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TECHNICAL NOTE

# Simple and reliable urea assay based on a signal accumulation type of ion-sensitive field-effect transistor

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**A simple urea assay was developed using a signal accumulation type of ion-sensitive field-effect transistor (SA-ISFET). Decreases in proton concentration resulting from urease-catalyzed hydrolysis of urea are detected by SA-ISFET as a change in potential. The method exhibits high sensitivity, linearity, and reproducibility when potential signals are accumulated 10-fold.**



[**Key words:** Ion-sensitive field-effect transistor; Signal accumulation; Urease; Urea assay; Proton]

Ion-sensitive field-effect transistors (ISFETs) are electronic devices that can be used to measure the density of protons in aqueous solutions as altered potentials (1–3). ISFET devices are smaller, and, if commercially produced, cheaper than other electronic devices. As such, ISFETs have been put into use as pH sensors (4). However, the sensitivity of ISFETs is low (theoretical maximum sensitivity determined according to the Nernst equation is approximately 60 mV/pH unit), and proton density cannot be determined with high precision. If the sensitivity of ISFETs can be improved, they could be used for biochemical measurements in applications such as enzyme-based assays. In fact, a signal accumulation type of ISFET (SA-ISFET) with improved sensitivity was recently developed (Fig. 1) (5–8). The SA-ISFET sensor can directly convert changes in ion abundance resulting from enzymatic reactions into electric signals. Thus, reactions can be monitored in real time, eliminating the need for complicated and troublesome colorimetric detection processes. The SA-ISFET is a highly sensitive semiconductor sensor requiring only a few microliters of sample.

In previous studies, we developed novel analytical methods based on SA-ISFET technology for measuring creatinine and cholesterol esters (5–7). These methods are remarkably simple, and only one enzyme is used, either creatinine deiminase or cholesterol esterase, whereas a conventional enzymatic assay requires at least three or four enzymes and a chromogen. However, the SA-ISFET-based assays we developed are not rapid, requiring 11 or more minutes, compared with 10 min for routine conventional assays. It should be also noted that the reproducibility of the previously developed SA-ISFET-based assays is not sufficient to measure actual samples of interest.

In this paper, we describe an SA-ISFET-based assay for measuring urea concentrations. Urea is the major end product of nitrogen metabolism and is produced by humans and many other mammals. Urea also plays an important role in the metabolism of nitrogen-containing compounds. Rapid and accurate estimation of urea is important for the diagnosis of renal and liver diseases, as well as in many other areas, such as food analysis, the nutritional management of cows, and the monitoring of soil bioremediation efforts, for example (9–12).

In the SA-ISFET-based urea assay described here, an aliquot of sample solution is added to a reagent composed of urease (EC 3.5.1.5) and buffer. Urea in the sample is hydrolyzed (reaction 1), and the decrease in proton abundance (reaction 2) is detected by the SA-ISFET as a change in potential. In contrast, conventional urea assays are based primarily on spectrophotometric procedures involving coupled enzymes (reactions 1 and 3). In these methods, the reagent is a complex mixture of urease, glutamate dehydrogenase (EC 1.4.1.2), NADH, 2-oxoglutarate, and a buffer. Compared with the reagent used in the SA-ISFET-based assay, the conventional assay reagent contains an additional enzyme and an expensive compound, NADH. In the present study, we demonstrate that the SA-ISFET-based assay is a simple and low-cost method for measuring urea. Moreover, reliable urea measurements require only 1–3 min.

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} CO_2 + 2NH_3 \quad (1)$$

$$NH_3 + H^+ \xrightarrow[(\text{pKa of } NH_4^+: 9.3)]{} NH_4^+ \quad (2)$$

$$\text{2-Oxoglutarate} + NH_3 + NADH + H^+ \xrightarrow{\text{Glutamate dehydrogenase}} \text{L-Glu} + H_2O + NAD^+ \quad (3)$$

The SA-ISFET system we used was obtained from Bio-X Inc. (AMIS-101; Kyoto, Japan), as reported previously (5–8). In order to

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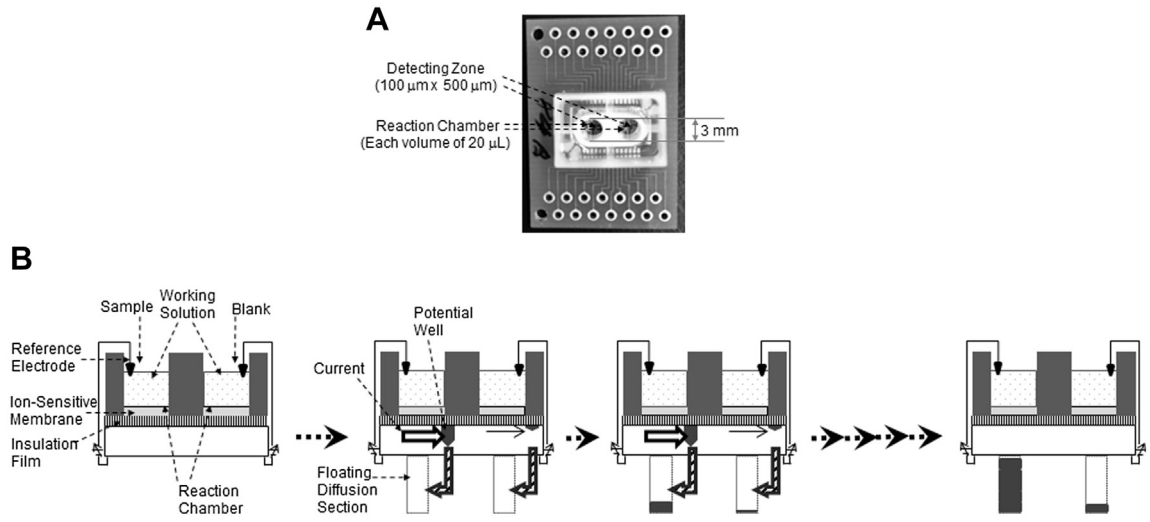


FIG. 1. (A) SA-ISFET sensor herein used. (B) Measurement principle. The working solution is placed into the reaction chamber. As the number of protons in the chamber changes over the course of a reaction, the electron polarization is altered in the semiconductor leading to a current change. The SA-ISFET sensor consists of CMOS device that stores and accumulates the signal in the floating diffusion section as electric charges. This allows the sensor to amplify the signal and to improve the signal-to-noise ratio.

maximize the sensitivity of the urea assay, the composition of the working solution was first optimized. The optimal buffer concentration was determined as 1 mM, according to previous studies (5,6). Increasing the buffering capacity above 5 mM greatly reduced the sensitivity. Based on evaluations of various buffers useful in the pH range 6.5–8.0 (e.g., GOOD buffers [PIPES, HEPES, and MOPS], Tris–HCl, and potassium phosphate), we chose MOPS (pH 7.0), which produced a significant potential change (data not shown). Addition of electrolytes was also investigated for maximizing assay sensitivity and stability. Potential changes were examined in buffer containing 10, 20, 50, and 100 mM NaCl, with 50 mM found to be optimal. Reaction temperature was also investigated. Creatinine and cholesterol ester were assayed using the SA-ISFET system at 37 and 30°C, respectively, similar to conventional spectrophotometric assays (5,6). Urea was assayed at 20°C, 25°C, 30°C, and 37°C, with 25°C producing the greatest potential increase (Table S1).

An 18-μL volume of working solution (composed of 1.0 unit/μL jack-bean urease [Toyobo Co., Ltd., Osaka, Japan], 1.0 mM MOPS [pH 7.0], and 50 mM NaCl) was placed into two reaction chambers on the sensor, each having 20-μL capacity (Fig. 1A), and then the reference electrode was set to the sensor. The temperature was stabilized at 25°C, and 2 μL of sample or water (control) was added to each reaction chamber. Potential was measured in real time for 1–3 min by accumulating and then subtracting output potential signals (Fig. 1B). Other settings were as follows: accumulation number, 10; averaging number, 10; measurement interval, 5 s; and end-point assay.

The time courses of the potential changes are shown in Fig. 2A. When 20 and 40 mg/dL urea solutions were used as calibrators, the potential increased proportionally to the urea concentration (0.22 V for 20 mg/dL and 0.42 V for 40 mg/dL). Hence, an end-point assay was adopted. In contrast, reactions without accumulation produced minimal signal (0.011 V for 20 mg/dL and 0.031 V for 40 mg/dL).

The standard curves for measurement times of 1–3 min are presented in Fig. 2B. The curves were linear at urea concentrations between 2 and 80 mg/dL, with correlation coefficients of 0.998–1.00. In contrast, standard curves for reactions without accumulation showed poor linearity due to low sensitivity. The urea concentrations assayed for evaluating linearity are important for general applications. For example, a mean urea concentration in milk of 27 mg/dL (range, 19–39 mg/dL) has been reported (11). The normal urea concentration in serum is 19–45 mg/dL, and increased concentrations may cause renal failure, urinary tract obstruction, and gastrointestinal bleeding. Moreover, below-normal urea concentrations may cause hepatic failure and nephritic syndrome.

For within-day and between-day precision studies, samples containing urea at 40 mg/dL were assayed 10 times per day and once per day for 7 days, respectively. The within-day and between-day calculated coefficients of variation for measurement times of 1–3 min were 3.5–4.5% and 3.9–4.4%, respectively (Table S2). These results indicate that our method is suitable for the assay of clinical or research samples.

Urea is a non-protein nitrogen compound and is commonly added to cattle feed because of its high nitrogen content. Urea measurement enables monitoring of the nutritional management of lactating dairy cows, as a high level of urea in milk may influence its production. High urea levels in milk can also affect cheese production. We determined the urea concentrations in different milk samples and in urea-added milk using the SA-ISFET-based assay (Fig. 2C). Good standard curve linearity was observed at adding urea concentrations up to 45 mg/dL. The urea concentration in four different milk samples was estimated by calibration from the standard curve as 24, 27, 30, and 35 mg/dL. The analytical recovery of urea added to milk samples was also determined. Addition of urea to a final concentration of 23 or 45 mg/dL to samples already containing 24–35 mg/dL of endogenous urea resulted in recoveries of 92–109 and 106–116%, respectively. The assay used water as a blank, similar to conventional assays. In addition, reagent without urease was assayed as a sample blank (data not shown). The use of one reaction chamber on the sensor as a sample blank minimizes the risk of skewed data due to sample interference.

The new method reported here was compared with a commercially available spectrophotometric method for food analysis (F-kit, no. 542946, Roche Diagnostics GmbH, Mannheim, Germany). A total of 46 samples of normal cow's milk were analyzed, and some of the samples contained added urea. Samples were assayed by each method, and a good correlation between the methods was observed (Fig. 3). The spectrophotometric method required approximately 35 min of measurement time. Moreover, this method required the pretreatment of samples using trichloroacetic acid, whereas the SA-ISFET-based method did not.

In conclusion, our SA-ISFET assay enables easy, reliable, and accurate determinations of urea concentrations. Measurement time is shorter as compared with previous SA-ISFET-based assays. This is