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Rapid real-time detection of procalcitonin using a microcontact imprinted surface plasmon resonance biosensor†

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Procalcitonin (PCT) is a promising biomarker for identification of the origin and severity of sepsis, which is a deadly body infection. In this work, we report the preparation of a surface plasmon resonance (SPR) biosensor which utilizes a molecular imprinted polymer surface for rapid and reliable detection of PCT. The molecular imprinted surface was prepared using a microcontact imprinting technique, in which PCT molecules were first immobilized onto a glass support and brought into contact with a solution of 2-hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) on a SPR sensor, then the polymerization process was performed. After removal of the PCT molecules, specific molecular recognition sites were obtained, where PCT molecules can selectively rebind, only at the surface of the polymer matrix. PCT detection studies were carried out using PCT solutions in phosphate buffer and simulated blood plasma (SBP) at different concentrations. The SPR biosensor can detect very low concentrations (9.9 ng mL^{-1}) of PCT within approximately 1 h, in both phosphate buffer and SBP. High selectivity of the biosensor against PCT was also demonstrated in the presence of several competitive proteins such as human serum albumin, myoglobin and cytochrome c.

Introduction

Sepsis is a severe body infection caused by microbial pathogens including bacteria, virus and fungi. If sepsis is not treated carefully, it may cause some adverse effects, leading to internal organ failure and death.[1] In order to give the appropriate medication to the patient, first, one must differentiate bacterial infection from viral and fungal infection and systemic sepsis from local infection.[2] So far, the most common and reliable method to identify the origin of sepsis and its severity is the pathogen culturing from body fluids such as blood plasma, urine, cerebrospinal fluid, or bronchial fluid. However, this method requires very long culturing times typically between 24 h and 48 h and it also needs intensive labor.[3] Therefore, rapid, reliable and simple methods for diagnosis of the origin of sepsis are highly demanded to treat patients properly and in time.[4] In addition, correct diagnosis can prevent unnecessary antibiotic use thus reducing antibiotic related cost to healthcare systems and preventing the promotion of antibiotic resistance of bacteria.[5,6]

Currently, some biomarkers such as C-reactive protein (CRP), serum decoy receptor 3 (DcR3), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and procalcitonin (PCT) have been investigated for the rapid identification of the origin and severity of sepsis.[7–11] PCT, a protein consisting of 116-amino-acids with a molecular weight of 13 kDa, is generally accepted as the most promising biomarker because of its better sensitivity and selectivity than other known biomarkers.[12,13] The PCT concentration in the blood of a healthy person is below 0.1 ng mL^{-1} and it can rise over 100 ng mL^{-1} for the bacteria originated sepsis, depending on the severity of the infection.[14] Consequently, several methods were developed to detect the PCT concentration in the blood mostly using chemiluminescence,[15,16] immunoluminometric assays[2,17] and SPR biosensors.[14] All of these methods recognize PCT upon selective binding of PCT to antibodies. However, these natural antibodies are very expensive and have poor stability which restricts their usage in hospitals. For widespread use of these methods in healthcare, replacement of natural antibodies with their synthetic counterparts (*i.e.* molecular imprinted polymers[18–22]) is required, which are cheap, robust and can be produced in large quantities.[23–26]

With these insights, here we report a facile method to prepare a synthetic antibody based SPR biosensor for rapid PCT detection. To prepare synthetic antibodies on the biosensor

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surface we used a microcontact imprinting method, where target molecules are immobilized onto a solid support, the so-called stamp, and then brought into contact with a monomer solution, then the polymerization process is performed.[27] After removal of the stamp, specific molecular recognition sites can be obtained only at the surface of the imprinted polymer matrix. This method has some significant advantages over conventional molecular imprinting methods especially when working with biomolecules,[18] such as reducing activity loss of imprinted biomolecules and requiring a very small amount of template molecules.[28] Accordingly, PCT microcontact imprinted polymer nanofilms on SPR sensor chips were prepared in order to create molecular cavities for PCT. The polymer nanofilm on the SPR sensor was prepared using HEMA and EGDMA. HEMA was selected to form a hydrophilic polymer nanofilm and EGDMA was selected as a crosslinker since it has a similar structure to HEMA.[29] PCT detection studies were carried out in phosphate buffer and simulated blood plasma (SBP) at different PCT concentrations. Selectivity of the biosensor was studied using several competitive proteins such as human serum albumin, myoglobin and cytochrome c. We observed a good limit of detection (9.9 ng mL^{-1}) and high selectivity for the PCT imprinted SPR biosensor in both media. In addition, all steps of this label-free assay can be completed in approximately 1 h which, we believe, makes this biosensor a promising candidate for rapid identification of the pathogenic origin of sepsis.

Materials and methods

Materials

Procalcitonin (PCT) was supplied from BRAHMS (Hennigsdorf, Germany). Myoglobin, cyctochrome c, 3-aminopropyl triethoxysilane (APTES) and allyl mercaptan were obtained from Sigma Chemical Co. (St. Louis, USA). Human serum albumin (HSA), HEMA, EGDMA and α,α′-azoisobutyronitrile (AIBN) were purchased from Fluka A.G. (Buchs, Switzerland). All other chemicals were of reagent grade and purchased from Merck A.G. (Darmstadt, Germany).

Preparation of the microcontact PCT imprinted polymer on the SPR sensor

The following experimental procedure was applied for the preparation of the protein imprinted polymer on the SPR sensor. PCT was immobilized onto an APTES modified glass slide (*ca.* 2.5 cm × 1.5 cm) and transferred onto the SPR sensor surface. All glass slides were cleaned with acidic piranha solution (3 : 1 H_2SO_4 : H_2O_2, v/v), then rinsed with distilled water, acetone, isopropanol and again with water and dried in an oven at 60 °C for 2 h. The cleaned glass slides were silanised by using 10% APTES solution (v/v) in ethanol at room temperature for 1 h. Noncovalently bound APTES molecules were removed from the surface by sonicating the glass slides in ethanol for 10 min and then immersed in ethanol and in deionized water, respectively. After that, they were dried in an oven at 60 °C overnight.[30] PCT molecules were adsorbed onto the APTES modified glass slides by immersing the glass slides in 0.1 mg mL^{-1} PCT solution (pH 7.4, phosphate buffer) at room temperature for 2 h. Finally, the glass slides were washed with deionized water and dried in a laminar flow hood at room temperature.

SPR sensor surfaces were cleaned with acidic piranha solution (3 : 1 H_2SO_4 : H_2O_2, v/v), then washed with deionized water and ethanol, respectively, and dried at room temperature prior to preparation of a microcontact PCT imprinted polymer nanofilm. After that, 5 μL of allyl mercaptan was dropped onto the SPR sensor and incubated for 12 h in a sealed container in order to introduce allyl groups onto the sensor surface. After allyl modification, the SPR sensor was rinsed with ethanol to remove unbound allyl mercaptan molecules and dried at room temperature. The microcontact PCT imprinted polymer nanofilm on the allyl mercaptan modified SPR sensor was prepared as follows: in the first step, stock monomer solution was prepared by mixing HEMA (0.25 mL), EGDMA (1 mL) and AIBN (5 mg). Then, 1.0 μL aliquots were taken from the stock solution and dropped onto the allyl mercaptan modified SPR sensor surface. Then, the PCT immobilized glass slide was brought into contact with the monomer solution and pressed. Polymerization was initiated by UV light at room temperature (100 W, 365 nm) and continued for 45 min. After the polymerization process, the glass slide was detached from the sensor surface carefully. The microcontact PCT imprinted polymer nanofilm coated SPR sensor was then washed first with deionized water and then with 1 M NaOH solution in order to desorb PCT from the surface and dried with nitrogen gas at room temperature. The non-imprinted SPR sensor was synthesized by applying the same procedure without immobilization of the template molecule, PCT, onto the glass slide.

Characterization of the SPR sensor surface

The PCT imprinted SPR sensor surface was characterized using a scanning electron microscope (SEM), an atomic force microscope (AFM) and a contact angle measurement system. SEM images were taken using a Quanta 200 (FEI, USA). AFM observations were carried out by using an AFM (Nanomagnetics Instruments, Oxford, UK) in tapping mode. Surfaces were analyzed using X-ray photoelectron spectroscopy (XPS, K-Alpha, Thermo Scientific). Water contact angles of surfaces were measured with a KRUSS DSA100 (Hamburg, Germany) instrument. The contact angle values of the surfaces were calculated from at least five separate measurements taken from different parts of the SPR sensor.

Kinetic studies with the microcontact PCT imprinted SPR sensor

Microcontact PCT imprinted SPR sensors prepared in this study were used for real-time PCT detection from aqueous solution by using a SPR system (GenOptics, SPRi-Lab, Orsay, France). 50 nm gold coated SPR chips (25 × 12.5 mm) were also supplied from GenOptics. The PCT imprinted and non-imprinted SPR sensors were washed with deionized water (50 mL) and equilibrated with phosphate buffer (pH 7.4, 50 mL) at a flow rate of 2.0 mL min^{-1}. Then, solution of the interested protein in the range of 20–4000 ng mL^{-1} was applied to the SPR system (5 mL) using a peristaltic pump and the change in resonance frequency was monitored online. Desorption of PCT was achieved by applying 5 mL of 1 M

NaOH solution. At the end of the desorption step, the microcontact PCT imprinted SPR sensor was washed with deionized water and equilibrated with phosphate buffer. In order to analyze the kinetic data obtained, SPR1001 software was used.[31]

PCT detection from SBP was also performed. In this respect, the concentrations of most abundant proteins in 1/10 diluted plasma were simulated by dissolving 80 mg of albumin, 25 mg of immunoglobulins, and 6 mg of fibrinogen into 20 mL phosphate buffer (pH 7.4) to establish their normal concentration in plasma.[32] Different amounts of PCT were spiked into SBP in order to give PCT concentrations in the range of 20–1000 ng mL^{-1}. The PCT spiked SBP samples were applied to the SPR system and SPR measurements were performed as given above.

In order to confirm the reliability of the biosensor proposed in this study, the PCT concentrations in SBP were simultaneously measured using a VIDAS system (The VIDAS BRAHMS PCT assay, bioMérieux, Marcy L'Etoile, France), which is based on automated heterogeneous sandwich immunoassay with fluorescence detection of calcitonin and katacalcin domains of the prohormone using antibodies, according to the literature and procedure's guide.[33]

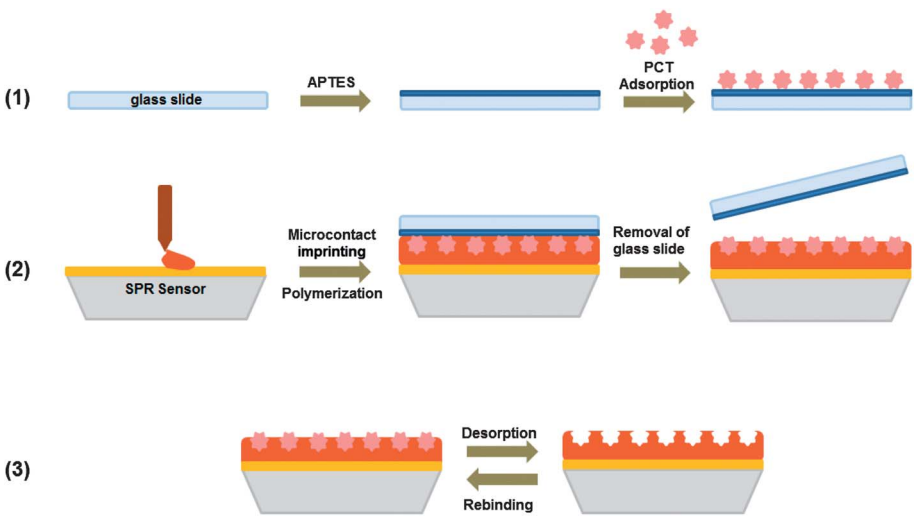
Results and discussion

Preparation and characterization of the SPR sensor

SPR is a promising biosensor platform in which the change in the refractive index of the metal surface through analyte introduction was measured.[34,35] It offers highly sensitive and selective real time measurement of analytes without the need for labeling. Also, the detection time is generally short (within a few minutes) and SPR signals are greatly reproducible.[36,37] Meanwhile, unlike natural antibodies, which are expensive and unstable, molecular imprinted polymers offer robust recognition of biomolecules in a cost effective manner.[23] Therefore, we aimed to prepare the microcontact imprinted SPR sensor surface as a biosensing platform for cost effective and rapid detection of PCT from both aqueous solutions and SBP.

The PCT imprinted SPR sensor was prepared using a microcontact printing technique. In the first step, PCT was adsorbed on previously aminopropyl modified glass surfaces. Then, the PCT adsorbed glass slide, the so-called protein stamp, was brought into contact with the allyl mercaptan modified SPR sensor in the presence of monomer solution containing HEMA and EGDMA. Polymerization of the monomer solution between two surfaces was performed under UV light and the glass slide was removed carefully after polymerization. Finally, PCT molecules on the polymer film surface were desorbed using 1 M NaCl solution to form PCT recognizing cavities on the SPR sensor surface. When the PCT molecules were introduced onto the imprinted SPR sensor surface they can easily rebind to the recognition cavities (Scheme 1).

The surface morphology of the microcontact imprinted SPR surface was investigated using SEM and AFM (Fig. 1). The SEM image of the SPR surface, which was taken from an intentionally scratched part of the surface, clearly shows the polymer film and the underlying gold surface (Fig. 1a). The thickness of the polymer film was measured to be around 230 nm from the tilted (40°) SEM image of the surface (Fig. 1b). AFM images of the polymer coated and bare SPR surfaces (Fig. 1c and d) revealed that the average surface roughness of the SPR chip was raised from 0.677 nm to 6.840 nm, which also indicates the formation of the polymer nanofilm. Furthermore, we investigated the chemical compositions of polymer film coated and bare SPR sensor surfaces using XPS (ESI, Fig. S1†). XPS analysis of the bare SPR sensor demonstrated the peaks related to the gold atoms as expected. On the other hand, the XPS spectrum proved the polymer film coated SPR sensor surface was mainly composed of carbon and oxygen atoms, which indicated the formation of the polymer film on the gold surface. In addition, water contact angle (WCA) measurements were performed for further characterization of the SPR surface (Table 1). The WCA of the bare SPR surface was 92.1°. After modification with allyl mercaptan, the WCA of the sensor surface was decreased to 85.4° because of introduction of the polar allyl groups onto the surface.



Scheme 1 Schematic representation of the microcontact PCT imprinted SPR biosensor fabrication.

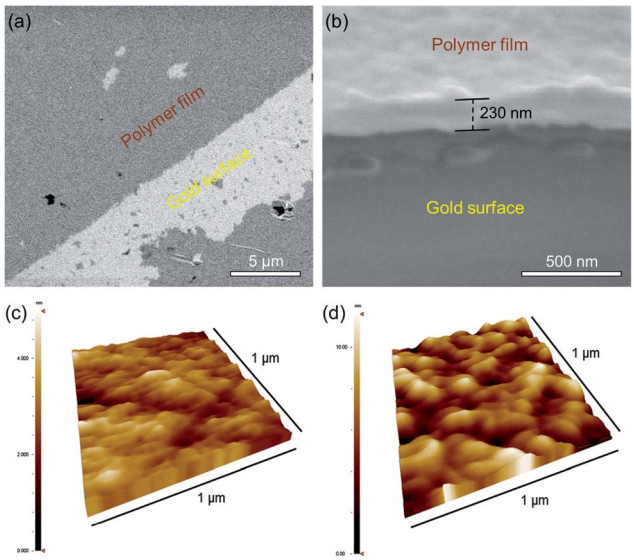


Fig. 1 (a and b) SEM images of a microcontact PCT imprinted polymer nanofilm. AFM images of (c) bare SPR sensor surface and (d) PCT imprinted SPR sensor surface.

Table 1 Water contact angles of the SPR sensor and glass surfaces

Surface	Water contact angle (°)
SPR sensor	
Bare	92.1
Allyl mercaptan modified	85.4
Microcontact imprinted	67.4
Glass slide	
Bare	38.5
APTES modified	80.9
PCT adsorbed	73.7

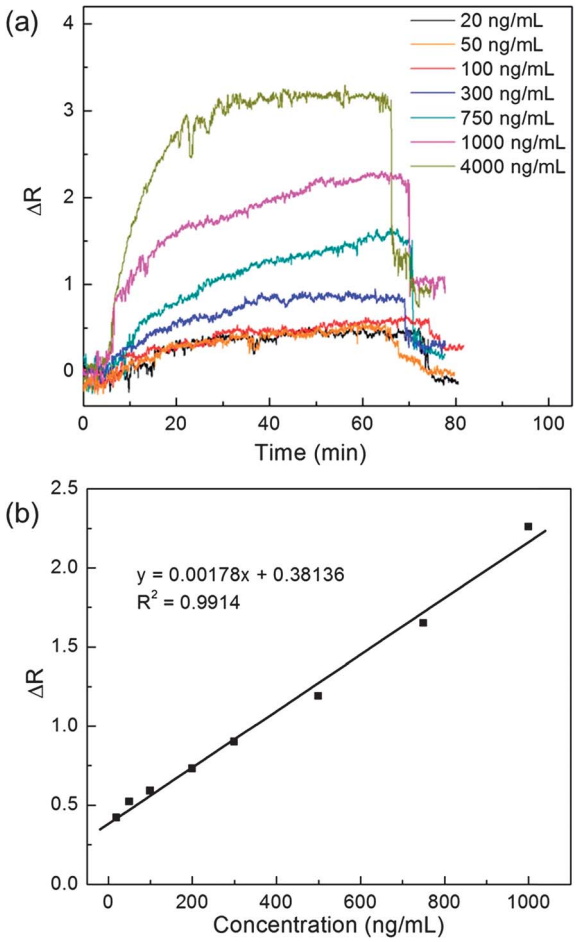


Fig. 2 (a) Real-time response of a SPR biosensor against several PCT solutions in phosphate buffer at different concentrations. (b) Concentration dependency of a PCT imprinted SPR biosensor showing the high linearity of the sensor response in the studied concentration range.

Preparation of the microcontact PCT imprinted polymer nanofilm on the SPR surface causes a further decrease in the WCA of the surface to 67.4° due to hydrophilic characters of HEMA and EGDMA monomers. These results prove the successful formation of the polymer thin film on the SPR chip surface. Also, the PCT adsorbed glass surface was characterized using WCA measurements (Table 1). The bare cleaned glass slide surface was very hydrophilic with a WCA value of 38.5°. After APTES modification, it raised to 80.9° as expected.[38] Finally, the WCA of the surface decreased to 73.7° after PCT adsorption.

Real-time PCT monitoring

Microcontact imprinted SPR sensors were used for real time PCT detection from aqueous solutions in a PCT concentration range of 20–4000 ng mL^{-1}. The variations in the SPR sensor response with respect to time after the injection of PCT solutions at different concentrations were online monitored and are given in Fig. 2a. PCT introduction onto the SPR sensor surface results in a rise in the reflectivity of the sensor, which increased immediately after PCT injection and reached a constant value typically in 30 min. Also, the sensor response reaches the plateau more quickly with increasing PCT concentration. All steps of PCT detection (equilibration, adsorption, desorption, and regeneration) were completed in around 80 min at all concentrations, which is very fast compared to the classical bacterial culture method.[13]

The difference in the reflectivity (ΔR) of the SPR sensor due to PCT injection gradually increases with increasing the PCT concentration as expected. It should be noted that, in order to determine ΔR values, first we took the average of R values, which are before PCT solution introduction (baseline), and after the R values reached a plateau (response) and then we subtracted the baseline value from the response. The concentration dependent sensor response is highly linear with a linear regression constant of 99.14% in the range between 20 and 1000 ng mL^{-1} (Fig. 2b). The microcontact imprinted SPR sensor surface can identify PCT at concentrations as low as 20 ng mL^{-1}, which indicates the high sensitivity of the microcontact imprinted SPR sensor against PCT. Using our SPR device, the limit of detection (LOD), the concentration of the analyte giving reflectivity shift equivalent to three average standard deviations obtained from

multiple blank measurements, and limit of quantification (LOQ), the concentration of the analyte giving reflectivity shift equivalent to ten averaged standard deviations of the blank, are determined to be 2.97 ng mL^{-1} and 9.90 ng mL^{-1}, respectively. It should be noted that LOD and LOQ values can be varied if different devices are used. In addition, a further improvement in the detection limit can be expected by decreasing the polymer film thickness since the evanescent field becomes more intense near the gold surface especially below 200 nm. Therefore, as future work, we will work on producing thinner PCT imprinted polymer films to achieve a more sensitive PCT sensor. It is worth noting that we can successfully reach the required PCT detection limit (<100 ng mL^{-1}) using cheap and robust molecular imprinted polymer (synthetic) antibodies instead of using their expensive and sensitive natural counterparts.[14]

Selectivity of the microcontact PCT imprinted SPR sensor

In order to demonstrate the selectivity of the microcontact imprinted SPR sensor against PCT, we used HSA, myoglobin and cytochrome c as competing proteins, which are proteins highly abundant in blood and/or similar in size/shape to PCT. Also, real-time measurements with a non-imprinted SPR sensor, which was prepared in the absence of PCT, were carried out using the proteins mentioned above to evaluate the imprinting efficiency of the microcontact imprinted SPR sensor. The changes in the reflectivity of the imprinted and non-imprinted SPR sensors after introduction of proteins at 1000 ng mL^{-1} concentration are given in Fig. 3. All competitive proteins resulted in an increase in the reflectivity of the sensor through non-specific interactions with the polymer surface and the PCT recognition cavities, yet changes in reflectivity were significantly lower than the PCT case. The selectivity factors for PCT, calculated from the results, are summarized in Table 2. As seen in the table, the selectivity constants for the microcontact PCT imprinted SPR sensor were 2.17, 3.96, and 11.30 for PCT against myoglobin, HSA, and cytochrome c, respectively. The constants for the non-imprinted sensor are 0.39, 2.19, and 2.33 for PCT against myoglobin, HSA and cytochrome c, respectively. The results show that the microcontact imprinting process allows

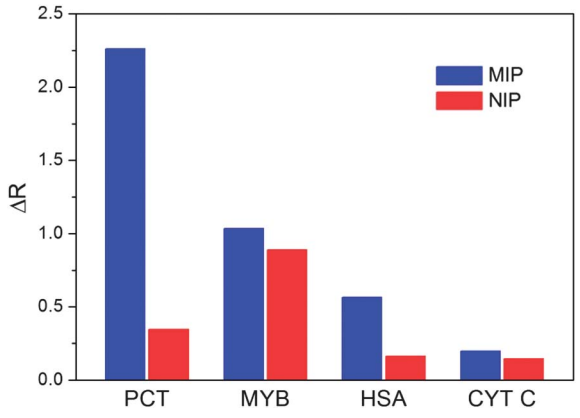


Fig. 3 Selectivity of PCT imprinted (MIP) and non-imprinted (NIP) SPR biosensors against myoglobin (MYB), human serum albumin (HSA) and cytochrome c (CYT C).

Table 2 Selectivity coefficients of the microcontact PCT imprinted SPR sensor

	SPR response, ΔR		Selectivity coefficient		
Protein	Imprinted	Non-imprinted	Imprinted	Non-imprinted	Imprinting efficiency
PCT	2.26	0.35	—	—	—
Myoglobin	1.04	0.89	2.17	0.39	5.56
HSA	0.57	0.16	3.96	2.19	1.81
Cytochrome c	0.20	0.15	11.30	2.33	4.85

the SPR sensor to recognize PCT molecules reversibly and selectively. The reflectivity change in sensor response in respect of the competitor proteins is almost the same for both the imprinted and the non-imprinted SPR sensor, which indicates that the interactions between the proteins and the sensor surface occur in a non-specific manner. In addition, the imprinting efficiency values, which show enhanced selectivity of the sensor according to competitors *via* microcontact imprinting of PCT, are calculated to be 5.56, 1.81, and 4.85 for PCT against myoglobin, HSA, and cytochrome c, respectively.

PCT detection from simulated blood plasma

The microcontact PCT imprinted SPR sensor was also used to detect PCT molecules from SBP, which comprises HSA, immunoglobulins and fibrinogen at their typical concentrations in human blood plasma. For this purpose, artificial blood plasma simulating 1/10 diluted one was freshly prepared in phosphate buffer (pH 7.4). To demonstrate the applicability of the microcontact PCT imprinted SPR sensor in complex biological solutions, SBP solutions were spiked with PCT with the final PCT concentration to be 20 ng mL^{-1}, 100 ng mL^{-1}, 300 ng mL^{-1}, and 1000 ng mL^{-1} and applied to the SPR sensor system while real-time monitoring of the SPR response. Fig. 4a shows the typical response of the microcontact PCT imprinted SPR sensor against SBP and PCT spiked SBP (1000 ng mL^{-1}). After SBP introduction to the SPR sensor, the reflectivity of the sensor is significantly increased because of the change in the refractive index of the medium and, then, reached equilibrium quickly. After reaching equilibrium, the reflectivity of the SPR sensor is slightly changed with time because of the non-specific binding of SBP proteins that compete with PCT onto the surface. When PCT spiked SBP is introduced to the sensor, the sensor response rapidly increased due to the specific binding of PCT onto the molecularly imprinted cavities having molecular recognition ability for the sensor surface. Fig. 4a (inset) shows the relationship between SPR sensor responses and PCT spiked SBP solution in the concentration range of 20–1000 ng mL^{-1}. The concentration dependency of the microcontact imprinted sensor response according to PCT spiked SBP is highly linear with a linear regression constant as high as 99.79%. Also, ELISA results also confirmed the reliability of the proposed biosensor. The linearity of ELISA results with respect to both PCT concentration and SPR response is as high as 99.92% and 99.66%, respectively. Here, we should mention that ELISA measurements had been performed under a convenient

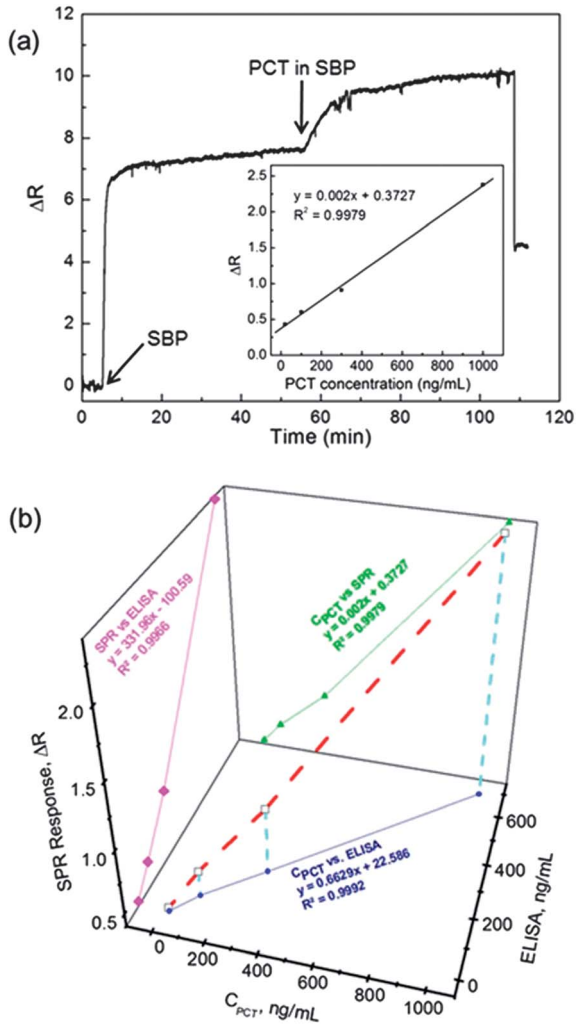


Fig. 4 (a) Typical real-time response of a SPR biosensor against PCT solutions in simulated blood plasma (SBP) at 1000 ng mL^{-1}. The inset shows the concentration dependency of a PCT imprinted SPR biosensor showing the high linearity of the sensor response in the studied concentration range. (b) Comparison of PCT detection results of the SPR biosensor with the results of the ELISA test.

dilution factor according to the producers' guide. In addition, space diagonal linearity between SPR response, ELISA results and PCT spiked SBP are highly linear (Fig. 4b). More importantly, the increases in sensor response with respect to the PCT spiked SBP and phosphate buffer are very similar to each other, which shows that the microcontact PCT imprinted SPR sensor could successfully operate without being affected by medium, whether aqueous or complex biological solution such as SBP, and the performance of the SPR sensor could compete with that of ELISA used.

Conclusion

In summary, we described the preparation of a novel microcontact imprinted SPR biosensor for rapid PCT detection from complex biological solutions. A microcontact PCT imprinted biocompatible polymer nanofilm, prepared using HEMA and EGDMA, was formed on the SPR sensor chip to create selective cavities for PCT molecules. The successful preparation of the polymer nanofilm on the sensor surface was demonstrated by AFM, WCA, SEM and XPS measurements. Microcontact imprinted SPR sensors were used for real time PCT detection from both aqueous solutions and SBP, which comprise HSA, immunoglobulins and fibrinogen at their typical concentrations in human blood plasma. The SPR biosensor can detect PCT at low concentrations (20 ng mL^{-1}) from both aqueous solutions and SBP. Sensor responses with respect to PCT in SBP and phosphate buffer are very similar to each other, which shows that the microcontact PCT imprinted SPR sensor can successfully operate without being affected by the medium, whether aqueous or complex biological solution such as SBP. The selectivity of the SPR biosensor against the PCT sensor was further demonstrated using competing proteins (HSA, myoglobin and cytochrome c). This robust and cost effective SPR biosensor holds great potential for rapid detection of the microbial origin and severity of sepsis. Finally, we believe that our method can be easily extended to other platforms, including quartz crystal microbalance (QCM), microcantilever, fiber-optics, waveguide and electrochemical based sensors.[37,39–42]

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