Doctoral Dissertation

Electrochemical lactate sensor using lactate oxidase from *Aerococcus viridans* and phenazine derivatives as the mediator

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Chapter 1. Introduction

1-1. General introduction

Biosensors are analytical devices used for the detection of target molecules or analytes by sensing biomolecules and recognizing the target through binding or reaction and converting the recognition signal (such as electrons, light, potential, heat, and mass) into an electrical signal using a transducer (Figure 1-1). Biosensors consist of biological component(s) that act as sensing molecules or recognition elements (such as enzymes, binding proteins, aptamers, and DNA) and transducers (amplifiers with electrodes) that convert the recognition signals into electrical signals. There are various types of biosensors. Enzyme sensors are popularly studied and are often used for clinical diagnosis. The enzyme sensor measures the target analytes using an electrochemical approach. Amperometric enzyme sensors are commonly used owing to their simplicity and ability to achieve high accuracy and sensitivity.

There are two well-known amperometric enzyme sensors. First, a disposable biosensor strip that was used to measure the levels of analytes. Moreover, it can be self-monitored by people without medical experience anytime and anywhere. For point-of-care testing (POCT), a disposable sensor is also used by medical technicians to measure the levels of analytes in patients on-site. Second, medical technicians and trainers often use continuous monitoring to monitor fluctuations in analyte levels in patients and athletes, respectively. It can be applied in flow injection analysis (FIA), wearable, and implantable biosensors.

In amperometric enzyme sensors, an electrode is employed as a solid support for the deposition and immobilization of biomolecules and for electron movement (that is generated by the reaction). The mechanism of the amperometric enzyme sensor utilizes

the redox principle, where the substrate or analyte is oxidized into a product and the enzyme cofactor is reduced during the reductive half-reaction, generating electrons. Then, in the subsequent oxidative half-reaction, the cofactor is reoxidized, and the electrons are transferred to the electrode (Karube *et al*., 1980; Matsunaga *et al*., 1982; Clark *et al*., 1984). Based on the electron transfer process, the amperometric enzyme sensor can be divided into three generations (Figure 1-2). In the 1st generation, molecular oxygen (O_2) is used as an electron acceptor in the oxidase reaction. When the reduced cofactor is reoxidized, O_2 is reduced, generating hydrogen peroxide (H_2O_2) (Ferri *et al*., 2011). Here, the formation of H_2O_2 is dependent on O_2, Thus, high O_2 fluctuations should be avoided (Hiraka *et al*., 2018). Furthermore, H_2O_2 is oxidized at the surface of the electrode with an appropriate potential, and the generated electrons are transferred to the electrode and can be measured electrochemically as a current signal or response. Thus, the obtained response currents were generated from the number of reduced O_2 molecules and represented as the amount of substrate present in the sample. The enzymatic reaction involving O_2 and the production of H_2O_2 commonly occurs in oxidases. However, the oxidation of H_2O_2 requires a high potential ($\geq$ +0.6 V vs Ag/AgCl; Clark *et al*.,1984; Rocchita *et al*., 2016; Monteiro and Almeida, 2018), which can cause the oxidation of other redox substances in blood and lead to an erroneous increase in the response current, and thus can cause serious problems (Monteiro and Almeida, 2018). The biosensors based on the 1st generation can be applied in disposable and continuous monitoring types.

The 2nd generation utilizes an artificial electron acceptor (also known as an electron mediator) to mediate electron transfer from the reduced cofactor to the electrode. This type of sensor is ideal for dehydrogenases because, it does not utilize O_2 as an electron acceptor. The utilization of a mediator allows a lower potential than that

necessary to oxidize H_2O_2 in the 1st generation sensors, which leads to fewer errors due to redox interference. Therefore, mediators are preferentially used, especially in disposable strip-type enzyme sensors. Here, the obtained response currents are generated from the number of reduced mediator molecules that are reoxidized at the electrode and represents the amount of substrate present in the sample. Currently, the widely studied and commercially available biosensors based on 2nd generation are disposable types, including for self-monitoring and POCT. The biosensors based on 2nd generation are not suitable for continuous monitoring systems because the mediators that are commonly deposited or immobilized together with enzymes often leak. Moreover, the effect can be dangerous especially in wearable and implantable biosensors because the mediator leakage can cause toxicity in human body.

The leakage problem in the 2nd generation is addressed in the 3rd generation when the enzyme is able to transfer electrons directly to the electrode, which is known as a direct electron transfer (DET)-type enzyme (Yamashita *et al*., 2013). Moreover, the potential required in the 3rd generation sensor is related to the redox potential of the enzyme cofactor. Because the redox potential of the enzyme cofactor is generally low (usually lower than -0.1 V vs Ag/AgCl), this sensor type can be used at a low potential of 0 V or even lower (vs Ag/AgCl). In this type of sensor, the reduced cofactor is directly reoxidized at the electrode, and the obtained response currents can represent to the actual amount of substrate present in the sample. Because of these advantages, the 3rd generation sensor is considered an ideal electrochemical enzyme sensor, allowing its application in continuous monitoring systems. Unfortunately, enzymes with DET ability are limited. Moreover, immobilization of DET-type enzymes is difficult, because they often inactivate or block the access of electrons to the electrode. Therefore, the

development of 3rd generation electrochemical enzyme sensors for various target molecules is challenging.

As an alternative, a 2.5th generation electrochemical enzyme sensor has been developed and studied since the 1980s. In this type of sensor, enzymes are modified with redox mediators to achieve quasi-DET ability. Commonly, redox mediators have a functional group such as a succinimidyl group for targeting the primary amine group of lysine residues or the maleimide group to target the thiol group in cysteine residues in proteins. Similar to the 1st and 3rd generation sensor, this sensor type can also be applied in a continuous monitoring system.

Lactate sensors have been widely studied as electrochemical enzyme sensors. The number of research articles on lactate sensors has gradually increased since the first study by Karube *et al*. (1980). Until 2010, the lactate sensor market share was $1.06 billion (Scognamiglio *et al*., 2010; Antonacci *et al*., 2016) and this value has been continuing increase along with the number of published papers about lactate sensors after 2010 has been obviously increasing (Hiraka, 2021). Moreover, studies on lactate sensors employing electron mediators have also been increasing (Figure 1-3). Most of the studies about lactate sensors utilized lactate oxidase (LOx) as the sensing element. LOx is a well-known enzyme widely used in the development of lactate sensors.

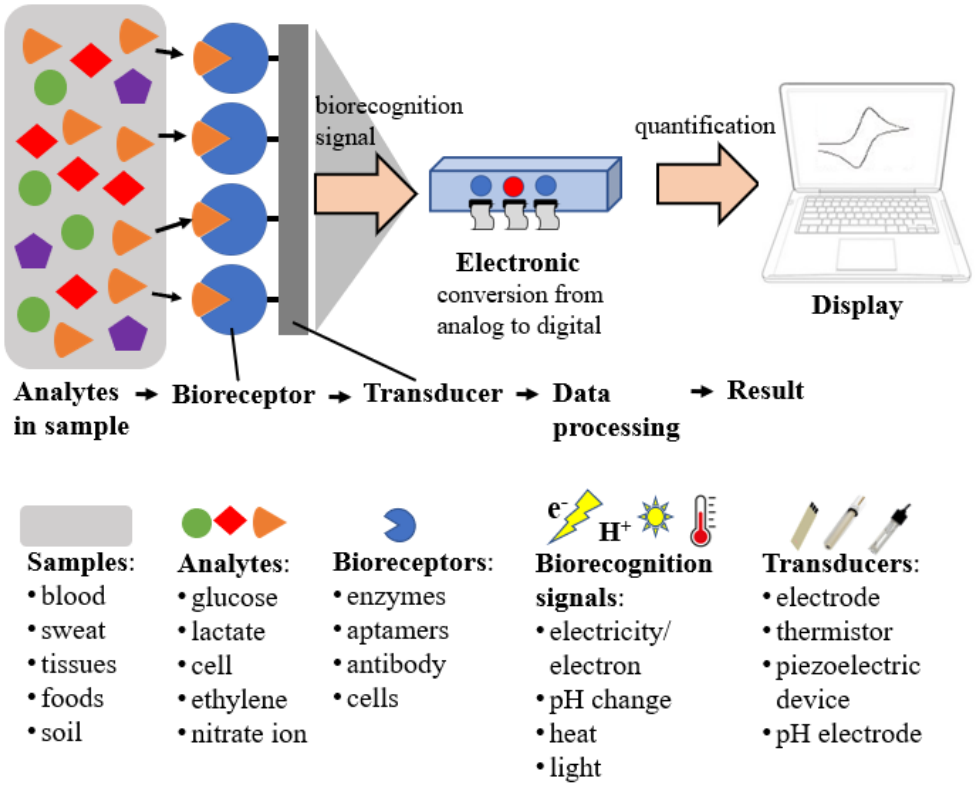


Figure 1-1. Illustration of biosensors.

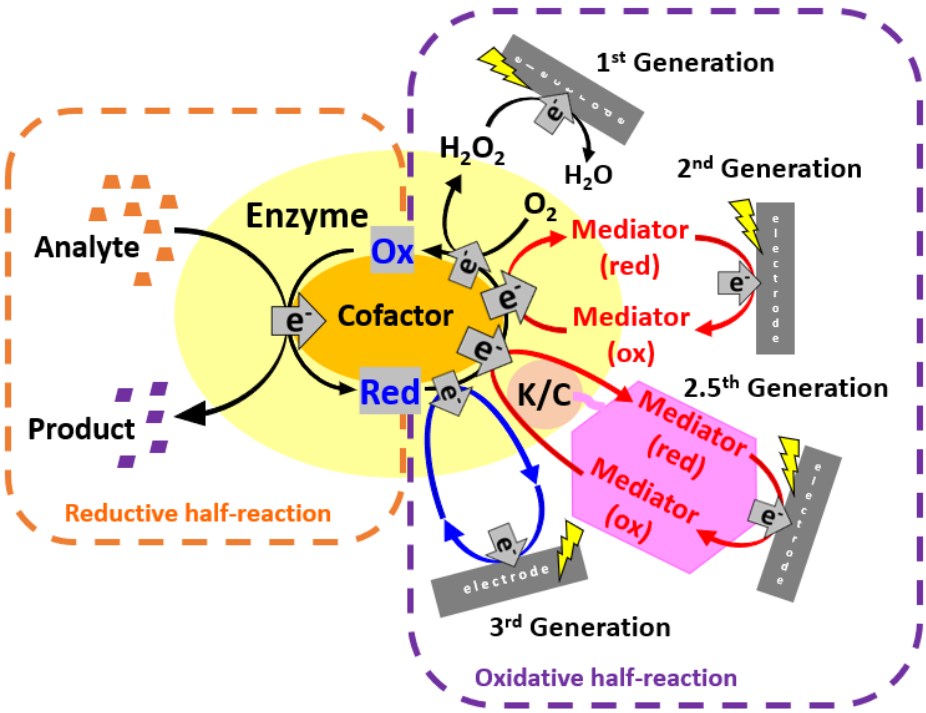


Figure 1-2. Principle of electrochemical enzyme sensors: first-, second-, third-, and in addition, two-point-fifth-generations.

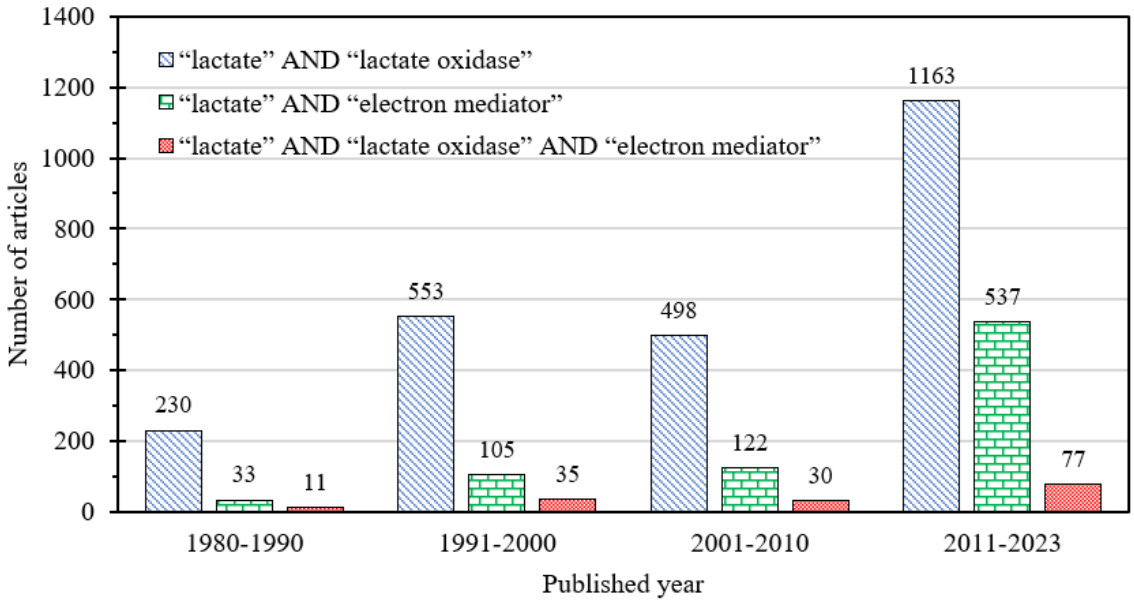


Figure 1-3. Lactate sensor article transition from ScienceDirect with keywords "lactate" AND "lactate oxidase", "lactate" AND "electron mediator", or "lactate" AND "lactate oxidase" AND "electron mediator".

1-2. L-Lactate detection

Measurement and monitoring of lactate levels have played significant roles in the medical care, sports medicine, and food industries. Determination of L-lactate concentration in the blood is widely used in clinical diagnosis; for example, it is important for the diagnosis of patient conditions in intensive care and during surgery (Pfeiffer *et al*., 1997; Yang *et al*., 1999). The blood lactate level under normal conditions (when people are at rest) is in the range 0.5–1.8 mM (Kruse, *et al*., 2011). The mentioned concentration can increase as an indicator of hypoxia, which can be related to health problems, or an increase after exercise. With extreme exercise, the blood lactate level can increase up to 25 mM (Kruse, *et al*., 2011; Goodwin *et al*., 2007). Therefore, blood lactate levels can also be used as an indicator for training status and fitness during exercises (training of athletes) (Pfeiffer *et al*., 1992; Iwuoha *et al*., 1999; Parra *et al*., 2006). In the food sector, L-lactate levels are also widely used for quality control of food, beverages and dairy products (Nikolaus *et al*., 2008).

Various conventional analytical methods are available for determining lactate levels. Among these, colorimetric tests and chromatographic analyses are the most important (Nikolaus *et al*., 2008). However, these methods are complicated, because they are laboratory-oriented (centralized methods using automatic machines) and expensive (Avramescu *et al*., 2002; Herrero *et al*., 2004). As an alternative, amperometric biosensors provide simple, direct and real-time measurements with quick responses at a low cost (Herrero *et al*., 2004; Miertu *et al*., 1998; Palleschi *et al*., 1991). Moreover, amperometric lactate sensors can exhibit high sensor performance, such as high sensitivity, high accuracy, and high specificity.

1-3. Lactate oxidase for lactate sensors

LOx is an enzyme that is widely used for development of lactate sensors. LOx is a homo-tetrameric enzyme that harbors flavin mononucleotide (FMN) as a cofactor and catalyzes the oxidation of L-lactate to pyruvate, wherein flavin is reduced (forming $FMNH_2$) in the reductive half-reaction (Duncan *et al*., 1989; Maeda-Yorita *et al*., 1995; Leiros *et al*., 2006; Umena *et al.,* 2006; Li *et al*., 2007; Taurino *et al*., 2013). In the subsequent oxidative half-reaction, the reduced flavin is reoxidized, reforming the oxidized flavin (FMN).

The flavoenzyme LOx is obtained from several bacteria such as *Tetrahymena pyriformis* (Esders *et al*., 1979), *Streptococcus faecalis* (Eichel and Rem, 1962), some species of *Pediococcus* (Mizutani *et al*., 1983, Mascini *et al*., 1984), and *Aerococcus viridans* (Duncan *et al*., 1989). Among these, LOx derived from *Aerococcus viridans* (*Av*LOx; EC. 1.1.3.15) are widely used to study lactate sensors (Figure 1-4) (Sphon *et al*., 1997; Moser *et al*., 2001; Thomas *et al*., 2012; Taurino *et al*., 2013; Bollella *et al*., 2019). *Av*LOx has been well characterized, and a high-resolution crystal structure has been reported (Leiros *et al*., 2006; Umena *et al*., 2006; Li *et al*., 2007). Moreover, *Av*LOx is easily recombinantly produced in *Escherichia coli* (*E. coli*) and is commercially available.

The subunit of the homotetrameric *Av*LOx has a molecular weight of 44 kDa. *Av*LOx is highly specific for L-lactate (Duncan *et al*., 1989). Hiraka *et al*. (2018) demonstrated that this enzyme was highly stable. The redox potential of *Av*LOx is -128 mV (Yorita *et al*., 2000). Like other oxidoreductases, enzymes that catalyze the transfer of electrons from a reductant (electron donor) to an oxidant (electron acceptor), native *Av*LOx has an oxidase (V_{max} 90 U/mg; K_m 0.56 mM) and dye-mediated dehydrogenase activity (V_{max} 130 U/mg; K_m 0.72 mM; Hiraka *et al*., 2018). For its oxidase activity, it

uses O_2 as an electron acceptor, whereas for its dye-mediated dehydrogenase activity, it does not require O_2 as an electron acceptor. Therefore, high oxidase and dye-mediated dehydrogenase activities are required to develop 1st and 2nd generation lactate sensors, respectively.

There is a common issue when oxidase is used for development of 2nd generation sensor: the reduction of electron mediators in the oxidative-half reaction competes with the reduction of O_2 (Figure 1-5) (Hiraka *et al*., 2018). My colleague from our research group engineered *Av*LOx A96L mutant, which has low reactivity toward O_2, thus decreasing oxidase activity (Hiraka *et al*., 2018). An enzyme with low oxidase activity reduces its dependency on O_2. Therefore, this mutant enzyme is suitable for development of 2nd generation lactate sensors.

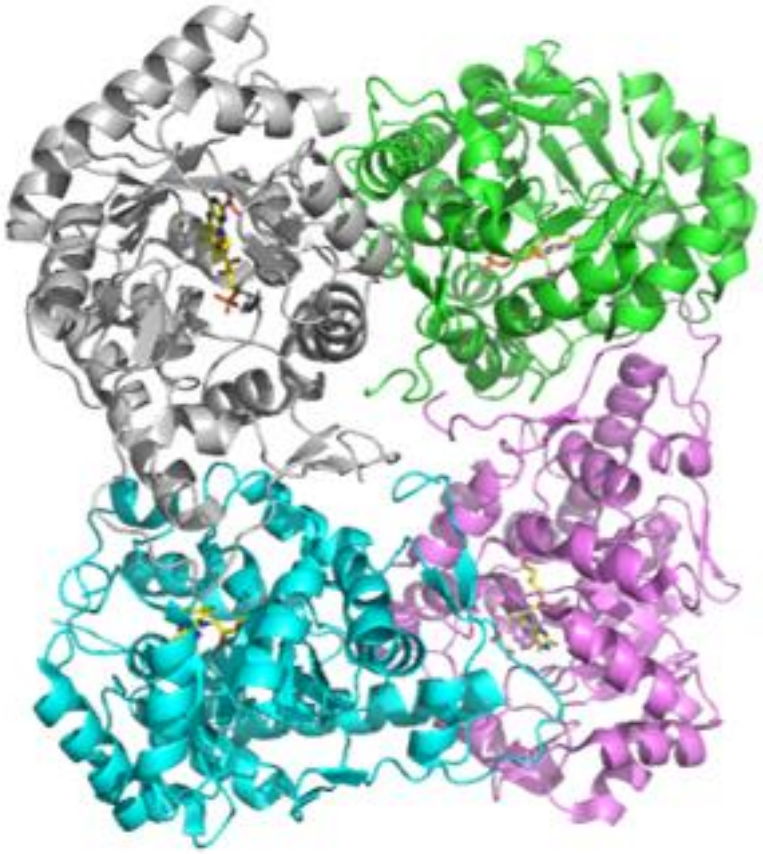


Figure 1-4. Structure of lactate oxidase derived from *Aerococcus viridans* (PDB code: 2E77). The enzyme is a homo-tetramer and harbors FMN as a cofactor in each subunit.

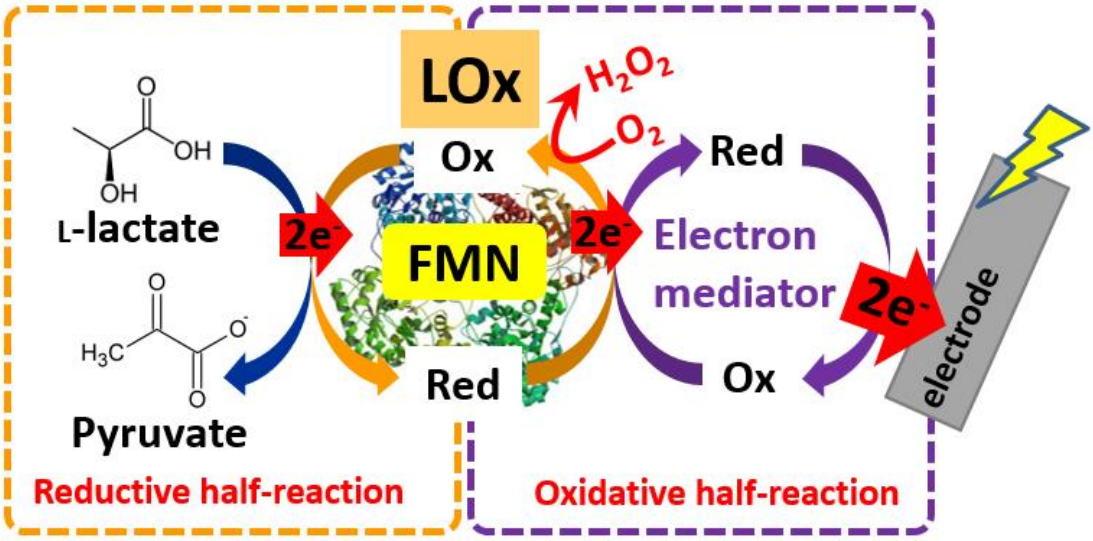


Figure 1-5. Principle of 2nd generation electrochemical lactate sensors based on lactate oxidase.

1-4. Electron mediators for enzyme-based sensors

Lactate sensors based on 2nd generation are commercially available. In this type of sensor, the electron mediator is one of the important elements next to the enzyme. An electron mediator is a redox species with a low molecular weight that can mediate electron transfer from the reduced cofactor of the enzyme to the electrode (Chaubey *et al*., 2002). However, not all mediators can be utilized by enzymes. Enzymes have a preference or suitability for certain mediators. Studies have shown that the suitability, or rather non-suitability, of a mediator may be influenced by electrostatic interactions between the mediator and the enzyme surface (Loew *et al*., 2017; Okurita *et al*., 2018) or by steric hindrance between the mediator and the enzyme (Nieh *et al*., 2013). The suitability or preference of a mediator for an enzyme affects the efficiency of the mediator in transferring electrons from the cofactor to the electrode.

Electrochemical enzyme sensors that utilize electron mediators as electron acceptors are also known as mediated electron transfer (MET)-type sensors. There are several criteria for a good mediator for biosensing applications, some of which are as follows: (1) The mediator should have fast reversible electron transfer kinetics, because its redox function is the main principle used in electrochemical sensors. (2) The mediator should have a low redox potential; thus, biosensors can be used at a low potential to avoid the oxidation of redox substances present in the samples (Chaubey *et al*., 2002), minimizing the response currents that are obtained from redox interferences. (3) The mediator should have high stability under various conditions and, thus, will not deteriorate owing to the biosensor fabrication process (such as after deposition, immobilization, and storage). Furthermore, when matching with an enzyme, (4) a mediator should be well-utilized by the enzyme as a mediator. By employing excellent

mediators, enzyme sensors can exhibit their best performance, resulting in an accurate measurement of analytes levels.

1-5. Objectives

The development of lactate sensors is increasing along with their applications, especially in clinical diagnosis and sports medicine. Therefore, high-performance lactate sensors are required for this purpose. MET-type lactate sensors are also increasingly being studied. MET-type enzyme sensors are primarily used for the development of disposable sensors for self-monitoring and POCT. Quasi-DET- and DET-type enzyme sensors are commonly used for the development of continuous monitoring systems. Currently, disposable lactate sensors for POCT are commercially available, whereas for self-monitoring, they are expected to become popular in the market soon, similar to self-monitoring blood glucose (SMBG). Furthermore, studies on continuous lactate monitoring (CLM) are attractive, and CLM for wearable and implantable sensors is currently under progress. The main objective of this study was to develop an electrochemical L-lactate sensor that utilizes a novel stable mediator derived from phenazine for a continuous monitoring system.

There are no reports evaluating a suitable mediator of *Av*LOx. Once a mediator is applied to enzyme sensors, biosensors should retain their stability. The suitability of mediators to enzymes and stability of biosensors are important for the development of high-performance biosensors. In this dissertation, I identified a suitable mediator for *Av*LOx and succeeded in constructing a disposable lactate sensor. Furthermore, quasi-DET lactate sensors that can be applied in CLM were also developed.

The main contents of this study are described in Chapters 2, 3, and 4.

Chapter 2, titled "Characterization of electron mediator preference of *Aerococcus viridans*-derived lactate oxidase for use in disposable enzyme sensor strips"

This study aimed to identify a suitable mediator for *Av*LOx. The preference of *Av*LOx for popular mediators in electrochemical enzyme sensors was characterized. Three mediators used in commercially available enzyme sensors (ferricyanide, 1-methoxy-5-methyl phenazinium methyl sulfate and hexaammine ruthenium[III]) were evaluated using screen-printed carbon electrodes. To this end, *Av*LOx was recombinantly produced. A mimicked-disposable blood lactate sensor with an end-point assay was used to evaluate the of sensor response. The preference of *Av*LOx for mediators is also discussed.

Chapter 3, titled "Employing a novel phenazine derivative mediator 1-methoxy-5-ethyl phenazinium ethyl sulfate as a stable electron mediator for disposable lactate sensor"

The second study aimed to develop a stable, disposable enzyme sensor. A recently commercially available and stable mediator, mPES, and an *Av*LOx A96L, a mutant with low O_2 reactivity, were used to construct a high-performance disposable lactate sensor. The storage stability and effect of redox substances were evaluated. Screen-printed carbon electrodes with end-point assay measurements were used to evaluate the response currents of the constructed lactate sensors. In addition, biosensors employing mPES and other oxidoreductases such as glucose dehydrogenase (GDH), glucose oxidase (GOx), and fructosyl peptide oxide (FPOx) were also evaluated. The constructed biosensors employing mPES were then discussed.

Chapter 4, titled "Application of a novel redox mediator from a phenazine derivative for the development of a quasi-direct electron transfer-type lactate sensor"

The last part of this study aims to develop a quasi-DET-type lactate sensor for continuous monitoring systems. A novel redox mediator, thiol-reactive PES

(trPES), was used to modify *Av*LOx. Moreover, trPES harbors a maleimide group and thus can bind to the thiol group of the cysteine residue in the proteins. *Av*LOx cysteine mutants were constructed and modified using a single molecule of trPES near the cofactor at the enzyme surface. A rod-type electrode was used to evaluate the response current. An *Av*LOx cysteine mutant that exhibited the highest response current was then modified with both trPES and amine-reactive PES (arPES), another redox mediator that modifies the amine group of the lysine residues of proteins. This increases the response currents by increasing the number of PES molecules attached to the enzyme. In addition, the versatility of trPES was evaluated using GDH.

Finally, the achievements obtained in Chapter 2-4 are summarized in Chapter 5, "Conclusion," and future perspectives of this study are described.

Chapter 2. Characterization of electron mediator preference of *Aerococcus viridans*-derived lactate oxidase for use in disposable enzyme sensor strips

2-1. Introduction

Electron mediator is one of the important elements in 2nd generation electrochemical enzyme sensors next to the enzyme. An effective mediator is needed for the development of an accurate biosensors. Each enzyme has its own preference toward mediator as the electron acceptor, therefore, suitable mediator is important to achieve highly accurate measurements. Several mediators are used in commercially available lactate biosensors, however, scientific explanations related to the mix-matching of the mediators and enzymes for lactate sensors are still limited.

Various mediators for LOx-based sensors have been studied, such as ferrocene derivatives (Taniguchi *et al*., 1988; Kulys *et al*., 1992; Hirano *et al*., 2002a; Boujtita *et al*., 1996; Kobayashi *et al*., 2001; Hirano *et al*., 2002b; Hirano *et al*., 2001), indophenol derivatives (Taniguchi *et al*., 1988; Hirano *et al*., 2002a), indoaniline derivatives (Hirano *et al*., 2002b; Hirano *et al*., 2001), phenoxazine derivatives (i.e., Meldola blue) (Kulys *et al*., 1992), tetrathiafulvalene (Liu *et al*., 1995), hydroquinone (Taniguchi *et al*., 1988), and metal complexes including ferricyanide (Taniguchi *et al*., 1988), Ni(II)-cyclam (Taniguchi *et al*., 1988), and Os complexes (Hirano *et al*., 2002a; Hirano *et al*., 2001; Nieh *et al*., 2013). Some of them studied comparison of different mediators regarding their suitability for LOx when utilized in lactate sensors (Taniguchi *et al*., 1988; Kulys *et al*., 1992; Boujtita *et al*., 1996; Hirano *et al*., 2001; Nieh *et al*., 2013). However, factors that influence interaction of mediator and enzyme other than local

mediator concentration at electrode only discussed by few papers (Taniguchi *et al*., 1988; Hirano *et al*., 2001; Nieh *et al*., 2013).

Some of mediators are widely studied and used in commercially available enzyme sensors, such as potassium ferricyanide, hexaammine ruthenium(III) chloride, and 1-methoxy-5-methyl phenazinium methyl sulfate (mPMS) (Pharmaceuticals and Medical Devices Agency, *online*). Potassium ferricyanide is one of mediators commonly used in commercially available enzyme sensors. However, the sensitivity of ferricyanide to light irradiation makes them easily reduced, which leads to inaccurate measurement results due to the increasing signal responses from ferricyanide reduction. Development of biosensors employing hexaammine ruthenium(III) chloride as a mediator is increasing in recent times due to their lower application potential and also their ability to be more stable under light irradiation than ferricyanide. Another one, mPMS, is an light-stable form of phenazinium methyl sulfate (PMS, an electron acceptor often used in spectrophotometric assays) employed in commercial blood lactate sensors (Pharmaceuticals and Medical Devices Agency, *online*). Both ferricyanide and hexaammine ruthenium(III) are inorganic metal complexes while mPMS is an organic material and all have reversible electron transfer kinetics (Dojindo Molecular Technologies Inc.a, *online*; Hisada *et al*., 1977). Although these mediators are commonly used, however there is no report which compares these mediators scientifically.

In this study, we evaluated the three popular mediators used in commercially available enzyme sensors in combination with *Av*LOx. The evaluation was carried out using disposable film electrodes mimicking enzyme sensor strips with end-point assay type measurements. The preference of *Av*LOx for these three mediators is discussed.

This is the first attempt to evaluate mediator preference of *Av*LOx scientifically and showing mediator specificity of *Av*LOx toward a mediator.

2-2. Materials and methods

2-2-1. Materials and devices

Dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), 4-Aminoantipyrine (4AA), 5-methyl-phenazinium methyl sulfate (PMS), potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium chloride (KCl), and Coomassie brilliant blue (CBB) staining packet were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 2,6-dichlorophenol indophenol (DCIP) was purchased from Merck (Darmstadt, Germany). 3-(N-ethyl-3-methyl anilino)-2-hydroxy-propane sulfonate (TOOS) and mPMS were purchased from Dojindo Laboratories Inc. (Kumamoto, Japan). Peroxidase (POD) was obtained from Amano Enzyme Inc. (Gifu, Japan). Bovine Serum Albumin (BSA) 2 mg/ml and DC protein assay were obtained from Thermo Scientific (Rockford, USA). BlueStar pre-stained protein marker was purchased from Nippon Genetics Europe GmbH (Tokyo, Japan). SuperSep ^{TM}Ace 10-20% agar was obtained from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). Sodium L-lactate, hexaammine ruthenium(III) chloride ($[Ru(NH_3)_6]Cl_3$), and kanamycin (Km) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amicon Ultra-15 centrifugal filter unit (30-KDa cutoff) was purchased from Merck Millipore Ltd. (Carrigtwohill Co., Cork, Ireland). The rod type glassy carbon (GC) working electrode (WE) 3.0 mm diameter (2.997 mm-2.972 mm), the silver/silver chloride (Ag/AgCl) glass reference electrode (RE) and electrode polishing kit (consisting of 1 μm polishing diamond and 0.05 μm polishing alumina) were purchased from BAS Inc. (Tokyo, Japan). A platinum (Pt) wire counter electrode (CE) was purchased from Tanaka

Kikinzoku Kogyo K.K. (Tokyo, Japan). Screen-printed carbon electrodes (SPCEs; WE: Carbon 2.4 mm^2, RE: Ag/AgCl, CE: Carbon) were kindly supplied by i-SENS (Seoul, Republic of Korea) (Figure 2-2). All other chemicals were of reagent grade. All solutions were prepared using pure water from a Milli-Q water purification system (Millipore Corporation, Burlington, MA, USA).

The enzyme was purified using Fraction Collector F9-C and Resource Q column from GE Healthcare Japan Corp. (Tokyo, Japan). Visualization of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Gel Doc ^{TM}Imager from Bio-Rad Laboratories Inc. (Tokyo, Japan). Colorimetric evaluations were performed using a UV-1289 UV-Vis spectrophotometer (Shimadzu Corp., Kyoto, Japan). All electrochemical measurements were carried out with a VersaSTAT4 potentiostat from Princeton Applied Research (AMETEC Inc., Pennsylvania, USA).

2-2-2. Production of an engineered *Av*LOx

The LOx was prepared by recombinant expression in *Escherichia coli* (*E. coli*) following the method by Maeda-Yorita *et al*., (1995) until obtained the purified LOx. Briefly, the cells of *E. coli* harboring *Av*LOx were cultivated in 100 mL of ZYP-5052 medium (0.5% glycerol, 0.05% glucose, 0.2% lactose, 50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 50 mM Na_2HPO_4, and 1 mM $MgSO_4$) containing 50 μg/ml Km (final concentration, fc.) using 500 ml baffle flasks (Studier, 2005) and incubated at 30 ^oC and 120 rpm for 36 hours (h). After cell harvesting by centrifugation, wet cells were suspended in potassium phosphate buffer (PPB) pH 7.0 and disrupted by ultrasonication. The lysate was centrifuged and ultracentrifuged to obtain soluble fraction. The soluble fraction containing LOx were purified by anion exchange chromatography with a linearly increasing gradient of 0–500 mM KCl in PPB (pH 7.0). The purified protein solution was dialyzed overnight against 20 mM PPB (pH 7.0).

The method of DC protein assay was used for determination of protein with BSA as the protein standard. The oxidase activity of the purified LOx was measured spectrometrically using 1.5 mM 4AA, 1.5 mM TOOS, 2 U/ml POD, and 0–25 mM lactate in 20 mM potassium phosphate buffer (PPB; pH 7.0) at 25 °C (Table 2-1). The enzymatic reaction was initiated by the addition of lactate (Figure 2-1). One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzes production of 1 μmol H_2O_2 per minute using 20 mM lactate at 25 °C (absorbance change at 555 nm due to the formation of quinone imine dye was monitored). Dye-mediated dehydrogenase activity was measured using 4 mM PMS, 0.06 mM DCIP, and 0–25 mM lactate in 20 mM PPB (pH 7.0) at 25 °C. One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzes the reduction of 1 μmol DCIP per minute using 20 mM lactate at 25 °C (the absorbance change at 600 nm was monitored). These assays were performed in triplicate for each enzyme sample. Kinetic parameters from oxidase and dye-mediated dehydrogenase activity were determined using Hanes-Woolf plots. The SDS-PAGE analysis was then performed using 10–20% agar by applied 0.5 mg LOx sample per lane and furthermore stained with CBB.

Table 2-1. Mixture composition in evaluation of the enzyme activity*.

Oxidase activity		Dye-mediated dehydrogenase activity	
Reagents (fc.)	Volume	Reagents (fc.)	Volume
1.5 mM 4AA	20 µl	4 mM PMS	20 µl
1.5 mM TOOS	20 µl	0.06 mM DCIP	20 µl
2 U POD	20 µl	-	-
20 mM L-Lactate	20 µl	20 mM L-Lactate	20 µl
LOx	20 µl	LOx	20 µl
20 mM PPB (pH 7.0)	(adjusted) µl	20 mM PPB (pH 7.0)	(adjusted) µl
Total volume	200 µl		200 µl
Monitored absorbance	555 nm		600 nm

* The mixture 180 µl was applied to the spectrophotometer to monitor the absorbance once lactate is added to the premix.

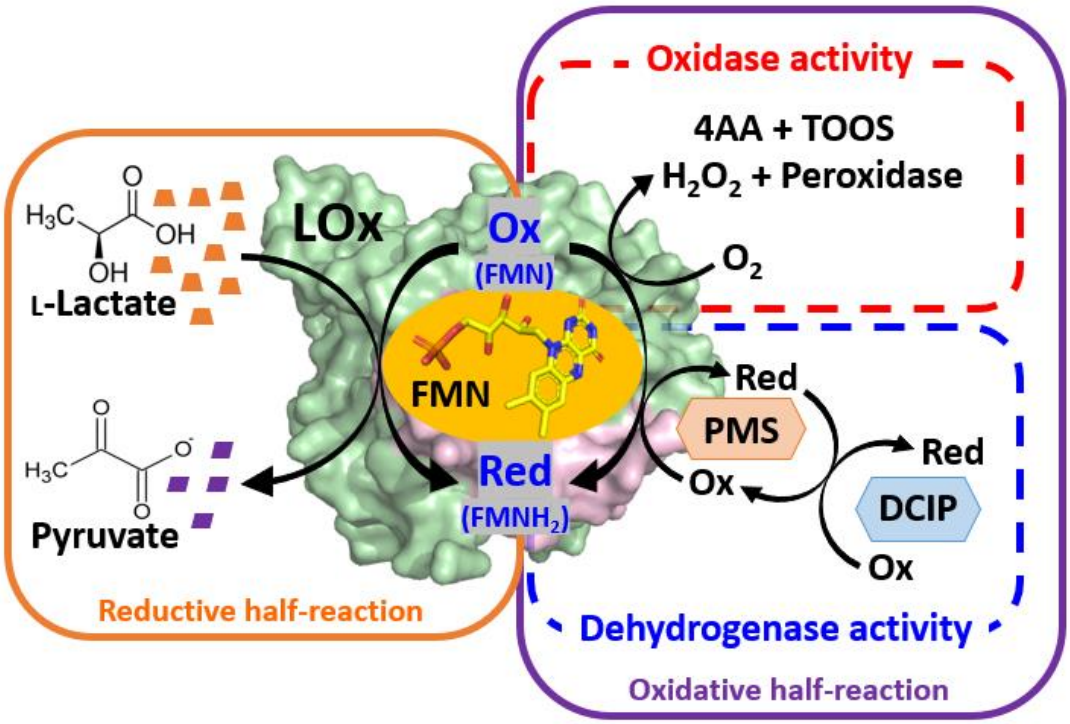


Figure 2-1. Principle of the evaluation of oxidase and dye-mediated dehydrogenase activity of LOx.

2-2-3. Investigation of electrochemical behavior of mediators

The cyclic voltammetry (CV) measurements were carried out for each of the 10 mM of mediator solutions (ferricyanide, mPMS or hexaammine ruthenium(III)) in 10 ml of 100 mM PPB (pH 7.0) with a scan rate 0.1 V/s (potential range from –0.4 V to +0.6 V vs Ag/AgCl). The GC electrode and Pt wire were used as WE and CE, respectively.

2-2-4. Employing the popular mediators for mimicked disposable lactate sensor based on lactate oxidase

The response currents of mimicked disposable lactate sensors were evaluated by chronoamperometry (CA) measurements. The bare SPCE (Figure 2-2) were prepared by attaching a spacer and cover to form a μL-volume capillary space above the electrodes. A premix (4 μL) was prepared containing a mediator ferricyanide and enzyme LOx in buffer 100 mM PPB (pH 7.0). The lactate (1 μL) was added to the premix and the enzyme reaction was started. Furthermore, 1.8 μL of the mixture was loaded onto an SPCE which was connected to the potentiostat, thus the final concentration (fc.) of lactate in the SPCE was 0, 0.5, 1, 2, 5, 10, 20, 30, or 50 mM, the enzyme was 0.1 U/sensor and the ferricyanide was 100 mM. After 60 s with the addition of lactate into the mixture (the waiting time), a potential of +0.4 V was applied, and the response current was monitored for the next 60 s. The current at 10 s (sampling time) after application of the potential was plotted against the lactate concentrations to determine the standard curve. The corresponding measurement was carried out for other concentration of ferricyanide (25, 50 or 200 mM) and also with another mediators, mPMS or hexaammine ruthenium(III) in the same set of concentrations. A potential of +0.2 V and 0 V was applied for mPMS and hexaammine ruthenium(III), respectively.

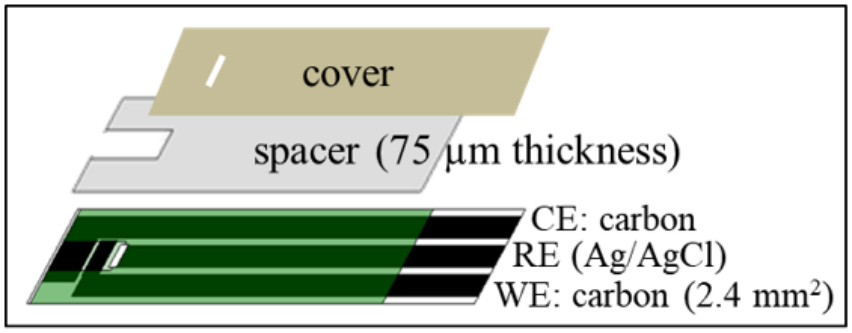


Figure 2-2. Screen-printed carbon electrode (SPCE) used in this study.

2-3. Results

2-3-1. Enzymatic activity of LOx

Recombinant *E. coli* successfully expressed soluble and active LOx. The purified LOx was confirmed by perform SDS-PAGE analysis and showed the prominent band slightly lower than 48 kDa (Figure 2-3), suggesting a size of LOx in approximately 44 kDa (Duncan *et al*., 1989). The properties of purified LOx were determined following Hanes–Woolf plots, obtaining the K_m value of 0.61 mM and the V_{max} value of 101 U/mg based on the oxidase activity (Table 2-2), which are comparable values to those in literature (Maeda-Yorita *et al*., 1995; Taurino *et al*., 2013). The K_m and V_{max} value of dye-mediated dehydrogenase activity were 0.58 mM and 135 U/mg, respectively.

2-3-2. Electrochemical behavior of the electron mediators

Cyclic voltammograms of each mediator solution were determined as can be seen in Figure 2-3. The half-wave or midpoint potential ($E_{1/2}$) was calculated to be +0.25 V, –0.15 V, and –0.2 V for ferricyanide (Figure 2-4a), mPMS (Figure 2-4b) and hexaammine ruthenium(III) (Figure 2-4c), respectively.

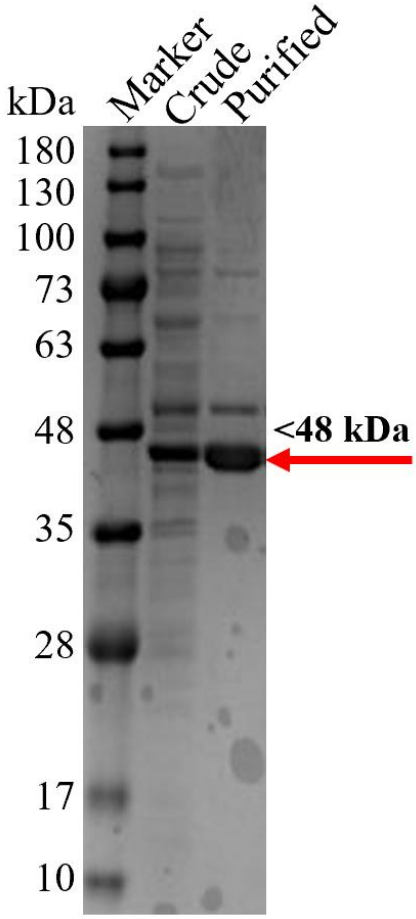


Figure 2-3. SDS-PAGE analysis result of purified *Av*LOx. Purified LOx showing the prominent band slightly lower than 48 kDa, suggested to the size of LOx in approximately 44 kDa.

Table 2-2. Properties of purified *Av*LOx.

Kinetic parameters	Oxidase activity	Dye-mediated dehydrogenase activity
K_m (mM)	0.61	0.58
V_{max} (U/mg)	101	135
V_{max}/K_m (U/mg•mM)	167	232

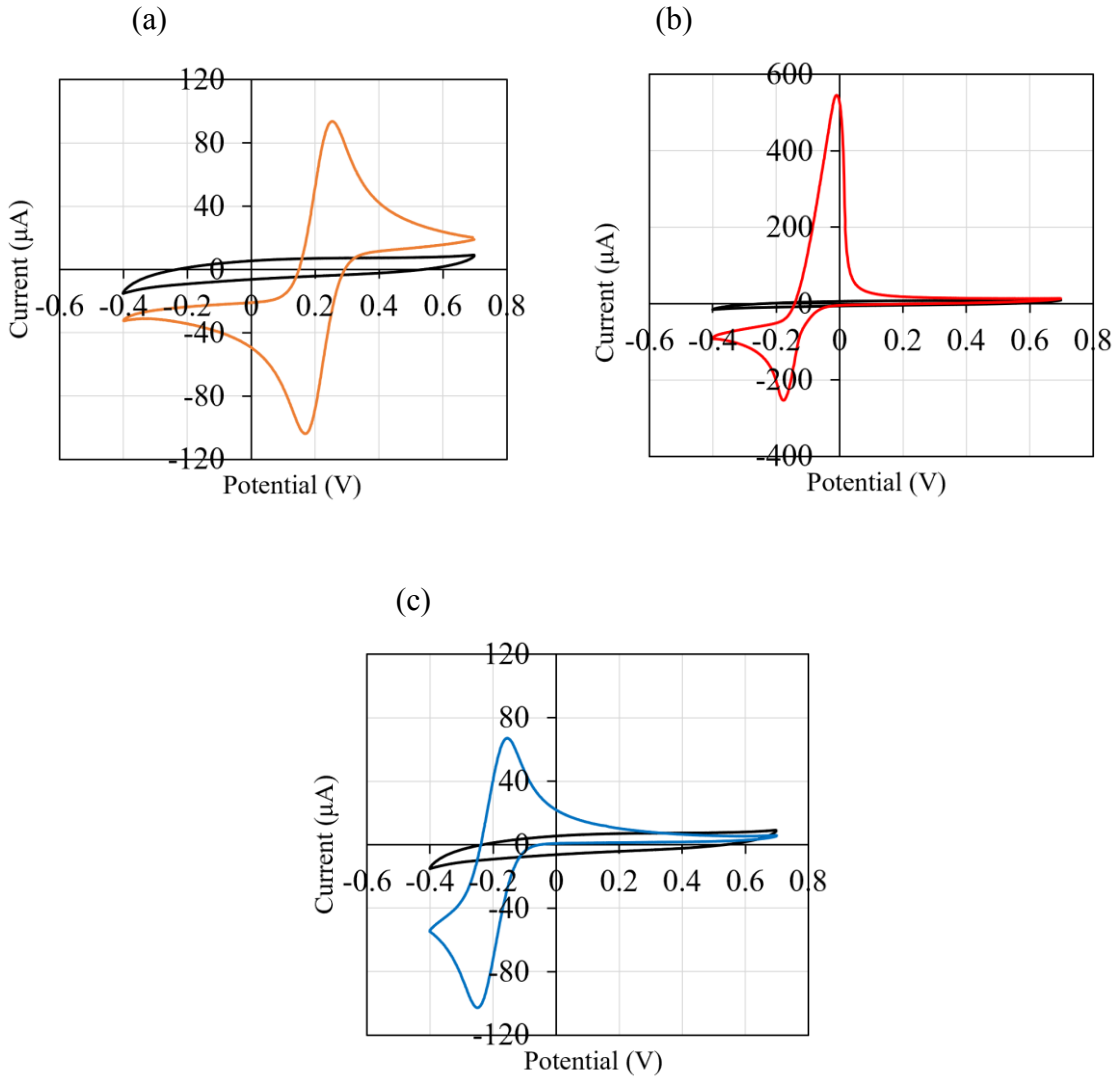


Figure 2-4. Cyclic voltammograms of electron mediators: (a) ferricyanide; (b) mPMS and (c) hexaammine ruthenium(III). Black line: background (100 mM PPB (pH 7.0)); 10 mM mediators in 10 ml of 100 mM PPB (pH 7.0); scan rate: 0.1 V/s; WE: GC; CE: Pt wire; RE: Ag/AgCl.

2-3-3. Response currents of mimicked disposable lactate sensors employing ferricyanide as a mediator

CA measurements were carried out to evaluate the response currents of mimicked lactate sensors. In this type of lactate sensor, the enzyme reaction occurs in the microtube once the lactate is added into the premix while loading this mixture into the blank SPCE that is connected to the potentiostat. After 60 seconds from the addition of lactate, the potential is applied to start the monitoring of response currents. Thus, the results of these end-point assays were plotted as the time course of response currents of mimicked lactate sensors (Figure 2-5a).

The response currents of lactate sensors employing ferricyanide (100 mM) as a mediator were clearly observed and increases depended on lactate concentration (Figure 2-5). This is suggesting that the lactate sensor employing ferricyanide as a mediator was successfully constructed, and lactate can be measured with this system. Furthermore, the response currents of various concentrations of ferricyanide (0, 25, 50, 200 mM) were observed (Figure 2-6). In concentrations tested, response currents were clearly observed, except in the lactate sensors without ferricyanide (0 mM), thus the sensitivities (slope) and linear range were achieved. The sensitivities obtained with different concentrations of ferricyanide are similar, while the linear ranges are different. Employing 100 mM ferricyanide observed the best response currents than other concentrations, achieving a good sensitivity (0.64 μA/mM) with a wide linear range (up to 30 mM lactate) (Figure 2-5b). A higher ferricyanide concentration resulted in a wider linear range obtained. With 25 and 50 mM ferricyanide, obtained linear ranges were up to 10 mM and 20 mM lactate, respectively (Figure 2-6). While, with 200 mM ferricyanide, the linear range was up to 20 mM

lactate, the same as with 50 mM ferricyanide, this was even lower than obtained with 100 mM ferricyanide.

2-3-4. Response currents of mimicked disposable lactate sensors employing mPMS as a mediator

The standard curve of response currents of lactate sensors employing mPMS showed that with mPMS as a mediator, higher currents were achieved than what was observed with ferricyanide (>2 fold) (Figure 2-7). With 100 mM mPMS achieved a linear range up to 50 mM lactate. Furthermore, the linear range for lower concentrations, the same phenomena was observed as well as with ferricyanide. In addition, the background current was high and increased with increasing mPMS concentrations.

2-3-5. Response currents of mimicked disposable lactate sensors employing hexamine ruthenium(III) as a mediator

Employing hexaammine ruthenium(III) as a mediator in the mimicked lactate sensors showed that no response currents were obtained for all concentrations tested (Figure 2-8) which is totally contrary with the other two mediators mentioned previously.

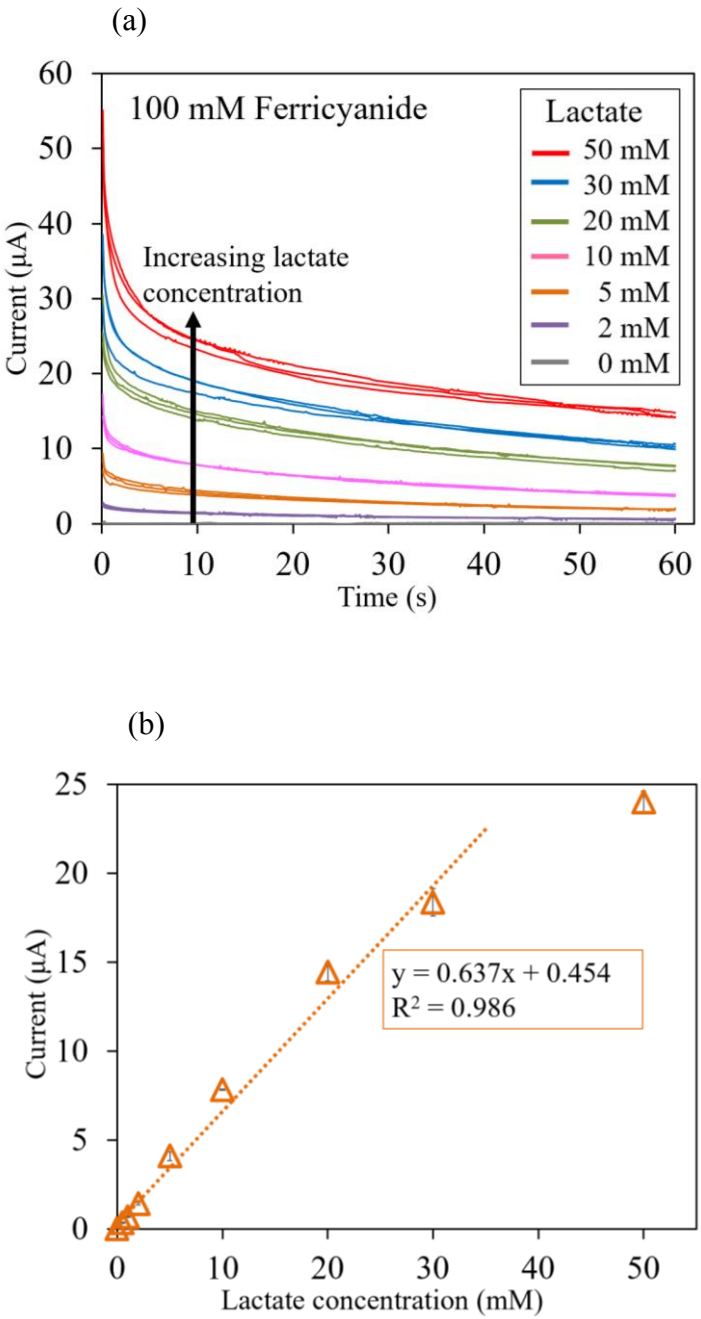


Figure 2-5. Response currents of mimicked lactate sensors: (a) time courses of response currents for various lactate concentrations and (b) standard curves of response currents vs lactate concentrations. Sensitivity: 0.64 µA/mM; linear range: 0–30 mM lactate. Conditions per sensor strip: 0.1 U *Av*LOx, 100 mM ferricyanide. Waiting time: 60 s; applied potential: +0.4 V; sampling time: at 10 s (black arrow); n = 3.

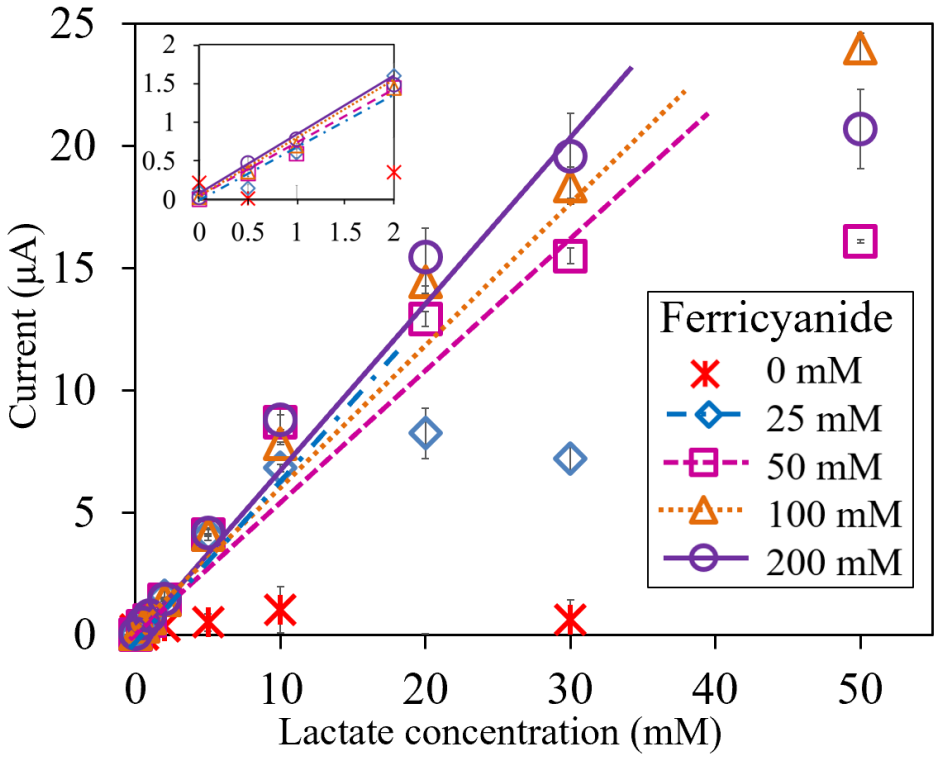


Figure 2-6. Standard curves of response currents vs lactate concentration for various ferricyanide concentrations. Conditions per sensor strip: 0.1 U *Av*LOx, 0–200 mM ferricyanide. Waiting time: 60 s; applied potential: +0.4 V; sampling time: at 10 s; $n = 3$. Inset: blow-up of low concentrations of lactate.

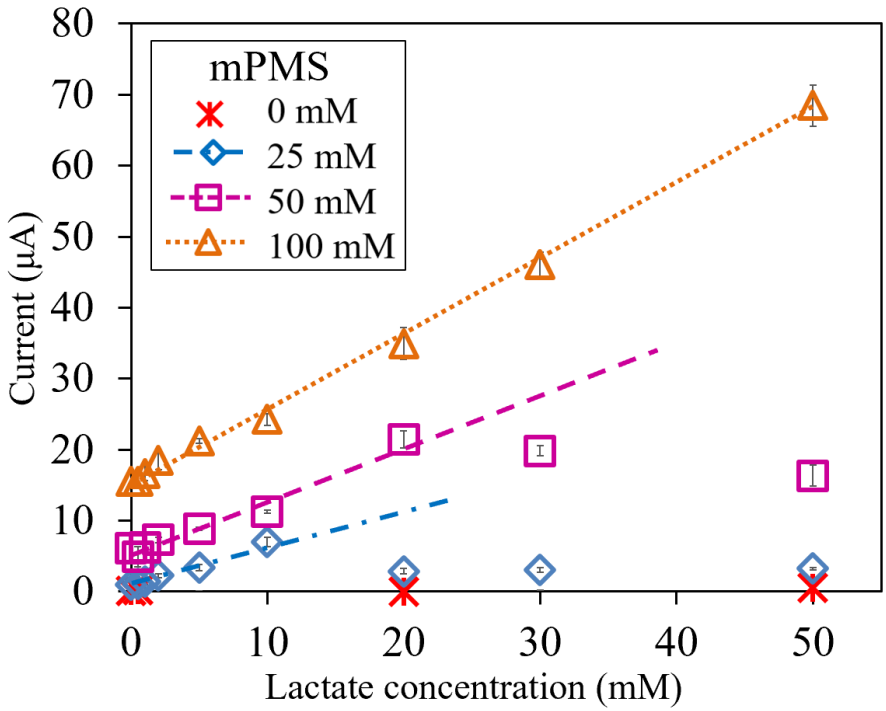


Figure 2-7. Standard curves of response currents vs lactate concentration for various mPMS concentrations. Conditions per sensor strip: 0.1 U *Av*LOx, 0–100 mM mPMS. Waiting time: 60 s; applied potential: +0.2 V; sampling time: at 10 s; $n = 3$.

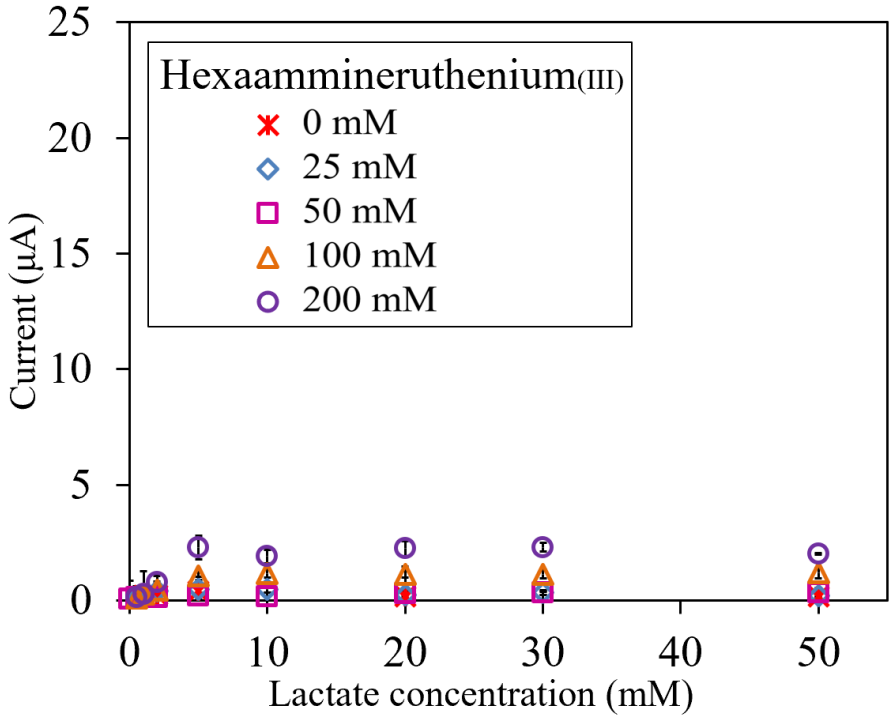


Figure 2-8. Standard curves of response currents vs lactate concentration for various hexaammine ruthenium(III) concentrations. Conditions per sensor strip: 0.1 U *Av*LOx, 0–200 mM hexaammine ruthenium(III). Waiting time: 60 s; applied potential: 0 V; sampling time: at 10 s; $n = 3$.

2-4. Discussion

Enzyme activity and purity

Purified *Av*LOx was obtained. It was confirmed by SDS-PAGE analysis that showed the prominent band in approximately 44 kDa (Figure 2-3), as well as the molecular weight reported of *Av*LOx (Duncan *et al*., 1989). Although it is not showing a single band, the purity of LOx is relatively high. This is also supported by the high activity of LOx obtained for both the oxidase activity and the dye-mediated dehydrogenase activity, thus it can be further applied for sensor applications.

Electrochemical behavior of mediators

The CV measurements were performed to characterize the electrochemical behavior of the mediators used in this study. All three mediators clearly showed the reversible redox behavior in their cyclic voltammograms as well as strong characteristics as a mediator (Figure 2-4). Furthermore, the $E_{1/2}$ was calculated for each mediator. This potential was used to decide the potential application used in CA measurements, which applied a higher potential than $E_{1/2}$ obtained to allow the complete oxidation of a mediator during the CA measurement. As $E_{1/2}$ ferricyanide is more positive (+0.25 V) than mPMS (–0.15 V) and hexaammine ruthenium(III) (–0.2 V), thus the higher potential application of +0.4 V was decided for CA measurements and showed a lower potential for mPES (+0.2 V) and hexaammine ruthenium(III) (0 V).

Response currents of mimicked disposable lactate sensors

The response currents of lactate sensors were evaluated mimicked blood lactate sensors. In the microtube, the enzyme reaction was firstly start by mix the enzyme, mediator and lactate in the sufficient time (the waiting time) to allow the complete conversion of the substrate, while then this mixture is loading onto the sensor strip

during the enzyme reaction process. A potential was applied to start the response currents recording. Thus, the end-point assay was used in the evaluation of response currents to lactate. The amount of enzyme was chosen for 60 s, so that complete conversion of all lactate concentrations tested was achieved within that time. Therefore, in this type of electrochemical measurement, it is measuring the specific amount of reduced mediator, which reflects the initial amount of substrate. The results show as the time courses or response currents which initially the current is high and decreases with time.

Figure 2-4 was shows that lactate sensors with ferricyanide as a mediator clearly observed the response currents. With increasing lactate concentration, the response current increased, indicating that a lactate sensor could be constructed with ferricyanide as the mediator, as well as reported that ferricyanide has been shown to be an electron acceptor for LOx (Taniguchi, *et al*., 1988). Response currents of various concentrations of ferricyanide (25–200 mM) were also observed, while without ferricyanide (0 mM) no response currents were observed. This is confirming that ferricyanide transfers electrons from the enzyme to the electrode. As the sensitivities were observed similar in the different concentrations of ferricyanide, however the linear ranges were different. These are related to the amount of either mediators (ferricyanide) or substrate (lactate). Each oxidation of one molecule of lactate, the LOx reduces two molecules of ferricyanide to two molecules of ferrocyanide. As this is an end-point assay, the enzyme reaction is finished before the potential is applied (within 60 s, the waiting time), which means that there are either no more lactate molecules or no more ferricyanide molecules. When the initial lactate concentration is low (<20 mM), all lactate molecules are oxidized. The resulting amount of ferrocyanide molecules (reduced state of ferricyanide) only depends on the initial lactate concentration, which is evidenced in that the response currents do

not depend on the ferricyanide concentration. In the case of the initial lactate concentration exceeds half of the initial ferricyanide concentration, the amount of ferricyanide molecules becomes the limiting factor. This results in response currents depending on the ferricyanide concentration and not on the lactate concentration, and thus a loss of linearity. For high lactate concentrations (50 mM), the obtained response currents were somewhat lower than expected for 100 mM and 200 mM ferricyanide, indicating an incomplete turn-over of lactate. A possible reason for this incomplete turn-over is an insufficient amount of time. The time needed for complete turn-over of a given lactate amount (concentration) depends on the amount of enzyme. Therefore, further optimization (increasing) of the waiting time or of the amount of LOx might lead to a wider linear range. However, the linear range up to 30 mM lactate which achieved with 100 mM ferricyanide is cover the range of blood lactate levels measurement.

The same phenomena were observed when mPMS is used as a mediator. The similar sensitivities obtained with different concentration of mPMS, but the linear ranges were different depends on either mPMS or lactate concentration. Furthermore, with mPMS as a mediator, higher response currents were achieved than with ferricyanide (Figure 2-7). For each oxidation of one molecule of lactate, the LOx reduces only one molecule of mPMS. Thus, with lower concentration of mPMS could achieve a wide linear range. Furthermore, the waiting time of 60 s was more than enough to allow the complete turn-over of lactate for up to 50 mM. This is indicating that the electron transfer from the FMN of the LOx to mPMS was faster than that to ferricyanide. However, the high background current was observed and increased depending on mPMS concentration, which suggests that it may contained some interfering substance in the mPMS sample used and could be oxidized at the electrode.

Although mPMS is more stable than PMS (Hisada *et al*., 1977), it is known to degrade in non-acidic solutions (Dojindo Molecular Technologies Inc.a, *online*). Therefore, the interfering substance might be coming from a degradation product. Another possibility is that a partially of the mPMS was in the reduced state. In addition, mPMS was used as a primary electron acceptor (mediator) in a commercial lactate sensor based on LOx (Pharmaceuticals and Medical Devices Agency, *online*). Thus, this study provides the information of the employing of mPMS as a single electron acceptor in lactate sensor based on LOx.

The mediator hexaammine ruthenium(III) was also evaluate in this study, which known also used as one of the electron acceptors in a commercial lactate sensor based on LOx (Pharmaceuticals and Medical Devices Agency, *online*), but in this study hexaammine ruthenium(III) was used as a final electron acceptor. The results obtained showed that no response currents observed when utilizing hexaammine ruthenium(III) as a mediator, indicate that this mediator did not transfer the electron from FMN of LOx to the electrode. Thus, hexaammine ruthenium(III) is not suitable to employed as a primary electron acceptor for LOx.

It has been known that a mediator can accept electrons from an enzyme should depend on the redox potential, which is the mediators with a high redox potential should accept electrons more readily. Indeed, the mediator with the highest redox potential in this study, ferricyanide, does accept electrons readily from the LOx. However, although the redox potentials of mPMS and hexaammine ruthenium(III) are similar, mPMS accept electrons from the LOx even more effective than ferricyanide, while hexaammine ruthenium(III) does not. Nevertheless, the redox potential of hexaammine ruthenium(III) should be high enough to accept electrons from the active center of LOx,

FMN (redox potential: –0.42 vs Ag/AgCl (Okamoto, *et al*., 2013)). Thus, the redox potential does not explain the phenomena observed in this study.

The factors influence the suitability of a mediator toward an enzyme other than the redox potential still unrevealed fully. Taniguchi *et al.* (1988) mentioned about the impact of some specific interaction", but did not reveal further information. Another report stated that the chemical structure of the mediator is important, but also, did not no further explanation (Hirano *et al*, 2001). Finally, Nieh *et al.* (2013) in their study in comparison of metal complexes bound to polymers, stated that the electrostatic repulsion from the negative ligands and the local surface charge of the enzyme could affect to less efficient mediators, as well as steric hindrance.

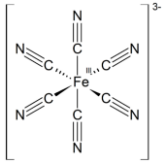
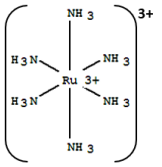
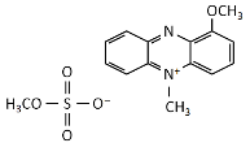
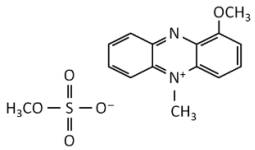
Ferricyanide and hexaammine ruthenium(III) are a small inorganic compound but have different charge. Ferricyanide has a strong negative charge and their redox potential of +0.23 V vs Ag/AgCl (O'Reilly *et al*., 1973). While, hexaammine ruthenium(III) is a strong positive charge with the redox potential of –0.11 V vs Ag/AgCl (Metzker *et al*., 2014). Another mediator used in this study is mPMS, an organic compound with either a weak positive charge (oxidized form) or no charge (reduced form), and have a similar redox potential to hexaammine ruthenium(III) (–0.14 V vs Ag/AgCl, Hisada *et al*., 1977) (Table 2-3).

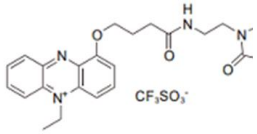
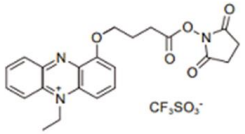
All mediators used in this study are small and of similar size, so steric hindrance does not influence in the phenomena observed in all the three mediators. Considering the unique charge that inherent in each mediator, thus the most likely related is the electrostatic interaction of the mediators and the local surface charge of the *Av*LOx. Especially for the small mediators with strong charges, the charge of the mediator, and the sign of the charge, has a great influence on the suitability as a mediator. The relatively uncharged mPMS is the most effective mediator for *Av*LOx in this study. The

ferricyanide, which has strong negatively charged, does function as mediator for LOx, however it is not as effective as mPMS. Therefore, LOx can tolerate negative charges fairly well, although the negative charge of the ferricyanide seems to hinder the electron transfer slightly, which is in accordance with Nieh *et al*. (2013) on the other hand, the strong positively charged hexaammine ruthenium(III) is not utilized as an electron acceptor by LOx. This suggests, that the access of the hexaammine ruthenium(III) to the active center of the LOx is blocked, despite the small size of the mediator.

Finally, I compare the standard curve of the lactate sensors utilizing 100 mM ferricyanide or mPMS (Figure 2-9) and stated that the mPMS is the most effective mediator for *Av*LOx achieving high sensitivity (1.00 µA/mM) and wide linear range (0–50 mM lactate) than obtained with ferricyanide (sensitivity 0.64 µA/mM; linear range 0–30 mM lactate).

Table 2-3. Properties of mediators used in electrochemical evaluation in this study. All mediators are water soluble.

Electron Mediator	Type	Structure/Formula (Oxidized Form)	Redox Potential (vs Ag/AgCl)	Charge (Ox/Red) [1]
Potassium ferricyanide	small inorganic metal complex	 $K_3[Fe_3(CN)_6]$	+0.23 V (O'Reilly *et al*., 1973)	(–3)/(–4)
Hexaammine ruthenium(III) chloride	small inorganic metal complex	 $[Ru(NH_3)_6]Cl_3$	–0.11 V (Metzker *et al*., 2014)	(+3)/(+2)
mPMS	small organic compound (phenazine derivative)		–0.14 V (Hisada *et al*., 1977)	(+1)/(0)
mPES	small organic compound (phenazine derivative)		–0.14 V (Fitriana *et al*., 2020)	(+1)/(0)

trPES	small organic compound (phenazine derivative-carrying thiol group)		–0.037 V [2] (Dojindo Molecular Technologies Inc.d)	(+1)/(0)
arPES	small organic compound (phenazine derivative-carrying amine group)		–0.026V [2] (Dojindo Molecular Technologies Inc.c)	(+1)/(0)

[1] oxidized form / reduced form [2] Converted from NHE

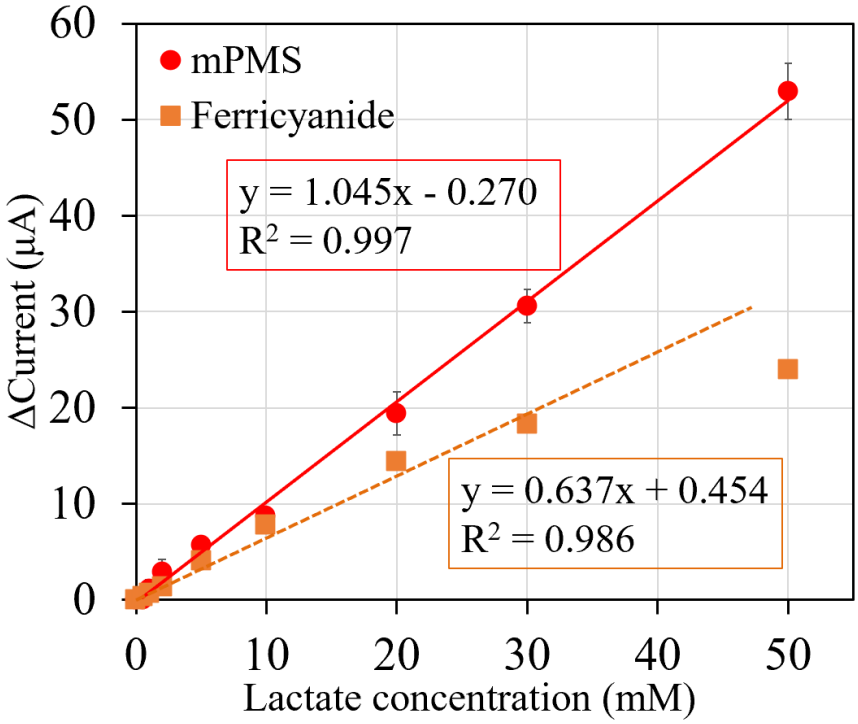


Figure 2-9. Calibration curves of mimicked lactate sensors with 100 mM ferricyanide or mPMS as a mediator.

2-5. Conclusion

The preferences of *Av*LOx for three popular mediators were investigated electrochemically. There are a strong negative charge ferricyanide, the relatively uncharged mPMS and a strong positive charge hexaammine ruthenium(III). The mPMS was most effective, with 100 mM mPMS, near perfect linearity was achieved up 50 mM lactate. However, mPMS is known to be unstable, so that such high mPMS concentrations are impractical for commercial use (Dojindo Molecular Technologies Inc.a, *online*). The ferricyanide was less effective than mPMS but did successfully transfer electrons from the LOx to the electrode. This suggests that *Av*LOx tolerates negative charges well enough. The linear range for up to 30 mM and the sensitivity of 0.64 μA/mM were achieved with 100 mM ferricyanide. It is sufficient for blood lactate sensors (normal range 0.5–1.8 mM and maximal with exercise 25 mM) (Kruse *et al*., 2011; Goodwin *et al*., 2007), as well as ferricyanide is already the most common mediator used in commercial enzyme sensors. On the other hand, hexaammine ruthenium(III) was not utilized as an electron acceptor by *Av*LOx. This is suggesting that the access of the positively charged hexaammine ruthenium(III) to the FMN in the catalytic center of the *Av*LOx is blocked by the protein scaffold. Thus, hexaammine ruthenium(III) cannot be used as the primary electron acceptor in electrochemical enzyme sensor strips utilizing *Av*LOx. However, recently the hexaammine ruthenium(III) is the most desirable mediator for commercial use due to their characteristic mentioned. Further study in structural investigations of the *Av*LOx might reveal the possible ways of the hexaammine ruthenium(III) could access the active center of *Av*LOx without it blocked, and this is could be strategies to engineer the enzyme to be able to utilize hexaammine ruthenium(III) as mediator. This study is the

first in attempts to evaluate mediator preference of *Av*LOx scientifically and showed the mediator specificity of *Av*LOx toward a mediator.

Chapter 3. Employing a novel phenazine derivative mediator 1-methoxy-5-ethyl phenazinium ethyl sulfate as a stable electron mediator for disposable lactate sensor

3-1. Introduction

Second-generation electrochemical biosensors for lactate are currently commercially available for several applications, including self-monitoring (Pharmaceuticals and Medical Devices Agency, *online*) and POCT (Woodley Equipment Company Ltd., *online*) of blood lactate. Blood lactate levels are a relevant parameter in clinical diagnosis and sports medicine, and the availability of disposable enzyme sensors for the determination of blood lactate is increasing.

*Av*LOx is widely used in lactate enzyme sensors (Spohn *et al*., 1997; Moser *et al*., 2002; Thomas *et al*., 2012; Taurino *et al*., 2013; Andrus *et al*., 2015; Bollella *et al*., 2019; Hiraka *et al*., 2018; Loew *et al*., 2017), including in commercially available lactate sensor strips (Woodley Equipment Company Ltd., *online*). However, LOx utilizes O_2 as an electron acceptor, which causes inherent problems in 2nd generation sensors and results in a lower sensor signal. This problem is common to 2nd generation biosensors utilizing oxidases and can be solved by utilizing dehydrogenases, which do not utilize O_2 as an electron acceptor. Our group recently engineered *Av*LOx to have a very low reactivity to O_2, thus converting the enzyme into a dehydrogenase (Hiraka *et al*., 2018). By utilizing this engineered *Av*LOx, an A96L mutant, the influence of O_2 is minimized. Thus, utilizing this mutant enzyme can increase the accuracy of lactate sensors.

Attempts are continuing to improve the performance of 2nd generation lactate sensors, including improving their accuracy and shelf life. In addition to the enzyme,

the mediator is a key element in 2nd generation electrochemical enzyme sensors, so the choice of mediator to be employed in enzyme sensors is one way to improve their performance. When choosing the mediator, the preference of the enzyme for the mediator as its electron acceptor must to be considered, as it affects the efficiency of the electron transfer from the cofactor of the enzyme to the electrode surface and thus determines the suitability of the mediator for sensor construction. Studies have shown that the availability of a mediator may be influenced by electrostatic interactions between the mediator and the enzyme surface or by steric hindrance between the mediator and the enzyme (Loew *et al*., 2017; Okurita *et al*., 2018; Nieh *et al*., 2013; Loew *et al*., 2017; Tsuruoka *et al*., 2017). To avoid interference from the oxidation of redox substances present in the sample and thus minimize errors in the response of the biosensor, the biosensor should be operated at a low potential, which can be achieved with a mediator with a low redox potential (Chaubey and Malhotra, 2002). Another parameter to be considered in the search for a good mediator is its stability under the sensor fabrication and storage of sensors, where the mediators will be exposed under several conditions, i.e., at room temperature, high humidity, and under the light illumination.

In Chapter 2, the mediator preference of *Av*LOx was evaluated. The light stable form of phenazinium methyl sulfate (PMS), a popular redox dye in spectrometric assays, mPMS, was shown to be a more effective mediator for *Av*LOx than potassium ferricyanide (Loew *et al*., 2017), a popular mediator commonly used in commercial enzyme sensors. Hexaammine ruthenium(III) chloride, a mediator that is currently gaining attention in the development of enzyme sensors due to its stability, i.e., to light irradiation, and low redox potential (−0.11 V vs Ag/AgCl) (Metzker *et al*., 2014) compared to potassium ferricyanide (redox potential +0.23 V vs Ag/AgCl) (O'Reilly,

1973), was not utilized as an electron acceptor by *Av*LOx (Loew *et al*., 2017). The lactate sensors employing mPMS as the mediator showed a higher sensitivity and a wider linear range than those employing potassium ferricyanide as the mediator. Due to the low redox potential of mPMS (−0.11 V vs Ag/AgCl) (Kimura and Niki, 1985), the lactate sensors can be operated at a low potential. Therefore, employing mPMS as the mediator for lactate sensors based on *Av*LOx improves the accuracy of the lactate sensors by avoiding interference from redox substances in the blood (Loew *et al*., 2017). A commercially available blood lactate sensor based on LOx that employs mPMS in combination with hexaammine ruthenium(III) chloride supports these findings (Pharmaceuticals and Medical Devices Agency, *online*). In this commercial lactate sensor, both the effectivity of mPMS as the primary electron acceptor for LOx and the low potential of hexaammine ruthenium(III) chloride are exploited by fabricating lactate sensor strips with a low amount of mPMS to act as the primary electron acceptor and mediator between the enzyme and secondary electron acceptor and a high amount of hexaammine ruthenium(III) chloride to act as the secondary electron acceptor and mediator between the primary electron acceptor and the electrode (Pharmaceuticals and Medical Devices Agency, *online*).

Despite its many advantages, however, hexaammine ruthenium(III) chloride contains rare metal ruthenium, the use of which, considering preservation and sustainability, should be avoided in the future. The organic mediator mPMS is easily synthesized and does not contain any rare elements. Unfortunately, mPMS is not stable in acidic to neutral solutions (Kimura and Niki, 1985; Jahn *et al*., 2020; Dojindo Molecular Technologies Inc.b, *online*). The low stability of mPMS can shorten the shelf life of enzyme sensors employing this mediator.

In 2018, 1-methoxy-5-ethyl phenazinium ethyl sulfate (mPES) became commercially available. This new mediator is stable in solution over a wide pH range (Dojindo Molecular Technologies Inc.b, *online*; Yomo *et al*., 1989). Therefore, *Av*LOx-based lactate sensors containing mPES are expected to have a longer shelf life than corresponding sensors containing mPMS. The similar structure and similar electrochemical characteristics of mPES and mPMS suggest that sensors with mPES as the mediator can be developed with negligible interference from redox substances and thus similarly high accuracy. However, the utilization of mPES as a mediator in enzyme sensors has yet to be reported.

In this study, disposable, strip-type enzyme sensors based on representative flavin oxidoreductases, *Av*LOx, *Aspergillus flavus*-glucose dehydrogenase (*Af*GDH), *Aspergillus niger*-glucose oxidase (*An*GOx) and *Phaeosphaeria nodorum*-fructosyl peptide oxidase (*Pn*FPOx), employing mPES as an electron mediator, were constructed to assess the universal applicability of mPES as a mediator in enzyme sensor strips. This is the first application of mPES for disposable enzyme sensors. The storage stability of enzyme sensor strip employing *Av*LOx and mPES was evaluated and showing the stability of the developed enzyme sensor strip.

3-2. Materials and methods

3-2-1. Materials and devices

Here are the new materials used in this study in addition to that mentioned in Chapter 2 (Section 2-2-1). Sucrose, Tween 20 and silica bead desiccant were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). D(+)-Glucose was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fructosyl valine was purchased from Santa Cruz Biotech, Inc. (Dallas, Texas, USA). L(+)-ascorbic acid was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Uric acid and 4′-hydroxyacetanilide were

purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), while mPES was kindly provided by Dojindo Molecular Technologies Inc. (Kumamoto, Japan). Amicon Ultra-50 centrifugal filter unit (30-KDa cutoff) was purchased from Merck Millipore Ltd. (Carrigtwohill Co., Cork, Ireland). All other chemicals were of reagent grade. All solutions were prepared using pure water from a Milli-Q water purification system (Millipore Corporation, Burlington, MA, USA). All kinds of measurement were carried out with the same devices used in Chapter 2 (Section 2-2-1).

3-2-2. Enzyme preparation and activity evaluation

The LOx A96L mutant was produced by recombinant expression in *E. coli* according to the methods by Hiraka *et al*. (2018) with the following minor modifications. The cells of *E. coli* harboring LOx A96L mutant were cultivated in 100 mL ZYP-5052 medium and incubated at 30^{o}C and 120 rpm for 36 h. Cell disruption to obtain crude enzyme, purification to obtain purified enzyme, and evaluation of enzyme activities of LOx A96L mutant was carried out following the methods described in Section 2-2-2.

Glucose dehydrogenase V149C/G190C mutant derived from *Aspergillus flavus* (*Af*GDH CC mutant) was prepared following the methods by Sakai *et al*. (2015). Glucose oxidase derived from *Aspergillus niger* (*An*GOx) was prepared following the methods by Horaguchi *et. al*. (2012) and Okurita *et al*. (2018). Fructosyl peptide oxide N56A mutant derived from *Phanaerous nodum* (*Pn*FPOx N56A mutant) was prepared following the methods by Kim *et al*. (2012).

3-2-3. Evaluation of electrochemical behavior of mPES

The CV measurements was carried following the methods mentioned in the previous study (Section 2-2-3). Solution of 1 mM mPES or mPMS in 4 mM of 100 mM

PPB (pH 7.0) was measured with scan rate 0.1 V/s using GC as WE, Pt wire as CE and Ag/AgCl as RE.

3-2-4. Fabrication of lactate and evaluation of the sensor response

The mixture of LOx, mPES, sucrose and Tween 20 was prepared in 100 mM PPB (pH 7.0). One microliter of the mixture, containing 1 U enzyme, 100 mM mediator, 4% sucrose and 2% Tween 20, was deposited onto the WE of an SPCE (Figure 3-1). The SPCE attached with the mixture solution was dried at RT at less than 1% relative humidity (RH) for 3 h. This lactate sensor resulted in an amount of 1 U enzyme, 100 nmol mPES, 40 μg sucrose, and 20 μg Tween 20 per sensor strip. A spacer (75 μm thickness) and a cover were attached to the dry modified SPCE to form a μL-volume capillary space above the electrode, resulting in disposable lactate sensors.

CA measurements were carried out to evaluate the response currents toward lactate by loading 1.0 μL lactate (0–50 mM) onto the sensor strip, which was connected to the potentiostat. A potential of +0.2 V (vs Ag/AgCl) was applied 60 s after the addition of lactate, and the response currents was monitored for another 60 s. The sensitivity, the linear range, the limit of detection (LOD), the limit of quantitation (LOQ), and reproducibility based on the relative standard deviation (RSD) value were calculated as follows.

Sensitivity (μA/mM) = the slope of the calibration curves obtained from plotting lactate concentrations versus response currents.

Linear range (mM) = the range of lactate concentration obtained from the linear line of the calibration curves of lactate concentrations versus response currents.

$$\text{LOD (mM)} = \frac{3.3 \times \text{standard deviation of background current (0 mM lactate)}}{\text{slope (sensitivity of lactate sensor)}}$$

$$\text{LOQ (mM)} = \frac{10 \times \text{standard deviation of background current (0 mM lactate)}}{\text{slope (sensitivity of lactate sensor)}}$$

$$\text{RSD (\%)} = \frac{100 \text{ x standard deviation of particular point (i.e.,for currents obtained with 2 mM lactate)}}{\text{mean of response current with 2 mM lactate } (n = 3)} 100\%$$

The lactate sensors were constructed by applying 25 or 50 mM mPES per sensor strip. The response currents obtained were compared with the lactate sensors applying 100 mM mPES.

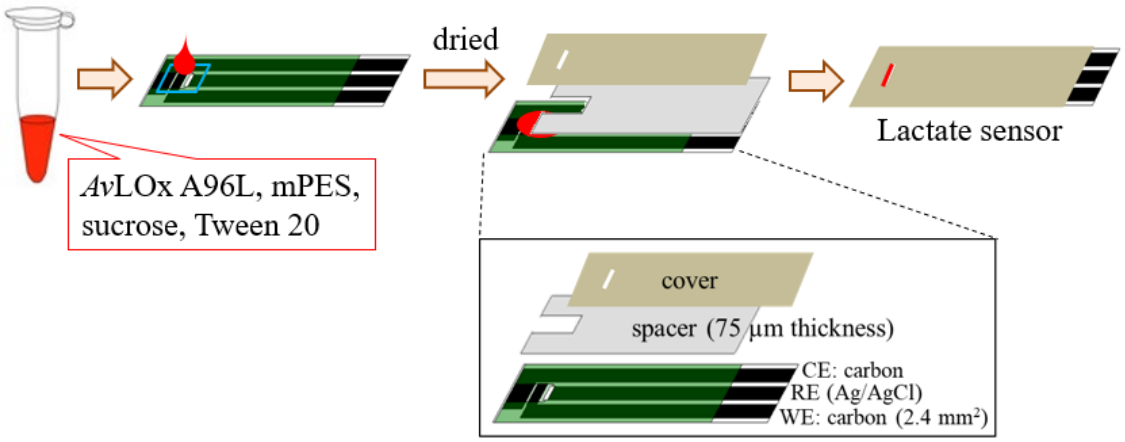


Figure 3-1. Construction of lactate sensor using screen-printed carbon electrode (SPCE). Inset: components of the SPCE.

3-2-5. Evaluation of response currents of lactate sensors with different application potentials

Lactate sensors were fabricated according to the method described in Section 3-2-4. The response currents were evaluated by applying a potential of 0 V, +0.05 V, +0.1 V, or +0.2 V vs Ag/AgCl.

3-2-6. Evaluation of lactate sensors toward interferences

Lactate sensors were fabricated according to the method described in Section 3-2-4. The sensors were evaluated using mixtures of 2 mM or 10 mM lactate containing 0.17 mM ascorbic acid, 0.1 mM uric acid, or 0.3 mM acetaminophen. As with pure lactate solutions, 1.0 μL of the mixture was loaded onto the lactate sensor, and the response current was monitored.

In other evaluation of lactate sensors, response currents to blank sample or 10 mM ascorbic acid, uric acid, or acetaminophen were measured and the results were compared with the response currents obtained with 10 mM lactate. Furthermore, response currents of lactate sensors toward 10 mM ascorbic acid were measured with applied potential 0 V, +0.05 V, +0.1 V or +0.2 V (vs Ag/AgCl).

3-2-7. Evaluation of storage stability of lactate sensors

Lactate sensors were fabricated following the method described in Section 3-2-4. At first, the storage stability evaluation in a relatively short period was carried out. For this study, 5 U enzyme per strip was applied. The lactate sensors were divided into several sets, containing a minimum of 18 sensors each. Each set was wrapped with aluminum foil. All sets were put in into a dark plastic box containing silica bead desiccant and stored at 25 ^{o}C until 50 days. After 2 days (prestorage), the response currents of one set of lactate sensor were evaluated with 0–50 mM lactate, and the

obtained data set was used as a control (Day-0). Next sets of lactate sensors were evaluated after 12, 28, and 48 more days of storage (Day 12, Day 28, and Day 48).

The storage stability of lactate sensors for a relatively long period was also evaluated. For this study, 1 U enzyme per strip was applied. The methods to prepare the lactate sensors and the storage are the same as mentioned above. The response currents of lactate sensors were evaluated after storage for 0 (without prestorage), 90, 100, 120 days.

3-2-8. Evaluation of stability of mPES electrode strips

One microliter of 100 mM mPES solution was deposited onto the SPCEs and dried for 2 h at RT with a very low humidity. The treated SPCEs were covered with a spacer and a cover resulting in the mPES/SPCE. As well as in lactate sensors, the mPES/SPCE were divided into several sets, containing a minimum of 18 strips each and stored at 25 or 45 ^{o}C for 30 days in a dark plastic box containing silica bead desiccant.

One set of mPES/SPCE was evaluated without storage. A premix containing LOx, sucrose and Tween 20 was prepared in 100 mM PPB (pH 7.0). Lactate solution (final concentration 0–50 mM) was then added to the premix. Soon after that, 1.0 μL of the mixture was loaded onto the SPCE which was connected to the potentiostat. Thus, the final concentration of mPES, LOx, sucrose and Tween 20 at sensor strip was 100 nmol, 5 U, 40 μg, and 20 μg, respectively. The CA measurement was started after 60 s from the addition of the mixture by applying the potential of +0.2 V (vs Ag/AgCl) for the next 60 s.

3-2-9. Employing mPES as an electron mediator for other enzyme sensors

The corresponding enzyme sensors were fabricated utilizing the enzymes GDH or FPOx (0.1 U per sensor strip) or GOx (0.05 U per sensor strip) following the same

method described in Section 3-2-4. The response currents were evaluated by loading the substrate solutions of 0–50 mM glucose for sensors based on GDH or GOx, or 0–10 mM fructosyl valine for sensors based on FPOx. The potential was applied 90 s after loading the solution.

3-3. Results

3-3-1. Electrochemical behavior of mPES

Cyclic voltammogram of 1 mM mPES was obtained from the CV measurement and the result was compared with the cyclic voltammogram of mPMS, the most effective mediator for *Av*LOx in our previous study (Chapter 2), which also a derivative of phenazine. The oxidation (anodic) and reduction (cathodic) peak potential of both mPES and mPMS were observed at similar potential, –0.11 V and –0.17 V, respectively (Figure 3-2). Thus, the calculated $E_{1/2}$ are the same, –0.14 V (vs Ag/AgCl). The oxidation and reduction peak current of mPES were observed at 170 μA and –40 μA, respectively. Whereas for mPMS, the lower peak currents were observed, 103 μA and –22 μA for the oxidation and reduction peak, respectively.

The electrochemical behaviors of mPES were considered to determine the redox potential application for CA measurement to evaluate the response currents of the enzyme sensors utilizing mPES as a mediator. The potential higher than the half-wave potential of the mediator is needed to oxidize the mediator at the electrode. Therefore, potential higher than –0.14 V (vs Ag/AgCl) was determined to apply in CA measurements.

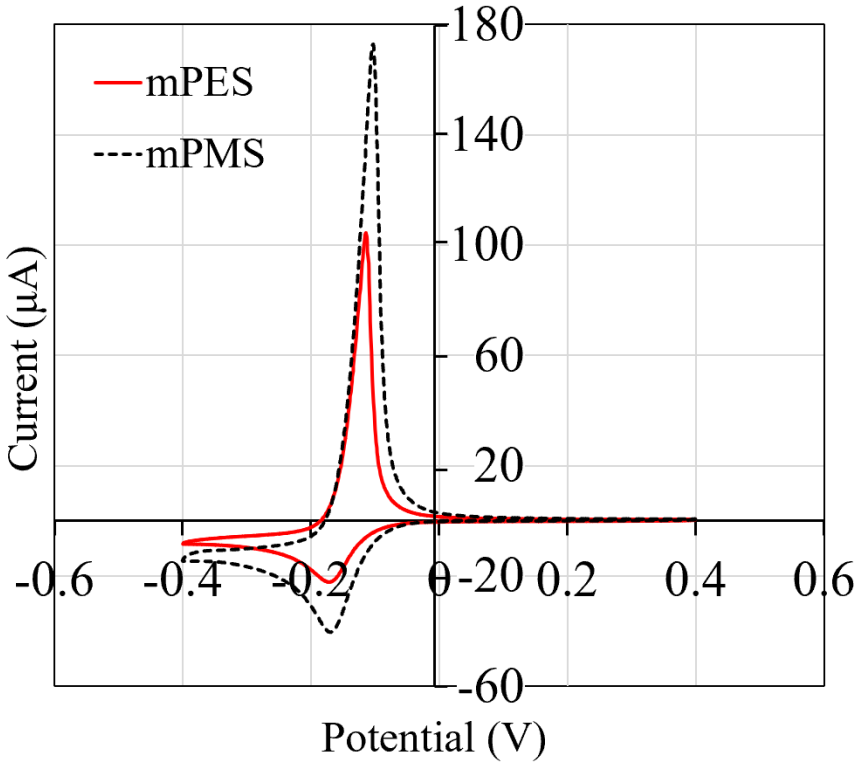


Figure 3-2. Cyclic voltammograms of 1 mM mPES (red line) or mPMS (black dash line) in 4 mL of 100 mM PPB pH 7.0). Potential range: 0.4 V to –0.4 V. Scan rate: 0.1 V/s; WE: GC; RE: Ag/AgCl; CE: Pt wire.

3-3-2. Enzyme activity of enzymes

The purified enzymes were obtained and the properties were determined (Table 3-1).

Table 3-1. Properties of the enzymes used in this study[1].

Enzymes	V_{max} (U/mg)	K_m (mM)	Reference
*Av*LOx A96L	140	0.81	Hiraka *et al*., 2018
*Af*GDH CC	n.i.	35	Sakai *et al*., 2015
*An*GOx	8.3	n.i.	Horaguchi *et. al*., 2012; Okurita *et al*., 2018
*Pn*FPOx	56	0.68	Kim *et al*., 2012

[1] From dye-mediated dehydrogenase activity; measured with PMS/DCIP system.
n.i.: no information.

3-3-3. Construction of disposable lactate sensor employing mPES as a mediator

In this study, the versatility of mPES was demonstrated by constructing a disposable type lactate enzyme sensor strip, employing SCPE as the representative disposable electrode. A solution containing mediator and enzyme was dried onto a film electrode, and a spacer and cover were attached to complete the disposable lactate sensor. The novel, stable mPES (Dojindo Molecular Technologies Inc.b) was employed as the mediator, and O_2 insensitive *Av*LOx A96L mutant was employed as the enzyme (Hiraka *et al*., 2018). When the sample lactate solution was loaded into the sensor strip, the LOx and the mediator dissolved, and the enzyme reaction began. The time between the start of the enzyme reaction and application of the potential (start of the electrochemical reaction) is known as the waiting time. The waiting time was optimized to 60 s to achieve complete oxidation of lactate by the enzyme and thus full turnover. The response currents observed after application of the potential were generated by oxidation of the reduced mediator at the electrode; the initial amount of the reduced mediator was proportional to the initial amount of lactate in the sample.

Construction of disposable lactate sensor employing 100 mM mPES

The response currents toward 0–50 mM lactate were monitored amperometrically at an operation potential of +0.2 V (vs Ag/AgCl), which was determined from cyclic voltammogram of the mediator (Figure 3-2). An initial high response current was observed, which gradually decreased with time until it reached a steady state. In Figure 3-3a, the time courses of the response currents, or amperograms, are shown. The current at 10 s after potential application was plotted against the lactate concentrations to obtain the calibration curve (Figure 3-3b). The observed response currents increased linearly depending on the lactate concentration. A wide linear range of up to 50 mM lactate was obtained. The sensitivity of the lactate sensor, defined as the slope of the linear range, was 0.73 ± 0.12 μA/mM. The lactate sensor also showed good reproducibility at each tested lactate concentration (RSD < 7%) (properties are summarized in Table 3-3). The LOD and LOQ was 0.5 mM and 1.8 mM, respectively. These results show that the lactate sensor was constructed successfully, and that lactate can be measured with this lactate sensor.

Evaluation of various mPES concentration for disposable lactate sensors

The lactate sensors employing various concentration of mPES (25-100 mM) were evaluated. It was found that the lactate sensors with 100 mM mPES showed better performances than applying lower concentration of mPES (25 or 50 mM), achieving highest sensitivity (0.58 μA/mM) with a wide linear range, up to 50 mM lactate (Figure 3-4). Lactate sensors with 50 mM mPES were obtained the same linear range, but the sensitivity is lower (0.48 μA/mM) and the reproducibility of the response currents showed not as good as the lactate sensors with 100 mM lactate. For lactate sensors with 25 mM mPES, the sensitivity is much lower (0.22 μA/mM), and so is the linear range of

the sensors, up to 30 mM lactate. As this is the end-point assay type evaluation, the concentration of 25 mM mPES is not enough to oxidize lactate higher than 30 mM within the waiting time (60 s), thus the linear range exceed 30 mM lactate cannot be reached. Therefore, concentration of 100 mM mPES was decided to use to fabricate the lactate sensors for further evaluation.

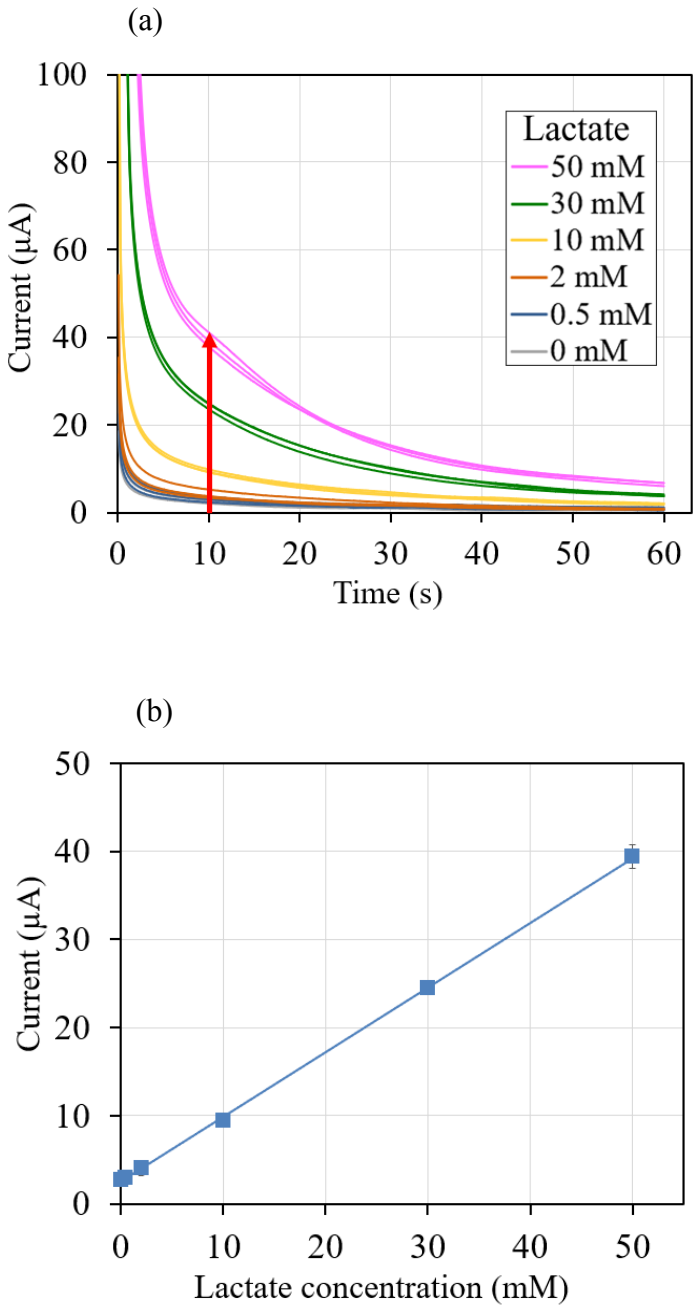


Figure 3-3. Response currents of lactate sensors; (a) time courses and (b) calibration curve (linear regression: $y = 0.73x + 2.5$; $R^2 > 0.99$). The arrow in (a) indicates increasing lactate concentration. Sensor composition: 1 U *Av*LOx A96L, 100 nmol mPES, 40 µg sucrose, and 20 µg Tween 20. Waiting time: 60 s; applied potential: +0.2 V (vs Ag/AgCl); $n = 3$. Sampling point for the calibration curve: at 10 s.

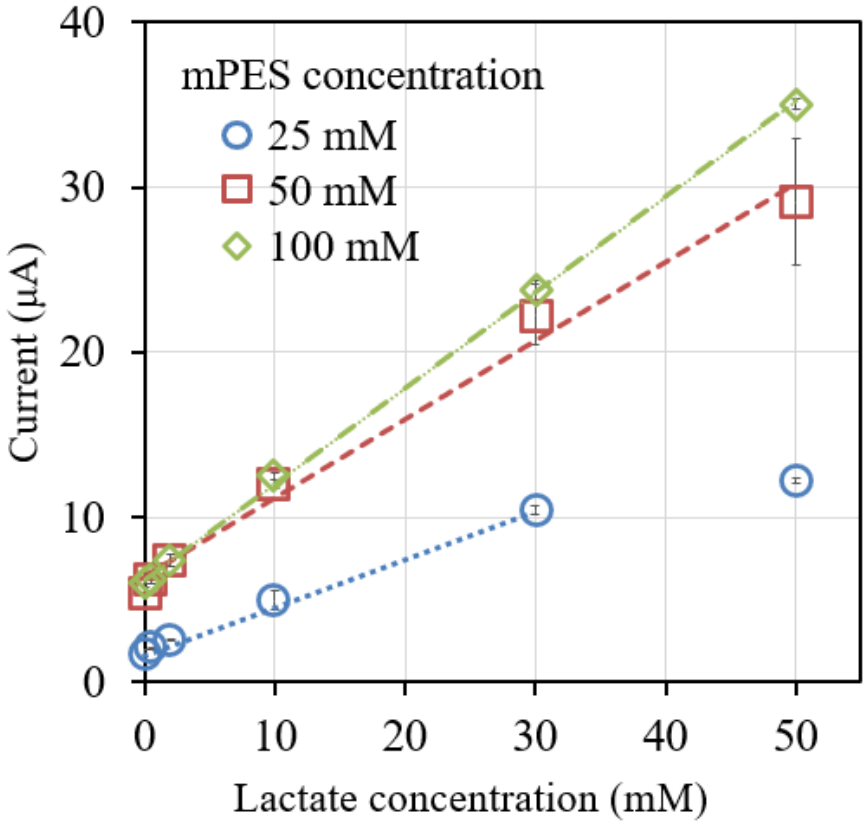


Figure 3-4. Response currents of lactate sensors with various concentration of mPES: 25 mM (linear regression: $y = 0.27x + 1.9$; $R^2 > 0.99$; liner range 30 mM), 50 mM (linear regression: $y = 0.48x + 6.4$; $R^2 > 0.99$; liner range 50 mM) and 100 mM (linear regression: $y = 0.58x + 6.2$; $R^2 > 0.99$; liner range 50 mM). Sensor composition: 1 U *Av*LOx A96L, 25–100 nmol mPES, 40 µg sucrose, and 20 µg Tween 20. Waiting time: 60 s; applied potential: +0.2 V (vs Ag/AgCl); $n = 3$. Sampling point for the calibration curve: at 10 s.

3-3-4. Optimization of operation potentials

To optimize the operation potential, response currents were evaluated with different applied potentials, from 0 to +0.2 V (vs Ag/AgCl) (Figure 3-5). A clear dependency of the response current on the lactate concentration was observed at all potentials. However, at 0 V applied, the linear range extended only up to 30 mM (Figure 3-5a). At higher operating potentials, the linear range extended up to 50 mM (Figure 3-5b–d). This is evidenced in the fact that the linear regression of the response currents obtained has the same slope and a high R^2 value with and without the response current value obtained with 50 mM lactate included when a potential higher than 0 V was applied (continuous vs dashed lines in Figure 3-5b–d). When 0 V was applied, the response current value obtained with 50 mM lactate significantly deviated from the linear regression line obtained with response current values up to 30 mM lactate (Figure 3-5a).

A linear range of up to 30 mM lactate, which was obtained at all tested operation potentials, is more than enough to cover the standard range of blood lactate levels in humans, which can increase to a maximum of 25 mM (Loew *et al*., 2017). The lactate sensor showed a good sensitivity of approximately 0.7 μA/mM, defined as the slope of the linear calibration, for the range of up to 30 mM lactate for all evaluated potentials, indicating that the sensor can be used at any operating potential between 0 V and +0.2 V (vs Ag/AgCl) for measurements of up to 30 mM lactate. If higher lactate concentrations are expected, an operation potential of +0.05 V or higher should be used.

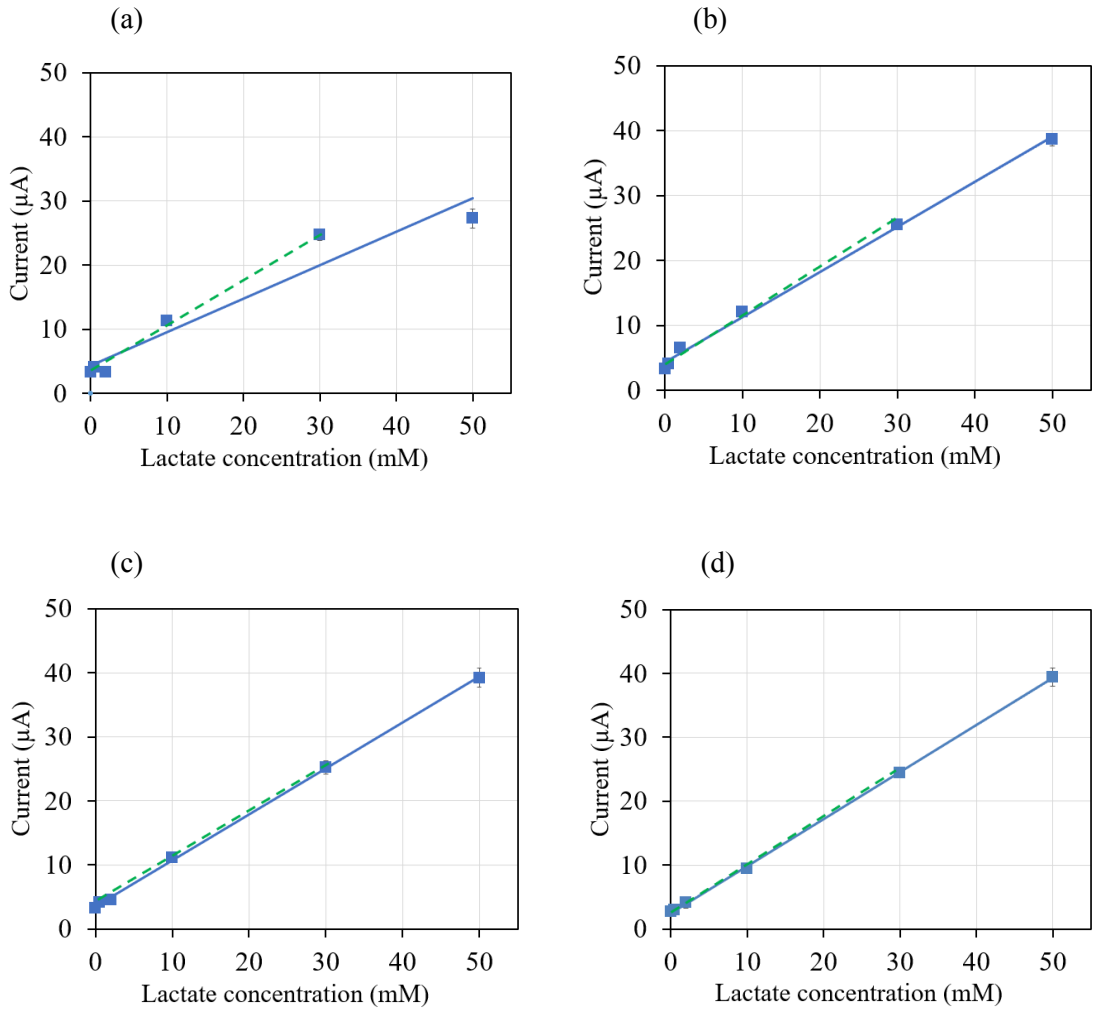


Figure 3-5. Response currents at different application potentials: (a) 0 V, (b) +0.05 V, (c) +0.1 V, and (d) +0.2 V (vs Ag/AgCl). Blue continuous line: linear regression for the range of up to 50 mM; green dashed line: linear regression for the range of up to 30 mM. Sensitivity for the range up to 50 mM: 0.52 µA/mM (R^2 = 0.93) in (a) and 0.69–0.73 µA/mM (R^2 > 0.99) in (b–d). Sensitivity for the range up to 30 mM: 0.69–0.73 µA/mM (R^2 > 0.99) in (a–d). Sensor composition: 1 U *Av*LOx A96L, 100 nmol mPES, 40 µg sucrose, and 20 µg Tween 20. Waiting time: 60 s; *n* = 3. Calibration curves at sampling point: 10 s.

3-3-5. Interference study of common redox substances in blood

The response of the lactate sensors in the presence of possibly interfering redox substances was evaluated. For this, mixtures of 2 mM and 10 mM lactate with either ascorbic acid, uric acid, or acetaminophen were loaded into the sensor strips. In the presence of ascorbic acid, the observed response currents were higher than the corresponding signals of samples without ascorbic acid (Figure 3-6). Student's unpaired t-test showed a p value of < 0.05, indicating a significant difference. In the presence of uric acid or acetaminophen, there was no significant difference in the response currents compared to corresponding currents in the absence of redox substances ($p > 0.05$). Measurements of samples with 0 mM lactate and 10 mM interferent were also carried out, with corresponding results (Figure 3-7). These results suggest that the lactate sensors are affected by ascorbic acid but not by uric acid or acetaminophen.

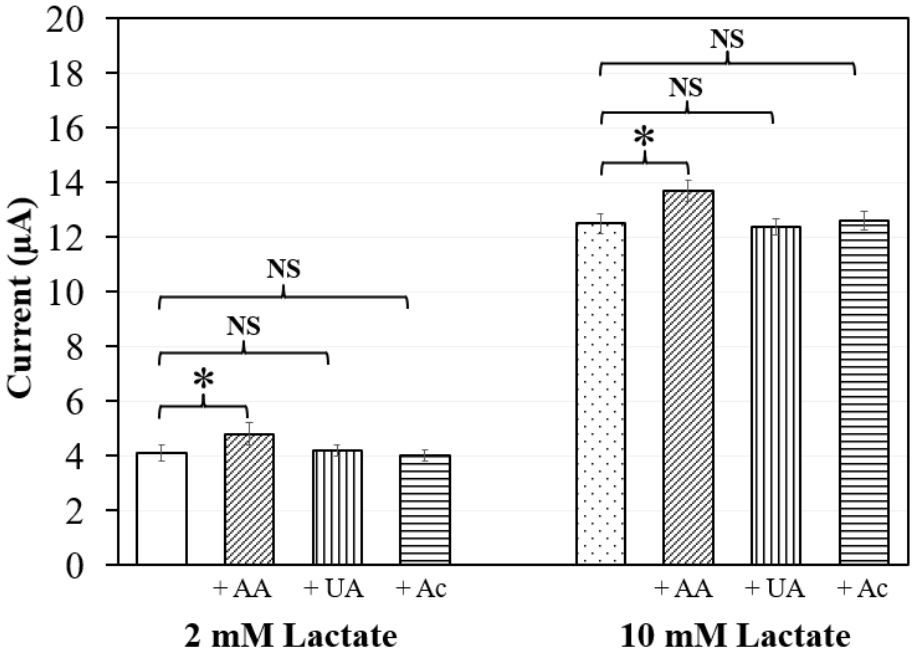


Figure 3-6. Response currents of lactate sensors to 2 mM or 10 mM lactate in the absence and presence of redox substances: 0.17 mM ascorbic acid (AA), 0.1 mM uric acid (UA) or 0.3 mM acetaminophen (Ac). * Student's unpaired t-test value $p < 0.05$ (suggesting a significant difference between data); NS: $p > 0.05$ (suggesting no significant differences between data). Sensor composition: 1 U *Av*LOx A96L, 100 nmol mPES, 40 µg sucrose, and 20 µg Tween 20. Waiting time: 60 s; applied potential: +0.2 V vs Ag/AgCl; $n = 3$. Sampling point: 10 s.

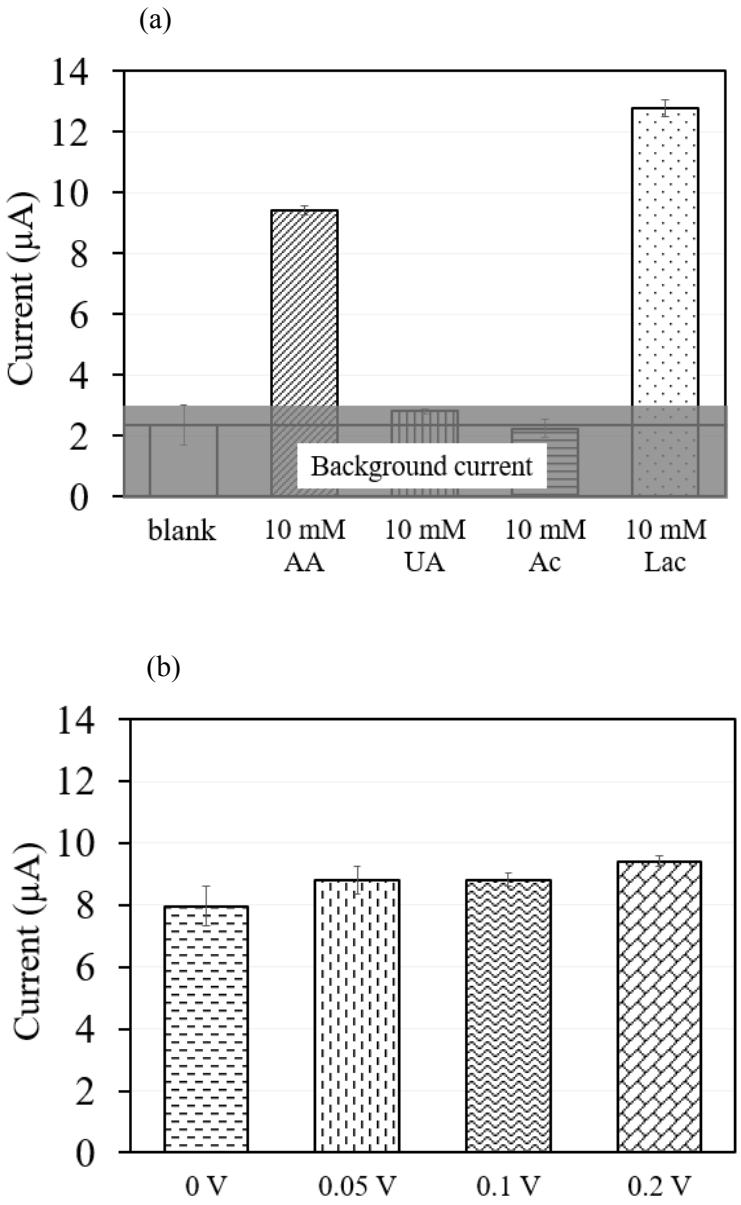


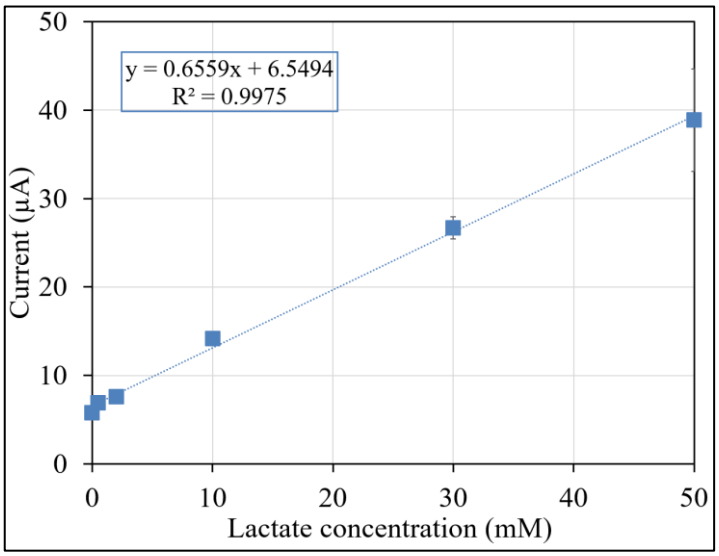
Figure 3-7. Response currents of lactate sensors toward common redox substances. (a) Response currents to blank sample and 10 mM ascorbic acid (AA), uric acid (UA), acetaminophen (Ac), and lactate (Lac). Applied potential: +0.2 V (vs Ag/AgCl). Gray area: background current. (b) Response currents to 10 mM ascorbic acid at various applied potentials. Sensor composition: 1 U *Av*LOx A96L, 100 nmol mPES, 40 μg sucrose, and 20 μg Tween 20. Waiting time: 60 s; $n = 3$. Sampling point: 10 s.

3-3-6. Storage stability of the lactate sensors

The storage stability of the lactate sensors was evaluated. Priory, lactate sensors with different amount of enzyme were optimized (Figure 3-8). Furthermore, lactate sensor with 5 U of LOx A96L per sensor strip was chosen for storage stability evaluation (Figure 3-9), to avoid the case that enzyme inactivation would be the major factor to show a decrease in the sensor performance. The stability of the sensor with 1 U of LOx A96L per sensor is shown in Figure 3-10, where no significant difference was observed from those of the sensor with 5 U of enzyme. The lactate sensors restored for up to 2 + 48 days at 25 °C in the dark, with the first 2 days designated as prestorage. Sets of sensors were evaluated at 0, 12, 28, and 48 days of main storage (Figure 3-8). The sensitivity for calibration of up to 30 mM lactate was approximately 0.4 μA/mM at 0 days of main storage. The lower sensitivity compared to the sensors in Sections 3-3-2 and Section 3-3-3 can be explained by the increased amount of protein. However, the sensitivity is still acceptably high. The detection limit of the sensor also did not change during the storage for 48 days at 25 °C (Table 3-2).

The sensitivity for calibration of up to 30 mM lactate did not change significantly for 48 days of storage (Figure 3-9). The response current obtained with 50 mM lactate, however, decreased slightly with storage time. To support these findings, electrode strips containing only mediator were prepared and evaluated using a mixture of LOx A96L mutant and lactate (Figure 3-11). The excellent storage stability of the mediator electrode strips at 25 °C and good storage stability at 45 °C suggests that the slight decrease in sensitivity to 50 mM lactate might be due to the enzyme. Therefore, this sensor can be used for measurements of up to 30 mM lactate after up to 48 days of storage at 25 °C in the dark without loss of sensitivity. For measurements of higher lactate concentrations, new lactate sensors should be used.

(a)



(b)

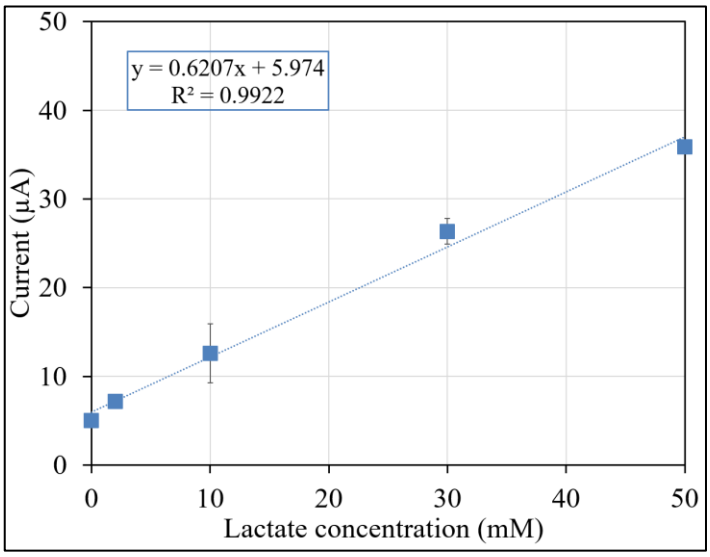


Figure 3-8. Calibration curves of lactate sensors with (a) 5 U and (b) 10 U enzyme. Linear regression: up to 50 mM lactate; sensitivities: 0.66 and 0.62 µA/mM ($R^2 > 0.99$) for 5 U and 10 U enzyme, respectively. Sensor composition: 5 or 10 U *Av*LOx A96L, 100 nmol mPES, 40 µg sucrose, and 20 µg Tween 20. Waiting time: 60 s; applied potential: +0.2 V (vs Ag/AgCl); n = 3. Sampling point for the calibration curve: at 10 s.

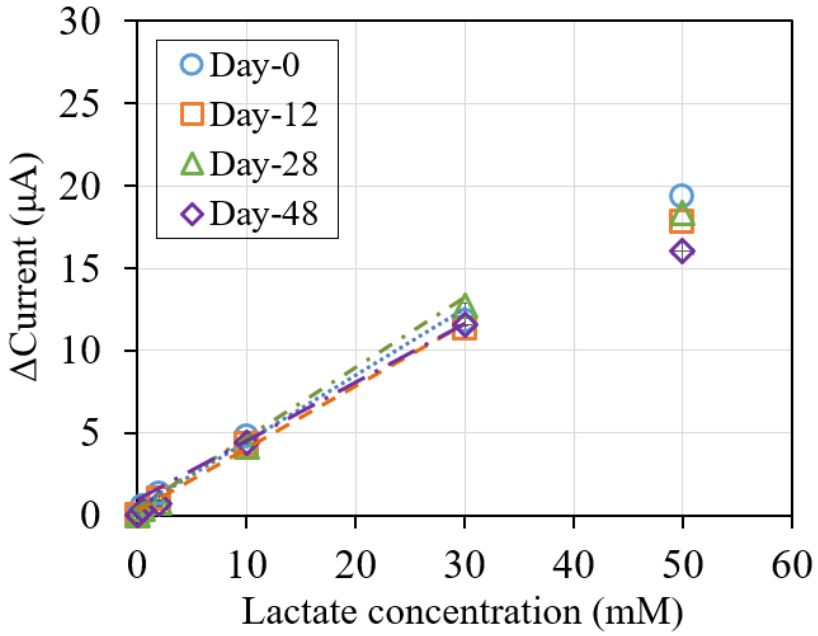


Figure 3-9. Calibration curves of lactate sensors stored at 25 °C in the dark, evaluated after storage for 2 days (prestorage) + 0, 12, 28, or 48 days. Linear regression was determined for up to 30 mM lactate; sensitivities were 0.38–0.42 μA/mM ($R^2 > 0.99$) for all calibration curves. Sensor composition: 5 U *Av*LOx A96L, 100 nmol mPES, 40 μg sucrose, and 20 μg Tween 20. Waiting time: 60 s; applied potential: +0.2 V (vs Ag/AgCl); $n = 3$. Sampling point: 10 s.

Table 3-2. The limit of detection (LOD) of the lactate sensor with 5 U *Av*LOx A96L during storage.

Day storage [1]	0	12	28	48
LOD (mM)	1.1	1.1	0.5	1.0

[1] with 2 days prestorage

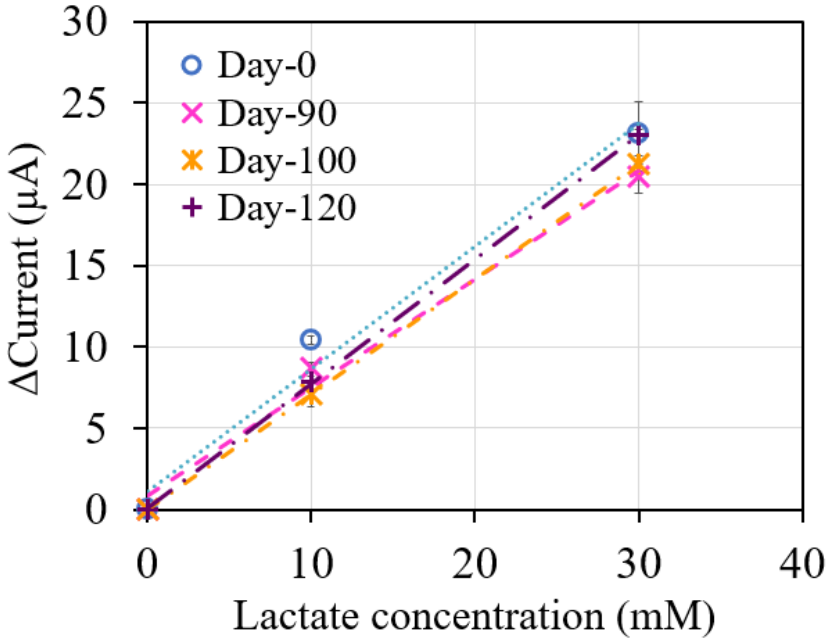


Figure 3-10. Storage stability of lactate sensors with 1 U of *Av*LOx A96L. Calibration curves of lactate sensors stored at 25 °C in the dark, evaluated after storage for 0, 90, 100 or 120 days. Linear regression was determined for up to 30 mM lactate; sensitivities were 0.68–0.77 μA/mM ($R^2 > 0.98$) for all calibration curves. Sensor composition: 1 U *Av*LOx A96L, 100 nmol mPES, 40 μg sucrose, and 20 μg Tween 20. Waiting time: 60 s; applied potential: +0.2 V (vs Ag/AgCl); $n = 3$. Sampling point: 10 s.

3-3-7. Storage stability of mPES at the electrode strip

The stability of the stored mPES in dry condition in SPCE was evaluated by monitoring the response currents of lactate oxidation catalyzed by LOx, following the same principle of lactate sensor evaluation. The mPES/SPCEs were stored at 25 ^{o}C, a representative of room temperature, or at 45 ^{o}C in aiming to accelerate the mPES deactivation.

The response currents of mPES/SPCE evaluated with lactate and LOx A96L mutant were clearly observed. The higher the lactate concentration higher response currents were obtained. The calibration curves showed that the linear dependencies were obtained (up to 30 mM lactate) and the sensitivities can be determined (Figure 3-11), suggesting that the lactate can be measured with this system. The sensitivities obtained did not show decreasing before and after 30 days storage both in 25 ^{o}C or 45 ^{o}C, thus showing the mPES/SPCEs are stable during that period even in such high temperature. An increasing of response currents stored at 45 ^{o}C after 30 days in the high lactate concentrations may be attributed to the manually evaluation process when applying the mixture containing LOx and lactate to the mPES/SPCE.

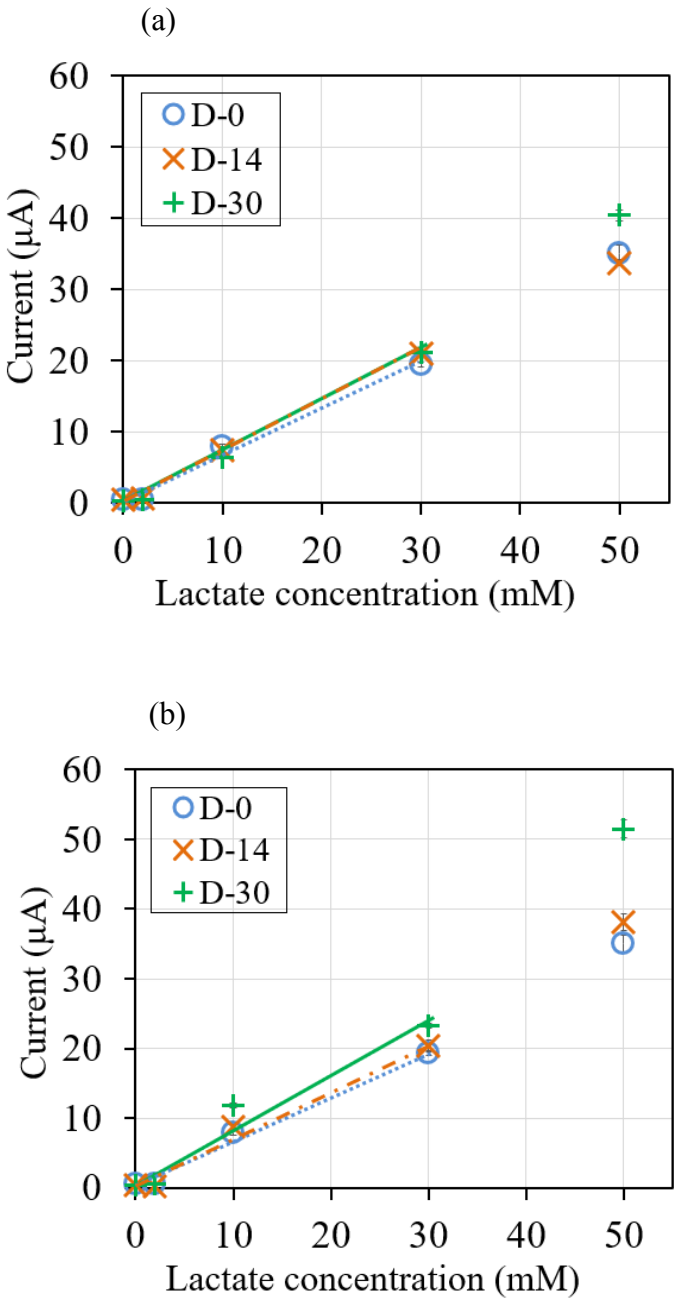


Figure 3-11. Stability of mPES electrode strips stored at (a) 25 °C or (b) 45 °C, evaluated with *Av*LOx A96L mutant. Sensor composition: 100 mmol mPES. Sample composition: 0–50 mM lactate, 5 U/µL *Av*LOx A96L, 4% sucrose, and 2% Tween 20 in 100 mM PPB (pH 7.0). Waiting time: 60 s; applied potential: +0.2 V (vs Ag/AgCl); $n = 3$. Sampling point: 10 s. Sensor strips were stored for 0, 14, or 30 days at (a) 25 °C or (b) 45 °C in the dark. Linear regression: 0–30 mM lactate; sensitivities: 0.65–0.78 µA/mM; $R^2 > 0.95$.

3-3-8. mPES as an electron mediator for other enzyme sensors

The enzyme sensor strips were constructed utilizing mPES and diagnostic enzymes *Af*GDH, *An*GOx, or *Pn*FPOx to investigate the usability of mPES as a mediator for other enzymes. Evaluation of the response currents toward glucose for the biosensors based on *Af*GDH or *An*GOx and fructosyl valine for the biosensors based on *Pn*FPOx were carried out. The response currents at 10 s were plotted against the analyte concentration, and calibration curves were obtained (Figure 3-12).

A clear linear dependency of the response currents on the analyte concentration was observed, with a linear range of up to 50 mM glucose for the sensor strips containing *Af*GDH or *An*GOx, and up to 10 mM fructosyl valine for the sensor strips containing *Pn*FPOx. These results show that *Af*GDH-, *An*GOx-, and *Pn*FPOx-based enzyme sensor strips were constructed successfully utilizing mPES as a mediator.

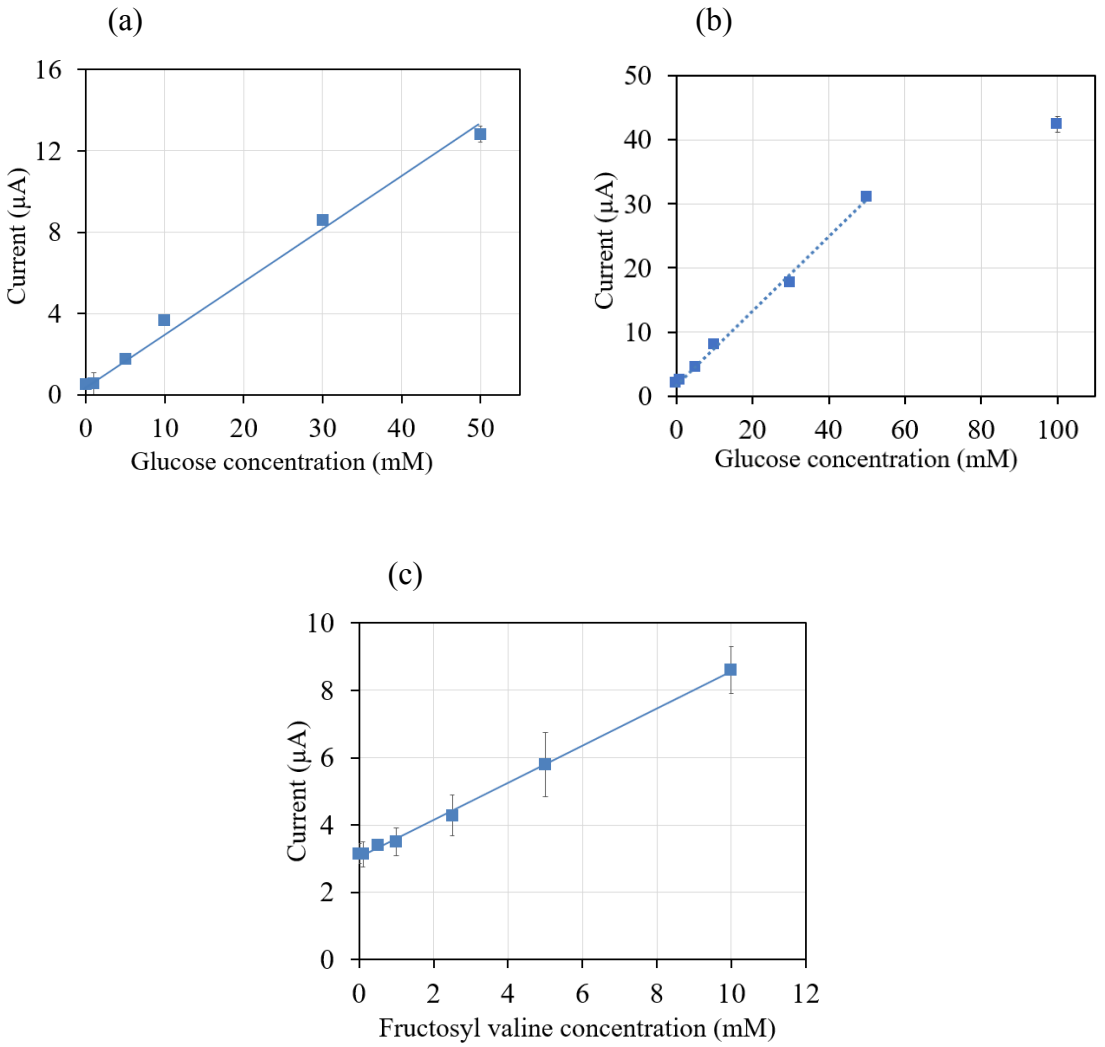


Figure 3-12. Calibration curves of biosensors utilizing mPES and (a) *Af*GDH CC mutant (linear regression: y = 0.25x + 0.65; R^2 = 0.99), (b) *An*GOx (linear regression: y = 0.57x + 1.8; R^2 = 0.99), or *Pn*FPOx N56A mutant (linear regression: y = 0.55x + 3.0; R^2 > 0.99). Sensor composition: 0.1 U enzyme, 100 nmol mPES, 40 µg sucrose, and 20 µg Tween 20. Waiting time: 90 s; applied potential: +0.2 V (vs Ag/AgCl); *n* = 3. Sampling point: 10 s.

Table 3-3. Properties of biosensors constructed in this study.

Enzymes	Enzyme per strip (U)	Linearity (mM)	Sensitivity (μA/mM)	R^2	LOD (mM)	RSD (%)
*Av*LOx A96L mutant	1	50 [a]	0.73 ± 0.12	0.997	0.5	< 7.8 [d]
*Af*GDH CC mutant	0.1	50 [b]	0.25	0.994	2.6	< 3 [e]
*An*GOx	0.1	50 [b]	0.57	0.996	0.2	< 9.5 [e]
*Pn*FPOx N56A mutant	0.1	10 [c]	0.55	0.998	1.1	< 8 [g]

Substrates: [a] lactate, [b] glucose, and [c] fructosyl valine

Substrate concentrations tested: [d] all; [e] 10 mM; [f] 1, 5, 10 mM; [f] 0.5 and 10 mM

3-4. Discussion

In this study, a novel electron mediator, mPES, was introduced as a versatile mediator for disposable enzyme sensor strips, employing representative flavin oxidoreductases, *Av*LOx, *Af*GDH, *An*GOx and *Pn*FPOx.

In the previous work, an oxygen insensitive mutant, *Av*LOx A96L, was constructed, and it was demonstrated the employment of this mutant was the solution to overcome the inherent problem of oxidase-based 2nd generation electrochemical biosensors that the sensor signal is affected by oxygen (Hiraka *et al*., 2018). However, the appropriate mediator for the lactate sensor employing *Av*LOx A96L was not evaluated. In the other achievement, the investigation of electron mediators for enzyme sensors employing *Av*LOx was reported, where concluded that mPMS was the best mediator for *Av*LOx (Loew *et al*., 2017). However, mPMS is recognized as an unstable mediator, especially in neutral condition (Kimura and Niki, 1985; Jahn *et al*., 2020; Dojindo Molecular Technologies Inc.b, *online*). Therefore, an alternate mediator for LOx-based enzyme sensors have been investigating. A novel mediator mPES, which is the derivative of mPMS, but is more chemically stable than mPMS, was used to construct a stable lactate sensor.

Priory, mPES as well also mPMS were characterized electrochemically by CV measurement. An identic $E_{1/2}$ of –0.14 V (vs Ag/AgCl) was obtained for both mPES and mPMS. This number is similar or even identic with the reported redox potential of mPMS (Kimura *et al*., 1985; Hisada *et al*., 1977). Therefore, the substitution of methyl group in mPMS to ethyl group in mPES did not change the electrochemical behavior of mPES. Furthermore, the potential to applied in CA measurement was decided, which should be higher than $E_{1/2}$. Thus, potential +0.2 V was used, it is the same potential with

those used to evaluate lactate sensors employing mPMS as a mediator in our previous study (Chapter 2).

Enzyme sensors employing the mentioned enzymes and mPES as mediator were constructed and the response currents were evaluated by CA measurements. The properties of the sensor strips investigated in this study are summarized in Table 3-1. All kind of enzyme sensors have a linear range that sufficiently covers the range needed for the measurement of blood lactate levels, blood glucose levels, and HbA1c values. All kind of enzyme sensors also have good sensitivity, a low LOD value, a high R^2 value for the linear regression, and a low RSD of the measurements. These parameters indicate the high accuracy of the sensors. In this study, to demonstrate the versatility of mPES, a SCPE was employed as the representative disposable electrode, which is widely utilized in the commercially available glucose enzyme sensor strips. Current results did not indicate any obstacle issues of mPES to be used for sensors with another electrode material, such as gold, platinum, and palladium, which are conventionally utilized for disposable sensor strips.

The method used in these enzyme sensors based on monitoring of lactate (*Av*LOx A96L), glucose (*Af*GDH or *An*GOx), and fructosyl amino acid (*Pn*FPOx) acid is an end-point assay, as has been utilized for commercially available glucose sensors for self-monitoring of blood glucose. Considering the amount of target molecule in 1 µl sample, 0.1 U of enzyme will be theoretically enough to oxidize an entire sample within the setting time for incubation (60 s for lactate and 90 s for glucose and fructosyl amino acid). In these types of enzyme sensors, the enzyme reaction reaches its equilibrium relatively fast, usually before the potential is applied. The response current is an indicator for the concentration of the reduced mediator at equilibrium. For example, in the current experiment with the sample volume of 1 µL with 50 mM of lactate sample,

there are 50 nmol of lactate and 1 U of LOx A96L. Considering the definition of 1 U unit of dye-mediated dehydrogenase activity was defined as the amount of enzyme necessary to catalyze the reduction of 1 μmol (1000 nmol) DCIP in 60 s at 25 °C, which corresponds to the oxidation of 500 nmol of lactate in 60 s, excess amount of LOx A96L existed on the electrode in this experiment. Usually, enzyme sensor strips contain an excess amount of enzyme, considering the shelf life of a sensor, as the enzyme gradually inactivates during the preservation. Enzyme of 1 U/sensor or 5 U/sensor of LOx A96L was chosen considering further investigation of mediator stability during the preservation, where enzyme stability would not be the limiting steps. For the investigation of the availability of mPES for *Af*GDH, *An*GOx, and *Pn*FPOx, where the stability investigation was not planned, 0.1 U/sensor of enzymes were employed with 90 s for enzyme reaction, which provided enough time to arrive at end points for each experiment.

A high mediator concentration (100 mM) is used to study the application of mPES as a mediator. This is important in the end point assay which is the enzyme reaction should be finished with in the short period. For that, the high concentration of mediator is required even higher than substrate concentration. When applying lower concentration of mediator (25 or 50 mM mPES), the lower sensitivities were obtained. These phenomena were similar as well as explained in the first study (Chapter 2), that the response currents observed are limit by mediator concentration (in condition of high lactate concentration).

In addition to the characteristics of the enzyme, the characteristics of the mediator are a key factor in determining the properties of 2nd generation electrochemical biosensors. A commonly considered characteristic of the mediator is its redox potential, which determines the possible operating potentials of the sensor and thus influences the

accuracy in terms of sensitivity to redox substance-type interference. Here, mPES has a low redox potential, which results in a low operating potential of +0.2 V. An even lower operating potential can be used with mPES as a mediator, although this might come at the cost of a narrower linear range (Figure 3-4).

A low operating potential generally means few interferences by redox substances that can be directly oxidized at the electrode. Three common redox substances in blood are ascorbic acid, uric acid, and acetaminophen, with the redox potential approximately +0.16 V, +0.45 V (Kamel *et al*., 2014), and +0.2 V (vs Ag/AgCl) (Wangfuengkanagul and Chailapakul, 2002), respectively. In interference tests using the LOx-based sensors with 2 mM and 10 mM lactate, which are within and above the physiological range of lactate concentration in blood, respectively, the results showed that the operating potential of +0.2 V is sufficiently low to eliminate interference by uric acid and acetaminophen (Figure 3-6). This was further supported by the fact that the sensors showed a response similar to the background (0 mM lactate) to 10 mM uric acid or acetaminophen (Figure 3-7).

The application potential below +0.4 V vs Ag/AgCl can minimize the effect of the interferences such as ascorbic acid, uric acid (Yuan *et al*., 2005) and acetaminophen (Cho *et al*., 2015). However, a small, yet significant, bias in the response current is observed in the presence of ascorbic acid at an operating potential of +0.2 V (Figure 3-6), despite the potential being lower than the redox potential of ascorbic acid. Furthermore, the response of the sensor strips to 10 mM ascorbic acid was almost as high as that to 10 mM lactate (Figure 3-7a). Moreover, the response current did not decrease much at lower operating potentials, although a large decrease in the direct oxidation of a substance with the redox potential of ascorbic acid would be expected (Figure 3-7b). Reports have shown that ascorbic acid interferes by reducing the

mediator rather than by being oxidized at the electrode (Pournaghi-Azar and Ojani, 1995; Murthy *et al*., 1998; Pournaghi-Azar and Ojani ,1999; Pournaghi-Azar and Ojani, 2000; Raoof *et al*., 2006), as exploited by a commercially available portable analyzer measuring reducing compounds, mainly ascorbic acid, by their ability to reduce the mediator, which is then quantified by oxidizing the mediator at the electrode (Arkray Pocket Chem VC, *online*). Therefore, it is feasible that ascorbic acid can reduce mPES, which is then oxidized at the electrode and thus leads to a false signal.

Another characteristic of the mediator to be considered is its stability. This is especially the case in disposable sensor strips that operate with the end-point principle, meaning that the enzyme reaction is completed before the electrode reaction begins. In contrast to the kinetic principle, in which the mediator is recycled between the enzyme and electrode, for the end-point principle, a large amount of mediator is needed, as no recycling occurs. Thus, the storage stability of the sensor strips relies significantly on the storage stability of the mediator in the strips. Reports show that mPES is more stable than similar mediators, such as mPMS, over a wide range of pH values (Dojindo Molecular Technologies Inc.b, *online*, Yomo *et al*., 1989). At highly alkaline conditions, decomposition of mPES due to ring-opening may occur (Yomo *et al*., 1989). Generally, neutral pH values are maintained during the fabrication and use of enzyme sensor strips, so that instability due to extreme pH conditions is not of concern. In this study, the storage stability of LOx-based sensor strips containing mPES was investigated. For this investigation, sensor strips containing more enzyme were used to avoid a decrease in the response current due to enzyme degradation, which led to a lower initial sensitivity due to the higher amount of protein. No significant decrease in the sensitivity in the range of 0–30 mM lactate was observed after 48 days of storage at 25 °C (Figure 3-8). The mediator itself proved to be stable at temperatures of at least

45 °C (Figure 3-10). Therefore, employing mPES as a mediator should be a successful step toward the development of sensor strips with an extended shelf life.

These results indicated that mPES is a promising organic mediator for strip-type disposable 2nd generation electrochemical biosensors, thanks to the superior characteristics of mPES, that is, the low redox potential, compatibility with various diagnostically relevant enzymes, and especially the high stability. The most common mediator, potassium ferricyanide, has a high redox potential (+0.23 V vs Ag/AgCl) (O'Reilly, 1973). Therefore, the amperometric enzyme sensor with potassium ferricyanide should be operated at high potential, otherwise consequently the signal will suffer from the oxidation current of electrochemically active ingredient potentially existing in samples. Potassium ferricyanide is also a labile molecule, which is easily inactivated during the preservation, especially under light exposure. Hexaammine ruthenium(III) has similar redox potential (−0.11 V vs Ag/AgCl) (Metzker *et al*., 2014) to mPES, however, hexaammine ruthenium(III) is not available as the mediator for native *Av*LOx and *Af*GDH as reported (Loew *et al*., 2017; Okurita *et al*., 2018). In addition, the use of the rare metal element ruthenium, which has limited existence in the earth, continuously in the future, should be reconsidered to maintain the sustainability. These inherent issues of using potassium ferricyanide or hexaammine ruthenium(III) as mediators will be overcome by substituting with mPES. PMS and mPMS have been widely utilized as the mediators of a variety of enzyme electrochemical investigations, thanks to their low redox potential (−0.11 V vs Ag/AgCl) (Kimura and Niki, 1985). Considering that PMS is a labile molecule toward light exposure, mPMS was developed; however, mPMS is not stable at neutral to alkaline condition. Another derivative, mPES, maintains low redox potential (−0.14 V vs Ag/AgCl) (Figure 3-2) and the stability under light exposure, but with improved stability over a wide pH range

(Dojindo Molecular Technologies Inc.b). This study demonstrated that mPES can be used as the electron mediator of a variety of oxidoreductases, and thus, it is the most promising mediator for further studies of electrochemical sensors.

3-5. Conclusion

In this study, a novel electron mediator, mPES, was introduced for disposable enzyme sensor strips, employing representative flavin oxidoreductases, *Av*LOx, *Af*GDH, *An*GOx, and *Pn*FPOx, by demonstrating versatility of this mediator including applying potential for the amperometric enzyme sensors, impact of potential redox active ingredients, storage stability, and availability as the electron acceptor for various enzymes. The successfully constructed lactate sensor had a high sensitivity (0.73 ± 0.12 μA/mM) and a wide linear range (0–50 mM lactate). Thanks to the redox potential of mPES, the sensor was operated by applying a low operation potential of +0.2 V vs Ag/AgCl, or even at low as low as 0 V, without significant loss in sensitivity, and consequently, no interference was observed in the presence of uric acid and acetaminophen. Ascorbic acid reacts with mPES, and thus, precautions need to be implemented to account for ascorbic acid in the sample. Furthermore, the lactate sensors were stable for at least 48 days of storage at 25 °C in the dark, which is unusually long for an organic mediator without the addition of stabilizing agents. This is the first application of mPES for disposable enzyme sensors and showing the stability of the developed enzyme sensor strip, thus it shows that mPES is stable in dry condition. The compatibility of mPES with other representative diagnostic enzymes, such as GDH, GOx and FPOx, was also approved. Thus, compared to potassium ferricyanide, or hexaammine ruthenium(III) chloride, mPES might be a good alternative mediator to consider for future developments of 2nd generation electrochemical enzyme sensor strips.

Chapter 4. Application of a novel redox mediator from phenazine derivative for the development of a quasi-direct electron transfer-type lactate sensor

4-1. Introduction

Modification of proteins and peptides using chemical probes is a general experimental technique used to functionalize these molecules. The modification is performed to analyse the functions and dynamics of proteins and peptides, and also to develop biosensing systems using a combination of spectroscopic or image analyses and/or electrochemical analyses. Similar to other chemical probes in general, proteins and peptides are covalently modified by redox probes directly via amine or thiol groups. The established modification processes are simple because the chemical probes have functional groups such as (1) the succinimidyl group for targeting primary amine groups at the side chain of lysine residues (Degani and Heller, 1987; Degani and Heller, 1988; Bartlett *et al*., 1987; Bartlett *et al*., 1991; Ryabov *et al*., 1992; Badia *et al*., 1993; Schuhmann, 1995; Sampath and Lev, 1996; Bartlett *et al*., 1997; Ban *et al*., 2001; Ban *et al*., 2003; Krikstopaitis *et al*., 2004; Mogharrab and Ghourchian, *et al*., 2005; Hatada *et al*., 2018; Hiraka *et al*., 2020; Suzuki *et al*., 2020; Hatada *et al*., 2021; Takamatsu *et al*., 2021) and the N-termini in proteins and peptides; or (2) the maleimide group for targeting the thiol group in cysteine residues (Trammell *et al*., 2001; Benson *et al*., 2001; Sandros *et al*., 2005; Sandros *et al*., 2006; Morón *et al*., 2011).

A variety of redox probes with different redox potentials have been used as redox mediators to develop biosensing systems. Most studies on protein modification using redox mediators targeted the amine group of lysine residues of oxidoreductases. In those studies, proteins were modified with derivatives of ferrocene (Degani and Heller, 1987;

Degani and Heller, 1988; Bartlett *et al*., 1987; Bartlett *et al*., 1991; Ryabov *et al*., 1992; Badia *et al*., 1993; Schuhmann, 1995; Sampath and Lev, 1996), a ruthenium complex (Degani and Heller, 1988), tetrathiafulvalene (Bartlett *et al*., 1997), phenothiazine (Ban *et al*., 2001; Ban *et al*., 2003), phenoxazine (Krikstopaitis *et al*., 2004), quinone (Mogharrab and Ghourchian, *et al*., 2005), and, as recently reported by our group, phenazine (Hatada *et al*., 2018; Hiraka *et al*., 2020; Suzuki *et al*., 2020; Hatada *et al*., 2021; Takamatsu *et al*., 2021). In contrast, studies of redox mediators targeting the thiol group of cysteine residues have been limited to the modification of binding proteins with a ruthenium complex (Trammell *et al*., 2001; Benson *et al*., 2001; Sandros *et al*., 2005; Sandros *et al*., 2006; Morón *et al*., 2011), and no studies on the modification of oxidoreductases with redox mediator targeting the thiol group of cysteine have been reported.

Protein modification targeting lysine residues is used for the modification of proteins with multiple redox molecules (Degani and Heller, 1987; Degani and Heller, 1988; Bartlett *et al*., 1987; Bartlett *et al*., 1991; Ryabov *et al*., 1992; Badia *et al*., 1993; Schuhmann, 1995; Sampath and Lev, 1996; Bartlett *et al*., 1997; Ban *et al*., 2001; Ban *et al*., 2003; Krikstopaitis *et al*., 2004; Mogharrab and Ghourchian, *et al*., 2005; Hatada *et al*., 2018; Hiraka *et al*., 2020; Suzuki *et al*., 2020; Hatada *et al*., 2021; Takamatsu *et al*., 2021), and protein modification targeting cysteine residues is used for the modification with single molecules (Trammell *et al*., 2001; Benson *et al*., 2001; Sandros *et al*., 2005; Sandros *et al*., 2006; Morón *et al*., 2011). This is because lysine has a high prevalence in proteins (~6%) (Brinkley, 1992; Hacker *et al*., 2017), whereas cysteine has a low prevalence (<2%) (Marino and Gladyshev, 2012). The modification with multiple redox molecules is generally used to facilitate the direct communication of enzymes and an electrode for the construction of reagentless biosensors. However, the

modified proteins have not been analyzed in detail because of the complexity of analyzing a large number of attached redox molecules in the proteins. For the detailed analysis of protein functions, bioelectrochemical analyses of redox enzymes, protein dynamics studies, and biosensing, modification with a single redox molecule at the desired position using a redox mediator targeting cysteine can be used.

Oxidoreductases harboring a flavin cofactor require electron mediators to transfer electrons to the electrode because flavin is buried inside the enzyme. Therefore, many studies on the development of biosensors using oxidoreductases in MET-type biosensors have been reported. However, the ideal electron transfer system for bioelectrochemical sensing is DET-type, in which electrons released by substrate oxidation are directly transferred from flavin to the electrode, but this is not feasible for oxidoreductases harboring a flavin cofactor without the presence of an electron mediator. The mediator can enter the cavity of the enzyme and reach the flavin, then be reoxidized at the electrode, thereby mediating electron transfer from flavin to the electrode.

Our research group has reported the application of a novel phenazine derivative redox mediator, 1-[3-(succinimidyl oxycarbonyl) propoxy]-5-ethyl phenazinium trifluoromethane sulfonate or amine-reactive phenazine ethosulfate (arPES), with proteins (Hatada *et al*., 2018; Hiraka *et al*., 2020; Suzuki *et al*., 2020; Hatada *et al*., 2021; Takamatsu *et al*., 2021). Phenazine derivatives are suitable mediators for biosensing applications because their redox potential is sufficiently low to avoid the appearance of bias signals originating from redox substance interference. Phenazine ethosulfate (PES), one of the derivatives of phenazine and which is the redox component of arPES, has high stability (Ghosh and Quayle, 1979; Yomo *et al*., 1989; Jahn *et al*., 2020; Dojindo Molecular Technologies, Inc.b); Fitriana *et al*., 2020;

Dojindo Molecular Technologies, Inc.c), and its versatility has been shown for oxidoreductase-based biosensors (Fitriana *et al*., 2020). Through its succinimidyl group, arPES can modify the amine group of lysine in oxidoreductases and provide quasi-DET to the enzyme (Dojindo Molecular Technologies, Inc.c). Enzyme modification with this commercially available redox mediator allows electron transfer from flavin to the electrode via the attached arPES. By immobilizing the modified enzyme on the electrode, the substrate can be monitored by electrochemical analysis such as chronoamperometry without the addition of a free mediator (Hatada *et al*., 2018; Hiraka *et al*., 2020; Suzuki *et al*., 2020; Hatada *et al*., 2021). The application of arPES lead to the successful development of a glucose sensor based on flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenase derived from *Botryotinia fuckeliana* (*Bf*GDH) (Hatada *et al*., 2018); however, no amperometric response was observed when using *Av*LOx (or LOx) modified by arPES (Hatada *et al*., 2018; Hiraka *et al*., 2020). Some lysine residues in *Bf*GDH might be located at a suitable position to mediate electron transfer from flavin to the electrode, thus enabling amperometric response currents to be observed. The position of the lysine residue in *Bf*GDH, which has a major impact on electron transfer from the reduced flavin, cannot be determined. Suzuki *et al*. (2020) predicted the position of this lysine residue. Then, Hiraka *et al*. (2020) introduced a lysine residue near the entrance to the active site on a LOx surface, and a lactate sensor was successfully developed using an arPES-modified LOx lysine mutant. In this case, multiple arPES molecules were attached and the response currents observed were based on the electrons mediated by these multiple arPES molecules. Here, the response currents mediated by a single arPES molecule attached on the introduced lysine cannot be determined.

Recently, another novel phenazine derivative redox mediator, 1-[3-(2-maleimidoethyl carbamoyl) propoxy]-5-ethyl phenazinium triflate or thiol-reactive phenazine ethosulfate (trPES) (Scheme 4-1), has become commercially available (Dojindo Molecular Technologies, Inc.d). Because trPES contains a maleimide group, it can be used to modify cysteine residues in proteins, thus enabling the modification of an enzyme with a single trPES. In 1993, Badia *et al*. reported that the locations of a few key ferrocene groups attached to the enzyme in the vicinity of the cofactor and also the enzyme surface are critical for electrocatalytic activity, rather than the number of ferrocene groups loaded onto the enzyme. However, the modification of protein with a single redox mediator molecule targeting cysteine in oxidoreductases, including the application of trPES for this purpose, has not been reported.

In this study, the application of the novel redox mediator trPES for targeting cysteine in well-known oxidoreductases, LOx, was demonstrated, and the impact of the modification of redox enzyme with a single redox mediator molecule near the cofactor was characterized. *Av*LOx is widely used in the development of L-lactate biosensors. *Av*LOx harbors the flavin mononucleotide (FMN) as the cofactor and catalyzes the oxidation of L-lactate. Since *Av*LOx does not have a cysteine residue, a single cysteine mutation enables one-point modification of the enzyme with trPES. The results obtained in this study were compared with reported studies of the modification of *Av*LOx with multiple redox mediator molecules of arPES. Following the common strategy of modifying proteins with a single redox molecule at a specific position by site-directed mutagenesis, a cysteine residue was introduced near the active site at the enzyme surface, which allowed quasi-DET via the attached trPES. This strategy is expected to clearly show the impact of attaching a single redox mediator at a specific position in an enzyme. Furthermore, *Aspergillus flavus*-derived FAD GDH (*Af*GDH) was modified

with trPES to evaluate the versatility of trPES for modifying other oxidoreductases. This is the first application of trPES in the development of lactate sensor utilizing LOx. Here, the trPES was used to understand the bioelectrochemical property of LOx.

4-2. Materials and methods

4-2-1. Materials and devices

Here are the new materials and devices used in this study in addition to that mentioned in Chapter 2 (Section 2-2-1) and Chapter 3 (Section 3-2-1). trPES and arPES were provided by Dojindo Molecular Technologies Inc. (Kumamoto, Japan). Poly(ethylene glycol) diglycidyl ether (PEGDGE Mn 500) and tricine buffer were purchased from Sigma-Aldrich (Missouri, USA). Triton X was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Morpholinepropanesulfonic acid (MOPS) and sodium chloride (NaCl) were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Serine protease inhibitor (Pefabloc® SC, Roche, Basel, Switzerland). Bradford reagent was purchased from Bio-Rad Laboratories (USA). All chemicals were of reagent grade. All solutions were prepared using pure water from a Milli-Q water purification system (Millipore Corporation, Burlington, MA, USA).

The 3D structure analysis of *Av*LOx was performed using PyMOL Molecular Graphics System software. Enzymes were incubated with trPES and/or arPES in an Eppendorf ThermoMixer R (Thermo Fisher Scientific, Tokyo, Japan). Microplate reader Varioskan Flash from Thermo Scientific was used to determine the protein concentrations. All electrochemical measurements were carried out using PG580RM potentiostat-galvanostat from UniScan Instruments Ltd., (Buxton, UK).

4-2-2. Preparation of engineered LOx mutants

Enzyme preparation and enzymatic activity evaluation

Cysteine mutation sites are designed and a cysteine residue was introduced in LOx A96L, a mutant with low reactivity toward oxygen (Hiraka *et al*., 2018), through site-directed mutagenesis (Table 4-1) by Dr. Kentaro Hiraka. *E. coli* BL21 (DE3) was used for engineered LOx production following the methods of Hiraka *et al.* (2020). The recombinant enzymes were prepared according to the method of Hiraka *et al.* (2018) with minor modifications (Fitriana *et al*., 2020). The oxidase activity and dye-mediated dehydrogenase activity evaluation of LOx mutants were carried out using 4AA/TOOS/POD system and PMS/DCIP system, respectively, following the same method stated in Section 2-2-2. Kinetic parameters were calculated using the Hanes–Woolf plot. These assays were performed in triplicate for each enzyme sample.

Determination of protein concentration using Bradford assay

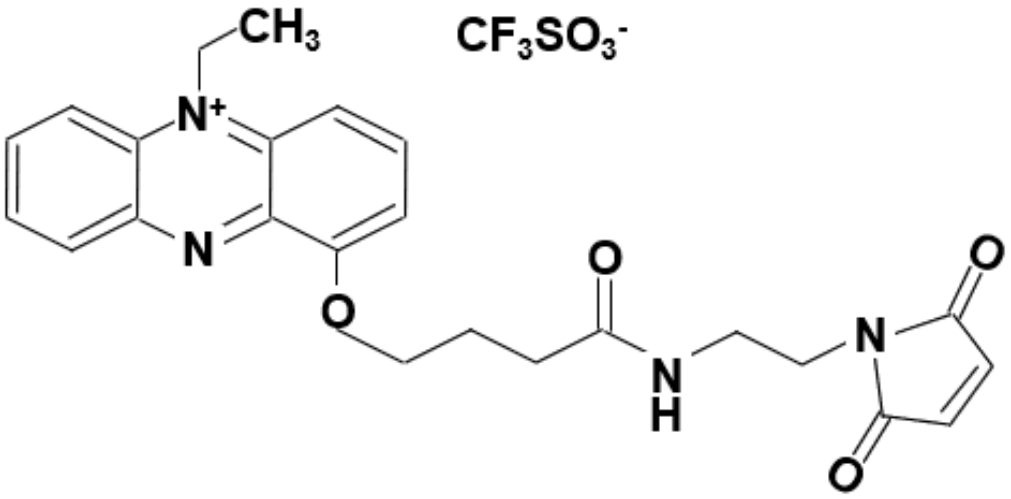
In this study, the protein concentration was measured with microplate reader using Bradford assay and BSA was used as the protein standard solution. BSA solution was prepared in concentration range 100–1000 μg/ml. For the measurement, BSA solution or LOx solution (5 μl) was added to a NUNC 96-well microplate in triplicate. Bradford reagent (250 μl) was then added. After 5-10 minutes of incubation, the absorbance at 595 nm was monitored using the microplate reader. The calibration curve was determined by plot the absorbances of the standard protein BSA vs BSA concentration, thus the function of slope of line is suggesting as the absorbance of the samples. The protein concentration of LOx mutants was then calculated by substituting this slope with the absorbance of the LOx sample. SDS-PAGE analysis was performed following the methods described in Section 2-2-2 by applied 0.2 mg LOx sample per lane.

4-2-3. Modification of LOx mutants with redox mediators

Purified LOx mutant (0.55 μM) was mixed with trPES (80 μM) in 20 mM PPB (pH 7.0) (Table 4-2) and subsequently incubated at 25 ℃ in a thermomixer with 1200 rpm shaking for 2 h. The unbound trPES was removed by ultrafiltration of protein concentrate at 14,000 g and 4 ℃ for 5 min. The centrifugation was repeated 10 times with 20 mM PPB (pH 7.0) added to obtain trPES-modified LOx (trPES-LOx).

In further evaluation of LOx cysteine mutant, one of LOx cysteine mutant which exhibited the best response currents, and the control mutant, were modified with both trPES and arPES. The LOx modification with trPES and arPES was carried out following the same steps and conditions above. The same concentration of LOx cysteine mutant (0.55 μM) and trPES (80 μM) were mixed with arPES (400 μM) and 20 mM Tricine buffer pH (8.3). Volume of the mixture was adjusted with 20 mM PPB (pH 7.0) (Table 4-2). The pH of the mixture changed during the removal of the unbound mediators, and trPES- and arPES-modified LOx (tr.arPES-LOx) with neutral pH was obtained. LOx mutants were also modified with arPES only (Hiraka *et al*., 2020) for comparison with other modified enzymes.

Dye-mediated dehydrogenase activity of modified LOx was evaluated using PMS/DCIP system (Section 2-2-2) with modification in volume used for each reagent. The mixture with total volume of 160 μl was prepared in 20 mM PPB (pH 7.0) with premix containing 16 μl of 4 mM PMS, 16 μl of 0.06 mM DCIP, and 4 μl of modified LOx. 16 μl of lactate (0–20 mM) was added to the premix and the absorbance monitoring was started by soon applying 150 μl of mixture in the spectrophotometer. The protein concentration was determined using Bradford assay following the methods described in Section 4-2-2. SDS-PAGE analysis was performed by applied 0.3 mg modified LOx per lane following the methods described in Section 2-2-2.



Scheme 4-1. Structure of trPES.

Table 4-1. Primers used to design the cysteine mutants.

Enzymes	Forward primers*	Reverse primers*
*Av*LOx cysteine mutants		
A96L (Hiraka *et al*., 2018)	-	-
A96L/V20C	5'- AAGTACATTGATGTTtgc AATACTTACGACTTA -3'	5'- TAAGTCGTAAGTATTgca AACATCAATGTACTT -3'
A96L/S178C	5'- TGACTCAACTGTTtgt GGAAACCGTGACC -3'	5'- GGTCACGGTTTCCaca AACAGTTGAGTCA -3'
A96L/V185C	5'- AACCGTGACCGTGATtgc AAGAATAAATTCGTT -3'	5'- AACGAATTTATTCTTgca ATCACGGTCACGGTT -3'
A96L/S210C	5'- ACAGCAGAAGGTATGtgt TTAAACAATATCTAC -3'	5'- GTAGATATTGTTTAAaca CATACCTTCTGCTGT -3'
A96L/N212C	5'- GAAGGTATGTCATTAtgc AATATCTACGGTGCT -3'	5'- AGCACCGTAGATATTgca TAATGACATACCTTC -3'
*Af*GDH cysteine mutant		
K477C	5'- GAGACCtgtCCAGGCCTG TGAGATCCCAGCTACCGC AGCGGATG -3'	5'- GCCTGGacaGGTCTCCTTC GCGATCAGTTTGTTCAGTG GTGCGC -3'

*Lowercase codons were the mutation sites.

Table 4-2. Mixture composition in modification of LOx with redox mediators.

LOx modification with trPES		LOx modification with trPES and arPES	
Reagents (fc.)	Volume	Reagents (fc.)	Volume
0.55 µM *Av*LOx	45 µl	0.55 µM *Av*LOx	45 µl
80 µM trPES	3.6 µl	80 µM trPES	3.6 µl
-	-	400 µM arPES	3.6 µl
-	-	20 mM Tricine (pH 8.3)	180 µl
20 mM PPB (pH 7.0)	(adjusted) µl	20 mM PPB (pH 7.0)	(adjusted) µl
Total volume	450 µl		450 µl

4-2-4. Cyclic voltammetry measurement of redox mediators

The CV analysis measurements were performed for 0.1 mM trPES or arPES in 2 ml of 100 mM PPB (pH 7.0), with potentials range from –0.6 V to +0.6 V at a scan rate 0.1 V/s. The GC and Pt wire were used as WE and CE, respectively.

4-2-5. Application of trPES for other enzymes

*Af*GDH (NCBI reference sequence: XP_002372599.1) and its mutant were also produced by almost the same method as in a previous report (Sakai *et al*., 2015). The amino acid sequence of *Af*GDH without a signal peptide was expressed using a plasmid (pET30C) to encode the *Af*GDH gene in *E. coli* BL21 (DE3) cells. A cysteine mutation was introduced into *Af*GDH K477 through site-directed mutagenesis (Table 4-1) by Mr. Yuma Hatano. The transformed *E. coli* that inserted with *Af*GDH wild-type (WT) or *Af*GDH K477 plasmid were cultivated at 20 °C, 160 rpm for 44 h in 100 mL of ZYP-5052 medium containing 50 μg/mL Km (Studier, 2005). After cell harvesting by centrifugation, wet cells were suspended in MOPS buffer (pH 6.5) and disrupted by ultrasonication with serine protease inhibitor. The lysate was centrifuged and ultracentrifuged to obtain soluble fraction. The soluble fraction containing *Af*GDH WT and K477C mutant were purified by anion exchange chromatography with a linearly increasing gradient of 0–100 mM NaCl in MOPS buffer (pH 6.5). The purified protein solution was dialyzed overnight against 10 mM PPB (pH 6.5).

*Af*GDH WT or *Af*GDH K477C mutant was modified with trPES by incubating trPES (80 μM) with the enzyme (1.33 μM) in 20 mM PPB buffer (pH 6.5) following the methods described in Section 4-2-3. Enzyme electrodes based on modified trPES-*Af*GDH WT or trPES-*Af*GDH K477C were prepared according to the methods described in Section 4-2-5 and the electrochemical response current toward glucose was evaluated in 20 mM PPB buffer with pH 6.5.

4-2-6. Construction of enzyme electrodes utilizing modified PES-LOx mutants

Construction of the enzyme electrodes

GC electrodes were polished and sonicated in pure water prior to use. The modified enzymes were immobilized through cross-linking with PEGDGE following the method of Vasylieva *et al*. (2011). A mixture (6 µl) containing 1 mg/mL modified enzyme (trPES-LOx mutants or tr.arPES-LOx mutants) and 10 mg/mL PEGDGE was deposited on the GC electrode for 5 layers. The enzyme electrode was dried at 25 ℃ for 15 min before the next layer was added. After adding the last layer, the enzyme electrode was dried at RT (around 25 ℃) with very low humidity (less than 1% relative humidity) for 2 h. Three different electrodes were prepared for each modified LOx variant. The enzyme electrodes were equilibrated in 20 mM PPB (pH 7.0) at RT for at least 15 min before use.

Evaluation of response currents of the enzyme electrodes by CV and CA measurements

CV measurements were performed in 2 mL of 100 mM PPB (pH 7.0) with a sweep range from –0.7 V to +0.3 V and scan rate of 0.05 V/s. CA measurements were performed to monitor the response currents with successive addition of lactate (0–20 mM) under constant stirring and with an operating potential of 0 V (vs Ag/AgCl) under atmospheric conditions. Finally, CV measurements in the presence of lactate were performed to record the behavior of the enzyme electrodes. The performances of the constructed biosensors such as sensitivity, range of linear detection, and limit of detection (LOD) were determined following the formula described in Section 3-2-4 and the previous studies (Hiraka *et al*., 2020; Fitriana *et al*., 2020).

4-3. Results

4-3-1. Design, construction, and characterization of LOx cysteine mutants

A cysteine residue was introduced in LOx to enable the modification with trPES. The residues to be substituted with cysteine were selected on the basis of two criteria (Hiraka *et al*., 2020): (1) they were located on the surface of LOx to provide accessibility to the electrode surface; (2) there was a short distance between the residue (alpha carbon, Cα) and the isoalloxazine ring of FMN (N5) (<30 Å) (Stuchebrukhov, 2010), thus enabling optimal electron transfers from flavin to the electrode through the attached trPES. Five residues, V20, S178, V185, S210, and N212, were chosen for substitution (Figure 4-1). Six LOx mutants were produced as recombinant proteins and purified: A96L/V20C, A96L/S178C, A96L/V185C, A96L/S210C, A96L/N212C and A96L, where A96L was used as a control.

SDS-PAGE analysis result showed the prominent band of purified LOx mutants at slightly lower than 48 kDa (Figure 4-2), suggested the size of LOx in approximately 44 kDa (Duncan *et al*.,1989). The oxidase activity of the LOx mutants was determined. All the LOx mutants showed very low oxidase activity (lower than 3 U/mg, Figure 4-3) because they maintained the characteristic of the A96L mutation, which exhibited low reactivity to the molecular oxygen (Hiraka *et al*., 2018; Hiraka *et al*., 2020). The kinetic parameters of the LOx mutants were determined from dye-mediated dehydrogenase activity (Table 4-3). The V_{max} values indicated the dye-mediated dehydrogenase activity of all LOx mutants, indicating that the LOx mutants were in an active form. LOx A96L/S210C exhibited the highest activity (162% of the control), whereas LOx A96L/N212C exhibited the lowest activity among the LOx mutants (38% of the control). The low oxidase activity and high dye-mediated dehydrogenase activity makes the LOx mutants suitable for MET- and quasi-DET-type sensor applications.

In a previous study (Hiraka *et al*., 2020), residues S178 and N212 were substituted with lysine to design the arPES modification site, resulting in inactivated LOx A96L/SI78K but high enzyme activity in LOx A96L/N212K. The cysteine mutation in this study appeared to markedly decrease the enzyme activity in LOx A96L/N212C.

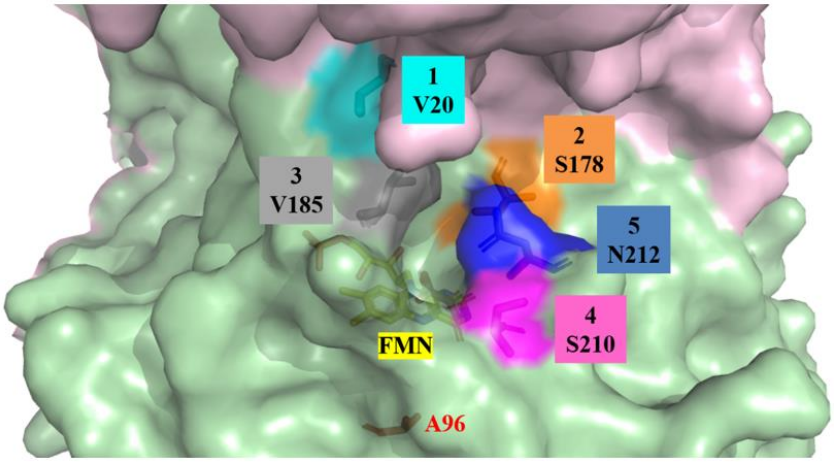
4-3-2. Catalytic activity of cysteine mutants after modification with trPES

The LOx mutants were modified by incubation with trPES. The modified trPES-LOx mutants were expected to have one molecule of trPES covalently attached to each monomer of the homo-tetrameric LOx, except for trPES-LOx A96L, which lacks a cysteine residue. SDS-PAGE analysis was performed to check the band of the modified LOx (Figure 4-4). The band of trPES-LOx A96L (control) showed at size slightly lower than other trPES-LOx mutants, possibly due to the lack of attached trPES in the trPES-LOx mutants, and thus may confirm that the trPES was attached to the LOx cysteine mutants. The kinetic parameters of the trPES-LOx mutants were determined (Table 4-3). All modified enzymes retained dye-mediated dehydrogenase activity, as shown by their V_{max} values, although all the V_{max} values after modification with trPES decreased to 72% of the values before modification or lower. trPES-LOx A96L/V185C showed the highest activity and trPES-LOx A96L/S178C showed the lowest activity (6% of its initial activity before modification). The K_m values were also changed by the modification. All K_m values after modification increased, except for trPES-LOx A96L, for which it decreased almost to half of that before modification.

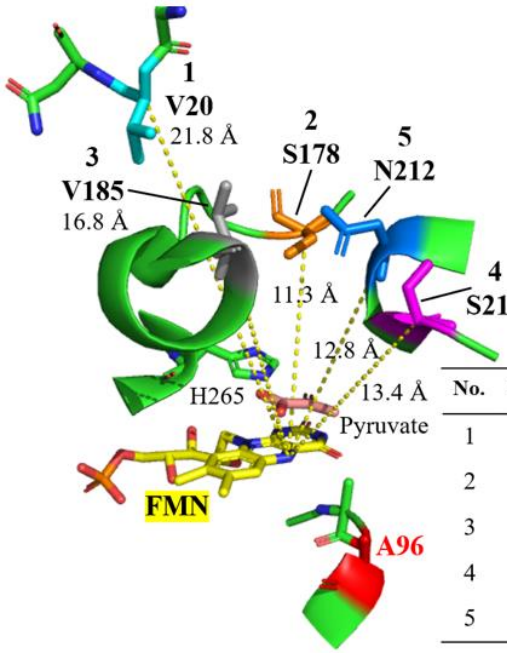
4-3-3. Electrochemical behavior of the redox mediators

Cyclic voltammograms of trPES or arPES was obtained (Figure 4-5). Both trPES and arPES showed similar redox peak with calculated $E_{1/2}$ at approximately -0.18 V.

(a)



(b)



No.	Residue	Distance (Å)
1	V20	21.8
2	S178	11.3
3	V185	16.8
4	S210	13.4
5	N212	12.8

Figure 4-1. Structure of *Aerococcus viridans*-lactate oxidase (*Av*LOx) (PDB code: 2E77) showing the mutation sites of *Av*LOx A96L/cysteine mutants: (a) location of mutated residues at the active site entrance on the enzyme surface and (b) native amino acid residues that were substituted with cysteine and their distance from the isoalloxazine ring of FMN (N5). 1: Valine20 (V20); 2: Serine178 (S178); 3: Valine185 (V185); 4: Serine210 (S210); 5: Asparagine212 (N212). A96 (red letter): residue mutated to decrease the oxygen reactivity. FMN: *Av*LOx cofactor. Pyruvate: product of L-lactate oxidation catalyzed by LOx. All residues in the model are in chain D (light green area) of the homo-tetrameric *Av*LOx, except V20, which is in chain B (pink area).

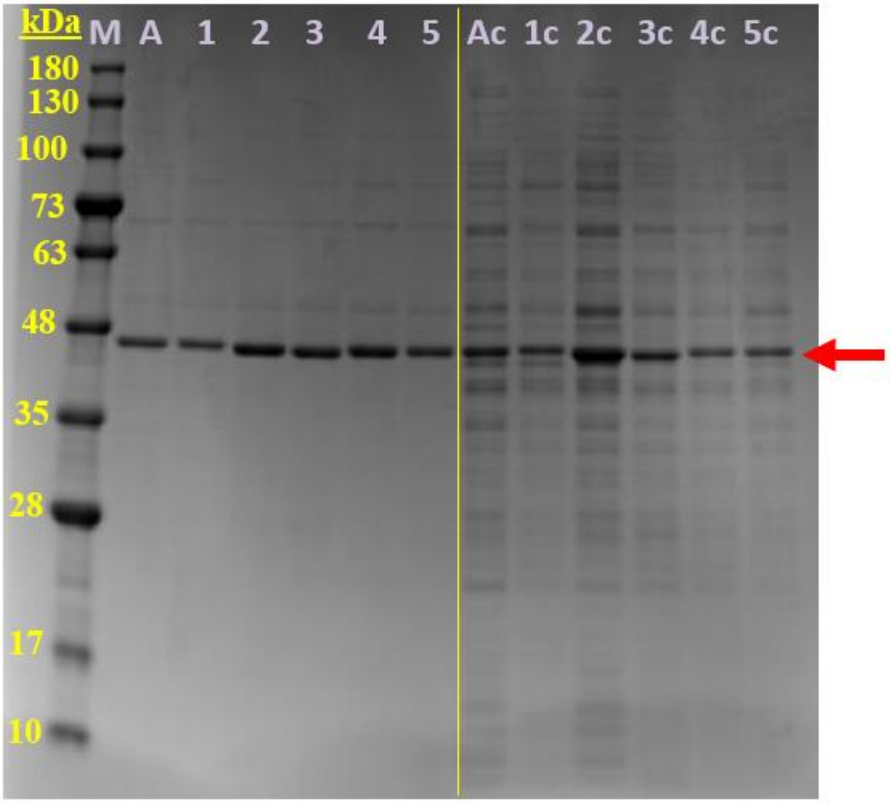


Figure 4-2. SDS-PAGE analysis result of LOx mutants. Purified enzymes (left side) – M: Marker. A: A96L. 1: A96L/V20C. 2: A96L/S178C. 3: A96L/V185C. 4: A96L/S210C. 5: A96L/N212C. – and crude enzymes (right side) – Ac: A96L crude. 1c: A96L/V20C crude. 2c: A96L/S178C crude. 3c: A96L/V185C crude. 4c: A96L/S210C crude. 5c: A96L/N212C crude –. Red arrow: band for LOx at molecular weight <48 kDa.

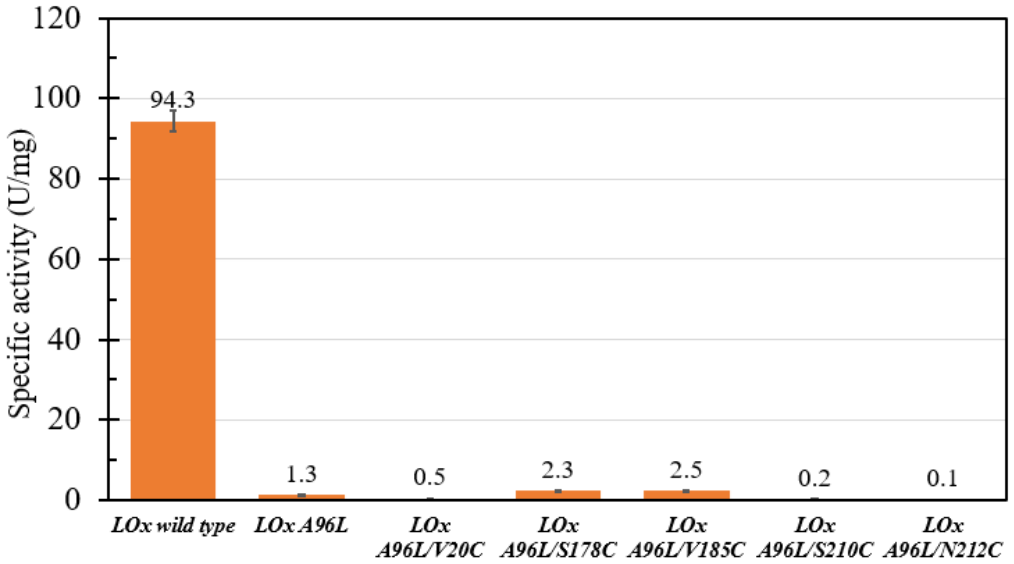


Figure 4-3. Oxidase activity of purified LOx cysteine mutants.

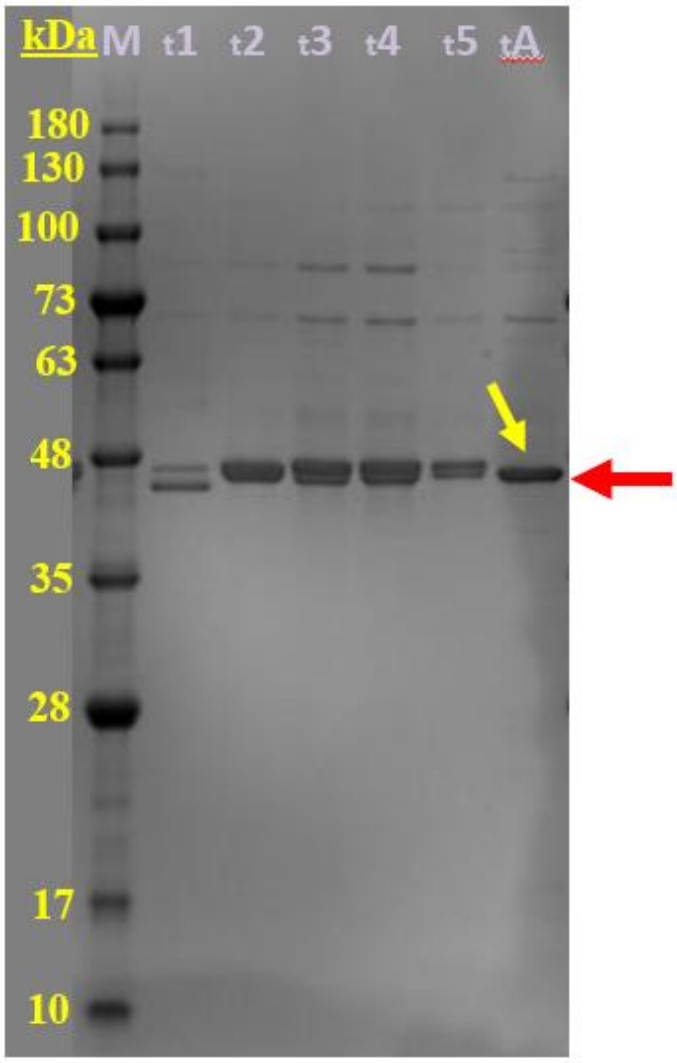


Figure 4-4. SDS-PAGE analysis result of trPES-LOx mutants. M: Marker. t1: trPES-A96L/V20C. t2: trPES-A96L/S78C. t3: trPES-A96L/V185C. t4: trPES-A96L/S210C. t5: trPES-A96L/N212C. tA: trPES-A96L. Red arrow: band for LOx at molecular weight <48 kDa. Yellow arrow: band of trPES-LOx A96L (control) showed lower band than other trPES-LOx mutants.

Table 4-3. Kinetic parameters of LOx mutants before and after modification with trPES.

LOx mutants	Kinetic parameters*			
	V_{max} (U/mg)		K_m (mM)	
	LOx	trPES-LOx	LOx	trPES-LOx
A96L	98 ± 6	50 ± 3	0.79 ± 0.10	0.44 ± 0.06
A96L/V20C	74 ± 4	33 ± 2	0.63 ± 0.03	6.80 ± 0.60
A96L/S178C	78 ± 3	5 ± 1	0.60 ± 0.03	2.58 ± 0.40
A96L/V185C	118 ± 9	85 ± 3	0.73 ± 0.11	1.19 ± 0.10
A96L/S210C	159 ± 1	76 ± 1	1.26 ± 0.12	2.50 ± 0.28
A96L/N212C	37 ± 3	22 ± 5	1.20 ± 0.08	1.34 ± 0.54

*V_{max} and K_m were determined using a Hanes–Woolf plot

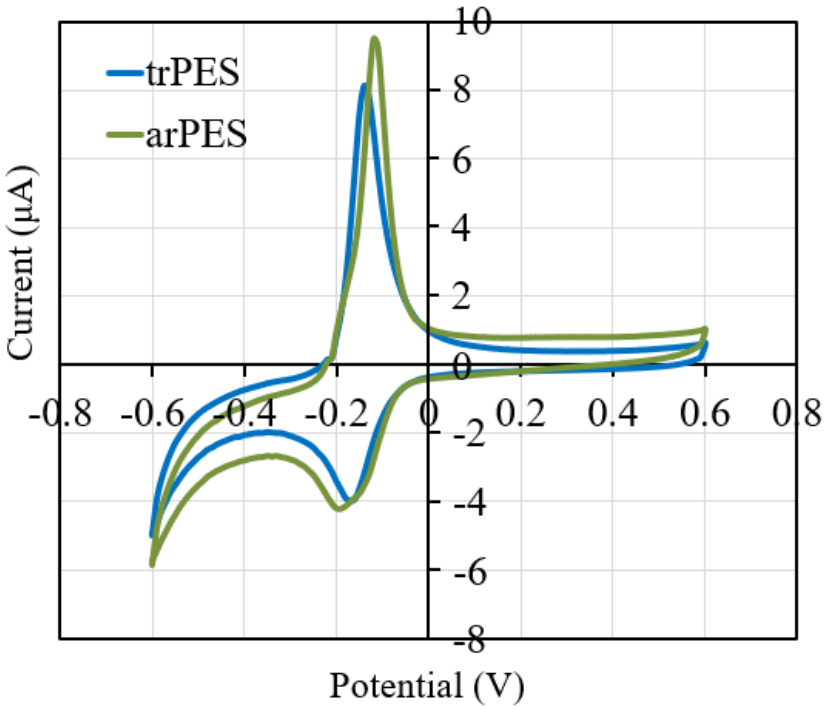


Figure 4-5. Cyclic voltammograms of 0.1 mM trPES or arPES in 2 ml of 100 mM PPB (pH 7.0). Scan rate: 0.1 V/s.

4-3-4. Electrochemical evaluation of enzyme electrodes using LOx modified with single mediator molecule

The enzyme electrodes constructed using the trPES-LOx mutants were evaluated by CV (Figure 4-6). All cyclic voltammograms showed redox peaks in both the absence and presence of lactate. These redox peaks are attributed to trPES because they are similar to the redox peaks of the trPES solution (Figure 4-5). This confirms that trPES attached on LOx and that the modified enzymes were electrochemically active. The $E_{1/2}$ are approximately –0.10 V, calculated from the oxidation and reduction peaks, which were observed at approximately –0.06 V and –0.14 V, respectively. Catalytic currents were observed in the presence of lactate, suggesting that the electrons were transferred from the attached trPES to the electrode upon substrate oxidation. trPES-LOx A96L/V185C (Figure 4-6d), trPES-LOx A96L/S210C (Figure 4-6e), and trPES-LOx A96L/N212C (Figure 4-6f) clearly showed high catalytic current. Therefore, the CA measurement was carried out and the lactate concentration dependence was evaluated for each modified LOx mutant.

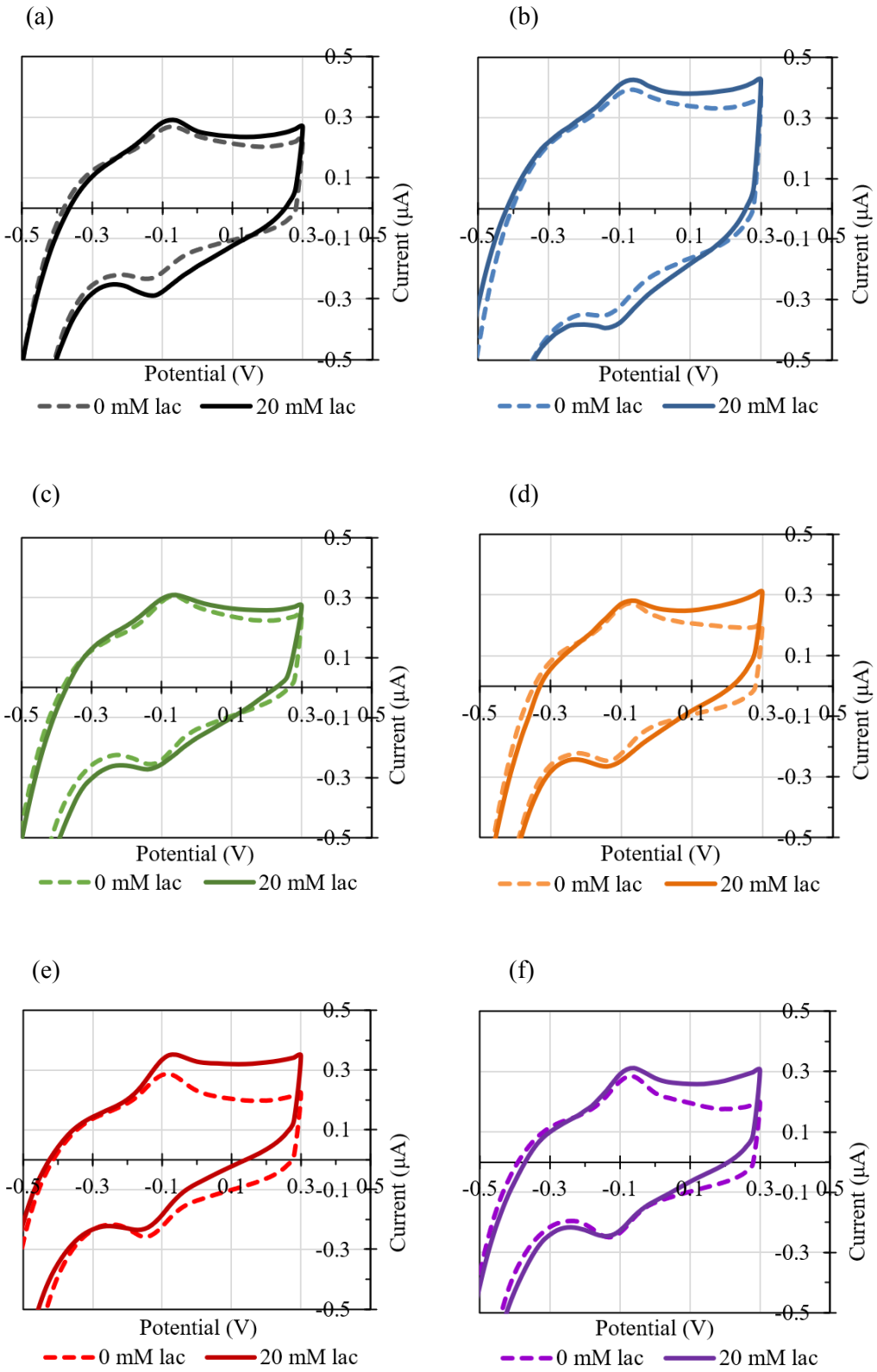


Figure 4-6. Cyclic voltammograms of modified enzymes: (a) trPES-LOx A96L, (b) trPES-LOx A96L/V20C, (c) trPES-LOx A96L/S178C, (d) trPES-LOx A96L/V185C, (e) trPES-LOx A96L/S210C, and (f) trPES-LOx A96L/N212C in 2 ml of 100 mM PPB (pH 7.0) in the absence (dashed line) or presence (solid line) of lactate. Scan rate: 0.05 V/s.

4-3-5. Amperometric response of enzyme electrodes using LOx modified with single mediator molecule

CA measurements were performed to evaluate the electron transfer from flavin to the electrode through the attached trPES. The response currents were recorded with successive addition of 0–20 mM lactate in a buffer solution without a free mediator (Figure 4-7a). The steady-state currents obtained with each addition of lactate were plotted against lactate concentration (Figure 4-7b).

The enzyme electrodes utilizing trPES-LOx A96L/V20C, trPES-LOx A96L/V185C, trPES-LOx A96L/S210C, and trPES-LOx A96L/N212C clearly showed response currents during the addition of lactate. In contrast, trPES-LOx A96L/S178C and the control, trPES-LOx A96L, did not show response currents. The observed response currents in the trPES-LOx A96L/cysteine mutants increased with increasing lactate concentration. The highest response current was observed with trPES-LOx A96L/S210C. The response currents in trPES-LOx A96L/V20C, trPES-LOx A96L/V185C, and trPES-LOx A96L/N212C were approximately 16, 24, and 35%, respectively, of that observed in trPES-LOx A96L/S210C (calculated from I_{max}). The response currents suggested that when lactate was oxidized, the electrons were successfully transferred to the electrode surface through the attached trPES on the LOx cysteine mutants, indicating successful quasi-DET in the trPES-LOx A96L/cysteine mutants. Although the dye-mediated dehydrogenase activity of trPES-LOx A96L/S210C was 89% of that obtained by trPES-LOx A96L/V185C, trPES-LOx A96L/S210C exhibited a higher response current than trPES-LOx A96L/V185C. In this case, the observed response current depends on the electron transfer ability of the modified enzymes, not on the enzyme activity, and the optimum electron transfer occurs in trPES-LOx A96L/S210C. Residue S210 in LOx is thus probably the best position for

the modification with trPES because it appears that residue S210 plays an important role in accessing the electrons from flavin to transfer them to the electrode. In the case of trPES-LOx A96L/N212C, the low enzyme activity of LOx A96L/N212C appears to be the reason for the low response currents observed, because the enzyme activity of LOx A96L/N212C before and after modification with trPES is approximately 23 and 29%, respectively, of that of LOx A96L/S210C (calculated from V_{max}). The low enzyme activity after modification with trPES also resulted in the low response currents of trPES-LOx A96L/V20C (approximately 10–15% of the response current of trPES-LOx A96L/S210C), and even trPES-LOx A96L/S178C showed no response. For trPES-LOx A96L, no response current was observed because no trPES was attached near the isoalloxazine ring of FMN.

4-3-6. Modification of LOx with trPES and arPES

To improve the performance of the lactate sensor, LOx A96L/S210C and the control mutant, were modified with both trPES and arPES, obtaining tr.arPES-LOx A96L/S210C and tr.arPES-LOx A96L, respectively. The amperometric response of tr.arPES-LOx A96L/S210C markedly increased to five-fold higher than that of trPES-LOx A96L/S210C (Figure 4-8). This due to that the modification of arPES increased the number of redox mediators attached in the enzyme surface, thus improved the quasi-DET ability of the tr.arPES-LOx A96L/S210C. This is because the number of attached PES in LOx A96L/S210C increased, resulting in an increased response current. In contrast, the response current of tr.arPES-LOx A96L was 58% of that obtained for trPES-LOx A96L/S210C, suggesting that although there are many arPES attached to LOx A96L, a high response current can be achieved if at least one PES is attached in a position accessible for electron transfer from flavin, and thus the electrons can be

transferred to either the electrode or another attached arPES. For comparison, LOx A96L and LOx A96L/S210C were also modified with only arPES to obtain arPES-LOx A96L and arPES-LOx A96L/S210C, respectively (Figure 4-8). Both showed similar response currents of approximately 46% of that obtained by trPES-LOx A96L/S210C. Such a low response current for arPES-LOx A96L was also observed in a previous study (Hiraka *et al*., 2020). This result supports the above assumption that PES should be attached to a crucial position to achieve a high response current. The properties and performances of lactate sensors were determined from the observed response currents (Table 4-4). As expected, the lactate sensor based on tr.arPES-LOx A96L/S210C exhibited the best performances.

In addition, the response currents of tr.arPES-LOx A96L/S210C were evaluated in the presence of mPES in the buffer solution (Figure 4-9). The response currents obtained 3.2-fold higher than that observed in tr.arPES-LOx A96L/S210C without free mediator in the solution (Figure 4-10). The presence of free mediator increased the number of redox molecules that involved in the electron transfer. The lactate saturation was observed in concentration >20 mM.

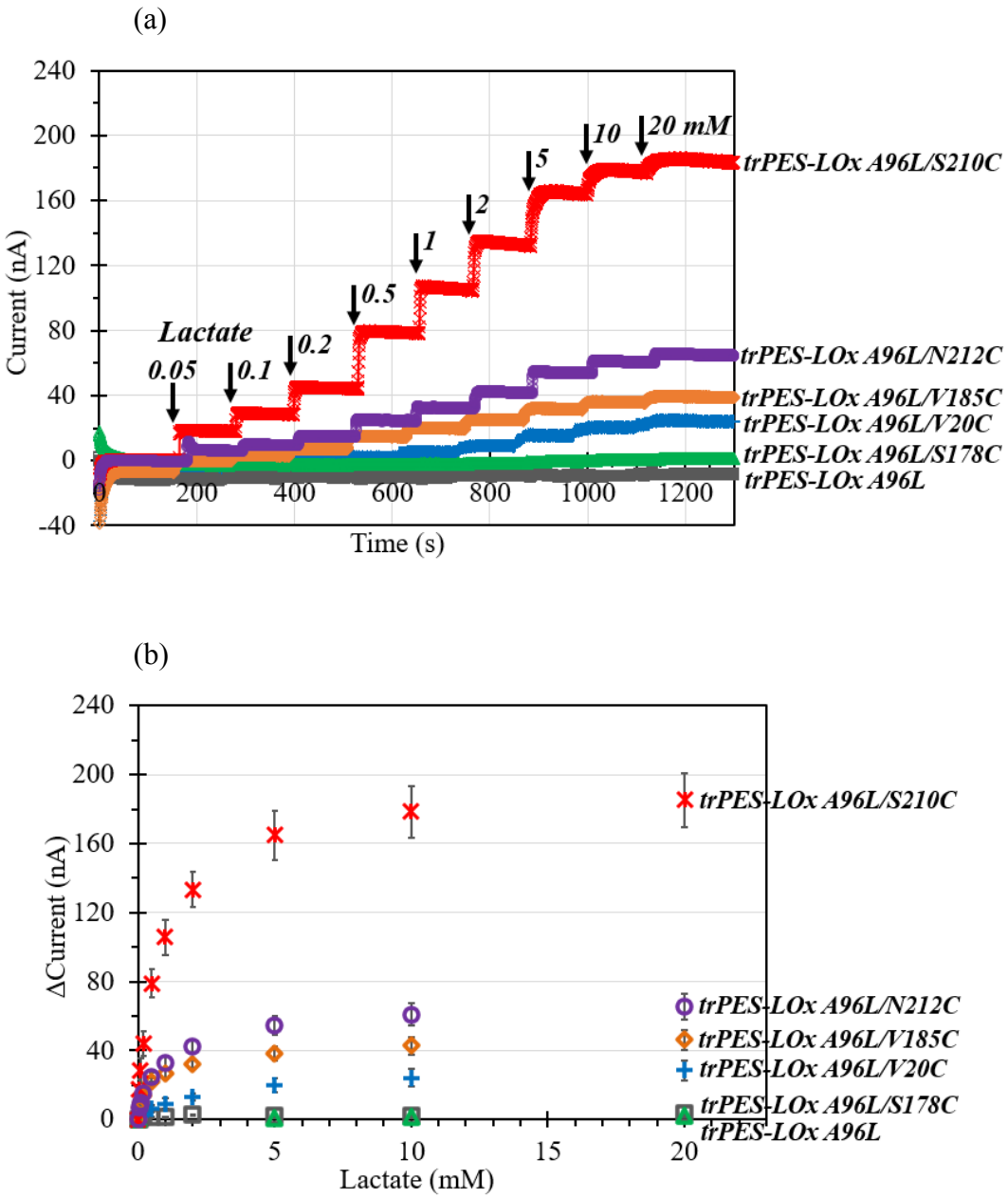


Figure 4-7. Response currents of enzyme electrodes utilizing trPES-LOx mutants: (a) representative time courses of response currents, and (b) steady-state currents of the trPES-LOx mutants at various lactate concentrations (n = 3). Applied potential: 0 V (vs Ag/AgCl).

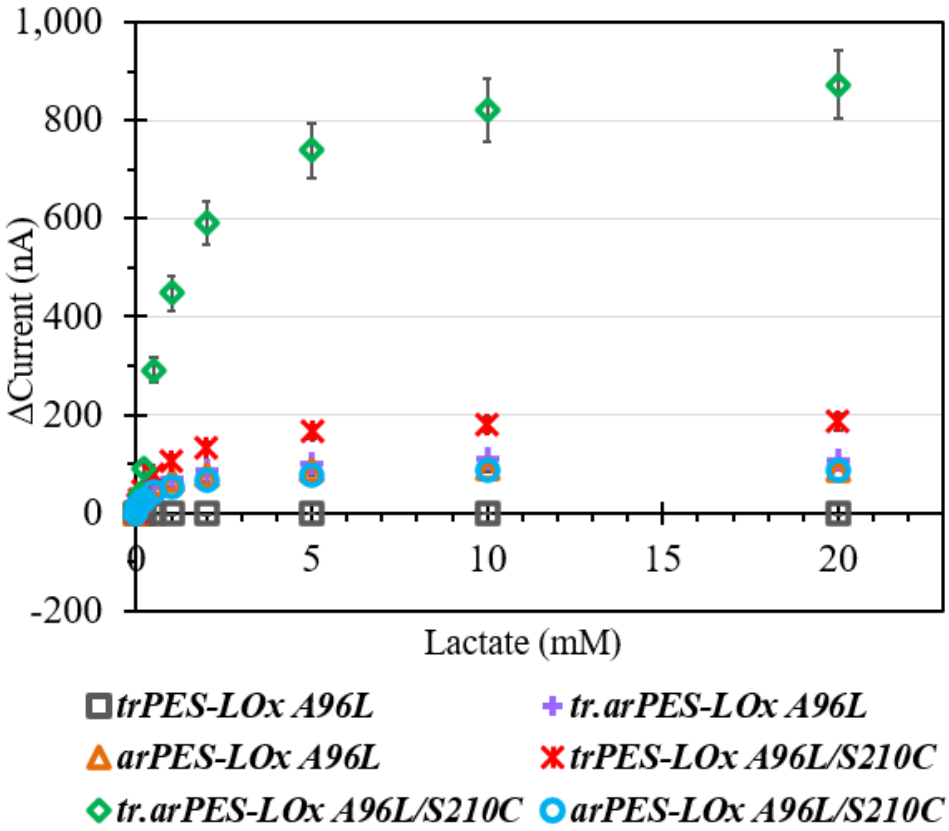


Figure 4-8. Steady-state response currents of LOx modified with multiple PES molecules at various lactate concentrations (n = 3). Applied potential: 0 V (vs Ag/AgCl).

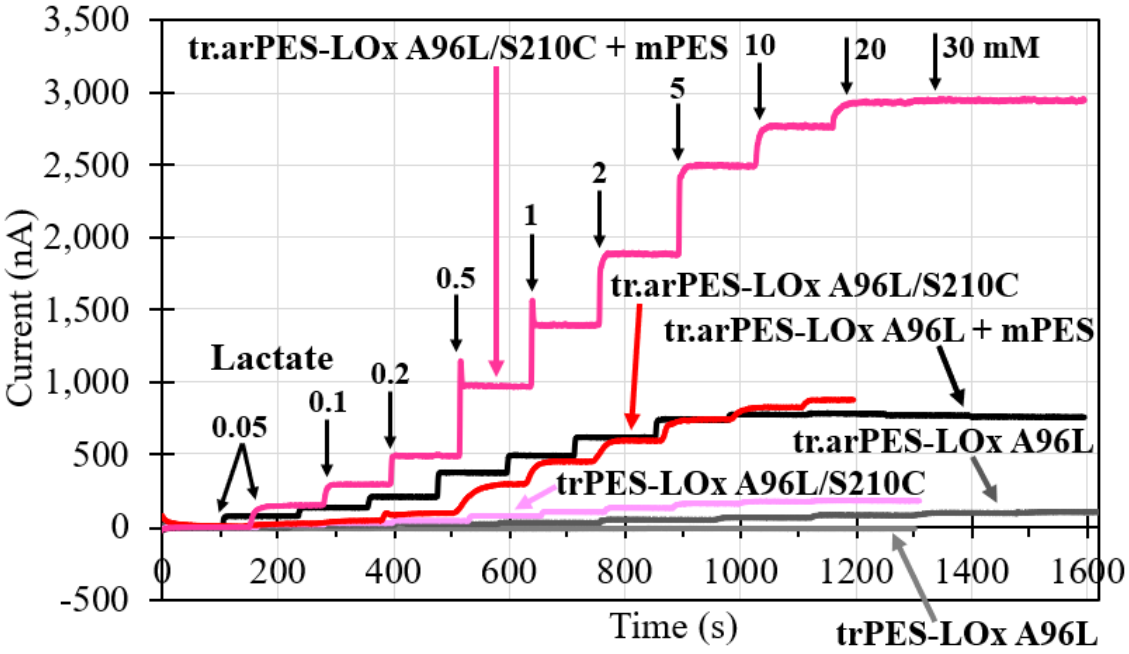


Figure 4-9. Response currents of LOx A96L or LOx A96L/S210C modified with trPES or trPES+arPES in the absence or presence of free mediator (mPES) in the solution. Lactate: 0-20 or 0-30 mM. Applied potential: 0 V (vs Ag/AgCl).

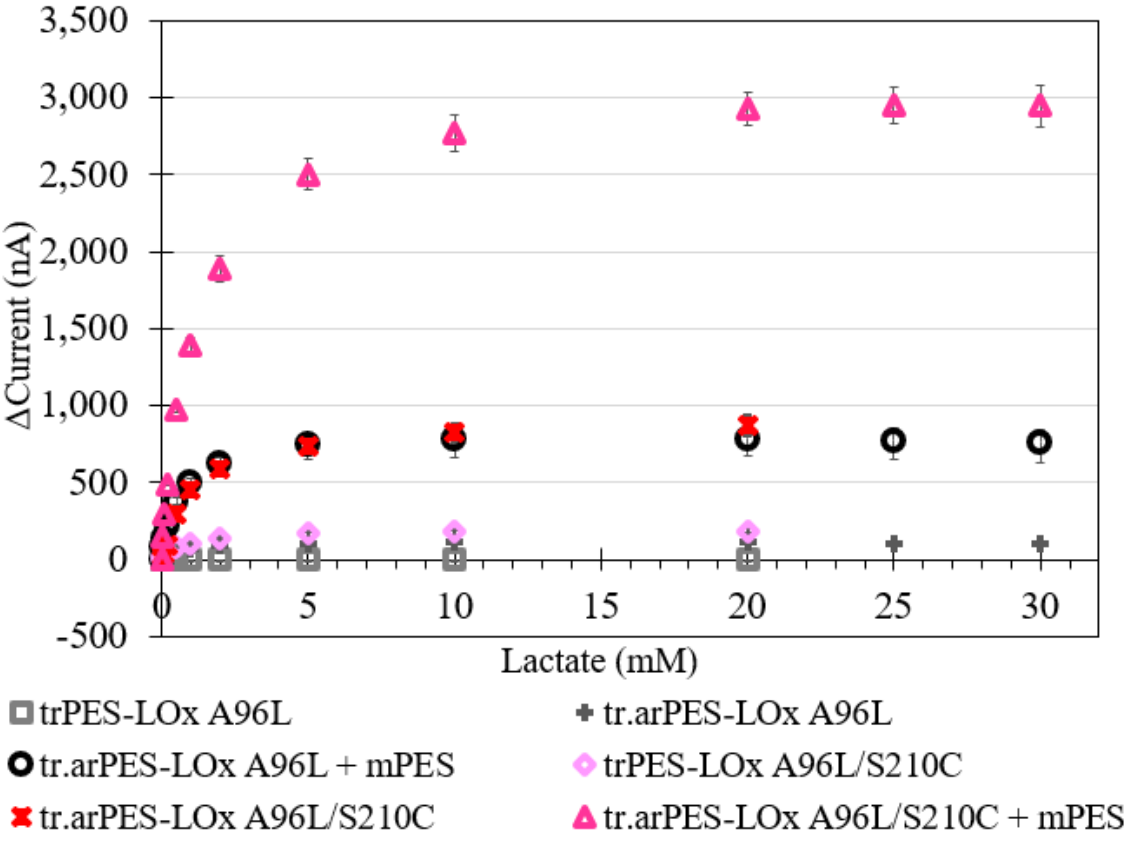


Figure 4-10. Steady-state response currents of LOx A96L or LOx A96L/S210C modified with trPES or trPES+arPES in the absence or presence of free mediator (mPES) in the solution. Lactate: 0-20 or 0-30 mM (n = 3). Applied potential: 0 V (vs Ag/AgCl).

4-3-7. Modification and electrochemical investigation of *Af*GDH modified with trPES

To evaluate the application of trPES for other enzymes, *Af*GDH WT (control) and *Af*GDH K477C were modified with trPES. Residue K477 was predicted as a suitable position to attach a redox mediator that could allow electron transfer from the cofactor to the electrode through the attached mediator (Suzuki *et al*., 2020). As in the chosen residues in LOx for cysteine substitution, residue K477 is also located close to the *Af*GDH cofactor, FAD, on the enzyme surface. *Af*GDH K477C mutant has one cysteine residue located in the enzyme surface; thus, the same approach as for LOx is acceptable. The response currents of modified trPES-*Af*GDH WT and trPES-*Af*GDH K477C were obtained (Figure 4-11). trPES-*Af*GDH K477C clearly showed a response current with the current depending on the glucose concentration, whereas no response current was observed from trPES-*Af*GDH WT. The results suggest that the attached trPES at residue 477 successfully mediated the electron transfer from the flavin to the electrode.

Table 4-4 Properties of lactate sensors.

Lactate sensor	K_m (mM)	I_{max} (nA)	Sensitivity (μA/mM.cm^2)*	LOD (μM)
trPES-LOx A96L	N.A.	N.A.	N.A.	N.A.
tr.arPES-LOx A96L	0.43	111	0.89	21.5
arPES-LOx A96L	0.23	89	0.82	22.8
trPES-LOx A96L/S210C	0.69	192	1.40	18.3
tr.arPES-LOx A96L/S210C	1.80	1000	6.62	9.9
arPES-LOx A96L/S210C	0.57	90	0.68	26.8
trPES-LOx A96L/N212C	0.86	68	0.42	27.5

*Sensitivity for detection range of 0.05–1 mM lactate
LOD: Limit of detection
N.A.: Not available
K_m and I_{max} were determined using a Hanes–Woolf plot

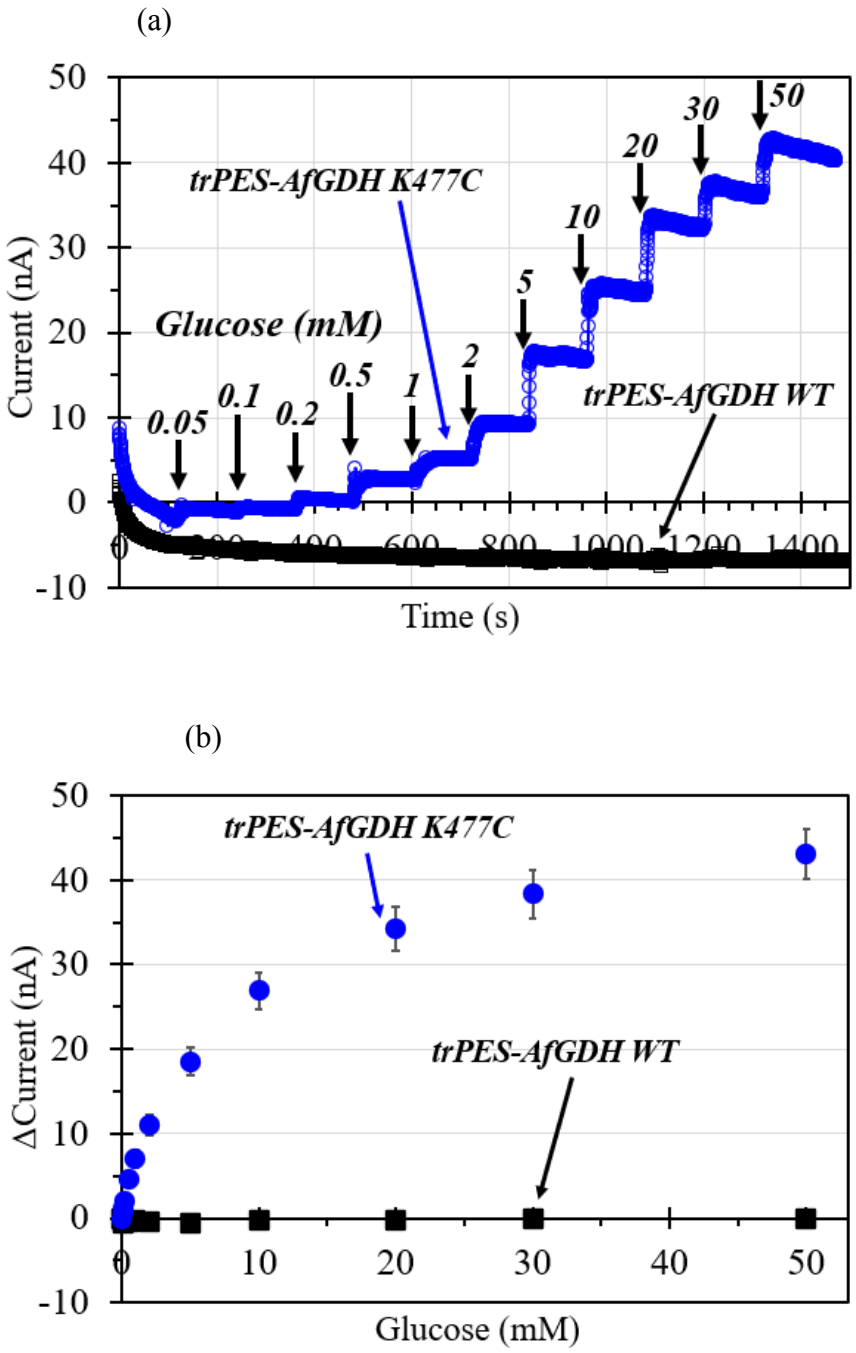


Figure 4-11 Response currents of *Af*GDH modified with trPES: (a) representative time course of response currents and (b) steady-state currents of trPES-*Af*GDH WT (black) and trPES-*Af*GDH K477C (blue) at various glucose concentrations (n = 3). Applied potential: 0 V (vs Ag/AgCl).

4-4. Discussion

In this study, LOx was modified with trPES to achieve a quasi-DET enzyme. LOx does not have a cysteine residue. Therefore, to enable the modification with trPES, a cysteine residue was introduced at the active site entrance on the LOx surface. Thus, the modified trPES-LOx cysteine mutant should have one trPES molecule attached near the flavin and the electrode to realize electron transfer. Here, the clear impact of modification with a single mediator molecule on a redox enzyme is described. The best location for the attachment of redox mediator on oxidoreductases to enable electron transfer is then confirmed.

The oxygen-insensitive mutant, LOx A96L, a suitable enzyme for MET-type sensors (Hiraka *et al*., 2018), was used as a template to design LOx cysteine mutants. Five variants of LOx A96L/cysteine mutant were prepared, and all were active, although they exhibited various enzyme activities upon cysteine substitution (Table 4-3). After chemical modification with trPES, the enzyme activity was observed, although the activity was decreased by the modification. In a previous study, residues S178 and N212 were substituted with lysine to obtain LOx A96L/S178K and LOx A96L/N212K, respectively (Hiraka *et al*., 2020), LOx A96L/S178K lost its activity as a result of mutation (Hiraka *et al*., 2020), whereas in this study, LOx A96L/S178C maintained its activity. However, trPES-LOx A96L/S178C showed a drastic decrease in activity. In contrast to LOx A96L/N212K, which has been reported to exhibit high enzyme activity (Hiraka *et al*., 2020), the activity of LOx A96L/N212C in this study dramatically decreased after mutation. A LOx cysteine mutant with high enzyme activity that is maintained after modification with trPES was expected to show a high response current in an amperometric evaluation. For this, LOx A96L/V185C and LOx A96L/S210C are potentially suitable mutants because they successfully maintained their high activity

after modification with trPES (Table 4-3). Additionally, differences in K_m values of all LOx cysteine mutants before and after modification might have been caused by the modification with trPES, which might alter the accessibility of the substrate (lactate) to the active center of enzymes (Okurita *et al*., 2018; Suzuki *et al*., 2020).

The LOx mutants were successfully modified with trPES because redox peaks attributed to trPES were observed in the cyclic voltammograms of all trPES-LOx mutants. Because redox peaks were also observed in trPES-LOx A96L even though this mutant does not possess a cysteine residue, trPES might have attached on its surface during the incubation with the enzyme. The modification with trPES was performed at neutral pH, which is the optimum condition for the reaction of maleimide with a thiol group. However, the maleimide group is also known to react slightly with the amino group at this pH (Brinkley, 1992; Baslé *et al*., 2010; Renault *et al*., 2018; Quanta Biodesign Limited; ThermoFisher Scientific.a; ThermoFisher Scientific.b). Therefore, trPES might modify not only the cysteine residue but also the lysine residue(s) on the LOx surface. This may occur in enzymes that possess multiple lysine residues on their surface such as LOx. Nevertheless, the amperometric results showed no response current for trPES-LOx A96L, indicating that spontaneous modification of the lysine surface with trPES does not affect electron transfer from flavin to the electrode. Therefore, trPES can be used to observe the impact of modification with a single trPES molecule on the active site entrance to enhance electron transfer, thus revealing the best position for attaching the mediator on a LOx surface.

The response currents toward lactate were observed in CA, indicating that PES can accept electrons from flavin and successfully transfer them to the electrode. Among the trPES-modified enzymes, only trPES-LOx A96L/cysteine mutants showed response currents toward lactate, suggesting that only the trPES-modified cysteine residue took

part in electron transfer. Since flavin, the redox center of the enzyme, is deeply buried in the enzyme structure, the electrons from the cofactor are rarely directly transferred to the electrode. To realize electron transfer, an electron mediator is used as a relay. In this case, the electron transfer occurs in two steps: (1) intramolecular electron transfer from the enzyme redox center to the electron relay and (2) transfer of electrons to the electrode (Degani and Heller, 1987).

In redox proteins, a high intramolecular electron transfer rate is achieved within a distance of 14 Å (Page *et al*., 1999). The distance between the isoalloxazine ring of FMN and trPES, which acts as the electron relay center (Degani and Heller, 1987) in the modified trPES-LOx, can be assumed to be the distance from N5 of flavin to Cα of cysteine (Figure 4-1). Since all the cysteine residues are close to flavin, all the trPES-LOx A96L/cysteine mutants were expected to show electron transfer, especially trPES-LOx A96L/N212C, because arPES-LOx A96L/N212K showed high response currents in a previous study (Hiraka *et al*., 2020), trPES-LOx A96L/N212C, trPES-LOx A96L/S178C, and trPES-LOx A96L/S210C were expected to achieve higher response currents in the amperometric evaluation because the distance between flavin and residues N212, S178, and S210 is shorter (<14 Å) than that between flavin and residues V185 and V20.

On the other hand, considering that all the mutations were designed at residues located at the enzyme surface, the electrons can be readily transferred from the relay to the electrode in all the trPES-LOx mutants. In fact, only trPES-LOx A96L/S210C exhibited a high response current and showed clear dependence to 0–20 mM lactate, which can be attributed to the highly efficient intramolecular electron transfer in the modified enzyme. LOx A96L/N212C showed low enzyme activity because of the impact of cysteine mutation; thus, trPES-LOx A96L/N212C exhibited a low response

current. trPES-LOx A96L/S178C showed no response current, mostly because of the drastic decrease in the enzyme activity after modification with trPES. trPES-LOx A96L/V185C exhibited a lower response current than trPES-LOx A96L/S210C, even though both mutants showed equally high activity both before and after modification with trPES. This may be due to the longer distance of residue V185 from flavin (>14 Å), which decreases the electron transfer rate (Degani and Heller, 1988; Marcus and Sutin, 1985). The longer distance of residue V20 from flavin may also be the reason for the low response currents in trPES-LOx A96L/V20C, in addition to their low activity after modification with trPES.

Furthermore, LOx A96L/S210C was modified with trPES and arPES to increase the number of PES molecules attached on LOx. For tr.arPES-LOx A96L/S210C, the response current was five-fold higher than that for trPES-LOx A96L/S210C. In this case, the response current was obtained from complex electron transfer, which included intramolecular electron transfer as the first step of the process. Thus, the response currents in tr.arPES-LOx A96L/S210C obtained from intramolecular and complex electron transfer were in the ratio 1:4. To elucidate details of the complex electron transfer, further in-depth study is necessary. Other modified enzymes with many attached PES molecules, i.e., tr.arPES-LOx A96L, arPES-LOx A96L, and arPES-LOx LOx A96L/S210C (Figure 4-8), exhibited similar response currents, which were lower than that obtained for trPES-LOx A96L/S210C with a single attached PES molecule. These results support the previous statement (Section 4-3-6) that a high response current can be achieved if at least one PES molecule is attached in a crucial position that enables electron transfer from the reduced flavin. Increasing the number of attached PES molecules can thus increase the response current, as observed in tr.arPES-LOx A96L/S210C. In addition, the increasing response currents due to the increasing number

of mediator molecules was shown by the addition of free mediator in the solution (Figure 4-9 and Figure 4-10). Here, a lactate sensor with high response currents and markedly improved performances was successfully developed using tr.arPES-LOx A96L/S210C (Table 4-4). For the same detection range (up to 1 mM lactate) as the reported lactate sensor using arPES-LOx A96L/N212K (Hiraka *et al*., 2020), the LOD was improved to less than 9.9 μM, suggesting that our lactate sensor can be used to detect such low lactate concentrations. Although the sensitivity of the lactate sensor (6.6 $\mu A/mM.cm^2$) is only half that of the sensor reported in study of Hiraka *et al*. (2020), because lactate can be detected at low concentrations owing to the low LOD, this lactate sensor can be used with confidence.

Residue S210 is found as the best position for modification with trPES because high electron transfer was achieved using the LOx modified with trPES attached to the residue. In a previous study, residue N212 was assumed as the best position for attaching arPES (Hiraka *et al*., 2020). In the 3D structure of LOx, residue S210 is located next to residue N212 at the active site entrance on the LOx surface, and both residues are located at a similar distance from flavin (<14 Å) (Figure 4-1). Combining our findings and the result of a previous study showing that residue N212 is important for electron transfer accessibility (Hiraka *et al*., 2020), the positions of residues S210 to N212 are obviously important for mediator access. When PES is attached at a position near residues S210 to N212 on LOx, the PES has high accessibility to electrons from the reduced flavin; thus, highly efficient electron transfer can be achieved.

In addition, *Af*GDH, a representative FAD-dependent oxidoreductase, was modified with trPES and its electrochemical performances were evaluated to demonstrate the versatility of trPES. Response currents were successfully observed with trPES-*Af*GDH K477C (Figure 4-10), suggesting that, with a similar approach, trPES can

be applied to other redox enzymes and thus to other proteins. On the basis of our structural analysis, FAD (N5) and residue K477 (Cα) are separated by a distance of 21.8 Å. This relatively long distance may be the reason for the response current observed in trPES-*Af*GDH K477C being lower than that observed in trPES-LOx A96L/S210C. However, this distance is still in the range of long-distance electron tunneling in proteins (<30 Å); thus, electron transfer can occur from the enzyme cofactor to the electrode (Stuchebrukhov, 2010). This distance is similar to the distance between FAD (N5) and residue I489 (Cα) in *An*GOx (21.7 Å), a crucial residue for mediator access in *An*GOx (Suzuki *et al*., 2020). These results indicate that quasi-DET is possible in a modified enzyme-redox mediator provided the mediator is attached within an acceptable distance range and in an accessible position for electron transfer.

Initial studies on protein modification using redox mediators mostly targeted the amine group of the lysine residue, and they were performed without considering protein structural analysis (Degani and Heller, 1987; Degani and Heller, 1988; Bartlett *et al*., 1987; Bartlett *et al*., 1991; Ryabov *et al*., 1992; Schuhmann, 1995) because the protein crystal structures were not known at that time. Hence, the modification of a protein with a single redox mediator molecule was not possible. In 1993, Badia *et al*. reported the use of a ferrocene derivative as a redox mediator to modify the amine group of lysine residues in *An*GOx by considering the X-ray structure, which had been reported in the previous year (Badia *et al*., 1993), The authors stated the importance of a few key ferrocene groups being attached to the enzyme close to the enzyme cofactor and the protein surface for electrocatalytic activity, rather than the number of ferrocene groups attached to the enzyme. This was one of the most important considerations in designing our study for the modification of oxidoreductases with a single redox mediator molecule using trPES.

The immobilized lactate sensors can be used in a continuous monitoring system or for flow injection analysis, as indicated in the study of Hatada *et al*. (2021), and the use of quasi-DET enzymes based on modified enzyme-redox mediators could prevent the leakage of mediators. However, some consequences of applying redox mediators for enzyme modification may occur, such as decreased enzyme activity due to enzyme mutation or chemical modification (Degani and Heller, 1987; Degani and Heller, 1988; Badia *et al*., 1993; Ban *et al*., 2003; Suzuki *et al*., 2020). Therefore, to anticipate any decrease in enzyme activity, the enzyme should have high activity before modification with redox mediators so that the activity is not decreased after enzyme modification, enabling appropriate response currents to be achieved in amperometric evaluation (Hatada *et al*., 2018).

The application of trPES can be expanded to a wider range of applications in bioelectrochemical analysis to develop better electrochemical sensor systems and to analyze the functions and dynamics of proteins, peptides, and nucleotides. trPES can be applied to other redox enzymes whose structures are well characterized. For enzymes that already possess native cysteine residues on their protein surface, it may be necessary to substitute these cysteines with other residues to introduce a new cysteine residue in the selected position to attach the trPES. trPES can also be conjugated with other proteins and peptides, as shown previously for binding proteins (Trammell *et al*., 2001; Benson *et al*., 2001; Sandros *et al*., 2005; Sandros *et al*., 2006; Morón *et al*., 2011) or nucleotides in recent studies on the application of arPES to aptamer-based sensors (Nagata *et al*., 2022; Kim *et al*., 2022), trPES is an alternative to the only previously reported redox mediator targeting cysteine, a ruthenium complex derivative (Trammell *et al*., 2001; Benson *et al*., 2001; Sandros *et al*., 2005; Sandros *et al*., 2006; Morón *et al*., 2011). This is because both trPES and the ruthenium complex derivative

have low redox potential (Trammell *et al*., 2001; Dojindo Molecular Technologies, Inc.d), and some redox enzymes were reported not to react with hexaammine ruthenium(III) because of its cationic property (Loew *et al*., 2017a; Loew *et al*., 2017b; Okurita *et al*., 2018). In addition, the use of the rare metal ruthenium raises problems related to sustainable usage. Therefore, the organic compound trPES is more suitable for large-scale and continuous applications. Moreover, trPES is now easy to obtain because it has become commercially available.

4-5. Conclusion

In this study, a novel and now commercially available redox mediator, trPES, was applied for the first time for the development of a quasi-DET-type lactate sensor. LOx, a well-characterized redox enzyme that is widely used to study lactate sensors, was successfully modified with trPES to produce a quasi-DET enzyme. The maleimide group of trPES was covalently bound to the thiol group of the cysteine residue in LOx after introducing a cysteine residue near the LOx cofactor FMN at the entrance to the active site on the LOx surface. Because native LOx does not have cysteine, the LOx cysteine mutant was modified with trPES to attach a single trPES molecule at the selected position on LOx. One of the modified enzyme variants, trPES-LOx A96L/S210C, exhibited high response currents in an amperometric evaluation, indicating the efficient transfer of electrons from flavin to the electrode through the attached trPES. This result suggests that the modified enzyme was successfully immobilized to realize a lactate sensor. Highly efficient electron transfer was observed in the LOx cysteine mutant with cysteine introduced at a residue with distance <14 Å from FMN (N5). A high response current can be achieved provided the LOx cysteine mutant maintains its dye-mediated dehydrogenase activity after mutation and after modification with trPES. Here, the position around residues S210 to N212 on the LOx surface (distance <14 Å from FMN (N5)) is obviously important to ensure the accessibility of the mediator to the reduced flavin. The attachment of a single PES molecule at these positions resulted in highly efficient electron transfer from the reduced flavin to the electrode. Furthermore, the number of attached PES molecules was increased by modifying LOx A96L/S210C with trPES and arPES, which are redox

mediators used to modify the lysine residue of proteins, to obtain tr.arPES-LOx A96L/S210C. The response current of the enzyme electrode based on tr.arPES-LOx A96L/S210C was five-fold higher than that of trPES-LOx A96L/S210C, thus improving the performances of the lactate sensor. The lactate sensor had a detection range of up to 1 mM with a sensitivity of 6.62 μA/mM.cm^2 and a LOD of 9.9 μM. This is the first application of trPES in the development of lactate sensor utilizing LOx for future continuous lactate monitoring system. Here, the trPES was used to understand the bioelectrochemical property of LOx. Finally, the versatility of trPES for another redox enzyme was evaluated. Following the same strategy, *Af*GDH and the mutant *Af*GDH K477C were successfully modified with trPES to obtain response currents, indicating that trPES can be applied to other oxidoreductases. *Af*GDH has one cysteine residue buried in the enzyme structure; thus, the same approach as that for LOx, which does not have a cysteine residue, is acceptable. For enzymes that already possess native cysteine residues on their protein surface, it may be necessary to substitute these cysteines with other residues to introduce a new cysteine residue in the selected position to attach trPES. The application of the new material trPES as a redox chemical for biological materials was reported. The study can be expanded by applying trPES to other proteins, peptides, or nucleotides for its wider application in sensing systems and bioelectrochemical analyses.

Chapter 5. Conclusion

5-1. Conclusive remarks

Electron mediator is one of major components for MET-type enzyme sensor next to the enzyme. The mediators are employed in 2nd and 2.5th generation electrochemical enzyme sensors. To develop enzyme sensors which can exhibit optimum performances, selection of the mediators for enzymes should be highly considered. The chosen mediators should have high preference with the enzymes used, thus the developed enzyme sensors can achieve maximum sensor responses, and this can increase the accuracy of the biosensors. Furthermore, the mediators, as well as the enzymes, should have appropriate stability to maintain the accuracy of the biosensors after being stored for some period of time. One of the popular mediators, mPMS, is a phenazine derivative and has been employed for commercial lactate sensors strip (Pharmaceuticals and Medical Devices Agency, *online*). However, mPMS is known to have a problem with the stability. A novel mediator from phenazine derivative, mPES, has higher stability than mPMS. Therefore, mPES is potential to be employed for the development of 2nd generation enzyme sensors in the future.

MET-type sensors based on 2nd generation has been used for the development of disposable enzyme sensor strips for self-monitoring and POCT. Whereas MET-type sensors based on 2.5th generation can be applied for continuous monitoring system or in flow injection analysis (FIA)-type sensors (Hatada *et al*., 2021). Although there is lack information of that the 2.5th generation sensor type is already employed for commercial enzyme sensors or not, studies on this sensor type have been reported since 1980s. In the last four years, our research group reported some studies in the development of biosensors based on 2.5th generation employing a novel redox mediator from phenazine

derivative, arPES. This arPES has been commercially available. Proteins or enzymes can be modified with arPES through their lysine residues and provides quasi-DET ability to the enzymes. If the enzymes have quasi-DET ability, the electrons are able to be transferred from reduced cofactor to electrode through the attached arPES. Although the prevalence of lysine in proteins is high, so that the modified enzyme-arPES has lot of arPES attached in the enzyme, arPES should be attached in certain location in the enzyme surface to allows an effective electron transfer. Therefore, most of the studies reported by our research group are focusing on engineering the enzymes by introducing a lysine residue in the location that expected to achieve high quasi-DET ability. Recently, another novel redox mediator from phenazine derivative, trPES, has been also commercially available. Enzymes can be modified with trPES through their cysteine residues. Because the prevalence of cysteine in proteins is low, it allows the attachment of a single molecule of trPES at enzymes. For the first time, I reported the application of trPES for the development of quasi-DET lactate sensor for future continuous monitoring system.

Summary of the achievements presented in this dissertation is provided below

Chapter 2, titled "Characterization of electron mediator preference of *Aerococcus viridans*-derived lactate oxidase for use in disposable enzyme sensor strips"

Three popular mediators which have been used in commercially available electrochemical enzyme sensors, ferricyanide, mPMS and hexaammine ruthenium(III), were coupled with *Av*LOx to evaluate the response currents of mimicked disposable blood lactate sensor. The mimicked lactate sensors employing mPMS as mediator showed the most effective for LOx, achieving high sensitivity along with wide linear range when applied 100 mM mPMS per sensor strip. When employing ferricyanide in

the same concentration, the results showed less effective than those obtained with mPMS, but it showed response currents, and it is acceptable for blood lactate levels measurement. Whereas, when employing hexaammine ruthenium(III), no response current was observed, suggesting that hexaammine ruthenium(III) is not utilized as a mediator by *Av*LOx.

The key factor that influences those results are the electrostatic interaction between small mediator compound with local surface charge of LOx. The relatively uncharged mPMS is easily interact with LOx surface without any repulsion; the strong negative charge ferricyanide is less repulsed but does transfer the electrons from cofactor to electrode, suggesting that LOx can tolerate negatively charge of ferricyanide fairly well; and the strong positive charge hexaammine ruthenium(III) cannot mediate the electron transfer due to the access of hexaammine ruthenium(III) to the cofactor is blocked by the local surface charge of LOx.

These results reveal the evident that the electrostatic interaction between small molecule mediators with the local charge of enzyme surface is the important factor in allowing electron transfer from cofactor to electrode that mediated by the mediators. The results in this study can be a strong consideration in choosing a suitable mediator when coupling it with enzymes in for the development of electrochemical enzyme sensors. Additional consideration for the future usage, especially for mass production, mPMS in an organic based mediator, derived from phenazine, can be easier to produce than inorganic based mediator. Hexaammine ruthenium(III) is an inorganic and metal-based mediator with the main component is a rare metal ruthenium, thus their usage for mass production and continuously should be reconsidered to maintain the sustainability.

Chapter 3, titled “Employing a novel phenazine derivative mediator 1-methoxy-5-ethyl phenazinium ethyl sulfate as a stable electron mediator for disposable lactate sensor”

A novel mediator, mPES, was employed for the construction of disposable lactate sensor. mPES has higher stability in wide range of pH than the phenazine derivative used in Chapter 2, mPMS. This mPES has been commercially available. A LOx mutant which has low O_2 reactivity, A96L mutant, was used to construct the lactate sensor. The lactate sensor exhibited good performances such as high sensitivity and wide linear range. The lactate sensor showed good storage stability at 25 ºC for up to 120 days (± 4 months). The lactate sensor can be used at low potential as 0 V vs Ag/AgCl, thus minimizing the effect of common electroactive interferences in blood, such as uric acid and acetaminophen. Furthermore, the versatility of mPES was evaluated in other oxidoreductases such as *Af*GDH, *An*GOx, and *Pn*FPOx.

Chapter 4, titled "Application of a novel redox mediator from phenazine derivative for the development of a quasi-direct electron transfer-type lactate sensor"

A novel redox mediator, trPES, was used to modify *Av*LOx through its cysteine residue. *Av*LOx A96L/cysteine mutants were engineered by introducing a cysteine residue in the entrance active at the enzyme surface, thus the introduced cysteine is close to the cofactor and electrode surface. Then, the LOx A96L/cysteine mutants were modified with a single molecule of trPES. Some of LOx A96L/cysteine mutants maintained their enzyme activity after modified with trPES. A variant of modified LOx, trPES-LOx A96L/S210C, showed the highest response currents in amperometric evaluation among others. This is suggesting that the location to attach trPES in residue S210 is the best among other locations, allowing highly efficient electron transfer in trPES-LOx A96L/S210C. Apparently, the observed response currents was obtained from intramolecular electron transfer because there is only one molecule trPES attached per monomer of LOx and it was successfully mediate the electron transfer from cofactor to electrode.

Furthermore, LOx A96L/S210C was modified with two kinds of novel redox mediator, arPES and trPES, obtaining tr.arPES-LOx A96L/S210C. The response current was increase 5-fold higher than that obtained in trPES-LOx A96L/S210C. In this case, $1/5^{th}$ (20%) of response current was obtained from intramolecular electron transfer and the rest, $4/5^{th}$ (80%) was obtained from other complex electron transfer mechanisms. The increasing of response currents in tr.arPES-LOx A96L/S210C are due to the increasing of electron transfer rate since the number of attached PES molecule increased. The ratio of 1:4 also represents the ratio of whole electron transfer (from cofactor to electrode) of trPES-LOx A96L/S210C : tr.arPES-LOx A96L/S210C. In the case of trPES-LOx A96L/S210C, electron transfer rate can represent the rate of intramolecular electron transfer; whereas in tr.arPES-LOx A96L/S210C, electron transfer rate represents the whole rate of electron transfer involving intramolecular electron transfer and other complex electron transfer mechanisms.

Finally, the versatility of trPES was evaluated with *Af*GDH. The amperometric response was clearly observed in the modified cysteine mutant, trPES-*Af*GDH K477C, whereas the modified wild type, trPES-*Af*GDH WT, did not show the response current. These results indicate that trPES can be applied in other oxidoreductases such as *Af*GDH.

The following table is the summary of the properties of lactate sensors constructed in this study.

Table 5-1. Summary of the properties of lactate sensors constructed in this study.

No.	Lactate sensor based on:	Sensitivity	Dynamic range	LOD
1	LOx-mPMS	1.04 μA/mM	0.5-50 mM	0.5 mM
2	LOx A96L-mPES	0.73 μA/mM	0.5-50 mM	0.5 mM
3	trPES-LOx A96L/S210C	1.40 μA/mM.cm^2	0.05-1 mM	0.0183 mM
4	tr.arPES-LOx A96L/S210C	6.62 μA/mM.cm^2	0.05-1 mM	0.0099 mM

No. 1 is mimicked lactate sensor (2^{nd} generation), No. 2 is disposable lactate sensor (2^{nd} generation), and No. 3 and 4 are CLM (2.5^{th} generation).

5-2. Implications and perspectives

Lactate sensors based on 2nd generation electrochemical enzyme sensor are widely used to develop disposable lactate sensors for self-monitoring and POCT. Many studies attempt to develop lactate sensors to achieve high accurate enzyme sensors. Initial studies in characterization of potential mediators are important since mediator is one of the major components for 2nd generation enzyme sensors next to the enzyme. This study can be used as a strong reference for further study in the development of enzyme sensors with better performance than that currently exist.

On the other hand, 2.5th generation electrochemical enzyme sensors has been studying since 1980s as an alternative to the 3rd generation sensors. As well as the 3rd generation sensors, 2.5th generation sensors can be applied for continuous monitoring systems. 2.5th generation enzyme sensors, which employed modified enzyme-mediators to achieve quasi-DET enzymes, is an alternative to the 3rd generation sensors, which the limitation of number of DET-type enzyme is the current issue.

Trend in utilization of phenazine derivative-based mediators in the future

Along with the development of human civilization, people are increasingly aware of the quality of their lives and their health. On the other hand, the development of human civilization also makes people become busier due to that many activities that they can do as the impact of science and technology development. Then, junk food becomes the best alternative for busy people since it is very simple and affordable but often not healthy. Then the degenerative diseases become increase and more variative, and so do the patients. Combination of awareness and busyness leads to the increasing demand of self-monitoring analyzers for various analytes, which are markers for specific body conditions. The self-monitoring analyzers are used as an early detection to

a disease or specific body condition; or used to monitor the body condition for people whose have been diagnosed to a disease that require routine monitoring. For further early detection by medical technician which require quick results, POCT analyzers is required. Moreover, monitoring of analytes continuously and real-time are also another demand.

Lactate sensors for self-monitoring and POCT are examples of hand-held analyzers that has been commercially available. The performance of lactate sensors are becoming better from time to time. Studies on the development of lactate sensor strips are increasing along with increasing demand of high accurate lactate measurements using lactate sensors. Therefore, studies related to the mediators for used in lactate sensor strips in MET-type sensors are required.

Lactate sensors for continuous monitoring are also another demand being increased. The 3rd generation electrochemical enzyme sensors are considered as an ideal continuous monitoring sensor system. However, because the number of enzymes which has DET ability is limited. Alternatively, 2.5th generation electrochemical enzyme sensors are developed. Enzymes are modified with redox mediators to gain quasi-DET ability to the enzymes. Then, the modified enzyme-mediators can be applied in continuous monitoring system without worrying the leakage of mediators in sample.

On the other hand, the selection of chemicals for the development of biosensors should reconsider the environmental issue and works with the environmental-friendly compounds is highly recommended to maintain the sustainability. For example, The utilization of hexaammine ruthenium(III) is gaining attention because it allows low application potential and has high stability toward light irradiation. However, this inorganic mediator is formed from a rare metal compound, ruthenium, which is rather difficult to obtained and nonrenewable. This makes the compounds contain ruthenium

pricy. Therefore, the future trend of the utilization of organic-based mediator is predicted will be more massive due to their relatively easy to synthesize and environmentally friendly.

Along with that, in the last few years, new organic-based mediators have been commercially available. mPES, which has been commercially available in 2018, is a derivative of PES, a stable version of phenazine derivative. mPES has higher stability in wide range of pH than the previous existing one, mPMS, which also a derivative of phenazine. Then, arPES and followed by the newest one, trPES, has been commercially available in 2019; both arPES and trPES are redox mediator from PES derivative. Employment of arPES and trPES for biosensors and bioelectroanalysis is predicted as a new trend in developing electrochemical enzyme sensors, as well as reported by our research group in the last four years (Hatada *et al*., 2018; Hiraka *et al*., 2020; Suzuki *et al*., 2020; Hatada *et al*., 2021). Furthermore, trPES can be used for wider application of only for biosensing application, also for bioelectroanalysis.

Development of an accurate mediated enzyme sensors for disposable type sensors and continuous monitoring systems

The demand of lactate sensors in market are increasing. One of the implications of it is that the commercially available mediators are becoming more variative since the studies in the development of lactate sensors increase. For examples in phenazine derivatives mediator, PMS has been using as a mediator in colorimetric assays, then PES was found more stable than PMS toward light irradiation. Next, mPMS, the electroactive form of PMS, was used in electrochemical analysis. Then, mPES, which more stable than mPMS, was then introduced and commercialized. Furthermore, arPES, a redox mediator which can modify lysine residues in proteins, was also introduced and commercialized. The later one, trPES, which can modify cysteine residues in proteins

and also has been commercialized, was introduced in this study. A series of innovations from these phenazine derivatives shows the concern of the mediator development. Finally, the excellent mediator will be used for the development of excellent biosensors for disposable sensors and continuous monitoring systems.

In the future, the demand of lactate sensor in market will continuing increase as well as other biosensors for detecting glucose, HbA1c, and uric acid. Therefore, an excellent lactate sensor and other biosensors are required. Furthermore, the development of biosensors for detecting multi analytes is becoming a trend currently. The multi analyte biosensors offering simplicity usage and lower cost to obtain multi analyte level results. Therefore, excellent multi analyte biosensors are required.

Moreover, the results of the analytes detection levels can be integrated with Internet of Things (IoT), an integrated internet-based information system that is currently gaining massive attraction in the development of biosensor applications along with increasing the application of smart living solution platform and IoT. The biosensors are the source of biological data continuously that can provide useful information about a person's condition and its health history. The application of IoT in collecting and transmitting information subsequent integrate the results of analyte levels detection from the biosensors is very useful to provide better monitoring and treatment (Pateraki *et al*., 2020).

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