RESEARCH PAPER

An immunoassay using an electro-microchip, nanogold probe and silver enhancement

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Abstract This paper presents a novel immunoassay using an electro-microchip to detect the immuno-reaction signal, gold nanoparticles (ANPs) as a label of antigen or antibody and as a catalyst for silver precipitation, and the silver enhancement reaction to magnify the detection signal. This study is based on the direct immunoassay (two-layer format) and the sandwich immunoassay (three-layer format). The ANPs are introduced to the electro-microchip by the specific binding of the antibodies–ANPs conjugates and then coupled with silver enhancement to produce black spots of silver metal. The silver precipitation constructs a "bridge" between two electrodes of the electro-microchip allowing the electrons to pass, and the variation of the impedance can be easily measured with a commercial LCR meter. Different gap sizes (20, 50, 100, and 200 μm) of the electrodes of electro-microchips were designed for the sensitivity study. The experimental data show that a chip with a 200 μm gap has the highest sensitivity. There is a significant difference in impedance between the experiment and the negative control after 10 min reaction time. The proposed method requires less time and fewer steps than the conventional enzyme-linked immunosorbent assay. In addition, it shows a high detection sensitivity [10 μg/mL of 1st antibody (IgG) immobilized on slides and 10 ng/mL of antigen (protein A)], and there is a clear distinction between the signal intensity and the logarithm of the sample concentration. This new immunoassay has potential applications in proteomics research and clinical diagnosis.

1 Introduction

Immunoassays are based on the specific reaction of antibodies with the target substances (antigens), and play an important role in clinical diagnoses (Hage 1999). Based on the material used for protein labeling, they are classified as radioimmunoassay (Mayer and Neuenhofer 1994), enzyme-linked immunosorbent assay (ELISA) (Lai et al. 2004; Rossier and Girault 2001), fluorescence immunoassay (Hildebrandt et al. 2005), chemiluminescence immunoassay (Tanaka and Matsunaga 2000), and others. Many studies have, in different ways, attempted to improve the efficiency of the immunoassay, such as the use of a new or different label, different detection method and the amplification of the detection signals (MacBeath and Schreiber 2000; Zhu et al. 2001; Mezzasoma 2002; Hildebrandt et al. 2005; Chumbimuni-Torres 2006).

The immunogold labeling technique (colloid gold-labeled proteins) is commonly used for diagnosis because the high electron density of the gold nanoparticles (ANPs) exhibits a dark brown spots under an electron microscope, resulting in a distinct image for labeling (Hayat 1989; Lee 2002). In addition, this image response can be further enhanced through immunogold silver staining (Hayat 1995;

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Kühn et al. 1997; Peng 2007; Xu 2004), a technique by which silver particles are precipitated on the surface of the ANPs (Ma and Sui 2002; Nam et al. 2002). Recently, Liang et al. and Taton et al. used a simple commercial flatbed scanner as a detector for the colorimetric detection of protein and DNA microarrays, which used a nanogold probe coupled with the silver enhancement method (Liang et al. 2004; Taton et al. 2000). Based on the same principle, Kaler and colleagues show how to create arrays of biosensors by in situ assembly of colloid gold (Velev and Kaler 1999) and Yi and colleagues designed a colorimetric metal sensors based on DNAzyme-directed assembly of ANPs and their use for sensitive detection and quantification of metal ions (Liu and Lu 2003). In addition, Chu et al. (2005) used silver-enhanced ANP labels for the electro-chemical stripping metalloimmunoassay.

Electrical detection is a relatively new technique for protein microarray analysis. For example, Van Gerwen et al. (1998) used nanoscaled interdigitated gold electrodes for an impedimetric immunosensor. Porter et al. (2001) used self-assembled monolayer modified electrodes for an electro-active system of immunoassay. Park et al. (2002) used an electrical detection device coupled with an ANP probe and the silver enhancement method for DNA array which yielded a similar sensitivity in comparison with fluorescence detection. In addition, Cui and colleagues have developed a fluoroimmunoassay based on the fluorescence quenching caused by ANPs coated with antibody, where the sandwich type was separated by a magnetic field (Ao et al. 2006).

However, we have found no studies that attempted to employ a combination of the electro-microchip, the nanogold probe, and silver enhancement for constructing an immunoassay. Therefore, the aim of this study is to develop a novel immunoassay using an electro-microchip to detect the immuno-reaction signal, using ANPs as a label of antigen or antibody and as a catalyst for silver precipitation, and use the silver enhancement reaction to magnify the detection signal. The relationship between the gap size of the electro-microchip and the detection sensitivity are discussed as well.

2 Materials and methods

2.1 Reagents

Rabbit immunoglobulin G (rIgG), protein A (PrA), 20 nm protein A-ANPs conjugates, employed as a marker of the IgG–ANPs conjugates, silver enhancement solutions A and B and hydrogen tetrachloroaurate (III) were all purchased from Sigma (St Louis, MO, USA). Bovine serum albumin (BSA) V was purchased from Roche (Mannheim,

Germany). All other chemicals were of analytical grade and were used without further purification.

2.2 Principle of the electro-immunoassay

2.2.1 Principle of the proposed model

The principle of the proposed method was based on (1) the direct immunoassay (two-layer format) which was designed for qualitative analysis (Fig. 1a), and (2) the sandwich immunoassay (three-layer format) which was designed for qualitative and quantitative analysis (Fig. 1b). For the direct immunoassay, the test antigens were immobilized on the glass slides, and the gold-conjugated antibodies were then bound to the antigens, resulting in the formation of a two-layer complex. In this way, we could analyze the relationship between concentrations of test antigen and variation of the impedance to achieve a qualitative analysis.

The proposed sandwich immunoassay can provide qualitative and quantitative analysis. The 1st antibodies
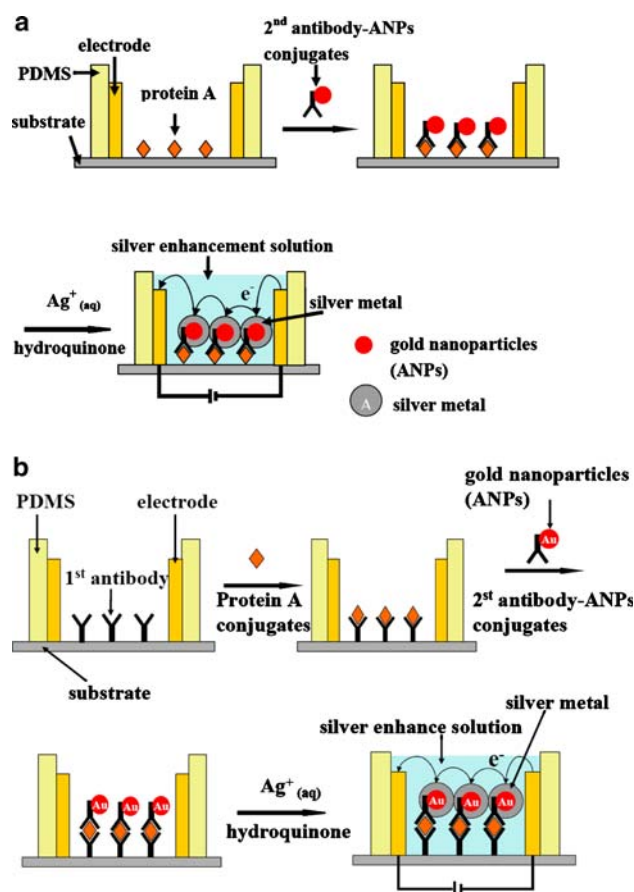
Fig. 1 Schematic illustrations of the antibody-ANPs conjugate recognition and signal amplification with silver enhancement: **a** direct immunoassay (two-layer format) and **b** sandwich immunoassay (three-layer format)

were immobilized on the glass slides, and the test antigens were then bound to the 1st antibodies. Excess unbound antigens were removed by washing with doubly distilled water. When the gold-conjugated 2nd antibodies were added to the chip, they would bind to the test antigens which were already bound to the 1st antibodies, resulting in formation of a sandwich-like complex. In this way, the qualitative information of relationship would be detected more precisely.

2.2.2 Principle of the signal amplification

In the signal amplification, the silver enhancement process was be used. The silver ions in the silver enhancement solution were reduced by the promotion of the nanogold, and large amounts of silver metal were precipitated on top of the gold [this is called "silver enhancement" (Peng 2007)]. These silver precipitations constructed a "bridge" to make short-circuited between two electrodes of the electro-microchip allowing the electrons to pass, and so further decreased the impedance. The variation of the impedance could be easily measured with a commercial LCR meter. In addition, the Lab-VIEW® program was employed to acquire experimental data for analysis.

### 2.3 Labeling of IgG to gold nanoparticles

Five nanometers of ANPs were prepared by a modified citrate-reduction process (Li et al. 2006). An aqueous tetrachloroaurate solution (1.0 mM, 100 mL) was cooled at near 0°C in an ice bath, and then trisodium citrate (0.17 M, 10 mL) was added to the solution. $NaBH_4$ solution (0.011 M, 20 mL) was then added dropwise to the Au(III)-citrate solution. The color change of the solution from blue to red indicated the formation of monodispersed spherical particles (~ 5 nm). The surfaces of the ANPs, covered with citrate anions in a solution at pH 6.2, indicated a negative charge. The value of pI of IgG is close to 7.0, and therefore, in a pH 6.0 solution IgG should bear a net positive charge. Thus, when 1 mL of IgG solution (100 µg/mL, pH 6.0, suspended in phosphate-buffered saline (PBS)) mixed with 10 mL of ANPs solution called IgG–ANPs solution was kept at 4°C for 120 min, then the IgG adsorbs onto the surfaces of ANPs through ionic interactions. We then added 1 mL of 2% BSA in the IgG–ANPs solution. The mixture was preserved at 4°C overnight, then centrifuged at 21,000 rpm ($23,100 \times g$) for 60 min, and the IgG–ANPs conjugates was then observed in the bottom of the mixed solution. We discarded the supernatant and added a resuspended solution of pH 7.2 (0.1% Triton X-100, 5% sucrose, 1% BSA, 10 mM PBS) to increase the stability of the IgG–ANPs conjugates.

### 2.4 Design and fabrication of an electro-microchip

The proposed electro-microchip shown in Fig. 2 consists of two components: an immuno-reaction well and the thin-film parallel electrodes. The electro-microchip was fabricated by the micro-electro-mechanical system technology on glass slides (Ao et al. 2006). Microfabrication processes, such as thermal evaporation, photolithography and wet etching were used to fabricate thin-film micro-electrodes as electrical probes. Gold and chromium thin films were thermally evaporated onto microscopic glass slides. Photolithography was used to transfer the electrode pattern to the thin films. The parallel electrodes were formed after chemical wet etching. Besides, a 3.0 mm thick layer with 2.5 mm diameter holes of poly-dimethyl siloxane (PDMS) was bonded to the glass surface with patterned parallel electrodes to form the fixed-volume (40 µL) immuno-reaction wells. The electrodes were made of 20 nm chromium and 200 nm gold, and the gap size ranged from 20 to 200 µm between the parallel electrodes. Glass slide and PDMS are suitable to be the reaction well for immunoassay due to they are both hydrophobic material. PDMS can naturally adsorb with glass slide to avoid leaking by pressing close to each other. In researches related utilizing PDMS and glass, the bond between these two materials is nature and simple, as for the reference (Pei et al. 2004; Adrien and Yong 2007).

2.5 Experimental procedure of electro-immunoassay

The immunoassay was conducted by following the typical procedure for the direct immunoassay (Fig. 3a) and the sandwich immunoassay (Fig. 3b). In the case of the sandwich immunoassay, rIgG was used the same way as the direct immunoassay test antigens. In each case, 40 µL of
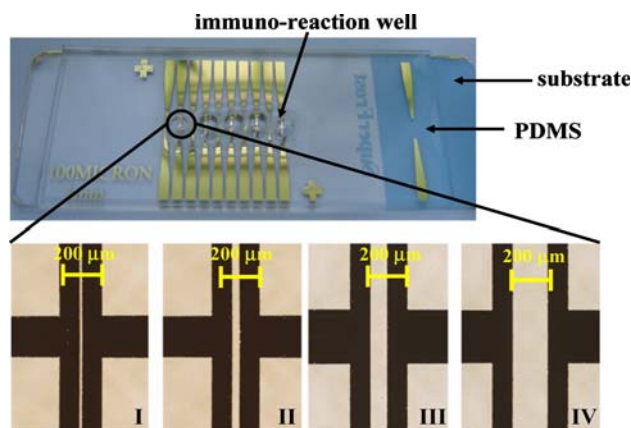
Fig. 2 Pictures of the impedance immunoassay electro-microchip include PDMS immuno-reaction well and parallel electrodes [a close-up view of the electrode gaps: (I) 20 µm, (II) 50 µm, (III) 100 µm and (IV) 200 µm]
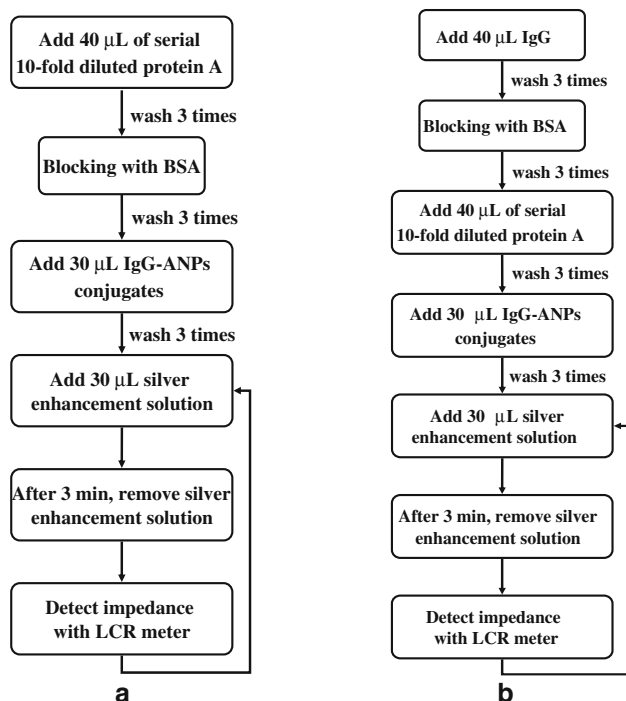
Fig. 3 Typical procedure for the immunoassay: **a** procedure for the direct immunoassay and **b** procedure for the sandwich immunoassay

IgG solution (10 μg/mL total antibody) in 50 mM PBS buffer (KH_2PO_4/K_2HPO_4, 150 mM NaCl, pH 7.6) was pipetted onto the PDMS reaction area on the electro-microchip and allowed to react for 2 h at 37°C. After being rinsed with copious amounts of PBST (PBS + 0.05% Tween 20) and deionized water, the sample was exposed to 40 μL of serial tenfold diluted protein A (diluted in PBS containing 2% BSA) for 3 h. Finally, 30 μL IgG–ANPs conjugates were added to the well and allowed to react for 2 h. All samples were then rinsed with deionized water.

The measurement system included: (1) an LCR meter, which was the main measurement instrument to measure the variation of the impedance, (2) a Lab-VIEW® program for acquiring the data automatically, (3) an electro-microchip to detect the immuno-reaction signal, and (4) ANPs as a label of antigen or antibody and as a catalyst for silver precipitation. The entire detection procedure was shown in Fig. 4.

3 Results and discussions

3.1 IgG–ANP conjugates

The TEM image and the UV-Visible spectrum were used to prove the IgG–ANPs conjugates. A 20 nm colloid gold-labeled protein A, purchased from Sigma Chemical Co., was employed as a marker of the IgG–ANPs conjugates. From Fig. 5 we find specific spaces between the 5 nm
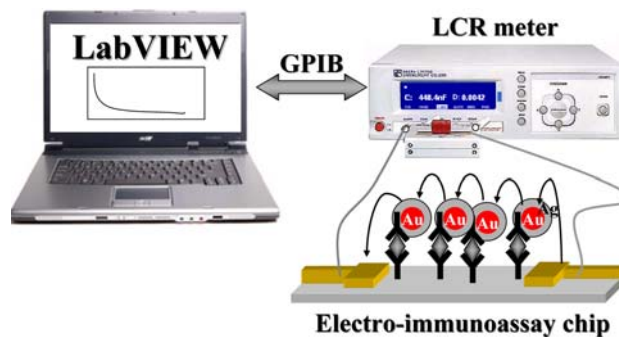
Fig. 4 Electrical detection system including computer, LCR meter and electro-micro chip. The GPIB protocol will be used to link the computer and LCR meter and the Lab-VIEW® program is used to intercept the data automatically

probe (IgG–ANPs conjugates) and the 20 nm marker (colloid gold-labeled protein A), which indirectly proved the conjugation of the ANPs and IgG. Due to the specific conjugation characteristic between IgG and protein A, when protein A-Au (20 nm) was added into IgG-Au (5 nm), the conjugation took place. Thus, 20 nm Au and 5 nm Au were bound together because of IgG and protein A which lied between two Au nanoparticles. However, we cannot see the IgG and protein A in TEM images because they are both protein. Therefore, a specific space would exist between the 5 nm ANPs and 20 nm ANPs in TEM images on the condition that IgG was bound successfully with 5 nm Au. On the other hand, if the bound between IgG and 5 nm Au was not successful, the Au nanoparticle of different sizes will aggregate naturally and no specific space could be observed in TEM images. The IgG–ANPs conjugates were also supported by their UV-Visible absorption spectroscopy, and two specific absorption peaks at 270 nm (IgG) and a red shift in the peak from 520 nm (5 nm ANPs) to 530 nm indicated a successfully constructed nanogold probe (Fig. 6) (Hayat 1989).

3.2 The relationships between gap size and sensitivity

Generally speaking, when the silver was reduced and coated on the ANPs, the impedance would be decreased.
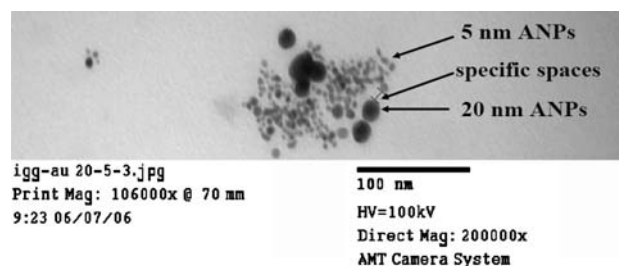
Fig. 5 TEM images of IgG-ANPs (5 nm) conjugates binding with protein A-ANPs (20 nm) conjugates
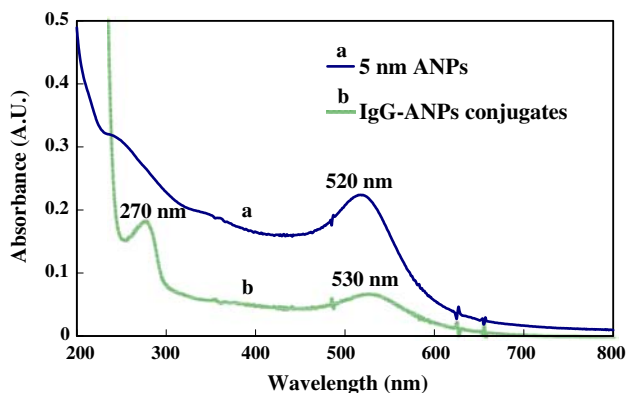
Fig. 6 The UV-Vis spectrum of 5 nm ANPs and IgG-ANPs conjugates. Two specific absorption peaks at 270 and 530 nm indicate a successfully constructed nanogold probe

The degree of impedance variation provided an interesting and a meaningful determination for the quantitative analysis of our proposed immunoassay. The different gap size (from 20 to 200 µm) of the electro-microchip was designed to facilitate a discussion on the relationship between the gap size of the electrode and the sensitivity. The experiments were carried out under the condition of 10 µg/mL antigen and 10^{-9} M IgG–ANPs conjugates. The experimental group is to add silver enhancement solution after detecting the IgG–ANPs conjugates adsorbed on the impedance chip, while controls group is to add silver enhancement solution onto the blank impedance chip. As a mixture of Hydroquinone and Ag^+ solution, the silver enhancement solution will spontaneously change in an environment without nanoparticles, therefore, we chose the silver enhancement solution as the control experiment to compare with the nanoparticle experiment. The results

of Fig. 7a shows that the variation in impedance between experiment and control were very similar because of the background interference. Nevertheless, the 200 µm gap of the electro-microchip has a better capability to discriminate the experiment and the control (the impedance variations between 300 and 660 s were higher than 150 ohm, blue line of Fig. 7b). Based on the results of Fig. 7a, b, we determined that the 20, 50, and 100 µm gaps of the electro-microchips were not an ideal sensor and transducer for our proposed immunoassay system. A 200 µm electrode gap size for the electro-microchip had the higher sensitivity, so we chose it for our follow-up experiment.

3.3 Specificity and detection limit experiment

The proposed sandwich model is similar to commonly sandwich of the ELISA. Just difference in the materials of protein labeling (enzymes vs. ANPs) and signal amplify process. Experiments were under the condition of 1st antibody 10 µg/mL, antigen $0.01 \sim 10$ µg/mL and IgG–ANPs conjugates 10^{-9} M. Non-specific antigen BSA was employed as the negative control. Based on Fig. 7c, we find an apparent decrease of the impedance in the experiments, but not in the negative control, meaning that the proposed immunoassay approach can distinguish the detected antigen from negative control. In addition, the results show that the degree of impedance variation was in direct proportion with the concentration of the detected antigen, and that the antigen detection limit was 10 ng/mL. We also subtract the negative control from the impedance of the experiment (Fig. 7d) to evaluate the optimized time of measurement. The impedance shows an obvious

Fig. 7 The impedance spectrum of **a** the direct immunoassay in different gap sizes (from 20 to 200 µm), **b** the experiment deducted from the control of **a**, **c** the sandwich immunoassay in different concentrations of antigen (from 0.01 to 10 µg/mL), and **d** the experiment deducted from the negative control of **c**
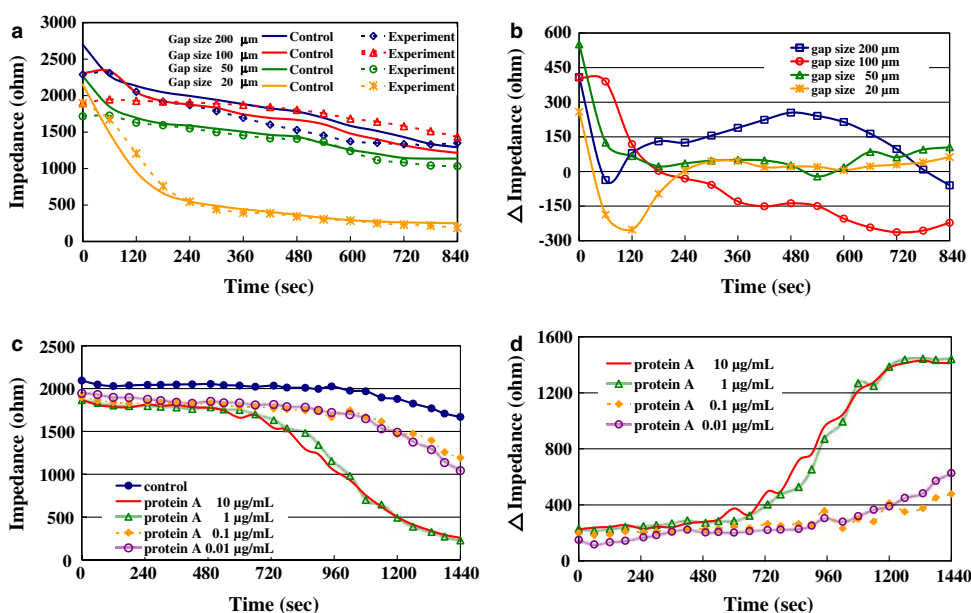
diversity after the ANPs reacted with the silver enhancement solution 720 s (12 min). We propose that the optimized time of measurement is between 720 s (12 min) and 1,200 s (20 min). This shows that the results of the immunoassay can be appraised faster than the conventional ELISA.

4 Conclusions

We have provided a novel immunoassay based on an electro-microchip to detect the immuno-reaction signal, using ANPs as a label of antigen or antibody and as a catalyst for silver precipitation, and using the silver enhancement reaction to magnify the detection signal. Experimental data show that an electro-microchip with a 200 μm gap have the highest sensitivity. The detection limit is 10 ng/mL of antigen (protein A) with 10 μg/mL of 1st antibody (IgG) immobilized on slides. Our results indicate that the proposed electro-immunoassay method can provide a highly accurate in aqueous solution which protein immobilized on slides with patterned parallel electrodes. This method provides several advantages for the implementation of an assay including easy to perform, only a small amount of the reagent is required (as little as 40 μL sample protein per well), fast (within 30 min), and it is convenient as well as low-cost. This approach has many potential usages for protein microarray research and clinical diagnosis.

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