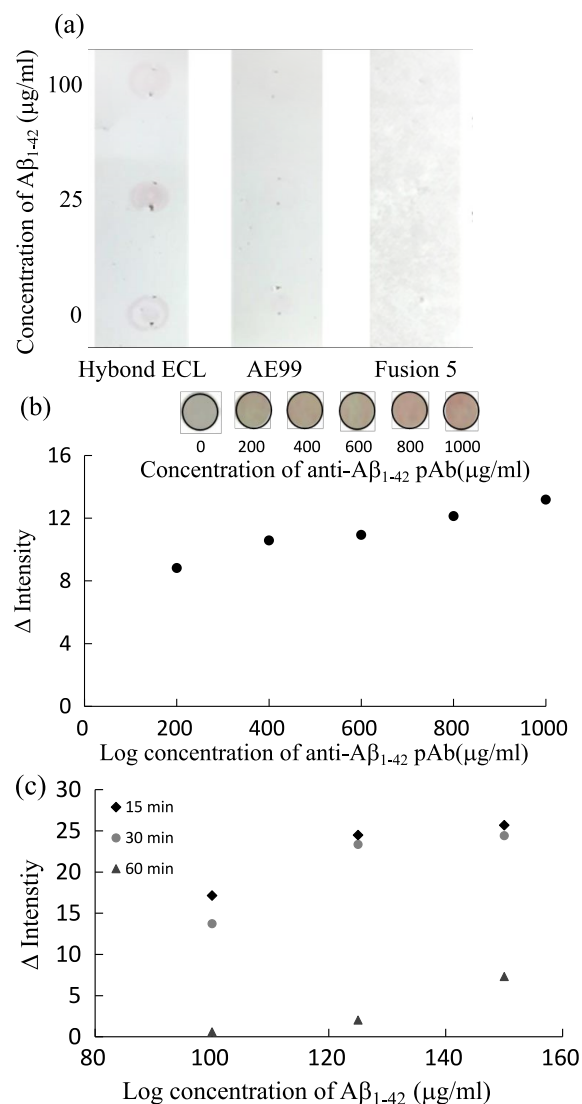
Fig. 4 Optimization of dot-blot sandwich immunoassay: (a) effect of paper-type: image results of $A\beta_{1-42}$ detection on different materials; (b) Effect of concentration of capture antibody: (above) the image results of $A\beta_{1-42}$ with concentration of capture antibody at 200, 400, 600, 800, and 1000 µg/ml, and (below) plot of intensity versus concentration of $A\beta_{1-42}$; (c) Effect of incubation time on antigen/antibody binding.

Sensitivity of the dot-blot was significantly higher due to the silver enhancer with the limit of detection of $A\beta_{1-42}$ falling to 1.0 µg/ml by naked eye. To assist in using the developed assay for detection of real samples, a colour chart was constructed under optimized conditions (Fig. 5c). Additionally, semi-quantitative analysis was performed using an image processing program in order to obtain more

reliable measurements. The calibration plot shown in Fig. 5d uses the equation $y = 0.470x^2 - 6.800x + 13.243$ ($r^2 = 0.991$). The limit of detection (LOD) by semi-quantitative analysis using this image processing program (at 3 SD of blank + mean value) was 0.3 µg/ml. The factors that could affect the sensitivity of detection include aggregation of $A\beta$ peptides to form unpredictable copies of oligomers. $A\beta$ oligomers with multiple copies may decrease the efficiency of both the conformation-specific capturing and the detection antibodies used in the study. These aggregates can also interfere with the accuracy of determining the LOD. Long incubation times helped to increase the binding of $A\beta$ with the antibodies, but long incubation periods also increase the background signal and so make more difficult the quantitation of $A\beta$ in samples. Furthermore, the LOD obtained has higher value compared to the previous work (1 ng/ml) by silver enhancement system 20, due to the assay conditions. First, the sandwich immunoassay[20] was performed in PDMS-glass slide wells. The amount of antibodies, sample and volume of the silver enhancement solution used are 10–20 times higher than that of this work due to the usage of an NC membrane. Second, a long incubation time (1–2 h) for each solution provides the good mixing of each reaction. Additionally, the sensitivity may be decreased by dry mixing of each reagent on membrane performed in this work.

For these reasons, in order to make this method applicable to detect the levels of amyloid beta 1-42 in human plasma, the dot-blot immunoassay should be further improved by adding pre-concentrated of an analyte on the detection zone and the wet system during of the experiment. Furthermore, the interpretation of the results during wet condition by UV-Vis equipment may also improve the sensitivity.

Detection of human plasma

The performance of the dot-blot immunoassay for $A\beta_{1-42}$ detection was verified using plasma samples and prepared $A\beta_{1-42}$. The results are shown in Table 1. The values of $A\beta_{1-42}$ in original plasma samples were lower than 0.1 µg/ml. Furthermore, the spiked samples were tested at concentrations of 1 and 5 µg/ml of $A\beta_{1-42}$ in prepared solution and sample. The colour development of the results was compared to a colour chart. The results obtained were in the range of 1–5 and 5–100 µg/ml for the samples with 1 and 5 µg/ml of $A\beta_{1-42}$ added, respectively. For the semi-quantitative analysis by image processing, the $A\beta_{1-42}$ value of samples 1, 2, and 3 were found at 1.5, 1.5, and 1.2 µg/ml,
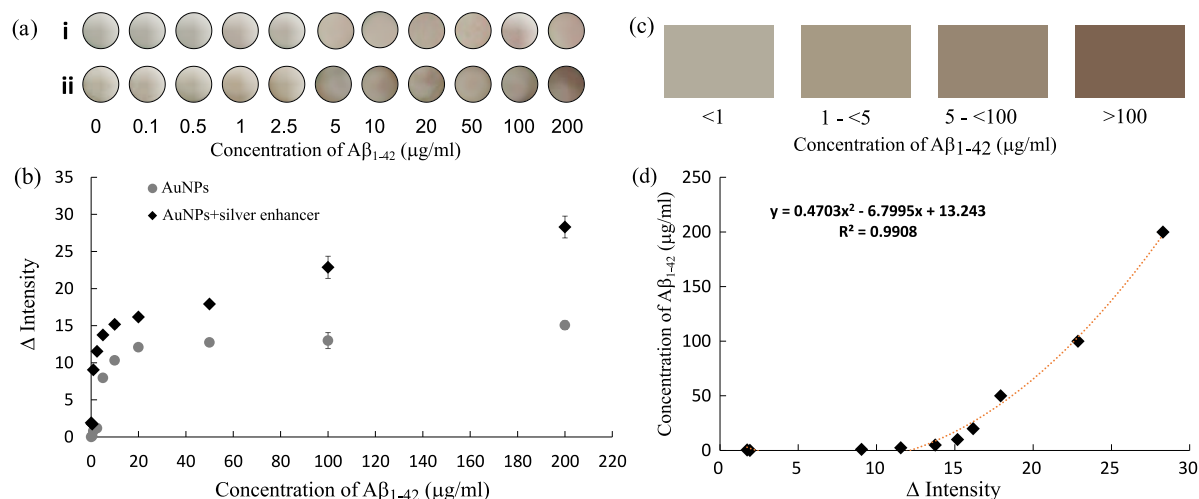
Fig. 5 Dot-blot immunoassay of Aβ$_{1-42}$ detection: (a) image results, (i) before and (ii) after silver enhancement; (b) plot of Δ intensity (intensity of sample - intensity of background) determined by digital - image analysis using Adobe PHOTOSHOP (Red and RGB channels for before and after silver enhancement, respectively); (c) the colour chart of Aβ$_{1-42}$ detection; (d) plot of concentration of Aβ$_{1-42}$ versus Δ intensity determined by digital-image analysis. The data were derived from 3 replicates.

Table 1 Determination of Aβ$_{1-42}$ in samples spiked with 1 or 5 µg/ml.

Sample	Found (µg/ml)	Added (µg/ml)	Colorimetric Found (µg/ml)	Image processing Found (µg/ml)	% Recovery	% RSD[*]
1	< 0.1	1	1–<5	1.5	150	3.7
		5	5–100	7.9	158	4.2
2	< 0.1	1	1–<5	1.5	150	3.9
		5	5–100	7.4	148	4.1
3	0	1	1–<5	1.2	120	2.8
		5	5–100	5.6	112	3.1

[*] RSD = relative standard deviation.

respectively, for adding 1 µg/ml of Aβ$_{1-42}$, and 7.9, 7.4, and 5.6 µg/ml, respectively, for adding 5 µg/ml of Aβ$_{1-42}$. The recoveries were in the range of 112–158%. These results indicated that the developed assay provided simple and rapid test for Aβ$_{1-42}$ detection.

CONCLUSIONS

A dot-blot immunoassay using gold nanoparticles with silver enhancement was developed for use as a simple semi-quantitative test for soluble Aβ$_{1-42}$. The assay required mixing of the sample with an anti-Aβ mAb conjugated to AuNPs followed by incubation for 10 min. After washing, a silver enhancer was applied to the assay. The total analysis time was about 20 min, and the assay was simple and more rapid than ELISAs. The limits of detection were

found to be 1.0 µg/ml and 0.3 µg/ml of Aβ$_{1-42}$ assessed by naked eye and calculation, respectively. For semi-quantitative analysis, an image processing program generating a calibration plot was utilized. This new dot-blot method was applied to detect spiked Aβ$_{1-42}$ in plasma with recoveries in the range of 148–158%. The current dot-blot detection system should be further improved for greater sensitivity of amyloid beta 1-42 levels measurement in human plasma by adding pre-concentration step and the wet system using UV-Vis equipment.

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