



A central composite face-centered design for optimizing the detection of *Salmonella typhi* with a fluorescence nanobiosensor using the microcontact method

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Abstract

The rapid detection of *Salmonella typhi* can be accomplished by developing nanobiosensor kits. Accordingly, in the present study, a molecularly imprinted polymer (MIP) was developed, formed via covalent bonds among methacrylic acid (MAA) monomers and hydrogen bonds between MAA and antibodies. Using this MIP, *S. typhi* antigen was detected with a fluorescence converter. The *S. typhi* concentration could be determined at 10 colony/mL (minimum) in polluted water. A spectrofluorometer was used to quantify the findings. In addition, *Escherichia coli* was inseminated in an aqueous environment containing *S. typhi*, and no interference with the sensor function was found. The sensor sensitivity was measured for 60 days, and the sensor performance was verified for 56 days. Thereafter, the performance diminished. The present findings indicated the sensitive and precise role of the sensor in detecting *S. typhi* antigens at concentrations of 10^{-1} – 10^9 .

Keywords Nanosensor · Microcontact · *Salmonella typhi* · Spectrofluorometry

Introduction

Spoiled and unstable products can cause food-borne infections, toxicity, and morbidities via microorganisms such as *Salmonella* and are health threats to humans. In the USA, *Salmonella* is responsible for almost 76 million cases of food poisoning every year. Therefore, a rapid and accurate method should be designed to detect this bacterium (Marotta et al. 2014).

Salmonella typhi, the pathogen responsible for typhoid fever, can be transmitted through contaminated water in sewage or from one person to another. Most non-typhoid *Salmonella* are transferred through contaminated food products (Kumar et al. 2013). Therefore, compliance with food hygiene standards and principles can prevent food-borne *Salmonella* infections (Yoshida et al. 2013).

The detection limits and linear detection ranges were 10^{-8} – 10^{-2} and 2×10^{-8} , respectively. Furthermore, Kryscio and Peppas (2012) used an MIP film sensor to examine the melanin content in milk. The detection limits and linear detection ranges were 10^{-6} – 10^{-2} and 5×10^{-6} , respectively.

To measure chlortetracycline content in blood serum and urine, Lin et al. (2007) modified a sensor via molecular imprinting. The linear range was 10^{-3} , 4.93×10^{-5} colony/mL. In addition, Anvar et al. (2014) used a potentiometer with an MIP to detect *Staphylococcus aureus* exotoxin. The sensor could detect dilutions up to 10^{-3} colony/mL, and assessment for up to 60 days confirmed its sensitivity for 28 days. Javanbakht et al. (2008) used a modified carbon paste electrode with MIP to evaluate the level of cetirizine in blood serum. The linear range was indicated as 10^{-8} , 10^{-2} and 7×10^{-7} , respectively.

Biosensors play a vital role in detecting pathogens, infectious factors, and toxins. The importance of these sensors is

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further highlighted with an increase in the quantity of microbial pollutants in the environment. In this study, we aimed to determine the most hazardous environmental pollutants based on a biosensor simulation. The main valuable features of biosensors include their reuse capacity and compatibility with almost all macromolecule biomarkers. Accordingly, these systems can be efficient in environments with poor hygienic infrastructures (e.g., developing countries) or after natural disasters.

Materials and methods

The antiserum of *S. typhi* belongs to the category of IgG antibodies, which are Y-shaped with a molecular weight of 150 KD. IgG contains significant amounts of carboxyl and amino acid groups. Amino acids such as tryptophan, tyrosine, and phenylalanine are located at the end of protein and show fluorescent properties. Nevertheless, their fluorescence intensity is majorly reduced owing to the complicated structure of antibodies and conformation changes.

The antibody was detected with a fluorescence convertor and MIP. Methacrylic acid (MAA) polymers were synthesized with EGDMA, which was cross-linked on the surface of silica particles. The thickened amino acid dilution (50 mg/mL) and 5% sucrose dilution were sequentially absorbed on the surface of silica plates. After adding the monomer dilution, a poly(methyl methacrylic) slide was used to cover it. Then, it was polymerized with UV-generated free radicals. Finally upon completion of the reaction, a covalent bond was formed between the polymer particles and MAA monomers. Additionally, a hydrogen bond between amino acids and MAA resulted in selective absorption.

Subsequently, polymerization of the lens surfaces was carried out using a protein stamp and a polymer slide. The stamp was formed via physical surface absorption on a cover surface, using a protein dilution and drying with N₂ gas. After pipetting the monomer dilution on the protecting slide, the stamp was added. Finally, a thin polymer membrane was formed using a UV polymer with two-dimensional signs of the sample.

Sensor simulation

The MIP-based sensor was designed by preparing a thin MIP film, with fluorophore used as the fluorescence monomer in the cover glass. The sensor was developed in the following steps:

1. Preparing the surface for the support and cover glass.
2. Activating the support glass.
3. Incubating the cover glass in the antibody solution.
4. Preparing the MIP solution with a fluorescence monomer and MMA.
5. Adding allyloxy coumarin, GDMA network builder, and azobisisobutyronitrile (AIBN) initiator.
6. Pouring the MIP solution on the support glass and coating the cover glass with antibody, followed by UV polymerization (Kryscio and Peppas 2012).

To prepare the support glass, piranha solution containing H₂SO₄:H₂O₂ was used. The glasses were immersed in the solution, washed, and then dried. The support glass was smeared with saline derivatives before MIP film preparation. Additionally, 0.5 mL of 3-methoxypropyl silyl methacrylate was used in 1.5 mL of water, 20 mL of ethanol, and 72 mL of acetic acid. To dry the glasses, nitrogen gas was used.

For the preparation of the cover glass, sonication was performed in sodium dodecyl sulfate (SDS), ethanol, H₂SO₄ (5% w/w), propanol, and distilled water (30 min per stage); nitrogen gas was also used for drying. The glass was immersed for 2 h in an antibody solution at moderate temperature and was then dried twice with nitrogen gas.

MMA, 50% multiethylene glycol, EGDMA, and 10% multi-AIBN initiator were used in MIP solution. Then, 10–20 µL of 4-allyloxy coumarin was poured on the support glass. Subsequently, the support glass was stamped with the antibody-covered glass. Polymerization was performed in a UV cabin for 20 h at 350 nm. The glasses were then separated. These processes were repeated without an antibody to make the control (Lin et al. 2007).

Antibody removal

To remove the antibody, MIP film was rinsed with NaOH 0.6 and 5% w/w SDS solution for 30 min at 85 °C in 1 colony/mL of NaCl solution and distilled water. To verify complete antibody removal from the film surface, a spectrophotometer was used to analyze the washing solution's absorption at 280 nm.

The microbial culture was prepared using *S. typhi* strain, supplied by the Iranian Research Organization for Science & Technology (PTCC code, 1609). Afterward, it was transferred from the solid phase to brain–heart infusion (BHI) broth and stored for 1 day at 37 °C in an incubator to obtain a 0.5 McFarland dilution with 10⁸ colony/mL (as determined by a spectrophotometer).

Centrifugation of the liquid broth with the bacterial mass was performed for 9 min at 3300 rpm. Then, the upper liquid layer was discarded, and the bacterial pellet was rinsed with saline. The pellet was resuspended in saline in the Falcon tube, and centrifugation was repeated. This washing process was repeated three times. The washed bacteria were finally placed in a water bath for 20 min to ensure capsule



deactivation; failure to deactivate the bacteria in this way impedes the biosensor detection process (Foroshani et al. 2013).

Microbial dilutions

After extracting the content of Falcon tubes containing the bacteria, the following steps were followed:

1. The spectrophotometer was calibrated at 620 nm for reading. Distilled water was added to the cuvette and read against the blank set at 0 optical density (OD).
2. When the Falcon tube with the bacterium mass was vortexed and transferred to the cuvette, the set should be read at 0.08–0.1 at 600 nm. However, the tube used was thick, so it was read in a higher range. It was diluted to 0.09 for preparing 10^8 dilutions. Other dilutions were prepared accordingly.
3. Saline (20 mL) was poured into the Falcon tube (based on $C_1 \times V_1 = C_2 \times V_2$ formula). C_1 was 10^8 , and we determined the amount required to obtain 10^5 dilutions for the next tube. C_2 was 10^5 and had to be multiplied by 20 mL ($V_1 \times 10^8 = 10^5 \times 20$ $V_1 = 0.02$ mL = 20 μ L). First, 20 μ L of the Falcon tube content (20 mL serum) was collected. Afterward, 20 μ L was taken from the 10^8 dilution and poured into the next tube from which 20 μ L was collected to prepare 10^5 dilutions (Kumar et al. 2013).
4. To prepare a 10^3 dilution, the Falcon tube containing 10^5 dilution was first vortexed. Subsequently, 200 μ L was removed from the Falcon tube (10^3 dilution) and replaced with 200 μ L of 10^5 dilution; consequently, a 10^3 dilution was prepared.
5. For preparing a 10^1 dilution, the 10^3 dilution tube was vortexed, and the same process as in step 4 was followed. Then, the dilutions were ready to be placed on the nanosensor kit (Sankar et al. 2014).

Fluorescence intensity

The fluorescence distribution was examined on MIP and non-imprinted polymer (NIP) sensors with and without antibodies. Afterward, both sensors were incubated in an antibody solution (10 ppm) for 60 min at pH of 7.4. An increase in MIP intensity versus NIP was observed, based on the fluorescence spectrum distribution in the presence of analyte.

Sensor lifetime

The analysis of the electrode life of the sensor examined the duration of the sensor's sensitivity in detecting features based on MIPs bound to the bacteria at 0, 4, 8, 12, 16, 20,

24, 28, 32, 36, 40, 44, 48, 52, and 60 days after the production of the sensor (Ahari et al. 2014).

Particle size analyzer

The distribution of size and formation of capsules was evaluated using the particle size analyzer. The nanoparticles were added to ionized water, and 2 mL of the solution was added following calibration with ionized water. The results were presented with respect to the average diameter and volume of nanoparticles (0.1 diameter, 10% nanoparticles; 0.5 diameter, 50% nanoparticles; and 0.9 diameter, 10% nanoparticles). Additionally, a light microscope (Leica DHLB) was used to examine 10 samples using a Leica Qwin 550 image analysis system (Ahari et al. 2014).

Results and discussion

According to the electron microscopic images, the particle distribution was in the range of 1–10 μ m at 15 kV and 1.00 Kx, indicating an average MIP size of 20–45 nm and NIP particle size of 80–100 nm. Since the NIP and MIP samples were not completely dissolved in acetonitrile, dark matter had accumulated, as presented in the images (Figs. 1, 2). Moreover, pictures acquired by TEM are depicted in Fig. 3.

The lifetime analysis

The sensor's response to binding with antigens decreased after 52 days (Fig. 4).

Analysis of sensor sensitivity

As reported in Table 1, the antibody binding to MIP has a scattering intensity of 273 in the absence of antigen, whereas in the presence of 10^1 and 10^5 dilutions of antigen, fluorescence intensity reaches 325 and 448 nm, respectively. The fluorescence intensity peaked at 745 nm for the 10^8 dilution. Therefore, fluorescence scattering enhanced owing to an increase in concentration (Fig. 5).

As illustrated in the table, the increase in fluorescence intensity and surge in antigen concentration were linked, as the fluorescence intensity was 234 at 10^1 , 263 at 10^3 , 341 at 10^5 , and 658 at 10^8 . These findings are also indicated in the figures.

Sensor sensitivity and specificity

The sensor sensitivity ranged from 10^1 to 10^8 . In addition, *Escherichia coli* (PTCC2216) was mixed with *S. typhi* in aquatic environments; however, no changes were made in the kit performance. The results presented in Tables 1 and

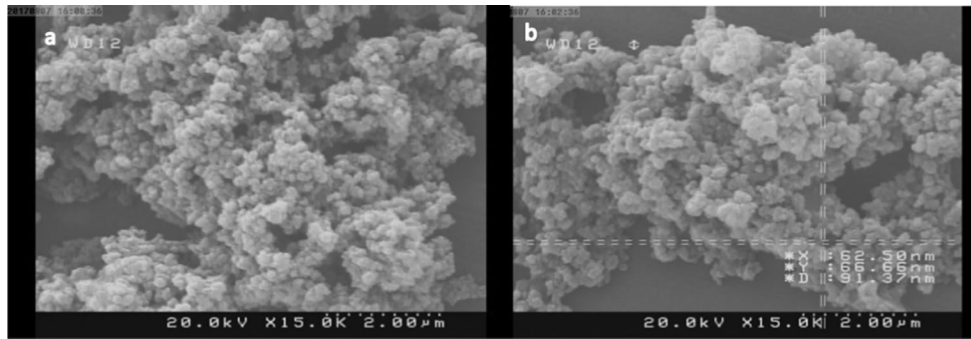


Fig. 1 MIP ($\times 15.0$ K magnification of particles with 1 mm diameter)

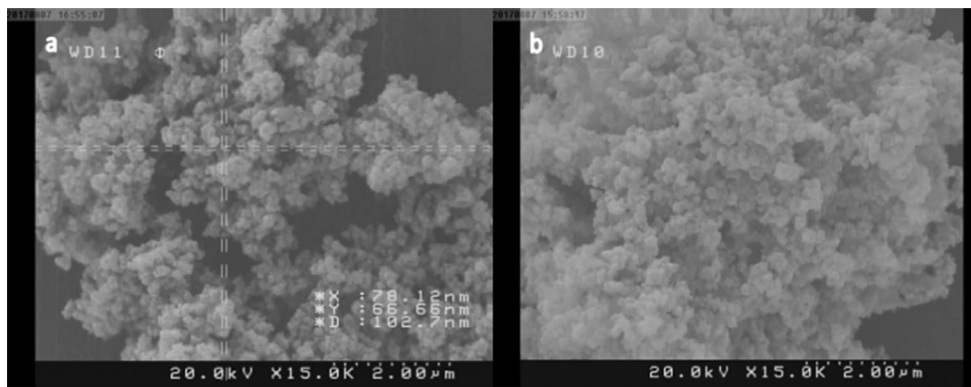


Fig. 2 NIP image acquired via electron microscopy ($\times 15.00$ K magnification; particle diameter, 10 mm)

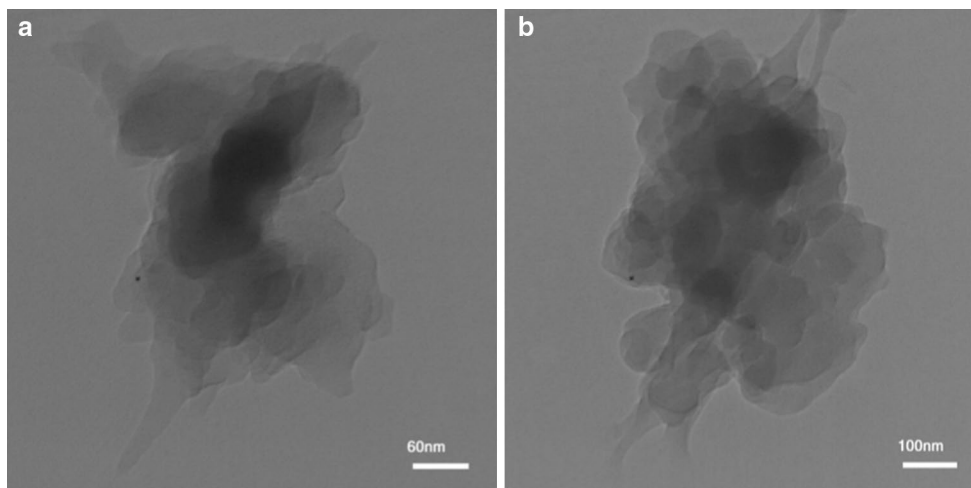


Fig. 3 MIP image acquired by TEM

2 indicate a generally increasing trend in the fluorescence intensity. In contrast, the decreasing trend presented in Table 2 is observed to day 52. Therefore, sensor sensitivity was lower than acceptable; in fact, after 52 days, the

detection results were no longer desirable. It should be noted that since the design of this kit and its introduction into the market, no other similar kits have been developed. Therefore, this kit cannot be verified yet.



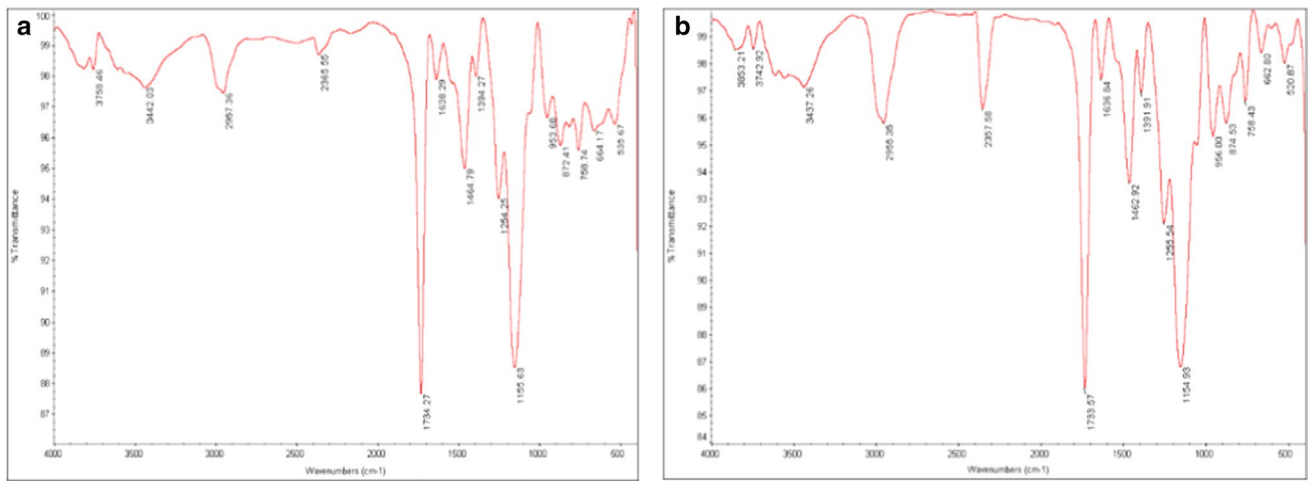


Fig. 4 FTIR picture of MIP (left) and NIP (right)

Table 1 Changes in sensor responses (fluorescence intensity) with respect to antigen dilution on the first day

Sample	Fluorescence intensity
Antibody binding to MIP in the absence of antigen	273
Binding in the presence of antigen 10 ¹	325
Binding in the presence of antigen 10 ³	350
Binding in the presence of antigen 10 ⁵	448
Binding in the presence of antigen 10 ⁸	745

Table 2 Sensor response changes to fluorescence intensity with respect to antigen dilution on day 52

Sample	Fluorescence intensity
Antibody binding to MIP in the absence of antigen	182
Binding in the presence of antigen 10 ¹	234
Binding in the presence of antigen 10 ³	263
Binding in the presence of antigen 10 ⁵	341
Binding in the presence of antigen 10 ⁸	658

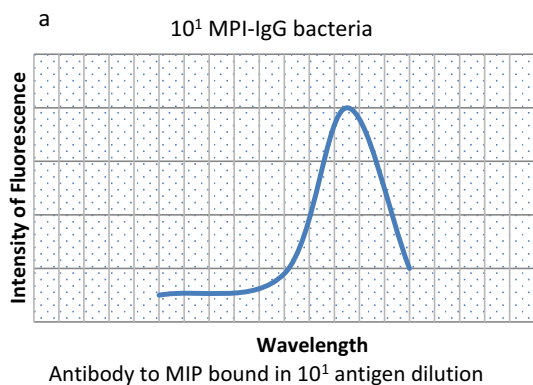


Fig. 5 Analysis of fluorescence intensity with respect to light absorption

Evaluation of fluorescence intensity reduction in the sensor

According to the results presented in Tables 3 and 4, a higher concentration of bacteria in the nanosensor is associated with a reduced fluorescence intensity percentage from day 1 to day 52; moreover, these results reveal the optimal

performance of the sensor. As most dilutions were above 10³ (risky dose) and approached 10⁸, the reduction in fluorescence intensity was less significant, which in turn indicates the proper function of the sensor.

The conventional use of culture media for detecting bacteria is labor-intensive and time-consuming. Sensitive and rapid detection methods of food-borne pathogens are of great significance today. If the concentration of pathogens is very low in food products, the applied method should be rapid and sensitive to ensure food safety. Generally, methods based on biochemical tests and culture studies (taking at least 4–7 days) are used to detect bacteria and toxins (Rani et al. 2014).

Overall, the use of biosensors or nanobiosensors can be helpful, considering the time required (2 days) to obtain initial results in microbial culture studies on food quality control. In fact, primary results can be obtained after 7–10 days in strains that require proenriching and enriching (e.g., *Salmonella*). Generally, in many food industries, the time required to reject or approve a product is quite long in research and development (R&D) and quality control (QC) sections. This, in turn, reduces the maintenance time and can impose great costs on the manufacturers

Table 3 Reduction in sensor sensitivity as determined by fluorescence intensity on the first day versus day 52

Sample	Percentage of reduction in fluorescence scattering (%)
Antibody binding to MIP in the absence of antigen	33.33
Antibody binding in the presence of antigen 10^1	28
Antibody binding in the presence of antigen 10^3	24.85
Antibody binding in the presence of antigen 10^5	23.88
Antibody binding in the presence of antigen 10^8	11.67

Table 4 Results from Design–Expert 8 (central composite face-centered design)

Std	Run	Factor 1 Concentration of bacterial cells (colony/mL)	Factor 2 B: Wavelength (nm)	Response 1 R1 Intensity of fluorescence
7	1	3.00	180.00	263
1	2	1.00	180.00	234
8	3	3.00	700.00	550
3	4	1.00	700.00	525
6	5	5.00	440.00	330
9	6	3.00	440.00	290
11	7	3.00	440.00	260
2	8	5.00	180.00	
12	9	3.00	440.00	250
13	10	3.00	440.00	175
5	11	1.00	440.00	235
10	12	3.00	440.00	258
4	13	5.00	700.00	30

indirectly. Time is a particularly important factor in QC systems and to obtain returns on investment for meat and dairy products, which are not similar to grains, cereal, oil, or canned foods (Meemken et al. 2014).

As depicted in Fig. 6, the capacity to detect the initial stages of infection at low antigen concentrations (below the infectious level) is an important feature of detection methods. The nanobiosensor designed in our study could detect antigens even below the infectious level. The online direct processing of the results in food QC systems is another advantage of this method, which can save time when assessing product quality. Overall, the features of this method include its low cost, high sensitivity, small size, broad frequency range, repeatability, linear function, and zero temperature quotient (Meemken et al. 2014).

Anvar et al. (2014) designed an MIP sensor for the detection of *S. aureus* exotoxin. The lifetime of the sensor was 52 days, and it could detect up to 10^{-4} dilutions. Moreover, the sensor could detect 10^1 – 10^8 colony/mL with the same lifetime in less time (Ahari et al. 2014).

Kryscio and Peppas (2012), using microcontact MIPs, designed a biosensor for detecting serum albumin in cow blood serum. In this design, it was not necessary to add protein to the monomer dilution before polymerization; this is consistent with the present method. Moreover, according to their results, renewability was introduced as a major feature. In addition, optimization of thin films against the sample proteins versus similar rival proteins caused high selectivity. The present results are in line with the mentioned findings.

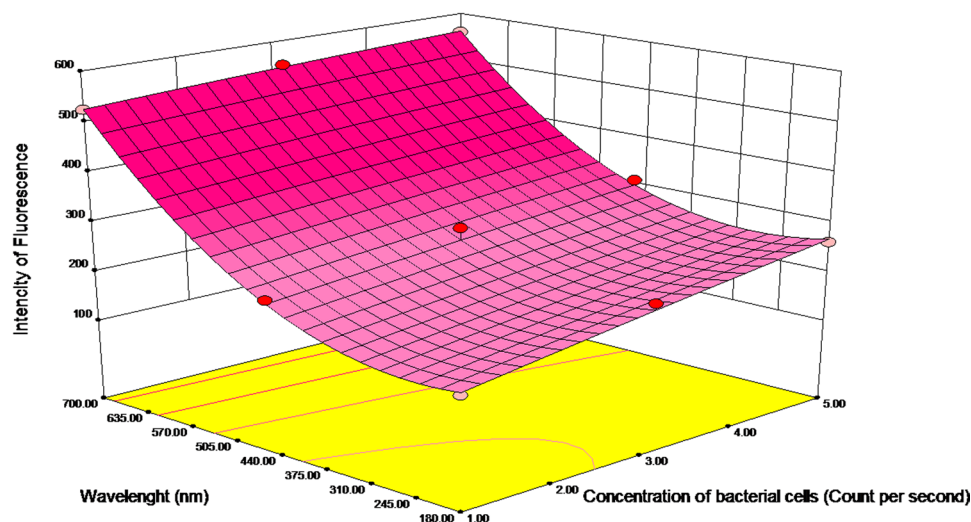
Kan et al. (2012) designed an efficient molecularly imprinted polymers (MIPs) based electrochemical sensor and prepared by electropolymerization of pyrrole in the presence of bovine hemoglobin (BHb) in an aqueous solution. The fabrication process of the sensor was characterized by differential pulse voltammetry and electrochemical impedance spectroscopy. The experimental results showed that the MIPs based sensor possessed a fast rebinding dynamics and an excellent recognition capacity to BHb. The current results were in Accordance to that of ours and application of MIP sensors could detect target cells (Kan et al. 2012).

Novel methods can be applied to produce imprinted proteins. The present method employed the microcontact imprinting method to improve efficiency via protein stabilization on the surface and the lack of a solvent. In traditional methods, a solvent consisting of the function molecule, pattern molecule, network builder, and initiator was used. Subsequently, a polymer was formed via alluvial or massive polymerization (Frimat et al. 2014).

In recent years, molecular imprinting has become increasingly popular as an effective separation technique for producing recognition elements that function as biological receptors. MIP can be applied for different functions in organic solvents and is appealing, considering the low cost of components in comparison with enzymes, mechanical resistance at high temperatures, long lifetime, and resistance in high- or low-acid environments. Moreover, one of the advantages of MIP is a polymer obtained from the opposite mixtures for antibodies.

Over the past two decades, MIP has attracted researchers' attention to the development of biological and chemical sensors (Moradi et al. 2014; Shin et al. 2012). MIPs are required for the design of chemical and biochemical sensors based on different photoelectric, electrochemical, and piezoelectric

Design-Expert® Software
 Factor Coding: Actual
 Intensity of Fluorescence
 ● Design points above predicted value
 ○ Design points below predicted value
 560
 156
 X1 = A: Concentration of bacterial cells
 X2 = B: Wavelength



Design-Expert® Software
 Intensity of Fluorescence

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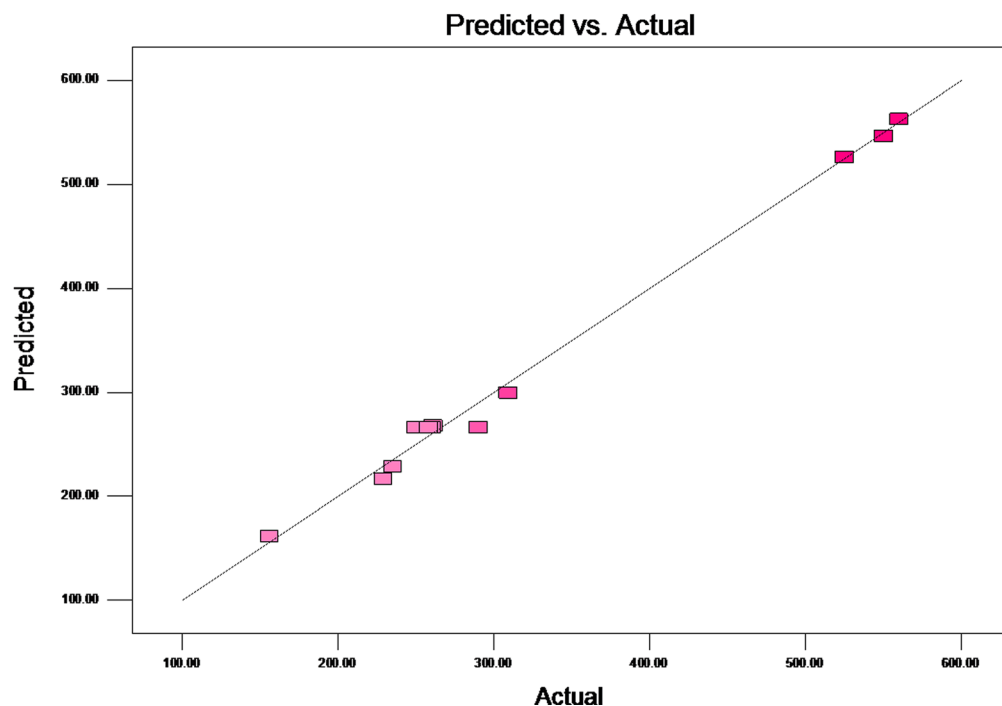


Fig. 6 Fluorescence intensity based on the 3D model of response surface and light absorption

characteristics. By applying MIPs in electrochemical methods, various voltmeter, transductometer, potentiometric, and amperometric sensors have been developed.

In the transductometer approach, a film with a molecular mold function was used between the electrolyte solvents. Piezoelectric crystals can be applied as molecular mold functions and a chemical interval in other transducers. In

our study, the molecular mold was prepared on the surface of piezoelectric crystal as a thin layer. Different types of sensors were used to bind MIP to various piezoelectric tools for analysis in liquid and gas phases.

Today, to improve sensor selectivity, optical sensors also use MIPs (e.g., fluorescence, low-luminescence, and infrared absorption-based sensors). In the present study,

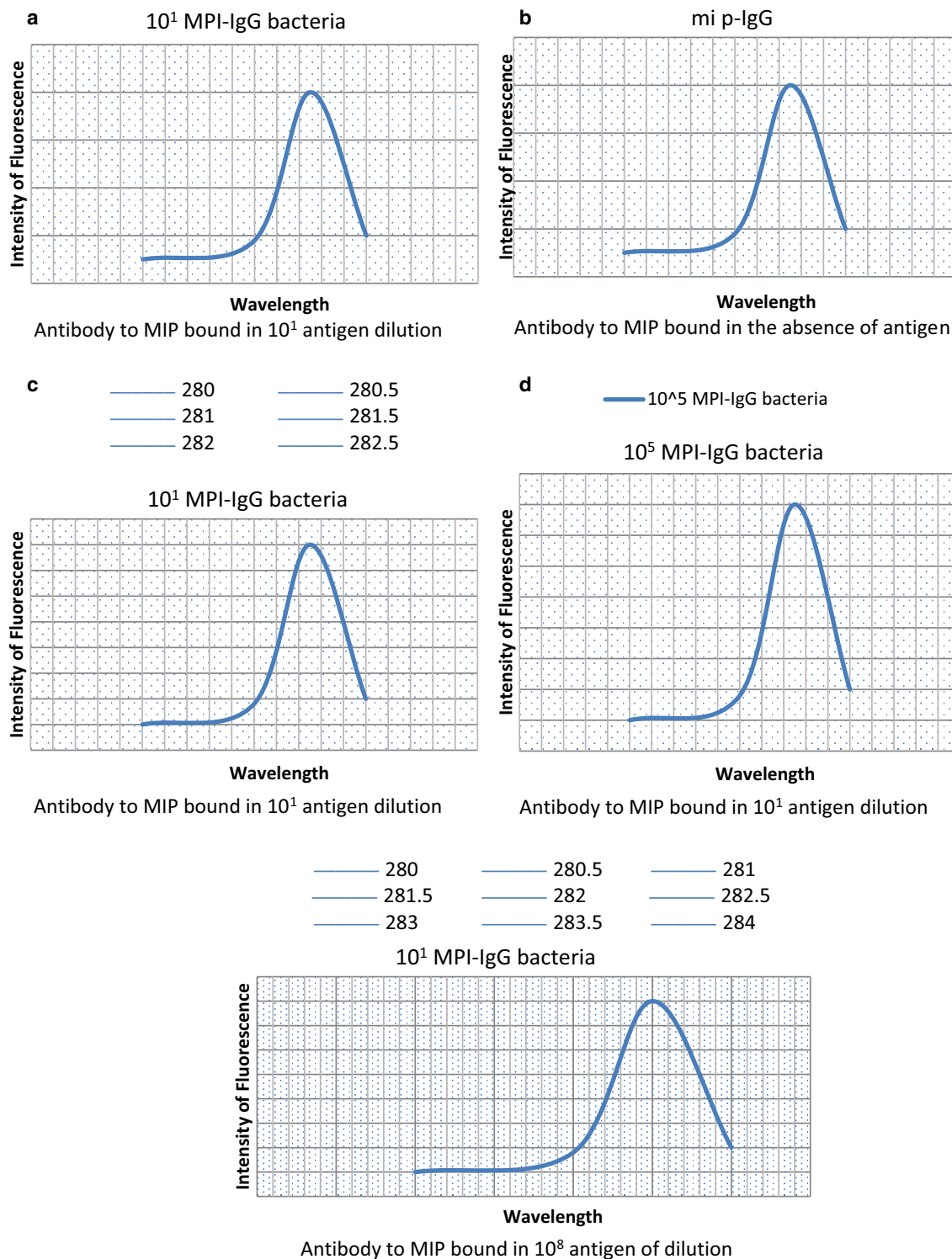


Fig. 7 Changes in the sensitivity of sensors

the fluorescence approach was applied, where a network builder or function monomer with fluorescence features was employed for MIP synthesis and targeting molecules with fluorescence features. In addition, optic sensors can

be designed based on MIPs (Frimat et al. 2014; Shin et al. 2012).

As presented in Tables 1 and 2, the changes in sensor responses on days 1 and 52 indicate that a higher dilution

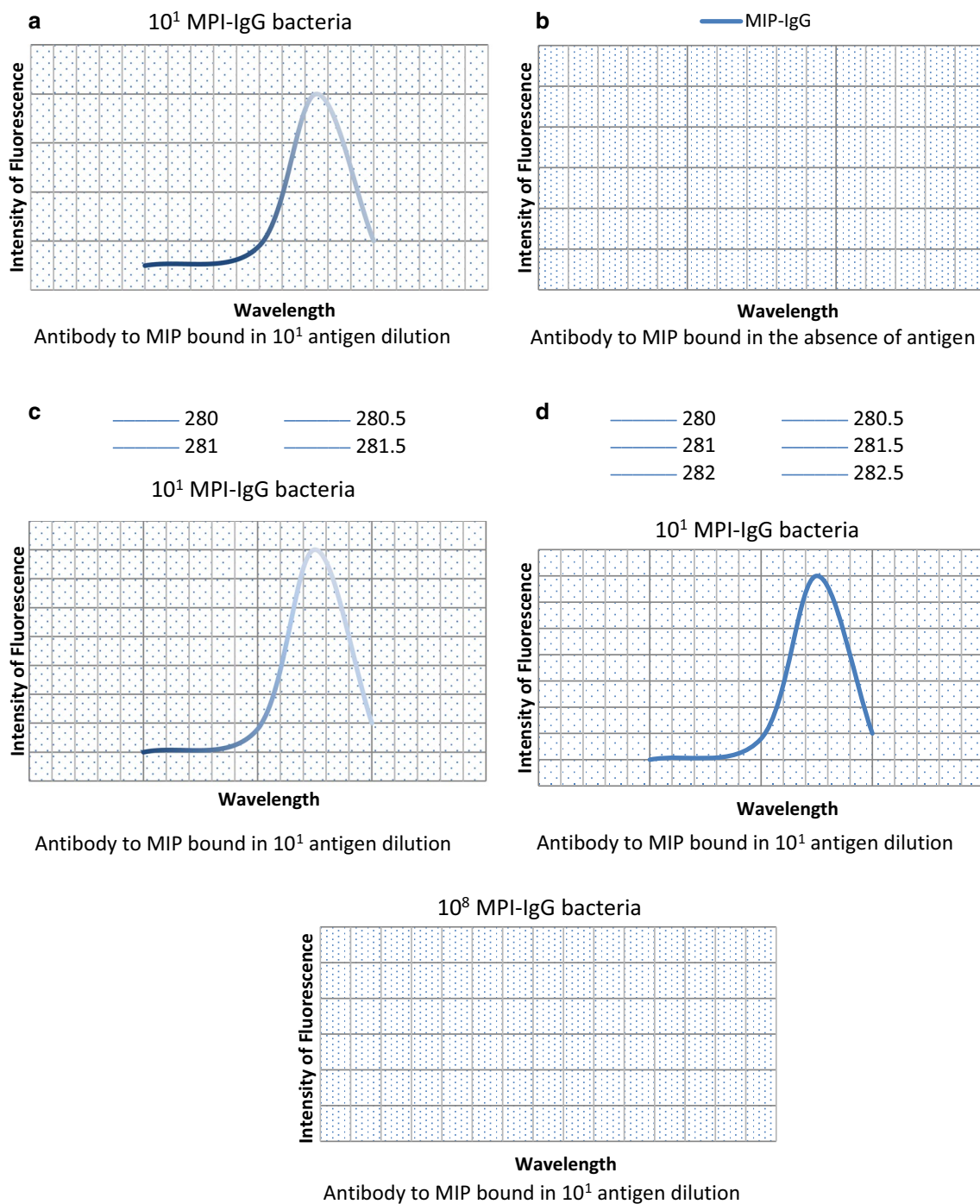


Fig. 8 Decreased intensity of fluorescence scatter from the sensor

of antigen is associated with a higher fluorescence intensity; the other conditions remained unchanged. Furthermore, according to the results presented in Table 2, a reduction in sensitivity was detected on day 52, but no decline was observed prior to that day. The following graphs confirm this finding (Fig. 7).

The following charts (Fig. 8) demonstrate a decreased intensity of fluorescence scatter from the sensor as antigen dilution increased; this actually boosts the fluorescence intensity.

Conclusion

To conclude, in 40 samples from aquatic environments containing different dilutions of *S. typhi*, 34 were examined via quantitative and qualitative approaches to assess their fluorescence intensity. The sensitivity of the designed nanobiosensor ranged from 10^1 to 10^8 mL.

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Compliance with ethical standards

Conflict of interests All authors declare that they have no conflict of interests.

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