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Electrochemical determination of total alkaline phosphatase in human blood with a micropatterned ITO film

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Abstract

A voltammetric determination of alkaline phosphatase (ALP), a well-known enzyme in human blood, was investigated to survey the feasibility for a health diagnosis sensor. Simple photolithography with an office printer and transparency film was employed to construct an electrochemical sensor with an Indium tin oxide (ITO) film on glass. ITO films can be easily patterned with a resolution of 50 μm, and an integrated sensor consisting of working, counter and reference electrodes on a single ITO plate was constructed by this method. We used *p*-nitrophenyl phosphate (PNPP) as the substrate for ALP. PNPP was hydrolyzed enzymatically, and the product *p*-nitrophenol was detected by cyclic voltammetry and square wave voltammetry at an oxidation potential of +1.1 V (versus a fabricated Ag|AgCl reference electrode) on a bare ITO electrode. According to this method, ALP can be detected in various media including fetal bovine serum, human serum and untreated human blood. The linear dynamic range of ALP was 5–180 units per liter (U/L). This method showed a good enough performance for applications and was sufficiently reproducible. Results were further examined via comparison with medical standard colorimetric analysis.

Keywords: Alkaline phosphatase; Electrochemical determination; ITO; Biosensors

1. Introduction

Alkaline phosphatase (ALP) is a well-known enzyme existing in human blood. It is a non-specific phosphomonoesterase, which exhibits optimum activity at alkaline pH [1,2]. The hydrolysis of phosphomonoesters in the presence of such an enzyme yields inorganic phosphate and the corresponding alcohol, phenol, etc. ALP in blood is mainly derived from liver and bone, and its activity increases if there is a problem in these organs. The rapid and convenient analysis of ALP in blood is important since the level of ALP activity is used as a preliminary diagnosis for many diseases such as hepatitis, cirrhosis and tumors [3]. As well as the role of a health marker, ALP and its conjugates are one of the most commonly used enzyme labels in enzyme-linked immunosorbent assay (ELISA) due to their high stability and wide variety of substrates [4].

Various substrates such as phenylphosphate, *p*-aminophenyl phosphate, *p*-nitrophenyl phosphate, and 3-indoxyl phosphate [5–8] have been employed. With regards to the detection system, the formation of the product is generally followed by spectrophotometry, fluorescence or chemiluminescence [9–13]. However, in recent years, analyses with electrochemical detection constitute a methodology used extensively [14–17]. The advantages of this approach include the speed, accuracy, and precision with which many electrochemical measurements can be made.

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Indium tin oxide (ITO) is a widely used conducting metal-oxide, and is known to be a material, which inherently withstands metal deposition and organic adsorption [18,19]. ITO does not adhere strongly with metal adatoms due to weak metal-oxide bond strength [18]. It also resists the adsorption of organic molecules because of poor surface nucleophilicity [19]. ITO has another advantage for ALP sensing. As an already oxidized form, ITO does not react at positive potential and exhibits little water oxidation current [20] at the potentials needed to achieve the electrochemical analysis of *p*-nitrophenyl phosphate (PNPP), the most commonly used ALP substrate [4]. We selected ITO as a candidate material for ALP sensor for such useful properties.

Simple photolithography with an office laser printer and transparency film was employed to construct an electrochemical sensor with an ITO film on glass. As in previous approaches to replace photolithography with low-cost printing techniques, such as micro contact printing (μCP) [21], inkjet printing [22] or photographic reduction [23], this patterning method is a convenient, low-cost route to patterned conducting oxide films, useful for the fabrication of electrodes and electrical connections in electrochemical sensors that do not require sub-micrometer scale patterning. This technique demonstrates its usefulness by producing patterned ITO films without any expensive instruments or facilities. It is especially appropriate for use in chemical and biochemical laboratories that do not have access to the facilities used to make photomasks to the standard of microelectronics, because it bypasses the requirement for chrome masks. The method reported here does not represent new science: we focused on the exploitation of the most broadly available techniques for forming patterns with features useful in functional microstructures. This method extends the capability for microfabrication to laboratories that have no (or limited) access to the facilities required to fabricate conventional chrome masks or to carry out high-resolution printing.

An integrated sensor consisting of working, counter and reference electrodes on a single ITO plate is constructed. With this sensor, ALP can be detected in various media including fetal bovine serum (FBS), human serum and untreated human blood. We could observe sufficiently reproducible results for this application. Results were further examined via comparison with medical standard colorimetric analysis.

2. Experimental

2.1. Reagents

Ag_2SO_4, $FeCl_3$, $K_3[Fe(CN)_6]$ and NaCl were purchased from Aldrich Chemical Co. Alkaline phosphatase (ALP) and its substrates, *p*-nitrophenyl phosphate disodium salt hexahydrate (PNPP) and *p*-nitrophenol (PNP) were obtained from Sigma. Acids such as HCl and HNO_3 were obtained from Junsei Chemical Co. Dialyzed fetal bovine serum (FBS, molecular cutoff: 1000) was purchased from Welgene Inc. in a frozen form. The health care center at KAIST prepared and donated human blood samples from some volunteers. All chemicals were used without additional purification. Aqueous solutions were prepared with thrice-distilled water from a Modulab water system (US Filter Corp.) unless otherwise mentioned.

A negative photoresist, SU-8, was purchased from Microchem Co., who also provided developer and stripper for SU-8. All the experiments were carried out at room temperature.

2.2. Instrumentation

The BAS 100B PC-controlled electrochemical analyzer (BioAnalytical Systems Inc.) with a three-electrode system was used for all electrochemical measurements. The electrodes used, including a gold disk electrode, a glassy carbon disk electrode, an RE-5B Ag|AgCl reference electrode and a platinum wire counter electrode were also purchased from BioAnalytical Systems Inc.

Branson 2210 ultrasonic cleaner (Branson Ultrasonics Corp.) was used to clean the ITO substrate. A homemade spin coater and a 235-nm ultraviolet lamp (Woongjin UV Co.) were used for photolithography. A commercial office printer, ML-6080 was purchased from Samsung Electronics and used with transparency film (3M Co.).

2.3. Device fabrication

Methods used to prepare the ITO microelectrodes were the following. First, ITO-coated glass (ITO thickness, ~250 nm; resistance, ~25 Ω/square; Hoya Glass Co.) was cleaned with sonication in methanol and acetone [21]. Su-8, a negative photoresist was then spin-coated onto the ITO. Next, the photoresist layer was patterned by illumination with a 235-nm light through a mask (a laser-printed transparency film) designed with a computer graphics program. These patterns were then transferred to the substrate by developing the photoresist and etching the ITO surface with an aqueous acid solution (20% HCl and 5% HNO_3) [24]. The remaining photoresist was removed with sonication in a stripper solution. The geometry and dimensions of the ITO patterns could be controlled easily by changing the patterns on the monitor screen. The resolution of this method was about 50 μm.

3. Results and discussion

3.1. Fabrication of the ITO sensor

A photograph of the fabricated ITO electrode is shown in Fig. 1. We could easily obtain patterned ITO films with minimum feature sizes of ~50 μm. Since the patterning of the ITO film could be achieved easily, we integrated the working, counter and reference electrodes on a single ITO plate. All three kinds of electrode can be fabricated on a single piece of ITO with an area of less than 1 cm^2. The easy fabrication of the miniaturized ITO sensor enables convenient experiments and a wide range of applications through connection with computers or other electronic devices. An example of an integrated ITO sensor is presented in Fig. 1(b). The 100 μm wide band microelectrode was used for ALP analysis, and was tested with cyclic voltammetry (CV) via the reaction of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox couple in aqueous solution before the application. Only electrodes proven to be functional were used. Meanwhile, the Ag|AgCl reference electrode was constructed on the ITO surface similarly to a previously reported method [25]. Briefly, silver was electrodeposited on ITO with electrochemical reduction of Ag^+ in Ag_2SO_4 solution. By dipping the silver-coated ITO electrode in 10 mM $FeCl_3$ solution for 30 s, we can obtain an Ag|AgCl reference electrode on an ITO substrate. The characteristics of the Ag|AgCl electrode are recorded versus a commercial Ag|AgCl reference electrode and presented in Fig. 2. As expected, the fabricated Ag|AgCl electrode exhibited a linear potential against log[Cl^-]. The value of the slope was about −55 mV/dec, which corresponds well to the theoretical value of −58.5 mV/dec. The fluctuation of potential over 1 h was less than ±4 mV in 0.1 M sodium chloride solution. Despite of its physical weakness due to poor adhesion of the ITO and silver layer [18], the fabricated solid-state Ag|AgCl electrode appears to be effective as a reference electrode in a solution with a known concentration of chloride ion (i.e. serum or blood). The fabricated reference electrode showed a stable potential against a commercial reference electrode in various media such as FBS, human serum and human whole blood. Because of the uniform chloride concentration of body fluids, the potential of the fabricated electrode was about +45 mV in all three media. All reported potentials are referenced to this fabricated, solid-state electrode.

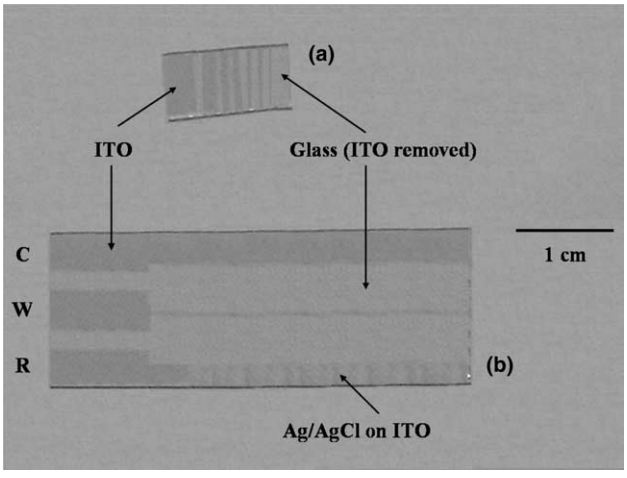

Fig. 1. Photograph of patterned ITO films: (a) lines with various widths; (b) an integrated ITO sensor consisting of a 100 μm width band microelectrode, Ag|AgCl reference electrode and ITO counter electrode.

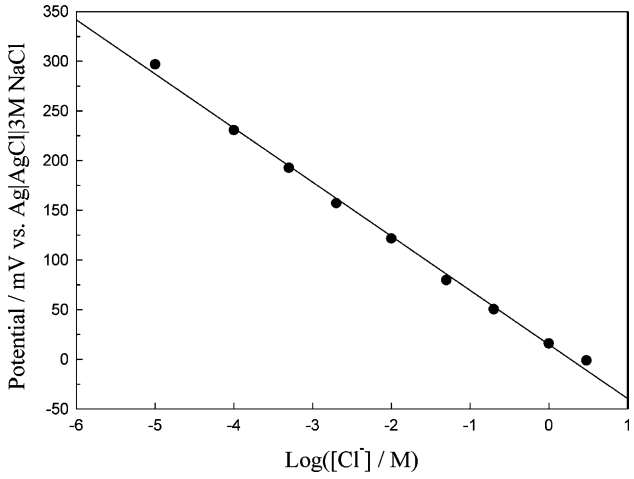

Fig. 2. Potential change of the fabricated Ag|AgCl reference electrode with varying concentrations of Cl^-.

3.2. Electrochemical determination of ALP activity

As a preliminary verification, the adsorption behavior of serum on various electrodes was investigated. Since the serum contains many kinds of organic adsorbent proteins including serum albumin, a non-specific adsorption occurs at the surface of the electrode [26]. Two kinds of widely used electrode material, gold and glassy carbon were selected, in addition to ITO. Fig. 3 shows the results from the adsorption survey. Fig. 3(a) shows cyclic voltammograms acquired in pure FBS (scan rate: 50 mV/s). No electrochemical signal should appear in FBS, but gold and the glassy carbon electrode (GCE) exhibited evidence of surface adsorption. Besides the unclean voltammograms from adsorption, there is a current from gold oxide formation and its reduction at the gold electrode. In contrast to other materials, there was no significant current signal at the ITO electrode. A clean baseline, except for a current near +1.3 V due to solution electrolysis, could be obtained. An FBS sample containing 30 mM of PNP was employed to examine the influence of surface adsorption on the electrochemical oxidation of PNP. The peak current ratio from the

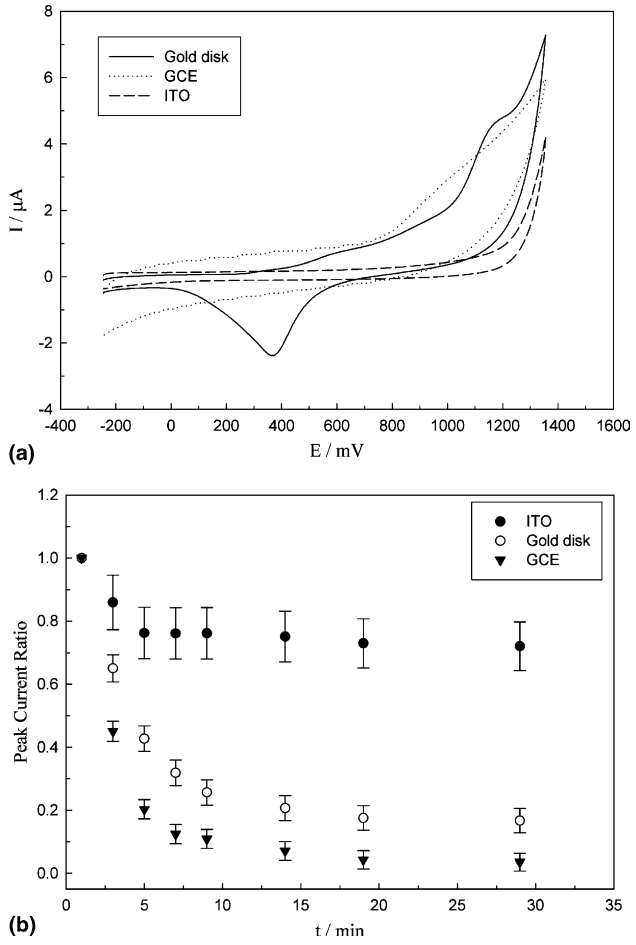

Fig. 3. Comparison of surface adsorption to various electrode materials in fetal bovine serum (FBS): (a) cyclic voltammograms in pure FBS and (b) variation of peak current with respect to time in FBS containing 30 mM *p*-nitrophenol.

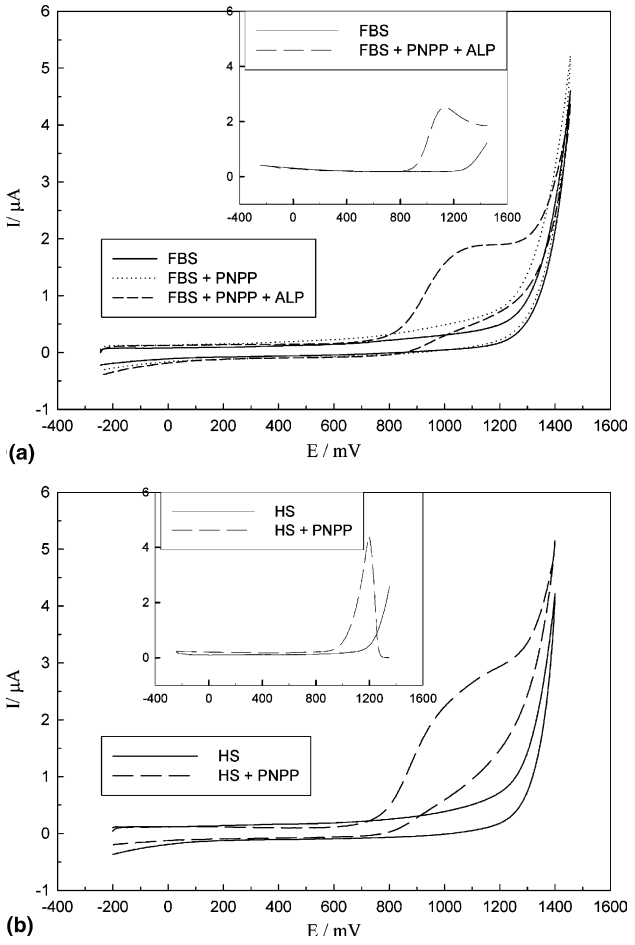

Fig. 4. Cyclic voltammograms from determination of ALP in (a) fetal bovine serum (FBS) and (b) human serum (HS): Insets in each figure represent results from square wave voltammetry under the same conditions.

oxidation of PNP is recorded versus time, and is shown in Fig. 3(b). As expected from the results in Fig. 3(a), there is a rapid decrease of the peak current at gold disk and GCE. Meanwhile, the ITO electrode shows stable properties. The peak current reaches equilibrium in about 5 min, and it remains at more than 75% for 30 min of experiment. With these results, ITO is confirmed to be resistive to adsorption.

ALP in two different types of sera, FBS and human serum (HS) was analyzed electrochemically with ITO electrodes. The results are shown in the voltammograms in Fig. 4. In the case of FBS (Fig. 4(a)), it has been already dialyzed by the provider and hence contains little ALP. Addition of PNPP to FBS does not play any role because there is no significant amount of ALP in the FBS sample. Almost the same voltammograms from pure FBS (solid line) and PNPP-added FBS (dotted line) prove the absence of ALP. Once ALP is added to FBS, enzymatic hydrolysis of PNPP to PNP takes place in solution, and the product, PNP can be detected electrochemically. The dashed line in Fig. 4(a) indicates the electrochemical oxidation of hydrolyzed PNP. A broad but clear peak from the oxidation of PNP [27] appears near +1.1 V. In contrast to the case of FBS, there is no need for ALP addition to HS because it contains a sufficient amount of ALP. As shown in Fig. 4(b), PNP is produced by simply adding PNPP to HS without the addition of ALP. Although FBS and HS are different in composition, the ITO electrode can withstand adsorption in both types of sera. Square wave voltammetry (SWV) was employed to enhance the analytical performances. The insets in Fig. 4(a) and (b) present the results from SWV in the same samples as in cyclic voltammetry, respectively. In SWV, well-defined peaks, as well as a clear baseline without any influence of solvent electrolysis, can be obtained. Quantitative analysis of ALP was performed with the SWV technique, and the parameters for SWV were $E_{SW} = 25$ mV, $\Delta E_S = 5$ mV and $f = 25$ Hz.

The results from quantitative analysis of ALP activity are shown in Fig. 5. The relationship between the peak currents of hydrolyzed PNP and two parameters:

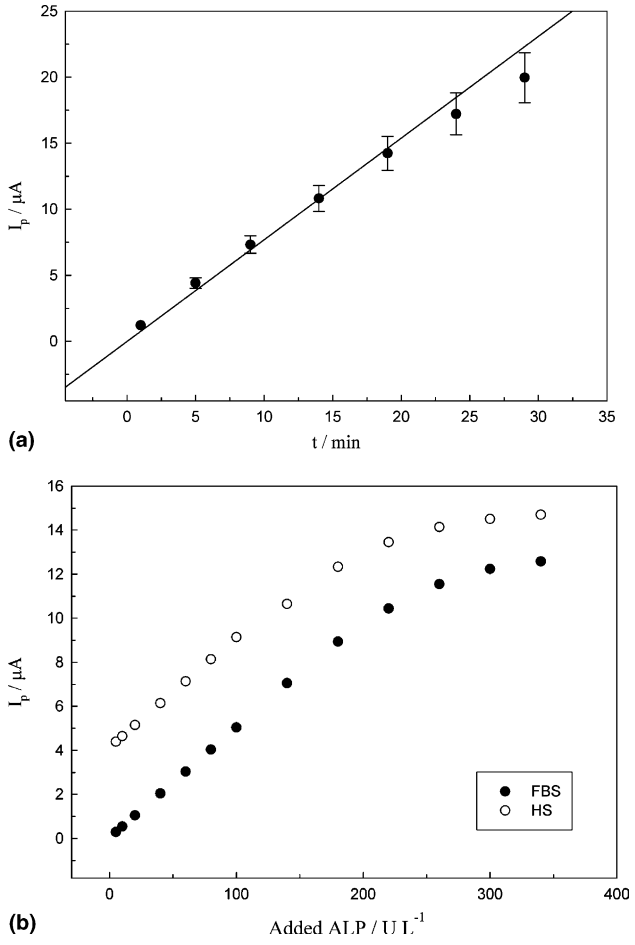

Fig. 5. Quantitative analysis of ALP activity: (a) peak currents proportional to exposure time to sample FBS containing 80 U/L ALP and (b) result of standard addition of ALP.

(a) hydrolysis time and (b) activity of ALP, are presented. Since the peak current at the ITO electrode reaches adsorption equilibrium in 5 min (Fig. 3(b)), we applied a 5 min pre-exposure step to the blank serum for each electrode before the analysis. Fig. 5(a) shows the peak current from an FBS sample containing 80 U/L of ALP acquired at various times. The peak current is proportional to the hydrolysis time, and the correlation coefficient r is about 0.93 (0.99 for the first five data). Although the linear relationship between peak current and hydrolysis time is obvious, there is a tendency to a decrease of hydrolysis activity after 20 min (i.e. the amount of current increase during same time interval is slowly diminished). This phenomenon is thought to happen for two reasons. One is a reduced ALP activity due to prolonged enzymatic reaction [28], and the other is an inhibition of oxidation by the formation of a polymeric side product at high concentrations of the PNP substrate [29]. The decrease of ALP activity is an interesting subject for study, but it is not considered here, since the effect of the activity decrease is insignificant in a short-time experiment like the system described in this article. On the basis of the results in Fig. 5(a), the relation between the peak current and ALP activity was investigated with standard additions of ALP. The hydrolysis time at each addition of ALP was set to 5 min in the presence of excess substrate, PNPP. Fig. 5(b) shows that the current response is proportional to the activity over a wide range while the correlation begins to deviate at high ALP activity. According to this method, the linear dynamic range of ALP is 5–180 U/L in both FBS and HS. The detection limit of ALP activity ($S/N = 3$) was 4.3 U/L for CV and 0.6 U/L for SWV, respectively. The system becomes more sensitive with increasing hydrolysis time, but the hydrolysis time was fixed to 5 min for fast analysis because a sensitivity of below 1 U/L is sufficient for practical application.

3.3. Determination of ALP in untreated blood sample

Since the preparation of serum with a centrifuge is a time consuming step in realizing an ALP enzyme sensor, we tried the determination of ALP in human whole blood without any pretreatment. ALP in blood was determined by the following steps. First, a few drops of concentrated PNPP solution were added to fresh human blood. The mixed solution was kept for 5 min for hydrolysis, and SWV was performed. Because the total procedure was concluded within 6 min, human blood could be analyzed before coagulation of the sample. Fig. 6 shows the ALP analysis of HS (solid line) and whole blood (dashed line) from the same person. As shown in the figure, the analysis in whole blood is similar to that in HS despite the broadening of the peak. Samples were analyzed by a medical standard method, the colorimetric detection of hydrolyzed PNP, and compared with the result of SWV to validate the capability of this electrochemical

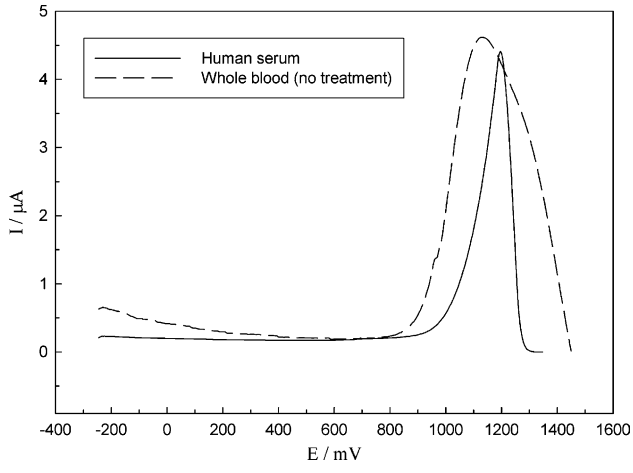

Fig. 6. Determination of ALP in untreated human blood: whole blood shows a result similar to that of refined serum.

Table 1
Comparison of results from colorimetric and electrochemical determination

	Colorimetric determination	Electrochemical determination
	ALP activity (U/L)	
FBS	2.5	1.0 ± 0.4
Human serum A	90	87.4 ± 9.3
Human serum B	113	109.6 ± 14.0
Human blood A	n.a.	91.5 ± 10.8
Human blood B	n.a.	116.3 ± 15.9

method. The results from each sample by different analysis techniques are given in Table 1. It is clear that the two methods give similar results and hence SWV with an ITO-based sensor can provide an acceptable method for the sensing of ALP. Moreover, the electrochemical method can be performed in both human serum and whole blood, while the traditional colorimetric method is useful only with serum.

Compared to other methods [9–17], this method has the advantages of a simple procedure, short experiment time, wide linear range and applicability to whole blood. The detection limit is not attractive, yet the linear range of 5–180 U/L is wide enough for health diagnoses since the standard activity of ALP in blood for a healthy male is 55–125 U/L (cf. 40–100 U/L for a female) [3].

For further application, the prevention of hemolysis should be considered because the red blood cell contains a high level of ALP – more than six times that of serum [3]. The temperature dependence of ALP activity is also under evaluation.

4. Conclusions

We observed an excellent performance in analysis of ALP in serum and whole blood by combining square wave voltammetry with a micropatterned ITO sensor. Simple photolithography with an office printer and transparency film was capable of constructing various patterns on ITO with a resolution of 50 μm. With *p*-nitrophenyl phosphate as a substrate for hydrolysis of ALP, ALP was detected in various media including fetal bovine serum, human serum and untreated human blood. ALP can be detected with a detection limit of 0.6 U/L and a linear range of 5–180 U/L. This method showed a good enough performance for applications and was sufficiently reproducible. The results were further examined via comparison with a medical standard colorimetric analysis.

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References

[1] L. Stryer, Biochemistry, fourth ed., W.H. Freeman, New York, 1995.
[2] J.E. Frew, N.C. Foulds, J.M. Wilshere, N.J. Forrow, M.J. Green, J. Electroanal. Chem. 266 (1989) 309.
[3] C.A. Burtist, E.R. Ashwood, Tietz Textbook of Clinical Chemistry, second ed., W.B. Saunders, Philadelphia, 1994.
[4] C.P. Price, D.J. Newman, Principles and Practice of Immunoassay, Stockton Press, New York, 1991.
[5] K.R. Wehmeyer, H.B. Halsall, W.R. Heineman, C.P. Volle, I.-W. Chen, Anal. Chem. 58 (1986) 135.
[6] H.T. Tang, C.E. Lunte, H.B. Halsall, W.R. Heineman, Anal. Chim. Acta 214 (1988) 187.
[7] E.M. Abad-Villar, M.T. Fernández-Abedul, A. Costa-García, Anal. Chim. Acta 409 (2000) 149.
[8] P. Fanjul-Bolado, M.B. González-García, A. Costa-García, Talanta 64 (2004) 452.
[9] S. Girotti, E. Ferri, S. Ghini, R. Budini, D. Patrono, L. Incorvara, A. Roda, Anal. Lett. 27 (1994) 323.
[10] C.R. Tillyer, S. Rakhorst, C.M. Colley, Clin. Chem. 40 (1994) 803.
[11] T. Kawakami, S. Igarashi, Analyst 120 (1995) 539.
[12] W. Withold, U. Schulte, H. Reinauer, Clin. Chem. 42 (1996) 210.
[13] J.M. Lin, A. Tsuji, M. Maeda, Anal. Chim. Acta 339 (1997) 139.
[14] A.L. Lasalle, B. Limoges, C. Degrand, J. Electroanal. Chem. 379 (1994) 281.
[15] L. Dellaciana, G. Bernacca, F. Bordin, S. Fenu, F. Garetto, J. Electroanal. Chem. 382 (1995) 129.
[16] K. Jiao, W. Sun, H.Y. Wang, Chinese Chem. Lett. 13 (2002) 69.
[17] R.E. Gyurcsanyi, A. Bereczki, G. Nagy, M.R. Neuman, E. Lindner, Analyst 127 (2002) 235.
[18] J.H. Hsieh, T.M. Wu, J.Z. Tong, J. Adhes. Sci. Technol. 17 (2003) 2085.
[19] K. Chen, W.B. Caldwell, C.A. Mirkin, J. Am. Chem. Soc. 115 (1993) 1193.
[20] D.H. Johnston, K.C. Glasgow, H.H. Thorp, J. Am. Chem. Soc. 117 (1995) 8933.
[21] Y. Xia, G.M. Whitesides, Angew. Chem., Int. Ed. 37 (1998) 551.
[22] A. Kumar, H.A. Biebuyck, G.M. Whitesides, Langmuir 10 (1994) 1498.
[23] T. Deng, H. Wu, S.T. Brittain, G.M. Whitesides, Anal. Chem. 72 (2000) 3176.
[24] M. Scholten, J.E.A.M. Van Den Meerakker, J. Electrochem. Soc. 140 (1993) 471.
[25] K.-S. Yun, H.-J. Kim, S. Joo, J. Kwak, E. Yoon, Jpn. J. Appl. Phys. 39 (2000) 7159.
[26] M. Heyrovsky, Electroanalysis 16 (2004) 1067.
[27] P. Canizares, C. Saez, J. Lobato, M.A. Rodrigo, Ind. Eng. Chem. Res. 43 (2004) 1944.
[28] M.S. Alaejos, F.J.G. Montelongo, Chem. Rev. 104 (2004) 3239.
[29] M. Gattrell, D.W. Kirk, J. Electrochem. Soc. 140 (1993) 1534.