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SHORT COMMUNICATION

Colorimetric immunoassay chip based on gold nanoparticles and gold enhancement

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Abstract A colorimetric immunoassay chip has been developed based on gold nanoparticles for indicating the antibody–antigen binding activity and gold enhancement for amplifying the specific binding signal. Our investigations showed that the results of immunoassay can be represented by the level of color intensity. They were easily observed by a regular camera or naked eye, which is not needed of sophisticated laboratory equipment. Optimization of experimental conditions was carried out and the colorimetric detection had been compared to the standard chemifluorescent detection. Under the optimized conditions, colorimetric immunoassay chip had been demonstrated to detect different amount of immobilized antigens, i.e., human IgG. The results, i.e., color intensity, were mapped to the concentration of immobilized antigens in a dynamic range of 1–5,000 ng/ml. The proposed detection method does not require any sophisticated optical systems; therefore, it is possible to be miniaturized and integrated into a microfluidic system for developing a portable immunoassay device.

Keywords Immunoassay · Gold nanoparticles · Colorimetric immunoassay · Microfluidics

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1 Introduction

With the recent developments in micro-fabrication technology, microfluidic systems have been rapidly developed from early single channel devices (Harrison et al. 1993) to current complex analysis systems (Melin and Quake 2007). Because of the mature fabrication techniques, researchers actively explore the possible applications using microfluidic technology. One of the applications is to integrate immunoassay into the microfluidic system (Sato et al. 2002; Cho et al. 2006). Immunoassay is commonly used in many clinical, pharmaceutical, and scientific research laboratories. This bio-analytical technique is capable of detecting the specific antigens through the binding of the corresponding antibodies. The immunoassay is used to measure the specific reacted signal from the label attaching on either the antibody or antigen quantitatively. According to the labeling material used, immunoassays are classified as enzyme immunoassay (Yakovleva et al. 2002; Kurita et al. 2006; Arenkov et al. 2000), radioimmunoassay (Ma et al. 1996), magnetic immunoassay (Chemla et al. 2000), fluorescent immunoassay (Li et al. 2005), metalloimmunoassay (Fan et al. 2005), etc. Among these techniques, the most widely used format of immunoassay is enzyme-linked immunosorbent assay (ELISA). An unknown amount of antigens is immobilized on a solid support (surface) and the antigens bind with the specific enzyme labeled antibodies. The enzyme, such as horseradish peroxidase (HRP), can produce a fluorescent signal by adding suitable reactive substrate. Then, the amount of antigens can be determined by the magnitude of the signal. However, the process of conventional immunoassay is laborious and requires various equipments and long processing time. Also, most of the immunoassay systems are based on fluorescent detection. It has the advantage of high sensitivity, but requires dedicated

optical equipment which has the limitation of miniaturization. Therefore, immunoassay is difficult to conduct in the point-of-care (POC) applications. The proposed colorimetric immunoassay chip has been developed to determine the immunoassay result using a regular camera or even by the naked eye. Therefore, it is possible to be miniaturized and integrated into a microfluidic system for developing a miniaturized immunoassay detector.

Recently, a number of research groups have reported the use of metallic nanoparticles, e.g., gold and silver colloids, for the labeling materials of protein or DNA in various analyses or detections. Conventionally, silver deposition on metallic nanoparticles is widely used in histochemical microscopy. For example, antibodies labeled by gold nanoparticles are bound to the antigens in biological tissues. Gold nanoparticles serve as catalysts to reduce silver ions to metallic silver; this process could enlarge the size of gold nanoparticles. The protein or DNA labeled by gold nanoparticles becomes visible under optical or electron microscope (Danscher and Rytter Norgaard 1983; Seopsi et al. 1986). This technique could identify the distribution of the target antigens over the cell surface under microscope. For the applications of immunoassay and DNA array, there are several reasons of using metallic nanoparticles: (1) the availability of a wide range of sizes; (2) unique optical and electrical properties; and (3) high biocompatibility. The pioneering works of using silver enhanced gold nanoparticles for colorimetric and electrical DNA array detection have been demonstrated (Taton et al. 2000; Alexandre et al. 2001; Park et al. 2002). The DNA hybridization on solid support was indicated by gold nanoparticles and amplified by silver enhancement process. The results were detected by a conventional flatbed scanner (Taton et al. 2000; Alexandre et al. 2001) or measured by conductivity changes across the micro-electrode (Park et al. 2002). For the detection of immunoassay, a label-free visual immunoassay on the glass slides has been reported (Ling et al. 2008). Antibody was first immobilized on the glass slides and silver nanoparticles were electrostatically adsorbed on the glass slides under particular conditions. A white light-emitting diode (LED) torch was employed to illuminate the glass slides; visual detection of the antibody can be detected by the naked eye. Moreover, colorimetric immunoassay using antibody-gold nanoparticle conjugate and silver enhancement has been demonstrated (Yeh et al. 2009). A flatbed scanner was utilized for the optical scanning and measuring of the immunoreaction signal on the glass slides. Electrical detection of immunoassay using silver enhanced gold nanoparticles has also been reported (Velev and Kaler 1999). In situ assembly of colloidal particles has been developed across the microelectrodes. The proteins were captured by the colloidal particles and their concentration can be determined by the conductivity changes across the micro-electrodes. Moreover, electrochemical detection of protein binding activity based on gold nanoparticles has been reported (Dequaire et al. 2000; Chu et al. 2005). The immunoassay was determined by anodic stripping voltammetry (ASV) via oxidative gold metal dissolution in the acidic solution. Also, chemiluminescent metalloimmunoassay has been developed based on luminol chemiluminescent reaction (Li et al. 2006). Furthermore, gold nanoparticles can also be applied to enhance the signal of surface plasmon resonance (SPR) biosensor (Mitchell et al. 2005). The SPR signal enhancement of sandwich immunoassay can be achieved by labeling the secondary antibodies using gold nanoparticles.

There are a number of studies of chemiluminescent and electrochemical immunoassay, but a few studies have discussed the immunoassay detected by visible color. In this work, a colorimetric immunoassay chip was described, and the analysis results could be visualized readily without the need of sophisticated equipment. The present protocol used gold nanoparticles for the indication of antibody–antigen binding activity and gold enhancement for the signal amplification. Gold nanoparticles were bound to the detection antibodies via biotin-streptavidin linkage. Then, gold enhancement process was performed to enlarge and fuse the gold nanoparticles. The enhanced metallic gold particles became visible and the level of color intensity represented to the concentration of the antibodies. Here, we utilized gold enhancement instead of silver enhancement for the signal amplification. Silver enhancement is commonly used in the literatures; however, it has a disadvantage of autonucleation. The time control of silver enhancement is critical for a sensitive detection with a low background signal. It suffers from shortcomings in reliability, accuracy, and signal quantification. Comparatively, gold enhancement presents much better capability in signal amplification because autonucleation is minimal even after 1–2 h.

Optimization of experimental conditions, including dilution of nanogold solution and gold enhancement time, was carried out. The antibody–biotin conjugates were directly immobilized on a 3-aminopropyltriethoxysilane (APTES)–glutaraldehyde (GA) modified glass surface. The dilution of nanogold solution was optimized for binding gold nanoparticles to the antibodies via biotin–streptavidin linkage. Also, the time required for gold enhancement process was figured out. These gold precipitations became visible and increased color intensity gradually with time. By using the optimized conditions, the color intensity can represent the concentration of the immobilized antibodies. Negative control (no immobilized antibody) was also set in the optimization process. It was noted that the color intensity of negative control can be minimized even after 20-min gold enhancement process. The results had been also compared to the chemifluorescent detection, and the colorimetric detection showed a promising performance. Moreover, a model

protein pair, i.e., human IgG/anti-human IgG, detection on the chip had been also demonstrated. Different concentrations of the target antigens, i.e., human IgG, were immobilized on the APTES–GA-modified glass surface and the detection antibodies, i.e., anti-human IgG (Fc specific)–biotin conjugates, which were applied and bound to the target antigens. Then, nanogold solution was applied and the amount of gold nanoparticles was in proportion to the amount of antigen–antibody complex. After gold enhancement process, the enhanced metallic gold particles became visible and the level of color intensity can represent the concentration of the target antigens. The proposed immunoassay chip has the advantage of the detection without sophisticated laboratory equipment. It has the potential to be integrated into a microfluidic system and developed to a portable immunoassay device.

2 Materials and methods

2.1 Chemicals and reagents

Anti-mouse IgG–biotin conjugates, human IgG, anti-human IgG (Fc specific)–biotin conjugates, and bovine serum albumin (BSA) were purchased from Sigma, USA. Anti-mouse IgG-HRP conjugates were purchased from Santa Cruz Biotechnology, USA. 3-aminopropyltriethoxysilane (APTES), glutaraldehyde (GA), and chemifluorescent HRP substrate were purchased from Pierce, USA. Nanogold-streptavidin conjugates and gold enhancement solution were purchased from Nanoprobes, USA (http://www.nanoprobes.com). Buffer used in this study was phosphate-buffered saline (PBS; 50 mM phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.6). Distilled water was used throughout the experiments.

2.2 Preparation and surface modification of the immunoassay chip

The surface modification of glass slide for immobilizing protein was followed as the previously reported method (Williams and Blanch 2004). A regular glass slide was first cleaned in a 70:30 (v/v) mixture of H_2SO_4 and H_2O_2 for 30 min at room temperature. Then, it was washed several times in distilled water and dried in oven. The glass slide was immersed in 5% (v/v) APTES in acetone for 30 min at room temperature. Then, it was rinsed thoroughly in acetone and distilled water, dried in nitrogen flow, and baked in oven at 80°C for 2 h. This process could form a reactive amine on the glass surface. The APTES-treated glass surface is hydrophobic, supporting the fact that the surface contact angle is around 60°. Then, a 1-mm-thick poly-dimethyl siloxane (PDMS) material with 14 circular openings in 2-mm diameter was bound to the treated glass surface and formed the reaction wells, as shown in Fig. 1. All experiments were performed in the reaction wells on glass surface. The reaction wells can control the reaction surface area and eliminate the cross-contamination among different wells. 2.5% (v/v) GA in PBS was pipetted to the reaction wells for 2-h incubation at room temperature. The GA reacted with amino group on glass surface and worked as a cross-linker. After carefully rinsing with distilled water, the APTES–GA-treated areas were ready to be covalently immobilized by the protein for further experiments.

2.3 Immunoassay procedure

The quantitative colorimetric immunoassay chip was based on gold nanoparticle indication followed by gold enhancement process. The schematic representation of the protocol is shown in Fig. 2. The target antigen and detection antibody were human IgG and anti-human IgG (Fc specific)–biotin conjugates, respectively. After the surface modification of the glass slide, the APTES–GA-treated areas in the reaction wells were exposed to the target antigen suspended in PBS buffer and incubated for 1 h at 37°C or overnight at 4°C. The target antigens were covalently immobilized on the glass surface via the GA cross-linker. The chip was treated with blocking buffer (1% BSA suspended in PBS) for 1 h at room temperature to block the active site of the unreacted GA. Then, the detection antibody suspended in PBS buffer was added to the reaction wells for another 1 h at 37°C to bind the target antigen. After each reaction, the glass slide was thoroughly washed in PBS containing 0.05% Tween 20.

The detection was carried out by tagging the antibody–biotin conjugates to the gold nanoparticles. Nanogold–streptavidin conjugates with 1% BSA suspended in PBS buffer (nanogold solution) in a certain dilution were added to the reaction wells and incubated for 1 h at room temperature. Gold nanoparticles were bound to the detection antibodies via biotin–streptavidin linkage. Theoretically, the more antibody–biotin conjugates immobilized on glass surface, the more gold nanoparticles could bind to the glass surface. Because the gold nanoparticles are of 1.4-nm diameter, they are too tiny to generate detectable visual signal. Therefore, gold enhancement process was performed, and the gold

Fig. 1 Glass slide with 14 reaction wells

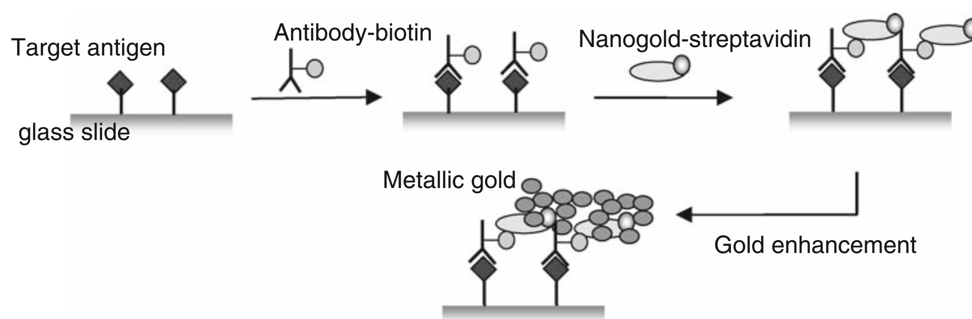

Fig. 2 Schematic representation of the colorimetric immunoassay chip. The detection was based on gold nanoparticle indication and gold enhancement process

nanoparticles could be enlarged. In gold enhancement process, gold ions in solution were catalytically deposited onto the gold nanoparticles and aggregated to metallic gold precipitations. The density of the gold precipitations was based on the density of gold nanoparticles. These gold precipitations became visible and darkened the color gradually with time. After completion of the process, the glass slide was rinsed in water and dried. The results, i.e., color intensity of the reaction wells, were captured by a regular camera with an appropriate illumination. They were analyzed by commercial software and converted to 8-bit gray level values for representation.

3 Results and discussion

3.1 Optimization of the experimental conditions

The experimental conditions, including the dilution of nanogold solution and gold enhancement time, were investigated and optimized. Because the detection signal was directly related to the concentration of the detection antibodies, the antibody–biotin conjugates were immobilized on the glass surface for the optimization in order to eliminate the biological variation, e.g., protein binding strength, specificity, etc. Different concentrations, i.e., 5,000, 500, 160, 80, 60, and 40 ng/ml, of anti-mouse IgG-biotin conjugates suspended in PBS buffer were pipetted to the APTES–GA-modified glass surface in the reaction wells. To ensure the specific signal, a negative control (NC), i.e., 0 ng/ml of immobilized protein, was also set by applying PBS buffer only. Four identical glass slides were prepared for investigating different dilutions of nanogold solution and gold enhancement time.

3.2 Dilution of nanogold solution

The detection sensitivity and signal-to-noise ratio of colorimetric immunoassay chip were mainly based on the concentration of nanogold solution. Applying high concentration of nanogold solution to the immobilized proteins in the reaction wells can enhance the color intensity after gold enhancement process. This could improve the detection sensitivity, but may also increase the possibility of fault positive signal in this process. Hence, dilution of nanogold solution was needed to be optimized for improving the signal-to-noise ratio. Furthermore, the washing step before gold enhancement process was also important to remove all unbound gold nanoparticles.

After immobilized the antibody–biotin conjugates under different concentrations on the respective reaction wells as described above, nanogold solutions in the dilution of 1:10, 1:50, 1:100, and 1:200 were pipetted to the corresponding reaction wells for 1-h incubation at room temperature. After thoroughly washing, gold enhancement was performed and set to 20 min. The experimental results are shown in Fig. 3. The color intensity decreased with the protein concentration and dilution of nanogold solution, respectively. In order to optimize the dilution of nanogold solution, the specific signal (antibody in 5,000 ng/ml) and the background signal (negative control) were converted to 8-bit gray level values and plotted in Fig. 4. High value of gray level represented light color intensity. The gray level of the specific signal decreased with the increasing concentration of nanogold solutions. For the background signal, the gray level values kept maximal after incubation in 1:200, 1:100, and 1:50 nanogold solution followed by 20-min gold enhancement. However, the gray level decreased after the incubation in 1:10 nanogold solution followed by 20-min gold enhancement. This is probably due to some gold nanoparticles were not able to wash away because of a high concentration of nanogold solution. The highest signal-to-noise ratio was obtained using 1:50 nanogold solution. This condition was used in further experiments.

3.3 Gold enhancement time

After gold nanoparticles were bound to the immobilized antibody via the streptavidin–biotin linkage, gold enhancement process was performed to enlarge the size of gold nanoparticles and generate a detectable visual signal. Gold enhancement has an advantage of minimal autonucleation even after 1–2 h. This could improve the signal-to-noise ratio and presents a much better capability in signal

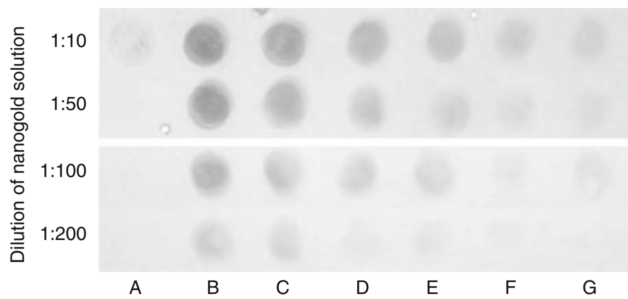

Fig. 3 Experimental results of colorimetric detection of protein concentration. Anti-mouse IgG–biotin conjugates were immobilized on the APTES–GA glass surface and incubated in different dilutions of nanogold solution (from 1:10 to 1:200). The gold enhancement time was 20 min. The concentrations of protein were (from *A* to *G*) 0, 5,000, 500, 160, 80, 60, and 40 ng/ml, respectively

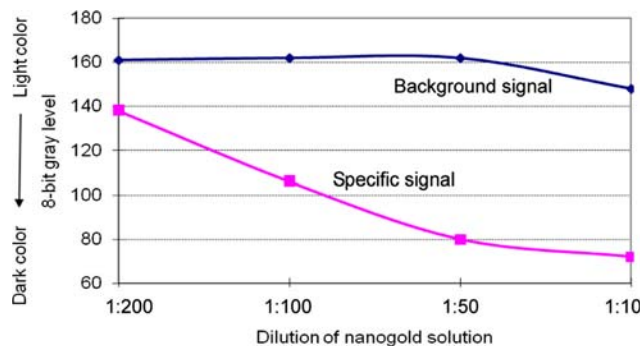

Fig. 4 Specific signal (immobilized antibody in 5,000 ng/ml) and background signal (negative control) after the incubation in different dilutions of nanogold solution followed by 20-min gold enhancement

amplification compared to silver enhancement, which is commonly used. The antibodies in different concentrations were immobilized on glass surface and incubated in 1:50 nanogold solution. After thoroughly washing, gold enhancement solutions were added to all reaction wells in the same volume. The color intensities of all reaction wells were captured by a camera every 2 min and converted to 8-bit gray level values for representation afterward. The kinetics of color intensity during gold enhancement process was plotted in Fig. 5. In Fig. 5, the gray level values decreased (increasing of color intensity) with the enhancement time. It was noticed that the gray level values became steady when the time was around 10 min. This indicated that the gold enhancement process was completed. The protein concentrations were, respectively, mapped to the gray level values, providing for the relation between the protein concentrations and the detection signals. Moreover, the gray level values kept unchanged until 20-min gold enhancement for the negative control. This is explained by the fact that the gold ions were not nucleation when gold nanoparticle was absent during gold enhancement process. Hence, the background signal can be minimized and the signal-to-noise ratio can be enhanced.

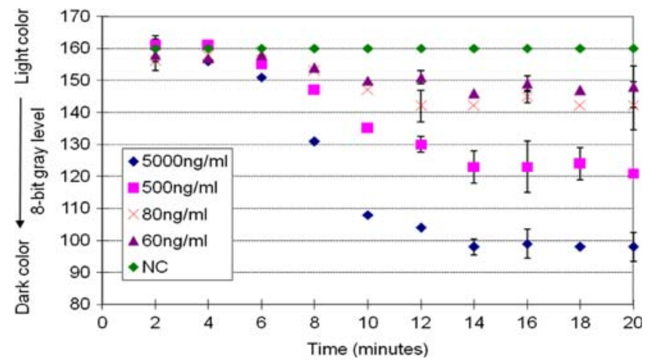

Fig. 5 Kinetics of color intensity during gold enhancement process. Different concentrations of the antibody–biotin conjugates were immobilized on the APTES–GA-modified glass surface. Incubation of 1:50 nanogold solution was performed before the gold enhancement process

3.4 SEM characterization

Scanning electron microscope (SEM) was utilized to characterize the particle size and distribution of gold nanoparticles. Two reaction wells immobilized antibody–biotin conjugates in different concentrations, i.e., 5,000 and 80 ng/ml, and one NC reaction well had been selected for the SEM characterization. The nanogold solution was in 1:50 dilution and the gold enhancement time was set to be 10 min for all reaction wells. After the incubation of nanogold solution and gold enhancement process, the immobilized protein became visible. The gold nanoparticles were to indicate the concentration of protein immobilized on the glass surface. The SEM images are shown in Fig. 6. The original size of gold nanoparticles was 1.4-nm diameter before the gold enhancement process. In Fig. 6, the nanoparticle size was enlarged to 50–200 nm after 10-min gold enhancement process. The distribution density of nanoparticles was significantly decreased with the protein concentration from 5,000 to 80 ng/ml. High density of nanoparticles on the glass surface showed a dark color visually. For the NC reaction well, there were a few nanoparticles on the glass surface, although no proteins were immobilized on it. This might be due to that the washing process cannot remove all unbound gold nanoparticles after the incubation of nanogold solution. However, the density of nanoparticles in NC reaction well was still low enough that showed visually colorless on the glass surface.

3.5 Comparison of colorimetric detection and chemifluorescent detection

The colorimetric detection signal and chemifluorescent detection signal were compared for the detection of the immobilized antibody on glass surface, as shown in Fig. 7. Anti-mouse IgG–biotin conjugates and anti-mouse

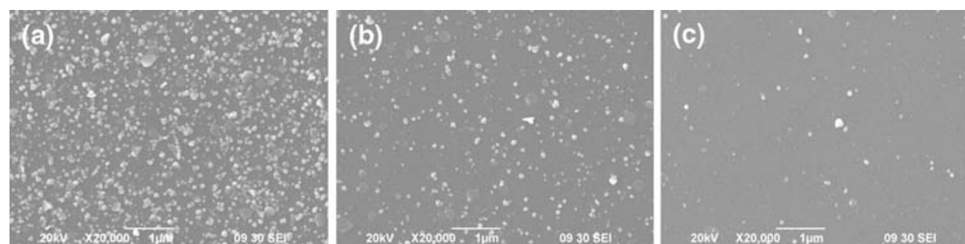

Fig. 6 SEM images of gold nanoparticles bound to anti-mouse IgG–biotin conjugates. The protein concentrations were **a** 5,000 ng/ml, **b** 80 ng/ml, and **c** 0 ng/ml, i.e., negative control. The images were taken at a magnification of 20K

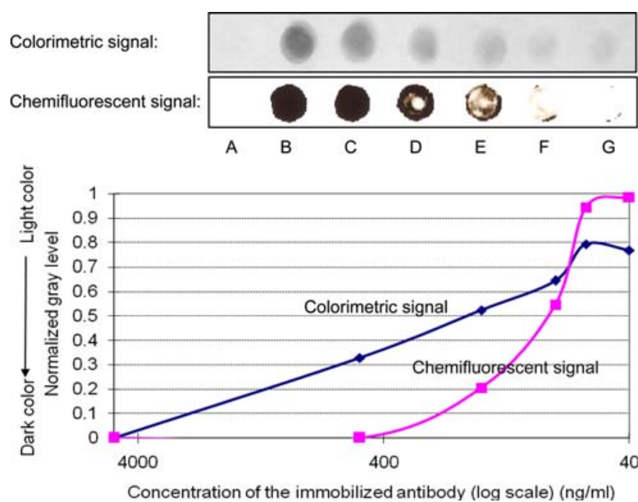

Fig. 7 Comparison of colorimetric detection and chemifluorescent detection of the immobilized antibody in different concentrations. The concentrations were (from *A* to *G*) 0, 5,000, 500, 160, 80, 60, and 40 ng/ml, respectively. Quantification of the color intensity represented by normalized gray level was plotted in the figure. The gray level was normalized between the mean of NC spot and 5,000 ng/ml spot

IgG–HRP conjugates in different concentrations (0, 5,000, 500, 160, 80, 60, and 40 ng/ml) were immobilized, respectively, on the reaction wells. For the colorimetric detection, the experimental procedures were followed by the above described protocol. For chemifluorescent detection, chemifluorescent HRP substrate was added to the reaction wells in the same volume after thoroughly washing. Then, the signals were captured by the Lumi-Imager with the exposure time of 20 s. Both colorimetric and chemifluorescent signals were quantified to 8-bit gray level values by a commercial software and normalized between the maximum gray level (the mean of NC spot) and minimum gray level (the mean of 5,000 ng/ml spot). The value of 1 in normalized gray level means totally white color (no signal), whereas the value of 0 means totally black color (specific signal). Both detection methods showed the mapping of protein concentration and detection signal. In Fig. 7, the dynamic range of colorimetric detection signal was from 60 to 5,000 ng/ml, and chemifluorescent signal was from 60 to 500 ng/ml. For the concentration below 60 ng/ml, colorimetric detection method still generated a visible color for determining the presence of protein. However, in chemifluorescent detection method, the color intensity was not significant compared to the negative control.

3.6 Direct immunoassay

Under the optimal experimental conditions, colorimetric immunoassay chip was demonstrated using human IgG (target antigen) and anti-human IgG (Fc specific)–biotin conjugates (detection antibody). The target antigens were immobilized on the APTES–GA-modified glass surface under different concentrations, i.e., 5,000, 1,000, 300, 200, 100, 10, and 1 ng/ml. Detection antibodies (1:2,000) were applied and bound to the target antigens. Theoretically, the more immobilized target antigens, the more detection antibodies could bind to the target antigens. However, it also depends on the protein binding strength and specificity. After the nanogold solution incubation and gold enhancement process, the colorimetric signal can be captured by a regular camera or observed by naked eye with an appropriate illumination. Different concentrations of immobilized antigens can be represented by different levels of color intensity, as shown in Fig. 8. Quantification of the color intensity represented by 8-bit gray level values was also plotted in the Fig. 8. The amount of metallic gold was depended on the amount of detection antibody bound to the target antigen, the color intensity captured from the reaction wells can be used to visually estimate the concentration of target antigen. The immobilized target antigen over the range between 1 and 5,000 ng/ml can be detected quantitatively using a regular camera.

4 Conclusion

Colorimetric immunoassay chip has been developed based on gold nanoparticles for indicating the protein binding activity and gold enhancement process for amplifying the specific signal. The results of immunoassay were represented by the color intensity on the reaction wells, and can be detected by a regular camera or observed by naked eye. Optimization of the experimental conditions, including the dilution of nanogold solution and gold enhancement time,

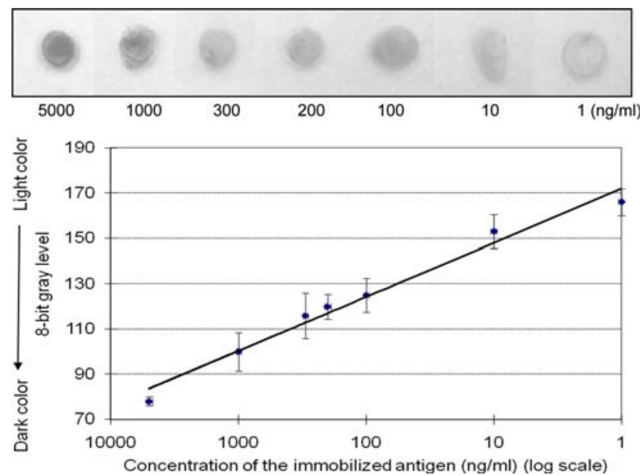

Fig. 8 Experimental results of colorimetric detection of immunoassay. Human-IgG antigens were immobilized on the APTES–GA-modified glass surface under different concentrations (5,000, 1,000, 500, 300, 200, 100, 10, and 1 ng/ml). Anti-human IgG–biotin conjugates were applied and bound to the human-IgG. Colorimetric detection was performed for the visual detection. Quantification of the color intensity represented by 8-bit gray level values was plotted in the figure

had been investigated. The results of the colorimetric detection were compared to the standard chemifluorescent detection. Under optimized conditions, colorimetric immunoassay chip had been demonstrated to detect the amount of target antigens, i.e., human IgG, immobilized on the reaction wells. The concentration of target antigens in a dynamic range from 1 to 5,000 ng/ml can be detected quantitatively using a regular camera. This detection method has a great potential to be integrated into a microfluidic system because there is no dedicated equipment involved, direct readable result, and easy implementation.

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