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PAPER

Fluid-permeable enzymatic lactate sensors for micro-volume specimen

Hiroyuki Kai*, Yuto Kato, Ryoma Toyosato, and Matsuhiko Nishizawa*

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Sensing of lactate in perspiration provides a way to monitor health and control exercise. The volume of perspiration is miniscule, and the efficient collection of perspiration is desired for its effective sensing. We developed mesh-type enzymatic electrodes fabricated on textile meshes and integrated the meshes into an enzymatic biofuel cell. We tested them as self-powered lactate sensors for a small volume of lactate solution. A fluid-permeable enzymatic anode was fabricated based on an insulating textile mesh that was coated with carbon nanotubes (CNTs) and lactate oxidase. The anode was further coated with polyurethane to increase the linear range by limiting the diffusion of lactate while maintaining the advantages of the original textile mesh, such as flexibility, stretchability, and permeability. Permeability of the mesh-type lactate-oxidizing anode allowed a vertically stacked structure of the anode and a previously developed air-breathing cathode. This resulted in a small overall device size (1 cm2). The mesh-type sensor was tested using a small flow rate of lactate solution, and a moderate linearity of amperometric response for a wide concentration range (5 to ≥20 mM) was confirmed. The fluid-permeable anode and enzymatic biofuel cell show the potential of the sensor for continuous monitoring of lactate in perspiration on skin.

Introduction

Collecting vital information from body fluids such as blood, perspiration, saliva, tears, and the interstitial fluid of the skin has been an effective method for monitoring health because these body fluids have rich biochemical information on vital conditions. The method for collecting perspiration is less invasive than the methods used to collect all other body fluids. It has chemical species that reflect conditions of the body[1] such as ethanol (an indicator of intoxication),[2] ammonia (exercise intensity),[3] and potassium (muscle activity and hypo- and hyperkalemia).[4,5] Lactate in perspiration has been suggested as an early indicator of pressure ischemia[6] and has clinical importance. It has also been suggested that the lactate concentration in perspiration is correlated with exercise intensity,[7,8,9,10] although this idea is controversial.[11] Quantification of lactate in solution has been carried out using enzymatic electrodes with lactate oxidase (LOx) and lactate dehydrogenase, taking advantage of the enzyme kinetics that depend on substrate concentration.[12] A self-powered amperometric sensor that is made of a LOx anode and a bilirubin oxidase (BOx) cathode has also been developed, which eliminates the external power source and is potentially useful for wearable applications.[13] Joseph Wang's group has pioneered an epidermal lactate sensor and a wearable enzymatic biofuel cell (EBFC) that is powered by lactate in perspiration.[14,15,16,17]

Every enzymatic electrode has a specific concentration profile, the relationship between substrate concentration and the generated electric current. The whole electrode reaction can be limited either by redox reactions of the enzyme that follows the Michaelis-Menten kinetics or substrate diffusion to the electrode surface; the combination of these factors determines the concentration profile of the electrode. The LOx has a native Michaelis constant of 1.0 mM, which causes 90% saturation of the electric current at 9.0 mM. This is too low and therefore unsuitable for sensing lactate in perspiration, which may reach a concentration of several tens of millimolars.[11] When the whole process is redox limited, the profile follows the Michaelis-Menten equation and shows a saturating, nonlinear curve. For LOx, Therefore, the increase in the linear range from the native LOx is crucial. By limiting substrate diffusion to the electrode surface, the concentration profile is modulated and shows linearity for a wider concentration range. Coatings such as polyurethane and Nafion® (acidic perfluorocarbon polymer) have been used to coat the electrode surface and modify the concentration profile to achieve a wider linear range than the original LOx electrode.[14,18,19] Among the available coatings, we adopted polyurethane for the LOx anode to achieve a wide linear range because polyurethane is stretchable and not harmful to the living body, which makes it suitable for wearable sensors.

In addition to the wide linear range, sampling of a small volume of perspiration is also important for successful lactate sensing on the skin. The rate of perspiration is only 1.3 μL min^{-1} cm^{-2} even for high perspiration conditions such as running,[20] so efficient interfacing of the device with perspiration on the skin is necessary. There have been efforts to collect and analyze perspiration on the skin in an efficient manner.[21] Rogers and

Department of Finemechanics, Tohoku University, 6-6-1 Aramaki Aoba, Aoba-ku, Sendai 980-8579, Japan. E-mail: kai@tohoku.ac.jp, nishizawa@biomems.mech.tohoku.ac.jp

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colleagues developed skin-mounted microfluidic devices to collect perspiration[22], and demonstrated the chronological sampling of perspiration.[23] The Heikenfeld group applied mineral oil on the skin to suspend and collect aqueous droplets of perspiration, which avoided contamination from the skin surface.[24] We recently developed an open-channel microfluidic film that efficiently collects aqueous droplets from a large area of the film, which is potentially useful for collecting perspiration on the skin.[25] All these efforts have been focused on developing an external device to manipulate liquid an droplets that can be integrated with a sensor such as an enzymatic electrode.

A self-contained EBFC-type lactate sensor can be effective for measuring perspiration on the skin if it satisfies a few requirements: a good fit to the body contours, a sufficient concentration range for lactate sensing, and the ability to measure lactate in a small volume of perspiration. To achieve these requirements, we here report our fabrication of a flexible mesh textile-based LOx anode that permits the permeation of perspiration through the electrode. Combining the LOx anode with a bilirubin oxidase (BOx)-based air-breathing cathode fabricated on CNT-coated fabric,[26] we examined its potential capability as a self-powered amperometric sensor for lactate in perspiration. The mesh structure of the fabric-based substrate of the LOx anode allowed for the permeation of perspiration through the anode face, and therefore the vertically stacked structure of the anode and cathode was possible (Fig. 1). The anode is placed in the middle of the directional flow of perspiration from skin to a drain at the periphery of the electrodes (Fig. 1b). This vertical arrangement has the advantageous such as the smaller total area of the device and the smaller electrical resistance between the anode and cathode compared with a side-by-side arrangement.

Experimental procedures

Materials

Stretchable fabric was cut from the a heel of pantyhose (SP723) purchased from Gunze Limited (Osaka, Japan). Carbon fiber fabric (TCC-3250) was donated from Toray Industries, Inc. CNTs (Baytubes C 70 P) were purchased from Bayer and treated with mixed acid prior to use. Bilirubin oxidase (BOx; Bilirubin Oxidase "Amano" 3, EC1.3.3.5) was purchased from Amano Enzyme. Lactate oxidase (LOx; diagnostic reagent grade, EC1.1.3.2) was purchased from Toyobo Enzyme. Tetrathiafulvalene (TTF) was purchased from Sigma-Aldrich. Polyurethane (Gumthane AR650) was a kind gift from Okada Engineering. All other chemicals were purchased from Wako Pure Chemicals and Sigma-Aldrich and used as received. Track-etched polycarbonate membranes (1000M25/851N201/47, it4ip) were a kind gift from AR BROWN, Co., Ltd.

Fabrication of a LOx anode

Aqueous CNT suspension was prepared by sonicating acid-treated CNTs (40 mg) in 1 wt% Triton-X solution in water (4 mL) using a homogenizer (Sonifier Analog Series 250, Branson) in an ice bath for 10 min with 30% amplitude and 50% duty cycle. Next, 50 µL of the resulting CNT suspension was painted on a 1 cm x 1 cm piece of stretchable fabric, and dried in an oven at 70 °C for 15 min. Each side of the fabric were painted with two coats of the CNTs. The painted fabric was washed in distilled water at room temperature and 50 kPa for 1 h to remove the Triton-X, followed by drying at 70 °C. After drying, 50 µL of tetrathiafulvalene (TTF) solution in methanol (10, 50, or 100 mM) was added to the CNT-coated fabric in a dropwise manner, followed by additional drying at room temperature for 20 min. Then, 50 µL of a solution of LOx (40 mg/mL) and BSA (10 mg/mL) in 50 mM phosphate buffer (pH 7) was added in a dropwise manner and dried in an oven at 35 °C to immobilize the LOx on the CNTs by physical adsorption. The resulting enzymatic anode was briefly immersed in polyurethane solution (1, 2, 5, or 10 wt%) in tetrahydrofuran (THF), and the excess polyurethane solution was wiped off, followed by drying at room temperature. The coating with polyurethane was repeated 1, 2, 5, or 10 times.

Fabrication of a BOx cathode

A BOx cathode was fabricated according to our previous report.[26] Aqueous CNT suspension was prepared in the same way as that for the anode. Next, 50 µL of the resulting CNT suspension was painted on a 1 cm x 1 cm piece of carbon fiber fabric, and dried in an oven at 70 °C for 15 min. Four coats of

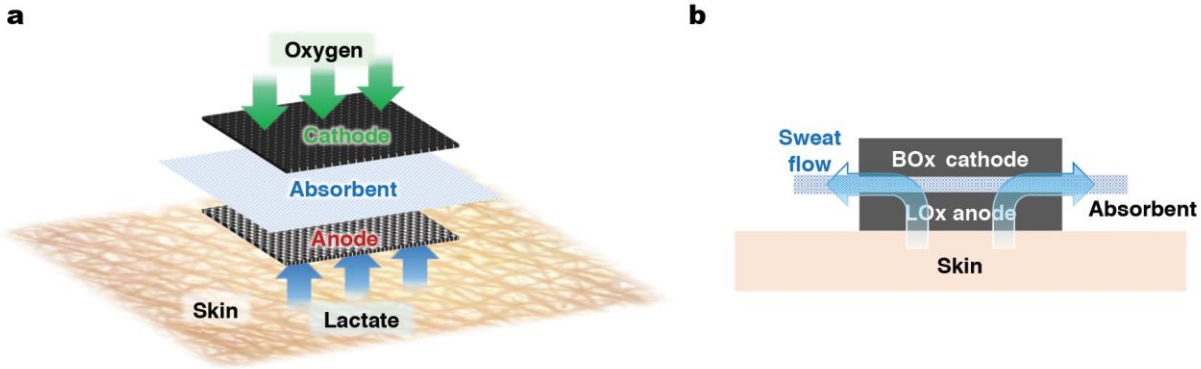

Fig. 1 Schematic structure of the mesh-type enzymatic biofuel cell for lactate sensing in perspiration. (a) Perspective view. (b) Cross sectional view.

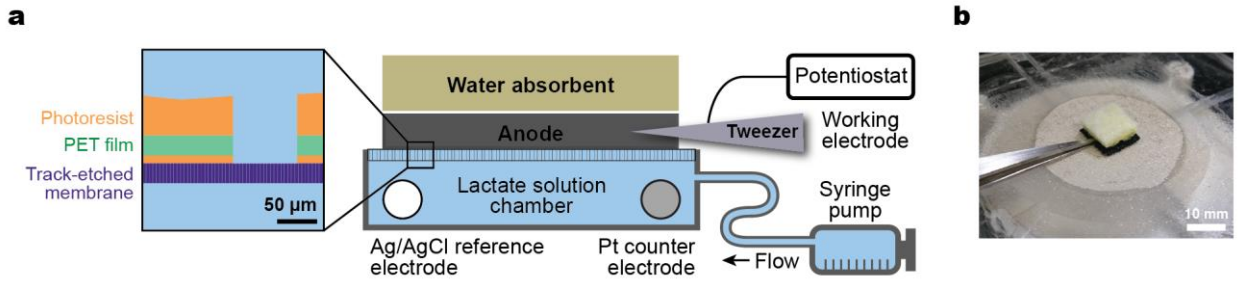

Fig. 2 (a) Schematic diagram of the perspiration device and the experimental setup for measuring the anode with a continuous supply of lactate solution. (b) Photograph of the LOx anode placed on the surface of the perspiration device.

the CNTs were painted onto one side. This fabric was washed in isopropanol at room temperature and 1 atm for 1 h to remove the Triton-X, followed by drying at 70 °C. Next, the CNT-coated carbon fiber fabric prepared above was set in a polydimethylsiloxane mold, and 500 µL of BOx (Bilirubin Oxidase "Amano" 3, Amano Enzyme) solution in 1x McIlvaine buffer solution (10 mg/mL, pH 5) was poured over it. The solution was dried at 35 °C and 50 kPa overnight. Another CNT suspension was prepared by sonicating acid-treated CNTs (16 mg) in ethanol (4 mL) using the homogenizer in an ice bath for 10 min. The fabric was further coated with 100 µL CNT solution in ethanol twice, followed by drying at 35 °C.

Electrochemical measurements

Cyclic voltammetry was carried out in aqueous solution with an Ag/AgCl reference electrode and a platinum counter electrode using a potentiostat (Model DY2323, ALS), where a potential sweep was done between 0 V and 0.6 V with a scan rate of 10 mV/s. Amperometric measurement was carried out at 0.2 V in aqueous solution with an Ag/AgCl reference electrode and a Pt counter electrode using the potentiostat. The potential was reported against Ag/AgCl for all measurements. The measurements were carried out in pH 7.0 100 mM phosphate buffer solution with various concentrations of lactate. The Michaelis constant was calculated by a Lineweaver–Burk plot. The linear range in a concentration profile was defined as the concentration range where linear regression on all the data points gives $R^2 > 0.99$.

Fabrication of an artificial perspiration device

For testing the LOx with a limited solution volume, a microfluidic skin mimic device was fabricated according to a previous report[27] with a modification to integrate reference and counter electrodes into the solution chamber to enable three-electrode electrochemical measurement (Fig. 2). A PET film of 25 µm thickness was coated with SU-8 photoresist and was milled with a laser cutter (VLS 3.50, Universal Laser Systems) operated at 0.7% power and 0.8% speed to make cylindrical holes with an average diameter of 80 µm at a density of 2 pores/mm^2. This film was bonded with a track-etched membrane made of polycarbonate with a thickness of 25 µm, a pore size of 0.2 µm, and a pore density of 5×10^8 pores/cm^2 by UV irradiation to cure the photoresist. Acrylic plates were machined by the laser cutter, and the Ag/AgCl reference and Pt counter electrodes were integrated into the solution chamber.

Measurement of fluid permeability of the LOx anode

The polyurethane-coated LOx anode was placed on top of the perspiration device fabricated above, and a piece of water absorbent material was placed on top to drain permeated solution through the LOx anode. Water was continuously supplied from the perspiration device with a flow rate of 1 µL cm^{-2} min^{-1}, controlled by a syringe pump, and the weight was measured at various times after placing the LOx anode and the piece of water absorbent material.

Evaluation of the sensing capability on the artificial perspiration device

The LOx anodes that showed high electric current (>30 µA/cm^2 by amperometry for 30 mM lactate) were selected from a batch of the fabricated LOx anodes before the lactate sensing experiment. For three-electrode amperometric measurement, the LOx anode was placed on top of the perspiration device and connected to a working electrode of the potentiostat via metal tweezers. A piece of water absorbent material was placed on the LOx anode. Phosphate buffer solution (pH 7.0, 100 mM) with 100 mM KCl and various concentrations of lactate was continuously supplied from the perspiration device with a flow rate of 1 µL cm^{-2} min^{-1}, controlled by a syringe pump, and chronoamperometric measurement at 0.2 V was carried out. For measuring a self-powered sensor, a piece of water absorbent material was sandwiched between the LOx anode and the BOx cathode, and placed on the perspiration device with a flow rate of 1 µL cm^{-2} min^{-1}, controlled by a syringe pump. The electric current through the resistance was calculated from the potential drop at a fixed external resistance of 20 kΩ.

Results and discussion

Preparation of a mesh-type LOx anode

The enzymatic anode with LOx was prepared by physical adsorption of TTF as a mediator followed by LOx on a CNT-modified mesh textile, according to a similar method to our previous report.[28] Characteristics of the pristine LOx anode fabricated above, without any coating, were first investigated. Cyclic voltammetry of the anode showed an oxidative current above 0.1 V (against Ag/AgCl), which agrees with the redox potential of lactate oxidation by LOx (Fig. 3a). Amperometric responses of the anode were measured for various concentrations of lactate in pH 7.0 phosphate buffer solution by chronoamperometry at 0.2 V with a three-electrode system (Fig. 3b). We selected the buffer solution according to the previous study[14]; pH of human perspiration ranges from 4.5 to 7.0. The electrolyte concentration in human perspiration may vary by one order of magnitude[1,29] and it is difficult to define a single composition that represents them. Therefore, we adopted the simplest formulation that simulates only pH and lactate concentration in human perspiration. The concentration profile, the relationship between lactate concentration and electric current, fitted well with a Michaelis-Menten kinetic equation with the maximum current density of 586 ± 4 (standard error of mean) μA cm^{-2} and the Michaelis constant of 0.974 ± 0.019 mM. The Michaelis constant was similar to those of LOx in solution and LOx enzymatic electrodes previously reported.[13] This indicated that the fabricated pristine LOx anode had LOx in a native state and the whole electrode process was limited by the enzymatic reaction.

Preparation of a mesh-type LOx anode with an adjustable linear range

The concentration profile of the LOx anode was modulated by polyurethane coating to increase the linear range. The surface morphologies of the electrodes before and after polyurethane coating were observed by scanning electron microscopy (SEM)

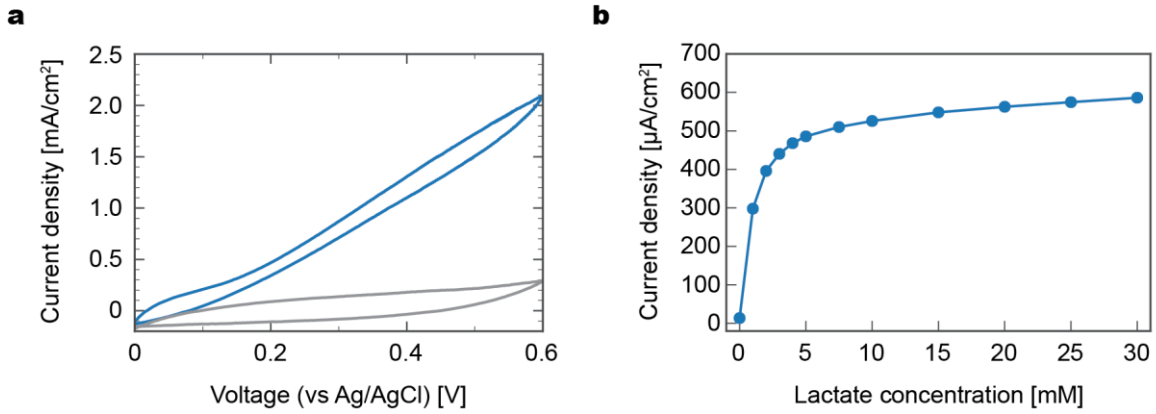

Fig. 3 Characteristics of a pristine lactate anode without polyurethane coating. (a) Cyclic voltammetry of an anode with 20 mM lactate (blue) and without lactate (gray) in 100 mM phosphate buffer solution (pH 7.0). (b) Current versus lactate concentration in 100 mM phosphate buffer solution (pH 7.0).

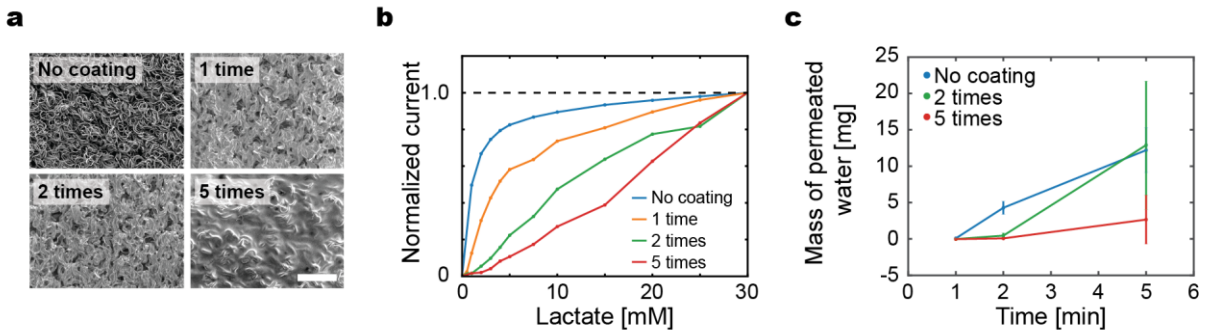

Fig. 4 Effects of polyurethane coating at various times. (a) Surface morphology observed by scanning electron microscopy (SEM). Scale bar: 1 mm. (b) Normalized current density of the lactate anode versus the lactate concentration. No coating (blue), one coating (orange), two coatings (green), and five coatings (red). Current density is normalized between the values at 0 mM and 30 mM. (c) Water permeation measured by weight increase of water absorbent material placed on the polyurethane-coated electrodes. No coating (blue), two coatings (green), and five coatings (red).

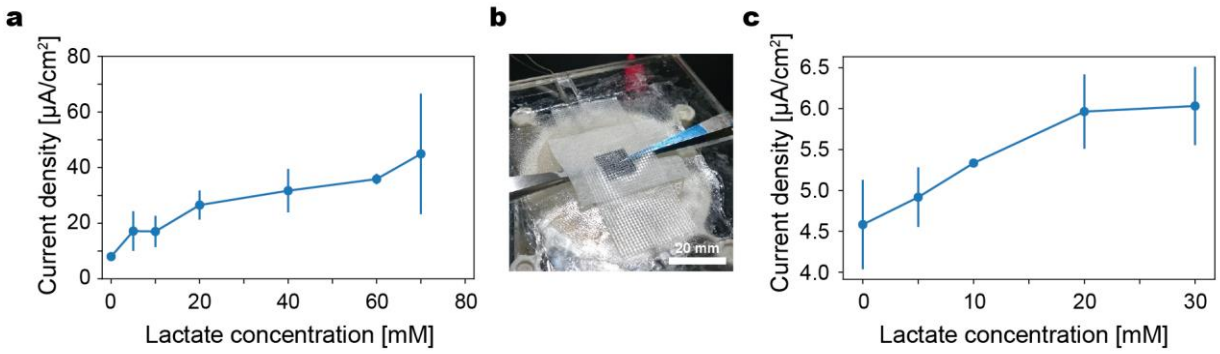

Fig. 5 (a) Amperometric response of the LOx anode to solution with various concentrations of lactate tested on the perspiration device. (b) Photograph of the self-powered EBFC sensor assembled on an artificial perspiration device. Error bars are standard deviation of 2 or 3 LOx anode samples. (c) Generated electric current of the self-powered EBFC sensor for solution with various concentrations of lactate tested on the perspiration device. Error bars are standard deviation of 2 EBFC samples.

at 20x magnification (Fig. 4a). The surface of the CNT-coated fabric had the highly porous structures with hierarchical meshes that existed in the original stretchable fabric. When the coating with 10 wt% polyurethane solution was applied once or twice, the surface of the treated fabric maintained the porous structures. However, after coating with 10 wt% polyurethane solution five times, there was apparent clogging of the mesh. Next, the concentration profiles of the LOx anodes with various times of coating were measured (Fig. 4b). As the number of polyurethane coatings increased, the linear range increased, which reached 0-70 mM at five coats (Table S1). Polyurethane has limited permeability to lactate, thus the diffusion rate of lactate into the electrode surface is reduced without completely blocking the diffusion. The limited diffusion causes the change in the concentration profile. A thicker polyurethane coating caused by repeated dipping led to an increase in the linear range but a decrease in the sensitivity (the slope of the concentration profile). The appropriate balance between these trade-offs can be chosen for specific applications. The investigations above as a whole indicated that the LOx anode with two coats of 10 wt% polyurethane combines sufficient solution permeability and a wide linear range. It should also be noted that in this condition, physically adsorbed TTF and LOx as well as CNTs were well contained in the polyurethane coating and no leakage of these was observed by eye, which makes the LOx anode safe to a human body.

We also compared the amperometric responses of the LOx anode in a phosphate buffer solution of pH 7.0 and pH 5.5 (Fig. S1a, b). The concentration profile in pH 5.5 showed inferior linearity to that in pH 7.0, probably because the LOx activity was different. Some calibration mechanism for different pH may be helpful for more precise measurement in actual perspiration with varying pH. When the LOx was denatured by heating, the slope in the concentration profile completely disappeared (Fig. S1c), which indicated that the amperometric sensing of lactate was actually done by enzymatic oxidation by LOx. When common organic molecules in the perspiration such as glucose (30 mg/mL) and urea (6 mM) were added to the solution, the change in the amperometric response was negligible, which suggested the robustness of the LOx anode in the presence of these chemicals (Fig. S2).

Permeability of the LOx anode

Solution permeability is required for the LOx anode to realize the vertically stacked structures of the anode and cathode. Solution permeability of the original stretchable fabric needs to be maintained after coating with CNTs and polyurethane. To examine the solution permeability, the amount of aqueous solution passing through the electrode into the water absorbent material placed on top was quantified in the presence of continuous supply of the solution (1 μL cm^{-2} min^{-1}) from the bottom using the perspiration device (Fig. 4c). The perspiration device has the LOx anode, the reference electrode, and the counter electrode, which are ionically connected through solution in the chamber and the micropores in the membrane. This setup enables three-electrode electrochemical measurement on an electrode placed on the device surface. The rate of the syringe pump that supplies lactate solution to the chamber was adjusted to 1 μL cm^{-2} min^{-1}, which is comparable to high perspiration rate on the human skin.

When two coats of 10 wt% polyurethane solution was applied, the electrode still showed solution permeability. Further coating with 10 wt% polyurethane (five times) caused clogging of the mesh structure and blocking of solution permeation. These results agreed with the macroscopically porous structures observed by SEM as described above. It should be noted that the actual flow rate of solution from the pores of the perspiration device was higher (2 μL min^{-1} cm^{-2}) than the rate of solution supply by the syringe pump. This is because the uppermost membrane is flexible and the suction force of the water absorbent material causes active drawing of solution from the chamber. This led to an outgoing solution flow that was faster than the incoming flow that accompanied air intake into the chamber from the surrounding pores. This was indicated by the appearance of air bubbles in the chamber during the water

absorption testing. Nonetheless, relative permeability for different samples can still be evaluated with this method.

Amperometric response of the lactate sensor on the perspiration device

Testing a lactate sensor for perspiration using a living body presents difficulties in controlling the perspiration rate and lactate concentration; therefore, it is preferable to use an artificial platform that simulates perspiration. Actual lactate sensing on the living body should be carried out using the tiny volume of perspiration that is continuously secreted from sweat glands on the skin surface, and an experimental setting that simulates such a condition in a controlled manner is desirable for testing a wearable perspiration sensor. For that purpose, an artificial perspiration device with microfluidic sweat glands that mimics the slow continuous flow of perspiration on skin[27] was used for measuring the response of the lactate sensor at various lactate concentrations in a controlled manner.

The amperometric sensing of lactate by the LOx anode and the self-powered EBFC at various lactate concentrations was examined on the artificial perspiration device. The amperometric sensor using the LOx anode was set up on the perspiration device, and lactate solution was continuously supplied to the sensor with a syringe pump at a rate of 1 μL min^{-1} cm^{-2}. Then, the amperometric response of the LOx anode at 0.2 V (against Ag/AgCl) was measured by a three-electrode system integrated in the perspiration device (Fig. 5a). The LOx showed a monotonically increasing amperometric response to the lactate concentration in the solution between 5 and 70 mM supplied from the perspiration device. This result indicated that the developed LOx anode generated an electric current even with a small volume of lactate solution absorbed in the sensor and worked as an amperometric sensor.

Finally, the self-powered lactate sensor was assembled using the LOx anode, a carbon fabric-based BOx cathode that had been previously developed,[26] and a piece of water absorbent material between them, which, combined, formed an EBFC (Fig. 5b). Prior to the measurement on the artificial perspiration device, the polarization curves of the EBFC were measured on hydrogels with various concentrations of lactate (Fig. S3a). The slope of the polarization curve increased with the increase of the lactate concentration, which suggested that the EBFC is limited by the LOx anode. The external resistance of 20 kΩ was chosen for the perspiration device, because it showed best linearity of the electric current against various concentrations of lactate (Fig. S3b). When the lactate concentration from the perspiration device was varied, the electric current generated by the EBFC showed linearity to the lactate concentration between 0 and 20 mM (Fig. 5c). The minimum sample volume required for measurement can be estimated to be ~25 μL (when electrodes of 5 mm x 5 mm are used).

Conclusions

We have demonstrated lactate sensing in a small volume of solution by a LOx anode and self-powered EBFC-type lactate sensor, taking advantage of a fluid-permeable enzymatic electrode using a stretchable textile mesh with a stretchable polyurethane coating to adjust the sensing range of electrochemical reactions by LOx. The amperometric response of the lactate sensor showed linearity against the lactate concentration on the artificial perspiration device. The device had a skin-mimic artificial membrane with microfluidic pores, which provided a similar environment to actual perspiration on skin. The lactate sensor worked with a small volume of lactate solution and has potential for quantifying lactate in perspiration on human skin.

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