Using competitive protein adsorption to measure fibrinogen in undiluted human serum

Seokheun Choi, Ran Wang, Arad Lajevardi-Khosh, and Junseok Chae
School of Electrical, Computer and Energy Engineering, Arizona State University, Tempe, Arizona 85287, USA

(Received 30 September 2010; accepted 19 November 2010; published online 20 December 2010)

We report a unique sensing mechanism based on competitive protein adsorption to measure fibrinogen, a cardiovascular biomarker, in undiluted human serum. The method uses physical adsorption of proteins to a surface rather than complex and time-consuming immobilization procedures. Two fibrinogen concentrations were differentiated in spiked in human serum [3.0 mg/ml (normal concentration) versus 3.2 mg/ml (abnormal concentration with heart disease)]. Real-time surface plasmon resonance signals were monitored as fibrinogen displaced a preadsorbed protein, IgM, on a hydrophobic gold surface. The relatively strong-affinity protein, IgM, was displaced primarily by fibrinogen and much less by other proteins in human serum. © 2010 American Institute of Physics. [doi:10.1063/1.3529445]

The quest for early diagnosis requires improved methods for identifying and quantifying disease biomarkers in complex mixtures such as human serum.1 Surface plasmon resonance (SPR) affinity-based biosensors are a promising technique for biomarker detections, and in recent years, a large number of SPR studies have shown that these sensors enable direct and label-free specific detection of various analytes below the nanomolar level with real-time monitoring of various biomolecular interactions.2 Also, the physical behaviors of proteins on a surface have long been monitored by using SPR.3 The assays, however, are quite complex; they require multicomponent, multistep configurations that are time-consuming and labor intensive, and this is often the bottleneck for high yield sensors.4,5 Furthermore, SPR-based, label-free biosensors are prone to high background noise from nonspecific binding in the complex mixtures (e.g., serum contains 40–80 mg/ml protein).6,7 Until now, the key to allowing surface sensors to be used in complex media has been reducing the nonspecific binding of proteins to the surface.8–10 Protein adsorption to surfaces, however, is practically unavoidable since every surface is hydrophobic, ionic, or polar, and proteins have hydrophobic, ionic, and polar domains.11 Here, we present a fundamentally different sensing technique that exploits the nature of protein adsorption itself to detect a target molecule in a complex mixture of undiluted human serum. When more than one type of protein is present in solution, a competitive adsorption/exchange process occurs between the different proteins and the surface.11,12 We previously reported that the adsorption and protein exchange occurred on four different proteins and that their adsorption strengths are a function of the surface properties.5 More recently, we demonstrated that the nature of protein adsorption could be used as a protein sensing mechanism;13 based on the exchange reaction, a cancer biomarker, thyroglobulin, was selectively detected in a controlled protein cocktail.

Here, we report that competitive protein adsorption can be designed to detect fibrinogen in undiluted human serum. Fibrinogen is one disease biomarker of cardiovascular disease.14,15 High fibrinogen levels are strongly correlated with the frequency of cardiovascular disease. Many studies have reported that fibrinogen concentrations increase by 200 μg/ml for those with heart diseases.15 Two different concentrations of fibrinogen spiked in human serum [3.0 mg/ml (normal concentration) versus 3.2 mg/ml (abnormal concentration with heart disease)] were differentiated by monitoring real-time SPR signals as fibrinogen displaced a preadsorbed protein, IgM. The technique provides a selective detection of fibrinogen; the relatively strong-affinity protein, IgM, was dominantly displaced by fibrinogen and significantly less by other proteins in human serum.

Human serum from platelet-poor human plasma as well as albumin and IgM were purchased from Sigma-Aldrich. The human serum fibrinogen was received as lyophilized powders from CalBioChem and used without further purification. Albumin and IgM concentrations were up to 0.1% (w/v) in phosphate-buffered saline (PBS) 1× (1.15 g/l Na2HPO4, 0.20 g/l KCl, 0.20 g/l KH2PO4, 8.0 g/l NaCl, and 4 g/l DMSO).

![FIG. 1. (Color online) Operating principle of measuring fibrinogen in human serum based on the competitive protein adsorption. (a) When weak-affinity proteins are preadsorbed on a surface, many proteins in serum tend to displace the preadsorbed proteins. (b) When the surface is preadsorbed by a strong-affinity protein, only several types of proteins, including fibrinogen, can displace the preadsorbed protein, increasing the chance of measuring fibrinogen.](image-url)
pH 7.4 immediately prior to preadsorption. The two human serum samples were prepared by spiking fibrinogen 3.0 and 3.2 mg/ml. Glass slides (BK7, n=1.517, 150 µm) were first cleaned in piranha solution (a 3:1 ration of H₂SO₄ and H₂O₂) for 10 min. The slides were then rinsed sequentially with de-ionized water and ethanol and were dried under a N₂ stream. Using 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 then cleaned by a hydrogen flame for several seconds. The bare gold surface was moderately hydrophobic (82.6° ± 0.77° of contact angle). Using a refractive index matching liquid, the glass slide was mounted to the semicylindrical prism of the SPR instrument (Bi-2000, Biosensing Instrument Inc.). The flow cell with two microfluidic channels was mounted on top of the surface. We then monitored the angle shift in real time as sample solution driven by an external syringe pump flowed through the channels. The experimental setup was equipped with a computer-controlled data acquisition system. SPR was calibrated each time to maintain the sensitivity, 60 mdeg at 1% ethanol solution. Throughout the experiments, room temperature was maintained at 25 °C.

Fibrinogen is known to be a “sticky protein” on hydrophobic surfaces for displacing other serum proteins. Our previous study showed that fibrinogen (340 kDa) has such high adsorption strength on a gold surface that it displaces thyroglobulin, which has higher molecular weight of 660 kDa. It is essential to design a preadsorbed surface that fibrinogen displaces yet other proteins do not; the higher the chance of being displaced by other proteins, the lower the selectivity of the sensor for fibrinogen. Figure 1 illustrates this sensing scheme. When weak-affinity proteins are preadsorbed on a surface, the complex mixture of many proteins in serum tends to displace the preadsorbed proteins. By contrast, when the surface is preadsorbed by a strong-affinity protein, we hypothesized that only several types of proteins, including fibrinogen, could displace the preadsorbed protein, thereby increasing the chance of measuring fibrinogen.

To verify this hypothesis, we selected two proteins as preadsorbed known proteins: albumin (67 kDa) and IgM (900 kDa). The exchange process is led by thermodynamics: proteins with different dimensions and morphologies adsorb differently to a surface based on their thermodynamic energy preferences and behave in ways that minimize the overall system energy. This can be interpreted as a natural outcome of surface reorganization to achieve the equilibrium interphase composition. Therefore, the change in the free energy of the protein adsorption process on a certain surface reflects...
the protein exchange reaction, which can be expressed as
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\Delta G = \Delta G^\circ + RT \ln \left( \frac{[AB]}{[A][B]} \right) = -RT \ln K_A = RT \ln K_D,
\]
where \(\Delta G\) and \(\Delta G^\circ\) represent the changes in adsorption free energy and in standard-state adsorption free energy, \(R\) is the ideal gas constant (1.985 cal/K mol), and \(T\) is the absolute temperature (298 K). \([A]\) is the molar concentration of the protein in solution and \([B]\) and \([AB]\) are the mole fractions of surface sites occupied by bare surface and adsorbed protein, respectively.\(^\text{17,18}\) The equilibrium association constant \(K_A\) and dissociation constant \(K_D\) can be acquired from SPR kinetic analysis. \(K_D\) of albumin, IgM, and fibrinogen were 13.2, 9.2, and 2.0 \(\mu\)M, respectively, and their respective \(\Delta G\) were \(-6.6\), \(-6.9\), and \(-7.8\) kcal/mol. Albumin has relatively small Gibbs free energy, which means that the albumin preadsorbed surface has a high chance of being displaced by other proteins in serum and thus may not offer any selectivity for fibrinogen measurements. However, IgM has large Gibbs free energy, which may offer high selectivity of fibrinogen measurements in serum.

Adsorption of proteins and their subsequent displacement behaviors were monitored by SPR in real time. We prepared two samples: 3.0 and 3.2 mg/ml of fibrinogen in undiluted human serum. 3.0 and 3.2 mg/ml suggest, respectively, normal and abnormal fibrinogen levels in human blood. Figure 2 shows SPR sensograms of displacement behaviors upon injection of the two serum samples. Each sample was introduced onto two surfaces at the speed of 30 \(\mu\)l/min and preadsorbed by two different proteins (albumin and IgM). The experiments were repeated six times. Initially, PBS was circulated until the SPR signal stabilized. Once it stabilized, the gold surfaces were preadsorbed by flowing albumin and IgM at a concentration of 0.1% (w/v), generating SPR angle shifts, as shown in Fig. 2.

When the protein adsorption was complete, we let PBS wash the surfaces to remove excess weakly bound proteins to establish the baseline. When fibrinogen-spiked samples flew onto the two preadsorbed surfaces, the SPR angle values rapidly increased on both surfaces and reached an equilibrium plateau. Finally, PBS washed out all proteins not involved in the interaction or displaced by serum samples. When the angle shift stabilized, we measured the final angle shift from the baseline where albumin and IgM preadsorbed on the surface. The final angle shift measurements were recorded all six times, and these are shown as inserts in Figs. 2(a) and 2(b), respectively. The inserts indicate how sensitively and selectively two fibrinogen-spiked samples can be differentiated by the measurements. The albumin preadsorbed surface does not provide distinct SPR angle shifts between 3.0 and 3.2 mg/ml, and this suggests that many serum proteins, including fibrinogen, were involved in the displacement reaction. On the other hand, the surface covered by IgM shows enough sensitivity and selectivity to differentiate two different fibrinogen concentrations in serum samples. SPR produced averages of 310.3 and 381.3 mdeg for 3.0 and 3.2 mg/ml, respectively, which correspond to \(4.58 \times 10^{11}\) and \(5.63 \times 10^{11}\) molecules/cm\(^2\) (120 mdeg corresponds to 1 ng/mm\(^2\)). The limit of detection and dynamic range were characterized by measuring the angle changes over a range of fibrinogen loadings (200 \(\mu\)g/ml–3.2 mg/ml), as shown in Fig. 2(c). The biosensor has a detection limit of \(\sim 200\ \mu g/ml\) and a sensitivity of 63.0 mdeq/ml. The selectivity of the biosensor was characterized by spiking 1 mg/ml albumin and transferrin in human serum samples [Fig. 2(d)].

We evaluated the IgM preadsorbed surface after exposing it to fibrinogen-spiked serum samples. By flowing antifibrinogen antibodies onto the surface, permanent SPR angle shifts were generated on both 3.0 and 3.2 mg/ml samples, as shown in Fig. 3. This permanent shift demonstrates that a majority of proteins displacing the IgM were fibrinogen.

The authors would like to thank Ms. Steele for valuable discussions. This work is partially supported by the NSF (Grant No. ECCS-#0846961).

\(^2\)Y. Li, J. Xiang, and F. Zhou, Plasmonics 2, 79 (2007).

FIG. 3. (Color online) SPR sensograms of the interaction of antifibrinogen and fibrinogen after the competitive protein adsorption/exchange. By flowing antifibrinogen antibodies to the surface, permanent SPR angle shifts were generated on both 3.0 and 3.2 mg/ml samples.