FAST WHOLE BLOOD TESTING FOR DETECTING BIOMARKERS
BY SIZE-EXCLUSION SPR SENSING
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ABSTRACT
This paper presents a simple and fast protein detection from a whole blood sample using an SPR (surface plasmon resonance) sensor chip integrated with in situ blood cell separation function. We demonstrated the separation of plasma from a whole blood just by dispensing the single drop on the sensor chip and the detection of IgG molecules in it. This sensor chip allows the detection of IgG molecules of a concentration lower than the physiological value and the fast detection time (<12 min).

KEY WORD
SPR, Blood separation, Blood testing.

INTRODUCTION
A whole blood testing is commonly used for early detection of diseases by detecting biomarker proteins in blood serum (ex. AFP and PSA). Conventional methods involve a time consuming procedure of blood cell separation using external equipments such as a centrifuge and filtration device, preventing the miniaturization of the system. Although various kinds of cell separation techniques have been developed in LOC field, including a microfluidic and electrostatic cell separations[1], most of them still require external devices such as a pump, tube, valve, and power supply. To overcome these drawbacks, we propose a novel technique for protein detection from a whole blood sample using a “Size-exclusion SPR sensor chip” (filter SPR chip) that we have previously developed for detecting particle aggregation[2].

A filter SPR chip has a microslit array on a flat gold surface, with the gap of 3 µm smaller than a cell size (Figure 1). Because the SPR sensing area is limited to about 300 nm above the gold surface, the walls of 5 µm height exclude blood cells from the sensing area, thus only a serum can touch the sensor surface, where ligands are immobilized to capture analytes, allowing the specific detection of proteins in the serum as the SPR angle shift. This method needs no external equipment for cell filtration, because it is driven by their sedimentation and diffusion. In addition, the short distance between the filtration and the sensing area enables us to detect proteins immediately after applying samples. Non-labeling of SPR sensing also allows the one-step procedure; just applying sample to a ligand-immobilized chip.

Fabrication
Glass chips (BK7, refractive index 1.515, 15×15×1 mm, NTT-AT Corp.) were cleaned with a piranha solution (H2SO4: H2O2 = 3:1) for 10 min at 85°C, and washed with DI water three times. Titanium was deposited to a 5 nm thickness as the adhesive layer, followed by the deposition of a 45-nm thick gold layer with a thermal deposition system. Micro-slits(width: 3 µm, height: 3 µm, gap: 3 µm) were fabricated by standard UV-lithography of SU-8 3005 (MicroChem Corp.) on the chips (Figure 2).

Cell separation test
Horse whole blood (Nippon Biotest Laboratories Inc.) was employed as the test sample. To verify the cell separation function, we used chips with microslit array (filter chip) and without it (flat chip). First, the blood plasma was measured using an SPR sensing system (Smart-SPR, NTT-AT Corp.) for the determination of the base line. Then, the whole blood sample was dispensed on the chips, when we monitored the shift of the SPR angle to check the exclusion effect of the microslits.

**SPR-based immunoassay for IgG detection**

Anti-IgG antibodies (goat monoclonal) were covalently immobilized on a sensor surface as follows. A carboxy-terminated SAM layer was formed on the gold surface of the sensor chip by 4, 4’-Dithiodibutyric acid (DDA). Then, 0.1 M 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.05 M N-Hydroxysuccinimide (NHS) in phosphate buffer (PBS) were dispensed onto the chip and incubated for 12 min to activate the carboxyl groups. After that, the anti-IgG solution (0.5 mg/ml) was applied there and incubated for 12 min for covalent immobilization. To block a nonspecific adsorption and deactivate unreacted NHS ester groups on the surface, we used 0.1 M ethanolamine/PBS. Then, an IgG solution (rabbit monoclonal, 0.0005 - 5 mg/ml) was dispensed to the chip, where we monitored the SPR angle shift. As a negative control, the same procedures were also performed using anti-BSA antibodies (0.5 mg/ml).

**Detection of IgG in whole blood**

Horse whole blood containing IgG molecules of 800 mg/dl concentration was applied to the ligand-immobilized sensor chip, and IgG was detected by the SPR angle shift. The concentration of IgG used here (800 mg/dl) is a physiological concentration of human blood [3]. In whole blood testing, various biomolecules in blood potentially cause nonspecific adsorption on sensor chip, preventing the determination of IgG concentration. Thus, we set reference channel to mask the nonspecific signals. Detection channel was covered with anti-IgG molecules signals, whereas reference channel was with anti-BSA molecules to obtain nonspecific signals. IgG signal were calculated by subtraction of the signal of the reference channel from that of the detection channel.

![Figure 2: Microslit structure of a filter SPR chip. a) Filter SPR chip b) SEM image.c) 3D profile.](image)

**RESULTS AND DISCUSSION**

**Cell separation test**

The in situ cell filtration was evaluated by a horse whole blood sample (Figure 3). The time course of the SPR angle (sensorgram) shows the increase of the signal after the addition only to a conventional flat chip, indicating that blood cells reached sensing area. In contrast, a filter chip shows no change, indicating that cells were successfully excluded. These results shows that a filter chip can separate plasma from a whole blood sample only by dispensing of the sample on it.

**SPR-based immunoassay for IgG detection**

As the demonstration of the detection of proteins, we used IgG molecules and anti-IgG antibodies as an analyte and ligand molecules, respectively. The sensorgrams in the ligand immobilization and analyte binding (Figure 4) show that a filter chip allows the immobilization and the detection as well as that in a conventional flat chip.

SPR angle shifts induced by IgG solution of various concentration were shown in Figure 5. SPR angle shift of a filter chip shows a curve similar to the flat chip one. This suggests that the microslits do not prevent the immunoassay used here. The SPR angle increased in accordance with the concentration of IgG, showing sensing performance equivalent to that of the flat chip.

**Detection of IgG in whole blood**

We detected IgG molecule in horse whole blood with filter and flat chips (Figure 6). Table 1 shows the SPR angle shifts of filter chip, flat chip and a value calculated from the calibration curve in Figure 5. The filter chip gives accurate IgG concentration because of the cell filtration effect, whereas the flat chip shows a large difference.
These results indicate that the signal of the filter chip agrees with the correct value calculated from the calibration curve, whereas that of the flat chip lowered due to the mask effect by blood cells. This suggests that the size-exclusion SPR sensing will bring a fast and simple whole blood testing for detecting biomarkers.

Figure 3: Time-courses of SPR angles after applying blood cells to a conventional flat chip and a filter chip. The black arrow indicates the timing of sample loading to the chips.

Figure 4: Time-courses of SPR angles from ligand immobilization (anti-IgG antibody) through analyte protein binding (IgG/PBS buffer).

Figure 5: Concentration dependence of SPR angle shift (IgG/PBS buffer).

Figure 6: Time course of SPR angle shift in IgG/whole blood detection.

Table 1. Comparison of SPR angle shift induced by binding of analyte protein (IgG) in whole blood sample.

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<tr>
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<th>SPR Angle shift value</th>
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<tr>
<td>Filter Chip</td>
<td>0.20 ± 0.05 deg.</td>
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<tr>
<td>Flat Chip</td>
<td>0.10 ± 0.03 deg.</td>
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<tr>
<td>Calculated from calibration curve</td>
<td>0.21 deg.</td>
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REFERENCES:
3. THE MERCK MANUAL SIXTEEN EDITION, MERCK & CO., INC., Section 9, Chapter 18.