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A Thiol-reactive Phenazine Ethosulfate – A Novel Redox Mediator for Quasi-direct Electron-transfer-type Sensors

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A novel redox mediator, thiol-reactive phenazine ethosulfate (trPES), was used to modify an enzyme for the first time for biosensor development. *Aerococcus viridans*-lactate oxidase (LOx), widely used to study lactate biosensors, was modified with a single trPES molecule. A cysteine mutation was introduced into the vicinity of the LOx cofactor to enable the modification by trPES. LOx cysteine mutants were then successfully modified using trPES, thus acquiring quasi-direct electron transfer ability. An electrode immobilized with the trPES-LOx A96L/S210C mutant showed the highest amperometric response currents among the modified LOx cysteine mutants, indicating efficient electron transfer. The position around residues S210 to N212 on the LOx surface (distance <14 Å from FMN N5) is important for the mediator to access the reduced flavin. Then, the performances of the lactate sensor were improved by utilizing LOx A96L/S210C modified with trPES and arPES, another redox mediator used to modify a lysine residue. The lactate sensor has a detection range of up to 1 mM, a sensitivity of 6.62 μA/mM.cm^2, and a limit of detection of 9.9 μM. Furthermore, *Aspergillus flavus*-derived FAD glucose dehydrogenase was successfully modified with trPES and the response currents were obtained, showing the versatility of trPES for modifying other oxidoreductases.

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 analyze 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. Similarly to

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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[1–18] and the N-termini in proteins and peptides; or (2) the maleimide group for targeting the thiol group in cysteine residues.[19–23]

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 probes targeted the amine group of lysine residues of oxidoreductases. In those studies, proteins were modified with derivatives of ferrocene,[1–8] a ruthenium complex,[2] tetrathiafulvalene,[9] phenothiazine,[10,11] phenoxazine,[12] quinone,[13] and, as recently reported by our group, phenazine.[14–18] In contrast, studies of redox probes targeting the thiol group of cysteine residues have been limited to the modification of binding proteins with a ruthenium complex,[19–23] and no studies on the modification of oxidoreductases with a 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,[1–18] and protein modification targeting cysteine residues is used for the modification with single molecules.[19–23] This is because lysine has a high prevalence in proteins (~6%),[24,25] whereas cysteine has a low prevalence (<2%).[26] 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, biosensing, and modification with a single redox molecule at the desired position using a redox probe targeting cysteine can be used.

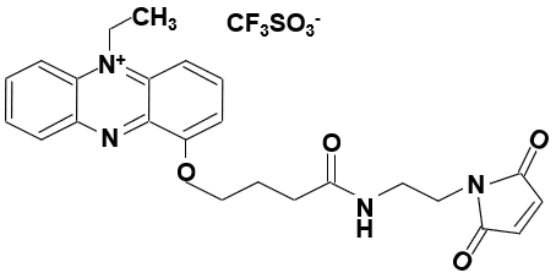
Oxidoreductases harboring a flavin cofactor require electron mediators to transfer electrons to the electrode because flavin is buried inside the enzyme. Therefore, many studies on the development of biosensors using oxidoreductases in mediated electron transfer (MET)-type biosensors have been reported. However, the ideal electron transfer system for bioelectrochemical sensing is the direct electron transfer (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 re-oxidized 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 probe, 1-[3-(succinimidyl oxycarbonyl) propoxy]-5-ethyl phenazinium trifluoromethane sulfonate or amine-reactive phenazine ethosulfate (arPES), with proteins.[14–18] 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 the redox component of arPES, has high stability,[27–31] and its versatility has been shown for oxidoreductase-based biosensors.[31] Through its succinimidyl group, arPES can modify the amine group of lysine in

oxidoreductases and provide quasi-direct electron transfer (quasi-DET) to the enzyme.[32] 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.[14–17] The application of arPES led to the successful development of a glucose sensor based on flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenase derived from *Botryotinia fuckeliana* (*Bf*GDH);[14] however, no amperometric response was observed when using *Aerococcus viridans*-lactate oxidase (*Av*LOx or LOx) modified by arPES.[14,15] 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.*[16] predicted the position of this lysine residue. Then, Hiraka *et al.*[15] 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 probe, 1-[3-(2-maleimidoethyl carbamoyl) propoxy]-5-ethyl phenazinium triflate or thiol-reactive phenazine ethosulfate (trPES) (Scheme 1), has become commercially available.[33] 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.*[6] 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 a 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, we demonstrated the application of the novel redox mediator trPES for targeting cysteine in well-known oxidoreductases, LOx, and we characterized the impact of the modification of the redox enzyme with a single redox mediator molecule near the cofactor. *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



Scheme 1. Structure of trPES.

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.

2. Materials and Methods

2.1 Materials and devices

trPES and arPES were provided by Dojindo Laboratories Inc. (Kumamoto, Japan). Sodium L-lactate and poly(ethylene glycol) diglycidyl ether (PEGDGE Mn 500) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,6-Dichloroindophenol (DCIP) was purchased from Merck (Darmstadt, Germany). 5-Methyl-phenazinium methylsulfate (PMS) and D(+)-glucose were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). An Amicon Ultra-0.5 centrifugal filter unit (30-KDa cutoff) was purchased from Merck Millipore Ltd. (Carrigtwohill Co., Cork, Ireland). A glassy carbon (GC) working electrode (WE, 3.0 mm diameter), 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). 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).

A 3D structure analysis of *Av*LOx was performed using PyMOL Molecular Graphics System software. Colorimetric evaluations were performed using a UV-1289 UV-Vis spectrophotometer (Shimadzu Corp., Kyoto, Japan). Enzymes were incubated with trPES and/or arPES in an Eppendorf ThermoMixer R (Thermo Fisher Scientific, Tokyo, Japan). All electrochemical measurements were performed using a PG580RM potentiostat-galvanostat from UniScan Instruments Ltd. (Buxton, UK).

2.2 Preparation of engineered LOx mutants

A cysteine mutation was introduced in LOx A96L, a mutant with low reactivity toward oxygen,[34] through site-directed mutagenesis (Supplementary Information Table S1). *Escherichia coli* (*E. coli*) BL21 (DE3) was used for LOx engineering following the methods of Hiraka *et al.*[15] The recombinant enzymes were prepared according to the methods of Hiraka *et al.*[34] with minor modifications.[31] Protein concentration was measured using the Bradford method.

The dye-mediated dehydrogenase activity of LOx mutants was measured using the PMS/DCIP system. Each enzyme sample was mixed with a premix solution containing (final concentrations) 4 mM PMS, 0.06 mM DCIP, and 20 mM lactate in 20 mM potassium phosphate

buffer (PPB, pH 7.0). DCIP reduction was determined by monitoring absorbance at 600 nm. One unit of enzyme was defined as the amount of enzyme that catalyzes the reduction of 1 μmol DCIP per min using 20 mM lactate at 25 ℃, based on the molar absorption coefficient at pH 7.0 (16.3 $mM^{-1}cm^{-1}$). Kinetic parameters were calculated using the Hanes–Woolf plot. These assays were performed in triplicate for each enzyme sample.

2.3 Modification of LOx mutants with redox probes

Purified LOx (0.55 μM) was mixed with trPES (80 μM) in 20 mM PPB (pH 7.0) and subsequently incubated at 25 ℃ in a thermomixer with 1200 rpm shaking for 2 h. The unbound trPES was removed by ultrafiltration 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).

LOx mutants were modified with both trPES and arPES. A mixture containing LOx (0.55 μM), trPES (80 μM), and arPES (400 μM) was incubated in 20 mM tricine buffer (pH 8.3). The modification steps and other conditions were the same as for the above methods. The pH of the mixture changed during the removal of the unbound mediators, and we obtained trPES- and arPES-modified LOx (tr.arPES-LOx) with neutral pH. LOx mutants were also modified with arPES only[15] for comparison with other modified enzymes.

2.4 Construction and evaluation of enzyme electrodes utilizing modified LOx enzymes

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.*[35] A mixture (6 μl) containing 1 mg/mL modified LOx and 10 mg/mL PEGDGE was deposited on the GC electrode layer by layer. 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 room temperature (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.

Cyclic voltammetry (CV) measurements were performed in 2 mL of 100 mM PPB (pH 7.0) with a sweep range from −0.7 to +0.3 V (vs Ag/AgCl) and a scan rate of 0.05 V/s. Chronoamperometry (CA) measurements were performed to monitor the response currents with the 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 previous studies.[15,31]

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.[36] 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*

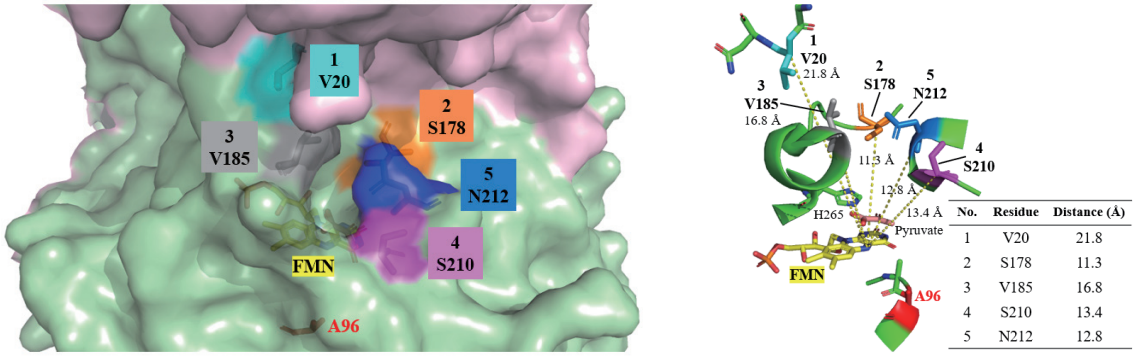
BL21 (DE3) cells. A cysteine mutation was introduced into *Af*GDH K477 through site-directed mutagenesis (Supplementary Information Table S1). *Af*GDH wild-type (WT) or *Af*GDH K477C mutant was modified with trPES by incubating trPES (80 μM) with the enzyme (1.33 μM) in 20 mM PPB buffer (pH 6.5) following the methods described in Sect. 2.3. Enzyme electrodes based on modified trPES-*Af*GDH WT or trPES-*Af*GDH K477C were prepared according to the methods described in Sect. 2.4 and the electrochemical response current toward glucose was evaluated in 20 mM PPB buffer with pH 6.5.

3. Results

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:[15] (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),[37] thus enabling optimal electron transfer from flavin to the electrode through the attached trPES. Five residues, V20, S178, V185, S210, and N212, were chosen for substitution (Fig. 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.

The kinetic parameters of the LOx mutants were determined (Table 1). The V_{max} values indicated the dye-mediated dehydrogenase activity of all LOx mutants, suggesting 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). In a previous study,[15] residues S178 and N212 were substituted with lysine to



(a) (b)

Fig. 1. (Color online) 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 letters): 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 located in chain B (pink area).

Table 1
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

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. In addition, all LOx cysteine mutants showed very low oxidase activity (Supplementary Information Fig. S1) because they maintained the characteristic of the A96L mutation, which exhibited low reactivity to the molecular oxygen,[15] making them suitable for MET- and quasi-DET-type sensor applications.

3.2 Catalytic activity of LOx 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. The kinetic parameters of the trPES-LOx mutants were determined (Table 1). 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, and the K_m values were also changed by the modification. trPES-LOx A96L/V185C showed the highest activity and trPES-LOx A96L/S178C showed the lowest activity. All K_m values after modification increased, except for trPES-LOx A96L, for which it decreased almost to half of that before modification.

3.3 Electrochemical evaluation of enzyme electrodes using LOx modified with single mediator molecule

3.3.1 Cyclic voltammetry

The enzyme electrodes constructed using the trPES-LOx mutants were evaluated by CV measurement (Fig. 2). 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 (Supplementary Information Fig. S2). This confirms that trPES attached on LOx and that the modified enzymes were electrochemically active. The midpoint potentials ($E_{1/2}$) were approximately −0.10 V (vs Ag/AgCl), as calculated from the oxidation and reduction peaks, which were observed at approximately −0.06 and −0.14 V, respectively.

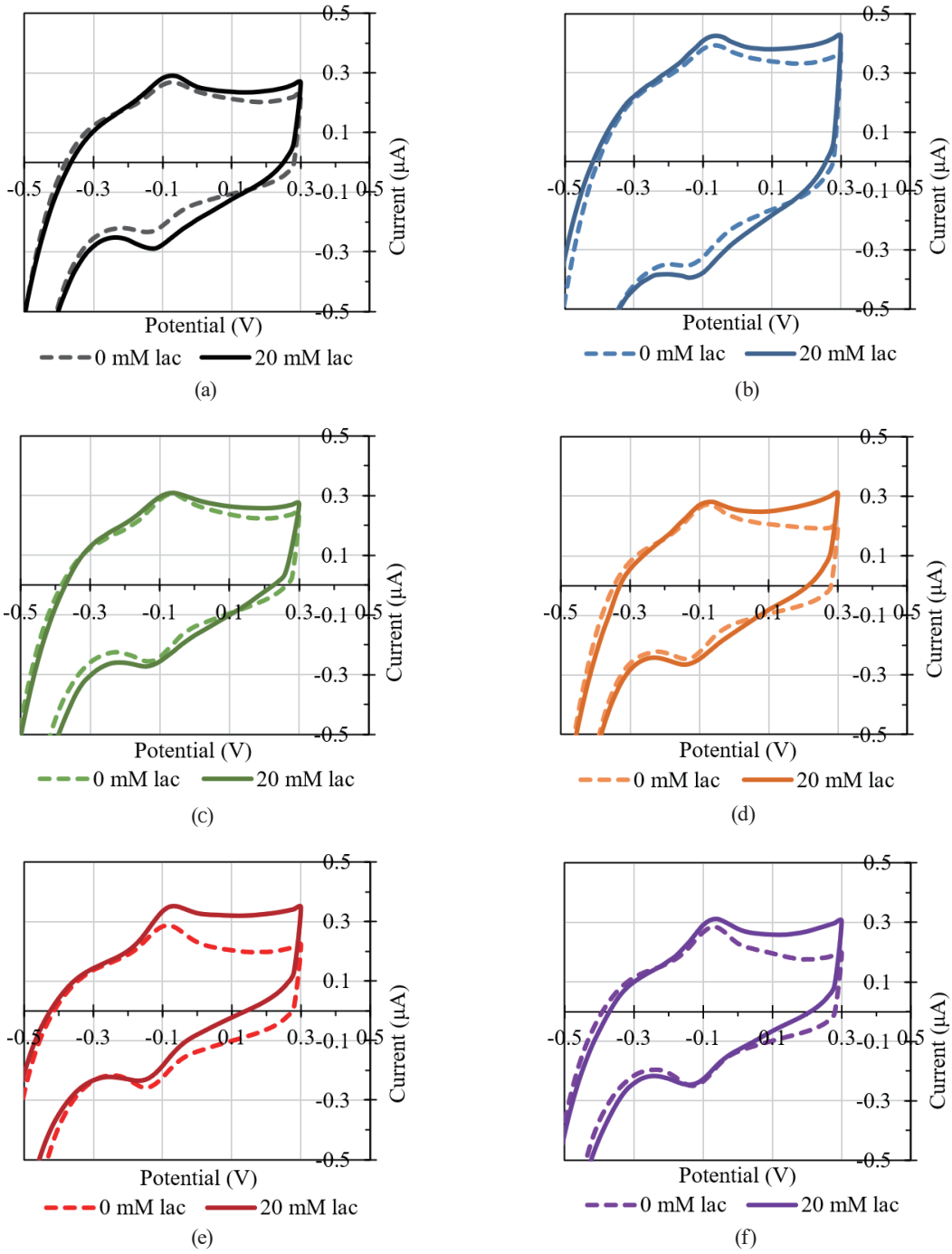


Fig. 2. (Color online) Cyclic voltammograms of modified enzymes: (a) trPES-LOx A96L, (b) trPES-LOx A96L/V20C, (c) trPES-LOx A96L/S178C, (d) trPES-LOx A96L/V185C, (e) trPES-LOx A96L/S210C, and (f) trPES-LOx A96L/N212C in 2 ml of 100 mM PPB (pH 7.0) in the absence (dashed line) and presence (solid line)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

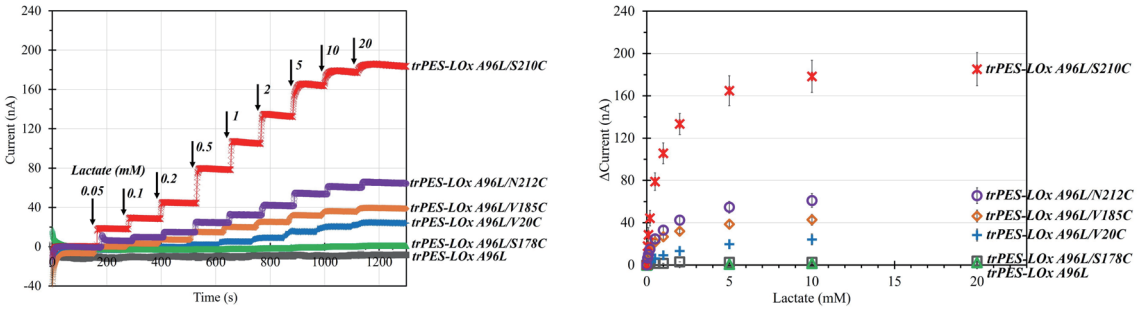
Catalytic currents were observed in the presence of lactate, suggesting that the electrons were transferred from the attached trPES to the electrode upon substrate oxidation. trPES-LOx A96L/V185C [Fig. 2(d)], trPES-LOx A96L/S210C [Fig. 2(e)], and trPES-LOx A96L/N212C [Fig. 2(f)]

clearly showed high catalytic current. Therefore, CA measurement was carried out and the lactate concentration dependence was evaluated for each modified LOx mutant.

3.3.2 Chronoamperometry

CA measurements were performed to evaluate the electron transfer from flavin to the electrode through the attached trPES. The response currents were recorded with the successive addition of 0–20 mM lactate in a buffer solution without a free mediator [Fig. 3(a)]. The steady-state currents obtained with each addition of lactate were plotted against lactate concentration [Fig. 3(b)].

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



(a) (b)

Fig. 3. (Color online) (a) Representative time course 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).

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, 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.

3.4 Response currents of LOx modified with multiple mediator molecules

To improve the performance of the lactate sensor, LOx A96L/S210C and the control mutant, LOx A96L, 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 fivefold that of trPES-LOx A96L/S210C (Fig. 4). 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. 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.[15] 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 2). As expected, the lactate sensor based on tr.arPES-LOx A96L/S210C exhibited the best performances.

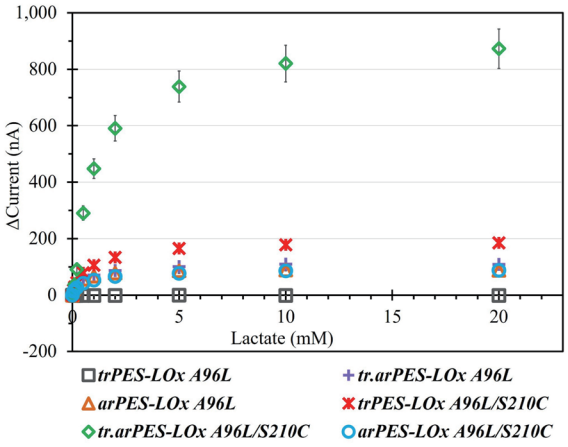


Fig. 4. (Color online) Steady-state response currents of LOx modified with multiple PES molecules at various lactate concentrations (n = 3). Applied potential: 0 V (vs Ag/AgCl).

Table 2
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 up to 1 mM lactate
K_m and I_{max} were determined using a Hanes–Woolf plot
LOD: Limit of detection
N.A.: Not available

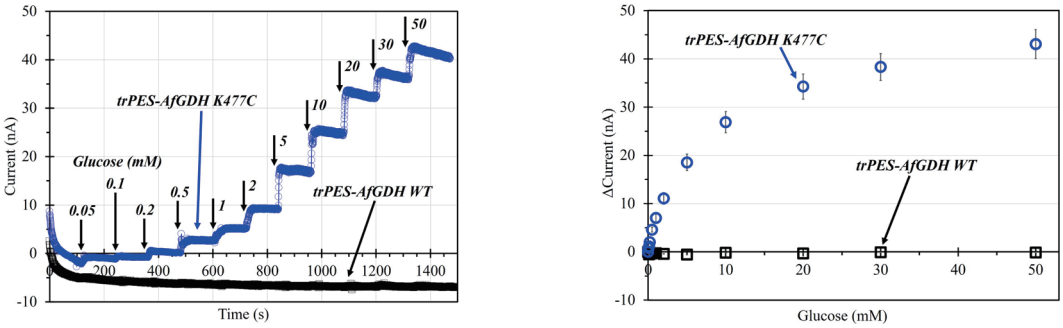
3.5 Modification and electrochemical investigation of *Af*GDH modified with trPES

To evaluate the application of trPES for other enzymes, we modified *Af*GDH WT (control) or *Af*GDH K477C mutant 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.[16] 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 (Fig. 5). 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 K477 successfully mediated the electron transfer from the flavin to the electrode.

4. Discussion

In this study, we modified LOx 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, we show the clear impact of modification with a single mediator molecule on a redox enzyme. The best location for the attachment of the redox mediator on oxidoreductases to enable electron transfer is then confirmed.

The oxygen-insensitive mutant, LOx A96L, a suitable enzyme for MET-type sensors,[34] 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 1). After chemical modification with trPES, the enzyme activity was obtained, 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,



(a) (b)

Fig. 5. (Color online) (a) Representative time course of response currents and (b) steady-state currents of *Af*GDH modified with trPES at various glucose concentrations (n = 3). Applied potential: 0 V (vs Ag/AgCl).

respectively.[15] LOx A96L/S178K lost its activity as a result of mutation,[15] 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,[15] 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 1). Additionally, the differences in the 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.[16]

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.[25,38–42] 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.[1]

In redox proteins, a high intramolecular electron transfer rate is achieved within a distance of 14 Å.[43] The distance between the isoalloxazine ring of FMN and trPES, which acts as the electron relay center[1] in the modified trPES-LOx, can be assumed to be the distance from N5 of flavin to Cα of cysteine (Fig. 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.[15] 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.[2,44] 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 fivefold 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, 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 (Sect. 3.4) 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. Here, a lactate sensor with high response currents and markedly improved performances was successfully developed using tr.arPES-LOx A96L/S210C (Table 2). For the same detection range (up to 1 mM lactate) as the reported lactate sensor using arPES-LOx A96L/N212K,[15] 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 μA/mM.cm^2) is only half that of the sensor reported in Ref. 15, because lactate can be detected at low concentrations owing to the low LOD, this lactate sensor can be used with confidence.

We found that residue S210 is 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.[15] 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) (Fig. 1). Combining our findings and the result of a previous study showing that residue N212 is important for electron transfer accessibility,[15] we conclude that the positions of residues S210 to N212 are 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, 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.[37] 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.[16] These results indicate that quasi-DET is possible in a modified enzyme-redox probe 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 probes mostly targeted the amine group of the lysine residue, and they were performed without considering protein structural analysis[1–5,7] because the protein crystal structures were not known at that time. Hence, the modification of a protein with a single redox probe molecule was not possible. In 1993, Badia *et al.* reported the use of a ferrocene derivative as a redox probe 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.[6] 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 probe molecule using trPES.

The immobilized lactate sensors can be used in a continuous monitoring system such as for flow injection analysis, as indicated in Ref. 17, and the use of quasi-DET enzymes based on modified enzyme-redox probes could prevent the leakage of mediators. However, some consequences of applying redox probes for enzyme modification may occur, such as decreased enzyme activity due to enzyme mutation or chemical modification.[1,2,6,11,16] Therefore, to anticipate any decrease in enzyme activity, the enzyme should have high activity before modification with redox probes so that the activity is not reduced after enzyme modification, enabling appropriate response currents to be achieved in amperometric evaluation.[14]

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[19–23] or nucleotides in recent studies on the application of arPES to aptamer-based sensors.[45,46] trPES is an alternative to the only previously reported redox probe targeting cysteine, a ruthenium complex derivative.[19–23] This is because both trPES and the ruthenium complex derivative have low redox potential,[19,33] and some redox enzymes were reported not to react with hexaammine ruthenium(III) because of its cationic property.[47–49] 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.

5. Conclusions

In this study, a novel and now commercially available redox probe, 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, we found that the position around residues S210 to N212 on the LOx surface [distance <14 Å from FMN (N5)] is 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, we increased the number of attached PES molecules 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 fivefold 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. 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. We reported the application of the new material trPES as a redox chemical for biological materials. The study can be expanded by applying trPES to other proteins, peptides, or nucleotides for its wider application in sensing systems and bioelectrochemical analyses.

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Appendix

Table S1
Primers used to design the cysteine mutants.

Enzymes	Forward primers*	Reverse primers*
*Av*LOx cysteine mutants		
A96L[34]	–	–
A96L/V20C	5'- AAGTACATTGATGTTtgcAATACT TACGACTTA -3'	5'- TAAGTCGTAAGTATTgcaAACATC AATGTACTT -3'
A96L/S178C	5'- TGACTCAACTGTTtgtGGAAAC CGTGACC -3'	5'- GGTCACGGTTTCCacaAACAGT TGAGTCA -3'
A96L/V185C	5'- AACCGTGACCGTGATtgcAAGAAT AAATTCGTT -3'	5'- AACGAATTTATTCTTgcaATCACG GTCACGGTT -3'
A96L/S210C	5'- ACAGCAGAAGGTATGtgtTTAAAC AATATCTAC -3'	5'- GTAGATATTGTTTAAacaCATACC TTCTGCTGT -3'
A96L/N212C	5'- GAAGGTATGTCATTAtgcAATATC TACGGTGCT -3'	5'- AGCACCGTAGATATTgcaTAATGA CATACCTTC -3'
*Af*GDH cysteine mutant		
K477C	5'- GAGACCtgtCCAGGCCTGTGAGAT CCCAGCTACCGCAGCGGATG -3'	5'- GCCTGGacaGGTCTCCTTCGCGATC AGTTTGTTCAGTGGTGCGC -3'

*Lowercase codons were the mutation sites.

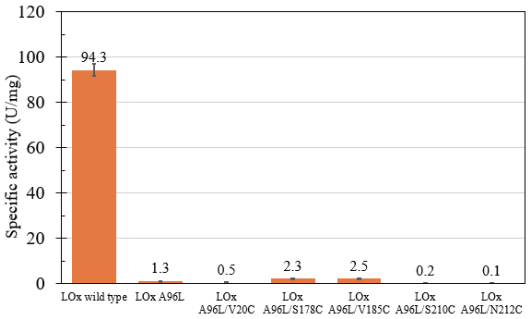

Fig. S1. (Color online) Oxidase activity of LOx mutants measured in 20 mM potassium phosphate buffer (PPB; pH 7.0) containing 1.5 mM 4AA, 1.5 mM TOOS, 2 U/ml POD, and 20 mM L-lactate. The oxidase activity was determined by monitoring the formation of quinone imine dye at 555 nm based on the molar absorption coefficient of TOOS at pH 7.0 (39.2 mM^{-1} cm^{-1}). One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the production of 1 μmol H_2O_2 per min using 20 mM lactate at 25 °C. 4AA: 4-aminoantipyrine. TOOS: 3-(*N*-ethyl-3-methylanilino)-2-hydroxy-propanesulfonate. POD: peroxidase. $n = 3$.

All LOx cysteine mutants showed very low oxidase activity compared with the LOx wild type.[34,47] They maintained the characteristic of the A96L mutation of low reactivity to molecular oxygen;[34] thus, they are suitable for MET- and quasi-DET-type sensor applications.

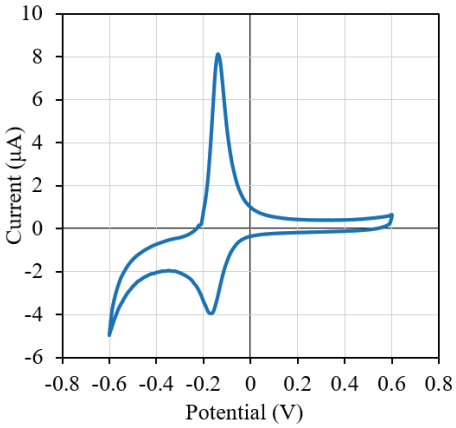

Fig. S2 (Color online) Cyclic voltammograms of 0.1 mM trPES in 100 mM PPB (pH 7.0). Scan rate: 0.1 V/s. Potential range: −0.6–+0.6 V (RE: Ag/AgCl; WE: GC; CE: Pt wire).

The oxidation and reduction peak potentials (E_{pc}: cathodic peak potential and E_{pa}: anodic peak potential) were observed at approximately −0.13 and −0.19 V (vs. Ag/AgCl), respectively; thus, the calculated midpoint potential ($E_{1/2}$) is −0.16 V. These redox behaviors are similar to those observed in other PES derivatives: arPES, which was used to modify *An*GOx,[16] and mPES.[31] A potential higher than $E_{1/2}$ was applied in the CA evaluation of the modified electrodes utilizing modified trPES-enzymes.