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Molecularly imprinted nanofilms for endotoxin detection using an surface plasmon resonance sensor

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ABSTRACT

In this study, a simple, fast, sensitive and selective surface plasmon resonance (SPR) sensor has been prepared using molecular imprinting method for endotoxin detection. Endotoxin imprinted and non-imprinted poly (hydroxyethyl methacrylate-N-methacryloyl-(L)-histidine methyl ester) based nanofilms were synthesized on the SPR chip surfaces using ultraviolet polymerization. Endotoxin imprinted and non-imprinted SPR sensors were characterized by using contact angle, atomic force microscopy and ellipsometry. After characterization studies, kinetic studies were carried out in the concentration range of 0.5–100 ng/mL. The limit of detection and quantification were obtained as 0.023 and 0.078 ng/mL, respectively. The response time for the equilibration, adsorption and regeneration was approximately 14 min. The selectivity studies with cholesterol and hemoglobin of endotoxin imprinted SPR sensor were examined. Validation studies were carried out via limulus amebocyte lysate (LAL) in order to demonstrate the applicability of the SPR sensor.

1. Introduction

Endotoxins are member of the outer membrane of the cell wall of gram-negative bacteria and part of lipopolysaccharides (LPS) [1]. Endotoxin consist of large peptides weighing an average of 200–1000 kDa. Although endotoxins are tightly adherent to the cell walls of gram negative bacteria, they are continuously released to the environment [2]. Release of endotoxins not only takes place after cellular death, but also during cellular growth and division phases. Besides being mandatory for the survival of bacteria, endotoxins play a significant role in the protection of bacteria from cellular host defense systems, bile acids and hydrophobic antibiotics in the infected host during severe infections, trauma and shock states. Endotoxins do not directly affect cells or organs. They exert their effects through immune system, over particularly macrophage and monocyte activation [3].

Presence of endotoxins in the circulation during severe gram negative bacterial infections (particularly following antibiotic applications) or translocation of enterobacteria from the intestines causes various pathophysiologic reactions such as fever, leucopenia, tachycardia, tachypnea, hypotension, disseminated intravascular coagulation, multiple organ failure and septic shock [4–6]. Septic shock is a potentially fatal clinical condition if not diagnosed in the early phases and treated promptly, which may cause the collapse of the immune system [7]. Early treatment and rapid treatment of sepsis have a critical role in order to decrease death rates. Hence, early and accurate detection of endotoxin in the medical devices, injectable drugs, food and blood is of great importance. According to the world health organization, the endotoxin limit depends on the administration method of the product and specified in the monograph. In intravenous application, the suggested value per kg body weight is 5 IU endotoxin. In intravenous application for radiopharmaceuticals, the desired value per kg body weight is 2.5 IU endotoxin. Finally, in intrathecal application, the expected value per kg body weight is 0.2 IU endotoxin [8].

Clinically detection of endotoxin is performed by limulus amebocyte lysate (LAL) test which is developed in the 1960's [9]. In the following years some advanced techniques were introduced to improve the traditional LAL test; such as chromogenic [10], turbidimetric [11] and viscosity [12]. In addition, various commercial endotoxin detection kits have been developed. LAL tests are both expensive and complicated and they can also be affected from the proteases and other impurities which may be present in the environment. In order to overcome these problems, studies have been carried out in recent years to develop endotoxin sensors using biomolecules and artificial endotoxin probes [13,14].

Surface plasmon resonance (SPR) sensor attracted attention to

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alternative techniques by facilitating simple and fast analysis in the clinical applications [15,16]. SPR measures the variability of the refractive index which is formed on the surface of the metal film that carries surface plasmon. SPR sensors have a lot of advantages in the investigation of the detection of biomolecules, such as being real time and extremely sensitive, having a cheap and small recognition part, enabling measurement with very small amounts of sample [17,18].

Molecular imprinting method is an effective method to prepare the special and selective cavities for identification of target molecules in polymeric matrix [19]. The aim of this method is arrange the functional monomers with covalent and non-covalent interactions around a model molecule and to produce chemically functional polymeric material with a suitable process [20,21]. After removal of the model molecule, three dimensional specific spaces peculiar to the model molecule are formed in the structure. Molecular imprinted polymers are applied as alternative materials in various investigations as being cheap, showing resistance in high and low pH ranges, easier preparation, revealing high selectivity against target molecule and suitability for repeated use [22,23]. In this study, advantages of SPR sensor and molecular imprinting method are combined for real time detection of endotoxin.

In this study, endotoxin imprinted (MIP) and non-imprinted (NIP) nanofilm consisting of poly (hydroxyethyl methacrylate-N-methacryloyl-(L)-histidine methyl ester) (PHEMAH) based SPR sensors with high selectivity and sensitivity were prepared for real time detection of endotoxin. Endotoxin imprinted and non-imprinted SPR chip surfaces were characterized by contact angle measurements, ellipsometer and atomic force microscopy. Kinetic studies were performed by using aqueous endotoxin solutions with different concentrations. Adsorption isotherm parameters were calculated using Freundlich, Langmuir, Langmuir-Freundlich isotherm models. Moreover, kinetic studies are performed with cholesterol and hemoglobin solutions in order to identify the selectivity of endotoxin imprinted SPR sensors. Using endotoxin imprinted SPR sensors, kinetic analysis was performed from artificial plasma samples. Validation studies were carried out via limulus amebocyte lysate (LAL) in order to demonstrate the applicability of the SPR sensor.

2. Experimental studies

2.1. Chemical materials

2-hydroxyethyl methacrylate (HEMA), 2,2′-azobisisobutyronitrile (AIBN), ethylene glycole dimethacrylate (EGDMA) endotoxin use as a model molecule, cholesterol and hemoglobin used in the selectivity studies were purchased from Sigma Chemical Company (ABD). Other chemical materials used in the study were obtained from Merck AG (Darmstadt, Germany) in analytical purity.

2.2. Preparation of endotoxin imprinted and non-imprinted SPR sensors

2.2.1. Surface modification of gold surfaces with allyl mercaptan

Surface modification of SPR chip gold surface was performed using allyl mercaptan (CH_2CHCH_2S). The gold surface is immersed into 20 mL acidic piranha solution and rinsed after waiting for 20 s. The chips taken out of the solution were washed with pure ethyl alcohol and dried for 3 h in vacuum oven (200 mmHg, 40 °C). 5 μL allyl mercaptan was dripped over SPR chip surface and kept waiting for 12 h. In the last stage, SPR chips were washed with ethyl alcohol and dried in N_2 atmosphere. As a result of surface modification, allyl group bound gold plated SPR chip surfaces are obtained [18].

2.2.2. Preparation of endotoxin imprinted and non-imprinted SPR chips

First of all, endotoxin:MAH pre-complex was prepared using template molecule endotoxin and ligand monomer (MAH). The synthesis procedure of MAH functional monomer was reported in our previous study [24]. Stoichiometric complex ratio of endotoxin:MAH pre-complex was prepared in different molar ratios as 1:0.5, 1:1, 1:2 and 1:4 μM. The effect of increased ratio of functional monomer (MAH) over production of complex was investigated using 1240 mini SHIMADZU UV-1601 model spectrophotometry (Tokyo, Japan). The absorbance of endotoxin:MAH pre-complex was measured in 200–700 nm wavelength. As shown in Fig. S1, absorption of endotoxin:MAH is increased with the increased MAH mole ratio. Maximum adsorption mole ratio was observed in 1:2 μM of endotoxin:MAH. Hence, in the synthesis of endotoxin imprinted nanofilm, endotoxin:MAH pre-complex was used in 1:2 μM.

Endotoxin imprinted SPR sensors are produced from polymerization endotoxin:MAH pre-complex with 2-hydroxyethylmethacrylate (HEMA) as functional monomer, ethylene glycol dimethacrylate (EGDMA) and 2,2-azobisisobutyronitrile (AIBN) as cross linker. Endotoxin imprinted nanofilm was produced using endotoxin:MAH pre-complex containing stock monomer solution (210 μL), HEMA (6 μL), EGDMA (18.8 μL) and initiator AIBN (4.0 mg). 5 μL of this polymer solution is dropped over the surface of SPR chip modified with allyl mercaptan and UV light was used to initiate polymerization in 24 °C (100 W, 365 nm). The prepared SPR chip was placed under UV light and polymerized by 40 min photopolymerization reaction. Endotoxin non-imprinted SPR sensors are prepared using the same procedure except for adding endotoxin into the polymer solution. Ethyl alcohol was used to remove the unreacted monomers. Schematic illustration of the preparation of endotoxin imprinted SPR sensor is shown in Fig. 1.

2.3. Characterization of SPR sensors

Characterization of unmodified, endotoxin imprinted and non-imprinted SPR chips was used with contact angle, atomic force microscopy and ellipsometry analysis.

Contact angle measurements was calculated using KRÜSS DSA100 (Hamburg, Germany) device. To determine the hydrophobicity of SPR chip surfaces, contact angle was identified by dropping one drop of water over SPR chip surface by sessile drop method. Ten photographs were obtained after dripping water to different sites of gold surfaces and was calculated a different contact angle. The mean of 10 contact angle values obtained from unmodified, endotoxin imprinted and non-imprinted SPR chip surfaces were calculated [25].

Characterization of SPR chip surfaces were obtained using atomic force microscopy (AFM) in the semi-contact mode (Nanomagnetics Instruments, Oxford, England). SPR chip was placed into the sample holder with double face carbon tape. Imaging studies were performed in aerial media and in the semi-contact mode. Vibration amplitude was 1 VRMS and empty vibration amplitude was 2 VRMS. The samples of 1 × 1 μm area were taken in the 1 μm/s scanning speed and 256 × 256 pixel resolution [26].

Thickness measurements of the nanofilm on the SPR chip surface was characterized using EP3-Nulling Ellipsometer (Göttingen, Germany). SPR chip is placed under laser light source, thickness measurements are performed in 532 nm wavelength and 62° angle of incidence. The measurements were repeated 3 times in 6 different sites of SPR chip surface. The results are reported as the mean of these values [17].

2.4. Kinetic analysis

Real time detection of endotoxin from the aqueous solution was performed using endotoxin imprinted and non-imprinted SPR sensors. Firstly, endotoxin imprinted SPR sensors were balanced with 0.1 M phosphate buffer (pH 7.4). Kinetic studies were performed with 0.5–100 ng/mL in endotoxin aqueous solutions prepared with pH 7.0 phosphate buffer. % change in reflectivity (%ΔR) of SPR sensor was determined using surface plasmon curves by kinetic analysis. SPRview software (GWC Technologies, WI, USA) of the kinetic imaging program was used for kinetic analysis studies. Removal of endotoxin bounded on the SPR sensor sensor surface was used with in 0.1 M NaCI as desorption agent

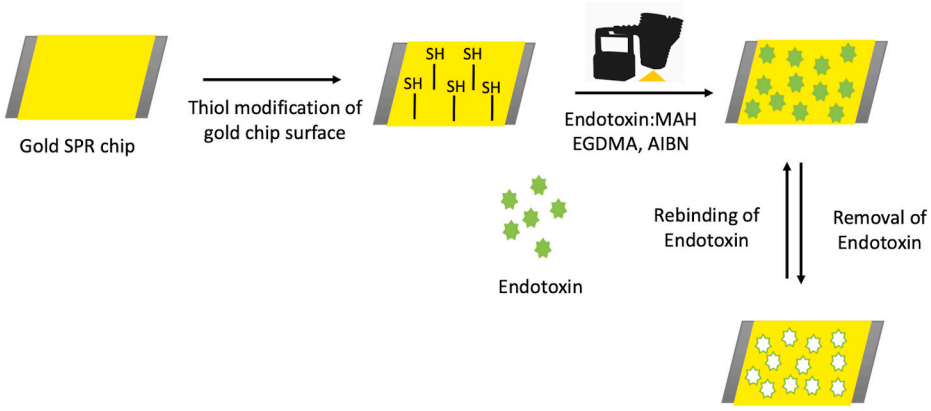


Fig. 1. Schematic illustration of the preparation of endotoxin imprinted PHEMAH based SPR chips.

for 10 min. After the desorption step, endotoxin imprinted SPR sensor was cleaned with deionized water and pH 7.4 phosphate buffer. The equilibration-adsorption-regeneration cycles were performed for all kinetic studies. In order to detect the adsorption isotherm parameters, SPR sensors were examined by using Langmuir, Freundlich, and Langmuir-Freundlich isotherm models.

The linearized forms of equations for SPR sensors are given as follows;

$$\text{Langmuir } \Delta R = \Delta R_{max}\, C/\, K_D + C \quad (1)$$

$$\text{Freundlich } \Delta R = \Delta R_{max}\, C^{1/n} \quad (2)$$

$$\text{Langmuir–Freundlich } \Delta R = \Delta R_{max}\, C^{1/n}/\, K_D + C^{1/n} \quad (3)$$

ΔR is the resonance frequency change. Concentration of endotoxin is C (nM).. K_A (1/nM) and K_D(nM) are the association and dissociation equilibrium constants, respectively. Freundlich exponent is described with 1/n. The equilibrium, maximum and experimental subscripts refer eq, max, and ex, respectively [27].

To investigate the repeatability of endotoxin imprinted SPR sensors in the same day, endotoxin solutions prepared in 50 ng/mL concentration are given 5 times to the SPR system. Moreover, repeatability kinetic analysis was performed at different days in order to identify the shelf lives of SPR sensors. Endotoxin solution prepared with 50 ng/mL endotoxin concentration is given to SPR system, sensorgrams are obtained and productivity of the sensors are identified.

2.5. Selectivity studies

In order to reveal the selectivity of endotoxin imprinted SPR sensors, endotoxin non-imprinted SPR sensors are prepared. To determine the selectivity of endotoxin imprinted and non-imprinted SPR sensors were used as competitive molecules cholesterol and hemoglobin, which resembles molecular weights and structures to endotoxin. For selectivity studies, endotoxin imprinted and non-imprinted SPR sensors was determined by applying at 50 ng/mL of each competitive molecules. The selectivity coefficients and relative selectivity coefficients were calculated for endotoxin imprinted (MIP) and non-imprinted (NIP) SPR sensors. The selectivity and relative selectivity coefficients are k and k', respectively. The selectivity coefficient (k) is described by the following equations:

$$k = \Delta R_{template} / \Delta R_{competitor} \quad (4)$$

$$k' = k_{MIP} / k_{NIP} \quad (5)$$

2.6. Clinical analysis

To determine the reliability and selectivity of endotoxin imprinted SPR sensor, clinical analysis was performed with artificial plasma. For that purpose, artificial plasma sample was diluted in with 10 mM phosphate buffer (pH 7.4) in the ratio of 1/10 (v/v). 10 and 25 ng/mL endotoxin aqueous solutions are spiked to 1/10 diluted artificial plasma solution. In order to dilute the samples, an isotonic solution (%1.0 NaCl solution) was applied. Firstly, endotoxin imprinted SPR sensor was equilibrated with 10 mM phosphate buffer (pH 7.4) for kinetic analysis. The spiked endotoxin artificial plasma samples were applied to the SPR system. The removal of endotoxin molecules on the endotoxin imprinted SPR sensors was carried out with 0.1 M NaCI solution. The kinetic data obtained from the SPR system were analyzed using SPR101 software. Taking LAL test as reference, the high accuracy and determination of endotoxin imprinted SPR sensor is determined.

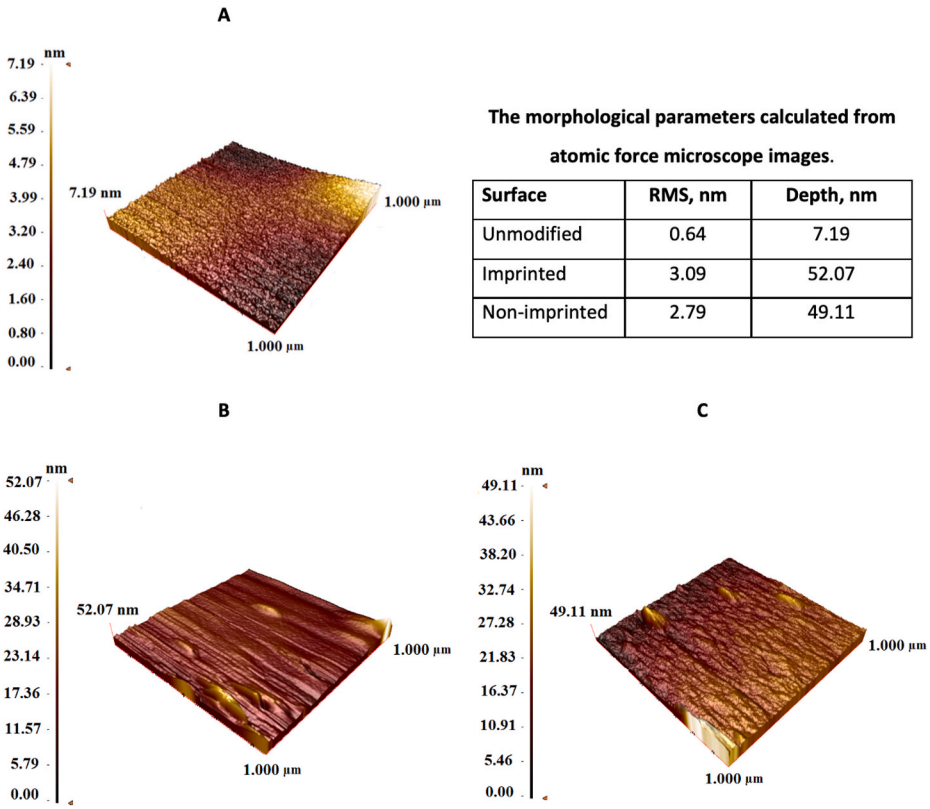
3. Results and discussion

3.1. Characterization studies

The surface morphology of unmodified, endotoxin imprinted and non-imprinted SPR sensors were evaluated with AFM device in the tapping mode. AFM images of SPR chip surfaces are shown in Fig. 2. The surface thickness values of SPR chip surface were 7.19, 52.07 and 49.11 nm for unmodified, endotoxin imprinted and non-imprinted SPR chip surface, respectively (Fig. 2A, 2B and 2C). Root mean square (RMS) values of the gold surface were also determined. RMS value of the unmodified gold SPR chip surface were determined as 0.64 nm. After the polymerization process, RMS values of endotoxin imprinted and non-imprinted SPR chip surfaces increased to 3.09 nm and 2.79 nm, respectively. AFM images indicate that nanofilm was homogenously created on the SPR chip surfaces.

To measure the nanofilm thickness of endotoxin imprinted and non-imprinted SPR sensors, EP3-Nulling Ellipsometer (Göttingen, Germany) is used. Ellipsometric measurements are performed in 658 nm wavelength and 65° angle of incidence. The nanofilm thickness of SPR chip surfaces were obtained as 51.4 and 46.4 for endotoxin imprinted and non-imprinted SPR chip surfaces, respectively (Fig. 3A1 and 3A2). All characterization results showed that SPR chip surfaces were synthesized nanofilm homogeneously.

Contact angle values of unmodified, endotoxin imprinted and non-imprinted SPR chip surfaces were obtained as 75.1° ± 0.69, 69.8° ± 1.42 and 64.8° ± 1.76, respectively (Fig. 3B1, 3B2 and 3B3). The observed decrease in contact angles depended on the increase of hydrophilic property of nanofilm on the SPR chip surfaces. The increase of hydrophilic of SPR chip surface is expected due to the hydrophilic functional groups of MAH monomer.



The morphological parameters calculated from atomic force microscope images.

Surface	RMS, nm	Depth, nm
Unmodified	0.64	7.19
Imprinted	3.09	52.07
Non-imprinted	2.79	49.11

Fig. 2. AFM images of the SPR chip surfaces in the surface morphology (A: unmodified, B: endotoxin imprinted and C: non-imprinted SPR chip surfaces).

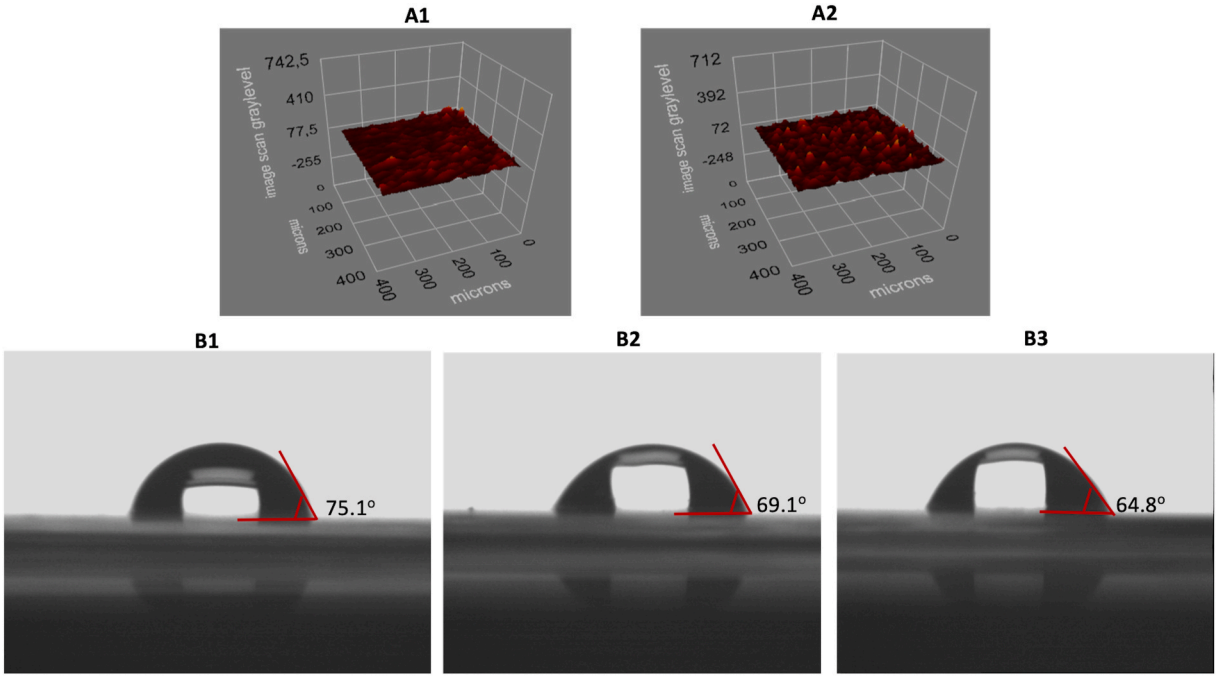


Fig. 3. Morphology of SPR chip surfaces (Elipsometry images; A1: endotoxin imprinted SPR chip, A2: non-imprinted SPR chip and contact angles; B1: unmodified, B2: endotoxin imprinted SPR chip, B3: non-imprinted SPR chip). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Kinetic analysis

SPR sensors are prepared by endotoxin imprinted and non-imprinted nanofilm synthesis for the detection of endotoxin from aqueous solutions and artificial plasma samples. Firstly, endotoxin imprinted SPR sensor was stabilized with pH 7.4 phosphate buffer as equilibration buffer. After, endotoxin imprinted SPR sensor was performed with aqueous endotoxin solutions in different concentration of 0.5–100 ng/

mL prepared with pH 7.0 phosphate buffer (Fig. 4A)With the interaction of endotoxin solutions and endotoxin imprinted SPR sensor, kinetic data are calculated using SPRview software. The figure shows % refractive index (%ΔR) vs time graphics of different concentrations of endotoxin solutions analyzed by SPR sensor. The removal of endotoxin molecules on the endotoxin imprinted SPR sensor was carried out with 0.1 M NaCI solution. In all measurements, these cycles for kinetic analysis were realized in 14 min for endotoxin detection.

In the interaction of endotoxin imprinted SPR sensor and endotoxin solution, %ΔR values are also increased with the increased endotoxin concentrations. The linear equation in the linear range of 0.5–100 ng/mL concentration is $y = 0.0604x + 0.1456$ with a coefficient of 0.984. Using the kinetic data of endotoxin imprinted SPR sensor, the limit of detection (LOD = 3.3 S/m) and the limit of quantification (LOQ = 10 S/m) were calculated from the slope of the calibration curve of endotoxin molecules. The standard deviation of the intercept is S and the slope of the regression line is m [16]. The LOD and LOQ for endotoxin were 0.023 ng/mL and 0.078 ng/mL, respectively. The comparative sensor studies in the literature on the determination of endotoxin are given in Table 1.

To determine the homogeneity of endotoxin imprinted SPR sensor, Langmuir, Freundlich, and Langmuir-Freundlich isotherm models can be applied to the adsorption isotherm equations. For endotoxin imprinted SPR sensor, the linearity of the Langmuir isotherm model was better than that of Freundlich and Langmuir–Freundlich isotherm models (Fig. S2). The calculated parameters for all isotherms are given in Table S1. The Langmuir model with ΔR_{max} equals 4.368 is found to be the most appropriate model to identify the interaction between endotoxin molecules and endotoxin imprinted SPR sensor. These result shows that the endotoxin binding properties on the endotoxin imprinted SPR sensor surface is monolayer, equivalent energy and lowest lateral interaction and homogenously distributed.

3.3. Selectivity studies

In order to detect the selectivity of endotoxin imprinted SPR sensor against endotoxin (ET), cholesterol (CHO) and hemoglobin (Hb) solutions were used as competitive molecules. These competitive molecules, which are close to endotoxin in terms of both molecular weight and structural are used in the selective studies. For selectivity studies, endotoxin with endotoxin imprinted and non-imprinted SPR sensors were kept constant as 50 ng/mL of each competitive molecules. As the results showed that the responses of SPR sensors for cholesterol and hemoglobin were lower than endotoxin in Fig. 5. The relative selectivity coefficients (k′) of endotoxin imprinted SPR sensor for endotoxin/cholesterol and endotoxin/hemoglobin were 5.73 and 1.08 times, respectively (Table 2). It is observed that selectivity for endotoxin is higher than that of other competitive molecules.

Table 1
Comparison of the most sensitive platform technologies for real time detection of endotoxin.

Method	Linear Range	LOD	Ref
Optical sensor	15.6–500 ng/mL	15.6 ng/mL	[28]
Nanobiocomposite sandwich immunosensor	10^{-5}-10^{5} ng/mL	4×10^{-6} ng/mL	[29]
Aptasensor	0.001–10 ng/mL	29×10^{-5} ng/mL	[30]
Electrochemical Impedance Biosensor	1×10^{-5}-1.0 ng/mL	2×10^{-6} ng/mL	[31]
Electrochemical sensing	–	0.0001 ng/mL	[32]
Biosensor	0–100 ng/mL	3.53 ng/mL	[33]
Magnetoelastic sensors	–	1.05×10^{-2} ng/mL	[34]
Electrochemical sensor	0.01–3 ng/mL	3.5×10^{-3} ng/mL	[35]
Electrochemical sensor	0.0001–0.1 ng/mL	0.0001 ng/mL	[36]
Electrochemical sensor	0.0015–750 ng/mL	6.1×10^{-4} ng/mL	[37]
Electrochemical aptasensor	5×10^{-5} - 0.01 ng/mL	$3.3\ 10^{-3}$ ng/mL	[38]
SPR biosensor	–	32.5 ng/mL	[39]
Biosensors	15.6–500 ng/mL	0.44 ng/mL	[40]
SPR sensor	0.5–100 ng/mL	0.023 ng/mL	This work

3.4. Detection of endotoxin from artificial plasma

In order to detect the accuracy of endotoxin imprinted SPR sensor in the clinical analysis, different concentration endotoxin was spiked to artificial plasma samples. Endotoxin solutions of 10 and 25 ng/mL concentrations are added to 1/10 diluted artificial plasma samples. Dilution of the samples were performed using an isotonic solution (1.0% NaCl solution). Firstly, endotoxin imprinted SPR sensor was equilibrated with 10 mM phosphate buffer (pH 7.4). Then, 10 and 25 ng/mL concentrations of endotoxin solutions which are prepared in 1/10 diluted artificial plasma samples are applied to SPR sensor systems. After, 1.0 M NaCl solution was applied to the system as a desorption solution and the bound endotoxin is removed from endotoxin imprinted SPR sensor surface. In this study, the LAL method used as the reference method was carried out by using the chromogenic technique based on the color change caused by the cleavage of the synthetic peptide-chromogen complex [41]. To determine the reliability and accuracy of endotoxin imprinted SPR sensor and LAL test were calculated the recovery (%) (Table 3). The compatibility of the obtained LAL results with SPR sensor results indicates that SPR sensor provides a reliability, accurate, quantitative and sensitive for the determination of endotoxin in artificial plasma samples.

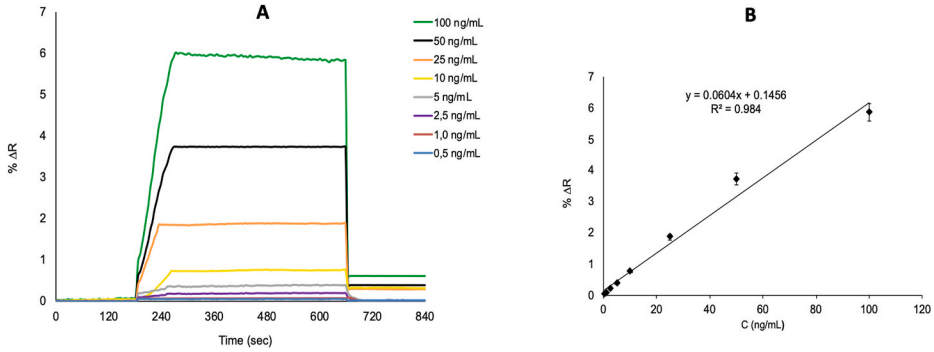


Fig. 4. Real-time responses (A) and linear regions (B) of endotoxin imprinted SPR sensor aqueous solutions of endotoxin at different concentrations.

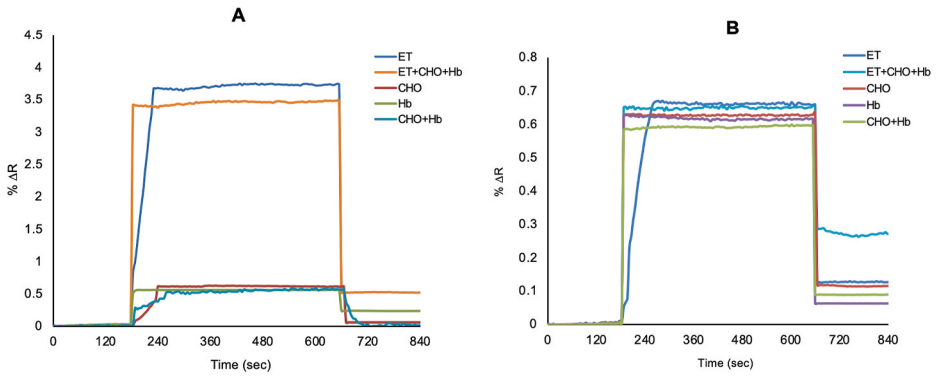


Fig. 5. The selectivity studies of endotoxin imprinted (A) and non-imprinted (B) SPR sensors (C: 50 ng/mL in all measurements).

Table 2
The selectivity and relative selectivity coefficients for competitive molecules for SPR sensors.

	MIP sensor		NIP sensor		
Molecules	ΔR	k	ΔR	k	k'
ET	3.721	–	0.661	–	–
CHO	0.617	6.031	0.628	1.053	5.729
Hb	0.561	1.099	0.617	1.018	1.081
CHO+Hb	0.563	0.996	0.594	1.039	0.959
ET+CHO+Hb	3.463	0.163	0.595	0.998	0.163

*ΔR: Resonance frequency change, MIP sensor: molecularly imprinted polymeric sensor, NIP: non-imprinted polymeric sensor, k: the selectivity coefficients, k': relative selectivity coefficients, ET: endotoxin, CHO: cholesterol, Hb: hemoglobin.

Table 3
The recoveries of endotoxin in artificial plasma samples (n:3).

Added endotoxin (ng/mL)	Found endotoxin (ng/mL)		Recovery (%)	
	SPR	LAL	SPR	LAL
10	9.87 ± 0.0096	9.78 ± 0.0082	98.73 ± 0.0957	97.80 ± 0.0816
25	24.59 ± 0.0163	24.18 ± 0.0129	98.36 ± 0.0653	96.7 ± 0.0516

***SPR:** Surface Plasmon Resonance**; LAL:** Limulus Amebocyte Lysate.

3.5. Repeatability of SPR sensors

SPR sensors having long shelf life and repeatability properties show high resistance to harsh environmental conditions. In order to investigate the repeatability of endotoxin imprinted SPR sensors, endotoxin solutions are given 5 times to the SPR sensor system (Fig. 6A). The repeatability of endotoxin imprinted SPR sensors was examined for endotoxin at 50 ng/mL concentration by using same SPR chip. Repeatability of the endotoxin imprinted SPR sensors in different times were also tested. Endotoxin imprinted SPR sensors were kept at 4 °C and there was no significant instability in the SPR responses against endotoxin after 27 months keeping. When applied in the presence of 50 ng/mL endotoxin during first day and different time periods after 27 months, the efficiency value of the SPR sensors were identified as 88.68% (Fig. 6B). These results suggest that the designed SPR sensors have high repeatability and may have value in the clinical applications. Precision studies were carried out to prove the repeatability of the method. Precision studies were performed by the relative standard deviation calculations (RSD%) should be better 2% RSD according to validation guideline of biological specimens [42]. Precision studies were performed by calculating the standard deviation (SD) and relative standard deviation (%RSD) which are 0.0153 and 0.412, respectively. The intraday studies were performed by 5 times replications and RSD% values prove that the system can detect the desired molecules with high repeatability.

4. Discussion

Endotoxin are lipopolysaccharides, composed of polysaccharides and a lipid component, that play a significant role in the protection of bacteria from cellular host defense systems, bile acids and hydrophobic

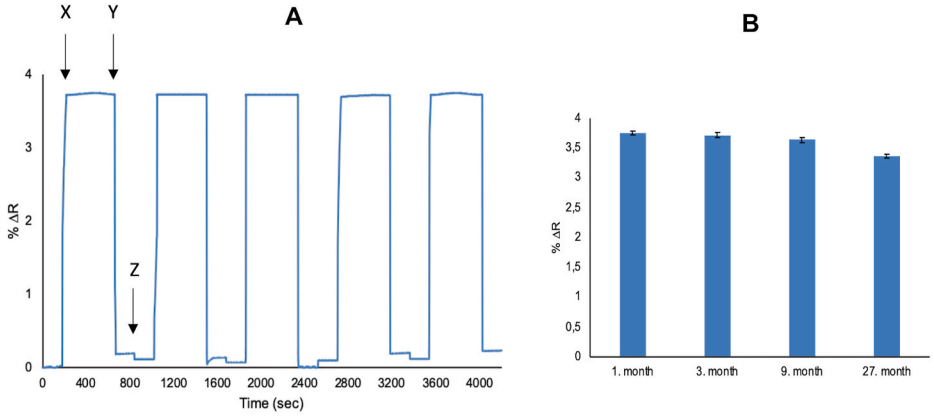


Fig. 6. Repeatability (A) and short-term and long-term stability (B) of endotoxin imprinted SPR sensors (C: 50 ng/mL in all measurements, X: equilibration, Y: adsorption, Z: regeneration).

antibiotics during severe infections, trauma and shock states in the infected host. Endotoxin imprinted SPR sensors are prepared in order to be used in clinical applications. Kinetic analysis of the endotoxin imprinted SPR sensors were performed from both endotoxin aqueous solutions and artificial plasma. The limit of detection and quantification for endotoxin in the linear range of 0.5–100 ng/mL were achieved as 0.023 and 0.078 ng/mL, respectively. Langmuir, Freundlich, and Langmuir-Freundlich isotherm models can be applied to determine the homogeneity of endotoxin imprinted SPR sensors. In addition, selectivity of SPR sensors was tested by using several competing proteins or a mixture of competing proteins. The repeatability of endotoxin imprinted SPR sensors were identified as 88.68%. According to the results obtained, it shows that endotoxin imprinted SPR sensors prepared for endotoxin detection can be used in clinical applications with their advantages such as high sensitivity and selectivity, short analysis time and long shelf life.

CRediT authorship contribution statement

Duygu Çimen: Writing, Conceptualization, Methodology, Formal analysis, Software, Visualization. **Sevgi Aslıyüce:** Writing, Conceptualization, Methodology, Formal analysis, Software, Visualization. **Tuğçe Deniz Tanalp:** Writing, Conceptualization, Methodology, Formal analysis, Software, Visualization. **Adil Denizli:** Investigation, Validation, Resources, Funding acquisition, Supervision, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2021.114221.

References

[1] C. Galanos, M.A. Freudenberg, Bacterial endotoxins: biological properties and mechanisms of action, Mediators, Inflamm. 2 (1993) 11–16.
[2] D. Heumann, T. Roger, Initial responses to endotoxins and Gram-negative bacteria, Clin. Chim. Acta. 323 (2002) 59–72.
[3] E.S. van Amersfoort, T.J.C. van Berkel, J. Kuiper, Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock, Clin. Microbiol. Rev. 16 (2003) 379–414.
[4] M. Levi, M. Schultz, T. van der Poll, Disseminated intravascular coagulation in infectious disease, Semin. Thromb. Hemost. 36 (2010) 367–377.
[5] L. Tan, Y. Huang, X. Pan, S. Quan, S. Xu, D. Li, L. Song, X. Zhang, W. Chen, J. Pan, Administration of bone marrow stromal cells in sepsis attenuates sepsis-related coagulopathy, Ann. Med. 48 (2016) 235–245.
[6] N. Semeraro, C.T. Ammollo, F. Semeraro, M. Colucci, Sepsis-associated disseminated intravascular coagulation and thromboembolic disease, Mediterr. J. Hematol. Infect. Dis. 2 (2010), 2010024.
[7] D.J. Stearns-Kurosawa, M.F. Osuchowski, C. Valentine, S. Kurosawa, D.G. Remick, The pathogenesis of sepsis, Annu. Rev. Pathol. 6 (2011) 19–48.
[8] 3.4 TEST for BACTERIAL ENDOTOXINS Final Text for Revision of the International Pharmacopoeia, 2012.
[9] J.F. Cooper, H.D. Hochstein, E.B. Seligmann, The Limulus test for endotoxin (Pyrogen) in radiopharmaceuticals and biologicals, Bull. Parenter. Drug Assoc. 26 (1972) 153–162.
[10] R. Nachum, R.N. Berzofsky, Chromogenic Limulus amoebocyte lysate assay for rapid detection of gram-negative bacteriuria, J. Clin. Microbiol. 21 (1985) 759–763.
[11] T.J. Novitsky, P.F. Roslansky, G.R. Siber, H.S. Warren, Turbidimetric method for quantifying serum inhibition of Limulus amoebocyte lysate, J. Clin. Microbiol. 21 (1985) 211–216.
[12] S.P. Sakti, R. Lucklum, P. Hauptmann, F. Bühling, S. Ansorge, Disposable TSM-biosensor based on viscosity changes of the contacting medium, Biosens. Bioelectron. 16 (2001) 1101–1108.
[13] P. Swain, S.K. Nayak, P.K. Nanda, S. Dash, Biological effects of bacterial lipopolysaccharide (endotoxin) in fish: a review, Fish Shellfish Immunol. 25 (2008) 191–201.
[14] J. Zhang, I. Khan, Q. Zhang, X. Liu, J. Dostalek, B. Liedberg, Y. Wang, Lipopolysaccharides detection on a grating-coup.led surface plasmon resonance smartphone biosensor, Biosens. Bioelectron. 99 (2018) 312–317.
[15] G. Ertürk, H. Özen, M.A. Tümer, B. Mattiasson, A. Denizli, Microcontact imprinting based surface plasmon resonance (SPR) biosensor for real-time and ultrasensitive detection of prostate specific antigen (PSA) from clinical samples, Sensor. Actuator. B Chem. 224 (2016) 823–832.
[16] S. Koyun, S. Akgönüllü, H. Yavuz, A. Erdem, A. Denizli, Surface plasmon resonance aptasensor for detection of human activated protein C, Talanta 194 (2019) 528–533.
[17] D. Çimen, N. Bereli, S. Günaydın, A. Denizli, Detection of cardiac troponin-I by optic biosensors with immobilized anti-cardiac troponin-I monoclonal antibody, Talanta 219 (2020), 121259.
[18] M. Jalilzadeh, D. Çimen, E. Özgür, C. Esen, A. Denizli, Designing and preparing imprinted surface plasmon resonance (SPR) nanosensor for detection of Zn(II)-ions, J. Macromol. Sci. 9 (2019) 877–886.
[19] C.I.L. Justino, A.C. Freitas, R. Pereira, A.C. Duarte, S.T.A.P. Rocha, Recent developments in recognition elements for chemical sensors and biosensors, Trends Anal. Chem. 68 (2015) 2–17.
[20] S. Asliyuce, B. Mattiasson, G. Mamo, Synthesis and use of protein G imprinted cryogel as affinity matrix to purify protein G from cell lyaste, J. Chromatogr. B 1021 (2016) 204–212.
[21] J. Li, M. Li, B. Yang, Strategies to improve the sensitivity of molecularly imprinted sensors, Smart Polymer Catalysts and Tunable Catalysis (2019) 149–175.
[22] A. Chiappini, L. Pasquardini, A.M. Bossi, Molecular imprinted polymers coupled to photonic structures in biosensors: the state of art, Sensors 20 (2020) 5069.
[23] L. Chen, X. Wang, W. Lu, X. Wu, J. Li, Molecular imprinting: perspectives and applications, Chem. Soc. Rev. 45 (2016) 2137.
[24] B. Garipcan, A. Denizli, A novel affinity support material for the separation of immunoglobulin G from human plasma, Macromol. Biosci. 2 (2002) 135.
[25] E.E. Bormashenko, Contact angles of rotating sessile droplets, Colloid. Surface. 432 (2013) 38–41.
[26] B.O. Payton, A.R. Champneys, M.E. Homer, L. Picco, M.J. Miles, Feedback-induced instability in tapping mode atomic force microscopy: theory and experiment, Proc. R. Soc. A 467 (2011) 1801–1822.
[27] S.K. Papageorgiou, F.K. Katsaros, E.P. Kouvelos, N.K. Kanellopoulos, Prediction of binary adsorption isotherms of Cu^{2+}, Cd^{2+} and Pb^{2+} on calcium alginate beads from single adsorption data, J. Hazard Mater. 162 (2009) 1347–1354.
[28] M.J. Abdin, Z. Altintas, I.E. Tothill, In silico designed nanoMIP based optical sensor for endotoxins monitoring, Biosensor, Biosens. Bioelectron. 67 (2015) 177–183.
[29] C. Liu, J. Xu, J. Qi, Y. Liu, J. Kang, H. Wang, S. Chen, Z. Huang, B. Lu, A novel nanobiocomposite sandwich immunosensor based on molecularly imprinted nano-membrane for endotoxin detection, Sensor. Actuator. B 290 (2019) 1–8.
[30] Y. Duan, N. Wang, Z. Huang, H. Dai, L. Xu, S. Sun, H. Ma, M. Lin, Electrochemical endotoxin aptasensor based on a metal-organic framework labeled analytical platform, Mater. Sci. Eng. C 108 (2020), 110501.
[31] X. Fan, Z. Li, S. Wang, L. Liu, P. Liu, F. Chen, X. Zheng, Electrochemical impedance biosensor for the determination of lipopolysaccharide using peptide as the recognition molecule, J. Braz. Chem. Soc. 30 (2019) 1762–1768.
[32] N. Yu, X. Zhang, Y. Gao, H. You, J. Zhang, P. Miao, Highly sensitive endotoxin assay combining peptide/graphene oxide and DNA-modified gold nanoparticles, ACS Omega 4 (2019) 14312–14316.
[33] J. Ji, Y. Pang, D. Li, Z. Huang, Z. Zhang, N. Xue, Y. Xu, X. Mu, An aptamer-based shear horizontal surface acoustic wave biosensor with a CVD-grown single- layered graphene film for high-sensitivity detection of a label-free endotoxin, Microsyst. Nanoeng. 6 (2020) 4.
[34] K.G. Ong, J.M. Leland, K. Zeng, G. Barrett, M. Zourob, C.A. Grimes, A rapid highly-sensitive endotoxin detection system, Biosensor, Biosens. Bioelectron. 21 (2006) 2270–2274.
[35] H. Jiang, J. Yang, K. Wan, D. Jiang, C. Jin, Miniaturized paper-supported 3D cell-based electrochemical sensor for bacterial lipopolysaccharide detection, ACS Sens. 5 (2020) 1325–1335.
[36] M. Cho, L. Chun, M. Lin, W. Choe, J. Nam, Y. Lee, Sensitive electrochemical sensor for detection of lipopolysaccharide on metal complex immobilized gold electrode, Sensor. Actuator. B 174 (2012) 490–494.
[37] Z. Li, G. Dai, F. Luo, Y. Lu, J. Zhang, Z. Chu, Q. Wang, An electrochemical sensor for bacterial lipopolysaccharide detection based on dual functional Cu^{2+} modified metal–organic framework nanoparticles, Microchim. Acta. 187 (2020) 1–10.
[38] N. Wang, H. Dai, L. Sai, H. Ma, M. Lin, Copper ion-assisted gold nanoparticle aggregates for electrochemical signal amplification of lipopolysaccharide sensing, Biosens. Bioelectron. 126 (2019) 529–534.
[39] J. Zhang, I. Khan, Q. Zhang, X. Liu, J. Dostalek, B. Liedberg, Y. Wang, Lipopolysaccharides detection on a grating-coupled surface plasmon resonance smartphone biosensor, Biosens. Bioelectron. 99 (2018) 312–317.
[40] Z. Altintas, M.J. Abdin, A.M. Tothill, K. Karim, I.E. Tothill, Ultrasensitive detection of endotoxins using computationally designed nanoMIPs, Anal. Chim. Acta 935 (2016) 239–248.
[41] EUROPEAN PHARMACOPOEIA 5.0 2.6.14. (Bacterial endotoxins).
[42] G.A. Shabir, Step-by-step analytical methods validation and protocol in the quality system compliance industry, J. Valid. Technol. 10 (2004) 314–324.