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Methylene Blue/Perfluorosulfonated Ionomer Modified Microcylinder Carbon Fiber Electrode and Its Application for the Determination of Hemoglobin

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The fabrication and surface morphology of methylene blue (MB)/perfluorosulfonated ionomer (Nafion) modified microcylinder carbon fiber electrode were studied. The stability of the modified electrode is good. The extraction coefficient K of MB between Nafion film and solution is ~110, and the diffusion coefficient of MB in Nafion film is ~5.3×10^{-9} cm^2/s. The MB/Nafion modified microcylinder electrode shows significant electrocatalytic activity for the oxidation of hemoglobin in weak acid media; it can be used for the determination of hemoglobin in the range of 5.0–50 μM with a relative standard deviation of 3.5% at 6.0 μM HB. A satisfactory result has been obtained for the determination of hemoglobin in clinical blood samples.

Microelectrodes have received considerable attention in some applications due to their many advantages compared with conventionally sized electrodes[1–4] and have offered dramatic improvements in the qualities of electrochemical data.[4–6] Recently, the study of heterogeneous catalytic reactions at a microelectrode modified with mediators or promoters has attracted great interest for the determination of biomolecules in special small environments.[7–9] However, because of the high mass transport rate at a microelectrode, the product of the electrode reaction rapidly diffuses away from the electrode surface;[4,10] thus, the catalytic efficiency of homogeneous or heterogeneous reactions declines greatly.[7] Generally, it is not easy to study a catalytic reaction with a low reaction rate constant at a microelectrode unless exceptionally high scan rates are employed or the electrode is coated with polymer to fix the mediators or promoters at the surface.

Hemoglobin (HB) is an important respiratory protein in red blood cells. Reduced hemoglobin is the carrier for oxygen transport. It is involved in many clinical diseases such as anemia, leukemia, excessive loss of blood, heart disease, etc. Thus, the determination of HB is very important in clinical medicine. In general, it is carried out with spectrophotometry by using cyanidation (KCN) or alkali hydroxylation (AHD-575). These methods often require highly purified reagents and very toxic KCN (in the KCN method) and are also very time-consuming. HB is composed of ferrous protoporphyrin-9 (heme) and four peptide chains. Although it has insufficient electrochemical response at a bare electrode because of its bulky structure and passivates the electrode surface, it should be possible to determine HB by an electrochemical method if the electrode can be chemically modified. Hawkridge et al.[11,12] have characterized the electrochemical activity of myoglobin at the methyl viologen (MV) surface modified gold minigrid electrode. Studies of the electrocatalytic reduction or oxidation of hemoglobin at a modified electrode have been reported.[13–16] Although Song et al.[15–20] had studied the properties of MB at a normal bare or Nafion modified electrode, little kinetic data about the diffusion of MB in Nafion film and the distribution of MB between film and solution have been reported. To our knowledge, up to now there has been no report of MB/Nafion modified carbon fiber microcylinder electrodes for the direct determination of hemoglobin.

The application of modified electrodes has been greatly restricted due to the poor reproducibility as a result of the adsorption of biomolecules. However, will the effect be even more serious with the small sized electrodes and high mass transport rate? In this work, we selected methylene blue as an electron mediator immobilized by Nafion on the surface of a carbon fiber microcylinder to construct the chemically modified electrode. The MB/Nafion modified electrode not only eliminates the loss of MB, but also accelerates the oxidation of hemoglobin in a weak acid medium. As a consequence, some properties such as duration,

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reproducibility, and sensitivity are improved. The dependence of catalytic currents on the experimental parameters and the reaction mechanism at this modified electrode were studied. The MB/Nafion modified microelectrode has been applied to the determination of hemoglobin in clinical human blood samples without special treatment. The results show that direct electrochemical determination of hemoglobin using properly modified microelectrodes is possible.

EXPERIMENTAL SECTION

Materials and Reagents. Methylene blue was of analytical reagent grade (produced by the Shanghai Third Chemical Reagent Factory, in China). Hemoglobin (oxen blood) was produced by Shanghai Biochemistry Research Institute. Nafion (5 wt % ethanol solution) was obtained from Aldrich Co. Carbon fiber (PAN type) came from the Shanghai Synthetic Fiber Research Institute. Epon 812 epoxy resin (New York) was used to seal the carbon fiber electrode. All other chemicals were of AR grade and used as received without further purification. Twice-quartz-distilled water was used in all experiments.

Instruments. Electrochemical measurements were carried out with a Model-366 bipotentiostat (EG & G) and BAS-100B electrochemical analyzer with a PA-1 preamplifier, which was used to amplify current and filter out noise. An X-560 electron scanning microscope (Hitachi) was used for SEM photos. The temperature was controlled at 20 ± 0.1 °C.

Preparation of MB/Nafion Modified Electrodes. The fabrication of the carbon fiber microcylinder electrode was usually similar to that given in a previous paper.[21] The carbon fiber, 6–8 μm diameter, was sealed into capillary glass with epoxy resin. Prior to Nafion coating and modification of MB, the fabricated carbon fiber electrode was first washed thoroughly with acetone and distilled water in an ultrasonic bath, dried under an infrared lamp, and then electrochemically pretreated in 1.0 M H_2SO_4 solution with a triangular-wave potential sweep from -1.0 to $+2.0$ V at a scan rate of 20 000 mV/s for 5 min. After the electrode was washed and dried, it was dipped into 5% Nafion solution for several seconds, and then placed under an infrared lamp. The thickness of the coated Nafion depends upon the number of coating steps. Coating twice is suitable for the formation of a well-defined Nafion film (see below). After coating with Nafion, the electrode was dipped into 1.0 mM MB solution, prepared with pH 7.2 phosphate buffer solution, for 20 min. The Nafion film cannot be protonated easily and facilitates the incorporation of MB ions by ion exchange to form a MB/Nafion film. The electrode was stored in a pH 7.2 buffer solution.

Procedure. A three-electrode system was employed in which a MB/Nafion modified electrode (with a length of ~8.0 mm) was used as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a Pt wire as the counter electrode. After deaerating with pure N_2 for 10 min, the electrochemical measurements were carried out under a N_2 atmosphere.

RESULTS AND DISCUSSION

Surface Morphology of the Modified Electrode. The Nafion modified electrodes were viewed with a electron microscope to ensure that the perfluorinated ionomer, Nafion, was distributed uniformly on the electrode surface. Figure 1A showed that the electrode dip-coated only once in 5% Nafion solution is not satisfactory. Dipping the electrode more than three times also gave a poor surface. Dip-coating twice was suitable for uniform coverage (Figure 1B). The surface morphology and electrochemical response of MB/Nafion modified electrodes were greatly dependent upon the immersion time of the coated-Nafion electrode in pH 7.2 buffer solution containing 1.0 mM MB. SEM pictures for different immersion times in MB solution are shown in Figure 1C,D. When the immersion time was much longer than 1 h, the MB/Nafion layer became a multiisland surface (see Figure 1D), resulting in a decrease of electrochemical response and also bad cyclic voltammograms. An immersion time of ~20 min was suitable (Figure 1C). To obtain the SEM pictures, the electrode surface was treated by spraying with gold.

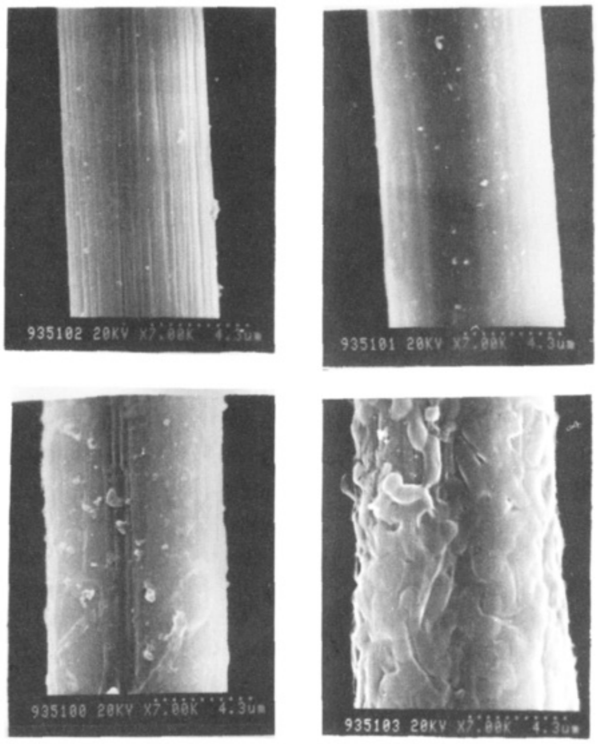

Figure 1. Scanning electron micrographs of modified carbon fiber microcylinder electrodes: (A, top left) dip-coated in Nafion solution once; (B, top right) dip-coated in Nafion solution twice and then dipped in 1.0 mM MB (C, bottom left) for 20 min and (D, bottom right) for 1 h.

Stability of MB/Nafion Modified Electrode. The voltammograms obtained in pH 7.2 buffer solution at a scan rate of 100 mV/s from 0.10 to -0.60 V at a MB modified electrode indicated that the cathodic and anodic peak currents decreased with the repetitive sweeps (Figure 2A). The cathodic and anodic peak potentials were -0.32 and -0.24 V, respectively. However, the peak currents of both cathodic and anodic processes of cyclic voltammograms at the MB/Nafion modified electrode with the same scan rate and sweep range decreased relatively slower with repetitive sweeps and then became constant (Figure 2B). Both the cathodic and anodic peak potentials shifted toward more negative values to -0.41 and -0.34 V, respectively. The cathodic peak currents at both MB/Nafion modified and MB modified electrodes followed a first-order kinetic expression (Figure 2C). The half-life measured was 52 h and 87 s, respectively. No change of peak currents appeared after the MB/Nafion modified electrode was dipped in pH 7.2 phosphate buffer solution for 1 week,

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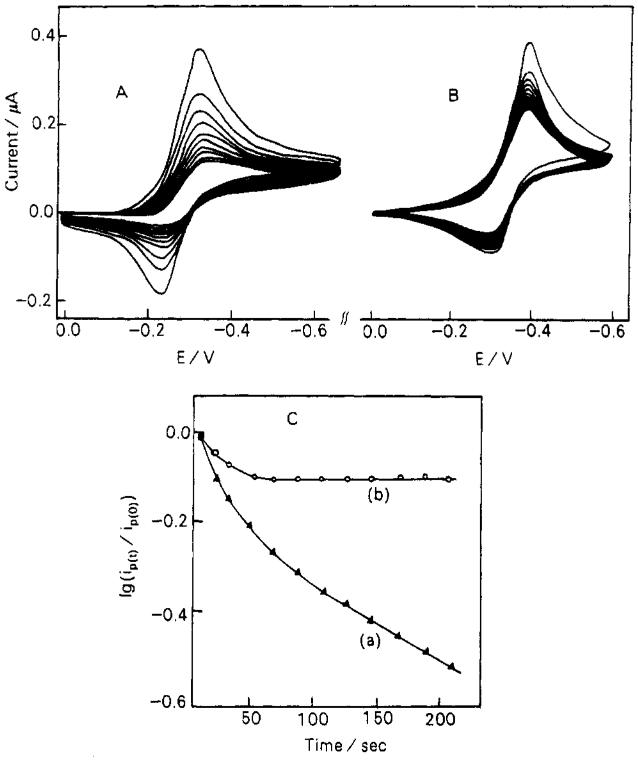

Figure 2. Cyclic voltammograms at MB (A) and MB/Nafion (B) modified carbon fiber microcylinder electrodes in pH 7.2 buffer solution, and the relation between log($i_p/i_{p,0}$) and scan time (C); (a) and (b) for MB and MB/Nafion modified electrode, respectively. The $i_{p,0}$ is the peak current of the first sweep, and the i_p is the peak current determined at time t, v = 100 mV/s, pH 7.2.

indicating the lack of dissolution of MB molecules from the polymer film into the aqueous solution. It suggested that the mediater, MB, was strongly immobilized on the electrode surface by means of coated Nafion film.

Dependence of Peak Currents on Scan Rate and pH. The dependence of cathodic peak currents, $i_{p,c}$, on scan rate at different treated electrodes is shown in Figure 3. At a bare carbon fiber electrode that was just immersed in MB solution, the relation between $i_{p,c}$ and $v^{1/2}$ showed good linearity (Figure 3A), suggesting that the electrode process was controlled by MB diffusion in the solution. However, $i_{p,c}$ at a bare electrode that had been dipped in MB solution for 1 h is proportional to v (Figure 3B). Thus, the electrode process was controlled by MB adsorption. Because the adsorption of oxidized or reduced forms of the depolarizer had a marked effect on the capacity of the double layer, the ac base current at a more positive or negative potential than the peak potential of the ac voltammogram would differ from the base current in the absence of the depolarizer.[22] In comparison with the base lines of ac voltammograms with or without MB, the evidence indicated that both MB and its reduced product adsorbed on the electrode surface, but the latter adsorbed less strongly than the former, because the reduction form of MB became more negative, decreasing its interaction with the negatively charged functional groups of the Nafion film. The linear dependence of $i_{p,c}$ on v for MB/Nafion modified electrode in different pH solutions in the absence of MB (shown in Figure 3C) revealed that the electrode process was similar to that in a thin-layer cell. Comparing all the curves in Figure 3 (A–C), it was found that the slopes of curves and the currents were affected by the pH of the solution except for those at the MB/Nafion modified electrode (curve b in Figure 3C was also an exception). It showed that the electrochemical properties of the electrode were affected by the pH more seriously at a bare electrode than a Nafion modified electrode.

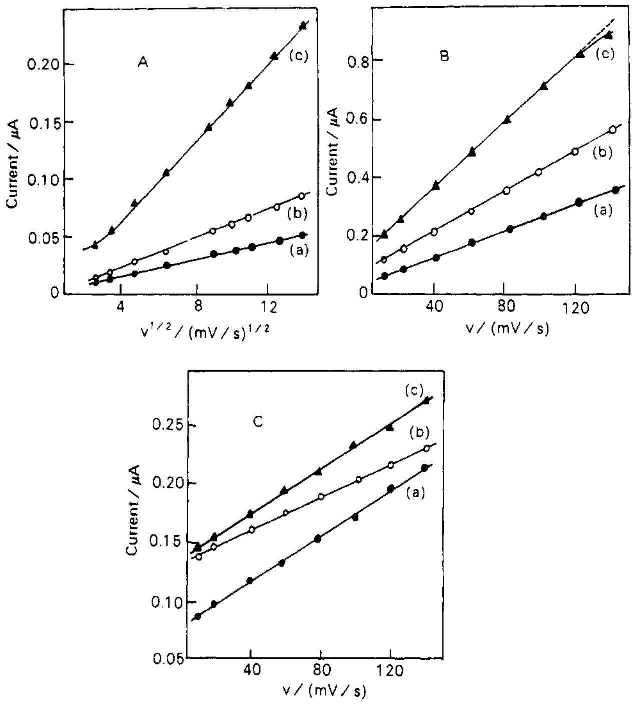

Figure 3. Dependence of peak currents on scan rate at pH 2.2., 6.6, and 10.7 solution for (a), (b), and (c), respectively: (A) at a bare electrode dipped in 1.0 mM MB; (B) at the same electrode after dipping in 1.0 mM MB for 60 min; (C) at a MB/Nafion electrode in buffer solution.

Distribution of MB in the Nafion Film and Solution. Because it was difficult to quantitatively determine and calculate the thickness of the Nafion film at a carbon fiber microcylinder electrode, we used a glassy carbon electrode with a radius of 0.25 cm to study MB distribution between the Nafion film and the solution. A glassy carbon electrode was coated uniformly with 3 μL of Nafion solution, dried, and then dipped into pH 7.2 buffer solution containing various concentrations (c^*) of MB for different immersion times. It is assumed that MB incorporated into the Nafion film was well distributed. MB concentration (c_p) in the Nafion film could be chronocoulometrically determined and expressed as follows:

$$c_p = Q/nFAL \qquad (1)$$

Where Q is the charge quantity consumed coulometrically in complete oxidation of film species, A is electrode area, L is average thickness of the Nafion film (the density of Nafion solid is 2.0 g/cm^3,[23,24] L = 0.5 μm was obtained from the density and the quantity used), and n = 2.[25] The dependence of c_p on immersion time at different MB concentrations in solution is shown in Figure 4. Initially, c_p increased with increasing immersion time and then

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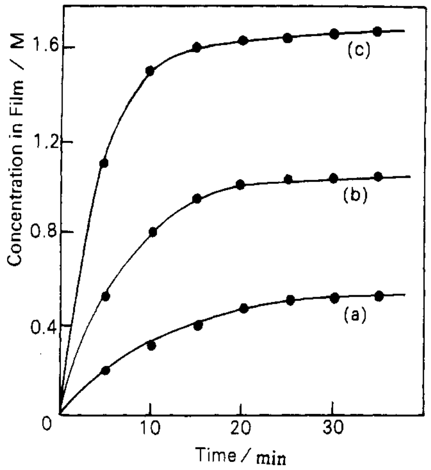


Figure 4. Dependences of the concentration of MB in Nafion film on the immersion time in different MB concentrations: (a) 0.5, (b) 1.0, and (c) 2.0 mM, in pH 7.2 buffer solution.

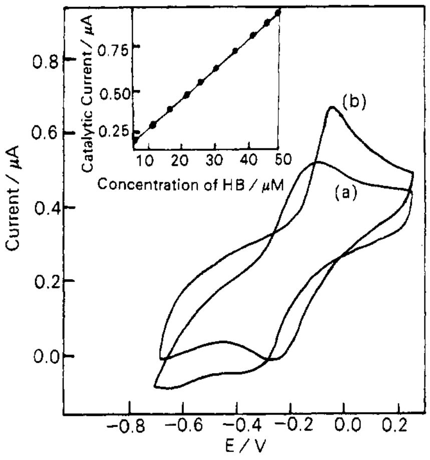


Figure 5. Cyclic voltammograms of MB/Nafion modified microelectrode: (a) in pH 5.5 buffer solution; (b) in (a) + 10 µM HB. Inset: dependence of catalytic currents on concentration of HB.

trended to a constant value, $c_{p,max}$. The times to achieve the approximate $c_{p,max}$ decreased with an increasing c^*. It meant that the rate of equilibrium of MB between the two phases, solution and solid, increased with increasing MB concentration in solution. The ratio (K) of $c_{p,max}$ to c^*, defined as the extraction coefficient,[24] was constant for the concentration range of 10^{-3}–1.5 mM. Beyond 1.5 mM, the ratio decreased with increasing c^*, suggesting the saturation of MB in the Nafion film. When the MB concentration in solution was 1.0 mM, the determined $c_{p,max}$, i.e., saturation concentration of MB in Nafion film, was 0.11 M; the extraction coefficient K was 0.11 M/1.0 mM = 110. Therefore, the MB concentration in the Nafion film is 100 times greater than that in the solution, and this value should be used for calculating the apparent diffusion coefficient D_{app} of MB in the Nafion film by means of the Cottrell equation. If the MB concentration in solution was taken instead of that in film to calculate D_{app} in film, a great error would occur. This is why the D_{app} in this work so greatly differs from that in ref 20. The K value was relatively large, and the equilibrium rate of MB between the Nafion film and the solution was relatively slow. It suggested that the loss of MB by diffusion from film to solution was very little for short periods of time. Thus, the MB/Nafion modified carbon fiber microcylinder electrode should be quite stable.

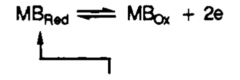
Diffusion of MB in the Nafion Film. The D_{app} of MB in the Nafion film at both microcylinder and normal planar electrodes can be determined according to the following chronoamperometric equation (2)[26] and Cottrell equation (3), respectively.

$$i = 2nFAD_{app}c_p/[r\ln(4\theta)] \qquad (A = 2\pi rl) \qquad (2)$$

$$i = nFAc_pD_{app}^{1/2}(\pi t)^{-1/2} \qquad (3)$$

Where c_p, the MB concentration in the Nafion film at the microcylinder electrode, can be calculated by the MB concentration in solution and the extraction coefficient mentioned above, $\theta = D_{app}t/r^2$ and l and r are the length and radius of microcylinder electrode, respectively. The others symbols have their usual meanings. Here a microcylinder electrode with a length of 8.0 mm and a radius of 4.0 µm, which was modified by Nafion film and then dipped in 1.0 mM MB solution for 20 min, was used for the chronoamperometric experiments in pH 7.2 buffer solution for the determination of D_{app}. A value of 5.3×10^{-9} cm^2/s was obtained by formula 2, while a D_{app} of 5.0×10^{-9} cm^2/s was also obtained for a Nafion modified glassy carbon electrode by formula 3. Both values were in good agreement and ~1000 times less than that in solution (7.6×10^{-6} cm^2/s[25]). The results are similar to those obtained from other species in polymer or solution.[23,24] The species with a lower diffusion coefficient would have a much smaller mobility within the Nafion film, facilitating the fast immobilization of MB in the film. Thus, the Nafion film played an important role in preventing the loss of the mediator MB.

Catalytic Oxidation of Hemoglobin at a MB/Nafion Modified Electrode. The present work shows that the MB/Nafion modified electrode can electrocatalyze the oxidation of HB ($HbFe^{II}$) in weak acid media through the electron transfer reaction at the heterogeneous boundary layer between MB and HB. The MB^+/MB redox couple has a high self-exchange rate, facilitating the charge transport. The regeneration of electroactive reactant MB_{red}, which was produced by $HbFe^{II}$ from its oxidized form MB_{ox}, enhances the anodic peak currents (oxidation process) and decreases the cathodic peak currents in cyclic voltammetry due to the electrocatalysis at the film boundary. The peak potentials of this redox couple MB_{ox}/MB_{red} for both anodic and cathodic processes shift in the positive direction by ~80 mV as HB_{red} sample solution was added to the pH 5.5 buffer solution (Figure 5). The catalytic mechanism could be expressed as follows:



and may be schematically represented in Figure 6.

At the same MB/Nafion modified electrode, no changes of the cyclic voltammograms in buffer solution occurred even though the electrode had been used for catalyzing the oxidation of HB_{red}. It suggested that the MB concentration in the Nafion film did not decrease and also that HB did not permeate into the Nafion film, nor did it adsorb on the electrode surface to passivate the redox reaction. The experimental results showed that the catalytic

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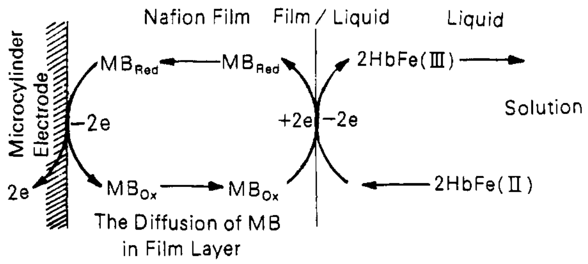

Figure 6. Schematic diagram of the mass transfer and electron exchange precesses at the MB/Nafion modified microelectrode in HB solution.

anodic peak currents were proportional to $v^{1/2}$, and the peak potential was independent of v, indicating the electrode processes were controlled by the HB diffusion in solution. However, the electrocatalytic peak currents depended greatly on the solution pH, because the interaction between the Nafion film and HB was apparently affected by the solution pH. When the pH was >6.8, there was a repulsion between HB and the Nafion film, because both HB, with an isoelectric point of 6.8, and Nafion, with functional group of SO_3^-, carries negative charges; thus, no catalytic currents appeared. When the pH was <6.8, the interaction between HB and SO_3^- strengthened greatly due to the protonation of HB; the catalytic currents increased with decreasing solution pH. However, the experiments showed that the catalytic currents would decrease if the pH went lower than 5.5. This phenomenon probably resulted from the overprotonation of the functional group SO_3^-, increasing repulsion with the decrease of pH. A suitable pH value of 5.5 was chosen for the determination of HB.

Application of the MB/Nafion Modified Electrode in the Determination of HB in Blood. In a pH 5.5 buffer solution, the catalytic currents of HB at the MB/Nafion modified electrode increased with increasing concentration of HB. The catalytic peak currents were proportional to the HB concentration in the range of 5.0−50 μM with the correlation coefficient of 0.998 (inset in Figure 5). The detection limit was ~2.0 μM. The relative standard deviation of results was 3.5% for six successive determinations at 6.0 μM.

The amount of hemoglobin in normal human blood samples is ~0.14−0.17 g/mL (i.e., 2.2−2.6 mM). A clinical human blood sample that was diluted with pH 5.5 buffer solution 100-fold has been directly tested by using the MB/Nafion modified electrode without any interference. The average value of three determinations was 2.4 mM. The result was close to the value of 2.5 mM obtained by spectrophotometry (KCN method) at a 540 nm wavelength.

The duration of chemically mediator modified electrode especially used for the determination of biological samples is one of the most interesting challenges because the electrode suffers from contamination by the adsorption of biomolecules. In fact, our experiments showed that the catalytic currents of HB_{red} only fell by 4% after the electrode had been used for 2 h. It showed that the feature of mediator (MB) modified microelectrode could be greatly improved by the use of Nafion and may be used quite well in practice.

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