Rapid Detection of *Mycobacterium bovis* in Milk by a Nanobiosensor

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ABSTRACT

Detection of *Mycobacterium bovis* (*M bovis*) is important for prevention of a cattle tuberculosis outbreak. In this study, a nanobiosensor modified by gold nanoparticles for detection of *M bovis* in milk was developed. The biosensor was fabricated firstly by dropping the mix solution of chitosan (Ch), Au nanoparticles (AuNPs), and alkaline phosphatase labeled goat anti-mouse IgG (ALP-IgG) on glassy carbon electrode (GCE) to form a robust film with good biocompatibility, then antibody against Mycobacterium tuberculosis (anti-M.TB), which could identify M bovis, was immobilized on the composite film through the reaction with ALP-IgG. The biosensor was able to detect *M bovis* with the detection limit 3.5×103 cfu/ml and a linear range from 104 to 106 cfu/ml. This strategy could be applied as a rapid platform for detection of M. bovis for cattle tuberculosis infection.

INTRODUCTION

Detection of Mycobacteriu*m bovis* (M bovis) in milk is a good solution to prevent cattle TB outbreak, which can also provide a guarantee for food safety and human health. Some studies have reported that Pasteurization and short-time high temperature sterilization cannot completely kill the Mycobacterium tuberculosis.[1] *M bovis* was found in commercial milk, raw milk, and the cow lung tissue in Nepal, Spain, and other countries.[2,3] However, the detection of *M bovis* is cumbersome and time-consuming due to their slow growth rate, which may take 4 to 12 weeks for culture.[4] Recently, the electrochemical immunosensor has been applied for detection of pathogenic bacteria, because it possesses the promising properties of specific and simple detection, short assay time, and suitable miniaturization.[5,6]

For the design of an electrochemical immunosensor, a crucial step is the immobilization of the immunoreagent onto the electrode surface. Chitosan (Ch), a kind of natural polysaccharide, has been extensively applied as immobilization matrices for preparation of biosensors[7,8] due to its attractive properties of excellent film-forming ability, biocompatibility, non-toxicity, high mechanical strength, and affordability. Au nanoparticles (AuNPs), with unique chemical and physical properties, have been widely used for constructing electrochemical biosensors due to their high electron-transfer ability between biomolecules and electrode surface, and with enhancement of the signal response. [9-11] In addition, AuNPs could retain the proteins conformation and make them free in orientation, thus improve the lifespan of the immunosensor. [12]

In this work, an immunosensor for the determination of *M bovis* was fabricated on the surface of glassy carbon electrode (GCE). The electrode was firstly modified by dropping the mix solution of the Ch, AuNPs, and alkaline phosphatase labeled goat anti-mouse IgG (ALP-IgG) on the electrode surface, and subsequently, monoclonal mouse antibody against M tuberculosis (anti-M.TB) was adsorbed onto the composite film through the specific binding with ALP-IgG. The enzyme ALP catalyzed the substrate 1-naphthyl phosphate (1-NP) in the detection buffer, and the resulting enzymatic product 1-naphthol provided electrochemical signal on GCEs that could be detected. [13] When anti-M.TB immunoreacted with *M bovis* to form immunocomplex after incubation, the active site of ALP was shielded and the access of substrate molecules to ALP was also partially shielded. Consequently, the electrochemical signal of the immunosensor changed correspondingly with the concentration of M bovis.

MATERIALS AND METHODS

Reagents and Apparatus

Soluble Ch powder, AuNPs, Tris, and 1-NP were purchased from Sigma, USA. Goat anti-mouse IgG labled with ALP (ALP-IgG), monoclonal mouse antibody to M tuberculosis (anti-M.TB), which could also react with M. bovis, were purchased from Abcam, USA. All these antibodies were diluted in 0.1 M Tris buffer pH 7.4 containing 1.0% (w/v) BSA and 0.05% (w/v) Tween 20. The washing buffer was 0.01 M pH 7.4 PBS.

Test target *M bovis* strain using Freeze dried Bacille Calmette-Guérin (BCG) was purchased from Shanghai Institute of biological Products (Shanghai, China). Escherichia coli (E coli) and Staphylococcus aureus (S aureus) were acquired from Institute of Environmental Medicine of Tongji medical College (Wuhan, China). *M bovis* was dissolved in 0.01 M pH 7.4 PBS.

Electrochemical experiments were performed on an electrochemical workstation CHI-660C (Shanghai Chenhua Instrument Co, China). GCEs were purchased from Hengsheng Technology Co. (Beijing, China). A three compartment electrochemical cell contained a saturated calomel reference electrode, a platinum wire auxiliary electrode, and the modified GCE (Φ=3.0 mm) as working electrode. Ultraviolet and visible (UV-Vis) absorption spectra were recorded with a Lambda 35 UV/Vis spectrometer (Perkin–Elmer instruments, USA). The measurements were carried out at room temperature (25 ± 0.5 □).

Fabrication of the Immunosensor

The mix solution ALP-IgG/AuNPs/Ch for modification of the electrodes was prepared according to the literature. 14 The concentration of ALP-Ig in the mixed solution was 0.125 mg/ml. After dropping the mix solution 8 µl on the surface of clean electrode GCE, the electrode was kept at 4□ for 8 h, and then rinsed with PBS. Then, 8 µl of 100 ng/ml anti-M.TB was dropped on the surface of the electrode modified by ALP-IgG/Au/Ch and kept at 4□ for 4 h to immobilize the anti-M.TB on the film through the reaction with ALP-IgG. And in this step, anti-M.TB could block the possible remaining active sites of Au nanoparticles to avoid the nonspecific adsorption. After washed with 1 ml PBS, the immunosensor anti-M.TB/

ALP-IgG/Au/Ch was stored at 4□ when not in use.

The modified electrodes were characterized by the method of electrochemical impedance spectroscopy (EIS) in 5 mM Fe(CN)64−/3− containing 0.1 M KCl with frequencies ranging from 100 kHz to 0.1 Hz with an amplitude of 5 mV. The cyclic voltametry (CV) was performed in 0.1 M pH 8.0 Tris-HCl containing 1 mM 1-NP.

Electrochemical Assay

Differential pulse voltammetry (DPV) was used for recording the analytical signal, which was performed in the testing buffer 0.1 M pH 8.0 Tris-HCl containing 1 mM 1-NP at room temperature. The instrumental parameters for DPV were as follows: amplitude 0.05 V, pulse width 0.01 s, pulse period 0.02 s, and potential range 0 to 0.6 V. The detection of *M bovis* level was based on detecting the inhibition of the access of ALP active center to 1-NP, which resulted from the immunoreaction anti-M.TB with M bovis.

The current response of the immunosensor was evaluated as the following equation: ΔI=I1-I2, where I1 represented the current response in the testing buffer before the immunoreaction, and I2 was the current response after incubating with *M bovis* for 30 min. When antigens were captured by immobilized antibodies, the antigen-antibody complex coating on the surface of the electrode inhibited the electron-transfer. Therefore, the amperometric reduction current response (ΔI) increased proportionally to the concentration of antigen in antibody-antigen reaction.

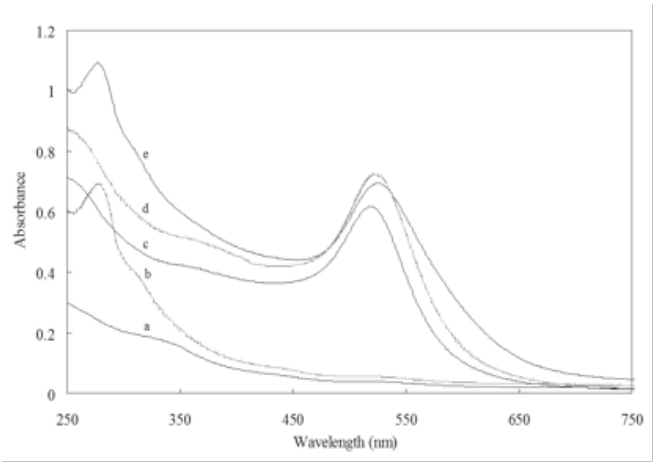
Milk Ssamples Preparation

Milk was purchased from a grocery store. Spiked samples were obtained by mixing *M bovis* and 1 ml of sterile milk together. A series of milk samples spiked with different concentration of *M bovis* (ranging from 103 to 106 cfu/ml) were prepared. According to the literature,15 diluted milk sample (10 ml) was centrifuged at 1,900 g for 10 min. After discarding the supernatant, the sediment of *M bovis* cells was washed three times. Finally, discarding the supernatant, 1 ml PBS was added to the sediment and mixed it for detection.

Statistical Analysis

Linear regression was used to determine the relationship between current shift and *M bovis* concentration in the samples. The consistency of current response signals between PBS samples and milk samples in the range of all concentrations were analyzed by correlation analysis. And t-test was used to determine the significance of differences between the signals produced by the two kinds of samples in each concentration separately. Statistical analyses were performed with SPSS software. A significance level of $P < 0.05$ was applied in all statistical tests.

***Figure. 1**. UV-Vis absorption spectra of (a) Ch solution, (b) Ch mixed with ALP-IgG, (c) AuNPs, (d) Ch mixed with AuNPs, and (e) Ch mixed with ALP-IgG and AuNPs.*



RESULTS AND DISCUSSION

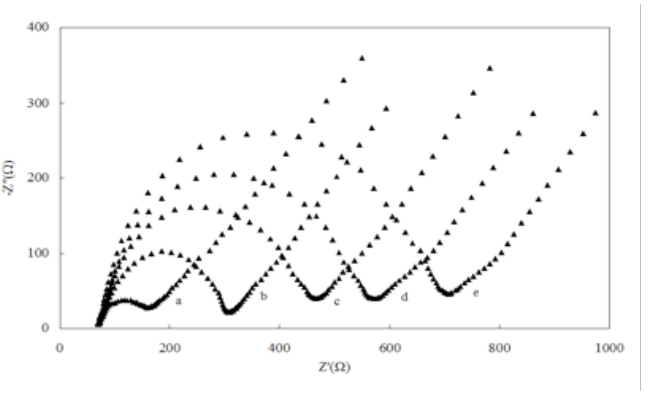
Characterization of the Ch/Au/ALP-IgG Mix Solution

It is well known AuNPs can be stabilized with –NH2- or –SH-terminated polymers,16 and through the amine groups and cysteine residues in the protein.17 As Ch is an

amine-rich polysaccharide, it is mixed with ALP-IgG and AuNPs to form stable solution for modification of the GCE. As shown in Fig 1, the UV-Vis absorption spectra of Ch solution (a) showed a flat curve (Fig1 a). After Ch mixed with ALP-IgG, a distinct peak of absorption was observed at about 280 nm (Fig 1b), which are similar to the absorption of IgG.18

AuNPs exhibited a distinct surface plasmon absorption band, with the maximum absorbance at about 518 nm (Fig 1c), which was the characteristic of plasmon absorbance of unaggregated AuNPs.19 When mixed with Ch and again with ALP-IgG, the maximum absorbances of AuNPs in the two mixtures were respectively found red shift (523 nm for Fig 1d and 524 nm for Fig 1e) compared to the bare AuNPs (Fig 1c), due to the change in the refractive index as a result of the addition components.[20]

Figure.2. *EIS of the different electrodes in 5 mM Fe(CN)64−/3− containing 0.1 M KCl: (a) bare GCE, (b) ALP-IgG/AuNPs/Ch, (c) ALP-IgG/Ch, (d) anti-M.TB/ALP-IgG/AuNPs/Ch and (e) after anti-M.TB/ALP-IgG/AuNPs/Ch modified electrode incubated with 105 cfu/ml M. bovis. The frequency range was from 10-2 to 106 Hz with perturbation amplitude of 5 mV.*



Electrochemical Characteristics of the Modified Electrode

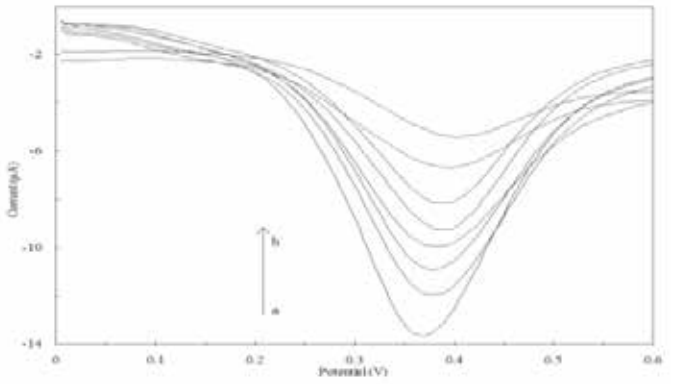
EIS is an effective method to investigate the interface properties of surface-modified electrodes. Fig 2 showed the EIS results of the stepwise fabrication of the immunosensor on the bare GCE (a), ALP-IgG/Ch (b), ALP-IgG/AuNPs/Ch (c), anti-M.TB/ALP-IgG/AuNPs/Ch (d), and M bovis/anti-M.TB/ALP-IgG/AuNPs/Ch (e) in 5 mM Fe(CN)64−/3− containing 0.1 M KCl. It could be seen that the bare GCE exhibited a small semicircle at high frequencies and a linear part at low frequencies, implying the characteristic of a diffuse limiting step of the electrochemical process on a bare GCE in the electrolyte solution (Fig 2a). After ALP-IgG/Ch layer formed onto the GCE surface, a remarkable increase in the semicircle diameter was observed (Fig 2c), which implied the successful formation of ALP-IgG/Ch membrane hindering diffusion of ferricyanide toward the electrode surface.

When AuNPs was present, the semicircle diameter of the ALP-IgG/AuNPs/Ch modified electrode decreased comparing to that of ALP-IgG/Ch (Fig 2b), indicating that AuNPs facilitated the diffusion of ferricyanide toward the electrode surface, as AuNPs acted as a conducting wire or an electron communication relay.21 After immobilization of anti-M.TB, the semicircle diameter of anti-M.TB/ALP-IgG/AuNPs/Ch obviously increased again (Fig 2d), suggesting that anti-M.TB was successfully immobilized on the surface of the electrode. Especially, after *M bovis* was coupled onto the anti-M.TB/ALP-IgG/AuNPs/Ch modified electrode through the reaction with anti-M.TB, a remarkable increase of interfacial resistance was obtained (Fig 2e). This increase was attributed to the immunocomplex layer on the electrode, which acted as the mass-transfer blocking layer, thus, a further increase resistance was observed. [22]

Detection of M. bovis in PBS and Milk Samples

After the electrodes of anti-M.TB/ALP-IgG/AuNPs/Ch were incubated to various concentrations of *M bovis* diluted in PBS.

Figure 3. *DPV curves of the anti-M.TB/ALP-IgG/AuNPs/Ch electrodes in pH 8.0 Tris-HCl solution containing 1mM 1-NP after incubating with various concentrations of M. bovis, and curves a–h represent 0, 1×104, 2.5×104, 5×104, 1×105, 2.5×105, 5×105, 1×106 cfu/ml M. bovis (from bottom to top).*



DPV curves were separately scanned to record the current response (Fig 4). When *M bovis* was captured by the anti-M.TB/ALP-IgG/AuNPs/Ch biofilm on the electrode, there would be an additional layer, which became a barrier for partly shielding the active center of immobilized ALP-IgG and decreased the catalytic capability of ALP.22 Consequently, a significant decrease in the DPV peak current was observed with the increment of *M bovis* concentration (Fig.3).

The pH value of test solution affected the performance of the immunosensor. Since highly acidic or alkaline surroundings would damage the immobilized protein and decrease the stability of Ch, pH 8.0 was selected as the optimum pH value for *M bovis* detection. Also, the amperometric response of the antigen-antibody reaction greatly depends on the incubation temperature and time.

Room temperature was employed as the optimum incubation temperature throughout the experiment in order to adapt to practical application. The incubation time of 30 min was adopted throughout the experiment, since longer incubation time was not able to improve the response. Under the optimal conditions, the calibration curve of the DPV peak current shift ΔI to *M bovis* in PBS was shown in Fig 4 line a. The ΔI increased proportionally to the logarithm of M. bovis concentration from 104 to 106 cfu/ml with a detection limit of 3.5×103 cfu/ml (S/N=3). The linear regression equation was ΔI = 3.344×logC-11.727, with a correlation coefficient of 0.994.

This immunosensor was also used to detect *M bovis* spiked milk samples. The current shift ΔI increased with *M bovis* concentration, the calibration curve was shown in Fig 4 line b. These results appeared to

Figure 4. *The calibration linear curves of DPV peak current shift ΔI for PBS samples (line a ❑) and milk samples (line b ❑) with different concentrations of M. bovis.*

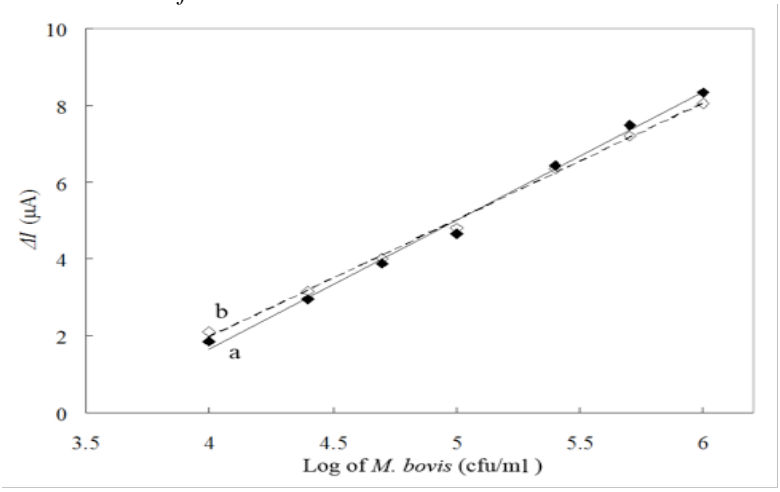
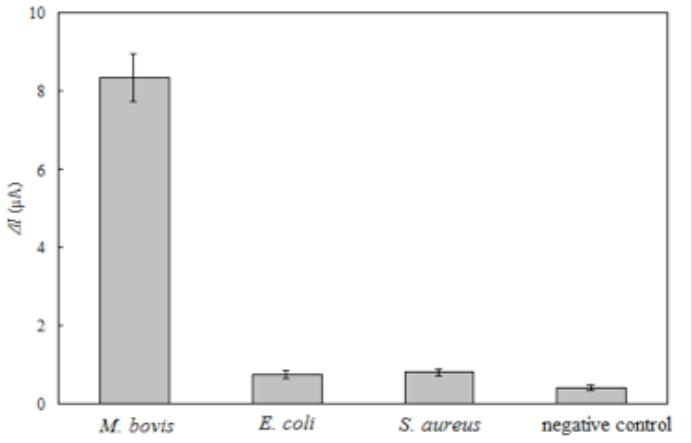


Figure 5. *The specificity of the immunosensor. The bars show the current shift ΔI of the immunosensor, respectively incubated with M. bovis (106 cfu/ml), E. coli (106 cfu/ml), S. aureus (106 cfu/ml) and negative control (0 cfu/ml) for 30 min at room temperature.*



agree well with the results obtained from *M bovis* in PBS (Fig. 4 line a). After statistical analysis, milk samples showed consistent signals with pure PBS samples in the range of all concentrations (r = 0.999), and there was no significant difference between them (P>0.05). Furthermore, the detection limit for milk samples was equal to that for PBS samples. In contrast with routine *M bovis* testing analysis and other supplementing methods,[23,24] this method is faster and only requires simple sample preparation.

Specificity, Repeatability and Stability

In order to evaluate the specificity of the immunosensor, possible interfering pathogenic bacteria in food such as Escherichia coli O157:H7 (E coli) and Staphylococcus aureus (S aureus) were chosen in the testing experiment. As shown in Fig 5, the current shift induced by *M bovis* was much larger than that induced by other pathogens, demonstrating a good selectivity of the immunoassay.

The repeatability of the immunosensor was evaluated from the response to *M bovis* (105 cfu/ml) at five different electrodes, and the relative standard deviation (R.S.D) was 8.3%. When the immunosensor was stored at 4 □ for a week, the amperometric response was 90% of the initial response. The results indicated that the immunosensor had acceptable reproducibility and stability.

CONCLUSION

In this paper, an amperometric immunosensor for rapid detection of *M bovis* has been developed based on immobilization of anti-M.TB on glassy carbon electrode modified by ALP-IgG/AuNPs/Ch composite membrane. *M bovis* was detected based on the inhibition of enzymatic activity to the oxidation reaction of 1-NP due to the formation of immunocomplex on the surface of the immunosensor. When the immunosensor was applied to milk spiked samples, good results were obtained. The fabrication of the sensor provides a simple and rapid method for detection of *M bovis* comparing to conventional analysis. [23, 24]

REFERENCES

1. Cvetnic Z, Katalinic-Jankovic V, Sostaric B, Spicic S, Obrovac M, Marjanovic S, Benic M, Kirin BK, Vickovic I: Mycobacterium caprae in cattle and humans in Croatia. *Int J Tuberc Lung Dis* 2007;11:652-8.
2. Gutierrez Garcia JM: Milk as a vector of transmission of bovine tuberculosis to humans in Spain: a historical perspective. *Vet Herit* 2006;29:41-44.
3. Leite CQ, Anno IS, Leite SR, Roxo E, Morlock GP, Cooksey RC: Isolation and identification of mycobacteria from livestock specimens and milk obtained in Brazil. *Mem Inst Oswaldo Cruz* 2003;98:319-23.
4. Iwamoto T, Sonobe T, Hayashi K: Loop-mediated isothermal amplification for direct detection of Mycobacterium tuberculosis complex, M. avium, and M. intracellulare in sputum samples. *Journal of clinical microbiology* 2003;41:2616-22.
5. Vijayalakshmi VK, Olga K, Kamila O, Catherine A: An overview of foodborne pathogen detection: In the perspective of biosensors. *Biotechnology advances* 2010;28:232-254.
6. Paul LH, Joanne B, Lynsey D, John Q, Trinad C, Richard O: Advances in biosensors for detection of pathogens in food and water. *Enzyme and Micro-*

bial Technology 2003;32:3-13.

7. Ding L, Hao C, Xue Y, Ju H: A bio-inspired support of gold nanoparticles-chitosan nanocomposites gel for immobilization and electrochemical study of K562 leukemia cells. *Biomacromolecules* 2007;8:1341-6.
8. Tkac J, Whittaker JW, Ruzgas T: The use of single walled carbon nanotubes dispersed in a chitosan matrix for preparation of a galactose biosensor. *Biosensors & bioelectronics* 2007;22:1820-4.
9. Kumar SJ, Phani, KL.: Novel Method for Deposition of Gold-Prussian Blue Nanocomposite Films Induced by Electrochemically Formed Gold Nanoparticles: Characterization and Application to Electrocatalysis Chemistry of Materials 2007;19:4722-4730.
10. Xia W, Li YY, Wan YJ, Chen T, Wei J, Lin Y, Xu SQ: Electrochemical biosensor for estrogenic substance using lipid bilayers modified by Au nanoparticles. *Biosensors and Bioelectronics* 2010;25:2253-2258.
11. Yang WJ, Li XB, Li YY, Zhao LF, He WL, Gao YQ, Wan YJ, Xia W, Chen T, Zheng H, Li M, Xu SQ: Quantification of microRNA by gold nanoparticle probes. *Analytical biochemistry* 2008;376:183-8.
12. Weiwei Yang YL, Yu Bai, Changqing Sun: Hydrogen peroxide biosensor based on myoglobin/colloidal gold nanoparticles immobilized on glassy carbon electrode by a Nafion film. *Sensors and Actuators B: Chemical* 2006;115:42-48.
13. Wang J, Kawde AN, Jan MR: Carbon-nanotube-modified electrodes for amplified enzyme-based electrical detection of DNA hybridization. *Biosensors & bioelectronics* 2004;20:995-1000.
14. Luo XL, Xu JJ, Du Y, Chen HY: A glucose biosensor based on chitosan-glucose oxidase-gold nanoparticles biocomposite formed by one-step electrodeposition. *Analytical biochemistry* 2004;334:284-9.
15. Jha VC, Morita Y, Dhakal M, Besnet B, Sato T, Nagai A, Kato M, Kozawa K, Yamamoto S, Kimura H: Isolation of Mycobacterium spp. from milking buffaloes and cattle in Nepal. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science* 2007;69:819-25.
16. Li BS, Zhao LF, Zhang C, Hei XH, Li F, Li XB, Shen J, Li YY, Huang Q, Xu SQ: Ultra-sensitive colorimetric method to quantitate hundreds of polynucleotide molecules by gold nanoparticles with silver enhancement. *Anal Sci* 2006;22:1367-70.
17. Anand Gole SV, Sumant Phadtare, Anil Lachke, Murali Sastry: Studies on the formation of bioconjugates of Endoglucanase with colloidal gold. *Colloids and Surfaces B: Biointerfaces* 2002;25:129-138.
18. Gulay B, M. Yakup A: A dye-ligand immobilized poly(2-hydroxyethylmethacrylate) membrane used for adsorption and isolation of immunoglobulin G. *Biochemical Engineering Journal* 2007;34:147-155.
19. Han X, Cheng W, Zhang Z, Dong S, Wang E: Direct electron transfer between hemoglobin and a glassy carbon electrode facilitated by lipid-protected gold nanoparticles. *Biochimica et biophysica acta* 2002;1556:273-7.
20. Zhang SB, Wu ZS, Guo MM, Shen GL, Yu RQ: A novel immunoassay strategy based on combination of chitosan and a gold nanoparticle label. *Talanta* 2007;71:1530-5.
21. Tangkuaram T, Ponchio C, Kangkasomboon T, Katikawong P, Veerasai W: Design and development of a highly stable hydrogen peroxide biosensor on screen printed carbon electrode based on horseradish peroxidase bound with gold nanoparticles in the matrix of chitosan. *Biosensors & bioelectronics* 2007;22:2071-8.
22. Jian-Ding Qiu R-PL, Rui Wang, Li-Xia Fan, Yi-Wang Chen, Xing-Hua Xia: A label-free amperometric immunosensor based on biocompatible conductive redox chitosan-ferrocene/gold nanoparticles matrix. *Biosensors and Bioelectronics* 2009;25:852-857.
23. Flores LL, Pai M, Colford JM, Jr., Riley LW: In-house nucleic acid amplification tests for the detection of Mycobacterium tuberculosis in sputum specimens: meta-analysis and meta-regression. *BMC microbiology* 2005;5:55.
24. Noordhoek GT, Kolk AH, Bjune G, Catty D, Dale JW, Fine PE, Godfrey-Faussett P, Cho SN, Shinnick T, Svenson SB, et al.: Sensitivity and specificity of PCR for detection of Mycobacterium tuberculosis: a blind comparison study among seven laboratories. *Journal of clinical microbiology* 1994;32:277-84.