Designing an immunosensor for detection of *Brucella abortus* based on coloured silica nanoparticles

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**ABSTRACT**

Brucellosis has always been a threat to the health and economics of societies. We report a new colorimetric immunoassay based on colored silica nanoparticles for detection of *Brucella abortus*. An immunosensor was designed based on blue-SiNPs and paramagnetic nanoparticles (PMNPs). The synthesized immunosensor was conjugated with a polyclonal antibody against *B. abortus*, which was activated by 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to form detection and capture probes, respectively. After adding the conjugates to the bacterial suspension, sandwich structure of PMNPs *B. abortus*-blue-SiNPs was formed and then separated by a magnet. The blue dye was released from the silica structure and its absorbance was measured at 670 nm with a spectrophotometer. Under optimal conditions, results showed a wide dynamic range from \(1.5 \times 10^3\) to \(1.5 \times 10^5\) cfu mL\(^{-1}\) with a detection limit of 450 cfu mL\(^{-1}\). The specificity of the sensor was confirmed in comparison with 5 other bacteria. Also, during the 120-days period, the complex was stable. The results suggested that it can be used in real samples (\(R^2 = .9865\)). This designed colorimetric immunoassay strategy can be used as an alternative, user-friendly and on-site tool for the rapid detection of *Brucella* spp. compared to other common methods with high sensitivity and specificity in a short time.

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**Introduction**

Brucellosis (Malta fever) is an ancient disease and one of the five most common bacterial zoonosis in the world that is caused by the *Brucella* spp. and threatens the health and the economy of the community [1,2]. It reduces lactation and power of fertility in animals. Infection is contagious and there is no effective treatment for bacteria. Also, it can infect various organs in human and causes a wide range of asymptomatic clinical manifestations until meningitis and endocarditis [3,4]. The survival power within phagocytosis cells is an important factor in the pathogenicity of the bacterium [5].

Human brucellosis is rarely fatal and the transmission of the disease is not person-to-person but it can be transmitted through respiration with a small infective dose. Wide geographical distribution, use of multiple antibiotic and long-term treatment, lack of effective vaccine, as well as the limitations of common detection methods, are factors that describes the *Brucella* spp. as a threat to the health and the economy of the community [6]. Various methods have been used for the detection of *Brucella* spp. from a long time such as culture-based methods, immuno-serologic tests, molecular and complex methods. Time-consuming, deficiency or lack of high sensitivity or specificity, need for expensive equipment, and trained personnel are their weaknesses [7]. Using an immunosensor based on nanoparticles can be helpful. Since the selection of a suitable antibody is of importance, three characteristics should be considered in selecting antibodies for the identification of pathogen bacteria: First, the ability of the antibody to detect the smallest number of target bacterial cells (sensitivity). Second, identification of a particular strain in a mixture of different bacteria (specificity) and finally, high affinity for bonding with target bacteria antigen [8]. Polyclonal antibodies can be a good choice for this purpose. They are a complex of immunoglobulin molecules that can react against a specific antigen and secrete through various B cells lineages [9,10].

Colorimetric immunoassay is a technique, which is based on the quantitative identification of the analytic by measuring the amount of light absorbed at a particular wavelength. In fact, changing the color is the key to this technique. It is used for environmental monitoring, detection of pathogenic bacteria and food safety [11,12]. Enzymes and gold nanoparticles are mostly used in colorimetric technique but the critical operating situation, high price and short lifetime prevent them from being used as a routine test in laboratories [13,14]. Paramagnetic nanoparticles and Silica nanoparticles
with fast kinetics in solution, quick and easy separation by the magnet, excellent compatibility and ease of synthesis, low cost of production and the presence of a large hydrophilic surface, have more applicability [15]. In most studies, SiNPs have been used in white or colourless, which are not suitable for signal amplification and signal conversion [16]. Therefore, organic dyes like C.I. Blue 21 can be used to compensate for this deficiency. These dyes are stable and do not fade in unfavourable conditions such as acid, alkali, light and heat. Wenchow Dou et al. used, successfully, magnetic and blue-silica nanoparticles in several studies for detection of Salmonella pullorum, Brucella abortus nosensor based on coloured silica nanoparticles and using Salmonella gallinarum,

Entamoeba histolytica blue-silica nanoparticles in several studies for detection of heat. Wenchow Dou et al. used, successfully, magnetic and fade in unfavourable conditions such as acid, alkali, light and heat. Therefore, organic dyes like C.I. Blue 21 can be used to compensate for this deficiency. These dyes are stable and do not fade in unfavourable conditions such as acid, alkali, light and heat. Wenchow Dou et al. used, successfully, magnetic and blue-silica nanoparticles in several studies for detection of Salmonella pullorum, Brucella abortus nosensor based on coloured silica nanoparticles and using Salmonella gallinarum, Entamoeba histolytica blue-silica nanoparticles in several studies for detection of heat. Wenchow Dou et al. used, successfully, magnetic and fade in unfavourable conditions such as acid, alkali, light and heat.

Materials and methods

Materials

Triton X-100, cyclohexane, hexanol, ammonia (25–28 wt%) were purchased from Merck (Merck, Germany). 3-[2-(Aminooethylamino)ethylamino]propyl-trimethoxysilane (APTMS), Tetraethyl Orthosilicate (TEOS), Bovine Serum Albumin (BSA), Fe₂O₃, Fe₃O₄, 1-Ethyl-3-(3-dimethyloxiranylmethyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich (Sigma-Aldrich, Merck, USA). C.I. Reactive Blue 21 was purchased from Biorbyt, orb10564 (Biorbyt, USA). C.I. Reactive Blue 21 was desorbed from the complex with 10 mL acetone was added for 30 min. Finally, the blue-SiNPs were washed several times with ethanol and water.

Introduction of amino groups onto the surface of blue-SiNPs was done overnight at room temperature. 30 mg of blue-SiNPs, 10 mL ethanol, 150 μL APTMS and 150 μL ammonia were mixed and stirred. Amino-modified blue-SiNPs (blue-SiNPs-NH₂) collected after 10 min centrifugation at 8000 rpm, were washed three times with 0.01 M phosphate buffered saline solution (PBS, pH 7.3). They were dispersed in 1 mL of PBS and stored at 4 °C. Nanoparticles were characterized by Fourier transform infrared (FTIR) and scanning electron microscope (SEM) [14,18,19].

Synthesis of PMNPs

1.6 g Fe₂O₃ and 0.8 g Fe₃O₄ dissolved in 20 mL of distilled water. The mixture was stirred at 80 °C for 20 min. 2 mL propanol and 2 mL ethanol were added to the complex for half an hour. Also, 20–30 mL ammonia was added dropwise for over an hour. The mixture condition was kept constant for 2 h after adding 10 mL TEOS and finally, PMNPs were formed by adding 5 mL APTMS after 2 h. PMNPs were washed 5–6 times with distilled water and characterized by FTIR and SEM and kept at 4 °C [20].

Immobilization of polyclonal antibodies on the surface of blue-SiNPs-NH₂ and PMNPs

0.0005 g EDC, 0.0002 g NHS, 5 μL polyclonal antibodies against Brucella abortus and 500 mL PBS were added to 30 mg blue-SiNPs-NH₂ and PMNPs, respectively. After incubating at 37 °C and washed by PBS, IgG-blue-SiNPs and IgG-PMNPs were again incubated with 1 mL BSA 1% for 1 h. During this period, unreacted active groups were blocked on nanoparticles. Antibody modified nanoparticles were washed by PBS and stored at 4 °C [19].

Culture of bacteria

At first, Brucella spp. were incubated for 72 h at 37 °C on Brucella broth medium and then transferred to Brucella agar medium for 48 h at 37 °C, and re-incubated. Other bacteria were cultured in nutrient-agar. After 24 h incubation at 37 °C, colonies were confirmed by a biochemical test, and stored at −70 °C before use [21].

Colorimetric immunoassay and optimization

10 mg IgG-blue-SiNPs, 5 mg IgG-PMNPs with 1 mL microbial suspension containing Brucella abortus were mixed and incubated for 1 h at 37 °C with gentle shaking. During this period, IgG-blue-SiNPs and IgG-PMNPs formed a sandwich structure with bacteria by immune reaction. The final sandwich immune complexes were separated by a magnet and washed several times to remove unbound IgG-nanoparticles. C.I. Reactive Blue 21 was desorbed from the complex with 100 μL of NaOH aqueous solution (5 mol L⁻¹) for 15–60 min. Afterwards, supernatants including an organic dye were separated. The absorbance of the dye was measured with a spectrophotometer at 670 nm [14,16]. The colorimetric
immunoassay was repeated with different concentrations of antibody, coloured silica and PMNPs to achieve optimal analytical performance. Also, all incubation times were evaluated for conjugation of each nanoparticle with activators and bacteria.

Sensitivity and specificity

According to the 0.5 McFarland standard, (mixing 0.05 mL of 1.175% barium chloride dehydrate (BaCl₂ 2H₂O) with 9.95 mL of 1% sulfuric acid (H₂SO₄); ~cell density of 1.5 × 10⁶ cfu mL⁻¹ and the absorbance at a wavelength of 625 nm should be 0.08 to 0.13 ), serial dilutions of the B. abortus, which were descending from 1.5 × 10⁸ to 1.5 × 10⁵ cfu mL⁻¹, were prepared. Sensitivity was evaluated by dilutions assay. In order to determine the specificity, a colorimetric immunoassay was done by B. abortus, B. melitensis, E. coli O:157, S. typhimurium, Y. enterocolitica O:9, S. aureus, S. maltophilia and PBS as a negative control [14].

Stability and applicability

Performance of PMNPs B. abortus-coloured-SiNPs complex was measured on days 7, 15, 30, 60, 90 and 120 to investigate the stability of the conjugated nanoparticles with the antibody. Applicability was evaluated by real samples of whey and milk. Serial dilutions of B. abortus, 1.5 × 10⁸ to 1.5 × 10⁵ cfu mL⁻¹ were prepared and added to each sterilized sample. The colorimetric immunoassay was repeated. Dye absorbance was recorded separately for each dilution and the performance was calculated finally [14].

Results

Characterization of nanoparticles

Coloured silica nanoparticles were physically bright with uniform dispersion rate in aqueous solution and according to the SEM had a smooth surface, spherical shape with an average size of 43–56 nm (Figure 1(A)).

On the other hand, the FTIR spectrum was used for confirming the presence of amine groups on the surface of these nanoparticles. Dried samples from blue-SiNPs and blue-SiNP-NH₂ were prepared by KBr pellet method in the range of 400–4000 cm⁻¹. The band at the region 980–1220 cm⁻¹ is corresponding to the Si–O–Si of the silica core. The band at 2945 cm⁻¹ in the blue-SiNP-NH₂ is assigned to the N–H of the silica (Figure 1(B)).

Based on the SEM results, size of PMNPs was determined to be 58–72 nm with uniform, spherical, and smooth surface dispersion, which is consistent with the findings of Amini et al. (Figure 1(C)).

Also, FTIR analysis was done for these particles in the region 400–4000 cm⁻¹. The band at 580–630 cm⁻¹ is corresponding to the Fe₃O₄–TEOS vibration, which is related to the magnetite phase, Fe–O. The band at 2923 cm⁻¹ is assigned to C–H of the Fe₃O₄–TEOS–APTMS (Figure 1(D)).

The band at 3450 cm⁻¹ is referred to the N–H stretching vibration. Bands around 3430.7 and 1630 cm⁻¹ were assigned to amide groups. Bands at 1017.3 and 1030 cm⁻¹ were

Figure 1. (A) The SEM image of blue-SiNPs. Spherical shape and 43–56 nm; (B) FTIR spectrum of blue-SiNPs (top) and blue-SiNP-NH₂ (bottom). The band at 2945 cm⁻¹ in the blue-SiNP-NH₂ is assigned to the N–H of the silica; (C) The SEM image of PMNPs, 58–72 nm, uniform and spherical; (D) FTIR spectrum of Fe₃O₄–TEOS (top) and Fe₃O₄–TEOS–APTMS (bottom). The band at 2923 cm⁻¹ is assigned to C–H of the Fe₃O₄–TEOS–APTMS. The band at 3450 cm⁻¹ refers to the N–H stretching vibration. Bands around 3430.7 and 1630 cm⁻¹ are assigned to amide groups.
assigned to the Si–O stretching vibration of 3-aminopropyltrimethoxysilane (APTES) on the surface of PMNPs.

**Optimization**

Different amounts of IgG-PMNPs (0.25, 0.5, 1, 3, 5 and 7 mg), IgG-blue-SiNPs (1, 5, 10 and 15 mg) and antibody (1, 3, 5, 7 and 10 µL) were prepared and the experiment was repeated. According to the results, the best concentration was determined for IgG-PMNPs 5 mg, IgG-blue-SiNPs 10 mg, and antibody 5 µL. (Figure 2(A,B)).

No significant changes were observed in the results with increasing of concentrations. Incubation times for antibody with EDC/NHS were optimized. Antibody and activators were incubated in 37°C for periods of time 15, 30, 45, 60, 120, 240, 360, 480, 600 and 720 min. Binding between the antibody and activators increased at 240 min and the trend remained constant with a slight decrease in 600 and 720 min (Figure 2(C)). The 240 min period was chosen as the optimal time for conjugation between the antibody and EDC/NHS. Nanosensors and microbial suspension were incubated at 37°C under an optimized situation for 15, 30, 45, 60 and 75 min. Results at 60 min showed that maximum interaction occurred between bacteria and nanoparticles. Then the curve decreased slightly and finally stayed linear (Figure 2(D)).

**Sensitivity**

To explore the sensitivity of nanosensors, serial dilutions of B. abortus were prepared according to 0.5 McFarland standard from $1.5 \times 10^8$ to $1.5 \times 10^6$ CFU mL$^{-1}$. A colorimetric assay was performed based on optimal conditions with serial dilutions (Figure 3(A)).

Figure 2. Condition optimization results for rapid colorimetric detection of B. abortus, (A) PMNPs and blue-SiNP-NH$_2$ optimization results, optimum concentration of PMNPs 5 mg and blue-SiNP-NH$_2$10 mg were determined; (B) 5 µL was the optimized concentration of the antibody; (C) Time optimization results, best time for conjugation of antibody with EDC/NHS complex was calculated as 240 min; (D) Optimized time for conjugated of bacteria with nanoparticles was 60 min.

Figure 3. (A) Visual results of the colorimetric assay by serial dilutions of the B. abortus from $1.5 \times 10^8$ to $1.5 \times 10^6$ CFU mL$^{-1}$, by reducing the concentration of bacteria the intensity of the color decreases; (B) A linear dependence between the dye absorbance and logarithm of B. abortus concentration.
Absorption was measured for each dilution and compared with each other (Figure 3(B)). Based on results, maximum detection was in the range of $1.5 \times 10^2$ to $1.5 \times 10^8$ cfu mL$^{-1}$, ($y = 0.0197x + 0.0306; R^2 = 0.9841$), a linear dependence between the absorbance and logarithm of $B$. abortus concentration. Limit of detection (LOD) area was between two dilutions $1.5 \times 10^2$ and $1.5 \times 10^2$ cfu mL$^{-1}$. This area was divided into smaller components. New dilutions were prepared from 100–900 cfu mL$^{-1}$ and the test was repeated. LOD was determined at $1.5 \times 300$ (450) cfu mL$^{-1}$.

**Specificity**

Identification of specific bacteria was evaluated in a mixture of different bacteria. For this, the assay was performed in the presence of $B$. abortus 544, $B$. melitensis 16M, $E$. coli, $S$. typhimurium, $Y$. enterocolitica, $S$. aureus, $S$. maltophilia and PBS as a negative control, respectively. The results demonstrated the highest rate of absorption belonged to the $B$. abortus and $B$. melitensis, although a slight increase was seen in the absorption of $E$. coli and $Y$. enterocolitica samples (Figure 4).

**Stability**

Stability of conjugated nanoparticles with antibody and organic dye in the long-term can be considered as one of the important factors in the evaluation of biosensor performance. Therefore, these factors were investigated in a 4-month period. The colorimetric assay was repeated on days 7, 15, 30, 60, 90 and 120. There was no significant change in performance of immunosensor (Figure 5).

**Applicability**

Biosensor efficiency was calculated by different dilutions of $B$. abortus according to 0.5 McFarland standard. After the preparation, different dilutions were added to sterile samples of whey and milk. Colorimetric assay results showed the correlation coefficient ($R^2$) was equal to $0.9865$, ($y = 0.019x + 0.030$).

**Discussion**

The colour changing is our strategy in identification of pathogen in this study, on the other hand, transfer of dye into silica particles has always been a major challenge because of hydrophilic environments that do not have the ability of inserting the hydrophobic dye into the particles, so in this study, C.I. Reactive Blue 21, a polar and organic dye was used to increase the electrostatic attraction of the dye molecules with a negative charge in the silica matrix [22]. Among the various available methods for the synthesis of coloured silica nanoparticles, w/o method was selected for easy control of the particles size, cost-effectiveness, thermodynamic and isotropic properties [14,23]. The particle size in this method is influenced by the nature of the surfactant molecules, the concentration of tetraethyl orthosilicate and ammonium, as well as, the molar ratio of water to the surfactant [18]. Our sanitized blue silica nanoparticles were consistent with the above methods. FTIR spectrum analysis confirmed that blue-SiNPs have been successfully coated with amine silica shells and APTMS have been successfully introduced onto the surface of the silica modified nanoparticles. Comparison of the absorbance spectra showed a significant difference in the region 2900–3450 cm$^{-1}$. These results are in accordance with the descriptions by Yu et al. and Sun et al. [14,16,19,24].

Synthesis of PMNPs was also done by a co-precipitation method, ease of work and economic viability of this method are important advantages. Temperature plays a significant role in this method. At below 60°C the Fe$_3$O$_4$ production is higher but Fe$_2$O$_3$ will produce more at temperatures higher than 80°C. Here, our manufactured nanoparticles are consistent with Bordbar et al. and Hariani and Amini et al’s descriptions [20,25,26]. By investigating the FTIR spectra results, the introduction of amine groups onto the surface of paramagnetic nanoparticles was confirmed.

The sensitivity of immunosensor was compared with other similar studies. According to this, Wen et al., by colorimetric technique and immunomagnetic capture, detected Shewanella oneidensis with a wide dynamic range between $5.0 \times 10^3$ and
5.0 × 10^6 cfu mL⁻¹ toward target cells [27]. Sun et al. detected *S. pullorum* by polyclonal antibody and blue-silica nanoparticles ranging from 4.4 × 10^4 to 4.4 × 10^5 cfu mL⁻¹ [14], and also detected *S. pullorum* and *S. gallinarum* by silica and magnetic nanoparticles in milk powder 8.8 × 10^6 cfu mL⁻¹ [16]. Song et al. detected *Brucella* by using polyclonal antibody-conjugating quantum dots and antibody modified magnetic beads, 10²–10⁵ cfu mL⁻¹ [28]. Wu et al. determined *B. melitensis* by sensitivity method and using gold nanoparticles-screen-printed carbon electrode 1 × 10⁴ to 4 × 10⁵ cfu mL⁻¹ [29]. Bayramoglu et al. detected *Salmonella* in milk samples by Fe₃O₄/SiO₂/pGMA and MCM-41 particles equal to 10⁴ cfu mL⁻¹ [30].

Li et al. based on immunomagnetic beads and quantum dots detected *Brucella* spp. through a sandwich immunoassay [31]. Wu et al. were able to detect as low as 1 × 10⁴ and 4 × 10⁵ cfu mL⁻¹ of *Brucella melitensis* in pure culture and milk samples by a label-free impedance immunosensor based on a gold nanoparticle-Modified Screen-Printed Carbon Electrode [29]. Liu et al., by colorimetric immunoassay and using core gold nanoparticles, silver nano-clusters as oxidase mimics, and aptamer-conjugated magnetic nanoparticles, detected *Listeria monocytogenes* in the10 to 10⁶ cfu mL⁻¹ concentration range without pre-enrichment [32]. In the present work, we used a polyclonal antibody against *B. abortus*, blue-SiNPs, PMNPs and UV spectroscopy detection range was from 1.5 × 10³ to 1.5 × 10⁸ cfu mL⁻¹ and LOD determined 450 cfu mL⁻¹. Some of these methods are better for identification of bacteria but they are not recommended for routine laboratory because of limitations such as complexity, expensive prices and inability to use them at any location and any time with the minimum equipment and trained personnel.

In the evaluation of specificity, the highest level of reaction was related to *B. abortus* and *B. melitensis*, which is an important advantage for the prepared immunosensor because both species are the most prevalent cause of Brucellosis and they have similar preventive and therapeutic measures. On the other hand, a slight increase occurred in the absorbance of *E. coli* and *Yersinia* because of similarity in the O-antigenic side chain of LPS of *Brucella* and other organisms like *Y. enterocolitica* O:9, *Vibrio cholerae*, *E. coli* O:157, and *Francisella tularensis* [33,34]. The nearest non-specific absorbance to *Brucella* spp. was *Yersinia* O:9. So, based on OD, diagnostic cut off in this method was calculated to be equal to 0.078877 (Mean ± SD for *Yersinia* O:9 and *B. abortus* determined to be equal to 0.056188 and 0.078877, respectively).

Compared to the enzyme-coated nanoparticles, immuno-nano-biosensor had better stability and its performance did not change significantly on day 120 compared to the first day. The important reasons are the formation of covalent bonds on the surface of the nanoparticles with anti-*Brucella* antibodies, as well as the replacement of labelled enzyme antibodies with modifying antibodies in the silica surface [14,16,24].

**Conclusions**

Conventional methods for diagnosis of *B. abortus* requires a laboratory and they are not capable of detecting the bacteria in a short time period and some of them lack high sensitivity and specificity despite economic efficiency; while the immuno-nano-biosensor, which was designed, has high sensitivity and specificity for detection of *B. abortus*. In addition, qualitative and quantitative identification is carried out, which is the greatest advantage and an important factor in situations where there is no access to a lab. The maximum time for detection of *B. abortus* in real samples was 90 min. In summary, this designed colorimetric immunoassay strategy can be used as an alternative, user-friendly and on-site tool for the rapid diagnosis of *Brucella* spp. compared to other common methods, with high sensitivity and specificity in a short time.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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