

COVALENT CONJUGATION OF ANTIBODY AND GOLD NANOPARTICLE FOR DEVELOPMENT OF LATERAL FLOW IMMUNOASSAY TEST STRIP

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ABSTRACT

Nanotechnology is one of the fastest growing technologies in this era. Gold nanoparticle (AuNP) based immunoassays have been performed on the basis of antigen-antibody interaction using AuNP antibody conjugates. Lateral flow immunoassays(LFA) which are also based on AuNP antibody conjugates are useful innovation in nanotechnology and widely applied in medicine and research fields. However, there are some limitations of the present LFA kits such as sensitivity and stability. In the study, we showed the result of covalent conjugation of anti-rotavirus antibody and AuNP for generating a lateral flow immunoassay strip to detect rotavirus in fecal samples. The suitable conditions for coating polyethylene glycol (PEG) on the surface of AuNP were 10.0 μ M PEG for 3 hours at room temperature (25 °C). Optimized conditions for covalent conjugation of antibody and AuNP were pH 4.0, 0.1 μ g antibody/conjugate, 0.01 mM reactant EDC/NHS [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/(N-hydroxy sulfosuccinimide)]. The coupling reaction was carried out at room temperature for 90 min. The conjugate pad, antibody immobilized nitrocellulose membrane strip were created with investigated conditions for generating an LFA test strip. The limit of detection of LFA test strip was determined by 1.6×10^5 virus particles/ml, three times lower than that of Rotaclone kit (UK). The generated strip could be used to detect rotavirus in fecal sample of patient.

Keywords: covalent conjugation, gold nanoparticle (AuNP), lateral flow immunoassay, rapid test, rotavirus.

1. INTRODUCTION

In recent years, advances in nanotechnology have opened a new era in the diagnostic analysis of biological agents. Several diagnostic tools were developed to detect biothreat agents on the basis of genetic techniques and immunosensor techniques [1, 2]. Among them, the lateral flow immunoassay test strip (LFA) has been well-established diagnostic tool. This technology offers several advantages such as simple, rapid and cost-effective. The format in LFA is based on the interaction of antigen and antibody on the surface of the nitrocellulose membrane

(NC).The NCmembrane is immobilized with capture binding protein (antibody or antigen) [3]. Several different labels have been employed in LFA development such as colloidal gold nanoparticle, latex, nanocarbon [4]. The label will be conjugated to antibody and embedded into conjugate pad. There are various methods available for cross-linking antibody to AuNP [5, 6]. The electrostatic adsorption of the antibodies to the AuNPs surfaces is the most commonly used strategies due to its simplicity in practice. However, this linkage is weak therefore the protein-AuNP conjugate is less stable. The EDC/NHS coupling is considered as an alternative method for preparation of protein-AuNP conjugate. For the preparation of biocompatible AuNP, thiol-containing polyethylene glycol (PEG) with a terminated amine or carboxylic group is commonly used as a surface capping ligand. The thiol group of PEG binds to the gold surface, the terminal carboxy or amine group serves as a binding site to conjugate functional antibody. The PEG unit will provide larger space for coupling of antibody on the surface of AuNP and therefore increase the number of antibody bound to AuNP. Therefore, this study conducted experiments forcovalent coupling of rabbit anti-rotavirus antibody and gold nanoparticle (Fig. 1) for development of lateral flow immunoassay test strip to detect rotavirus in fecal sample.

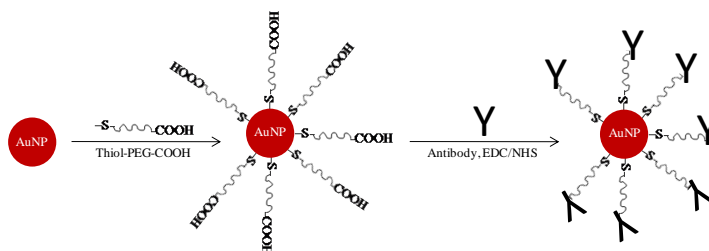


Figure 1. Schematic representation of amide bond formation between antibody and PEG-coated AuNP. Thiol-PEG-COOH molecule is coated to gold nanoparticles through SH-group. Antibody is coupled to a PEGylated gold nanoparticle through EDC/NHS reaction.

2. MATERIALS AND METHODS

2.1. Materials

Standard rotavirus sample from ProSpecT Rotavirus Microplate Assay (Oxoid Ltd, UK). Rabbit anti-rotavirus IgG and Guinea pig anti-rotavirus IgG were purified from sera samples provided by POLYVAC – Ministry of Health. Goat anti-rabbit IgG was purchased from Arista Biological (USA). Materials for making lateral flow strip were purchased from Shanghai JY-Biotech™. Chemicals for preparing buffers were purchased from Sigma-Aldrich (USA), Merck (Germany), Bio-rad (USA)...

2.2. Methods

2.2.1. Pegylation of colloidal gold nanoparticle

The procedure for coating PEG on the surface of AuNP was carried out as described by Manson et al. [7]. Briefly, polyethylene glycol with two ends of the -SH and -COOH group and colloidal gold nanoparticles were prepared in 0.01 % citrate buffer and mixed together. The mixture was incubated for three hours at room temperature. After three washes with deionized water, the pellet of PEG-coated gold nanoparticles (PEG-AuNP) was dissolved in deionized water and keep in dark at 4°C for several months.

2.2.2. Conjugation of IgG and PEG-coated colloidal gold nanoparticle [8]

In a typical reaction, a solution of the IgG (10 μ l, 1 mg/ml) was added to 1 ml of PEG-AuNP and mixed. To this solution, aliquot solutions of coupling reagents EDC (10 μ l, 1 mM) and sulfo-NHS (10 μ l, 1 mM) were added simultaneously. Then, the mixture was incubated in dark for 90 min at 25 $^{\circ}$ C. The particles were washed three times by 1X phosphate buffered saline, pH 7.4 and redispersed in 20 mM sodium borate buffer containing 2 % BSA, 3 % sucrose, 0.6 M NaCl, 0.2 % tween 20 to generate a conjugate solution.

2.2.3. Preparation of conjugate pad and blotting membrane [9]

The conjugate pad is generated by embedding the empty pad in the IgG-AuNP conjugate solution and then drying at 42 $^{\circ}$ C for 30 min. The conjugate pad should be stored at 4 $^{\circ}$ C for further investigations. The NC membrane was cut into pieces with appropriate sizes and assembled on the CAMAG Linomat 5 automatic sampler platform. The antibody solutions at experimental concentrations were dispensed onto the membrane at 1.0 μ l/cm. The blotted membrane then is dried at 42 $^{\circ}$ C for 30 min in an air drying oven. The membrane could be stored at 4 $^{\circ}$ C for several months.

3. RESULTS AND DISCUSSION

3.1. Polyethylene glycol functionalized gold nanoparticles

The surface of gold nanoparticle will be functionalized by pegylation with HS-PEG-COOH. With initial reaction conditions, the generated PEG-AuNP conjugate was checked by the wavescan method. In the range of wavelength of 450 - 600 nm, the absorption spectrum of PEG-AuNP conjugate was different from that of AuNP (Fig. 2A). The PEG-AuNP was also checked by agarose gel electrophoresis and the result showed that appearance of a strong band on the agarose electrophoresis pattern (Fig 2B, lane 2) whereas a smearing pattern was observed with AuNP sample (Fig. 2B, lane 1). The AuNP is very sensitive therefore it will be oxidized by the electrophoresis buffer and resulted in no appearance of signal band on the electrophoresis pattern. The PEG-coated AuNP was more resistant to medium and resulted in the appearance of a signal band on the electrophoresis pattern. This result was also obtained by Bartczak et al. [8]. The observed results indicated that AuNP was successfully coated with PEG molecules.

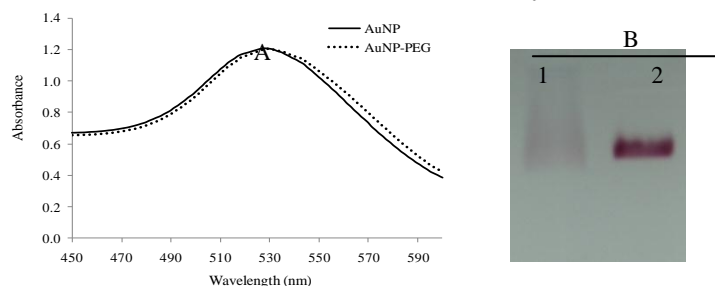


Figure 2. Pegylation of gold nanoparticles. (A) The absorbance of AuNP and PEG functionalized AuNP (AuNP-PEG) was determined by the wavescan method. (B) Agarose gel electrophoresis pattern of AuNP (lane 1) and AuNP-PEG (lane 2).

To obtain the best condition for coating PEG on the surface of AuNP, some reaction conditions were optimized. Firstly, different concentrations (1, 5, 10, 20, 25 μ M) of PEG were

investigated and the result showed that suitable concentration of PEG for conjugation was 10 μM (Fig. 3A). The incubation time of 3 and 4 hours showed higher efficiency of PEG conjugation (Fig. 3B). The efficiency of PEG-AuNP conjugation at two temperatures of 25 $^{\circ}\text{C}$ and 37 $^{\circ}\text{C}$ were the same and higher than that of 4 $^{\circ}\text{C}$ (Fig. 3C). Suitable conditions for coating the PEG on the surface of AuNP were 10 μM PEG, 3 hours and 25 $^{\circ}\text{C}$ with OD_{530} of AuNP of 10.

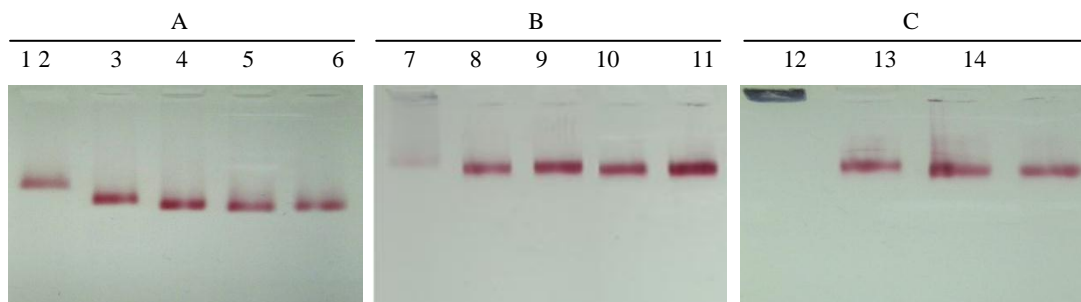


Figure 3. Different conditions for pegylation of gold nanoparticles. (A) Different concentrations of polyethylene glycol (1, 5, 10, 20, 25 μM PEG in lanes 1-5 respectively); (B) different incubation times (0, 1, 2, 3, 4 hours incubation in lanes 6-10 respectively); (C) different temperatures (4 $^{\circ}\text{C}$, 25 $^{\circ}\text{C}$, 37 $^{\circ}\text{C}$ in lanes 12-14 respectively), lane 11: AuNP

3.2. IgG linking to functionalized gold nanoparticles

The IgG molecules can be immobilized on the surface of AuNP by two ways: absorption through electrostatic interaction and covalent linkage through functionalized groups. Conjugation of IgG and AuNP through the covalent bonds will improve the stability of conjugate. In this study, the initial condition for covalent conjugation of IgG and PEG-coated AuNP was set up including 1 μg of IgG, 10 μl of PEG-coated AuNP, 1 mM of EDC/NHS at 25 $^{\circ}\text{C}$ for 2 hours. The result of wavescan showed that the absorption spectrum of IgG-PEG-AuNP was significantly different from AuNP, AuNP-PEG and AuNP-IgG, especially in the range of wavelength of 530 - 600 nm (Fig. 4A). The change in absorption after conjugation was also observed by Sosibo et al. [10].

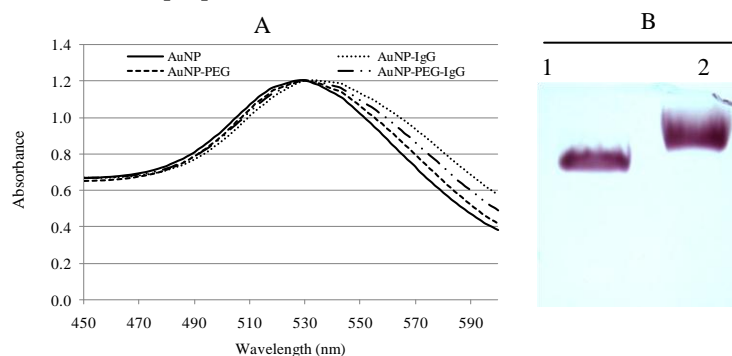


Figure 4. Immobilization of IgG on the functionalized gold nanoparticles. (A) Wavescanning of different conjugates of AuNP-PEG, AuNP-PEG-IgG, AuNP-IgG and uncoated AuNP; (B) Agarose electrophoresis pattern of AuNP-PEG (lane 1) and AuNP-PEG-IgG (lane 2)

Agarose electrophoresis analysis of IgG-PEG-AuNP conjugate showed that the electrophoretic mobility of IgG-PEG-AuNP conjugate is significantly lower than those of the PEG-AuNP (Fig. 4B). This observation implicated that the IgG molecules were covalently linked to PEG-coated AuNP leading to increase the molecular weight of conjugate.

3.3. Effect of different conditions on the covalent conjugation of IgG and AuNP-PEG for development of lateral flow immunoassay test strip

As described above, the EDC/NHS coupling reaction can be performed in several steps, however in this study the coupling reaction was carried out in a single step. The successful coupling for the different reaction parameters was evaluated by the lateral flow immunoassay. In the first set of experiments, the pHs of reaction were varied.

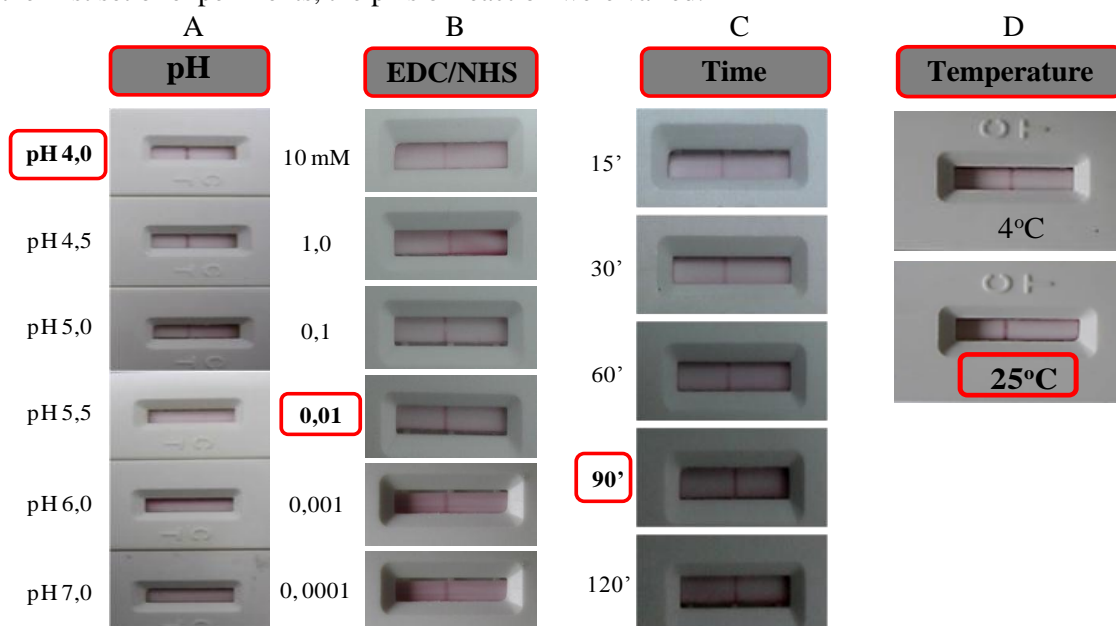


Figure 5. Optimization of conditions for immobilizing IgG on the gold nanoparticles. Effect of pH (4.0 – 7.0), concentration of EDC/NHS (0.0001 – 10 mM), time (15 – 120 min) and temperature (4 °C and 25 °C) on the conjugation of IgG and coated AuNP.

As can be seen in Fig. 5A, the strongest signal band is observed for the coupling reaction at pH 4.0. Figure 5B shows that the most effective coupling conditions are observed for 0.01 mM of EDC/NHS. The coupling yield seems to decrease similarly by increasing or decreasing the concentrations of EDC and NHS. Bartczak et al., 2011 also showed that the coupling efficiency was not proportional to the concentration of EDC and NHS [8]. It is possible that a competition event takes place at higher concentration. The high amounts of EDC and NHS may cause the polymerization of protein, thus decreasing the available proteins for coupling to the surface of gold nanoparticle. For further optimization of coupling conditions, we investigated the influence of reaction time and temperature. In a range of incubation time of 15, 30, 60, 90 and 120 min, the signal was the strongest after 90 min incubation (Fig. 5C), implying that a larger number of IgG is conjugated to the nanoparticles longer than 90 min incubation. Similarly, Figure 5D indicates that suitable temperature for coupling reaction is 25 °C.

3.4. Generation of the lateral flow immunoassay test strip

Generation of conjugate pad

The amount of antibody in the conjugate pad will influence the detectability and also the cost of lateral flow test strip. In this study, a range of rabbit anti-rotavirus antibody amount of 0.1, 0.3, 0.5, 0.7 and 1.0 μg was used to generate the conjugate pad. Figure 6 shows that there is a clear signal band in the observing window of the test strip with the lowest amount of antibody (0.1 μg).

0.1 μg 0.3 μg 0.5 μg 0.7 μg 1.0 μg



Figure 6. Conjugation of different amounts of rabbit anti-rotavirus IgG with gold nanoparticles to generate the conjugate pad.

Generation of antibody immobilized nitrocellulose membrane strip

Similarly, the different amounts of guinea pig anti-rotavirus antibody (0.1, 0.3, 0.5, 0.7, 1.0 μg) were loaded on the nitrocellulose membrane to determine the appropriate IgG amount for development of LFA strip. The result showed that a significant signal band was observed at 0.3 μg immobilized IgG (Fig. 7), implying that the appropriate amount of IgG for immobilization on the nitrocellulose membrane strip should be 0.3 μg .

0.1 μg 0.3 μg 0.5 μg 0.7 μg 1.0 μg



Figure 7. Immobilization of different amounts of guinea pig anti-rotavirus IgG on the nitrocellulose membrane as T-line of LFA strip.

** Generation of lateral flow immunoassay test strip*

In the present study, the LFA test strip was designed to detect rotavirus in the fecal sample. Rabbit anti-rotavirus antibody and goat anti-rabbit IgG antibody were immobilized on the nitrocellulose membrane strip at two different positions of T and C line, respectively. Above generated components were assembled in a typical order to produce the LFA strip.



Figure 8. Generation of lateral flow immunoassay test strip. The test strip was tested with positive sample (A) and negative sample (B)

The test strip was checked by both positive and negative samples (Fig. 8) and showed the appearance of two and one signal bands for positive and negative samples respectively. This result indicated that the test strip was successfully developed.

The limit of detection (LOD) of the test strip was also checked with 10-fold serial dilutions of standard rotavirus sample and showed that the test strip could detect rotavirus with the titer of 1.6×10^5 particles/ml (Fig. 9). This LOD is three times lower than that of Rotaclone kit (UK)

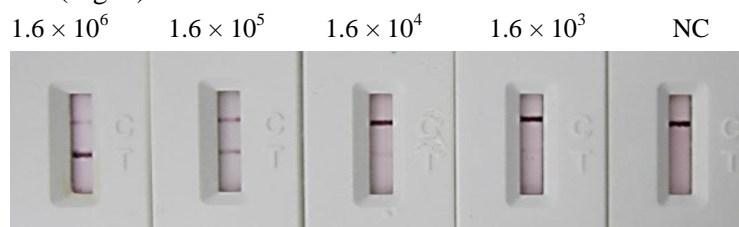


Figure 9. The limit of detection of the test strip for detecting rotavirus. A 10-fold serial dilution of standard rotavirus sample (1.6×10^6 to 1.6×10^3) and negative control (NC) was used to check the test strip

4. CONCLUSIONS

The present study has successfully investigated conditions for covalent coupling of antibody on the surface of the gold nanoparticles. Antibody was covalently conjugated to gold nanoparticle through a PEG linker. The antibody-AuNP conjugate was applied to generate the lateral flow test strip for detecting rotavirus in fecal sample.

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TÓM TẮT

NGHIÊN CỨU CỘNG HỢP CỘNG HÓA TRỊ KHÁNG THỂ VÀ NANO VÀNG ỨNG DỤNG PHÁT TRIỂN QUE THỬ NHANH

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Công nghệ nano là một trong những công nghệ phát triển nhanh nhất trong kỷ nguyên này. Nhiều kỹ thuật miễn dịch đã được thực hiện dựa vào tương tác kháng nguyên kháng thể sử dụng cộng hợp kháng thể và hạt nano vàng. Que thử hoạt động dựa trên nguyên tắc sắc ký miễn dịch sử dụng cộng hợp kháng thể - nano vàng là một sáng kiến hữu ích trong công nghệ nano và đã được sử dụng rộng rãi trong nhiều lĩnh vực như y học và nghiên cứu. Một số kit dạng que thử thương mại hiện nay còn có một số hạn chế về độ nhạy và độ bền. Trong nghiên cứu này, chúng tôi trình bày kết quả nghiên cứu về việc tạo cộng hợp cộng hóa trị bền vững giữa kháng thể và hạt nano vàng để phát triển que thử phát hiện virus rota trong mẫu phân. Điều kiện thích hợp để gắn polyethylene glycol (PEG) lên bề mặt của hạt nano vàng là 10 μ M PEG trong 3 giờ ở nhiệt độ phòng (25 °C). Điều kiện tối ưu cho phản ứng gắn cộng hóa trị kháng thể với hạt nano vàng là pH 4,0; 0,01 mM chất xúc tác EDC/NHS [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/(N-hydroxysulfosuccinimide)]. Phản ứng cộng hợp được thực hiện ở nhiệt độ phòng trong thời gian 90 phút. Các thành phần của que thử như miếng cộng hợp, thanh màng nitrocellulose được cố định kháng thể đã được tạo ra ở điều kiện thích hợp lựa chọn. Que thử đã được kiểm tra đánh giá với cả mẫu dương tính (chứa virus rota) và mẫu âm tính và cho kết quả rất tốt. Giới hạn phát hiện của que thử là 1.6×10^5 hạt virus/ml, nhạy hơn 3 lần so với kit Rotaclone của Anh. Que thử được tạo ra hoàn toàn có thể được sử dụng để phát hiện virus rota trong mẫu phân bệnh phẩm.

Từ khóa: cộng hợp cộng hóa trị, hạt nano vàng (AuNP), sắc ký miễn dịch, test nhanh, virus rota.