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High Performance of Enzymatic Glucose Sensor with Polymeric Mediator for Blood Analysis

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Abstract

To produce micro blood glucose sensor on analysis chip, a series of hydrophilic and hydrophobic copolymers, poly(vinylferrocene-*co*-2-hydroxyethyl methacrylate) (poly(VFc-*co*-HEMA)) and poly(vinylferrocene-*co*-*n*-butyl methacrylate) (poly(VFc-*co*-BMA)) was prepared as a polymeric electron transfer mediator for preparing amperometric glucose sensors. The poly(VFc-*co*-HEMA) was observed redox current clearly, however, poly(VFc-*co*-BMA) was not observed due to its hydrophobic nature. The glucose sensors were consist of the poly(VFc-*co*-HEMA), enzyme glucose oxidase (GOD), crosslinked with 1,6-hexamethylenediamine and glutaraldehyde. To avoid an effect of adhesion of plasma proteins on the surface of GOD-polymer layer, poly(2-methacryloyloxyethyl phosphorylcholine-*co*-*n*-butyl methacrylate) was applied to cover the membrane. Since GOD-polymer layer indicated color response after immobilization, and approximately 70% of GOD activity remained when GOD mixed with glutaraldehyde, glutaraldehyde is a conducive crosslinking agent for GOD-copolymer. We observed response current over 360mg/dL of glucose concentration.

Key words: vinylferrocene, glucose sensor, glutaraldehyde

1. INTRODUCTION

Glucose oxidase (GOD) combined amperometric glucose electrodes have been widely investigated for blood glucose measurement [1]. Glucose react with oxygen in the presence of GOD, as formula (1).

$$\text{D-glucose} + O_2 \Rightarrow \text{D-gluconolactone} + H_2O_2 \quad \ldots(1)$$

Primary glucose sensors were based on peroxidase sensors which GOD immobilized membrane patched on. However, the reaction is limited by oxygen, measurement value of the sensors is affect by oxygen concentration. Thus, new series of glucose electrodes is applied electron transfer mediators instead of oxygen for transporting electrons between GOD and electrode, therefore, designing and selection of mediator is as important as holding GOD activity.

Since we are planning to produce blood glucose sensor, it is essential to avoid affects of interfering substrates such as ascorbate, uric acid and acetaminophen. By applying ferrocene, we can avoid such effects whereas redox potential of ferrocene group is low; around 0.35V (vs.SCE). In addition, to observe reproducible results, the concentration of GOD and mediator should be completely immobilized on the electrode to be an identical condition with every measurement in the sample solution. Considering the glucose sensor to an application of micro-blood-analysis, for instance, healthcare chip having multi-item measurement functions[2], there are some problems peculiar to this system. To obtain high sensitivity and accuracy in small dimentions, the electrodes require stability of enzyme activity and electron transfer mediator more rigidly. Moreover, GOD and electron transfer mediator must not contaminate sample solution. Thus we prepared the copolymer, poly (vinylferrocene - *co* - 2-hydroxyethyl methacrylate) (poly(VFc-*co*-HEMA)) expecting redox reactivity, hydrophilicity and water insolubility. As a finishing scheme, we coated the electrodes with excellent biocompatible and hydrophilic polymer, poly(2-methacryloyloxyethyl phosphorylcholine -*co*-*n*-butyl methacrylate) (PMB30) [3] to avoid adhesion of blood plasma proteins. In this communication, we will discuss the performance of the sensor with attention to the chemical structure and composition of the polymeric electron transfer mediator to enhance the sensitivity.

2. MATERIALS AND METHODS

2.1 Materials

Vinylferrocene (VFc) was reagent grade, purchased from Aldrich Co. (Milwaukee, U.S) and used without further purification. 2-Hydroxyethyl methacrylate (HEMA) and *n*-butyl methacrylate (BMA) were purchased from Wako Pure Chemical Co., Osaka, and distilled at 54 ℃, 1.5 mmHg or at 60℃, 30 mmHg respectively to remove inhibitor. 2,2'-Azobis(isobutyronitrile) (AIBN, Wako Pure Chemical Co.) was used without further purification. Freeze dried blood plasma (Nissui Co. Tokyo) was used by dissolving purified water.

2.2 Synthesis of polymeric mediator

Poly(VFc-*co*-HEMA) (PVH) and poly(VFc-*co*-BMA) (PVB) were synthesized by a conventional radical copolymerization[4]. Corresponding monomers, solvent, and initiator were put into a glass ampule. The ampule was deoxidised by argon bubbling for 10 min, then sealed. The polymerization temperature was 60 ℃.

Fig. 1 shows chemical structures of PVH and PVB as polymeric electron transfer mediators. The VFc mole fraction in copolymer was determined by absorbance at 440nm based on the adsorption of ferrocenyl group, molecular weight was determined by gel permeation chromatography (GPC). *N,N*-dimethylformamide (DMF) was used as an eluent of the GPC measurement.

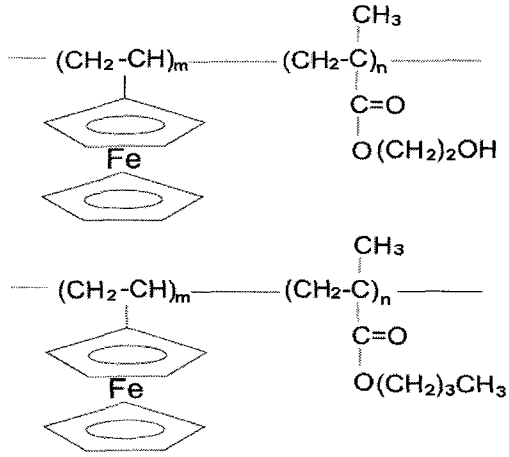

Fig.1 Chemical structure of PVH (above) and PVB (below), Polymeric electron transfer mediators.

2.3 Electrochemical measurement in cyclic voltammetry

3 wt% of PVH in DMF or 3 wt% of poly(VFc -*co*-BMA) in methanol was dropped on glassy carbon electrode (BAS, outer & inner diameter; 3.0 & 1.0 mm). Then cyclic voltammetry (CV) measured in phosphate buffered saline (PBS, pH 7.4).

2.4 Evaluation of immobilized GOD activity

Sample 1: GOD was immobilized on the glassy carbon plate (BAS Co. Tokyo, 15 mm×30 mm×2 mm) with the same method of preparing a glucose sensor. Then the layer was scraped and the activity of GOD was evaluated.

Sample 2: 2 μL of 1 % glutaraldehyde was added to 2 μL of 15 mg/mL GOD solution in PBS. The mixture was used to evaluate GOD activity.

Evaluation method was as follows; 24.0 mL of 0.10 mol/L phosphate buffer (PB) (pH7.0) and 0.25 mL of 0.20 mmol/L *o*-dianisidine aqueous solution were mixed and oxygenated for 5 minutes. Then 5.0 mg of peroxidase from horseradish in 1.0 mL of the PB and 5.0 ml of 10 wt% D-glucose with distilled water were added to the mixture. GOD was dissolved 1 mL of the PB, and the sample in 0.10 mL PB was added to the mixture. We measured the absorbance change in 436 nm of wavelength for 2 minutes and calculated the absorbance change per minutes (△A) between the first linear range. The activity of the enzyme was calculated from eq. (2).

$$\text{units/mg} = (\Delta A_{test} - A_{control}) \times \frac{1}{8.3} \times 30.1 \times \frac{1}{S} \quad \cdots (2)$$

$A_{control}$: Absorbance change of control that 0.10ml PB was added instead of the sample

8.3: Milli-molar extinction coefficient of quinine dye

30.1: Total volume (mL)

S: Sample mass (mg)

One unit is the amount of enzyme which produce 1 μmol of D-gluconolactone per minutes.

2.5 Preparation of GOD immobilized electrode

3 wt% of PVH in DMF was dropped on glassy carbon electrode. The PVH modified glassy carbon electrode was stored in the 1,6-hexamethylenediamine aqueous solution of 10 wt% for 20 min, washed by purified water and dried. Then 2 μL of GOD solution (Wako Pure Chemical Co., 3000unit/mL of PBS) was dropped on it. After drying at 4 ℃, 1 % and 3 % of glutaraldehyde aqueous solution (Kanto Chemical Co., Tokyo) dropped on the electrode. After drying at 4 ℃, the electrode was washed by PB and coated by 0.3 wt% PMB30 in ethanol.

2.6 Electrochemical measurement in steady-state mode

Sample was prepared in different glucose concentration in PBS, or blood plasma. The glucose response current was measured at 0.35 V vs. SCE from low glucose concentration.

3. RESULT AND DISCUSSION

Table I shows the synthetic result of polymers containing VFc units. Three of the polymers containing HEMA units (PVH 1~3) were polymerized in benzene. Though separated out during polymerization, the polymerization proceeded successfully. The PVH could be dissolved in DMF. Thus, we tried to make polymerization in DMF, however, its polymerization was scarcely success.

Monomer concentration was also important factor to determine the polymer properties. Comparing with PVH1 and PVH3, the higher monomer concentration related larger molecular weight, higher yield and higher VFc mole fraction in the obtained polymer. That is, the VFc is more difficult to polymerize than HEMA. The composition of VFc in polymer was lower than that in feed. The higher mole fraction of VFc, related to low molecular weight and low yield.

Fig.2 shows typical CV curves of PVH1 and ferrocene-poly(HEMA) mixture in PBS.

Although the PVH1 contained only 15 % of VFc, the redox current was observed clearly. The peak currents of the PVH1 and ferrocene-poly(HEMA) mixture were around 0.40 V and 0.35 V, respectively. In case of polymeric electron transfer mediator, both reduction and oxidation potentials of the copolymer shifted to higher value.

On the other hand, PVH3 had small peak around 0.40 V on the 1st cycle. Then the peak grows, up to 7th cycle sharp peaks were observed. Fig.3 shows CV curves of PV3.

Table I Results of polymerization of PVH and PVB

Abb.	Co-monomer	VFc mole fraction In feed /	In copolymer	Solvent	Total monomer concentration (mol/L)	Time (h)	Yield (%)	Number-averaged molecular weight	Molecular weight distribution
PVH1	HEMA	0.50	0.15	Benzene	2.0	16	23	5.7×10^4	1.2
PVH2	HEMA	0.75	0.21	Benzene	2.0	72	15	3.7×10^4	1.2
PVH3	HEMA	0.50	0.23	Benzene	5.0	21	54	1.9×10^5	1.3
PVH4	HEMA	0.50	N.D.	DMF	5.0	22	39	Very low	N.D.
PVB	BMA	0.50	0.29	Methanol	1.0	26	26	N.D.	N.D.

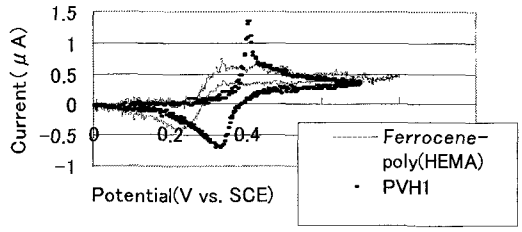

Fig.2 A typical CV of PVH1 and ferrocene-poly(HEMA). Scan rate: 10mV/sec.

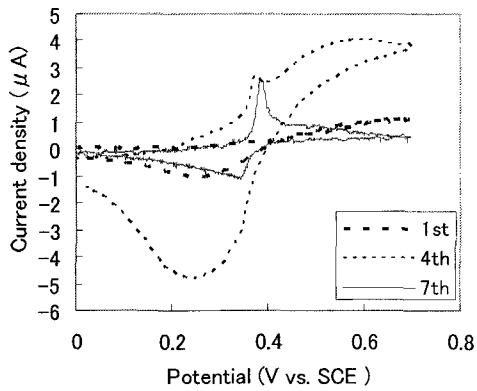

Fig.3 A typical CV of PVH3. Scan rate: 10mV/sec.

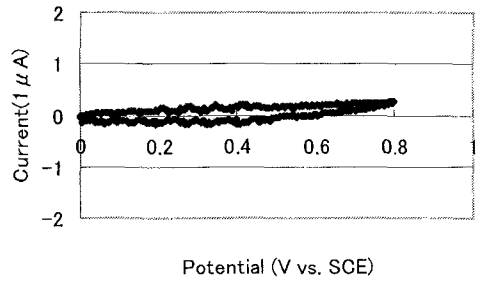

Fig.4 Typical CV of PVB. Scan rate: 50mV/sec.

Fig.4 shows CV of PVB. The scan late was 50 mV/sec, and the faster scan late relates the larger redox current in the same substance. However, unlike PVH, it was not observed redox current. Since the poly(BMA) is a hydrophobic polymer, water could not permeate to the PVB and electron transfer hardly occured even when VFc mole fraction was 0.29. To observe redox current on the PVB, higher VFc composition should be required.

Table II : Activity of GOD..

Raw GOD	Sample 1	Sample 2
200 unit/mg	--	139unit/mg

Table II shows that the result of GOD evaluation. Sample 1 was strongly immobilized GOD and the sample did not dispersed in aqueous solution. Therefore we could not determine the enzyme activity of Sample 1, but we could identify color reaction around Sample 1 due to oxidation of substrate *o*-dianisidine. The GOD was completely immobilized to PVH by glutaraldehyde and its activity remained on the polymer membrane. We needed measurement of Sample 2 to identify affect of glutaraldehyde. GOD was mixed with 1 % glutaraldehyde , and 70 % of enzyme activity compared with that of raw GOD remained.

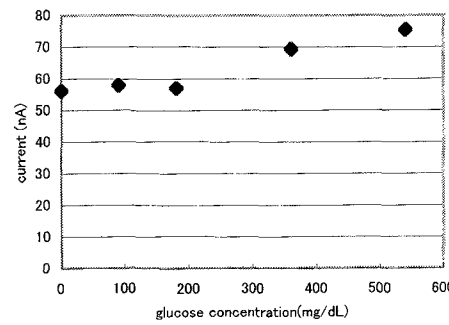

Fig.5 Correlation between glucose concentration and anodic current. Glutaraldehyde: 1 %.

Fig.5 shows the relationship between the glucose concentration and the anodic current measured at +0.38 V vs. SCE at 20 sec after applying the potential. We expected that the current rises linearly. However, although we observed glucose response currents over 360 mg/dL, we could not observe around 90-180 mg/dL.

Normal blood glucose concentration is in the range of 60 -110 mg/dL (fasting) and 60-160 mg/dL (full), we need further research to improve sensitivity of less than 180 mg/dL. We need to enhance the immobilization density of GOD on the electrode to improve sensitivity and magnitude of response current.

Fig.6 shows the result of the same experiment of Fig.5 in blood plasma. The blood plasma contains 778-906 mg/dL sodium chloride, PBS contains 800 mg/dL. The blood plasma also contains phospholipids, plasma proteins such as albumin and γ -globulin. These proteins adsorb on the surface of the sensor, and can lower its sensitivity.

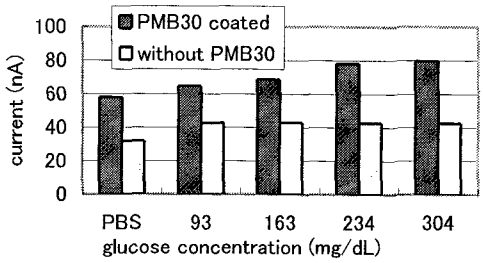

Fig.6 Correlation between glucose concentration and anodic current in blood plasma. Glutaraldehyde: 1%.

The 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers are known for their excellent functions to reduce protein adsorption, even when they contacted with whole blood. We applied a type of ethanol soluble and water insoluble MPC polymer, PMB30, to coat the electrodes. Fig.6 shows the effect of the MPC polymer coating to the sensitivity of the glucose electrode. The current increased with an increase in the glucose concentration when the electrode was covered with the PMB30. That is due to the PMB30 avoided the adsorption of plasma protein on the GOD-PVH layer and enabled to detect glucose concentration of blood plasma. From this results, when the glucose will be measured from blood or plasma directly, the directly, the surface of the electrode should be covered with biocompatible and hydrophilic polymer such as PMB30.

There are a report that a MPC polymer coated glucose sensor could monitor 14 days in vivo [5].

4. CONCLUSION

We modified glassy carbon with poly(VFc-*co*-HEMA), and redox current was observed clearly in cyclic voltammetry. On the other hand, it was not observed in poly(VFc-*co*-BMA) modified electrode. We also prepared glucose sensor with poly(VFc-*co*-HEMA), GOD and glutaraldehyde. We determined that GOD was completely immobilized on the poly(VFc-*co*-HEMA) layer by glutaraldehyde, and enzyme activity remained. We observed glucose response current with the glucose electrode over 360 mg/dL, we still need to improve sensitivity of less than 180 mg/dL. The surface coating with the MPC polymer was to stabilize the response of the electrode.

5. REFERENCES

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