

Nano-ELISA using gold nanoparticle-DNA complex for the detection of pathogenic microbe

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ABSTRACT

In this study, we report on the rational design of a nano-complex that consists of gold nanoparticles (AuNP), antibody, oligonucleotides and horseradish peroxidase (HRP) for the detection of pathogen existing in contaminated environment. The Digoxigenin(DIG)-labeled oligonucleotides that HRP labeled anti-DIG was bound to DIG to amplify the signal were covalently attached on the AuNP. The interaction forces that resulted from the immune reaction between the functionalized MMP containing target pathogen and AuNPs was used to separate unbound AuNPs. For detection of pathogen, we exploited the reaction of HRP enzyme immobilized on Au probes with tetramethylbenzidine (TMB) that is a substrate of HRP. Using this method, we could detect down to 10^2 CFU/mL of target pathogen within a hour. This method is very simple and highly sensitive relative to established pathogen biosensors and can be used to detect specific protein markers associated with tumors, bacterial infections or other disease.

Keywords: gold nanoparticle, magnetic particle, biosensor, enzyme amplification, digoxigenin-labeled oligonucleotide

1 INTRODUCTION

Escherichia coli (*E. coli*) O157:H7 is a major health problem and a cause of foodborne illness. Infection with *E. coli* O157:H7 should be considered in all patients with bloody diarrhea, the hemolytic uremic syndrome, or thrombotic thrombocytopenic purpura because the infection can disguise as gastrointestinal bleeding of noninfectious cause, the antecedent diarrhea may be resolved and forgotten by the time the hemolytic uremic syndrome or thrombotic thrombocytopenic purpura is diagnosed and the detection of *E. coli* O157:H7 requires specific stool culture techniques[1]. According to these requirements, the development of rapid, sensitive detection method is a priority because cultural methods require at least 3 days to provide results.

High sensitive bioassays for protein, pathogen are of central importance for many applications, including medical diagnostics, pharmaceutical development and warning against bio-warfare agents[2-5]. Such biodetection commonly relies on immune-reaction, and requires proper transducers to the achievement of ultrasensitive measurements. Colorimetric transducers are very attractive for such bioassays because of their high sensitivity, simplicity, miniaturization, and low cost. The use of enzyme labels to produce colorimetric signals has been extremely useful for ultrasensitive bioassays of pathogens.

In this study, we report on the rational design of a nano-complex that consists of AuNPs, polyclonal anti-*E.coli* O157:H7 and HRP for the detection of target *E.coli* O157:H7. AuNPs were modified to polyclonal anti-*E.coli* O157:H7 and DIG-labelled DNA for the subsequent binding of HRP labelled anti-DIGs. The localized binding of multiple HRP enzymes to the AuNP was used to amplify the signal of the target probe. Afterwards, MMP were functionalized with monoclonal anti- *E.coli* O157:H7 and used in a sandwich reaction with the target pathogen. The MMPs containing the target pathogen were then bound to AuNPs via immunoreaction. The interaction forces that resulted from the immunoreaction between the functionalized MMP containing target pathogen and AuNPs was used to separate unbound AuNPs; a magnetic field was used to effectively remove unbound AuNPs. For detection of *E.coli* O157:H7, we exploited the reaction of HRP enzyme immobilized on AuNPs with tetramethylbenzidine (TMB) that is a substrate of HRP, followed by stopping the reaction with 2M H₂SO₄. The resulting end products were analyzed by UV-vis spectroscopy (Figure 1). Using this method, we could detect down to 10^2 CFU/mL of target pathogen a short time.

2 EXPERIMENT

2.1 Reagents and Apparatus

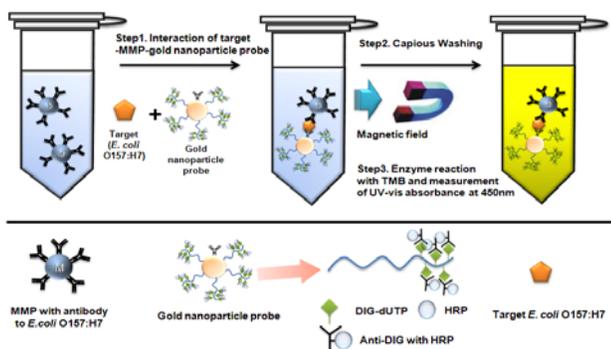


Figure 1: Schematic diagram of Nano-ELISA using gold nanoparticle-DNA complex for the detection of pathogenic microbe

Tosylactivated 2.8-um-diameter MMPs were purchased from Dynal Invitrogen Corp. Oligonucleotide 5'-Thiol-TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG-3' was synthesized by Genotech Corp. *E. coli* O157:H7 antibodies were obtained from Abcam Corp. DIG Oligonucleotide Tailing Kit, Anti-Digoxigenin-POD were purchased from Roche Corp. TMB (3,3',5,5'-tetramethylbenzidine) were purchased from Pierce Corp. The buffer solutions involved in this study are as follows. Blocking buffer consisted of PBS buffer solution (10mM, pH 7.4), 6% BSA, 0.025% Tween20 Washing buffer consisted of PBS buffer solution(10mM, pH 7.4), 0.1% BSA, 0.05% Tween20. Assay buffer, storage buffer consisted of PBS buffer solution (10mM, pH 7.4), 0.1% BSA.

2.2 Preparation of magnetic particle probes

The MMPs (1ml stock solution) were activated by incubating with borate buffer (0.1M, pH9.5), at room temperature for 2 minutes. The hydroxyl groups are activated by reaction with p-toluensulphonyl chloride. The resulting can subsequently react covalently with other ligands containing amino groups. After applying the magnet and pipetting off the supernatant, resuspend the washed beads in the same volume of borate buffer. The antibody(Mouse monoclonal to *E.coli* O157:H7) was dissolved in borate buffer (3ug antibody per 10^7 MMP). The antibody was added in MMPs solution, continued to vortex for 1 minute. It was incubated for 20 hours at 4°C with slow tilt rotation. After incubation, the MMPs were washed 4 times with the washing buffer (10mM PBS pH7.4 with 0.1% BSA) on the magnet. The remaining active sites on MMPs were blocked by incubating the washed MMPs with the blocking buffer (0.2M Tris pH8.5 with 0.1% BSA) for 4h at 37°C. The MMPs were stored in washing buffer at 4°C.

2.3 Preparation of DIG oligonucleotide Tailing

Oligonucleotides are enzymatically labeled at their 3'-end with terminal transferase either by incorporation of a single digoxigenin-labeled dideoxyuridine-triphosphate (DIG-dUTP) or by the addition of a longer nucleotide tail. For the generation of tailed oligonucleotide probes, a mixture of deoxynucleotide-triphosphates (dNTP) and DIG-dUTP is used in a template-independent reaction. Briefly, 100 pmol oligonucleotide(5'-Thiol-TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG-3') and sterile double distilled water to a final volume of 9uL to a reaction was added. The reaction buffer (100uL, 1M potassium cacodylate, 0.125M Tris-HCL, 1.25mg/mL bovine serum albumin, pH 6.6), CoCl_2 -solution (100uL CoCl_2 -solution, 25mM), DIG-dUTP solution(25uL DIG-11-dUTP, in double dist. Water, 1mM), dATP solution(25uL dATP, in double dist. Water, 10mM), 400U Terminal transferase(25uL terminal transferase, in 60mM K-phosphate pH7.2, 150mM KCL, 1mM 2-Mercaptoethanol, 0.5% Triton X-100, 50% glycerol) was added and mixed and centrifuge briefly on ice. It was incubated at 37°C for 15min, then place on ice, to stop the reaction by adding 2uL 0.2M EDTA.

2.4 Preparation of Gold nanoparticles probes

First of all, gold nanoparticle-antibody was fabricated by an appropriate concentrate of antibody (Goat polyclonal to *E.coli* O157:H7) because of gold nanoparticle had characteristic color to be aggregation. If all antibody was immobilization on gold nanoparticle surface, when NaCl solution added that, gold nanoparticle looked just a red, and if not, the color was an almost black. In this study, gold nanoparticle probe was fabricated to add antibody and Oligonucleotide tailing, so the antibody must be attached an apt concentration to be conjugated others. After the different concentrate of antibody (0uL, 0.1uL, 0.25uL, 0.5uL, 0.75uL, 1uL, 2.8mg/mL) per gold nanoparticle(1ml, 2.6×10^{10} No./ml) was prepared and added phosphate buffer to set volume and NaOH solution of 5uL. After they were incubated for 15 minutes, at room temperature, added slowly the minimum NaCl solution. We decided the concentration of antibody at 0.375uL because of the gold nanoparticle color middle of black and red color. (Figure 2) For the probe of the detection, fabrication of gold nanoparticle-antibody complex was carried out. In this study, mixed solution of AuNP and polyclonal antibody (pab) was prepared after dilution with water, Pab against *E. coli* O157:H7 (0.375uL of 1mL gold nanoparticle), and final AuNP volume was 3mL. The prepared solution was incubated with NaOH solution of 15uL for 20 minutes at room temperature. After DIG oligonucleotide tailing solution (24uL) was included the prepared solution, they were incubated for 30 minutes at room temperature. The AuNP complexes were measured using UV/vis. Spectrophotometry. After blocking solution with 6% BSA, 0.05% tween20 in PBS (7.4 pH, 10mM) was added to the prepared solution, they incubated for 2 hours 30 minutes at

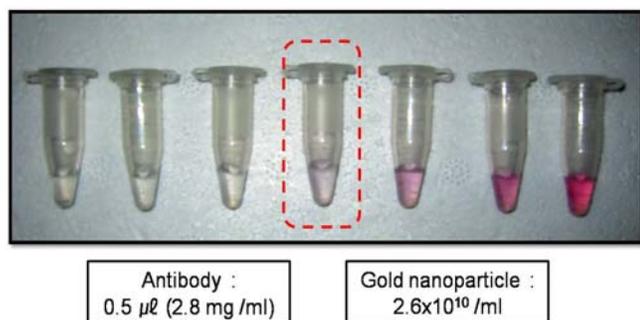


Figure 2. Concentration of Gold nanoparticle of antibody to conjugation with Gold nanoparticle

room temperature. Then it need to neutralization. So to neutralization (300uL) was prepared after the solution made 10mM phosphate buffer(150uL), 2M NaCl(90uL), 0.2% tween20(60uL). The prepared solution incubated for 20 minutes at 4°C. To prepare the AuNP probes was centrifuged for 10 min at 13,000 rpm, and the supernatant was removed. The particles were resuspended in PBS solution (pH 7.4, 10mM) with 0.1% BSA, 0.05% tween20 and the centrifugation step was repeated four times more. The resulting AuNP probes were resuspended in PBS solution with 0.1% BSA. Finally Anti-Digoxigenin-POD (2uL/microtube) with HRP was added to the prepared solution. In like manner, the washing step was repeated several times. To store the AuNP probes were resuspended in PBS solution with 0.1% BSA.

2.5 Enzyme amplification based bioassay for detection of *E. coli* O157:H7

The enzyme amplification based bioassay method (Figure 1) was tested with *E. coli* O157:H7 in assay buffer as a target over the 10^2 CFU/mL to 10^7 CFU/mL concentration range. In a typical assay, a sample solution with a set of target concentration was added to 50 µl of MMP probes functionalized with a capture antibody (2.5 mg/ml), and the solution was shaken on an orbital shaker at room temperature for 20 minutes. The AuNP probes were then added to the solution and given 20 minutes to form a sandwich structure. After immune-reaction, MMPs with target-linked AuNP probes can be easily separated from supernatant by applying a magnetic field. To effect magnetic separation, a 1.5 ml tube containing the assay solution was placed in a BioMag® microcentrifuge tube separator at room temperature. After 15 seconds, the unreacted solution components were washed away with PBS buffer. This washing step was repeated several times to effect removal of AuNPs that were not specifically bound to the MMPs through immune-reaction. After then the immobilized enzyme on AuNP probes was reacted with TMB for 15 minutes, followed by stopping the reaction with 2M H₂SO₄. The resultant end product was analyzed by UV-vis spectroscopy. In the experiment, a negative control (with no target present) was run for comparison purposes.

3 RESULTS AND DISCUSSION

3.1 Activity conjugation of DIG oligonucleotide Tailing

Gold nanoparticle size was decided to 60 nm. Each 40 nm and 60 nm gold nanoparticle with DIG-oligonucleotide tailing was reacted to Anti-Digoxigenin-POD reacted to substrate. 60 nm gold nanoparticle probes is more stability than 40nm gold nanoparticle (data not shown). Furthermore, 80nm gold nanoparticle probes was occasionally aggregation in experiment. AuNP probe was evaluated by AuNP-antibody comparison conjugated oligonucleotide tailing to oligonucleotide. The control (just conjugated oligonucleotide) was reacted with 2.0uL/1ml anti-DIG (150U/ml) for 30 minutes at room temperature, as above experiment and detected by UV-vis spectroscopy at 450 nm absorbance. The control was no signal at 450 nm absorbance, but signal of AuNP-antibody conjugated oligonucleotide tailing was the more increased AuNP probe the more signal.

The activity of indirectly immobilized enzyme on AuNPs via DIG-oligonucleotide tail (this method was mentioned in above) was compared with that of directly immobilized enzyme on AuNPs using physical binding events (data not shown). Enzyme functionalized AuNPs was reacted with TMB for 10 minutes, followed by stopping the reaction with 2M H₂SO₄. The activity of immobilized enzyme was studied by UV-vis spectroscopy by measuring the absorbance of the substrate end product at 450 nm. The activity of indirectly immobilized enzyme via DIG-oligonucleotide tail was much higher than that of directly immobilized enzyme on AuNPs because of minimized chemical renaturation and increased No. of immobilized enzyme.

3.2 Enzyme amplification based immunosensor for detection of *E. coli* O157:H7

The selection of antibody with high specificity is important in developing the immunosensor, because the specificity for the measurement of analytes in all immunosensor system is dependent on used antibody. In this study, commercial antibody to *E. coli* O157:H7 was used. In order to investigate the cross-reaction between commercial commercial antibody to *E. coli* O157:H7 and related pathogens existed in contaminated water, indirect ELISA was performed (data not shown). As a result, it was observed that antibody to *E. coli* O157:H7 had the high specificity with corresponding pathogen and did not react with various related pathogens such as *S. typhimurium*, *Yersinia* spp., *Shigella* spp., and *Vibrio* spp. As such, it was deemed to be appropriate as an antibody for the detection of *E. coli* O157:H7.

In a typical assay, the AuNP probes and MMP were formed a sandwich structure with the target via immune

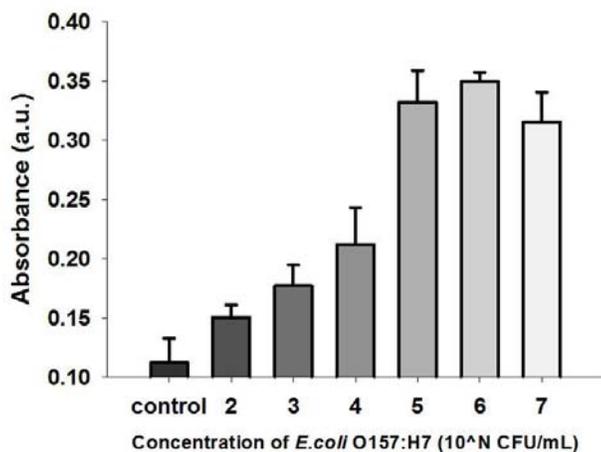


Figure 3. UV-vis absorbance intensity at 450 nm as a function of target pathogen concentration

reaction. A magnetic field was used to effectively remove unbound AuNPs. For detection of pathogen, we exploited the reaction of HRP enzyme immobilized on Au probes with TMB that is a substrate of HRP, followed by stopping the reaction with 2M H₂SO₄. The resulting end products were analyzed by UV-vis spectroscopy. All target concentrations over the 10² CFU/mL to 10⁶ CFU/mL concentration range could be easily differentiated from the negative control, Figure 3. The assay exhibits a linear relationship between target concentration (notice that the x-axis is a log scale) and UV absorbance intensity over a four order of magnitude concentration range. Under the conditions studied, UV absorbance intensity starts to saturate above 10⁶ CFU/mL target concentration. Importantly, the total assay time for enzyme amplification based bioassay is within a hour, and the required steps and instruments for the entire assay were very simple compared with the typical bioassay. The lowest detection limit for the immunosensor based on enzyme amplification method was 10² CFU/mL, plus the assay was four orders of magnitude more sensitive than a standard ELISA[7]. Accordingly, it was concluded that an immunosensor based on enzyme amplification method can be used to monitor *E. coli* O157:H7.

4 CONCLUSION

In this study, we developed a rational design of a nano-complex that consists of AuNP, antibody, oligonucleotides and HRP for the detection of pathogen existing in contaminated environment. The DIG-labeled oligonucleotides that HRP labeled anti-DIG was bound to DIG to amplify the signal were covalently attached on the AuNP. The interaction forces that resulted from the immune reaction between the functionalized MMP containing target pathogen and AuNPs was used to separate unbound AuNPs. For detection of pathogen, we exploited

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REFERENCES

- [1] Roger P. Johnson. *et al.*, "Detection of *Escherichia coli* O157:H7 in Meat by an Enzyme-Linked Immunosorbent Assay, EHEC-Tek.", *Applied and environmental microbiology*, Vol. 61, p. 386–388, 1995
- [2] Han, M.S., Lytton-Jean, A.K.R., Oh, B.-K., Heo, J. & Mirkin, C.A., "Colorimetric assay method for double strand DNA binding molecule using DNA linked gold nanoassembly.", *Angew. Chem., Int. Ed.* **45**, 1807-1810, 2006.
- [3] Parak, W.J. *et al.*, "Biological applications of colloidal nanocrystals.", *Nanotech.* **14**, R15-R27, 2003.
- [4] Besteman, K. & Dekker, Cees., "Enzyme-coated carbon nanotubes as single-molecule biosensors.", *Nano Lett.* **3**, 727-730, 2003.
- [5] Qhobosheane, M., Santra, S., Zhang, P. & Tan, W., "Biochemically functionalized silica nanoparticles.", *Analyst*, **126**, 1274-1278, 2001.
- [6] Nam JM, Thaxton CS, Mirkin CA., "Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins.", *Science*, 301(5641), 1884-6, 2003
- [7] Kim, J. W., Jin, L. Z., Cho, S. H., Marquardt, R. R., Frohlich, A. A., Baidoo, S. K., "Use of chicken egg-yolk antibodies against K88+fimbral antigen for quantitative analysis of enterotoxigenic *Escherichia coli* (ETEC) K88 by a sandwich ELSIA" *J. Sci. Food Agric.*, **79**, 1513-1518, 1999.