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Rational engineering of *Aerococcus viridans* L-lactate oxidase for the mediator modification to achieve quasi-direct electron transfer type lactate sensor

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ABSTRACT

The L-lactate oxidase (LOx) based lactate sensors are widely used for clinical diagnostics, sports medicine, and food quality control. However, dissolved oxygen interference and electroactive interferent effects are inherent issues of current lactate sensors. In this paper, a quasi-direct electron transfer (quasi-DET) type lactate sensor was developed using rationally engineered *Aerococcus viridans* LOx (AvLOx) modified with amine-reactive phenazine ethosulfate (PES). Since the modification of wild type AvLOx by PES did not result quasi-DET, engineered AvLOx with additional Lys residue was designed. The additional Lys residue was introduced by substituting residue locating on the surface of AvLOx, and within 20 Å of the isoalloxazine ring of FMN. Among several constructed mutants, Ala96Leu/Asn212Lys double mutant showed the highest dye-mediated dehydrogenase activity with negligible oxidase activity, showing quasi-DET properties after PES modification, when the enzyme was immobilized on screen printed carbon electrode. The constructed electrode did not show oxygen interference in cyclic voltammetric analysis and distinct catalytic current with 20 mM L-lactate. The sensor performance of a chronoamperometric L-lactate sensor employing PES modified Ala96Leu/Asn212Lys AvLOx, marked with linear range between 0 and 1 mM, with sensitivity of 13 μA/mM·cm^2, and a limit of detection of 25 μM for L-lactate. By applying −200 mV vs. Ag/AgCl, L-lactate could be monitored with negligible interference from 170 μM ascorbic acid, 1.3 mM acetaminophen, 1.4 mM uric acid or 20 mM glucose. These results indicated that a quasi-DET type lactate sensor was developed that did not suffer from the interference of oxygen and representative electroactive ingredient compounds.

1. Introduction

L-lactate is an important biomarker for clinical diagnostics (Bakker et al., 2013), fitness monitoring in athletes (Billat, 1996), and food quality control (Mello and Kubota, 2002). The L-lactate concentration can reflect lactic acidosis which is caused by tissue hypoxia or other underlying diseases such as liver disease or sepsis (Mizock and Falk, 1992). In addition, monitoring the L-lactate concentration can indicate the lactate thresholds of athletes, an indicator of endurance (Faude et al., 2009). L-Lactate biosensors employing L-lactate oxidase (LOx) as a molecular recognition element have been studied since the first report of enzyme glucose sensors because of the high specificity and stability of LOx (Matsunaga et al., 1982; Palleschi and Turner, 1990; Ohara et al., 1994). LOx (EC: 1.1.3.15) is a member of the flavin mononucleotide (FMN)-dependent α-hydroxyacid oxidizing flavoprotein family (Duncan et al., 1989; Maeda-Yorita et al., 1995; Leiros et al., 2006; Umena et al., 2006; Li et al., 2007). This enzyme oxidizes L-lactate to pyruvate by FMN reduction in the reductive half-reaction and uses oxygen as a natural electron acceptor to reoxidize FMN and generate hydrogen peroxide in the oxidative half-reaction.

Two types of electrochemical principles have been reported in L-lactate biosensors using LOx: the first-generation principle employs oxygen as an electron acceptor, and the second-generation principle employs artificial electron acceptors. First generation L-lactate sensors

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have been reported as part of a microbial sensor system for the measurement of phenylalanine by the combination of LOx and an oxygen sensor (Karube et al., 1980) to detect a decrease in oxygen concentration depending on the L-lactate concentration. Then LOx was combined with a platinum electrode to detect hydrogen peroxide generated from the oxidation of L-lactate (Matsunaga et al., 1982; Clark et al., 1984). However, fluctuations in oxygen concentration directly affected the sensor signal, and the high potential applied for the oxidation of hydrogen peroxide also allows the oxidation of some electroactive interferents in blood, such as ascorbic acid, acetaminophen, and uric acid (Cass et al., 1984). Second-generation L-lactate sensors utilize artificial electron acceptors as electron mediators between LOx and the electrode (Palleschi and Turner, 1990). Another inherent issue of second-generation enzyme sensors employing oxidases is that the dissolved molecular oxygen competes with the electron mediator in the oxidative half-reaction of LOx and affects the sensor signal (Palleschi and Turner, 1990; Kenausis et al., 1996). To minimize the oxygen interference effect in second-generation lactate sensors, we have reported the development of an oxygen-insensitive L-lactate enzyme sensor by developing an LOx mutant (Hiraka et al., 2018). By using the biochemical and structural information of LOx derived from *Aerococcus viridans* (AvLOx) (Duncan et al., 1989; Maeda-Yorita et al., 1995; Stoisser et al., 2015a, 2015b, 2016), our group engineered AvLOx, by predicting the oxygen accessible channel of AvLOx and introducing a mutation. Thus, the constructed mutant, Ala96Leu, showed drastically low oxidase activity without losing dye-mediated lactate dehydrogenase activity, thermal stability, and substrate specificity. Consequently, the enzyme electrode with the immobilized AvLOx Ala96Leu mutant showed a much lower oxygen interference effect in potassium ferricyanide solution as an electron mediator compared with wild-type AvLOx under atmospheric conditions.

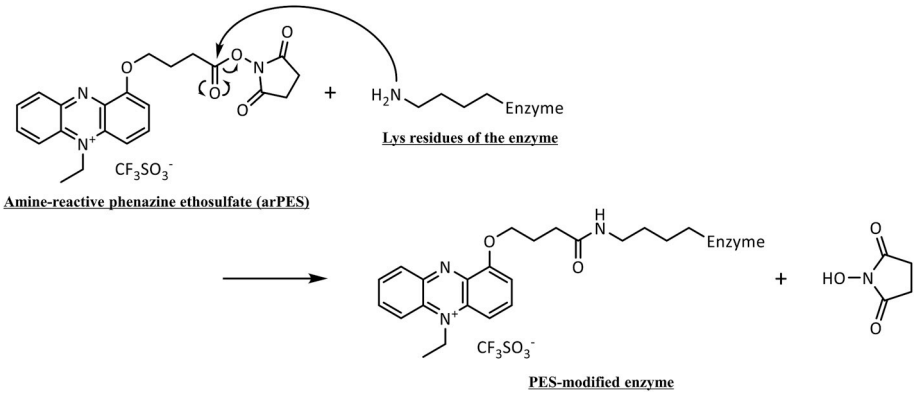
On the other hand, third-generation enzyme sensors that utilize direct electron transfer (DET)-type enzymes, have been considered ideal because the sensor signal is not affected by the fluctuations and limited diffusion of oxygen or mediators in the solution. In addition, low-oxidation-potential operation will resolve the potential problems in first- and second-generation enzyme sensors caused by the presence of interferents such as ascorbic acid and acetaminophen.

Besides, since the first-generation principle based glucose sensor was invented, the development of glucose sensors goes hand in hand with the history of discovery and engineering of glucose sensing enzymes to be mainly dedicated for diabetic patient (Okuda-Shimazaki et al., 2020; Ferri et al., 2011). As results, not only glucose oxidase, but several glucose dehydrogenases and their engineered enzymes were developed and available for first- and second-generation principle based commercial glucose sensors. Recently, glucose dehydrogenases capable of DET with electrode are paid attention to realize third-generation based continuous glucose sensors. In contrary, the technological progress of lactate sensor is relatively slow because the availability of lactate sensing enzymes is limited. LOx shows an oxygen interference effect since the primary electron acceptor of LOx is oxygen, and is not able to show DET. Consequently, the limitation of the enzyme availability restricted the progress of the development of lactate sensing principles. For that reason, further developments of lactate sensing enzymes, such as the reduction of the impact of oxygen interference and the addition of DET ability, are strongly desired to foster and accelerate technology development for the future lactate sensors.

In the 1980s, several groups started to modify redox enzyme by covalently attaching redox mediators to improve the electron transfer between the redox center and the electrode, to achieve "quasi-DET" type sensors, using such as ferrocene derivatives (Bartlett et al., 1987, 1991; Degani and Heller, 1987, 1988; Schuhmann et al., 1991; Ryabov et al., 1992; Badia et al., 1993; Tsai and Cass, 1995; Schuhmann, 1995; Sampath and Lev, 1996; Carolan et al., 2007), phenothiazine derivatives (Ban et al., 2001), ruthenium derivatives (Degani and Heller, 1987; Ryabova et al., 1999), tetrathiafulvalene derivative (Bartlett et al., 1997), and phenoxazine derivative (Krikstopaitis et al., 2004). By covalently attaching of redox mediators to the surface of redox enzymes, the electron transfer distance is reduced, and the intramolecular electron transfer from enzyme redox center to attached redox mediator, consequently, the intermolecular electron transfer from redox mediator to electrode was achieved. However, these methods for the modification of enzymes with mediators include relatively complex procedures, stepwise chemical reactions to introduce functional groups into redox mediator to be modified enzyme, mainly primary amine of Lys residue, which require long modification times. To propose versatile and simple method for enzyme modification with mediator with low redox potential, we previously reported a revival of the concept of mediator modification, which can be applied to several types of redox enzymes, for the development of quasi-DET type enzyme based sensors (Hatada et al., 2018). Flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenase (FADGDH) was modified with 1-[3-(succinimidyloxycarbonyl) propoxy]-5-ethylphenazinium trifluoromethanesulfonate or amine-reactive phenazine ethosulfate (arPES). Amine-reactive PES, which is currently commercially available, is composed of two parts, methoxy PES which is a stable, relatively low redox potential mediator, and a succinimide group. In this modification scheme, the primary amine groups of LOx, such as Lys residue, attack the carboxylate ester carbon of the succinimide group of arPES and form amide bonds in slightly alkaline conditions at room temperature (Scheme 1).

Therefore, PES-modified enzymes can be prepared by simply mixing arPES and enzymes. PES-modified FADGDH showed electron transfer from the enzyme to the electrode via the modified PES, which was described as a quasi-direct electron transfer (quasi-DET) principle. Thanks to the low redox potential of PES (−320 mV vs. Ag/AgCl), glucose sensor employed PES-modified FADGDH electrode could be operated by applying 0 mV vs. Ag/AgCl without any additional free electron mediator, even though FADGDH is not DET type enzyme. This achievement makes us possible to prepare the quasi-DET type sensors easily. The study also reported the PES modification of AvLOx. However, PES-modified AvLOx did not show quasi-DET. The reason was attributed to the oxygen interference effect and/or the fact that the modified positions in AvLOx, which were the position of Lys residue, might not be accessible at the electrode surface to achieve quasi-DET. These observations and interpretation inspired us to design AvLOx being suitable for PES modification by introducing Lys residue at a position located near the active center to achieve highly efficient electron transfer from FMN and should be accessible to the electrode to realize quasi-DET. Such approach, that is the rationally designing and introducing residue to be modified by mediators based on the structure of redox enzymes, has not been achieved in the previous achievements reported the modification of the enzyme with the redox active group to electron transfer mediated by the immobilized derivative.

In this paper, a quasi-DET type L-lactate sensor was developed by using an engineered oxygen-insensitive AvLOx mutant, which was rationally designed to be modified by arPES modification. To construct novel engineered AvLOx, we used the structural gene of AvLOx Ala96-Leu mutant (Hiraka et al., 2018), which showed decreased oxidase activity, as the template molecule. The residues for PES modification, which were substituted by Lys, were selected according to two criteria: 1) they should be located on the surface of AvLOx to facilitate electrode accessibility, and 2) they should be located within 20 Å of the isoalloxazine ring of FMN. The residues to be substituted by Lys were rationally proposed to be on the surface of AvLOx; subsequently, Lys-substituted mutants were evaluated colorimetrically and electrochemically. Eventually, the AvLOx Ala96Leu/Asn212Lys double mutant, which was successively modified with PES, showed a quasi-DET response toward L-lactate, while the wild type or Ala96Leu mutant showed almost no response. Unexpectedly, the double mutant showed almost negligible oxidase activity. Consequently, this developed L-lactate sensor employing PES-modified Ala96Leu/Asn212Lys double mutant AvLOx did not show a bias signal in the presence of



Scheme 1. The modification of primary amine of Lys residue by succinimide group of amine-reactive phenazine ethosulfate (arPES) during the preparation of PES modified enzymes.

acetaminophen, ascorbic acid, or uric acid when it was operated at −200 mV vs. Ag/AgCl, as a result of the low redox potential of PES, that is, −320 mV vs. Ag/AgCl.

2. Materials and methods

2.1. Chemicals and materials

Sodium L-lactate and poly(ethylene glycol) diglycidyl ether (PEGDGE) Mn500 were purchased from Sigma-Aldrich (MO, USA). 4-Aminoantipyrine (4-AA) and phenazine methosulfate (PMS) were obtained from Kanto Kagaku (Tokyo, Japan). *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt (TOOS) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Dojindo Laboratories (Kumamoto, Japan), and 1-[3-(succinimidyloxycarbonyl)propoxy]-5-ethylphenazinium trifluoromethanesulfonate (arPES) was kindly donated by Dojindo Laboratories (Masuki-cho, Kumamoto, Japan). 2,6-Dichloroindophenol (DCIP) (Merck, Darmstadt, Germany) and peroxidase (Amano Enzyme, Gifu, Japan) were purchased. The screen-printed carbon electrodes (SPCE) (DEP-EP-P, WE: Carbon, 2.64 mm^2; RE: Ag/AgCl; CE: Carbon) were obtained from Bio Device Technology Ltd. (Ishikawa, Japan). All chemicals were reagent grade.

2.2. Bacterial strains and plasmids

Escherichia coli BL21 (DE3) was transformed with expression vectors for wild-type (NCBI protein ID: WP_003142047) and mutant AvLOxs. These genes were inserted into the expression vector pET30c(+) (Merck KGaA, Darmstadt, Germany). Site-directed mutagenesis was conducted using the Quikchange method with PfuUltra High-fidelity DNA polymerase (Agilent Technology, Santa Clara, CA, USA) and two primers (Supplementary data, Table SI). Template DNA was digested with *Dpn*I (Roche Diagnostics GmbH, Mannheim, Germany) at 37 °C for 2 h. After amplification, the plasmid sequences of were confirmed by the ABI Prism BigDye Terminator cycle sequencing kit v3.0 on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. Preparation and PES modification of enzymes

Enzyme recombinant production and purification were performed according to a previous paper (Hiraka et al., 2018). As a screening for mutants, *E. coli* BL21 (DE3) was transformed with the constructed plasmids and inoculated into 3.0 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 kanamycin as the main culture. *E.coli* were cultivated for 24 h at 30 °C with shaking. Cells were harvested from 2.0 mL of each culture and disrupted by BugBuster™ (Merck Millipore, Darmstadt, Germany) containing 20 mM potassium phosphate buffer (P.P.B. (pH 7.0)). After disruption for 30 min, the supernatants after centrifugation were evaluated as enzyme samples.

For further investigation, transformed *E. coli* BL21 (DE3) was grown aerobically for 12 h in 5.0 mL of LB medium containing 50 μg/mL kanamycin at 37 °C as a preculture. Subsequently, 1 mL of the precultures were inoculated into 100 mL 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 kanamycin as the main culture and cultured aerobically for 36 h at 30 °C. Cells were harvested by centrifugation, washed twice with 0.85% NaCl, and disrupted by a French pressure cell in 20 mM PPB (pH 7.0). After centrifugation and ultracentrifugation, the supernatants were dialyzed overnight in 20 mM PPB (pH 7.0). Wild-type AvLOx and mutants were purified by anion exchange chromatography. The purified enzyme solutions were stored at 4 °C.

PES modification of the purified enzymes was conducted as described by Hatada et al. (2018). Enzymes (6 μM) and 0.4 mM arPES were mixed in 20 mM Tricine buffer (pH 8.3) and incubated at 25 °C in a thermomixer with shaking for 2 h. After incubation, the mixtures were ultrafiltered 10 times at 4 °C at 14,000 g for 5 min to remove excess arPES. Before each ultrafiltration, 20 mM potassium phosphate buffer (P.P.B.) at pH 7.0 was added to exchange the buffer.

2.4. Enzyme assays

Enzyme assays were conducted according to previous papers (Hiraka et al., 2018). Oxidase activity measurements were conducted in 20 mM PPB (pH 7.0) containing 1.5 mM 4-AA, 1.5 mM TOOS, 2.0 U/mL peroxidase, and various concentrations of sodium L-lactate. The oxidase activities were determined by monitoring the formation of quinoneimine dye at 555 nm based on the molar absorption coefficient of TOOS at pH 7.0 (39.2 $mM^{-1}cm^{-1}$). One unit of oxidase activity was defined as the amount of enzyme necessary to catalyze the production of 1 μmol H_2O_2 per minute at 25 °C. The dye-mediated lactate dehydrogenase activities were measured in 20 mM PPB (pH 7.0) containing 0.06 mM DCIP, 4 mM PMS, and various concentrations of sodium L-lactate. The dehydrogenase activities were determined by monitoring the reduced DCIP at 600 nm based on the molar absorption coefficient of DCIP at pH 7.0 (16.3 $mM^{-1}cm^{-1}$). One unit of dehydrogenase activity was defined as the amount of enzyme necessary to catalyze the reduction of 1 μmol DCIP per minute at 25 °C. These assays were performed in triplicate. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, CA, USA).

2.5. Fabrication and characterization of enzyme sensors

After the preparation of PES-modified wild type and mutant AvLOx,

enzyme electrodes were fabricated with a cross-linking method using PEGDGE (Vasylieva et al., 2011). PES-modified or unmodified enzyme solution (1 mg/mL) and 20 mg/mL PEGDGE were mixed in equal volumes. One microliter of mixed solution was deposited onto the working electrode of a SPCE (DEP-EP-P, WE: Carbon; 2.64 mm^2, RE: Ag/AgCl; CE: Carbon). After the solution had dried, SPCEs were put in 10 mM Tris-HCl (pH 7.4) to block unreacted epoxy groups for 15 min at room temperature. After equilibration in 20 mM P.P.B. (pH 7.0), the SPCEs were stored in the same buffer until use. Cyclic voltammetry and chronoamperometry were performed using these SPCEs and an HZ-5000 electrochemical measurement system. Cyclic voltammetry was conducted in 2 mL of 20 mM P.P.B. (pH 7.0) with or without 20 mM L-lactate with a sweep potential range from −0.7 V to 0.3 V and scan rate of 20 mV/s. Chronoamperometric investigations were carried out using 2 mL of 20 mM P.P.B. (pH 7.0) with the successive addition of sodium L-lactate at 0 mV vs. Ag/AgCl with continuous stirring.

2.6. Interferent study

To investigate the interferent effect, chronoamperometry was performed using the constructed electrodes and 2 mL of 20 mM P.P.B. (pH 7.0) with the successive addition of sodium L-lactate at 0 or −200 mV vs. Ag/AgCl with continuous stirring. Ascorbic acid (170 μM), acetaminophen (1.3 mM), uric acid (1.4 mM), and glucose (20 mM) were used to evaluate the interferent effect. These concentrations were based on the FDA (USA) guidance draft for blood glucose sensing systems (U.S. Food and Drug Administration, 2016).

3. Results

3.1. Rational design and characterization of engineered AvLOx for PES modification

The residues for PES modification, which were substituted by Lys, were selected according to two criteria: 1) they should be located on the surface of AvLOx to facilitate electrode accessibility, and 2) they should be located within 20 Å of the isoalloxazine ring of FMN. Accordingly, Glu26, Glu48, Ser178, Arg183, Asp184, Glu207, and Asn212 were selected and were substituted by Lys residues in combination with the Ala96Leu mutant (Fig. 1A–B).

These mutant enzyme samples were recombinantly prepared, and their dye-mediated lactate dehydrogenase and oxidase activities were analyzed to investigate the impact of Lys substitution on the enzymatic activities (Table 1). The expression level analyses by SDS-PAGE revealed that there was no significant difference in the expression level of the double mutant enzymes compared with that of the Ala96Leu single mutant (Supplemental data Fig. S1). Among these mutants, the Ala96Leu/Glu26Lys, Ala96Leu/Glu48Lys, Ala96Leu/Ser178Lys and Ala96Leu/Asp184Lys double mutants showed drastic decrease in both oxidase and dehydrogenase activities. indicating that Lys substitution was not applicable at these residues. In contrast, the Ala96Leu/Arg183Lys, Ala96Leu/Glu207Lys and Ala96Leu/Asn212Lys double mutants showed equivalent or higher dehydrogenase activity compared with that of the Ala96Leu single mutant. Unexpectedly, the double mutant, Ala96Leu/Asn212Lys showed 1.2-fold higher dye-mediated dehydrogenase activity and markedly decreased oxidase activity compared with those of Ala96Leu. Therefore, the AvLOx Ala96Leu/Asn212Lys mutant, together with Asn212Lys single mutant, were further analyzed using purified enzyme samples.

Fig. 2 and Table 2 show the oxidase and dye-mediated dehydrogenase activities of purified wild type, Ala96Leu, Asn212Lys, and Ala96Leu/Asn212Lys AvLOx. The oxidase activity of the Ala96Leu/Asn212Lys double mutant showed a drastic decrease compared with that of the wild type (0.16% using 20 mM L-lactate) and Ala96Leu mutant (1.9% using 20 mM L-lactate). Regarding single amino acid substitution, Asn212Lys alone also showed decreased oxidase activity

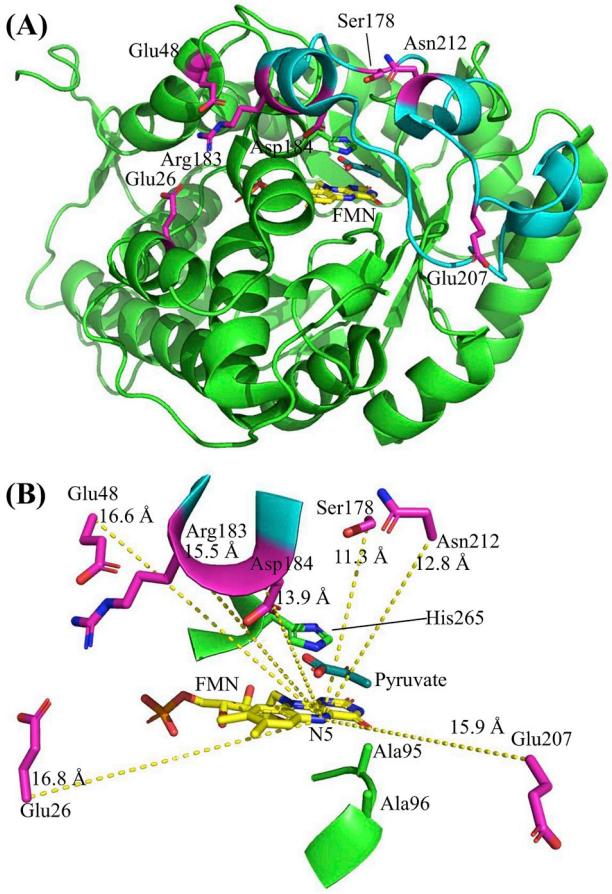


Fig. 1. AvLOx structure and Lys mutation site. (A) The structure of the AvLOx monomer (PDB code: 2E77 chain D) and (B) the structure of the active site. Cyan-colored residues are shaped to allow substrate entrance. Magenta-colored residues are substituted with Lys. The yellow dotted line indicates the distance between Cα of the mutation site and FMN N5. The image was prepared using The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

compared with that of the wild type (15% using 20 mM L-lactate); however, the impact was smaller than that obtained with Ala96Leu substitution. A marked increase in dehydrogenase activity was observed in both Asn212Lys and Ala96Leu/Asn212Lys compared with that of the wild type and Ala96Leu single mutant. The dehydrogenase activity to oxidase activity ratio was calculated from the specific activity for 20 mM L-lactate. The wild type enzyme, Ala96Leu and Asn212Lys showed 1.1-fold, 80-fold and 15-fold higher dehydrogenase activity compared with the oxidase activity, respectively. However, the double mutant Ala96Leu/Asn212Lys showed 1100-fold higher dehydrogenase activity compared with its oxidase activity.

The substrate specificities of all mutant AvLOxs were identical to those of the wild type and were specific to L-lactate (Fig. 3A). The thermal stabilities of Ala96Leu and Ala96Leu/Asn212Lys were almost the same, and these two mutants were more stable than the wild type and the Asn212Lys single mutant (Fig. 3B).

3.2. Cyclic voltammetry

Enzyme electrodes immobilized with PES-modified wild type or mutant AvLOx were evaluated by cyclic voltammetry (Fig. 4A). The solid lines in Fig. 4A are the voltammograms for the enzyme electrodes in the presence of L-lactate, and the dotted lines are the ones in the absence of L-lactate. Redox peaks were observed in all PES-modified

Table 1
The oxidase and dehydrogenase activities of Lys mutants of AvLOx Ala96Lys from crude preparations.

Activity (U/mg)	Oxidase activity (A) 20 mM L-lactate	Dehydrogenase activity (B) 20 mM L-lactate	B/A	(%)	Expression level (%)[b]
Wild Type[a]	22	31	1.4	100	17
Ala96Leu[a]	0.23	33	150	11,000	16
Ala96Leu	0.14	20	150	11,000	21
Ala96Leu/Glu26Lys	0.029	4.1	140	10,000	8.3
Ala96Leu/Glu48Lys	0.019	0.091	4.7	340	13
Ala96Leu/Ser178Lys	0.069	3.1	45	3200	19
Ala96Leu/Arg183Lys	0.16	20	120	8800	19
Ala96Leu/Asp184Lys	0.018	0.085	4.7	330	19
Ala96Leu/Glu207Lys	0.13	18	130	9400	20
Ala96Leu/Asn212Lys	0.0015	25	16,000	1,200,000	15

[a] Previous reported value (Hiraka et al. (2018)) for comparison with present data.
[b] These expression levels were calculated from image analyses of the corresponding bands observed by SDS-PAGE (Supplementary data, Fig. S1).

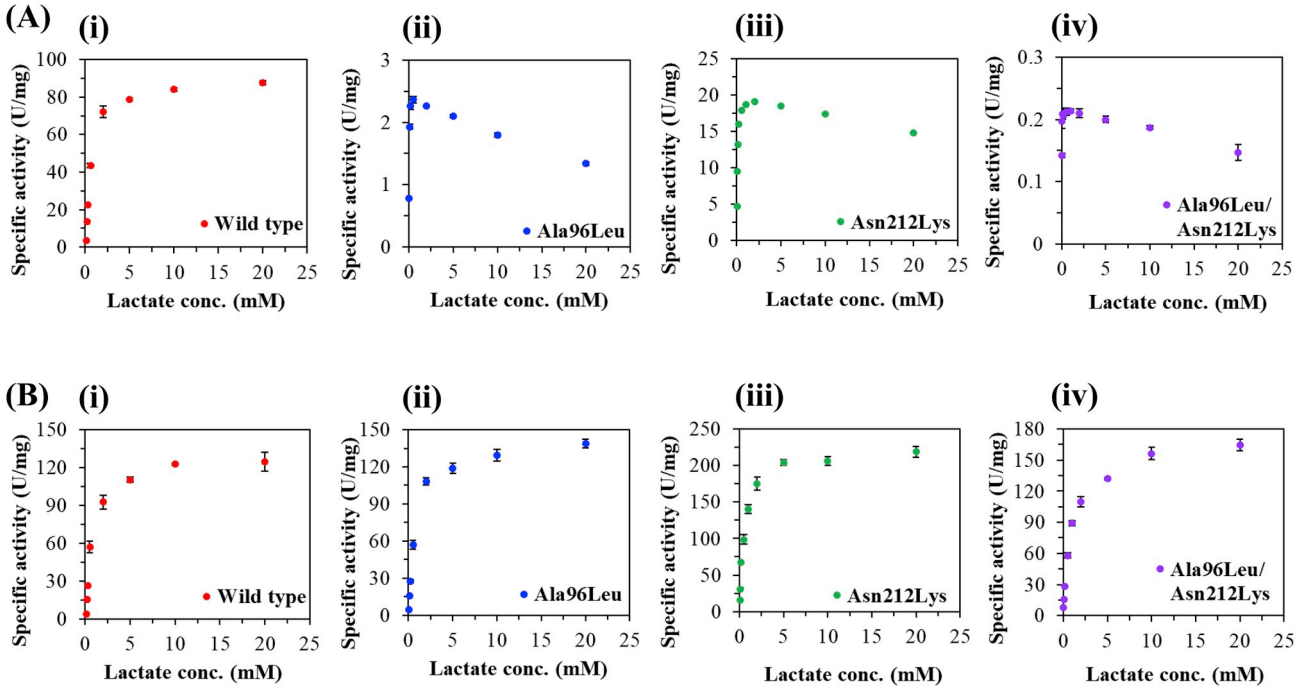


Fig. 2. Enzyme activities of purified AvLOx mutants. (A) Oxidase activities. (B) Dye-mediated dehydrogenase activities. (i) Wild type, (ii) Ala96Leu, (iii) Asn212Lys, and (iv) Ala96Leu/Asn212Lys were evaluated. Each experiment was conducted in triplicate, and the error bars show the standard deviation. The data of wild type and Ala96Leu mutant are derived from previous report (Hiraka et al. (2018)).

Table 2
Kinetic parameters of each AvLOx mutant and wild type.

	Oxidase activity (A)			Dehydrogenase activity (B)			
	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (U/mg· mM)	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (U/mg· mM)	B/A for 20 mM L-lactate
Wild type[a]	0.56	90	160	0.72	130	180	1.1
Ala96Leu[a]	N.A.	N.A.	N.A.	0.81	140	170	80
Asn212Lys	N.A.	N.A.	N.A.	0.54	220	410	15
Ala96Leu/Asn212Lys	N.A.	N.A.	N.A.	1.0	170	170	1100

N.A.: not available.
[a] The data of wild type and Ala96Leu mutant are derived from previous report (Hiraka et al. (2018)).

AvLOx immobilized electrodes. These redox peaks can be attributed to PES bound to the enzyme because the peak potentials are almost the same as those in the cyclic voltammogram of PES (Hatada et al., 2018). Comparing with PES-modified AvLOx wild type electrodes, a slight increase in the oxidation peak was observed in the presence of L-lactate with the enzyme electrode modified with Ala96Leu. In contrast, the electrodes with Asn212Lys and with Ala96Leu/Asn212Lys showed a marked increase in the oxidation peak in the presence of L-lactate. The reduction currents from −0.5 to −0.7 V, which corresponded to the reduction of oxygen, were increased after the addition of L-lactate in the wild type or Asn212Lys mutant immobilized electrodes. These results were caused by oxygen reduction current changes due to the

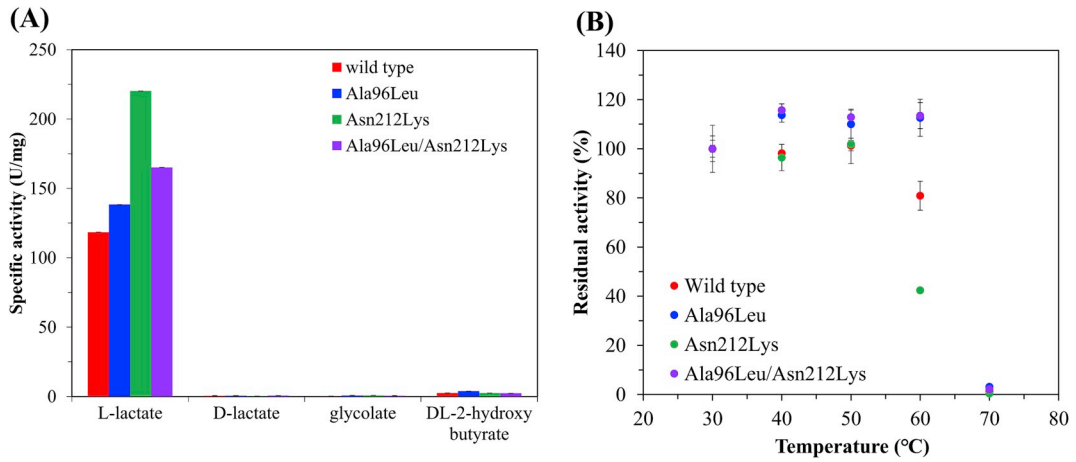


Fig. 3. Substrate specificity and thermal stability. (A) The substrate specificity of each mutant was evaluated by dehydrogenase activity assay using 20 mM L-lactate, D-lactate, glycolate, and DL-2-hydroxy butyrate as substrates. (B) The thermal stability of each mutant was evaluated by dehydrogenase activity assay after 10 min incubation at each temperature in a water bath. Red: wild type, blue: Ala96Leu, green: Asn212Lys, purple: Ala96Leu/Asn212Lys. Each experiment was conducted in triplicate, and the error bars show the standard deviation. The data of wild type and Ala96Leu mutant are derived from previous report (Hiraka et al. (2018)).

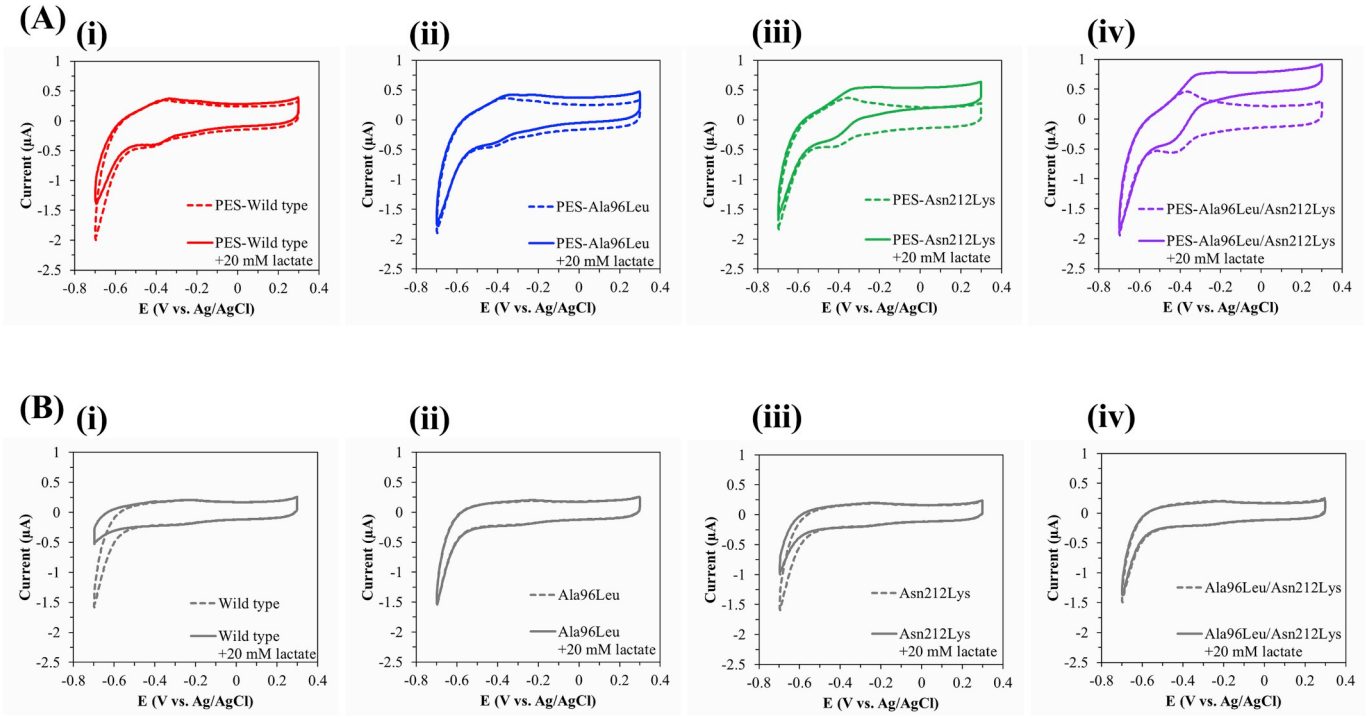


Fig. 4. Cyclic voltammograms of electrodes with PES-modified or unmodified enzymes. (A) PES-modified mutant and wild type AvLOx were evaluated by cyclic voltammetry ((i) wild type, (ii) Ala96Leu, (iii) Asn212Lys, and (iv) Ala96Leu/Asn212Lys). The dashed line indicates the absence of lactate, and the solid line is after the addition of 20 mM lactate. (B) Unmodified mutant and wild type AvLOx were also evaluated. The dashed and solid lines show data before and after the addition of 20 mM lactate, respectively. All experiments were conducted by using three different electrodes with 20 mV/s as the scan rate. All voltammograms showed a third cycle.

consumption of dissolved molecular oxygen by the oxidation of L-lactate which is derived from the oxidase activities of the wild type and Asn212Lys mutant. In contrast, the Ala96Leu and Ala96Leu/Asn212Lys mutants did not show a change in the peak current for oxygen reduction because of their very low reactivity for oxygen. These results indicated that the Asn212Lys mutation improved the quasi-DET ability of AvLOx after PES modification. In particular, the Ala96Leu/Asn212Lys double mutant modified with PES showed the highest oxidation peak in the presence of L-lactate without a change in the reduction peak of oxygen. The SDS-PAGE analysis of PES modified wild type and mutant AvLOxs revealed that the enzyme samples after the PES modification showed bands at higher molecular weight than unmodified enzymes (Fig. S3). Considering the molecular weight of PES as 294.35, AvLOx wild type and Ala96Leu mutant monomers were presumably modified by 6–7 PES molecules, whereas Ala96Leu/Asn212Lys mutant monomers were presumably modified by 8 PES molecules. Together with the observation of redox peaks from the electrode with PES modified enzymes, these results showed that the wild type and mutant AvLOxs were successively modified with PES.

3.3. The quasi-DET type L-lactate sensor employing PES modified Ala96Leu/Asn212Lys AvLOx

L-Lactate sensor employing PES modified Ala96Leu/Asn212Lys AvLOx was constructed and L-Lactate measurement was carried out based on chronoamperometry, by comparing with the sensors employing PES-modified wild type or mutant AvLOx (Fig. 5) in the absence of any mediators in the reaction solution. The sensors with PES-modified enzymes showed a current increase after the addition of L-lactate depend lactate concentration (Fig. 5A and B, Table 3), although the sensors with the PES-modified wild type and Ala96Leu mutant showed very low responses. Among them, the sensor with PES-modified Ala96Leu/Asn212Lys double mutant LOx showed the highest response, more than 12 times that of with the wild type and 5 times that with the Ala96Leu mutant based on the I_{max} value evaluation (Table 3). In contrast, in the sensors with PES-unmodified enzymes, no current increase was observed after the addition of L-lactate (Fig. 5C and D). These results indicated that by employing PES-modified rationally designed AvLOx, a lactate sensor based on the quasi-DET principle was developed. The sensor performance towards L-lactate was marked with linear range between 0 and 1 mM, sensitivity of 13 μA/mM·cm^2, and a limit of detection of 25 μM (3.3 × standard deviation).

The investigation of electrochemically interfering compounds was carried out with the sensor employing PES-modified Ala96Leu/Asn212Lys double mutant (Fig. 6). Ascorbic acid, acetaminophen, and uric acid are well-known electroactive substances often observed in human blood samples (Sylvester et al., 1994). The enzyme sensors were tested by applying 0 mV vs. Ag/AgCl; the interference was negligible for acetaminophen, but obvious interferences were observed with ascorbic acid. However, this interference by ascorbic acid was minimized by decreasing the applied oxidation potential to −200 mV vs. Ag/AgCl without losing the response for L-lactate. Similarly, the negative bias observed with the addition of uric acid was less than 5% of the 1 mM L-lactate signal, and negligible. Therefore, the constructed quasi-DET type lactate sensor with the PES-modified Ala96Leu/Asn212Lys AvLOx mutant was revealed to be an interference-free lactate sensing

Table 3
Electrochemical kinetic parameters of each PES-modified AvLOx mutant and wild type.

	K_m (mM)	I_{max} (μA)	I_{max}/K_m (μA· mM)
Wild type	1.1	56	51
Ala96Leu	0.52	130	250
Asn212Lys	0.65	430	660
Ala96Leu/Asn212Lys	0.93	710	760

K_m and I_{max} values were obtained from the calibration curve, which was obtained from Fig. 5B. These parameters were calculated using the following equation: $I = I_{max}[S]/([S]+K_m^{app})$.

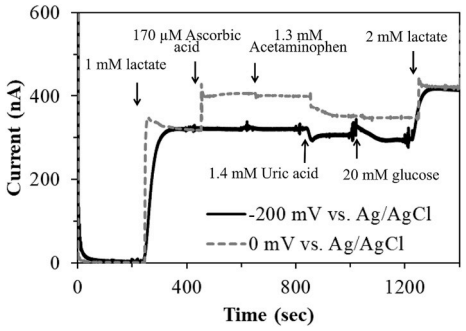


Fig. 6. Investigation of the electrochemical interference of compounds on L-lactate monitoring using the electrode immobilized PES-modified Ala96Leu/Asn212Lys double mutant. The impact of 170 μM ascorbic acid, 1.3 mM acetaminophen, 1.4 mM uric acid, and 20 mM glucose as interferents was investigated in the presence of 1 mM L-lactate by applying an oxidation potential of either 0 (dashed line) or −200 mV (solid line) vs. Ag/AgCl. Arrows indicate the timing of the addition of L-lactate or interferents and the final concentration of these substances.

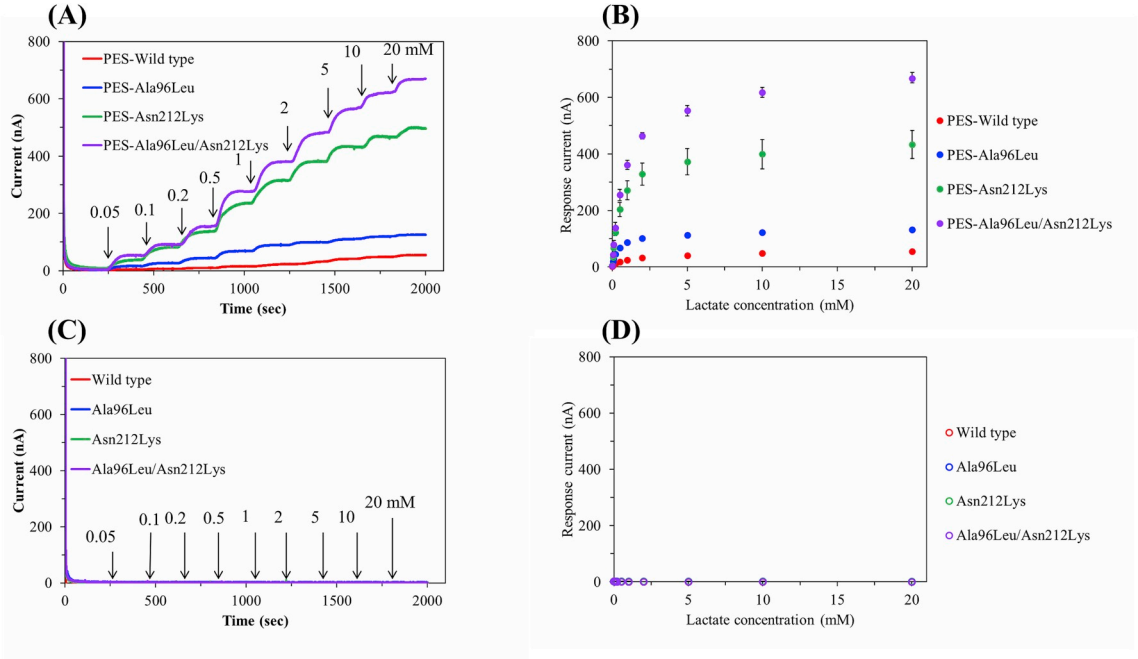


Fig. 5. L-Lactate measurements based on chronoamperometry. Amperograms using the constructed electrodes with PES modified enzymes (A) and unmodified enzymes (C). Correlation between current increase and L-lactate concentration from amperograms using PES-modified enzymes (B) and unmodified enzymes (D). Red: wild type, blue: Ala96Leu, green: Asn212Lys, purple: Ala96Leu/Asn212Lys. Arrows indicate the timing of the addition of L-lactate and the final concentration of L-lactate. These experiments were performed by using three different electrodes at 0 mV vs. Ag/AgCl. Error bars show the standard deviation.

system when it was operated at −200 mV vs. Ag/AgCl.

4. Discussion

In this study, the PES modification site, which is the residue substituted with Lys, was designed to develop a quasi-DET type lactate sensor. Considering that the distance from where electron transfer will occur to the co-factor FMN is less than 15–30 Å (Stuchebrukhov, 2010), we picked up polar residues on the surface of the enzyme; Glu26, Glu48, Ser178, Arg183, Glu207 and Asn212 were selected as the residues to be substituted by Lys for PES modification. Among these mutations, Glu26Lys, Glu48Lys, Ser178Lys and Asp184Lys resulted in the inactivation of the enzyme, probably because these amino acid substitutions caused unfavorable structural changes in AvLOx. In contrast, Arg183-Lys, Glu207Lys and Asn212Lys showed equal or even higher enzyme activity compared with that of the wild type. Unlike our previously reported engineered AvLOx Ala96Leu mutant, which was rationally designed by predicting of oxygen accessible tunnel, Asn212Lys was designed to be modified by arPES, therefore, this mutation was designed at residue located at the surface of enzyme and the reduction of oxidase activity was unexpected. The predicted oxygen accessible tunnel might not be directly affected by the amino acid substitution of at Asn212 residue (Fig. S2). The possible impact of Asn212Lys mutation might be the result of the formation of a new electrostatically interaction between Lys212 side chain with that of Asp184, consequently might cause the move of main chain including Leu211 which might obstruct the predicted oxygen accessible tunnel (Fig. S2). Consequently, the Ala96-Leu/Asn212Lys mutant showed higher dehydrogenase activity than the Ala96Leu mutant, with a drastic decrease in oxidase activity (Table 1). The distance between FMN N5 and the Cα of Asn212 is 12.8 Å and is the shortest except for that of the Ser178 residue (11.3 Å) among the mutants constructed in this study. Considering that Asn212 is located relatively close to the surface of the enzyme where the electrode material is easily accessible, modified PES at Lys212 can accept electrons from reduced FMN and transfer electrons to the electrode.

The impact of the presence of oxygen on the PES-modified electrode was observed from cyclic voltammograms (Fig. 4). The change in the reduction peak of oxygen due to the addition of L-lactate was observed in electrodes with PES-modified or unmodified wild type and Asn212Lys AvLOx. Considering that the Asn212Lys single mutant showed higher dye-mediated dehydrogenase activity than the Ala96Leu/Asn212Lys double mutant, a higher oxidation peak current was expected at the electrode with the PES-modified Asn212Lys mutant; however, the electrode with the PES-modified Ala96Leu/Asn212Lys double mutant showed a higher oxidation peak and a higher response in the chronoamperometry investigations. These results indicated that AvLOxs showing oxidase activity might be subject to competition between electron transfer from FMN to oxygen and from FMN to PES and consequently show a decreased quasi-DET response.

From another point of view, as Ala96Leu/Asn212Lys double mutant, was strongly repressed its oxidase activity (0.1% of oxidase activity of wild type), and could not liberate hydrogen peroxide at the oxidative half-reaction, the sensor with this double mutant does not work in the first-generation type sensing system based on hydrogen peroxide.

Comparing with the previously reported LOx based lactate sensors (Nikolaus and Strehlitz, 2008; Rathee et al., 2016), this sensor did not require excess quantity of mediator in the disposable sensor strip, nor in the solution for flow injection analyses, which are economically and environmentally advantageous. Employment of double mutant AvLOx, which was virtually “lactate dehydrogenase” realized the lactate sensing with minimized impact of oxygen on signal. Operation at low applied potential realized the lactate sensing which was not biased by the interferents. These features are the identity of the lactate sensor, which could be achieved by employing PES modified Ala96Leu/Asn212Lys AvLOx.

Current sophisticated industrial technologies in the blood glucose monitoring provide the configuration of disposable enzyme sensor strips and the protocols, such as correction of the impact of hematocrit effect, which are necessary to construct a disposable lactate sensor strips for whole blood sample monitoring. Therefore, by combining the engineered AvLOx reported in this study together with the established industrial technology will realize lactate sensor which is not affected neither by oxygen nor potential interferents, which will be acknowledged in the future clinical market.

5. Conclusion

A quasi-DET type lactate sensor was developed by rational engineering of AvLOx for PES modification. The residues for PES modification, which were substituted by Lys, were selected according to two criteria: 1) they should be located on the surface of AvLOx to facilitate electrode accessibility, and 2) they should be located within 20 Å of the isoalloxazine ring of FMN. The mutagenesis studies revealed that Asn212Lys showed the highest dye-mediated dehydrogenase activity compared with the wild type and Ala96Leu mutant. After modification by PES, the Ala96Leu/Asn212Lys double mutant showed the highest oxidation peak in the presence of L-lactate, whereas the electrodes with the PES-modified wild type or Ala96Leu mutant did not. The electrode with the PES-modified Ala96Leu/Asn212Lys mutant did not show oxygen interference. The sensor with PES-modified Ala96Leu/Asn212Lys double mutant LOx showed the highest response, more than 12 times that with wild type and 5 times that with Ala96Leu mutant. The sensor performance towards L-lactate marked with linear range between 0 and 1 mM, sensitivity of 13 $\mu A/mM \cdot cm^2$, and a limit of detection of 25 μM. The investigation of electrochemically interfering by applying an oxidation potential of −200 mV vs. Ag/AgCl, revealed that the interference was negligible for acetaminophen, ascorbic acid, and uric acid. These results indicated that by employing PES-modified rationally designed AvLOx, a lactate sensor based on the quasi-DET principle was developed which did not suffer from the interference of oxygen and representative electroactive ingredient compounds.

Author contribution

Hiraka, Kojima, Tsugawa, Asano, Ikebukuro, Sode: Study conception and design.
Hiraka: Acquisition of data.
Hiraka, Tsugawa, Sode: Analysis and interpretation of data.
Hiraka, Sode: Drafting of manuscript.
Hiraka, Sode: Critical revision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.111974.

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