京都大学 博士(農学) 氏名 聶 其華

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<th>論文題目</th>
<th>Study on Oxidase/Peroxidase-based Biosensors with Pentacyanoferrate-bound Polymer</th>
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<td>（論文内容の要旨）</td>
<td>H₂O₂-producing oxidase and peroxidase (POD)-coupled system has been widely utilized for detection of the oxidase's substrate(s) in spectrophotometric methods, since POD reacts with several kinds of reduced reagents that can be oxidized into fluorescent or colored product for detection, thanks to poor substrate specificity of POD. However due to the property, the POD system is labile to cause negative interference by oxidizing electroactive compounds such as ascorbic acid (AA) or uric acid in physiological fluids, especially in solution reactions. Such interference reaction catalyzed by POD decreases the H₂O₂ concentration to cause underestimation of the H₂O₂ concentration. To solve the problem, oxidase-immobilized electrochemical biosensors covered with perm-selective membrane have been developed with the advantages of low cost, rapid and simple operation. In such enzyme-based amperometric biosensors, the concentration of analyte can be directly determined based on the detection of oxygen consumption or generated H₂O₂. In addition, the usage of some membrane on the top of immobilized enzyme layer can in part eliminate the interference effect based on perm-selectivity. Interferences can be blocked because of size exclusion or electrostatic repulsion. However, in the detection of oxygen consumption based on the reduction of oxygen, the signal response is seriously influenced by the change in the concentration of dissolved oxygen in samples and the diffusion rate of oxygen from the bulk solution to the surface of working electrode. On the other hand, the direct oxidative electrochemical detection of H₂O₂ requires high operating potential, which leads to decrease in the signal/noise ratio. Therefore, the methods may not be utilized for practical purpose required high sensitivity. In order to overcome these problems, H₂O₂-producing oxidase/POD-based biosensors have been proposed, in which H₂O₂ generated from an oxidase reaction is reductively detected at low operating potentials around 0 V vs. Ag/AgCl with the aid of POD in the mediated-electron-transfer (MET) system. Since the low potential operation and the usage of an outer-membrane decrease the background current and noise levels, and minimize the undesirable oxidation of electroactive interferences, such bienzyme biosensors are considered to show high sensitivity and stability. Nevertheless, the other thing which needs to be concerned is that the mediator oxidized by POD may be reduced not only at electrode but also by oxidase, because most of oxidases show dehydrogenase activity to transfer electrons to artificial mediators. Such cross-reaction diminishes the cathodic current to cause a decrease in the electrochemical response of mediator reduction.</td>
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By considering the following three factors: 1) the possibility of cross-reaction of mediators with POD and oxidases, 2) the interference effect of O₂ reduction around −0.2 V (vs. Ag|AgCl), and 3) the occurrence of interference oxidation at positive potentials, the author has focused her attention to select an appropriate mediator with high selectivity for POD alone and a suitable operating potential in the narrow range from −0.2 to 0 V.

To find the suitable mediator, in Chapter 1, the MET activity of the three kinds of metal complex-containing polymers: PVI[Os(dmebpy)2Cl] with neutral ligands, PVI[Os(dcbpy)2Cl] and PVI[Fe(CN)5] with negatively charged ligands (PVI = poly(1-vinylimidazole); dmebpy = 4,4'-dimethyl-2,2'-bipyridine; dcbpy = 4,4'-dicarboxy-2,2'-bipyridine), has been estimated for four H₂O₂-producing flavoenzymes (glucose oxidase, choline oxidase, sarcosine oxidase and lactate oxidase). The results reveal that the MET reactivity of the negatively charged polymers is very low, which is strongly related to the electrostatic repulsion between the local surface charge of the flavoenzymes and the polymer’s charge as well as the steric hindrance. Especially, in PVI[Fe(CN)5], the polymerization of Fe(CN)₅ via PVI increases the negative charge density and then enhances the electrostatic repulsive effect. However, PVI[Fe(CN)5] retains its high MET activity in the POD reaction to reduce H₂O₂. This enzyme-specific catalytic property of PVI[Fe(CN)5] is very convenient to construct H₂O₂-producing oxidase/POD-based bienzyme biosensors.

For the application, in Chapter 2, PVI[Fe(CN)5] has been utilized for constructing the creatinine biosensor which is based on the reductive H₂O₂ detection. PVI[Fe(CN)5] is appropriate for the enzyme immobilization in this biosensing system and it shows the superiority of being a mediator, since it only mediates the POD reaction but not against the sarcosine oxidase reaction. The interference effect due to the poor specificity of POD has been successfully eliminated by the Nafion film and the proposed method is applicable for clinical diagnosis.

To construct highly sensitive biosensors mediated by PVI[Fe(CN)5], in Chapter 3, D-amino acid oxidase (DAAO)/POD bienzyme biosensor mediated by PVI[Fe(CN)5] has successfully been constructed for the D-amino acid determination. There is no cross reaction between PVI[Fe(CN)5] and DAAO, and the severe interference effect resulted from high concentrations of interference has been eliminated by the Nafion films and ascorbate oxidase. The stability of DAAO has successfully been improved by adding free flavin adenine dinucleotide (FAD), and the Nafion film prevents the considerable leakage of free FAD. The biosensor has low detection limit and high sensitivity, which indicates its applicability for clinical diagnosis.
H₂O₂生成型酸化酵素(OxD)とペルオキシダーゼ(POD)を併用した発色系酵素分析法は汎用されているが、試薬との反応にPODの基質特異性の低さを利用していているので、本質的に共存還元剤による妨害を受けやすい欠点がある。これを克服するひとつの手段として、膜を用いたメディエータ電子移動(MET)型電気化学バイオセンサが提案されている。しかし、後者のMET型OxD/PODでは、メディエータが介在する交差反応による妨害を受けやすくするという本センサ特有の欠点もある。

本研究では、上記のようなOxDを基軸とする分析法の問題点を克服する目的で、ペンタシアノ鉄錯体を配位させたポリビニルイミダゾール(PVI[Fe(CN)₅])をメディエータとして導入することにより、交差反応の影響を極めて小さくすることに成功した。また本系を用いて、MET型OxD/POD二酵素電気化学バイオセンサを構築し、尿の検査に応用できることを示した。評価できる点は以下の通りである。

1. 本ポリマー（酸化型）は多くのOxDに対して静電反発あるいは立体障害を示すため、これらのOxDの電子受容体としては機能しないことを見出した。その結果として、本ポリマーを利用することによりMET型OxD/POD反応に特有なOxDが関連する交差反応による妨害を回避できることを示した。

2. 応用として、本反応系をクレアチニンおよびD-アミノ酸センサに組み入れた。クレアチニンバイオセンサではナファイオン膜にて還元性アニオンの妨害を低減するとともに、四酵素連結系が有効に機能することを示した。またD-アミノ酸バイオセンサでは、アスコルビン酸酸化酵素も併用し妨害物質の影響を極限まで抑え、高感度化を実現した。両センサとも、尿を対象とした実分析に適用できることを示した。

以上のように本論文は、負電荷を持つPVI[Fe(CN)₅]とOxDやPODとの相互作用を考慮することにより、交差反応のない高感度バイオセンサを設計したものである。また、物質透過選択性の保護層で電極を被覆することによって、高選択性かつ実用的な電気化学バイオセンサの開発へと展開したものであり、分析化学および電気化学の発展に寄与するところが大きい。

よって、本論文は博士（農学）の学位論文として価値あるものと認める。

なお、平成25年7月11日、論文並びにそれに関連した研究にかわり試問した結果、博士（農学）の学位を授与される学力が十分あるものと認めた。