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Functionalization of anti-Brucella antibody based on SNP and MNP nanoparticles for visual and spectrophotometric detection of *Brucella*

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ABSTRACT

An Immuno-Nano-Biosensor with high sensitivity was designed based on iron and silica nanoparticles to detect *B. abortus*. Briefly explain, primary polyclonal antibody (IgG_1) was conjugated on surface magnetic nanoparticles (MNPs) to form MNP-IgG_1. Secondary polyclonal antibody (IgG_2) and Horseradish Peroxidase enzyme were conjugated on silica nanoparticles (SNPs) to form HRP-SNP-IgG_2. HRP-SNP-IgG_2. MNP-IgG_1 and HRP-SNP-IgG_2 were added to *B. abortus*. The MNP-IgG_1-*B.abortus*-IgG_2-SNP-HRP complex was isolated from the reaction mixture using a magnet. After that, tetramethylbenzidine was added to the complex. The reaction was stopped with HCl and investigated using UV–Vis spectrophotometry. The nanoparticles' structure and size were investigated using SEM and DLS. Immuno-Nano-Biosensor sensitivity and specificity were determined. The SEM and DLS results indicated that the SNPs, MNPs, HRP-SNP-IgG_2 and MNP-IgG_1 size and structure were 35, 44, 60 and 56 nm, respectively. In addition, a good linear correlation was observed at 10^2–10^7 CFU mL^{-1} concentrations, which their linear equation and regression were $Y = 0.3\times + 0.18$ and R^2 0.982, respectively. The limitation of detecting *B. abortus* was 160 CFU mL^{-1}. Finally, the results demonstrated that those designed Immuno-Nano-Biosensor could be specifically detected *B. abortus* and *B. melitensis* in real samples.

1. Introduction

Brucella bacteria are the negative germ, without any movement and anaerobic. *Brucella* is distributed into a class of pathogen three for transmission risk and its transmission paths to humans. Its important species are identified as *B. abortus* and *B. melitensis*. The *Brucella* geographical spread is extensive and is almost everywhere in all over the world [1]. *Brucellosis* is identified as one of the common diseases between humans and livestock, which humans randomly encounter. Human beings have usually infected with contaminated animals like cows, sheep, goats, pigs, and dogs, or nostrils dairy products like milk, cheese and cream [2]. *Brucellosis* disease is very important in livestock, due to its results in mortality and miscarriage. Consequently, its rapid diagnosis is essential to prevent disease spread and economic losses [1–4].

There are many ways for *Brucella* diagnosis. Nowadays, bacterial cultures, blood culture [5–8] and biochemical methods [9–12] are used for the detection of *Brucella*. However, these tests are time-consuming and have limitations in use. Furthermore, if the disease is chronic or undergoing medical treatment, the sensitivity will decrease [13–16]. At any time that the result of microbial culture is negative, utility serology tests applying could be helpful. However, it can still be relied on serology tests, because they can be considered as positive tests in some conditions like recovery after treatment [17–21]. Therefore, for low sensitivity and efficiency, introducing new methods for disease diagnose are necessary. One of the new methods for bacteria detection is Nanobiotechnology methods. Nanobiosensors, due to their higher levels of volume, have more efficiency for microorganisms' connection and detection [22–28]. In the 1990 s, many types of research were conducted on SNPs structures and factors, which affect its structure like some factors of temperature, pH, and solvents [29–31]. Some additives such as salts are lead to different types of SNPs production. Moreover, SNPs have unique properties that can be attributed to the hydrophobic surface, the ability to change surface with the chemical groups, excellent biocompatibility; easy synthesis and also low-cost production [32–36].

Sun et al., (2015) applied blue-SNPs and magnetic nanoparticles (MNPs) basic antibodies to detect *Salmonella* [36]. *Amini* et al., (2017) utilized MNPs and gold nanoparticles (AuNPs) based on DNA biosensors for detecting *P. aeruginosa*. This method sensitivity was 1.2 ng mL^{-1} [7].

Abbreviations: MNPs, Iron nanoparticles; LOD, limit of detection; LB-broth, Luria Bertani broth; TEOS, Tetraethoxysilane; APTES, 3-aminopropyltriethoxysilane; NHS, Sulfo-NHS acetate; DLS, Dynamic Light Scattering; NHS, N-Hydroxysuccinimide; EDC, N-3-Dimethylaminopropyl-N′-ethyl carbodiimide hydrochloride; HRP, Horseradish Peroxidase enzyme; D.W, Distilled Water; TMB, 3, 3′, 5, 5′-tetramethylbenzidine; BHI, Brain Heart Infusion.

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Shahbazi et al., (2018) used AuNPs based on DNA biosensor to detect *S. aureus*, in a situation that the method sensitivity was determined as 9.8 ng mL^{-1} [6]. *Amini* et al. in 2019 applied DNA bio barcode technology-based on MNPs and AuNPs for detecting *S. aureus*, in which the method sensitivity was 86 CFU mL^{-1} [11]. In these study, SNPs and MNPs were utilized to detect *B. abortus* for the first time. The most important advantage of using MNPs and SNPs is omitted stage of the centrifuge, and also using a magnet for bacteria collecting. To achieve this goal, SNPs and MNPs were synthesized under optimal conditions. The MNPs were conjugated with primary polyclonal antibody (MNP-IgG_1) *B. abortus*. On the other hand, the SNPs were conjugated by the use of a secondary polyclonal antibody (IgG_2) *B. abortus* (HRP-SNP-IgG_2), and also horseradish peroxidase enzyme (HRP). The MNP-IgG_1 and the HRP-SNP-IgG_2 were added to *B. abortus* under the optimized condition to form HRP-SNP-IgG_2-*B.abortus*-IgG_1-MNPs complex. The HRP-SNP-IgG_2-*B.abortus*-IgG_1-MNPs complex was isolated using a magnet. After that, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) was added to the mixture to produce a blue color. At the final stage, the reaction was stopped using HCl and were read by the use of UV–Vis spectrophotometry. This method sensitivity was determined in *B. abortus* different concentrations. This method specificity was determined by the negative and positive control bacteria presence. Also, this method sensitivity was determined in the real samples in cheese water and milk.

2. Methods and Materials

2.1. Materials

The polyclonal antibody (IgG) *B. abortus* was obtained from the Biorbyt, Company, U.K. NHS (N-Hydroxysuccinimide), EDC (N-(3-Dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride), TEOS (Tetraethyl orthosilicate), APTES (3-Aminopropyl tri ethoxy silane), $FeCl_2$, Fe_2Cl_3, Brucella Agar, Brucella broth, acetone, ammonia (25%, V/V), ethanol, Horseradish Peroxidase enzyme (HRP) and other materials used in this study were purchased from Merck, Company, Germany.

2.2. Bacterial Strains

This study obtained *B. abortus S19 and B. abortus 544 biovars* as positive control from Razi Vaccine and Serum Research Institute, Tehran, Iran. Other bacteria strains including *S. aureus* ATCC 27217, *E. coli* ATCC 25922, *P. aeruginosa* ATCC27853, *Klebsiella pneumoniae* ATCC 7881 were prepared as negative control from Pasteur Institute, Tehran, Iran.

2.3. Bacteria Culture

B. abortus was cultured in 5 mL of Brain Heart Infusion (BHI) broth for 12 h at 37 °C. After that, it was cultured in Brain Heart Infusion (BHI) Agar for 24 h at 37 °C. Then, the bacteria were investigated applying biochemical tests like catalase, oxidase, urease, and enol.

2.4. MNPs Synthesis

MNPs were synthesized utilizing the *Stober* method with some modifications. Briefly explain, 0.8 g $FeCl_2$ and 1.6 g Fe_2Cl_3 (1: 2) were dissolved in 15 mL distilled water (D.W). As a next step, 2 mL propanol and 2 mL ethanol were added to the reaction mixture at 80 °C, whit 700 rpm. When the color of the solution has been changed to a pale brown, the ammonia (25%, 15 mL, V/V) was added to the reaction mixture drop by drop. This mixture was incubated with 700 rpm for 3 h at 80 °C, when the solution color transferred to completely black. 2 mL Tetraethoxysilane (TEOS, 98%, V/V) was added to the reaction mixture and it was incubated with 700 rpm for 2 h at 80 °C. The MNPs were produced using a magnet was isolated and washed with distilled water three times. The MNPs were dissolved in 20 mL of ammonia (25%, V/W). After that 0.5 mL, 3-amino-propyl-tri-ethoxy-silane (APTES) was added to the reaction mixture and was incubated with 700 rpm for 2–3 h at 80 °C. Finally, MNPs were rinsed by D.W and maintained at 4 °C [7 and 11].

2.5. SNPs Synthesis

The water-oil homogeneous method was applied to synthesis the SNPs. Briefly; TEOS (2 mL, 98%, V/V) was added to 20 mL D.W at 80 °C with 700 rpm. After that, 3 mL of methanol and 0.5 mL Triton X100 were added to the reaction mixture. Also, APTES (0.5 mL, 98%, V/V) was added to the reaction mixture. The reaction was started by adding 100 μl of ammonia (25%, V/V), for 24 h at 80 °C with 700 rpm. At the final stage, when the color of nanoparticles was turned into white, the SNPs were separated by the use of centrifuge at 6000 rpm and also was raised three times by D.W. The SNPs were dissolved in 10 mL D. W, and after that stored at 4 °C [36].

2.6. Conjugation of MNPs

For obtaining this purpose, 200 μg mL^{-1} antibodies (IgG_1) were added to 50 ng mL^{-1} and 25 ng mL^{-1} of EDC and NHS, respectively, and after that, the reaction mixture was incubated for 5 min at 25 °C to activate antibodies. *B. abortus* activated polyclonal antibodies were added to 50 mg mL^{-1} of MNPs for 12 h at 25 °C. Conjugated MNPs with IgG_1 were separated from the supernatant using a magnet. The supernatant solution was stored at 4 °C for measuring the conjugation rate of those antibodies with MNPs. The isolated MNP-IgG_1 was washed three times with phosphate buffer (PBS, 0.1 M). Afterward, 1 mL of albumin (0.1%) was added to MNP-IgG_1 for 12 h at room temperature, for the goal of albumin reacting with free amine agents that have not reacted with antibodies. The Alb-MNP-IgG1 was washed three times with PBS (0.1 M) and after that stored at 4 °C. Those synthesized MNPs and Alb-MNP-IgG_1 size and the structures were investigated using SEM and DLS.

2.7. Conjugation of SNPs

For achieving this purpose, Horseradish Peroxidase enzyme (HRP) (100 unit) and polyclonal antibodies (IgG_2, 200 μg mL^{-1}) were added to EDC (50 ng mL^{-1}) and NHS (20 ng mL^{-1}) linkers and incubated for 30 min at room temperature. After that, 30 mg of synthesized SNPs were added to the reaction mixture that contains enzyme and antibodies, the reaction was incubated for 12 h, at the temperature of 25 °C. After that, the conjugated HRP-SNP-IgG_2 was centrifuged at 6000 rpm and perception was washed three times with PBS buffer (0.1 M). Also, 1 mL albumin (Alb) (0.1%, V/V) was added to the HRP-SNP-IgG_2, and incubated for 12 h, at 25 °C. The HRP-SNPs-IgG_2-Alb was washed three times with PBS buffer (0.1 M) and later was stored at 4 °C. The SNPs and HRP-SNPs-IgG_2-Alb size and shape were investigated using DLS and SEM.

2.8. Efficiency of Conjugation

ELISA and Bradford's methods were utilized to increase the efficiency of conjugation. To obtain this goal, 200 μg mL^{-1} of polyclonal antibodies (IgG_1 and IgG_2) *B. abortus* and 2 μL of HRP were diluted before and after the conjugation of IgG and HRP with MNPs in 1 mL of D.W. IgG_1, IgG_2 and HRP concentrations and SNPs were measured using ELISA and Bradford methods, based on manufacturer protocols. Due to the HRP activity reduction after conjugation, the Bradford method was used to determine HRP concentration. Regarding the following equation, antibodies and HRP concentrations were calculated. In this method, before and after conjugation, 50 μL of diluted peroxidase enzyme was added to 950 μL of Bradford solution. The absorbance rate

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was measured at 540 nm wavelength. Also, the conjugation efficiency was calculated to the following equation:

$$\text{Efficiency} = \{(\text{IgG total} - \text{IgG unconjugated})/\text{IgG total}\} \times 100$$

2.9. Detection of Bacteria

For detecting the *B. abortus* bacteria, 2.5 mg mL^{-1} of MNP-IgG_1 and 1 mg mL^{-1} of HRP-SNP-IgG_2 were added to a microtube containing *B. abortus* (10^6 CFU mL^{-1}) and were incubated for 45 min at room temperature. After the bacteria bonding to the MNP-IgG_1 and HRP-SNP-IgG_2, they were formed MNP-IgG_1- *B. abortus*-IgG_2-SNP-HRP complex, also the complex was extracted from the reaction mixture by the use of a magnet, and later was washed three times with PBS buffer (0.1 M). 100 μL TMB as a substrate was added to the complex and incubated for 10 min at room temperature. Finally, the reaction was stopped by HCl (0.1 M), and the absorbance rate was evaluated at 450 nm wavelength using spectrophotometry and ELISA.

2.10. Sensitivity

To investigate the Immuno-Nano-Biosensor sensitivity, different *B. abortus* bacteria concentrations were prepared in microtubes based on McFarland solution, from 10^1 up to 10^8 CFU mL^{-1}. 2.5 mg mL^{-1} MNP-IgG_1 and 1 mg mL^{-1} HRP-SNP-IgG_2 were added to each one of the microtubes that contained bacteria and were incubated for 45 min at room temperature. The complex (MNP-IgG_1-*B. abortus*-IgG_2-SNP-HRP) was separated using a magnet and washed three times with PBS buffer (0.1 M). Finally 100 μL TMB was added to the complex and incubated for 10 min at room temperature. Finally, the reaction was stopped by HCl (0.1 M) and the absorbance rate was measured at 450 nm wavelength using spectrophotometry.

2.11. Specificity

To obtain this purpose, different bacterial strains were applied. *B. abortus* and *B. melitensis* were used as control positive. *S. aureus*, *E. coli*, *P. aeruginosa*, *Klebsiella pneumonia* were utilized as control negative. To achieve this purpose, control positive and control negative bacteria were prepared in concentrations 1×10^6 CFU mL^{-1}. 2.5 mg mL^{-1} MNP-IgG_1 and 1 mg mL^{-1} HRP-SNP-IgG_2 were added to each microtube contain bacteria and were incubated for 45 min at room temperature. The complex of MNP-IgG_1-Brucella abortus-IgG_2-SNP-HRP was separated by a magnet and washed three times with PBS buffer (0.1 M). At the final stage, 100 μl TMB was added to the complex and was incubated for 10 min at room temperature, after that reaction was stopped using HCl (0.1 M). The absorbance intensity was measured at 450 wavelengths using spectrophotometry (Fig. 1).

3. Results

3.1. Nanoparticles Characterization

In this study, the characterization of nanoparticles was determined using UV-VIS Spectrophotometry, SEM and DLS.

3.1.1. SEM

To accomplish that, 1–3 mg mL^{-1} of MNPs, SNPs, MNP-IgG_1 and HRP-SNP-IgG_2 were dried in an incubator for 1-2 h at 50 °C. The samples form SEM imagines were referred to Sharif University, Tehran, Iran. The SEM image's results indicated that the MNPs and the SNPs sizes were 14–25 nm and 41–50 nm, respectively (Fig. 2 A, G). After adding the amine groups (MNPs-APTES), MNPs sizes were enhanced up to 40–48 nm (Fig. 2 C). Besides, after antibodies conjugation, MNPs and SNPs sizes were increased to 47–60 nm and 44–56 nm, respectively

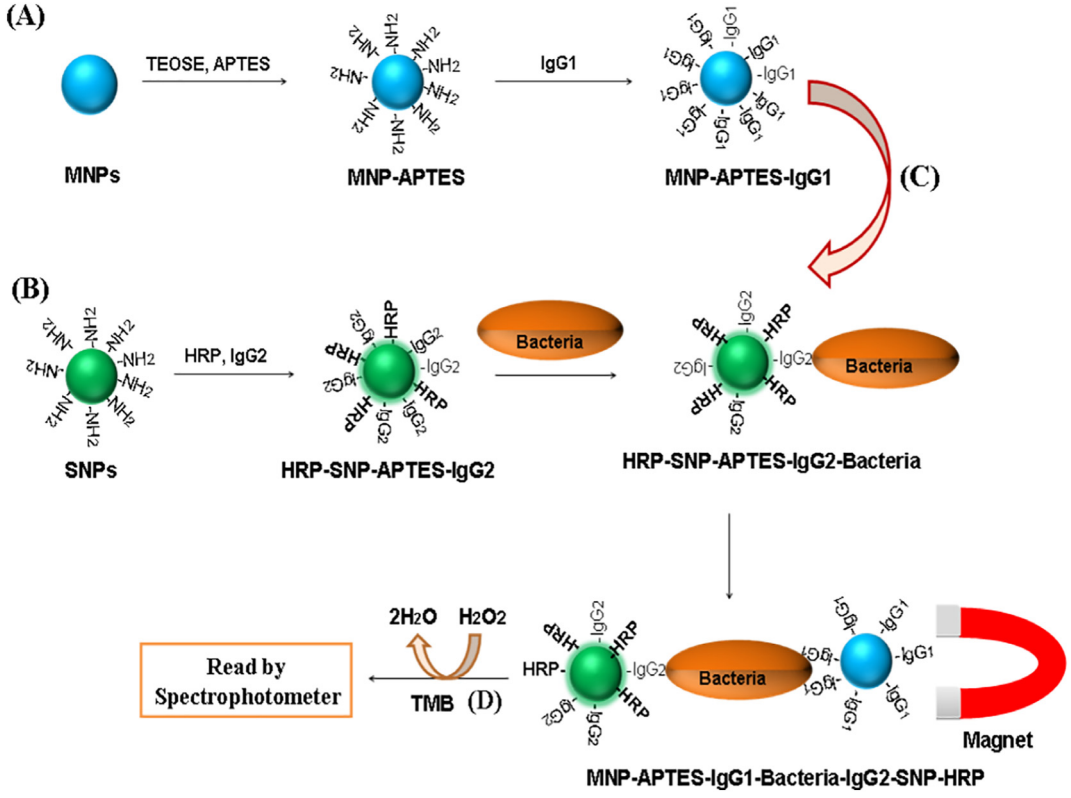

Fig. 1. Schematic detection of *B. abortus*, (A) synthesis and conjugation of MNP-IgG_1, (B) synthesis and conjugation of HRP-SNP-IgG_2 (C) MNP-IgG_1 and HRP-SNP-IgG_2 were added to *B. abortus*, (D) detection of *B. abortus* by UV–Vis spectrophotometry.

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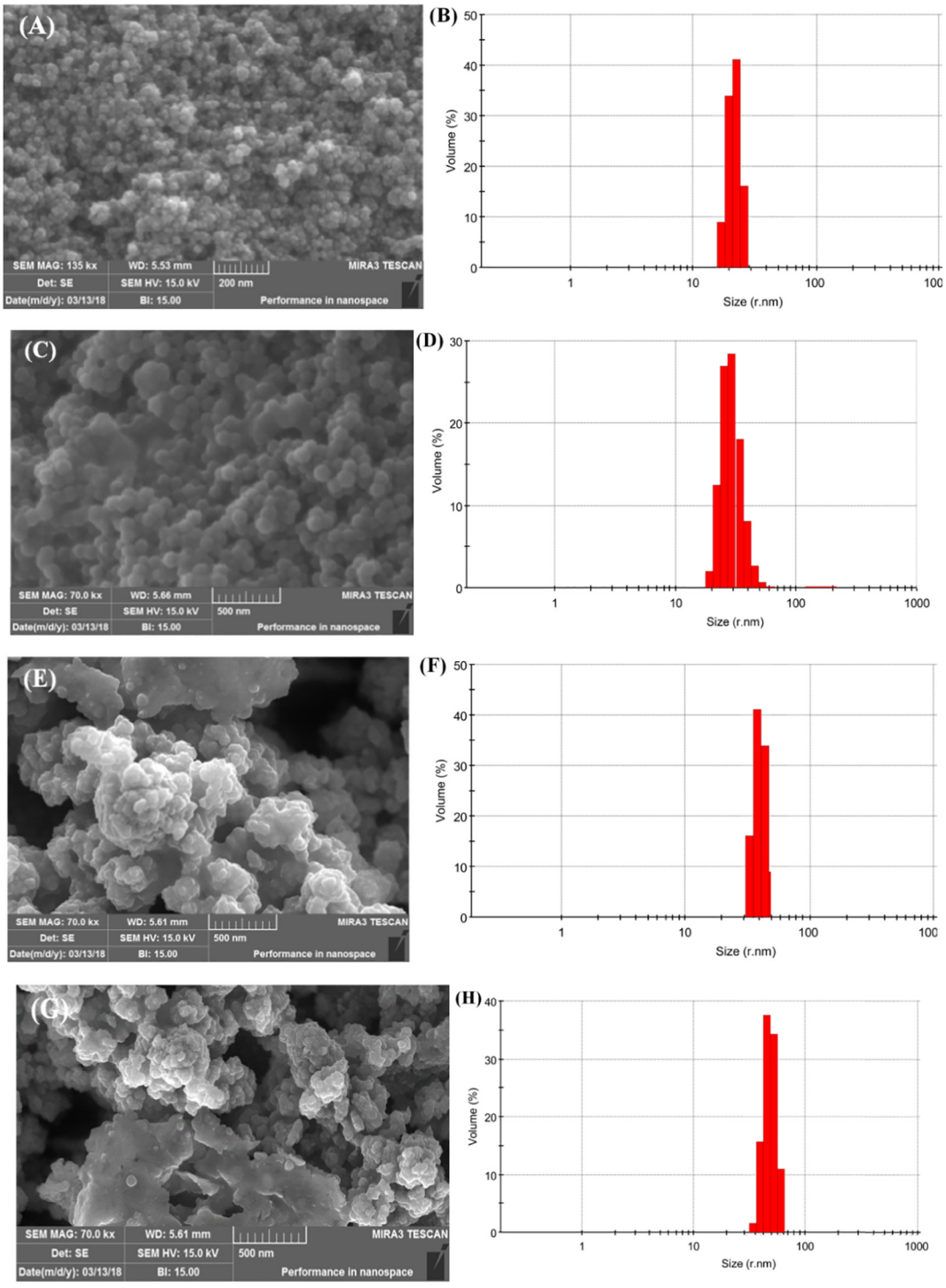

Fig. 2. Electronic microscope SEM and DLS, (A, B) MNPs, (C, D) MNP-APTES, (E, F) MNP-APTES-IgG_1, (G, H) SNPs, (J, K) nanoparticles SNP-IgG_2.

(Fig. 2 E, J). Increasing the size of nanoparticles was demonstrated antibodies conjugation with nanoparticles (Fig. 2).

3.1.2. DLS

DLS was applied to define the MNPs, MNP-IgG_1, SNPs and HRP-SNP-IgG_2 size and the potential. In this method, nanoparticles were dissolved in distilled water and measured sizes and potential with DLS device. This study results indicated that the MNPs, MNP-APTES, and SNPs sizes were 25, 35 and 44 nm, respectively (Fig. 2 B, D, and H). After MNPs and SNPs conjugation with Anti *B. abortus* antibodies (IgG), the MNPs diameters were increased from 35 to 56 nm, and SNPs were increased from 44 to 60 nm that appears at Fig. 2 K and F.

3.1.3. UV-VIS Spectrophotometry

For determining HRP enzymes bounding on SNPs surface, UV–Vis spectrophotometry was utilized. Briefly explain, 1 mg mL^{-1} of HRP-SNP-IgG_2 and 100 μL TMB (0.2 M) was added to a microtube, and they were as positive control incubated for 10 min at room temperature. For control negative, non-conjugated with HRP (SNP-IgG_2) and 100 μL, TMB (0.2 M) was added to a microtube and was incubated for 10 min

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at room temperature. In positive control, microtubes present of conjugated peroxidase enzymes (HRP-SNP-IgG_2) was acted on the substrate, and then blue color was produced. After stopping the reaction by HCl (100 μL, 0.1 M), the absorbance rate was measured at 450 nm wavelength (Fig. 3 a and Curve A). Also, the blue color was not observed in the negative control microtube that nanoparticles (SNP-IgG_2) were not conjugated with HRP. Consequently, the absorbance rate was not increased at 450 nm wavelength (Fig. 3 a and curve B). These study results were confirmed the HRP enzyme conjugation on SNPs surface.

3.2. Efficiency of Conjugation

The performance of antibodies (IgG) conjugation with MNPs and SNPs was accomplished by the use of the ELISA method. Also, for evaluating the efficiency of HRP enzyme conjugation, the Bradford method was utilized. These achieved results indicated the best efficiency of HRP and antibody conjugation with SNPs in optimum concentration that was 83% and 73%, respectively. Moreover, the efficiency of antibody conjugation on surface MNPs was 72% in optimum concentration (Fig. 3 a).

3.3. Optimization

3.3.1. The Concentration of NHS and EDC Linkers

EDC and NHS linkers were used for MNPs and SNPs conjugation with IgG and HRP enzyme. In this method, different EDC and NHS concentrations (10, 25, 50, 75 and 100 ng mL^{-1}) were used for 200 μg mL^{-1} antibody conjugation with MNPs (50 mg mL^{-1}), SNPs (30 mg mL^{-1}). The reaction was performed for 12 h at room temperature. The supernatant was separated, and nanoparticles were washed three times with PBS buffer (0.1 M). The conjugated concentration of IgG with nanoparticles was determined using ELISA and spectrophotometry methods. This study results indicated that the optimum concentrations of EDC and NHS for MNPs and SNPs conjugation were 50 ng mL^{-1} and 25 μg mL^{-1}, respectively (Fig. 3 b).

3.3.2. MNPs and SNPs

The concentrations of MNPs (1, 2.5, 5, 7.5 and 10 mg mL^{-1}) were used to determine the MNPs optimum concentration. Different concentration of MNPs was added to each microtube. After that, a polyclonal antibody of *B. abortus* (IgG_1), EDC and NHS linkers were added and conjugation was performed for 12 h, at room temperatures. The MNPs-IgG_1 complex was separated using a magnet and was washed three times with PBS buffer (0.1 M). The conjugated concentration of IgG_1 with MNPs was measured using ELISA and spectrophotometry methods. This study results indicated that the optimum concentration of IgG_1 for MNPs was 2.5 mg mL^{-1}, and also the absorbance rate for this concentration was the highest in comparison with other concentrations (Fig. 3 c). This process was accomplished for SNPs different concentrations (0.1, 0.5, 1.0, 1.5, and 2 mg mL^{-1}). The results demonstrated that the optimum concentration of SNP-IgG_2 was 1 mg mL^{-1} for *B. abortus* detection (Fig. 3 d).

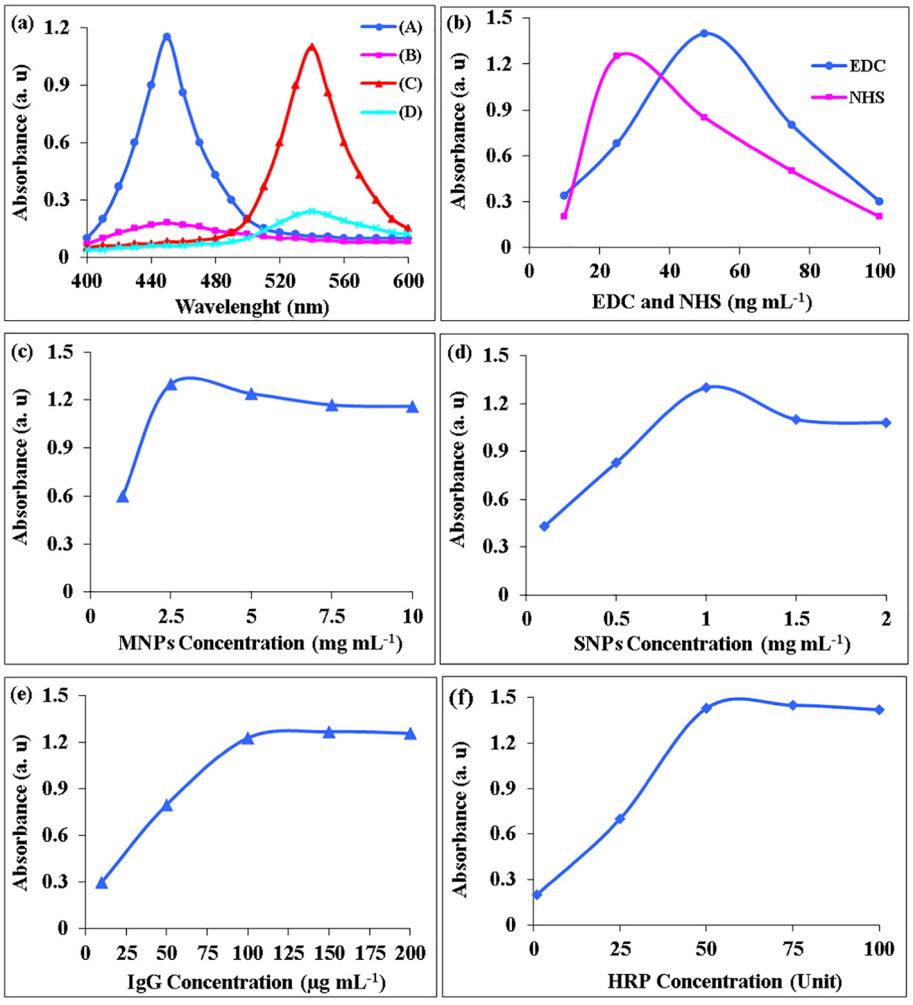

Fig. 3. (a) Efficiency of conjugation HRP and IgG, curve A, IgG before conjugation, curve B, IgG after conjugation, curve C, HRP before conjugation, curve D, IgG after conjugation, (b) concentration of NHS and EDC linkers, (c) concentration of MNPs, (d) concentration of SNPs, (e) concentration of IgG, (f) concentration of HRP.

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3.3.3. The Concentration of Antibody (IgG)

Different polyclonal antibody (IgG) of *B. abortus* concentrations (10, 50, 100, 150 and 200 μg mL^{-1}) were added to each micro-tube containing MNPs (50 mg mL^{-1}) and SNPs (30 mg mL^{-1}). The reaction was performed for 12 h at room temperature. The MNP-IgG_1 and SNP-IgG_2 were separated using a magnet and centrifuge, respectively. The MNP-IgG_1 and SNP-IgG_2 were washed three times with PBS buffer. At the final stage, IgG conjugated concentrations were determined to apply ELISA kit and were read at 450 nm wavelength. These results presented that the antibody optimum concentrations were 50 and 100 μg mL^{-1} for conjugation with MNPs and SNPs nanoparticles, respectively (Fig. 3 e).

3.3.4. Concentration of HRP

For HRP conjugation with SNPs, different concentrations of HRP were used (1, 25, 50, 75 and 100 unite). To accomplish that, 30 mg mL^{-1} of SNPs were added to each one of the microtubes continuing different HRP concentrations, and after that, they were incubated for 12 h at room temperature. Also, the SNP-HRP was washed three times with PBS buffer (0.1 M). The SNP-HRP conjugation rate was investigated by the use of the Bradford solution and spectrophotometry. These results demonstrated that optimum concentration HRP was 50 unite for conjugation of 30 mg mL^{-1} of SNPs (Fig. 3 f).

3.4. Bacterial Detection

2.5 mg mL^{-1} MNP-IgG_1 and 1 mg mL^{-1} HRP-SNP-IgG_2 were added to a microtube containing *B. abortus* 10^6 CFU mL^{-1} and incubated for 45 min at room temperature, for bacteria detection. After that, the MNP-IgG_1- *B. abortus* -IgG_2- SNP-HRP complex was extracted using a magnet and washed three times with PBS buffer. 100 μL TMB was added to the complex and incubated for 10 min at room temperature. The formed blue color complex has indicated the bacteria's presence. Finally, the reaction was stopped by adding HCl (0.1 M), and the blue color of the complex was changed to yellow. Afterward, the MNP-IgG_1- *B. abortus* -IgG_2-SNP-HRP complex was separated using a magnet, and the supernatant was investigated at 450 nm wavelength using ELISA and spectrophotometry.

3.5. Sensitivity

To achieve this purpose, different *B. abortus* concentrations from 2.3×10^1 up to 2.3×10^8 CFU mL^{-1} were prepared in terms of McFarland standard. After that, conjugated MNP-IgG_1 and HRP-SNP-IgG_2 were added to each concentration in microtube for 45 min at room temperature. Following, the MNP-IgG_1- *B. abortus* -IgG_2-SNP-HRP complex were washed and 100 μL TMB was added to each concentration for 10 min at room temperature. The color solutions were studded visually and spectrophotometry. These results indicated that there was a linear association between bacterial concentration and absorbance rate (2.3×10^2–2.3×10^6 CFU mL^{-1}). The absorbance rate in concentrations of 2.3×10^2–2.3×10^6 CFU $mL^{-1}$was increased, along with the bacterial concentration increasing, however, there was no significant change in absorbance rate in concentrations of 2.3×10^7–2.3×10^8 CFU mL^{-1} (Fig. 4). Linear equation and regression of reaction were $Y = 0.3\times + 0.18$ and R^2 0.982, respectively (Fig. 4). Limit of detection (LOD) of this method for *B. abortus* diagnosis was 160 CFU mL^{-1}. All of the tests were checked five times. Table 1 displayed the results, in which the recovery was 98–103%. The matrix effect was much reduced in real samples (Table 1).

3.6. Specificity

For evaluating designed Nano-biosensor specificity, *B. abortus* and *B. melitensis* were utilized as a positive control. *S. aureus, E. coli, P. aeruginosa and K. ponomonieh* were applied as a negative control. First, control negative and control positive bacteria (10^6 CFU mL^{-1}) were added to a mixture containing MNP-IgG_1 (2.5 mg mL^{-1}) and HRP-SNP-IgG_2 (1 mg mL^{-1}). After that, the MNP-IgG_1- *B. abortus* -IgG_2-SNP-HRP complex was separated using a magnet and washed three times with PBS buffer, and also 100 μL TMB was added to this mixture and were incubated for 10 min at room temperature. Finally, the reaction was stopped by adding HCl, and the supernatant was separated and investigated using spectrophotometry. The results demonstrated that the absorbance rate increased in the positive control specimens' presence (Fig. 5 a, curve A, B). The absorbance rate in *B. abortus* and

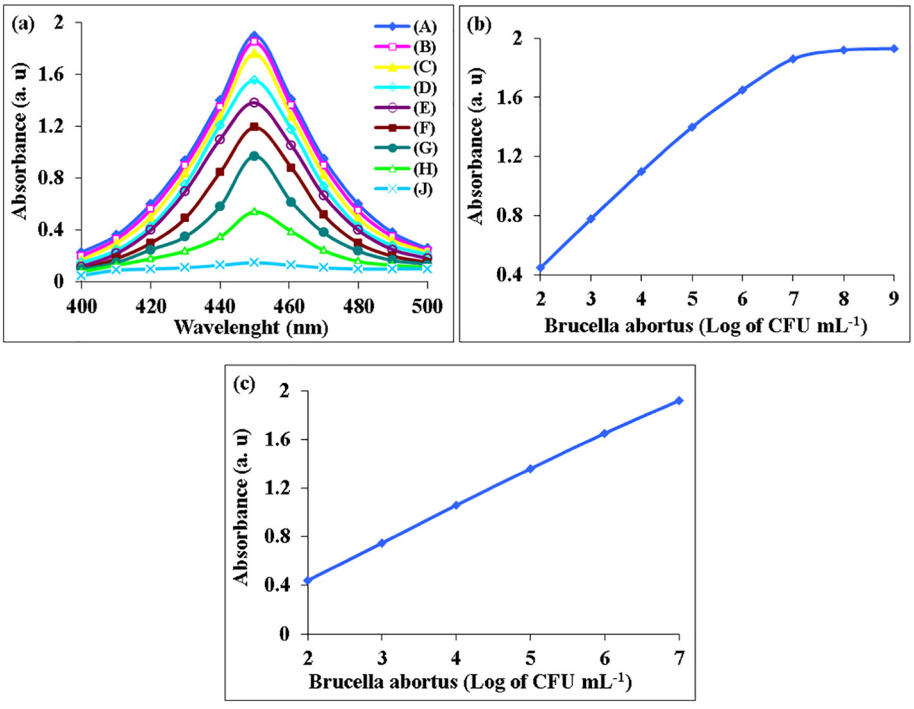

Fig. 4. (a) Sensitivity of *B. abortus* from 10^1 up to 10^9 CFU mL^{-1}, (b) liner related between absorbance rate and concentration of *B. abortus* from 10^2 up to 10^9 CFU mL^{-1}, (c) standard curve of *B. abortus* from 10^2 up to 10^7 CFU mL^{-1}.

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Table 1
The recovery and RSD value of detecting *B. abortus* in real sample ($n = 5$).

Analyses number	*B. abortus* (CFU mL^{-1})	Found (CFU mL^{-1})	Recovery (%)	RSD (%)
1	2.3×10^2	2.32×10^2	100.8	3.8
2	2.3×10^3	2.42×10^3	102.5	4.3
3	2.3×10^4	2.5×10^4	104	4.5
4	2.3×10^5	2.4×10^5	102.3	4.8
5	2.3×10^6	2.2×10^6	98	4.1

B. melitensis were almost identical (Fig. 5 a, curve A and curve B), which indicates the nano-biosensor specificity for both bacterial. However, in control negative specimens, *S. aureus, E. coli, P. aeruginosa and K. ponomonieh* indicated no increase in absorbance rate and color changing, which presented the specificity of designed biosensors for *B. abortus* detection (Fig. 5 a curves C, D, E, and G).

3.7. Real Samples

Milk and cheese water were used as real samples to evaluate the designed Nano-biosensors in real samples. For achieving this purpose, 10^6 CFU mL^{-1} *B. abortus* was added to the milk and cheese water. 2.5 mg mL^{-1} MNP-IgG_1 and 1 mg mL^{-1} HRP-SNP-IgG_2 were added to the micro tubes containing milk, cheese water, and *B. abortus*, under optimum condition. The reaction was accomplished for 45 min at room temperature. Also, milk and cheese water were added to other microtubes without bacteria at the same condition as a negative control. After performing the reaction, the MNP-IgG_1-*B. abortus*-IgG_2-SNP-HRP complex was separated from the reaction mixture by the use of a magnet and was washed three times with PBS buffer. 100 μL TMB was added to each sample, and they were incubated for 10 min at room temperature. Finally, the reactions were stopped by HCl and investigated at 450 nm wavelength using spectrophotometry or ELISA (Fig. 5). The results demonstrated that the absorbance rate was not increased in those samples without bacteria (Fig. 5 b, curve C and D). However, in the samples containing *B. abortus*, the absorbance rate had to increase (Fig. 5 b, curve A and B). The blue color production and absorbance rate increasing were specifically indicated by designed Nano-biosensor for *B. abortus* detection. Moreover, the absorbance rate was increased in both samples (milk and cheese water) (Fig. 5 b, curves A and B).

4. Discussion

Many methods were used for *B. abortus* detection as followings: ELISA, serological, immunological and PCR methods. However, nowadays nano-biotechnology methods based on nanoparticles were applied for microorganism detection. *Sun* et al., (2015) utilized blue-SNPs and magnetic nanoparticles (MNPs) basic antibodies to detect *Salmonella* [36]. *Amini* et al., (2017) applied MNPs and gold nanoparticles (AuNPs) based on DNA biosensors for detecting *P. aeruginosa.* This method sensitivity was 1.2 ng mL^{-1} [7]. *Shahbazi* et al., (2018) accomplished a study by the use of AuNPs based on DNA biosensor to detect *S. aureus*, in a situation that the method sensitivity was determined as 9.8 ng mL^{-1} [6]. *Amini* et al. conducted a study in 2019 that was used DNA bio barcode technology based on MNPs and AuNPs for *S. aureus* detecting, in which the method sensitivity was 86 CFU mL^{-1} [11]. In this study, a nano-biosensor based on MNPs and SNPs were designed for *B. abortus* detection. At first, the MNPs were synthesis with respect to Amini et al. method [7]. The amine groups were in the MNPs surface and they were increased along with APTES and TEOS rising during MNPs synthesis. Also, SNPs white was synthesis. The SNPs color was dependent on methanol rate during synthesis. The increased methanol to mixture reaction has changed the color of nanoparticles. However, reducing that resulted in the colorless SNPs. The IgG and HRP enzyme was conjugated with nanoparticles. The IgG, HRP, SNPs, MNPs and EDC and NHS linkers' concentration were optimized. Conjugation IgG and HRP efficiency were checked using the ELISA and the Bradford methods. After HRP enzyme conjugation, the HRP activity was reduced and did not apply ELISA method, and then the Bradford method was utilized for investigating conjugation efficiency. Also, the EDC and NHS linkers were applied for IgG and HRP conjugation. The linkers' concentrations were optimized. The concentration of more and less than optimum concentration resulted in the HRP activity decreasing. Due to that, the EDC and NHS linkers were reacted with amine groups on the HRP surface, consequently, the amino acid active site was changed and decreased the HRP activity. Finally, the *B. abortus* and *B. melitensis* were detected with designed nano-biosensors in real samples under optimum condition. The results indicated the absorbance rate for *B. abortus* a little more than *B. melitensis*. Limit of detection was determined 160 CFU mL^{-1} similar to other reporters [6,23,37,and].

5. Conclusion

In this study, a novel method was introduced based on MNPs and SNPs for *B. abortus* detection. The SNPs were as a signal reporter and MNPs were as separated of *B. abortus* from the reaction mixture. The conjugated HRP-SNP-IgG_2 and the conjugated MNP-IgG_1 were well reacted with *B. abortus*, and they formed MNP-IgG_1-*B. abortus*-IgG_2-SNP-HRP complex as a result. After that, the complex was separated from the reaction mixture using a magnet and the product was washed three times with PBS buffer. Finally, 100 μL of TMB was added to this mixture and were incubated for 10 min at room temperature. The reaction was stopped by HCl. The supernatant was separated and investigated using spectrophotometry. The results indicated the good linear related that was between absorbance rate and *B. abortus* concentration

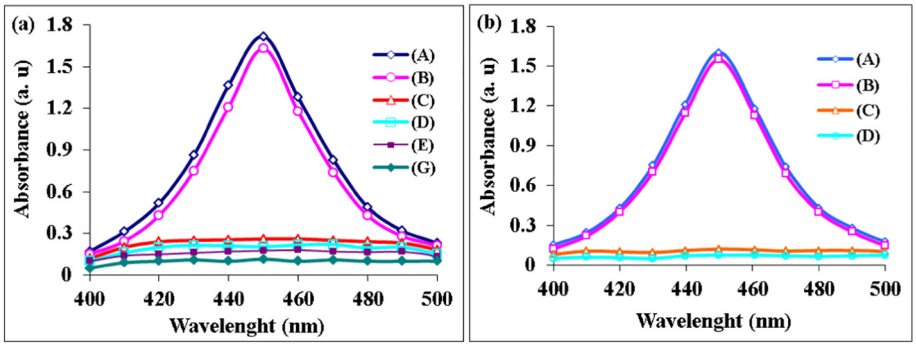

Fig. 5. (a) Specificity of designed Nano-biosensor for detection of *B. abortus*, (Curve A and B) *B. abortus* and *B. melitensis*, respectively, (Curve C to G) *S. aureus, E. coli, P. aeruginosa and K. ponomonieh* respectively. (b) Specificity of designed Nano-biosensor for detection of *B. abortus* in real samples, (Curve A and B) *B. abortus* in milk and cheese water, (Curve C and D) milk and cheese water without *B. abortus.*

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in 10^2–10^7 CFU mL^{-1}. Detection of *B. abortus* linear equation and regression were Y = 0.3× + 0.18 and R^2 0.982, respectively. Also, limited detection was 160 CFU mL^{-1} for *B. abortus* detection. Additionally, the designed Nano-biosensor was very specific for *B. abortus* detection in PBS buffer and also in real samples.

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