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Development of a ratiometric fluorescent urea biosensor based on the urease immobilized onto the oxazine 170 perchlorate-ethyl cellulose membrane

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ABSTRACT

In this work, the oxazine 170 perchlorate (O17)-ethyl cellulose (EC) membrane was successfully applied in the fabrication of a urea-sensing membrane. The urea-sensing membrane was a double layer consisting of the O17-EC membrane and a layer of the enzyme urease entrapped into EC matrix. The sensing principle of urea was based on the hydrolysis reaction of urea under the catalysis of the urease to produce ammonia in water and also on the binding of ammonia with the dye O17 to create the shift in the emission wavelength from λ_{em}=630 nm to λ_{em}=565 nm. The data collected from the ratio of the fluorescence intensities at λ_{em}=630 nm and λ_{em}=565 nm was proportional to urea concentration. The urea-sensing membrane with the ratiometric method was used to measure the concentrations of urea in the range of 0.01–0.1 M with a limit of detection (LOD) of 0.027 mM and 0.1–1.0 M with LOD of 0.224 mM. It showed fast response time, high reversibility and long-term stability in this concentration range. The recovery percentage of urea concentrations of the urea-sensing membrane for two kinds of biological urine solutions (BU1, BU2) was around 85–118%. The measured results were in good agreement with standard urea concentrations in the range of 0.06 M to 1.0 M.

1. Introduction

Urea is one of the most important analytes to be used in clinical and biological chemistry with a biosensor [1]. Therefore, quantitative analysis methods for urea have been developed depending on the sample matrix. Literature reviews on the development of the urea analysis methods have been also reported in many papers [2].

Urea can be determined by the direct analysis of the molecule (urea) or by the indirect analysis of its reaction products. In the direct analysis methods, urea reacts with specific chemicals and produces color products which absorb light at certain wavelengths and are measured with a spectrophotometer. Some examples for these methods are as follows: the reaction of urea with butane-2,3-dione monoxime and thio semicarbazide in strongly acidic conditions produce a chromophore with a maximum absorption at 525 nm [3], or the reaction of urea with 1-phenyl-1,2-propanedione-2-oxime produces a chromophore with a maximum absorption at 540 nm [4], and both methods have a detection limit of 1 mg/l.

The indirect analysis methods are based on the selective hydrolysis of urea with urease to produce ammonia and carbon dioxide. In the enzyme-based analysis system, a substrate active enzyme layer is embedded on a suitable surface of a classical sensor which measures the concentration of products formed during the enzymatic reaction. These measurements can be based on potentiometry [5–7], voltammetry [8,9], conductometry [10–12], ion selectivity [13] and spectrophotometry [14,15]. Immobilization of the enzyme urease onto a suitable matrix is also varied, which can be nanomaterials [14], sol–gels [10,15,16] or polymers [5–7,12,17].

Even though many urea biosensors [2] are highly developed based on direct and indirect analysis techniques, some drawbacks still exist that have yet to be overcome. That is, the analysis system is vulnerable to interference and the detection range for urea in many studies is still narrow [5,6,10]. Apart from clinical applications with a detection range for low urea concentrations, there is a demand for robust, reliable urea sensors to be used in the measurement of high urea concentrations in hydrogen production using urea-rich wastewater [18]. An optical urea sensor with a linear detection range of 0.01–0.5 M urea has been developed by immobilizing the enzyme urease and a pH indicator (neutral red) onto a tri-acetyl cellulose membrane [19].

To the best of our knowledge, there are no published studies about ratiometric fluorescent urea biosensors. Therefore, we have

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fabricated a ratiometric urea biosensor to improve the sensor quality in terms of the detection range, selectivity, long term stability, and vulnerability. The major advantage of the ratiometric method is its ability to normalize the change in the fluorescence intensities that are not related to the change of the target concentration. For example, the temporal and spatial distribution of the measured fluorescence intensity can typically fluctuate due to a non-uniform distribution of fluorophores within the sensor, the variation in dynamics of fluorophores in different mediums, or noise in the measurement system such as variations in the illumination intensity. The fabrication of the ratiometric urea biosensor was based on our previous research [20] of a ratiometric ammonia-sensing membrane, called the O17-EC membrane. In this work the enzyme urease was immobilized in ethyl cellulose matrix and embedded on the surface of the O17-EC membrane. Ammonia (or ammonium ions) produced from the hydrolysis reaction of urea under the catalysis of urease was bound with the dye O17 to change the emission fluorescence intensity as well as the emission wavelengths of the dye O17 (a decrease of the fluorescence intensity at λ_{em}=630 nm and an increase of the fluorescence intensity at λ_{em}=565 nm). The changes in the fluorescence intensities at two emission wavelengths were proportional to urea concentrations, so that a ratiometric calculation was carried out. The properties of the urea-sensing membrane were investigated, and the reaction kinetics of urea in the urea-sensing membrane as well.

2. Materials and methods

2.1. Materials

Oxazine 170 perchlorate (O17), ethyl cellulose (EC), urease, and urea were purchased from Sigma Aldrich Chemical Co. (Seoul, Korea). Other analytical-grade chemicals such as sodium phosphate, sodium chloride, sodium hydroxide, hydrochloric acid were used without further purification.

2.2. Preparation of the urea-sensing membrane

The O17-EC membranes were prepared as in our previous study [20] by mixing 15 μl of O17 stock (2 mg/ml) with 300 μl of 10 wt% EC in ethanol. The mixture was incubated for 4 h at room temperature and then 50 μl of the mixture were coated on the bottom of one well in the 24-well microtiter plate (NUNC Co., Copenhagen, Denmark). Afterwards, the O17-EC membrane was dried at 60 °C for 12 h. In the next step, 100 μl of 10 wt% EC in ethanol were mixed with 80 units (U) of urease, and then coated onto the O17-EC membrane and incubated for 24 h at 4 °C. The surface morphologies of the O17-EC membrane and the urea-sensing membrane were identified by atomic force microscopy (AFM).

2.3. Urea measurements

The urea concentrations for measurements was in the range of 0.01–1.0 M. Data was collected from the fluorescence intensity of the urea-sensing membrane at two emission wavelengths (λ_{em}=565 nm and λ_{em}=630 nm) with an excitation wavelength of 470 nm (λ_{ex}=470 nm). The fluorescence spectra for detecting urea were measured with a multifunctional fluorescence microplate reader (Safire^2, Tecan Austria GmbH, Wien, Austria).

The immobilization efficiency of urease into the urea-sensing membrane was calculated by dividing the amount of immobilized urease by the total amount of urease used for immobilization. The amount of the immobilized urease was determined by subtracting the amount of the unimmobilized urease from the total amount of urease used. The unimmobilized urease was separated from the immobilized urease in one well by washing several times with 4.5 ml of 10 mM phosphate buffer (pH 7.4). The protein values of the washed, unimmobilized urease were determined by the Bradford method. The optimization of urease amount for immobilization was performed with 40 U, 60 U, 80 U, 100 U and 120 U of urease. The urea-sensing membranes immobilized with various amounts of urease were used for the measurements of different urea concentrations. The sensitivity of the membrane was evaluated through the slope value (SI), i.e. the ratio of the fluorescence intensities at two emission wavelengths (λ_{em}=565 nm and λ_{em}=630 nm) with respects to urea concentration, to get a conclusion for optimum amount of urease for immobilization.

The kinetic parameters (V_{max} and K_m) of the immobilized urease were determined from the Lineweaver–Burk plot based on the ratio of the fluorescence intensities at λ_{em}=565 nm and λ_{em}=630 nm.

Reversibility of the urea-sensing membrane was performed at 0.1 M urea and distilled water. The urea-sensing membrane was first exposed to 0.1 M urea solution, followed by distilled water. The microplate reader was set for fluorescence measurements against time with interval of 30 s.

The effects of pH and temperature on the urea measurement were investigated. 0.1 M urea solutions in the range of pH 5.0 to pH 9.0 were exposed to the urea-sensing membrane. The urea-sensing membrane was also tested with different temperatures (25, 30, 33, 37, 40 °C) at urea concentrations ranging from 0.01 M to 1.0 M. The long-term stability of the urea-sensing membrane with various urea concentrations was evaluated through the determination of its repeatability by measuring the fluorescence intensity obtained initially and after 2 months.

The selectivity of the urea-sensing membrane was investigated with an alkaline compound as sodium hydroxide (NaOH) and ions (K^+, Na^+). The urea-sensing membrane was measured with various concentrations (0, 10, 20, 40, 60, 80, 100, 200, 300 mM) of NaOH solution. Different potassium concentrations (0, 10, 50, 100, 150 mM) and sodium concentrations (0, 10, 50, 100, 300 mM) were also tested with the urea-sensing membrane at various urea concentrations.

Two types of biological urine solutions containing various urea concentrations were prepared, and the urea concentrations were determined with the urea-sensing membrane. First biological urine solution (BU1) included 2.5 mM $CaCl_2$, 45 mM NaCl, 3.5 mM KH_2PO_4, 3.5 mM K_2HPO_4 and urea in the concentration range of 0.01–1.0 M at pH 7.2. Second biological urine solution (BU2) was prepared by adding 2.5 mM $NaHCO_3$, 1 mM $MgSO_4$ and 2.5 mM Na_2SO_4 to the components of BU1.

2.4. Ratiometric method

A ratiometric method for the urea biosensor was based on the ratio of the fluorescence intensities of the urea-sensing membrane at two emission wavelengths (λ_{em}=630 nm (I_{630}), λ_{em}=565 nm (I_{565})) as follows:

$$R=\frac{I_{565}}{I_{630}}$$

2.5. Data analysis

For repeated measurements of the urea-sensing membrane with different levels of pH, temperature, ions (K^+, Na^+), the differences of the fluorescence intensities were assessed by one-way analysis of variance (ANOVA). Significant differences between samples were accepted with p-value <0.05. Statistical tests were

performed using the software InStat (vers.3.01, GraphPad Software Inc., San Diego, USA).

The fluorescence intensity normalized at an emission wavelength was calculated by dividing the fluorescence intensity (I) measured at a given urea concentration by the maximum fluorescence intensity (I_{max}), i.e., I/I_{max} at λ_{em}. Ratio of the normalized fluorescence intensities was obtained from the equation, (I/I_{max} at λ_{em}=565 nm)/(I/I_{max} at λ_{em}=630 nm).

3. Results and discussion

3.1. Properties of the urea-sensing membrane

The properties of the fluorescent dye O17 and the O17-entrapped EC membrane (i.e., O17-EC membrane) were presented in our previous study [20].

The urea-sensing membrane included two layers, the below layer was the O17-EC membrane and the above layer was the membrane of the enzyme urease immobilized into EC matrix. EC was employed as supporting material for both layers so that the enzyme layer is strongly attached on the below layer after coating the mixture of EC and urease onto the surface of the O17-EC membrane (Fig. 1).

When urea solution was added to the well containing the urea-sensing membrane, the enzyme urease catalyzes the hydrolysis of urea into ammonia and carbon dioxide as in the following equation:

$$(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2 \quad (1)$$

Ammonia produced from this reaction was detected by using the O17-EC membrane through the shift in the emission wavelength of the fluorescent dye O17 from λ_{em}=630 nm to λ_{em}=565 nm. The shift in the emission spectrum of the O17 is an interesting property to make a ratiometric fluorescent sensor.

Fig. 1 showed AFM images for the surface morphology of the O17-EC membrane and the urea-sensing membrane. A smooth surface with a surface mean roughness (R_a) of 0.555 nm and a root mean square roughness (R_q) of 1.438 nm was observed with the O17-EC membrane, while the surface of the urea-sensing membrane had higher roughness, i.e. R_a of 1.889 nm and R_q of 2.598 nm. This indicated that the enzyme urease was successfully immobilized into the EC matrix since the urease was homogenized completely in this matrix.

According to data collected from the Bradford protein assay, the immobilization efficiency of urease into the urea-sensing membrane was very high at all amounts of urease used. Only 11.08% was released from the EC matrix for 40 U of urease amount used for immobilization, about 11.12% for 60 U of urease used, 12.95% for 80 U of urease used, 12.90% for 100 U of urease used and 15.37% for 120 U of urease used. This is attributed to good urease-capturing capacity of the EC matrix. The response of the urea-sensing membrane with different amounts of immobilized urease is shown in Fig. 2. This indicates the sensitivity of the urea-sensing membrane to various urea concentrations in the range of 0.01 M to 0.1 M. Among various amounts of urease used, 80 U of urease showed the highest sensitivity (slope value, SI_{80U}=3.133) in term of the response of the urea-sensing membrane to urea concentrations. However, use of higher amounts of urease (e.g. 100 U or 120 U) did not increase the sensitivity of the urea-sensing membrane since their slope values (SI_{100U}=2.847, SI_{120U}=2.847) were lower than that of 80 U of urease. Moreover, the large amounts of urease immobilized into the EC matrix could increase the hydrolysis reaction of urea but may cause an obstruction of the NH_4^+ transport to contact with the O17-EC membrane, whereas the lower amounts of urease (e.g. 40 U) decreased the sensitivity of the O17-EC membrane at low urea concentrations. Thus, for our further experiments, 80 U of urease was chosen to fabricate the urea-sensing membrane based on the O17-EC membrane.

In case of using different concentrations of EC to immobilize a given urease amount, the results from Bradford protein assay indicated that the amount of urease released from 10% EC is lowest (9.11%) as compared with that released from 2.5% EC (21.78%) or 5% EC (20.23%) (data not shown). It is easy to understand high

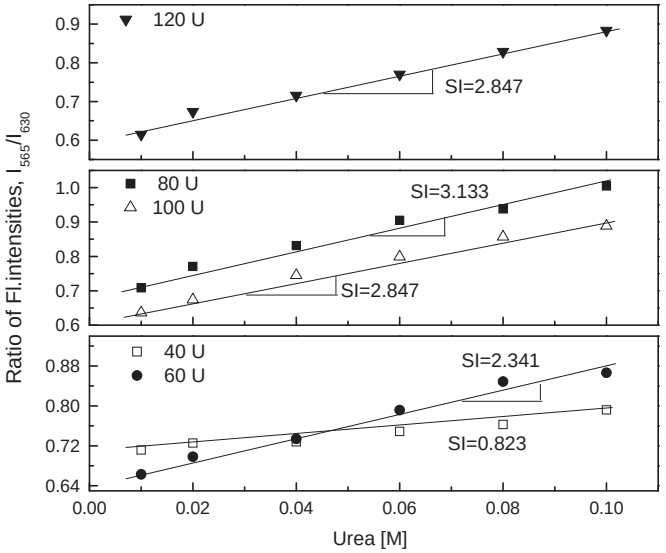

Fig. 2. The response of the urea-sensing membrane with various amounts of immobilized urease in the urea hydrolysis reaction. SI represents slope value and indicates the sensitivity of the membrane.

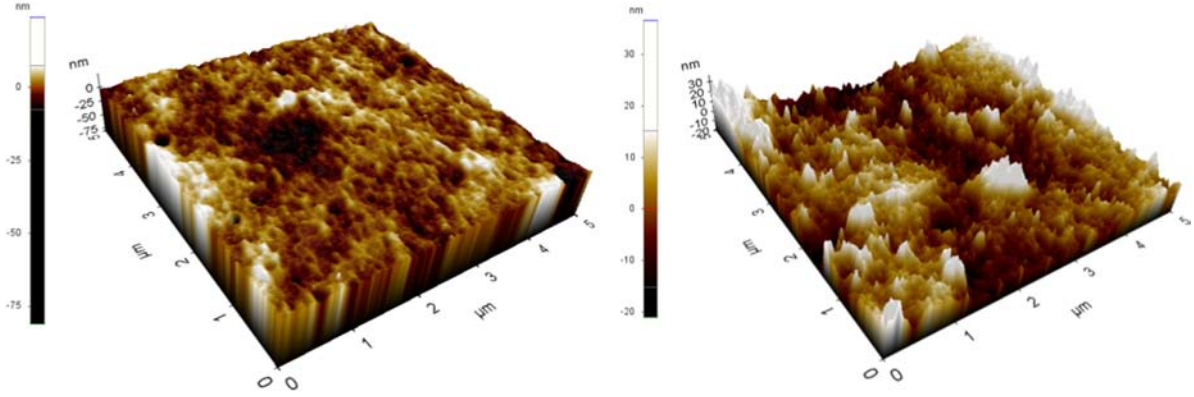

Fig. 1. AFM images of the O17-EC membrane (left), and the urea-sensing membrane (right).

immobilization efficiency of 10% EC as supporting material, because 10% EC is a concentrated matrix that captured urease in its matrix more tightly than 2.5% EC or 5% EC.

In addition, the EC concentrations for urease immobilization can affect the response time of the urea-sensing membrane. But our results showed that the response times (t_{95}∼4.8 min) of the urea-sensing membrane with the urease-immobilized layer of 5% EC or 10% EC seemed to be not much different at various urea concentrations, whereas the urea-sensing membrane with the enzyme layer of 2.5% EC had longer response time (t_{95}∼7.6 min) (data not shown). This means that the thickness of the enzyme layer is not a decisive factor affecting the response time of the urea-sensing membrane. The response time is more related to the protein-capturing ability of the enzyme layer according to EC concentrations. Herein, the amounts of the immobilized urease with 2.5% EC and 5% EC are quite equal, i.e. 78.25% for 2.5% EC and 79.77% for 5% EC before urea measurements. But the leakage of the immobilized urease from the enzyme layer of 2.5% EC and 5% EC caused to a significant difference in the response times during urea measurements. Similar response times of the enzyme layer of 5% EC and 10% EC can attribute to the thickness and structure of the supporting material. That is, the enzyme layer of 10% EC is not too thick to hamper the reaction rate and its structure helps to immobilize more amount of the enzyme within the layer.

3.2. Response of the urea-sensing membrane

As shown in Fig. 3, the O17-EC membrane is very sensitive to ammonia produced from the hydrolysis reaction of urea even though a urease-immobilized EC matrix was laid over on it. The fluorescence emission spectra of the urea-sensing membrane with various urea concentrations are similar to those of the O17-EC with ammonia in our previous research [20]. The fluorescence intensity at λ_{em}=565 nm increased and decreased at λ_{em}=630 nm with increasing the concentrations of urea.

Fig. 4a shows a calibration curve for urea calculated by the ratio of two fluorescence intensities at λ_{em}=565 nm and at λ_{em}=630 nm. The detection range of urea based on the ratiometric calculation method could be divided into two linear ranges: 0.01–0.1 M and 0.1–1.0 M with high regression coefficient values of $r^2_{0.01-0.1}$=0.989 and $r^2_{0.1-1.0}$=0.981. The detection limit (LOD) was 0.027 mM for the range of 0.01–0.1 M urea and 0.224 mM for the range of 0.1–1.0 M urea. Compared with our previous research [16] using CdSe/ZnS quantum dots as a urea indicator and sol–gel matrix as supporting material for urease immobilization, this ratiometric urea-sensing membrane based on the O17-EC membrane could be successfully used to detect urea at higher concentrations.

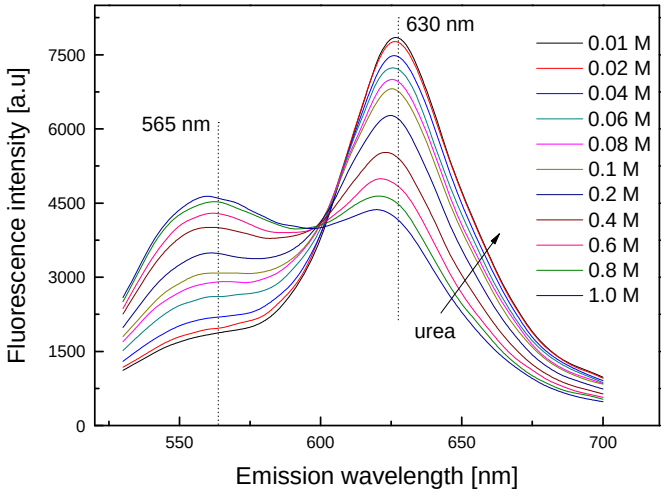

Fig. 3. Fluorescence emission spectra of the urea-sensing membrane to urea concentrations in the range of 0.01 M to 1.0 M, monitored at an excitation wavelength of 470 nm.

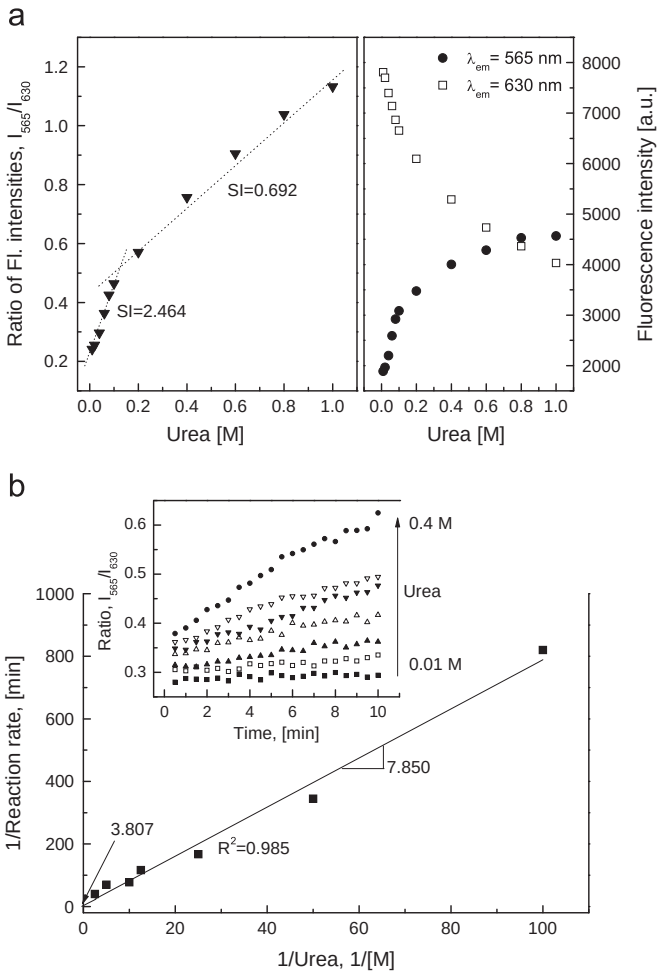

Fig. 4. (a) Calibration curve for urea calculated by the ratio of two fluorescence intensities measured at emission wavelengths of 565 nm and 630 nm, (b) Lineweaver–Burk plot with the changes in the ratio of two fluorescence intensities at λ_{em}=565 nm and λ_{em}=630 nm of the urea-sensing membrane at different urea concentrations as a function of time.

The enzyme urease immobilized on the O17-EC membrane was evaluated via Michaelis–Menten kinetics. Kinetic parameters were calculated from the ratio of two emission fluorescence intensities at λ_{em}=565 nm and λ_{em}=630 nm. The maximal reaction rate (V_{max}) of 0.263 1/min and Michaelis–Menten constant (K_m) of 2.062 M were obtained from the Lineweaver–Burk plot in Fig. 4b. Here, K_m value was about 1.2×10^4 times higher than that (0.1698 mM) of the double layer consisting of the urease-immobilized membrane and the QD-entrapped sol–gel membrane in our previous research [16], whereas V_{max} was slower than that (0.416 1/min) of the double layer with the QD-entrapped membrane. The slow reaction rate of this urea-sensing membrane based on the O17-EC membrane may result from thicker layer of the membrane.

It is clear that the hydrolysis reaction of urea under the catalysis of urease has strongly occurred with large amounts of the urease immobilized. In general, use of a large amount of an enzyme can lead to increase the reaction rate and final products that make a sensor more sensitive. But, the sensitivity of the sensor becomes rapidly limited to a narrow detection range due to reaching fast saturation of the hydrolysis reaction. However, in this

case, the sensitivity was still high at high concentrations of urea. This is attributed to the effect of the supporting material (i.e. ethyl cellulose) on the transport of ammonia in solution reacting with the O17-EC membrane. The EC matrix did not release ammonia produced from the hydrolysis reaction of urea rapidly nor slowly. Therefore, the response of the O17-EC membrane was still high at both low and high concentration ranges of urea, however, the response time was long (t_{95}~4.8 min) as compared with other membranes [16,20]. The response time included both the hydrolysis reaction time of urea to produce ammonia and the transport time of ammonia passing through the urease-immobilized EC membrane to contact with the O17-EC membrane.

Kinetic parameters (V_{max} and K_m) also indicated indirectly the binding process between ammonia and the dye O17 as predicted in the following equations:

$$NH_3 + H_2O \rightleftharpoons NH_4^+ OH^- \quad (2)$$

$$NH_4^+ OH^- + H^+ Dye^- \rightleftharpoons NH_4^+ Dye^- + H_2O \quad (3)$$

$$NH_4^+ Dye^- \rightleftharpoons H^+ Dye^- + NH_3 \quad (4)$$

The higher values of V_{max} and K_m correspond to more ammonia being produced and bound with the dye O17.

The high reproducibility of a fluorophore-entrapped sensing membrane means little leakage of dye, no photobleaching and high contact with the target analyte. These parameters could be satisfied and proved with the O17-EC membrane.

As shown in our previous study [20], when O17 donates a proton to the weak base, the hydroxyl radical (OH^-) abstracts a loosely bound proton, leaving the positively charged ammonium cation (NH_4^+) to counterbalance the perchlorate anion (Dye^-). This reaction produces significant fluorescence band shifts and leads to a color change of the dye O17 from blue to red. When the NH_4^+ is reduced and the Dye^- is protonated, the color of the dye O17 is fully restored from red to blue. With urea, the ammonia produced from the hydrolysis reaction of urea reacted with the dye O17 captured in the O17-EC membrane and led to a change in the fluorescence intensity at both emission wavelengths (λ_{em}=565 nm and λ_{em}=630 nm). Moreover, the urea-sensing membrane demonstrated the ability to restore the fluorescence intensity when it was exposed in the presence or absence of ammonia. This was easy to recognize when the urea-sensing membrane was repeatedly exposed to 0.1 M urea and to distilled water (DW).

In Fig. 5, the reversibility of the urea-sensing membrane could be expressed at the ratio of the fluorescence intensities at λ_{em}=565 nm and λ_{em}=630 nm. The fluorescence intensities at both emission wavelengths (λ_{em}=565 nm and λ_{em}=630 nm) had very low RSD (relative standard deviation), i.e. 0.9% at DW and 1.9% for 0.1 M urea at λ_{em}=630 nm and 2.5% at DW and 2.6% for 0.1 M urea at λ_{em}=565 nm.

The performance of the biosensor with enzyme is usually influenced with pH and temperature. That is, pH can make the enzyme activity high or low, and as a consequence it results in high or low efficiency of catalytic reaction in the biosensor. As the same thing with the pH, temperature can increase or decrease the catalytic reaction rate of the enzyme.

In this case, the urease immobilized into EC matrix preferred alkaline medium (i.e. pH range of pH 7.0–pH 9.0) to weak acid medium (pH 5.0–pH 6.0). The fluorescence intensity of the urea-sensing membrane at 0.1 M urea did not change significantly (p-value=0.9923 for λ_{em}=565 nm, p-value=0.9932 for λ_{em}=630 nm) in the pH range of pH 7.0–pH 9.0, whereas it decreased considerably at λ_{em}=565 nm at pH 5.0 (Fig. 6a). These pH effects of urea solution in the pH range of pH 7.0–pH 9.0 are the same as those of ammonia solution [20]. Therefore, the response of the urea-sensing membrane was not influenced with ammonia produced from the hydrolysis

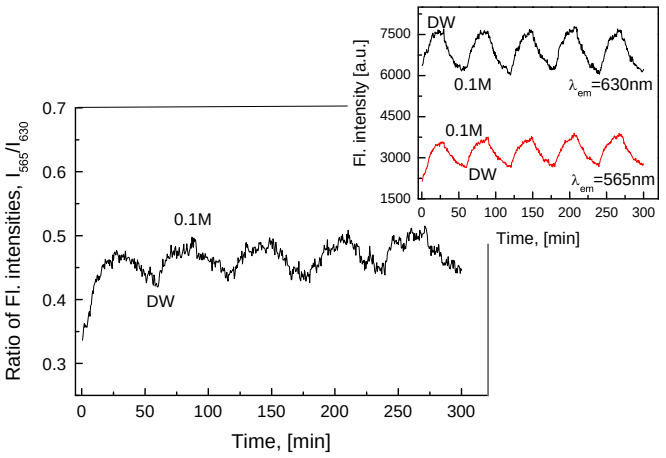

Fig. 5. Reversibility of the urea-sensing membrane at 0.1 M urea and distilled water (DW).

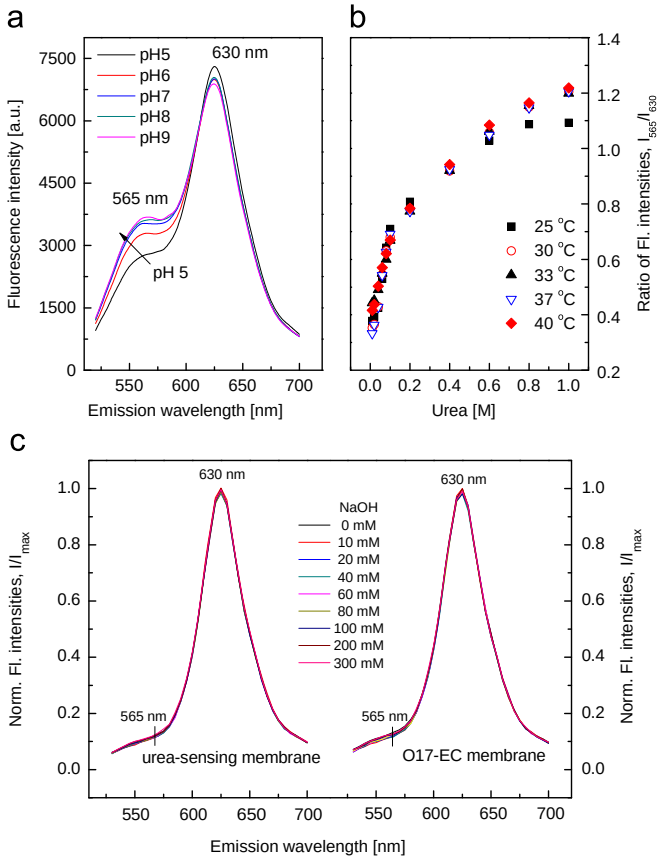

Fig. 6. (a) Response of the urea-sensing membrane in 0.1 M urea in the pH range of pH 5.0 to pH 9.0, (b) Response of the urea-sensing membrane with different temperatures at various urea concentrations, (c) response of the urea-sensing membrane and the O17-EC membrane at various concentrations of NaOH solution.

reaction of urea in the pH range of pH 7.0–pH 9.0. In addition, neutral or alkaline medium are the favorite medium for this urease strain.

Fig. 6b shows the response of the urea-sensing membrane with different temperatures at various urea concentrations. The urea-sensing membrane seems to be not considerably influenced by temperature in the range of 25–40 °C at the urea concentration range of 0.01–1.0 M. However, in the urea range of 0.1–1.0 M the sensitivity (SI=0.43) of the urea-sensing membrane at 25 °C was a little lower than that of the membrane at higher temperatures. Therefore, for the lifetime of the urease as well as the quality,

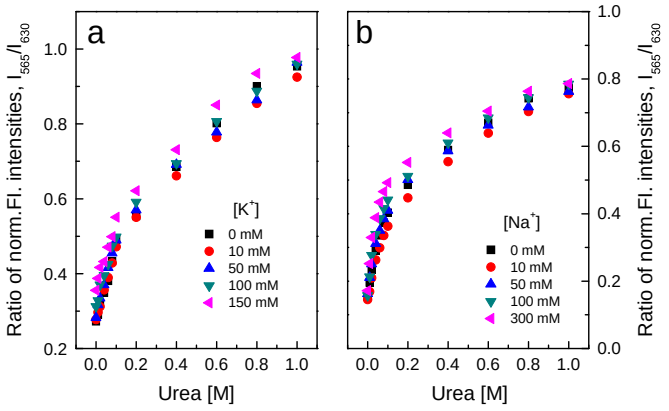

Fig. 7. Response of the urea-sensing membrane at the urea concentration range of 0–1.0 M in the presence of different amounts of K^+ and Na^+.

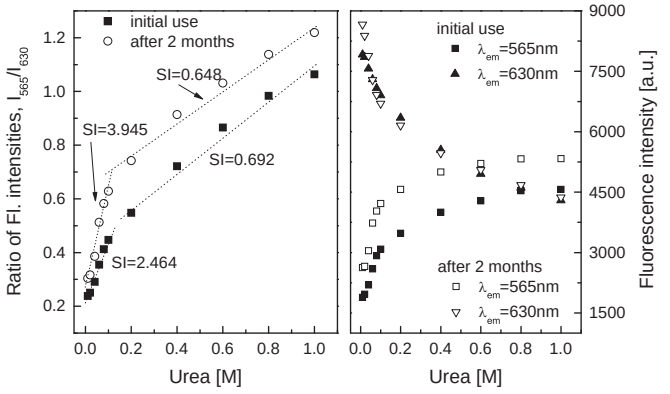

Fig. 8. Long-term stability of the urea-sensing membrane in the urea concentration range of 0.01 M to 1.0 M at initial use and after 2 months of use (left), and two emission fluorescence intensities at λ_{em}=565 nm and λ_{em}=630 nm (right).

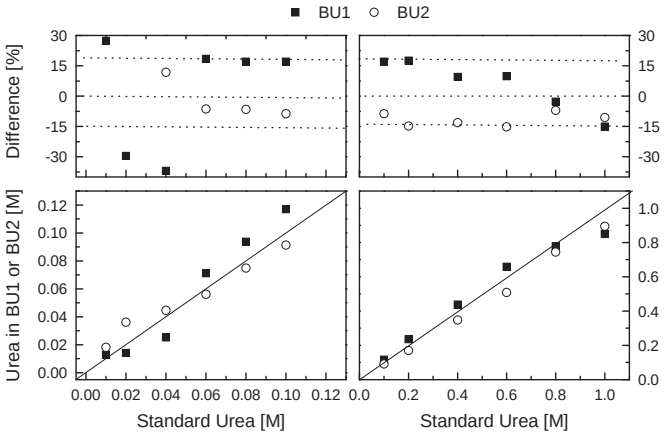

Fig. 9. Correlation between standard urea concentrations and urea concentrations dissolved in two biological urine solutions (BU1, BU2) measured with the urea-sensing membrane, and their difference plots. The difference represents 100 × (urea in BU1 or BU2 − standard urea)/(standard urea). The dashed lines indicate the recovery percentage of urea concentrations in BU1 or BU2 samples around 85% to 118%.

sensitivity and reproductivity of the urea-sensing membrane for long time in use, a temperature of around 30 °C was used for urea measurements in this work.

To demonstrate the selectivity of the dye O17 and the effects of alkaline compounds on the urea-sensing membrane, the urea-sensing membrane was investigated with different concentrations of sodium hydroxide (NaOH). There was neither significant decrease in the fluorescence intensity of the dye O17 at λ_{em}=630 nm nor increase in the fluorescence intensity at λ_{em}=565 nm for the urea-sensing membrane and the O17-EC membrane at various concentrations of NaOH solution (Fig. 6c).

For the evaluation of some interferences on the urea-sensing membrane, potassium (K^+) and sodium (Na^+) cations were chosen as competitive factors for the cation, NH_4^+. In fact, potassium (K^+) and sodium (Na^+) cations maintain water (amounts of fluid inside and outside body's cells) and electrolyte balance of the body. The level of potassium often varies with the level of sodium. When the sodium levels go up, the potassium levels go down and vice versa. Levels of potassium or sodium that are too high or too low can represent a serious problem. The normal concentration range of potassium in urine varies from 25 mM to 100 mM, and that of sodium varies from 20 mM to 220 mM.

Fig. 7 showed that the urea-sensing membrane was not influenced with potassium and sodium ions (K^+, Na^+) in the concentration range of 0–150 mM and 0–300 mM, respectively. The calibration curves of the urea-sensing membrane did not change significantly in the presence of these cations. All p-values for the ratio of the normalized fluorescence intensities at λ_{em}=565 nm and λ_{em}=630 nm were greater than 0.05 at different amounts of K^+ and Na^+ (e.g., $p-value_{10\ mM\ (K^+)}=0.8249$; $p-value_{50\ mM\ (K^+)}=0.9020$; $p-value_{100\ mM\ (K^+)}=0.9181$; $p-value_{150\ mM\ (K^+)}=0.7710$; $p-value_{10mM\ (Na^+)}=0.7299$; $p-value_{50mM\ (Na^+)}=0.9519$; $p-value_{10mM\ (Na^+)}=0.7609$; $p-value_{300mM\ (Na^+)}=0.4749$).

After the urea-sensing membrane was tested about 266 times during 2 months, the sensitivity of the membrane was found to be quite good (Fig. 8). Moreover, the slope value of the linear curve in the urea concentration range of 0.01–0.1 M increased from SI=2.464 (initial use) to SI=3.945 (after 2 months). This could be attributed to the formation of more cavities inside EC matrix, so that the urea-sensing membrane becomes more versatile and softer, which led to fast interaction between the produced ammonia and the dye O17. However, in the urea concentration range of 0.1–1.0 M, the slope value of the urea-sensing membrane after 2 months was little changed (about 6%) as compared with that at initial use.

It could conclude that the urea-sensing membrane consisting of the O17-EC membrane and the urease-immobilized EC membrane has shown an excellent stability for long time in use. Ethyl cellulose (EC) was a good supporting material for the fluorescent dye O17 as well as for the immobilization of urease. It prevented the leaking-out of the dye O17 and the enzyme urease, but it still created an open environment for the hydrolysis reaction of urea and the sensing reaction of the O17 with ammonia produced as well.

3.3. Detection of urea in biological urine solution

For the evaluation of the recovery capability, the developed urea-sensing membrane was used to determine the concentrations of urea dissolved in two types of biological urine solutions (BU1, BU2). Using standard urea solution prepared in distilled water, two linear calibration curves based on the ratiometric calculation method were obtained as follows: $y=3.7846x+0.3175$ ($r^2 \geq 0.976$) for the urea concentration range of 0.01–0.1 M and $y=0.5744x+0.6688$ ($r^2 \geq 0.958$) for the urea concentration range of 0.1–1.0 M. Here, x represents urea concentration and y is the ratio of the fluorescence intensities at λ_{em}=565 nm and λ_{em}=630 nm. Fig. 9 shows the measurement results of urea concentrations in BU1 and BU2, which were determined using two calibration equations. The difference plot in Fig. 9 also showed the recovery percentage of urea concentrations in BU1 and BU2. The recovery percentage of the urea-sensing

Table 1
Summary of some urea biosensors for urea detection in biological samples.

Principle of detection	Sensing matrix	Urea concentration range	Response time	Recovery (%)	Reference
Ratiometry of fluorescence intensity	Ethyl cellulose	0.06–1.0 M	4.8 min	85–118	This work
Potentiometry	Poly(carbamoylsulfonate) hydrogel	0.072–21 mM	2 min	100 ± 5–8	[5]
Potentiometry	Poly(*o*-phenylene diamine) film	0.01–100 mM	20 s	na	[6]
Potentiometry	Polyaniline coated by a layer by layer film	0.01–1 mM	2 min	100 ± 11	[21]
Conductometry	Sol–gel tetramethylorthosilicate (TMOS)	6.25–23.5 mM	8.3 min	100 ± 5	[10]

* na: not available.

membrane was quite good (85–118%) in the urea concentration range of 0.06–1.0 M in BU1 and BU2. But the membrane seems to be less sensitive at low urea concentrations (0.01–0.04 M). This may be mainly due to low linear regression coefficient values of two calibration equations and also to the presence of anions such as SO_4^{2-} in BU2.

In comparison with other urea biosensors for urea measurement in biological samples as shown in Table 1, the ratiometric fluorescent urea biosensor in this work showed large linear detection range, i.e. up to 1.0 M of urea. The response time of the sensor was relatively faster than other urea biosensors, which was attributed to the structure of the supporting material used for the immobilization of the urease. With a wide detection range and a fast response time, the ratiometric urea-sensing membrane can be applied to detect urea in various industrial fields. A considerable amount of urea is produced from human or animal urine (containing about 2–2.5 wt% urea) and from wastewater during the industrial synthesis of urea. Recently, a urea fuel cell was successfully produced by using urine or wastewater [22]. A ratiometric urea-sensing membrane with a detection range of 0.1–1 M urea can be applied to detect high concentrations of urea in the manufacturing process of urea fuel cells. In addition, with a detection range of 0.01–0.1 M urea, it can be used in food quality control and clinical diagnostics. However, Francis [23] mentioned that among many methods for the determination of urea, each one may be better suited to the desired application and instrumentation available in any particular laboratory.

4. Conclusion

The urea-sensing membrane with the ratiometric method showed a high sensitivity to urea in two linear concentration ranges of 0.01–0.1 M and 0.1–1.0 M with LOD of 0.027 mM and 0.224 mM, respectively. The reproducibility of the urea-sensing membrane was also excellent with very low RSD (less than 3%). Moreover, the lifetime of this sensing membrane was so long, that its sensitivity to urea remained for at least 2 months. The urea-sensing membrane with fast response time was applied to determine the concentrations of urea dissolved in biological urine solutions including some cations and anions. High recovery percentage indicated that the urea-sensing membrane with the ratiometric method would have great significance in clinical chemistry as well as in food chemistry and environmental monitoring. In addition, the successful fabrication of the urea-sensing membrane based on the ratiometric ammonia-sensing membrane can offer a method to develop a few optical sensing membranes in detecting some analytes which can produce ammonia in their reactions from different metabolic pathways.

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References

[1] B.R. Eggins, Biosensors: An Introduction, John Willey & Sons Ltd and B.G. Teubner (1996).
[2] M. Singh, N. Verma, A.K. Garg, N. Redhu, Sens. Actuators, B: Chem. 134 (2008) 345.
[3] A.J. Taylor, P. Vadgama, Ann. Clin. Biochem. 29 (1992) 245.
[4] J. Almy, C.S. Ough, J. Agric. Food Chem. 37 (1989) 968.
[5] C. Eggenstein, M. Borchardt, C. Diekmann, B. Gruendig, C. Dumschat, K. Cammann, M. Knoll, F. Spener, Biosens. Bioelectron. 14 (1999) 33.
[6] D. Chirizzi, C. Malitesta, Sens. Actuators, B: Chem. 157 (2011) 211.
[7] B. Lakard, G. Herlem, S. Lakard, A. Antoniou, B. Fahys, Biosens. Bioelectron. 19 (2004) 1641.
[8] F. Mizutani, S. Yabuki, Y. Sato, Biosens. Bioelectron. 12 (1997) 321.
[9] Y.C. Luo, J.S. Do, Biosens. Bioelectron. 20 (2004) 15.
[10] W.Y. Lee, S.R. Kim, T.H. Kim, K.S. Lee, M.C. Shin, J.K. Park, Anal. Chim. Acta 404 (2000) 195.
[11] M.M. Castillo-Ortega, D.E. Rodriguez, J.C. Encinas, M. Plascencia, F.A. Mendez-Velarde, R. Olayo., Sens. Actuators, B: Chem. 85 (2002) 19.
[12] P.S. Chaudhari, A. Gokarna, M. Kulkarni, M.S. Karve, S.V. Bhoraskar, Sens. Actuators, B: Chem. 107 (2005) 258.
[13] B. Kovacs, G. Nagy, R. Dombi, K. Toth, Biosens. Bioelectron. 18 (2003) 111.
[14] C.P. Huang, Y.K. Li, T.M. Chen, Biosens. Bioelectron. 22 (2007) 1835.
[15] H.C. Tsai, R.A. Doong, Biosens. Bioelectron. 23 (2007) 66.
[16] H.D. Duong, J.I. Rhee, Anal. Chim. Acta 626 (2008) 53.
[17] E. Cevik, M. Senel, M.F. Abasiyanik, J. Solid State Electrochem. 16 (2012) 367.
[18] B.K. Boggs, R.I. King, G.G. Borte, Chem. Commun. 32 (2009) 4859.
[19] M. Krysteva, M.A. Hallak, Sci. World J 3 (2003) 585.
[20] H.D. Duong, J.I. Rhee, Sens. Actuators, B: Chem. 190 (2014) 768.
[21] B. Lakard, D. Magnia, O. Deschaume, G. Vanlancker, K. Glinel, S. Demoustier-Champagne, B. Nysten, A.M. Jonas, P. Bertrand, S. Yunus, Biosens. Bioelectron. 26 (2011) 4139.
[22] R. Lan, S. Tao, J.T.S. Irvine, Energy Environ. Sci 3 (2010) 438.
[23] P.S. Francis, Aust. J. Grape Wine Res 12 (2006) 976.