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Regenerative Surface Plasmon Resonance (SPR) biosensor: Real-time measurement of fibrinogen in undiluted human serum using the competitive adsorption of proteins

Ran Wang*, Arad Lajevardi-Khosh, Seokheun Choi, Junseok Chae

School of Electrical, Computer, and Energy Engineering, Arizona State University, AZ 85287, USA

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ABSTRACT

Epidemiological studies suggest that elevated plasma fibrinogen levels are associated with an increased risk of cardiovascular disorders. Normal fibrinogen level is in the range of 1.5–4.5 mg/mL, depending upon both genetic (intrinsic) and environmental (extrinsic) factors. An increase of 0.25 mg/mL from the normal level can often be correlated with a high risk of cardiovascular disease. Thus, it is useful to monitor fibrinogen level in serum of a patient for clinical diagnosis. We report a regenerative biosensor that measures real-time fibrinogen levels in undiluted serum. The biosensor uses Surface Plasmon Resonance (SPR), highly sensitive optical technique. The biosensor does not use bio-receptors (i.e., antibodies, enzymes, DNA, etc.) unlike conventional biosensors, and deploys the nature of competitive adsorption of proteins to achieve selective detection of fibrinogen. We measured fibrinogen-spiked serum samples with a concentration of 1.5–4.5 mg/mL, and repeated six measurement trials to obtain statistical distribution of the measurements using the regeneration method of the sensing surface. The SPR biosensor has a sensitivity of 42 mDeg/(mg/mL) for a fibrinogen concentration in the range of 0.5–2.5 mg/mL, whereas it was hard to correlate the measurements to the spiked-fibrinogen samples of above 2.5 mg/mL.

1. Introduction

Biosensors are used in many areas such as medicine, biotechnology, environmental monitoring, food industry, and military technology (Choi and Chae, 2010). In particular, a biosensor used for protein detection is essential for clinical diagnostics and drug development (Ly and Tao, 2006). Protein microarray chips, as actively used in protein biosensors, allow analysis of thousands of molecules of interest simultaneously, thus deliver high throughput protein analysis (Glokler and Angenendt, 2003). Nevertheless, limitations persist due to the protein array chips' difficulty in detecting very low concentration of biomarker proteins and finding appropriate bio-receptors which have high specificity to immobilize the biomarker proteins (Han et al., 2006; Daniels and Pourmand, 2007).

Extensive research efforts focus on developing a protein biosensor possessing both high selectivity and sensitivity (Choi et al., 2008). Sensitivity is determined by transducers that convert the immobilization process to an alternate form of signal, such as an optical, mechanical, or electrical signal. Transducers may be broadly categorized as a labeled or label-free mechanism. The label-free mechanism is more frequently preferred over the labeled mechanism as the labeling process is time-consuming, labor-intensive, often difficult to achieve accurate quantification, and vulnerable to modifying target proteins characteristics and altering their behavior (Haab, 2003; Yu et al., 2006). Among various label-free mechanisms, SPR (Surface Plasmon Resonance) is a surface-sensitive analytical tool responding to minute changes in refractive index occurring adjacent to a metal film, offering detection limits up to few ppt ($pgmL^{-1}$). In this way, immobilization of proteins on the surface and their subsequent adsorption interactions may be monitored in real-time without labeling (Bally et al., 2006).

While many transducers, including SPR, offer high sensitivity, the low selectivity of these protein biosensors is a persistent challenge. Selectivity is determined by the bio-receptors, such as antibodies, protein lysates, lectins, peptides, and aptamers (Chaerkady and Pandey, 2008), that selectively capture target entities. However, these bio-receptors are often constrained by their weak binding affinity with analytes, non-specific adsorption, low reproducibility, and difficulty with integration on to the transducer (Han et al., 2006; Daniels and Pourmand, 2007; Choi et al., 2008, 2010). We utilize the competitive adsorption of proteins, which is a process driven by thermodynamics, to aim for a high selectivity for the SPR biosensor without the need for any bio-receptors. The competitive adsorption of proteins, termed the Vroman effect, was reported by Vroman and Adams in 1969, and many researchers

* Corresponding author. Tel.: +1 4804065818.
E-mail address: rwang32@asu.edu (R. Wang).

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have studied the Vroman effect since then (Jung et al., 2003; Willems et al., 1991; Turbill et al., 1996; Krishnan et al., 2004; Noh and Vogler, 2007). The Vroman effect has been studied from many different aspects, including the adsorption from complex mixtures, protein sensing, the interplay of substrate surface to proteins, and the interaction artificial materials to the blood (Choi et al., 2010; Slack and Horbett, 1989; Leonard and Vroman, 1992). The competitive nature of protein adsorption onto the surface, primarily relies upon a protein's molecular weights (Vroman and Adams, 1969). Thus, low-molecular weight proteins are displaced by high-molecular weight proteins and/or more strongly interacting proteins.

Substantial evidence from epidemiological studies suggests that elevated plasma fibrinogen levels are associated with an increased risk of cardiovascular disorders, including ischaemic heart disease (IHD), stroke and other thromboembolism (Meade et al., 1986; Wilhelmsen et al., 1984). Currently deployed methods to measure fibrinogen can be classified into two groups: 'functional' and 'direct'. Functional methods measure the coagulation time of blood, which is proportional to the fibrinogen concentration. The most widely used method in functional fibrinogen assay in most clinical laboratories is the Clauss method, total clottable protein assay, which records the elapsed time to reach the coagulation end point. Direct methods quantify fibrinogen concentration directly, either immunologically, gravimetrically or by precipitation (by heat or salting out) (Nieuwenhuizen, 1995; Kamath and Lip, 2003). From dissimilar epidemiological studies that used different measure methods, the fibrinogen concentration in healthy individuals was identified to be in the range of 1.5–4.5 mg/mL. The fibrinogen concentration is dependent upon both genetic (intrinsic) and environmental (extrinsic) factors that include gender, age, body mass index and body habitus, metabolic syndrome, physical exercise, seasonal changes, vitamin C, infection, psychosocial factors, hormonal status, smoking and alcohol use (Kamath and Lip, 2003).

Quantifying fibrinogen concentrations has been typically executed by a series of measurements with disposable sensor units (Asanov et al., 1998). However, variation occurs due to batch-to-batch irreproducibility and uncontrollable experimental conditions, which generates unalterable false-positive/negative responses (Choi and Chae, 2009). It is necessary to perform a series of measurements from a patient's blood sample to reduce these variations; this motivates our research to design a fibrinogen biosensor of having a regenerative sensing surface.

In this paper, we present a regenerative fibrinogen biosensor using the competitive adsorption of proteins in a microfluidic environment. The target protein, fibrinogen, is adsorbed selectively on the sensing surface following the competitive process of protein adsorption/exchange. This adsorption and exchange process results in a refractive index change that is monitored by SPR. Fig. 1 illustrates SPR sensorgram of the biosensor. A protein with relatively strong adsorption strength, IgM, is dominantly displaced by fibrinogen, yet is displaced much less significantly by other proteins in fibrinogen-spiked human serum samples (Choi et al., 2010). Using the nature of competitive protein adsorption, we obviate the need to rely on complex assays and their attachment to transducers.

Once the SPR angle shift is stabilized, all the proteins on the surface including fibrinogen, IgM and other proteins are washed away by bleach. We use 0.9% sodium hypochlorite solution as the bleach that is effective to sanitize the sensing surface. It is relatively harmless to the surface of the biosensor because 0.0075% sodium hydroxide is used to delay the breakdown of sodium hypochlorite into sodium chloride and sodium chlorate. The regeneration enables multiple measurements of fibrinogen level in human serum on the same biosensor chip that can reduce irreproducibility and enhance controllability of multiple measurements. This regenerative biosensor potentially may be used to diagnose risk of cardiovascular disease.

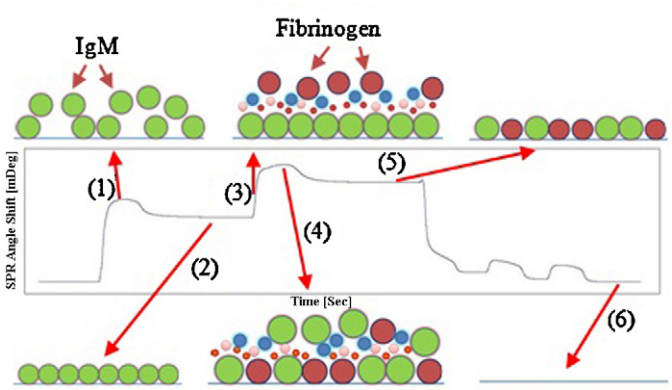

Fig. 1. SPR angle profile of the competitive adsorption of proteins and regeneration of the sensing surface to measure fibrinogen in serum (1) injection of IgM to pre-adsorb on the surface (2) saturation of IgM and washing away of excess proteins (3) injection of fibrinogen-spiked undiluted serum sample (4) replacing IgM based on the competitive nature of protein adsorption/exchange (5) adsorption of fibrinogen and washing away excess proteins (6) regeneration of the surface.

2. Materials and methods

2.1. Agents

Human serum and human IgM (Solution in 0.05 M Tris–HCl, 0.2 M sodium chloride, pH 8.0, containing 15 mM sodium azide) were purchased from Sigma–Aldrich. Fibrinogen was received as lyophilized powders from La Jolla Inc. and used without further purification. Bleach containing 6.0% sodium hypochlorite was diluted to 15% with DI water.

2.2. Sensing surface formation

Glass slides (BK7, $n = 1.517$, 150 μm thick, 18 mm × 18 mm) were first immersed in ethanol (Proof 190) and cleaned in a Ultrasonic Cleaner for 5 min. The slides were then rinsed sequentially with DI water and ethanol and were dried under a N_2 stream. Using a sputter, a Cr layer was first coated on the glass substrates to a thickness of 2 nm followed by Au to a thickness of 48 nm. The slides were cleaned in a plasma cleaner for 1 min.

2.3. Experimental method

After the refractive index matching liquid was applied to the prism face to reduce beam scatter at the interface, the biosensor chip was mounted on the semi-cylindrical prism of the SPR instrument (Bi-2000, Biosensing Instrument Inc.). A flow cell with two microfluidic channels was mounted on top of the chip. We operated the SPR instrument under the serial mode: protein samples flowed through channel 1 first, and then reached channel 2.

Initially, the biosensor surface was cleaned by bleach flowing through the microfluidic channel driven by an external syringe pump. Once the angle shift was stabilized, IgM was injected to pre-adsorb the biosensor surface at rate of 30 μL/min. The adsorption of IgM produces SPR angle shift. After the adsorption, phosphate-buffered saline (PBS) was used to wash away any weakly bound IgM on the surface. This completes pre-adsorption process. We ensure the surface is saturated by IgM before we flow fibrinogen-spiked serum samples (Choi et al., 2010). IgM has a molecular weight of approximately 900 kDa; thus it is one of the proteins with highest adsorption strength in serum. That means IgM is unlikely replaced

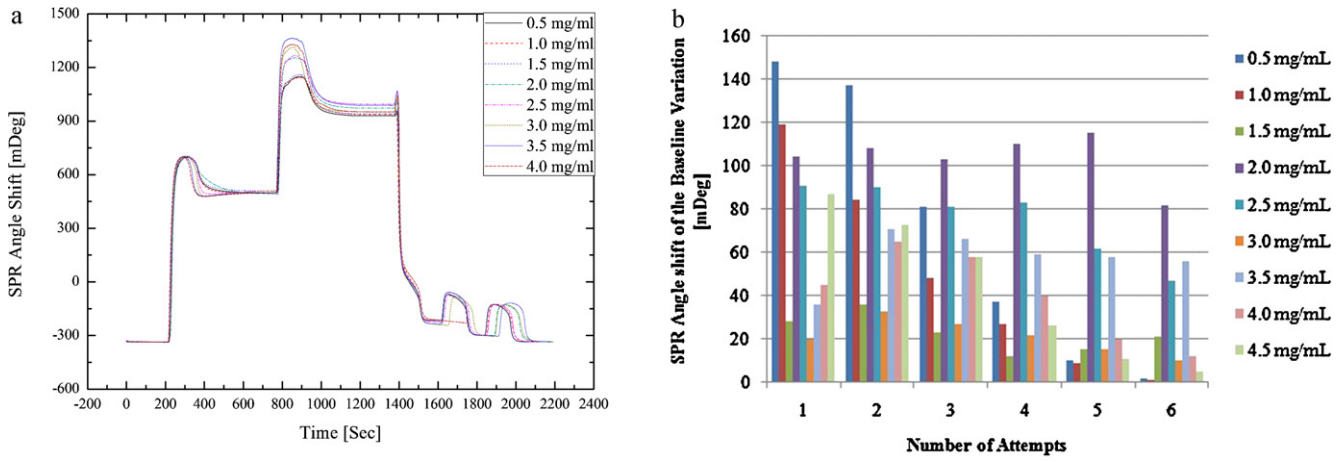

Fig. 2. (a) SPR sensorgrams of a series of fibrinogen-spiked serum samples ranging from 0.5 to 4.0 mg/mL (b) SPR angle shift of the baseline variation of fibrinogen-spiked serum ranging from 0.5 to 4.0 mg/mL.

by proteins with weaker adsorption strength in serum. It will be replaced by proteins with very strong adsorption strength such as fibrinogen.

Fig. 2(a) illustrates SPR sensorgram of regeneration at different concentrations of fibrinogens on a chip, ranging from 0.5 to 4.0 mg/mL at 0.5 mg/mL intervals. Once the angle shift of IgM stabilized, the fibrinogen-spiked serum sample was injected at 30 μL/min and flowed through the surface that is pre-adsorbed by IgM. After washing away any weakly bound protein on the surface, we recorded the final angle shift from the baseline, which can be correlated to the concentration of fibrinogen. Finally, bleach was used to regenerate the biosensor chip at 50 μL/min three times to ensure removing all proteins on the surface till the baseline recovered. The SPR baseline variations from having different concentrations of fibrinogen is shown in a supplementary material.

In addition to the attempts of different concentrations on a chip, we also conducted sets of experiments regenerating the same concentrations of fibrinogen on a chip as well. Each concentration set of fibrinogen-spiked solutions was repeated six times and the SPR baseline variations as a function of the number of regeneration attempts are shown in Fig. 2(b). The regeneration solution cannot completely remove all the absorbed proteins on the surface. The data shows a general trend of decreasing the baseline variations as the number of attempts increased. After six attempts of regeneration, the baselines return nearly back to the original.

Fig. 3(a) plots a summary of the two types of experiments showing SPR angle variations as a function of different concentrations of fibrinogen-spiked serum from 0.5 to 4.0 mg/mL.

3. Results and discussion

In Fig. 2(b), we demonstrate that we can differentiate different concentrations of fibrinogen human serum samples from 0.5 to 2.5 mg/mL. Each data point represents the average measured angle shift over 6 repeated measurements for a particular concentration of fibrinogen. In order to scrutinize the possibility of coagulation of fibrinogen-spiked serum samples, we recorded SPR angle shifts of both channel 1 and 2.

Fibrinogen concentrations of less than 2.5 mg/mL exhibit a linear relationship to SPR angle shift, with a slope of 42 mDeg/(mg/mL). However, above a concentration of 2.5 mg/mL, the angle shift actually decreases and fluctuates. There are three hypothetical reasons for the discernible nonlinear phenomena that occur at concentrations greater than 2.5 mg/mL: (i) saturation, (ii) coagulation, and (iii) decrease in sensitivity.

The first hypothesis, that the sensor surface saturates for concentrations above 2.5 mg/mL, seems unlikely to rationalize the observed nonlinear phenomena. If the surface was saturated, the SPR angle shifts observed for increasing concentrations above 2.5 mg/mL, should remain constant, rather than fluctuating. The SPR instrument includes two channels that are sequentially connected; fibrinogen-spiked samples flow through channel 1 and then through channel 2. If the sensing surface in channel 1 was saturated, the SPR angle shift of channel 2 for a fibrinogen concentration of 3.0 mg/mL should keep increasing until as high as the angle shift of channel 1, but instead follow a similar trend to that of channel 1. This is because the slopes of the two curves (channels 1 and 2) are nearly identical (42 mDeg/(mg/mL) and 44 mDeg/(mg/mL) for channels 1 and 2, respectively), which confirms that both channels possess the same sensitivity.

Coagulation, the second hypothetical explanation for unforeseen phenomena observed at fibrinogen concentrations above 2.5 mg/mL, forms faster and more profusely if the concentration of fibrinogen is higher for a longer period (Kamath and Lip, 2003). As coagulation increases in the human serum, fewer proteins dissolve in the sample, leading to a lower fibrinogen concentration. This hypothesis is suggested by the measurement of the reduced angle shifts in channel 2 as compared to channel 1. As a result, there exist two factors determining the final concentration of fibrinogen: the initial concentration and coagulation level. Higher initial fibrinogen concentration results in a greater amount of coagulation, which may lead to a lower final concentration of fibrinogen. Consequently, a higher initial concentration may not necessarily produce a higher SPR angle shift. Further evidence to attribute coagulation to the unexpected nonlinear phenomena is the shape deformation observed during real-time SPR angle shift measurements, as encircled in Fig. 3(b). The maximum angle shifts in channel 2 are noticeably deformed as compared to the angle shifts measured in channel 1. Although it is difficult to entirely avert fibrinogen coagulation due to the clotting nature of fibrinogen, preventative measures may be taken to reduce coagulation; reducing sample preparation time and washing the micro-channels immediately after samples are processed through them. Without these anticipatory measures, the experimental data is likely to deviate and succumb to error and the microfluidic system in the SPR instrument will need to be disassembled to clean out the clot due to coagulation.

The third hypothetical reason is a reduction of sensitivity due to the damage on the surface of the biosensor chip caused by multiple regeneration cycles using bleach. In order to assess

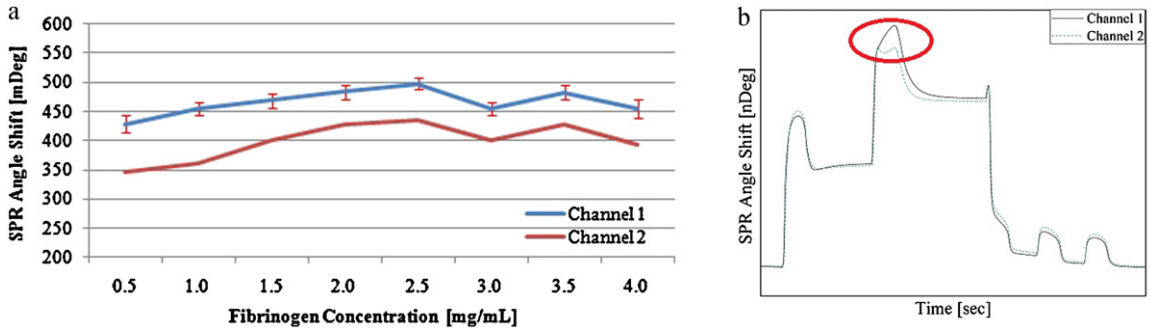

Fig. 3. (a) SPR angle shifts as a function of fibrinogen concentration from 0.5 to 4.0 mg/mL. The plot was generated for 6 repeated trials at each concentration. SPR angle shifts of two channels were compared to examine the possibility of coagulation of fibrinogen-spiked samples (b) two real-time monitored SPR angle shift plots were compared to illustrate the deformation of channel 2.

the decrease in sensitivity, we started the experiments with the fibrinogen concentration of 1.5 and 2.5 mg/mL separately. We increased the fibrinogen concentration by 0.5 mg/mL per regeneration cycle for the two sets of experiments. If the sensitivity of the biosensor chip had been reduced due to multiple bleach washing sequences and this reduced sensitivity was the cause for the angle shift to decrease above a fibrinogen concentration of 2.5 mg/mL, in these two experiments, the first regeneration cycles should not be affected significantly and the angle shift should keep increasing, because the bleach does not damage the biosensor surface seriously in the first regeneration cycles. Nevertheless, the angle shift observed at a concentration of 2.5 mg/mL was still the maximum angle shift measured for all concentration levels and the angle shift still fluctuated in the range of 2.5–4.5 mg/mL. For all of the aforementioned reasons, a possible reduction in sensitivity of the biosensor chip fails to substantiate the SPR angle shift decrease and fluctuations observed above concentrations of 2.5 mg/mL.

As a result, the first and third possibilities, saturation and possible sensitivity reduction, respectively, are unlikely to be attributable to the unexpected angle shift decrease and fluctuations seen at concentrations greater than 2.5 mg/mL. Instead, the second purported hypothesis, coagulation, remains as the most probable grounds for the nonlinear phenomena.

4. Conclusion

In this work, a reusable SPR biosensor was developed to detect the fibrinogen concentration in undiluted human serum. The surface of the biosensor chip was regenerated for reuse by bleach, which reduces batch-to-batch irreproducibility and uncontrollable experimental conditions, which generates unalterable false-positive/negative responses. Using the competitive adsorption/exchange nature of proteins along with a regeneration procedure, this SPR biosensor provides a label-free and high throughput measurement technique for fibrinogen of multiple samples in real time. From our experiments, it was demonstrated that the concentration levels could be differentiated in the range of 0.5 to 2.5 mg/mL.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.07.036.

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