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# Mixed monolayer decorated SPR sensing surface for thrombin detection

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ABSTRACT

The development of surface plasmon resonance (SPR) based immunosensor for thrombin detection was aimed. For this purpose, 3,3′ Dithiodipropionic acid di (N-hydroxysuccinimide ester) (DSP):6-mercapto-1-hexanol (MCH) mixed self-assembled monolayers (mSAMs) were formed on gold surfaces for immobilization of anti-thrombin antibody. The performance of the immunosensor was determined against the target protein thrombin at various concentrations using flow cell coupled SPR. The linear detection range of the immunosensor was 30.0–100.0 nM with an $R^2$ value of 0,992. Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined to be 6.0 nM and 30.0 nM, respectively. The selectivity of the immunosensor was tested against a non-target model protein, human serum albumin (HSA) and the obtained ΔRU value was found to be below the ΔRU value corresponding to the LOQ concentration for thrombin. The immunosensor's capability to detect thrombin in diluted complex serum matrix was also tested and the obtained ΔRU value ($159 \pm 16$) was compared with ΔRU value obtained for thrombin detection in PBS solution ($137 \pm 19$). Based on the results, it was shown that DSP:MCH interface is a promising immobilization platform for binding biological recognition elements for the development of biosensors.



## 1. Introduction

Enhancing the performance of biosensors (optic, mass-sensitive, amperimetric etc.) has become an important task for the development of biosensing platforms [1–3]. Some of the requirements for improving the sensitivity and selectivity of these systems include overcoming the steric hinderance issue, providing resistance to nonspecific binding, stable biological recognition layer formation and providing upright position while retaining the biological activity of the biological recognition element during the surface immobilization process [4,5]. Last mentioned property is important for maintaining the affinity and the selectivity of the biological recognition element exhibited in solution. Immobilizing the biological recognition element on the surface involves noncovalent or covalent binding [5]. Covalent binding involves attachment of the biological recognition element via covalent bond formation [5]. Although this type of modification may result in a change in the surface properties and decrease in the biological activity of the biological recognition element, it contributes to generation of stable biological recognition layer, due to strong covalent interactions [5]. In addition, because covalent bond formation is highly selective, there is less chance of nonspecific binding (via amine groups) of the biological recognition element to the surface, which may result in hindering the active site for recognizing the target molecule. Because of these two important features, covalent modification is commonly preferred over noncovalent modification. Some of the examples for covalent modification include thiol / disulfide on metals such as Au, Ag and Cu and semiconductors (CdS, CdSe, ZnS) [6] or modifying the surface with carboxyl groups and activating the surface via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hyrdoxy succinimide (NHS) to form NHS-esters to link with an amine terminated biological recognition element or with the amine groups of a protein [7]. For the covalent attachment of the biological recognition element lacking the required functional groups such as thiols, linker molecules are commonly used [8,9]. These types of molecules form an intermediate

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layer between the biological recognition element and the surface. In addition, linker molecules provide stable and specific binding of the biological recognition element while orienting the biological recognition element towards solution, increasing its chance of capturing the target molecule [10]. 3,3′ Dithiodipropionic acid di (N-hydroxysuccinimide ester) (DSP) is one of the homobiofunctional cross-linkers used for surface functionalization [9]. It is a succinimidyl alkanedisulfide having a disulfide bridge within its structure, which cleaves upon binding to gold while N-hydroxy succinimide (NHS) ester forms a peptide bond to free amino groups of lysine residues of protein to be immobilized. It is a commonly preferred linker molecule, for enzyme and antibody immobilization to gold surfaces [8,9]. The importance of using DSP as a linker molecule is stated not only as providing the specific immobilization of the biological recognition element, but also for increasing the surface density of protein to be immobilized and orienting the biological recognition element towards solution compared to direct immobilization [10]. In addition, due to its NHS ester groups, DSP does not require EDC/NHS activation and is capable of direct biological recognition element immobilization. Although DSP is used to form an intermediate layer with the mentioned advantages, increasing the surface density of the protein or biological recognition element to be immobilized might result in reduced target binding due to steric hinderance. There were studies where $CH_3$-terminated and longer chain alkanethiols were used to dilute DSP and NHS-esters, respectively for enzyme immobilization and imaging single protein by AFM [11,12]. However, a study where DSP is diluted with a short-chain hydroxyl-terminated spacer with a similar length to DSP has not been encountered in literature. For biosensor applications, to enhance the transducer property, formation of mixed self-assembled monolayers (mSAMs) on gold surfaces via mixing short chain hydroxyl-terminated alkanethiols with thiol modified biological recognition elements, and testing the sensor properties with the target molecule is commonly used [13]. 11-Mercapto-1-Undecanol (MUO) and 6-mercapto-1-hexanol (MCH) are utilized short-chain hydroxyl-terminated alkanethiols for forming mSAMs and are used for two main reasons; 1) to avoid nonspecific DNA and protein bindings on surface 2) to be used as spacers between the biological recognition elements on surface so as to reduce the risk of steric hinderance and increase the target molecule recognition [4]. Although in previous studies, DSP alone or together with longer chain alkanethiols was used as an intermediate layer for biological recognition layer formation, DSP dilution with and short-chain hydroxyl-terminated spacers such as MCH, has not been encountered in literature. In this study, DSP:MCH mSAMs was used as an intermediate layer for the first time for the detection of thrombin using SPR. In our first study, DSP:MCH mSAMs formed with different molar ratios over the gold surface for developing an effective biosensor platform. BSA and lysozyme were used as model proteins to test the protein binding efficiency of mSAMs by using flow-cell coupled Surface Plasmon Resonance (SPR). These proteins at different concentrations were immobilized on maximum protein binding interface [DSP:MCH (2.0:2.5) mSAM] to deduce the optimum BSA and lysozyme concentration. The DSP:MCH mSAMs were also characterized simultaneously with SPR experiments using Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), X-ray photoelectron spectroscopy (XPS) and Atomic Force Microscopy (AFM). The results of this study were reported elsewhere [14]. In this study, to develop an SPR based immunosensor for thrombin detection, optimum mSAM interface [DSP:MCH (2.0:2.5) mSAM] was formed on gold surface. Then, anti-thrombin antibody was immobilized over the mSAM and the developed immunosensor was tested with the target protein thrombin. Biosensor preparation involving chemical and biological activation of the gold surface and testing the performance of the immunosensor with thrombin within a concentration range of 0.0–150.0 nM were performed by the continuous flow cell coupled to a SPR system. The developed biosensor with the DSP:MCH interface were able to detect thrombin within the physiologically relevant range of 1.0–500.0 nM, where thrombin is generated within this concentration range during the blood coagulation cascade for blood clotting. This model study was aimed to prove that the developed immunosensor can detect thrombin in the matrix of the blood serum environment. Therefore it has a great potential to detect thrombin during the blood coagulation cascade in the future.

## 2. Materials and methods

### 2.1. Materials

3,3′ Dithiodipropionic acid di(N-hydroxysuccinimide ester) (DSP), 6-mercapto-1-hexanol (MCH), dimethyl sulfoxide (DMSO) ≥99.9%, Human Serum Albumin (HSA), [2-(N-morpholino)ethanesulfonic acid] (MES), ethanolamine (≥ %98), thrombin from human plasma, human blood serum, diethyl pyrocarbonate, DEPC) ≥ %97, centrifugal filter unit with a cut-off value (MWCO) of 100 kDa, were provided from Sigma Aldrich (St. Louis, MO, USA). 0.2- μm cellulose filter was provided from Merck KgaA (Cork, Ireland). Absolute ethanol (EtOH) 99.5% was provided from Merck (Darmstadt, Germany). Thrombin polyclonal anti thrombin antibody (70 kDa) was obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

### 2.2. Methods

#### 2.2.1. Preparation of buffer and washing solutions

For preparing DSP:MCH mSAM solution in a molar ratio of DSP:MCH (2.0:2.5), DMSO:EtOH (20%:80%) was used as a solvent. Same solvent and 0.2-μm cellulose filtered deionized water were both used as wash buffers to remove nonspecific bindings from the biosensor surface. For anti-thrombin antibody solution, 0.05 M MES buffer was prepared and the pH was adjusted to 6.5 using 3 M NaOH. The same buffer was used as a wash buffer. For blocking the unreacted NHS-ester groups on the surface, 1.0 M ethanolamine prepared in 0.05 M MES buffer was used. For the preparation of thrombin and HSA solutions and a wash buffer to remove nonspecific bindings from the biosensor surface, 10 mM PBS buffer including 10 mM KCl, 50 mM NaCl (pH 7.4) was used, which will be referred to as PBS solution throughout the article. All the buffer solutions and deionized water were filtered with 0.2-μm cellulose filter, autoclaved at 121 °C for 15 min. The details of mSAM preparation by DSP:MCH over the gold surface were given in our previous study [14].

#### 2.2.2. SPR measurements

SPR measurements were performed by Spreeta SPR system (Texas Instruments, Inc., Dallas, Texas, USA) The system contains an SPR gold chip placed on a plastic prism with an integrated photodiode array detector (model TSPR1K23), flow cell with three channels, flow block assembly and a control box. Plastic prism used for coupling of the light to the surface plasmon is covered with the glass surface coated with 50 nm layer of gold, which is the sensing surface. LED is used as a light source emitting near-infrared light (830 nm peak wavelength) directed to the surface and reflected from the surface. Changes in the reflectivity observed due to an increase in mass on the sensor surface as a result of biomolecule adsorption, is measured in terms of change in the refractive index (RI) versus time. The system displays the changes in the refractive index within the range of 1.320–1.368, with a sensitivity of $5 \times 10^{-6}$ RI. Changes in the Response Units ($\Delta$RU) (1RU = $10^{-6}$ RI) versus time are obtained as a sensogram. Goldman syringe pump

system (Biasis Ltd. Sti, Ankara, Turkey) controlled by 4 port switching, three-way flow valves (Upchurch Scientific, Oak Harbor, USA), was coupled to Spreeta SPR system for gold surface modifications.

*2.2.3. Preparation of DSP:MCH mSAM interface*

The mSAM was formed on SPR gold chips by injecting the DSP:MCH (2.0:2.5) solution, through three channels with a flow rate of 10 μL $min^{-1}$, for 1,5 h. Following the formation of mSAM, the surface was first washed with DMSO:EtOH (20%: 80%) for 15 min followed by deionized water (1 h) at a flow rate of 50 μL $min^{-1}$ to remove nonspecific bindings. Subsequently the flow rate was decreased to 20 μl $min^{-1}$ and 0.05 M MES buffer (pH = 6.50) was introduced and the baseline was formed. In the following steps, 20 μl $min^{-1}$ was used as the flow rate.

*2.2.4. Anti-thrombin antibody immobilization on the DSP:MCH interface*

For preparing the immunosensor, 0.4 mg $mL^{-1}$ anti-thrombin antibody solution in 0.05 M MES buffer (pH = 6.50) was passed through the surface for the covalent attachment of anti-thrombin antibody to DSP:MCH (2.0:2.5) mSAM. The surface was then washed with the same buffer to remove nonspecific bindings. In order to block the unreacted carboxyl groups on the surface, 1.0 M ethanolamine solution prepared in 0.05 M MES buffer (pH = 6.50) was flown over the surface for 50 min. As the next step, PBS solution was passed through the surface and baseline was formed. The maximum protein binding DSP:MCH mSAM and anti-thrombin antibody concentration was selected according to the results of our previous study [14]. The anti-thrombin antibody (70 kDa) concentration was selected according to the interpolation results of BSA (67 kDa) and lysozyme (14 kDa) concentrations studied in the same study. The procedure for preparation of the biosensor surface (Sections 2.2.3 and 2.2.4) was followed before testing the performance of the immunosensor, selectivity experiment, and detection of thrombin in thrombin spiked blood serum samples.

*2.2.5. Testing the performance of the immunosensor*

Different concentrations of 500 μl thrombin solutions (0.0–150.0 nM) were injected over the immunosensor surface for 30 min until RU reached a plateau. Subsequently the surface was washed with the PBS solution to remove nonspecific bindings. The difference in RU between the baseline before thrombin injection and last washing step following thrombin injection gave the Change in the Response Unit (ΔRU) value. The calibration curve was obtained using mean triplicate measurement for each thrombin concentration.

*2.2.6. Testing the selectivity of the immunosensor*

In order to test the selectivity of the immunosensor, non-target protein HSA (66 kDa) was chosen as the model protein, which constitutes ≈50–60 of the total protein in human serum. The same protocol mentioned in Sections 2.2.3 and 2.2.4 was used for immunosensor preparation and 50.0 nM HSA was injected over the biosensor surface with a flow rate of 20 μl $min^{-1}$ for 30 min until RU reached a plateu. Subsequently the surface was washed with the PBS solution to remove nonspecific bindings. The ΔRU values obtained for the non-target protein 50.0 nM HSA and 50.0 nM target protein thrombin were compared.

*2.2.7. Detection of thrombin by the immunosensor in spiked blood serum samples*

Prior to detection of thrombin in blood serum samples, thrombin spiked blood serum samples were prepared. For testing the immunosensor, human blood serum was filtered using centrifugal filter unit with a cut-off value (MWCO) of 100 kDa and diluted with PBS solution (1:4) for a final concentration of 30.0 nM of thrombin.

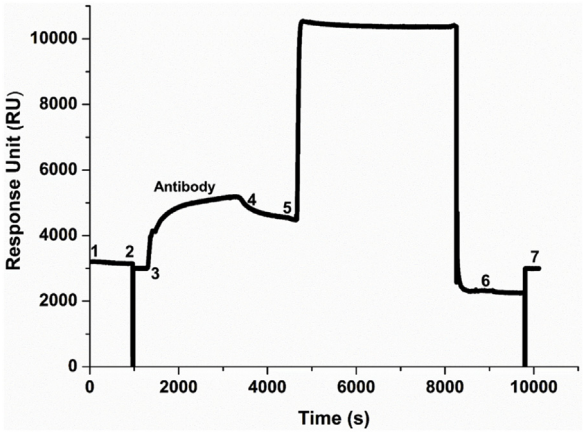


**Fig. 1.** Sensogram demonstrating the different steps of biosensor surface preparation after DSP:MCH mSAM formation (1) 0.05 M MES, pH = 6.50 buffer (2) baseline formation with 0.05 M MES, pH = 6.50 (3) immobilization of antibody (0.4 mg $mL^{-1}$) in 0.05 M MES, pH = 6.50 (4) Washing step with 0.05 M MES, pH = 6.50 (5) Ethanolamine in 0.05 M MES, pH = 6.50 (6) 10 mM PBS including 10 mM KCl, 50 mM NaCl (pH 7.4) (PBS solution) **(7)** baseline formation with PBS solution.

The same protocol mentioned in Sections 2.2.3 and 2.2.4 was used for immunosensor preparation and thrombin spiked blood serum sample was injected over the biosensor surface with a flow rate of 20 μl $min^{-1}$ for 30 min until RU reached a plateau. Subsequently the surface was washed with the PBS solution to remove nonspecific bindings. The ΔRU values obtained from the immunosensor for the thrombin spiked human blood serum sample (30.0 nM) and 30.0 nM thrombin in PBS solution were compared.

## 3. Results

### 3.1. Thrombin biosensor

*3.1.1. Anti-thrombin antibody immobilization on the DSP:MCH interface*

The anti-thrombin antibody concentration was selected according to the results of our previous study [14], as mentioned above in Section 2.2.4. The limit concentrations for lysozyme and BSA were found to be $5.7 \times 10^{-5}$ M and $8.96 \times 10^{-6}$ M respectively. In the light of these data, the interpolation results showed that optimum binding concentration for the anti-thrombin antibody (MW =70 kDa) was $6.36 \times 10^{-6}$ M (0.4 mg $mL^{-1}$). The different steps of immunosensor preparation following the formation of DSP:MCH (2.0:2.5) mSAM is demonstrated in a sensogram format **(**Fig. 1**)**.

The sensogram shows the RU versus time and the lettered arrows refer to different stages for biosensor surface preparation. In the first step, 0.05 MES buffer (pH = 6.50) was injected and the baseline was formed. Anti-thrombin antibody immobilization led to an increase in the RU. There was a decrease in the signal after washing with 0.05 MES buffer (pH = 6.50) due to the removal of loosely bound molecules. Ethanolamine solution prepared in 0.05 M MES buffer was then injected to block the unreacted carboxyl groups. Following the blocking step, PBS solution was injected and there was a decrease in the signal due to a buffer switch from MES to PBS. Finally at the last step, the baseline was formed with PBS prior to thrombin injection.

*3.1.2. Testing the performance of the immunosensor*

Following the preparation of the biosensor surface, immunosensor was tested for its capability to bind the target protein thrombin. The ΔRU values obtained from the immunosensor as a result of thrombin binding at a concentration range of 0.0–150.0 nM is shown in Fig. 2a. According to the results of the calibration curve,

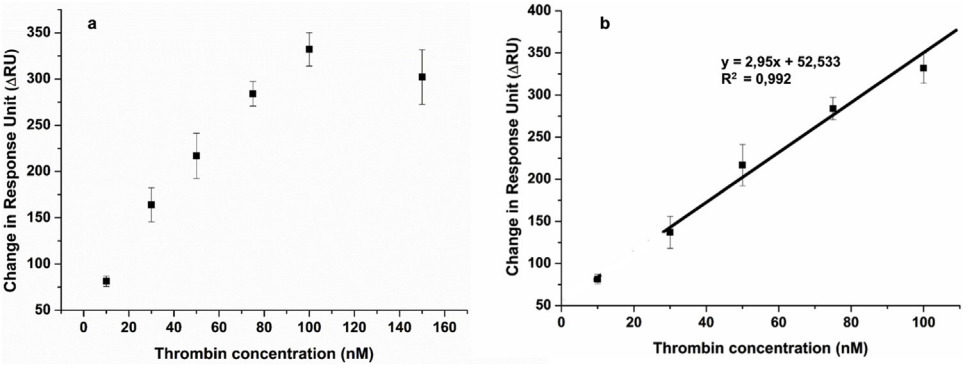


**Fig. 2.** a) ΔRU of immunosensor for thrombin at a concentration range of 0.0–150.0 nM (n = 3). b) Linear detection range for the immunosensor for thrombin.

the linear detection range was found to be 30.0–100.0 nM showing a good correlation between the thrombin concentration and the ΔRU values ($R^2$ =0,992) (Fig. 2b). The saturation concentration for the prepared immunosensor was 150.0 nM.

The limit of detection (LOD) for thrombin was calculated according to Eqs. (1) and (2) [15] and determined to be 6.0 nM.

$$Sm == S_{bl} + ks_{bl,} \quad (1)$$

Sm: minimum signal distinguished from noise
$\bar{S}_{bl}$: average ΔRU value from blank samples measurements
k (constant) = 3
$s_{bl}$: standard deviation from blank samples measurements

$$c_m = (Sm- = S_{bl})/m = ks_{bl}/m = 3s_{bl}/m \quad (2)$$

$c_m$: LOD concentration, m: slope

Limit of Quantification (LOQ) was determined as 30.0 nM according to the following equation [15],

$$LOQ = 10s_{bl}/m \quad (3)$$

### 3.1.3. Testing the selectivity of the immunosensor

Following the preparation of the biosensor surface, selectivity of the immunosensor was tested with a non-target protein, HSA. In the initiation phase of thrombin generation under physiological conditions, thrombin concentration can range from 1.0 nM to 500.0 nM. The selected concentration (50.0 nM) for thrombin is within this range [16]. HSA constitutes %50 of the total protein content of human serum. Although 50.0 nM concentration for HSA does not reflect the real concentration of HSA in human serum, the selectivity experiment was performed to make a comparison of the ΔRU values obtained from the immunosensor for thrombin and non-target HSA at the same concentration. ΔRU values obtained from the immunosensor for thrombin and HSA at the same concentration were 217 ± 24 ΔRU and 76 ± 7 ΔRU, respectively (Fig. 3).

Although some nonspecific binding of HSA in PBS solution occurred, ΔRU value obtained for HSA is below the ΔRU values corresponding to the LOQ concentration for thrombin. The results showed that the designed immunosensor was shown to have higher selectivity towards thrombin in PBS solution, therefore the immunosensor was also tested in blood serum samples to observe how thrombin was competing with HSA in the complex environment of the blood serum.

### 3.1.4. Detection of thrombin by the immunosensor in spiked blood serum samples

There is not a technique which directly quantitates endogenous thrombin in blood serum sample, whether the blood serum is drawn from an healthy individual or a thrombotic patient due to the dynamic nature of thrombin. It is a protein that occurs

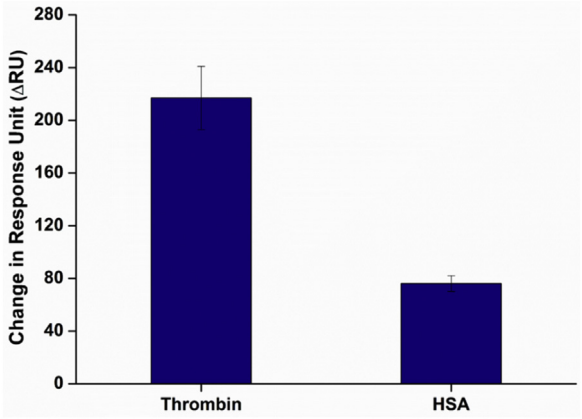


**Fig. 3.** Selectivity of the immunosensor for thrombin by comparing ΔRU values for 50.0 nM target protein (thrombin) and 50.0 nM non-target protein (HSA) in PBS (n = 3).

at the site of vascular injury to initiate blood coagulation cascade. *Instead of direct quantitation of thrombin,* INR (International Normalized Ratio for blood clotting time) tests or PTT (Prothrombin time test) may be applied to measure the duration of blood coagulation in an individual for determining a possible risk for thrombosis (personal communications with medical doctors and biochemists of Faculty of Engineering). The results of these INR tests may then be correlated with thrombin concentration. As will be discussed in detail in the discussion part, the circulating thrombin in the body of an healthy individual, is only at picomolar (pM) concentrations under noncoagulation state. The linear detection range of the immunosensor that was developed in this study was 30.0–100.0 nM. Therefore, with this particular immunosensor inorder to detect thrombin in blood serum; the blood serum is required to be spiked with nM concentration of thrombin within the linear detection range and the immunosensor may be tested with this sample. Therefore the immunosensor was tested using thrombin spiked blood serum samples. The steps of thrombin addition to the human blood serum is explained below in detail.

In order to minimize the complexity of the human blood serum, the blood serum was passed through a centrifugal filter unit with a MWCO of 100 kDa, to remove large non-target molecules. In this form, some other blood serum components with molecular weights < 100 kDa still existed in blood serum. The filtered blood serum was then diluted with PBS solution in 1:1, 1:4, 1:8 proportions. Sensor response for each blood serum dilution was then compared with PBS solution. Since PBS is devoid of any proteins that human blood serum contains, no binding of any blood serum protein to the surface is expected upon its injection to surface. The sensor response difference between the blood serum:PBS(1:4) and PBS solution was

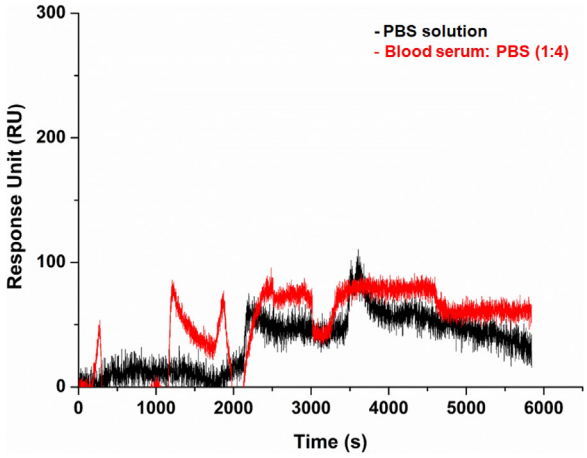


**Fig. 4.** Comparison of the sensor response of the immunosensor for PBS (-) and blood serum diluted with PBS in 1:4 proportion (blood serum:PBS (1:4)) (-).

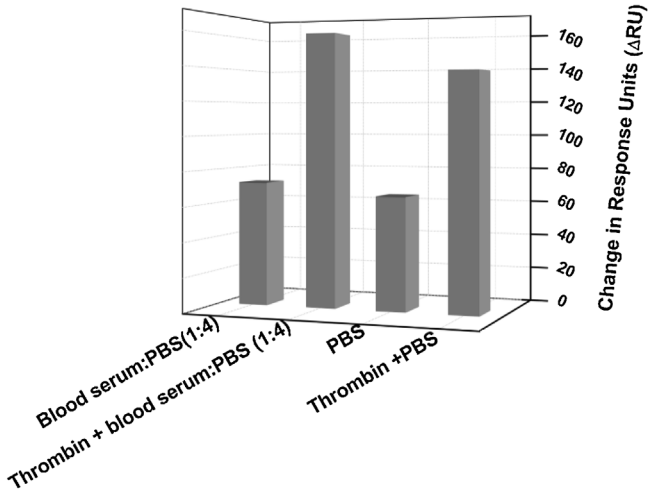


**Fig. 5.** Comparison of the sensor response for PBS solution, blood serum:PBS(1:4) dilution (without thrombin), thrombin spiked PBS solution and thrombin spiked blood serum:PBS(1:4) dilution (n = 3).

observed to be negligible (Fig. 4), indicating nonspecific binding of the blood serum matrix components from the blood serum:PBS (1:4) to the immunosensor was minimum.

Therefore this blood serum:PBS dilution was used in the preparation of thrombin spiked human blood serum samples. The human blood serum was filtered with centrifugal filter and thrombin was spiked in blood serum and diluted with PBS solution for a final concentration of 30.0 nM. Following the preparation of biosensor surface, baseline was formed with PBS solution and 30.0 nM thrombin spiked blood serum:PBS(1:4) solution was injected and sensor response was obtained (Fig. 5).

Fig. 5 compares the sensor response for PBS and blood serum:PBS(1:4) solution in addition to thrombin added PBS and thrombin added blood serum:PBS(1:4) solution (both solutions for a final concentration of 30.0 nM) in three replicates. ΔRU values for PBS and blood serum:PBS(1:4) solution prior to thrombin addition were 65 ± 13 and 72 ± 5 respectively. ΔRU values for thrombin added PBS and thrombin added blood serum:PBS(1:4) solution were 137 ± 19 and 159 ± 16 respectively. As mentioned above, human blood serum was filtered using centrifugal filter unit with a cut-off value (MWCO) of 100 kDa. Therefore proteins with a molecular weight above 100 kDa such as fibrinogen, and high molecular weight globulins were removed from the human blood serum, where as proteins with a molecular weight of 66–67 kDa such as HSA and hemoglobin remained in the human blood serum. In spite of the existence of blood serum components in blood serum:PBS(1:4), the sensor response for PBS and blood serum:PBS(1:4) was found to be very similar, showing that similar amounts of interference were coming from PBS and blood serum:PBS (1:4). When PBS and blood serum:PBS (1:4) were spiked with the same concentration of thrombin, the increase in the sensor response was again similar, ~47% and ~45% respectively (Fig. 5). The results indicated that the immunosensor showed high selectivity for thrombin regardless of the complexity of the blood serum matrix. The reason for the 22 unit difference between the sensor responses for thrombin added PBS solution and thrombin added blood serum:PBS(1:4) solution could be the nonspecific interaction of thrombin with other blood serum matrix components in blood serum:PBS(1:4) solution. HSA, hemoglobin or other blood serum matrix components found in blood serum:PBS (1:4) solution could be associating with thrombin and binding to anti-thrombin antibody as a molecular complex leading to a slightly higher ΔRU signal (Fig. 5). The nonspecific interaction of the analyte with other proteins in blood serum has been discussed extensively in a recent review [17]. The review discusses two possible situations. The first possibility is that the nonspecific interaction of the analyte with other proteins in blood serum may block the antibody binding site of the analyte, decreasing its binding to the antibody. This leads to a smaller response of the SPR sensor for analyte detection in blood serum, in comparison to analyte detection in PBS. The second possibility is that the nonspecific interaction of the analyte with other proteins in blood serum results in a molecular complex, yet it does not affect the binding of the analyte to the antibody. However due to the formation of this molecular complex, a slightly larger response of the SPR sensor is obtained for analyte detection in blood serum in comparison to analtye detection in PBS. Therefore in this study, the slight SPR response difference between the thrombin added PBS solution and thrombin added blood serum:PBS(1:4) solution (Fig. 5) is explained by the second case scenario.

## 4. Discussion

In this study, we developed an immunosensor for the detection of thrombin in PBS solution and thrombin spiked blood serum samples. A new interface was used for the immobilization of anti-thrombin antibody. Polyclonal antibody was used as the biological recognition element for the preparation of the immunosensor. Although this anti-thrombin antibody showed some binding to non-target protein HSA in PBS solution, it was highly selective towards thrombin in thrombin spiked blood serum samples (Fig. 5). The designed immunosensor had a linear detection range of 30.0–100.0 nM with an $R^2$ value of 0,992. The LOD and LOQ values were 6.0 nM and 30.0 nM respectively. A comparison is made between previously developed label-free biosensors and label based and/or label based sandwich detection format biosensors for detection of thrombin and our immunosensor (Table 1).

To our knowledge, for thrombin detection, aptamers are commonly prefered as biological recognition element for label-free biosensors [7,13,18,19] as well as for label based [20–22] (Table 1) and/or for label based sandwich detection format biosensors [23–27,29] (Table 1). Instead, antibodies are usally preffered in designing label based sandwich detection format biosensors [28] as shown in Table 1.

**Label-free biosensors:** Label-free detection involves the detection of the target biomolecule - biological recognition element interaction without labeling the biological recognition element or the target biomolecule [7]. The label-free biosensors were listed in Table 1 for comparison of their LOD value and linear detection range with the currently developed immunosensor The study of Polon-

**Table 1**
Comparison of the previously developed biosensors and the currently developed immunosensor and aptasensor for thrombin detection.

| Target molecule | Biological recognition element | Detection format | Biosensor type | Linear detection range | LOD | Reference |
|---|---|---|---|---|---|---|
| Thrombin | Aptamer | Label-free | SPR | – | 2.5 -3.0 nM<br>5.0 -5.5 nM | [18] |
| Thrombin | Aptamer | Label-free | SPR | 5.0 -1000.0 nM | – | [7] |
| Thrombin | Aptamer | Label-free | SPR | 50.0-200 nM (thrombin spiked serum samples) | 25.0 nM (buffer)<br>50.0 nM (serum/buffer) | [19] |
| Thrombin | Aptamer | Label-free | Electrochemical | 5.0 -35.0 nM | 2.0 nM | [13] |
| Thrombin | **Antibody** | **Label-free** | **SPR** | **30.0 -100.0 nM** | **6.0 nM** | **Currently developed immunosensor** |
| Thrombin | AuNP/ peptide/ recombinant protein-luciferase | Label based | Bioluminescence based | 8.0 – 8.0 × $10^3$ nM(thrombin spiked urine and buffer samples) | 80.0 pM | [20] |
| Thrombin | Aptamer-complementary DNA strand/QD | Label based | Electrochemical | 2.7 aM-27.0 nM | 2.7 aM | [21] |
| Thrombin | QD/aptamer | Label based | Electrochemical | 0.5-12.5 pM | 0.5 pM | [22] |
| Thrombin | Gold surface /aptamer (1) and AgNP/aptamer (2) | Label based sandwich assay | Absorbance based | 20.0 – 5000.0 pM | 20.0 pM | [23] |
| Thrombin | AuNP /$Fe_3O_4$ nanohybrid / aptamer (1) and glucose labelled aptamer (2) | Label based sandwich assay | LSPR | 0.5 -10.0 nM | 0.2 nM | [24] |
| Thrombin | QD-aptamer (1) QD-aptamer (2) | Label based sandwich assay | Fluorescence based | 5.0 nM-500.0 nM | 2.6 nM | [25] |
| Thrombin | Gold surface /aptamer (1) and AuNP/aptamer (2) | Label based sandwich assay | SPR | 0.1-75.0 nM | 0.1 nM | [26] |
| Thrombin | Gold surface /aptamer (1) and gold nanoparticle /aptamer (2) | Label based sandwich assay | SPR | 1.0 fM- 1.0 pM (cages)<br>10.0 aM-10.0 fM (rods)<br>1.0 aM- 1.0 fM (quasi-spherical NPs) | 1.0 aM (quasi-spherical NPs)<br>10.0 aM (rods) | [27] |
| Thrombin | Gold surface /antibody and gold nanorod /aptamer | Label based sandwich assay | SPR | 0.1 - 2.0 aM | 0.1 aM | [28] |
| Thrombin | Gold surface /aptamer (1) and /aptamer (2) | Label based sandwich assay | SPR | 1.0 aM- 0.1 pM | 1.0 aM (with RCA amplification) | [29] |

schii 's group demonstrates one of the few studies involving the comparison of the newly developed interfaces with the commonly utilized interfaces [18]. SPR gold chips modified with carboxyl end cross-linked BSA (cBSA) films and commercially available carboxylmethyl dextran were both used for immobilizing thrombin aptamer and compared in terms of their thrombin detection performance. Biotin-labelled thrombin aptamer were immobilized on streptavidin and avidin coated cBSA film and commercially available carboxylmethyl dextran modified surfaces. LODs of the streptavidin coated cBSA film and streptavidin coated carboxylmethyl dextran modified surfaces were 3.0 nM and 2.5 nM respectively. The LODs of the avidin coated surfaces were determined in the range of 5.0–5.5 nM. Zheng et al. used a well known procedure for SPR biosensor development for direct detection of thrombin, where SAM modified surfaces were activated by EDC/NHS activation [7]. [3-mercaptopropionic acid (MPA)] SAMs were activated with EDC/NHS and amine-modified thrombin aptamer was immobilized. The developed biosensor was tested for thrombin detection at various concentrations. The linear detection range and the LOD value were determined to be 5.0–1000.0 nM and 5.0 nM, respectively. Mani et al. used bi-cell SPR platform for thrombin aptamer immobilization where samples were passed through a low volume flow cell for directly detecting thrombin [19]. The LOD for thrombin detection in buffer and blood serum diluted buffer was 25.0 nM and 50.0 nM respectively. Radi et al. generated a biological recognition layer by forming SAM layers on gold electrode using thiol modified aptamer and 2-mercaptoethanol and added target protein thrombin [13]. Using the $[Fe(CN)_6]^{4-/3-}$ redox couple, the electron transfer resistance at each modification step, including thrombin binding was measured by electrochemical impedance spectroscopy. The LOD of the developed biosensor was 2.0 nM with a linear detection range of 5.0–35.0 nM.

As a summary, the LOD values of previously developed label-free biosensors ranged within the concentrations of 2.0–50.0 nM [7,13,18,19].The LOD value of the present immunosensor was 6.0 nM and is within this range. The linear detection ranges of two of these label-free biosensor were comparable [13,19] (Table 1) with the linear detection range of the currently developed immunosensor (30.0–100.0 nM), whereas only one of these label-free biosensors had a linear detection range of nearly 3 orders of magnitude [7] (Table 1).

The LOD and linear detection ranges of the previously developed label-based and label-based sandwich detection format biosensors were also listed and compared with the currently developed immunosensor (Table 1).

**Label-based biosensors:** Label-based detection involves the detection of the target biomolecule - biological recognition element interaction by labeling the biological recognition element

or the target biomolecule via fluorescent, nanoparticle, enzymatic labelling of the target or biological recognition element [21]. Chen et al. developed a thrombin biosensor using bioluminescence energy transfer (BRET) technique [20]. Luciferase enzyme linked to a short peptide was immobilized on AuNP and AuNP quenched the luciferase catalyzed bioluminescence. In the presence of thrombin, short peptide was cleaved due to thrombin's enzymatic activity, releasing luciferase enzyme and resulting in recovery of bioluminescence. There was a direct correlation with bioluminescence signal and thrombin concentration. The linear detection range of the developed biosensor in both spiked human urine and buffer samples was 8.0 nM-8.0 μM with an LOD of 80.0 pM. Lu et al.2015 developed a bi-functionalized aptasensor for thrombin detection [21]. Thiol-terminated TBA15 was hybridized to its biotin labelled complementary ssDNA to form a dsDNA and immobilized to gold surface. Then the distal end of dsDNA labelled with biotin was bound to avidin labelled QD via biotin-avidin interaction. Thrombin addition and its interaction with TBA15 dissociated the avidin labelled QD from the surface resulting in a decrease in the electrochemical signal. The developed biosensor had an LOD of 2.7 aM with a linear detection range of 2.7 aM-2.7 nM. Hansen et al. developed a label-based biosensor where thrombin was labelled instead of the biological recognition element. Thrombin aptamer was covalently immobilized on gold surface and QD-labelled and non-labelled thrombin was injected on the surface and change in the electrochemical signal was measured. The developed biosensor had an LOD of 0.5 pM with a linear detection range of 0.5–12.5 pM [22]. According to the results of the previous studies summarized in Table 1, as expected, when biological recognition elements were labelled with a fluorescent marker or a nanoparticle, lower LOD (LOD values ranging from aM to pM) were observed [20–22] due to amplification of the signal and increase in signal to noise (S/N) ratio. The linear detection ranges were extended to 2–3 orders of magnitude [20,22] compared to our immunosensor (1 order of magnitude), with the exception of one study where the linear detection range of 10-orders of magnitude was reported [21].

**Label-based sandwich detection format biosensors:** Sandwich detection format is commonly used for improving the detection limit of SPR biosensors consisting of two steps. One biological recognition element is immobilized on the surface and the target solution is brought in contact with the surface where target-biological recognition element binding occurs. The sensor surface is then incubated with a second biological recognition element with a different binding site to the target. The binding of the second biological recognition element to the previously captured target increases the number of bound biomolecules and increases the SPR sensor response [23]. Zhao et al. developed a colorometric assay based thrombin biosensor depending on color change due to silver nanoparticle (AgNP) aggregation [23]. Biotin labelled TBA15 was immobilized on streptavidin modified microfluidic channels. The mixture of thrombin and TBA29 modified AgNPs were injected in microfluidic channels. The sandwich formation between TBA15 and TBA29 due to thrombin also resulted in AgNP aggregation and a color change visible by naked eye or flatbed scanner. The LOD of the developed biosensor was 20.0 pM with a linear detection range of 20.0–5000.0 pM. Yan et al. combined AuNP/ $Fe_3O_4$ nanohybrid and enzyme labelled aptamer for SPR signal amplification [24]. TBA29 was covalently attached to AuNP/ $Fe_3O_4$ nanohybrid structure and captured thrombin in solution. Glucose oxidase enzyme labelled TBA15 recognized another epitope binding region on thrombin and formed a sandwich complex. The surface was then incubated with glucose and $HAuCl_4$ starting the $Au^{+3}$ to $Au^0$ conversion leading to an increase in AuNP size due to $Au^0$ deposition on the AuNP surface, resulting in an absorption peak shift and color change. The developed biosensor had a linear detection range of 0.2–10.0 nM with an LOD of 0.2 nM for thrombin. Yin et al. developed a thrombin biosensor using QD as a fluorescence marker [25]. Thrombin binding aptamers TBA1 and TBA2 with bioaffinity to thrombin at different recognition sites were labelled with QDs forming QD-TBA1 and QD-TBA2, respectively. In the presence of thrombin, QD dimers or oligomers were formed with bigger sizes resulting in an increase in the characteristic diffusion time of QDs in the detection volume of fluorescence correlation spectroscopy (FCS). The developed biosensor had a linear detection range of 5.0–500.0 nM with an LOD of 2.6 nM. Bai et al. developed a thrombin biosensor using AuNPs and SPR gold films [26]. The AuNP and SPR gold films were utilized together for SPR signal amplification aiming to decrease the LOD. TBA15 was covalently attached to streptavidin modified gold surface and TBA29 was covalenty attached to AuNP. Thrombin addition resulted in double aptamer sandwich structure and enhanced the SPR signal due to plasmonic coupling between the AuNP and SPR gold films. The enhancement in the SPR signal was directly proportional to thrombin concentration. The developed thrombin biosensor had a linear range of 0.1–75.0 nM with an LOD of 0.1 nM. Kwon et al. compared the effect of different nanoparticles(NPs), plasmonic coupling of gold surface and nanoparticles of different sizes and shapes on SPR signal amplification [27]. Thrombin aptamer covalently attached to gold surface and anti-thrombin antibody labelled with NPs with different sizes and shapes were used in the sandwich assay format for thrombin detection. The LOD and the linear detection range of the developed SPR biosensors ranged according to the size/shape of the NP used. The LOD values were 1.0 aM and 10.0 aM respectively when (quasispherical NPs and rod shaped NPs were used. The linear detection ranges of the developed SPR biosensors when nanocages, nanorods and quasispherical NPs were used were 1.0 fM-1.0 pM, 10.0 aM-10.0 fM, 1.0 aM-1.0 fM, respectively. Baek et al. tested dual nanoparticle enhancement approach for its SPR signal amplification capacity [28]. Following thrombin capture on anti-thrombin antibody modified gold surface, thrombin specific DNA aptamer with a polyadenine (A30) tail bound to captured thrombin in a sandwich assay format. Controlled hybridization adsorption of polythymine-NP conjugates (T20-NPs) folllowed by polyadenine-NP conjugates (A30-NP) resulted in thrombin detection at subattomolar concentrations. The LOD of the biosensor was 0.1 aM with a narrow linear detection range (0.1–2.0 aM). He et al. developed an aptasensor where rolling circle amplification (RCA) technique and bio-bar-coded AuNP enhancement was used to detect human α-thrombin [29]. Sandwich assay format was used where the first aptamer was covalently attached to gold surface as a capture probe for thrombin. The second aptamer modified with a primer sequence recognized another region on thrombin and bound to captured thrombin. The primer sequence served as the primer for the RCA reaction. A linear RCA reaction was then initiated with Phi29 DNA polymerase and deoxyribonucleotide triphosphate (dNTPs), producing hunderds of tandem-repeat sequences named as the RCA product. A DNA sequence complementary to primer (probe) was labellled with AuNP and incubated with these RCA products. AuNP labelled bio-bar-coded probes were assembled on the RCA product, which resulted on SPR signal enhancement due to increasing the mass and refractive index. The LOD of the developed biosensor without the amplification was 1 nM, with bio-bar-coded AuNPs alone the LOD decreased to 0.1 pM, and with bio-bar-coded AuNPs-RCA amplification the LOD decreased even further to 1.0 aM.

The detection limits were further decreased with the development of biosensors with sandwich detection formats (LOD values ranging from lower aM to pM) [23,27,28](Table 1), although some of these biosensors had higher LOD values in the low nM range [24–26] (Table 1). The linear detection range in most of the mentioned studies were to 2–3 orders of magnitude [24–28] (Table 1), similar to the label-based biosensors where only one label was

used. However in one study, the linear detection range extended to 5 orders of magnitude [29].

The steepness of the calibration curve define the sensitivity of an instrument or a method. If the tool for sensing is a bioensor for example, as the calibration curve is steeper, by definition the biosensor has a better capacity to discriminate between closer concentrations of the antigen to be detected and hence a better sensitivity (lower LOD) with a compromise from linear detection range [15]. On the contrary, the decrease in the slope of the calibration curve increases the linear detection range although this time the sensitivity decreases. For the label-based/label-based sandwich detection format biosensors listed in Table 1, although the LOD values are lower than the LOD values obtained from label-free biosensors, the linear detection ranges were unexpectedly extended to 2–3 orders of magnitude. However, it should be stated that, in many of these studies linear detection ranges has the lower limit as equal to LOD, while this number should correspond to LOQ not LOD. In some other works, on the other hand, the lower limit seems to be 100-fold of LOD; actually this number should be close to LOQ, which is 3.3-fold of LOD [20].

The sensitivity of the present immunosensor may be further improved with trials of different detection formats. For example, the LOD values of the currently developed immunosensor using the DSP:MCH interface may be improved by using sandwich detection format where the second biological recognition element may be labelled with an enzyme, QD or a gold nanstructure such as a gold nanorod and incubated with the thrombin bound biological recognition element immobilized sensor surface. In future a DSP:MCH interface may also be prepared on nano-surfaces (AuNPs, AgNPs, nanorods etc.) to increase the surface area for more biological recognition element binding, which may extend the linear detection range of the developed immunosensor.

Previous studies have investigated the effect of utilizing different biological recognition elements, detection formats and labels on the improvement of LOD values and the linear detection range [20–26]. On the contrary, fewer studies involve the comparison of different interfaces for improving the biosensing platform [18], although different types of mSAMs are characterized with surface characterization techniques [4]. The biological recognition elements are either directly immobilized on the sensor surface or immobilized with the commonly utilized interfaces such as carboxy methyl dextran or mercaptoundecanoic acid (MUA) both requiring EDC/NHS activation [26,27], where additional experimental steps increases the surface preparation time. Considering the fact that, interface determines the maintenance of biological activity of the biological recognition after immobilization, the proximity of the biological recognition element to the transducer and the stability of the recognition layer, the comparison of the newly developed interfaces with the commonly utilized interfaces are required for improving the biosensor applications. This study was focused on improving the sensor performance of thrombin biosensors using our previously developed DSP:MCH interface. The selectivity of the developed immunosensor to the target protein thrombin in a blood serum matrix was also compared with thrombin detection in PBS solution and discussed. Although the sensor responses of the developed immunosensor towards thrombin spiked blood serum:PBS (1:4) and thrombin added PBS solution were close to each other, there was some nonspecific binding of HSA in PBS solution to the immunosensor surface. The selectivity of immunosensor may be increased by increasing the duration time for blocking the unreacted NHS-ester groups on the surface or changing the type of the agent used for blocking.

During the blood coagulation process, thrombin generation occurs in three different phases: initiation, propagation and termination at the site of vascular injury [16]. During the initiation phase, prothrombin (inactive form of thrombin) is converted to thrombin via Factor Xa (FXa) and thrombin is produced in minute amounts. This process activates platelets and procofactors for generating more thrombin, the so-called thrombin burst via positive feed-back mechanisms in the propagation phase. At the termination phase, thrombin concentration declines via inhibitors and thrombin generation is terminated [16]. The initiation phase occurs when thrombin concentration reaches 10.0 nM and is followed by the propogation phase. During the stage of initiation and propogation phase of the blood coagulation cascade, the physiological thrombin concentration can range within 1.0–500.0 nM [16]. In healthy individuals, thrombin is circulating in the body at picomolar (pM) concentrations as a precaution to initiate thrombin generation through the thrombin burst mechanism at the site of vascular injury [30]. In the case of vascular injury the levels of thrombin reaches nanomolar (nM) levels to start the blood coagulation cascade. There is a risk of thrombosis, if unhibited thrombin in the range of 5.0–20.0 nM escapes the injury site and enters the blood circulation. The circulation of thrombin in blood above the upper limit of this range (> 20.0 nM), thrombosis occurs [30]. The designed immunosensor has a linear detection range of 30.0–100.0 nM for thrombin that is within the physiologically relevant range of 1.0–500.0 nM during the blood coagulation cascade. Therefore this immunosensor may be informative about abnormalities in thrombin generation during coagulation and also diseases such as hemophilia or thrombosis in future.

## 5. Conclusion

The aim of this study was to test the performance of a new interface as a detection platform and thrombin was used as a model for detection. DSP:MCH interface was formed on gold surfaces for anti-thrombin antibody immobilization for the development of an immunosensor. The designed immunosensor was tested with various concentrations of thrombin. The LOD of the immunosensor was determined to be 6.0 nM, with a comparable sensitivity to previously designed SPR sensors using label-free detection format. The results of this study showed that the sensor responses obtained from both PBS and blood serum:PBS(1:4) were similar. Following the addition of same concentration of thrombin, the increase in sensor response for both solutions were also comparable. This demonstrated that the designed immunosensor was capable of detecting thrombin with high and similar selectivity regardless of the matrix of the solution, whether it is PBS or the complex blood serum matrix consisting of variety of proteins. The selectivity of the immunosensor may be improved via increasing the duration for injection of the blocking solution and hence nonspecific binding may be minimized. With further improvement, this interface may also be used for examining the binding interactions of other biological recognition elements and their target molecules for the development of new biosensors.

## Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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