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A microfluidic biosensor based on competitive protein adsorption for thyroglobulin detection

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ABSTRACT

We report a microfluidic sensing platform for the detection of thyroglobulin (Tg) using competitive protein adsorption. Serum Tg is a highly specific biomarker for residual thyroid tissue, recurrence and metastases after treatment for differentiated thyroid cancer (DTC). Conventional Tg detection techniques require complicated immobilization of antibodies and need to form a sandwich assay using additional secondary antibodies to enhance the sensitivity. We present a fundamentally different sensing technique without using antibody immobilization on a microfluidic platform. We engineer two surfaces covered by two known proteins, immunoglobulin G (IgG) and fibrinogen, with different affinities onto the surfaces. The microfluidic device offers a selective protein sensing by being displaced by a target protein, Tg, on only one of the surfaces. By utilizing the competitive protein adsorption, Tg displaces a weakly bound protein, IgG; however, a strongly bound protein, fibrinogen, is not displaced by Tg. The surface plasmon resonance (SPR) sensorgrams show that five human serum proteins, albumin, haptoglobin, IgG, fibrinogen and Tg, have different adsorption strengths to the surface and the competitive adsorption of individuals controls the exchange sequence. The adsorption and exchange are evaluated by fluorescent labeling of these proteins. Tg in a protein mixture of albumin, haptoglobin, and Tg is selectively detected based on the exchange reaction. By using the technique, we obviate the need to rely on antibodies as a capture probe and their attachment to transducers.

1. Introduction

A recent advance in molecular biology has led to our better understanding of biomolecular interactions. The interactions of specific ligands, such as DNA–DNA, RNA–DNA, antibody–antigen, enzyme–substrate, and protein–aptamer, involve formation of a strong and specific chemical bond, allowing biosensors in detecting target biomolecules (Mohanty and Kougianos, 2006; Powner and Yalcinkaya, 1997; Skottrup et al., 2008; Wu et al., 2007). A specific bioelement immobilized on a sensing surface recognizes a specific target molecule and a sensor element transduces the change in the biomolecular interactions into an electrical signal. While the extensive research on the transducers has allowed the application of extremely high sensitivity up to the level of fM concentration (Kim et al., 2008), the relatively slow advancements in techniques for bioelements cause an enduring problem in creating widespread commercial protein sensor (Pavlickova et al., 2004; Predki et al., 2005; Saerens et al., 2008; Taussig and Landegren, 2003). First, a large diversity of bioelements is not available for detection of various diseases (Glokler and Angenendt, 2003). Second, general immobilization strategies for high capture capacity are not well defined (Taussig and Landegren, 2003). Third, some bioelements do not have high specificity and affinity (Taussig and Landegren, 2003). Besides these limitations, integrating them on to the transducer is a time-consuming and labor intensive process and often becomes the bottle neck of high yield sensors. In affinity-based sensing, developments of new bioelements and their attachment techniques are currently being pursued to overcome the limitations (Kusnezow and Hoheisel, 2002). To date, few alternative platforms for the protein detection have been active in biosensor communities. Here, we report a fundamentally different protein detection method that relies on the competitive nature of protein adsorption onto a surface. The method can be a complementary solution to the existing affinity-based biosensor.

It is known that a smaller-size protein initially covering the surface is displaced by a larger-size protein, namely the Vroman effect (Green et al., 1999; Noh and Vogler, 2007; Vroman and Adams, 1969). Since the effect is first introduced by Vroman and Adams (1969), the Vroman effect has been studied extensively in the biochemistry community for competitive protein adsorption to materials having different surface chemistries and surface energies (Horbett and Brash, 1995). The adsorption/exchange reaction depends on a size and concentration of proteins, pH of solution, substrate properties and environmental temperature (Latour, 2005).

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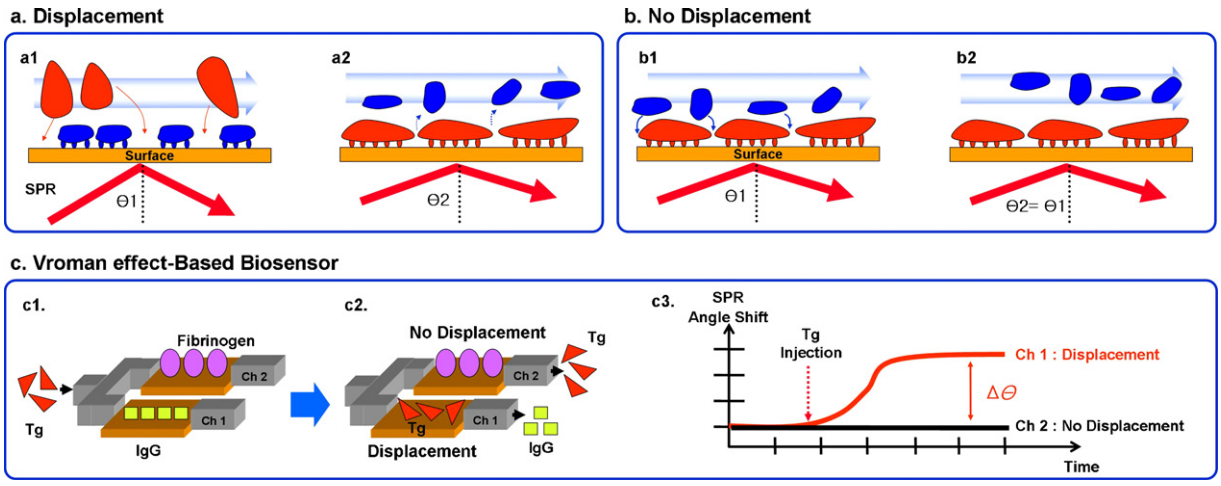

Fig. 1. A schematic of Vroman effect (a and b) and Vroman effect-based Biosensor (c). (a) When a surface is covered by weakly bound proteins, higher interacting proteins, arriving subsequently, displace the pre-adsorbed proteins. This event induces SPR angle shift ($\theta 2 - \theta 1$). (b) However, the reverse sequence does not induce any displacement of proteins. Thus, no angle shift exists ($\theta 2 = \theta 1$). (c1 and c2) One can engineer two different proteins which have different attractions to two surfaces; one is covered weakly bound proteins (IgG) and the other is covered by strongly bound proteins (fibrinogen). When moderate interaction proteins (Tg; thyroglobulin) arrive the pre-adsorbed surfaces, IgG is displaced by Tg but fibrinogen remains on the surface without exchange reaction. (c3) Therefore, the differential SPR angle shift from channel 1 and 2 provides quantitative information of a selective protein biosensor.

Most proteins have amphiphilic surfaces; they have hydrophobic residues buried within their core and their hydrophilic residues facing outside or visa versa (Latour, 2005). The adsorption of any protein onto a solid surface is highly dependent on hydrophobic attraction. Hydrophilic residues form the orientation of the adsorbed protein and do not take part in the adsorption process itself. Therefore, proteins rapidly adsorb onto a hydrophobic surface, unfold and spread their hydrophobic core over the surface (Wertz and Santore, 2001). When more strongly interacting proteins arrive at the surface, they displace pre-adsorbed proteins (Fig. 1a). However, the reverse sequence does not occur (Fig. 1b) (Choi et al., 2008). This process is led by thermodynamics; proteins that have different dimensions and morphologies adsorb differently to a surface based upon their thermodynamic energy preferences and behave to minimize the overall system energy. This can be interpreted as a natural outcome of surface reorganization to achieve the equilibrium interphase composition (Krishnan et al., 2004). Our group reported that the adsorption and exchange of proteins occur on four different non-human proteins (aprotinin, lysozyme, streptavidin, and isolectin) and their adsorption strengths vary according to the surface properties (Choi et al., 2008). In this paper, we present that a specific human serum protein, thyroglobulin (Tg), in a protein mixture of albumin, haptoglobin and Tg, can be selectively detected based on the adsorption/exchange reaction. The concept is that one can engineer a pair of surfaces covered by two known proteins; one is covered by a weaker-affinity protein and the other is covered by a stronger-affinity protein than the target protein. Then, the pair of the surfaces becomes a selective protein sensor since one is displaced and the other is not displaced by the target protein (Fig. 1c). By using the technique, we demonstrate that a specific target protein in a controlled proteins cocktail can be identified without using the conventional affinity-based method.

Tg is a widely used cancer biomarker in human serum for the presence of residual thyroid tissue or cancer recurrence in patients treated for differentiated thyroid cancer (DTC) (Iervasi et al., 2004). Tg is unique to thyroid tissue, therefore, Tg is primarily used as a cancer biomarker test for DTC. Currently, two different methodologic approaches are used to detect Tg; the competitive radioimmunoassay methodology and noncompetitive immunometric assay methods (Spencer and Lopresti, 2008). These techniques have lengthy and complicated procedures and need to form a sandwich assay to enhance the sensitivity. Moreover, since Tg is a large glycoprotein (660 kDa), many antibodies are required to more effectively capture a Tg molecule through recognizing different antigenic regions on the Tg molecule (Iervasi et al., 2004). This imposes more complicated immobilization process for optimal orientation and good degree of freedom for the antibodies (Skottrup et al., 2008). The sensing technique we report here obviates the need to rely on the antibodies and their attachment to the sensing surface.

We first show that the five different human serum proteins, albumin, haptoglobin, immunoglobulin G (IgG), fibrinogen and Tg, have different adsorption strengths to a surface and the competitive adsorption/exchange controls the displacement of individual proteins. Then, we present Tg in a controlled proteins cocktail can be selectively detected based on the exchange reaction. In this work, the sensing surfaces are integrated with microfluidic systems and we monitor protein behaviors occurred in microfluidic channels by fluorescent detection and surface plasmon resonance (SPR). SPR has been one of the leading transducer techniques due to its extremely high sensitivity, offering detection limits up to few ppt (pg mL^{-1}) (Li et al., 2007). SPR is a surface-sensitive analytical tool responding to slight changes in refractive index occurring adjacent to the metal film, approximately within 200 nm. Thus, adsorption of proteins on the surface and their subsequent displacement interactions could be extensively monitored in real-time.

2. Experimental

2.1. Materials

We used five human serum proteins to characterize the sensor: albumin (66 kDa), haptoglobin (86 kDa), IgG (150 kDa), fibrinogen (340 kDa), and thyroglobulin (660 kDa) (Sigma–Aldrich and CalBIOCHEM). All the chemicals were received as lyophilized powders and used without further purification. The proteins were made up to 0.05% (w/v) concentrations in PBS 1× (1.15 g/L–Na_2HPO_4, 0.20 g/L–KCl, 0.20 g/L–KH_2PO_4, 8.0 g/L–NaCl, pH 7.4) immediately prior to analysis. The concentration of mixed proteins is 0.05% (w/v). Fluorescent dyes (Alexa Fluor 488 (green) and 594 (red)) were purchased from Invitrogen, Inc.

2.2. Sensing surface formation

Glass substrates (BK7, $n = 1.517$, 150 μm) were first cleaned in piranha solution (a 3:1 ration of H_2SO_4 and H_2O_2) for 10 min. The substrates were then rinsed with water and ethanol sequentially and were dried under N_2 stream. Using thermal evaporator, Cr layer was coated first on the glass substrates to a thickness of 2 nm followed by Au to a thickness of 48 nm. Then, the substrates were cleaned by oxygen plasma (Harrick Plasma Inc.) at 18 W for 1 min.

2.3. Microfluidic device for detecting fluorescent intensity

The microfluidic device was fabricated to evaluate the protein displacement using fluorescent labeling. The device consists of three layers. A 1 mm-thick top glass was mechanically drilled to have two holes; an inlet and an outlet. Bottom glass substrate (100 μm-thick) has Cr/Au (2 nm/10 nm) electrode, which was evaporated and patterned for inverted microscopy. The spacer between the two substrates was made of polydimethylsiloxane (PDMS). These layers were bonded all together after oxygen plasma treatment. The PDMS layer was prepared to be 1 mm in thickness and mechanically cut to have a channel.

2.4. Fluorescent intensity measurement

The fabricated microfluidic device was mounted onto inverted microscope (Nikon, TE2000-U) with a CCD camera (Photometric). Using a sample injection valve (6-port M-461, Upchurch Scientific), protein and buffer solution selectively flowed through the microfluidic channel driven by two external syringe pumps. Each solution flowed at the speed of 10 μl/min. The captured fluorescent image was analyzed on Image J software provided by NIH Image.

2.5. Microfluidic device for SPR measurement

We used soft-lithography to fabricate the microfluidic device. For a top layer, photoresist, AZ4330, was spin-coated on a silicon wafer and patterned for channels. Then the pattern was transferred to silicon by Deep RIE, etched ~100 μm. The width of the channel is 2.1 mm and two channels are separated by 1.3 mm. PDMS solution was poured and cured on the silicon wafer and we peeled off the molded PDMS. The thickness of the layer is approximately 1 cm. Intel/outlet tubes (Upchurch Scientific) (inner diameter: 25 μm, outer diameter: 360 μm) were inserted through the PDMS using a syringe needle and fixed by adhesive. A bottom substrate has patterned Cr/Au (2 nm/47 nm) pads on the glass substrate, which are two sensing surfaces for detection of the protein exchange. The size of the sensing surfaces is 1.8 mm wide and 8.0 mm long. The width of the channel is slightly wider than that of the gold surface to completely bond two substrates by using oxygen plasma.

2.6. SPR setup

The fabricated microfluidic device was mounted to the semi-cylindrical prism of SPR instrument (Bi SPR 1000, Biosensing Instrument Inc.) by using a refractive index matching liquid. The experimental setup is equipped with a computer-controlled data acquisition system. The SPR produces two sensorgrams in real time; one on a reference channel and the other on a sensing channel. Throughout the experiments, room temperature was maintained at 25 °C.

3. Results and discussion

3.1. Protein displacement

In its first implementation, we demonstrate that five human serum proteins, albumin (66 kDa), haptoglobin (86 kDa), IgG (150 kDa), fibrinogen (340 kDa) and thyroglobulin (660 kDa), have different adsorption strengths onto the hydrophobic gold surface (contact angle measurement: $83.2° \pm 0.75°$). The different adsorption strengths induce an exchange reaction among them. First, a protein is pre-adsorbed on the surface (row in Table 1). Then another protein (column in Table 1) subsequently reaches to the surface to interact with the pre-adsorbed protein. The surface was saturated using 0.05% (w/v) proteins to form a fully packed monolayer (Green et al., 1997), verified by re-injecting the same sets of protein (Table 1). Since we used neutral PBS (phosphate buffered solution, pH 7.4) and all the proteins used for experiments have the p*I* (isoelectric point) less than pH 7.0, multilayer formation due to protein-protein electrostatic interaction does not occur (Van der Veen et al., 2004). For example, when IgG is pre-adsorbed on the surface, fibrinogen and Tg displace the IgG producing 142 mDeg and 62 mDeg, which correspond to 2.10×10^{11} molecules/cm^2 and 4.72×10^{10} molecules/cm^2 (120 mDeg corresponds to 1 ng/mm^2 for the SPR employed here). However the SPR angle change is almost negligible when fibrinogen and Tg are exposed to IgG, hence demonstrating that the angle changes are not due to the multi-layer formation but due to the protein displacement. Table 1 shows all the cases of the displacement and the monolayer formation among five different proteins. The displacement strength is ranked in the following order; fibrinogen (340 kDa) > thyroglobulin (660 kDa) > IgG (150 kDa) > haptoglobin (86 kDa) > albumin (66 kDa). The Vroman effect does not always follow the size of proteins; for instance, thyroglobulin is larger than fibrinogen and yet is being displaced by fibrinogen. We may possibly explain this phenomenon because of their structures and potential conformation at the surface. Fibrinogen has a triad structure tethering two D domains to a central E domain by triple-stranded α-helical-coiled coils (αC) (Holden and Cremer, 2005). On the neutralized Au surfaces, D and E domains are involved in the strong adsorption and even positively charged αC domains in the neutralized PBS does not have any electrostatic repulsion against the surface. On the other hand, thyroglobulin has

Table 1
Summary of interaction strengths of five different proteins. Row proteins are pre-adsorbed to the surface. Column proteins arrive to the pre-adsorbed surfaces. Weakly bound proteins are displaced by higher interacting proteins. Generally, larger size proteins have stronger interaction than smaller size proteins, except fibrinogen (340 kDa) and thyroglobulin (660 kDa).

Second	First				
	Albumin	Haptoglobin	IgG	Fibrinogen	Thyroglobulin
Albumin	X (1 mDeg)	X (3 mDeg)	X (−2 mDeg)	X (2 mDeg)	X (−4 mDeg)
Haptoglobin	O (40 mDeg)	X (−1 mDeg)	X (2 mDeg)	X (−1 mDeg)	X (0 mDeg)
IgG	O (90 mDeg)	O (75 mDeg)	X (3 mDeg)	X (0 mDeg)	X (−2 mDeg)
Fibrinogen	O (320 mDeg	O (249 mDeg)	O (142 mDeg)	X (4 mDeg)	O (90 mDeg)
Thyroglobulin	O (200 mDeg	O (87 mDeg)	O (62 mDeg)	X (3 mDeg)	X (3 mDeg)

O: displacement occurs; X: no displacement.

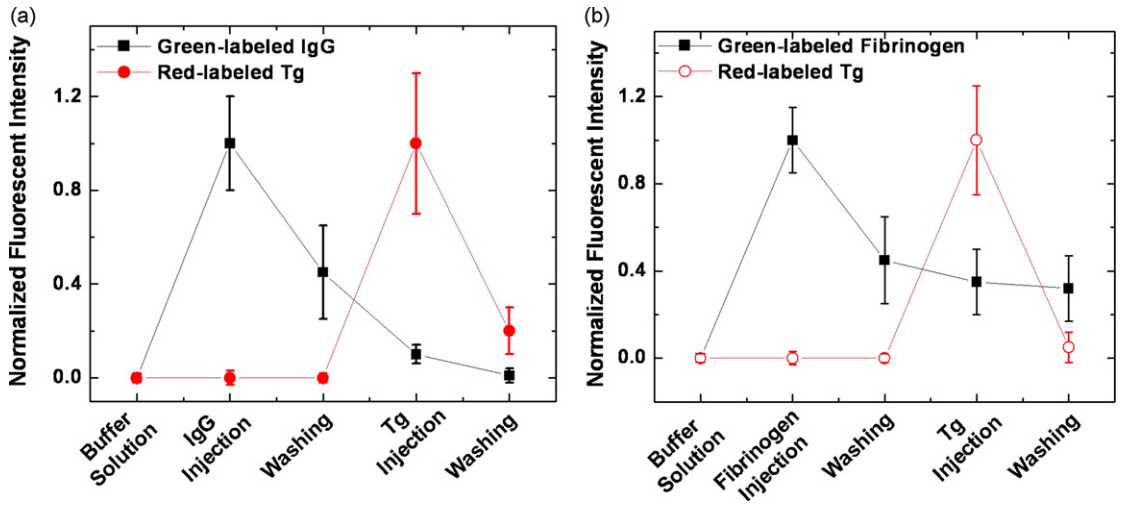

Fig. 2. Real-time fluorescent intensity changes demonstrate proteins adsorption/exchange, the Vroman effect. (a) IgG (green) is displaced by Tg (red), showing the proteins exchange. (b) Tg (red) does not displace pre-adsorbed fibrinogen (green); showing the reverse sequence does not induce the proteins exchange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

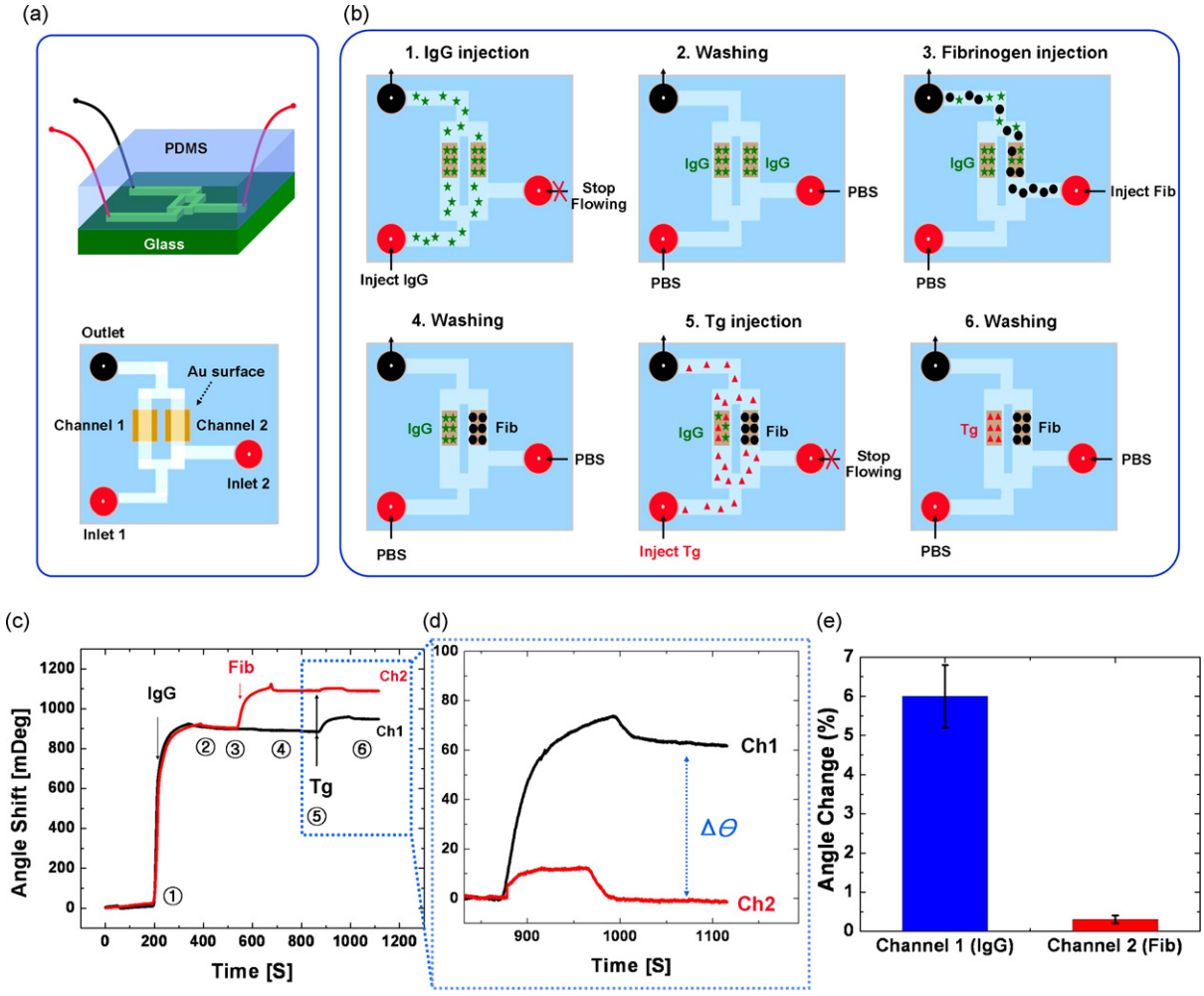

Fig. 3. (a) A custom-made microfluidic device to demonstrate the Vroman effect-based protein biosensor. (b) A schematic of operating principle. (1) IgG is injected from the inlet 1 to cover both surfaces. (2) Washing process to remove unbound IgG (3) fibrinogen flows from inlet 2 and displaces the pre-adsorbed IgG on one surface. (4) Washing process to remove any residue on the surface. (5) A target protein (Tg) flows from inlet 1. (6) Tg displaces IgG in channel 1 while it does not displace fibrinogen in channel 2. (c) SPR sensorgram of the displacement event; Tg detection of two engineered surfaces, pre-adsorbed by IgG and fibrinogen. (d) Normalized close-up SPR sensorgram after the Tg injection (e) final angle changes (%) on both surfaces (angle change/previous angle value × 100). Each has selectivity to a specific protein to be detected.

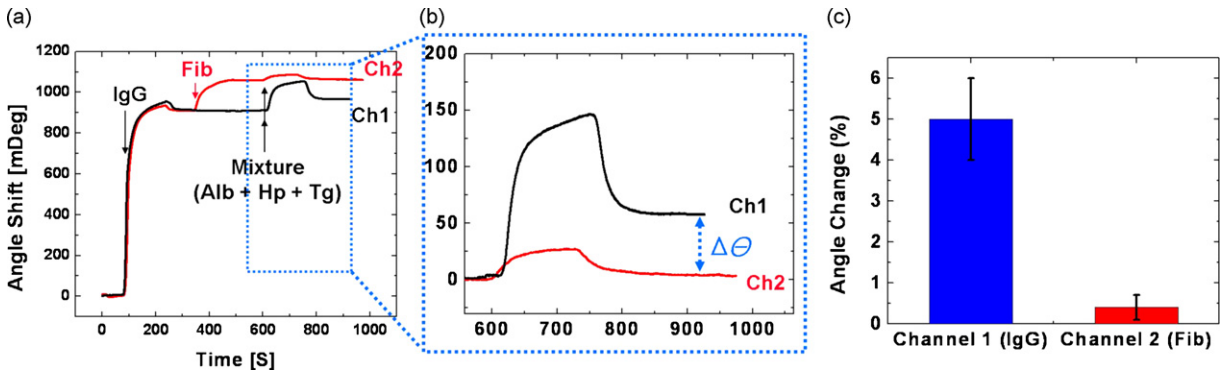

Fig. 4. SPR sensorgram of displacement event upon a mixture of proteins. (a) Tg detection of pre-adsorbed IgG and fibrinogen surfaces in a mixture of albumin, haptoglobin, and Tg. (b) Normalized close-up view of the Tg detection (c) SPR angle changes (%) of channel 1 (IgG) and channel 2 (fibrinogen) (angle change/previous angle value × 100). Each has selectivity to a specific protein to be detected.

a rather high degree of spherical symmetry and compactness. We expect that thyroglobulin is involved in the smaller hydrophobic attraction than fibrinogen because thyroglobulin acts as a compact particle (Bloth and Bergquist, 1968; Deshpande and Venkatesh, 1999).

3.2. Evaluation

The displacement events were evaluated by using fluorescent labeling of proteins (Fig. 2). A custom-made microfluidic device was prepared with a 10 nm-thick gold film on a glass slide to observe the proteins adsorption/exchange in real time. The thin film was to form hydrophobic properties and to be transparent to detect fluorescent intensity using inverted microcopy. IgG/fibrinogen and Tg were labeled by green and red fluorescent dyes, respectively. Those dyes are assumed not to interfere in protein displacement process because of their relatively small size compared to proteins used in the experiment. When Tg flows on the surface of pre-adsorbed by IgG, IgG fluorescent intensity decreases (from 0.453 to 0.015) and Tg intensity remains high (0.202) after washing (Fig. 2a). On the other hand, Fig. 2b shows the reverse consequence; fibrinogen is pre-adsorbed on the surface and Tg arrives later. Fibrinogen fluorescence intensity remains high (0.304) and Tg drops significantly on the washing step (0.081). Tg fluorescent intensity has a larger positive response in Fig. 2a than it does in Fig. 2b, which can be interpreted as a displacement reaction. The fluorescent detection of proteins adsorption/exchange is in good agreement with the results of Table 1, demonstrating the Vroman effect.

3.3. Target protein detection

Based on the data set (Table 1), we identify specific target proteins without using the conventional affinity-based method. Fig. 1c shows how to detect Tg using a pair of surfaces pre-adsorbed by two known-sized proteins; IgG and fibrinogen. Tg displaces IgG in channel 1 but flows through the fibrinogen-covered surface (channel 2) without any exchange reaction. The differential measurement of the SPR angle changes from channel 1 and 2 allows the detection of Tg, and the angle changes indicate the number of Tg molecules displacing IgG (Fig. 1c-3). The sensor is enclosed by microfluidic channels/chambers in order to facilitate potential integration within microfluidic systems (Fig. 3a). The device has two inlets and one outlet unlike our previous approach that has two inlets and two outlets (Choi et al., 2008). A mixture of sample is injected from only one of the two inlets. The three ports microfluidic device allows minimizing baseline discrepancy generated by having the sample injected from two inlets. We monitor the angle shift in real time as protein solution flows through the microfluidic channels driven by two external syringe pumps from the two inlets. First, PBS is circulated for approximately 20 min until the angle stabilizes. Once the angle stabilizes, the first protein sample is injected from inlet 1 and flows through both microfluidic channels at 10 μl/min, generating angle changes on both surfaces (Fig. 3b-1 and c). When the protein adsorption completes on both gold surfaces, we let PBS wash the surface to remove excess weakly bound proteins (Fig. 3b-2). Then, the second protein sample is injected from inlet 2 and flows only through the channel 2 at the speed of 5 μl/min (Fig. 3b-

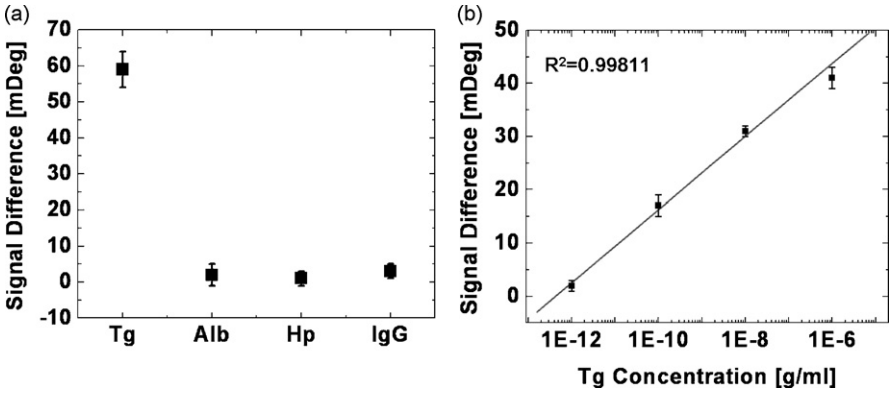

Fig. 5. (a) Selectivity and (b) standard curve of Vroman effect-based biosensor in a case of thyroglobulin detection. One surface is covered by IgG and the other is by fibrinogen. (a) The differential SPR angle shifts are shown when the following proteins are injected; Tg, albumin, haptoglobin and IgG. Tg has 59 mDeg angle shifts while the others generate insignificant angle change. (b) Plot of differential SPR angle vs. Tg concentration. The LOD for Tg is estimated to be 1 pg/ml and the linear range is determined to be up to 1 μg/ml.

3). During the process, PBS keeps flowing from inlet 1 at 10 μl/min to prevent the second protein from flowing back to the channel 1. The second protein is selected to displace the first one in channel 2. For instance, to detect Tg (Fig. 3b), IgG was first introduced from inlet 1 and adsorbed on both gold surfaces. Then, we injected fibrinogen into inlet 2 while PBS flows from inlet 1. The fibrinogen, then, displaced the pre-adsorbed IgG in the channel 2. When the target molecule, Tg, was introduced from inlet 1, it flowed through both channels and channel 1 had an angle change of 60.1 mDeg and channel 2 showed negligible angle change (Fig. 3d). This selective displacement offers the selectivity of the sensor. During the surface preparation, fibrinogen completely displaced IgG in channel 2; no angle change occurred after the Tg injection. If IgG residue remained on the surface, while not being completely displaced by fibrinogen, Tg should have displaced the IgG residue and produced angle change in channel 2. Fig. 3e shows the final angle changes (%) of a series of experiments on both surfaces, which indicates that the sensor has selectivity in the controlled cocktail of proteins.

When a mixture of proteins solution was injected, the sensor only responded to a specific target protein. Fig. 4 shows that the sensor responds to the target protein, Tg, in a mixture of albumin, haptoglobin and Tg. Upon the mixture reaching the surfaces channel 1 showed 58.9 mDeg angle change and the channel 2 had little angle change (3 mDeg) (Fig. 4b). This set of experiment demonstrates that fibrinogen (channel 2) remained on the surface whereas IgG (channel 1) was displaced by the mixture. Since albumin and haptoglobin in the mixture cannot displace IgG (Table 1) and multi-layer is not formed in the process, only thyroglobulin generates the SPR angle change in the channel 1 (Fig. 4a and b). In a series of experiments, we verified that the sensor has a selectivity (Fig. 4c).

When the exchange reaction by the mixtures occurs, many weakly bonded proteins are easily detached by subsequent PBS washing flow because few proteins are involved in the displacement. Also, it is noteworthy to observe the behavior of proteins in channel 2. Although target molecules do not displace pre-adsorbed proteins on the surface in channel 2, the SPR shows a small increase with moderate slope. However, their interactions to the surface are very weak and they are easily detached by subsequent PBS flow, resulting in a very small hill of SPR angle change. After waiting to be stabilized, the permanent angle change is almost negligible (Figs. 3d and 4b).

3.4. Selectivity and calibration curve in Tg detection

Fig. 5a shows the selectivity of Tg on the pre-adsorbed surfaces by IgG and fibrinogen. The selectivity was measured with respect to albumin, haptoglobin and IgG. When Tg was injected, 59 mDeg angle was generated while others had insignificant angle changes, all less than 5 mDeg. This indicates that the Vroman-effect-based biosensor can be configured to selectively detect Tg. Fig. 5b shows a calibration curve by measuring the signal magnitude over a range of Tg loadings (1 pg/ml to 1 μg/ml). The plot demonstrates that the assay is linear over 6 orders in concentration (1 pg/ml to 1 μg/ml).

4. Conclusion

In this paper, we demonstrated that the competitive nature of protein adsorption allows selective detection of a cancer biomarker, Tg, in a protein mixture (albumin, haptoglobin, and Tg) without using the antibody immobilization. The sensing method offers multiple advantages over many existing affinity-based detection methods: (i) rather expensive, time-consuming and labor-intensive immobilization process can be avoided using hydrophobic physical adsorption of proteins and (ii) diverse target molecules can be detected by choosing an appropriate combination of pre-adsorbed protein pairs. The proof-of-concept protein sensor has many challenges to be addressed. The sensor requires extensive sample preparation steps to separate proteins including size exclusion chromatography and electrophoresis since the sensor alone cannot detect a protein of interest among a large mixture of proteins with similar adsorption properties. These sample preparation steps are currently deployed in analytical instruments, which the sensor needs to be interfaced with.

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