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การสังเคราะห์และการปรับแต่งเพื่อเชื่อมติดชิ้นส่วน Fab แอนติบอดีที่จำเพาะต่ออะฟลาท็อกซินบี1 บนอนุภาคทองคำขนาดนาโน

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บทคัดย่อ

การสังเคราะห์และการปรับแต่งเพื่อเชื่อมติดชิ้นส่วน Fab แอนติบอดีที่จำเพาะต่ออะฟลาท็อกซินบี1 บนอนุภาคทองคำขนาดนาโน

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บทนำ: อนุภาคทองคำขนาดนาโนเป็นหนึ่งในโลหะที่มีคุณสมบัติทางแสงที่ดีและนำมาประยุกต์ใช้อย่างมากในงานชีวแพทย์ศาสตร์ เทคโนโลยีชีวภาพและงานตรวจวิเคราะห์ต่างๆ ชิ้นส่วน Fab แอนติบอดีมีความจำเพาะต่อสารอะฟลาท็อกซินบี 1 ถูกสร้างขึ้นให้ห้องปฏิบัติการเรา มีหลายปัจจัยที่ส่งผลต่อการเชื่อมชิ้นส่วน Fab เข้ากับอนุภาคทองคำ การศึกษาสภาวะที่เหมาะสมจึงมีความจำเป็นต่อการเชื่อมชิ้นส่วน Fab เข้ากับอนุภาคทองคำ ซึ่งสามารถนำไปใช้ตรวจหาสารอะฟลาท็อกซิน บี1 ได้ โดยอาศัยคุณสมบัติทางแสงของอนุภาคทองคำ **วัสดุและวิธีการ:** การศึกษานี้ ผู้วิจัยทำการสังเคราะห์อนุภาคทองคำขนาดนาโน ตามวิธีของเทอร์วิทช์ เราศึกษาผลของ pH และความเข้มข้นของชิ้นส่วน Fab แอนติบอดีต่อการเชื่อมติดบนอนุภาคทองคำ หลังจากเหนี่ยวนำการตกตะกอนด้วยเกลือ จะสังเกตเห็นสีที่เปลี่ยนไปและใช้คุณสมบัติการดูดกลืนแสงเป็นตัวกำหนดสภาวะที่เหมาะสม Indirect-ELISA ถูกใช้วัดปริมาณแอนติบอดีที่เหลือจากการติดบนอนุภาคเพื่อยืนยันผลการเชื่อมติด **ผลการศึกษา:** ค่าการดูดกลืนแสงสูงสุดของอนุภาคที่ทำการเชื่อมติดแล้วเพิ่มจาก 520 เป็น 532 นาโนเมตร การเพิ่ม pH ไม่มีผลต่อขนาดและสี อัตราส่วนความเข้มข้นของชิ้นส่วน Fab-AFB1 ต่ออนุภาคทองคำ คือ 0.71 อัตราส่วนที่น้อยกว่าไม่สามารถป้องกันการรวมกลุ่มตกตะกอนของอนุภาคทองคำได้ indirect-ELISA เผยให้เห็นว่าจุดอิ่มตัวของการเชื่อมติดชิ้นส่วน Fab-AFB1 บนอนุภาคทองคำเกิน กว่า 3.53 **สรุปผล:** สามารถเชื่อมชิ้นส่วน Fab ที่จำเพาะต่อสารอะฟลาท็อกซิน บี1 ลงบนอนุภาคทองคำได้สำเร็จสามารถนำไปประยุกต์ใช้ได้หลากหลายและมีประสิทธิภาพ

คำสำคัญ: อนุภาคทองคำขนาดนาโน, การเชื่อมต่อ, ชิ้นส่วน Fab แอนติบอดี

Abstract

Synthesis and Optimization of Fab Antibody Specific to Aflatoxin B1 Conjugated to Gold Nanoparticles

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Introduction: Gold nanoparticles was one of the perfect metals for studies the optical properties and most widely applied in biomedicines, biotechnology and diagnostic applications. Fab fragments of antibody specific to aflatoxin B1 were preformed in our laboratory. The Fab-AFB1 coated gold is affected by multiple factors. The optimal conditions were studied for preparation the Fab-AFB1 conjugated gold nanoparticles. The conjugated particles could be used to detect aflatoxin B1 on the basic of the optical property of gold nanoparticles. **Materials and methods:** In this study, we synthesized the colloidal gold solution by Turkevich method. We studied the effect of pH and concentration of Fab-AFB1 antibody on conjugated in the gold

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nanoparticles. After inducing aggregation with salt, color changes and UV-absorbance were used to determined for the optimal conditions. Indirect-ELISA was used to measured the unconjugated antibody in conjugation solution for confirming conjugation reaction. **Results:** The maximum absorption spectra (λ_{max}, $A_{520,}$) of coated gold nanoparticle increases from 520 to 531 nm. Increasing pH does not affect on size and the color shown. The optimum concentration was conjugation in molar ratio 0.71. Less conjugated antibody could not prevent salt-induced aggregation. Indirect-ELISA reveal the saturation concentration points above 3.53 in molar ratio. **Conclusion:** Gold nanoparticles conjugated with Fab-AFB1 antibody specific to aflatoxin B1 is successfully prepared and could be potentially used for versatile application.

Keyword: gold nanoparticles, conjugation, Fab antibody

Introduction

Colloidal gold nanoparticles (suspensions of gold nanoparticles) are widely used in the immunolabeling technique. It has a very strong ability conjugated with biomolecule through electrostatic or hydrophobic interactions. Turkevich J. *et al.* in 1951 and Frens G. in 1972 introduced a sodium citrate reduction of chloroauric acid ($HAuCl_4$) (Frens, 1972; Kimling *et al.*, 2006; Turkevich *et al.*, 1951). The basic principle of Turkevich method is that the gold ions are reduced to gold atoms by adding a certain amount of reducing agent to a gold solution (Turkevich, 1985). It is available to get gold nanoparticles in different sizes by changing the proportion of chloroauric acid to reducing agent during the reaction (Sau *et al.*, 2001). Immunolabel gold conjugate plays an important role in clinical observation or diagnosis, and is widely used (Miura *et al.*, 2002; Nghiem *et al.*, 2010; Nichtl, 2004; Wang *et al.*, 2005).

A high affinity antibody specific to aflatoxin B1 can be prepared in our laboratory (Putkam and Tangkeangsirisin, 2007). Therefore, In this study, the gold nanoparticles will be used to conjugate with such an antibody for further application. Optimal conditions are important for conjugate developers to give consideration to the ways in which antibodies bind to gold nanoparticles. The conjugation conditions (pH and concentration of antibody) depend on the isoelectric point of the proteins to be conjugated. It is a general practice to adjust the pH of the reaction buffer to slightly more basic than the isoelectric point of antibody to be conjugated as this may maximize protein density on the gold nanoparticle surface (Geoghegan, 1988; Hermanson, 2008). In this study, the different pH and concentration of antibody in conjugation reaction were optimized.

Materials and method

Preparation of gold nanoparticles

Gold nanoparticles of 9 nm in diameter were prepared with the standard citrate method, which was pioneered by Turkevich J. *et al.* in 1951 (Turkevich *et al.*, 1951) and refined by Frens G. in the 1972 (Frens, 1972). Typically, 1 ml of 12.7 mM Gold (III) chloride solution ($HAuCl_4$, Aldrich) in 49 ml ultra-pure water. The solution was boiled and stirred vigorously in a flask connected to a water-cooling column and subsequently adding 0.98 ml of 38.8 mM sodium citrate under constant stirring. After the color had changed from blue to ruby red. It was boiled for another 20 min. Then the heating source was removed. The solution was continuously stirred until it had cooled to room temperature. The particles were stored until used at 4°C.

Preparation of Fab monoclonal antibody against Aflatoxin B1 (Fab-AFB1 antibody)

The aflatoxin B1 antibody clone was selected by Rutairat Putkam (Putkam and Tangkeangsirisin, 2008) using phage display technology. The bacterial culture was grown using standard scale up culturing conditions to produce 1 liter in 2xTY containing 100 µg/ml of ampicillin, incubated for 8 hours in 37°C shaker (160 rpm). The culture were induced by adding 0.25 mM IPTG and incubated for 16 hours in the same conditions. The pellet of cellular debris were centrifuged at 8,000 rpm for 90 minute at 4°C. The supernatants concentrated to approximately 100 ml by ultrafiltration using 10 kDa-cutoff Diaflo® ultrafilter. (Amicon, USA) Concentrated supernatant were subjected for further.

Purification Fab-AFB1 antibody using affinity chromatography

The Fab-AFB1 antibody was purified from 90 ml of concentrated supernatant using 1 ml HiTrap® Protein G HP column (Amersham, UK). Filtration the sample through 0.2 µm size of Supor® filter membrane before pass through the affinity column. Proteins were eluted with elution buffer (0.1M glycine-HCl, pH 2.7) then *neutralization buffer (1M Tris*-HCl, pH 9) *were added to each fraction tube.* The eluted fractions were monitored the Fab-AFB1 antibodies purity by 12% SDS-PAGE and detected by silver staining. The eluted fractions were also checked for the presence of antibodies by ELISA. The concentration of Fab-AFB1 antibodies was determined by the Bicinchoninic Acid (BCA) method (MicroBCA™ Protein Assay Kit, PIERCE).

Stability of conjugation conditions in salt-induced aggregation test

The pH value of the colloidal gold nanoparticle for Fab-AFB1 antibody conjugation was adjusted to 7, 9 and 11 with 0.2 M Na_2CO_3. The dilution series was made of the Fab-AFB1 antibody (0-30 µg) in the row of the microtiter plate. After 10 min reaction time at room temperature, the difference in absorbance at 562 nm and 630 nm was recorded with the microplate reader. The optical properties of colloidal gold nanoparticles were monitored using the NaCl method. Upon addition of 10% NaCl, the color of the colloidal gold solution changes from red to blue, indicating the aggregation formation. UV-visible spectrophotometer was also used to observe the change of gold nanoparticles aggregates.

Indirect-ELISA for unconjugated Fab-AFB1 antibody detection

Each well of Costar EIA/RIA 96-well half area plates (Corning, NY) were coated with aflatoxin-BSA antigen in 120 ng/well and incubated overnight at 4°C. The coated plates were blocked with 170 µl of 3% BSA/PBS. After 1 hour incubation at 37°C, the wells were emptied, and incubated with series of Fab-AFB1 antibody standards (performed in our lab) in PBS pH 7.4 (50 µl each; 0-4 nM) for 1 hour at 37°C. Unbound antibody was removed and washed from the plate. The plate was incubated for 1 hour at 37°C with HRP conjugated goat anti-Human IgG antibodies (GE Healthcare) diluted 1 : 2,500 in 3% BSA/PBS (50 µl per well). Plate was washed with distilled water, added 50 µl of ABTS substrate solution and incubated in dark room for 15 minutes. Absorbance was read in the microplate reader (BIOHIP BP800) in 405 nm. For various experimental samples, the unconjugated antibody in conjugation solution can be added to each well in the same manner as the Fab-AFB1 antibody standard.

The unconjugated Fab-AFB1 antibody of each conjugation solutions were determined using a standard curve (A_{405nm} vs. Fab-AFB1 antibody standard, (0-4 nM)). Each standard concentration was tested in triplicate for individual experiment.

Results and discussion

Gold nanoparticles

The gold nanoparticles were prepared according to Amornpun *et al.* (Sereemaspan *et al.*, 2009). UV-visible spectra were recorded using a Agilent 8453 UV-Visible spectrophotometer, USA in the range 200-800 nm. The maximum absorbance wavelength (λ_{max}) for the colloidal gold nanoparticles is 520 nm (Figure 1.), and the λ_{max} for the Fab-AFB1 antibodies coated gold nanoparticles solution is 531 nm, an 11-nm increase. The red shift of the absorption peak indicated a successful conjugation between Fab-AFB1 molecules and gold nanoparticles (Figure 2.).

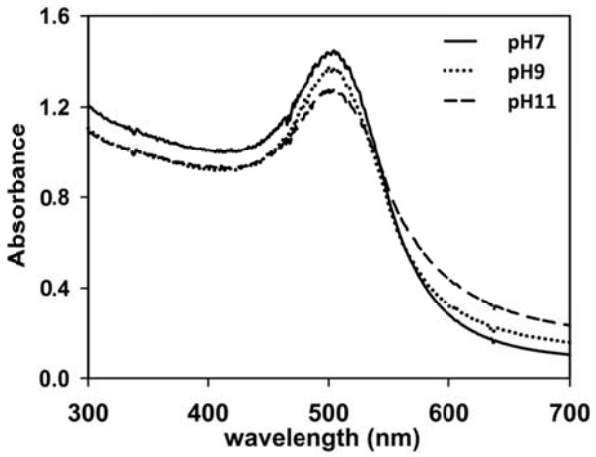

Figure 1: The maximum absorption spectra of colloidal gold nanoparticles at pH7, 9 and 11. Every peak showed the maximum absorption spectra at 520 nm

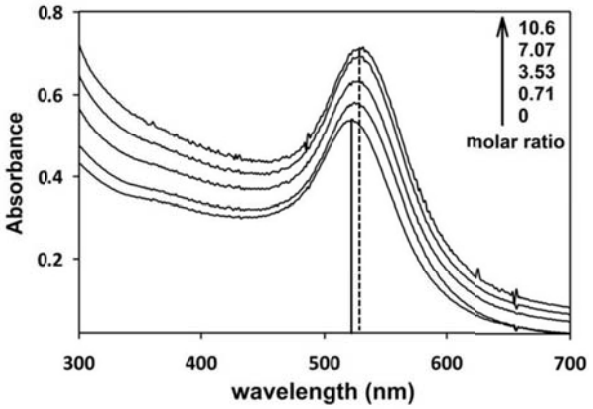

Figure 2: The maximum absorption spectra of gold nanoparticles prepared in the presense of Fab-AFB1 antibodies at different molar ratio.

Effect of pH and concentration of Fab-AFB1 antibody on conjugated in the gold nanoparticles

Typically, gold nanoparticles in nano ranges is ruby red. Larger turn particles blue or purple. After pH was adjusted with Na_2CO_3 to 7, 9 and 11, the color of solution does not obviously change virtually. The λ_{max} of colloidal gold nanoparticles at pH 7, 9 and 11 are 520 nm. (Figure 1), suggesting that increasing pH does not affect the size of gold nanoparticles.

The aggregation of gold nanoparticles leads to the color change from red to purple (A_{600}-A_{750}). Figure 3A and 3B show the absorbance at A_{600}-A_{750} in pH 7 and 9 conjugated solution increase by comparing the A_{600}-A_{750} of the unconjugated gold nanoparticles, indicating that the particle are aggregates. The molar ratio 0.34 of conjugated solution at pH 11 does not change the color (data not shown) and the absorbances are lower than the unconjugated gold nanoparticles, indicating that the surface of gold nanoparticles contain enough proteins to prevent salt-induced aggregation (Figure 3C). Taken together, elevation of pH increases conjugation of Fab-AFB1 antibody surface of gold nanoparticles.

The optimal antibody concentration were usually determined in a series of dilutions in a titration experiment, which gives the minimum amount of Fab-AFB1 antibody that is necessary to stabilize the conjugated particles. Optimal Fab-AFB1 antibody concentration was selected as a color appearing after an equal volume of 10% NaCl was added to conjugation solution. Antibodies adsorbed to the surface of nanoparticles with the effect of inhibiting aggregation, even strong electrolyte (10% NaCl) is added. Figure 4 shows the molar ratio of Fab-AFB1 to gold nanoparticles less than 3.5 could not protect the conjugated particles from salt-induced aggregation.

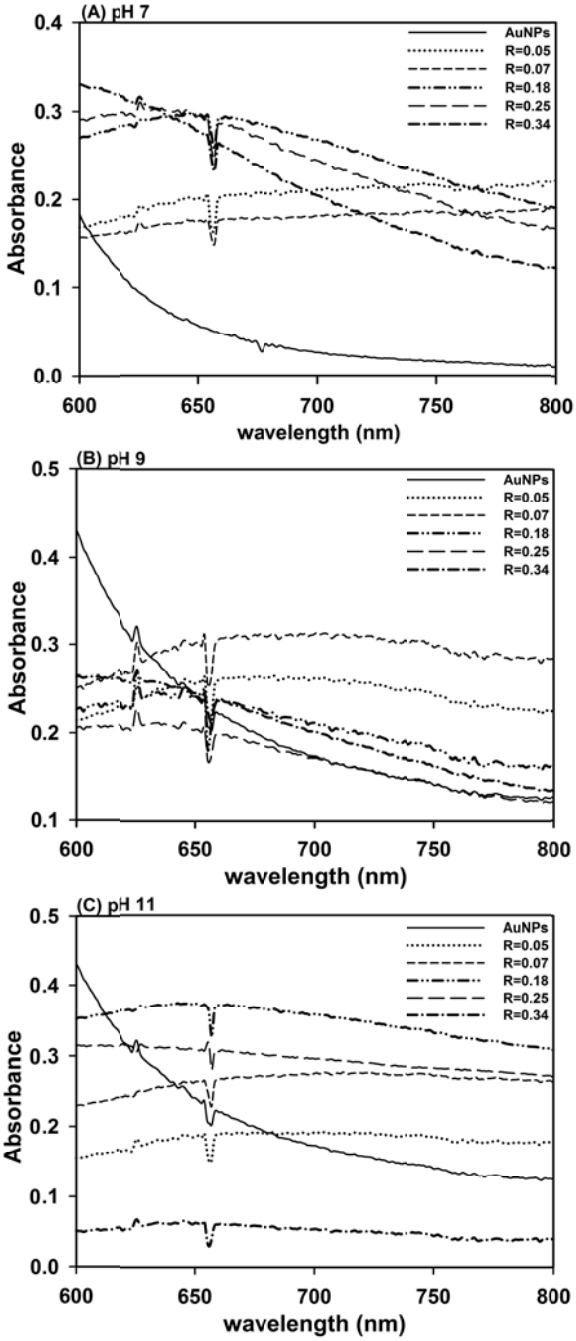

Figure 3: The absorption spectra of the aggregation formation of conjugated gold nanoparticles in different molar ratio (R) : (A) pH 7, (B) pH 9, and (C) pH 11.

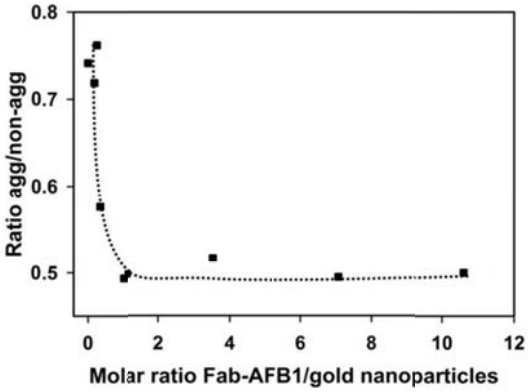

Figure 4: Ratio of absorbance of aggregated forms at 630 nm vs. non-aggregated forms at 562 nm after adding 10% NaCl vs. the molar ratio of Fab-AFB1 antibody on gold nanoparticles.

Indirect-ELISA for unconjugated Fab-AFB1 antibody detection

In this study, indirect-ELISA is an analytical method for the detection and quantification of the unconjugated antibody in conjugated solution. The unconjugated antibody was determined according to the equation that obtained from the slope of standard curve and plotted vs. molar ratio of conjugated gold nanoparticles. The saturation concentration points above 3.53 in molar ratio (Figure 5.).

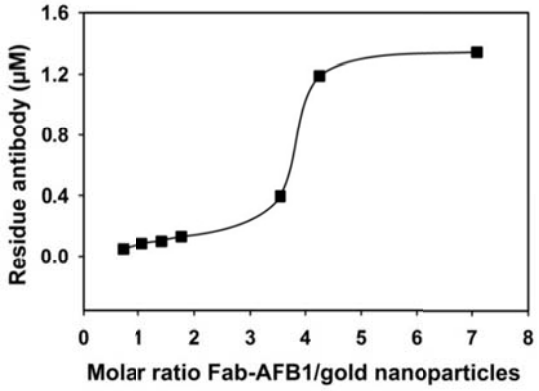

Figure 5 : The residue antibodies detection in conjugation solutions using indirect-ELISA.

Conclusion

The results demonstrate that synthesized gold nanoparticles with Turkevich method can be coated with Fab fragments of aflatoxin B1 antibody by direct conjugation method. An important parameter to consider when preparing gold nanoparticle conjugates is the amount of antibody bound to surface of gold nanoparticles. After adding 10% NaCl into the conjugation solution, the color change could be observed with the naked eyes immediately. The conjugated gold nanoparticles in 0.71 molar ratio effectively inhibit the color change and can be used for formation stable antibody layers on gold nanoparticles. Although the surface of gold nanoparticles can be coated at higher concentrations, but 0.71 molar ratio is enough for immunoreactions between aflatoxin B1 antigen and Fab-AFB1 antibodies coated gold (data not shown). Our results indicate that the optimal conditions can be used for preparing the Fab-AFB1 coated on gold nanoparticles. To confirm these results, indirect-ELISA is the method of choice for measuring residue antibodies after the conjugation antibodies on gold nanoparticles occurs. All that results provide only a preliminary, for applied to the design of materials for Aflatoxin B1 antigen detection and versatile application of antibody.

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