Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioelectrochemistry 63 (2004) 257–259
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Amperometric measurement of ds-DNA content using a peroxidase-modified electrode

Fumio Mizutani*, Soichi Yabuki, Yukari Sato, Seiichiro Iijima

National Institute of Advanced Industrial Science and Technology, Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566 Japan

Received 23 June 2003; received in revised form 21 October 2003; accepted 24 October 2003

Abstract

An enzyme electrode with a chemically amplified response for methylene blue (MB) was constructed from a glassy carbon electrode and a layer containing immobilized horseradish peroxidase (HRP). MB is reduced on the electrode but regenerated through the HRP-catalyzed reaction in the presence of H_2O_2. The electroreduction/regeneration cycle for MB resulted in an amplified electrode response. The enzyme electrode was applied to the highly sensitive measurement of ds-DNA. The current for MB decreased in association with its complexation with DNA, and the current response caused by DNA was also amplified through the recycling processes. The detection limit of ds-DNA (from salmon testes) was as low as 5 ng ml^{-1}.

Keywords: Amperometry; Peroxidase; Methylene blue; DNA; Chemical amplification

1. Introduction

Various continuous cell lines are used for producing recombinant DNA products, such as protein and protective antigen. These cellular products are contaminated with their oncogenes in the residual host cell DNA. Such contaminants must be removed from the products used for intravenous administration, in order to avoid the possible cellular transformation of the oncogenic DNA. A World Health Organization consultative group recommended reducing the cellular DNA content in such biopharmaceutical products to be <10 ng ml^{-1} [1,2]. Therefore, the sensitive, simple and rapid determination of DNA content is necessary in industrial biopharmaceutical laboratories. Electrochemical sensing systems can accomplish this function.

Nucleic acids have been studied by means of electrochemical techniques for about four decades [3,4]. Cathodic and anodic stripping analysis methods have been applied to the highly sensitive determination of DNA, and the detection limits around 20 ng ml^{-1} have been achieved [5,6]. However, the cathodic detection system used poisonous mercury as the working electrode material [5]. On the other hand, carbon paste electrodes have been used for the anodic detection, but the oxidation current for DNA was observed around 1 V vs. Ag/AgCl [6]: in such a high potential region, species in biological samples (e.g., proteins with cysteine residues) may be oxidized to cause a positive error. Therefore, the development of alternative method is still desired.

The use of redox-active intercalators would be a suitable approach [3,4,7]. Electrochemical sensor for DNA can be prepared by using such redox-active species as methylene blue (MB) [8–10]. The current response for MB is considered to reduce remarkably in association with its complexation with DNA (ds-DNA) in the sample solution to be measured, owing to the steric hindrance of MB in the complexes as well as to the lowering of the diffusion coefficient of the electroactive species after the complexation. This means that the DNA content can be estimated from the current decrease for the electrochemical label. However, our preliminary experiments showed that the sensitivity for such a sensing system was not high enough for monitoring DNA whose concentration range was less than 10 ng ml^{-1}.

We have shown that an electron transfer mediator could be determined with high sensitivity by using an enzyme electrode [11]. That is, the mediator is oxidized (or reduced) through the enzymatic reaction in the presence of the

* Corresponding author. Tel.: +81-29-861-6167; fax: +81-29-861-6177.
E-mail address: mizutani.fumio@aist.go.jp (F. Mizutani).

1567-5394/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2003.10.023

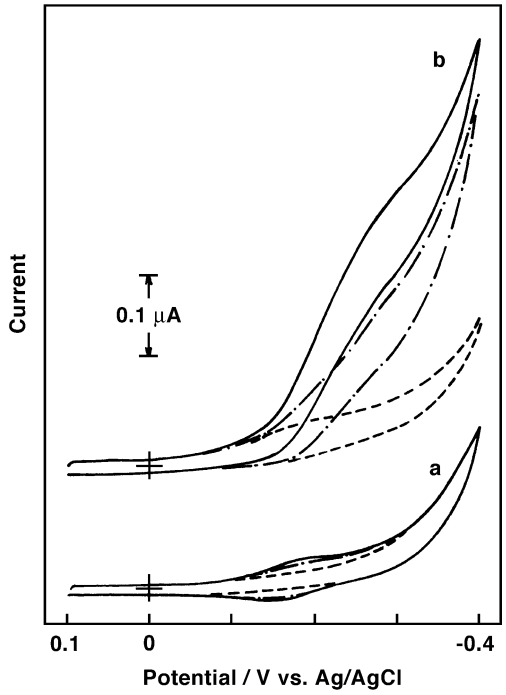

Fig. 1. Cyclic voltammograms on the HRP-modified electrode in 0.1 M phosphate buffer (pH 6.0) containing 0 (a) and 2 mM (b) H_2O_2, before (---) and after the successive addition of 5 μM MB (—) and 10 μg ml^{-1} DNA (-·-).

enzyme substrate, and regenerated through the electrochemical reaction: the consumption/regeneration cycle for the mediator results in an amplified electrode response, which provides an inherent gain in sensitivity. MB has been reported to act as an electron transfer mediator for horseradish peroxidase (HRP, EC 1.11.1.7) [12,13], which means that an HRP-based electrode is useful for the high sensitive measurement of MB, and, accordingly, for that of DNA. This paper describes the preliminary results for the measurement of DNA with an HRP-modified electrode.

2. Experimental

HRP (216 U mg^{-1}) and MB were obtained from Toyobo (Osaka) and Wako (Osaka), respectively. Bovine serum albumin (BSA) and ds-DNA (from salmon testes) were purchased from Sigma (St Louis, MO). Other reagents were of analytical reagent grade (Nacalai, Kyoto). Deionized, doubly distilled water was used throughout.

A grassy carbon disc electrode (Bioanalytical Systems, West Lafayette, IN), 1 mm in diameter, was polished with 0.05-μm alumina slurry, rinsed with water and then sonicated in water for 2 min. On the grassy carbon electrode, solutions containing HRP (2% (w/v), pH 7, 0.6 μl), BSA (2% (w/v), pH 7, 1.2 μl) and glutaraldehyde (0.5% (w/v), pH 7, 0.6 μl) were successively placed. Then the electrode was allowed to dry for 4 h. Thus, an HRP/BSA-crosslinked membrane was formed on the electrode surface. The enzyme electrode, an Ag/AgCl electrode (saturated with KCl) and a platinum wire were employed as the working, reference and auxiliary electrodes, respectively, in a three-electrode configuration. The testing buffer solution used was a 0.1 M phosphate buffer (20 ml; pH 6.0) whose temperature was kept at 25.0 ± 0.2 °C. The potential of the enzyme electrode was usually set at -0.3 V vs. Ag/AgCl. The potential was negative enough for the reduction of MB as described later.

3. Results and discussion

3.1. Current-potential profile

Fig. 1 shows cyclic voltammograms on the enzyme electrode in the buffer solution containing (a) 0 and (b) 2 mM H_2O_2, respectively, before and after the successive addition of 5 μM MB and 10 μg ml^{-1} DNA. The addition of H_2O_2 to the buffer solution without MB did not bring about a discernible current response on the glassy carbon-based enzyme electrode in the potential range examined. As shown in Fig. 1a, cathodic and anodic peaks corresponding to the electron transfer between MB and its reduced form, $(MB)_{red}$ was observed around -0.2 V vs. Ag/AgCl on the enzyme electrode, indicating that MB permeated through the HRP/BSA-membrane to undergo the electrochemical reaction on the glassy carbon surface. The addition of DNA caused a slight decrease in the redox peak heights, resulting from the complexation of MB with DNA.

On the other hand, the addition of H_2O_2, the substrate of HRP, caused a remarkable increase in the cathodic current

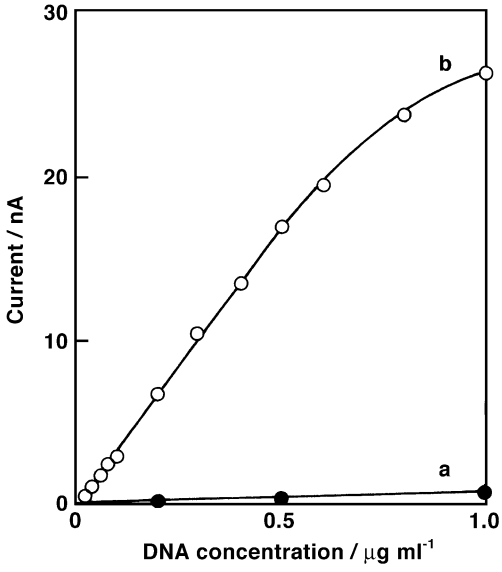

Fig. 2. Calibration curves for DNA of the HRP-modified electrode in 0.1 M phosphate buffer solutions containing 5 μM MB, and 0 (a) and 2 mM (b) H_2O_2 at -0.3 V vs. Ag/AgCl.

so as to give a catalytic limiting current (Fig. 1b) for the MB-recycling sequences:

$$2MB + 2e \rightarrow 2(MB)_{red}$$
$$H_2O_2 + HRP \rightarrow H_2O + HRP\ I$$
$$HRP\ I + (MB)_{red} \rightarrow HRP\ II + MB$$
$$HRP\ II + (MB)_{red} \rightarrow HRP + MB$$

Furthermore, much larger current decrease was given by the addition of DNA than that obtained in the hydrogen peroxide-free solution, as shown in Fig. 1a and b. This is inherently advantageous to increasing the sensitivity for the DNA measurement.

3.2. Amperometric measurement of DNA

Fig. 2 shows relationships between the amount of DNA and the steady-state current decrease of the enzyme electrode in the solution containing 5 μM MB (a) and that containing 5 μM MB and 2 mM H_2O_2. The time required for obtaining a steady-state response after the addition of DNA was less than 2 min in each solution system. The response of DNA of the enzyme electrode in the MB/H_2O_2-solution was proportional to the DNA concentration only up to 0.5 μg ml^{-1}. In the linear range, however, the DNA response was amplified by a factor of 45 compared to that in the H_2O_2-free, MB solution. This resulted in a much higher sensitivity of the recycling system: the detection limit for the salmon testes DNA was 5 ng ml^{-1} (signal-to-noise ratio, 5) in the MB/H_2O_2-system, although that was 0.2 μg ml^{-1} in the H_2O_2-free solution. The sensitivity of the present recycling system is high enough for applying to the detection of DNA in biopharmaceutical products. The relative standard deviation for 10 successive measurement of 10 ng ml^{-1} DNA was 3.7%. The HRP-modified electrode could be used for a week.

The extent of signal amplification for DNA, an inhibitor of the electrochemical mediator, depends on the parameters of the enzyme membrane used, such as the enzyme activity, membrane thickness and diffusion coefficient of the mediator in the membrane [11,14]. The study for enhancing the amplification factor, as well as the application of the present recycling system to the biological samples, is now in progress.

References

[1] Report on the WHO Expert Committee on Biological Standardization, in: WHO Weekly Epidemiological Report vol. 72, (1997) 141–145.
[2] M. Sakata, K. Matsumoto, N. Obaru, M. Kunitake, H. Mizokami, C. Hirayama, Removal of DNA from a protein solution with cross-linked poly(ethyleneimine) spherical particles, J. Liq. Chromatogr. 26 (2003) 231–246.
[3] E. Palecek, Past, present and future of nucleic acids electrochemistry, Talanta 56 (2002) 809–819.
[4] E. Palecek, M. Fojta, DNA hybridization and damage, Anal. Chem. 73 (2001) 75A–83A.
[5] E. Palecek, M. Tomschik, V. Stankova, L. Havran, Chronopotentiometric stripping of DNA at mercury electrodes, Electroanalysis 9 (1997) 990–997.
[6] X. Cai, G. Rivas, P.A.M. Farias, H. Shiraishi, J. Wang, M. Fojta, E. Palecek, Trace measurement of plasmid DNAs by adsorptive stripping potentiometry at carbon paste electrodes, Bioelectrochem. Bioenerg. 40 (1996) 41–47.
[7] J. Wang, Towards genoelectronics: electrochemical biosensing of DNA hybridization, Chem. Eur. J. 5 (1999) 1681–1685.
[8] R. Rohs, H. Sklenar, R. Lavery, B. Roeder, Methylene blue binding to DNA with alternating GC base sequence: a modeling study, J. Am. Chem. Soc. 122 (2000) 2860–2866.
[9] A. Tani, A.J. Tomson, J.N. Butt, Methylene blue as an electrochemical discriminator of single- and double-stranded oligonucleotides immobilized on gold substrates, Analyst 126 (2001) 1756–1759.
[10] D. Ozkan, P. Kara, K. Kerman, B. Meric, A. Erdem, F. Jelen, P.E. Nielsen, M. Ozsoz, DNA and PNA sensing on mercury and carbon electrode by using methylene blue as an electrochemical label, Bioelectrochemistry 58 (2002) 119–126.
[11] F. Mizutani, S. Yabuki, M. Asai, Highly-sensitive measurement of hydroquinone with an enzyme electrode, Biosens. Bioelectron. 6 (1991) 305–310.
[12] J. Qian, Y. Liu, H. Liu, T. Yu, J. Deng, An amperometric new methylene blue N-mediating sensor for hydrohen peroxide based on regenerated silk fibroin as an immobilization matrix for peroxidase, Anal. Biochem. 236 (1996) 208–214.
[13] J.-Z. Xu, J.-J. Zhu, Q. Wu, Z. Hu, H.-Y. Chen, An amperometric biosensor based on the coimmobilization of horseradish peroxidase and methylene blue on a carnon nanotubes modified electrode, Electroanalysis 15 (2003) 219.
[14] F. Mizutani, T. Yamanaka, Y. Tanabe, K. Tsuda, An enzyme electrode L-lactate with a chemically-amplified response, Anal. Chim. Acta 177 (1985) 153–168.