Sensors and Materials, Vol. 34, No. 8 (2022) 3173–3183
MYU Tokyo

S & M 3033

Development of Small-sized Lysine Enzyme Sensor for Clinical Use

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(Received March 31, 2022; accepted July 6, 2022)

Keywords: lysine, enzyme sensor, amperometric sensor

We have developed an electrochemical biosensor to measure the lysine concentration in blood. The lysine biosensor has a simple design and can complete measurements of a 5 μL sample in 2 min, enabling rapid measurement in clinical practice. The target molecules are catalyzed by enzymes encapsulated in carboxymethyl cellulose on the working electrodes to produce potassium ferrocyanide as an electron mediator that is detected on the electrodes amperometrically. In a clinical application, the sensor was used to measure blood lysine concentrations in 47 pediatric surgical patients. The results showed that the lysine level was correlated with the transthyretin level, a conventional nutritional indicator, confirming its usefulness for nutritional assessment. This sensor can be used for postoperative nutritional management and treatment of rare diseases such as lysinuric protein intolerance. The platform also has a versatile design for the detection of other amino acids in addition to lysine.

1. Introduction

Many patients with short bowel syndrome or inflammatory bowel disease require long-term nutritional management.(1) For these patients, regular assessment of their nutritional condition is necessary to prescribe the appropriate choice of enteral or parenteral nutrition, resulting in the need for a convenient method of nutritional measurement. Monitoring the concentration of lysine, an essential amino acid that is consumed through food intake, is central to this effort. Blood amino acid levels have been monitored for a variety of diseases. One of these diseases, lysinuric protein intolerance, is the excessive excretion of lysine in urine, which lowers the concentration of lysine in the blood.(2) In addition, monitoring of blood lysine levels is also essential for hyperlysinemia(3) and hypercitrullinemia. These diseases are congenital diseases

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https://doi.org/10.18494/SAM3914

ISSN 0914-4935
https://myukk.org/

that occur in young children. In the design of a detection method, it is necessary to reduce the sample volume required for routine testing. However, sensors that can measure quickly with a low sample volume and are suitable for point-of-care testing (POCT) have not yet been developed.

Amino acid analysis can be reliably performed by ion exchange chromatography using automated analyzers and ninhydrin detection in laboratory settings.[4,5] Derivatized amino acids can also be determined by high-performance liquid chromatography (HPLC) methods based on spectrophotometric or fluorometric determination.[5] However, both methods require labor and expensive equipment that are not easily mobilized in a clinical setting. To adapt these methods for wider use, a portable, low-cost, and easy-to-use biosensor is necessary. Four different enzymes have been used to develop biosensor systems for L-lysine analysis: L-lysine-decarboxylase,[6,7] L-lysine-dehydrogenase,[8] L-lysine-2-monooxygenase,[9] and L-lysine-(α)-oxidase.[10-19] L-lysine-decarboxylase was used with a CO_2 sensor to determine the lysine concentration in aqueous solutions. L-lysine-dehydrogenase was used for a flow injection analysis system and showed a high throughput of 40 samples/h. L-lysine-2-monooxygenase was used with an O_2 sensor to determine the lysine concentration in aqueous solutions. Finally, L-lysine-(α)-oxidase has been used for many types of sensors and immobilized on a membrane such as porous glass. Although some of these sensors used as little as 0.025 mL of sample, none of them have been designed for single use in a clinical setting or to target human body fluids.

In this paper, we aim to expand the portfolio of POCT diagnostics to biomarkers that are routinely monitored at the clinical level. We prototyped and evaluated a device for quantifying the lysine (Lys) concentration in a biologically relevant sample, which can be deployed in a clinical setting. For example, blood Lys level is high in patients with hyperlysinemia and low in patients with undernutrition or lysinuric protein intolerance. Currently, the device is designed to quantify lysine using an enzyme (L-lysine-(α)-oxidase)-generated electrochemical signal. L-lysine-(α)-oxidase (lysine oxidase) was adopted for this sensor because it showed relatively fast electron transfer with ferricyanide ions. When designing a disposable biosensor with a configuration similar to that of this sensor, the dynamic range is limited by the dissolved oxygen concentration without an electron mediator. Therefore, when designing biosensors that measure substances with concentrations above that of dissolved oxygen, it is important to use an electron mediator that rapidly transfers electrons such as ferricyanide ions to the active site of the enzyme. Leveraging this design will improve the sensitivity of lysine detection and refine the dynamic range of detection.

The sensors introduced in this article were designed with the aim of being suitable for any of the following practical requirements. In addition, the platform discussed in this paper can be used to develop other amino acid sensors.

- Small sample volume — The device only requires 5 μL of sample per run.
- Fast detection — Results are available in 2 min for detection and analysis.
- Cost-effective — The device was designed using low-cost synthetic materials and to have low production costs.
- Adaptable — Lys Ox can be swapped for enzymes targeting other amino acids and biomolecules as desired.

2. Materials and Methods

2.1 Materials

Polyethylene terephthalate film was purchased from Toray Industries, Inc., Japan. Double-sided adhesive tape was purchased from Lintec, Japan. Microgranular cellulose was purchased from Whatman, UK. Carboxymethyl cellulose (CMC) was purchased from Dai-ichi Kogyo Seiyaku, Japan. Lysine oxidase (EC 1.4.3.14)[20] was manufactured in our laboratory. All other reagents were of analytical grade.

2.2 Sensor chip and apparatus

The lysine biosensor was designed to measure the concentrations of lysine and interfering substances simultaneously in a sample. The sensor was assembled by combining a flow path for the sample solution and a flexible printed conductive pattern supporting the reaction layer. The sensor layers are shown in Fig. 1(a), which shows that the flow path of the sample solution was formed by stacking a 0.125-mm-thick polyethylene terephthalate film, a 0.15-mm-thick double-sided adhesive tape, and a flexible printed conductive pattern in this order.

The sensor has two sample inlets: A and B. Sample inlet A is for introducing blood from a fingertip directly into the sensor, and sample inlet B is for the dropwise addition of the sample using an instrument such as a micropipette. Figure 1(b) shows a top view of the sensor. The sample enters through sample inlet A or B and moves by capillary action until it fills the cavity of the device. In Fig. 1(b), the arrow indicates the sample flow direction. As the sample moves laterally, it reaches the electrode surface to start the enzyme reaction.

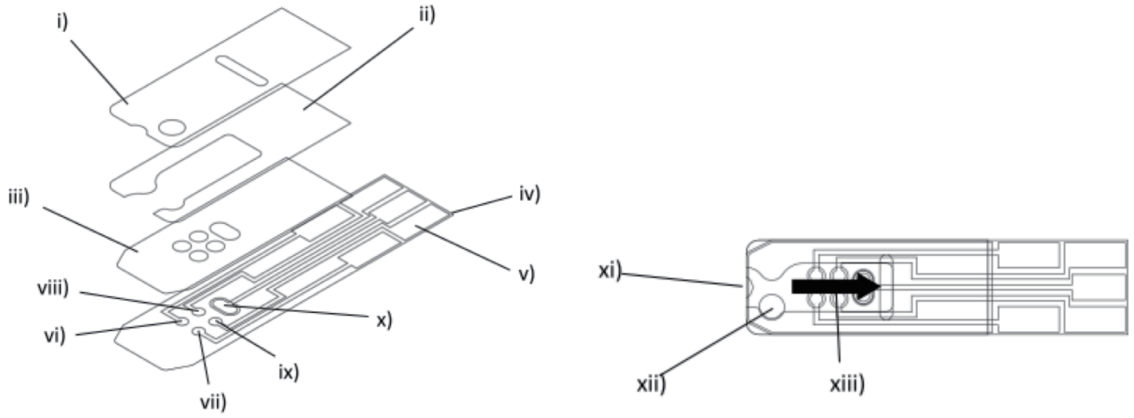


Fig. 1. Structure of lysine sensor (a) and top view of lysine sensor (b). (a) (i) Polyethylene terephthalate film, (ii) double-sided tape, (iii) electric insulating layer, (iv) flexible printed conductive pattern, (v) gold thin layer, (vi) and (vii) sub-working electrodes for measuring interfering substances, (viii) and (ix) main working electrodes, (x) screen-printed Ag/AgCl electrode. (b) (xi) Sample inlet A for blood injection from finger, (xii) sample inlet B for blood injection by dropwise addition, (xiii) arrow indicating sample direction.

After the sample stops at the electrode, a voltage of 0.545 V is applied between each working electrode and the reference electrode intermittently, so that the circuit is off for 6.8 s and voltage is applied for 0.7 s, which is repeated for 120 s. The progress of the enzymatic reaction is monitored by tracking the final current of each voltage application. To exclude the influence of interfering substances such as ascorbic acid, the difference between the mean value of the electric currents of electrodes (viii) and (ix) in Fig. 1(a) and the mean value of the electric currents of electrodes (vi) and (vii) is calculated. The difference in electric currents is substituted into the calibration formula to calculate the lysine concentration. The calibration formula has a coefficient specific to the manufacturing lot of the sensor. The current is the sum of the Faradaic current and non-Faradaic current. It is desirable to sample the current after the non-Faradaic current has sufficiently attenuated. Therefore, the current was sampled 0.7 s after the start of voltage application, at which it was estimated that the non-Faradaic current has sufficiently attenuated.

2.3 Electrodes

Figure 1(b) also shows the structure of the flexible printed conductive pattern. The electrodes on the polyethylene terephthalate film consist of four working electrodes and a counter electrode. The surface of the working electrode is formed with gold ion plating, and the counter electrode is covered with a mixture of silver and silver chloride. The electric circuit is covered with an insulation layer deposited by screen printing except for the four working electrodes, the counter electrode used to form the two-electrode system, and the terminals. The functions of the four working electrodes are as follows:

(vi) and (vii) Gold working electrodes for the measurement of interfering substances around the working electrode during lysine measurement.

(viii) and (ix) Gold working electrodes for the measurement of lysine concentration.

The electric currents induced by working electrodes (viii) and (ix) were integrated and used to calculate the lysine concentration to improve the measurement results. The electric currents induced by working electrodes (vi) and (vii) were also integrated and calculated for the same purpose.

The Ag/AgCl layer was screen-printed on the gold layer to form a reference electrode. A terminal for connecting the working and reference electrodes to the equipment was formed on the edge of the conductive pattern. The potential of the Ag/AgCl electrode mainly depends on the chloride ion concentration around the electrode. In this sensor, plasma is present around the Ag/AgCl electrode. The physiological variation of the chloride ion concentration in plasma is narrow; thus, the potential of the Ag/AgCl electrode is controlled within a narrow range. We designed the sensor to require a sample volume of only 5 μL for clinical measurements by limiting the flow channel and device size. A layer of ink was formed on the exposed gold electrodes, except for the four working electrodes and reference electrodes, to minimize unexpected noise signals.

2.4 Enzyme reaction

Before designing the sensor, the fundamental properties of lysine oxidase were evaluated by absorption spectrophotometry. Reagents contained 0.05–1.5 mM lysine, 0.03 U/mL lysine oxidase, 5 U/mL peroxidase, 20 mM N, N-bis (4-sulfobutyl)-3-methylaniline (Dojindo Laboratories, Japan), 20 mM 4-aminoantipyrine, and 0.1 M NaCl. Phosphate buffer (0.1 M, pH 7.5) was used as the solvent. The time course of absorbance at 550 nm was evaluated. The enzyme activity U was defined such that 1 U causes a decrease of 1 μM of L-lysine per minute under the conditions described above.

2.5 Reaction layer

The enzyme mixture was drop-cast on working electrodes (viii) and (ix) to form an enzyme reaction layer. Reaction mixtures contained 0.015 units of lysine oxidase, 4 ng of CMC, 10 ng of microgranular cellulose, and 30 μg of potassium ferricyanide as an electron mediator. Reaction mixtures except for lysine oxidase were cast on working electrodes (vi) and (vii). The above concentrations were adjusted to complete the enzymatic reaction within 120 s. Sodium phosphate buffer (10 mM, pH 7.5) was used as the solvent for each mixture. The reaction used for lysine determination is described as follows:

$$\text{L-Lysine} + \text{Ferricyanide ion} \xrightarrow{\text{Lysine oxidase}} \text{2-Keto-6-aminocaproic acid} + \text{Ferrocyanide ion} \quad (1)$$

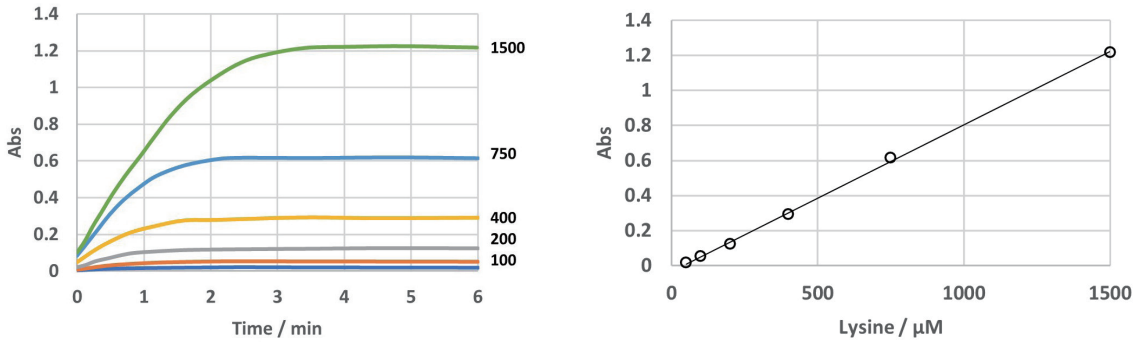
2.6 Clinical use

To confirm the practicality of the proposed sensor in a clinical site, we performed measurements on subjects in a hospital. The subjects were patients with pediatric surgical disease who were treated at the University of Tsukuba Hospital from January 2015 to March 2017. In addition to measuring blood lysine levels using the proposed sensor, the transthyretin level was measured by a blood test as a conventional nutrition indicator. We compared the correlation between the blood lysine level and the transthyretin level. Spearman's rank correlation coefficient was used to evaluate the correlation between the two groups, and the Mann–Whitney U test was used to evaluate the difference between the two groups. A p value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1 Enzyme reaction

Figures 2(a) and 2(b) show the reactivity of lysine oxidase to lysine in the reagent. Absorbance was proportional to the concentration of lysine in the solution, and the reaction reached equilibrium within 4 min [Fig. 2(a)]. The absorbance 6 min after the start of the reaction is



(a) (b)

Fig. 2. (Color online) (a) Time courses of absorbance of 1500, 750, 400, 200, 100, and 50 µM lysine. (b) Absorbance of 1500, 750, 400, 200, 100, and 50 µM lysine at 6 min.

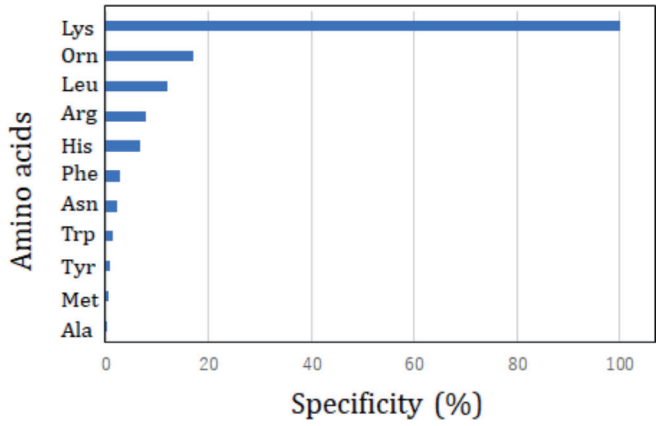


Fig. 3. (Color online) Reaction rate of each amino acid against reaction rate of lysine.

plotted in Fig. 2(b). The lysine concentration and absorbance in the sample show a linear correlation. Figure 3 shows the substrate specificity of lysine oxidase for amino acids. The concentration of each amino acid except for tyrosine was 1 mM, and the concentration of tyrosine was 0.01 mM.

Table 1 shows the relative activity of lysine oxidase for each amino acid, the physiological variation of each amino acid, and the effect on the error obtained by multiplying them. Since the concentrations and the physiological variation of the relatively reactive amino acids in blood were low, lysine oxidase was recognized as usable for the selective measurement of the lysine concentration in blood.

Next, the electron transfer between the enzyme and ferricyanide ions was measured using the electrode. Figure 4 shows cyclic voltammograms obtained with and without lysine oxidase to assess the electron transfer of ferricyanide ions and lysine oxidase. An HA-501 potentio-galvanostat (Hokuto Denko Corporation, Japan) and an HB-104 function generator (Hokuto Denko Corporation, Japan) were used for the measurement. A platinum plate electrode was used as the working electrode and a platinum wire was used as the counter electrode. The scan range

Table 1
Relative activity of lysine oxidase for each amino acid, physiological variation of each amino acid, and effect on the error.

Amino acids	(1) Relative activity (% vs Lys)	(2) Physiological variation (±%)	(3) = (1) × (2) Effect on error (±% vs Lys)	Effect
Lys	100	—	—	—
Orn	17	7.2	1.2	Ignorable
Leu	12	10	1.2	Ignorable
Arg	7.6	9.5	0.72	Ignorable
His	6.7	8.9	0.60	Ignorable
Phe	2.6	3.3	0.086	Ignorable
Asn	2.0	4.7	0.094	Ignorable
Trp	1.2	6.9	0.083	Ignorable
Tyr	0.84	5.9	0.050	Ignorable
Met	0.42	2.7	0.011	Ignorable
Ala	0.15	45	0.68	Ignorable

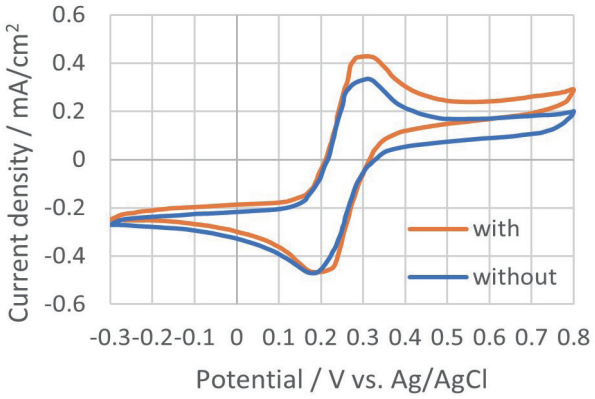


Fig. 4. (Color online) Cyclic voltammograms obtained with and without lysine oxidase to assess electron transfer of ferricyanide ions and lysine oxidase.

was −300 to 800 mV and the scan speed was 50 mV/s. The lysine concentration was 5 µmol/dL, the ferricyanide ion concentration was 5 mM, the lysine oxidase concentration was 1 U/mL, and 50 mM phosphate buffered saline (pH 7.5) was used as the solution. AgCl wire prepared by electrochemically chlorinating the surface of a Ag wire was dipped in KCl saturated solution to form a reference electrode. A salt bridge filled with agarose gel and KCl in a glass tube was used for electrical connection between the sample solution and the KCl saturated solution. The voltammograms indicate the electron transfer of ferricyanide ions and lysine oxidases.

3.2 Amperometric determination and evaluation of lysine biosensor

We developed prototype equipment consisting of an amperometric measurement system (Fig. 5). The prototype sensor, consisting of an amperometry measurement system, could perform evaluations with a 5 µL sample, and the total measurement time was ca. 2 min from the injection of the sample to the display of the measurement results.

Fig. 5. (Color online) Prototype equipment consisting of an amperometric measurement system.

Lysine and ferricyanide ions were respectively converted to 2-Keto-6-aminocaproic acid and ferrocyanide ions by lysine oxidase. The oxidative current of the ferrocyanide ions was measured on the surface of the gold electrode. The concentrations of lysine were calculated using calibration curves derived from currents using the equipment's data processor.

3.3 Correlation with standard method

Figure 6 shows the correlation between the lysine concentration in the model blood and the electric current 120 s after sample injection. The lysine concentration ranged from 125 to 1000 μM. To prepare the model blood, 150 mM NaCl and 7% bovine serum albumin (BSA) were dissolved in 100 mM phosphate buffer (pH 7.4) and lysine was added.

The current was 57 nA for a lysine concentration of 125 μM and 128 nA for a lysine concentration of 1000 μM, and the current increased linearly between these concentrations. The y-intercept in Fig. 6 does not cross the origin because of the impurities contained in lysine oxidase, and the purification of lysine oxidase is the next task.

The samples used to obtain this data were model samples; thus, there was little influence of interfering substances. However, when this sensor is applied to blood containing various concentrations of interfering substances, the effect of the sub-working electrodes is expected to be significant.

3.4 Stability of sensor

The storage stability of the sensor chip was measured using a commercialized machine equipped with the performance of the demo machine. The long-term storage stability of the sensor was investigated by assessing its response to lysine using a sensor chip stored at room temperature and sealed with a desiccant. As shown in Fig. 7, the sensor chip showed good

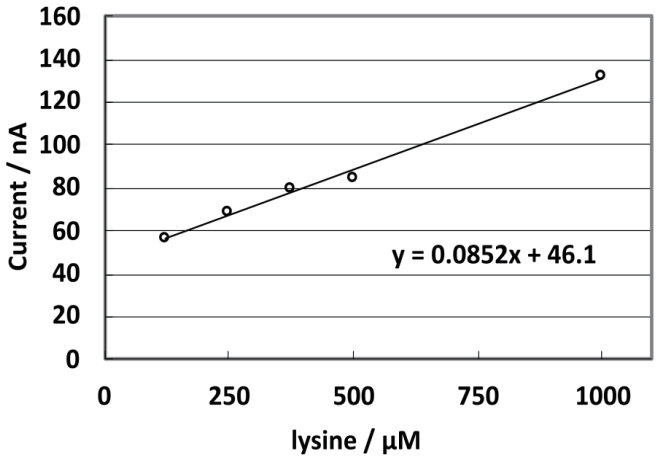


Fig. 6. Correlation between the lysine concentration in the model blood and the measurement results obtained by the sensor (5 samples).

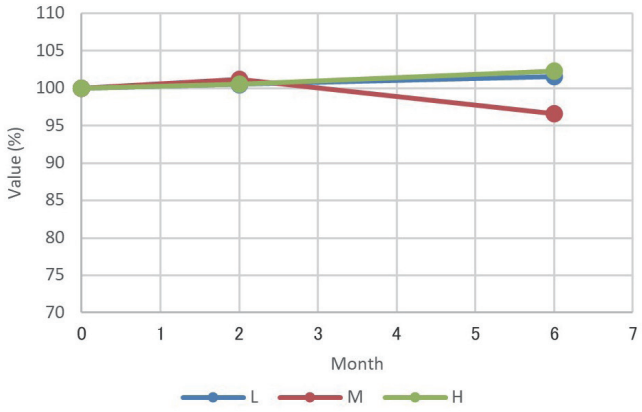


Fig. 7. (Color online) Storage stability of sensor chip measured using a commercialized unit equipped with the performance of the demo machine.

stability and detection efficacy at low (200 mg/dL), medium (300 mg/dL), and high (500 mg/dL) target concentrations, and there was no noticeable change in the response after storage for more than 6 months. The variation of the values compared with the start of the storage test ranged from −3.5% to +2.3%.

3.5 Clinical use

We used the proposed sensor to measure blood lysine levels in pediatric surgical patients and tested its usefulness in nutritional assessment (Fig. 8). Blood lysine levels were measured in 62 pediatric surgical patients, including those with short bowel syndrome and Hirschsprung's disease. The mean blood lysine level was 145.7 µM, with 30 patients presenting a blood lysine

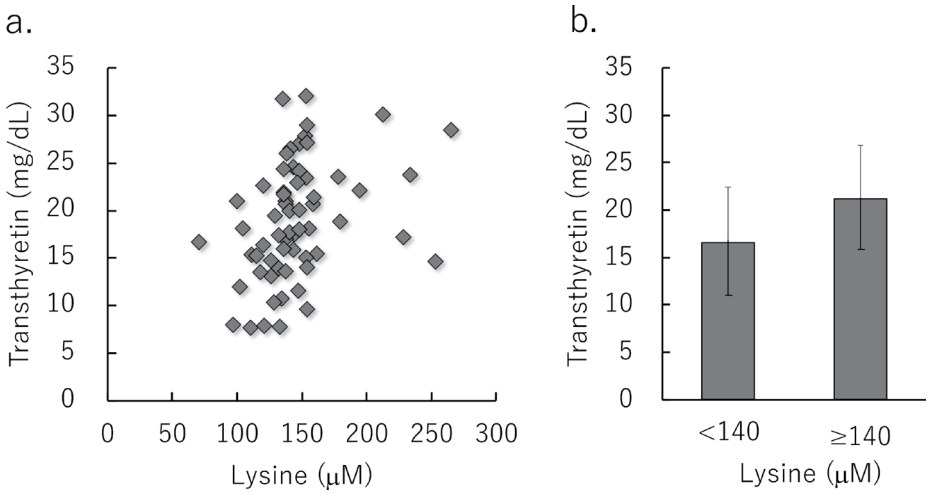


Fig. 8. (Color online) (a) Correlation of blood lysine and transthyretin levels in pediatric surgical patients. Spearman's rank correlation coefficient 0.438, $^{*}p = 0.0006$. (b) Comparison of transthyretin levels with a lysine level of 140 μM as the cut-off. $^{*}p = 0.002$. $^{*}p < 0.05$.

level lower than the cut-off level of 140 μM, the concentration that indicates hypolysinemia. The lysine level was correlated with the transthyretin level, which is a conventional nutrition indicator (Spearman's rank correlation coefficient of 0.438, $p = 0.0006$). Thus, the lysine level is considered to be useful for nutritional assessment.

4. Conclusions

In this study, we have developed a system that can determine the blood lysine concentration in 2 min. The substrate specificity of the enzyme and its adaptability to the electrochemical sensor were confirmed. A linear relationship was observed between the lysine concentration in model blood and the electric current, and the stability of the sensor was observed over 6 months of storage. Finally, we reported the application of the lysine sensor to obtaining clinical evaluation data, showing its usefulness as a nutritional assessment tool. The sensor is fully usable at the clinical level and can also be used for monitoring the blood of patients with the rare diseases hyperlysinemia and hypercitrullinemia.

In the future, we would like to develop new devices based on this platform for the clinical testing market, especially for the POCT market. Furthermore, by integrating the proposed system with IT, we can use the advantages of electrochemical sensors for the health management of individuals, for telemedicine, and for home care for elderly patients.

Contributions

Tohoku University, AIST, and TechnoMedica designed the sensor and improved its measurement performance (no human blood was used). The clinical evaluation was conducted at the University of Tsukuba Hospital.

Use of Human-derived Samples

Approval for clinical use was obtained from the Institutional Review Board of University of Tsukuba Hospital (R03-318). Informed consent was obtained in the form of an opt out on the website (https://www.md.tsukuba.ac.jp/clinical-med/ped-surg/).

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