Iranian Journal of Microbiology

Volume 14 Number 6 (December 2022) 802-812

ORIGINAL ARTICLE

Design of an optical nanobiosensor for detection of *Legionella pneumophila* in water samples

Raheleh Karimiravesh[1], Ashraf Mohabati Mobarez[1*], Mehrdad Behmanesh[2], Maryam Nikkhah[3], Amin Talebi Bezmin Abadi[1], Saber Esmaeilli[4,5]

[1]Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[2]Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[3]Department of Nanobiotechnology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[4]National Reference Laboratory for Plague, Tularemia and Q fever, Research Centre for Emerging and Reemerging Infectious Diseases, Pasteur Institute of Iran, Akanlu, Kabudar Ahang, Hamadan, Iran
[5]Department of Epidemiology and Biostatistics, Research Centre for Emerging and Reemerging Infectious Diseases, Pasteur Institute of Iran, Tehran, Iran

Received: May 2022**, Accepted:** November 2022

ABSTRACT

Background and Objectives: *Legionella* spp. is a causative agent of Legionnaires' disease that creates public health problems. Isolation of these bacteria from water sources is essential to identify outbreak origins and prevent disease. Diagnostic biosensors for water quality control to protect consumers from water-borne infections can predict many outbreaks. Gold nanoparticles conjugated probes are a new generation of diagnostic tools. In this study, an optical nano biosensor was designed and characterized to detect *Legionella pneumophila* in water samples rapidly.

Materials and Methods: Thiolated probes designed for the *mip* gene were attached to gold nanoparticles and then water samples containing *Legionella pneumophila* were examined.

Results: The limit of detection for PCR and biosensor was 10^4 and 10^3 copy numbers/µl, respectively. Biosensor sensitivity and PCR were reported to be 90% (18 out of 20) and 85% (17 out of 20), respectively. Specificity 100% has been reported for both methods.

Conclusion: According to the obtained results, this method has the potential to diagnose *L. pneumophila* with high sensitivity and specificity. This system can be employed as a practical tool for rapid, accurate, high-sensitivity, and acceptable detection of *Legionella pneumophila* in contaminated water, which is cost-effective in terms of cost and time.

Keywords: Biosensor; Probe; *Legionella pneumophila*; Water; Nanoparticles

INTRODUCTION

Legionella pneumophila is a Gram-negative and fastidious bacterium that causes Legionaries' disease and Pontiac fever (1). The *Legionellaceae* includes 53 species and 70 distinct serogroups. 85% of human infections are caused by serogroups 1, 4, and 6. Reports indicate that 1-5% of cases of community-ac-

*Corresponding author: Ashraf Mohabati Mobarez, Ph.D, Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. Tel: +98-21-82883862 Fax: +98-21-82884555 Email: mmmobarez@modares.ac.ir

quired pneumonia and 30% of hospital-acquired pneumonia were caused by *Legionella* species and mortality in immunocompromised patients reaches 50% (2). Therefore, the presence of species of this environmental microorganism is of particular importance in hospital water resources as the outbreak of infection has been reported in both inpatient wards and intensive care units (3). Rapid diagnosis of *Legionella* is very important in public health care and reduction of mortality due to it (4).

Since *Legionella* is a fastidious bacterium, DNA-based molecular detection can be very important. Molecular techniques based on gene 16S-rDNA to detect bacteria at the genus level along with other diagnostic markers including 5S and 23S-rDNA, dot A, and *mip* gene which is a diagnostic marker of *L. pneumophila* species have been given special attention in order to study different samples (5-7).

Culture-based diagnostic methods in *Legionella pneumophila*, in addition to being time-consuming and having low sensitivity, are not able to detect viable but not culturable forms (8, 9).

PCR and Real-time PCR based are also used to diagnose *Legionella pneumophila*, but are time-consuming and require equipment (8, 10). Gold nanoparticles based on optical sensing due to their unique optical properties have recently attracted much attention in detection cases (11, 12). Their distinctive optical properties, called surface plasmon resonance (SPR), is due to the resonant excitation of their conducting electrons (13). Surface plasmon resonance (SPR) biosensors belong to label-free optical biosensor technologies. An optical surface plasmon resonance (SPR) biosensor can measure the refractive index changes associated with the binding of analyte molecules in a sample to bio-detect molecules stabilized on the SPR sensor (14, 15). Aggregation-based sensors are able to detect biological or chemical compounds, including DNA and proteins, and metal ions (16-20).

There are two categories of nanoparticle aggregation methods for detecting nucleic acids (DNA and RNA). Cross-Linking (CL) is the most common method used to detect nucleic acid in recent decades. It uses two nucleic acid nanoprobes (Np-DNA), each of which complements a portion of the target gene. These nanoprobes hybridize in the presence of the target gene and form a polymeric network of nanoparticles and nucleic acid as they approach each other, resulting in a change in the color of the solution from red to purple (21, 22). In the Non-Cross-Linking (NCL), one (Np-DNA) is used, which alone complements the entire identified gene sequence. Salt induction is also used in this method to aggregate nanoparticles. This nanoprobe, in the presence of its complementary target sequence in the sample, hybridizes with it and prevents the aggregation and discoloration of nanoparticles even at high concentrations of the induction agent (23, 24).

Gold nanoparticles conjugated probes are a new generation of diagnostic tools. Its optical properties change based on the binding to specific molecules and allow the detection and quantification of the desired analyte (25). Active groups, especially thiols, have a high capacity for strong binding to gold nanoparticles and are used to immobilize probes on the surface of nanoparticles. Hybridization of the target strand with the probes results in the formation of an extended polymeric Au nanoparticle/ polynucleotide aggregate, which triggers a red to purple color change in the solution (26). This contributes greatly to the increased sensitivity of gold nanoparticles. Different studies have been performed with the aim of designing biosensors in the field of detection of various microorganisms (16, 27-29). In this study, a gold nanoparticle-based biosensor was designed to detect *Legionella pneumophila* using the *mip* gene.

MATERIALS AND METHODS

Design of primers and probe. The *mip* gene, which is a conserved gene and a diagnostic marker for *L. pneumophila* species, was considered the target gene in PCR and biosensor test (30). The recorded sequences of the *mip* gene were taken from the NCBI database and then the alignment of the *Legionella mip* gene sequences was performed by gene runner software. Gene Runner and Amplicon software were used to design primers forward and reverse in PCR and biosensor thiolated probes. To double-check and ensure the specificity of the designed primers and probes, the IDT website and primer blast, and nucleotide blast of the NCBI database was used. Primer forward (GCATAGATGTTAATCCGGAAGCAATG) and reverse (GCCATCAAATCTTTCTGAAACTTGTT) designed for PCR and also designed thiolated probes for biosensor assay were synthesized by Metabion, Germany.

The thiolated probes for the biosensor assay were

the following: Thiolated probe 1: 5′-SH-CGCTATGAGTGGCGCTCAATT. Thiolated probe 2: TTAACCGAACAGCAAATGAAAG-SH-3′.

Chemical and reagents. Trisodium citrate dihydrate [$Na_3C_6H_5O_7 2H_2O$], Dithiothreitol (DTT), sodium chloride (NaCl), and ethyl acetate were purchased from Merck. Hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4 \cdot 3H_2O$) was obtained from Environment Research Center, Razi University, Kermanshah, Iran, and phosphate buffer (PBS; 10 mM, pH 7.4) was obtained from Biobench, Iran. All glassware was cleaned with Aqua regia cleaning solution [HCl – HNO3 (3: 1)], rinsed with water, and dried. FavorPrep Tissue Genomic DNA Extraction Mini Kit for DNA extraction of *L. pneumophila* was obtained from Favorgene, Taiwan.

Preparation and characterization of gold nanoparticles. Au-NPs were synthesized using the seeded growth method (31). Briefly, 1.1 ml of 60 mM Tri-sodium citrate dihydrate was added to 30 ml of Milli-Q water in a round bottom flask and heating for 15 min under powerful stirring. Two hundred µl of $HAuCl_4$ solution (25 mM) was quickly added to the flask. The solution was cooled to 90°C. Then, 200 µl of $HAuCl_4$ (25 mM) was rapidly injected into the solution. After 30 minutes, 200 µl of $HAuCl_4$ (25 mM) was added again. And after 30 minutes the reaction was finished. The colloidal solution was stored overnight at room temperature and then refrigerated. During the gold nanoparticle fabrication reaction, a color change to cherry red was observed (32). Determination of the size, and size distributions of nanoparticles using Zetasizer Nano Series device (Malvern, England) were characterized. The characteristics of SPRs from GNPs were evaluated in the wavelength range of 400-700 nm by a UV-visible spectrophotometer (Dual Beam Peybord, Iran). Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) are widely considered the gold standard for nanoparticle characterization. As TEM can provide a higher resolution, it may be better suited for particle size analysis in the nano to sub-nano region (33). The size and morphology of the nanoparticles by Zeiss EM900 TEM operating at 50 kV (ZEISS, Germany) were analyzed.

Conjugation of AuNPs with probes (nanoprobe). Thiolated probes were treated with 1.0 N dithiothreitol (DTT) at room temperature for 15 minutes to reduce the disulfide bonds and activate probes. Then to remove DTT, the mixture was washed with ethyl acetate (5 times the volume of dithiothreitol). This process was repeated 3 times and each time the upper layer was removed after vortexing. Two hundred fifty µl of gold nanoparticles were centrifuged for 10 minutes at 7000 rpm and then dissolved in an equal volume of distilled water after removal of the supernatant. One nM of the reduced thiolated probes was added to the gold nanoparticles, and finally, 0.1 M of NaCl was added and incubated for 45 minutes in an orbital shaker incubator (20°C at 60 rpm). Two hundred fifty µL of each nanoprobe 3′ and 5′ were mixed together and incubated again for 30 minutes at room temperature. Thiolate probes formed a self-assembled monolayer on the surface of gold nanoparticles (34). Immobilization of the probe on the GNPs was analyzed by UV–visible spectra, Dynamic Light Scattering (DLS) by using Zetasizer Nano ZS, (Malvern, England).

Preparation of standard positive control. *Legionella mip* gene amplification was performed using PCR, then TA cloning was performed using PCR product to provide a standard positive control for gold nano biosensor (35). For the ligation reaction, the PCR product was purified using the PCR purification kit (Vivantis). Vector cloning of pTZ57RT was used for cloning of *mip* gene (Fermentas). The ligation reaction was performed according to the protocol of the InsTAclone PCR Cloning kit (Fermentas), in which a purified fragment of *mip* gene with a length of 124 bp was ligated into pTZ57R/T vector using 1 unit of T4 DNA ligase. The product of the ligation reaction was transformed into *E. coli* DH5α strain. Plasmids of the selected clones were extracted by Plasmid DNA Extraction kit (Vivantis), and recombinant plasmids containing *mip* gene were confirmed by PCR with general primers (M13F and M13R) and sequencing using primers M13F (GTAAAACGACGGCCAGT) and M13R-pUC-40 (CAGGAAACAGCTATGAC) (36).

Hybridization of AuNP-probes with pTZ57R/T/*mip* plasmid and extracted DNA. Bacterial genomic DNA was extracted from *Legionella pneumophila* ATCC33152 cultured on BCYE agar containing amino acid supplements using the FavorPrep genomic DNA extraction kit (Favorgene, Taiwan). A 10-fold serial dilution of the pTZ57R/T/*mip* plasmid stock

with a concentration of 35 ng was investigated. The diluted DNA concentration was determined from 3.5 to 3.5 $\times 10^{-10}$ ng (~10^9 to 1 copy number/µl). The amount of ~3.5 ng plasmid was sided in water and serial dilutions were prepared. DNA extracted from the *L. pneumophila* ATCC33152 and recombinant plasmid and water sample containing *Legionella pneumophila* were heated at 96°C for 15 minutes and immediately placed on ice for 5 min to remain single-stranded. Four µl of target single-stranded DNA was added to 500 µl of biosensor and at the same time and after 30 and 60 minutes, the absorption peak changes were examined using UV-visible spectrophotometry. The SPR spectra of DNA extracted from standard bacteria and recombinant plasmids were compared. Also, after hybridization with the target DNA, the aggregation of GNPs was analyzed using a transmission electron microscope at 50 kV and the DLS biosensor results.

Detection of *L. pneumophila* by PCR. *Legionella pneumophila* ATCC 33152 strain was used as a reference strain in this method. PCR assay was performed on DNA extracted from *L. pneumophila* using designed forward primer GCATAGATGTTAATCCGGAAGCAATG and reverse primer GCCATCAAATCTTTCTGAAACTTGTT. The PCR was done by the following reagents: 12.5 µL of master mix (2×), 1 µL of mip forward and reverse primer (10 pmol/µL), 1 µL DNA template, and sterile deionized water in a final volume of 25 µL.

The cycling conditions were as follows: first cycle at 95°C for 5 min, followed by 34 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 30 s, and final extension step at 5 min for 72°C in a thermocycler. The sensitivity and specificity of the gold nano biosensor for *L. pneumophila* were compared with the PCR detection of the bacteria.

Specificity, sensitivity, and limits of detection of designed PCR and nanobiosensor. Bacteria that share a common ecological status with *Legionella*, including *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* PTCC 1919, and *Klebsiella pneumoniae* ATCC 13883 were used to determine specificity. DNA extraction of strains was performed using the Favor Prep Tissue Genomic DNA Extraction Mini Kit (Favorgene, Taiwan). About 3.5 ng of extracted DNA was used in all assays. The specificity and sensitivity of both methods for the detection of *L. pneumophila* in water samples were calculated based on the formula proposed by Parikh et al. (37). In sensitivity tests, the real-time PCR method was used as the gold standard. The lowest concentration detectable by each technique is considered the limit of detection (LOD). The LOD was assessed using a 10-fold dilution of plasmid pTZ57R / T / *mip* at a concentration of 35 ng. ~ 3.5 ng of plasmid was sided in water and serial dilutions were prepared. After extraction of DNA, LOD of biosensor and PCR were determined.

Statistical analysis. Each test was performed in independent triplicates and errors were presented as the mean standard deviation. LOD quantification was performed by drawing standard curves and linear regression using the Excel program.

RESULTS

Characterization of AuNPs. Spherical nanoparticles have a peak in the visible region. The presence of an absorption peak at 520 nm indicates the formation of a spherical gold nanoparticle (Fig.1a). In addition to spectroscopic examinations, the use of TEM confirmed the spherical morphology of the synthesized nanoparticles and the mean diameter of the GNPs was 32 nm. Size of the nanostructures was estimated by ImageJ Software (Fig.1b). Dynamic light scattering analysis showed that about 99% of AuNPs are in the range of 35 nm (Fig. 1c).

The hydrodynamic radius of nanoparticles is measured by dynamic light scattering analysis (DLS) and differs from the radius measured by TEM. Because, in DLS, the volume of solvent (opposite charge ion) which is located on the surface is also part of the particle's hydrodynamic radius. Therefore, the size obtained from the electron microscope is smaller than the DLS.

Optimization of ssDNA-GNPs nano-bioconjugates. Different concentrations of probes (1 to 200 nM) were combined with nanoparticles and the reduction of SPR bands was investigated. At a concentration of 1 nM of the probe, the SPR band showed the greatest decrease in intensity. ssDNA functionalized gold nanoparticle has been previously reported (38). Such optimization is very significant in the design process

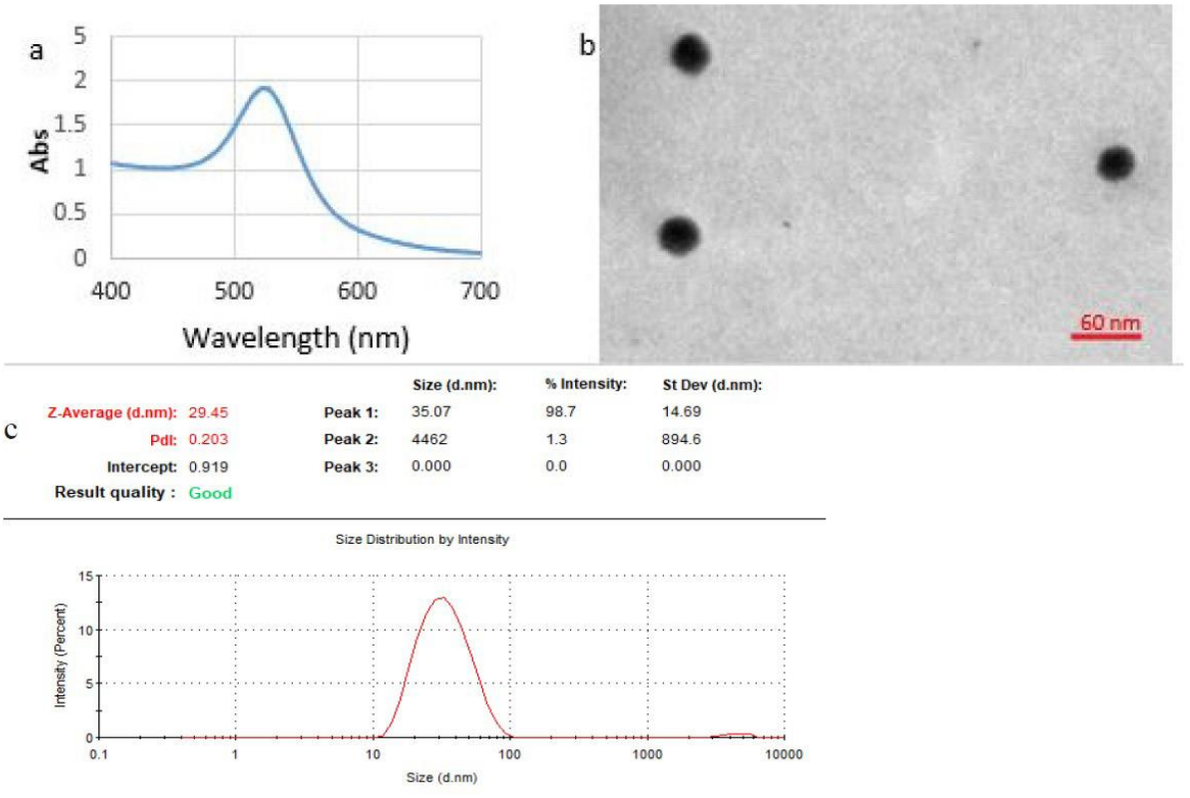

Fig. 1. a: Synthesized nanoparticles. a characteristic surface plasmon resonance bands of GNPs; b TEM microscope image of gold nanoparticles; c DLS analysis of nanoparticles

of surface plasmon resonance-based nano biosensors (39). In other words, after conjugation of the probe to the nanoparticles and forming nanoprobes, changes in the SPR spectrum were observed, including a decrease in the absorption spectrum and a shift to higher wavelengths due to a change in the refractive index of the environment around the nanoparticles (Fig. 3a). DLS results also showed an increase in the hydrodynamic diameter of nanoparticles conjugated to probes to 39 nm (Fig. 2).

The nanoprobes were centrifuged after the conjugation process and the concentration of the unbound ss-DNAs was measured to be ~ 1.4 pM. The concentration of the GNPs can also be calculated by Lambert–Beer law (33).

$A = \varepsilon bC$

Therefore, the ratio of the bound ss-DNAs/GNPs was calculated to be 255.

Hybridization of the biosensor with target DNA. After hybridization of the target DNA to the biosensor, reduction of the SPR band due to a change in the refractive index of the surface layer of GNPs (Fig. 3a) as well as a shift of the absorption peak curve towards higher wavelengths and also the aggregation of gold nanoparticles and discoloration of the solution to the purple was observed with increasing time (Fig. 3b).

The results were also similar regarding recombinant plasmid as the target and indicated the biosensor efficacy for the detection of *mip* gene of *L. pneumophila* in water samples (Fig. 4). Therefore, it seems that the current biosensor can be directly applied for the detection of *L. pneumophila* in the water samples. No reduction was observed in *Acinetobacter baumannii* DNA SPR bands as a negative control (Fig. 5). Conjugation of the nanoprobe with the target DNA by adding a hydrodynamic layer to the surface of the gold nanoparticles increased the average diameter of the GNPs to 60.7 nm (Fig. 6a), which actually confirmed the conjugation of the nanoprobe to the target DNA. Aggregation of nano biosensors after hybridization with target DNA was observed by transmission electron microscope and the size of conjugated nanoprobes with target DNA was determined to be ~ 50 nm (Fig. 6b).

			Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm):	33.19	Peak 1:	39.04	95.0	13.60
PdI:	0.318	Peak 2:	4411	5.0	915.5
Intercept:	0.924	Peak 3:	0.000	0.0	0.000
Result quality :	Refer to quality report				

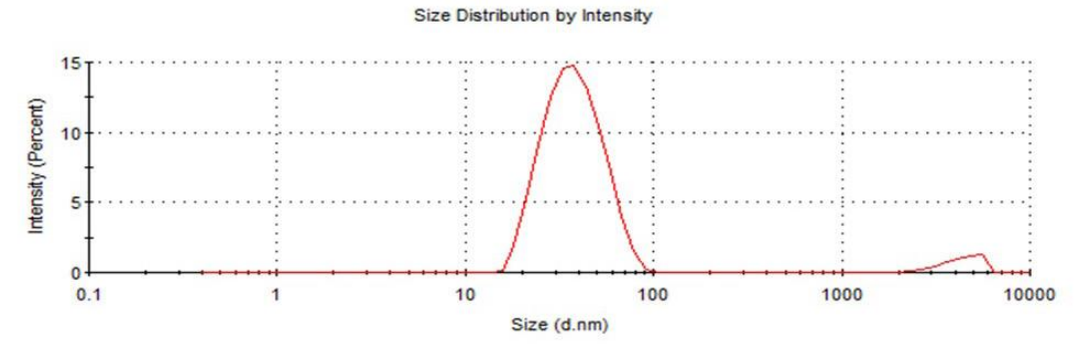

Fig. 2. A DLS analysis of nanoprobes

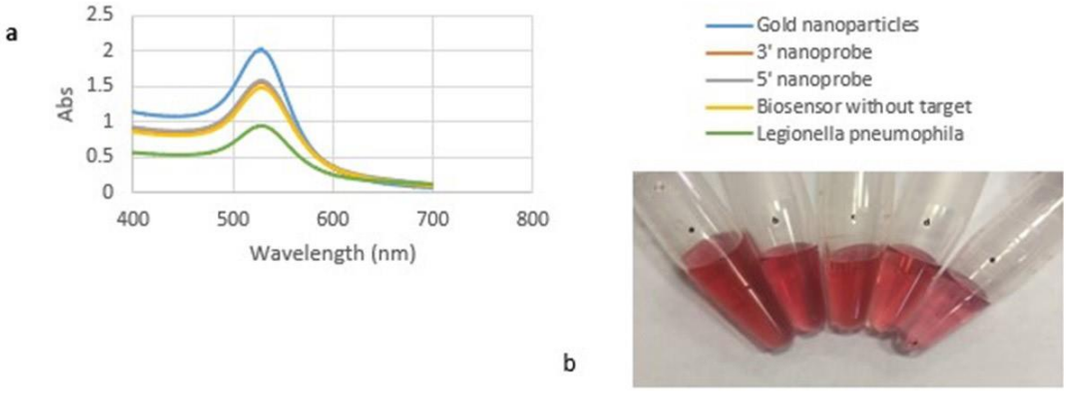

Fig. 3. a: Use of 1nM concentration and 5 µl volume of probe and proximity of biosensor to target; b Color change from red to purple in the presence of target DNA.a Gold nanoparticles; b: 3′nanoprobe; c 5′nanoprobe; d nano biosensor without target; e *L. pneumophila* ATCC 33152.

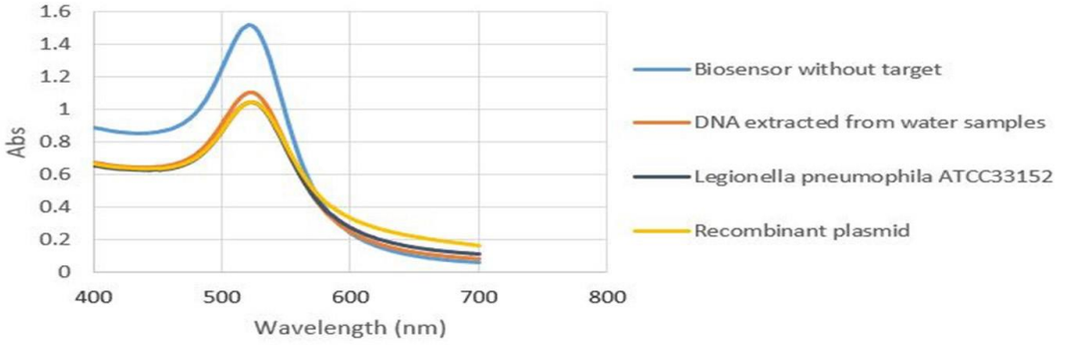

Fig. 4. SPR bands of the nano biosensor after hybridization with ss-DNA of standard strain, recombinant plasmid, and DNA extracted from water sample

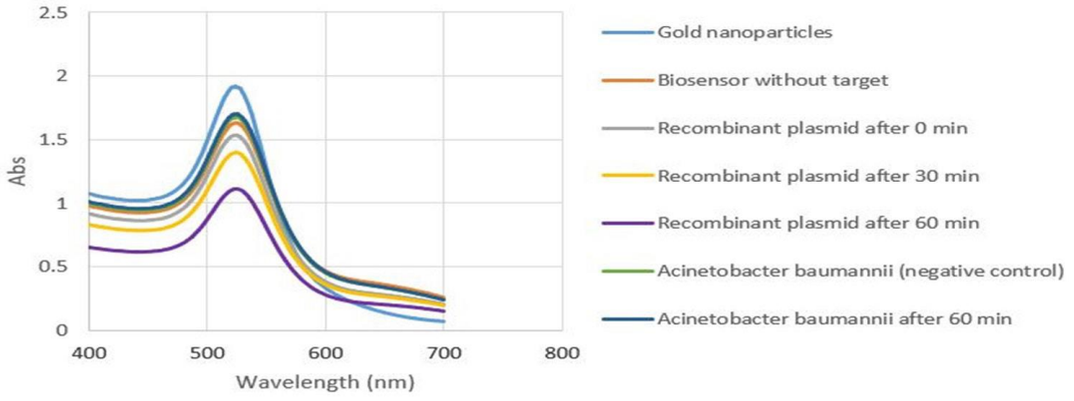

Fig. 5. Addition of recombinant plasmid as target to nanobiosensor.

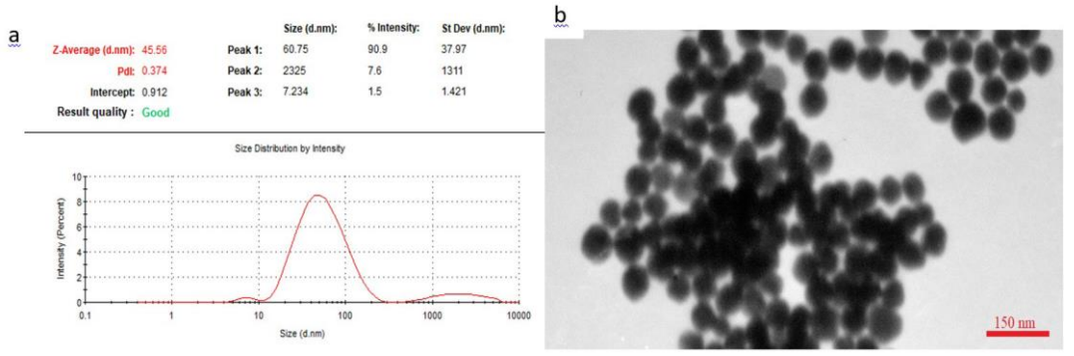

Fig. 6. A: DLS analysis of nanobiosensor; b: transmission electron microscopy image.

Limit of detection of designed gold nano biosensor. Serial dilutions of pTZ57R/T/*mip* plasmid stock were prepared and analyzed using a biosensor assay to determine the limit of detection. Although the biosensor was stable when the recombinant plasmid concentrations were 35×10^{-8} to 35×10^{-10} ng, the aggregation and change of color from red to purple of the gold nano-biosensor took place after adding 35×10^{-1} to 35×10^{-7} ng of DNA was done. Therefore, the 10^{-7} dilution (10^3 copy number/µl) was determined as the LOD of the biosensor.

The standard linear regression curve of the SPR position against the log copy number/reaction shows that there is a significant difference in the SPR intensity between 10^9 and 10^3 copies of the recombinant plasmid (Fig. 7). The decrease in SPR band intensity is observed in the presence of target DNA. The changes in the SPR position were linearly observed when the plasmid increased with a correlation coefficient of ~ 0.98. Considering the increased concentration of *L. pneumophila* in contaminated water samples, the biosensor can efficiently detect the target with a remarkable change in the SPR absorption peak.

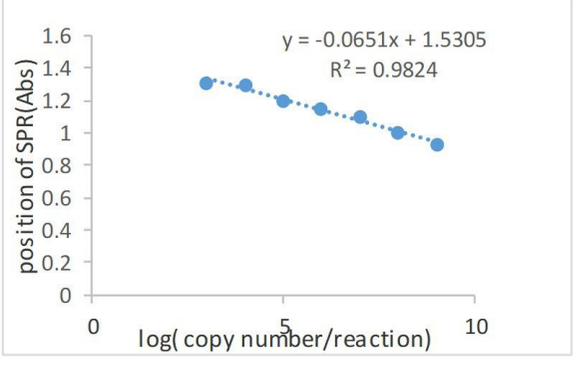

Fig. 7. Standard linear regression curve of log (copy number/reaction) against position of SPR

Limit of detection of PCR for detection of *L. pneumophila*. To evaluate the sensitivity of PCR, serial dilutions of recombinant plasmid with an initial concentration of 35 ng were used as the template, and

dilution of 10^{-6} (10^4 copy number/µl) was detected as LOD (Fig. 8).

Specificity of PCR for detecting *L. pneumophila*. Using the genomic DNA extracted from bacteria with a common ecological status with *L. pneumophila* including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, PCR was performed and no nonspecific bands were observed, indicating the high specificity of primers for the detection of *Legionella pneumophila* (Fig. 9).

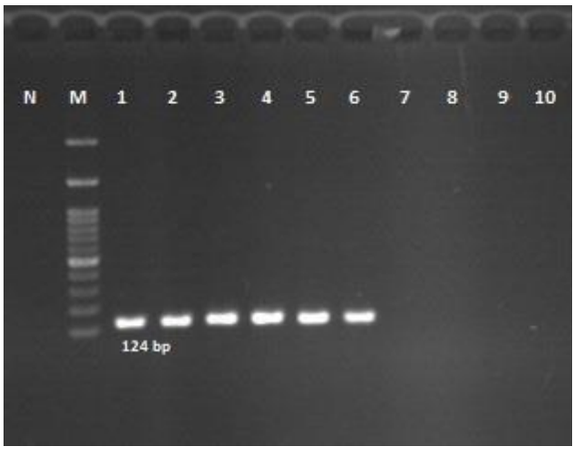

Fig. 8. LOD image of PCR products using recombinant plasmid
Lane M: DNA ladder,1 to 10: 10 serial dilutions of recombinant palmsid, N: Negative control.

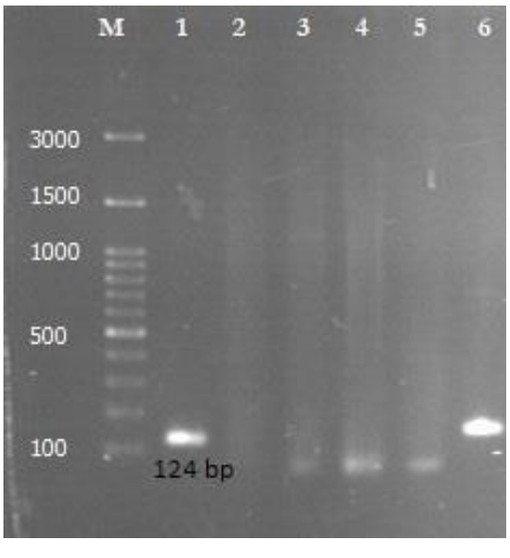

Fig. 9. Specificity of PCR to detect *L. pneumophila*. Lane M: DNA ladder, 1: *L. pneumophila*, 2: *P. aeruginosa* ATCC 27853, 3: *A. baumannii* PTCC 1919, 4: *K. pneumonia* ATCC 13883, 5: Negative control, 6: Positive control.

DISCUSSION

Previous studies reported that densities above 10^4-10^{10} *Legionella* CFU/L represent a potential increased threat to human health. Isolation of these bacteria from water sources is essential to identify outbreak origins and prevent disease (34). On the other hand, various methods based on nucleic acid amplification have already been proposed to identify environmental and clinical specimens. But these methods are expensive and time-consuming (40, 41).

Because of unpredictable outbreaks and economic losses to the country's health system, the design of a rapid, highly sensitive, and specific diagnostic system is a top priority for the evaluation and quality control of hospital waters. Because with the emergence of the diagnostic method and the widespread monitoring of hospital water we will be able to manage this bacterium and its resulting diseases. Hence, we developed a biosensor based on gold nanoparticles to identify the *Legionella pneumophila* genome in the water sample. In this study, gold nanoparticles were used owing to their prominent optical properties due to their deformation, size, and composition, as well as low toxicity and high biocompatibility. Citrate-stabilized Au-NPs were synthesized using the seeded growth method. The loosely bound citrate layers can be easily replaced with the thiolated probe. Furthermore, the presence of probes on the surface of Au-NPs increases the surface negative charge and leads to more stability of the colloidal nanoparticles (42). In different studies, the Aggregation of gold nanoparticles in the colorimetric method including cross-linking (CL) and non-cross linking (NCL) together has been compared, and the CL assay has shown higher sensitivity and specificity than the NCL method. In contrast, the NCL assay has shown a higher response speed compared to the CL method. However, both methods are cost-effective and easy to perform (20). In Nuthong's study, a DNA-AuNP probe was used to detect *L. pneumophila* DNA and the detection limit was 1.5×10^4 copy number/µl (43) which was lower than the LOD obtained in our study, which can be attributed to the use of the NCL method.

In our study, the CL method was employed for DNA detection. After mixing nanoprobes 3 and nanoprobes 5 and then adding the target, a polymer network was formed and changed the color of the solution from red to purple or blue. One of the advantages of this technique is to determine results

in a short period of time. The detection time for the biosensor can be considered to be about 30 minutes, which is a fast process compared to culture methods (about 10 days or more) and PCR (about 2 to 3 hours) (44).

In addition, the low cost of biosensors, the required equipment, and their use in places outside the laboratory can prove the advantage of this method over molecular methods. On the other hand, the biosensor is not an amplification-based method, so it is not affected by the inhibitor present in the sample like other molecular methods. Another advantage of the biosensor method is its visual evaluation. visual observation of positive and negative samples in the form of color changes from red to purple and the accumulation of nanoparticles in the form of black particles, which were consistent with the results of reading the samples using the UV-Visible device. Therefore, in the absence of a device, a biosensor can be used. LOD values for biosensors vary in published studies. In our study, the biosensor detection limit was 10^3 copy number/µl. While the study of Oh et al. LOD biosensor 10^7 copy number/µl in the diagnosis of *S. typhimurium* and in the study of Wang et al. 10^4 copy number/µl *E. coli* O157: H7 were reported (45, 46). In the case of PCR amplification, no specific band was revealed in negative samples; thus, the primers are highly specific for *L. pneumophila*. In addition to *Legionella*, functionalized gold nanoparticles with specific probes have been used to detect bacterial pathogens such as *Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium* sp. (29, 43, 47-49).

CONCLUSION

In summary, we have developed an optical nano biosensor based on gold nanoparticles to detect the *L. pneumophila mip* gene, with a detection level of 10^3 copy number/µl, which was less than PCR. This system can be used as a practical and accurate tool with rapid screening and acceptable sensitivity and specificity for the diagnosis of *Legionella pneumophila* in contaminated water. High specificity and acceptable sensitivity of biosensor compared to the PCR method, high speed, and easy use of this technique is suitable and efficient for screening and diagnosis of *Legionella pneumophila* and will be able to become a useful tool in laboratories.

ACKNOWLEDGEMENTS

We wish to thank the Research Council of Tarbiat Modares University for developing the project.

REFERENCES

1. Papian S, Mohabati Mobarez A, Khoramabadi N, Mehdi Abdol M, Talebi Bezmin Abadi A. Investigating the role of *L. pnuemophila* LPS derivatives in formation of specific cell-mediated immune responses against the pathogen. *Microb Pathog* 2020; 147: 104396.
2. Grúas C, Llambi S, Arruga MV. Detection of *Legionella* spp. and *Legionella pneumophila* in water samples of Spain by specific real-time PCR. *Arch Microbiol* 2014; 196: 63-71.
3. Spiegelman J, Pedutem T, Francisco MJ. Legionnaires' disease cases at a large community hospital—common and underdiagnosed. *Int J Environ Res Public Health* 2020; 17: 332.
4. Li J, Qin T, Jia XX, Deng AH, Zhang X, Fan WH, et al. Rapid identification of *Legionella* pathogenicity by surface-enhanced raman spectroscopy. *Biomed Environ Sci* 2015; 28: 437-444.
5. Den Boer JW, Yzerman EPF. Diagnosis of *Legionella* infection in Legionnaires' disease. *Eur J Clin Microbiol Infect Dis* 2004; 23: 871-878.
6. Moosavian M, Moradzadeh M, Ghadiri A, Saki M. Isolation and Identification of *Legionella* spp. in environmental water sources based on macrophage infectivity potentiator (*mip*) gene sequencing in southwest Iran. *AIMS Microbiol* 2019; 5: 223-231.
7. Yáñez MA, Carrasco-Serrano C, Barberá VM, Catalan V. Quantitative detection of *Legionella pneumophila* in water samples by immunomagnetic purification and real-time PCR amplification of the *dotA* gene. *Appl Environ Microbiol* 2005; 71: 3433-3441.
8. Joly P, Falconnet P-A, André J, Weill N, Reyrolle M, Vandenesch F, et al. Quantitative real-time *Legionella* PCR for environmental water samples: data interpretation. *Appl Environ Microbiol* 2006; 72: 2801-2808.
9. Niu C, Zhang Y, Zhang Y. Evaluation of a most probable number method for detection and quantification of *Legionella pneumophila*. *Pathogens* 2022; 11: 789.
10. Islam MA, Hassen WM, Ishika I, Tayabali AF, Dubowski JJ. Selective detection of *Legionella pneumophila* serogroup 1 and 5 with a digital photocorrosion biosensor using antimicrobial peptide-antibody sandwich strategy. *Biosensors (Basel)* 2022; 12: 105.
11. Kaminker R, Lahav M, Motiei L, Vartanian M, Popovitz-Biro R, Iron MA, et al. Molecular structure–func-

tion relations of the optical properties and dimensions of gold nanoparticle assemblies. *Angew Chem Int Ed Engl* 2010; 49: 1218-1221.
12. Padmavathy B, Vinoth Kumar R, Jaffar Ali BM. A direct detection of *Escherichia coli* genomic DNA using gold nanoprobes. *J Nanobiotechnology* 2012; 10: 8.
13. Wang J, Lin W, Cao E, Xu X, Liang W, Zhang X. Surface plasmon resonance sensors on Raman and fluorescence spectroscopy. *Sensors (Basel)* 2017; 17: 2719.
14. Rasooly A, Herold KE. Biosensors and Biodetection: Methods and protocols. Preface. *Methods Mol Biol* 2009; 503: v-ix. 10.1007/978-1-60327-567-5.
15. Ma X, Song S, Kim S, Kwon M-S, Lee H, Park W, et al. Single gold-bridged nanoprobes for identification of single point DNA mutations. *Nat Commun* 2019; 10: 836.
16. Bahavarnia F, Pashazadeh-Panahi P, Hasanzadeh M, Razmi N. DNA based biosensing of *Acinetobacter baumannii* using nanoparticles aggregation method. *Heliyon* 2020; 6(7): e04474.
17. Pavlov V, Xiao Y, Shlyahovsky B, Willner I. Aptamer-functionalized Au nanoparticles for the amplified optical detection of thrombin. *J Am Chem Soc* 2004; 126: 11768-11769.
18. Huang C-C, Huang Y-F, Cao Z, Tan W, Chang H-T. Aptamer-modified gold nanoparticles for colorimetric determination of platelet-derived growth factors and their receptors. *Anal Chem* 2005; 77: 5735-5741.
19. Liu J, Lu Y. A colorimetric lead biosensor using DNAzyme-directed assembly of gold nanoparticles. *J Am Chem Soc* 2003; 125: 6642-6643.
20. Liu J, Lu Y. Accelerated color change of gold nanoparticles assembled by DNAzymes for simple and fast colorimetric Pb2+ detection. *J Am Chem Soc* 2004; 126: 12298-12305.
21. Heidari Z, Rezatofighi SE, Rastegarzadeh S. Development and comparison of cross-linking and non-cross-linking probe-gold nanoparticle hybridization assays for direct detection of unamplified bovine viral diarrhea virus-RNA. *BMC Biotechnol* 2021; 21: 30.
22. Wang J, Wu ZL, Zhang HZ, Li YF, Huang CZ. Selective colorimetric analysis of spermine based on the cross-linking aggregation of gold nanoparticles chain assembly. *Talanta* 2017; 167: 193-200.
23. Hakimian F, Ghourchian H, Hashemi AS, Arastoo MR, Behnam Rad M. Ultrasensitive optical biosensor for detection of miRNA-155 using positively charged Au nanoparticles. *Sci Rep* 2018; 8: 2943.
24. Wang G, Akiyama Y, Shiraishi S, Kanayama N, Takarada T, Maeda M. Cross-linking versus non-cross-linking aggregation of gold nanoparticles induced by DNA hybridization: a comparison of the rapidity of solution color change. *Bioconjug Chem* 2017; 28: 270-277.
25. Wang Z, Ma L. Gold nanoparticle probes. *Coord Chem Rev* 2009; 253: 1607- 1618.
26. Sun Y, Harris NC, Kiang C-H. Phase transition and optical properties of DNA–Gold nanoparticle assemblies. *Plasmonics* 2007; 2: 193-199.
27. Pal D, Boby N, Kumar S, Kaur G, Ali SA, Reboud J, et al. Visual detection of Brucella in bovine biological samples using DNA-activated gold nanoparticles. *PLoS One* 2017; 12(7): e0180919.
28. Andreadou M, Liandris E, Gazouli M, Taka S, Antoniou M, Theodoropoulos G, et al. A novel non-amplification assay for the detection of *Leishmania* spp. in clinical samples using gold nanoparticles. *J Microbiol Methods* 2014; 96: 56-61.
29. Kalidasan K, Neo JL, Uttamchandani M. Direct visual detection of *Salmonella* genomic DNA using gold nanoparticles. *Mol Biosyst* 2013; 9: 618-621.
30. Chen DJ, Procop GW, Vogel S, Yen-Lieberman B, Richter SS. Utility of PCR, culture, and antigen detection methods for diagnosis of legionellosis. *J Clin Microbiol* 2015; 53: 3474-3477.
31. Bastús NG, Comenge J, Puntes V. Kinetically controlled seeded growth synthesis of citrate-stabilized gold nanoparticles of up to 200 nm: size focusing versus Ostwald ripening. *Langmuir* 2011; 27: 11098-11105.
32. Karimi S, Fouani MH, Moshaii A, Nikkhah M, Hosseinkhani S, Sheikhnejad R. Development of dual functional nucleic acid delivery nanosystem for DNA induced silencing of Bcl-2 oncogene. *Int J Nanomedicine* 2020; 15: 1693-1708.
33. Liu X, Atwater M, Wang J, Huo Q. Extinction coefficient of gold nanoparticles with different sizes and different capping ligands. *Colloids Surf B Biointerfaces* 2007; 58: 3-7.
34. Wilson DA, Yen-Lieberman B, Reischl U, Gordon SM, Procop GW. Detection of *Legionella pneumophila* by real-time PCR for the mip gene. *J Clin Microbiol* 2003; 41: 3327-3330.
35. Motohashi K. A novel series of high-efficiency vectors for TA cloning and blunt-end cloning of PCR products. *Sci Rep* 2019; 9: 6417.
36. Nguyen TTT, Voigt K, Santiago ALCMA, Kirk PM, Lee HB. Discovery of novel Backusella (Backusellaceae, Mucorales) isolated from invertebrates and toads in Cheongyang, Korea. *J Fungi (Basel)* 2021; 7: 513.
37. Parikh R, Mathai A, Parikh S, Chandra Sekhar G, Thomas R. Understanding and using sensitivity, specificity and predictive values. *Indian J Ophthalmol* 2008; 56: 45-50.
38. Caprara D, Ripanti F, Capocefalo A, Sarra A, Brasili F, Petrillo C, et al. DNA-functionalized gold nanoparticle assemblies for Surface Enhanced Raman Scattering. *Colloids Surf A Physicochem Eng Asp* 2020; 589: 124399.

39. Liang P, Canoura J, Yu H, Alkhamis O, Xiao Y. Dithiothreitol-regulated coverage of oligonucleotide-modified gold nanoparticles to achieve optimized biosensor performance. *ACS Appl Mater Interfaces* 2018; 10: 4233-4242.
40. Collins S, Jorgensen F, Willis C, Walker J. Real-time PCR to supplement gold-standard culture-based detection of *Legionella* in environmental samples. *J Appl Microbiol* 2015; 119: 1158-1169.
41. Yang G, Benson R, Pelish T, Brown E, Winchell JM, Fields B. Dual detection of *Legionella pneumophila* and *Legionella* species by real-time PCR targeting the 23S-5S rRNA gene spacer region. *Clin Microbiol Infect* 2010; 16: 255-261.
42. Li H, Nelson E, Pentland A, Van Buskirk J, Rothberg L. Assays based on differential adsorption of single-stranded and double-stranded DNA on unfunctionalized gold nanoparticles in a colloidal suspension. *Plasmonics* 2007; 2: 165-171.
43. Nuthong B, Wilailuckana C, Tavichakorntrakool R, Boonsiri P, Daduang S, Bunyaraksyotin G, et al. One step for *Legionella pneumophila* detection in environmental samples by DNA-gold nanoparticle probe. *J Appl Microbiol* 2018; 125: 1534-1540.
44. Saleh M, El-Matbouli M. Rapid detection of Cyprinid herpesvirus-3 (CyHV-3) using a gold nanoparticle-based hybridization assay. *J Virol Methods* 2015; 217: 50-54.
45. Oh SY, Heo NS, Shukla S, Cho HJ, Vilian ATE, Kim J, et al. Development of gold nanoparticle-aptamer-based LSPR sensing chips for the rapid detection of *Salmonella typhimurium* in pork meat. *Sci Rep* 2017; 7: 10130.
46. Wang Y, Alocilja EC. Gold nanoparticle-labeled biosensor for rapid and sensitive detection of bacterial pathogens. *J Biol Eng* 2015; 9: 16.
47. Suaifan GA, Alhogail S, Zourob M. Rapid and low-cost biosensor for the detection of *Staphylococcus aureus*. *Biosens Bioelectron* 2017; 90: 230-237.
48. Wen J, Liu J, Wu J, He D. Rapid measurement of waterborne bacterial viability based on difunctional gold nanoprobe. *RSC Adv* 2022; 12: 1675-1681.
49. Liandris E, Gazouli M, Andreadou M, Čomor M, Abazovic N, Sechi LA, et al. Direct detection of unamplified DNA from pathogenic mycobacteria using DNA-derivatized gold nanoparticles. *J Microbiol Methods* 2009; 78: 260-264.