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One-chip biosensor for simultaneous disease marker/calibration substance measurement in human urine by electrochemical surface plasmon resonance method

Kohei Nakamoto[a,b], Ryoji Kurita[b], Osamu Niwa[a,b,*]

[a] Graduate School of Pure and Applied Science, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8573, Japan
[b] National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba 305-8566, Japan

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ABSTRACT

We have developed a miniaturized electrochemical surface plasmon resonance biosensor for measuring two biomolecules that have very different molecular sizes, one is transferrin (MW = 75 kDa) as a disease marker protein, the other is creatinine (MW = 113) as a calibration marker for the accurate measurement of human urinary samples. The sensor has a PDMS based microchannel that is 2 mm wide and 20 μm deep. Two gold films were integrated in the microchannel; one was modified with anti-transferrin antibody for immuno-reaction, and the other was modified with osmium-poly-vinylpyridine wired horseradish peroxidase (Os-gel-HRP). We further immobilized a tri-enzyme layer of creatininase, creatinase and sarcosine oxidase in order to measure creatinine by converting it to hydrogen peroxide in the upstream channel. We measured the transferrin concentration from the refractive index change involved in an immuno-complex formation, and we were simultaneously able to measure creatinine by employing the refractive index change in the Os-gel-HRP caused by oxidation with the hydrogen peroxide produced from creatinine by the tri-enzyme. The effects of ascorbic acid and uric acid in urine samples were sufficiently eliminated by adding ascorbate oxidase and uricase to the urine samples during sampling. We were able to measure two analyte concentrations within 15 min by one simple injection of 50 μL of diluted human urine into our sensor. The detectable transferrin and creatinine ranges were 20 ng/mL to 10 μg/mL, and 10 μM to 10 mM, respectively, which are sufficient levels for clinical tests. Finally, we compared the results obtained using our sensor with those obtained with a conventional immunoassay and the Jaffe method. We obtained a similar trend that can reduce the fluctuation in the urinary transferrin concentration from three different samples by calibrating the creatinine concentration.

1. Introduction

Various health conditions and disease symptoms have been monitored by utilizing biological substances such as DNA, proteins, and hormones in blood, urine, and saliva samples (Barratt and Topham, 2007). Disease markers in blood samples have been most widely utilized in clinical diagnosis because they have a homeostatic property and provide a quick response regarding the acuteness of a symptom. Since blood sampling is often invasive, it is more suitable to use urinary samples for point of care testing (POCT) or daily monitoring after surgery (Price, 2001).

However, diagnosis is difficult solely from measurements of the disease marker concentration in a urinary sample (Wang et al., 2009). This is because the urinary concentration fluctuates greatly as a result of the diaphoresis condition and the amount of water intake. To overcome this problem, creatinine is detected to calibrate the urinary concentration because almost the same amount of creatinine is excreted every day (Narayanan and Appleton, 1980). The Jaffe reaction between creatinine and picric acid has been the most widely used approach for creatinine measurement owing to its simplicity. The Jaffe reaction occurs only in an alkaline solution, therefore it is difficult to measure creatinine and protein simultaneously in a sample due to the denaturalization of the analyte protein in alkaline solution. In addition, it is reported that the Jaffe method has poor selectivity because of a cross-reaction with pyruvate, ascorbate, etc. Siangproh et al. (2009) measured the albumin/creatinine ratio using spectrophotometric sequential injection analysis with dyes. However, the method stains not only the analytes (albumin and creatinine) but also other proteins and

* Corresponding author at: National Institute of Advanced Industrial Science and Technology (AIST), Division of Biological Resources and Functions, Central 6, 1-1-1 Higashi, Tsukuba 305-8566, Ibaraki, Japan. Tel.: +81 29 861 6158; fax: +81 29 861 6177.
E-mail addresses: r.kurita@aist.go.jp (R. Kurita), niwa.o@aist.go.jp (O. Niwa).

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substances with structures similar to creatinine owing to the poor selectivity of the dyes. Therefore, a positive error was induced in real samples in clinical assays.

An enzymatic method for measuring creatinine with high selectivity has also been reported. Generally, enzymatic products such as NH_3 or H_2O_2 converted from creatinine have been detected using an electrochemical method (Gutierrez et al., 2008; Mizutani et al., 2004; Suzuki and Matsugi, 2004). However, there have been no reports regarding the simultaneous measurement of creatinine using an enzymatic reaction and a disease marker protein in a real urinary sample because of the difficulty involved in the electrochemical determination of protein.

We previously reported the simultaneous detection of a disease marker, transferrin, and a calibration marker, creatinine, with the surface plasmon resonance (SPR) method (Nakamoto et al., 2008). Of the various available detection techniques, the SPR method has attracted a lot of attention as a POCT format since it provides real-time and non-label measurement with a miniaturized system (Homola et al., 1999; Miura et al., 2003). The SPR method determines the analyte concentration from a refractive index change induced by the formation of an immuno-complex between an analyte antigen and an antibody immobilized on a sensing surface. Although it is difficult to detect small molecules with the SPR method owing to the small change in the refractive index, we could successfully detected creatinine in a microfluidic channel using an electrochemical SPR method, which detects the refractive index change caused by an oxidation state change in redox polymer film (Iwasaki et al., 2001).

Another remaining problem as regards measuring real samples is the existence of ascorbic acid, uric acid and urinary albumin, which are considered to interfere with electrochemical SPR. This is because oxidizable acid reduces the redox polymer, and the non-specific adsorption of urinary albumin induces a refractive index change on a sensing surface. Also internal creatine reduces the accuracy of the results by creatinine calibration because creatine is an intermediate substance of creatinine. To realize a POCT device that maintains high accuracy with a simple detection system, we should detect these two different sized molecules using a single injection without a separation step.

In this paper, we report a procedure for fabricating a one-chip biosensor for transferrin and creatinine. We investigated the efficiency with which we could eliminate interfering substances by mixing enzymes to decompose these interferences in a sample solution and by immobilizing them in a channel. Then we were able to obtain a correlation with a conventional method under optimized conditions. We succeeded in the on-site measurement of transferrin and creatinine in real human urine samples with a single injection by combining portable SPR equipment.

2. Experimental

2.1. Chemicals

Phosphate buffered saline, catalase, creatininase, creatinase, and sarcosine oxidase were purchased from Sigma. Ascorbate oxidase and uricase were purchased from Oriental Yeast Co., Ltd. Osmium-poly(vinylpyridine)-wired horseradish peroxidase (Os-gel-HRP) was purchased from Bioanalytical Systems (West Lafayette, IN). Block Ace (a blocking agent made mainly from milk) was purchased from Yukijirushi Nyugyo (Tokyo, Japan). A QuantiChrom™ Creatinine Assay Kit was purchased from Bioassay Systems (Hayward, CA, USA). 10-Carboxy-1-decanethiol (10-CDT) was obtained from Dojindo (Kumamoto, Japan). N-Hydroxysuccinimide (NHS) and N-ethyl-N-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) were purchased from Pierce (Rockford, IL). 2-Aminoethanol and glycine were purchased from Nakalai Tesque, Inc. (Kyoto, Japan).

2.2. Chip fabrication

Fig. 1 shows our sensor chip. It has two circular gold thin films (A and B) formed on a BK7 glass plate ($n = 1.525 \pm 0.0015$, Seiken Precision Glass, Tokyo, Japan) covered with poly(dimethylsiloxane) (PDMS) with a straight microflow channel (2 mm wide and 20 μm deep) as shown in Fig. 1(c).

The fabrication process is as follows. First, we sealed the glass plates using an adhesive sheet (18 mm × 18 mm) with two round 2 mm-diameter holes. We then deposited a 2 nm chromium layer on the glass plates as an adhesion layer using electron beam (EB) evaporation (EB-680, Eiko Engineering, Tokyo, Japan) and then formed gold film without breaking the vacuum. The total thickness of the gold and chromium was about 45 ± 2 nm. For the transferrin measurement, we formed a self-assembled monolayer of carboxyl decanethiol on film B by immersing 1 μM carboxyl decanethiol in ethanol. We then modified gold film B with anti-transferrin antibody as follows by the well-known carbodiimide coupling reaction provided by an EDC/NHS system. We immersed gold film B with a carboxyl decanethiol monolayer in 10 mM acetate buffer (pH 5.0) that contained 0.4 mg/mL EDC and 1.1 mg/mL NHS. Then anti-transferrin antibody (6E6) was added to the buffer to give a

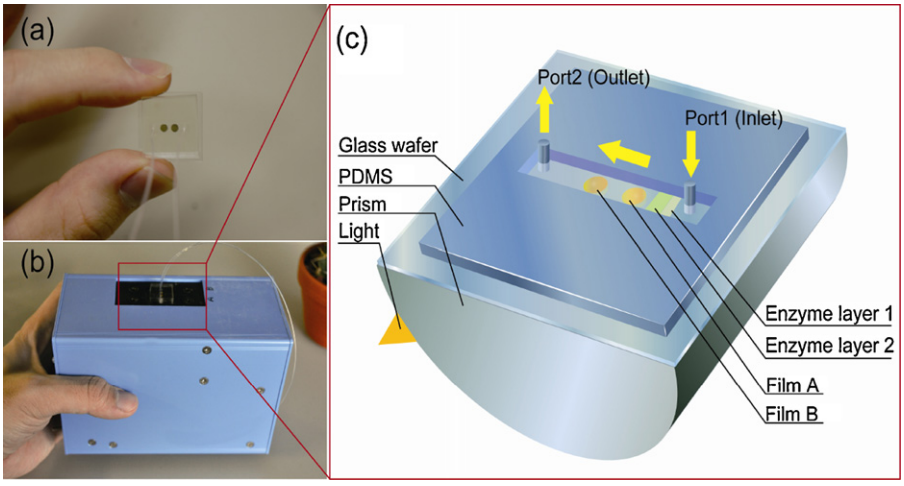


Fig. 1. Photograph of (a) SPR sensor chip and (b) portable SPR device. (c) Illustration of sensor device. Glass wafer and prism are connected via matching oil.

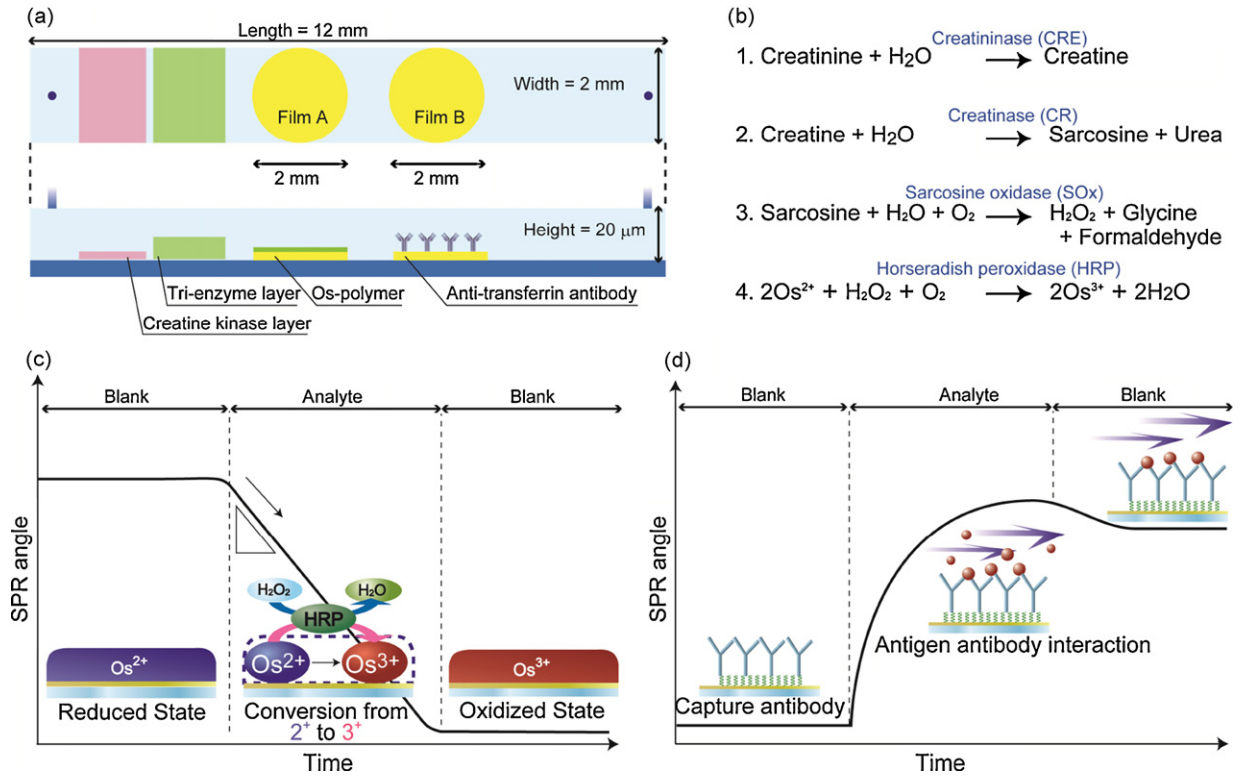


Fig. 2. Schematic illustrations of (a) channel modification, (b) enzyme reactions from creatinine to hydrogen peroxide, (c) mechanism of electrochemical SPR, and (d) immuno-reaction between captured anti-transferrin antibody and transferrin.

final concentration of 20 μg/mL, and the solution was incubated for 15 min at room temperature. For the creatinine measurement, we cast osmium-poly(vinylpyridine)-wired horseradish peroxidase (32 μL/cm^2) (Os-gel-HRP) on film A. The Os-gel-HRP layer was dried in a refrigerator overnight. We immobilized creatininase, creatinase, and sarcosine oxidase in the upstream region in the microchannel by covering it with 1 μL PBS solution containing 2 wt.% BSA and 0.2 wt.% glutaraldehyde, 0.5 unit of creatininase, creatinase, and sarcosine oxidase and then dried it at room temperature overnight. We also immobilized 0.5 unit of creatine phosphokinase in the upper region of the microchannel, which we called Enzyme layer 1. Subsequently we employed a 0.1% block ace as a blocking substance on both sensor surfaces and incubated them for 15 min to suppress non-specific adsorption. After removing any excess blocking reagent, we covered the PDMS on the glass slide. A CMA 102 syringe pump (CMA Microdialysis, Stockholm, Sweden) was used to introduce the sample solutions into the channel with the sensor surfaces in the suction mode. The sensor surface can be used repeatedly by introducing glycine–HCl buffer (pH 3.0) for antibody dissociation, and a creatinine sensor with 100 μM ascorbic acid to reduce the Os-gel-HRP (Os^{3+} to Os^{2+}).

2.3. Measurement procedure

Before starting the measurements, we peeled the adhesive sheet from the glass plate, and then we attached the plate to the PDMS cover with a straight microchannel. We connected two tubes, 1 and 2, to ports 1 and 2, respectively, on the PDMS cover. We also connected the other end of tube 2 to a syringe, and the syringe was installed in a syringe pump.

First, the urine sample was diluted 10 times in PBS buffer. We also added 1 unit each of ascorbate oxidase, uricase, and catalase. A transferrin and creatinine mixture solution was immediately introduced from port 1 at a flow rate of 3 μL/min for 10 min via the

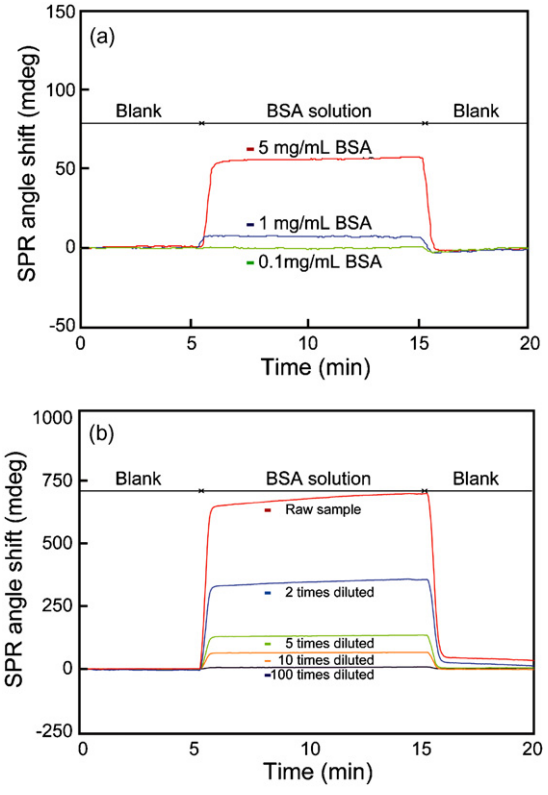


Fig. 3. SPR sensorgrams when injecting (a) various concentration of BSA solutions and (b) diluted urine samples.

syringe pump. In this step, ascorbic acid and uric acid were pre-treated with an enzymatic reaction. When the solutions transited the phosphocreatine kinase layer, the creatine in the urine sample was converted to phosphocreatine. When the solution flowed across the transferrin sensing layer, an antigen antibody reaction occurred on the gold film and the refractive index increased. On the other hand, the SPR angle for the creatinine sensing part decreases because the Os complex was oxidized by the hydrogen peroxide converted by tri-enzymes as shown in Fig. 2.

3. Results and discussion

3.1. Transferrin measurement

With SPR measurement, non-specific adsorption is a major problem with respect to protein detection. Various proteins are naturally included in urine samples. Most of the urinary protein is albumin, therefore we investigated the non-specific adsorption on our sensor with BSA and a diluted human urine sample. Fig. 3(a) shows SPR sensorgrams when solutions of BSA at various concentrations in PBS buffer were injected into a microchannel. Non-specific adsorption was not observed after we changed the injection solution from BSA to blank buffer even if we injected 5 mg/mL BSA. Next we injected a real urine sample and monitored the SPR angle. SPR sensorgrams are shown in Fig. 3(b). As a result, we found that the non-specific adsorption was negligible when the urine was diluted more than 5 times as shown by the SPR angles obtained before and after injecting the sample solution. In contrast, when the urine sample was diluted less than 2 times, non-specific adsorption was observed as shown in Fig. 3(b). For example, an SPR angle shift of about 50 mdeg was observed when we injected human urine without dilution. This indicates that about 0.5 ng/mm^2 of urinary protein is adsorbed on the sensing surface because a SPR angle shift of 1° is reported to correspond to a 10 ng/mm^2 protein adsorption (Fagerstam et al., 1992; Stenberg et al., 1991). These findings revealed that we can obtain accurate results by diluting urine samples more than 5 times to prevent non-specific adsorption.

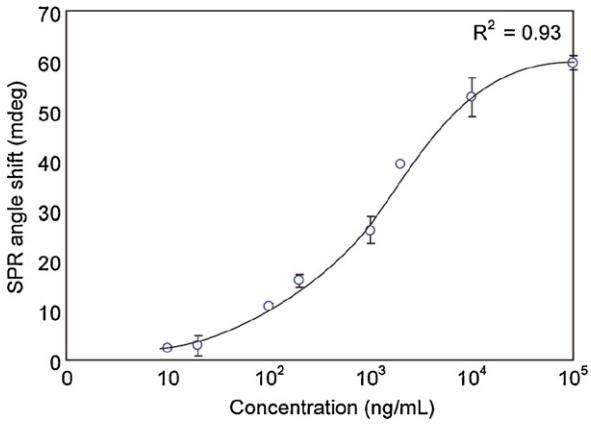


Fig. 4. Calibration curve for transferrin from 10 ng/mL to 100 μg/mL with 1 mg/mL BSA.

Fig. 4 shows the calibration curve for transferrin from 10 ng/mL to 100 μg/mL. The linear range was from 100 to 10,000 ng/mL

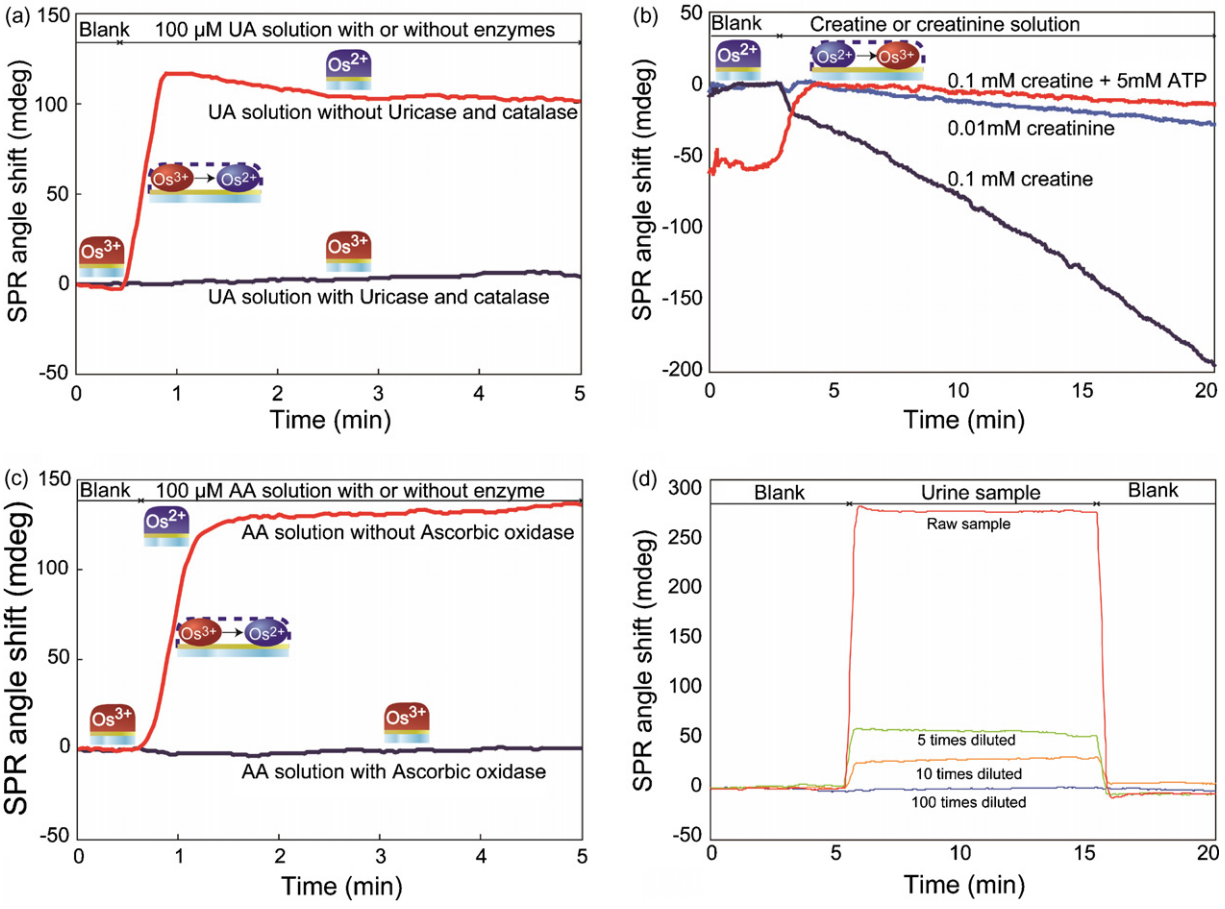
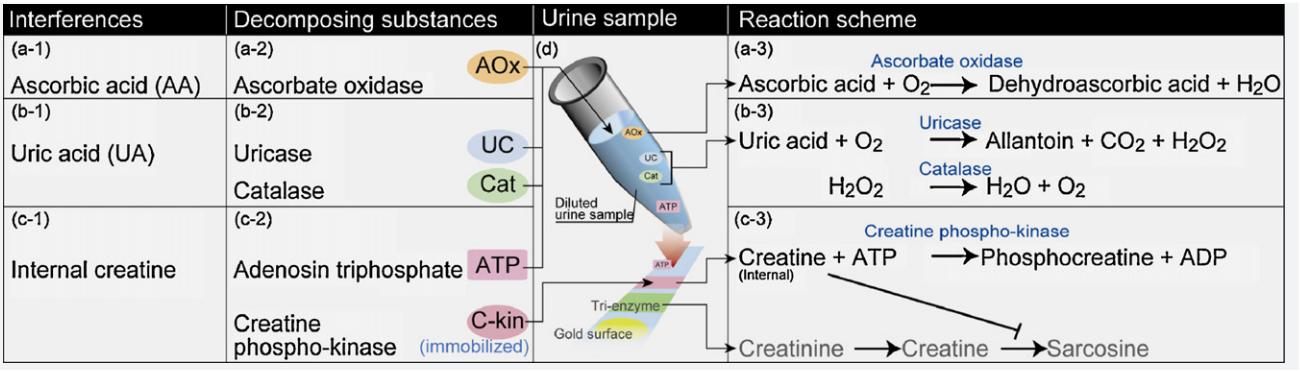


Fig. 5. SPR sensorgrams for interference substances of (a) 100 μM uric acid, (b) 100 μM ascorbic acid, (c) creatine, and (d) 5, 10, 100 times diluted and raw urine samples collected from a healthy person.

Table 1
Interference substances (a-1, b-1, c-1), decomposing substances (a-2, b-2, c-2) and their decomposition scheme (a-3, b-3, c-3). (d) Schematic illustration of sample preparation and flow process.



(R^2 = 0.93), and the detection limit was 20 ng/mL. The usual cut-off value for urinary transferrin is about 700–10,000 ng/mL (Wan et al., 1994). Thus our sensor had sufficient sensitivity even if the urine sample were diluted about 10 times due to non-specific adsorption.

3.2. Creatinine measurement

When we detect the creatinine, interference substances such as ascorbic acid (AA) and uric acid (UA) become a critical problem. We solved this problem by employing several enzymes to remove interferences from the sample solution. Table 1 shows the schemes used to remove each interference. We added ascorbate oxidase for ascorbic acid, uricase and catalase for uric acid in the sample solution, and immobilized creatine phosphokinase for internal creatine for removing ascorbic acid, uric acid and internal creatine. Adenosine triphosphate (ATP) was added to the sample solution to decompose the internal creatine.

Fig. 5(a) and (b) shows SPR sensorgrams when 100 μM UA and AA were injected onto the sensor surface, respectively. Before the measurements, the Os complex was maintained in an oxidized state (Os^{3+}) to allow us to evaluate the decomposing efficiency of enzymes. When uric acid solution without uricase was injected, the SPR angle was rapidly increased as shown by the red trace in Fig. 5(a) owing to the reduction of Os polymer from 3^+ to 2^+. In contrast, the SPR angle of the blue trace in Fig. 5(a) did not shift when we injected 100 μM UA solution with uricase and catalase (1 unit/mL). This is because the uric acid was decomposed by these two enzymes as shown in Table 1(b-3). The blue trace in Fig. 5(b) did not shift due to the decomposition of ascorbic acid. Ascorbic acid was converted to dehydroascorbate, which does not affect Os complex reduction as shown in Table 1(a-3). Although about 10 μg/mL ascorbate oxidase and uricase was added to the injection solution, the non-specific adsorption of these enzymes was not monitored from the SPR angle shift in Fig. 5(a) and (b) the blue line.

Creatine is an intermediate substance when creatinine is converted to hydrogen peroxide as shown in Fig. 2(b). Human urine samples include both creatinine and creatine. By eliminating creatine, we can obtain a calibration curve for creatinine with high selectivity. Here we converted creatine to phosphocreatine enzymatically. Creatine phosphokinase was immobilized in the upstream of a tri-enzyme layer as shown in Table 1(d). This pre-reactor layer had no effect on creatinine detection because it was located on the upper layer of the tri-enzyme layer as shown in Fig. 2(a). 5 mM adenosine triphosphate (ATP) was previously added to the diluted sample solution. Fig. 5(c) shows the SPR sensorgrams we obtained when we injected 0.1 mM creatine (blue line), 0.1 mM creatine and 10 mM ATP (red line), and 0.01 mM creatinine (light blue line). The slope of the blue line was about 0.2 mdeg/s. And the slope of the light blue line was about 0.015 mdeg/s. In contrast the slope of the red line was about 0.026 mdeg/s. By converting creatine to phosphocreatine as shown in Table 1(c-3), the SPR slope decreased less than one tenth. And the estimated residual creatine concentration was less than 0.01 mM as shown by the red line and the light blue line in Fig. 5(c). We basically detect creatinine at concentrations of 0.1–10 mM. Thus the efficiency with which we convert creatine to phosphocreatine is practically acceptable for creatinine detection in a urine sample. The blocking effect of the sensor surface was evaluated in the same way as that shown in Fig. 3(b). Fig. 5(d) shows SPR sensorgrams when diluted or raw urine samples collected from one healthy person were injected into the system for 10 min. The SPR angles before and after injection were almost the same, indicating that non-specific adsorption on

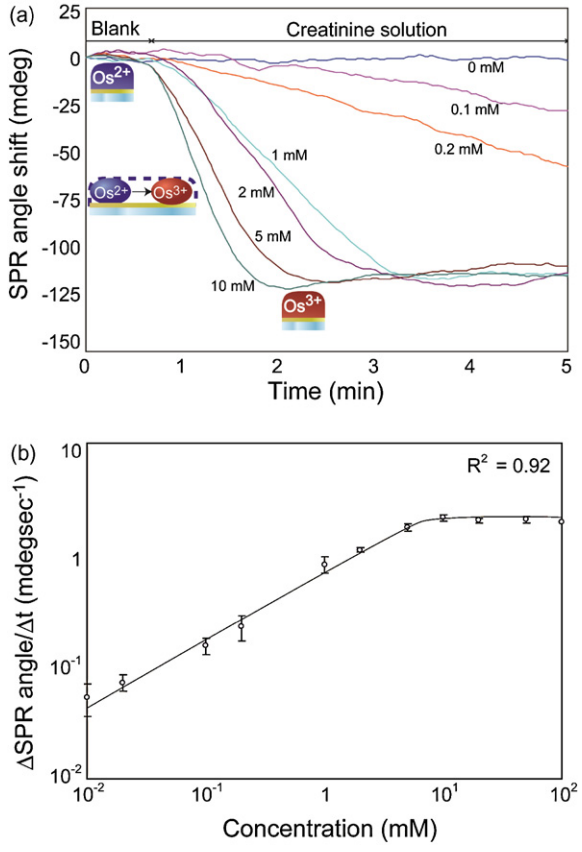


Fig. 6. (a) SPR sensorgram for creatinine when injecting various concentration samples. (b) Calibration curve for creatinine (n = 3).

the gold film A was also blocked in the same way as with gold film B shown in Fig. 3(b).

Fig. 6(a) shows the SPR sensorgrams we obtained when we injected various concentrations of creatinine solution. The SPR angle shift is around 100 mdeg, but the slope of the SPR sensorgram increased with increasing creatinine concentration. Thus we estimated the creatinine concentration by measuring the slope of the SPR angle shift. We performed the detection repeatedly by regenerating the Os complex layer by injecting 100 μM AA solution as a reductant. Fig. 6(b) shows the calibration curve for creatinine. We obtained a linear range of 10 μM to 10 mM (R^2 = 0.92). When the creatinine concentration was more than 10 mM, the slope of the SPR angle saturated. This is because the dissolved oxygen was insufficient when sarcosine was converted to hydrogen peroxide as shown in Fig. 2(b-3). Usually up to about several tens of mM of creatinine is found in a human urine sample. Therefore, when we dilute urine samples about 10 times, urinary creatinine remains in the detectable range.

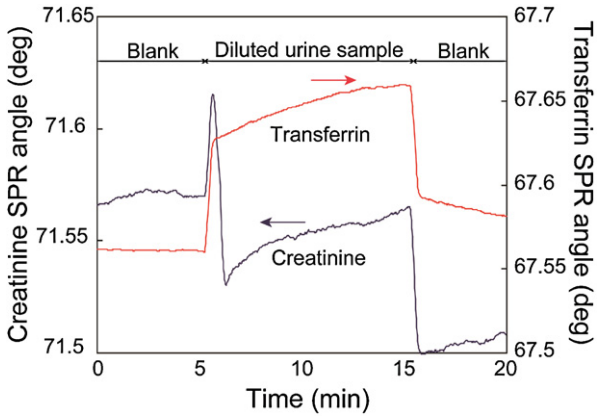


Fig. 7. Simultaneous detection of transferrin and creatinine in real urine sample (10 times diluted).

3.3. Real urine sample measurement

On the basis of previous experimental results, we diluted the urine samples 10 times to suppress non-specific adsorption. Fig. 7 shows a simultaneous measurement of urinary transferrin and creatinine when we injected a prepared sample solution. Immediately after changing the solution, both sensorgrams rapidly increased by about 50 mdeg because the refractive index of the urinary solution was higher than that of the blank solution. Then the SPR sensorgram of creatinine decreased due to the oxidization of Os^{2+} polymer to Os^{3+} on gold film A as shown in Fig. 2(c). For the creatinine measurement, we calculated the slope of the SPR angle after the refractive index change induced by the solution change had finished. Here we measured an SPR angle shift of about 100 mdeg and a slope of about 0.96 mdeg/s. This value indicates that about 1 mM creatinine was included in the diluted urinary sample. As a result we estimated that about 10 mM creatinine was included in the raw urine sample. On the other hand, the SPR sensorgram of transferrin gradually increased owing to the antigen antibody interaction on gold film B. When we injected the sample, the SPR sensorgram for transferrin gradually increased due to the immuno-reaction on gold film B as shown in Fig. 2(d). We could obtain an SPR angle shift

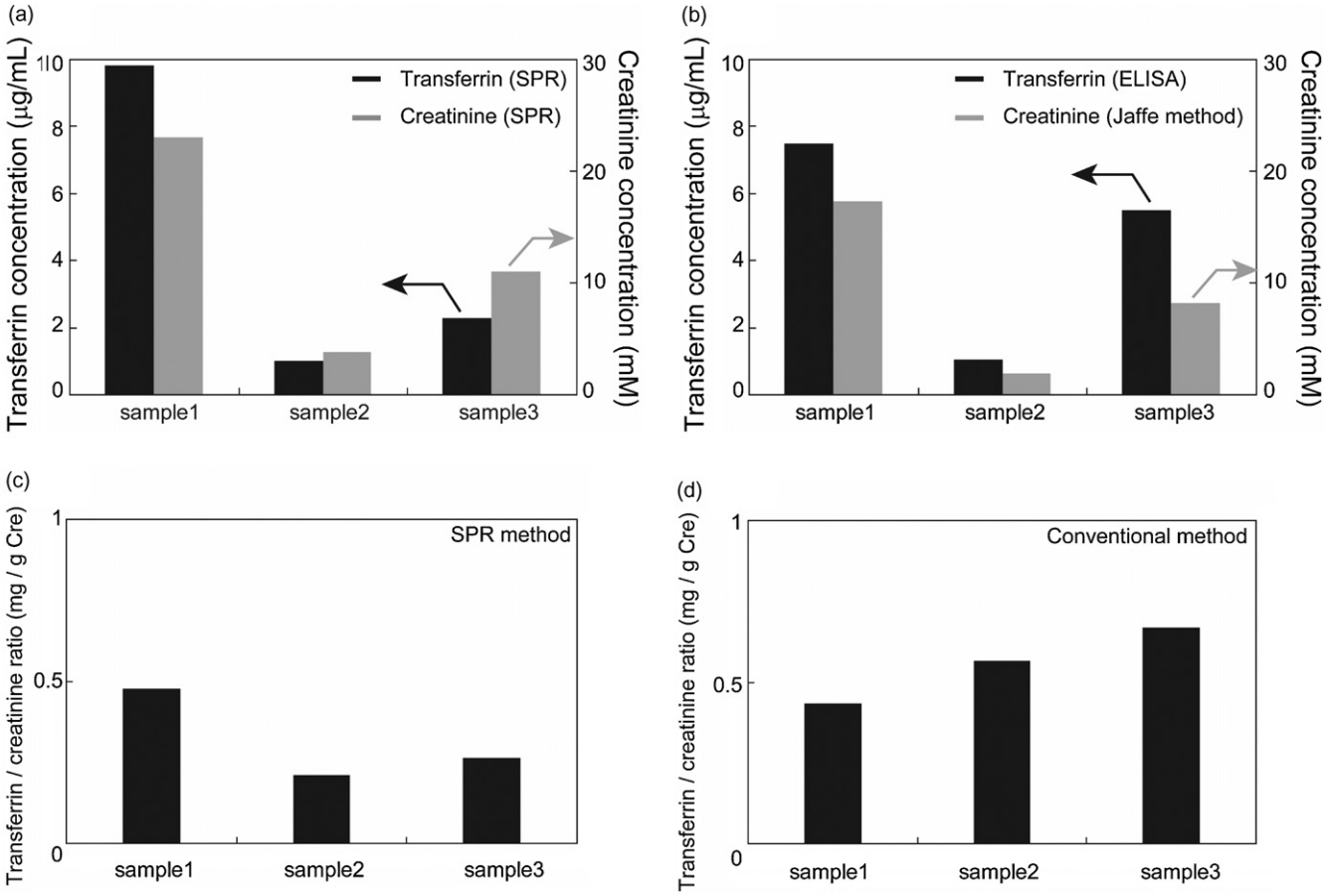


Fig. 8. Simultaneous detection results for transferrin and creatinine of three real samples by (a) SPR method, (b) conventional ELISA and Jaffe method. (c) and (d) Calibration results of urinary transferrin obtained by SPR and conventional method, respectively. These three urine samples were collected from one healthy person at the different day.

by subtracting the SPR angle of before and after sample solution injection. In this case, we observed an SPR angle shift of 32.5 mdeg. From our calibration curve for transferrin in Fig. 4, this value indicates that about 11 μg/mL transferrin was contained in the raw sample. Finally we could obtain the calibration value for transferrin by dividing the transferrin concentration by the creatinine concentration. The total sensing time is about 15 min and it can be completed in one microfluidic chip with no separation step. This is useful for detecting both the diabetic marker, transferrin, and other urinary disease markers by changing the antibody immobilized on the sensing surface.

We applied this detection method to three real samples obtained from one healthy man. Transferrin is a diabetic marker and its concentration does not change very much in a short-term checkup. By applying our detection method to three different samples obtained over several days, we were able to show the efficiency of our sensor. Also we compared our sensor with the conventional detection methods by employing ELISA and the Jaffe reaction to measure transferrin and creatinine, respectively. Fig. 8 shows (a) SPR measurement results and (b) ELISA and Jaffe method results for transferrin and creatinine. Fig. 8(c) and (d) shows calibration results obtained using our sensing chip based on the SPR method and microtiter plates based on the ELISA and Jaffe reaction, respectively.

Before calibrating the transferrin results, the values provided by both the SPR and ELISA methods fluctuated greatly. However the values became moderate by calibrating the transferrin value with the creatinine results because the urine concentrations varied among the three samples. The CV value of the three samples obtained with the SPR method decreased from 78.9% to 14.2%. On the other hand, the value obtained with the ELISA method decreased from 64.1% to 21.2%. In this case our sensor realized smaller a CV value than the conventional method. This was because of the poor selectivity of the Jaffe method since picric acid reacts with the ascorbic acid and glucose in urine not only the creatinine. Although we could obtain moderate CV value for transferrin with our method by calibrating creatinine value, where the trend is comparable to that obtained with the conventional method, there were significant differences between the values for each sample, especially with sample 2. We can consider a number of possible reasons for this difference. The first is that the dilution conditions are not optimized since sample 1, which has the highest concentration, exhibits a relatively good correlation. In contrast, with sample 2 there are very large differences between different measurements. Since our proposed method uses the slope of SPR response for the creatinine measurement, accurate measurements may be difficult to obtain for low concentration samples such as sample 2. Improved results should be obtained by optimizing dilution condition.

Another reason concerns reproducibility of the modified redox polymer layers for each device since we modified the redox polymer and the tri-enzyme layers on the glass channel for the creatinine measurement manually and also performed the antibody immobilization manually. The sensitivity fluctuation is also observed by relatively large error bars in Figs. 4 and 6(b)Figs. 6(b) and 4. In addition, we operated the conventional ELISA manually. There are many steps including rinsing and tapping to complete the measurement, and this results in a larger deviation than when automatic operation is employed.

4. Conclusion

We have developed a one-chip sensor for simultaneously detecting the diabetic markers, transferrin and creatinine, by utilizing the SPR method. We were able to measure the two concentrations in real human urine in less than 15 min with the sensor. By calibrating the transferrin concentration with the creatinine concentration as an indicator of urinary concentration, we could realize less variation in three urine samples from a healthy man, than that obtained with the conventional method. Our proposed device will provide a useful and simple POCT sensor once we have minimized the signal fluctuations between individual devices by optimizing the experimental conditions such as surface modification and urine dilution.

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