Electrochemical Biosensor for Simplified Determination of Salivary Uric Acid

Hiroyuki Kudo* and Yuki Takagi

Department of Electronics and Bioinformatics, School of Science and Technology, Meiji University
1-1-1 Higashi-Mita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan

A uric acid biosensor aimed at a simplified determination of salivary uric acid was fabricated and utilized for the measurement of the diurnal variation of salivary uric acid. The biosensor measures uric acid as the change in the amount of hydrogen peroxide produced in the uricase reaction. Because uric acid is oxidized as easily as hydrogen peroxide, the osmium-HRP redox reaction was employed. The sensitivity of the biosensor was 170 nA/mM, which was sufficient for salivary uric acid determination. For simplified measurement of a saliva sample, a paper-based saliva sampling device, which enables the sample collection of a regulated amount of saliva in 5 s, was used. As a test using an actual sample, the diurnal variation of salivary uric acid was measured. The result indicated that salivary lactic acid increased in the morning. The total measurement time for the saliva measurement was approximately 3 min, which was sufficiently fast for the purpose of daily health management. The proposed method is expected to be used not only in gout treatment but also possibly in the measurement of other substances contained in saliva.

1. Introduction

Gout is a kind of arthritis caused by the crystallization of uric acid in serum, which can be associated with hyperuricemia (high levels of uric acid in the blood), in the joints. This causes an attack of sudden severe pain, stiffness, and swelling. The favorite site for gouty arthritis is the first metatarsophalangeal joint (the joint on the thumb of the foot) but any joint may be involved in a gout attack. Uric acid is the end product of the metabolic breakdown of purine nucleotides. Although uric acid contained in food is less than that synthesized in the body, the ingestion of foods rich in purines, such as cooked or processed food especially from animals and seafood, is a key element in the increase of uric acid precursors.[1] Alcohol intake also leads to an increase in the blood uric acid level. Alcoholic beverages enhance the synthesis of uric acid in the body although they do not contain much purine. The deposition of monosodium urate crystals in tissues can be found in hyperuricemia (6.8 mg/dl).[2] Because gout occurs mainly in middle-aged or elderly men, items related to their blood uric acid levels and dietary

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habits are one of the common daily topics in Japan. In addition, gout has recently been seen in young people, and it has been reported that the number of patients with gout is increasing.[3]

In the treatment of gout, dietary therapy[4,5] and medication to suppress the uric acid levels[1,2,6] are commonly recommended. To estimate the efficiency of a treatment, blood uric acid measurements, which require blood sampling by needle puncture, are often used. Considering the risks and inconveniences of needle puncture (e.g., infection, pain), a noninvasive and convenient method to assess blood uric acid levels would be useful to improving the quality of life for patients with gout. Hence, the purpose of our study is to develop a simplified self-check device capable of noninvasive blood uric acid assessment. Usually, noninvasive assessment of blood contents can be made by measuring other body fluids that reflect the blood content. For this purpose, we focused on uric acid in saliva, which is reported to be positively correlated with blood uric acid levels in the recent literature.[7–11] Although many of the components of saliva, including uric acid, can be analyzed using conventional analyzers such as high-performance liquid chromatography (HPLC),[12–15] those methods are not suitable for daily personal use because they usually take much time and call for expensive equipment. Owing to the advantages of the simple measurement procedure, short response time, and sufficient sensitivity and selectivity, enzyme-based biosensors[16,17] have been competitive devices for routine analysis.[18] Previously, several types of uric acid biosensors using the redox reactions of uricase were reported.[19–22] For simplified measurements, we developed an electrochemical biosensor with a reaction cell for measuring uric acid contained in saliva that does not require any pretreatment. The novelty of our simplified salivary uric acid biosensor is the capability of fast self-check owing to the use of a paper-based saliva sampling device. With our paper-based sampling device, a certain amount of a saliva sample can be collected by contacting the sampling device to the salivary grand. In this paper, we report the structure and characteristics of our device. Also, the first result of salivary uric acid determination is reported.

2. Materials and Methods

2.1 Reagents and materials

Uricase from *Candida* sp. (218-00721, 3.0–6.5 units/mg, EC. 1.7.3.3) and osmium-wired horseradish peroxidase (Os-HRP) redox polymer (002096) were obtained from Wako (Japan) and Bioanalytical Systems (USA), respectively. Polydimethylsiloxane (PDMS: SYLGARD184 Silicone Elastomer Kit) was purchased from Dow Corning Co. (USA). For the purpose of enzyme immobilization, an UV curable polymer (Biosurfine-AWP-MRH, Tokyo Gosei Kogyo Co., Japan) was employed. A standard uric acid solution was prepared by diluting uric acid (216-00222, Wako, Japan) with phosphate buffer saline [PBS: pH 7.4, 50 mM (PO_4)]. Both carbon graphite paste (C2030519P4) and silver/silver chloride paste (C2130809D5) were purchased from Gwent Electronic Materials Co. (UK). Polyethylene terephthalate (PET) sheets (thickness: 100 μm, N0791400) and polyvinyl chloride (PVC) adhesive sheets (SX-00Z) were products of Featherfield Co. (Japan) and Europort Co. (Japan), respectively. Mesh screens for screen printing (SR-260, #225) were obtained from Sunhayato Co. (Japan).

2.2   Fabrication and characterization of uric acid sensor

2.2.1   Uric acid biosensor

As shown in Fig. 1, uricase catalyzes the oxidation of uric acid to allantoin, and hydrogen peroxide and carbon dioxide are also produced at the same time. Therefore, the biosensor measures uric acid as changes in hydrogen peroxide concentration. However, uric acid is oxidized as easily as hydrogen peroxide. Therefore, hydrogen peroxide is specifically converted to water by the redox reaction of HRP, and an osmium complex is used as a mediator to transfer electrons from the electrode to HRP. By this reaction, the operating potential is reduced so that uric acid is not oxidized unexpectedly by the potential of the electrodes.

The uric acid biosensor was fabricated by conventional screen printing techniques. Carbon graphite and silver/silver chloride pastes were printed on a 100-µm-thick PET substrate using a 225-mesh screen. After printing, the pastes were cured at 130 °C for 30 min. Os-HRP redox polymer (0.3 µL) was coated on the sensing region of the working electrodes, and uricase was immobilized on the sensing region by adding 0.2 µL of PBS that contained uricase (50 units/mL). Then, a reaction cell fabricated by conventional PDMS (inner diameter: 8 mm) molding was set on the electrode and the reaction cell was tightly fixed with a polymethyl methacrylate (PMMA) casing (Fig. 2).
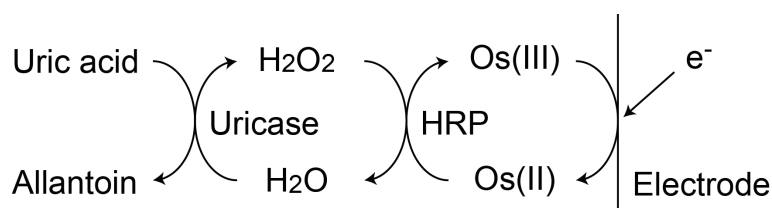

Fig. 1.   Principle of salivary uric acid determination.

(a)   (b)   (c)
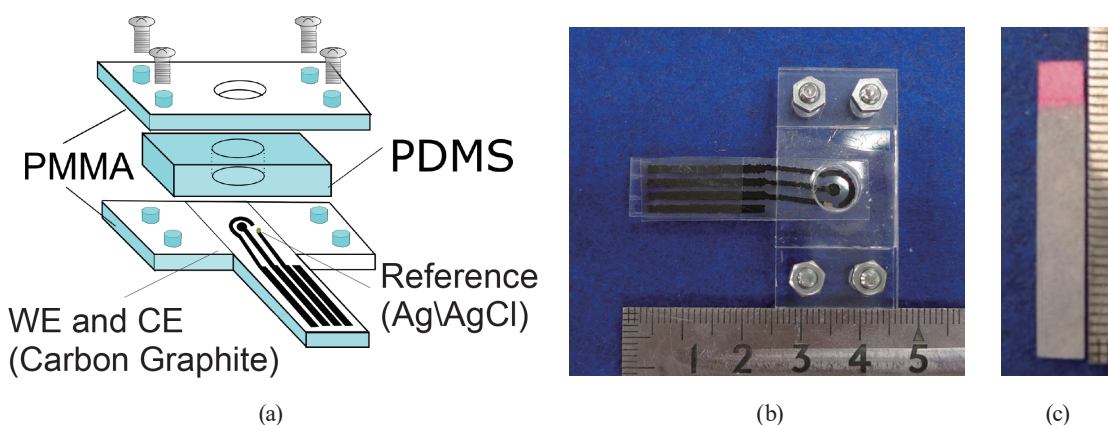
Fig. 2.   (Color online) (a) Structure, (b) appearance, and (c) paper-based sampling device of the salivary UA sensor. The contact pads were arranged from the left in order of the counter electrode, the working electrode, the reference electrode and the dummy. To make it easier to visualize the region used in sampling, the sampling device was wetted with a colored solution.

2.2.2 Sampling device

The most important requirements for the purpose of salivary uric acid measurement are the accuracy of sampling volume and a simplified sampling method. To satisfy these requirements, we developed a paper-based sampling device. Prior to preparing the sampling device, materials suitable for saliva sampling were investigated. Materials used for saliva collection in our system are required to absorb a specific volume with sufficient reproducibility rather than merely absorbing a large amount of water. Hence, we compared the water contents of a commercially available coffee filter (VCF-01-100MK, Hario Co.) and two kinds of paper filters with similar particle retention for scientific experiments: Grade 3 (Whatman Co., particle retention: 6 μm) and No 1 (Advantec Co., particle retention: 6 μm). The sampling device was 5 mm wide × 3 cm long and the sampling region was 5×5 mm^2, as shown in Fig. 2(c). The size of the sampling device was determined for ease of saliva collection from the sublingual gland. To control the collecting region, the filter paper was coated with PDMS, except for the sampling region.

2.2.3 Characterization of biosensor

The characterization of the biosensor was carried out using amperometric techniques. First of all, the reaction cell was filled with 60 μL PBS and a constant potential of 0 V vs Ag/AgCl was applied to the working electrode. Fresh biosensors were used for every measurement. In order to assess the effective characteristics in the measurement of a saliva sample, a standard uric acid sample was added using the sampling device. The sampling device was wetted in standard uric acid solution for 5 s and immediately put into the reaction cell. The output current was recorded continuously throughout the measurement.

2.3 Sampling and measurement of salivary uric acid

With the approval of the ethical committee of Meiji University (No 17-534), the measurement of salivary uric acid was demonstrated. Saliva samples were collected from adult male volunteers. The sampling procedure is quite simple, as shown in Fig. 3. The sampling region of the device was gently placed on the salivary gland for 5 s. The sampling device containing
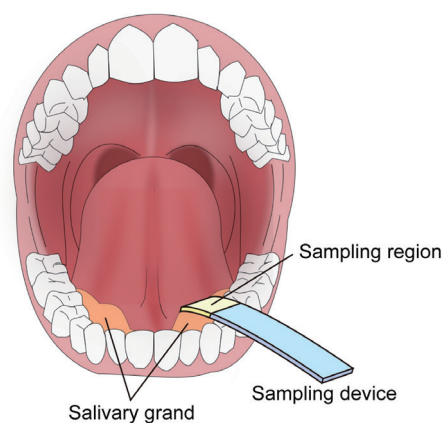

Fig. 3.   (Color online) Saliva collection method using the paper-based sampling device.

the saliva sample was then directly inserted into the reaction cell so that the corners of the rectangular sampling device were aligned with the boundary between the bottom and the side of the cylindrical reaction cell. To ensure accuracy of measurement, the output currents for standard uric acid solution (100 µM) were also measured with the same sensor, before and after the measurement of the saliva sample. Eating and drinking were restricted for subjects 1 h before the measurement.

3.   Results and Discussion

3.1   Evaluation of uric acid biosensor

3.1.1   Selection of material for sampling device

Figure 4 shows the deionized (DI) water contents of the coffee filter and two kinds of paper filters with similar particle retention for scientific experiments. The filters were cut into five square pieces (5×5 mm^2). The *x*-axis represents the identification number of the filter paper before being cut into five pieces. The weight of the square pieces of the filter were measured as soon as they were taken from the water. As presented in the figure, the Whatman filter showed high reproducibility [64.0 ± 4.6 mg/cm^2 (8.0 ± 1.2 µL)] compared with the others [52.6 ± 12.3 and 56.4 ± 14.8 mg/cm^2 (13.2 ± 3.1 and 14.1 ± 3.7 µL)]. Because of this result, we chose the Whatman filter as the saliva collecting material. Specifications and water absorptions of different grades of Whatman filters are also investigated and shown in Table 1. As clearly indicated in Table 1, the grade 4 filter had acceptable reproducibility and relatively high water content. From these results, we chose the grade 4 filter as the material for saliva collection.

3.1.2   Fundamental characteristics of uric acid sensor

The characteristics of the biosensor were investigated using amperometric techniques. Figure 5 shows typical responses of the biosensor towards immersion of the sampling device containing various concentrations of uric acid, and the calibration curve for uric acid. Each plot
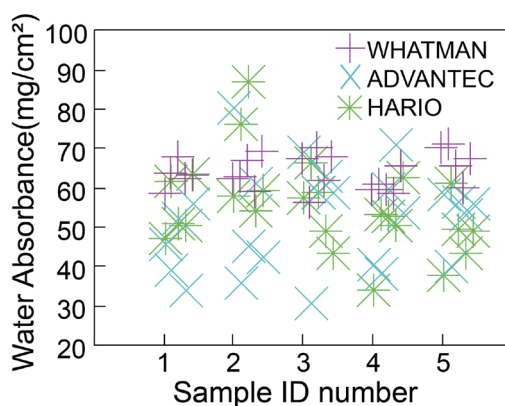

Fig. 4.   (Color online) Water absorbance of various filter papers. Five numbered filter papers (ID: 1 to 5) were cut into five 5×5 mm^2 square pieces.

Table 1
Water content of Whatman filters with various particle retention capacities.

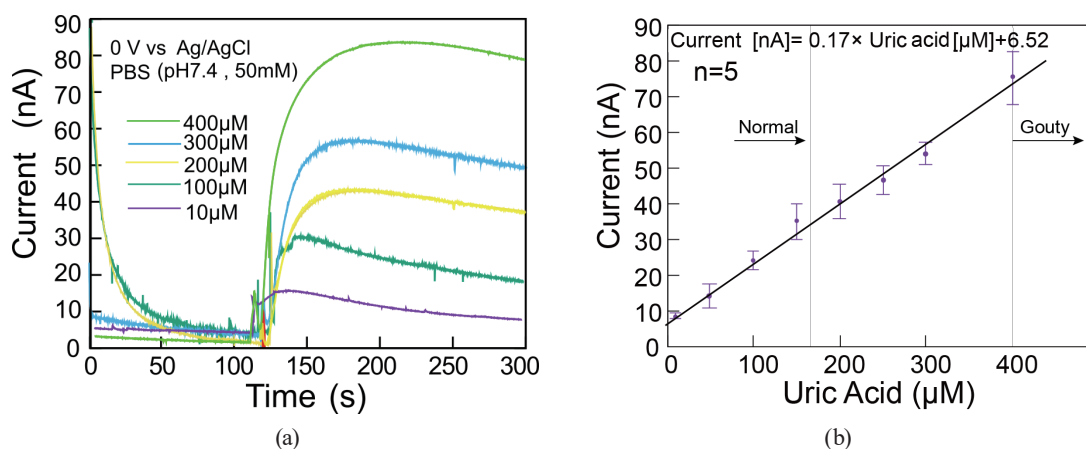| Grade	Particle retention (µm)	Thickness (mm)	Weight (mg)	Water content (mg/cm^2)
1	11	0.18	2.1	42.1 ± 3.4
2	8	0.19	2.6	43.3 ± 3.8
3	6	0.39	5.5	64.0 ± 4.6
4	20	0.21	2.6	52.9 ± 0.8
5	2.5	0.20	3.0	41.0 ± 1.6
6	3	0.18	2.5	40.7 ± 2.2

Fig. 5.   (Color online) (a) Typical responses of the biosensor when the sampling device containing uric acid solution was inserted into the reaction cell and (b) calibration curve for uric acid obtained using the sampling device. The calibration range involved salivary uric acid levels of both patients with and without gout.

shown in the calibration curve was the average from 120 to 150 s after immersion of the filter paper. The error bars represent standard deviations for five different samples. As shown in the figure, current increased immediately after immersion of the sampling device and reached its peak in approximately 30 s. The temporal change of the current is thought to reflect the process in which the uric acid contained in the sampling device diffused into the reaction cell and the concentration reached equilibrium. The region of the sampling device containing uric acid sank automatically into the bottom of the reaction cell. This also was considered to be one of the reasons why the current peaked. The response time can be improved by miniaturizing the reaction cell. The linear range (10–400 µM) included salivary uric acid levels of patients both with and without gout. The sensitivity for the use of the sampling device was 170 nA/mM, which was sufficient for salivary uric acid determination. Selectivity to possible substances found in saliva was also investigated. As a result, current changes for 50 mM glucose, creatinine, and lactic acid were 7.49, 4.25, and 2.67%, respectively compared with that of 50 mM uric acid. This suggests that our system is sufficiently specific for uric acid as a result of the specific activity of uricase.

3.2   Results of salivary uric acid measurement

On the basis of the fundamental characteristics discussed above, salivary uric acid was also measured.   First, fluctuation in the quantity of collected saliva owing to individual differences was evaluated.   In this test, each of five male volunteers collected their own saliva five times using fresh sampling devices each time.   Measured quantities of saliva contained in the sampling devices of each subject were 28.4 ± 4.0, 28.1 ± 2.0, 26.8 ± 3.5, 29.2 ± 1.2, and 29.6 ± 3.2 mg/cm^2 (7.1 ± 4.0, 7.0 ± 0.5, 6.7 ± 0.9, 7.3 ± 0.3, and 7.4 ± 0.8 µL).   The values were approximately 53% of the water content shown in Table 1.   Despite the difference in the specific weight of saliva and water being less than 1%, it is an interesting result that such a large difference in collected amount was found.   Upon further investigation, we considered the reason to be as follows.   When evaluating water absorption, the filter paper was soaked in water. Therefore, water was absorbed from both sides of the filter paper.   In contrast, when saliva was collected, it was brought into contact with the sublingual gland, which was moistened with saliva, thus absorbing saliva from only one side.   Consequently, water was stored on the surface of the filter paper owing to surface tension, whereas absorption of saliva for 5 s does not progress to that extent.   We also confirmed that by turning over the filter paper and collecting saliva again after the first sampling, the amount of saliva collected reached about 90% that of water.   However, we concluded that collecting saliva from only one side was sufficient for our purposes from the point of view of simplicity.

The measurement of salivary uric acid was then carried out.   Figure 6(a) represents a typical response to a saliva sample.   Although the states of insertion were slightly uneven depending on the user, the currents after 120 s converged to a certain value depending on the concentration of the uric acid contained in the sampling device.   In contrast to the response to a standard solution, the current increased slowly and showed a peak 45 s after the sampling device was inserted.   One of the reasons is the effect of saliva viscosity on initial diffusion.   Saliva secreted
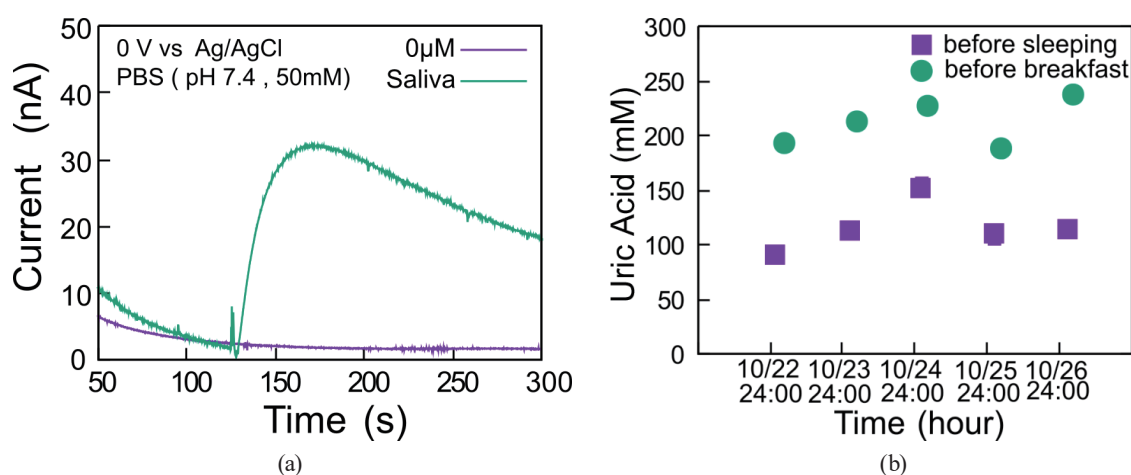

Fig. 6.   (Color online) (a) Typical response to a saliva sample collected from the salivary grand of a healthy adult subject using the paper-based sampling device and (b) the typical diurnal variation of salivary uric acid measured using our system.

from the sublingual gland is relatively rich in mucin.[23,24] Therefore, it is more viscous than saliva derived from the parotid gland. The concentration of salivary uric acid was estimated to be 92 μM, which is consistent with the salivary uric acid levels reported in the literature.[25] In addition, the diurnal variation of salivary uric acid was measured and the results indicated that the level of salivary uric acid was relatively high in the morning compared with that at night, as shown in Fig. 6(b). This is also consistent with the previous report as well as similar to the diurnal variations of serum uric acid.[7]

From these results, the feasibility of our simplified method to assess salivary uric acid, which would be useful for daily self-checking, was successfully demonstrated. Salivary uric acid is an attractive biomarker that can be measured noninvasively. This method is expected to be acceptable to gout patients and to enhance the quality of their lives.

4. Conclusions

A simplified determination method of salivary uric acid was demonstrated using a uricase-based biosensor and a paper-based sampling device. With the low operating potential derived from the redox reaction of osmium and HRP, a highly selective determination of salivary uric acid was enabled without any pretreatment. The sensor had a sufficient sensitivity of 170 nA/mM and a calibration range that included uric acid levels of both patients with and without gout. Utilizing the sampling device, saliva was easily collected in 5 s and the amount of uric acid contained in saliva was successfully measured (92 μM). The results were consistent with previous reports. The total time for measurement was 3 min and we expect to shorten it by miniaturizing the components. Thus, the proposed method has the potential for use in daily self-checks.

Acknowledgments

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About the Authors

Hiroyuki Kudo received both his M.E. and Ph.D. degrees from the Department of Electronics and Communications of Waseda University in 1999 and 2004, respectively. He was a research officer of the MEMS Laboratory, Tokyo Metropolitan Industrial Technology Research Institute, from 2003 to 2007. He worked at Tokyo Medical and Dental University as an assistant professor from 2005, a junior associate professor from 2007 to 2011, and an associate professor from 2011 to 2013. Currently, he is an associate professor in the Department of Electronics at Meiji University. His research interests include biomicrosystems based on enzymatic biosensors and immunosensors for life science applications.

Yuki Takagi received his B.S. degree from Meiji University, Japan, in 2018. His research interests are biomicrosystems aimed at Internet of Things in healthcare and enzyme-based sensors, particularly uric acid sensors based on uricase.