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Determination of Serotonin Concentration in Single Human Platelets through Single-Entity Electrochemistry

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ABSTRACT: This research introduces a method to directly detect serotonin in a single platelet through single-entity electrochemistry. Platelets isolated from human blood were analyzed by cyclic voltammetry and current-time measurements. When a single platelet collides with an ultramicroelectrode, serotonin inside the platelet is oxidized at the electrode surface, and an anodic current peak is consequently observed during measurement. The concentration of serotonin can be determined by integrating this peak current. In addition, this method can be used to determine the platelet concentration. Analysis of the collision frequency of platelets can provide information about the platelet concentration in the blood. As a result, platelet levels and serotonin concentrations in single platelets can be measured quickly and easily.

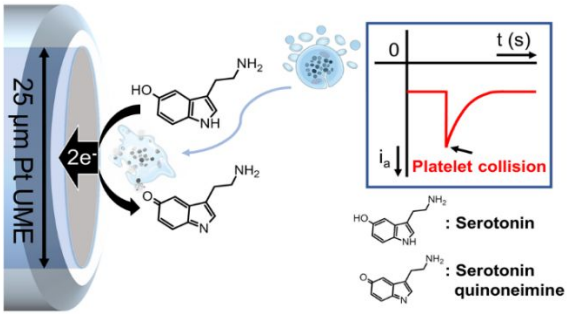
Platelets are a blood cell component that play an important role in the initial stages of hemostasis. In the quiescent state, before being charged with hemostatic stimuli, platelets assume a disk shape with a typical diameter ranging from 2 to 4 μm. The platelets form a hemostatic plug to fill the hole in the vasculature at the site of tissue damage and prevent further blood loss. This simple physical hemostatic function is significantly hampered if the platelet count in blood decreases below a certain level. Low platelet count often presents a serious medical problem and complicates various medical and/or surgical conditions. Any invasive medical procedure should be withheld until the platelet count is restored to a normal level to avoid the risk of iatrogenic bleeding. Thus, the platelet count is screened beforehand and closely monitored if a patient is continuously exposed to bleeding risk associated with low platelet count. The most popular methods of measuring platelet count in clinical laboratories are based upon an automated Coulter cell counting method or flow cytometry. Distinction between platelets and white blood cells (WBC) and/or red blood cells (RBC) is possible by applying a certain cutoff to the size-related electric signal between platelets and WBCs and removing the RBCs by treating the whole blood sample with a RBC-specific lysis buffer. Though these methods are widely adopted in numerous automated hematology analyzers and currently produce a massive quantity of blood cell count results in daily clinical practice, but they are by no means perfect. Because the distinction between blood cells and platelets is mainly made based on size, small non-platelet particles such as fragmented RBCs are often mistaken as platelets, and falsely elevated platelet counts are produced in several relevant clinical conditions. Conversely, large platelets, which are produced in certain diseases and/or conditions, can be missed in platelet counting, resulting in a falsely low platelet count. According to the standard laboratory practice, any suspicion of either instance should trigger a burdensome series of laboratory responses, including blood film preparation, the Romanowsky stain, and microscopic observation to verify the automatically produced platelet count. An inaccurate platelet count can lead to a missed opportunity to prevent problematic bleeding or arouse unnecessary medical alarm. Thus, the inherent limitation in the accuracy of current automated platelet counting methods is a factor that increases the health care budget.

Given this background, a more specific method to detect platelets has practical value in improving medical practice. The specificity of the assay can be enhanced in various ways, one of which is to exploit flow cytometric immunophenotyping and use a fluorescent probe conjugated to a platelet specific antibody such as CD61.[1,2] However, this kind of method still involves time consuming manual preparation of blood and expensive equipment, which is by no means suitable for use in a high throughput test setting. In contrast, an electrochemical method is an attractive alternative if a proper platelet-specific redox substance is chosen as an assay target.

Serotonin is the right choice in this regard. This well-known neurotransmitter also possesses hemostatic properties and is involved in platelet activation.[3] Serotonin is synthesized mostly in intestinal enterochromaffin cells, secreted into the blood stream, and absorbed by other cells, including platelets, through the action of serotonin transporter (SERT).[4,5] Serotonin uptake by platelets is so active that it may be stated that the blood serotonin is mainly carried by platelets. Within the platelet, serotonin is stored in dense granules at high concentration (65 mM) along with other substances essential for platelet function, such as adenosine diphosphate (ADP) and/or bivalent calcium ions.[6,7] When presented with a hemostatic challenge, platelet releases serotonin into the surrounding environment, which acts upon itself and/or neighboring platelets.[3] This in-and-out property of serotonin in platelet biology has been explored and adopted in a standard clinical laboratory assay to diagnose heparin-induced thrombocytopenia, with a somewhat different approach from ours. The serotonin-release assay measures the gross change in serotonin concentration of the reaction mixture containing platelets, thus probing platelet activation in response to a pathological autoantibody.[8,9] Consequently, it is necessary to detect the presence and concentration of serotonin in platelet granules within a significant range for continuous monitoring of human health.

Recently, much research has been conducted on the individual detection and analysis of soft particles (droplets,[10–14] vesicles,[15–17] and cells[18–20]) using single-entity electrochemistry (SEE, also known as the stochastic electrochemical collision, or nano-impact method). These studies are important not only to detect the analyte itself, but also to measure the properties of individual particles, such as size, surface charge, and diffusion coefficient. Thus, the SEE method was applied to the detection of serotonin in a single platelet. The first measurement of serotonin in granules using an ultramicroelectrode (UME) was demonstrated by the Haynes group.[21] They immobilized a platelet on the coverslip surface, positioned an UME on the top layer of the platelet, and performed amperometry to detect serotonin in rabbit blood granules. To do this, a complex three-dimensional (3D) positioning system, which involved two optical microscopes and a piezo-controlled micromanipulator, is required. In addition, delicate adjustments are required to position the UME above the top layer of the cell, so only experts can conduct these experiments. Therefore, it is necessary to develop a new method for detecting not only platelets in human blood, but also serotonin inside the platelet by an electrochemical method using a simple and easily manipulated arrangement.

This study aims to investigate the serotonin concentration of a single human platelet as this has not been explored by single particle collision so far. We also introduced the detection of serotonin concentration in individual platelets based on the oxidation reaction of serotonin. This was achieved by placing the UME in a solution containing the platelets to be detected and then measuring the current-time (i-t) curve. When the platelet collides with the electrode, serotonin in the platelet is oxidized, and an anodic current is generated by the reaction (Scheme. 1). This spike-type current is well known to be due to the electrochemical reaction of colliding particles.[11–14,22] The anodic peak current is integrated to calculate the concentration of serotonin in the individual platelet. The result is used to calculate the concentration distribution of serotonin within the platelets at the individual particle level. In addition, the frequency of platelet collision can be used to calculate the concentration of platelets.



Scheme. 1. Schematic diagram of serotonin oxidation using SEE in a single platelet. When a platelet collides with an ultramicroelectrode, serotonin in the platelet is oxidized on the electrode, and a peak current is observed as a result.

EXPERIMENTAL SECTION

Reagents. Serotonin (5-hydroxytryptamine), phosphate buffered saline (PBS, 0.01 M, pH 7.4), phorbol 12-myristate 13-acetate and ferrocenemethanol (97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tirofiban was purchased from Merch & Co. (Kenilworth, NJ, USA). Normal saline (NS) including 0.9 g sodium chloride per 100 mL was purchased from JW Life Science (Dangjin, Korea). Red blood cell lysis buffer (RLB) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Acetone (99.5%) and ethanol (94.5%) were purchased from Daejung Chemicals & Metals (Siheung, Korea). Pt wire (99.99%; 25 μm diameter) was supplied by Goodfellow (Devon, PA, USA). All materials were used without further purification and the buffer solution was prepared with Millipore water (> 18 MΩ·cm).

Sample preparation. Blood samples were acquired after the approval of institutional review board. With written consents, blood from healthy adult volunteers was drawn into dipotassium ethylenediaminetetraacetate anticoagulant (K2EDTA, BD Vacutainer, BD, Franklin Lakes, NJ, USA). Each experiment was performed with blood samples drawn from a single donor. To minimize the stimulation of the platelets, platelet suspensions with desired platelet concentrations were prepared with only a single step of centrifugation. The entire blood samples were centrifuged at 250 g for 15 min at room temperature. The supernatant, which was platelet rich plasma (PRP), was transferred to and pooled in another test tube to get the required volume, and the platelet count was measured with an automated hematologic analyzer. The pooled PRP was serially diluted into the desired platelet concentrations

with normal saline (154 mM NaCl) for each experiment. (The remaining PRP was centrifuged again at 1,000 g for 15 min at room temperature to get platelet poor plasma (PPP). The PPP was diluted in the same way as PRP and used as a control for the corresponding PRP dilutions.) The upper part of the blood cell column, which was the buffy coat layer, was aspirated and discarded to remove WBCs. The remaining lower part of the pellet was used as RBC rich plasma (RRP) or packed RBC.

Instrumentation. The electrochemical experiments were carried out with CHI 617B and CHI 721E potentiostats (CH Instrument Inc., Austin, TX, USA) equipped with a 3-electrode system. Pt wire was a counter electrode, a Ag/AgCl electrode (3 M NaCl) was used as a reference electrode, a 2 mm diameter Pt disk electrode or a 25 μm diameter Pt UME as working electrodes. These components were placed in a Faraday Cage (Wuhan Corrtest Instrument Co., Ltd, Wuhan, China). The RBC, WBC, platelet, PPP, PRP, and RRP levels were measured using an automated hematology analyzer, Advia2120i (Siemens, Germany).

Preparation of a Pt UME. The Pt UME was prepared according to the general procedure.[23] After the UME was prepared, the electrode was tested using 1 mM ferrocenemethanol-standard redox electrochemistry. Before performing each experiment, the working electrode (Pt UME) was polished sequentially using 3 μm and 0.1 μm diamond lapping films (Allied High Tech, Rancho Dominguez, CA, USA) until a mirror surface was observed.

Detection of serotonin in a single platelet using current-time (i-t) curve measurement. The PRP sample was added into the NS, and the number of platelets (C_{PRP}) in the final solution was set to ca. 6.05×10^3, 12.1×10^3, 24.2×10^3, and 60.5×10^3 cells/μL. When the number of cells (i.e., platelets) present per μL is changed into the molar concentrations using Equation 1, the corresponding values are 10.1, 20.1, 40.2, and 100 fM, respectively. The molar concentration of the diluted PRP in NS (C_{PLT}) was estimated using Equation 1:

$$C_{PLT} = \frac{V_{PRP} C_{PRP}}{(V_{PRP} + V_S) N_A} \quad (1)$$

where V_{PRP} is the volume of PRP sample, V_S is the volume of the NS, C_{PRP} is the number of platelets per μL, and N_A is Avogadro's constant. The clinical blood tests performed on the automated hematology analyzer were used to obtain C_{PRP} (Figure S5). The i-t curve was obtained for 200 s at an applied potential of +0.5 V (vs. Ag/AgCl) using a Pt UME of 25 μm diameter (used the i/E converter filter at 3.2 Hz).

RESULTS AND DISCUSSION

Determination of serotonin oxidation potential on a bulk Pt electrode. In order to determine the oxidation potential of serotonin present in a single platelet, cyclic voltammetry (CV) was measured using a 25 μm Pt UME. As shown in Figure S1a, CVs were collected at a +0.2 to +1.0 V potential window using an UME. In the CV results, the PPP sample (black line in Figure S1a) shows no particular anodic current around +0.4 V. Even in the case of the PRP sample, the CV (red line in Figure S1a) shows that the current slightly increases near +0.6 V (vs. Ag/AgCl) due to the various kinds of ions and molecules in human plasma, but no specific peak was observed. Because PRP solution contains only a small amount of serotonin, it is difficult to measure the serotonin oxidation via CV by immersing UME in PRP solution. Therefore, the platelet samples were coated on the disk Pt electrode (2 mm diameter) to detect the serotonin oxidation signal. (Note that, UMEs with a small diameter (25 μm) are not suitable for coating platelets on electrodes, so a 2 mm disk electrode was used.)

CV was performed using bare and PRP coated disk electrodes immersed in NS or 2 mM serotonin solution. Figure S1b shows the CV data collected in the +0.1 to +0.7 V potential range. Four different conditions were tested to determine whether platelets collide with the electrode surface and whether serotonin in the platelets was oxidized. Anodic current peaks were consequently observed during measurement. When using a bare disk electrode (black line in Figure S1b), no anodic peak currents were observed. On the other hand, a PRP coated disk electrode (red line in Figure S1b) showed oxidized peak of serotonin near +0.4 V. Because of the irreversible electrochemical oxidation of serotonin, no reduction peak current was observed. The oxidation peaks of 2 mM serotonin solution appeared at +0.23 and +0.31 V with bare 2 mm Pt disk electrode (green line in Figure S1b) and PRP coated 2 mm Pt disk electrode (blue line in Figure S1b), respectively. The PRP coated layer increased the resistance of the electrode surface, resulting in a shift in the oxidation potential of serotonin by 0.08 V. Therefore, when we measured serotonin inside the platelets, its oxidation potential was shifted toward a more positive direction (ca. 0.4 V, red line in Figure S1b).

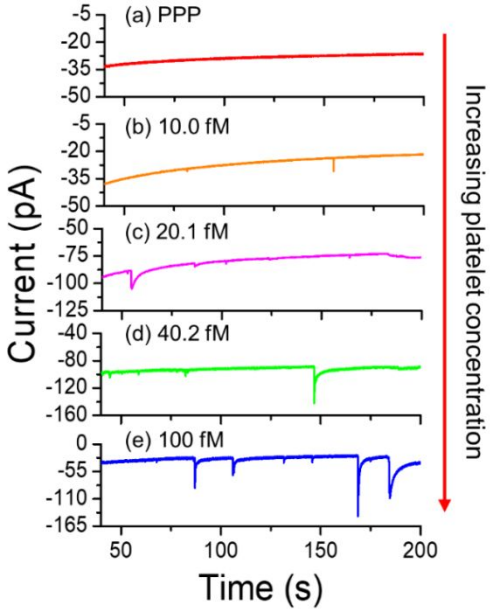


Figure 1. i-t curves of (a) PPP, (b) 10.0, (c) 20.1, (d) 40.2, and (e) 100 fM platelets in NS at a Pt UME (more data are shown in Figure S4).

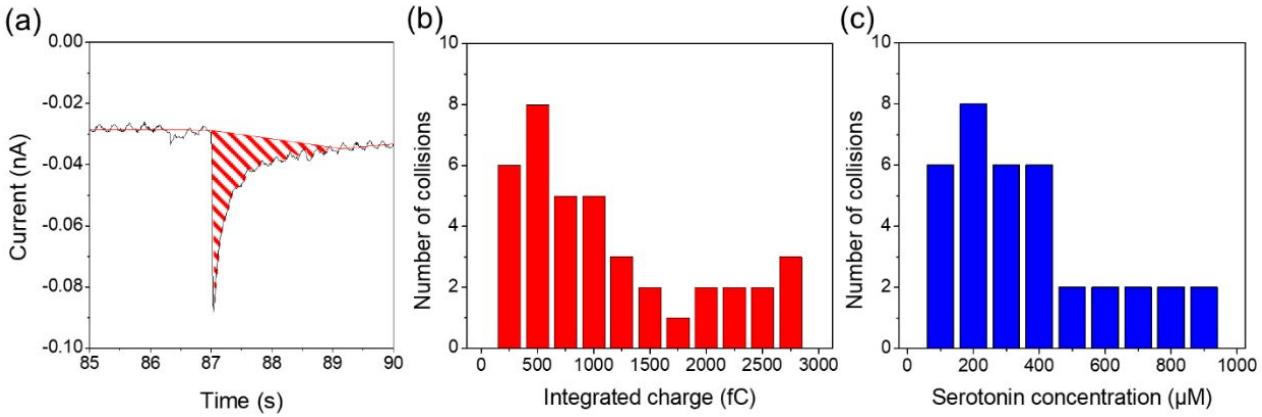


Figure 2. (a) The integral area (red dashed line) of a single current spike due to serotonin oxidation in the i-t curve. (b) The number of collisions vs. integrated charge distribution data. (c) The number of collisions vs. serotonin concentration, calculated by integrated-charge distribution using Equation 2.

Based on this result, the i-t curves were obtained at an applied potential of +0.5 V, indicating an anodic potential, to ensure complete oxidation of serotonin in the platelets at the UME surface.

Detection of serotonin in a single platelet on a Pt UME. For the various solutions, the i-t curves were measured at an applied potential of +0.5 V, using a 25 μm Pt UME. As shown in Figure 1a, in the PPP (i.e., without platelets), no current spike was observed. On the other hand, when human platelets were present in the solution, anodic current spikes were observed. These types of current spikes are generally obtained by tiny reactor systems with redox species in SEE. Here, platelets and serotonin act as tiny reactors and redox species, respectively. As the concentration of platelets increased to 10.0, 20.1, 40.2, and 100 fM, the frequency of collision signals also increased to 0.008 (± 0.004), 0.014 (± 0.007), 0.023 (± 0.012), and 0.045 (± 0.018) Hz, respectively (Figure 1b-e, see the detailed information about the different shaped spikes in Section 1 in the Supporting Information). The potential dependency was also confirmed by measuring the i-t curves of PRP at +0.2 V and +0.5 V on an UME (Figure S2). A current spike peak is only observed at +0.5 V, indicating that peaks are due to serotonin oxidation.

Once again, the experiment was conducted with the PRP, PPP, and RRP to confirm that the collision signal is due to only platelets and not other cells. As shown in Figure S3, the platelet collision signal clearly distinguishes between PRP with PPP and RRP. Note that, when the platelets were removed from the plasma, the PPP and RRP samples have very small amounts of platelets (the platelet concentrations of the PPP and RRP samples were ca. ~1 fM). These platelet concentrations are not enough to detect collision signals with this time interval, so no collision signal is observed even if sufficient potential is applied for serotonin oxidation (black and blue lines in Figure S3). This result confirms that the current peaks observed in the PRP experiments are not due to other blood cells (e.g., RBC and WBC) and plasma. Only in the case of PRP samples including 100 fM platelets (red line in Figure S3), a large number of collision signals was detected.

Determination of serotonin concentration in a single human platelet. Spike-type currents are considered to be caused by serotonin oxidation when platelets collide with the UME surface in response to electrochemical reactions. As shown in Figure 2a, the charge due to serotonin oxidation in each collision event is determined by integrating the area under the peak current in the i-t curve (see the detailed information about the signal determination in Section 2 in the Supporting Information). In addition, the amount of charge calculated as an integral from each collision is shown for the concentration distribution of serotonin in the platelets (Figure 2b and 2c).

The concentration of serotonin (C_{ST}) in a single platelet can be determined from the amount of charge integrated into the peak current. The C_{ST} can be calculated through the following Equation 2:

$$C_{ST} = \frac{6Q}{\pi n F (d_{PLT})^3} \quad (2)$$

where Q is the integrated charge from the peak current, n is the number of electrons transferred per molecule, F is Faraday's constant (96,485 C/mol), and d_{PLT} is the average platelet diameter (assuming that the shape of the platelet is spherical and the average diameter is 3 μm).[24] When one serotonin molecule is electrochemically oxidized, two electrons are generated, so n can be considered 2. The serotonin concentration in an individual platelet is 178 μM using integrated charge value (ca. 486 fC). The serotonin concentrations obtained by our proposed SEE method were compared with those obtained in previous studies. In the previous studies, only concentrations of serotonin present in dense granules were reported, and these values were used to calculate the concentration of serotonin present in a single platelet. The calculation was performed using some assumptions and known figures as follows. The reported serotonin concentration in a dense granule is 65 mM.[7] The average diameter of dense granules is 161 nm,[25] resulting in 1.42 × 10^{-19} mol of serotonin molecules per

granule. There are 6 dense granules per platelet,[7] so the number of serotonin molecules per platelet is 8.52×10^{-19} mol. Dividing this value by 9 fL (the average volume of platelets),[26] the concentration of serotonin in a single platelet is 94.7 μM. This value is quite similar to the median value of serotonin (178 μM) obtained by our suggested method. Note that, this 94.7 μM is calculated using the average values of several terms. In practice, various concentrations of serotonin can be measured from various platelets. The distribution of serotonin in platelets is of great clinical importance. As shown in Figure 2c, this method can easily identify serotonin distribution in platelets, and we believe that our proposed SEE method is the best way to achieve these results (see the detailed information about the serotonin analysis of platelets in Section 3 in the Supporting Information).

Determination of platelet concentration from collision frequency. This proposed method can also be used to determine platelet concentrations. Assuming that serotonin is present in all platelets (serotonin concentration varies among platelets) and is oxidized when the platelets collide with the electrode, the frequency of collision of the platelets observed in this experiment is related to the platelet concentration. Therefore, the relationship between the number of platelets and collision frequency was analyzed. The SEE method was performed for various concentrations, and regression curves were obtained from the collected data (Figure 3). The collision events were 1.6 (± 0.9), 2.7 (± 1.5), 4.7 (± 2.3), and 9.0 (± 3.5) at platelets concentrations of 10.0, 20.1, 40.2, and 100 fM, respectively. Based on the experimental results, we have derived an equation that uses regression analysis to convert the number of platelets to the frequency of collision (Equation 3).

$$f_{exp} = 1.18 \times 10^{-3} N_{PLT} - 7.13 \times 10^{-6} N_{PLT}^{2} \quad (3)$$

where f_{exp} is the frequency of collision events from experimental data and N_{PLT} is the number of platelets per μL. Equation 3 can be used to determine platelet concentration from the collision frequency. As the number of platelets increases, the frequency of collisions also increases. When the concentration of platelets is relatively high, the electrode surface is continuously struck by many platelets and the collision detectable surface area of the electrode is further decreased. Therefore, the frequency of collision does not follow a linear trend and decreases slightly as the detectable surface area is saturated.

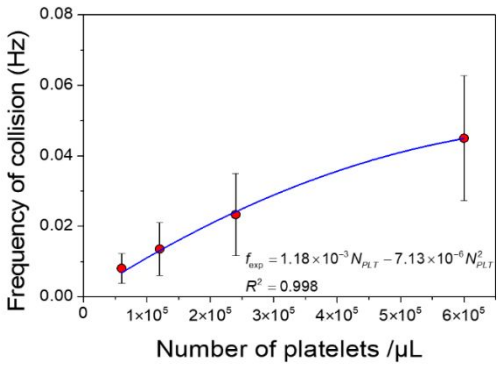


Figure 3. Regression plot of the collision frequency as a function of the number of platelets per μL. The error bars represent standard deviations from at least five measurements.

The collision frequency can be compared to the theoretical frequency. The theoretical frequency (f_{the}) by diffusion using an UME without migration effect was derived from the following Equation 4:

$$f_{the} = 4 D_{PLT} C_{PLT} r_e N_A \quad (4)$$

where D_{PLT} is the diffusion coefficient of the platelets, C_{PLT} is the concentration of platelets, r_e is the radius of the electrode, and N_A is Avogadro's number. Considering previously reported data,[27–30] the diffusion coefficient of platelets is 1.6×10^{-9} cm²/s in the plasma.[30] Assuming that the diffusion coefficient is 1.6×10^{-9} cm²/s, the f_{the} values obtained are 0.0005, 0.0009, 0.0019, and 0.0047 Hz at platelet concentrations of 10.0, 20.1, 40.2, and 100 fM, respectively. The f_{exp} values are 0.008 (± 0.004), 0.014 (± 0.007), 0.023 (± 0.012), and 0.045 (± 0.018) Hz. The f_{exp} values were measured to be ca. 10 ~ 16 times larger than the f_{the} values. This is due to the migration effect. The applied potential is +0.5 V but the zeta potential of platelets is –14.2 mV.[31] Therefore, migration between the positively induced electric field near the electrode surface and the negatively charged platelets could result in higher collision frequencies than theoretical values. Platelet concentrations play an important role in the diagnosis and monitoring of various diseases (e.g., leukemia, thrombocytopenia, and anemia). Using the SEE method proposed here, platelet concentration can be easily measured with short measurement time (ca. 3 min). In addition, this SEE method can be easily applied to a portable point-of-care-testing (POCT) device. These POCT devices allow patients to measure platelet levels when necessary at home and, as a result, they can quickly respond to emergencies. Further research is needed to develop portable POCT devices using this SEE method.

CONCLUSION

For the first time, the detection of serotonin in single platelets from human blood using a SEE method was demonstrated. Our method is useful in determining the level of platelets as well as the level of serotonin inside the platelets, without any modification of the electrode. Serotonin is a well-known marker of platelet activation, the secreted form of which is measured in many reference clinical laboratories for diagnostic purposes. It was proven that serotonin in platelets is oxidizable when platelets collide with the electrode surface. This method can be an easy and simple alternative to the current serotonin detection method used in clinics. In addition, the frequency of collision data can be used for the calculating the concentration of platelets. Compared to the method used in hospitals, our method can be applied to

electrochemical sensors because it can quickly and simply obtain the concentration of platelets present in the blood. Moreover, the equipment in hospitals (e.g., auto hematology analyzer) is expensive, lab-based, and not portable. This method is applicable to POCT devices because it uses electrochemical methods. This method also allows the study of cells with electrochemically active substances at the single cell level. It is a useful way to get a lot of important information without artificial modifications or destruction by external forces to the cell membrane. Therefore, we believe that this method will be actively used by many researchers studying clinical science, biology, and cytology.

ASSOCIATED CONTENT

SUPPORTING INFORMATION.

This material is available free of charge via the Internet at http://pubs.acs.org.

CVs of PPP and PRP with Pt UME, CVs of NS (with and without serotonin) with bare and PRP coated Pt disk electrode, i-t curves of PRP in RLB at Pt UME, i-t curves of PRP, PPP, and RRP with a Pt UME, description of different shaped spikes, signal determination considering S/N ratio, and serotonin analysis of platelets through the assumption.

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

WBC, White blood cells; RBC, red blood cells; SERT, Serotonin transporter; ADP, adenosine diphosphate; SEE, single-entity electrochemistry; UME, Ultramicroelectrode; 3D, three-dimensional; i-t, current-time; ST, Serotonin, 5-hydroxytryptamine; PBS, Phosphate buffered saline; NS, Normal saline; RLB, Red blood cell lysis buffer; EDTA, Ethylenediaminetetraacetic acid; PLT, Platelet; PPP, Platelet-poor plasma, PRP, Platelet-rich plasma; RRP, RBC-rich plasma; CV, Cyclic voltammetry; POCT device, Portable point-of-care-testing device

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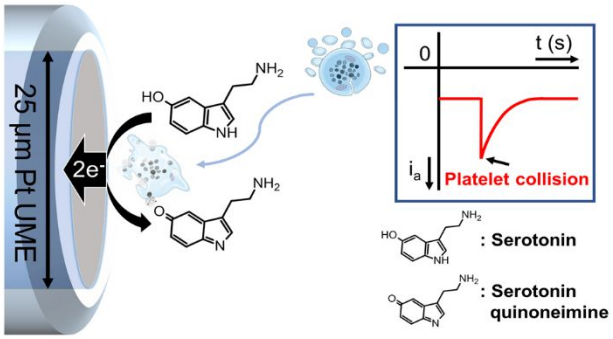
TOC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