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Short communication

Quantitative analysis of sialic acid on erythrocyte membranes using a photothermal biosensor

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ABSTRACT

The quantitative analysis of sialic acid (SA) at an erythrocyte membrane is becoming an important clinical parameter in diagnosing cancer and diabetes. In spite of such clinical importance, there are only a few, very expensive, time consuming and complicated quantifying methods established. To solve this problem, we demonstrate a novel and direct measurement technique for SA exposed to the cell membrane using a photothermal biosensing system in which the hemoglobin molecules in the erythrocyte absorb a specific wavelength of photons (532 nm) and convert it to a temperature change. For measuring the quantity of SA, we first modified the sensor surface of a micro-scaled thermometer using phenylboronic acid (PBA) containing a self-assembled monolayer (SAM) to capture the SA-expressing erythrocytes. Second, the sensor surface was thoroughly washed, and when more SA was expressed, tighter association of erythrocytes to the biosensor was expected. Thirdly, blood sample changes in temperature, heated by the 532 nm wavelength laser, were measured by the bottom layer's micron sized platinum thermometer. The temperature changes from the erythrocytes captured on the sensor surface could be estimated by the amount of SA expressed on the erythrocyte membrane. This novel SA analysis system can solve the problems raised by conventional methods such as multiple enzyme reactions and a time consuming process. We expect that this system will help provide a new tool in the quantitative analysis of SA expression level for the diagnosis of diabetes and cancers.

1. Introduction

Sialic acid (SA, N-acetylneuraminic acid), a negatively charged monosaccharide, is usually expressed at the termini of glycan chains on red blood cell (erythrocyte) membranes (Schauer, 2000). The quantitative analysis of SA on the cell membrane is an important clinical parameter because SA is an alternative indicator of malignant cancer (Raz et al., 1980; Dobrossy et al., 1981; Passaniti and Hart, 1988) and diabetes mellitus (Vahalkar and Haldankar, 2008; Mazzanti et al., 1997; Moretti et al., 2002). Overexpressed SA on the erythrocyte membrane surface is clinically recognized as a malignant and metastatic phenotype for many different types of cancer. On the contrary, underexpressed SA is recognized as an indicator of diabetes mellitus. Researchers have reported that SA expression in insulin-dependent diabetes mellitus patients is 38% less than that of healthy people (Vahalkar and Haldankar, 2008).

In spite of the clinical importance of SA, only a few, very expensive, time consuming, massive detection device required and complicated quantification methods have been established (Akimitsu and Toshifumi, 2001; Luca et al., 2004; Akira et al., 2009; Abdellah et al., 2011; Jessie et al., 2012). The commercialized SA quantification process uses N-acetylneuraminic acid aldolase to catalyze SA to pyruvic acid, pyruvic acid is reduced to lactic acid by β-nicotinamide-adenine dinucleotide (β-NADH), and β-NADH oxidation can be measured by spectrophotometric methods. This method has very high accuracy but requires more than 12 h to cleave all of the SA linkages, including α(2→3,6,8,9) linkages and branched SA. In addition, the assay costs are expensive, greater than $20 per assay, because multiple enzymes are needed. Moreover, it is difficult to quantify SA for narrow and low detection ranges (typically 1–200 nmol). Recently, the field effect transistor (FET)-based SA analysis method was developed (Akira et al., 2009). However, this method also requires a long analysis time and a very close distance between the SA and FET sensor surface, within several nanometers of the electrical double layer or Debye length for the counterion screening effect.

To overcome the limitations of the quantitative analysis of SA, we developed a thermal biosensor for SA analysis in which the

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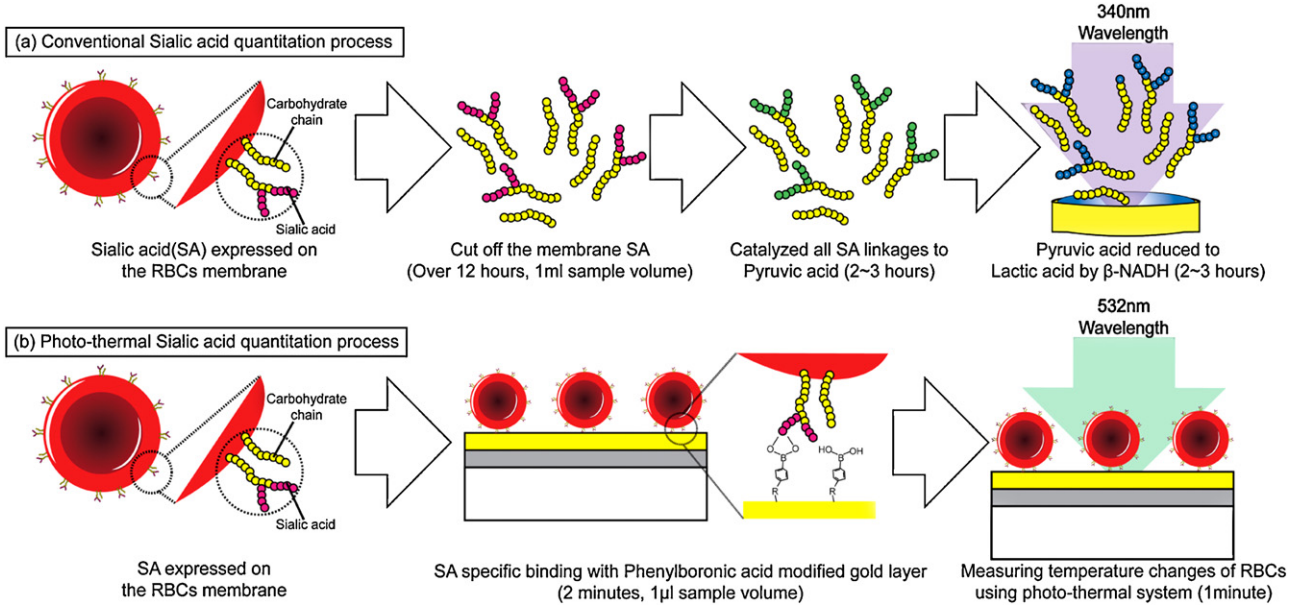

Scheme 1. Comparison of SA analysis using a photothermal sensing system with a conventional method. (a) The conventional methods consist of several enzyme based chemical reactions. Therefore, the total amount of analysis time is more than 16 h. (b) The photo-thermal biosensor used premade gold layer with a SAM and a PBA-modified layer for specific binding to the SA. This method can provide the SA quantity within a 5 min using extremely small (1 μl) amount of blood sample. The chip consists of a micro-thermometer with a gold layer at the bottom for measuring thermal changes and a SAM with a PBA-modified layer at the top for specific binding with SA on erythrocyte membranes. After the removal of unbound erythrocytes, thermal changes generated from the number of captured erythrocytes depending on SA concentration were measured.

gold surface was chemically modified for capturing erythrocytes according to the SA concentration. We subsequently measured the temperature changes of erythrocytes heated by photothermal phenomena using a micro-thermometer (Scheme 1).

Chromophores absorb photons of a specific wavelength and convert them to thermal energy (Anderson and Parrish, 1983). The hemoglobin molecule is a typical chromophore where a single erythrocyte contains 270 million hemoglobin molecules, and each hemoglobin molecule has four heme groups. Each heme group consists of an iron atom at the center of a large heterocyclic porphyrin. These iron ions absorb photons at wavelengths of 418 nm, 530–545 nm, and 577–595 nm and convert this energy to thermal energy. Specifically, the absorption peak for light is at a wavelength of 532 nm, and the photothermal parameters of erythrocytes at this wavelength have been well established (Lapotko and Lukianova, 2005; Lapotko, 2006; Almond and Patel, 1996). Previously, our group developed a thermal biosensor to exploit these phenomena and analyze the hemoglobin content of whole blood (Kwak et al., 2010a,b). In this paper, we expand the utility of our photothermal biosensor to precisely measure the quantity of SA expressed on the erythrocyte membrane to diagnose cancers and diabetes.

2. Materials and methods

2.1. Fabrication process for a thermal biosensor for SA analysis

A thermal biosensor for SA analysis was developed in this research. The SA chip consisted of three layers deposited on a Pyrex glass wafer. The bottom layer is a resistive temperature detector (RTD), which is a thermometer that functions by correlating the resistance changes of the RTD element with temperature changes. The middle layer, above the RTD, is silicon dioxide for electrical insulation between the top and bottom layers. The top layer is gold, on which the SAM and PBA are immobilized to capture SA on the membrane surfaces of red blood cells. The detailed thermal biosensor fabrication processes and photothermal sensing system are described in supplemental materials.

2.2. Surface modification of the sialic acid-mediated capture of erythrocytes

To capture the erythrocytes using SA-mediated cross-linking, the gold surface on a micro-thermometer with silicon dioxide was chemically treated to allow biomolecular interaction between PBA and SA. Dissociated PBA is a synthetic molecule capable of reversibly and selectively binding with SA in a stable way (Otsuka et al., 2003). For surface modification using PBA, we first coated the micro-thermometer with a self-assembled monolayer (SAM; 1-carboxy-10-decanthiol, Dojindo Laboratories). After SAM deposition, we modified the surface with PBA to facilitate the capture of SA on the erythrocytes. The diameter of the PBA-modified detection area was 1 mm^2, and, theoretically, more than 20,000 erythrocytes could be bound. To verify each surface modification process, the surface morphology and contact angle were measured. Fig. 1(a–c) shows scanning electron microscopy (SEM) images of the surface for each of the steps, and Fig. 1(d–f) shows the contact angle of de-ionized water for each step. To measure the contact angle, 10 μl of de-ionized water was used and analyzed using a graphic analysis program (ImageJ with the contact angle plug-in). The detailed surface modification processes are described in supplemental materials.

2.3. Blood sample preparation

A ficin enzyme reaction was used to produce erythrocytes with various concentrations of SA. Ficin is a cysteine protease that cleaves membrane proteins, including glycoprotein, and then release sugars, such as SA. We performed ficin treatment at varying times (30 s intervals for each sample), and the results of the SA contents, determined by a commercial quantification process for each sample, are shown in Fig. 2(a). The SA concentration of the whole

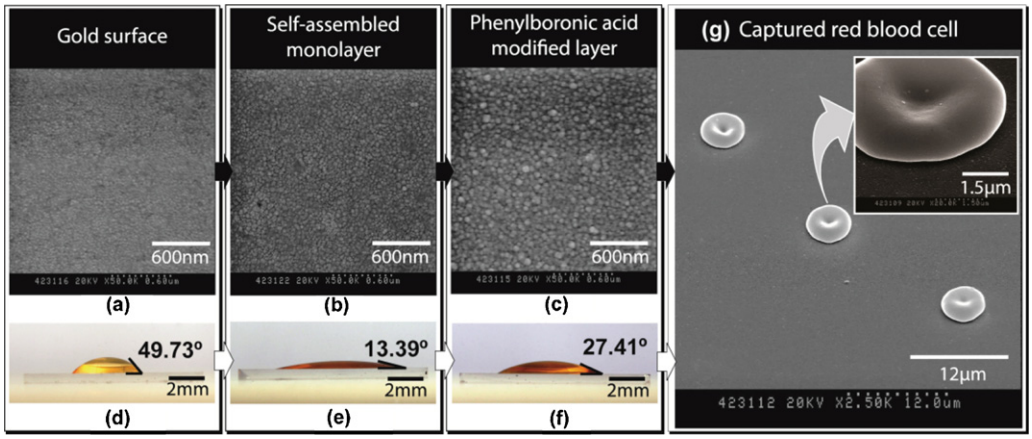

Fig. 1. Scanning electron microscopy images of the (a) gold surface, (b) SAM-deposited layer, and (c) phenylboronic acid-modified surface, and (d, e, and f, respectively) the contact angles of each surface. The gold, SAM, and PBA-modified layers were hydrophobic, hydrophilic, and semi-hydrophilic, respectively. After verification of each step, an (g) erythrocyte-capturing experiment was performed.

blood was 379 pmol/10^6 cells, and the reduced SA concentration percentages of 30 s, 60 s, 90 s, and 120 s ficin-treated samples compared to that in whole blood were 4.1%, 23.8%, 28.4%, and 51.6%, respectively. The SA concentrations of five blood samples, four treated with ficin and one untreated, were 183.6 ± 9.5, 271.6 ± 30.9, 288.9 ± 17.5, 363.7 ± 4.0, and 379.3 ± 25.9 pmol/10^6 cells, respectively. All blood samples were washed three times with PBS solution before use and the detailed SA quantification processes are described in supplemental materials.

3. Results and discussion

3.1. Calibration of the thermal biosensor for SA analysis

After fabrication of the chip, the calibration of the thermometer was performed via the resistance changes of the RTD and temperature changes. The temperature of the SA chip was measured using a J-type (Iron/Constantan) thermocouple (Omega Engineering, Inc.) bonded to the SA chip surface and connected to a digital

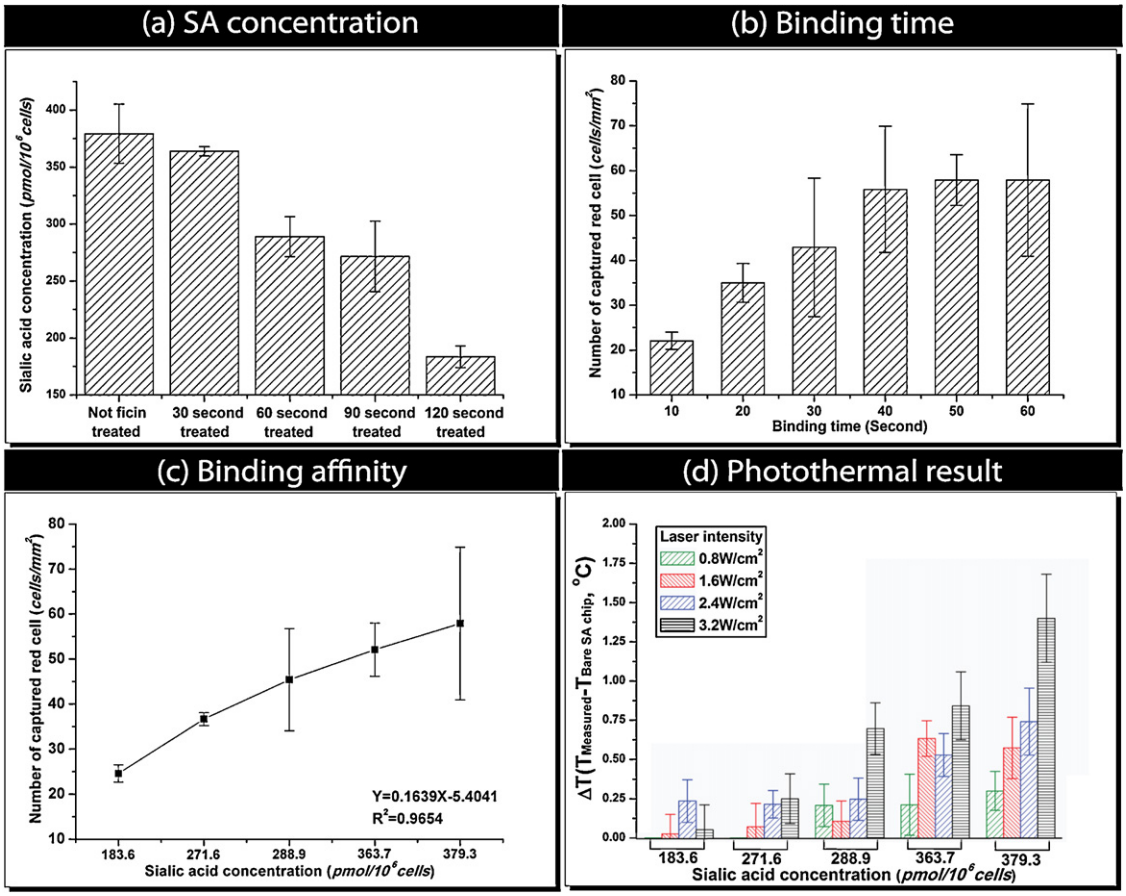

Fig. 2. (a) SA concentrations of whole blood that was treated with ficin for varying time intervals; (b) binding time optimization test; (c) binding affinity test using various concentrations of erythrocyte samples (4.90×10^6 cells/μl); (d) relative temperature changes between the bare PBA-modified gold surface and captured erythrocytes measured with the photothermal sensing system. The limit of detection of 0.8, 1.6, 2.4, and 3.2 W/cm^2 is 322.3, 246.6, 234.3, and 219.7 pmol/10^6 cells, respectively. Data shown is mean ± SD, $n = 3$.

multimeter (Agilent 34970A) for data acquisition. The resistance changes of the platinum RTD were measured by an *I–V* meter (PXI-4071 DMM, PXI-4130 power SMU, PXI-1033 chassis, National Instruments). For temperature control of the SA chip, a Peltier element (Ace Tec) and hot plate (Barnstead International) were used. The calibration was performed three times within a 10–100 °C range at 18 different points. The calibration results are shown in supplementary files (SFig. 1(i)). The calibration equation is as follows:

$$R(t) = 1.8459(t) + 601.34 \quad (1)$$

where $R(t)$ and t represent the resistance (Ω) at temperature t and the temperature in °C, respectively. All experimental results were calculated using Eq. (1).

3.2. Optimization of experimental procedure and feasibility test

Before the temperature changes from the erythrocytes were measured using photothermal phenomena, binding experiments and a feasibility test were performed with erythrocytes and a PBA-modified chip to determine the optimized conditions for erythrocyte capture. The binding kinetics were determined by the number of captured erythrocytes, and the results are shown in Fig. 2(b). The SA–PBA coupling was saturated after approximately 50 s of incubation. An incubation time of 60 s was optimal to obtain a high binding efficiency. After the binding experiments, different concentrations of SA samples were measured by an optical cell counting method. The results of the binding affinity of erythrocytes based on the SA concentrations are shown in Fig. 2(c). We placed 1 μl of a washed, whole blood sample on the PBA-modified gold surface and incubated the SA- and PBA-binding affinity test for 60 s. The diameter of the 1 μl volume of the whole blood sample was 5.3 mm^2, which corresponds to an area 6.8 times larger than the PBA-modified gold surface sensing area. After incubation, the test chip was immersed in PBS to remove unbound erythrocytes. The bound erythrocytes were counted with an optical image using a microscope (BX51, Olympus). In the case of the whole blood sample, which had a SA concentration of 379 pmol/10^6 cells, the bound erythrocyte concentration was 58 ± 17 cells/mm^2. The 30 s, 60 s, 90 s, and 120 s ficin enzyme-treated samples had concentrations of 52 ± 6, 45 ± 11, 37 ± 1 and 25 ± 2 cells/mm^2, respectively. The binding affinity test showed that the number of captured erythrocytes was proportional to the SA concentration (see Fig. 2 (c)). The erythrocytes bound with PBA are shown in Fig. 1(g).

3.3. Determination of sialic acid concentration using photothermal effects

The hemoglobin content of erythrocytes is the most important source for the analysis of the SA content of the erythrocytes. The number of erythrocytes, their hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were measured by a complete blood cell count machine (ADVIA®2120, Siemens) at 4.90×10^6 cells/μl, 13.5 g/dL, 79.5 fL, 27.5 pg, and 34.6 g/dL, respectively. We also performed a thermal sensing experiment using the captured erythrocytes for SA quantification analysis by inducing 532 nm wavelength light with differently powered lasers (see Fig. 2(d)). A power tunable, diode pumped, solid state, continuous wavelength laser module was used as the light source. The protocol for the thermal sensing experiment was the same as that used for the binding affinity test. Specific heating of the erythrocytes was induced with a 532 nm wavelength laser. Thermal changes caused by the hemoglobin molecules were transferred by conduction through the SAM layer from the erythrocytes to a platinum resistive temperature detector. Before measuring the temperature changes of the erythrocytes, we had to compensate for the photothermal effect of the gold layer. A 532 nm wavelength light is not the maximum absorption wavelength of the gold layer; however, it absorbs light energy and converts the light energy into thermal energy by surface plasmon resonance. The relative temperatures changes with and without erythrocytes based on the laser intensity and SA results are shown in Fig. 2(d). All experiments were performed three times. The temperature changes were proportional to the number of captured erythrocytes, which corresponds to the SA concentration. A thermal biosensor blocked with 0.01 M SA was prepared for verification of the specific binding between SA and PBA. After this preparation step, the same experimental procedure for the SA analysis method was tested. The result showed no significant temperature changes, and no bound erythrocytes were observed because all the PBA was bound to the free-form SA. The relative standard deviations (RSD) of Fig. 2(d) in detection rages are 0.8 W/cm^2: 91.9% (363.7 pmol/10^6 cells), 41.2% (379.3 pmol/10^6 cells), 1.6 W/cm^2: 209.9% (271.6 pmol/10^6 cells), 123.3% (288.9 pmol/10^6 cells), 17.9% (363.7 pmol/10^6 cells), 34.0% (379.3 pmol/10^6 cells), 2.4 W/cm^2: 40.6% (271.6 pmol/10^6 cells), 54.6% (288.9 pmol/10^6 cells), 25.8% (363.7 pmol/10^6 cells), 28.7% (379.3 pmol/10^6 cells), and 3.2 W/cm^2: 63.4% (271.6 pmol/10^6 cells), 23.7% (288.9 pmol/10^6 cells), 25.7% (363.7 pmol/10^6 cells), 20.1% (379.3 pmol/10^6 cells).

4. Conclusions

We have demonstrated a new method for measuring SA concentrations on erythrocyte membranes using a surface-modified micro-thermometer and photothermal system. The new method includes optimization of binding time and demonstration of binding affinity via the chemically modified sensor, as well as changes in temperature and light intensities to the SA expression level of erythrocytes. Temperature increases of the erythrocytes were both proportional to the SA express level and laser intensity. Our system has several advantages over previous analytical methods: it can directly measure the quantities of SA in the blood without requiring a multiple enzyme process, labeling, spectrophotometric devices, or concerns of the counterion screening effect. Furthermore, the system is relatively simple to implement and is cost-effective as no reagents are needed. The temperature alterations of samples with different SA concentrations were measured with the SA analysis biosensor, which suggests that our system can be used to diagnose diabetes mellitus. Future work will involve the analysis of samples from diabetes mellitus patients to understand the relationship between SA concentration and SA-related disease stage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.03.008.

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