Simultaneous On-chip Surface Plasmon Resonance Measurement of Disease Marker Protein and Small Metabolite Combined with Immuno- and Enzymatic Reactions

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Disease marker protein and a correction marker were simultaneously measured with a portable surface plasmon resonance (SPR) system and PDMS microchannel. Two gold films in the microchannel were modified with antibody and redox polymer containing horseradish peroxidase (HRP). Creatininase, creatinase, and sarcosine oxidase were immobilized in the upstream. We could measure glycoprotein, transferrin (MW: 75 kDa) and creatinine (MW: 113) both by SPR angle shifts caused by immuno- and redox reactions, indicating that urinary transferrin concentration could be normalized with creatinine concentration by single injection.

Recently, the on-site monitoring of disease markers with point of care testing (POCT) devices has become attractive for early diagnosis and treatment.[1,2] Many researchers have reported various clinical testing devices that mainly detect the markers in the blood. However, blood sampling is invasive and frequent sampling imposes huge stress on patients. In contrast, urine sampling is simple, easy, and non-invasive.[3] However, urine concentration fluctuates greatly depending on water intake or sweating and accurate measurement is difficult. Therefore, urine samples have been standardized by creatinine concentration since almost the same amount is excreted each day.[4,5] Although creatinine can be detected by spectroscopy[6] or HPLC,[4,7] these approaches are time-consuming and require additional equipment. More recently, electrochemical enzyme sensors have been introduced to detect creatinine since they constitute an easy, simple, and specific method.[8,9]

On the contrary, disease markers including proteins and peptides have been detected by enzyme-linked immunosolvent assay (ELISA),[10] and immunochromatography.[11] These methods are highly sensitive and provide a high throughput, but labeling is required. Recently, surface plasmon resonance (SPR) has become attractive as a non-label measurement technique.[12–14] This method is very effective for large molecules but not sensitive for small molecules, especially those less than 10 kDa, because of the small refractive index change. If we could detect the small substance by amplifying the SPR signal, we can obtain size-independent SPR measurement system that enables us to employ simple POCT.

Here, we show the simultaneous detection of both small metabolite, creatinine and urinary diabetes marker, transferrin[15,16] by SPR method in a microfluidic chip. Figure 1 shows a schematic representation of a poly(dimethylsiloxane) (PDMS)-based microfluidic sensing chip and a photograph of our portable dual SPR equipment. The chip consists of a glass wafer (18 × 18 mm) with two gold thin films ($d = 2$ mm) and a PDMS plate with a microchannel (2-mm wide, 20-μm deep). The two gold films were fabricated by photolithography, RF sputtering, and lift-off techniques. For the transferrin measurement, we formed a self-assembled monolayer of carboxyl decanethiol on one of the gold films, and then modified protein A with a carbodiimide coupling reaction. Subsequently, antitransferrin antibody was immobilized via protein A. For the creatinine measurement, we cast osmium-poly(vinylpyridine)-wired horseradish peroxidase (32 μL/cm^2) (Os–gel–HRP)[17] on the other gold film surface, and dried it overnight. Then, we cast a 0.1-mg/mL BSA solution on the Os–gel–HRP-modified film and incubated it for 15 min to suppress nonspecific adsorption. After removing excess BSA by rinsing, we immobilized creatininase, creatinase, and sarcosine oxidase in the upstream region in the microchannel. Finally, we attached the glass wafer and PDMS plate to inlet/outlet tubes. The creatinine detection mechanism is as follows. Hydrogen peroxide generated by the three-enzyme conversion of creatinine was reduced by HRP, which was then regenerated with Os complex. We can measure the creatinine concentration from the redox state change of Os–gel–HRP (Os^{2+} to Os^{3+}) by the SPR angle shift. Our sensor can be used repeatedly since we can regenerate the transferrin sensor by introducing glycine–HCl buffer (pH 3.0) for antibody dissociation, and creatinine sensor with 100-μM ascorbic acid to reduce the Os–gel–HRP (Os^{3+} to Os^{2+}).[18,19]

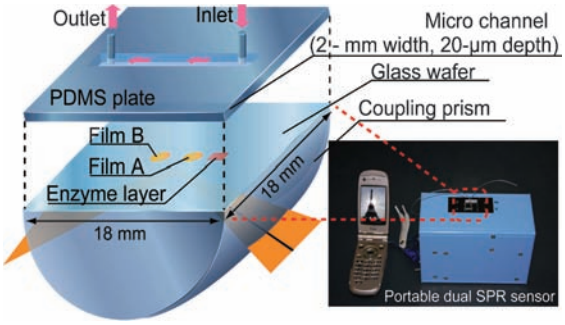

Figure 1. Schematic representation of a PDMS-based microfluidic sensing chip and a photograph of portable dual SPR sensor. Film A was modified with Os–redox polymer. Film B was modified with capture antibody via protein A.

Figure 2 shows the variations in SPR angles when we injected a mixture solution of 10-μg/mL transferrin and 1 mM creatinine. The SPR angle on the transferrin sensing surface gradually increased with time and saturated as a result of immuno-complex formation in 15 min (RSD = 7.7%, $n = 5$). In contrast, the SPR angle on the creatinine sensing surface decreased more rapidly and reached a steady state in 2 min. This indicates a fast enzymatic conversion from creatinine to hydrogen peroxide and a rapid flow from the tri-enzyme layer to the Os–gel–HRP-modified surface.[20] We could obtain reproducible responses of 6.4 mdeg/s for 1 mM creatinine (RSD = 7.1%, $n = 5$). Though

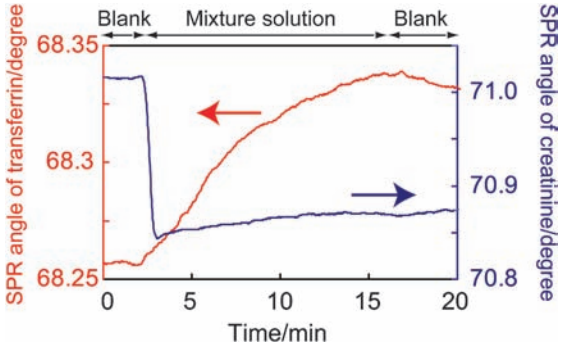

Figure 2. Variations in SPR angles when a mixture solution of 10 μg/mL transferrin, sufficiently high concentration of anti transferrin antibody, and 1 mM creatinine in 0.1 M phosphate buffer (pH 8.2) was injected for 13 min. Flow rate was 3 μL/min.

interference substances such as ascorbic acid could affect the sensitivity of creatinine, we could remove these substances successfully by integrating pre-reactor that we have developed previously.[21]

In a microfluidic system, the crosstalk of the signals at an adjacent sensing surface often causes a problem. In our system, creatinine has little effect on the transferrin sensing surface since the SPR angle change caused by the bulk effect of 1 mM creatinine was only 3 mdeg. In contrast, we modified BSA to prevent nonspecific adsorption of transferrin on the creatinine sensing surface to suppress nonspecific adsorption. Although the slope of the SPR sensorgram for creatinine decreased to 83% owing to the suppression of creatinine diffusion in the BSA layer, the sensitivity for real sample measurement was maintained since the urinary creatinine is above 2 mM. These results indicate that we could determine two biomolecules with very different molecular weights in a small volume sample (50 μL) without any crosstalk.

Figure 3 shows the calibration curves for transferrin and creatinine. The linear range of transferrin was 100 ng/mL–10 μg/mL with a detection limit of 20 ng/mL. Since the general cut off value for transferrin is around 800 ng/mL, the sensor has sufficient sensitivity to measure urine transferrin directly without preconcentration. On the contrary, the linear range of creatinine was 10 μM–5 mM. The calibration curve of creatinine gradually saturated when the concentration was above 5 mM because the sensitivity is affected by the dissolved oxygen concentration. Since the creatinine concentration in urine is around from 2 to 30 mM, we could obtain the concentration accurately by diluting the urine sample several times. Even if we dilute the sample solution 10 times, we can successfully detect transferrin at less than diluted cut off value by considering our detection limit. In addition, sample dilution will also suppress the bulk effect because the SPR sensor is affected by the refractive change in solutions. Although the detection limit of creatinine (10 μM, 1.1 μg/mL) was 55 times higher than that of transferrin (20 ng/mL), it is clearly enhanced by our method because the molecular size of creatinine is 1–6637th that of transferrin. Our device could be more suitable than conventional techniques for the following reasons. First, we can complete one assay within 15 min with one injection whereas ELISA needs several hours to complete multiple steps including labeling, substrate injection and rinsing. With our device it is also much easier to integrate multiple sensing points for a wider variety of analytes including not only large disease markers but also small biomolecules such as glucose and uric acid that cannot be integrated in immunochromatography.

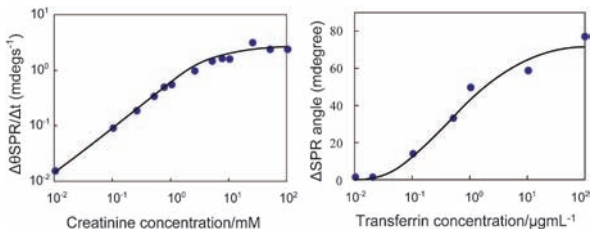

Figure 3. Calibration curves for (a) creatinine and (b) transferrin. The concentration range for creatinine and transferrin are from 10 μM to 100 mM and 10 ng/ml to 100 μg/mL, respectively. Other conditions are the same as those in Figure 2.

In conclusion, we have developed a miniaturized uric sensor for the simultaneous measurement of very different sized biomolecules by the SPR method. This sensor was achieved by integrating immuno- and enzyme sensing surfaces that could induce sufficient SPR angle shift even for small molecules. We could measure transferrin and creatinine with the concentration ranges required for clinical applications. This technique could be used to monitor various on-site biomarkers in urine by changing the capture antibodies.

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Published on the web (Advance View) May 31, 2008; doi:10.1246/cl.2008.698